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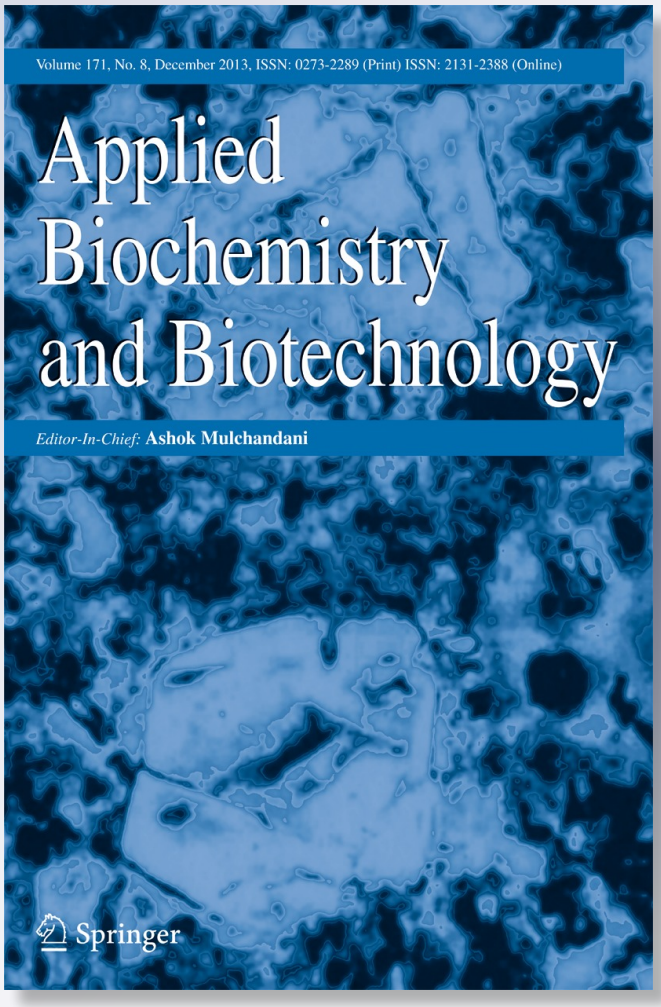

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Simultaneous Saccharification of Inulin and Starch Using Commercial Glucoamylase and the Subsequent Bioconversion to High Titer Sorbitol and Gluconic Acid

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Abstract A new bioprocess for production of sorbitol and gluconic acid from two low-cost feedstocks, inulin and cassava starch, using a commercially available enzyme was proposed in this study. The commercial glucoamylase GA-L NEW from Genencor was found to demonstrate a high inulinase activity for hydrolysis of inulin into fructose and glucose. The glucoamylase was used to replace the expensive and not commercially available inulinase enzyme for simultaneous saccharification of inulin and starch into high titer glucose and fructose hydrolysate. The glucose and fructose in the hydrolysate were converted into sorbitol and gluconic acid using immobilized whole cells of the recombinant *Zymomonas mobilis* strain. The high gluconic acid concentration of 193 g/L and sorbitol concentration of 180 g/L with the overall yield of 97.3 % were obtained in the batch operations. The present study provided a practical production method of sorbitol and gluconic acid from low cost feedstocks and enzymes.

Keywords Inulin · Starch · Glucoamylase · Sorbitol · Immobilized *Zymomonas mobilis*

Introduction

Sorbitol is one of the most important building block chemicals selected by the US Department of Energy [1]. The bioprocess technology for sorbitol production has been proposed by converting fructose and glucose into D-sorbitol and gluconic acid using glucose–fructose oxidoreductase (GFOR) in *Zymomonas mobilis* cells [2]. The use of low cost feedstock materials such as sugar cane molasses [3] or pineapple juice sugars [4] was an important consideration for cost reduction of the proposed bioprocess.

Inulin is a fructan sugar extracted from roots and tubers of Jerusalem artichoke, chicory and dahlia etc., and it has been considered as a potential feedstock for sorbitol and gluconic acid production because of its high fructose content [5], besides its applications in the

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production of bioethanol [6], lactic acid [7], and other value added products [8, 9]. Either whole cells of *Z. mobilis* or free GFOR enzyme can be used as the catalyst for bioconversion of fructose and glucose into sorbitol and gluconic acid [10–12]. Immobilized whole cells of *Z. mobilis* were employed but the conversion efficiency was decreased significantly [5, 13]. Since equimolar ratio of fructose and glucose is required in the stoichiometric conversion of fructose and glucose to sorbitol and gluconic acid and inulin hydrolysate contains mainly fructose, glucose supplementation is needed.

