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Sorbitol production using recombinant *Zymomonas mobilis* strain

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ABSTRACT

A recombinant *Zymomonas mobilis* strain harboring the plasmid pHW20a-*gfo* for over-expression of glucose–fructose oxidoreductase (GFOR) was constructed. The specific activity of GFOR enzyme in the new recombinant strain was at least two folds greater than that in the wild strain. The maximum GFOR activity achieved in terms of the volumetric, and the cellular were 2.59 U ml^{-1}, and 0.70 U mg^{-1}, respectively, in the batch cultures. A significant improvement of the bioconversion process for the production of sorbitol and gluconic acid from glucose and fructose was made using divalent metal ions which drastically reduced the ethanol yield and significantly increased the yield of target product. Among several divalent metal ions evaluated, Zn^{2+} was found to be most effective by inhibiting the Entner–Doudoroff pathway enzymes. The yield of the byproduct ethanol was reduced from 16.7 to 1.8 g l^{-1} and the sorbitol yield was increased to almost 100% from 89%. The Ca^{2+} enhanced the sorbitol yield and the formation of calcium gluconate salt made the separation of gluconate from the reaction system easier.

1. Introduction

Sorbitol is one of the top 12 high value-added building block intermediate chemicals that can be produced from renewable biomass resources (Werpy and Petersen, 2004). The current chemical process for sorbitol production is based on catalytic hydrogenation of glucose (Silveira and Jonas, 2004). The biological process for sorbitol production has been proposed but the industrially competitive bioprocess technology has not been well established (Silveira and Jonas, 2004). This bioprocess utilizes a periplasmic enzyme, glucose–fructose oxidoreductase (GFOR; EC 1.1.99.28) of *Zymomonas mobilis*, to convert fructose and glucose to sorbitol and glucono-δ-lactone (shortly converted to gluconic acid) (Zachariou and Scopes, 1986). NADPH and NADP are used as the cofactors in the reduction of fructose to sorbitol and the oxidation of glucose to gluconic acid by the GFOR enzyme, respectively. The NADPH and NADP are re-generated, recycled and self-sufficient and no cofactor addition is required for the conversion of fructose and glucose to sorbitol and gluconic acid.

GFOR has been cloned and expressed in *E. coli* and *Z. mobilis* strains (Loos et al., 1991; Kanagasundaram and Scopes, 1992). The GFOR expression in *E. coli* produced only the precursor form of GFOR (preGFOR), and the processing of preGFOR to the mature protein with GFOR activity was not successful (Wiegert et al., 1996). Thus, the GFOR enzyme to be used for bioconversion of fructose and glucose to sorbitol and gluconic acid was all from *Z. mobilis* cells, either in the form of whole cell or free enzyme separated. When *Z. mobilis* whole cells are used as catalyst for the production of sorbitol and gluconic acid, the ethanol yield is up to 11% (w/w) from glucose or fructose substrates (Viikari, 1984). To improve the sorbitol yield based on substrate consumed, various cell permeabilization methods were evaluated by releasing the soluble cofactors necessary for the activation of enzymes on the Entner–Doudoroff pathway. Chun and Rogers (1988) permeabilized *Z. mobilis* ZM4 using toluene to improve sorbitol yield, close to 95%. Ichikawa et al. (1989) used the dried *Z. mobilis* cells to reduce or eliminate the ethanol production and achieved only minimal ethanol production. Rehr et al. (1991) treated the *Z. mobilis* cells with cetyltrimethylammonium bromide (CTAB) to stop ethanol production and the sorbitol yield increased to 98%. Bringer-Meyer and Sahm (1991) permeabilized *Z. mobilis* cells by freezing at −20 °C and thawing at room temperature, and the sorbitol yield was increased to 100%. Silveira et al. (1999) found that the concentrated fructose and glucose syrup (up to 650 g l^{-1} or 1.8 mole each of equimolar glucose and fructose) resulted in a nearly complete conversion of substrate to sorbitol and gluconic acid without ethanol formation.

In this study, the enhancement of sorbitol production was carried out by the processes, the over-production of GFOR enzyme by the fermentation of the recombinant *Z. mobilis* cells, and the improvement of bioconversion from fructose and glucose to sorbitol and gluconic acid by a new cell treatment method. A

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recombinant *Z. mobilis* strain harboring a conjugation plasmid pHW20a-*gfo* was constructed and used for over-expression of GFOR enzyme. The fermentation parameters of the recombinant *Z. mobilis* were experimentally investigated to maximize the GFOR expression under the well-controlled bioprocess conditions. The cells were harvested as the whole-cell biocatalyst for the conversion of fructose and glucose to sorbitol and gluconic acid. A new method for eliminating or significantly reducing ethanol production without cell permeabilization using divalent metal ions was proposed. This study describes an efficient method for sorbitol production using a new recombinant *Z. mobilis* strain, and a new method of selectively inhibiting Entner–Doudoroff pathway enzymes thereby reducing the ethanol production and at the same time improving sorbitol and gluconate production.

2. Materials and methods

2.1. Chemicals

Fructose and sorbitol were obtained from Amresco Inc. (Solon, OH, USA). BSA, p-nitrophenol and N-morpholinoethanesulfonic acid (MES) were purchased from Acros Organics (Geel, Belgium). Tetracycline was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals including glucose, KH_2PO_4, $(NH_4)_2SO_4$, NaOH, $ZnSO_4{\cdot}7H_2O$, $CaCl_2$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, $CoCl_2{\cdot}7H_2O$ and $MgCl_2$ were of reagent grade and purchased from local suppliers (Lingfeng Chemical Reagent Co., Ltd.) in Shanghai, China.

2.2. Medium and strains

Zymomonas mobilis ZM4 (ATCC 31821) and ZM6 (ATCC 29191) were purchased from the American Type Culture Collection (ATCC, Washington, DC, USA); *E. coli* DH5α and S17-1 were purchased from Novagen (Madison, WI, USA). The shuttle conjugation plasmid pHW20a between *E. coli* and *Z. mobilis* was constructed in our laboratory.

