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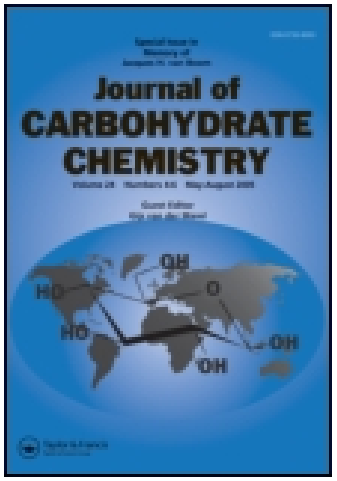

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Synthesis of D-Sorbose and D-Psicose by Recombinant Escherichia coli

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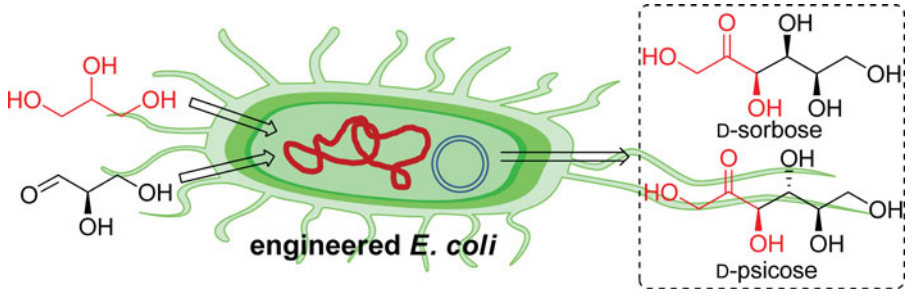
GRAPHICAL ABSTRACT



In the current work, the in vitro synthetic system for rare sugars was successfully transformed into an engineered *Escherichia coli* with a plasmid containing both the aldolase RhaD and phosphatase YqaB. By taking advantage of the inherent biosynthetic pathways in *E. coli*, this approach permits the use of simple and cheap glycerol for the synthesis of DHAP in vivo. Moreover, the introduction of the phosphatase into the *E. coli* system allows for the removal of the phosphate group on the synthetic intermediate to yield the neutral rare sugars, which can be readily secreted to the medium without accumulation in the cell.

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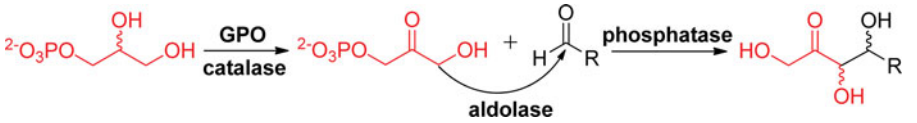
Keywords Biosynthesis; Biocatalysis; Biotransformations; in vivo synthesis; Rare sugars; *Escherichia coli*

INTRODUCTION

Aldolases from carbohydrate metabolism have been used as an important tool to perform enzymatic carbon-carbon couplings.[1,2] Mild reaction conditions and stereoselective formation of C−C bonds are some of the major advantages for these biocatalysts.[3–5] Usually aldolases have high specificity for the donor (nucleophile) substrate, which gives rise to the classification of aldolases.[6] Among the aldolase families, we have been focusing on dihydroxyacetone phosphate (DHAP)-dependent aldolases in recent years and investigating the stereochemistry of these enzymes in in vitro synthesis of rare sugars.[7–11] However, an important prerequisite for a suitable biocatalysis is that the substrate of the biocatalyst should be readily available in sufficient amount and relatively stable. Thus, the strict specificity for the donor substrate DHAP, a rather expensive and unstable compound, is disadvantageous for broad application of DHAP-dependent aldolases.[12,13] Fortunately, DHAP is an important metabolic intermediate that involves various pathways, which has inspired us to develop efficient ways to generate this compound for synthetic purposes. For example, the major route for glycerol processing in a number of microorganisms provides DHAP by the oxidation of L-glycerol 3-phosphate catalyzed by glycerol phosphate oxidase (GPO).[14] Not only have we followed this route, but also we have achieved the successful integration of GPO oxidation and aldolization into a one-pot four-enzyme system in which DHAP could be generated and used in situ, so that rare sugars could be produced on preparative scales without isolation of the intermediates (Sch. 1).[7–10]



Scheme 1: In vitro one-pot four-enzyme synthesis of rare sugars via DHAP.

Although this one-pot strategy could address the instability and availability issues of DHAP to some extent, a few fundamental problems still need to be considered, such as the assembly of the multienzyme system and the scalability of this approach. Several drawbacks like equilibrium shifts and enzyme deactivation should also be considered.

