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Metabolic engineering for bioproduction of sugar alcohols

Olubolaji Akinterinwa[1], Reza Khankal[1] and Patrick Carmen Cirino

Sugar alcohols find applications in pharmaceuticals, oral and personal care products, and as intermediates in chemical synthesis. While industrial-scale production of these compounds has generally involved catalytic hydrogenation of sugars, microbial-based processes receive increasing attention. The past few years have seen a variety of interesting metabolic engineering efforts to improve the capabilities of bacteria and yeasts to overproduce xylitol, mannitol, and sorbitol. Examples include heterologous expression of yeast xylose reductase in *Escherichia coli* for the production of xylitol, coexpression of formate dehydrogenase, mannitol dehydrogenase, and a glucose facilitator protein in *Corynebacterium glutamicum* for mannitol production from fructose and formate, and overexpression of sorbitol-6-phosphate dehydrogenase in lactate dehydrogenase-deficient *Lactobacillus plantarum* to achieve nearly maximum theoretical yields of sorbitol from glucose.

Address
Department of Chemical Engineering, Pennsylvania State University, University Park, PA 16802, USA

Corresponding author: Cirino, Patrick Carmen (cirino@engr.psu.edu)
[1] These authors contributed equally to this work.

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Introduction

Sugar alcohols are a class of polyols in which sugar's carbonyl (aldehyde or ketone) is reduced to the corresponding primary or secondary hydroxyl group. They have characteristics similar to sugar and are used to improve the nutritional profile of food products owing to health-promoting properties such as lower caloric content, noncariogenicity, and low glycemic index and insulin response [1,2]. Other auspicious qualities as food additives include high enthalpies of solution and lack of reactive carbonyls. Sugar alcohols additionally find many applications in pharmaceuticals, chemicals production, oral and personal care, and animal nutrition [3]. They are found naturally in fruits and vegetables and are produced by microorganisms, serving as carbohydrate reserves, storage of reducing power, translocatory compounds, and osmoprotectants.

Traditional industrial production of most sugar alcohols is accomplished by hydrogenating sugars over nickel catalysts under high temperature and pressure conditions [2]. Biosynthetic routes offer the potential for safer, environmentally friendly production with enhanced product specificity. Enzyme-based processes for the production of sugar alcohols via sugar reduction have been investigated but are not within the scope of this review. However, costs associated with enzyme preparations and cofactor regeneration for *in vitro* synthesis of sugar alcohols contribute to the general perception that the use of whole cells presents a more attractive biological approach to produce these compounds from crude sugar feedstocks. Here we review recent metabolic engineering efforts to improve microbial production of the common sugar alcohols xylitol, mannitol, and sorbitol.