The LB medium for the cultivation of *E. coli* DH5α and *E. coli* S17-1 contained 10 g l^{-1} of peptone, 5 g l^{-1} of yeast extract, and 10 g l^{-1} of NaCl (pH 7.0). The RM medium contained 20 g l^{-1} of glucose, 10 g l^{-1} of yeast extract, and 2 g l^{-1} of KH_2PO_4 (pH 6.0). The fermentation medium for the cultivation of wild and recombinant *Z. mobilis* strains contained glucose, 10–200 g l^{-1}; $(NH_4)_2SO_4$, 5 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1}; KH_2PO_4, 1 g l^{-1}; and yeast extract, 5 g l^{-1}. Tetracycline was added to the inoculum medium at 20 μg ml^{-1}. The concentrated glucose solution (500 g l^{-1}) was prepared and sterilized separately and added to the medium before inoculation. Both the medium and glucose solution were sterilized at 121 °C for 20 min.

2.3. Construction of recombinant Z. mobilis (pHW20a-gfo)

The genomic DNA of *Z. mobilis* ZM4 was prepared using Qiagen DNeasy Tissue Kit (Valencia, CA, USA). The sequence of the complete *gfo* gene of *Z. mobilis* ZM4 was obtained from the NCBI genomic DNA sequencing bank (access number: NC_006526) (Seo et al., 2005). The primers were designed using Primer v5.0 software and synthesized by Sangon Biotech Services (Shanghai, China). The *gfo* gene was amplified by polymerase chain reaction (PCR) using primers 5′-CGGAATTCTCGAAATTAACGATCACCCAC-3′ (forward with *Eco*RI and *Xba*I underlined) and 5′-GCTCTAGACCATGGTCAATAACCACCCTGAC GG-3′ (reverse with *Xba*I underlined). The PCR product was cleaved, gel purified, and ligated into the shuttle vector pHW20a to generate an expression plasmid pHW20a-*gfo* (Fig. 1). The plasmid pHW20a-*gfo* was transformed into *E. coli* DH5α and screened by blue/white selection. Then the plasmid was transformed into the competent *E. coli* S17-1 cells, and the recombinants were selected on LB plates containing tetracycline (20 μg ml^{-1}).

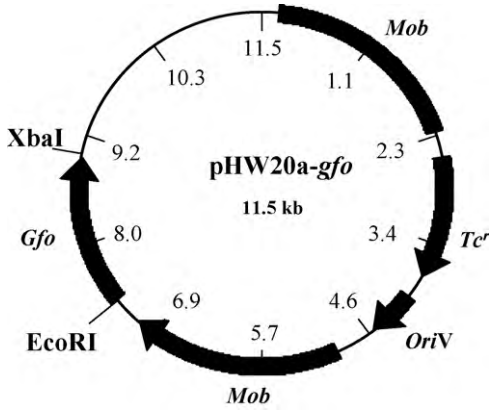

Fig. 1. Construction of recombinant *Zymomonas mobilis* (pHW20a-*gfo*) strain using shuttle conjugation plasmid for over-expression of GFOR enzyme.

The expression plasmid pHW20a-*gfo* was transformed into *Z. mobilis* ZM4 and ZM6 strains using a modified conjugation procedure on membrane filter (Conway et al., 1987) to generate a recombinant strain *Z. mobilis* (pHW20a-*gfo*) for over-expression of GFOR enzyme. *E. coli* S17-1 cell harboring pHW20a-*gfo* plasmid was cultured on LB medium containing tetracycline (20 μg ml^{-1}) and *Z. mobilis* in RM medium until the two cultures reached a similar cell concentrations. The combined cell suspension was placed on a 0.22-μm filter membrane. Then, these cells on the membrane were placed on RM agar plate and incubated for 12 h at 30 °C. The cells were washed from the membrane using the liquid RM medium, then transferred onto the RM agar plates containing tetracycline (20 μg ml^{-1}) and nalidixic acid (40 μg ml^{-1}), and incubated at 30 °C for 2–3 days. The *Z. mobilis* transconjugants would grow on these agar plates and were selected as the recombinant for GFOR over-expression.

2.4. Over-expression and activity assay of GFOR

The recombinant *Z. mobilis* (pHW20a-*gfo*) cells were cultivated in a 3-l fermenter which equipped with automatic control systems for temperature, pH and dissolved oxygen (Baoxing Biotech, Shanghai, China) at 200 rpm of agitation rate, 30 °C, and pH 6.0. The inoculation ratio was 10% by volume of the total culture volume. Samples were collected periodically every 2 h and then the cell growth, glucose consumption and ethanol production were calculated. μ_{max} was calculated using the data in the exponential growth phase. $Y_{X/S}$ was calculated using the data from the culture starts till the point of the complete consumption of glucose. The fermentation time was defined as the period from the point of the inoculum start and to the point of the complete consumption of glucose.

The cells were harvested in the late exponential growth phase and suspended in 10 mM K–MES buffer (pH 6.4) after five times repeated washing and resuspension with K–MES buffer. The cells harvested was sonicated in an ice bath for 4 min; then the mixture centrifuged at 16,000 × *g*, 4 °C for 20 min to separate the cell debris. The supernatants were collected for enzyme assay.

The GFOR activity was quantified using the same method as described by Zachariou and Scopes (1986) measuring the absorption change at 405 nm, at 25 °C and quantified by titration with HCl. The routine spectrophotometric assay solution contained 0.4 M glucose, 0.8 M fructose, 10 mM K–MES buffer, pH 6.4 and 0.29 mM p-nitrophenol/ml. One unit of GFOR activity is defined as one micromole of gluconic acid formed per minute at 25 °C.

Table 1
Effect of aeration rate on metabolic parameters of *Z. mobilis* ZM4 and ZM6 in the batch culture medium.

	0	0.21	0.42	0.83
ZM4 aeration rate (vvm)				
Maximum specific growth rate, μ_m (h^{-1})	0.46	0.37	0.38	0.40
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.032	0.032	0.032	0.033
Fermentation time (h)	13	15	15	15
ZM6 aeration rate (vvm)				
Maximum specific growth rate, μ_m (h^{-1})	0.36	0.43	0.41	0.32
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.028	0.032	0.030	0.030
Fermentation time (h)	13	15	15	15

3-l fermenter, 30 °C, 200 rpm, pH 6.0, and 10% inoculum (by volume). The medium contained: glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$.