Recently, green production from renewable resources via microbial fermentation has attracted increasing attention as microbial transformation has the advantages of using cheaper catalysts without purification and avoidance of cofactor recycling in vitro.[15–18] Therefore, we envisioned that both efficiency and

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practicality will be improved if DHAP could be generated from simple achiral reagents through microbial metabolic pathways and utilized in situ. We report here a transformation of the multienzymatic system for the synthesis of rare sugars into engineered *E. coli* assembly. As a proof-of-concept demonstration, L-rhamnulose-1-phosphate aldolase (RhaD) and YqaB phosphatase are overexpressed in *E. coli* with glycerol as the carbon source, which were assimilated through the inherent metabolic pathways to provide DHAP for in situ consumption. Meanwhile, we fed the bacteria with the acceptor D-glyceraldehyde (D-GA) for the production of D-sorbose and D-psicose.

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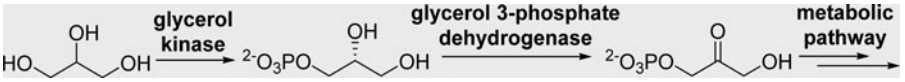
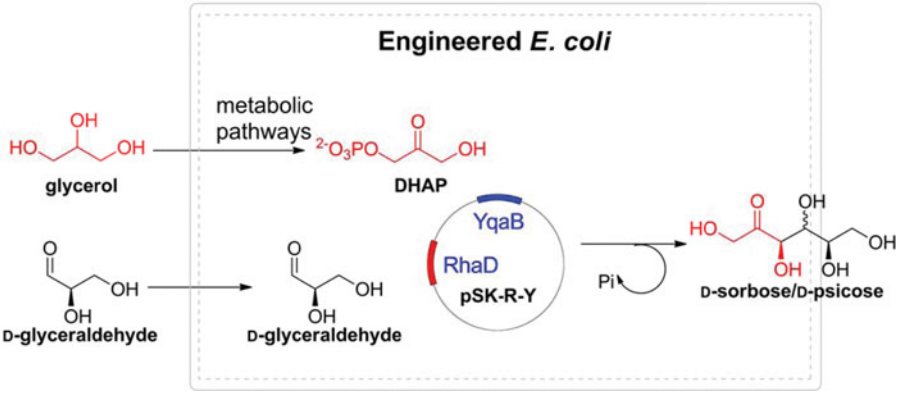
RESULTS AND DISCUSSION

Glycerol has become a very attractive carbon source, as a large surplus of glycerol is generated inevitably as a by-product during the production of biodiesel.[19–21] In wild-type *E. coli*, the process of glycerol dissimilation is as follows (Sch. 2): first, glycerol is transported into the *E. coli* cells by glycerol facilitator; then, glycerol is phosphorylated by glycerol kinase, producing *sn*-glycerol 3-phosphate (G3P); in the subsequent step, G3P is converted into DHAP catalyzed by glycerol 3-phosphate dehydrogenase, a flavoprotein with molecular oxygen as the electron acceptor.[22] The resulting DHAP is further metabolized through the glycolytic pathway. This straightforward biosynthetic route for DHAP from glycerol has already been successfully applied to the synthesis of nonnatural carbohydrates in vitro by Sheldon et al.[23,24] Motivated by this pathway and previous research, we visualized that the problem of DHAP synthesis could be solved through its native microbial generation. If in vivo DHAP generation is further coupled with in situ aldolization reaction catalyzed by aldolase followed by dephosphorylation, large-scale production could thus be realized by fermentation, and the desired products could be harvested and isolated from the culture media (Sch. 3).



Scheme 2: Glycerol dissimilation in *E. coli*.

L-Rhamnulose-1-phosphate aldolase (RhaD) is a class II DHAP-dependent aldolase that catalyzes the reversible cleavage of L-rhamnulose-1-phosphate to DHAP and L-lactaldehyde.[25,26] RhaD from *E. coli* ($RhaD_{E.coli}$) is a homotetramer of the rare C4-symmetric type, containing two molecules of Zn^{2+} per enzyme complex, and each subunit is composed of 274 amino acid residues with a molecular weight of 30,149 Da.[26,27] Interestingly, Fessner et al. first reported in 1991 that $RhaD_{E.coli}$ can also tolerate aldehyde with D-configuration, such as D-GA, as an acceptor, and we discovered in our previous study that



Scheme 3: Production of D-sorbose and D-psicose using engineered *E. coli*.

