Publication III

M. Helanto, K. Kiviharju, M. Leisola, and A. Nyyssölä. 2007. Metabolic engineering of *Lactobacillus plantarum* for production of L-ribulose. Applied and Environmental Microbiology, volume 73, number 21, pages 7083-7091.

Applied and Environmental Microbiology, Nov. 2007, p. 7083–7091 Vol. 73, No. 21
0099-2240/07/$08.00+0 doi:10.1128/AEM.01180-07

Metabolic Engineering of *Lactobacillus plantarum* for Production of L-Ribulose[▽]

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Received 25 May 2007/Accepted 2 September 2007

L-Ribulose is a rare and expensive sugar that can be used as a precursor for the production of other rare sugars of high market value such as L-ribose. In this work we describe a production process for L-ribulose using L-arabinose, a common component of polymers of lignocellulosic materials, as the starting material. A ribulokinase-deficient mutant of the heterofermentative lactic acid bacterium *Lactobacillus plantarum* NCIMB8826 was constructed. Expression of *araA*, which encodes the critical enzyme L-arabinose isomerase, was repressed by high glucose concentrations in batch cultivations. A fed-batch cultivation strategy was therefore used to maximize L-arabinose isomerase production during growth. Resting cells of the ribulokinase-deficient mutant were used for the production of L-ribulose. The isomerization of L-arabinose to L-ribulose was very unfavorable for L-ribulose formation. However, high L-ribulose yields were obtained by complexing the produced L-ribulose with borate. The process for L-ribulose production in borate buffer by resting cells was optimized using central composite designs. The experiment design suggested that the process has an optimal operation point around an L-arabinose concentration of 100 g liter^{-1}, a borate concentration of 500 mM, and a temperature of 48°C, where the statistical software predicted an initial L-ribulose production rate of 29.1 g liter^{-1} h^{-1}, a best-achievable process productivity of 14.8 g liter^{-1} h^{-1}, and a conversion of L-arabinose to L-ribulose of 0.70 mol mol^{-1}.

Despite being a common metabolic intermediate in different organisms, L-ribulose is scarce in nature. This ketopentose has therefore been classified as a rare sugar (3). The markets and interest for rare and unnatural sugars have been growing, especially in the sweetener and pharmaceutical industries (40). Microbial and enzymatic reactions are very suitable for converting D sugars to various L sugars (3).

Biotechnological L-ribulose production has been mainly investigated by using ribitol as the raw material. Most efficient L-ribulose production has been obtained by incubating ribitol in the presence of resting cells of acetic acid bacteria (4, 14, 29). The enzyme catalyzing the oxidation of ribitol to L-ribulose in these bacteria has been shown to be a membrane-bound NAD(P)-independent oxygenase (2). However, the volumes of production of ribitol are small, and it is expensive in pure form.

Unlike other L sugars, L-arabinose is fairly abundant in nature. It is a common component of polymers of lignocellulosic materials (20). For example, sugar beet pulp, which is a by-product of the sugar industry, has been reported to contain considerable amounts of L-arabinose (20%, wt/wt, of the deproteinated mass). L-Arabinose can easily be isolated from the pulp by acid or enzymatic hydrolysis, filtration, and acetone fractionation (41).

The L form of ribulose is an intermediate in the pathway for L-arabinose utilization in many bacteria. The L-arabinose taken up by the cells is first isomerized to L-ribulose, which is then phosphorylated to L-ribulose-5-phosphate. The phosphorylated form of L-ribulose is epimerized to D-xylulose-5-phosphate, which is metabolized further (10, 11, 21, 30, 31, 32, 33). The genes for the enzymes of the reaction sequence, the L-arabinose isomerase gene (*araA*), the L-ribulokinase gene (*araB*), and the L-ribulose 5-phosphate 4-epimerase gene (*araD*), have been reported to be arranged as an operon in bacteria (30, 31, 32, 33).

The L-enantiomers of nucleoside analogues have wide use as antiviral drugs (44). Since L-ribose can be used as a precursor for the synthesis of most of these compounds, there has been a growing interest in its production (45). L-Ribulose has been the precursor of choice in the reports on biotechnological L-ribose production (4, 14, 29). An L-ribose isomerase catalyzing the reaction from L-ribulose to L-ribose has been isolated and sequenced previously (34).

