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Short Communication

Complete oxidative conversion of lignocellulose derived non-glucose sugars to sugar acids by *Gluconobacter oxydans*

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ABSTRACT

Non-glucose sugars derived from lignocellulose cover approximately 40% of the total carbohydrates of lignocellulose biomass. The conversion of the non-glucose sugars to the target products is an important task of lignocellulose biorefining research. Here we report a fast and complete conversion of the total non-glucose sugars from corn stover into the corresponding sugar acids by whole cell catalysis and aerobic fermentation of *Gluconobacter oxydans*. The conversions include xylose to xylonate, arabinose to arabonate, mannose to mannonate, and galactose to galactonate, as well as with glucose into gluconate. These cellulosic non-glucose sugar acids showed the excellent cement retard setting property. The mixed cellulosic sugar acids could be used as cement retard additives without separation. The conversion of the non-glucose sugars not only makes full use of lignocellulose derived sugars, but also effectively reduces the wastewater treatment burden by removal of residual sugars.

1. Introduction

Hemicellulose is a heteropolymer of hexoses (D-glucose, D-galactose and D-mannose), pentose (D-xylose and L-arabinose) and some sugar acids. Approximately hemicellulose accounts for more than 40% of the total carbohydrates in lignocellulose biomass, in which D-xylan accounts for 34.1% of the total structural carbohydrate in corn stover, L-arabinan accounts for 5.1%, D-mannan accounts 0.5%, and D-galactan accounts for 2.7% (Humbird et al., 2011). The amorphous structure of hemicellulose can be easily hydrolyzed into monomeric sugars during various pretreatment processes (Sun and Cheng, 2005). The efficient utilization of the non-glucose sugars is the major focus of lignocellulose biorefining research for both the full use of lignocellulose derived sugars and the reduction of wastewater treatment burden by removal of residual sugars.

Most naturally occurring microorganisms only utilize glucose. Therefore, various fermentation strains had been metabolically engineered to boost the utilization of xylose and/or arabinose into ethanol and lactic acid (Zhang et al., 1995; Ho et al., 1998; Deanda et al., 1996; Peng et al., 2012). However, the wild *Gluconobacter oxydans* (*G. oxydans*) provides a unique route of oxidation conversion of various saccharide sugars into sugar acids. The membrane-bound dehydrogenases of *G. oxydans* not only convert glucose into gluconic acid, but also convert xylose into xylonic acid (Zhang et al., 2016a), D-galactose into D-galactonic acid (Svitel and Sturdik, 1994). The conversions of D-mannose and L-arabinose into the corresponding acids have been not reported, but are still possible. These sugar acids with hydroxy-carboxylate groups show the tricalcium silicate hydration retardation performance (Nalet and Nonat, 2016; Tan et al., 2011) as cement retard additive.

In this work, we investigated the oxidative conversion of all the non-glucose sugars derived from corn stover into the corresponding sugar acids by *G. oxydans* DSM 2003. In this work, we found that *G. oxydans* was capable of utilizing D-xylose, L-arabinose, D-mannose, D-galactose as carbon source for the cell growth and converting all these non-glucose sugars into the corresponding sugar acids (D-xylonate, L-arabonate, D-mannonate, and D-galactonate) by both aerobic fermentation and whole cell catalysis by *Gluconobacter oxydans*. The cement retard setting performance of the individual non-glucose sugar acids and the mixed acids was assayed as cement retard additive. The conversion of the non-glucose sugars not only makes full use of lignocellulose derived sugars, but also effectively reduces the wastewater treatment burden by removal of residual sugars.