$RhaD_{E.coli}$ actually lost its stereoselectivity in the aldolization to simultaneously synthesize D-sorbose and D-psicose.[7,28] As no RhaD expression (or at very low level) was observed under our culture condition, RhaD overexpression was performed with pBluescript SK-plasmid. It is also necessary to hydrolyze the phosphate on the C-1–OH of the aldol adduct (Sch. 1) inherent to the use of DHAP because it was assumed that phosphorylated intermediates from cellular metabolism were efficiently retained by the plasma membrane, leading to relatively low product yields. Meanwhile, accumulation of such products at high levels in vivo was assumed to be toxic or, at least, a burden to the cells.[17] More practically, sugar molecule without a highly polar phosphate group can be readily secreted into the culture medium, affording an easier product harvest process. Acid phosphatase, commonly used in vitro to remove the phosphate group of aldol products, is known to operate at a pH optimum of 4.6, which precludes its application in *E. coli*. Fortunately, we found that YqaB phosphatase from *E. coli,* belonging to the haloacid dehalogenase superfamily, shows a remarkably broad substrate spectrum, among which the dephosphorylation activity toward D-fructose-1-phosphate is the highest.[29] It was also discovered in our preliminary results that YqaB also showed comparable activity toward D-sorbose-1-phosphate, D-psicose-1-phosphate, and L-fructose-1-phosphate. Most importantly, this enzyme showed high activity under neutral or physiological conditions as tested by our in vitro reactions. Thus, to carry out the reaction sequence, the recombinant plasmid pSK-rhaD-yqaB was constructed for coexpression of both aldolase RhaD and phosphatase YqaB in *E. coli* for the in vivo rare sugar synthesis (Fig. S1).

E. coli DH5α-RY strain containing the plasmid pSK-rhaD-yqaB was used to carry out the synthesis of D-sorbose and D-psicose (Sch. 3) with glycerol added to the culture medium. The donor substrate DHAP was generated in this recombinant *E. coli* strain from glycerol through native metabolic pathways.

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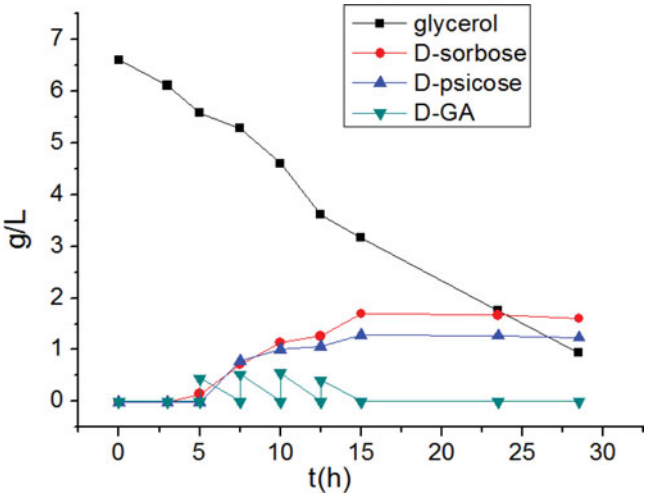

Figure 1: Monitor the in vivo reaction progress by HPLC.

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The acceptor substrate D-GA was supplied by adding into the medium continuously (discussed below), which could be transported into the cell through glycerol facilitator.[30] Then, intracellular DHAP was condensed with D-GA catalyzed by overexpressed RhaD to give the aldol product sugar-1-phosphate, which was subsequently dephosphorylated by YqaB phosphatase to provide the final products D-sorbose and D-psicose.

In a typical 200-mL fermentation experiment, the consumption of glycerol, D-GA, and the formation of products were monitored by HPLC (Fig. 1). The concentration of glycerol decreased steadily as it was constantly assimilated by the bacteria. It was noticed that, after the first addition at 5 h fermentation, D-GA would be almost consumed within 3 h, so we had to feed the bacteria D-GA continuously once it was exhausted. During the whole process of fermentation, 8 mL of D-GA (0.5 M, 2 mL $\times$ 4) was added to the medium. Consistent with our in vitro studies, two rare sugars D-sorbose and D-psicose were produced simultaneously (Fig. S2). It seemed that the concentration of D-GA was a very important limiting factor, as the yields of both products increased dramatically accompanied by the addition of D-GA. Interestingly, as the fermentation proceeded, the concentration of these two sugar products did not decrease even after we stopped supplying D-GA, evidencing that they were not consumed/metabolized by the bacteria, which probably do not possess the enzymes that can utilize these two rare sugars. Toward the end of the fermentation, the culture broth was centrifuged and the products in both the cells and supernatant were examined. Cells were disrupted by sonication and analyzed by TLC, but no product was detected. Meanwhile, the concentration of D-sorbose (1.6 g/L) and D-psicose (1.23 g/L) in the supernatant was determined

