Biosynthesis of L-Sorbose and L-Psicose Based on C—C Bond Formation Catalyzed by Aldolases in an Engineered Corynebacterium glutamicum Strain

Jiangang Yang, Jitao Li, Yan Men, Yueming Zhu, Ying Zhang, Yuanxia Sun, Yanhe Ma
National Engineering Laboratory for Industrial Enzymes, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, China

The property of loose stereochemical control at aldol products from aldolases helped to synthesize multiple polyhydroxylated compounds with nonnatural stereoconfiguration. In this study, we discovered for the first time that some fructose 1,6-diphosphate aldolases (FruA) and tagatose 1,6-diphosphate (TagA) aldolases lost their strict stereoselectivity when using L-glyceraldehyde and synthesized not only L-sorbose but also a high proportion of L-psicose. Among the aldolases tested, TagA from Bacillus licheniformis (BGatY) showed the highest enzyme activity with L-glyceraldehyde. Subsequently, a “one-pot” reaction based on BGatY and fructose-1-phosphatase (YqaB) generated 378 mg/liter L-psicose and 199 mg/liter L-sorbose from dihydroxyacetone-phosphate (DHAP) and L-glyceraldehyde. Because of the high cost and instability of DHAP, a microbial fermentation strategy was used further to produce L-sorbose/L-psicose from glucose and L-glyceraldehyde, in which DHAP was obtained from glucose through the glycolytic pathway, and some recombination pathways based on FruA or TagA and YqaB were constructed in Escherichia coli and Corynebacterium glutamicum strains. After evaluation of different host cells and combinations of FruA or TagA with YqaB and optimization of gene expression, recombinant C. glutamicum strain WT(pXFTY) was selected and produced 2.53 g/liter total ketoses, with a yield of 0.50 g/g L-glyceraldehyde. Moreover, deletion of gene cgl0331, encoding the Zn-dependent alcohol dehydrogenase in C. glutamicum, was confirmed for the first time to significantly decrease conversion of L-glyceraldehyde to glycerol and to increase yield of target products. Finally, fed-batch culture of strain SY14(pXFTY) produced 3.5 g/liter L-sorbose and 2.3 g/liter L-psicose, with a yield of 0.61 g/g L-glyceraldehyde. This microbial fermentation strategy also could be applied to efficiently synthesize other L-sugars.

Aldolases are remarkably useful and have been widely investigated for catalyzed C—C bond formation (1). The ability to catalyze aldol addition reactions from small chiral polyfunctional molecules and generate up to two new stereogenic centers allows aldolases to synthesize a broad range of both natural and novel polyhydroxylated compounds. Among the aldolase families, dihydroxyacetone-phosphate (DHAP)-dependent aldolases, which utilize DHAP as the donor substrate and accept a broad range of acceptor aldehydes, are widely investigated. Well-known members of this class include fuculose 1-phosphate aldolase (FucA), rhamnulose 1-phosphate aldolase (RhaD), fructose 1,6-diphosphate aldolase (FruA), and tagatose 1,6-diphosphate aldolase (TagA) (2). These enzymes have been used to synthesize several new deoxy or phosphorylated sugars and iminocycloits (3–5).

DHAP-dependent aldolases create two new stereogenic centers at C atoms 3 and 4 ([3S,4R], [3S,4S], [3R,4S], [3R,4R]). FucA, FruA, RhaD, and TagA generally form (3S,4R), (3S,4S), (3R,4S), and (3S,4S) stereoconfigurations, respectively (1, 2). However, some aldolases sometimes lost their strict stereoselectivity at the C-4 stereocenter with some aldehydes as substrates. For example, RhaD aldolase from Escherichia coli catalyzed DHAP and L-glyceraldehyde to form 1-fructose (3S,4S). However, D-sorbose (3R,4S) and D-psicose (3R,4R) were formed when D-glyceraldehyde (6–8). Moreover, FucA aldolase from E. coli catalyzed DHAP and L-glyceraldehyde to form the single product 1-tagatose (3R,4R). However, FucA from Thermus thermophilus HB8 generated 1-tagatose (3R,4R) and 1-fructose (3R,4S) simultaneously from the same substrates. This finding indicated that aldolases had a high level of stereocontrol at C-3; however, the configuration of the stereocenter at C-4 in some cases depended on the particular enzyme and acceptor structure. This aldolase property can be well utilized to synthesize various types of rare sugars. Thus far, no reports have shown this property for FruA aldolase. In addition, TagA aldolases form diastereomer mixtures of product, in which instead of the natural configuration (3S,4S), those of the configuration (3S,4R) predominated (>90%) (4).

L-Sugars, which are one large group of rare sugars, often hold enormous potential for applications in the pharmaceutical industry (9–11). For example, several L-sugars can be used to produce L-nucleoside analogues, which showed increased antiviral activity, better metabolic stability, and more favorable toxicological profiles (12, 13). L-Sorbose has been applied to produce the potent glycosidase inhibitor 1-deoxygalactonojirimycin and L-ascorbic acid, known as vitamin C (11, 14). Several (bio)chemical methods have been applied to synthesize L-sugars because of their low nat-
ural abundance. Ken Izumori presented a strategy to synthesize L-sugars from polyalcohol through oxidoreductases (15). Because of requirements for the expensive cofactor NAD(P)H, these oxidation reactions with oxidoreductases often were performed inside microbial cells so that the cellular metabolism could provide the reductive power. For example, recombinant E. coli harboring an NAD-dependent mannitol-1-dehydrogenase (MDH) from *Apium graveolens* had a significantly improved ability to convert several polys to their L-sugar counterparts, such as L-ribose (16). However, polysyns such as allitol and sorbitol also were valuable compounds.

Recently, DHAP-dependent aldolases have been applied successfully to the biosynthesis of some L-sugars. For example, RhaA aldolase was used to catalyze the aldol reaction of DHAP to L-glyceraldehyde to form L-fructose-1-phosphate; the latter could remove the phosphate group by acid phosphatase (AP) to produce L-fructose (8). By using a similar strategy, L-tagatose could be synthesized with FucA aldolase and L-sorbose through Frua from rabbit muscle aldolase (RAMA) (6, 17). However, another important ketohexose, L-psicose (3S, 4S), was scarcely produced in previous research by using aldolases. It was believed that L-psicose would be obtained if some Frua or TagA aldolases exhibited loose stereoselectivity at the C-4 stereocenter.

