Metabolic Engineering of *Lactobacillus fermentum* for Production of Mannitol and Pure L-Lactic Acid or Pyruvate

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Abstract: For production of mannitol in combination with pure L-lactic acid or pyruvate, the D- and L-lactate dehydrogenase genes (*ldhD* and *ldhL*) of a mannitol-producing *Lactobacillus fermentum* strain were cloned and stepwise inactivated. For inactivation of both *ldh* genes by a gene replacement technique, deletion constructs removing a 0.4-kb fragment from the promoter and the 5′ end region of the *ldh* genes were used. The first inactivation mutant, designated *L. fermentum* GRL1030, carried the deletion in *ldhD* (Δ*ldhD*). A double mutant, Δ*ldhD*-Δ*ldhL*, was constructed by the inactivation of the *ldhL* gene of strain GRL1030, resulting in strain *L. fermentum* GRL1032. The correctness of the both mutants was confirmed at the DNA level by polymerase chain reaction, as shown by the absence of *ldh* transcripts by northern blotting and as a lack of the corresponding enzyme activity. In bioreactor cultivations, the single mutant GRL1030 produced mannitol and L-lactic acid as expected. Mannitol and lactic acid yields and productivities were practically unaffected by deletion of the *ldhD* gene. The double mutant GRL1032 produced mannitol and pyruvate as expected. However, although the yield of mannitol from fructose remained high, its volumetric productivity was reduced. The double mutation negatively affected the glucose consumption rate, resulting in reduced cellular growth. In addition to pyruvate, the double mutant produced 2,3-butanediol. More surprisingly, some lactic acid was still produced. *Biotechnol Bioeng* **82**: 653–663, 2003.

Keywords: mannitol; mannitol dehydrogenase; metabolic engineering; lactate dehydrogenase; lactate; pyruvate

INTRODUCTION

D-Mannitol is a sugar alcohol — a hydrogenated form of fructose. It is produced commercially in large quantities by catalytic hydrogenation from glucose/fructose mixtures, and is used in the food and pharmaceutical industries and as medicine. Glucose and β-fructose are converted in the chemical hydrogenation process into sorbitol, whereas α-fructose is converted into mannitol (Soetaert et al., 1999). Thus, sorbitol is the main product in the chemical hydrogenation process and the yield of mannitol is only around 25% to 30% from the total sugars. Mannitol is recovered from the mixture either by chromatographic separation or directly by crystallization (Soetaert et al., 1999) due to its low solubility (180 g/L) in water compared with sorbitol (700 g/L) (Schiweck et al., 1994).

Many microorganisms, such as fungi (Hendriksen et al., 1988; Smiley et al., 1967), yeasts (Onishi and Suzuki, 1970), and especially heterofermentative lactic acid bacteria (LAB) (Soetaert, 1990), are known to produce mannitol from various carbon sources. Certain LAB can convert fructose almost quantitatively to mannitol when glucose is used as a cosubstrate (Korakli et al., 2000). Figure 1 shows how 1 mol of glucose is converted to lactic acid, acetic acid, and CO_2. Two moles of NADH are produced, which, in the presence of 2 mol fructose, is used by mannitol dehydrogenase to produce 2 mol mannitol. We have recently compared different mannitol-producing LAB (von Weymarn et al., 2002a) and optimized mannitol production in a membrane cell-recycle bioreactor using *Leuconostoc mesenteroides* (von Weymarn et al., 2002b).

In addition to mannitol, heterofermentative LAB produce a mixture of D- and L-lactate, acetate, and some ethanol. Commercial use of this acidic mixture is difficult because separation of the acids is a tedious process. In this investigation we approached the problem and aimed to develop a two-product concept. We first developed genetic tools to modify *L. fermentum*, which, according to our previous work (von Weymarn et al., 2002a), is a good mannitol producer. We then proceeded to inactivate first the *ldhD* gene

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and then the *ldhL* gene using principles taken from our previous work (Kylä-Nikkilä et al., 2000). With these gene inactivations we sought to create an organism that could produce mannitol and either pure L-lactate or pyruvate in a single process. Industrially, L-lactate is used in the production of, for example, valuable synthetic biopolymers, whereas pyruvate is used in the biosynthesis of pharmaceuticals such as L-tryptophan, L-tyrosine, and alanine (Li et al., 2001). Pyruvate is also used as an antioxidant, a fat-reducing agent, and in the production of polymers and cosmetics. We report the results of our conceptually and commercially interesting study.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All microbial strains were maintained in standard Lactobacilli MRS (Difco Laboratories, Detroit, MI) or GM17 growth medium supplemented 15% (v/v) with glycerol and stored at −80°C. The GM17 medium is standard M17 medium (Difco) containing 0.5% glucose. *L. fermentum* NRRL-B-1932 was grown in MRS medium at 30° to 42°C and *Lactococcus lactis* GRL71 in GM17 medium at 30°C.

Basic DNA Techniques, Transformation Methods, and Oligonucleotides

Basic molecular biology techniques were used essentially as described by Sambrook and Russel (2001). *L. fermentum* chromosomal DNA was isolated from protoplasts as follows: Cells grown to the early stationary phase were harvested by centrifugation (10,000*g*, 10 min), washed with phosphate-buffered saline (PBS), resuspended in a protoplasting buffer (10 m*M* Tris/HCl [pH 7.3], 20% sucrose, 10 m*M* $MgCl_2$, 450 U/mL mutanolysin, 2 mg/mL lysozyme, and 1 mg/mL proteinase K), and incubated at 37°C for 1 h, followed by harvesting of protoplasts by centrifugation (15,000*g*, 5 min) and isolation of DNA with the Nucleobond AX Kit (Macherey-Nagel, Germany). The Vectorette II System (Sigma Genosys, Ltd., UK) was used to create a genomic library of *L. fermentum*. Chromosomal DNA of *L. fermentum* was extensively digested either with *Cla*I, *Eco*RI, or *Hin*dIII, and ligated with the respective Vectorette units. Vectorette amplicons were sequenced by the dideoxy chain termination method of Sanger et al. (1977) using an ABI-Prism 310 Genetic Analyzer (Applied Biosystems, Inc.) with an ABI-Prism BigDye Terminator (v2.0) Cycle Sequencing Kit (Applied Biosystems). DNA sequence analyses were performed using the SEQUENCER 3.0 program (Gene Codes Corp.). Oligonucleotides used in this study are listed in Tables Ia and Ib.