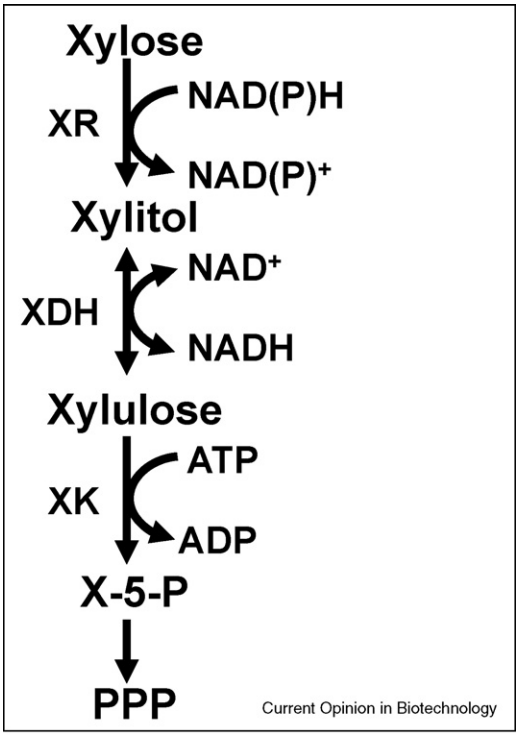
Xylitol

Xylitol is a five-carbon sugar alcohol obtained from xylose reduction. The annual xylitol market is estimated to be \$340 million, priced at \$4–5 kg^{-1} [4]. Xylitol has received the most recent attention of all the sugar alcohols, particularly as it pertains to microbial production and metabolic engineering. As depicted in Figure 1, yeasts naturally produce xylitol as an intermediate during D-xylose metabolism. Xylose reductase (XR) is typically an NADPH-dependent enzyme, while xylitol dehydrogenase (XDH) requires NAD^+ [1]. Cofactor imbalance results in the secretion of xylitol as a xylose fermentation by-product.

Among the yeast strains that naturally produce xylitol, *Candida* sp. have received the most recent attention for developing microbial-based xylitol processes [5,6]. Using a fed-batch submerged membrane bioreactor with cell recycle, *Candida tropicalis* produced 12 $g\,l^{-1}\,h^{-1}$ xylitol from xylose, with glucose as cosubstrate [7]. This is among the highest productivities reported. Ko *et al.* produced xylitol aerobically from xylose with glycerol as a cosubstrate, achieving a volumetric productivity of 3.2 $g\,l^{-1}\,h^{-1}$ and a yield of 0.98 mol xylitol (mol xylose)$^{-1}$ in a XDH gene (*XYL2*)-disrupted mutant of *C. tropicalis* [8]. While *Pichia stipitis* does not naturally overproduce xylitol, mutant strains with disruptions in alcohol dehydrogenase, XDH, or D-xylulokinase have been shown to produce xylitol [9].

Saccharomyces cerevisiae lacks native xylose-specific transporters and is not an efficient xylose-utilizing organism

Figure 1



Pathway for xylose metabolism in yeast. XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulokinase; X-5-P, xylulose-5-phosphate; PPP, pentose phosphate pathway.

[10]. The popularity of *S. cerevisiae* has led to research to improve xylose fermentation by this organism, yielding recombinant strains capable of producing high levels of xylitol [11]. Efforts to increase xylose transport and utilization have included expression of homologous and heterologous transporters [12,13]. While *S. cerevisiae* does possess at least one native aldose reductase capable of reducing xylose (NADPH-dependent GRE3) [14], endogenous expression or activity of this enzyme is apparently insufficient for appreciable xylitol production. Overexpression of endogenous glucose-6-phosphate dehydrogenase (G6PDH) encoded by *ZWF1* to increase NADPH availability in a recombinant strain of *S. cerevisiae* harboring *P. stipitis* XR gene (*XYL1*) [15] resulted in sixfold increased G6PDH activity and increased volumetric xylitol productivity (from 1.6 to 2.0 g xylitol l^{-1} h^{-1}) in glucose-limited, fed-batch cultivations containing xylose [16]. To further increase pentose phosphate pathway (PPP) flux, phosphoglucose isomerase (PGI) activity encoded by *PGI1* was reduced by replacing the *PGI1* promoter with the *ADH1* (encoding alcohol dehydrogenase I) promoter [17•]. Simultaneous overexpression of G6PDH and attenuation of PGI in a recombinant *S. cerevisiae* strain expressing *P. stipitis XYL1* resulted in a specific productivity of 0.34 g xylitol (g cdw h)$^{-1}$ in glucose-limited, fed-batch cultivations, which is 90% higher than the specific productivity of the parent strain expressing only *XYL1* [17•]. Glucose served as the primary source of reducing equivalents for xylose reduction, with 4.2 moles xylitol produced per mole glucose consumed in rich medium.

Xylitol production from glucose was also demonstrated using engineered *S. cerevisiae* [18]. The strain design included deleting transketolase (resulting in accumulation of D-xylulose-5-phosphate and secretion of ribitol and pentose sugars) followed by expressing XDH from *P. stipitis* (*XYL2*) and overexpressing *DOG1* encoding sugar phosphate phosphatase. Ribitol was a coproduct and the total sugar alcohol yield was at most 0.042 mol (mol glucose)$^{-1}$ [18].