2.5. Cell treatment

The permeabilized recombinant ZM4 cells were prepared by going through two cycles of freezing at −20 °C for 12 h and thawing at room temperature for 4 h (Bringer-Meyer and Sahm, 1991). These permeabilized cells were used as the control experiment in this work in order to compare the effects of metal ions on the yields of sorbitol and ethanol to the effect of permeabilization on the yields of these products.

Various divalent metal ions, including Zn^{2+} ($ZnSO_4{\cdot}7H_2O$), Ca^{2+} ($CaCl_2$), Fe^{2+} ($FeSO_4{\cdot}7H_2O$), Cu^{2+} ($CuSO_4{\cdot}5H_2O$), Co^{2+} ($CoCl_2{\cdot}7H_2O$), and Mg^{2+} ($MgCl_2$), were tested as inhibitors of the Entner–Doudoroff pathway enzymes in *Z. mobilis* cells during the bioconversion of fructose and glucose to sorbitol and gluconic acid.

2.6. Bioconversion of fructose and glucose to sorbitol and gluconic acid

The simultaneous bioconversion of fructose and glucose to sorbitol and gluconic acid as well as to ethanol was carried out in the same bioreactor (Baoxing Biotech, Shanghai, China). The bioreactor was filled with 500 ml substrate solution containing fructose and glucose at 150 $g\,l^{-1}$ each and 20 $g\,l^{-1}$ (dry weigh) of the *Z. mobilis* cells. The operating conditions were controlled at 150 rpm, pH 6.4 with 14 M NaOH solution and 39 °C.

2.7. Analyses

Cell growth was determined by measuring the optical density of cell suspensions at 600 nm. These measurements gave a linear relationship with dry cell mass concentration. Glucose, fructose, sorbitol and ethanol concentrations were analyzed using high performance liquid chromatography (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with a Bio-rad Aminex HPX-87H column at the column temperature 65 °C. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 ml min^{-1}. The protein concentration was determined using Bradford method with bovine serum albumin (BSA) as a reference standard.

3. Results

3.1. Effect of oxygen on the metabolism of the wild and recombinant Z. mobilis strains

Z. mobilis is a facultative anaerobic bacterium and different *Z. mobilis* strains showed different impact from dissolved oxygen level in the submerged liquid fermentation (Swings and De Ley, 1977). Table 1 shows cell growth of the two wild *Z. mobilis* strains, ZM4 and ZM6, under the different aeration condition. The maximum specific growth rate of ZM4 was obtained under anaerobic condition without oxygen input, and the cell yield was kept almost constant with changing aeration rate ($\mu_m = 0.46\,h^{-1}$, $Y_{X/S} = 0.032\,g\,g^{-1}$); for ZM6, the maximum specific growth rate and cell yield were obtained under the aeration rate of 0.21 vvm ($\mu_m = 0.43\,h^{-1}$, $Y_{X/S} = 0.032\,g\,g^{-1}$), and changed significantly with changing aeration condition.

Table 2 shows the effect of aeration rate on GFOR activity using the recombinant strains ZM4 (pHW20a-*gfo*) and ZM6 (pHW20a-*gfo*) in a 3-l fermenter batch culture. The results indicate that the GFOR activity by ZM6 (pHW20a-*gfo*) was more sensitive on aeration than that by ZM4 (pHW20a-*gfo*). The anaerobic condition provided the optimal cell growth for the recombinant ZM4 (pHW20a-*gfo*), while ZM6 (pHW20a-*gfo*) preferred suitable oxygen supply for better growth. The specific enzyme activity (A_E, enzyme units per mg of crude protein) for the two recombinant strains remained practically the same. The cellular specific enzyme activity ($A_{E/X}$, enzyme units per mg cell) for ZM6 (pHW20a-*gfo*) was a little higher than ZM4 (pHW20a-*gfo*) when the aeration rate was varied from 0.0 to 1.67 vvm. Table 3 shows the transformation efficiency of pHW20a-*gfo* to ZM4 and ZM6. The higher transformation efficiency of pHW20a-*gfo* to ZM6 than ZM4 might be the reason for little higher GFOR activity in ZM6 than in ZM4.

Table 3
Transformation efficiency of *Z. mobilis* strains by transconjugation.

Plasmid	Strain	Transformation efficiency
pHW20a-*gfo*	*Z. mobilis* ZM4	$5.5–6.9 \times 10^{-5}$
pHW20a-*gfo*	*Z. mobilis* ZM6	$9.3–10.1 \times 10^{-5}$

30 °C, pH 6.0. The RM medium contained: glucose, 20 $g\,l^{-1}$; KH_2PO_4, 2 $g\,l^{-1}$; yeast extract, 5 $g\,l^{-1}$; tetracycline, 20 $\mu g\,ml^{-1}$, and nalidixic acid, 40 $\mu g\,ml^{-1}$.

Table 2
Effect of aeration rate on GFOR activity of the recombinant *Z. mobilis* strains.

ZM4 (pHW20a-*gfo*) aeration rate (vvm)	0	0.42	0.83	1.67
Maximum specific growth rate, μ_m (h^{-1})	0.35	0.34	0.26	0.30
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.038	0.037	0.036	0.037
Specific GFOR activity, A_E ($U\,mg^{-1}$)	4.48 ± 0.15	4.45 ± 0.13	4.42 ± 0.01	4.43 ± 0.04
Cellular specific GFOR activity, $A_{E/X}$ ($U\,mg^{-1}$)	0.60 ± 0.00	0.65 ± 0.01	0.64 ± 0.01	0.61 ± 0.01
Fermentation time (h)	15.5	17.5	18.0	18.0
ZM6 (pHW20a-*gfo*) aeration rate (vvm)	**0**	**0.21**	**0.42**	**0.83**
Maximum specific growth rate, μ_m (h^{-1})	0.47	0.48	0.41	0.39
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.031	0.035	0.034	0.034
Specific GFOR activity, A_E ($U\,mg^{-1}$)	4.45 ± 0.01	4.49 ± 0.06	4.49 ± 0.08	4.43 ± 0.12
Cellular specific GFOR activity, $A_{E/X}$ ($U\,mg^{-1}$)	0.88 ± 0.01	0.76 ± 0.00	0.80 ± 0.03	0.81 ± 0.02
Fermentation time (h)	17.5	19.5	19.5	19.5

3-l fermenter, 30 °C, 200 rpm, pH 6.0, and 10% inoculation ratio. The medium contained: glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$.

