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Microbial synthesis of sedoheptulose from glucose by metabolically engineered *Corynebacterium glutamicum*

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Abstract

Background Seven-carbon sugars, which rarely exist in nature, are the key constitutional unit of septacidin and hygromycin B in bacteria. These sugars exhibit a potential therapeutic effect for hypoglycaemia and cancer and serve as building blocks for the synthesis of C-glycosides and novel antibiotics. However, chemical and enzymatic approaches for the synthesis of seven-carbon sugars have faced challenges, such as complex reaction steps, low overall yields and high-cost feedstock, limiting their industrial-scale production.

Results In this work, we propose a strain engineering approach for synthesising sedoheptulose using glucose as sole feedstock. The gene *pfkA* encoding 6-phosphofructokinase in *Corynebacterium glutamicum* was inactivated to direct the carbon flux towards the pentose phosphate pathway in the cellular metabolic network. This genetic modification successfully enabled the synthesis of sedoheptulose from glucose. Additionally, we identified key enzymes responsible for product formation through transcriptome analysis, and their corresponding genes were overexpressed, resulting in a further 20% increase in sedoheptulose production.

Conclusion We achieved a sedoheptulose concentration of 24 g/L with a yield of 0.4 g/g glucose in a 1 L fermenter, marking the highest value up to date. The produced sedoheptulose could further function as feedstock for synthesising structural seven-carbon sugars through coupling with enzymatic isomerisation, epimerisation and reduction reactions.

Keywords Seven-carbon sugars, Sedoheptulose, Metabolic engineering, *Corynebacterium glutamicum*

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Introduction

Rare sugars represent a substantial category of monosaccharides, which rarely exist in nature. Some known rare sugars, including D-allulose and D-tagatose, demonstrate diverse biological functions and find extensive application as food ingredients or drug synthesis precursors [1, 2]. Sedoheptulose and other seven-carbon-chain sugars are also hardly found in nature [3, 4]. This family of sugars has been widely used in food and pharmaceutical industries. Some of these sugars constitute the essential building blocks of secondary metabolites, such as septacidin and hygromycin B, in bacteria [5]. Recent research has revealed that heptuloses and their derivatives possess the ability to inhibit sugar metabolism, making them potential therapeutic agents for hypoglycaemia and cancer [6–8]. Heptuloses also serve as foundational elements for the synthesis of C-glycosides and novel antibiotics [9, 10]. Consequently, an efficient method for manufacturing those seven-carbon-chain sugars should be devised.

Various chemical and enzymatic methods have been developed for the synthesis of heptoses. Chemical methods require complex reaction steps, leading to diastereomeric mixtures and poor overall yields [11–14]. Transketolase, a well-known enzyme in the pentose phosphate pathway, naturally catalyses the reversible conversion of D-xylulose-5-phosphate (X5P) to D-ribose-5-phosphate (R5P), forming D-sedoheptulose-7-phosphate (S7P) and glyceraldehyde-3-phosphate (G3P). This enzyme class has been employed to transfer a two-carbon unit from β-hydroxypyruvate (HPA) to D-xylose or L-arabinose, generating kinds of heptuloses. However, this approach is constrained by the low enzyme activity of transketolase and necessitates expensive HPA as the ketone donor [15].

Recently, microbial fermentation has presented a promising approach for producing functional and unnatural sugars [16]. Metabolic engineering has been employed to engineer the cell metabolic network to increase the productivity and titre. For example, *Yarrowia lipolytica* has been engineered to produce erythritol and mannitol, natural sweeteners in the beverage industry, on an industrial scale [17–19]. In this work, we presented a strain engineering strategy for the *de novo* production of sedoheptulose with *Corynebacterium glutamicum* as host. We blocked the glycolytic pathway by inactivating the gene of 6-phosphofructokinase (*PfkA*) and redirected the carbon flux to the pentose phosphate pathway of the cell metabolic network. This gene modification successfully enabled the engineered strain to produce sedoheptulose using glucose as the sole carbon source. The transcriptome analysis confirmed the key enzymes in the sugar metabolic network for sedoheptulose synthesis. The overexpression of key genes increased the product concentration. This work demonstrated a promising approach for the industrial manufacturing of sedoheptulose in future.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Descriptions	Source
Strains		
E.coli DH5α	*endA1 supE44 recA1 gyrA96 relA1 deoR U169 Φ 80dlacZ Δ M15 mcrAΔ(mrr–hsdRMS–mcrBC)*	Invitrogen
C.glutamicum ATCC 13,032	Wild-type	ATCC
Sed 1	WT derivative, *ΔpfkA*	This study
Sed 2	*C. glutamicum* containing plasmid pEC-tkt	This study
Sed 3	*C. glutamicum* containing plasmid pEC-tal	This study
Sed 4	*C. glutamicum* containing plasmid pEC-tkt-tal	This study
Sed 5	*C. glutamicum* containing plasmid pEC-Cgl0761	This study
Plasmids		
pEC-XK99E	Kan^R; *C.glutamicum/E.coli* shuttle vector (P*trc*, *lacI*^{q}; pGA1, $OriV_{C.gl}$, $OriV_{E.c}$)	[39]
pH36SacB	Kan^R; pBR322 ori, H36 promoter, gene of *sacB*	[25]
pEC-tkt	pEC-XK99E containing *tkt* gene from *C. glutamicum*	This study
pEC-tal	pEC-XK99E containing *tal* gene from *C. glutamicum*	This study
pEC-tkt-tal	pEC-XK99E containing *tal* and *tkt* genes from *C. glutamicum*	This study
pEC-Cgl0761	pEC-XK99E containing *Cgl0761* gene from *C. glutamicum*	This study

Materials and methods

Strains, medium and cultivation

The strains and plasmids used in this study were listed in Table 1. *E. coli* DH5α was selected for gene cloning and plasmid construction. It was cultivated at 37 ℃, 200 rpm in Luria-Bertani medium. *C. glutamicum* ATCC 13,032 and its derivatives were cultivated in BHI (Brain Heart Infusion) at 30 ℃ and 200 rpm. To maintain the plasmid stable in cell, a finial concentration of 50 μg/mL kanamycin was added to culture medium. The CGXII minimal medium [20] supplemented with 10 g/L glucose was used for product formation. The initial pH for CGXII buffer was maintained to 7.4.