Bacteria offer several potential advantages over yeasts for the production of xylitol. Cirino and coworkers recently described approaches to produce xylitol from engineered *Escherichia coli* [19,20•]. The objective was to use glucose metabolism as source of reducing equivalents to drive either direct xylose reduction (via expression of an XR) or conversion of xylose to xylulose (via the native xylose isomerase) followed by reduction to xylitol (via expression of a XDH). A xylulokinase (*xylB*) deletion prevented xylose metabolism. To overcome glucose repression of xylose transporter gene expression, the native *crp* gene was replaced with a catabolite repression mutant (*crp**) [20•]. Several XRs and XDHs were screened in *E. coli*, and the NADPH-dependent XR from *Candida boidinii* (CbXR) allowed for the highest levels of xylitol production in batch cultures (38 g l^{-1} in 46 hours using minimal medium) [20•]. Use of resting cells allowed for a higher yield on reducing equivalents delivered to the reductase reaction: 1.7 moles xylitol were produced per mole glucose consumed in batch culture, compared to 4.7 moles xylitol produced per mole glucose consumed by resting cells in minimal medium (theoretically, ~7–10 moles xylitol can be produced from xylose per mole glucose consumed, depending on the mechanism of xylose transport [19]). The contribution of key enzymes in *E. coli* central metabolism toward NADPH supply for xylitol production was also examined [21]. NADPH availability limited xylitol production in resting cells, and was increased by increasing flux through PPP during glucose metabolism. Excess reducing equivalents in the form of NADH (resulting from glucose oxidation) did not translate into available NADPH for xylitol production [21].

An alternative to the use of *crp** in *E. coli* is plasmid-based overexpression of xylose transporters [19]. Overexpressing the ATP-dependent XylFGH xylose transport system from *E. coli* resulted in an average specific xylitol productivity of 0.33 g (g cdw h)$^{-1}$ in a fed-batch fermentation using mineral salts medium [19]. Alternate approaches to xylitol production from glucose–xylose mixtures (expression of *crp**, *xylE*, or *xylFGH*) were

compared in three common *E. coli* host strains (K-12 strains W3110 and MG1655, and wild-type *E. coli* B), and differences in host strain genetic background were found to significantly impact metabolic engineering strategies [22].

In a similar approach as described above, a xylitol-producing *E. coli* strain was constructed by chromosomal insertion of NADPH-dependent XR from *Kluyveromyces lactis* (*XYL1*) and the *E. coli* D-xylose permease (*xylE*) under the control of an IPTG-inducible promoter [23]. Transcriptome analysis of the strain under conditions where xylitol is produced versus not produced revealed that xylitol production down-regulated 56 genes, which were considered as factors related to reduced NADPH supply. The 56 individual gene deletions were studied and a *yhbC*-deficient strain showed the highest improvements in xylitol production (increasing xylitol productivity from 0.68 to 0.81 $g\,l^{-1}\,h^{-1}$). YhbC is uncharacterized, but is potentially a regulatory factor.

E. coli XylB was shown to phosphorylate xylitol to xylitol phosphate, resulting in inhibited growth on xylose and poor xylitol production [47]. By contrast, xylitol is a poor substrate for the xylulokinase Xyl3 from *P. stipitis*. To construct an *E. coli* strain capable of producing xylitol while metabolizing xylose as a source of carbon and energy, *xylB* was deleted and Xyl3 was expressed instead [47].

XRs naturally show relaxed sugar specificity and are able to reduce L-arabinose. This is problematic when xylitol is the desired product from plant hemicellulose raw materials containing both D-xylose and L-arabinose. Zhao and coworkers characterized an XR from *Neurospora crassa* (NcXR), and showed that it naturally had higher selectivity for D-xylose over L-arabinose (~2.4), compared to several other XRs [24]. Using both error-prone PCR and iterative targeted site-saturation mutagenesis (TSSM), combined with high-throughput screening and a clever genetic selection for specificity toward D-xylose reduction, they were able to enhance the substrate specificity of NcXR to ~16.5, with a moderate loss in catalytic efficiency [25••]. The mutant NcXR also increased selectivity toward xylitol production in recombinant *E. coli* resting cells. Xylitol production by engineered *E. coli* using hemicellulose feedstocks has potential to become a commercially viable process (zuChem; URL: http://www.zuchem.com/).

