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Single-step bioconversion for the preparation of L-gulose and L-galactose

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ABSTRACT

Both carbohydrate monomers L-gulose and L-galactose are rarely found in nature, but are of great importance in pharmacy R&D and manufacturing. A method for the production of L-gulose and L-galactose is described that utilizes recombinant *Escherichia coli* harboring a unique mannitol dehydrogenase. The recombinant *E. coli* system was optimized by genetic manipulation and directed evolution of the recombinant protein to improve conversion. The resulting production process requires a single step, represents the first readily scalable system for the production of these sugars, is environmentally friendly, and utilizes inexpensive reagents, while producing L-galactose at 4.6 g L^{-1} d^{-1} and L-gulose at 0.90 g L^{-1} d^{-1}.

1. Introduction

Pharmaceutical, food, and agrochemical products depend on the production of optically pure intermediates.[1–4] Carbohydrates are involved in cellular recognition, signaling, extra and intracellular targeting, and even in the development of disease states.[1,4,5] L-Carbohydrates in particular are essential in both pharmaceuticals and biochemical research. For example, L-sugars form the core of many clinically relevant antiviral treatments currently under developement or recently approved by the FDA.[6–8] Access to consistent, optically pure, and inexpensive carbohydrate starting materials are critical to pharmaceutical research, development, and production as well as to the continuation of basic biochemistry research. L-Gulose **1** and L-galactose **2** are sugars found in nature at extremely low frequency. Noteworthy among few examples, **1** is found in a glycolipid of *Thermoplasma acidophilum*[9] and in the potent anticancer compound bleomycin produced by *Streptomyces verticillus.*[10] Similarly, **2** is found in the natural product saponine[11] and as a minor constituent of various biopolymers.[12] Both **1** and **2** have been studied as a precursor in plant vitamin C biosynthesis.[13,14] Thus far only **1** has proven to be a useful starting material for the production of L-nucleoside-based antiviral medications,[15,16] but **2** might also fill this role given a consistent low cost supply. The main limitation to utilization of rare sugars such as **1** and **2** for R&D efforts and basic research is limited availability and extreme cost ($900/g and $240/g estimated, respectively). Herein we describe the production of both **1** and **2** by a method that (1) represents the first readily scalable system, (2) requires a single step, (3) is an environmentally friendly whole-cell bioconversion, and (4) utilizes inexpensive reagents (D-sorbitol **3** and galactitol **4**, respectively).

2. Results and discussion

2.1. Initial attempt at production of 1 and 2

The natural function of the unique NAD-dependent mannitol-1-dehydrogenase (MDH) from *Apium graveolens*[17–23] is oxidation at the 1 position of D-mannitol producing D-mannose as opposed to the more common 2-mannitol dehydrogenase, which interconverts D-mannitol and D-fructose.[17] The novel regioselectivity at the 1 position combined with stereoselectivity at the 2 position allows the MDH enzyme from *A. graveolens* to catalyze several other interesting conversions in vitro including **3** to **1**, **4** to **2**, and ribitol to L-ribose.[21] We previously created a recombinant *Escherichia coli* system expressing this MDH capable of producing >17 g L^{-1} d^{-1} of L-ribose from ribitol in vivo.[24] It would then stand to reason that this same system would readily convert **3** to **1**and **4** to **2**.

However, *E. coli* K12 is not proficient at metabolism of ribitol and does not have a dedicated transferase system for this carbon source, whereas there are specific metabolic pathways for *E. coli* K12 uptake and metabolism of both **3** and **4**. *E. coli* utilizes the phosphotransferase system (PTS) with a specific three-component permease made up of subunits IIA^{srl}, IIB^{srl}, and IIC^{srl} (left side green/cyan, Fig. 1) for the uptake of **3**. This permease combined with components PtsH and PtsI (Fig. 1, labeled H, I in grey) is responsible for the concomitant uptake and phosphorylation of **3** to D-sorbitol-6-phosphate **5**, which can then be oxidized to

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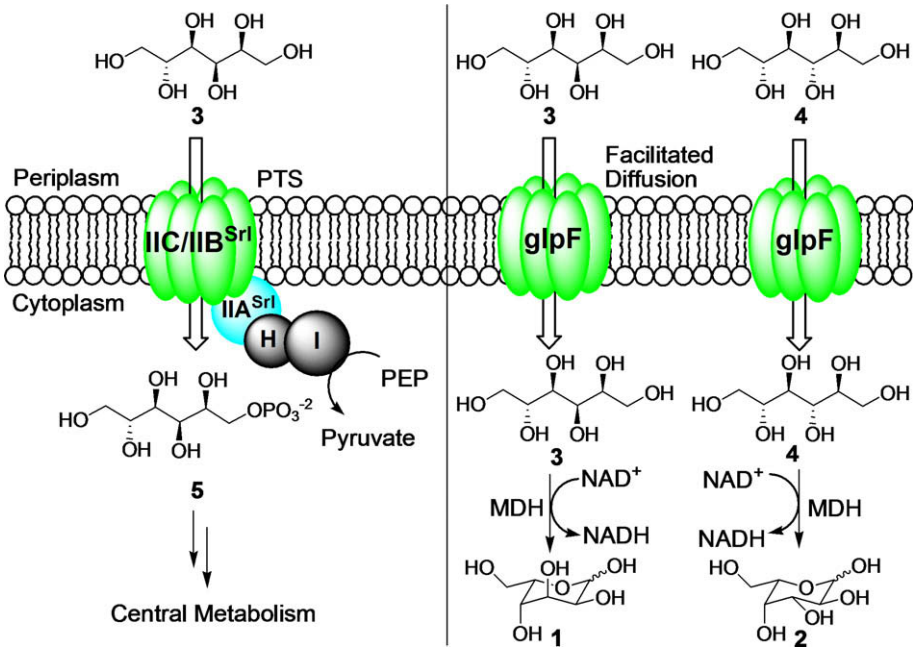

