JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b03423 • Publication Date (Web): 14 Nov 2016

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Pathway Construction in *Corynebacterium glutamicum* and Strain engineering to Produce Rare Sugars from Glycerol

Jiangang Yang[1], Yueming Zhu[1], Yan Men, Shangshang Sun, Yan Zeng, Ying Zhang, Yuanxia Sun*, Yanhe Ma

National Engineering Laboratory for Industrial Enzymes, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308,China.

1: These authors contributed equally to this work.

* Corresponding author： E-mail: syx0430@hotmail.com; Tel.: +86 22 84861960; fax: +86 22 84861961.

ABSTRACT

Rare sugars are valuable natural products widely used in pharmaceutical and food industries. In this study, we expected to synthesize rare ketoses from abundant glycerol using dihydroxyacetone phosphate (DHAP)-dependent aldolases. First, a new glycerol assimilation pathway was constructed to synthesize DHAP. The enzymes which convert glycerol to 3-hydroxypropionaldehyde and L-glyceraldehyde were selected and their corresponding aldehyde synthesis pathways were constructed in vivo. Four aldol pathways based on different aldolases and phosphorylase were gathered. Next, three pathways were assembled and the resulting strains synthesized 5-deoxypsicose, 5-deoxysorbose, and 5-deoxyfructose from glucose and glycerol and produce L-fructose, L-tagatose, L-sorbose, and L-psicose with glycerol as only carbon source. To achieve higher product titer and yield, the recombinant strains were further engineered and fermentation conditions were optimized. Fed-batch culture of engineered strains obtained 38.1 g/L 5-deoxypsicose with a yield of 0.91±0.04 mol product per mol glycerol and synthesized 20.8 g/L L-fructose, 10.3 g/L L-tagatose, 1.2 g/L L-sorbose, and 0.95 g/L L-psicose.

KEYWORDS: *rare sugars, aldolases, Corynebacterium glutamicum, metabolic engineering, glycerol*

INTRODUCTION

Rare sugars and their derivatives have been used as food additives or building blocks for drugs.[1-3] L-sugars, which are one large group of rare sugars, often hold enormous potential

for the applications in the pharmaceutical industry.[4,5] 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.[6,7] L-sorbose has been applied to produce the potent glycosidase inhibitor 1-deoxygalactonojirimycin and L-ascorbic acid, known as vitamin C.[8] L-fructose is known as a nonnutritive sweetener, an inhibitor of various glycosidases, and an insecticide for ants and house flies.[9,10] L-psicose can be used as a novel inhibitor of murine herpes simplex keratitis.[11] In nature, many plants or organisms have the ability to accumulate a slight amount of rare sugars.[12,13] However, low abundance of these compounds in nature restricts their potential applications.[14] Izumori et al. created an efficient "Izumoring" strategy to provide possible methods for the production of monosaccharides from cheap and widely available substrates.[15] Inspired by this strategy, many D/L-sugars, such as D-psicose and L-fructose, have been obtained using enzymatic transformations.[16,17]

DHAP-dependent aldolases show strict specificity to DHAP and catalyzed the aldol reaction between DHAP and a broad range of aldehydes to synthesize complex polyhydroxylated sugars.[18,19] In view of the high cost and instability of DHAP, the glycolytic pathway has been employed and engineered by deleting triose phosphate isomerase (TPI, EC 5.3.1.1) to increase accumulation of intracellular DHAP.[20] Several recombinant pathways based on aldolases and phosphorylases are constructed in *Escherichia coli* or *C. glutamicum* to produce rare sugars and their derivatives, such as D-sorbose, L-psicose, and D-fagomine, directly from glucose and aldehydes.[21,22] Nevertheless, the aldehydes used are unstable, which decreased the product yield.[23,24]

Glycerol is generated as a major byproduct in the biodiesel production process and has

been considered as an important carbon source for production of value-added bioproducts.[25-27] Glycerol not only can be converted to some C3 aldehydes such as L-glyceraldehyde and 3-hydroxypropionaldehyde catalyzed by alcohol dehydrogenases (ADHs) or glycerol dehydrogenases (GDHs) [28,29] but also be converted to DHAP through glycerol utilization pathway. Therefore, this substrate shows higher potential in microbial synthesis of rare ketoses.

In this study, we expected to produce rare ketoses from glycerol. The rare ketoses synthesis-related pathways would be divided into three modules, endogenous DHAP synthesis, heterologous aldehydes synthesis and aldol reaction pathways (Fig. 1). Systematic assembly and balance of such modules in *Corynebacterium glutamicum* successfully synthesize three kinds of 5-deoxysugars and four L-sugars. The optimal fermentation conditions were determined, and preferable rare ketoses yield and productivity were achieved by fed-batch culture of the engineered strains.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Materials. Nicotinamide adenine dinucleotide hydrogen (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), L-fructose, L-tagatose, L-sorbose, and L-psicose were purchased from Kagawa Rare Sugar Research Center (Takamatsu, Japan). All restriction enzymes and DNA ligase were purchased from Novagen (Darmstadt, Germany). Ni-NTA affinity chromatography column was purchased from QIAGEN (Hilden, Germany). Raw glycerol was taken from a concentrate coming directly from biodiesel production with the following composition: 80.5% (w/w) glycerol, 10.1% (w/w) water, 5.2% (w/w) sodium salts, 0.4% (w/w) potassium salts, 0.3% (w/w) other

89 salts, 0.5% (w/w) methanol etc.

90 All bacterial strains and plasmids were listed in Table 1. The *E. coli* strain DH5α was
91 used for plasmid construction. *C. glutamicum* ATCC13032 was used as the initial host strain.
92 Plasmid pET-21a(+) was used to express exogenous enzymes in *E. coli* BL21(DE3). Plasmids
93 pXMJ19 and pEC-XK99E were used to construct the recombinant pathways in *C.*
94 *glutamicum*.[30,31]