Using glucose as the energy source, xylitol was produced from xylose in a recombinant *Lactococcus lactis* strain by expressing XR from *P. stipitis* (*XYL1*) [26]. A glucose-limited, fed-batch fermentation produced 2.5 mol xylitol $(\text{mol glucose})^{-1}$ and 1 mol xylitol $(\text{mol xylose})^{-1}$ (xylose was not metabolized) at the rate of 2.7 g xylitol $l^{-1}\,h^{-1}$ over 20 hours. Coexpression of a xylose transporter with XR did not improve xylitol production.

Finally, Povelainen and Miasnikov reported xylitol (and ribitol) production from glucose in engineered strains of *Bacillus subtilis* [27]. Expression of D-xylitol phosphate dehydrogenase (XPDH) from *Lactobacillus rhamnosus* or *Clostridium difficile* in a pentulose-producing mutant of *B. subtilis* (GX7) [28] resulted in xylitol production with a yield of 0.26–0.27 mol xylitol $(\text{mol glucose})^{-1}$ in rich medium containing 10% glucose. Dephosphorylation of pentitol phosphates was presumably owing to an intracellular or membrane-associated sugar phosphate phosphatase [27].

Mannitol

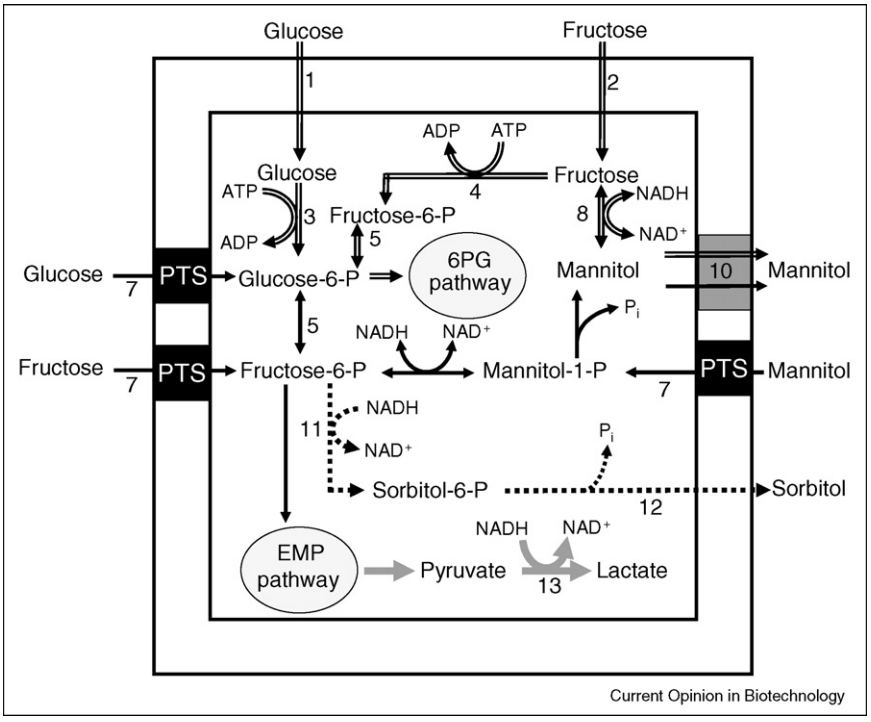
D-Mannitol is a six-carbon sugar alcohol with a variety of clinical applications, in addition to its use as a sweetener. It is produced by a variety of organisms including bacteria and plants, and *Candida magnoliae* has been used for the industrial production of mannitol [29]. Other organisms currently targeted as microbial hosts for mannitol production include lactic acid bacteria (LAB), *E. coli*, *Bacillus megaterium*, *S. cerevisiae*, and *Corynebacterium glutamicum*. Metabolic engineering efforts to improve mannitol production are discussed below.

