High-Level Production of the Low-Calorie Sugar Sorbitol by Lactobacillus plantarum through Metabolic Engineering

Victor Ladero, Ana Ramos, Anne Wiersma, Philippe Goffin, André Schanck, Michiel Kleerebezem, Jeroen Hugenholtz, Eddy J. Smid, and Pascal Hols

Sorbitol is a low-calorie sugar alcohol that is largely used as an ingredient in the food industry, based on its sweetness and its high solubility. Here, we investigated the capacity of Lactobacillus plantarum, a lactic acid bacterium found in many fermented food products and in the gastrointestinal tract of mammals, to produce sorbitol from fructose-6-phosphate by reverting the sorbitol catabolic pathway in a mutant strain deficient for both L- and D-lactate dehydrogenase activities. The two sorbitol-6-phosphate dehydrogenase (Srl6PDH) genes (srlD1 and srlD2) identified in the genome sequence were constitutively expressed at a high level in this mutant strain. Both Srl6PDH enzymes were shown to be active, and high specific activity could be detected in the overexpressing strains. Using resting cells under pH control with glucose as a substrate, both Srl6PDHs were capable of rerouting the glycolytic flux from fructose-6-phosphate toward sorbitol production with a remarkably high efficiency (61 to 65% glucose conversion), which is close to the maximal theoretical value of 67%. Mannitol production was also detected, albeit at a lower level than the control strain (9 to 13% glucose conversion), indicating competition for fructose-6-phosphate rerouting by natively expressed mannitol-1-phosphate dehydrogenase. By analogy, low levels of this enzyme were detected in both the wild-type and the lactate dehydrogenase-deficient strain backgrounds. After optimization, 25% of sugar conversion into sorbitol was achieved with cells grown under pH control. The role of intracellular NADH pools in the determination of the maximal sorbitol production is discussed.

Obesity is a growing problem in Western countries. Therefore, special diets and dietary ingredients for body weight control are of major interest to the food industry. Belonging to the family of low-calorie sugars, polyols such as mannitol and sorbitol are nonmetabolized sugar alcohols that can replace sucrose or lactose in food products, with a nearly equivalent sweetness and taste (28). Moreover, these compounds have a stabilizing effect on food by partially mimicking fat (7). The range of potential applications of polyols goes far beyond their use as low-calorie sweeteners or texturing agents. For instance, they have been shown to display an in vivo anticariogenic effect (29) since they are not fermented by Streptococcus mutans, the most potent cariogenic bacterium (15). Taking into consideration health benefits and industrial applications, the development of novel dairy products naturally enriched in polyols during fermentation processes offers interesting perspectives (16).

In the context of polyol production, Lactobacillus plantarum possesses some relevant characteristics. It is a food-grade microorganism belonging to the group of lactic acid bacteria. L. plantarum is a normal member of the human intestinal microbiota and can also be isolated from the oral cavity (27, 32). It is largely found as the dominant species in the last step of natural food raw-materi...
tion of other polyols through engineering of these organisms (16, 18).

Sorbitol, also referred to as d-glucitol, is naturally found in many fruits (e.g., berries, cherries, and apples) (3). The worldwide production of sorbitol is estimated to be higher than 500,000 tons/year, and the market is continuously increasing (30). This polyol has a relative sweetness of around 60% compared to that of sucrose and displays a 20-fold higher solubility in water than mannitol (8, 30). Based on these properties, sorbitol is widely used in a range of food products such as confectionery, chewing gum, candy, desserts, ice cream, and diabetic foods. In these products, it fulfills a role not only as a sweetener but also as a humectant, a texturizer, and a softener (8, 30). In addition, sorbitol is the starting material for the production of pharmaceutical compounds such as sorbose and ascorbic acid (3). Several industrial processes have been described for the production of sorbitol (8). However, only a few microorganisms have been suggested as potential sorbitol producers, including three yeast strains and the ethanol-producing bacterium Zymomonas mobilis (19, 30, 31).