95 Table 1. Strains and plasmids used in this study

Strain and plasmid	Relevant genotype or phenotype	Reference
Strain		
E. coli DH5 α	*endA1 supE44 recA1 gyrA96 relA1 deoR U169 Φ 80dlacZ Δ M15 mcrAΔ(mrr–hsdRMS–mcrBC)*	Invitrogen
E. coli BL21(DE3)	*Omp Thsd ThsdS (rB^-mB^-) gal(DE3)*	Invitrogen
C. glutamicum	Wild-type strain	ATCC 13032
SY6	Gene *tpi* deletion in *C.glutamicum*	22
SY14	Gene *cgl0331*deletion in *C.glutamicum*	24
SY39	Gene *tpi* and *cgl0331* deletion in *C.glutamicum*	This study
Plasmids		
pET21a(+)	Expression vector, Ap^R	Invitrogen
pETSADH	pET21a(+) derivative carrying gene *ADH1* from *Saccharomyces cerevisiae*	This study
pETGGDH	pET21a(+) derivative carrying gene of GDH from *Gluconobacter oxydans*	This study
pETNGDH	pET21a(+) derivative carrying gene of GDH from *Neurospora crassa*	This study
pETHLADH	pET21a(+) derivative carrying gene of ADH from *horse liver*	This study
pK18-*tpi*	pK18mobsacB derivate to delete gene *tpi* in *C.glutamicum*	22
pXMJ19	Cm^R; *C.glutamicum/E.coli* shuttle vector (P*tac*, *lacI*^{q}; pBL1,$OriV_{C.g}$, $OriV_{E.c}$)	30
pEC-XK99E	Kan^R; *C.glutamicum/E.coli* shuttle vector (P*trc*, *lacI*^{q}; pGA1, $OriV_{C.g}$, $OriV_{E.c}$)	31
pXRTY	pXMJ19 derivate with genes *rhaD* and *yqaB* overexpression	22
pXFucATY	pXMJ19 derivate with genes *fucA* and *yqaB* overexpression	This study
pXFTY	pXMJ19 derivate with genes *fbaA* and *yqaB* overexpression	24
pXBTY	pXMJ19 derivate with genes *gatY* and *yqaB* overexpression	24
pEDhaBA	pEC-XK99E derivate with operons *dhaB123* and *gdrBA* from *Klebsiella pneumoniae* overexpression	This study
pEFKD	pEC-XK99E derivate with genes *glpF, glpK, glpD* overexpression	This study

pEFDK	pEC-XK99E derivate with genes *glpF, dhaD, dhaK* overexpression	This study
pEFTH	pEC-XK99E derivate with genes *glpF* and *hladh* overexpression	This study
pEFDKTH1	pEFDK derivate, overexpression of gene *hladh* under additional *tac* promoter in anti-clockwise direction	This study
pEFDKTH3	pEFDK derivate, overexpression of gene *hladh* under additional *tac* promoter in clockwise direction	This study

Expression and Purification of His-tagged Enzymes. The genes of glycerol dehydrogenase (GDH) from *Gluconobacter oxydans* and *Neurospora crassa*, and ADH from *horse liver* were codon-optimized (http://www.jcat.de), synthesized by GeneScript (Nanjing, China) and ligated to plasmid pET-21a(+) at *Nde*I and *Hind*III sites to obtain pETGGDH, pETNGDH, and pETHLADH, respectively. The gene *ADH2* from *Saccharomyces cerevisiae* was cloned with SADH2-1/2 primer set and ligated into pET-21a(+) at *Nde*I and *Xho*I sites to obtain pETSADH.

E. coli BL21(DE3) strains harboring the expression plasmids were cultured at 37 ℃ in 1 L LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride) to an optical density OD_{600} of 0.6. 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture to induce protein expression and the temperature was adjusted to 16 ℃ 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 cells were then lysed by sonication and centrifuged at 14,000×*g* and 4 ℃ for 10 min. Clear supernatant was collected and loaded onto a Ni^{2+}-NTA-agarose column pre-equilibrated 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 using normal

saline, freeze dried using vacuum pump and stored at -20 °C. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.[32]

Enzyme Activity Analysis. Enzyme activity of ADHs and GDHs were determined in an assay volume of 0.2 ml containing TEA buffer (50 mM, pH 7.0), substrate (10 mM), $NADP^+/NAD^+$ (1 mM), and freeze dried enzyme (0.5 mg/L). The NADPH/NADH formation was measured by the increase in absorbance at 340 nm using a molar absorption coefficient of 6.22 mM^{-1} cm^{-1}. The enzymatic activity is measured as the amount of enzyme that catalyzes the formation of NADH/NADPH per min.

Recombinant Pathways Construction and Assembly. To construct plasmid pXEFucATY, the primers FucTY-1/2, FucTY-3/4, and FucTY-5/6 were used to amplify gene *fucA* from genome of *E. coli*, *tac* promoter from plasmid pXMJ19, and *yqaB* gene from *E. coli* DH5α, respectively. Three resulting fragments were fused using primers FucTY-7/8 to form the "RBS-*fucA*-*tac*-RBS-*yqaB*" fragment; the latter was further digested with *Pst*I and *Xba*I and ligated into pXMJ19 to obtain pXEFucATY.

For plasmid pEFKD construction, the operon *glpFK* from *E. coli* was firstly cloned using primer sets EFKD-1(*Sac*I)/EFKD-2(*Xba*I) and ligated into plasmid pEC-XK99E at *Sac*I and *Xba*I sites to obtain pEFK. Then, the gene *glpD* from *E. coli* was amplified using primer set EFKD-3(*Xba*I)/EFKD-4(*Pst*I) and ligated to plasmid pEFK to form pEFKD.

To construct plasmid pEFDK, gene *glpF* was fistly amplified with *E. coli* genome as template using primers EFDK-1(*Sac*I)/EFDK-2(*Sma*I) and ligated into the *Sac*I and *Sma*I sites of pEC-XK99E to form pEF. Then, the genes *dhaD* from *Klebsiella pneumonia* and *dhaK* from *Citrobacter freundii* were amplified using primer sets

137 EFDK-3(*Sma*I)/EFDK-4(*Afl*II and *Xba*I) and EFDK-5(*Afl*II) /EFDK-6(*Xba*I), respectively.
138 The resulting fragments of *dhaD* was inserted into plasmids pEF at *Sma*I and *Xba*I sites, and
139 then the *dhaK* was placed into *Afl*II and *Xba*I sites of the resulting pEFD to form pEFDK.

140 The genes of glycerol dehydratase (DhaB) and its reactivase (GdrAB) from *Klebsiella*
141 *pneumonia* were codon-optimized (http://www.jcat.de) and synthesized by GeneScript
142 (Nanjing, China). For plasmid pEFDhaB construction, the operon *dhaB123* was amplified
143 from pUC57-*dhaB123* (synthesized by GeneScript) using primers
144 EFDhaB-1(*Xba*I)/EFDhaB-2(*Xba*I) and inserted into pEF as mentioned above at *Xba*I site to
145 obtain pEFDhaB. Then, *tac* promoter and operon *gdrAB* were cloned from plasmid pXMJ19
146 and pUC57-*gdrAB* (synthesized by GeneScript) using primers EFDhaB-3/4 and EFDhaB-5/6,
147 respectively. The resulting fragments were fused using EFDhaB-3(*Pst*I)/EFDhaB-6(*Pst*I) to
148 form "*tac*-RBS-*gdrAB*" fragment; the latter was further ligated into pEFDhaB at *Pst*I site to
149 obtain pEFDhaBA.