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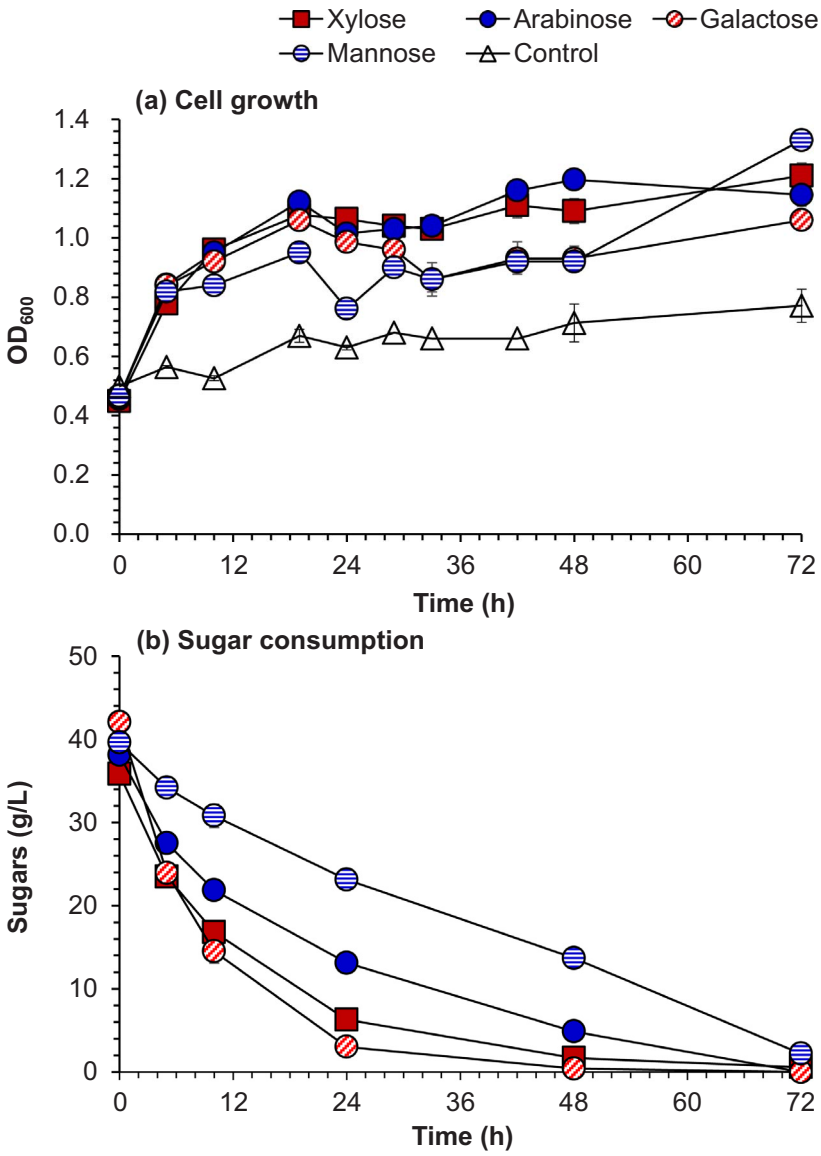

Fig. 1. Cell growth and utilization of non-glucose sugars by *G. oxydans* DSM 2003. (a) Cell growth indicated by the optical density at 600 nm; (b) Sugar consumption. The fermentation was carried out at 30 °C, 220 rpm with an inoculum size of 10% (v/v), in 250 mL flask containing 45 mL of the synthetic medium. Control is the synthetic fermentation medium without any sugar addition. The NaOH (5 M) was added every 6 h to neutralize the produced sugar acids for controlling the pH to a range of 5–6.

2. Material and methods

2.1. Raw materials and reagents

Corn stover was harvested from Bayan Nur, Inner Mongolia, China, in fall 2015. Corn stover was washed to remove field dirts, stones and metal pieces, then air dried. A hammer crusher was used to grind the dried corn stover to pass through the mesh with the circle diameter of 10 mm. The composition of corn stover was measured as 35.38% of cellulose, 24.62% of hemicellulose, 26.51% of lignin (dry base) using the two-step sulfuric acid hydrolysis according to the Laboratory Analytical Procedure (LAP) protocols of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008, 2012).

Commercial cellulase enzyme Youtell #7 was purchased from Hunan Youtell Biochem Co., Yueyang, Hunan, China. L-arabinose was purchased from Beijing HWRK Chem Co., Beijing, China. D-mannose was from Jiuding Chem Co., Shanghai, China. D-galactose was from Macklin Biochem Co., Shanghai, China. Sodium gluconate was from Xingwang Co., Shandong, China. All other chemicals including glucose, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, NaOH, and H_2SO_4 were of analytical reagent grade and purchased from the local supplier Lingfeng Chemical Reagents Co., Shanghai, China.