Here we describe a metabolic engineering approach to achieving high-level sorbitol production from L. plantarum by reversing the catabolic pathway for sorbitol utilization. Two operons potentially involved in sorbitol catabolism were identified in the genome of L. plantarum (20). The corresponding sorbitol-6P dehydrogenase genes were expressed at a high level, and sorbitol production was evaluated using both resting and growing cells. Analysis of the impact of culture conditions on sorbitol production, such as the carbon source, pH, and aeration, enabled optimization of production, which reached a maximum of 65% of sugar rerouting with resting cells, while a level of 25% was achieved with growing cells.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Escherichia coli TG1 was grown in Luria broth with aeration at 37°C (29). L. plantarum NCIMB8826 (National
were amplified by PCR using the following primer pairs: Stl1Nsi (5′-AGTAGCATAACAGATTGOTTGGG-3′) and Stl1Xba (5′-AGCTGTAGTTGATTATTCACAATACCTC-3′) for srlD1, and Stl2Nsi (5′-TTAGTGCAATACTTGATTATTATACATTGTTGTGATTATTATTCACAATACCTC-3′) and Stl2Xba (5′-TTAGTGCAATACTTGATTATTATTCACAATACCTC-3′) for srlD2, containing NsiI and XbaI restriction sites (underlined). The NsiI/XbaI-digested PCR products were cloned into plasmid pGIZ906 (12), digested with the same enzymes, yielding plasmids pGIVL201 (srlD1) and pGIVL202 (srlD2). In both constructs, the srlD open reading frame was translationally fused with the expression signals of the L. plantarum ldlh, gene. The absence of mutations in the translational fusions was confirmed by DNA sequencing. Both plasmids were transformed in L. plantarum strain NCIBM8826 (wild type) and VL103(ldhL, ldhD). The VL103 strain is a derivative of TF103 (ΔldhL ΔldhD) obtained by removal of the chloramphenicol resistance marker from its genome (V. Ladero, unpublished data).

Enzymatic assays. Cells were grown in MRS broth until mid-exponential phase (an optical density at 600 nm (OD600) of 2.0), harvested by centrifugation, and mechanically broken with glass beads, as previously described (13). Sorbitol-6P oxidation by sorbitol-6P dehydrogenase (SrlBPDH) was determined with sorbitol-6P as a substrate, as reported by Yebra et al. (39). Mannitol-1P dehydrogenase (Mtl1PDH) activity was assayed with mannitol-1P as a substrate, as described by Wisselink et al. (35). Mtl1PDH and Stl6PDH activities were determined from the rate of NADH formation by measuring the absorbance at 340 nm. One unit corresponds to 1 nmol of NAD+ reduced min⁻¹ mg total protein⁻¹. Total protein concentration in the crude cell extracts was measured using the Bradford method (2).

Small-scale cell suspensions without pH control. Cells were grown in MRS medium under microaerobic conditions (static cultures) until mid-exponential phase (OD600 2.0), harvested by centrifugation, and washed twice with 50 mM sugar (glucose, fructose, or an equimolar mixture of both) at an absorbance of 2.0, harvested by centrifugation, and washed twice with either potassium phosphate buffer (50 mM) or Tris-maleate buffer (50 mM), and resuspended in 1/10 the initial culture volume of the washing buffer supplemented with 50 mM sugar (glucose, fructose, or an equimolar mixture of both) at an initial pH ranging from 5.0 to 8.0. After 2 hours of fermentation, culture supernatants were collected and analyzed either by high-performance liquid chromatography (HPLC) or by ¹³C nuclear magnetic resonance (NMR). For ¹³C NMR analyses, the fermentation buffer was supplemented with 30 mM [¹³C]glucose.

Large-scale cell suspensions under pH control for in vivo NMR. Cells were collected at the mid-exponential growth phase, harvested, washed, and resuspended to a protein concentration of approximately 10 mg ml⁻¹ in potassium phosphate buffer (pH 6.5) as described for small-scale suspensions (see above). In vivo NMR experiments were performed under controlled pH (6.5) and gas atmosphere (argon), using the experimental system described previously (23). Twenty or 30 millimolar of [¹³C]glucose was supplied to the cell suspension, and the time course for its consumption and product formation was monitored in vivo. After substrate exhaustion and when no changes in the resonances due to end products were observed, an NMR total extract was prepared as reported previously (24). End products of glucose catabolism were quantified in the NMR total extract by ¹³C NMR, a repetition delay of 60.5 s was used. Carbon chemical shifts were referenced to the resonances of external methanol, designated at 49.3 ppm. ¹H NMR analysis of the fermentation products in total extracts was performed with a Bruker AMX300 spectrometer, using formate as a concentration standard as described by Neves et al. (23).