Genes expression and inactivation

The *E. coli-C.glutamicum* shuttle vectors pEC-XK99E was used for overexpression of genes *Cgl1574*, *Cgl1575*, and *Cgl0761* in *C. glutamicum*. Those genes were cloned from genomic DNA of *C. glutamicum* ATCC 13,032 and inserted into the pEC-XK99E vector and under the control of P*tuf* using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). A modified

suicide plasmid pH36SacB [21] was used to knockout the *pfkA* gene (*Cgl1250*) in *C. glutamicum* ATCC 13,032. The upstream and downstream neighboring region of 800 bp was used for homologous recombination.

Flask fermentation and dual-phase fermentation

To conduct the one-step fermentation for sedoheptulose formation, the strains wide-type, Sed 1, Sed 2, Sed 3, Sed 4, and Sed 5 were cultivated in 500 mL shake flasks containing 50 mL of BHI medium and 10 g/L glucose. The cultivation was performed at 30 °C for 72 h. To conduct the dual-phase fermentation for sedoheptulose synthesis, the strain Sed 2 were initially cultivated in 100 mL BHI medium in a 500-mL shake flask for 24 h at 30 °C and 200 rpm. Then, the medium was centrifugated at 8,000×g and 4 °C for 10 min. The harvested cells were then suspended in 10 mL CGXII medium with an initial OD_{600} of approximately 40. After that, glucose was added into the medium to achieve an initial concentration of 60 g/L and the cultivation was carried out at 30 °C and 200 rpm for 72 h. To measure the product formation, samples were captured every 6–12 h for HPLC analysis.

RNA isolation and transcriptome analysis

The wild-type ATCC 13,032 and Sed1 were inoculated in BHI medium containing glucose concentration of 10 g/L. Samples were collected at 10 h and 16 h then immediately placed into liquid nitrogen and stored at −80 °C. Total RNAs were extracted using RNAprep Pure Cell/Bacterial Kit. In order to minimize the DNA contamination, the total RNA samples were treated with RNase-free DNase I. The extracted total RNAs were analyzed with NanoPhotomete spectrophotometer (IMPLEN, CA, USA) by determining A230/260 and A260/A280. The library construction and transcriptome sequencing were performed by Novogene Biotech (Tianjin, China). The sequence raw data have been deposited in Gene Expression Omnibus of NCBI database with the accession number of GSE254540.To perform building index of reference genome and aligning clean reads to reference genome (GenBank Accession Number GCA_000011325.1), the Bowtie 2/2.2.3 software was employed. The expression analysis of strains under different conditions were performed using the DESeq R package (1.18.0). The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate [22].

MS and NMR analysis

To confirm the product of sedoheptulose, the collected samples after fermentation were conducted for MS analysis. To obtain purified sedoheptulose for NMR analysis, in this work, we employed the preparative HPLC system to separate and purify product. The desired product was monitored and collected in one tube. Then, the collected samples were evaporated under vacuum-freezing conditions. To perform NMR analysis, purified products were dissolved in D_2O and subjected to Bruker AVANCE III 600 MHz NMR (Bruker Daltonik, Bremen, Germany).

Results

Demonstration of the pathway for sedoheptulose synthesis

The S7P served as an important precursor in the pentose phosphate pathway of cellular metabolism [23]. We proposed that if S7P could be efficiently accumulated in the cell and an S7P-specific phosphatase for dephosphorylation of S7P to sedoheptulose was introduced into the intracellular sugar metabolic network, microbial synthesis of sedoheptulose could be achieved. We noted that two enzymes played the key role in synthesis and conversion of S7P. The first one, transketolase, catalyzed the transfer of a ketol group from X5P to R5P, forming S7P and G3P. Another enzyme, transaldolase, catalyzed the conversion of fructose-6-phosphate (F6P) and D-erythrose 4-phosphate (E4P) to S7P and G3P. It was known that X5P, R5P, E4P, and S7P are key intermediates in the pentose phosphate pathway, and F6P is the key intermediate in the glycolytic pathway. Therefore, the pentose phosphate pathway presented the key point for sedoheptulose production (Fig. 1).

According to the demonstrated pathway, three involved reactions were presented here. (1) 1 Xu5P + 1 Ru5P = 1 G3P + 1 S7P; (2) 1 F6P + 1 G3P = 1 Xu5P + 1 E4P; (3) 1 E4P + 1 F6p = 1 G3P + 1 S7P. Together, 2 F6P + 1 Ru5P = 1 G3P + 2 S7P. Given that F6P and Ru5P could be obtained from glucose through glycolytic pathway and pentose phosphate pathway, respectively, 3 glucose molecules could be converted to 1 G3P and 2 S7P. Therefore, the synthetic yield of designed pathway for S7P synthesis from glucose was 2/3 = 0.66 mol/mol. To achieve this end and redirect the carbon flux from glucose to S7P, the gene of *pfkA*, encoding 6-phosphofructokinase that catalyzes the conversion of F6P to fructose 1,6-diphosphate, should be inactivated (Fig. 1). Naturally, G3P was synthesized from cleavage of fructose-1,6-bisphosphate by fructose-bisphosphate aldolase in glycolysis. If the gene of *pfkA* was deleted, G3P would be obtained from E4P and Xu5P *via* pentose phosphate pathway. Under such condition, the G3P would be the major carbon input for cell growth and metabolism.