In this study, the Frua and TagA aldolases from three organisms initially were cloned and characterized. For the first time, we discovered that Frua and TagA aldolases could simultaneously synthesize L-psicose and L-sorbose from L-glyceraldehyde and DHAP in vitro. Subsequently, a microbial fermentation strategy, in which some recombinant pathways based on Frua or TagA aldolases and Yqab were constructed in *E. coli* and *Corynebacterium glutamicum* strains, was used to synthesize L-sorbose/L-psicose from glucose and L-glyceraldehyde. Furthermore, gene cgl0331, encoding the Zn-dependent alcohol dehydrogenase in *C. glutamicum*, was deleted to decrease conversion of L-glyceraldehyde to glycerol and increase target product yield.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and materials.** Acid phosphatase from potato (AP; 6 U/ml), D-fructose 1,6-bisphosphate aldolases from rabbit muscle (RAMA), dihydroxyacetone phosphate hemimagnesium salt hydrate, NADP (NADPH), dihydroxyacetone, D-fructose-1-phosphate barium salt, glyceraldehyde, L-glucose, D-fructose-1-phosphate, and L-sorbose were purchased from Sigma-Aldrich, D-erythrose were purchased from Sigma-Aldrich, D-erythrose, D-xylulose, L-sorbose, and L-psicose were purchased from Kagawa Rare Sugar Cluster. All restriction enzymes and DNA ligase were purchased from Novagen (Darmstadt, Germany). The nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column was purchased from Qiagen. Yeast extract and tryptone were purchased from Oxoix Ltd., and brain heart infusion (BHI) broth were purchased from Becton, Dickinson, and Company.

All bacterial strains and plasmids are listed in Table 1. The *E. coli* strain DH5α was used for plasmid construction and as the donor for *fbaA*, *kbaY*, and *Yqab* gene amplification. *Bacillus licheniformis* ATCC 14580 and *Klebsiella oxytoca* E718 were used as the donor for *gatY* and *yqab* gene amplification, respectively. *E. coli* BL21(DE3) was used for enzyme preparation. *C. glutamicum* ATCC 13032 was the wild-type parent strain that was engineered for the biosynthesis of target products. Plasmid pET-21a (+) was used as the foundation vector for expression of Frua, TagA, and Yqab in *E. coli* BL21(DE3). The vector pETDuet-1 and *C. glutamicum* *E. coli* shuttle vector pXMJ19 were used to construct the recombinant pathways in *E. coli* BL21(DE3) and *C. glutamicum*, respectively.

**Medium and culture conditions.** *E. coli* DH5α and *E. coli* BL21(DE3) were routinely cultured in Luria-Bertani (LB) medium as previously described (19). *C. glutamicum* strains were cultivated at 30°C in BHI medium and CGXII medium (20) supplemented with 10 g/liter glucose. Plasmid DNA was transferred into *C. glutamicum* ATCC 13032 via electroporation, and the recombinant strains were selected on brain heart infusion-sorbitol (BHIS) agar plates that contained 20 mg/liter chloramphenicol.

For cell growth of the *C. glutamicum* strains, a single clone was inoculated and incubated overnight at 30°C and 200 rpm in BHI medium. The preculture cells then were transferred to 50 ml BH medium in a 500-ml shake flask. The main cultures were grown at 30°C and 200 rpm.

**Expression and purification of His-tagged Frua, Taga, Yqab, and Cgl0331.** The *fbaA*, *kbaY*, and *yqab* genes from *E. coli* were amplified by PCR with the *fbaA* (Ndel)/FbaA2 (BamHI), KbaY1 (Ndel)/KbaY2 (HindIII), and EYqab1 (Ndel)/EYqab2 (HindIII) primer sets, respectively. The *gatY* gene from *B. licheniformis* ATCC 14580 was amplified by PCR with the *GatY*1 (Ndel)/GatY2 (HindIII) primers and the *yqab* gene from *K. oxytoca* E718 with KYqab1 (Ndel)/KYqab2 (HindIII) primers. The amplified fragments were cloned into the PET-21a (+) vector to obtain pETHisFbaAa, pETHisKbaY, pETHisGatY, and pETHisYqab. The cgl0331 gene from *C. glutamicum* was amplified by PCR with the 21cg0331 (Ndel)/2 (HindIII) primer set. The amplified fragments were cloned into the PET-21a (+) vector to obtain pETHis-Cgl0331. *E. coli* BL21(DE3) strains harboring expression plasmids were cultured at 37°C in 1 liter of LB medium containing 100 mg/liter ampicillin to an optical density at 600 nm (OD600) of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) was added into the culture to induce protein expression, and the temperature was adjusted to 16°C to avoid inclusion body formation. After incubation for an additional 20 h, cells were harvested, washed twice, and suspended in 50 mM triethanolamine (TEA) (pH 7.5) buffer. The suspension cells then were lysed by sonication and centrifuged at 14,000 × g and 4°C for 10 min. Clear supernatant was collected and loaded onto an Ni2+ -NTA-agarose column preequilibrated with binding buffer (50 mM TEA buffer, 300 mM NaCl, 20 mM imidazole, pH 7.5). The retained proteins were recovered with elution buffer (50 mM TEA buffer, 300 mM NaCl, 300 mM imidazole, pH 7.5). The eluted fraction containing purified protein was dialyzed and stored at −20°C. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

**Enzyme activity analysis.** To determine the activity of Frua and TagA, a mixture containing 50 mM TEA buffer (pH 7.5), 5 mM DHAP, 8 mM L-glyceraldehyde, and 40 μl (0.25 mg) of purified protein was shaken at 120 rpm and 25°C for 1 h. Subsequently, the pH of the mixture was adjusted to 4.8 to 5.2 with 4 M HCl, and then 2 U AP was added. The mixture was shaken at 120 rpm and 30°C for 2 h. Samples then were treated with 10 M NaOH, centrifuged (22,000 rpm, 20 min), and analyzed by high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the enzyme amount catalyzing the formation of 1 nmol total product per min.