The transformation procedure described for *L. fermentum* (Wei et al., 1995) was further optimized as follows. A 2% inoculum of an overnight culture was grown in MRS medium supplemented with 1% glycine at 37°C until the OD_{660} of the culture was 0.2 to 0.3. The cells were harvested and washed twice with cold washing buffer (5 m*M* sodium phosphate [pH 7.4] and 1 m*M* $MgCl_2$). The cells were then resuspended to 1% of the original culture volume in a cold electroporation buffer (0.9% sucrose, 3 m*M* $MgCl_2$). For electroporation, 45 μL of the cell suspension was mixed with 50 to 500 ng of plasmid DNA and subjected to a 2.5-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette by using a Genepulser II electroporation system (Bio-Rad Laboratories). After the pulse, 450 μL of cold MRS was immediately added to the cell suspension, kept on ice for 10 min, and incubated for 1 h at 30°C. Cells were then induced with 0.01 μg/mL erythromycin for 2 to 16 h at 30°C, followed by plating onto MRS agar plates containing 5 μg/mL erythromycin. The transformation procedure for *L. lactis* was performed essentially as described by Holo and Nes (1989).

RNA Isolation and Northern Hybridization

For isolation of total RNA, *L. fermentum* cells were grown at 37°C to an OD_{600} of 1.0. Cells (3 mL) were harvested, washed with water, and disrupted using glass beads in a Vibrogen cell mill (Edmund Bühler) for 1.5 min at 4°C. Total RNA was isolated by the RNeasy Mini-Kit (Qiagen, Germany). For northern blot, RNA (6 μg) was denatured at 75°C for 5 min (Pelle and Murphy, 1993) and run on a 1% agarose gel with 10 m*M* phosphate buffer (pH 6.8). The gel was blotted onto a positively charged nylon membrane (Boehringer Mannheim, Germany) and hybridized with [α-^{33}P]dCTP-labeled *ldhD*- or *ldhL*-specific polymerase chain reaction (PCR) probes (1080/1098 and 1096/1097, respectively; Tables Ia and Ib) at 50°C for 18 h. Washing of the filter was done twice in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 0.5× SSC and 0.1% SDS at 68°C for 15 min, followed by detection in a molecular imaging system (Model GS-525, Bio-Rad).

Construction of *L. fermentum* Δ*ldhD* and Δ*ldhD*-Δ*ldhL* Mutants

Integration vectors for gene inactivations were constructed essentially as described by (Kylä-Nikkilä et al. (2000). The inactivation construct for *ldhD* was synthesized by PCR amplifications of a 0.6-kb region upstream of the *ldhD* gene with primers 1077 and 1078 (Table Ia) and of a 0.6-kb fragment from the 3′ end region of *ldhD* with primers 1079 and 1080 (Table Ia). These PCR products were ligated at their *Hin*dIII sites, and the hybrid formed was PCR amplified with primers 1077 and 1080 and ligated as an *Xba*I–*Eco*RI fragment with pG^+host4. The recombinant plasmid formed was designated as pKTH5096 (Fig. 2a) and used to transform *L. lactis*, resulting in the strain *L. lactis* GRS1059.

The inactivation construct for *ldhL* was carried out in a manner similar to that of *ldhD*. The primer pairs used for the amplification of a 0.7-kb fragment from the *ldhL* upstream region and a 0.5-kb 3′ end region of *ldhL* by PCR were 1126/1127 and 1128/1129 (Table Ib), respectively. The

Table Ia. Primers used in this study: *ldhD* primers and pG$^+$host4 primers.

Primer	Sequence (5′ → 3′)	Specific use/restriction site
662	GGTGTGCTTAAATCGGGC	pG$^+$host4 primer
744	ATTTATACTGCAATCGGATGCG	pG$^+$host4 primer
745	CAGGGATAGACTGTAACATTCTCACG	pG$^+$host4 primer
867	CAATAATCGCATCCGATTGC	pG$^+$host4 primer
951	GTITAYCARCARCTIGAYTAYAC	Degenerated primer for *ldhD*
952	TIAGIAAYGTIGGIGTIGAYAA	Degenerated primer for *ldhD*
953	GTRTARAAIGCIGTRTGIGGIGT	Degenerated primer for *ldhD*
1004	GGGTCACATCGGTCAAGTCTTC	Sequencing of *ldhD*
1005	CGTCATTGATCATGTGGATCG	Control for Δ*ldhD*
1035	GCAGCTGGATTACACCGC	Sequencing of *ldhD*
1036	CAAGGAAACGTAGTAGCCC	Sequencing of *ldhD*
1048	CGTGTTCAGCGATGGCGTTCG	Control for Δ*ldhD*
1049	GCTGTGACGGAACTACTGTCGG	Sequencing of *ldhD*
1050	GCCAAGCTGACGTACATGTTACAGG	Deletion primer for *ldhD*
1077	TGCATCTAGACCTCTAGTGAATCAGTCGGC	Cloning of *ldhD*/*Xba*I
1078	GAGAAAGCTTGCTGGTGGAAGCTAGGTACG	Cloning of *ldhD*/*Hin*dIII
1079	CCGAAAGCTTCGTTACTAAGATGTCCTTGCG	Cloning of *ldhD*/*Hin*dIII
1080	CAGGGAATTCCTTACCTTGCC	Cloning of *ldhD*/*Eco*RI
1095	CGTCGGGCACTTCAACG	Control for Δ*ldhD*
1098	CGTTACTAAGATGTCCTTGCG	Primer for *ldhD* specific probe

Table Ib. Primers used in this study: *ldhL* primers.

Primer	Sequence (5′ → 3′)	Specific use/restriction site
954	GTIATITTIGTIGGIGAYGGIGCIGTIGG	Degenerated primer for *ldhL*
957	CCRTGYTCICCCATIATRTAIGC	Degenerated primer for *ldhL*
960	GGIGCICCICARAARCCIGGIGARACICG	Degenerated primer for *ldhL*
961	TCIAGIGAIGTICCIGAICCIATIACICG	Degenerated primer for *ldhL*
962	IATIGGRTARAAIGTIGCICCYTT	Degenerated primer for *ldhL*
1006	CGTCTGGACCTCGTTGACAAG	Sequencing of *ldhL*
1007	GCTTGGACAAACCAACCCG	Control for Δ*ldhL*
1047	GGTAGCGTCTTCAAGGTCGAGGG	Sequencing of *ldhL*
1071	GTCAATGATGGCAAATTCTTGG	Sequencing of *ldhL*
1074	CCGGTCTGATCCTAATCTTCC	Deletion primer for *ldhL*
1085	GCGTGGGAAGCCGGAG	Sequencing of *ldhL*
1086	CTTGTCAACGAGGTCCAGACG	Sequencing of *ldhL*
1089	CCTAAGGATTGGTTAAGTTCACCC	Sequencing of *ldhL*
1090	GGTGATTGACCACGTTTGC	Sequencing of *ldhL*
1091	CTCCGGCTTCCCACGC	Sequencing of *ldhL*
1096	CCATGGTTCAACAAGGGTTG	Primer for *ldhL* specific probe
1097	CAGATCGTACAGTGGTAAACCACC	Primer for *ldhL* specific probe
1126	GGTCTCTAGAGTGTCGGTCAAAATGTAGGAAAC	Cloning of *ldhL*/*Xba*I
1127	GAAGAAGCTTTGAACTTAACCAATCCTTAGGGAAG	Cloning of *ldhL*/*Hin*dIII
1128	AGGGAAGCTTCAAGAATTTGCCATCATTGAC	Cloning of *ldhL*/*Hin*dIII
1129	CGTAGAATTCGTTACGAACATCGTCTTCTAACTTG	Cloning of *ldhL*/*Eco*RI
1133	CGGGTCGACATTTTCGC	Control for Δ*ldhL*

fragments were ligated at their *Hin*dIII sites, amplified by PCR with primers 1126 and 1129 (Table Ib), and cloned as an *Xba*I–*Eco*RI fragment with pG$^+$host4 into *L. lactis*, resulting in the plasmid pKTH5097 (Fig. 2b) and *L. lactis* strain GRS1060.

