High titer gluconic acid fermentation by *Aspergillus niger* from dry dilute acid pretreated corn stover without detoxification

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HIGHLIGHTS

- Dilute acid pretreated corn stover was used for gluconic acid production without detoxification.
- 76.67 g/L of gluconic acid was obtained with 94.83% of the overall yield from cellulose.
- No extra nutrients were added to corn stover hydrolysate for gluconic acid fermentation.
- Zero wastewater discharge from pretreatment to gluconic acid fermentation.
- Sodium gluconate from corn stover is used as cement additive.

GRAPHICAL ABSTRACT
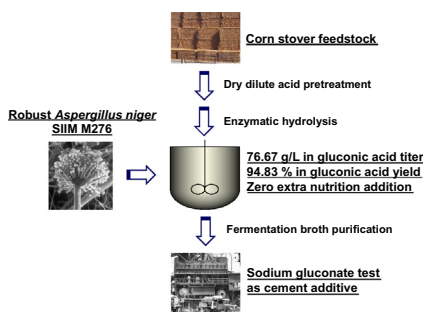
ABSTRACT

This study reported a high titer gluconic acid fermentation using dry dilute acid pretreated corn stover (DDAP) hydrolysate without detoxification. The selected fermenting strain *Aspergillus niger* SIIM M276 was capable of inhibitor degradation thus no detoxification on pretreated corn stover was required. Parameters of gluconic acid fermentation in corn stover hydrolysate were optimized in flasks and in fermentors to achieve 76.67 g/L gluconic acid with overall yield of 94.91%. The sodium gluconate obtained from corn stover was used as additive for extending setting time of cement mortar and similar function was obtained with starch based sodium gluconate. This study provided the first high titer gluconic acid production from lignocellulosic feedstock with potential of industrial applications.

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1. Introduction

Gluconic acid is an important biobased chemical used in food, feed, pharmaceuticals and construction industry. Sodium gluconate, the sodium salt of gluconic acid, is widely used as the cement additive to extend the setting time of cement (Ma et al., 2015). The recent booming of infrastructure investment in developing countries has stimulated the requirement of gluconic acid. Taking China as an example, the annual production of cement in 2012 was 2.1 billon tons (Li et al., 2015), the requirement of sodium gluconate as additive was estimated as 2.5–5.0 million tons if 0.1–0.2% of sodium gluconate is added. Currently, the industrial gluconic acid is produced by fermentation of filamentous fungus *Aspergillus niger* using starch or sucrose as feedstocks (Ramachandran et al., 2006; Wong et al., 2008). The future market expansion of sodium gluconate requires the alternative feedstock to substitute starch and sucrose. Sugar cane molasses (Rao and Panda, 1994), sugar beet molasses (Roukas and Harvey, 1988), sugar cane syrup (Purane et al., 2012), grape juice (Buzzini et al., 1993; Singh and Singh, 2006), fig fruit (*Figus carica*) juice

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(Roukas, 2000), and waste office paper (Ikeda et al., 2006) had been tested as feedstock for gluconic acid fermentation.

Among many feedstock options, lignocellulosic biomass such as corn stover, wheat straw, rice straw provides the most abundant carbohydrate material as potential feedstock for gluconic acid fermentation. However, the gluconic acid titer was pretty low comparing the starch based fermentation when lignocellulosic feedstock was used (Matsui et al., 2013), thus the potential of industrial applications was reduced. Ikeda et al. (2006) used waste office paper (WOP) as feedstock, first to hydrolyze into glucose and then ferment into about 80 g/L of gluconic acid by A. niger IAM2094 with only 60% of yield and 0.047 g/L/h of productivity. Although waste office paper is generally regarded as a specific lignocellulose, its high content of calcium carbonate (up to 20%, w/w) requires large amount of acid for neutralization before enzymatic hydrolysis (Wang et al., 2011). Its limited supply also made it less attractive as alternative of starch or sucrose feedstocks for industrial gluconic acid fermentation.

This study reported a high titer gluconic acid fermentation using a typical lignocellulosic biomass corn stover as feedstock after it was pretreated using dry dilute sulfuric acid method (DDAP) (Zhang et al., 2011; He et al., 2014) and enzymatically hydrolyzed into fermentable sugars. An industrial strain A. niger SIIM M276 was used as fermenting strain using the freshly prepared corn stover hydrolysate without inhibitor removal. High titer gluconic acid with the high overall yield was obtained and the obtained sodium gluconate was satisfied when it was used as cement additive. This study provided the first high titer gluconic acid production case from lignocellulosic feedstock with potential of industrial applications.

2. Materials

2.1. Raw materials

Corn stover (CS) was grown in Dancheng, Henan, China and harvested in fall, 2012. After collection, the materials were milled coarsely using hammer crusher and screened through a mesh with the circle diameter of 10 mm. The milled corn stover was washed to remove field dirt, stones and metals, then dried until constant weight. The raw corn stover contained 38.72% of cellulose, 20.55% of hemicellulose, 26.51% of lignin, 2.76% of ash on dry weight base (w/w) determined on ANKOM 220 Cellulose Analyzer (ANKOM Technology, Macedon, NY, USA).

2.2. Enzymes and reagents

Commercial cellulase enzyme Youtell #6 was purchased by Hunan Youtell Biochemical Co., Yueyang, Hunan, China. The filter paper activity was 145 FPU per gram determined using the NREL protocol LAP-006 (Adney and Baker, 1996), the cellobiase activity was 344 IU per gram according to the method by Ghose (1987), and the protein concentration was 90 mg/g cellulase determined by Bradford method using BSA as protein standard. Peroxidase horseradish was purchased from Sangon Biotech. Co., Shanghai, China, with the activity of 250 U/mg enzyme.