LAB are industrially important food-grade microorganisms used for the production of food and pharmaceutical products. Heterofermentative LAB naturally produce mannitol by direct reduction of fructose in an NADH-dependent reaction catalyzed by mannitol dehydrogenase (MDH), as shown in Figure 2 [30]. *Lactobacillus intermedius* strain NRR B-3693 [31] growing on high fructose syrup has the potential to become a new commercial process for mannitol production (http://www.confectionerynews.com). Efficient mannitol production by heterofermentative LAB is often achieved when NADH is supplied by coutilization of glucose [32]. Mutants of the heterofermentative LAB *Leuconostoc pseudomesenteroides* with improved mannitol yields have been isolated by chemical mutagenesis [33].

Mannitol can also be produced from a variety of substrates using homofermentative LAB deficient in lactate dehydrogenase (LDH). As shown in Figure 2 for the homofermentative case, fructose-6-phosphate (F6P) is converted to mannitol-1-phosphate (Mtl1P) in an NADH-dependent reaction catalyzed by mannitol-1-phosphate dehydrogenase (Mtl1PDH), and Mtl1P is subsequently dephosphorylated. Dephosphorylation occurs either via mannitol-1-phosphatase [34] or by protein EII^{Mtl} of the phosphoenolpyruvate-mannitol phosphotransferase system (PTS^{Mtl}) [35].

Upon glucose depletion, LDH-deficient *L. lactis* metabolizes previously produced mannitol [36]. This strain was therefore further engineered for mannitol production by inactivating the mannitol transport pathways to prevent mannitol reutilization [37]. Two genes of PTS^{Mtl} (*mtlA*

Figure 2



Pathways for mannitol production in heterofermentative (⟹) and homofermentative (⟶) LAB, and for sorbitol production in *L. casei* and *L. plantarum* (■ ■ ■▶). *Enzymes* — 1: glucose permease; 2: fructose permease; 3: glucokinase; 4: fructokinase; 5: phosphoglucose isomerase; 6: mannitol-1-phosphate dehydrogenase; 7: PEP-dependent PTS; 8: mannitol dehydrogenase; 9: mannitol-1-phosphatase*; 10: unspecified hexitol transport*; 11: sorbitol-6-phosphate dehydrogenase; 12: mechanism unspecified*; 13: lactate dehydrogenase. PTS, phosphotransferase system; 6PG, 6-phosphogluconate; EMP, Embden–Meyerhof–Parnas. *Reactions 9 + 10 and reaction 12 may occur via PTS [35].

and *mtlF*) were independently disrupted. Under growing conditions, the recombinant strains did not produce mannitol but resting cells of the double mutant strains (Δ*ldh*/Δ*mtlA* or Δ*ldh*/Δ*mtlF*) were able to produce mannitol, ethanol, 2,3-butanediol, and lactate as major end products with glucose conversion to mannitol close to 0.33 mol mannitol (mol glucose)$^{-1}$. Production of lactate was attributed to expression of alternate LDH activity. In the absence of NAD^+ regeneration via other fermentation pathways, the maximum theoretical conversion of glucose to mannitol is 0.67 mol mannitol (mol glucose)$^{-1}$ [38].

Another strategy to improve mannitol production in *L. lactis* involved expression of Mtl1PDH from *Lactobacillus plantarum* to promote conversion of F6P to Mtl1P [38]. Conversion of glucose to mannitol was 0.25 mol mannitol (mol glucose)$^{-1}$ in an LDH-deficient strain expressing Mtl1PDH under resting (nongrowing) conditions. Mannitol was not reutilized immediately after glucose depletion and this was attributed to lack of expression of genes involved in mannitol transport and utilization [38]. In a subsequent study mannitol production by *L. lactis* was further improved by expressing mannitol-1-phosphatase from *Eimeria tenella* [34]. A glucose to mannitol conversion of 0.50 mol mannitol (mol glucose)$^{-1}$ was achieved.