The integration/inactivation plasmids, pKTH5096 and pKTH5097, were isolated from their *L. lactis* hosts and used to inactivate the *L. fermentum ldhD* and *ldhL* genes by a gene replacement method described by Bhowmik et al. (1993). The first integration/inactivation event with pKTH5096 resulted in *L. fermentum* Δ*ldhD* strain GRL1030. The Δ*ldhL* gene of *L. fermentum* GRL1030 was then similarly inactivated, resulting in the double mutant *L. fermentum* Δ*ldhD*-Δ*ldhL*, named GRL1032. Both gene replacement events resulted in a 0.4-kb deletion in the promoter region of *ldhD* and *ldhL*.

Bioreactor Cultivations

For the first preculture, 10 mL of standard MRS growth medium, supplemented with 20 g/L fructose, was inoculated from a frozen glycerol stock and grown for 10 h at 30°C without mixing. For the second preculture, 100 mL of the same medium was inoculated with 5% (v/v) of the broth from first preculture and grown at 30°C without mixing to

the late exponential growth phase. This broth was used to inoculate the bioreactor cultures. The initial pH in standard MRS growth medium was 6.2.

A complex growth medium with the following composition was used in the bioreactor cultivations: 10 g/L tryptone (Lab M, International Diagnostics Group, UK); 5 g/L yeast extract (Lab M); 20 g/L fructose; 10 g/L glucose; 2 g/L K_2HPO_4; 400 mg/L $MgSO_4$; and 20 mg/L $MnSO_4$. Tryptone, yeast extract, and K_2HPO_4 were autoclaved with the bioreactor (121°C, 13 min). Stock solutions of fructose and glucose were autoclaved separately (121°C for 13 min) and stock solutions of $MgSO_4$ and $MnSO_4$ were sterilized by filtration (0.22 μm).

The bioreactor cultivations were performed in 2-L glass-vessel bioreactors (Biostat MD, B. Braun Biotech International, Germany). The bioreactors were equipped with three six-bladed Rushton turbines and the agitation rate was 200 rpm. The temperature was set at 37°C. The pH was controlled with 3 *M* NaOH. The bioreactor and the base flask were placed on balances. The growth media were sparged with pure nitrogen for 30 min prior to inoculation and flushing of the media was continued throughout the experiments, at a rate of 0.2 L/min.

Analytical Methods

Cell-free extracts for measuring lactate dehydrogenase (LDH) activities were prepared as follows. *L. fermentum* cells were disrupted with glass beads in a Vibrogen cell mill (Edmund Bühler) and then resuspended in cold 10 m*M* phosphate buffer (pH 6.8), followed by removal of cell debris by centrifugation (15,000*g*, 10 min). The supernatants were used in LDH assays. L-LDH activity was assayed in 40 m*M* HEPES buffer (pH 8.0) containing 10 m*M* NAD^+ and 150 m*M* L-lactate, and D-LDH activity was assayed in 40 m*M* Tris-HCl buffer (pH 9.0) containing 10 m*M* NAD^+ and 100 m*M* D-lactate at room temperature (RT). The reduction of NAD^+ was followed at 340 nm. Protein concentration was measured by the Bradford method (Bradford, 1976) using a protein assay (Bio-Rad). The D- and L-lactate concentrations were analyzed enzymatically using a D-lactic acid/L-lactic acid kit (Catalog No. 111 2821, Boehringer Mannheim).

Concentration of organic acids, sugars, ethanol, acetoin, 2,3-butanediol, and mannitol was determined by high-performance liquid chromatography (HPLC). Mannitol, acetoin, and 2,3-butanediol concentrations were analyzed using an Aminex HPX-87P column (Bio-Rad) at 70°C with distilled water as the mobile phase. Glucose, fructose, and sucrose concentrations were measured using an Aminex HPX-87C column at 70°C with distilled water as the mobile phase. Organic acid and ethanol concentrations were measured using an Aminex HPX-87H ion-exclusion column at 60°C with 5 m*M* H_2SO_4 as the mobile phase. Pyruvate was analyzed with an ultraviolet (UV) detector, whereas all the other components were analyzed with a refractive index (RI) detector. One precolumn (Deashing Micro-Guard, Bio-Rad) was used for the analysis of mannitol, acetoin, 2,3-butanediol, and all sugars, whereas another precolumn (Cation H Micro-Guard, Bio-Rad) was used for the analysis of organic acids and ethanol. The elution rate in all systems was 0.6 mL/min.

The optical density (OD) of the culture broths was measured at 600 nm against distilled water. The samples were diluted in such a manner that the absorbance values were in the range of 0.1 to 0.6. The cell dry weight (cdw) was measured as follows: A sample of culture broth was pipetted into a preweighted centrifuge tube followed by centrifugation at 6000*g* for 5 min. The cell pellet was washed with sterile saline (0.9% [w/v] NaCl), the centrifugation was repeated, and the cell pellet was dried at 80°C until a constant cdw was achieved. The correlation factor between cell dry weight and optical density was 0.50 ± 0.02 cdw/OD.

Calculations

The maximum specific growth rates (μ_{max}) were calculated with Microsoft EXCEL. A chart for the natural logarithm of cell dry weight vs. time was plotted. The maximum specific growth rate was the steepest slope of a linear trendline (three to five successive values) in the exponential growth phase.

The logarithmic mean of the biomass (X') was calculated as:

$$X' = (X_{t2} - X_{t1})/(\ln X_{t2} - \ln X_{t1})$$

where X is the biomass concentration (grams of cell dry weight per liter) and t is time.