In this study, a commercially available glucoamylase was found to demonstrate high inulinase activity and a new bioprocess was proposed for production of sorbitol and gluconic acid from inulin and starch. First, inulin and starch were simultaneously hydrolyzed into an equimolar fructose and glucose hydrolysate using a commercially available glucoamylase GA-L NEW, then converted into sorbitol and gluconic acid using immobilized whole cells of *Z. mobilis* strain. This study provided an important and practical process option of sorbitol and gluconic acid production from the low cost feedstocks and enzyme.

Materials and Methods

Reagents and Raw Materials

Both inulin and cassava starch were purchased from the agricultural produce stores. The inulin powder was extracted from Jerusalem artichoke tubers and purchased from Langrui Fine Chemical Co., Shanghai, China. The inulin composition was measured to be 0.78 g of fructose and 0.23 g of glucose obtained from 1 g of inulin (dry base) according to the method by Gao et al. [8]. Cassava starch was purchased from Qiongzhong Benlu Starch Co., Shanghai, China. The moisture of the commercial cassava starch was determined to be 14 % of the total weight, indicating 0.86 g dry starch existed in 1 g of the commercial cassava starch and theoretically 0.96 g glucose could be obtained after hydrolysis. By using the method of Thang et al. [14], 0.88 g glucose was obtained from 1 g of the commercial cassava starch, indicating approximately 92.5 % of the starch in the commercial cassava starch was converted into glucose.

All other general chemicals including glucose, fructose, KH_2PO_4, $(NH_4)_2SO_4$, yeast extract, $CaCl_2$, $CuSO_4{\cdot}5H_2O$, $MgSO_4{\cdot}7\ H_2O$, $ZnSO_4{\cdot}7\ H_2O$, $FeSO_4{\cdot}7\ H_2O$, $CoCl_2{\cdot}6H_2O$, $MnSO_4{\cdot}H_2O$, polyvinyl alcohol (PVA), sodium alginate and boric acid were purchased from Lingfeng Chemical Reagent Co., Shanghai, China. Gluconic acid and sorbitol were from Amresco (Solon, OH, USA). Tetracycline was from Sigma-Aldrich (St. Louis, MO, USA).

Enzymes and Kinetic Determination of Glucoamylase GA-L NEW for Inulin Hydrolysis

The α-amylase HTAA (lot number: 7201455498) and glucoamylase GA-L NEW (lot number: 7201417190) were purchased from Genencor Bio-Products (Wuxi, China). The enzyme activities of α-amylase HTAA and glucoamylase GA-L NEW using soluble starch as the substrate were 22,000 and 100,000 WU/mL, respectively (WU was a special unit used by the producer to characterize the two amylase enzyme activities). According to the producer's manual, one WU of α-amylase HTAA was defined as the amount of enzyme that liquefies 1 mg soluble starch per minute under the assay conditions (70 °C, pH 6.0); one WU of glucoamylase GA-L NEW was defined as the amount of enzyme that release 1mg glucose per hour under the assay conditions (40 °C, pH 4.6).

The inulinase activity of glucoamylase GA-L NEW using soluble inulin as the substrate was determined to be 20 U/mL. One unit (U) was defined as the amount of enzyme which produced 1 μmol reducing sugar per min in 1 % inulin solution (in 0.1 M sodium acetate buffer at pH 4.0) and incubated at 60 °C for 10 min. The apparent kinetic parameters of glucoamylase GA-L NEW for inulin hydrolysis were determined under the assumption that the commercial glucoamylase GA-L NEW was considered as one single inulinase enzyme.

Media and Strains

The recombinant *Z. mobilis* (pHW20a-*gfo*) strain was constructed in our previous studies [15, 16]. The media for seeds culture contained 20 g/L of glucose, 5 g/L of yeast extract, 1 g/L of KH_2PO_4, 5 g/L of $(NH_4)_2SO_4$, and 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 20 μg mL^{-1} of tetracycline at pH 6.0. The fermentation media contained 100 g/L of glucose, 5 g/L of yeast extract, 5 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, and 1 g/L of KH_2PO_4 without addition of tetracycline. The fermentation of *Z. mobilis* was carried out in a 3-L bioreactor (Baoxing Biotech 4-BG, Shanghai, China) followed the same procedure as in [15, 16].

The immobilized *Z. mobilis* whole cells were prepared by mixing the freshly harvested cells with the PVA-alginate gel solution containing 12.5 % (*w*/*v*) of PVA and 1.25 % (*w*/*v*) of sodium alginate. The fresh cells were harvested from the fermentation broth and re-suspended into the cells solution containing 75 g cells/L. The cell solution and the gel solution were mixed in the volume ratio of 1:4 and stirred until the complete homogenization. Then the homogenized suspension was dropped into the saturated boric acid solution contained 0.3 M $CaCl_2$ (pH 6.4) and stirred for 3 h to form the gel beads. One hundred gel beads were withdrawn from the beads collection, and the volume was measured then the average diameter of the gel beads was calculated to be approximately 4.0 mm.