Sodium gluconate and coniferaldehyde was purchased from Sigma–Aldrich, St. Louis, MO, USA. Yeast extract was from Angel Yeast Co., Yichang, Hubei, China. Furfural, 5-hydroxymethylfurfural (HMF) were from J&K Scientific Co., Beijing, China. Furfuralcohol and HMF alcohol were from Bide Pharmatech Co., Shanghai, China. Acetic acid, formic acid, and levulinic acid were from Sinopharm Chemical Reagent Co., Shanghai, China. Vanillin was from Aladdin Reagents Co., Shanghai, China. 4-Hydroxybenzaldehyde and syringaldehyde were from Sangon

Biotech. Co., Shanghai, China. Vanillic acid and 4-hydroxybenzoic acid were from Tokyo Chemical Industry, Tokyo, Japan. Syringate was from Alfa Aesar Co., Tianjin, China. The other reagents are all from Lingfeng Chemical Reagent Co., Shanghai, China.

2.3. Strains and medium

A. niger SIIM M276 was purchased from Shanghai Industrial Institute of Microbiology (SIIM), Shanghai, China. The medium used for A. niger SIIM M276 included:

(1) Activation medium contained 8.0 g of glucose, 2.0 g of Yeast extract, 0.2 g of $MgSO_4$, 0.1 g of NaH_2PO_4, 0.01 g of $MnSO_4$, 20.0 g of agar in one liter of deionized water.
(2) Seed medium contained 60 g of glucose, 2.0 g of yeast extract, 0.2 g of $MgSO_4$, 0.1 g of NaH_2PO_4, 0.1 g of KH_2PO_4, 0.01 g of $MnSO_4$ in one liter of deionized water.
(3) Synthetic medium for fermentation contained 100.0 g of glucose, 0.3 g of $MgSO_4$, 0.2 g of KH_2PO_4, 0.9 g of $(NH_4)_2HPO_4$, 0.06 g of $MnSO_4$ in one liter of deionized water.

2.4. Pretreatment and hydrolysate preparation

Dry dilute acid pretreatment method (DDAP) was used as described by Zhang et al. (2011) and He et al. (2014). Briefly, 2.5% (w/w) of sulfuric acid on dry corn stover weight was co-currently fed into the pretreatment reactor with corn stover material at a ratio of the solid (the dry materials) to the liquid (the sulfuric acid solution) of 2:1 (w/w). The pretreatment was operated at 175 °C for 5 min under helically agitation at 50 rpm. The pretreated corn stover contained approximately 50% (w/w) of dry DM (solid matter) and no free wastewater stream was generated from the pretreatment. The pretreated corn stover contained 39.47% of cellulose, 6.80% of hemicellulose, 6.57% of ash according to two-step acid hydrolysis method according to NREL protocols (Sluiter et al., 2012, 2008). The composition of inhibitors in the pretreated corn stover contained (mg/g dry pretreated corn stover): furfural 5.13, HMF 3.38, acetic acid 16.65, formic acid 1.97, levulinic acid 2.54, vanillin 1.27, syringaldehyde 0.67, 4-hydroxybenzaldehyde 0.18, coniferaldehyde 0.11.

Corn stover hydrolysate was prepared in a 5 L bioreactor (Zhang et al., 2010) equipped with helical ribbon impeller for mixing. Freshly pretreated corn stover was hydrolyzed using cellulase at dosage of 15 FPU/g DM at 50 °C, pH 4.8 for 48 h. The slurry was centrifuged at 10,000 rpm for 10 min to remove the water insoluble solids and get clear hydrolysate. Then the hydrolysate was autoclaved at 115 °C for 20 min and filtered to remove solids by filter paper before use. No any nutrients were added to corn stover hydrolysate in the fermentation step.

2.5. Gluconic acid fermentation

The spore suspension of A. niger SIIM M276 was maintained at −80 °C freezer in the 2 mL stock vials containing 30% (v/v) glycerol solution. 200 μL of one stock vial was inoculated into petri dish with activation medium and cultured at 30 °C for 72 h. The spores growing on petri dish was washed by sterile water and inoculated into a 500 mL flask containing 100 mL seed medium at ~1.0×10^5 per mL for seed culture at 30 °C, 200 rpm for 24 h. For flask culture, the seed broth was inoculated into a 250 mL flasks containing 50 mL fermentation medium with 10% (v/v) inoculation ratio at 30 °C, 200 rpm for 72–144 h. All flask cultures were carried out in duplicate.

For fermentor culture, the seed broth was inoculated into 3 L fermentor containing one liter corn stover hydrolysate with 10% (v/v) inoculation ratio at 33 °C, 500 rpm, 1.6 vvm of ventilation

volume for 48–96 h. 30 g of sterile CaCO$_3$ per 100 g of glucose was added to fermentation medium for pH control in flasks, and 5 M NaOH was used for pH control in fermentors. Medium and water used above were autoclaved at 115 °C for 20 min. 30 g CaCO$_3$ powder (equivalent to 0.28 mol considering the impurities in it) was added to flasks before the fermentation started by taking advantage of low solubility in water and the slow release to neutralize the 0.56 mol gluconic acid produced by 100 g glucose and maintain the pH at 6.0.

2.6. Analysis of sugars, gluconic acid and inhibitors

Samples were taken periodically and centrifuged at 13,000 rpm for 5 min followed by filtration through 0.22 μm filters before analysis. Glucose was analyzed using SBA-40D biosensor (Shandong Academy of Agriculture, Jinan, China). Gluconic acid was analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with a Shodex Rspak JJ50-4D column (Showa Denko, Tokyo, Japan) at 40 °C using the mobile phase of 12 mM NaHCO$_3$ at the rate of 0.5 mL/min.

Acetic acid, formic acid, levulinic acid, furfural, furfural alcohol, HMF and HMF alcohol were analyzed using HPLC (LC-20 AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) at 65 °C using the mobile phase of 5 mM H$_2$SO$_4$ at the rate of 0.6 mL/min.