In the current work we describe the construction of an L-ribulokinase-deficient mutant of the heterofermentative lactic acid bacterium *Lactobacillus plantarum* NCIMB8826. We show how resting cells of this mutant can be used for the efficient production of L-ribulose and report the optimization of the production process using central composite designs (CCD). The equilibrium in enzymatic isomerization of L-arabinose to L-ribulose has been reported to be very unfavorable for L-ribulose formation (21). We show how high L-ribulose yields can be obtained by complexing the produced L-ribulose with borate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. plantarum* NCIMB8826 and its L-arabinose isomerase-deficient mutant BPT197 were cultivated at 30°C in standard MRS growth medium (Lab M Limited) and in simplified MRS medium in bioreactor cultivations. The simplified MRS medium comprised 10 g liter^{-1} glucose, 10 g liter^{-1} Bacto peptone (Difco), 10 g liter^{-1} yeast extract (Lab M

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▽ Published ahead of print on 14 September 2007.

TABLE 1. Oligonucleotides used in this study

Primer	Sequence[a] (5′–3′)	Specific use
AraOPF	ATATGTCGACGCGGTAGTTTATCTGGAATTAATCA	Cloning of part of *L. plantarum araBAD* operon
AraOPR	ATATGGATCCTAACACCGGCTACCTTATCATTGTA	Cloning of part of *L. plantarum araBAD* operon
RKdelU	AATAGATCTAAGAAACTTTGCCAGTTTTAATC	Primer for pRKDEL deletion plasmid construct
RKdelD	ATTAGATCTTATTCAACGTTATCAAGCTGGCT	Primer for pRKDEL deletion plasmid construct
RKdelcheckF	TTGACTAATGCAACATTGGTTGGG	Confirming pRKDEL integration
RKdelcheckR	GTGCAATCTGCTCTTGCACATCTT	Confirming pRKDEL integration
RKdelsekvF	CCAGAAACTCGTGGTCACTCACTT	Sequencing of surroundings of *L. plantarum araB* deletion
RKdelsekvR	ACCAGATGGCTTGATCACGAATAG	Sequencing of surroundings of *L. plantarum araB* deletion
*araB*probeF	TCTGGCGTTTTCCCAATTGATGAAA	Primer for *araB* transcripts for RT-qPCR
*araB*probeR	GTTTCATATAATCGATCCGGTTTCAATT	Primer for *araB* transcripts for RT-qPCR
*araA*probeF	TTCGTGGTAAAGGCTGTGTAAC	Primer for *araA* transcripts for RT-qPCR
*araA*probeR	TTCGGATGCTGATGTTGATAAGG	Primer for *araA* transcripts for RT-qPCR
Ghost-F	TTTTCCCAGTCACGACGTTG	pGHOST4-specific primer
Ghost-R	CACACAGGAAACAGCTATGACC	pGHOST4-specific primer
M13–47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing of pGM-T Easy insert
RP	TTTCACACAGGAAACAGCTATGAC	Sequencing of pGM-T Easy insert

[a] Underlined sequences indicate restriction sites.

Limited), 2 g liter^{-1} K_2HPO_4, 0.2 g liter^{-1} $MgSO_4$, 0.05 g liter^{-1} $MnSO_4$, and 5 g liter^{-1} L-arabinose. Erythromycin was used at a concentration of 5 μg liter^{-1} for the selection of plasmid pRKDEL. *Escherichia coli* XL1-Blue strain (Stratagene) was used as the cloning host in sequencing studies. It was grown in standard Luria-Bertani medium (Pronadisa) at 37°C using a final concentration of 100 μg ml^{-1} of ampicillin for the selection of the pGEM-T Easy plasmid (Promega). *Lactococcus lactis* NZ9000, obtained from NIZO Laboratories (The Netherlands) (26), was grown at 30°C in M17 medium (Difco) containing 5 g liter^{-1} glucose.

Plasmid construction. Isolation of *L. plantarum* chromosomal DNA was performed as previously described (22). *L. plantarum* (1) and *L. lactis* (23) were transformed by electroporation as previously described.