Table 4
Comparison of GFOR activities between the wild *Z. mobilis* ZM4 and recombinant ZM4 (pHW20a-*gfo*) strains from flask and 3-l fermenter cultures.

GFOR activity from flask scale culture[a]	ZM4	ZM4 (pHW20a-*gfo*)
Volumetric GFOR activity, C_E (U ml^{-1})	0.09 ± 0.00	0.21 ± 0.00
Specific GFOR activity, A_E (U mg^{-1})	1.38 ± 0.02	4.10 ± 0.04
Cellular specific GFOR activity, $A_{E/X}$ (U mg^{-1})	0.15 ± 0.00	0.51 ± 0.00
GFOR productivity in 3-l fermenter culture[b]	ZM4	ZM4 (pHW20a-*gfo*)
Maximum specific growth rate, μ_m (h^{-1})	0.44 ± 0.02	0.39 ± 0.06
Cell yield based on glucose, $Y_{X/S}$ (g g^{-1})	0.035 ± 0.000	0.035 ± 0.001
Volumetric GFOR activity, C_E (U ml^{-1})	1.45 ± 0.16	2.59 ± 0.01
Specific GFOR activity, A_E (U mg^{-1})	2.40 ± 0.28	4.17 ± 0.15
Cellular specific GFOR activity, $A_{E/X}$ (U mg^{-1})	0.39 ± 0.04	0.70 ± 0.00
Fermentation time (h)	12.5	16.0

30 °C, pH 6.0, 150 rpm (flask, rotating), 200 rpm (3-l fermenter), and 10% inoculum (by volume). The culture medium used for both scale cultures of both strains contained: glucose, 10 g l^{-1} (in flask), 100 g l^{-1} (in fermenter); $(NH_4)_2SO_4$, 5 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1}; KH_2PO_4, 1 g l^{-1}; and yeast extract, 5 g l^{-1}.

[a] Glucose concentration: 10 g l^{-1}.

[b] Glucose concentration: 100 g l^{-1}.

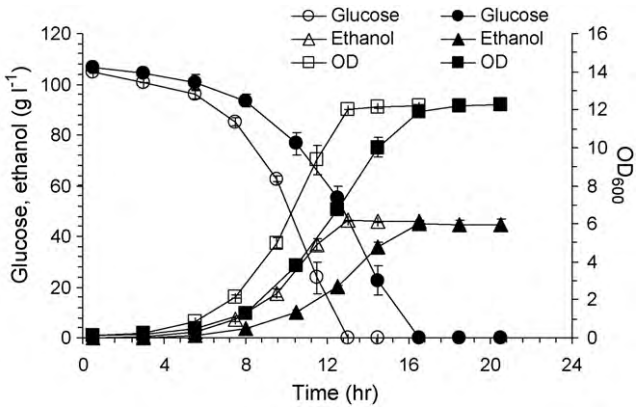

Fig. 2. Fermentation profiles for the wild *Z. mobilis* ZM4 strain (open legends) and the recombinant *Z. mobilis* ZM4 (pHW20a-*gfo*) (closed legends) strain. Fermentation conditions: cells were cultured at 30 °C, 200 rpm, pH 6.0, and 10% inoculum (by volume). The medium contains: glucose, 100 g l^{-1}; $(NH_4)_2SO_4$, 5 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1}; KH_2PO_4, 1 g l^{-1}; yeast extract, 5 g l^{-1} for both wild ZM4 and recombinant ZM4 (pHW20a-*gfo*) strains.

Based on the similar GFOR activity performance, the fermentation at anaerobic condition for expression of GFOR using ZM4 (pHW20a-*gfo*) provided less power consumption for uniformly distributing the air into the liquid medium, and less resource supply of the sterilized air. Therefore the ZM4 (pHW20a-*gfo*) was selected as the optimal fermentation strain for GFOR expression.

3.2. Comparison of GFOR activity of the wild and recombinant Z. mobilis ZM4 strains

Table 4 shows that the volumetric GFOR enzyme activity (C_E, enzyme units per ml), the specific enzyme activity (A_E, enzyme units per mg of crude protein), and the cellular specific enzyme activity ($A_{E/X}$, enzyme units per mg cell) obtained from the recombinant *Z. mobilis* ZM4 (pHW20a-*gfo*) strain were approximately 2.3 times, 3 times, and 3.4 times greater, respectively, as compared to those values obtained from the wild *Z. mobilis* ZM4 strain in flask scale culture.

In the 3-l fermenter, the value of C_E for the recombinant strain was 1.8 times greater than that of the wild strain. In the different culture types, the C_E value for the recombinant strain from the 3-l fermenter was about 12 times greater than that from the flask scale culture; for the wild strain, the C_E value from the 3-l fermenter was about 16 times greater than that from the flask.

In the flask culture, the A_E value from the recombinant strain was about three times greater than that from the wild strain. In the 3-l fermenter culture, the A_E value from the recombinant strain was about 1.7 times greater than that from the wild strain. However, the A_E value of the recombinant strain was about the same in both cultures, 4.1 U mg^{-1} crude protein. The reason might be that the over-expression of GFOR using the recombinant strain led to the accumulation of GFOR activity quickly, even the glucose concentration and the fermentation mode were different. For the wild strain, the A_E value in 3-l fermenter was about 1.7 times greater than that in the flask culture. The reason might be that the accumulation of GFOR activity increased with the cell growth performance because of GFOR is the compositional enzyme, while the cell growth was affected by the initial glucose concentration and fermentation mode.

The $A_{E/X}$ value obtained from the recombinant strain in 3-l fermenter was about 1.4 times greater than that cultivated in flask culture, while that of the wild strain cultivated in 3-l fermenter was about 2.6 times greater than that cultivated in the flask culture. The $A_{E/X}$ value obtained from the recombinant strain was about 1.8 times greater than that from the wild strain in the 3-l fermenter scale culture and about 3.4 times for the flask scale culture.