The carbon balances were calculated on a C-molar basis as the ratio between the sum of the end-products and consumption of sugars, as described by Curic et al. (1999). The consumption (in moles) of fructose and glucose and the formation of mannitol, ethanol, 2,3-butanediol, acetoin, acetate, lactate, pyruvate, and formate were determined by HPLC. The formation of carbon dioxide was calculated by:

$$n_{CO_2} = n_{H_{Ac}} + n_{EtOH} + 2 \times n_{BD}$$

where n_{CO2} is moles of carbon dioxide produced, n_{HAc} is moles of acetic acid produced, n_{EtOH} is moles of ethanol produced, and n_{BD} is moles of 2,3-butanediol produced.

The elemental composition of the biomass, $C_{4.63}H_{7.89}O_{2.35}N_{1.0}$, was taken from Novak et al. (1997). The fraction of glucose-6-phosphate channeled into formation of biomass was calculated by:

$$n_X = 6 \div 4.63 \times (n_{Fru} - n_{Mtol} + n_{Glu} - n_{HAc} - n_{EtOH})$$

where n_X is moles of biomass produced, n_{Fru} is moles of fructose consumed, n_{Mtol} is moles of mannitol produced, and n_{Glu} is moles of glucose consumed. The redox balances were calculated by:

$$\text{NAD/NADH} = (3 \times n_{\text{HAc}} + 3 \times n_{\text{EtOH}})/(n_{\text{Mtol}} + 2 \times n_{\text{EtOH}} + n_{\text{HLac}} + n_{\text{BD}})$$

The NADH formation/consumption from biomass production was assumed negligible and the equation considers only the NADH formed and consumed in the primary metabolism of the cells (Fig. 1). ATP yields on fructose were calculated from Figures 5 and 8. Hence, the formation and consumption of ATP was calculated according to the numbered reactions in Figure 1 (Reactions 3 + 4 * 2 − 1 − 2).

All data are given as the mean and standard deviation of two independent experiments.

RESULTS AND DISCUSSION

Cloning and Sequencing of *ldhD* and *ldhL* Gene Regions From *Lactobacillus fermentum*

The *Lactobacillus fermentum* D- and L-lactate dehydrogenase genes (*ldhD* and *ldhL*) were first isolated by PCR amplification using degenerated oligonucleotides (Tables Ia and Ib) designed according to the conserved DNA regions of the *ldh* gene sequences from different LAB. Both the *ldhD* primer pair (951 and 953; Table Ia) and the *ldhL* primer pair (954 and 962; Table Ib) generated 0.7-kb PCR fragments that were sequenced and found to share high sequence similarities to the *ldhD* and *ldhL* genes of other bacteria. For inactivation constructs, the upstream regions of the *ldh* genes were isolated using the Vectorette system followed by sequencing of these 1-kb regions found for *ldhD* and *ldhL*.

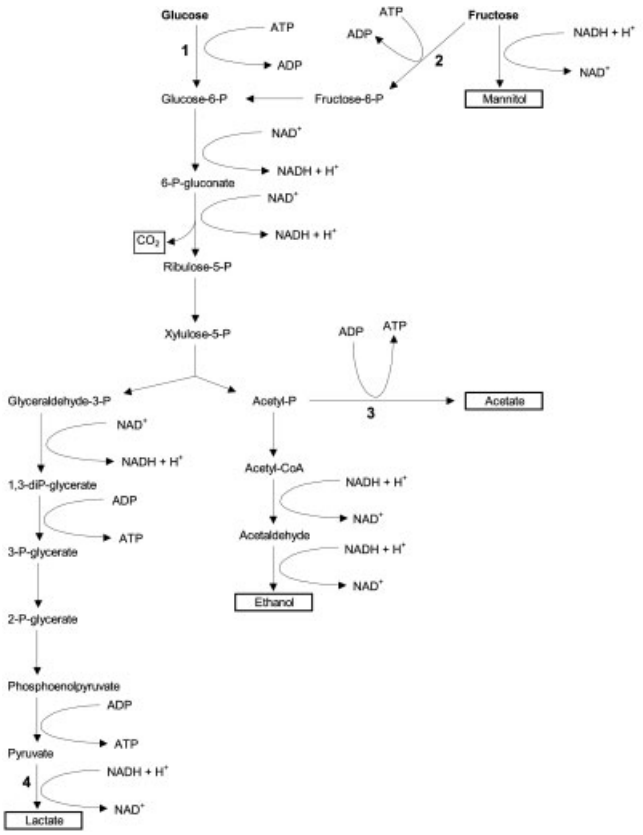

Figure 1. Cometabolism of glucose and fructose in heterofermentative LAB.

Inactivation of *L. fermentum ldhD* Gene

The inactivation of *ldhD* was carried out with the integration vector pKTH5096 (see "Materials and Methods" and Fig. 2a) in such a way that a 0.4-kb fragment from the promoter and the 5′ region of *ldhD* were deleted during gene replacement. The first homologous recombination step of pKTH5096 in *L. fermentum* took place by a temperature shift from 30° to 42°C under erythromycin selection. The second homologous recombination was achieved by growing the cells for 50 generations at 30°C without selection. One erythromycin-sensitive clone, designated *L. fermentum* GRL1030, showed a D(−)-lactic acid-negative phenotype. PCR amplification of GRL1030 DNA with *ldhD*-specific oligonucleotides (1048 and 1095; Table Ia) further confirmed that the size of the deletion was correct (Fig. 3, lane 3), whereas, with the oligonucleotides specific to the deleted region (1005 and 1050) (Fig. 3, lane 6) and to the integration vector (not shown) (744 and 745; Table Ia), no PCR amplification products could be detected. Inactivation of the *ldhD* gene in *L. fermentum* GRL1030 was also verified based on the absence of *ldhD* transcripts by northern blotting. An *ldhD*-specific probe did not hybridize to the total RNA isolated from GRL1030, whereas an *ldhL*-specific probe detected a transcript of approximately 1.1 kb (Fig. 4, lane 2 and 5). Both probes, however, detected their target transcripts (approximately 1.1 kb) from the total RNA isolated from the wild-type *L. fermentum* strain (Fig. 4, lanes 1 and 4).