150 To construct plasmid pEFTH, primer sets EFTH-1/2 and EFTH-3/4 were used to amplify
151 *tac* promoter from plasmid pXMJ19 and gene *HLADH* from pUC57-HLADH (synthesized by
152 GeneScript), respectively. Two resulting fragments were fused using primers EFTH-5/4 to
153 form the "*tac*-RBS-*HLADH*" fragment; the latter was further digested with *Sma*I and *Xba*I
154 and ligated into pEF to obtain pEFTH. Plasmids pEFDKTH were constructed by amplifying
155 fragment "*tac*-RBS-*HLADH*" from plasmid pEFTH using primers EFDKTH-1/2 and ligating
156 the resulting product into the *Xba*I site of plasmid pEFDK as mentioned above.

157 **Strain SY39 Construction.** The in-frame deletion of *tpi* gene in strain SY39 was
158 accomplished through a two-step homologous recombination procedure using the pK18-*tpi*

159 suicide vector.[22] The plasmid pK18-*tpi* was transferred into strain SY14 by electroporation.
160 Selection for the first and second recombination events and verification of positive clones
161 were performed as previously described.[33]

162 **"Dual-phase" Fermentation to Produce Rare Sugars.** *C. glutamicum* strains were
163 cultivated in brain heart infusion (BHI) medium or CGXII medium[34] supplemented with 10
164 g/L glucose. For precultivation of *C. glutamicum* ATCC 13032 and its recombinant
165 derivatives, a single clone was grown in 5 mL of BHI medium. After incubation for
166 approximately 15 h, cells were inoculated into a 500-mL shake flask containing 100 mL BHI
167 medium and cultivated at 25 °C in a rotatory shaker at 220 rpm. When the cell OD_{600} reached
168 to 0.8, 1 mM IPTG was added to induce enzyme expression for 12 h. Subsequently, the cells
169 were harvested by centrifugation (8,000×*g*, 10 min, 4 °C) and suspended in CGXII medium.
170 Then 10 mL cells were transferred into a 50-mL shake flask containing glucose (110 mM)
171 and glycerol (110 mM) with an initial OD_{600} of approximately 30. When appropriate, 10
172 mg/L chloramphenicol and 25 mg/L kanamycin were added.

173 For the fed-batch mode, 50 mL cells were transferred to a 500-mL shake flask containing
174 glucose (20 g/L) and glycerol (20 g/L) with an initial OD_{600} of approximately 40. When
175 necessary, additional glucose and glycerol was supplemented. Samples were collected every
176 two hours and centrifuged at 14,000 g for 20 min. The resulting supernatants were analyzed
177 by HPLC.

178 **Quantitative Measurement of Biomass, Glucose, Glycerol, Rare ketoses.** Cell growth
179 was followed by measuring the optical density at 600 nm (OD_{600}) with a UV-Vis
180 spectrophotometer (TU-1901, Persee, Beijing, China). Cell dry weight (CDW, g/L) of *C.*

glutamicum was calculated from OD_{600} values using the experimentally determined correlation factor of 0.25 g cells (dry weight [DW])/liter for an OD_{600} of 1. For sample analysis, 0.5 ml of culture was centrifuged at 13,000×g for 5 min and the supernatant was then filtered through a 0.22-μm syringe filter for HPLC analysis. The HPLC system equipped with a refractive index detector and a cation exchange column (HPX-87H, Biorad Labs, Hercules, CA, USA). A mobile phase of 0.5 mM H_2SO_4 solution was used at a flow rate of 0.5 mL/min and the column was operated at 55 °C. Standards of glucose, glycerol, and L-sugars were used to create calibration curves. 5-Deoxysugars were collected by combining the automatic fraction collector with HPLC system and analyzed by NMR. The purified 5-deoxysugars were also used to create calibration curves.

RESULTS AND DISCUSSION

Construction of Glycerol Utilization Pathway. DHAP was not only the precursor of aldolases but also a metabolic intermediate of glycolysis. Glycerol can be converted into intracellular DHAP through glycerol utilization pathway (Fig. 1). Naturally, *C. glutamicum* 13032 cannot utilize glycerol as carbon source. The expression of the *E. coli* glpFKD operon, which encoded glycerol facilitator (GlpF), glycerol kinase (GlpK), and glycerol-3-phosphate dehydrogenase (GlpD), allows the organism to grow on glycerol as sole carbon and energy source.[35] However, generated intermediate glycerol-3-phosphate in this pathway may inhibit cell growth[36]. In this study, we focus on another glycerol assimilation pathway which contained GlpF, glycerol dehydrogenase (DhaD) and ATP-dependent dihydroxyacetone kinase dihydroxyacetone kinase (DhaK) (Fig.1). The DhaD from *K. pneumonia* and DhaK from *Citrobacter freundii* were chosen for their higher enzyme activity[37,38] and employed to

construct the pathway (pEFDK) in *C. glutamicum*. To compare the glycerol consumption rate of those two pathways, the *glpFKD* operon from *E. coli* (pEFKD) was also introduced into *C. glutamicum.* The cell growth and glycerol consumption rates of resulting strain WT(pEFDK) in CGXII or BHI medium increased between 0.7- and 3.0-fold relative to strain WT(pEFKD) (Table 2, Fig. 2). The final cell mass of the strain WT(pEFDK) was also 3-fold higher than that of the strain WT(pEFKD). These results demonstrated the efficiency of the new glycerol assimilation pathway and indicated the application potential in the synthesis of other high-value chemicals from glycerol.

Table 2 Cell growth and glycerol assumption of two glycerol assimilation pathways in CGXII and BHI medium. [a] Growth rate and consumption rate were calculated for the exponential growth phase. [b] The final cell mass was calculated from OD_{600} value at the end of culture. Cell dry weight (CDW, g/L) of *C. glutamicum* was calculated from OD_{600} values using the experimentally determined correlation factor of 0.25 g cells (dry weight [DW])/liter for an OD_{600} of 1. Mean and standard deviation were calculated based on triplicate experiments.