Fig. 2 shows the fermentation time courses of glucose consumption, ethanol production and cell growth for both the wild and recombinant *Z. mobilis* strains in 3-l fermenter culture. The results indicate that the final cell and ethanol concentrations for both strains were about the same, respectively, at the end of batch cycle. However, the time for the complete consumption of the initial glucose using the wild strain was approximately 3 h shorter than that using the recombinant strain. These results are, at least in part, attributable to the metabolic burden on the recombinant cells in terms of the cell growth, plasmid replication and over-expression of the GFOR enzyme. The maximum specific growth rate of wild strain in 3-l fermenter scale cultivation was about 11% higher than that of the recombinant strain as shown in Table 4.

3.3. Maximizing GFOR expression using the recombinant Z. mobilis strain

In order to maximize GFOR expression, the effects of the initial glucose concentration and pH on the enzyme activity were studied and the optimal conditions were determined.

Table 5 shows that the maximum specific growth rate (μ_m = 0.34 h^{-1}) was found at the initial glucose concentration of 50–100 g l^{-1}. The values of A_E and $A_{E/X}$ obtained all showed an increasing trend with the increasing initial glucose concentration. On the other hand, the $Y_{X/S}$ values decreased with the increasing initial glucose concentration.

The recombinant strain did not grow outside the pH value greater than 7.0 and smaller than 4.0 after 72.5 h fermentation (data not shown). In Table 5, the optimal pH range for the maximum specific growth rate for GFOR expression was found to be about 5.0–6.0. $Y_{X/S}$ showed the maximum values in the pH range of 5.5–6.0.

3.4. Effect of metal ions on the sorbitol production

A new method using divalent metal ions in the reaction system was developed and evaluated for the purpose of altering the main flux from ethanol biosynthetic pathway to sorbitol producing pathway by inactivating or inhibiting the enzymes involved in ethanol biosynthesis in the Entner–Doudoroff pathway of *Z. mobilis*.

Table 6 shows the effect of zinc sulfate addition in the $ZnSO_4{\cdot}7H_2O$ concentration range of 0, 0.1, 1.0, and 2.0 g l^{-1}. The results show that the addition of Zn^{2+} effectively reduced the

Table 5
Effect of initial glucose concentration and pH on GFOR activity of the recombinant *Z. mobilis* ZM4 (pHW20a-*gfo*) strain in the batch culture medium.

	Initial glucose ($g\,l^{-1}$)			
	50	100	150	200
Maximum specific growth rate, μ_m (h^{-1})	0.34	0.34	0.19	0.15
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.054	0.035	0.026	0.017
Specific GFOR activity, A_E ($U\,mg^{-1}$)	3.84 ± 0.02	4.22 ± 0.01	4.32 ± 0.02	4.89 ± 0.10
Cellular specific GFOR activity, $A_{E/X}$ ($U\,mg^{-1}$)	0.55 ± 0.01	0.76 ± 0.01	0.72 ± 0.00	1.03 ± 0.00
Fermentation time (h)	12.5	16.5	22.5	52.5
pH	5.0	5.5	6.0	6.5
Maximum specific growth rate, μ_m (h^{-1})	0.29	0.28	0.33	0.17
Cell yield based on glucose, $Y_{X/S}$ ($g\,g^{-1}$)	0.037	0.039	0.035	0.027
Specific GFOR activity, A_E ($U\,mg^{-1}$)	4.40 ± 0.31	4.27 ± 0.20	4.23 ± 0.02	4.33 ± 0.01
Cellular specific GFOR activity, $A_{E/X}$ ($U\,mg^{-1}$)	0.64 ± 0.07	0.62 ± 0.01	0.66 ± 0.01	0.86 ± 0.01
Fermentation time (h)	17.5	16.5	16.5	18.5

3-l fermenter, 30 °C, 200 rpm, and 10% inoculum (by volume). In different initial glucose concentration fermentation process, the medium contained: varying amount of glucose, 50–200 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; yeast extract, 5 $g\,l^{-1}$; and pH 6.0. In the different pH fermentation process, pH range tested 4.0–7.0, and the medium contained: glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$.

ethanol production to 10.7% of the cell's full capacity and significantly improved the sorbitol yield by 16.5%. The sorbitol yield increased to almost a theoretical level (100%) from 89%. Fig. 3 shows the time course profiles of sorbitol and ethanol production using the recombinant whole cell and Zn^{2+}. The results were comparable to the profiles of sorbitol and ethanol produced by the permeabilized cells, indicating that both reaction systems had similar effects on the bioconversion.

The effect of calcium chloride ($CaCl_2$) addition was also studied and the results in Table 7 indicate that the sorbitol yield was

Table 6
Effect of $ZnSO_4{\cdot}7H_2O$ on the bioconversion of fructose and glucose to sorbitol and gluconic acid using recombinant ZM4 (pHW20a-*gfo*) cells at varying concentrations Zn^{2+} ions.

$ZnSO_4{\cdot}7H_2O$ ($g\,l^{-1}$):	0	0.1	1.0	2.0
Zn^{2+} ($g\,l^{-1}$):	0	0.023	0.23	0.46
Initial glucose concentration ($g\,l^{-1}$)	153.7	155.0	160.0	157.1
Initial fructose concentration ($g\,l^{-1}$)	153.5	154.7	158.5	158.9
Sorbitol produced ($g\,l^{-1}$)	138.3	152.8	159.1	161.1
Ethanol produced ($g\,l^{-1}$)	16.70	5.65	1.89	1.78
Sorbitol yield based on fructose (%)	89.0	97.6	99.3	100.3
Specific sorbitol productivity, $Q_{P/X}$ ($g\,g^{-1}\,h^{-1}$)	3.60	3.01	2.97	3.06
Reaction time (h)	2.0	2.5	2.5	2.5

Recombinant cells were prepared in 3-l fermenter at 30 °C, 200 rpm, pH 6.0, and with 10% inoculum (by volume). The medium contains: glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$. Fresh cells were harvested and collected by centrifugation at 8000 × *g* at 4 °C for 5 min. 20 $g\,l^{-1}$ (dry weight) fresh cells were used for bioconversion of fructose and glucose to sorbitol and gluconic acid in a bioreactor at 39 °C, pH 6.4, and agitation at 150 rpm. The GFOR activity in the 20 $g\,l^{-1}$ (dry weight) fresh cells equals to 28,000 U.