Phenolic compounds including vanillin, vanillic acid, syringaldehyde, syringate, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid and coniferaldehyde were analyzed using HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) at 35 °C and 270 nm at the flow rate of 1.0 mL/min. The detailed procedure was that the initial mobile phase was composed of 0.1% formic acid (pump A) and acetonitrile (pump B) at a ratio of 90 –10%. Acetonitrile was increased from 0% to 10% from 1 to 15 min, then acetonitrile was increased from 10% to 35% over 15 to 20 min, finally, acetonitrile was used at 10% over 20 to 30 min (Khoddami et al., 2013).

2.7. Glucose oxidase activity assay

Glucose oxidase activity was measured according to the method by Barham and Trinder (1972). Briefly, hydrogen peroxide produced in glucose oxidation was determined by coupling phenol and 4-aminoantipyrine to generate red quinone imine compound at 37 °C, pH 7.0 in a reaction solution containing 2 mL of 50 mM β-D-glucose, 1 mL of 25 mM 4-aminoantipyrine, 1 mL of 25 mM phenol, 0.5 ml of 0.05 mg/ml horseradish peroxidase, 0.5 mL of fermentation broth containing glucose oxidase in 5 mL then vigorously shaking for 30 s. The change of absorbance in 1 min was measured at 500 nm and calculated. One unit of glucose oxidase activity was defined as the amount of enzyme producing 1 μmol of hydrogen peroxide per milliliter per minute at 37 °C.

$$\text{Glucose oxidase activity (U)} = \frac{(A - A_0) \times k}{v \times t} \times n$$

where A_0 and A indicate initial and final absorbance of reaction solution at 500 nm, k is the conversion factor for absorbance of reaction solution at 500 nm to equivalent concentration of hydrogen peroxide, v is the volume of reaction solution (mL), t is the reaction time (min), n is the dilution ratio of fermentation broth.

2.8. Calculation of gluconic acid yield

The conversion yield of glucose to gluconic acid is defined as the ratio of the gluconic acid formed to the gluconic acid according to the theoretical value:

$$\text{Gluconic acid yield (\%)} = \frac{[GA] \times V - [GA]_0 \times V_0}{[Glu]_0 \times V_0 \times 1.089} \times 100\%$$

where $[GA]$ and $[GA]_0$ indicate the concentrations of gluconic acid at the end and the start of gluconic acid fermentation, respectively (g/L); $[Glu]_0$ indicate glucose concentration at the start of fermentation (g/L); 1.089 is the conversion factor for glucose to equivalent gluconic acid derived from the molecular weight ratio; V and V_0 indicate the volumes of fermentation broth at the end and the start of the gluconic acid fermentation, respectively (L).

## 2.9. Setting time and fluidity assay

Gluconic acid fermentation broth was purified by centrifugation, filtration, and decoloration to obtain the final sodium gluconate solution at 384 g/L. The setting time of the cement paste was determined on a Vicat apparatus according to Chinese Standard Protocol GB/T 1346-2011. The fluidity of the cement mortar was determined according to the Chinese Standard Protocol GB/T 2419-2005 using a cement mortar rheometer. Cement was loaded into the conical mortar and vibrating for 25 s at the frequency of 1 s^{-1} inside the rheometer. The circle diameter of the cement mortar flow was measured to give the average value as the fluidity of cement mortar (mm).

3. Results and discussion

3.1. Gluconic acid fermentation of A. niger SIIM M276 in corn stover hydrolysate

Gluconic acid fermentation of *A. niger* SIIM M276 was carried out in synthetic medium and corn stover hydrolysate as feedstocks (Fig. 1). The most significant difference was its long lag phase in corn stover hydrolysate: 48 h for cell growth starting, 96 h for gluconic acid formation, and 144 h for complete glucose utilization. As the comparison in synthetic medium, cells grew immediately after inoculation, glucose consumed completely and gluconic acid reached its maximum within 72 h. The inhibitor compounds in the initial corn stover hydrolysate were measured and high inhibitor concentrations were discovered at 3.24 g/L of acetic acid, 0.20 g/L of 5-HMF, 0.37 g/L of furfural, 0.16 g/L formic acid, 0.21 g/L levulinic acid, 0.11 g/L vanillin, 0.06 g/L syringaldehyde, 0.02 g/L 4-hydroxybenzaldehyde, 0.01 g/L coniferaldehyde. The three major inhibitor compounds from dilute acid pretreatment including furfural, HMF and acetic acid were monitored during the 144 h fermentation period in corn stover hydrolysate and an obvious inhibitor conversion was demonstrated (Fig. 1c): furfural decreased steadily from very beginning but the decreases of HMF and acetic acid were very slow; only when furfural entered its fast degradation stage from 72 h then HMF started its fast degradation correspondingly; only when furfural was completely degraded after 96 h, acetic acid started its observable degradation. Coincidentally, *A. niger* SIIM M276 started its fast growth when furfural entered its fast degradation stage at 72 h, and gluconic acid started to accumulate when furfural was completely consumed at 96 h. The results suggest that *A. niger* SIIM M276 was capable of inhibitor degradation or "detoxification" function while maintained its fermentability for gluconic acid production. On the other hand, although a long lag phase of *A. niger* SIIM M276 existed in corn stover hydrolysate, cell growth and gluconic acid accumulation were still satisfactory, 60% cell mass greater than in synthetic medium with the gluconic acid yield of 94.17% at the end of the 144 h fermentation.