The Yqab enzyme activity of both D-fructose-1-phosphate barium salt and the mixture of L-sorbose/L-psicose-1-phosphate was analyzed. D- Fructose-1-phosphate barium salt was purchased from Sigma-Aldrich, and the mixture of L-sorbose/L-psicose-1-phosphate was obtained from an aldol reaction which was catalyzed by Frua aldolase with DHAP and L-glyceraldehyde as substrates. The enzyme activity assay for Yqab contained 5 mM substrates, 50 mM TEA buffer (pH 7.5), and 20 μl of purified (0.1 mg) protein. The mixture was shaken at 120 rpm and 25°C for 30 min. Samples then were treated with 10 M NaOH, centrifuged (22,000 rpm, 20 min), and analyzed by HPLC. One unit of enzyme activity was defined as the enzyme amount catalyzing the formation of 1 nmol ketose per min.

To determine the activity of enzyme Frua, TagA, and Yqab in recombinant *C. glutamicum* strains, the preculture cells were induced by 1 mM IPTG for about 10 h at 25°C and 150 rpm, harvested by centrifugation (8,000 × g, 20 min, 4°C), and washed with 50 mM TEA buffer (pH 7.5). Cell disruption was performed by sonication, and crude extracts were
centrifuged at 14,000 × g and 4°C for 30 min. The measurement methods were similar to those for the corresponding purified enzymes described above.

Enzyme activity of Cgl0331 was determined in 0.2 ml of TEA buffer (50 mM, pH 7.0), containing 0.1 mM NADPH (1 mM), and 20 μl (60 μg/ml) of the purified enzyme Cgl0331. The reaction was initiated by addition of the enzyme and was monitored for 3 min with a SpectraMax M2e (MD, USA) at 30°C. The activity was determined by measuring the molar absorption coefficient of 6.22 mM−1 cm−1. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol NADPH per min.

In vitro aldol reactions with DHAP and aldehydes as substrates.

Aldehydes were dissolved in water to achieve a concentration of 500 mM. The reaction mixture (400 μl) contained 40 mM aldehydes, freshly neutralized 40 mM DHAP solution (pH 7.0), 200 mM TEA buffer (pH 7.5), 80 μl (~0.5 mg) purified aldolases, and 80 μl (~0.1 mg) fructose-1-phosphatases. The reactions were carried out at 25°C and 120 rpm for 3 h. Samples were captured every 1 h for HPLC analysis.

The “one-pot” reaction mixture (500 μl) contained 50 mM 1-glycerol-aldheyde, freshly neutralized 40 mM DHAP solution (pH 7.0), 200 mM TEA buffer (pH 7.5), 80 μl (~0.5 mg) purified aldolases, and 80 μl (~0.1 mg) fructose-1-phosphatases. The reactions were carried out at 25°C and 120 rpm for 3 h. Samples were captured every 1 h for HPLC analysis.

**TABLE 1** Strains and plasmids used in this study

<table>
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<tr>
<th>Strains and plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or source</th>
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<td>DH5o</td>
<td>endA1 supE44 recA1 gyrA96 relA1 deoR1 U169 F80lacZΔM15 ncrAΔ(mrr-hsdRMS-mcrBC)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>ompT hsdS2 (rha− mna−) gal(DE3)</td>
<td>Invitrogen</td>
</tr>
<tr>
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<td>Wild type</td>
<td>ATCC 13032</td>
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<tr>
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<td>This study</td>
</tr>
<tr>
<td>SY14</td>
<td>Gene cgl0331 deletion of C. glutamicum</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

- pET21a (+) Expression vector, Ap' | Invitrogen |
- pETHisFbaA pET21a (+) derivative carrying gene fbaA from E. coli | This study |
- pETHisKbaY pET21a (+) derivative carrying kbaY from E. coli | This study |
- pETHisGatY pET21a (+) derivative carrying gatY from B. licheniformis | This study |
- pETHisEYqaB pET21a (+) derivative carrying yqaB from E. coli | This study |
- pETHisKqYba pET21a (+) derivative carrying yqaB from Klebsiella oxytoca | This study |
- pETHisCgl0331 pET21a (+) derivative carrying cgl0331 from C. glutamicum | This study |
- pK18mobsacB Kan'; vector for in-frame deletions (RP4 mob; sacBbacillus subtilis lacZ; oriV_E. coli) | This study |
- pK18-cgl0331 pK18mobsacB cloning with the neighboring sequence of gene cgl0331 | This study |
- pETDuet-1 Double T7 promoters, pBR322 ori, Ap' | Invitrogen |
- pEFY pETDuet-1 carrying fbaA and yqaB | This study |
- pEKY pETDuet-1 carrying kbaY and yqaB | This study |
- pEBY pETDuet-1 carrying gatY and yqaB | This study |
- pXM19 Cm'; C. glutamicum/E. coli shuttle vector (PtaC, lacP; pBL1, OriV_c. glutamicum; OriV_E. coli) | 18 |
- pEC-XK99E Kan'; C. glutamicum/E. coli shuttle vector (Ptrc, lacP; pGA1, OriV_c. glutamicum; OriV_E. coli) | 21 |
- pXFTY pXM19 containing fragments fbaA-tac-yqaB | This study |
- pXBY pXM19 containing fragments gatY-tac-yqaB | This study |
- pXKTY pXM19 containing fragments kbaY-tac-yqaB | This study |
- pXYTF pXM19 containing fragments yqaB-tac-fbaA | This study |
- pXYTB pXM19 containing fragments yqaB-tac-gatY | This study |
- pXYRF pXM19 containing fragments yqaB-trc-fbaA | This study |
- pXYRB pXM19 containing fragments yqaB-trc-gatY | This study |
the fragment of src which was amplified from plasmid pEC-XK99E (21). The primer sets used in this study are shown in Table S1 in the supplemental material.