		WT(pEFKD)	WT(pEFDK)
CGXII	Growth rate (h^{-1}) [a]	0.09±0.01	0.15±0.03
	Consumption rate (g/L·h) [a]	0.31±0.02	0.54±0.02
	Final cell mass (g CDW/L) [b]	1.45±0.07	4.82±0.18
BHI	Growth rate (h^{-1})	0.11±0.01	0.19±0.02
	Consumption rate (g/L·h) [a]	0.33±0.02	0.96±0.04
	Final cell mass (g CDW/L) [b]	3.91±0.12	8.54±0.19

To testify the feasibility of the new constructed glycerol utilization pathway in DHAP formation and rare ketoses production, the plasmid pEFDK was transferred into the strain

222 WT(pXRTY). When the resulting strain WT(pXRTY/pEFDK) was cultured with glycerol and
223 L-glyceraldehyde as substrates, 4.3 g/L L-fructose with a yield of 0.32±0.03 mol L-fructose
224 per mol L-glyceraldehyde was obtained (Scheme S1 and Fig. S1). This L-fructose titer was
225 even identical with that of strain WT(pXRTY) which was cultured with glucose and
226 L-glyceraldehyde as substrates. This result indirectly confirmed the feasibility of the new
227 constructed glycerol utilization pathway in DHAP formation. However, the L-fructose
228 production rates of strain WT(pXRTY/pEFDK) was lower than that of strain WT(pXRTY). It
229 was probably due to the lower DHAP formation rates of glycerol consumption pathway. Thus,
230 we can use both glycolytic pathway and glycerol utilization pathway, which exhibited
231 different synthesis rate, to accumulate intracellular DHAP.

232 **Construction of Aldehydes Synthesis and Aldol Reaction Pathway.** To obtain acceptor
233 aldehydes in vivo, the enzymes with higher catalytic efficiency of conversion of glycerol to
234 aldehydes should be selected. Here we screened some ADHs from horse liver and
235 *Saccharomyces cerevisiae* and GDHs from *Gluconobacter oxydans* and *Neurospora crassa*
236 and tested their catalytic efficiency to glycerol.[39,40] The enzyme activities of the purified
237 ADHs and GDHs to glycerol were tested with NAD^+ or $NADP^+$ as cofactor. The ADH from
238 *horse liver* (HLADH) showed the higher activity to glycerol than other ADHs and GDHs
239 (Table 3). The glycerol dehydratase (DhaB) from *K. pneumonia* was also selected for its high
240 oxidization of glycerol to 3-hydroxypropionaldehyde.[41] Therefore, we chosen HLADH or
241 DhaB and constructed L-glyceraldehyde synthesis pathway (pEFTH) based on glycerol
242 facilitator (GlpF) from *E. coli* and HLADH and 3-hydroxypropionaldehyde synthesis
243 pathway (pEFDhaBA) based on GlpF from *E. coli*, DhaB and glycerol dehydratase reactivase

(GdrAB) from *K. pneumonia*.

In the previous studies, we have already constructed three aldol pathways using aldolases (rhamnulose 1-phosphate aldolase (RhaD), fructose 1,6-diphosphate aldolase (FruA), or tagatose 1,6-diphosphate aldolase (TagA)) and fructose-1-phosphatase (YqaB) and synthesized many C4-C7 rare ketoses in *C. glutamicum*.[14, 16] Here, the aldol pathway based on fuculose 1-phosphate aldolase (FucA) and YqaB from *E. coli* was further constructed and introduced into *C. glutamicum.*

Table 3 Enzyme activity data of ADHs and GDHs from different organisms.[a] NCRGDH: GDH from *Neurospora crassa*; GGDH: GDH from *Gluconobacter oxydans*; SADH: ADH from *Saccharomyces cerevisiae*; HLADH: ADH from *horse liver.* [b] The enzyme activity was calculated from the ratio of cofactor conversion value to enzyme amount used per minute. Mean and standard deviation were calculated based on triplicate experiments.

Enzyme [a]	Substrates	Cofactor	Products	Enzyme activity (nmol/mg·min) [b]
NCRGDH	Glycerol	NADP+	D-glyceraldehyde	9.60±1.24
GGDH	Glycerol	NADP+	D-glyceraldehyde	5.37±1.41
SADH	Glycerol	NAD+	Glyceraldehyde	ND
HLADH	Glycerol	NAD+	L-glyceraldehyde	35.10±2.53

Modules Assembly to Produce 5-deoxysugars from Glucose and Glycerol. Deoxysugars exhibit various interesting biological activities and are used as precursors for valuable antiviral agents, such as 2-deoxy-L-ribose and 2-deoxyglucose.[42] A combination of chemical and enzymatic transformation methods allows synthesizing 5-deoxyhexoses; however, such strategy suffers from unavailable substrates.[43] In this work, we presented a

new pathway to biosynthesize 5-deoxyhexoses from glucose and glycerol (Fig.1 and Scheme S2). The glycolytic pathway to produce DHAP, 3-hydroxypropionaldehyde synthesis pathway relying on DhaB, and four aldol reaction pathways (RhaD, FucA, FruA, TagA) were assembled in *C. glutamicum*, the resulting strains, WT(pXRTY/pEFDhaBA), WT(pXFucATY/pEFDhaBA), WT(pXFTY/pEFDhaBA), and WT(pXBTY/pEFDhaBA), were fermented with glucose and glycerol as substrates. New products were purified and structurally analyzed with NMR (Fig. 3A, S3, and S4). Strain WT(pXRTY/pEFDhaBA) simultaneously synthesized 5-deoxysorbose (3R,4S) and 5-deoxypsicose (3R,4R), and the content ratio of 5-deoxysorbose to 5-deoxypsicose was 1.8. Directed evolution based on saturation mutagenesis at sites lining the binding pocket could be applied to enhance the stereoselectivity of RhaD. Other strains WT(pXFucATY/pEFDhaBA) and WT(pXFTY/pEFDhaBA) synthesized 5-deoxypsicose (3R,4R) and 5-deoxyfructose (3S, 4R), respectively. The product configuration of the strain WT(pXBTY/pEFDhaBA) was identical to that of WT(pXFTY/pEFDhaBA). It was also discovered that glucose consumption rates of strain WT(pXFucATY/pEFDhaBA) and WT(pXRTY/pEFDhaBA) were 10% higher than that of strain WT(pXRTY/pEC) (Fig. 3B). However, the cell growth of those two strains decreased 16% and 20% relative to the control.