The temperature tolerance of *A. niger* SIIM M276 in corn stover hydrolysate was investigated (Table 1). In the range of 28–33 °C were suitable for *A. niger* strains, both glucose consumption rate
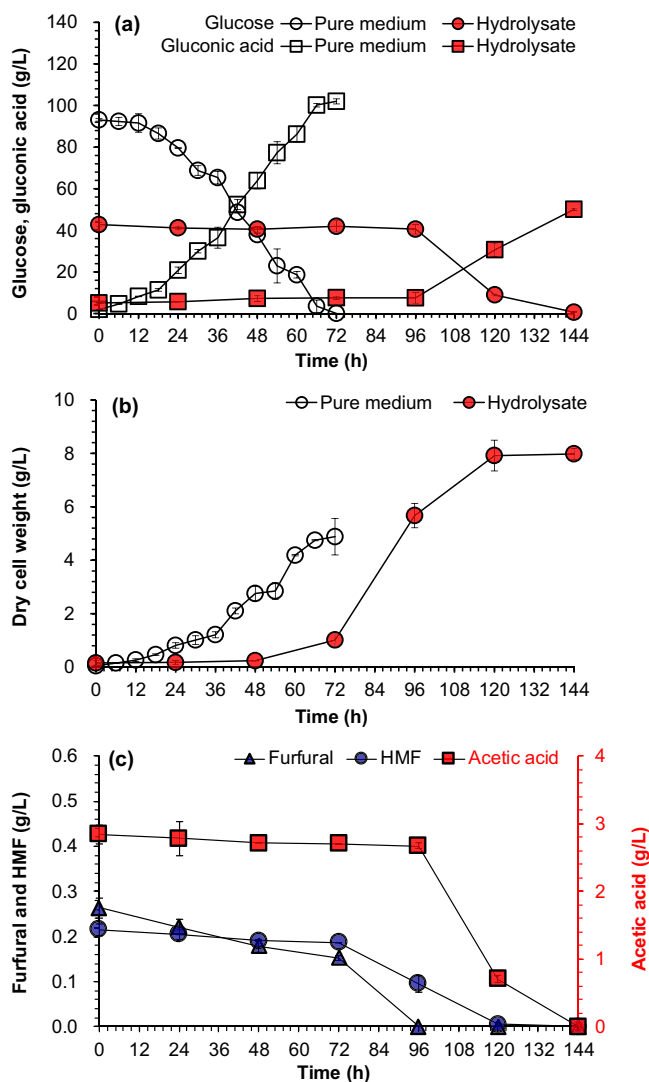
Fig. 1. Gluconic acid fermentation in synthetic medium and corn stover hydrolysate. (a) Glucose consumption and gluconic acid formation; (b) cell growth; (c) inhibitors degradation in corn stover hydrolysate. Corn stover hydrolysate contained 44.49 g/L of glucose, 21.84 g/L of xylose, 3.24 g/L of acetic acid, 0.20 g/L of 5-HMF, 0.37 g/L of furfural, 0.16 g/L formic acid, 0.21 g/L levulinic acid, 0.11 g/L vanillin, 0.06 g/L syringaldehyde, 0.02 g/L 4-hydroxybenzaldehyde, 0.01 g/L coniferaldehyde. The three major inhibitors including furfural, HMF and acetic acid were monitored during the fermentation period. The fermentation was carried out at 30 °C, pH 6.0, inoculum size 10%.

Table 1
Gluconic acid fermentation by *A. niger* SIIM M276 from corn stover hydrolysate under different temperature.

Temperature (°C)	Gluconic acid titer (g/L)	Gluconic acid productivity (g/L/h)	Glucose consumption rate (g/L/h)
28	34.23 ± 1.73	0.249 ± 0.014	0.298 ± 0.011
30	38.04 ± 3.28	0.284 ± 0.026	0.339 ± 0.041
33	43.49 ± 0.98	0.332 ± 0.008	0.363 ± 0.004
37	39.66 ± 2.08	0.300 ± 0.022	0.359 ± 0.000

The hydrolysate contained 44.49 g/L of glucose, 21.84 g/L of xylose, 3.24 g/L of acetic acid, 0.20 g/L of 5-HMF, 0.37 g/L of furfural. All the dates were collected at 120 h. The fermentation condition was 200 rpm, pH 6.0, liquid volume ratio 20% in 250 mL shaking flasks, inoculate size 10%.

and gluconic acid productivity increased with increasing temperature, then decreased with the further increase to 37 °C. By the time of complete consumption of glucose after 120 h, gluconic acid titer

and productivity were approximately the same, in which at 33 °C showed slightly greater gluconic acid titer. On the other hand, *A. niger* SIIM M276 was unable to utilize xylose and easy to aggregate in the prolonged culture in the inhibitor containing hydrolysate. These disadvantages should be the targets of future metabolic engineering on the fermenting strain of *A. niger*.

3.2. Tolerance of A. niger SIIM M276 to lignocellulose derived inhibitor compounds

Inhibitor existence in corn stover hydrolysate significantly inhibited gluconic acid fermentation of *A. niger* SIIM M276 by experiencing a long lag phase to degrade the inhibitor compounds (Fig. 1). Therefore, we examined the detailed inhibitor tolerance on cell growth, gluconic acid accumulation and glucose oxidase activity of *A. niger* SIIM M276 (Fig. 2). Nine inhibitors from dilute acid pretreatment were selected as representative model inhibitors including two furan derivatives, furfural and HMF; three weak organic acids, acetic acid, formic acid, and levulinic acid; and four phenolic compounds, 4-hydroxybenzaldehyde and coniferaldehyde representing *p*-hydroxyphenyl group (H), syringaldehyde representing syringyl group (S), and vanillin representing guaiacyl group (G) (Palmqvist and Hahn-Hägerdal, 2000; Klinke et al., 2004; Nichols et al., 2008).