The in-frame deletion of the cg0331 gene of *C. glutamicum* 13032 was accomplished through a two-step homologous recombination procedure using the pK18mobsacB suicide vector (22). The upstream and downstream fragments (approximately 800 bp each) of the cg0331 gene, amplified from the genome of *C. glutamicum* with primers cg0331-1/cg0331-2 and cg0331-3/cg0331-4, served as templates for overlap extension PCR with cg0331-5/cg0331-6 primers to form the cg0331-cg0331 fragment; the latter then was digested with PsiI and XbaI and cloned into pK18mobsacB to obtain pK18-cg0331. The resulting plasmid, pK18-cg0331, was further transferred into *C. glutamicum* by electroporation. Selection for the first and second recombination events was performed as previously described (23), and the deletion of the cg0331 gene was verified by PCR using the cg0331-1 and cg0331-4 primers.

**Fermentation conditions.** The recombinant *C. glutamicum* strains initially were cultivated in 100 ml BHI medium in a 500-ml shake flask at an OD600 of 0.8 and then induced with 1 mM IPTG for 12 h at 25°C and 200 rpm. Subsequently, the cells were harvested by centrifugation (8,000 × g, 10 min, 4°C), washed twice, and suspended in CGXII medium. Ten-milliiliter cell suspensions were transferred into 50-ml shake flasks (8,000 rpm) and the mixture was stirred at 200 rpm. When appropriate, 20 mg/liter chloramphenicol was added. The fermentation conditions for recombinant *E. coli* strains were similar to those for *C. glutamicum*. Notably, for recombinant *E. coli* strains, the BHI and CGXII culture media mentioned above were replaced with LB and M9.

For the fed-batch mode, 50-ml cell suspensions supplemented with glucose (20 g/liter) and *l*-glyceraldehyde (5 g/liter) were transferred to a 500-ml shake flask at an initial OD600 of approximately 30. The temperature was held at 30°C and the mixture was stirred at 200 rpm. Samples were captured every 2 h and centrifuged at 14,000 rpm for 20 min. The resulting supernatants were analyzed by HPLC.

**Quantitative measurement of biomass, glucose, aldehydes, dihydroxyacetone, and rare ketoses.** Growth was monitored by measuring the OD600 with a UV-visible spectrophotometer (TU-1901; Persee, Beijing, China). Cell dry weight (CDW; grams/liter) was calculated from the optical density according to a linear relationship between OD600 and CDW. Thus, the biomass concentration of *C. glutamicum* was calculated from OD600 values using the experimentally determined correlation factor of 0.25 g/liter *dry weight*/liter for an OD600 of 1. The concentrations of glucose, *l*-glyceraldehyde, *l*-sorbose, and *l*-psicose were quantified by HPLC using a Hitachi GL-C611 column (10.7 mm by 300 mm; Japan) with a refractive index detector. A mobile phase of 0.1 mM NaOH solution was used at a flow rate of 1 ml/min, and the column was operated at 60°C.

**Real-time quantitative PCR (RT-qPCR).** The recombinant *C. glutamicum* strains were grown to exponential phase and then harvested by centrifugation for 1 min at 14,000 × g and 4°C. Total RNA was extracted by using an RNeasy prep pure kit DP430 (Tiangen, Beijing, China). First, 500 ng of total RNA was transcribed into cDNA using Quanti reverse transcriptase with random primers (Tiangen, Beijing, China). Samples then were analyzed using a Light Cycler H 480 II (Roche, Basel, Switzerland) with Real master mix (SYBR green). The 16S rRNA gene was used as a reference for normalization, and the primers used for gene quantification are listed in Table S1 in the supplemental material.

**RESULTS**

**Expression and characterization of FruA and TagA aldolases.** TagA has been used widely for asymmetric synthesis of polyoxygcnated compounds. However, previous research showed that (3S,4R) instead of (3S,4S) stereoconfiguration predominated in aldol products of TagA aldolases (4). The aldol product with (3S,4S) stereoconfiguration has barely been synthesized thus far. We speculated that if TagA or FruA lost its strict stereoselectivity at aldol products when using some aldehyde acceptors, then the aim of synthesizing a (3S,4S) configuration product would be achieved.

In this study, the genes of class I TagA from *E. coli* (*kbaY*) and *B. licheniformis* (*gatY*) and the class II FruA from *E. coli* (*fba*) were cloned and overexpressed in *E. coli* BL21(DE3). In addition, FruA from Rama also was purchased. Sequence alignments showed that TagA from *B. licheniformis* ATCC 14580 (BGatY) and FruA from *E. coli* (EFruA) exhibited 56% and 22% identity to TagA from *E. coli* (EKbaY), respectively. To purify the enzyme in one step, N-terminal 6×His tag was added. SDS-PAGE of the final purified enzyme showed molecular masses of BGatY (31.8 kDa), EKbaY (32.1 kDa), and EFruA (39.9 kDa) that were in agreement with the sizes predicted from the DNA sequence (see Fig. S1 in the supplemental material). The gene class II TagA proteins from *E. coli* and *K. oxytoca* also were cloned and expressed in this study. However, no soluble target proteins were obtained (data not shown).

**In vitro** assays were conducted with DHAP and five kinds of polyhydroxy aldehyde, 1 to 6, as substrates. For in vitro experiments, 50 mM DHAP and 40 mM aldehydes initially were condensed by purified FruA and TagA, and the aldol products were dephosphorylated by commercial AP. Conversion rates were calculated with respect to aldehyde consumption. New products were identified by comparing them to standard substances. The authentic compounds of *l*-erythulose 11, *d*-xylulose 12, *d*-fructose 13, *l*-sorbose 14, and *l*-psicose 15 were purchased. Standard substance 16 was prepared by using FruA from RAMA with DHAP and *d*-erythrose 6 as substrates and was further structurally confirmed by NMR spectroscopy (see Fig. S2 in the supplemental material). As shown in Table 2, both FruA aldolases and TagA aldolases could accept many kinds of polyhydroxy aldehydes to synthesize the corresponding products 11 to 16 (Table 2; also see Fig. S3). The FruA from RAMA exhibited preferable product formation ability. For four aldehyde substrates 1, 2, 3, and 5, products with (3S,4R) stereoconfiguration still predominated (>90%); however, the percentage of product with (3S,4S) configuration in total aldol products was higher than that with (3S,4R) when *l*-glyceraldehyde was used as the substrate (see Fig. S3 and S4). Both EKbaY and BGatY could catalyze *l*-glyceraldehyde and DHAP to produce *l*-sorbose/*l*-psicose as well. In particular, the percentage of *l*-psicose (3S,4S) in aldol products of BGatY reached 67%. Furthermore, EFruA also exhibited the ability to synthesize *l*-psicose when accepting *l*-glyceraldehyde; however, this property did not happen for RAMA (Table 2).