Preparation of the Mixed Inulin and Starch Hydrolysate

The hydrolysate preparation is outlined in Fig. 1. Inulin was hydrolyzed at 60 °C and pH 4.0 by glucoamylase GA-L NEW at 1 U/g inulin for 48 h. Cassava starch was hydrolyzed at 90 °C and pH 6.0 by α-amylase HTAA at 11 WU/g starch for 1 h. The two slurries were mixed and the saccharification of starch and inulin was processed by glucoamylase GA-L NEW in the inulin slurry for another 12 h at 60 °C and pH 4.0 to obtain the clear hydrolysate. The inulin and starch used were in weight ratio of 1:0.63 to get the equimolar glucose and fructose in the mixed hydrolysate.

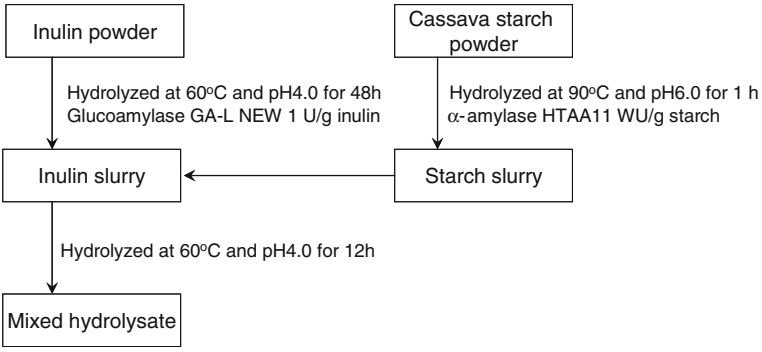

Fig. 1 Process outline for mixed hydrolysate preparation from inulin and cassava starch

Conversion of the Mixed Inulin and Cassava Starch Hydrolysate into Sorbitol and Gluconic Acid

Fructose and glucose in the hydrolysate were converted into sorbitol and gluconic acid using free whole cells of *Z. mobilis* was carried out in 3 L bioreactor (Baoxing Biotech 4-BG, Shanghai, China) at 39 °C, pH 6.4, and 150 rpm. The pH was maintained by adding 14 M NaOH solution during the bioconversion. The conversion using immobilized whole cells of *Z. mobilis* strain was carried out in a 500-mL glass reactor at 39 °C, pH 6.4, and 60 rpm. The pH was maintained using 4 M NaOH solution during the bioconversion in a relatively slow rate.

The change of GFOR activity in the immobilized whole cells of *Z. mobilis* was determined during the storage at 4 °C and the incubation at 39 °C, pH 6.4, and 60 rpm for 48 h. The immobilized whole cells were used and the maximum specific rate of sorbitol formation was determined after 1 h bioconversion, and taken the fresh prepared gel beads as control.

Calculation of Fructose and Sorbitol Yield

The fructose yield based on the inulin was calculated as follows:

$$\text{Fructouse yield} = \frac{[\text{Fructose}] \times V_0}{W_{\text{inulin}} \times 0.78} \times 100\%$$

where V_0 was the total volume of the mixed hydrolysate (L); [Fructose] was the fructose concentration in the mixed hydrolysate (g/L); W_{inulin} was the weight of inulin used in the hydrolysis (g); 0.78 was the weight in gram of fructose in per gram of dry inulin (g/g).

The sorbitol yield with respect to the fructose feedstock in the conversion of fructose and glucose to sorbitol and gluconic acid was calculated as follows:

$$\text{Sorbitol yield} = \frac{[\text{Sorbitol}]_t \times V_1}{[\text{Fructose}]_0 \times V_2} \times \frac{180}{182} \times 100\%$$

where V_1 and V_2 were the final and initial volumes of the solution (L); $[Sorbitol]_t$ was the sorbitol concentration in the final solution (g/L); $[\text{Fructose}]_0$ was the fructose concentration in the initial solution (g/L); 180 and 182 were the molecular weights of fructose and sorbitol, respectively.

Analysis Methods

Fructose, sorbitol, and ethanol were measured on HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with a Bio-rad Aminex HPX-87H column at the column temperature 65 °C using 0.005 M H_2SO_4 at 0.6 mL/min as the mobile phase. Glucose and gluconic acid have the very close retention time when Bio-rad Aminex HPX-87H column was used, therefore only their concentrations at the start and the end of fermentation operations were determined. During the bioconversion of fructose and glucose into sorbitol and gluconic acid by glucose–fructose oxidoreductase (GFOR) in *Z. mobilis* cells [2], gluconic acid was produced at the equimolar ratio with sorbitol, thus gluconic acid concentration in g/L unit was simply obtained by converting equimolar sorbitol produced considering molecular weight ratio of 196/182.