HPLC analyses. Organic acids were analyzed by HPLC as previously reported (35). Sugars were analyzed by HPLC using a chromatographic system consisting of a precolumn packed with a cation exchange resin, AG50W-X4, 400 mesh (Bio-Rad, Hercules, CA) and AG5-X4A, 200/400 mesh (in a proportion of 35:65). A cation exchanger in a prepacked column (RT 300-7.8 Polyphenyl CHPb, 300 by 7.8 mm; Merck, Darmstadt, Germany). The samples were eluted with an isotropic pump system (Shimadzu Corporation, Kyoto, Japan) using water as the mobile phase. Detection was carried out using a refractive index detector, ERC-7512 (Erma).

RESULTS

Cloning and overexpression of the sorbitol dehydrogenase genes. Glycolytic conversion of the available carbon source by the L. plantarum mutant strain deficient for both L-LDH and D-LDH generates an excess of NADH that is dissipated by the activation of metabolic routes capable of NADH oxidation in order to maintain an equilibrated redox balance (Fig.1A) (10). Although many activated metabolic routes use pyruvate as the initial substrate, production of manniol from fructose-6-phosphate (fructose-6P) opens the possibility of producing other related compounds from this glycolytic intermediate. One such possibility is the overproduction of sorbitol-6P dehydrogenase, with the aim of deviating the glycolytic flux from fructose-6P toward the end product sorbitol, while at the same time providing an additional NADH sink (Fig.1A).

The genome of L. plantarum WCFS1 (a single-colony isolate of strain NCIBM8826) contains two putative operons that could be involved in sorbitol catabolism (20). A sequence comparison of the two operons revealed a 65% identity at both the DNA and the protein levels. Both sorbitol operons have highly similar genetic organizations. The first gene (srlD) encodes sorbitol-6P dehydrogenase, followed by two regulatory genes (srlR and srlM) that encode a putative repressor and activator, respectively, and the components of a complete phosphotransferase sugar uptake system (pts37 and pts38, components IIa, IIB, and IIC, encoded by separate genes). Both srl operons are preceded by a putative promoter sequence and are enclosed by predicted transcription termination sequences (Fig.1B).

In order to evaluate their function as specific sorbitol-6P dehydrogenases, the two srlD coding regions were constitutively overexpressed by translational fusion to the strong expression signals of the L. plantarum ldhL gene (plasmids pGIVL201 and pGIVL202, containing srlD1 and srlD2, respectively). The recombinant plasmids were introduced into L. plantarum NCIBM8826 (wild type) and its LDH-deficient derivative, VL103. Stl6PDH overproduction was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein gel showed an additional band with the expected molecular mass of 29 kDa in crude cell extracts of VL103 (pGIVL201) and VL103(pGIVL202) that was absent from crude extracts of the control strain containing the empty overexpression vector [VL103(pGIZ906)] (data not shown). In order to confirm Stl6DH overproduction, Stl6PDH-specific activity was measured in crude extracts of the recombinant strains. High Stl6PDH-specific activity levels were detected in the overexpressing strains VL103(pGIVL201) (250.6 U/mg to...
Sorbitol and mannitol were produced from glucose by small-scale cell suspensions of *L. plantarum* strains NCIMB8826(pGIZ906), VL103(pGIZ906), VL103(pGIVL201), and VL103(pGIVL202). Samples were collected after 2 h of incubation at 37°C under low aeration and without pH control (initial glucose concentration, 50 mM; initial pH, 5.5).

The data in Table 1 show that sorbitol production using cell suspensions without pH control. In order to gain better insights into the kinetics of sugar consumption and formation of fermentation end products, a range of fermentations with large variations in parameters such as the initial pH and the carbon source (glucose, fructose, and an equimolar mixture of both) were performed.