Table 7
Effect of $CaCl_2$ on bioconversion of fructose and glucose to sorbitol and gluconic acid using recombinant ZM4 (pHW20a-*gfo*) cells at varying concentrations Ca^{2+} ions.

$CaCl_2$ ($g\,l^{-1}$):	0	5	20	50
Ca^{2+} ($g\,l^{-1}$):	0	1.82	7.28	18.2
Initial glucose concentration ($g\,l^{-1}$)	153.7	158.1	158.7	152.7
Initial fructose concentration ($g\,l^{-1}$)	153.5	159.3	158.9	150.4
Sorbitol produced ($g\,l^{-1}$)	138.3	157.9	158.1	153.2
Ethanol produced ($g\,l^{-1}$)	16.70	5.05	2.96	0.14
Sorbitol yield based on fructose (%)	89.0	98.0	98.4	100.8
Specific sorbitol productivity, $Q_{P/X}$ ($g\,g^{-1}\,h^{-1}$)	3.60	3.14	3.04	1.59
Reaction time (h)	2.0	2.5	2.5	5.0

Recombinant cells were prepared using 3-l fermenter at 30 °C, 200 rpm, pH 6.0, and 10% inoculum (by volume). The medium contains: glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$. Fresh cells were harvested and collected by centrifugation at 8000 × g at 4 °C for 5 min. 20 $g\,l^{-1}$ (dry weight) fresh cells were used for bioconversion of fructose and glucose to sorbitol and gluconic acid in a bioreactor at 39 °C, pH 6.4, and agitation at 150 rpm. The GFOR activity in the 20 $g\,l^{-1}$ (dry weight) fresh cells equals to 28,000 U.

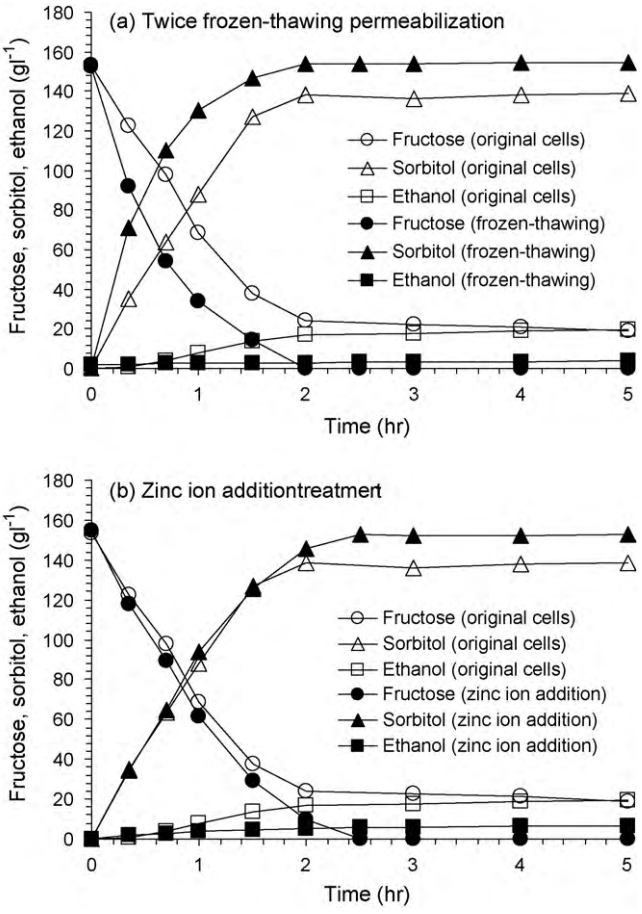

Fig. 3. Effects of zinc ion addition to the bioconversion bioreactor (b) and of permeabilized cells (a) on production of sorbitol and ethanol using the recombinant *Z. mobilis* ZM4 (pHW20a-*gfo*) cells. (a) Bioconversion reaction profiles obtained from a bioreactor using permeabilized cells prepared by repeated freezing–thawing treatment. Open symbols are for untreated fresh recombinant cells and the closed symbols are for the permeabilized recombinant cells. (b) Bioconversion reaction profiles obtained from a bioreactor where zinc ion was added at 0.1 $g\,l^{-1}$ of $ZnSO_4{\cdot}7H_2O$. Open symbols are for the reaction system without addition of metal ions and the closed symbols are for the reaction system with metal ion addition. Cell preparation condition: Recombinant cells were cultured at 30 °C, 200 rpm, pH 6.0, and 10% inoculum (by volume). The medium contained glucose, 100 $g\,l^{-1}$; $(NH_4)_2SO_4$, 5 $g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,l^{-1}$; KH_2PO_4, 1 $g\,l^{-1}$; and yeast extract, 5 $g\,l^{-1}$. The bioconversion reactions for fructose and glucose to sorbitol and gluconic acid were carried out in the 3-l bioreactor at 39 °C, pH 6.4, 150 rpm, and 20 $g\,l^{-1}$ (dry weight) recombinant cells with varying amount of substrates.

Table 8
Effects of divalent metal ions on bioconversions of fructose and glucose to sorbitol and gluconic acid using recombinant ZM4 (pHW20a-*gfo*) cells.