In this work, we endeavoured to construct an engineered strain for sedoheptulose production with *C. glutamicum* ATCC 13,032, a gram-positive soil bacterium with GRAS status, as model host [24]. The gene of *Cgl1250* encoding PfkA was knocked out using the two-step homologous recombination method [25]. The inactivation of the enzyme PfkA in strain Sed1 significantly

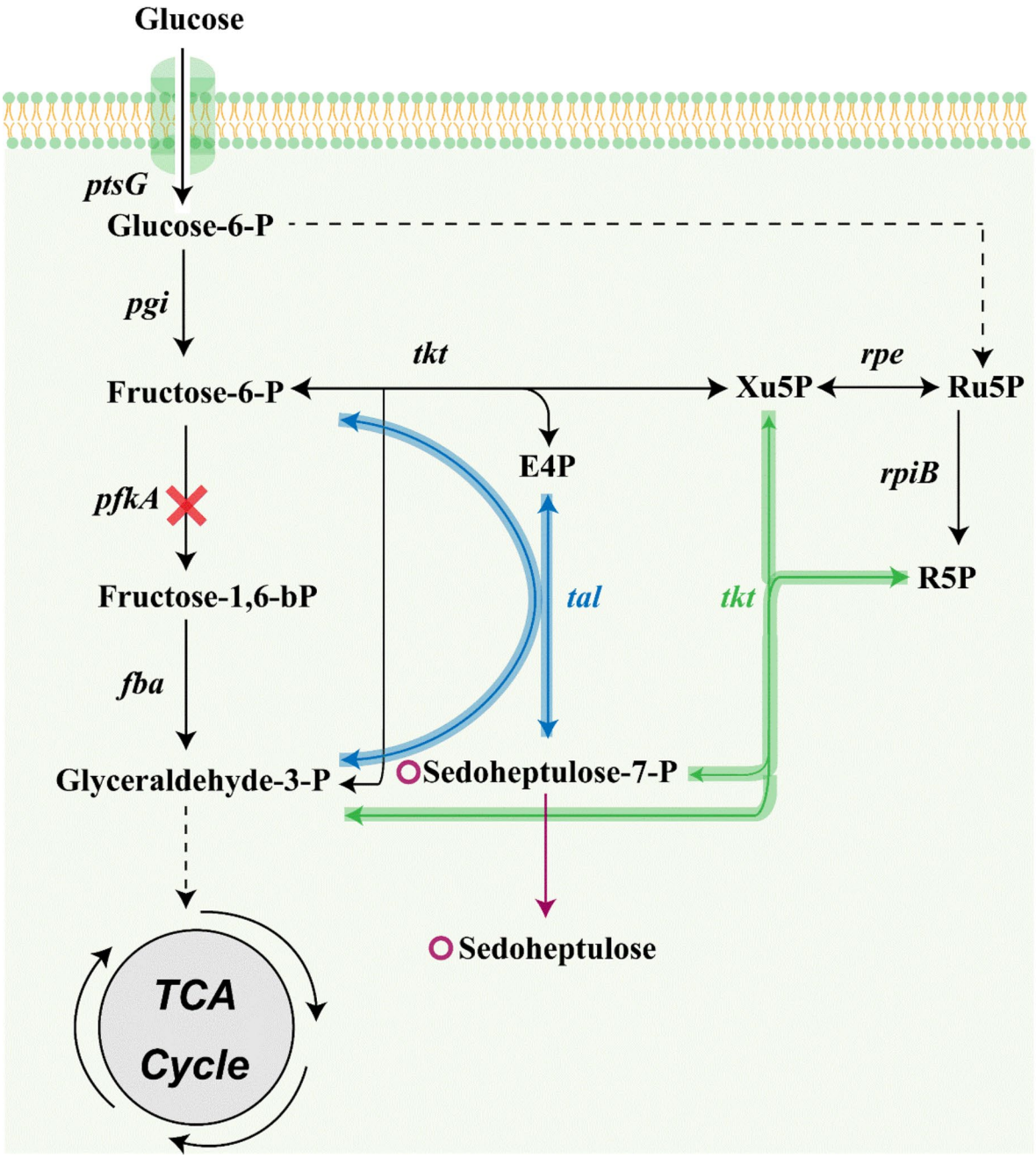

Fig. 1 The strain engineering strategy for sedoheptulose synthesis from glucose. *ptsG*, phosphotransferase system; *pgi*, glucose-6-phosphate isomerase; *tkt*, transketolase; *rpe*, ribulose-phosphate 3-epimerase; *fba*, fructose-bisphosphate aldolase; *tal*, transaldolase; *rpiB*, ribose 5-phosphate isomerase B; Xu5P, D-Xylulose 5-phosphate; Ru5P, D-Ribulose 5-phosphate; R5P, D-Ribose 5-phosphate; E4P, D-Erythrose 4-phosphate; Fructose-1,6-bP, fructose-1,6-bisphosphate

decreased the cell growth and glucose consumption in the CGXII minimal medium (Fig. 2a and b) but showed a slight decrease in the BHI-rich medium (Fig. 2c and d). A new product emerged for strain Sed1 after 72 h of fermentation (Fig. 3a). The MS analysis result showed that the molecular mass of the emerging product was 233 (Fig. 3b). We subsequently purified this product, confirmed its structure via NMR and verified that it was sedoheptulose. This product did not occur in the fermentation medium of the wild-type strain. Therefore, for the first time, we successfully synthesised 3.4 g/L of sedoheptulose from 10 g/L of glucose only through inactivating one enzyme of *pfkA* in the cell.