2.2. Strains and cultures

Gluconobacter oxydans DSM 2003 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Two synthetic culture media were used:

(1) Seed culture medium contained 80.0 g of sorbitol, 20.0 g of yeast extract, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1.5 g of KH_2PO_4, 1.5 g of $(NH_4)_2SO_4$ per liter of deionized water.
(2) Fermentation medium contained 40.0 g of sugar (D-xylose, L-arabinose, D-mannose, D-galactose), 10.0 g of yeast extract, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1.5 g of KH_2PO_4, 1.5 g of $(NH_4)_2SO_4$ per liter of deionized water. The monomeric sugars were added separately as carbon source.

Biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated in our previous work (Zhang et al., 2011) and stored in China General Microorganism Collection Center (CGMCC), Beijing, China with the registration number 7452. *A. resinae* ZN1 was maintained in potato dextrose agar (PDA) medium containing 200 g/L of potato extract juice, 20 g/L of glucose with 15 g/L of agar. *A. resinae* ZN1 spores were inoculated onto the pretreated corn stover solids from the PDA slant and

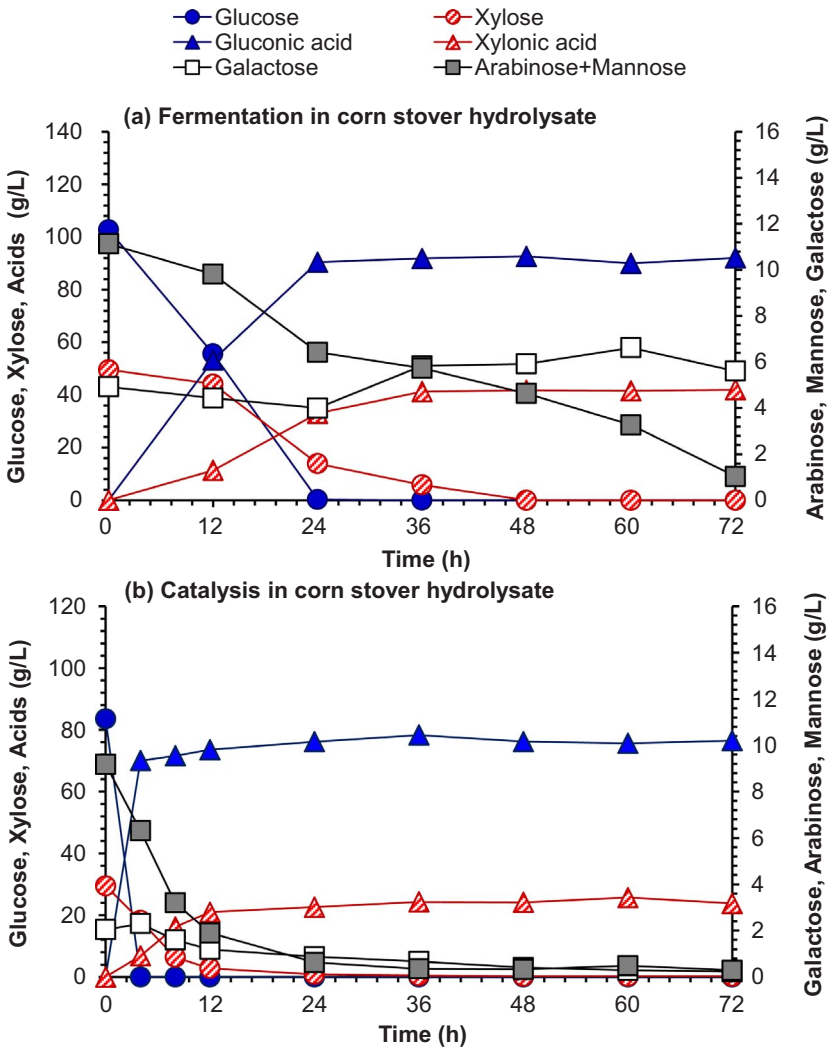

Fig. 2. Non-glucose sugar conversion by *G. oxydans* DSM 2003 in the corn stover hydrolysate containing glucose, xylose, arabinose, galactose, and mannose. (a) Fermentation. Conditions: pH 4.8, 35 °C, 700 rpm, 2.5 vvm of aeration rate, 10% (v/v) inoculum ratio, 1 L corn stover hydrolysate in 3 L fermentor. (b) Whole cell catalysis. The well prepared resting cells were set at an initial cell weight of 1.8 g/L (OD_{600} at 3.5). Catalysis conditions: pH 4.8, 35 °C, 700 rpm, 2.5 vvm, 1 L corn stover hydrolysate in 3 L fermentor.

incubated for five days at 28 °C as the seeds.