The integration vector for *araB* inactivation was constructed as previously described (28). A region extending 1.0 kb upstream and 1.0 kb downstream from the coding sequence of *araB* was amplified with primers AraOPF and AraOPR by PCR (Table 1). The PCR fragment was cloned into the integration vector pGHOST4 as a SalI/BamHI fragment. The resulting plasmid, pARAOP, was transformed into *L. lactis* NZ9000, which was used as the cloning host. The pARAOP plasmid was purified using the High Speed plasmid midikit (QIAGEN).

The deletion plasmid was constructed with PCR amplification of the whole pARAOP plasmid with primers RKdelU and RKdelD (Table 1). Amplification with these primers resulted in a fragment with BglII restriction sites at both ends of the fragment. Digestion and ligation of the PCR fragment resulted in plasmid pRKDEL, which had a 1.5-kb deletion in the middle of the *araB* insert. The pRKDEL plasmid was transformed into *L. lactis* NZ9000 and isolated using the High Speed plasmid midikit (QIAGEN). The pRKDEL plasmid was used to inactivate the *L. plantarum araB* gene by gene replacement as previously described (8).

Isolation of RNA and reverse transcription-quantitative PCR (RT-qPCR). The cells for RNA isolation were treated with the RNAprotect bacterial reagent (QIAGEN), harvested by centrifugation (5,000 × *g*, 10 min), and resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 5 mM EDTA, pH 8, 20 mg ml^{-1} lysozyme, 50 000 U ml^{-1} mutanolysin). Cells were incubated at 37°C for 10 min, and the RNA was isolated using the RNAeasy minikit (QIAGEN). The RNA for cDNA synthesis was treated with DNase I (Sigma).

The relative amount of *araBAD* mRNA from *L. plantarum* cells was determined by RT-qPCR using the comparative threshold cycle method. cDNA for the RT-qPCR assays was synthesized from 1 μg of RNA template with the StrataScript cDNA synthesis kit (Stratagene) using random hexamer primers. PCR amplification was performed in 20 μl (final volume) containing 1 μl of cDNA, 0.15 μM of each primer, 12.5 μl 2× Brilliant SYBR Green master mix (Stratagene), and 0.03 μM 6-carboxy-X-rhodamine reference dye. All the amplifications were carried out in optical grade eight-well strips using an Mx3000P real-time PCR system (Stratagene) with the following program: initial step, 95°C for 10 min, and then 40 cycles of 95°C for 30 s, 53°C for 1 min, and 72°C for 30 s (*araA*) or 1 min (*araB*). All samples were analyzed as duplicates. The primers used are presented in Table 1.

L-Arabinose isomerase activity assays. Cell extracts for L-arabinose isomerase activity assays were prepared as follows. *L. plantarum* cells were harvested by centrifugation (5,000 × *g*, 15 min), washed with 50 mM Tris-HCl, pH 7.5, and resuspended in cold sonication buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, 1 tablet/20 ml protease inhibitor [EDTA free; Roche]). Cells were disrupted by sonication, and the cell debris was removed by centrifugation (20,000 × *g*, 30 min). The L-arabinose isomerase activities were determined from the supernatants.

The L-arabinose isomerase activities were assayed at 30°C. The reaction mixture (475 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, and 300 mM L-arabinose. The reaction was started by adding 475 μl of cell extracts. The reaction was stopped after 15 min by adding 50 μl of 0.5 M H_2SO_4. L-Ribulose concentrations were determined from the reaction mixtures by high-performance liquid chromatography (HPLC) as described below. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmol min^{-1} of L-ribulose. Protein concentrations were determined using the Qubit fluorometer (Invitrogen) according to the instructions by the manufacturer.

Growth and L-arabinose isomerase production. *L. plantarum* cells were grown overnight in standard MRS medium at 30°C. A 50-ml culture of MRS supplemented with 5 g liter^{-1} of L-arabinose and 10 g liter^{-1} of glucose, fructose, galactose, lactose, maltose, mannose, or sucrose was inoculated with 2% of culture in late exponential growth phase and grown at 30°C for 10 h. Samples for determination of sugar concentrations were taken at 0 h and 10 h. The cells were harvested by centrifugation (5,000 × *g*, 15 min) at the end of the cultivation for L-arabinose isomerase activity assays. All experiments were performed as duplicates.