Results and Discussion

Inulinase Activity Assay and Apparent Enzyme Kinetics of Glucoamylase GA-L NEW

The commercial glucoamylase GA-L NEW enzyme was found to behave a strong inulinase activity of 20 U/mL when inulin was used as the substrate. Thus GA-L NEW was used as the inulinase enzyme for inulin hydrolysis, replacing the expensive inulinase from *Aspergillus niger* or other strains [17, 18]. The effect of pH and temperature on the inulinase activity of GA-L NEW was shown in Fig. 2. Figure 2a shows that GA-L NEW was active in the pH

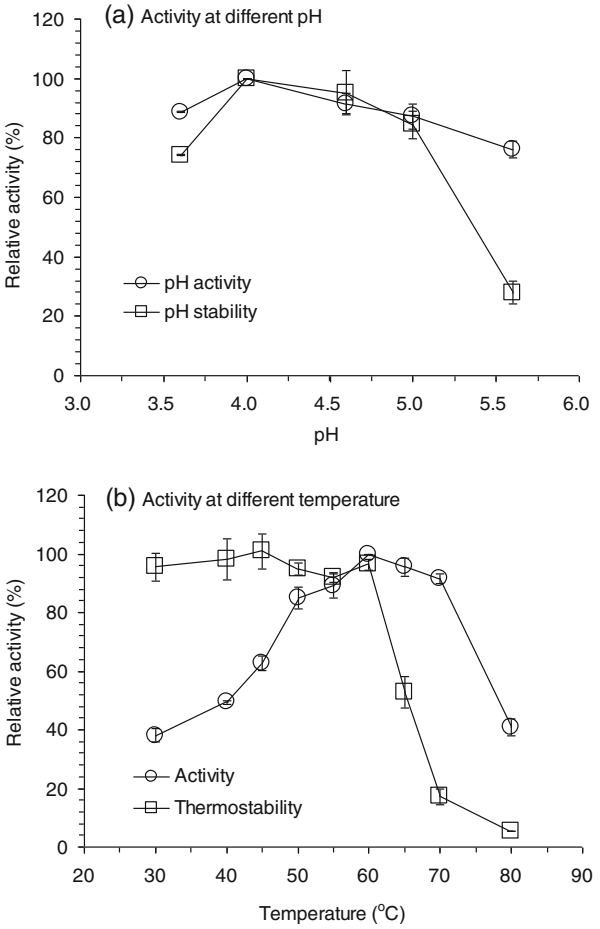

Fig. 2 Inulinase activity and stability of glucoamylase GA-L NEW at various pH and temperature. **a** Effect of pH. **b** Effect of temperature. Conditions: **a** 900 μL of 1 % inulin (dissolved in 0.1 M sodium acetate buffer at different pH) was reacted with 100 μL of the dilute enzyme at 60 °C for 10 min. pH stability was tested by preincubating the enzyme at different pH (diluted in 0.1 M sodium acetate buffer at different pH) for 2 h at 4 °C, then measuring the residual enzyme activity at standard condition (1 % inulin, pH 4.0, and 60 °C). The activity at pH 4.0 was set to be 100 %; **b** 900 μL of 1 % inulin was reacted with 100 μL of the dilute enzyme at different temperatures and pH 4.0 for 10 min. Its thermo stability was tested by preincubating the enzyme at different temperatures for 2 h, then measuring the residual enzyme activity at standard condition (1 % inulin, at pH 4.0 and 60 °C). The activity at pH 4.0 was set to be 100 %

range tested (3.6–5.6), with a maximum activity at pH 4.0. However, its pH stability was poor beyond the pH range of 4.0–5.0, indicating the practical pH range for GA-L NEW should be around 4.0–5.0. Figure 2b shows that the inulinase activity of GA-L NEW increased with increasing temperature in the range of 30 °C to 60 °C, then decreased quickly with the further increase from 60 °C to 80 °C. The thermo stability of the inulinase activity was similar: it was stable from 30 °C to 60 °C, then decreased sharply with the further increase and almost lost all the inulinase activity at 80 °C. The inulinase behaviors of GA-L NEW was very similar to the typical inulinase obtained from *A. niger* with a high activity in the temperature range of 45 °C to 60 °C and in the pH range from 4.35 to 5.35 [17, 19].

The effect of various divalent metal ions on the inulinase activity of GA-L NEW is shown in Table 1. The activity was not sensitive to most of the divalent ions tested such as Zn^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, etc. However, the activity was enhanced by Co^{2+} and Ca^{2+}, but inhibited by Cu^{2+} slightly.