To assay the inhibitor tolerance of *A. niger* SIIM M276, the inhibitor concentration range was selected based on the real concentration of the nine inhibitors in the corn stover hydrolysate from dry dilute acid pretreatment operation as described in Section 2, as well as the possible changes from varying feedstocks, solids loading, sulfuric acid dosage, operation time, and temperature. Furfural demonstrated the most toxic inhibition on gluconic acid fermentation of *A. niger* SIIM M276 (Fig. 2a), in which the cell growth and glucose oxidase activity were reduced by half at 0.5 g/L of furfural; when furfural was greater than 1.0 g/L, the metabolism of *A. niger* SIIM M276 almost completely stopped. On the other hand, another furan derivative HMF showed the less toxic inhibition on *A. niger* SIIM M276 (Fig. 2b). For weak organic acid inhibitors, formic acid showed the complicated effect with increased fermentability at lower concentration range (1.0–3.0 g/L), then decreased with increasing formic acid concentration (Fig. 2c). The fermentability of *A. niger* SIIM M276 decreased with increasing acetic acid and levulinic acid concentrations, but still maintained certain cell growth rate, gluconic acid productivity and glucose oxidase activity until 10 g/L of acetic acid and levulinic acid (Fig. 2d and e). For phenolic inhibitors, coniferaldehyde showed the strongest inhibition on fermentability of *A. niger* SIIM M276 in which 0.4 g/L of coniferaldehyde stopped the fermentation activity of the strain (Fig. 2i), then followed by vanillin, 4-hydroxybenzaldehyde, and syringaldehyde (Fig. 2f–h). The results indicate that *A. niger* SIIM M276 had outstanding tolerance to the typical inhibitors in corn stover hydrolysate, especially to organic weak acids. The most toxic inhibitor was furfural, and some phenolic inhibitors were also harsh to the fermenting strain.