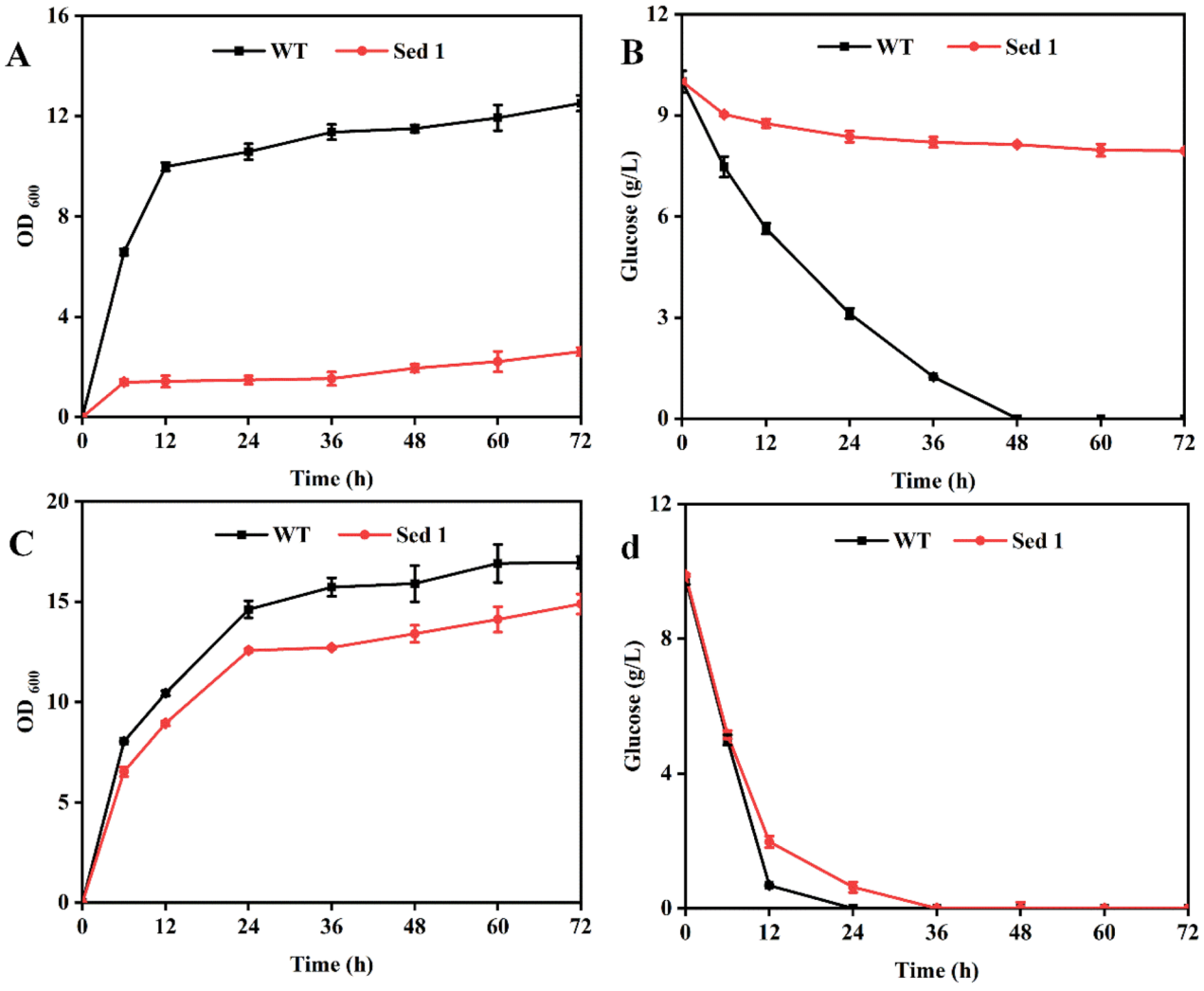

Fig. 2 The cell growth and glucose consumption of wide-type and Sed1. **A** and **B** represented the cell growth and glucose consumption under CGXII medium; **C** and **D** represented the cell growth and glucose consumption under BHI medium

Transcriptome analysis for identifying key enzymes

Transcriptome analysis was performed for a wild-type strain and strain Sed1 to assess the effect of *pfkA* inactivation on the gene expression of the cellular sugar metabolic network and identify the key enzymes for sedoheptulose production. Samples were collected after fermentation for 4, 10 and 16 h. Upregulated and downregulated genes were analysed. The results revealed a significant increase in the expression of genes in the pentose phosphate pathway when gene *pfkA* was knocked out (Fig. 4). We could deduce that the inactivation of enzyme *pfkA* led to the blockage of carbon flux in the glycolytic pathway. As a result, the cell increased the gene expression of the pentose phosphate pathway to redirect the carbon flux from G6P to G3P for cell growth. Specifically, the expression levels of genes *Cgl1574* encoding transketolase and *Cgl1575* encoding transaldolase exhibited 2.26- and 2.25-fold improvement compared with the wild-type strain, respectively (Fig. 5a). Hence, these two enzymes played a key role in sedoheptulose formation in Sed1. Notably, the expression of gene *Cgl2911* for L-lactate synthesis decreased because of *pfkA* knockout, resulting in lower lactate accumulation for strain Sed1 compared with that for the wild-type strain.

Improvement of sedoheptulose production through increasing the expression of key genes

The transketolase and transaldolase in the pentose phosphate pathway played important roles in sedoheptulose production. Therefore, we attempted to increase the expression level of those two genes, namely, *Cgl1574* and *Cgl1575*, resulting in Sed2 and Sed3, respectively. Subsequently, the cell growth, glucose consumption and product formation of Sed2 and Sed3 were measured. The results showed that an improvement in the expression level of *Cgl1574* in Sed2 increased the cell growth and