To characterize the catalytic efficiency of EFruA, BGatY, and EKbaY, the enzyme activity of *l*-glyceraldehyde also was measured. The results of in vitro assays showed that the specific activity of BGatY (43 U/mg) was 6- to 8-fold higher than that of EKbaY and EFruA. However, the conversion rate of BGatY was only 65% of that of EFruA (Tables 2 and 3).

**Expression and characterization of fructose-1-phosphatase.** DHAP-dependent aldolases catalyze DHAP and *l*-glyceraldehyde to form ketose-1-P. The latter should be dephosphorylated to obtain target ketoses. Fructose-1-phosphatase (*YqaB*) has been reported to catalyze dephosphorylation of fructose-1-phosphate to form fructose and potentially is effective for other C-1 phosphorylated carbohydrates (24). Therefore, fructose-1-phosphatases
from *E. coli* (EYqaB) and *K. oxytoca* (KYqaB) were cloned and overexpressed in *E. coli* BL21(DE3). Sequence alignment showed that EYqaB showed 85% identity to KYqaB. After one-step purification with an N-terminal 6×His tag, SDS-PAGE of the obtained proteins showed the desired molecular masses of EYqaB (21.6 kDa) and KYqaB (21.5 kDa) (see Fig. S1 in the supplemental material). YqaB enzymes also were characterized to test the catalytic efficiency of fructose-1-phosphate and the mixture of L-sorbose/L-psicose-1-phosphate, which was obtained from EFruA with DHAP and L-glyceraldehyde as substrates. The results shown in Table 3 indicated that both YqaB enzymes could perform dephosphorylation of the substrates used in this study, and enzyme activity of fructose-1-phosphate was higher than that of the mixture. Moreover, the catalytic efficiency of EYqaB for both substrates was better than that of KYqaB (Table 3).

### Table 2 In vitro synthesis of many kinds of rare ketoses based on FruA and TagA

<table>
<thead>
<tr>
<th>Aldehydes</th>
<th>Products (formula)</th>
<th>Conversion rates (%)</th>
<th>Ee values (%)</th>
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<tr>
<td></td>
<td></td>
<td>EFruA</td>
<td>RAMA</td>
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<td>Formaldehyde (1)</td>
<td>L-erythulose (11)</td>
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<td>D-erythrose (6)</td>
<td>Sedoheptulose (16)</td>
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</table>

a Conversion rates were calculated with respect to aldehyde consumption.

b Ee values were defined as the percentage of the ketose in total aldol products.

c DHAP and aldehydes were condensed with FruA and TagA aldolases to obtain rare ketose-1-phosphatase. These then were dephosphorylated with commercial acid phosphatase from potato to generate rare ketoses.

from *E. coli* (EYqaB) and *K. oxytoca* (KYqaB) were cloned and overexpressed in *E. coli* BL21(DE3). Sequence alignment showed that EYqaB showed 85% identity to KYqaB. After one-step purification with an N-terminal 6×His tag, SDS-PAGE of the obtained proteins showed the desired molecular masses of EYqaB (21.6 kDa) and KYqaB (21.5 kDa) (see Fig. S1 in the supplemental material). YqaB enzymes also were characterized to test the catalytic efficiency of fructose-1-phosphate and the mixture of L-sorbose/L-psicose-1-phosphate, which was obtained from EFruA with DHAP and L-glyceraldehyde as substrates. The results shown in Table 3 indicated that both YqaB enzymes could perform dephosphorylation of the substrates used in this study, and enzyme activity of fructose-1-phosphate was higher than that of the mixture. Moreover, the catalytic efficiency of EYqaB for both substrates was better than that of KYqaB (Table 3).

### Table 3 Enzyme activity data of purified FruA, TagA, and YqaB

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EFruA</td>
</tr>
<tr>
<td>D-Glyceraldehyde</td>
<td>85.30 ± 5.1</td>
</tr>
<tr>
<td>L-Glyceraldehyde</td>
<td>5.21 ± 0.8</td>
</tr>
<tr>
<td>D-Fructose-1-phosphate</td>
<td>649.86 ± 29.21</td>
</tr>
<tr>
<td>L-Sorbose/l-psicose-1-phosphate b</td>
<td>130.31 ± 18.14</td>
</tr>
</tbody>
</table>

a One unit of enzyme activity was defined as the enzyme amount catalyzing the formation of 1 nmol product per min. EFruA, class II fructose 1,6-diphosphate aldolase from *E. coli*; EKbaY, class I tagatose 1,6-diphosphate aldolase from *E. coli*; BGatY, tagatose 1,6-diphosphate aldolase from *B. licheniformis*; EYqaB, fructose-1-phosphatase from *E. coli*; KYqaB, fructose-1-phosphatase from *K. oxytoca*. Means and standard deviations were calculated based on triplicate experiments.

b The mixture of L-sorbose/l-psicose-1-phosphate was obtained by EFruA in vitro with DHAP and L-glyceraldehyde as substrates.
Although L-sorbose/L-psicose could be synthesized, achieved 378 mg/liter L-psicose and 199 mg/liter L-sorbose, were reaction for 3 h, increased concentrations of ketoses, which concentration ratio of BGatY to EYqaB (1:1, 3:1, 5:1, 7:1, 9:1, and 3:1). After reaction was further optimized by modulating the protein concentration. Thus, the one-pot reaction mixture (500 µl) contained 50 mM L-glyceraldehyde, freshly neutralized 40 mM DHAP solution (pH 7.0), 80 µl (~0.5 mg) pure aldolases, and 80 µl (~0.1 mg) fructose-1-phosphatase. The ratio of L-psicose to L-sorbose was calculated from the ratio of the percentage of L-psicose in total ketoses to that of L-sorbose. Means and standard deviations were calculated based on triplicate experiments. (C) Effect of different protein concentration ratios of BGatY to EYqaB on product formation.