A. niger SIIM M276 also demonstrated its strong capacity of inhibitor degradation into less toxic compounds for cell growth and gluconic acid fermentation (Fig. 3). For furan derivatives, 1.0 g/L of furfural was degraded to its alcohol derivative within 48 h and the higher furfural concentration significantly reduced the conversion capacity (Fig. 3a). On the other hand, *A. niger* SIIM M276 converted up to 2.0 g/L of HMF into HMF alcohol quickly (Fig. 3b). For weak organic acids, *A. niger* SIIM M276 degraded a high level of 4.0 g/L of formic acid, but only showed limited capacity to convert acetic acid even at a relatively low concentration range of 2.5 g/L, and no conversion was observed for levulinic acid (Fig. 3c–e). For phenolic aldehydes, *A. niger* SIIM M276 converted these phenolics into the corresponding acids at a low
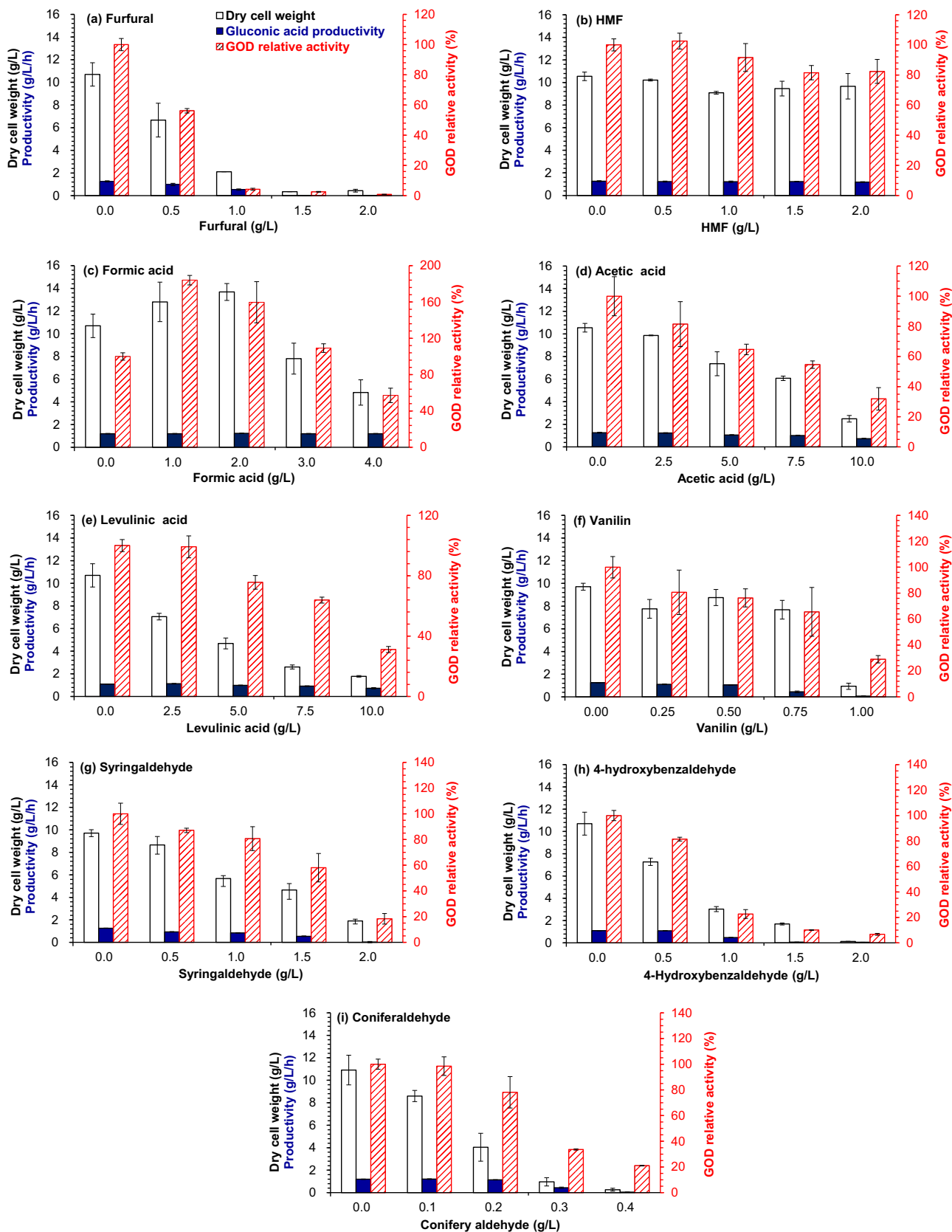
Fig. 2. Inhibitor tolerance on cell growth, gluconic acid productivity and gluconic oxidase activity of *Aspergillus niger* SIIM M276. (a) Furfural; (b) HMF; (c) formic acid; (d) acetic acid; (e) levulinic acid; (f) vanillin; (g) syringaldehyde; (h) 4-hydroxybenzaldehyde; (i) coniferaldehyde. Conditions: 30 °C, pH 6.0, liquid volume ratio 20%, inoculate size 10% for 72 h.
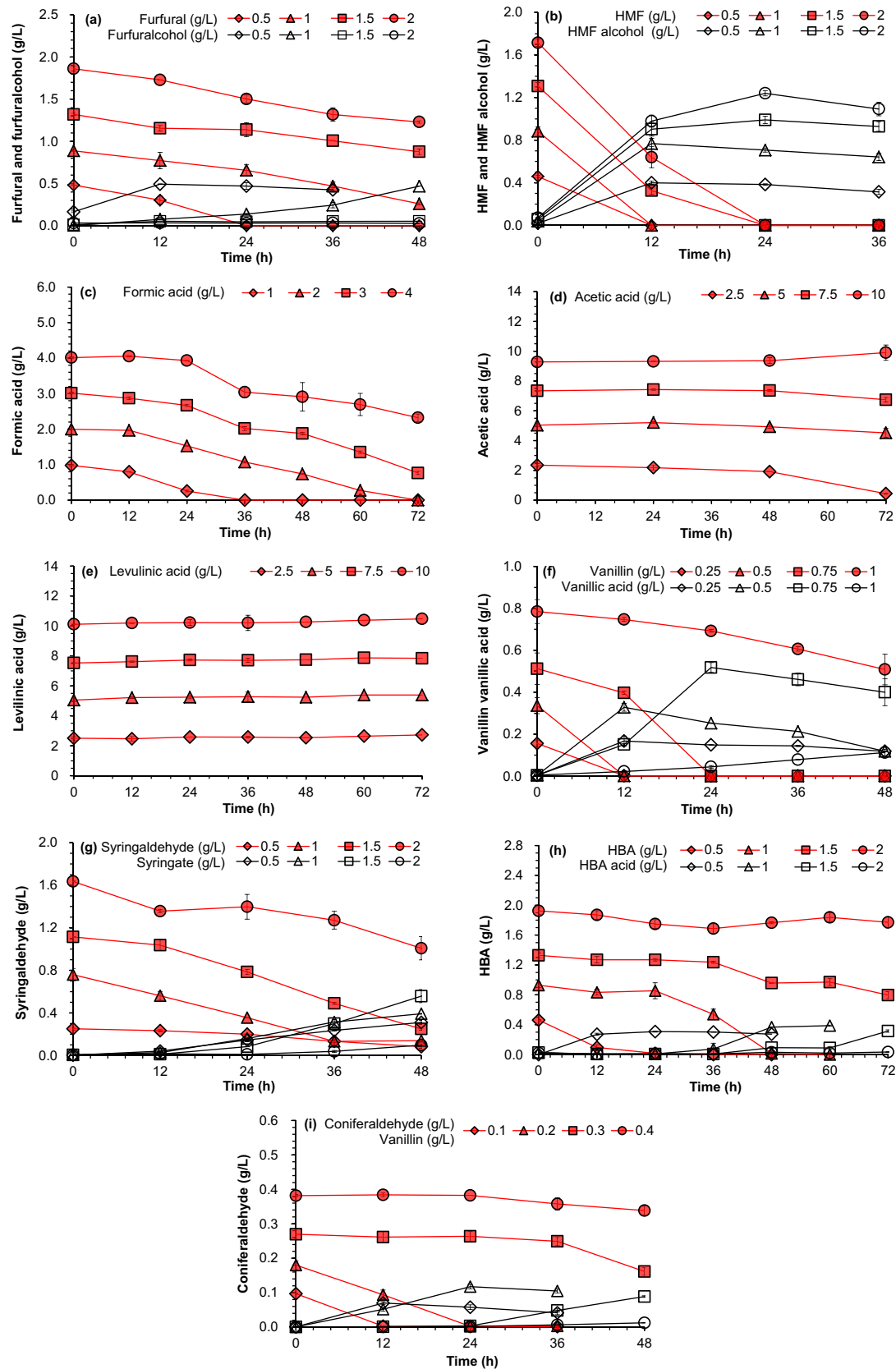
Fig. 3. Degradation of inhibitors by *Aspergillus niger* SIIM M276. (a) Furfural; (b) HMF; (c) formic acid; (d) acetic acid; (e) levulinic acid; (f) vanillin; (g) syringaldehyde; (h) 4-hydroxybenzaldehyde (HBA); (i) coniferaldehyde. Conditions: 30 °C, 200 rpm, pH 6.0, inoculate size 10%. Legends indicate the initial concentration of inhibitors in the culture broth.

concentration range, but higher phenolics inhibited the conversion capacity (Fig. 3f–i).

These results indicate that *A. niger* SIIM M276 survived in high toxic corn stover hydrolysate by its strong tolerance to various inhibitor compounds, as well as the capacity of converting the aldehyde inhibitors such as furfural, HMF and phenolics into the less toxic alcohols or acids to release the stress on cell growth and fermentability.