Effect of Gene *tpi* and *cgl0331* deletion on 5-deoxysugars Production. The gene *tpi* deletion resulted in a 20-fold increase in DHAP accumulation and 24% increase in D-sorbose/psicose production.[22] Here, to test the effect of this gene elimination on 5-deoxyhexulose production, we assembled the 5-deoxyhexulose synthesis modules in strain SY6. As expected, the 5-deoxysorbose and 5-deoxypsicose yields of strain

SY6(pXRTY/pEFDhaBA) improved by 18% to 0.92 mol product per mol glycerol (Fig. 3C).

To suppress cells consumption of obtained aldehydes, the related endogenous enzymes should be identified and inactivated. One Zn-dependent ADH (Cgl0331) which converted L-glyceraldehyde to glycerol has been confirmed and deleted to improve L-sorbose/L-psicose yield.[24] Then, the effect of gene *cgl0331* deletion on 5-deoxyhexuloses production was further studied. Inconsistent with our prediction, gene *cgl0331* deletion in strain SY14(pXRTY/pEFDhaBA) decreased the final 5-deoxyhexuloses titer and led to nearly 3-fold increase in 1,3-propanediol accumulation compared with that of the control (Fig. 3C). This result may be related to metabolic regulation, the clear mechanism remains unknown.

Subsequently, we further constructed the strains SY6(pXFucATY/pEFDhaBA) and SY6(pXFTY/pEFDhaBA) and cultured them in a fed-batch mode. The 5-deoxyhexuloses content of all strains rapidly increased within the initial fermentation stage, after that it nearly stagnated (Fig. 3D, 3E, and 3F). It was probably due to the cell toxicity or DhaB inhibition caused by the high concentration of intracellular 3-hydroxypropionaldehyde.[44] At the end of the fermentation, the strain SY6(pXRTY/pEFDhaBA) produced 14.5 g/L 5-deoxysorbose and 8.0 g/L 5-deoxypsicose with a yield of 0.85±0.03 mol product per mol glycerol (overall C-mol yield of 0.39±0.02 mol product per mol glycerol and glucose), and strain SY6(pXFucATY/pEFDhaBA) synthesized 38.1 g/L 5-deoxypsicose with a yield of 0.91 ±0.04 5-deoxypsicose per mol glycerol (overall C-mol yield of 0.43±0.02 mol 5-deoxypsicose per mol glycerol and glucose). The strain SY6(pXFTY/pEFDhaBA) produced a lower concentration of 5-deoxyfructose (10.8 g/L). This result was probably due to low enzyme activity of FruA which has been measured in our previous study.[16] Selection

of FruA with higher enzyme activity would increase 5-deoxyfructose production. A certain amount of byproducts such as DHA (4.1 g/L), 1,3-propanediol (1.8 g/L), lactate (1.6 g/L), and acetate (0.6 g/L) were also detected in the culture. HPLC analysis data showed that DHA was shown in strain SY6 but hardly in *C. glutamicum* wide-type. This result indicated that DHA was come from dephosphorylation of DHAP. Previous studies have confirmed that a HAD superfamily phosphatase (HdpA) may play an important role in the dephosphorylation of DHAP.[45] Byproduct 1,3-propanediol was probably derived from 3-hydroxypropionaldehyde conversion catalyzed by endogenous ADHs in *C. glutamicum*. Therefore, we believe that deletion of genes of byproducts formation pathway will help to increase intracellular DHAP and 3-hydroxypropionaldehyde contents and further increase 5-deoxysugars production.

Modules Assembly to Produce L-sugars from Glycerol as Sole Carbon Source. Given the importance of L-sugars, the development of their synthesis has been the subject of considerable recent interest. Here, we assembled L-glyceraldehyde synthesis pathway based on the obtained HLADH and GlpF, glycolytic pathway and one rare ketose formation pathway in *C. glutamicum* (Fig. 1). The resulting strain WT(pXRTY/pEFTH) could synthesize L-fructose with glucose and glycerol as substrates, but the L-fructose titer (0.83 g/L) was very low compared with that of 5-deoxysorbose and 5-deoxypsicose (Fig. 4). Cell metabolized glucose with high consumption rate through glycolytic pathway,[22] which indicated intracellular DHAP synthesis rate was concomitantly high. However, L-glyceraldehyde synthesis rate was low for lower HLADH enzyme activity (Table 3). The flux gap was formed between precursors DHAP and L-glyceraldehyde synthesis. To solve

this problem, some ADHs with higher conversion efficiency of glycerol to L-glyceraldehyde should be selected or the DHAP synthesis rate should be reduced. We have speculated that DHAP synthesis rate through glycerol assimilation pathway was lower than glycolytic pathway (Figure S1). In addition, glycerol could not only be converted into DHAP but could also be to L-glyceraldehyde. L-fructose could be then synthesized from glycerol as only carbon source. Therefore, we combined glycerol assimilation pathway (pEFDK), L-glyceraldehyde synthesis pathway, and one aldol pathway (pXRTY) in *C. glutamicum* (Fig. 1 and Scheme S3). Plasmids pEFDKTH1 and pEFDKTH3 were constructed, in which the direction of operon "*tac-HLADH*" was opposite (Fig. S5). Fig. 4 showed that the new combinatorial strains WT(pXRTY/pEFDKTH1) and WT(pXRTY/pEFDKTH3) led to higher titer of L-fructose than that of strain WT(pXRTY/pEFTH). Strain WT(pXRTY/pEFDKTH1) achieved 3.0 g/L L-fructose which was 62% higher than that of WT(pXRTY/pEFDKTH3).

To investigate the effect of the gene *cgl0331* deletion on L-fructose production, we introduced L-fructose synthesis modules into strain SY14. The L-fructose titer of the obtained strain SY14(pXRTY/pEFDKTH1) improved by 75% to 5.28 g/L relative to the control. Further deletion of the gene *tpi* in the strain SY39(pXRTY/pEFDKTH1) resulted in a 2.5-fold increase in L-fructose yield, which reached to 92% of the theoretical value (0.5 mol L-fructose per mol glycerol). However, the final L-fructose concentration decreased by 40% compared with that of SY14(pXRTY/pEFDKTH1) (Fig. 4). Thus, the preferable module combination for L-fructose production was in strain SY14(pXRTY/pEFDKTH1).