FIG 1 (A) One-pot reaction synthesis of L-sorbose/L-psicose. (B) EKbaY, BGatY, and EFruA were combined with EYqaB to synthesize L-sorbose/L-psicose. The one-pot reaction mixture (500 µl) contained 50 mM L-glyceraldehyde, freshly neutralized 40 mM DHAP solution (pH 7.0), 80 µl (~0.5 mg) pure aldolases, and 80 µl (~0.1 mg) fructose-1-phosphatase. The ratio of L-psicose to L-sorbose was calculated from the ratio of the percentage of L-psicose in total ketoses to that of L-sorbose. Means and standard deviations were calculated based on triplicate experiments. (C) Effect of different protein concentration ratios of BGatY to EYqaB on product formation.

Each aldolase (EFruA, BGatY, and EKbaY) was combined with 0.2 mg/ml of EYqaB in the one-pot reaction system. After reaction for 3 h, all three of the combinations could synthesize both L-sorbose and L-psicose (Fig. 1B). Moreover, the percentage of L-psicose in total ketoses was greater than 50%. The combination of enzymes BGatY and EYqaB produced 249 mg/liter L-psicose and 130 mg/liter L-sorbose, which was 1.5-fold higher than the levels of EFruA and EYqaB. The ratio of L-psicose to L-sorbose was 1.91, which was similar to the results shown in Table 2. Consequently, BGatY and EYqaB were chosen to further optimize the in vitro one-pot reaction.

The enzyme activity value of EYqaB for the mixture of L-sorbose/L-psicose was nearly 3-fold higher than that of BGatY for L-glyceraldehyde (Table 3), which indicated that the aldol reaction from L-glyceraldehyde to L-sorbose/L-psicose-1-P is the rate-limiting step in the one-pot reaction scheme. Thus, the one-pot reaction was further optimized by modulating the protein concentration ratio of BGatY to EYqaB (1:1, 3:1, 5:1, 7:1, 9:1, and 3:1). After reaction for 3 h, increased concentrations of ketoses, which contained 378 mg/liter L-psicose and 199 mg/liter L-sorbose, were produced when the protein ratio was controlled at 9:1. The percentage of L-psicose in total ketoses increased from 55% to 67% along with increasing protein concentration ratios from 1:1 to 7:1 (Fig. 1C).

Construction of recombination pathways in E. coli and C. glutamicum. Although L-sorbose/L-psicose could be synthesized from DHAP and L-glyceraldehyde via the one-pot reaction system, compound DHAP, which is unstable and currently very expensive, limited its application for large-scale production of L-psicose. Several enzymatic approaches from glycerol or dihydroxyacetone have been developed to synthesize DHAP; however, the yield has not advanced beyond small scale (25). The substance DHAP is also a natural intermediate of the glycolytic pathway, which indicates that DHAP can be obtained from glucose through the glycolytic pathway. Therefore, the recombination pathways based on FruA or TagA aldolase and YqaB phosphatase were constructed in E. coli BL21(DE3) and a C. glutamicum strain to produce L-sorbose/L-psicose with glucose and L-glyceraldehyde as substrates (Fig. 2). Moreover, the dual-phase fermentation process, in which cell and enzyme preparations both were performed in one stage and rare ketose production was in another stage (26), was used.

Given the lower enzyme activity of FruA and TagA aldolases and YqaB phosphatase, we initially expected to increase the expression level of the corresponding genes of those enzymes. Thus, plasmids pE FY, pEKY, and pEBY were constructed in which the expression of those enzyme genes was controlled with the tac promoter. The expression plasmids then were transferred into E. coli BL21(DE3) to obtain BL21(pEFY), BL21(pEKY), and BL21 (pEBY). When substrate glucose and L-glyceraldehyde were added into the culture, all of the recombinant E. coli strains exhibited the ability to produce L-psicose and L-sorbose. However, the final products and yield were extremely low (Fig. 3).

The recombinant pathways were further constructed in C. glutamicum based on plasmids pXKT, pXBTY, and pXFTY, in which the expression of every FruA, TagA, and YqaB gene was controlled with the tac promoter. The dual-phase fermentation conditions were similar to those of E. coli. As shown in Fig. 3, both L-sorbose/L-psicose could be synthesized by all recombinant C. glutamicum strains with glucose and L-glyceraldehyde as substrates. Furthermore, the final ketose concentration of recombinant C. glutamicum strains was nearly 6- to 20-fold higher than that of the corresponding recombinant E. coli strains. Interestingly, we discovered that the total ketoses from strain WT (pXBTY) were 20% lower than those from WT(pXFTY), although the enzyme activity of purified BGatY to L-glyceraldehyde was 8-fold higher than that of EFruA in vitro (Table 3). This phenomenon also occurred for recombinant E. coli strains. It was probably due to the higher conversion rate of EFruA to L-glyceraldehyde relative to that of BGatY (Table 2). The percentage of L-psicose in each aldolase (EFruA, BGatY, and EKbaY) was combined with 0.2 mg/ml of EYqaB in the one-pot reaction system. After reaction for 3 h, all three of the combinations could synthesize both L-sorbose and L-psicose (Fig. 1B). Moreover, the percentage of L-psicose in total ketoses was greater than 50%. The combination of enzymes BGatY and EYqaB produced 249 mg/liter L-psicose and 130 mg/liter L-sorbose, which was 1.5-fold higher than the levels of EFruA and EYqaB. The ratio of L-psicose to L-sorbose was 1.91, which was similar to the results shown in Table 2. Consequently, BGatY and EYqaB were chosen to further optimize the in vitro one-pot reaction.

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total ketoses was lower than that of L-sorbose because of L-psicose consumption (data not shown). After fermentation for 24 h, the strain WT(pXFTY) produced 1.30 g/liter L-sorbose and 1.23 g/liter L-psicose with a yield of 0.50 g/g L-glyceraldehyde (Fig. 3; also see Fig. S5 in the supplemental material). In addition, nearly 3 g/liter glycerol also was detected in the culture.