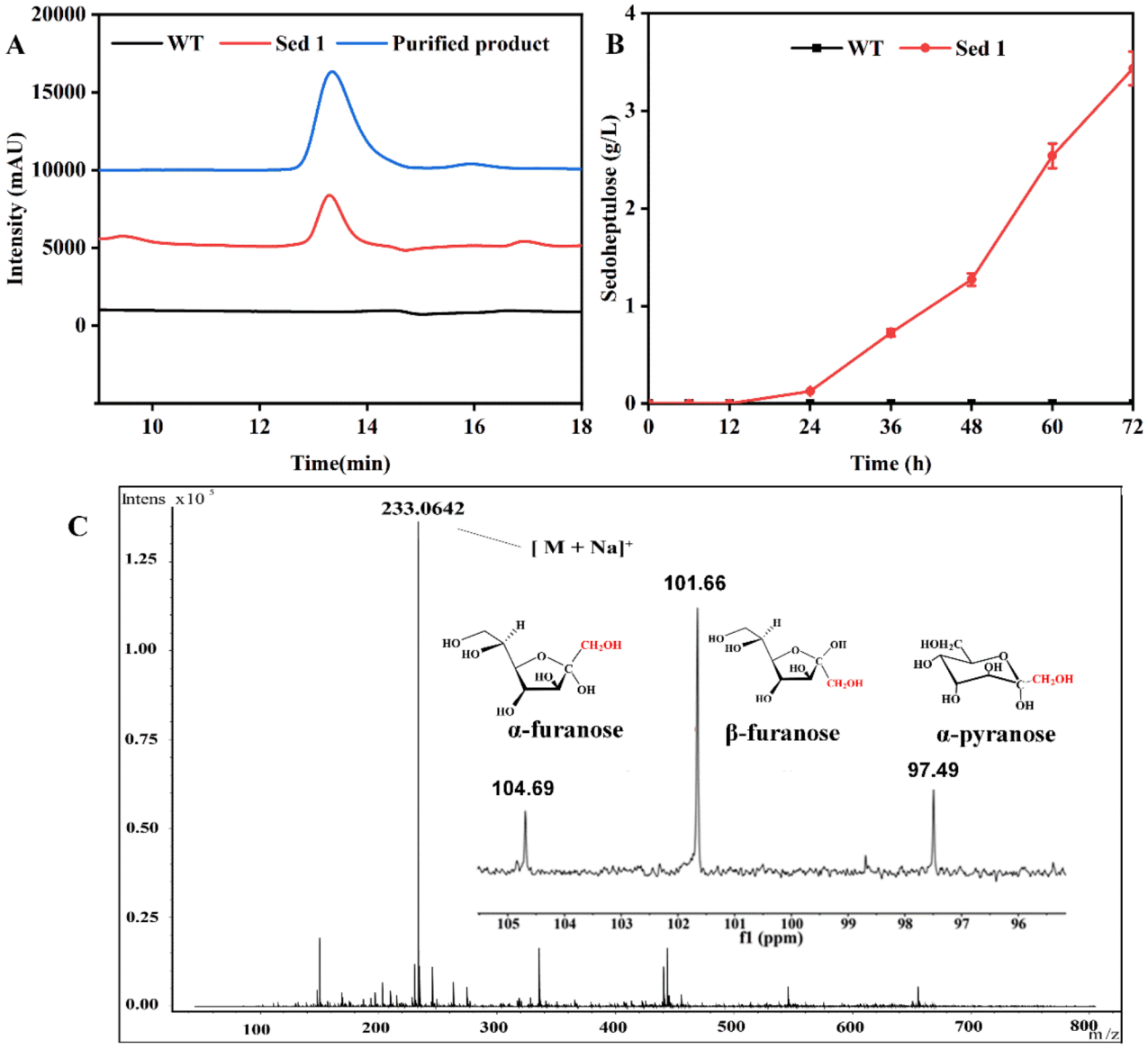

Fig. 3 The confirmation of sedoheptulose synthesis of strain Sed1. **A**) The HPLC results of wide-type and Sed1; **B**) The curve for synthesis of sedoheptulose; **C**) The MS and NMR results for sedoheptulose synthesis

glucose consumption (Fig. 5b and c). The sedoheptulose concentration of Sed2 was 20% higher than that of Sed1 (Fig. 5d). However, the overexpression of gene *Cgl1575* in Sed3 had no effect on cell growth and glucose consumption and even decreased the product formation compared with that in Sed1.

According to the transcriptome data analysis, the specific phosphatase for converting S7P into sedoheptulose has not been discovered. We noted that one enzyme encoded by gene *Cgl0761* may have the dephosphorylation function to substrate D-glycero-mannoheptose-1,7-bisphosphate [26]. The overexpression of this gene was performed in Sed1, resulting in strain Sed4. However, the overexpression of this gene did not increase the sedoheptulose production. In future work, the specific phosphatase for the dephosphorylation of S7P is still required.

Sedoheptulose synthesis with a 'dual-phase' fermentation process

In this study, we observed that the *pfkA* inactivation led to nearly no growth in the CGXII minimal medium; by contrast, in the BHI-rich medium, the cell growth of Sed1 significantly recovered. Therefore, a 'dual-phase' fermentation process was conducted for product synthesis [27]. In the first stage, cell growth was initiated in the BHI medium. Then, the cells were collected and transferred to the CGXII medium, with a certain cell density OD_{600}