The apparent kinetic parameters of GA-L NEW for inulin hydrolysis were determined under the assumption that all the enzyme components in the commercial glucoamylase GA-L NEW was treated as one single enzyme of inulinase. The Lineweaver-Burk double reciprocal plot of the initial reaction rate to the inulin substrate concentration was drawn for determination of the apparent maximum reaction rate V_{max} and the apparent Michaelis–Menten constant K_M as shown in Fig. 3. The apparent K_M and V_{max} values were determined as 19.29 g/L and 0.092 g/(L·min), respectively. The K_M value of GA-L NEW as an inulinase was very close the purified inulinase from *Cryptococcus aureus* (20.06 g/L) [20], *Pichia guilliermondii* (21.1 g/L) [21], and close to that from *Kluyveromyces marxianus* (11.9 g/L) [22]. On the other hand, the V_{max} values were rather scatted among the reports and no comparison basis was found because of difference in inulinase component purification [23].

Simultaneous Saccharification of Inulin and Starch and the Subsequent Conversion to Sorbitol and Gluconic Acid

The equimolar substrates of fructose and glucose were required for the conversion by GFOR enzyme inside the whole cells of *Z. mobilis*. Fructose was obtained from inulin hydrolysis but glucose should be supplemented by starch hydrolysis to balance the glucose shortage in the inulin hydrolysate, because only a small portion of glucose was produced from inulin.

Table 1 Effect of different metal ions on glucoamylase GA-L NEW activity

Compounds	Concentration (mM)	Relative enzyme activity (%)
Control	0	100.0
$CaCl_2$	10	110.6±5.5
$CuSO_4{\cdot}5H_2O$	10	96.7±0.5
$MgSO_4{\cdot}7H_2O$	10	102.2±0.4
$ZnSO_4{\cdot}7H_2O$	10	100.2±0.4
$FeSO_4{\cdot}7H_2O$	10	101.1±1.9
$CoCl_2{\cdot}6H_2O$	10	165.8±3.8
$MnSO_4{\cdot}H_2O$	10	101.2±2.4

The glucoamylase GA-L NEW was preincubated with various metal ions (1 mmol metallic compound dissolved in 100 mL 0.1 M sodium acetate buffer at pH 4.0) at 4 °C for 1 h. Then 900 μL of 1 % inulin was reacted with 100 μL of the dilute enzyme at 60 °C and pH 4.0 for 10 min

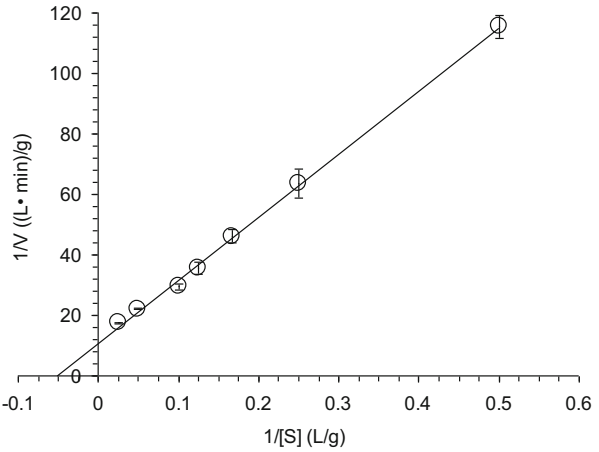

Fig. 3 Lineweaver-Burk plot for determination of K_M and V_{max} values of glucoamylase GA-L NEW. Conditions: 900 μL of 2 g/L, 4 g/L, 6 g/L, 8 g/L, 10 g/L, 20 g/L and 40 g/L inulin (dissolved in 0.1 M sodium acetate buffer at pH 4.0) was reacted with 100 μL of the dilute enzyme at 60 °C and pH 4.0 for 10 min

Figure 1 shows the outline of the simultaneous saccharification of inulin and starch to get the equimolar fructose and glucose at the total sugar concentration above 500 g/L.

Table 2 shows the fructose yield and concentration in the simultaneous saccharification step under different solids contents of inulin and starch. The fructose concentration increased but the yield decreased with the increasing solids content from 30 % to 50 %. Table 3 shows the conversion of fructose and glucose in the mixed hydrolysate to sorbitol and gluconic acid using the free whole cells of *Z. mobilis*. The results indicate that the formation of ethanol by Entner–Doudoroff pathway of *Z. mobilis* was completely inhibited by the osmotic stress of fructose and glucose at the concentration above 500 g/L. A satisfactory sorbitol yield close to the theoretical level (100 %) and the high specific sorbitol productivity of 6.80 g/(g cells·h)) were obtained. In this conversion, no any permeabilization or ions were used for inhibiting the ethanol formation thus the purification step and chemical contamination were avoided in the production of sorbitol and gluconic acid.