L-Ribulose production during growth on different sugars. L-Ribulose production was tested with growing cells of *L. plantarum* BPT197 in MRS medium supplemented with L-arabinose and glucose, fructose, or sucrose. The cells were grown overnight in standard MRS medium at 30°C. Fifty milliliters of MRS containing from 5 to 30 g liter^{-1} L-arabinose and from 5 to 30 g liter^{-1} glucose, fructose, or sucrose was inoculated with 2% (vol/vol) of the broth from the overnight culture. Cells were grown at 30°C for 10 h. Samples for determining glucose, fructose, sucrose, L-arabinose, and L-ribulose concentrations by HPLC were taken at 0 h and 10 h. All experiments were performed as duplicates.

Bioreactor cultivation. The cultivations were inoculated with 5% of culture in late exponential phase. All cultivations were carried out in a Biostat MD reactor (total volume, 2 liters; B. Braun Biotech International) at 1.5 liters with a stirring rate of 200 rpm (two six-blade Rushton-type impellers) at 30°C. pH was controlled at a minimum of 6.2 with 3 M NaOH. Glucose and L-arabinose concentrations and L-arabinose isomerase activities were determined from the cultivation samples. Cell dry weight was determined in triplicate by centrifuging, washing, and drying the cells at 80°C from 5-ml samples taken from the cultivations.

A slightly modified cultivation medium was used in fed-batch cultivations. The initial batch phase medium comprised 10 g liter^{-1} glucose, 4 g liter^{-1} L-arabinose, 20 g liter^{-1} Bacto peptone, 20 g liter^{-1}yeast extract, 4 g liter^{-1} K_2HPO_4, 0.2 g liter^{-1} $MgSO_4$, and 0.05 g liter^{-1} $MnSO_4$. The feed medium contained

TABLE 2. CCF experiment design for the investigation of L-ribulose production with resting cells of *L. plantarum* BPT197[a]

c_B (mM)	T (°C)	c_A (g liter^{-1})	r_i (g liter^{-1} h^{-1})	x (mol mol^{-1})
50	30	10	0.34	0.218
600	30	10	0.35	0.701
50	40	10	0.41	0.187
600	40	10	0.73	0.820
50	30	50	1.09	0.100
600	30	50	2.16	0.806
50	40	50	1.97	0.137
600	40	50	4.98	0.896
50	35	30	0.94	0.144
600	35	30	1.91	0.817
325	30	30	1.59	0.761
325	40	30	3.55	0.898
325	35	10	0.74	0.825
325	35	50	3.57	0.788
325	35	30	2.19	0.838
325	35	30	2.30	0.898
325	35	30	2.23	0.889

[a] c_B, T, and c_A are the factors; r_i and x are the responses.

TABLE 3. CCC experiment design for the optimization of L-ribulose production with resting cells of *L. plantarum* BPT197[a]

T (°C)	c_A (g liter^{-1})	r_i (g liter^{-1} h^{-1})	x (mol mol^{-1})	r_{max} (g liter^{-1} h^{-1})
38	100	9.40	0.781	3.64
46	100	20.39	0.779	10.85
38	300	20.98	0.203	15.70
46	300	42.51	0.195	29.06
36	200	16.73	0.391	10.89
48	200	48.32	0.397	37.73
42	50	6.13	0.846	2.33
42	350	27.83	0.148	22.66
42	200	26.78	0.412	12.05
42	200	26.33	0.416	11.83
42	200	27.03	0.410	12.05

[a] CCC, central composite circumscribed. T and c_A are the factors; r_i, x, and r_{max} are the responses.

150 g liter^{-1} glucose and 4 g liter^{-1} L-arabinose. The glucose concentration was controlled at a minimum of 2.5 g liter^{-1} using the YSI 2700 Select enzymatic analyzer (Yellow Springs Instrument Inc.) and a 101U/R pump (Watson-Marlow). The YSI analyzer's built-in controller was used in controlling the glucose feed. Glucose, L-arabinose, and mRNA concentrations as well as L-arabinose isomerase activities were determined from cultivation samples. Dry cell weight was estimated from online data obtained by using a TruCell cell density probe (Finesse Instruments), which was calibrated by measuring the true cell dry weight.