Inactivation of *ldhL* Gene of *L. fermentum* Δ*ldhD* Strain GRL1030

To construct a Δ*ldhD*-Δ*ldhL* double mutant for *L. fermentum*, the *ldhL* inactivation vector pKTH5097 (see "Materials and Methods" and Fig. 2b) was transferred to the D-lactate dehydrogenase-negative mutant strain GRL1030. The gene replacement resulting in a 0.4-kb deletion at the promoter and 5′ region of *ldhL* was carried out as described for construction of the Δ*ldhD* strain GRL1030. The second homologous recombination was achieved by growing the cells for 100 generations in MRS broth supplemented with 2% fructose without selection at 30°C. With one erythromycin-sensitive clone, designated *L. fermentum* GRL1032, an L(+)-lactic acid-negative phenotype was found. PCR amplification of GRL1032 DNA with *ldhL*- and *ldhD*-specific oligonucleotides (1007/1133 and 1048/1095, respectively; Table Ia and Ib) further confirmed that the size of both deletions was correct (Fig. 3, lanes 10 and 4). With the oligonucleotides specific to the deleted regions (Δ*ldhL*-specific oligonucleotide 1074 and Δ*ldhD*-specific oligonucleotide 1050; Fig. 3, lanes 7 and 13) and to the integra-

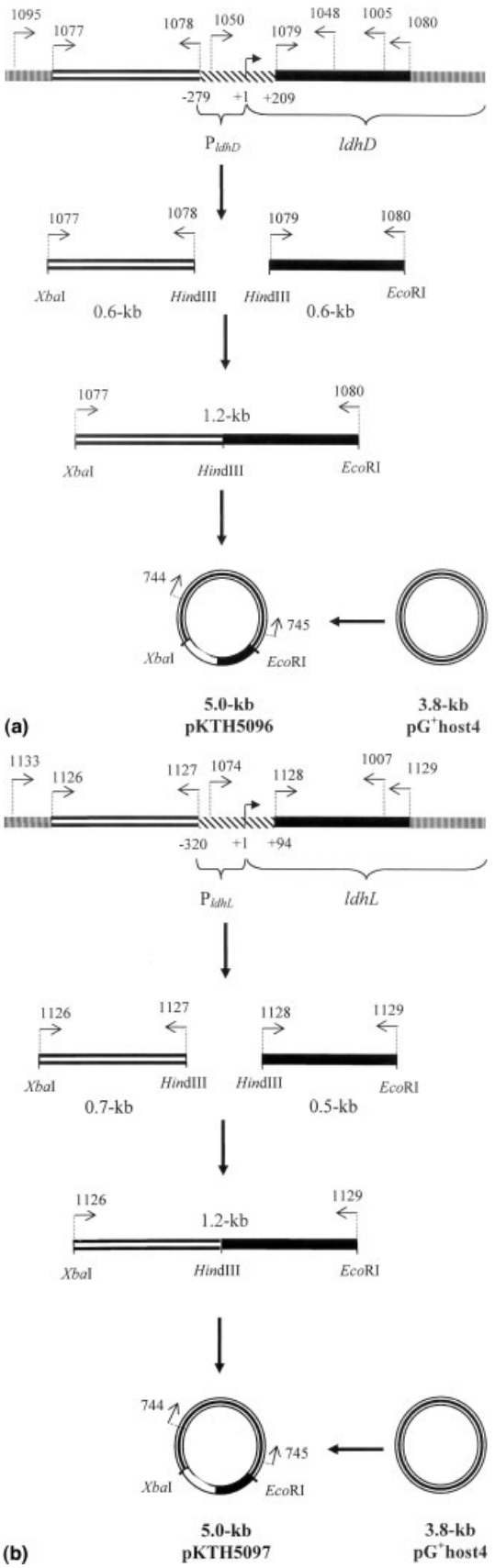

Figure 2. (a) Schematic representation of the Δ*ldhD* vector construction. (b) Schematic representation of the Δ*ldhL* vector construction.

Figure 3. Control of *ldhL* and *ldhD* inactivations by PCR. Chromosomal DNA from wild-type *L. fermentum* (lanes 2, 5, 8, and 11), Δ*ldhD* mutant strain GRL1030 (lanes (3, 6, 9, and 12), and Δ*ldhD*-Δ*ldhL* double mutant strain GRL1032 (lanes 4, 7, 10, and 13) was PCR amplified using the *ldhD*-specific primers 1048 and 1095 (lanes 2 to 4), primer 1050 specific for the deleted *ldhD* region (lanes 5 to 7), *ldhL*-specific primers 1007 and 1133 (lanes 8 to 10), and primer 1074 specific for the deleted *ldhL* region (lanes 11 to 13). Lanes 1 and 14 refer to the DNA ladder. The primer list is shown in Table I.

tion vector (not shown) (744 and 745; Table Ia) no PCR amplification products could be detected. Inactivation of the *ldhL* and *ldhD* genes in *L. fermentum* GRL1032 was also verified based on the absence of *ldhL* and *ldhD* transcripts by northern blotting. Neither the *ldhL*-specific probe nor the *ldhD*-specific probe hybridized to the total RNA isolated from GRL1032 (Fig. 4, lanes 3 and 6).

Coproduction of D-Mannitol and L-Lactate by *L. fermentum* Δ*ldhD* Strain 1030

The effect of inactivation of the D-lactate dehydrogenase gene in *L. fermentum* GRL1030 on mannitol production was studied. The lack of the D-lactate dehydrogenase activity in *L. fermentum* resulted in minor changes in primary sugar metabolism (Table II). The mutant cells, however, grew and consumed fructose slightly slower than the parent cells. The glucose-to-fructose consumption ratio between $t = 0$ h and $t = 5$ h was 0.51 ± 0.01 mol glucose/mol fructose for the parent cells and 0.49 ± 0.01 mol/mol for the mutant cells. The effect of the mutation on the yield of mannitol from fructose was negligible. Furthermore, the primary fluxes of the mutant, as shown in Figure 5, did not differ significantly from the respective fluxes of the parent strain, although the calculation of CO_2 formation instead of its direct measurement may have caused some uncertainties in the flux calculation. Surprisingly, the L-lactate dehydrogenase activity of the mutant cells was decreased to about 33% of that of

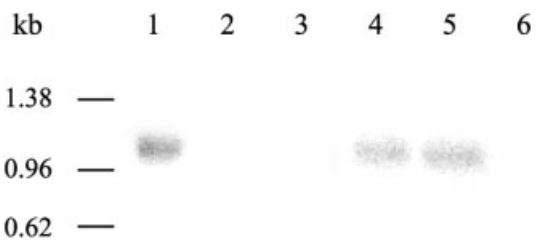

Figure 4. Northern hybridization of *L. fermentum* and *ldh* mutants. Total RNA isolated from wild-type *L. fermentum* (lanes 1 and 4) Δ*ldhD* mutant strain GRL1030 (lanes 2 and 5) and Δ*ldhD*-Δ*ldhL* double mutant strain GRL1032 (lanes 3 and 6) was hybridized with a 0.6-kb *ldhD*-specific probe (lanes 1 to 3) and with a 0.5-kb *ldhL*-specific probe (lanes 4 to 6).

Table II. *L. fermentum* NRRL-B-1932 compared to a D-lactate dehydrogenase negative mutant (GRL1030) in a growth medium containing both fructose and glucose at 37°C and pH 5.0.