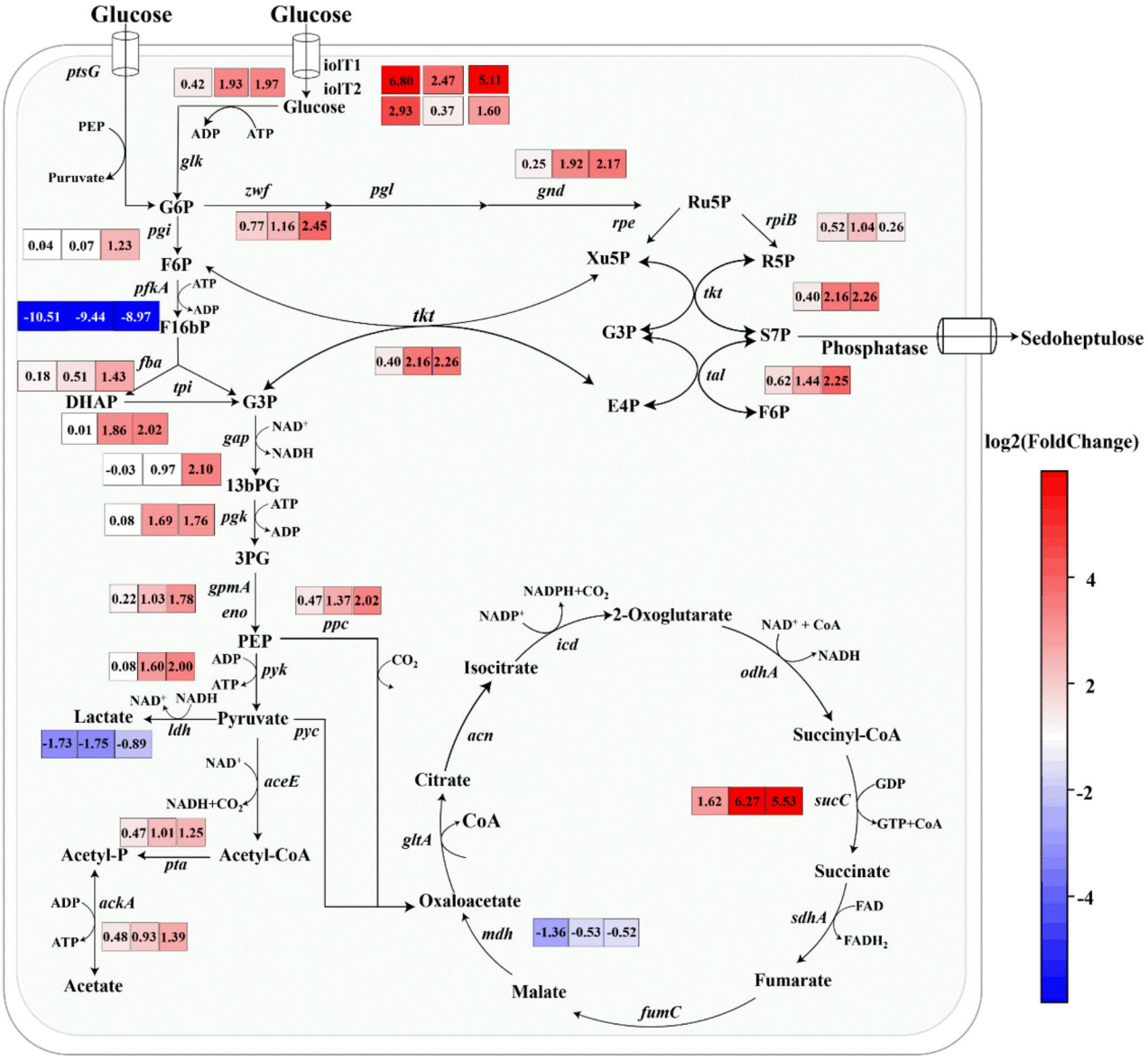

Fig. 4 The transcriptome analysis data of strain Sed1 compared with wide-type. *glk*, glucokinase; *zwf*, glucose-6-phosphate 1-dehydrogenase; *pgl*, 6-phosphogluconolactonase; *gnd*, 6-phosphogluconate dehydrogenase; *tpi*, triosephosphate isomerase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *gpmA*, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; *eno*, enolase; *pyk*, pyruvate kinase; *ldh*, L-lactate dehydrogenase; *pyc*, pyruvate carboxylas; *aceE*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *ackA*, acylphosphatase; *ppc*, phosphoenolpyruvate carboxylase; *icd*, isocitrate dehydrogenase; *sdhA*, succinate dehydrogenase; *fumC*, fumarate hydratase; *mdh*, malate dehydrogenase; *gltA*, citrate synthase; *acn*, aconitate hydratase; F16bP, D-fructose 1,6-bisphosphate; G3P, D-glyceraldehyde 3-phosphate; 13bPG, D-glycerate 1,3-diphosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate

of 30–40 and an initial glucose concentration of 60 g/L. As shown in Fig. 6, the glucose was completely after fermentation for 72 h for strain Sed2, and 24 g/L of sedoheptulose, with a yield of 0.4 g/g glucose, was produced at that time (Fig. 6). We further conducted the fermentation process in a 1 L fermenter, and a similar product concentration was obtained.

Discussions

Previous studies showed that wild-type *Flavobacterium* sp. and streptomycetes could produce 1.2 g/L of sedoheptulose from glucose [28]. *Bacillus subtilis* was also engineered by inactivating transketolase to produce 3.8 g/L of sedoheptulose from 100 g/L of glucose [29]. However, the production titre and efficiency were low. In this work, we demonstrated a metabolic engineering strategy to engineer a strain for the *de novo* synthesis of sedoheptulose from glucose. The engineered strain achieved higher titre