2.3. Pretreatment, biodetoxification and hydrolysate preparation

Corn stover was dry acid pretreated according to the following procedure: corn stover solids (dry base) and 5% (w/w) dilute sulfuric acid solution were co-currently fed into the 20 L helically agitated reactor with the solids-to-liquid ratio of 2:1 (w/w). The finally sulfuric acid usage was 2.5 g per 100 g of the dried corn stover (Zhang et al., 2011; He et al., 2016). Corn stover was maintained at 175 ± 1 °C for 5 min under the mild helical agitation (50 rpm). The pretreated corn stover contained 50% (w/w) of dry solid matter (DM) and another 50% (w/w) of water. The dry pretreated corn stover contained 37.34% of cellulose, 5.88% of hemicellulose. No free wastewater stream was generated during pretreatment.

The pretreated corn stover was neutralized with 20% (w/w) Ca $(OH)_2$ suspension slurry to pH 5.5 and then biodetoxified with *A. resinae* ZN1 to remove the inhibitors generated during the pretreatment operation (He et al., 2016). Briefly, the prepared *A. resinae* ZN1 seeds were inoculated onto the freshly pretreated corn stover at 10% (w/w) inoculation ratio and maintained at 28 °C at aerobic condition for 48 h in a 15 L bioreactor. The detoxified corn stover feedstock was disk milled to remove the residual long cellulose fibers before use.

The biodetoxified pretreated corn stover was hydrolyzed at 50 °C, pH 4.8 for 48 h at the cellulase dosage of 4 mg protein per gram of dry corn stover matter (DM) to obtain the hydrolysate slurry. The clear corn stover hydrolysate was obtained by centrifugation, autoclaving at 115 °C for 20 min and filtration through filter paper.

2.4. Aerobic fermentation and whole cell catalysis

One vial (2 mL) of *G. oxydans* DSM 2003 stock was inoculated into 20 mL of the seed medium in 100 mL flask and cultured at 30 °C, 220 rpm for 15 h. For flask fermentation, the seed broth was inoculated at 10% volume ratio into 45 mL of fermentation medium in a 250 mL flask and fermented at 30 °C, 220 rpm. The pH was controlled to a range of 5–6 by adding 5 M NaOH every 6 h. For fermentor fermentation, the seed broth was inoculated at 10% (v/v) inoculation ratio into fermentor containing corn stover hydrolysate. The fermentation was conducted at 35 °C, 2.5 vvm and 700 rpm in 3 L bioreactor (Baoxing Biotech Co., Shanghai, China). The pH was maintained at 4.8 by 5 M NaOH.

The resting cells of *G. oxydans* DSM 2003 used for whole cell catalysis were prepared according to Meyer et al. (2013). Cells in one liter of the seed culture broth were harvested at the optical density at 600 nm (OD_{600}) of 5.0 by centrifugation for 20 min at 2,500*g*. The cells were washed twice with one liter of 40 mM potassium phosphate buffer (KPB, pH 6) and then the cell pellets were re-suspended in the KPB buffer to the OD_{600} of 35 as the resting cells. The resting cell slurry was used at 10% (v/v) ratio into the 3 L bioreactor containing corn stover hydrolysate for whole cell catalysis at 35 °C, 2.5 vvm and 700 rpm. The pH was maintained at 4.8 by 5 M NaOH.