	Parent	Mutant
Time to fructose depletion (h)	4.75	5
Final cell dry weight (g/L)	4.7 ± 0.2	2.5 ± 0.1
Maximum specific growth rate (l/h)	0.91 ± 0.02	0.82 ± 0.02
Volumetric fructose consumption rate (g/L · h)[a]	3.6 ± 0.0	2.5 ± 0.0
Specific fructose consumption rate (g/g cdw/h)[a]	3.9 ± 0.1	2.3 ± 0.1
Yield of mannitol from fructose (mol%)	90.6 ± 1.2	89.5 ± 0.5
Volumetric mannitol productivity (g/L · h)	3.7 ± 0.1	3.7 ± 0.0
Fraction of D-lactate (%)	56.7 ± 0.5	ND[b]
Fraction of L-lactate (%)	43.3 ± 0.5	100
Specific D-lactate dehydrogenase activity (U/mg)	4.9 ± 0.4	ND
Specific L-lactate dehydrogenase activity (U/mg)	1.0 ± 0.0	0.3 ± 0.0
Mannitol (C-mol/C-mol sugar)	0.595 ± 0.000	0.599 ± 0.006
Lactic acid (C-mol/C-mol sugar)	0.185 ± 0.003	0.177 ± 0.006
Acetic acid (C-mol/C-mol sugar)	0.100 ± 0.001	0.091 ± 0.001
Ethanol (C-mol/C-mol sugar)	0.027 ± 0.001	0.033 ± 0.000
Carbon dioxide (C-mol/C-mol sugar)	0.063 ± 0.001	0.062 ± 0.001
Biomass (C-mol/C-mol sugar)	0.026 ± 0.005	0.027 ± 0.010
Carbon balance	0.996 ± 0.005	0.990 ± 0.005
NAD/NADH balance	1.01 ± 0.01	0.97 ± 0.01

[a]Between $t = 1$ and $t = 3$ h. The specific consumption rates were calculated using a logarithmic mean of the biomass (see *Materials and Methods*).
[b]ND = not detected.

the parent strain. Importantly, the mutant produced high levels of pure L-lactate without a slowdown in mannitol production. No novel end-products were detected. In 5 h, 40 g of fructose (220.3 mmol) and 20 g of glucose (108.6 mmol) were metabolized into 36 g of D-mannitol (197.1 mmol) and 10.5 g of pure L-lactic acid (116.4 mmol). Hence, about 77.6% (C-mol/C-mol sugar) of the total carbon present in the sugars was now recovered in the form of valuable end-products (D-mannitol and L-lactic acid). The respective value for the parent strain was 59.5 C-mol% (D-mannitol).

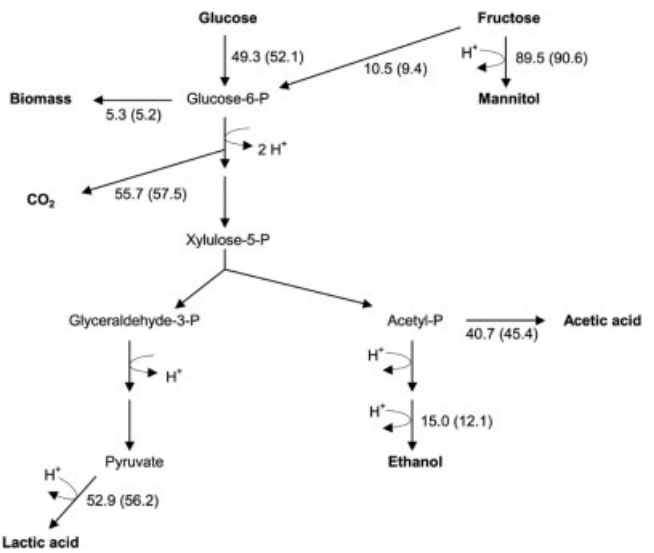

Figure 5. The primary metabolism of *L. fermentum* NRRL-B-1932 and a D-lactate dehydrogenase-negative mutant (GRL1030) at 37°C, pH 5.0. The values represent yields on fructose (mol/mol × 100). Fructose, glucose, mannitol, lactic acid, acetic acid, and ethanol were measured with HPLC, whereas biomass and carbon dioxide were calculated as described in "Materials and Methods." The respective values of the parent strain are shown in parentheses.

The mutation resulted in small changes of the NADH oxidation patterns. Although slightly less mannitol was produced per fructose consumed by the mutant, a small increase in ethanol production was observed. Moreover, the yield of ATP per fructose consumed was lower in the D-lactate dehydrogenase-negative mutant than in the parent strain (86.7 mol/mol compared with 96.3 mol/mol). This corresponded well with the lowered growth rate and final biomass of the mutant.

Use of metabolic engineering for the production of pure L-lactic acid has been studied previously in homofermentative LAB. For instance, using *Lactobacillus helveticus*, Bhowmik and Steele (1994) constructed a D-lactate dehydrogenase-negative mutant, which produced only pure L-lactate. The metabolism of the mutant cells was otherwise unchanged. Similar studies and results were reported by Kylä-Nikkilä et al. (2000). Applying the same target of inactivation in *Lactobacillus johnsonii* resulted in the production of pure L-lactate, and in this case some pyruvate was lost to other end-products (e.g., diacetyl and acetoin) (Lapierre et al., 1999). Lapierre and coworkers also detected a decrease in the remaining L-lactate dehydrogenase activity.

Coproduction of D-Mannitol and Pyruvate by *L. fermentum* ΔldhD-ΔldhL Mutant Strain GRL1032

Using the D-lactate dehydrogenase-negative mutant (GRL1030) as the starting point, a mutant deficient in both

lactate dehydrogenases was constructed (GRL1032). The hypothesis was that such a mutant would excrete pyruvate to the growth medium, thus giving rise to a novel microbial two-product process (D-mannitol and pyruvate).

The sugar consumption patterns of the double mutant (deficient in both lactate dehydrogenases) deviated clearly from the respective patterns of the *L. fermentum* parent strain (Fig. 6). Whereas the parent strain consumed glucose and fructose in a typical 0.5:1 ratio, and both fructose and glucose ran out at approximately the same time, the glucose consumption of the double mutant GRL1032 was severely decelerated compared with the fructose consumption. When the initial fructose (20 g/L) was consumed by the mutant cells (about $t = 7$ h), only about 55% of the initial glucose (10 g/L) was consumed. The glucose-to-fructose consumption ratio (at $t = 5$ h) was decreased to 0.28 ± 0.00 mol glucose/mol fructose for the mutant cells (parent cells = 0.48 ± 0.00 mol/mol). After fructose was depleted in the mutant cell cultivations, the cells consumed glucose very slowly.