L-Ribulose production with resting cells in borate buffer. The effect of borate on L-ribulose formation was studied by incubating resting cells of *L. plantarum* BPT197 in sodium tetraborate and sodium phosphate buffers as follows. Fifty milliliters of MRS medium supplemented with 5 g liter^{-1} L-arabinose and 20 g liter^{-1} glucose was inoculated with 2% of culture in late exponential phase, and the culture was grown at 30°C for 10 h. Cells were harvested by centrifugation (5,000 × *g*, 15 min) and washed with saline. Cells were suspended in 5 ml of 0.6 M sodium tetraborate buffer, pH 8, or in 50 mM sodium phosphate buffer, pH 8, supplemented with 20 g liter^{-1} L-arabinose, 5 g liter^{-1} glucose, and 5 mM $MnCl_2$ and incubated for 30 h at 30°C. Samples for the determination of glucose, L-arabinose, and L-ribulose concentrations were taken at 0 h and 30 h. All experiments were performed as duplicates.

Optimization of L-ribulose production. *L. plantarum* BPT197 cells were cultivated in fed-batch cultivation mode as described above. The cells were harvested by centrifugation (5,000 × *g*, 15 min) and washed with saline. The cell suspension was distributed in 20-ml aliquots to 50-ml reaction tubes containing the medium of the individual optimization point (30 ml). L-Ribulose concentrations were determined from samples by HPLC as described below.

A central composite face-entered (CCF) experiment design for three variables was used in the initial investigation of L-ribulose production with resting cells of *L. plantarum* BPT197. The variables were temperature (T), L-arabinose concentration (c_A), and borate concentration (c_B) at ranges from 30 to 40°C, from 10 to 50 g liter^{-1}, and from 50 to 600 mM, respectively. The experiment design is shown in Table 2. Initial L-ribulose production rate (r_i) and conversion of L-arabinose to L-ribulose (x) were used as responses. A further two-variable central composite circumscribed experiment design with a star distance of 1.5 was constructed in order to optimize T (from 38 to 46°C) and c_A (from 100 to 300 g liter^{-1}). This experiment design is shown in Table 3. In addition to the responses used in the previous experiment design, the best achievable process productivity (r_{max}) was used as the response. Modde 5.0 software (Umetrics) was used in constructing the experiment designs and in the calculations.

Growth model. The traditional Monod model was used for evaluating microbial growth rate (μ). Biomass and glucose consumption during batch cultivation are described by the equations $dX/dt = \mu X$, where X is the biomass concentration (g liter^{-1}) and t is time (h), and $dS/dt = -X[(\mu/Y_{XS}) + m_S]$, where S is the substrate concentration (g liter^{-1}), Y_{XS} is the biomass yield from substrate (g g^{-1}), and m_S is the maintenance coefficient (g g^{-1} h^{-1}). The model parameters were estimated using Matlab 6.0 software and the Simulink 4.0 software package (MathWorks) and a previously developed parameter estimation protocol (25).

Purification of L-ribulose. The cell suspensions from the optimization experiments were pooled, and the cells were separated by centrifugation at 16,000 × *g* for 20 min. The supernatant (520 ml) was evaporated under reduced pressure to 190 ml and filtered through a Whatman 1 filter paper and through a layer of activated carbon. The solution was evaporated to 30 ml, and 70 ml of ethanol was added. The resulting suspension was filtered, and the filtrate was concentrated by evaporation to 15 ml and applied to a column (2.6 by 78 cm) of Dowex 50WX4-400 (200 to 400 mesh) in the K^+ form. The column was eluted with 16 mM potassium tetraborate buffer, pH 8.0, at room temperature. L-Ribulose and L-arabinose were analyzed from the collected fractions by HPLC as described below.

L-Ribulose was recovered from the L-ribulose–borate complex using a modification of the method reported previously (16). Fractions containing the L-ribulose–borate complex were applied to Finex CS16 G H^+ ion-exchange column. The column was eluted with distilled water at room temperature. Collected fractions were dried with a freeze dryer (Christ Alpha 2-4; Martin Christ Gefriertrocknungsanlagen GmbH). Dried samples were dissolved in methanol, and the boric acid was removed as methyl borate under vacuum. L-Ribulose was dissolved in distilled water and analyzed by HPLC as described below.