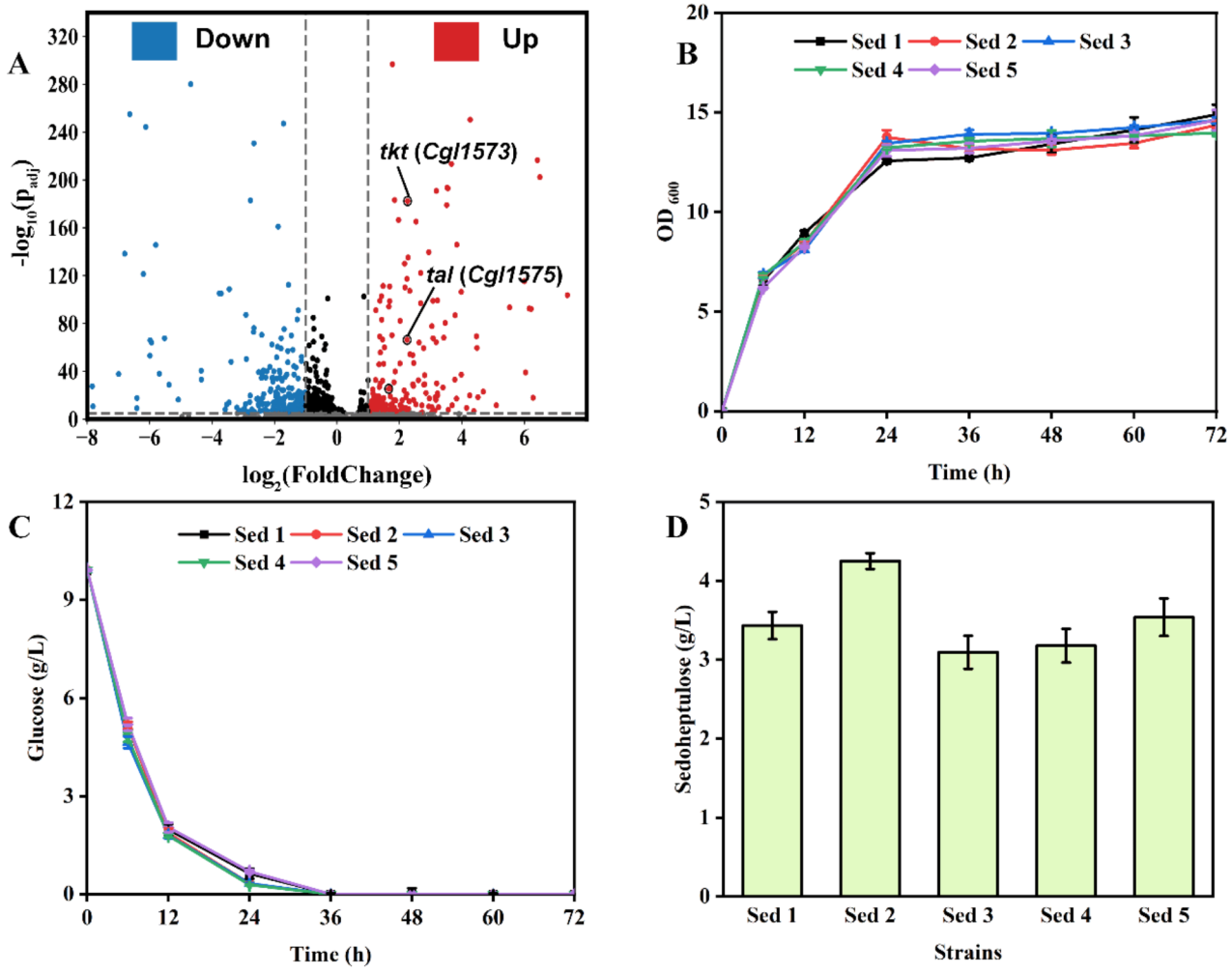

Fig. 5 Overexpression of the key genes to increase sedoheptulose production. **A**) The up and down expression of genes according to transcriptome analysis data; **B**), **C**) and **D**) The cell growth, glucose consumption, and product formation of different strains with overexpression of keys genes

(24 g/L) and yield (0.4 g/g glucose) than those in reported studies.

In our previous studies, we have constructed a heterogenous aldol pathway in strains to produce seven-carbon rare sugars from low-cost glucose and glycerol [27, 30]. Two C7 sugars, namely, 3R, 4 S, 5R, 6R-heptulose and 3R, 4R, 5R, 6R-heptulose, could be synthesised; however, this approach required supplementing high-cost C4 sugar D-erythrose into the medium, which restricts the large-scale manufacturing of seven-carbon sugars. The demonstrated method in this work confirmed the promising ability of producing sedoheptulose from low-cost glucose. The raw materials, such as glycerol and sucrose, could also serve as feedstocks for manufacturing [31, 32]. In addition, the sedoheptulose synthetic approach may have broad application for producing other seven-carbon sugars. For example, enzymatic isomerisation, epimerisation and redox reaction of sedoheptulose could be conducted to synthesise other structurally diverse ketoheptoses and aldoheptoses (Fig. 7).

In this work, the knockout of only one gene of *pfkA* successfully produced sedoheptulose from glucose. We have confirmed the corresponding synthetic pathway on the basis of the transcriptome data. However, the key phosphatase for catalysing the dephosphorylation of S7P to sedoheptulose is still unknown. Large amounts of HAD phosphatases catalyse the dephosphorylation of phosphorylated sugar to maintain the Pi recycle in cell [33]; however, most of them have not been annotated for specific substrates [34, 35]. Therefore, how to identify the key phosphatase and further increase its activity would be the next key research point. The cell central network should also be regulated to enhance the sedoheptulose production. For example, regulation of the gene *tal* expression level could be conducted using a Base Editor-Targeted and Template-free Expression Regulation

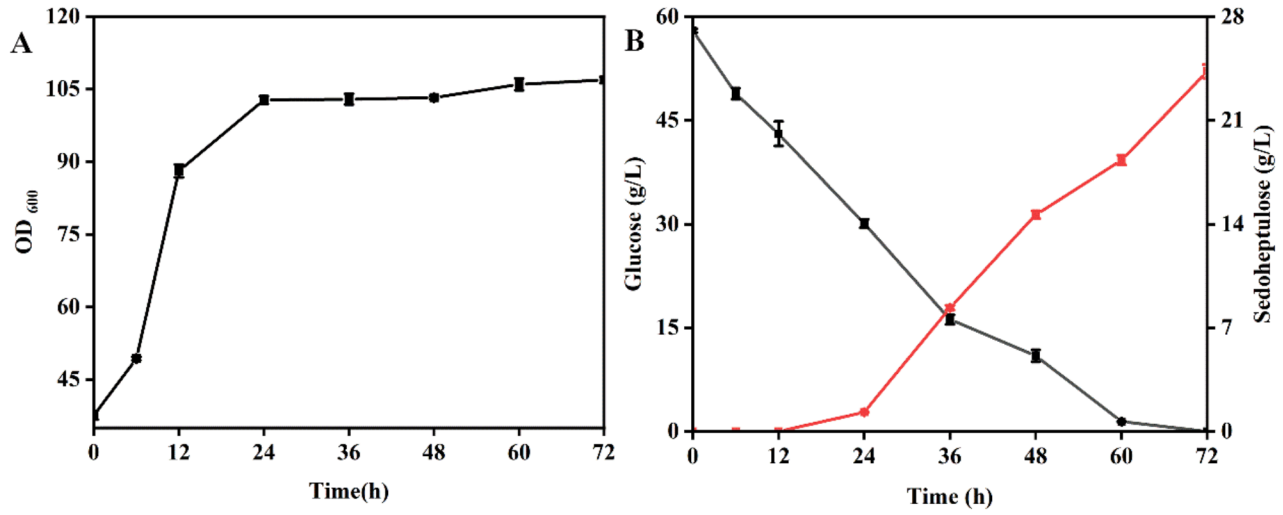

Fig. 6 The synthesis of sedoheptulose for strain Sed2 under "dual-phase" fermentation process. **A**) The cell growth for Sed2; **B**) The glucose consumption and sedoheptulose formation for strain Sed2