Divalents	Control	Cu^{2+}	Co^{2+}	Fe^{2+}	Mg^{2+}
Metal ions concentration ($g\,l^{-1}$)	0	0.5	0.5	2.0	2.5
Initial glucose concentration ($g\,l^{-1}$)	153.7	160.1	160.4	156.1	158.2
Initial fructose concentration ($g\,l^{-1}$)	153.5	159.1	159.5	156.8	158.1
Sorbitol produced ($g\,l^{-1}$)	138.3	161.0	158.8	154.5	156.6
Ethanol produced ($g\,l^{-1}$)	16.70	0.51	3.29	3.51	2.20
Sorbitol yield based on fructose (%)	89.0	100.1	98.5	97.4	97.9
Specific sorbitol productivity, $Q_{P/X}$ ($g\,g^{-1}\,h^{-1}$)	3.60	1.68	3.10	3.25	3.18
Reaction time (h)	2.0	5.0	2.5	2.5	2.5

Recombinant cells were prepared using 3-l fermenter at 30 °C, 200 rpm, pH 6.0, and 10% inoculum (by volume). The medium contains: glucose, $100\,g\,l^{-1}$; $(NH_4)_2SO_4$, $5\,g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$, $0.5\,g\,l^{-1}$; KH_2PO_4, $1\,g\,l^{-1}$; and yeast extract, $5\,g\,l^{-1}$. Fresh cells were harvested and collected by centrifugation at 8000 g at 4 °C for 5 minutes. $20\,g\,l^{-1}$ (dry weight) fresh cells were used for bioconversion of fructose and glucose to sorbitol and gluconic acid in a bioreactor at 39 °C, pH 6.4, and agitation at 150 rpm. The GFOR activity in the $20\,g\,l^{-1}$ (dry weight) fresh cells equals to 28,000 U.

increased to almost theoretical, 98–100% from 89% with increasing Ca^{2+}, and the ethanol yield showed decreasing trend with increasing calcium ion concentration. However, the specific sorbitol productivity ($Q_{P/X}$, gram sorbitol per gram cell per hour) decreased somewhat with increasing calcium chloride.

The effects of other divalent metal ions such as, Cu^{2+}, Co^{2+}, Fe^{2+}, and Mg^{2+}, were studied and the results are shown in Table 8. The concentrations of divalent metal ions were selected according their sensitivity to the enzymes on the ED pathway according to the reference (Chang et al., 2009). The sorbitol production was significantly enhanced and the ethanol production was reduced to about 3–21% level as compared to the control experiment where no metal ions were added. These results were similar to that obtained in the experiment in which the effect of Zn^{2+} and Ca^{2+} were studied.

4. Discussion

In this study, we focused on the improvement of the bioconversion of fructose and glucose to sorbitol and gluconic acid catalyzed by recombinant *Z. mobilis* cells while reducing ethanol production. For this purpose, the recombinant *Z. mobilis* strain capable of overexpression of GFOR enzyme was constructed and a new method of improving sorbitol productivity while reducing ethanol productivity was developed using divalent metal ions.

Z. mobilis is a facultative anaerobe with incomplete TCA cycle and respiration function (Belaich and Senez, 1965). Obviously the anaerobic culture mode of *Z. mobilis* for GFOR expression would be benefit to the cost reduction aim. Because GFOR is a constitutive enzyme in *Z. mobilis*, the better growth performance would naturally indicate the better GFOR expression performance. Table 1 shows that the growth of ZM4 strain preferred anaerobic condition, while ZM6 strain preferred the minimum oxygen supply for better growth. Table 2 shows that ZM4 (pHW20a-*gfo*) gave the maximum growth and GFOR activities under anaerobic condition, while ZM6 (pHW20a-*gfo*) was highly dependent on aeration condition. The metabolic characteristics of ZM4 (pHW20a-*gfo*) provide an economic advantage in terms of fermentation process cost. Thus ZM4 (pHW20a-*gfo*) was selected as the fermentation strain for GFOR expression.

A significant improvement of GFOR activity was demonstrated using the recombinant *Z. mobilis* ZM4 (pHW20a-*gfo*) strain, and the productivities of sorbitol and gluconic acid were significantly enhanced consequentially. The parameters such as aeration rate, initial substrate concentration, and pH values were tested to find the maximum GFOR activity in the experimental range, and the GFOR activity was improved. Based on this study, the typical and/or the maximum values of metabolic process parameters found and estimated are: cell yield based on glucose, $Y_{X/S} = 0.054\,g\,g^{-1}$; maximum specific growth rate, $\mu_m = 0.35\,h^{-1}$; specific enzyme activity, $A_E = 4.48 \pm 0.15\,U\,mg^{-1}$; and cellular specific enzyme activity, $A_{E/X} = 1.03 \pm 0.00\,U\,mg^{-1}$.

When the ZM4 (pHW20a-*gfo*) whole cells were used to catalyze fructose and glucose to sorbitol and gluconic acid, the cells had to be pretreated with permeabilization to block or inhibit some enzymes involved in the ethanol biosynthetic pathway in the Entner–Doudoroff pathway. Otherwise, considerable amount of substrate sugars (glucose and fructose) will be converted to ethanol instead of sorbitol. The cell permeabilization treatments, such as blanketing with or soaking in toluene and cetyltrimethylammonium bromide (CTAB), repeated freezing and thawing, drying, and treatments under high osmotic pressure or mild temperature, cause damages to the cell membrane or make the membrane more permeable, and allow some cellular components that are not essential to the desired specific reactions and/or soluble cofactors to be released from the cell (Bringer-Meyer and Sahm, 1991). GFOR enzyme activities are selectively retained from the harsh permeabilization treatments (Rehr et al., 1991). However, these harsh permeabilization treatments could cause many problems, such as having to manage use of toxic organic solvents, difficulty involved in removal of the cell debris, protein leakage in the reaction solution, and an energy intensive process.

In this work, we have developed a new method of selectively inhibiting certain enzymes involved in ethanol biosynthetic pathway by divalent metal ions in the Entner–Doudoroff pathway. A comprehensive survey on the inhibition effects of various metal ions, especially the divalent ions, to many enzymes in the Entner–Doudoroff pathway was carried out using Brenda Enzyme Database (Chang et al., 2009) as shown in Fig. 4. They include Zn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Ba^{2+}, Ag^{2+}, Be^{2+}, Cd^{2+}, Cr^{2+}, Hg^{2+}, Li^{+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zr^{2+}, and Sr^{2+}. On the other hand, GFOR was not affected by almost all these metal ions and again demonstrated its unique robustness. Tables 6–8 show the effects of selected divalent metal ions on the bioconversion of fructose and glucose to sorbitol and gluconic acid. The results have shown that an addition of predetermined amount of divalent metal ions to the bioreactor system is an efficient and economical bioprocess strategy. The new method successfully avoided many problems that have been mentioned above, such as the use of toxic organic solvents, difficulty involved in removal of the cell debris, protein leakage in the reaction solution, and an energy intensive process.