Fermentation Conditions Optimization to Increase L-sugars Titer and Yield. The fermentation conditions were further optimized by adjusting the initial cell density and

352 glycerol concentration. L-fructose production improved 2-fold when the cell density (OD_{600})
353 increased from 20 to 40 but decreased when it further increased from 40 to 80 (Fig. 4). The
354 final titer and yield of L-fructose increased by 2.7- and 3.1-fold to attain 14.64 g/L and
355 0.32±0.02 mol L-fructose per mol glycerol, respectively, along with glycerol concentration
356 increased from 20 to 80 g/L. Further investigations showed the catalytic activity of HLADH
357 to glycerol was not in a constant rate (data no shown). It decreased when reaction time was
358 prolonged; however, HLADH was not inactivated. Therefore, we speculated that HLADH
359 was inhibited by its generated cofactor NADH and equilibrium was reached between glycerol
360 and L-glyceraldehyde. A high glycerol concentration will help producing more
361 L-glyceraldehyde. HLADH should be mutated to decrease this inhibition. After fermentation
362 for 72 h, cell autolysis appeared when the initial glycerol concentration was 80 g/L. Thus, the
363 initial cell density (OD_{600}) and glycerol concentration were controlled at 40 and 60-80 g/L,
364 respectively. Microbial synthesis of L-fructose was further carried out using raw glycerol
365 coming directly from biodiesel production. Cell density (OD_{600}) and raw glycerol
366 concentration was controlled at 40 and 60 g/L, respectively. Final concentration of L-fructose
367 was 22% lower than that obtained with pure glycerol (Fig. 4).

368 Subsequently, to synthesize other types of L-hexuloses, we employed other two aldol
369 pathways and constructed the strains SY14(pXFucATY/pEFDKTH1) and
370 SY14(pXFTY/pEFDKTH1) (Scheme S3). Three recombinant strains were cultured in a
371 fed-batch mode. When necessary, additional glycerol was supplemented to maintain the
372 glycerol concentration above 40 g/L. L-fructose productivity of strain
373 SY14(pXRTY/pEFDKTH1) maintained at a constant value of 0.20 g/L·h, and 20.8 g/L

L-fructose with a yield of 0.21±0.03 mol L-fructose per mol glycerol was obtained after fermentation for 96 h. Eventually, the strain SY14(pXFucATY/pEFDKTH1) synthesized 10.3 g/L L-tagatose with a yield of 0.12±0.02 mol L-tagatose per mol glycerol, and SY14(pXFTY/pEFDKTH1) produced 1.2 g/L L-sorbose and 0.95 g/L L-psicose (Fig. 5). In addition, the cell growth and glycerol consumption rates decreased slightly during the entire fermentation process. These results indicated the application potential in large-scale production of L-hexuloses.

In summary, we synthesized three gram-scale 5-deoxysugars and four gram-scale L-sugars from glycerol. The selection of enzymes, such as ADHs and aldolases, with higher enzyme activity and balance of carbon flux between three modules through adjusting gene expression level would further increase the rare hexuloses production. The strategy used herein also opens the way for construction of cell factories for rare sugars production from other alcohols.

Supporting Information

The primers (Table S1), some experimental results (Fig. S1, S2, S5), HPLC and NMR analysis (Fig. S3, S5), and three rare ketoses synthesis scheme (Scheme S1, S2, S3)

AUTHOR INFORMATION

Corresponding Authors

Tel.: +86-22-84861960. E-mail: syx0430@hotmail.com

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National High Technology Research and Development Program of China (No. 2014AA022108) and Natural Science Foundation of China (No. 31571793).

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Fig. 1 The microbial strategy to synthesize rare ketoses from glycerol

Fig. 2 Cell growth and glycerol consumption of two glycerol assimilation pathway in CGXII and BHI medium.: Glycerol consumption (**A**) and cell growth (**B**) of strains WT(pEC)(■), WT(pEFKD)(●) and WT(pEFDK)(▲) in CGXII medium; Glycerol consumption (**C**) and (**D**) of strains WT(pEC)(■), WT(pEFKD)(●) and WT(pEFDK)(▲) in BHI medium. Mean and standard deviation were calculated based on triplicate experiments.

Fig. 3 Biosynthesis of 5-deoxysugars. (A): HPLC analysis of the supernatant of strains WT(pXRTY/pEC), WT(pXRTY/pEFDhaBA), WT(pXFucATY/pEFDhaBA), WT(pXFTY/pEFDhaBA). (B): The cell growth and glucose consumption of recombinant strains mentioned above. (C): The effect of gene *tpi* or *cgl0331* deletion on product formation; Cell growth (■), glucose (●) and glycerol (▲) consumption, and product formation (○) of strains SY6(pXRTY/pEFDhaBA) (**D**), SY6(pXFucATY/pEFDhaBA) (**E**), and SY6(pXFTY/pEFDhaBA) (**F**) in a fed-batch culture mode. Mean and standard deviation were calculated based on triplicate experiments. Gene *tpi* was deleted in SY6; Plasmid pEC means blank plasmid without any exogenous genes; Plasmid pXRTY contained genes of aldolase RhaD and phosphorylase YqaB; pXFucATY contained genes of aldolase FucA and YqaB; pXFTY contained genes of aldolase FruA and YqaB. pEFDhaBA contained genes of glycerol facilitator GlpF, glycerol dehydratase (DhaB123) and glycerol dehydratase reactivase (GdrAB).