**TABLE 1. Production of sorbitol and mannitol from glucose by small-scale cell suspensions**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose consumed (mM)</th>
<th>Sorbitol (mM)</th>
<th>Mannitol (mM)</th>
<th>% of sorbitol</th>
<th>Polyol activity*</th>
<th>Polyol activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCIMB8826(pGIZ906) (wild type)</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>135.0 ± 3.0</td>
</tr>
<tr>
<td>VL103(pGIZ906) (∆ldhL, ∆ldhD)</td>
<td>18.5</td>
<td>ND</td>
<td>ND</td>
<td>79 (42.3)</td>
<td>ND</td>
<td>157.0 ± 3.0</td>
</tr>
<tr>
<td>VL103(pGIVL201) (∆ldhL, ∆ldhD)</td>
<td>19.6</td>
<td>5.2 (26.7)</td>
<td>2.0 (10.1)</td>
<td>72</td>
<td>250.6 ± 15.9</td>
<td>145.0 ± 5.0</td>
</tr>
<tr>
<td>VL103(pGIVL202) (∆ldhL, ∆ldhD)</td>
<td>37.6</td>
<td>5.5 (14.5)</td>
<td>2.0 (5.4)</td>
<td>73</td>
<td>459.0 ± 31.7</td>
<td>119.0 ± 15.0</td>
</tr>
</tbody>
</table>

* Sorbitol and mannitol were produced from glucose by small-scale cell suspensions of *L. plantarum* strains NCIMB8826(pGIZ906), VL103(pGIZ906), VL103(pGIVL201), and VL103(pGIVL202). Samples were collected after 2 h of incubation at 37°C under low aeration and without pH control (initial glucose concentration, 50 mM; initial pH, 5.5).

** Data are representative of at least two experiments. Values in parentheses are percentages of glucose converted to the product. The percentage of sorbitol is from total polyol, ND, not detected.

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**Fig. 2.** 13C NMR spectra of fermentation products from supernatants of small-scale cell suspensions (Tris-malate buffer; 50 mM; initial pH, 5.5) of the VL103(pGIVL201) strain performed in the presence of 30 mM [1-13C]glucose under low (A) and high (B) aeration. Samples were analyzed after 2 hours of fermentation at 37°C. Spectra are presented from 10 to 95 ppm. x denotes an unidentified compound.
scale cell suspensions was performed under pH control (pH 6.5) with continuous measurement of metabolites by 13C NMR (in vivo NMR).

The glucose consumption rate of the wild-type strain was very high (0.08 mol min⁻¹ mg⁻¹ dry weight) in comparison to that of the three LDH-deficient strains, which metabolized glucose at a rate of 0.01 mol min⁻¹ mg⁻¹ dry weight (data not shown).

In the wild type, [1-13C]glucose catabolism by resting cells under pH control resulted almost exclusively in lactate production, with only minor amounts of acetate and succinate (Table 2 and data not shown). For strain VL103(pGIZ906), [1-13C]glucose (20 mM) was fermented with a mixture of 2,3-butanediol (8.8 mM), acetoin (2.6 mM), mannitol/mannitol-1P (4.0 mM), ethanol (4.8 mM), acetate (2.6 mM), and minor amounts of succinate (1.8 mM) and lactate (0.7 mM) (Table 2). For strain VL103(pGIVL202), the kinetics of glucose consumption and product formation are shown in Fig. 3. Similar results were obtained with VL103(pGIVL201) (data not shown). With VL103(pGIVL202), the major fermentation end products formed from [1-13C]glucose (20 mM) were sorbitol (13.1 mM) and acetoin (6.6 mM) (Fig. 3A), while minor amounts of mannitol/mannitol-1P (2.7 mM), acetate (1.8 mM), ethanol (0.7 mM), lactate (0.5 mM), succinate (0.3 mM), and pyruvate (0.3 mM) were detected (Fig. 3B and Table 2). The resonances of [1-13C]mannitol and [1-13C]mannitol-1P overlap in the in vivo 13C NMR spectra, and therefore their individual concentrations could not be calculated (24).