Silveira et al. [24] reported when the total glucose and fructose concentration reached 650 g/L, the maximum sorbitol yield obtained was 91 %; when the concentration reached to 500 g/L, the sorbitol yield was only 79 %, and the specific productivity was 1.8 g/(g cells·h). In the present work, the fructose and glucose concentration in the mixed hydrolysate reached

Table 2 Yield and concentration of fructose from inulin under different solids content of mixed hydrolysate

Solid content of the mixed hydrolysate (*w*/*w*)	Fructose yield (%)	Fructose concentration (g/L)
30 %	99.4	150.1
40 %	98.1	196.2
50 %	92.7	257.4

Inulin power under different content was hydrolyzed at pH 4.0 and 60 °C with the glucoamylase GA-L NEW dosage of 1 U/g inulin for 48 h, and then the liquefied cassava starch slurry with corresponding equivalent content was added to the inulin slurry. After that, the mixed inulin and cassava starch hydrolysate was saccharified for another 12 h

Table 3 Effect of solid content of the mixed hydrolysate on bioconversion using fresh cells

Solid content of the mixed hydrolysate (*w*/*w*)	30 %	40 %	50 %
Initial Fructose Concentration (g/L)	150.1	196.2	257.4
Sorbitol produced (g/L)	138.2	191.0	238.1
Specific sorbitol productivity (g/(g cells·h))	4.61	5.46	6.80
Sorbitol yield based on fructose[a] (%)	92.0 %	97.4 %	100.6 %
Sorbitol yield based on inulin (%)	91.4 %	95.5 %	97.5 %
Gluconic acid produced (g/L)	148.8	205.7	256.4
Specific gluconic acid productivity (g/(g cells·h))	4.96	5.88	7.32
Gluconic acid yield based on glucose[a] (%)	92.0 %	97.4 %	100.6 %
Ethanol produced (g/L)	2.82	2.56	0.00

Cells prepared condition: 3 L bioreactor at 30 °C, 150 rpm, pH 6.0, 10 % inoculum (*v*/*v*). After the culture was for 18 h, fresh cells were harvested by centrifugation at 8,000 rpm for 5 min. 10 g/L (dry weight) fresh cells were used for the bioconversion of mixed hydrolysate in a 3-L bioreactor (liquid media volume 500 mL) at 39 °C, pH 6.4, 150 rpm

[a] High concentration of fructose and glucose resulted in a minor volume shrink when the sugars were converted into sorbitol and gluconic acid. The small change resulted in a minor increase of conversion yield and sometimes a yield greater than 100 %, when the yield was close to the theoretical value

520 g/L (Table 3), the sorbitol yield was almost 100 % and the specific productivity was 6.80 g/(g cells·h) without any ethanol formation.

Table 4 shows that when the cell dosage was decreased from the full dosage (10 g cells/L) to the half of the original value (5 g cells/L), the concentration and yield of sorbitol were essentially high and no difference to that with the original full cell dosage; when the cell dosage was reduced to the one fourth of the original dosage (2.5 g/L), the sorbitol concentration, yield, and specific productivity were still in the considerably high range.

Production of Sorbitol and Gluconic Acid Using the Immobilized Whole Cells of Z. Mobilis

The recombinant whole cells of *Z. mobilis* were immobilized onto the PVA-alginate carrier and applied to the conversion to sorbitol and gluconic acid. The immobilized whole cells were recycled for four successive batches. Figure 4 shows that the concentration of gluconic acid and sorbitol were 193 g/L and 180 g/L (due to the dilution by 4 M NaOH), respectively.

Table 4 Effect of cell dosage on bioconversion of mixed hydrolysate using fresh cells

Fresh cell dosage (g/L)	10	7.5	5.0	2.5
Initial fructose concentration (g/L)	257.4	255.8	245.7	254.2
Sorbitol produced (g/L)	238.1	233.4	224.4	213.9
Specific sorbitol productivity (g/(g cells·h))	6.80	7.78	8.98	14.26
Sorbitol yield based on fructose (%)	100.6 %	99.3 %	99.4 %	91.6 %
Gluconic acid produced (g/L)	256.4	251.4	241.7	230.4
Specific gluconic acid productivity (g/(g cells·h))	7.32	8.38	9.67	15.36

Cells prepared condition: 3l bioreactor at 30 °C, 150 rpm, pH 6.0, 10 % inoculum (*v*/*v*). After the culture was for 18 h, fresh cells were harvested by centrifugation at 8,000 rpm for 5 min. Fresh cells of different dosages were used for the bioconversion of mixed hydrolysate under 50 % solids content in a 3l bioreactor (liquid media volume 500 mL) at 39 °C, pH 6.4, 150 rpm