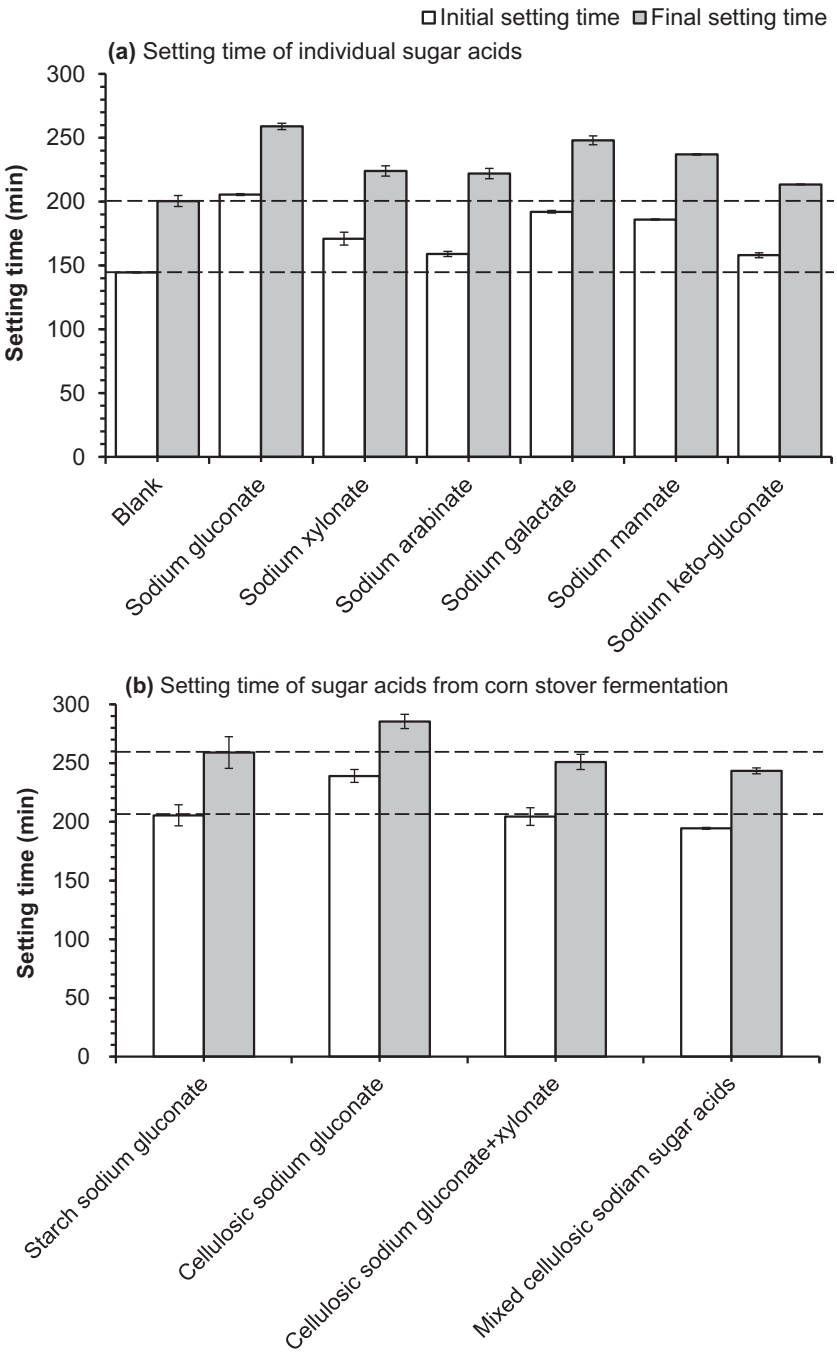

Fig. 3. Retard setting assay of individual sugar acid from corresponding single sugar conversion and cellulosic sugar acids from corn stover fermentation as cement retarding additive. (a) sodium salts of individual non-glucose sugar acids; (b) sodium salts of mixed sugar acids (starch sodium gluconate as control). All the addition dosages of sugar acid salts product into cement was 0.03% (w/w). Blank represents cement setting experiment without additives.

2.5. Analysis of sugars and acids

D-glucose, D-xylose, D-galactose, L-arabinose, D-mannose were analyzed by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with HPX-87P column (Bio-rad, Hercules, CA, USA) at 80 °C with the sterilized deionized water as mobile phase at a flow rate of 0.6 mL/min. D-gluconate, D-xylonate, 2-keto-D-gluconate were determined by HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) fitted with an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) at 55 °C using the mobile phase of 5 mM H_2SO_4 at the rate of 0.4 mL/min and the detection wavelength of 210 nm.

Sugar acids were identified and analyzed on Agilent 1200 HPLC with Quadrupole LC-MS 6120 detector (Santa Clara, California, USA). The LC system was fitted with Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5-μm particle size). The mobile phase was the mixture of 10 mM ammonium formate (pump A) and acetonitrile (pump B) with an initial ratio of 80%–20% at flow rate of 0.6 mL/min at 25 °C. Acetonitrile started at 20% and increased to 90% in 4 min and held for 6 min, then decreased to 20% in 2 min and held for 3 min. The analytes were ionized by an electrospray ionization (ESI) source in negative ion mode.