Among the divalent ions evaluated, Zn^{2+} was found to be most effective even at a low level addition ($0.023\,g\,l^{-1}$ Zn^{2+} ions). Ca^{2+} was also used for the precipitation of calcium gluconate as an *in situ* simultaneous separation of gluconic acid from the product mixture of gluconic acid–sorbitol. The amount of Ca^{2+} addition can be controlled by the amount of gluconic acid produced and calcium gluconate to be precipitated. This process strategy also increased the gluconate production by pushing the forward reaction with simultaneous *in situ* separation of calcium gluconate from the reaction solution. At the same time, the sorbitol production is enhanced by this coupled reaction with the gluconate production. When this calcium gluconate precipitation method is combined with the

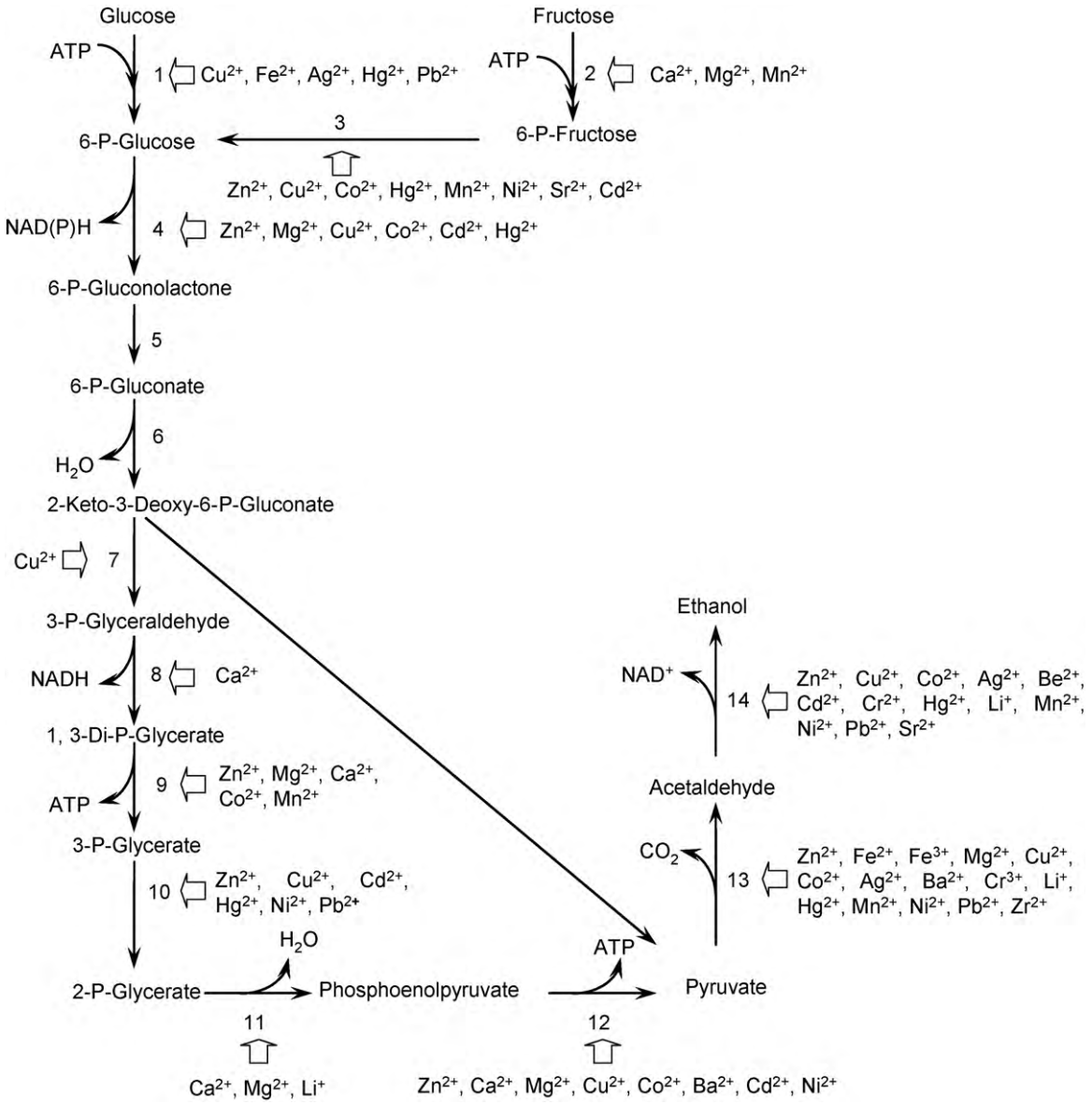

Fig. 4. Inhibition of enzymes in Entner–Doudoroff pathway by metal ions. The enzyme code numbers are: (1) glucokinase; (2) fructokinase; (3) 6-P-glucose isomerase; (4) 6-P-glucose dehydrogenase; (5) 6-P-gluconolactonase; (6) 6-P-gluconate dehydratase; (7) 2-keto-3-deoxy-6-P-gluconate aldolase; (8) 3-P-glyceraldehyde dehydrogenase; (9) phosphoglycerate kinase; (10) phosphoglycerate mutase; (11) enolase; (12) pyruvate kinase; (13) pyruvate decarboxylase; and (14) alcohol dehydrogenase.

simultaneous reactions for sorbitol and gluconic acid production in the bioreactor, the gluconate productivity is significantly greater than the gluconic acid productivity in soluble form without precipitation. The productivity of gluconic acid obtained by calcium gluconate precipitation method was 0.45 mole (50 g l^{-1} for $CaCl_2$) as compared to that of soluble form was only 0.29 mole under the reaction conditions, 40 °C and pH 5–6 (Bao et al., 2003). Further improvement for increased productivity is under investigation by manipulating the initial feed substrate concentrations, molar ratio of the two substrates, combined effects of cofactors and controlled *in situ* precipitation of calcium gluconate.

The bioprocess technology developed for the production of sorbitol and gluconic acid and the separation method of sorbitol from the reaction mixture is considered to be practical, economically competitive, and feasible in industrial scale. At the same time, the enabling technology developed in this work which the relative amount of ethanol and sorbitol to be produced can be controlled depending on the market demand is considered to be a very attractive biotechnology strategy.

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