551 Fig. 4 Production of L-fructose using different recombinant strains or fermentation
552 conditions. Mean and standard deviation were calculated based on triplicate experiments.
553
554 Fig. 5 Fed-batch culture of recombinant strains to produce L-hexuloses. (**A**): Cell growth
555 (■), glycerol consumption (▲), and L-fructose formation (●) of strain
556 SY14(pXRTY/pEFDKTH1); (**B**): Cell growth (■), glycerol consumption(▲), and L-tagatose
557 formation (●) of strain SY14(pXFucATY/pEFDKTH1); (**C**): Cell growth (■), glycerol
558 consumption(▲), and L-sorbose (●) and L-psicose (★) of strain SY14(pXFTY/pEFDKTH1).
559 Gene *cgl0331* was deleted in strain SY14. Plasmid pEFDKTH1 contained genes of *glpF*,
560 *dhaD*, *dhaK*, additional *tac* promoter, and *HLADH*.
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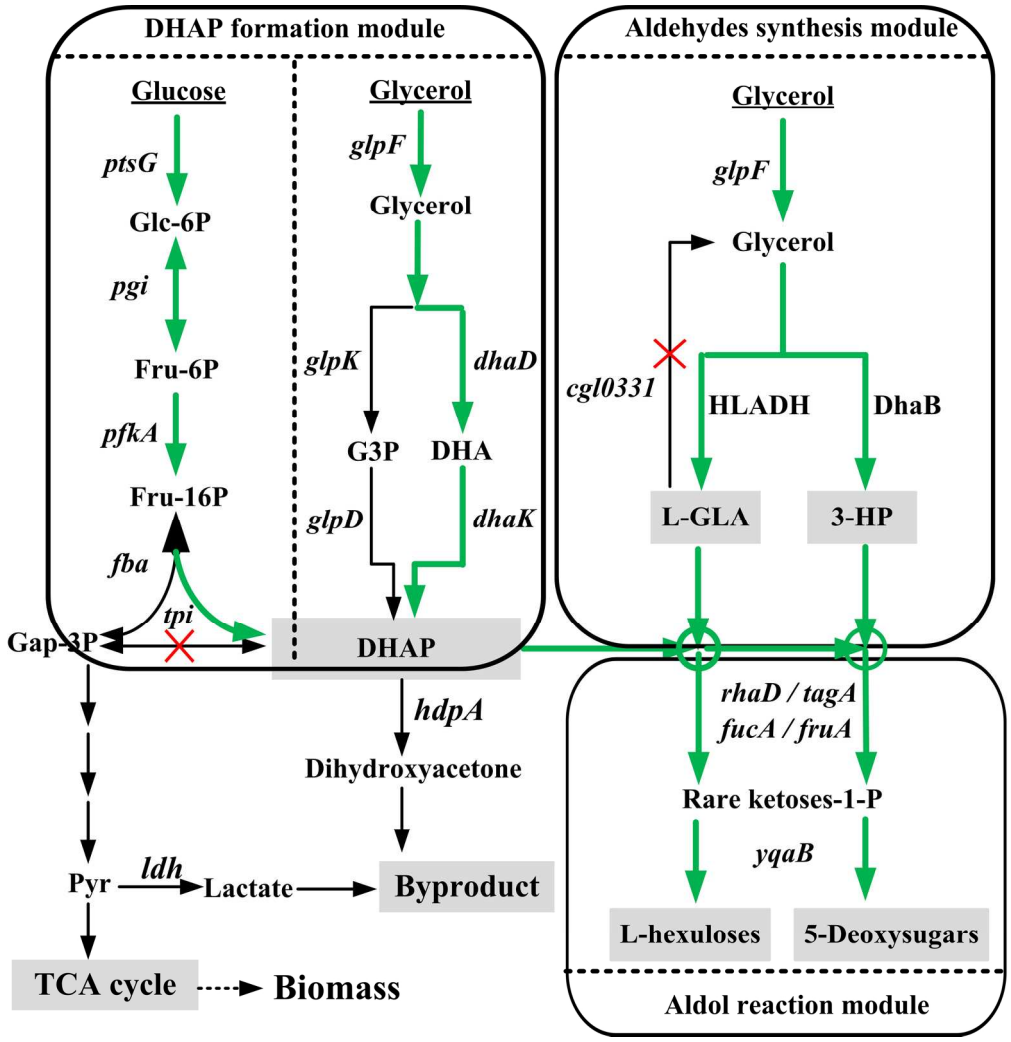

Fig. 1
Fig. 1
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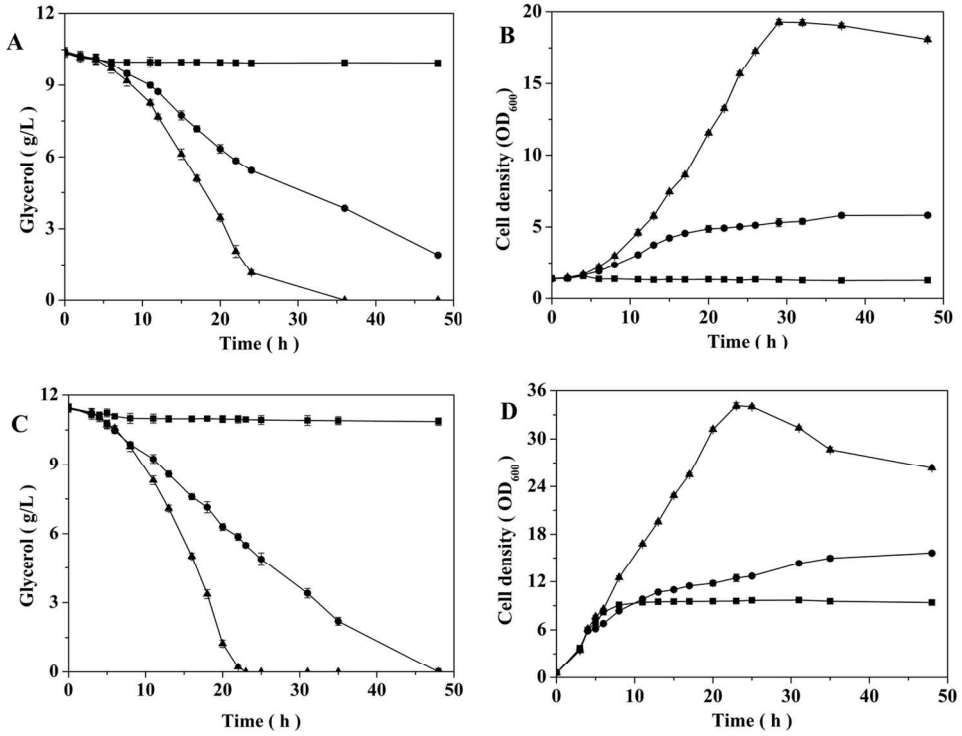