2.6. Analysis of setting time of cement paste

The setting time of the cement paste was determined by Vicat apparatus according to Chinese Standard Protocol GB/T 1346-2011. Briefly, 500 g of the standard cement was mixed by required amount of water and fermentation liquid into cement paste mixer (NJ-160, Wuxi Construction Co., Jiangsu, China). The fresh paste was stored in a curing box with the temperature of 20 °C and humidity of 95% for setting time measure.

3. Results and discussion

3.1. Utilization of non-glucose sugars and the conversion by G. oxydans

The cell growth of *G. oxydans* DSM 2003 using the non-glucose sugars of D-xylose, L-arabinose, D-mannose, D-galactose as carbon source was illustrated in Fig. 1. The obvious cell growth of *G. oxydans* on individual D-xylose, L-arabinose, D-mannose, D-galactose (Fig. 1a) indicates that *G. oxydans* is able to utilize the non-glucose sugars as carbon source. The utilization rate of the sugars by *G. oxydans* is in the order of D-galactose > D-xylose > L-arabinose > D-mannose (Fig. 1b). The conversion of xylose to xylonic acid by *G. oxydans* was already confirmed in the previous studies (Zhang et al., 2016a,b). The conversion products of D-galactose, L-arabinose, and D-mannose were identified on LC-MS by comparison of the correspondence of retention time and molecular weight (MW) with the pure sugar acids as controls, in which L-arabonate was MW of 165.1 at the retention time of 3.013, D-galactonate was MW of 195.1 at the retention time of 3.009, and D-mannonate was MW of 195.1 at the retention time of 3.019, respectively.

3.2. Conversion of non-glucose sugars in corn stover hydrolysate by G. oxydans

The utilization and conversion of the sugars in corn stover hydrolysate by *G. oxydans* was investigated in both fermentation and whole cell catalysis (Fig. 2). In the fermentation conversion, D-glucose, D-xylose, L-arabinose, D-mannose was completed converted within 72 h, and the D-galactose conversion was almost not converted in the fermentation (Fig. 2a). In the whole cell catalysis conversion with the resting cell density at 3.5 (OD_{600}), the conversion of the non-glucose sugars were significantly accelerated and all the sugars including D-galactose were quickly and completely converted into the corresponding acids within 48 h (Fig. 2b). The results further confirm the effectiveness of *G. oxydans* for the complete conversion of the lignocellulose derived non-glucose sugars into the corresponding sugar acids.

3.3. Cement retarding property of the mixed cellulosic sugar acids

The cement retarding property of the individual sugar acid produced from corresponding single sugar and mixed sugar acids produced from corn stover was assayed by retard setting time test (Fig. 3). The results show that the individual sodium salts of the non-glucose sugar acids had clear cement retarding property with extended setting time, similar to that of sodium gluconate (Fig. 3a). The mixed cellulosic sugar acids (sodium salts) without separation also demonstrated the strong retard setting capacity on cement (compared with blank experiment), similar to the commercial sodium gluconate produced from corn starch feedstock (Fig. 3b). When only gluconic acid content was taken into account and ignored the non-glucose sugar acids content, cellulosic sugar acids showed the better retarding property; when gluconic acid and xylonic acid contents were taken into account, the retarding property was similar to the commercial sodium gluconate; when all the sugar acids content were taken into account, the retarding property was slightly lower than that of the commercial sodium gluconate. The results indicate that the retarding property of cellulosic gluconic acid was enhanced by the existence of non-glucose sugar acids and perhaps the synergy among the multiple sugar acids.

4. Conclusion

The fast and complete oxidative conversion of non-glucose sugars to the corresponding acids by *G. oxydans* shows advantages over the reductive conversion to ethanol by *S. cerevisiae* or *Z. mobilis* in several ways including: (1) naturally occurring capacity of *G. oxydans* is faster and genetically more stable; (2) covers all the non-glucose sugars, not only xylose and arabinose, but also mannose and galactose; (3) membrane glucose dehydrogenase catalyzed reaction thus less affected by inhibitors and transport property of sugars. The obtained cellulosic sugar acids behave the strong cement retarding property with direct potentials on practical use as cement retarder additives.

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