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

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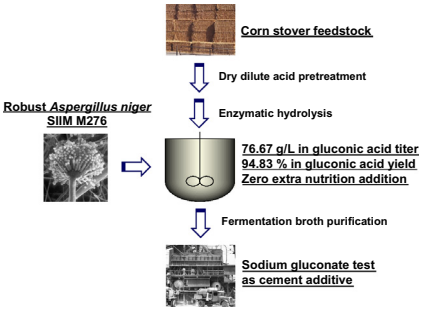
HIGHLIGHTS

- 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



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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.

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 $\sim 1.0 \times 10^5$ 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