The results of one-pot reactions in vitro indicated that the catalytic efficiency of FruA and TagA was the major rate-limiting step in recombinant pathways (Fig. 1C). Thus, the expression level of \( fbaA \), \( gatY \), and \( yqaB \) genes in the recombinant pathways was further optimized by changing the order of gene \( fbaA \) or \( gatY \) with that of \( yqaB \) (pXYTB and pXYTF) and by replacing the tac promoter of genes \( fbaA \) and \( gatY \) with trc (pXYRB and pXYRF). The obtained plasmids also were transferred into \( C. \) glutamicum. The enzyme activity of EFruA and BGatY slightly increased (Fig. 4). However, ketose formation decreased significantly compared to the level with the previous manner of gene expression (pXRTY and pXFTY). This phenomenon probably occurred because of a significant decrease (from 13.47 ± 1.21 U/mg to 4.28 ± 0.67 U/mg) in the enzyme activity of EYqaB. Consequently, the recombinant pathway based on EFruA and EYqaB, which was constructed in plasmid pXFTY, showed the best performance for L-psicose and L-sorbose production in this study.

**Influence of gene tpi deletion upon DHAP accumulation and L-sorbose and L-psicose formation.** Generally, compound DHAP scarcely accumulated in vivo because of triose phosphate isomerase (TPI), which catalyzed isomerization of DHAP to glyceraldehyde 3-phosphate (Gap-3P), which can be further metabolized to pyruvate, permitting the generation of ATP and NADH. We have described that gene tpi mutation helped to accumulate intracellular DHAP and increase rare ketose synthetic ability in \( C. \) glutamicum (26). Therefore, the recombinant pathway was introduced further into strain SY6, in which gene tpi was deleted, to obtain SY6(pXFTY). The effect of gene tpi mutation on cell growth, glucose and \( \beta \)-glyceraldehyde consumption, L-psicose/L-sorbose formation, and DHAP accumulation was tested. The dual-phase fer-
mentation process conditions of SY6(pXFTY) were similar to those for WT(pXFTY).

Although the glucose and l-glyceraldehyde consumption rates of strain SY6(pXFTY) were slightly lower than that for WT(pXFTY), the average productivity of strain SY6(pXFTY) after the initial 8 h was 27% higher than that of its control (Fig. 5). The L-psicose/L-sorbose yield of strain SY6(pXFTY) further increased to 0.62 g/g l-glyceraldehyde. The cell growth of strain SY6(pXFTY) decreased 42% compared to that of WT(pXFTY), which probably was due to only half of the glucose flux through glycolysis (Fig. 2). We also discovered that the dihydroxyacetone concentration of strain SY6(pXFTY) during the dual-phase fermentation process was nearly 10-fold higher than that of WT (pXFTY). Dihydroxyacetone in the culture was dephosphorylated from DHAP through phosphatase in vivo (27). Thus, this result to some extent indicated that the intracellular DHAP formation ability of strain SY6(pXFTY) was greater than that of WT(pXFTY). The dihydroxyacetone concentration of SY6(pXFTY) increased to the maximal level (74 mM) when glucose was exhausted, which indicated that a nearly 67.2% carbon flux from glucose was directed to DHAP formation.

Effect of gene cgl0331 mutation on product formation. A large amount of glycerol was synthesized as a by-product in culture medium of all recombinant C. glutamicum strains when glucose and l-glyceraldehyde were used as substrates. When a single glucose was added to the culture, only a small amount of glycerol was detected. Thus, glycerol probably was produced from l-glyceraldehyde. In a search of the protein database BRENDA for candidate enzymes, we discovered that the glycerol dehydrogenase (EC 1.1.1.72), Zn-dependent alcohol dehydrogenase (EC 1.1.1.2), and aldehyde reductase (EC 1.1.1.21) could catalyze the conversion of l-glyceraldehyde to glycerol. However, no gene homologous to the gene encoding glycerol dehydrogenase and aldehyde reductase has been annotated in C. glutamicum except for Zn-dependent alcohol dehydrogenase, which was encoded by gene cgl0331.

In this study, the gene cgl0331 from C. glutamicum was cloned and overexpressed in E. coli BL21(DE3). After one-step purification with an N-terminal 6X His tag, SDS-PAGE of the obtained proteins showed the desired molecular mass of Cgl0331 (37.3 kDa) (see Fig. S6 in the supplemental material). The enzyme Cgl0331 also was characterized to test the catalytic efficiency of D/l-glyceraldehyde and dihydroxyacetone. We discovered that enzyme Cgl0331 indeed showed enzyme activity with those substrates with NADPH as a cofactor. Moreover, the catalytic efficiency of Cgl0331 with l-glyceraldehyde was nearly 2-fold and 8-fold higher than that with l-glyceraldehyde and dihydroxyacetone, respectively (Fig. 6).

Thus, the gene cgl0331 was deleted from C. glutamicum to obtain strain SY14, and the effect of this mutation on glycerol and product formation was demonstrated. The recombination pathway (pXFTY) based on EFruA and EYqaB then was introduced into strain SY14 to obtain SY14(pXFTY), and the latter was further fermented to produce L-sorbose/L-psicose from glucose and l-glyceraldehyde. The cell growth and glucose consumption of strain SY14(pXFTY) were similar to those of WT(pXFTY) (Fig. 7). Moreover, the l-glyceraldehyde consumption rate of SY14(pXFTY) after an initial 4 h decreased 50% compared with that of WT(pXFTY). The glycerol formation rate also significantly decreased. This result confirmed that the Zn-dependent alcohol...
dehydrogenase played an important role in conversion of L-glyceraldehyde to glycerol. At the end of fermentation, strain SY14(pXFTY) accumulated 1.79 g/liter L-sorbose and 1.56 g/liter L-psicose with a yield of 0.67 g/g L-glyceraldehyde, which increased 34% compared with that of strain WT(pXFTY) (Fig. 7). However, nearly 1 g/liter glycerol still was synthesized as a by-product in the culture.

Fed-batch culture of strain SY14(pXFTY). The performance of strain SY14(pXFTY) was further characterized in a fed-batch culture mode. The initial glucose, L-glyceraldehyde, and cell OD600 were controlled at 20 g/liter, 5 g/liter, and 50, respectively. An additional 1 ml of glucose (500 g/liter) and 200 μl of L-glyceraldehyde (500 g/liter) were added to the fermentation culture at 3 h and 8 h, respectively. After fermentation for 36 h, 3.5 g/liter L-sorbose and 2.3 g/liter L-psicose were produced with a yield of 0.61 g/g L-glyceraldehyde (Fig. 8). The initial productivity within 2 h was 1.23 g/liter · h. The glucose and L-glyceraldehyde consumption rate decreased significantly after fermentation for 10 h because of acid accumulation (pH 4.6). Therefore, adjustment of pH environment to the neutral level would further increase production of L-sorbose/L-psicose. However, strain SY14(pXFTY) still accumulated 1.5 g/liter glycerol as a by-product in culture.