E. coli was engineered to produce mannitol from fructose by plasmid-based expression of MDH from *L. pseudomesenteroides* and formate dehydrogenase (FDH) from *Mycobacterium vaccae* N10 [39]. A recombinant oxidation/reduction cycle was established in which NADH produced from formate via FDH was used to drive MDH-catalyzed reduction of fructose to mannitol. The low-affinity glucose facilitator protein from *Zymomonas mobilis* (GLF) was also expressed to enable transport of free fructose into the cell. By employing pH-static conditions (by the addition of formic acid), mannitol productivity of ~4.1 g (g cdw h)$^{-1}$ was obtained after four hours by resting cells (3 g cdw l^{-1} of the recombinant *E. coli*). After eight hours, 66 g l^{-1} mannitol was obtained, with a yield of 0.84 mol mannitol (mol fructose)$^{-1}$. In a subsequent study, mannitol production from glucose was enabled in *E. coli* by additional expression of glucose isomerase [40]. Specific productivities of 0.63 g (g cdw h)$^{-1}$ (plasmid-based expression of glucose isomerase) and 2.1 g (g cdw h)$^{-1}$ (external addition of glucose isomerase) were obtained.

Table 1

Summary of metabolic engineering strategies discussed in this review

Organism	Genetic modifications	Substrate	Yield and/or productivity	Reference
Xylitol				
C. tropicalis	Disrupted *XYL2*	Xylose + glycerol	0.96 mol (mol xylose)$^{-1}$; 3.2 g l^{-1} h^{-1}	[8]
S. cerevisiae	Overexpressed G6PDH, expressed Xyl1, attenuated PGI activity	Xylose + glucose	0.34 g (g cdw h)$^{-1}$	[17•]
E. coli	Expressed *C. boidinii* XR, replaced *crp* with *crp**, deleted *xylB*	Xylose + glucose	4.7 mol (mol glucose)$^{-1}$	[20•]
E. coli	Expressed *C. boidinii* XR, deleted *xylB*, overexpressed XylE or XylFGH	Xylose + glucose	0.33 g (g cdw h)$^{-1}$	[19]
E. coli	Expressed XylE and *K. lactis* XR, deleted *xylA*, *yhbC*-deficient	Xylose + glucose	0.81 g l^{-1} h^{-1}	[23]
L. lactis	Expressed *P. stipitis* XR	Xylose + glucose	2.5 mol (mol glucose)$^{-1}$; 2.7 g l^{-1} h^{-1}	[26]
B. subtilis	Expressed XPDH, deleted *rpi*, transketolase-deficient	Glucose	0.27 mol (mol glucose)$^{-1}$	[27]
Mannitol				
L. lactis	LDH-deficient, disrupted PTS^{Mtl} gene *mtlA* or *mtlF*	Glucose	0.33 mol (mol glucose)$^{-1}$	[37]
L. lactis	Expressed Mtl1PDH and mannitol-1-phosphatase	Glucose	0.50 mol (mol glucose)$^{-1}$	[34]
E. coli	Expressed MDH, FDH, and GLF; glucose isomerase (XylA) expressed on a plasmid or added externally	Formate + glucose	2.1 g (g cdw h)$^{-1}$ (external XylA)	[40]
C. glutamicum	Expressed MDH, FDH, and GLF	Formate + fructose	1.25 g (g cdw h)$^{-1}$	[41•]
B. megaterium	Expressed MDH and FDH	Formate + fructose	0.28 g (g cdw h)$^{-1}$	[42]
Sorbitol				
L. plantarum	Overexpressed Stl6PDH, LDH-deficient	Glucose	0.65 mol (mol glucose)$^{-1}$	[44•]
L. casei	Expressed Stl6PDH, deleted *ldhL*	Glucose	0.04 mol (mol glucose)$^{-1}$	[46]