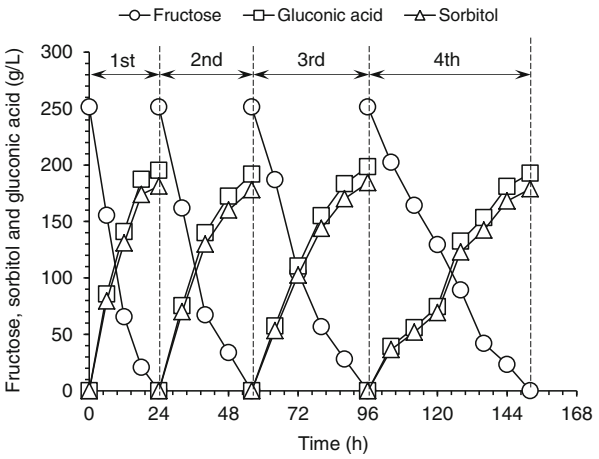

Fig. 4 Time course of sorbitol and gluconic acid production from the mixed hydrolysate using the immobilized whole cells. Conditions: Cells were cultured in a 3-L bioreactor at 30 °C, 150 rpm, pH 6.0, and 10 % inoculum (*v*/*v*). After the culture was for 18 h, fresh cells were harvested and suspended to a solution of 75 g cells/L, and then immobilized onto the mixed PVA and sodium alginate solution. The conversion was carried out in a 500-mL glass reactor containing the prepared fresh gel beads and 300 mL of the mixed hydrolysate under 50 % solids content at 39 °C, pH 6.4, and 60 rpm stirring

The yield and specific productivity of sorbitol were 97.3 %, and 0.98 g/(g cells·h), respectively. However, the conversion time of each batch increased from 24 h in the first batch to 48 h in the last, indicating the GFOR enzyme activity of the immobilized cells lost gradually during the successive batch operation. The use of glutaraldehyde treatment on the fresh cells did not help reserving the enzyme activity (data not shown).

The reasons for GFOR activity loss in the immobilized whole cells of *Z. mobilis* were investigated. Figure 5(a) shows that the stability of the GFOR enzyme during the storage at 4 °C declined steadily within few days. This phenomenon was very similar to that by Ferraz et al. [25] using the alginate as the carrier of the permeabilized whole cells of *Z. mobilis*, caused by the presence of gluconic acid acting as the Ca^{2+} chelating agent [26]. The result again revealed that sodium alginate was not a proper carrier material for *Z. mobilis* immobilization because of cell leaking from alginate entrapment when gluconic acid was produced in the conversion. Malvessi et al. [27] pointed out that the diffusional barrier of calcium alginate beads hindered the transport of gluconic acid from the inner space of the beads to the external media, resulting in the inactivation of GFOR and the crackdown of the beads structure.

On the other hand, if PVA was used as the only carrier material of *Z. mobilis* immobilization, the PVA gels swelled as soon as the catalytic conversion started and lost the GFOR activity quickly (data not shown). Therefore, a mixed material of 10 % sodium alginate plus 90 % PVA was used and both the swelling by PVA and the leaking by alginate were lessened and the conversion using the immobilized cells was improved.

The GFOR activity of the immobilized *Z. mobilis* at high concentration of the substrates and products were tested. Figure 5b shows that the GFOR activity of the immobilized *Z. mobilis* cells was not significantly affected by the high concentration of glucose, and the two products sorbitol and gluconic acid. However, the GFOR activity was strongly deactivated under the high fructose concentration environment. Almost half of the activity was lost in the fructose solution of 250 g/L for 48 h at 39 °C and never recovered in the following conversion process. Clearly the high fructose osmotic stress was one of the major factors