by HPLC (ratio: D-sorbose/D-psicose 1.3:1, Fig. S2). It indicated that D-sorbose and D-psicose produced were excreted by the bacteria, which enabled an easier purification process. After purification by silica gel and Bio-Gel P-2 column chromatography, a mixture of D-psicose and D-sorbose (259 mg) was obtained and the isolated conversion yield was 36% based on D-GA. Further separation by Ca^{2+} ion exchange resin at elevated temperature afforded D-sorbose (129 mg) and D-psicose (62 mg), which were separated according to their different coordination affinities to Ca^{2+}.[31]

To summarize, we established a method for the synthesis of rare sugars in engineered *E. coli* by fermentation. The bacteria were transformed with a plasmid containing both the aldolase RhaD and phosphatase YqaB. In our process, the donor substrate DHAP was generated in vivo from cheap and readily available glycerol through the native metabolic pathways in the host *E. coli* without the introduction of additional enzymes, cofactors, or ATP. Significantly, the final products could be released to the culture medium, affording a relatively easy harvest and purification process. The current method could also avoid the purification of the aldolase and phosphatase utilized in our in vitro studies and achieve the regeneration of the phosphate. We believe this approach could ultimately contribute to the large-scale synthesis of other carbohydrates and their derivatives.

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EXPERIMENTAL SECTION

Construction of the Recombinant Plasmids pSK-rhaD-yqaB

Primers pSK-rhaD-F and pSK-rhaD-R were used to amplify the gene encoding L-rhamnulose-1-phosphate aldolase with *E. coli* MG1655 as the template. The *rhaD* gene amplified was digested with *BamH*I and *Hind*III, then ligated into pBluescript SK (-) plasmid with the same enzymes digested to generate plasmid pSK-rhaD (Fig. S1). Primers pSK-yqaB-F and pSK-yqaB-R were used to amplify the gene encoding YqaB phosphatase with *E. coli* MG1655 as the template. The *yqaB* gene amplified was digested with *Hind*III and *Xho*I, then ligated into pSK-rhaD plasmid with the same enzymes digested to generate plasmid pSK-rhaD-yqaB (Fig. S1).

Analytical Method

The fermentation progress was monitored by HPLC. Samples were taken at regular intervals to monitor the consumption of D-GA and production of D-sorbose and D-psicose. After centrifugation, the supernatant was applied to HPLC column (Aminex HPX-87H, 300 × 7.8 mm) with 5 mM sulfuric acid as the mobile phase and detected with the Refractive Index Detector. The flow rate was 0.5 mL/min, and the column temperature was 60°C.

General Procedure for the *In Vivo* Synthesis and Purification of D-sorbose and D-psicose

E. coli DH5α was transformed with the plasmid pSK-rhaD-yqaB, resulting in the recombinant strain DH5α-RY, which was used to carry out the in vivo synthesis of D-sorbose and D-psicose. A 4-mL overnight culture of *E. coli* DH5α-RY was inoculated to 200 mL fresh LB medium supplemented with 6.5 g/L glycerol and 100 μg/mL ampicillin, and grown aerobically at 37°C, 225 rpm until the OD_{600} reached 0.8–1.0 ($\sim$3 h). IPTG was thus added at a final concentration of 1 mM to induce the coexpression of RhaD and YqaB for 2–3 h. Then D-GA (0.5 M, filter-sterilized stock solution) was added into the medium with a final concentration of 0.45 g/L. D-GA was added to the medium continuously when consumed as indicated by HPLC. The pH was adjusted to 7.0 using sodium hydroxide during the fermentation process. Upon completion, the fermentation broth was centrifuged at 4,000 $\times$ g for 20 min at 4°C. The supernatant was decolored with activated carbon and concentrated with rotary evaporator under reduced pressure at 40–45°C. The residue was purified by flash silica gel column chromatography (EtOAc/iPrOH/H_2O 9/3/1 (v/v/v)) to afford the crude products as a yellow syrup, which was further purified by P-2 gel filtration. The mixture of D-sorbose and D-psicose could be separated by Ca^{2+} ion exchange resin column (temperature 65°C, ddH_2O as the elutant, flow rate $\sim$1.5 mL/min).

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SUPPLEMENTARY DATA

Supplementary data related to this article can be accessed on the publisher's website.

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