DISCUSSION

DHAP-dependent aldolases create two new stereogenic centers at C atoms 3 and 4. In some cases, FucA and RhaD aldolases showed loose stereocontrol of configuration at C-4 when using some aldehydes. This property helped in synthesizing many kinds of rare ketoses (6, 7). In this study, we discovered that FruA from E. coli could synthesize not only the natural product L-sorbose (3S,4R) but also L-psicose (3S,4S) from DHAP and L-glyceraldehyde. Furthermore, the TagAs from both E. coli and B. licheniformis created a major (3S,4S) configuration product (L-psicose) instead of a (3S,4R) configuration (L-sorbose), which was different from previous discoveries. Therefore, all four types of DHAP-dependent aldolases showed loose product stereoselectivity when using some specific aldehydes and could synthesize multiple rare ketoses. For example, RhaD could synthesize D-sorbose and D-psicose simultaneously from D-glyceraldehyde, FucA generated L-tagatose and L-fructose together from L-glyceraldehyde (6, 7), and FruA and TagA produced L-sorbose and L-psicose from L-glyceraldehyde. Based on these discoveries, two conclusions were obtained. First, for one aldolase, the change of product stereoselectivity may be related to the configuration of the hydroxy group adjacent to the aldehyde group, such as D/L-glyceraldehyde. Second, for one substrate, this change may be determined by the aldolases that were used, such as FruA from E. coli and RAMA. The mechanism for this phenomenon should be explored in the future.

In this study, the one-pot reaction system containing aldolases and phosphatase was used to produce L-sorbose/L-psicose from DHAP and L-glyceraldehyde. However, the cost and instability property of DHAP limited the large-scale production of target products. We have presented a rare ketose biosynthesis strategy in which DHAP was accumulated from glucose through the glyco-
lytic pathway in *C. glutamicum* and a recombinant pathway based on RhaD aldolase, and YqaB was constructed to produce D-sorbose and D-psicose from glucose and D-glyceraldehyde (26). With this in mind, the recombinant pathways based on FruA or TagA aldolase and YqaB phosphatase were constructed in both *E. coli* BL21(DE3) and *C. glutamicum* strains to produce L-psicose and L-sorbose from glucose and L-glyceraldehyde. Both the recombinant *E. coli* and *C. glutamicum* strains showed the expected ability to produce L-sorbose/L-psicose; however, the synthetic efficiency of recombinant *E. coli* strains was extremely low compared with that of the corresponding *C. glutamicum* strains. Therefore, the efficiency of the recombinant pathway could be increased by changing the host cells. In summary, the recombinant *C. glutamicum* strains containing FruA or TagA aldolase and YqaB phosphatase could synthesize L-psicose from glucose and L-glyceraldehyde, which was produced from allitol by *Glucobacter frateurii* IFO 3254 previously (28).

We further introduced the recombinant pathway based on FruA and YqaB from *E. coli* into a *tpi* gene deletion strain. Although the inactivation of the *tpi* gene increased intracellular DHAP formation ability and the yield of L-psicose/L-sorbose, the final concentration of total ketoses still was lower than that of our previous study (26). We attribute this to the lower enzyme activity of FruA from *E. coli*. Therefore, further investigations to screen another type of FruA or TagA with higher activity are under way. Large amounts of dihydroxyacetone were produced from DHAP as a by-product during dual-phase fermentation processes not only in this study but also in our previous research (26). Thus, the phosphatase that catalyzes dephosphorylation of DHAP to produce dihydroxyacetone should be identified and deleted to further increase intracellular DHAP concentrations during dual-phase fermentation processes.

Although strain WT(pXFTY) showed the ability to produce L-sorbose/L-psicose, the yield still was low. Substrate L-glyceraldehyde in culture was depleted in three ways: (i) the equilibrium between L-glyceraldehyde and dihydroxyacetone via Lobry De Bruyn-Van Ekenstein transformation (29); (ii) the conversion of L-glyceraldehyde to glycerol by alcohol dehydrogenase; and (iii) the aldol reaction from DHAP and L-glyceraldehyde to synthesize L-sorbose/L-psicose. A large percentage of glycerol instead of target ketoses was produced, which indicated that the formation of glycerol from L-glyceraldehyde should be decreased. Fortunately, the Zn-dependent alcohol dehydrogenase in *C. glutamicum* encoded by gene cgl0331 was identified for the first time in the conversion of L-glyceraldehyde to glycerol. Although mutation of gene cgl0331 significantly decreased glycerol formation and increased target keto production, a small amount of glycerol still was detected as a by-product in culture. Therefore, other enzymes in *C. glutamicum*, which were capable of catalyzing L-glyceraldehyde to glycerol, should be explored and deleted to further increase the yield.

In summary, we discovered for the first time that both FruA and TagA lost their strict product stereoselectivity when using L-glyceraldehyde. This property has been applied further to synthesize L-sorbose/L-psicose simultaneously. We believe that this phenomenon also exists extensively in other aldolases. Thus, products with nonnatural configurations could be produced by changing the structure of the acceptor or screening other aldolases. Furthermore, the microbial strategy used to produce L-psicose in this study also could be applied to the large-scale synthesis of other L-sugars, such as L-fructose. Some research presented that substrate L-glyceraldehyde could be obtained from glycerol, which was more inexpensive and abundant (8, 30). Therefore, further investigations to produce L-sugars from low-cost resources, such as glucose and glycerol, would be significant.

**ACKNOWLEDGMENTS**

This work was supported by the National High Technology Research and Development Program of China (no. 2013AA102105), Science and Technology Projects of Tianjin (no. 13JCZDSY05600), and Chinese Academy of Sciences Visiting Professorship for Senior International Scientists (no. 2010T154).

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