Importantly, similar mannitol production levels were obtained with both strains (Fig. 7). In contrast to expectations, the D/L-lactate dehydrogenase-negative mutant also produced some D-lactate (0.5 g/L compared with 6.4 g/L of total lactate produced by the parent strain). Furthermore, pyruvate (2.0 g/L) and 2,3-butanediol (0.4 g/L) were also produced. The formation of lactate in the mutant cultivations was unexpected. Inactivation of the lactate dehydrogenase genes had been checked thoroughly: First, no lactate dehydrogenase activities were detected in cell lysates from mutant cell cultures (Table III). Second, using PCR and several different sets of primers, it was assured that correct inactivation had occurred at the DNA level. Third, by applying northern blotting, the mRNAs encoding for both lactate dehydrogenases, clearly detected in the parent cells, were not detected in the mutant cells. Although lactic acid

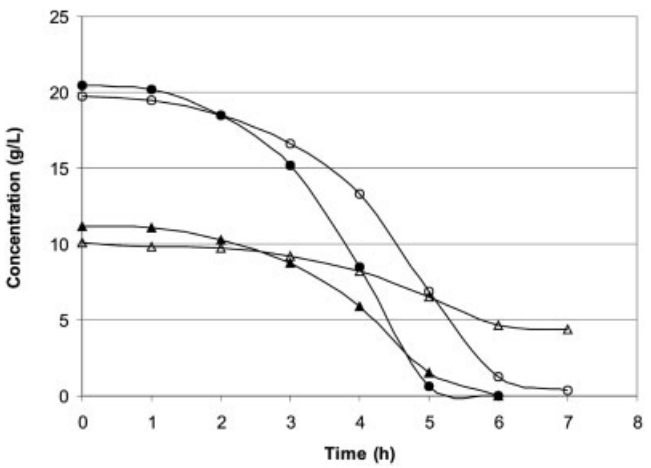

Figure 6. Sugar consumption by *L. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase-negative mutant (GRL1032). The temperature, pH, and agitation were set at 37°C, 7.0, and 200 rpm, respectively. Circles: fructose (g/L); triangles: glucose (g/L). Filled symbols indicate parent strain, open symbols indicate mutant strain.

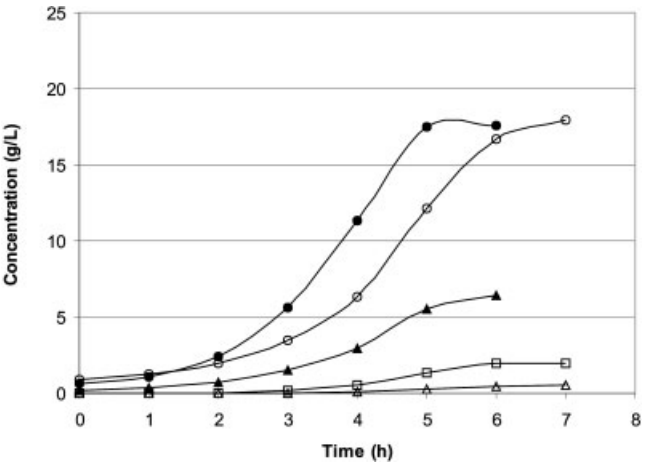

Figure 7. Concentration of mannitol, pyruvic acid, and lactic acid in cultivations with *L. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase-negative mutant (GRL1032) (run 1 of 2). The temperature, pH, and agitation were set at 37°C, 7.0, and 200 rpm, respectively. Circles: mannitol (g/L); triangles: lactic acid (g/L); squares: pyruvic acid. Filled symbols indicate parent strain, open symbols indicate mutant strain.

could be produced from media components (e.g., from citrate by malolactic fermentation or from leucine), the accuracy of the carbon and redox balances (Table III) supported the fact that the lactate formed was actually produced from pyruvate. Also, in *Lactobacillus plantarum*, an *ldh*-negative mutant was found to produce small amounts of lactic acid, suggested to be due to D-hydroxyisocaproate dehydrogenase activity (Ferain et al., 1996). This enzyme, related to D-lactate dehydrogenases, has been detected in several lactic acid bacteria; however, we could not detect this activity from the double *ldh* mutant strain under the assay conditions used. Thus, the formation of D-lactate could not be explained, but it was speculated that activation of another enzyme, with D-lactate dehydrogenase side activity, was responsible for reducing a fraction of the pyruvate to lactate in these cells. Recently, it has also been observed that the *ldh*-negative mutant of *Lactococcus lactis* produces small amounts of lactic acid (G. J. Grobben, personal communication, 2002).

Furthermore, the double mutant GRL1032 grew significantly slower than the parent strain (Table III). Although the specific fructose consumption rate was not affected, the lack of lactate dehydrogenases drastically affected the glucose catabolism and the redox balance of the cells. In the parent cells, all NADH needed for reduction of fructose to mannitol was formed when glucose-6-P metabolized to ribulose-5-P (upper branch). All excess NADH formed in this branch of the primary metabolism was reoxidized to NAD^+ in the production of ethanol from acetyl-coenzyme A. As shown in Figure 8, 88.1 mol of mannitol per mole of fructose consumed was produced by the mutant cells. An equal amount of NADH was required for the reaction to proceed, but only 66.8 mol NADH per mole fructose was produced in the upper branch. Therefore, to complete the fructose-to-mannitol reaction, NADH (21.3 mol/mol) typically used for

Table III. *L. fermentum* NRRL-B-1932 compared with a D/L-lactate dehydrogenase-negative mutant (GRL1032) in a growth medium containing both fructose and glucose at 37°C and pH 7.0.

	Parent	Mutant
Time to fructose depletion (h)	5.5	7
Final cell dry weight (g/L)	3.8 ± 0.0	2.7 ± 0.1
Maximum specific growth rate (l/h)	0.81 ± 0.01	0.60 ± 0.02
Volumetric fructose consumption rate (g/L · h)[a]	2.5 ± 0.1	1.5 ± 0.1
Specific fructose consumption rate (g/g cdw · h)[a]	4.2 ± 0.0	4.1 ± 0.1
Yield of mannitol from fructose (mol%)	89.0 ± 1.3	88.1 ± 0.5
Volumetric mannitol productivity (g/L · h)	3.5 ± 0.0	2.6 ± 0.0
Fraction of D-lactate (%)	53.2 ± 1.0	100
Fraction of L-lactate (%)	46.8 ± 1.0	ND
Specific D-lactate dehydrogenase activity (U/mg)	4.1 ± 0.1	ND
Specific L-lactate dehydrogenase activity (U/mg)	0.1 ± 0.0	ND
Mannitol (C-mol/C-mol sugar)	0.601 ± 0.010	0.683 ± 0.002
Lactic acid (C-mol/C-mol sugar)	0.187 ± 0.000	0.021 ± 0.001
Acetic acid (C-mol/C-mol sugar)	0.099 ± 0.001	0.080 ± 0.001
Pyruvic acid (C-mol/C-mol sugar)	ND[b]	0.080 ± 0.002
Ethanol (C-mol/C-mol sugar)	0.023 ± 0.000	0.006 ± 0.000
2,3-Butanediol (C-mol/C-mol sugar)	ND	0.021 ± 0.001
Carbon dioxide (C-mol/C-mol sugar)	0.061 ± 0.000	0.053 ± 0.001
Biomass (C-mol/C-mol sugar)	0.032 ± 0.012	0.058 ± 0.007
Carbon balance	1.004 ± 0.001	1.003 ± 0.000
NAD/NADH balance	0.99 ± 0.00	0.98 ± 0.02

[a]Between $t = 1$ and $t = 3$ h. The specific consumption rates were calculated using a logarithmic mean of the biomass (see *Materials and Methods*).