Comparison of the end-product formations measured by in vivo NMR for VL103(pGIVL201) and VL103(pGIVL202) revealed very similar profiles in which sorbitol (61 to 65% glucose rerouting) and acetoin (32 to 33%) were the major fermentation products (Table 2). In contrast, strain VL103 (pGIZ906) produced a considerable amount of mannitol (20%) but did not display sorbitol production, which corroborates the observations with small-scale cell suspensions (Table 2). In the latter strain, the redox balance appeared to be maintained by the production of mannitol, 2,3-butanediol, and, to a lesser extent, ethanol. In strains VL103(pGIVL201) and VL103(pGIVL202), the NADH used to produce sorbitol via the Stl6PDH was derived mainly from the carbon that flowed through the glycolytic pathway, leading to 2,3-butanediol and ethanol (Table 2).

Optimization of sorbitol production with growing cells. In order to optimize sorbitol production with growing cells, different fermentations were performed under pH-controlled conditions (pH 6.5). Using modified MRS supplemented with 2% glucose, the final cell yield obtained with srlD-overexpressing strains was 36% relative to that of the LDH-positive wild type but equivalent to the LDH-deficient parental strain.

### Table 2. End-product amounts from large-scale cell suspensions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose consumption (mM)</th>
<th>End-product amounts (mM)</th>
<th>Carbon balance (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sorbitol</td>
<td>Mannitol/ mannitol-1P (%)</td>
<td>Acetoin</td>
</tr>
<tr>
<td>NCIMB8826(pGIZ906)</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VL103(pGIZ906)</td>
<td>20</td>
<td>ND</td>
<td>4.0 (20.0)</td>
</tr>
<tr>
<td>VL103(pGIVL201)</td>
<td>20</td>
<td>12.2 (61.0)</td>
<td>1.8 (9.0)</td>
</tr>
<tr>
<td>VL103(pGIVL202)</td>
<td>20</td>
<td>13.1 (65.5)</td>
<td>2.7 (13.5)</td>
</tr>
</tbody>
</table>

*End-product concentration values (mM) from [1-13C]glucose (mM) fermentation as determined by 1H and 13C NMR analyses from large-scale cell suspensions under controlled conditions (pH 6.5, 30°C, argon atmosphere) of L. plantarum strains NCIMB8826(pGIZ906), VL103(pGIZ906), VL103(pGIVL201), and VL103(pGIVL202). Samples were collected after glucose exhaustion. Data are representative of at least two experiments.

Values in parentheses are percentages of glucose converted to the product. ND, not detected.

#### Fig. 3. Kinetics of glucose consumption and end-product formation by cell suspensions (30°C, controlled pH 6.5, argon atmosphere) of strain VL103(pGIVL202) as determined by in vivo 13C NMR using 20 mM [1-13C]glucose as a substrate. (A) Major metabolites were sorbitol and acetoin. (B) Minor metabolites were mannitol/mannitol-1P (Mtl-1P), succinate, pyruvate, acetate, lactate, and ethanol.
The generation time of the three LDH-deficient strains (between 120 and 144 min) was twofold higher than that of the LDH-positive wild type (57 min). Sorbitol production of strains VL103(pGIVL201) and VL103(pGIVL202) was evaluated with modified MRS supplemented with 2% each of different carbon sources (glucose, fructose, a mixture of glucose and fructose, maltose, and sucrose) (Table 3). The relative conversion rate of the available carbon source to sorbitol from strain VL103(pGIVL201) appeared to be consistently higher than that from strain VL103(pGIVL202). Both strains produced the highest sorbitol levels when grown on maltose. VL103(pGIVL201) could convert up to 5.5% of the maltose consumed into sorbitol (6.0 mM) (Table 3). Besides sorbitol, these strains also produced minor amounts of mannitol. VL103(pGIVL202) converted 3.4% of the maltose consumed to mannitol (3.7 mM), while in VL103(pGIVL201), mannitol production levels appeared to be lower (1.1 mM) (Table 3).