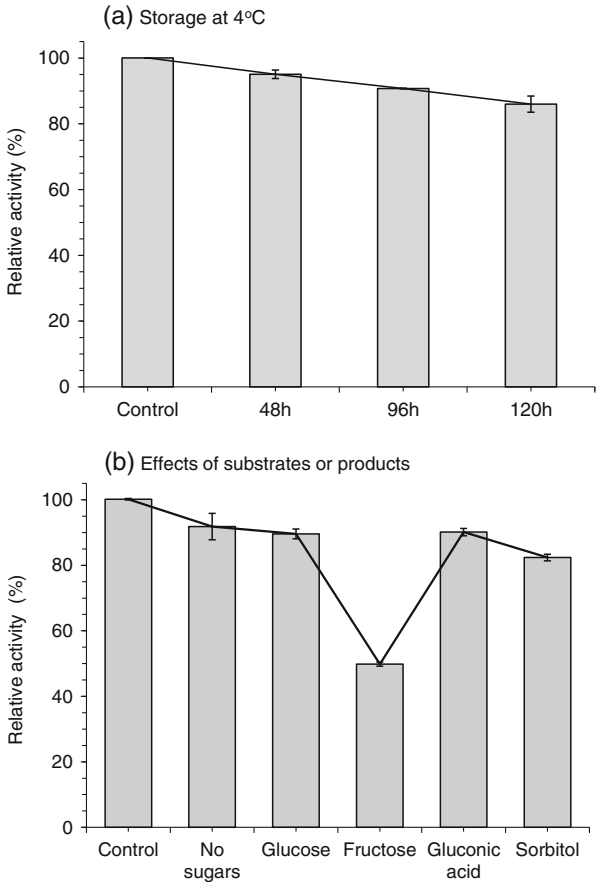

Fig. 5 Change of GFOR activity in immobilized whole cells of *Z. mobilis* strain. **a** During the storage at 4 °C for 48, 96, and 120 h. **b** During the incubation with 300 mL solution (no substrate media; at the 250 g/L of glucose, fructose, gluconic acid, and sorbitol) at 39 °C, pH 6.4, and 60 rpm for 48 h. Conditions: After the storage or preincubation, the immolibilized *Z. mobilis* cells were used for the bioconversion under the conditions as described (300 mL of the mixed hydrolysate under 50 % solids content, at 39 °C, pH 6.4, and 60 rpm). The maximum specific rate of sorbitol formation was determined during the first hour of the bioconversion, and taken the fresh prepared gel beads as control

for the loss of the immobilized *Z. mobilis* cells. According to the previous studies on immobilization of GFOR, the major problem is the generation of gluconic acid inside the

Table 5 Comparison of sorbitol production performance using immobilized whole cells of *Z. mobilis* and inulin substrate between [5] and this study

	Kim and Kim [5]	This study
Hydrolysis enzyme	Inulinase	Glucoamylase
Maximum fructose concentration (g/L)	76.0	254.2
Sorbitol yield based on inulin (%)	44.4 %	97.5 %
Maximum sorbitol productivity (g/L/h)	21.3	25.4

gel beads, which caused the decomposition of calcium alginate and the structure damage of gel beads [25–27]. The result in Fig. 5(b) suggests that fructose was another major factor to cause the activity decrease, the synergetic effect of gluconic acid and fructose will cause more severe reduction of GFOR activity in immobilized whole cell in the gel beads.

Kim and Kim [5] reported a continuous production of gluconic acid and sorbitol from glucose and inulin from Jerusalem artichoke using the co-immobilized *Z. mobilis* and inulinase with the cells of 48.9 g/L and the inulinase of 88,666 U/L, respectively, in a packed-bed reactor. The maximum sorbitol yield was only 44 % and a significant portion of sugars remained unused. As a comparison, the present study used a commercial glucoamylase, the cheapest industrial enzyme, for the co-saccharification of inulin and cassava starch to obtain the high titer fructose and glucose; and the immobilized recombinant *Z. mobilis* cells with dosage of 5 g/L for production of sorbitol and gluconic acid. The high sorbitol yield of 97.3 % and the high concentration of 180 g/L were obtained in the batch operation. The detailed comparison in Table 5 indicates that all the major bioconversion data including maximum fructose concentration from inulin, the sorbitol productivity, and sorbitol yield in this study were improved significantly comparing to that in [5].

Glucoamylase is widely used in starch processing industry and considered as one of the cheapest industrial enzymes, if not the only cheapest one: the cost was $2/kg of glucoamylase with the similar activity to GA-L NEW from Genencor [28] According to the process procedure in Fig. 1, the hydrolysis of 20 kg inulin and 12 kg starch required 1 kg glucoamylase. The cost for glucoamylase was approximately only $0.1 for producing 2 kg of mixed fructose and glucose. Comparing to inulinase enzyme, which was not commercially available and the reagent price is extremely expensive (www.sigma-aldrich.com), there is huge margin in using the two enzymes in industrial scale.

Conclusion

A new process of sorbitol and gluconic acid production from inulin and cassava starch was proposed using the commercial glucoamylase GA-L NEW and the immobilized whole cells of recombinant *Z. mobilis* strain. GA-L NEW demonstrated high inulinase activity and used to replace commercially not available inulinase for hydrolysis of inulin and starch into high titer glucose and fructose. The process was operated for multiple successive batches and a high sorbitol concentration of 180 g/L and a high yield of 97.3 % were obtained using the inulin and cassava starch hydrolysate.

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