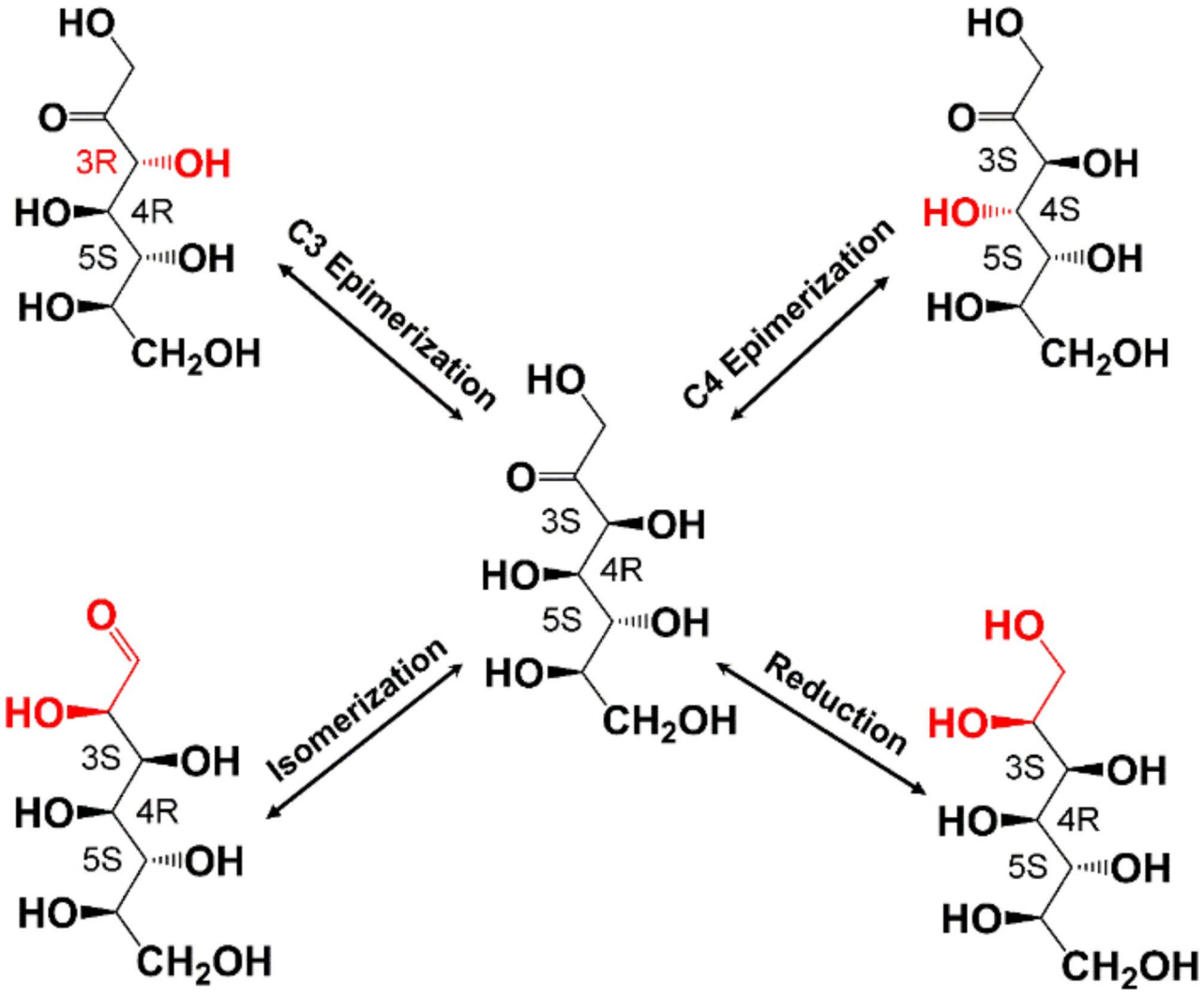

Fig. 7 The scheme for synthesis of other seven-carbon sugars with sedoheptulose as precursor

method [36]. The large production of sedoheptulose was realised from the high concentration of S7P in strain Sed2. Given that S7P also served as a key precursor in the shinorine synthesis pathway for mycosporine-like amino acid synthesis [37, 38], the gene of *pfkA* may be a candidate engineering target to increase the production of those products during the strain engineering process.

Conclusion

In this work, we demonstrated a metabolic engineering strategy to engineer strains for the *de novo* synthesis of sedoheptulose from glucose. The key enzymes according to sedoheptulose production in the central metabolic network have been confirmed. The engineered strain achieved higher titre and yield than those in reported studies and produced 24 g/L of sedoheptulose in a 1 L fermenter, demonstrating high potential in large-scale applications. In future work, several strategies, including metabolic engineering of the pentose phosphate pathway, identification and overexpression of key enzymes for dephosphorylation of S7P and optimisation of fermentation process, could be pursued to further increase the product formation. The generated sedoheptulose could serve as a substrate to synthesise other structurally diverse seven-carbon sugars (ketoheptoses and aldoheptoses) through coupling with enzymatic isomerisation, epimerisation and reduction reactions. The synthetic seven-carbon sugars may present new physiological functions and have application potential in food and pharmaceutical areas.

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Author contributions
Y. L and Q. D conducted most of the experiments, analyzed the results, and wrote the manuscript. W. S and W. P conducted the transcriptome analysis. Y. Z analyzed the structure of sedoheptulose. M. W and Y. S analyzed the data and provided the useful suggestions for experiments. Y. M and J. Y designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest
All authors declares that he/she has no conflict of interest.

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