For all fermentations performed during the optimization procedure, the formation of other fermentation end products was monitored. The metabolic end-product profiles showed that part of the acetate that was present in modified MRS was consumed, while concomitant and equimolar production of ethanol was observed (Fig. 4A). Similar results were previously reported in an LDH-deficient strain of *L. lactis* that converted acetate to ethanol via acetyl-P and acetyl coenzyme A (Fig. 1A), thereby contributing to the redox balance via NADH consumption (17). Since the electron sink provided by acetate conversion to ethanol could reduce the efficiency of polyol production, the effect of acetate on sorbitol production in strain VL103(pGIVL201) was investigated using MRS – Ac supplemented with various sugars (Table 3 and Fig. 4B). Notably, higher biomass yields were obtained for all carbon sources analyzed when acetate was omitted from the media, with the exception of maltose (Table 3 and Fig. 4B). With maltose as the carbon source, sorbitol production was more than fourfold higher in MRS – Ac compared to that in MRS with acetate. Analogously, fermentations performed in the absence of acetate dramatically improved sorbitol production for all carbohydrate sources evaluated (Table 3). Sorbitol and mannitol formation during the course of the fermentation on MRS – Ac supplemented with 2% maltose was monitored.

**Table 3. Influence of the carbon source and acetate on the production of polyols during fermentations performed with growing cells**

<table>
<thead>
<tr>
<th>Carbon source(s)</th>
<th>Maximum polyol formation (mM) from strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VL103(pGIVL201)</td>
</tr>
<tr>
<td></td>
<td>Sorbitol</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td>Glucose + fructose</td>
<td>1.2 (1.1)</td>
</tr>
<tr>
<td>Fructose</td>
<td>ND</td>
</tr>
<tr>
<td>Maltose</td>
<td>6.0 (5.5)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.8 (0.7)</td>
</tr>
</tbody>
</table>

a Data show influences of the carbon source and acetate on the production of polyols (sorbitol and mannitol) during fermentations performed with growing cells of *L. plantarum* strains VL103(pGIVL201) and VL103(pGIVL202). Cells were grown in reconstituted MRS broth in the presence or absence of acetate at 37°C under low aeration (120 rpm) and pH control (pH 6.5) conditions.

b Glucose + fructose, equimolar mixture.

c Values in parentheses are percentages of sugar(s) converted to the product. The maximum (max) value of OD<sub>600</sub> was measured at the stationary growth phase. ND, not detected.

d Grown in modified MRS broth without acetate supplemented with 2% (wt/vol) sugar.
TABLE 4. Influence of pH on the maximal production of polyols during fermentation with growing cells

<table>
<thead>
<tr>
<th>pH value</th>
<th>Maximum polyol formation (mM)</th>
<th>OD_{600} max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sorbitol</td>
<td>Mannitol</td>
</tr>
<tr>
<td>5.5</td>
<td>6.1 (5.6)</td>
<td>0.05 (0.05)</td>
</tr>
<tr>
<td>6.0</td>
<td>6.3 (5.7)</td>
<td>0.6 (0.55)</td>
</tr>
<tr>
<td>6.5</td>
<td>16.0 (14.5)</td>
<td>0.2 (0.15)</td>
</tr>
<tr>
<td>7.0</td>
<td>27.7 (25.2)</td>
<td>4.8 (4.4)</td>
</tr>
</tbody>
</table>

* Data show the influence of pH on the maximal production of polyols (sorbitol and mannitol) during fermentation experiments performed with growing cells of *L. plantarum* VL103(pGIVL201). Cells were grown in modified MRS medium supplemented with maltose (2% wt/vol) in the absence of acetate at 37°C under low aeration (120 rpm) and pH control.

* Values in parentheses are percentages of maltose converted to the product.

* Maximum (max) values of OD_{600} were measured at the stationary growth phase.

(FIG. 4B). Sorbitol production appeared to start at the end of the exponential growth phase and coincided with the time at which more than 75% of the available maltose had been consumed.