Fig. 2
Fig. 2
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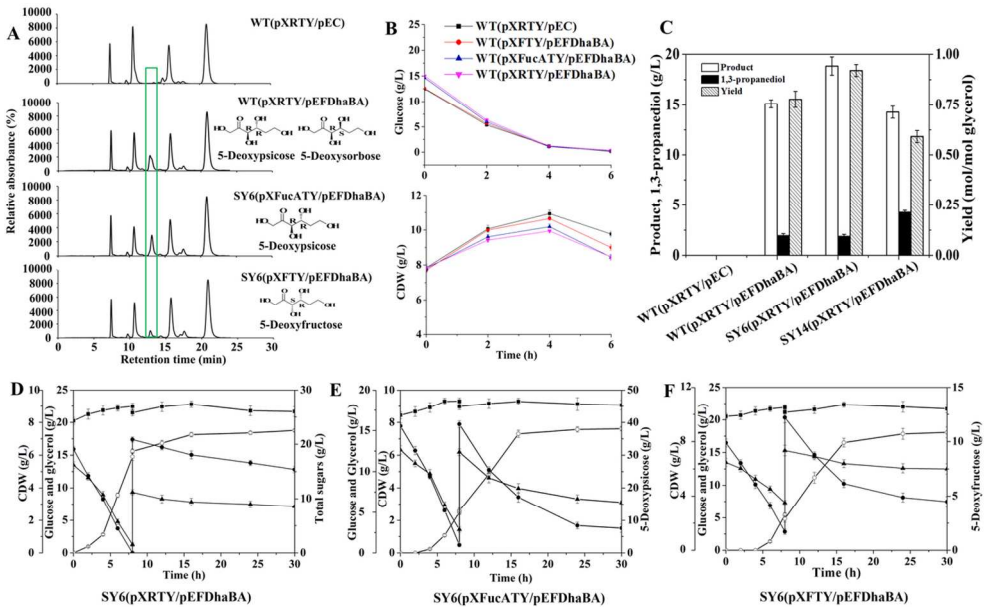

Fig.3
Fig.3
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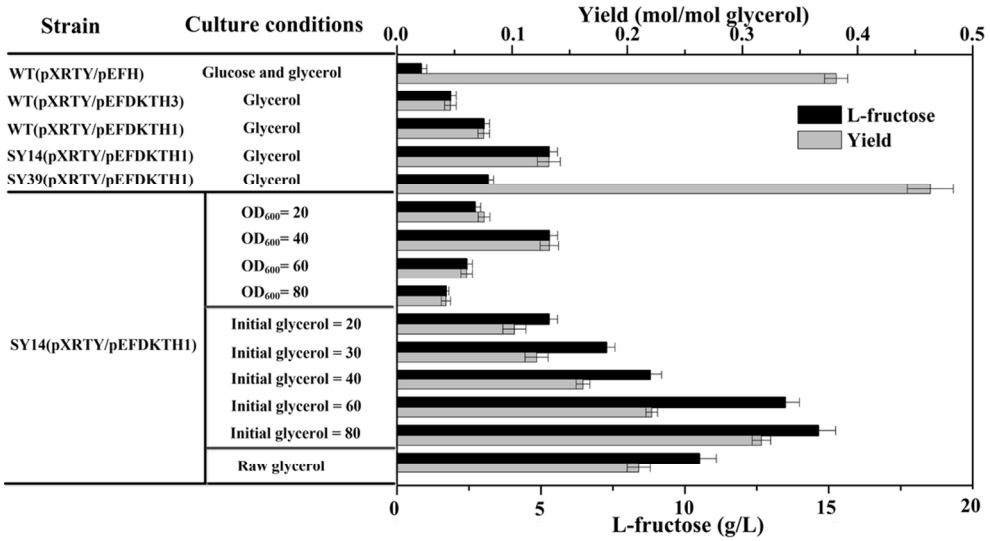

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Fig.4
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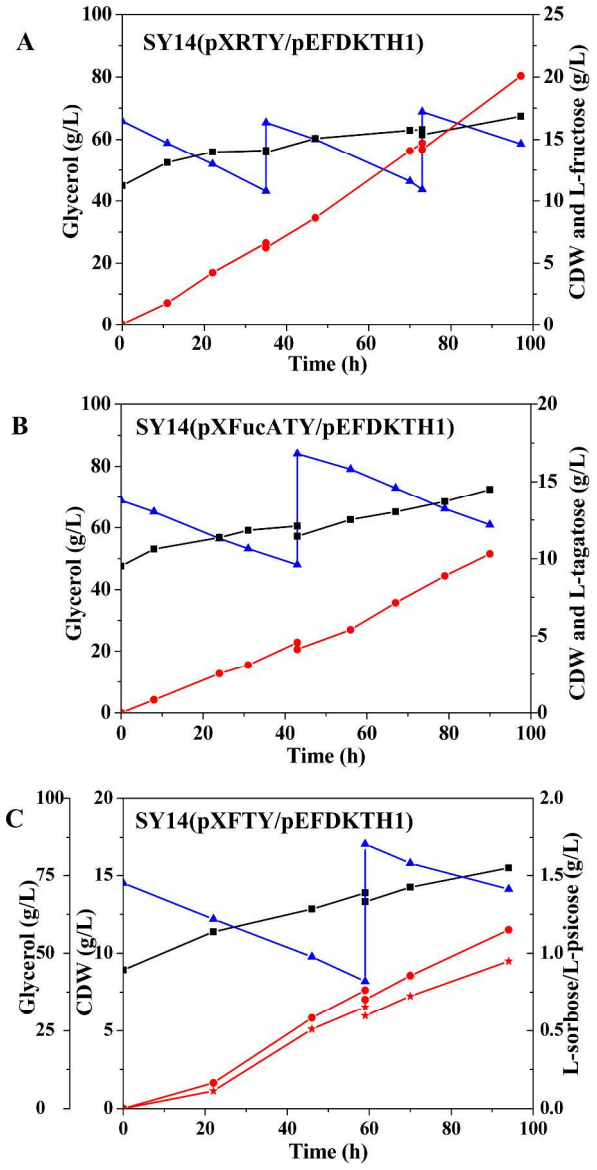

Fig.5.
Fig.5
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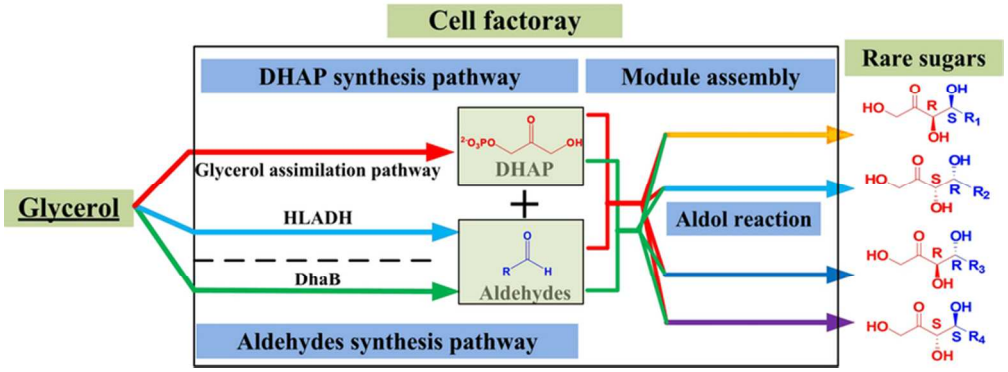

TOC grapfic
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