[b]ND, not detected.

lactate production (i.e., formed when glyceraldehyde-3-P is metabolized to pyruvate) was now reoxidized by mannitol dehydrogenase. Due to the lack of lactate dehydrogenases, pyruvate accumulated into the growth medium. Also, 2,3-butanediol was produced from pyruvate. To fulfill the remaining redox imbalance, a small amount of ethanol was produced, whereas acetyl-P was mainly dephosphorylated into acetate. In this work, we have assumed that NAD,

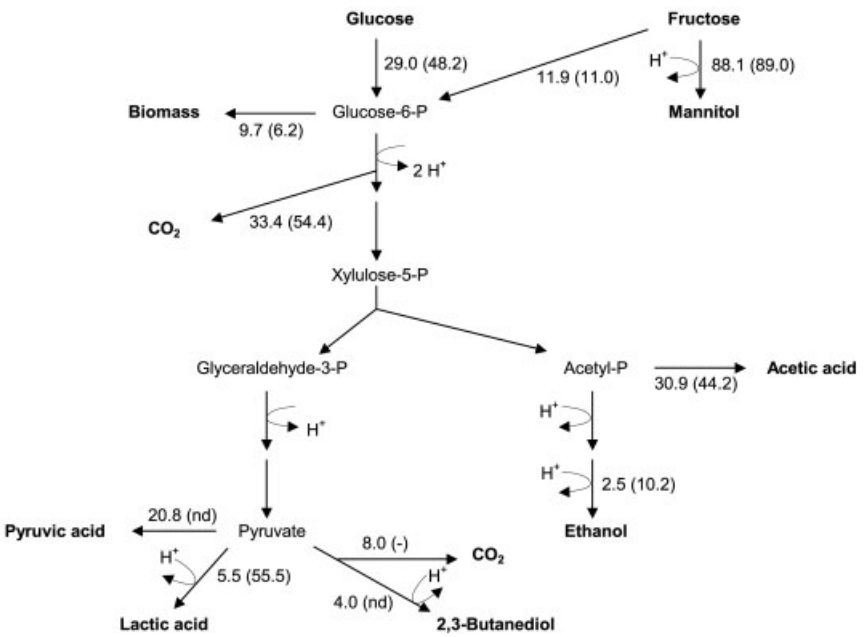

Figure 8. The primary metabolism of *L. fermentum* NRRL-B-1932 and a D/L-lactate dehydrogenase-negative mutant (GRL1032) at 37°C, pH 7.0. The values represent yields on fructose (mol/mol × 100). Fructose, glucose, mannitol, lactic acid, acetic acid, and ethanol were measured with HPLC, whereas biomass and carbon dioxide were calculated as described in "Materials and Methods." The respective values of the parent strain are shown in parentheses. nd, not detected.

instead of NADP, is the cofactor of the key enzymes. The previously uncharacterized *L. fermentum ldh* genes revealed distinct NAD-binding sites in their deduced sequence (data not shown). Regarding *L. fermentum* MDH, the affinity for NADP has not yet been tested but we have recently shown that MDHs from heterofermentative lactic acid bacteria (e.g., *L. fermentum*, *Leuconostoc mesenteroides*, and *Leuconostoc pseudomesenteroides*) are likely to form a structurally and functionally separate mannitol dehydrogenase group (Aarnikunnas et al., 2002). *L. mesenteroides* MDH is known to require NAD for its activity and has little affinity toward NADP (Sakai and Yamanaka, 1968).

The production of 2,3-butanediol is common among bacteria. A low-affinity acetolactate synthase first converts pyruvate into α-acetolactate, which is then converted into acetoin by α-acetolactate decarboxylase. Two moles of pyruvate is needed for the production of 1 mol α-acetolactate. Finally, acetoin is reduced to 2,3-butanediol by acetoin reductase, whereas NADH is simultaneously oxidized to NAD^+. By nonenzymatic conversion, diacetyl can also be produced from α-acetolactate. This important aroma compound is usually formed in trace amounts, but was not detected in the samples using HPLC analysis.

The metabolism of the double-mutant GRL1032 was also studied at pH 5.0. The external concentration of pyruvate increased until the mutant cells reached the late exponential growth phase, whereafter the pyruvate produced (0.9 g/L) was rapidly consumed by the cells (data not shown). At the lowered pH the mutant cells produced even more D-lactate (2.7 g/L). No lactate dehydrogenase activities were detected. Furthermore, the glucose-to-fructose consumption ratio was now 0.4:1. This cultivation pH was tested with the double Δ*ldh* mutant only, because it was the standard pH in all other cultivations. To maximize the amount of ionized pyruvate in the medium, and to prevent the free backflow of nonionized acid, the pyruvate fermentation was performed at pH 7.0.

In conclusion, at pH 7.0, 38.5 g of fructose (213.7 mmol) and 11 g of glucose (61.9 mmol) were metabolized into 34 g of D-mannitol (188.2 mmol) and 4 g of pyruvic acid (44.3 mmol) in 7 h. Hence, in this process, about 76.3% (C-mol/C-mol sugar) of the total carbon present in sugars was recovered in valuable end-products (D-mannitol and pyruvic acid). Similar levels were obtained earlier with the mannitol and L-lactic acid two-product process. However, the concentration of pyruvate obtained was not as high as expected, which complicates the purification steps. The low level of pyruvate formed was primarily due to the severe slowdown of glucose catabolism or transport and the formation of "waste products" such as 2,3-butanediol and lactate.

In earlier studies, the inactivation of both lactate dehydrogenases in *Lactobacillus plantarum* resulted in major rerouting of glucose catabolism, which led to the production of novel end-products such as acetoin, ethanol, acetate, mannitol, and succinate (Ferain et al., 1998). Moreover, Neves et al. (2000) studied the disruption of lactate dehydrogenase in *L. lactis* and found that, in a resting state, the mutant cells produced the same novel end-products as previously noted. Mannitol was transiently produced and metabolized once glucose was depleted. In these cells, mannitol was formed from fructose-6-P via mannito1-P. In addition, lactate dehydrogenase deficiency can also be lethal to certain LAB at a high glucose concentration (*Streptococcus mutans;* Hillman et al., 2000).

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