3.3. Oxygen requirement of A. niger SIIM M276 for gluconic acid fermentation in corn stover hydrolysate

Common *A. niger* strains require sufficient oxygen for its cell growth and metabolism. When *A. niger* M276 is used for gluconic acid fermentation, extra oxygen is also required for oxidizing glucose into gluconic acid besides its cell growth requirement. In this study, dissolve oxygen level on cell growth and gluconic acid productivity was examined in both flasks and fermentors.

In flask fermentation, dissolved oxygen level in corn stover hydrolysate increased with increasing rotation rate and reducing liquid volume ratio in flasks. Table 2 shows that the gluconic acid titer, yield and productivity significantly increased with increasing rotation rate and decreasing liquid volume ratio in the range of experiment, indicating dissolved oxygen level was an important on elevating gluconic acid fermentability of *A. niger* SIIM M276 in corn stover hydrolysate.

In fermentor fermentation with accurate control of pH and temperature, when dissolved oxygen level was regulated by changing rotation rate of 300–800 rpm at constant aeration flowrate

(1.6 vvm), glucose consumption rate and gluconic acid productivity significantly increased (Table 3). The cell growth rate increased but the dissolved oxygen level maintained at high level of about 60% of saturation, even at the 72 h with a large number of gluconic acid formation. Among the three major inhibitors, furfural quickly degraded, then HMF, acetic acid degraded slowly and had the similar conversion rates at different rotation rate at 72 h. Similarly, when dissolved oxygen level was regulated by changing aeration flowrate of 0.8–2.4 vvm at constant rotation rate (500 rpm), glucose consumption rate and gluconic acid productivity increased significantly except at the lowest aeration flowrate of 0.8 vvm. Dissolved oxygen level changed with cell growth and gluconic acid formation but still maintained about 60% of saturation although at the highest value of aeration flowrate. Furfural showed the fast degradation except the lowest aeration flowrate, and HMF and acetic acid conversions were approximately the same as at differ-

Table 2
Gluconic acid fermentation from corn stover hydrolysate under changing dissolved oxygen levels in flasks.

Parameters		Titer (g/L)	Yield (%)	Productivity (g/L/h)
Rotation rate (rpm)	0	3.30 ± 0.02	3.67 ± 0.18	0.018 ± 0.000
	100	21.44 ± 0.85	43.12 ± 1.77	0.207 ± 0.008
	200	38.32 ± 3.74	87.34 ± 4.79	0.415 ± 0.021
	250	42.70 ± 1.09	88.08 ± 2.60	0.426 ± 0.010
Liquid volume ratio (%)	10	40.86 ± 0.26	80.42 ± 0.21	0.391 ± 0.002
	20	39.12 ± 0.17	76.25 ± 1.02	0.367 ± 0.005
	30	8.44 ± 2.61	10.73 ± 5.26	0.054 ± 0.028
	40	5.67 ± 0.04	4.21 ± 0.12	0.021 ± 0.002

Corn stover hydrolysate contained 44.49 g/L of glucose, 21.84 g/L of xylose, 3.24 g/L of acetic acid, 0.20 g/L of HMF, 0.37 g/L of furfural. All the dates were collected at 96 h. The rotation rate test was carried out at 30 °C, pH 6.0, liquid volume ratio 20% in 250 mL shaking flasks, inoculate size 10%. The liquid volume ratio test was carried out 30 °C, 200 rpm, pH 6.0, inoculate size 10%, in 250 mL shaking flasks.

Table 3
Gluconic acid fermentation under different agitation rate and aeration rate in 3 L bioreactor.

	Glucose (g/L)	Gluconic acid (g/L)	Dry cell mass (g/L)	Furfural (g/L)	HMF (g/L)	Acetic acid (g/L)
At varying rotation rate (rpm) at 33 °C, pH 6.0, airflow rate 1.6 vvm						
300	39.50	10.58	3.45	0.00	0.04	1.81
500	11.10	30.64	4.05	0.00	0.02	1.76
800	0.00	52.50	6.45	0.000	0.00	1.45
At varying aeration rate (vvm) at 33 °C, pH 6.0, rotation rate 500 rpm						
0.8	49.40	2.47	0.52	0.17	0.32	2.56
1.6	18.30	33.60	5.73	0.00	0.03	2.05
2.4	0.00	51.76	5.90	0.00	0.00	2.15