For further optimization, a range of pH values (5.5, 6.0, 6.5, 7.0 and 7.5) was evaluated using the most effective sorbitol production strain, VL103(pGIVL201), in MRS – Ac supplemented with 2% maltose. This strain was unable to grow at pH 7.5, and the highest sorbitol production was observed at pH 7.0. Under these conditions, some mannitol was also detected (Fig. 4C and Table 4), but the production was largely in favor of sorbitol (up to 87% of total polyols). Notably, considerable amounts of mannitol (20% rerouting) were produced by the parental LDH-deficient strain VL103, while sorbitol production depended strictly on the plasmid-based expression of the Stl6PDH enzyme. By analogy, no intrinsic Stl6PDH activity could be detected, suggesting tight control of *srlD* expression, which may involve one or more of the two putative transcription regulators that are present in both *srl* operons and/or the *milR*-encoded transcriptional regulator identified in the mannitol catabolic operon (20). Interestingly, inactivation of the *ldh* gene in *L. lactis* resulted in an enhanced *Mtl1PDH* activity (24), while similar levels of *Mtl1PDH* activity were present in all *L. plantarum* strains used in this study, including the wild-type strain. This observation suggests that mannitol production is not subjected to a strict control, which is in apparent contrast to sorbitol production.

Interestingly, production of sorbitol and/or mannitol by cell suspensions was not observed under conditions of strong aeration. Since sorbitol production depends strictly on the availability of NADH as a cofactor, this effect is most likely explained by oxidation of the NADH pool by the NADH oxidase in the presence of molecular oxygen (NADH-oxidase reaction: \( \text{O}_2 + \text{NADH} \rightarrow 2\text{H}_2\text{O} + \text{NAD}^+ \)). A similar effect of high aeration was previously shown to strongly reduce mannitol production by an LDH-deficient strain of *L. lactis*, which was suggested to be the consequence of NADH oxidase activation (24). Analogously, NADH oxidase activity is known to be strongly induced in *L. plantarum* under aerobic conditions (22) and effectively dissipates NADH in the presence of molecular oxygen and thereby interferes with polyol production via the *Mtl1PDH* and *Stl6PDH* enzymes by competing for their mutual cofactor NADH (Fig. 1A). The importance of the availability of high levels of NADH for the production of sorbitol was corroborated by the negative effect of NAD\(^+\) regeneration via the acetate-to-ethanol conversion, which was observed for growing cells. Taken together, these observations indicate that a relatively high level of NADH accumulation is a prerequisite for activation of the *Stl6PDH* enzyme and sorbitol formation.

Although a reasonably high level of sugar rerouting toward polyol (sorbitol and mannitol) was achieved with growing cells (up to 30%), this level is significantly lower than the maximal rerouting level obtained with resting cells, which corresponds to the theoretical maximum percentage of conversion (67%) (36). This difference may be caused by a higher ATP demand for biomass production in growing cells (21). In resting cells as well as in the stationary phase of growth, NAD\(^+\) regeneration and maintenance of redox balance probably exert a more dominant metabolic control than ATP generation.

The high rerouting levels obtained show that *L. plantarum* is a promising candidate host for polyol production. By comparison, higher mannitol production levels (50%) were recently reported with growing cells of *L. lactis* (36). However, the
metabolic engineering strategy employed in that study was relatively complicated and included multiple gene overexpressions and deletions, which were required to avoid mannitol consumption and to increase mannitol-1P dephosphorylation (11, 35, 36). Notably, such complex engineering strategies are not required to achieve relatively effective polyol production in L. plantarum. Nevertheless, a high capacity for polyol production does not seem to be general among lactobacilli, since only low levels of sorbitol production (3% compared to 65% in L. plantarum) were recently obtained with resting cells of Lactobacillus casei using a similar strategy (26).

Our results show that metabolic engineering of L. plantarum for high sorbitol production was successfully achieved by a simple two-step strategy that does not require any heterologous gene expression. However, the use of L. plantarum as a cell factory for polyol production at this stage would be restricted to that of a resting cell bioreactor, while production in growing cultures would require further optimization of conversion efficacy. Nevertheless, considering the consumer health-related properties of sorbitol, the moderate level of polyol production obtained here offers opportunities for the future use of L. plantarum for in situ sorbitol production in fermented food products, since in that case, a high efficient polyol production would not be necessarily required.

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References