Figure 1. *E. coli* uptake and conversion of D-sorbitol 3 and galactitol 4. Cellular uptake of **3** (and **4**) is normally achieved by the PTS (left side) resulting in intracellular D-sorbitol-6-phosphate **5** (or D-gal-6P, not shown), which is metabolized and not a substrate of recombinant MDH. When the PTS is inactivated, **3** and **4** can diffuse without phosphorylation and be oxidized to **1** and **2**, respectively, by recombinant MDH.

D-fructose-6-phosphate and then enter central metabolism. A homologous system exists for uptake/phosphorylation and metabolism of **4** (not shown for clarity). It was therefore more disappointing than surprising that our recombinant conversion system, when tested in shaken flasks in the presence of **3** or **4**, produced no **1** or **2** as observed by HPLC. The polyols are directed into central metabolism (as evidenced by acid production, Supplementary data) and the phosphorylated intermediates are no longer MDH substrates.

2.2. Phosphotransferase deletion

Since the phosphorylated intermediates of the PTS uptake of **3** and **4** do not appear to be substrates for MDH and metabolism of the starting material is certainly not beneficial, we sought to modify the *E. coli* production strain such that its PTS was inactive. This was accomplished by PCR-based genetic deletion[25] of phosphocarrier protein *ptsH*, phosphotransferase system enzyme I *ptsI*, and the glucose specific enzyme IIA PTS component *crr*.

The resulting *E. coli* strain (Zuc174) was no longer able to metabolize **3** or **4** (Supplementary data) when grown in a flask as indicated by HPLC of spent medium and the absence of acid production due to overflow metabolism. The possible route of entry for **3** and **4** into *E. coli* would now be glycerol facilitator (GlpF), which has been previously shown to accept these polyols (Fig. 1, right).[26] To determine if Zuc174 was capable of taking up and converting **3** or **4**, and exporting products **1** and **2**, it was transformed with a vector for constitutive MDH expression (pTrp-MDH)[24] resulting in *E. coli* Zuc175. Zuc175 was tested for the production of **1** and **2** from **3** and **4**, respectively, in shaken flasks. Media was then analyzed at different time points by HPLC. As shown in Figure 2, Zuc175 was capable of converting **4** to **2**, but not **3** to **1**. This was an extremely encouraging result and the first such production reported in recombinant *E. coli*. However, the conversion (~2%) and volumetric productivity for **2** (0.16 g L^{-1} d^{-1}) were significantly lower than our previously described system for L-ribose production (>50% conversion and >17 g L^{-1} d^{-1})[24] and the production of **1** was not detectable. Therefore, further improvements to this system were sought.

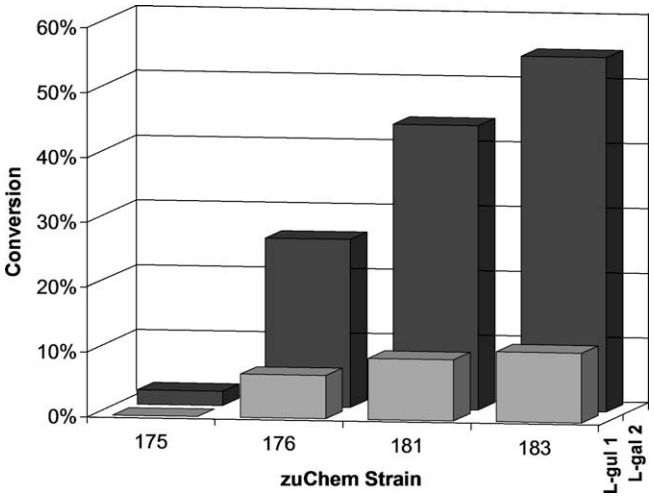

Figure 2. *E. coli* whole-cell production of L-gulose **1** (grey) and L-galactose **2** (black). Bioconversion from 50 g/L **3** or **4** as detected by HPLC at 144 h was carried out by Δ*ptsH-crr* strains expressing recombinant MDH (Zuc175) and MDH mutants (Rnd 1—Zuc176, Rnd2—Zuc181, and Rnd3—Zuc183.

There were two likely issues for the reduced production of **1** and **2** in comparison with that of L-ribose: (1) the transport of **3** and **4** into the cell and the transport of **1** and **2** out of the cell are slower than that of ribitol and L-ribose, respectively, and (2) lower specific activity of recombinant MDH on **3** and **4** as compared to that of L-ribose.[24] Therefore both issues were considered for improvement. Transport was targeted by screening libraries of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG)-mutagenized Zuc175 with the hope of creating a mutant with better polyol permeability. On the other hand, MDH activity was targeted using directed enzyme evolution with random error prone-PCR mutagenesis. In both cases, the resulting libraries were screened using a high