All the data were collected after 72 h fermentation in the bioreactors. Glucose, furfural, HMF, and acetic acid were residual concentrations after 72 h, and gluconic acid and dry cell mass were the produced after 72 h. The composition of hydrolysate: 48.97 g/L of glucose, 26.11 g/L of xylose, 3.16 g/L of acetic acid, 0.45 g/L of HMF, 0.91 g/L of furfural. Liquid volume 1 L, inoculum size 10%.
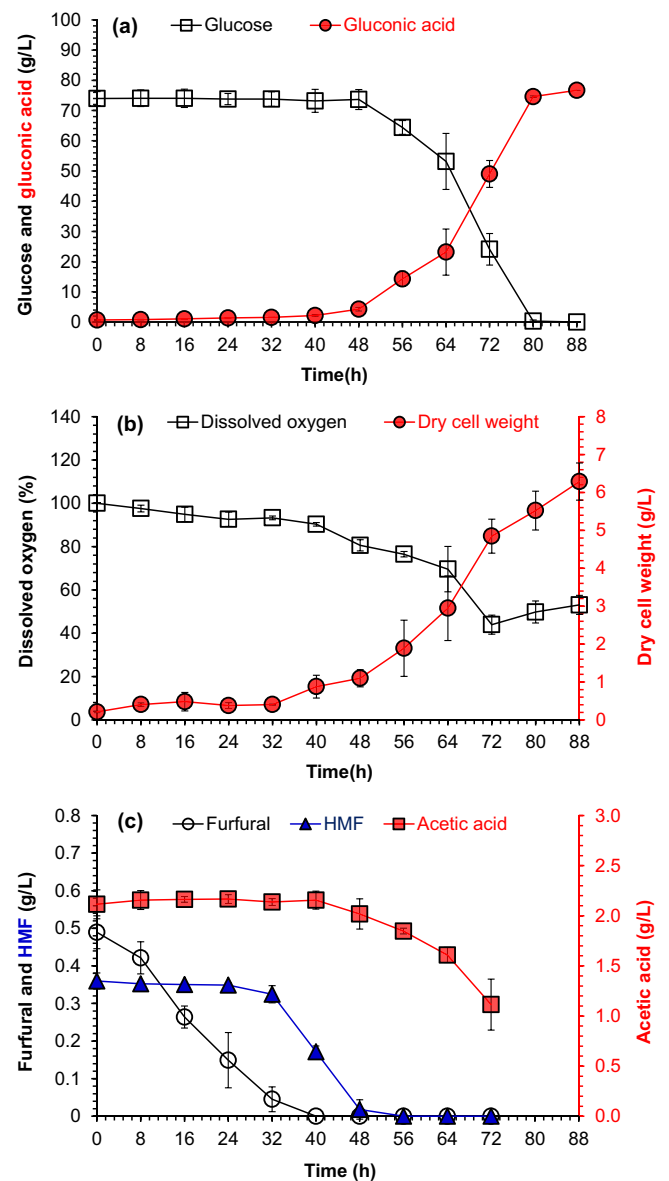
Fig. 4. Production of gluconic acid by high solid loading corn stover hydrolysate. (a) Glucose and gluconic acid profiles; (b) dry cell weight and dissolved oxygen profiles; (c) inhibitor profiles. Conditions: 33 °C, pH 6.0, liquid volume 1 L, inoculum size 10%, 500 rpm, 1.6 vvm. The composition of the hydrolysate: 80.21 g/L of glucose, 35.20 g/L of xylose, 2.29 g/L of acetic acid, 0.49 g/L of HMF, 0.60 g/L of furfural.

Table 4
Setting time and fluidity of cement mortar at different sodium gluconate addition.

Additive	Final setting time (min)	Fluidity (mm)
Without sodium gluconate addition	200	265
Commercial sodium gluconate	222	260
Cellulosic sodium gluconate	226	295

P.O 42.5R cement in this test was from Shandong Qiyin Co., Zibo, Shandong, China, the commercial sodium gluconate produced from corn was from Xiwang Group, Zouping, Shandong, China. Lignocellulosic sodium gluconate from this study. The dosage of two sodium gluconate were both 0.2% (w/w) for testing final setting time and 0.06% (w/w) for testing the fluidity.

ent rotation rate. These results indicate that aeration flowrate should be maintained at the minimum level of 0.8 vvm for *A. niger* SIIM M276, and enhanced oxygen supply elevated gluconic acid productivity in a way of maintaining high oxygen saturation level, instead of oxygen consumption requirement. The aeration also facilitated the removal of volatile inhibitors such as furfural for enhancing gluconic acid formation by *A. niger* SIIM M276. In this experiment, only one parameter (rotation rate or aeration rate) was changed in the small step size, each was regarded as controls for others in the data series.

Fig. 4 demonstrated a gluconic acid fermentation operation with high product titer using corn stover hydrolysate prepared by hydrolyzing 20% (w/w) of pretreated corn stover solids containing 80.21 g/L of glucose. Finally 76.67 g/L glucose acid was obtained with the productivity of 0.86 g/L/h and the yield of 94.83% based on the glucose in corn stover hydrolysate. The final gluconic acid concentration also have the potential to be increased by adding more pretreated corn stover solids for hydrolysate preparation.

3.4. Assay of sodium gluconate from corn stover as cement additive

Gluconic acid produced from corn stover hydrolysate existed in the form of sodium gluconate. The obtained sodium gluconate was used as cement additive to prolong the setting time of cement mortar by measuring its setting times and fluidity. The commercial sodium gluconate produced from starch feedstock was used as control. Final setting time indicates the time of cement mortar to completely losing the plasticity and behaving rigorous structural strength. Table 3 shows that the final setting time of cement mortar with cellulosic sodium gluconate addition was almost same to that with starch sodium gluconate (222 and 226 min, respectively). Fluidity indicates the uniformity and stability of cement additive in cement mortar indicated by circle diameter of cement mortar flow. Table 4 shows that the fluidity of cellulosic sodium gluconate was 13.5% greater than the commercial sodium gluconate from starch. The results indicate that the cellulosic sodium gluconate from corn stover was comparable to the starch based sodium gluconate as the cement additive for extending the setting time and fluidity of cement mortar.

This study reported a dry dilute acid pretreated corn stover as feedstock of high titer gluconic acid fermentation, in which no free wastewater was generated from pretreatment operation, no detoxification step was applied to inhibitor removal, and no extra nutrient addition was required. The preliminary test of the obtained cellulosic sodium gluconate demonstrated the competitive property as cement additive to starch based commercial sodium gluconate for extending the setting time and fluidity of cement mortar. This environment friendly technology provided a promising options for replacing starch feedstock for gluconic acid production with lignocellulosic biomass.

4. Conclusion

A. niger SIIM M276 showed an exciting inhibitor tolerance and gluconic acid fermentability from dry dilute acid pretreated corn stover hydrolysate without detoxification. Increasing oxygen supply shortened lag phase and 76.67 g/L gluconic acid with the overall yield of 94.91% was obtained from corn stover hydrolysate. The cellulosic sodium gluconate showed similar property as cement additive for extension of setting time and fluidity of cement mortar. The study suggested that lignocellulose could be an important alternative feedstock used for future industrial gluconic acid production.

Acknowledgements

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