A similar recombinant oxidation/reduction cycle for the conversion of fructose to mannitol was constructed in the Gram-positive soil bacteria *C. glutamicum* and *B. megaterium* [41•,42]. Resting cells of recombinant *C. glutamicum* strains expressing FDH and MDH showed a specific mannitol productivity of 0.22 g (g cdw h)$^{-1}$. Additional expression of GLF resulted in a fivefold increase in specific D-mannitol productivity (1.25 g (g cdw h)$^{-1}$). To successfully express FDH and MDH in *B. megaterium*, codon optimization of the FDH gene sequence and adaptation of the MDH ribosome binding site were required [42]. Resting cells of *B. megaterium* expressing FDH and MDH showed a specific mannitol productivity of 0.28 g (g cdw h)$^{-1}$ and exhibited high stability in terms of specific productivities and cofactor retention. GLF expression was not required because D-fructose is naturally transported without phosphorylation.

Sorbitol

D-Sorbitol (D-glucitol) is a popular six-carbon sugar alcohol (a stereoisomer of mannitol) with an estimated annual production of over 500,000 tons and finding applications in the food industry and as a building block for pharmaceutical products [3]. Early studies of biotechnological production of sorbitol primarily focused on the bacterium *Z. mobilis*, which naturally can convert fructose and glucose to sorbitol (via glucose–fructose oxidoreductase) for osmoprotection [3,43].

The ability of the fermentative food-grade LAB *L. plantarum* and *Lactobacillus casei* to use different electron acceptors for NAD^+ regeneration via dehydrogenases makes them valuable platforms for polyols production through metabolic engineering [30,35]. Ladero *et al.* produced sorbitol from F6P in an engineered strain of *L. plantarum* by reversing the sorbitol catabolic pathway [44•]. Figure 2 depicts the reactions involved in sorbitol production in this organism. Two native sorbitol-6-phosphate dehydrogenase (Stl6PDH) genes were identified and overexpressed in a mutant strain of *L. plantarum* lacking LDH. Using resting cells with glucose as substrate, the mutant strain produced sorbitol with a yield up to 0.65 mol (mol glucose)$^{-1}$, which is near the maximum theoretical value of 0.67 mol (mol glucose)$^{-1}$ [44•]. Sorbitol was not detected when Stl6PDH was not overexpressed. Mannitol was also produced owing to natively expressed Mtl1PDH activity competing for the common substrate F6P.

In a similar study, a recombinant strain of sorbitol-producing *L. casei* was constructed by integrating a Stl6PDH-encoding gene (*gutF*) [45] into the chromosomal lactose

operon [46]. While the parent strain produced negligible amounts of sorbitol, the engineered strain produced 0.024 mol sorbitol (mol glucose)$^{-1}$ from glucose in resting cells pregrown on lactose. Deletion of lactate dehydrogenase (*ldhL*) increased sorbitol production to 0.043 mol (mol glucose)$^{-1}$, presumably because of elevated NADH/NAD$^+$ ratios. The mechanism of sorbitol-6-phosphate dephosphorylation is unknown, but may occur via a hexitol phosphate-specific EII of the PTS.

Conclusions

Microbial production of xylitol, mannitol, and sorbitol has received considerable attention in recent years, and the metabolic engineering strategies addressed in this review are summarized in Table 1. Although many organisms naturally produce these compounds, genetic modification strategies have allowed for their enhanced production. LAB have been particularly exploited in that regard. Alternately, organisms that do not naturally produce a sugar alcohol have been provided the facilities to do so through metabolic engineering. *E. coli* has proven an effective host organism in that category. As biorefining technology improves and renewable, agricultural-based chemicals continue to gain importance, so will the demand and applications of sugar alcohols grow. Biological production routes are increasingly attractive, and some microbial-based processes for sugar alcohols are already commercially viable.

Acknowledgement

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- • of special interest
- •• of outstanding interest

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