HPLC analysis. Concentrations of sugars in screening, batch, and fed-batch cultivations, after breaking the L-ribulose–borate complex, and in L-arabinose isomerase activity assays were analyzed using the Aminex HPX-87P column (Bio-Rad) at 70°C with distilled water as the mobile phase. Glucose, L-arabinose, and L-ribulose concentrations from incubations in sodium and potassium tetraborate buffers were analyzed using HPX-87K column (Bio-Rad) at 85°C with borate buffer as a mobile phase as previously described (13). All components were analyzed with a refractive index detector. The elution rate was 0.6 ml min^{-1}.

RESULTS

Inactivation of *L. plantarum araB* gene. Inactivation of *araB* was carried out using the integration vector pRKDEL. A 1.5-kb fragment, starting 48 bp downstream from the start of the *araB* coding sequence and ending 33 bp upstream from the end of *araB* coding sequence, was successfully deleted during gene replacement. The first homologous-recombination step using pRKDEL was performed by using a temperature shift from 30°C to 42°C under erythromycin selection. The integration of pRKDEL was confirmed by PCR amplification with primers specific to the flanking region of the insert (RKdelcheckF and RKdelcheckR; Table 1). The second homologous-recombination step was achieved by growing the cells for 100 generations at 30°C without selection. The clones were analyzed by PCR with *araB*-specific oligonucleotides (RKdelsekvF and RKdelsekvR; Table 1), and the clones having the deletion of the right size were isolated. No

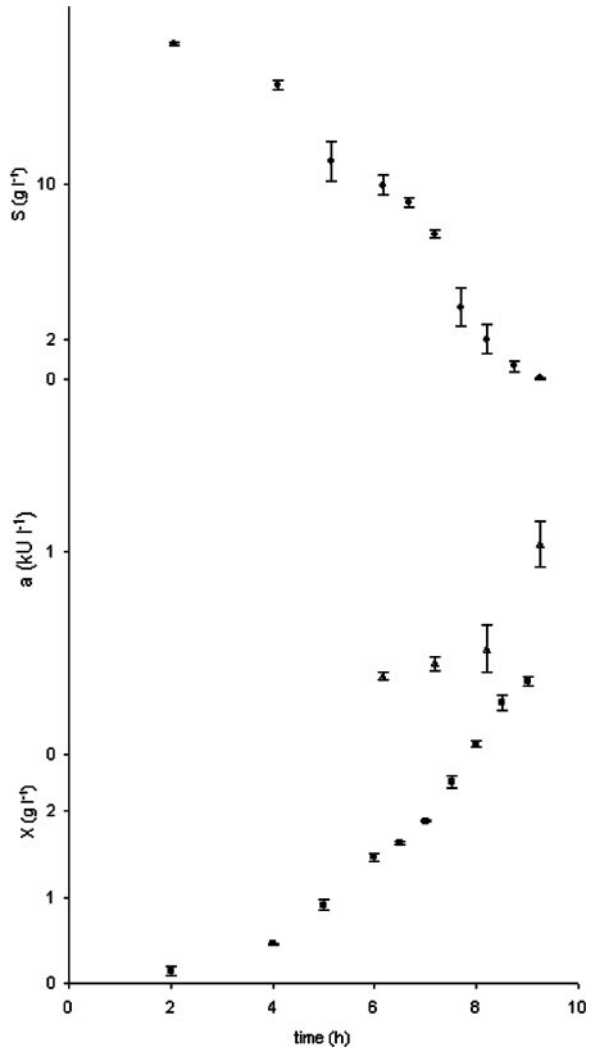

FIG. 1. Glucose concentration (S), L-arabinose isomerase activity (a), and cell density (X) in a batch cultivation of *L. plantarum* BPT197.

amplification products could be detected by PCR amplification with oligonucleotides specific to the integration vector (GhostF and GhostR; Table 1). The clones that contained the deletion were tested to be L-arabinose negative, and the strain named BPT197 was selected for further investigation. Sequencing the regions flanking the deletion site with oligonucleotides (RKdelsekvF and RKdelsekvR; Table 1) confirmed that the deletion had occurred. Furthermore the *araB*-specific RT-PCR product was detected from the arabinose-induced wild-type *L. plantarum* strain but not from the mutant *L. plantarum* BPT197.

***L. plantarum* BPT197 growth.** The growth of the L-ribulokinase-deficient mutant and the native *L. plantarum* strain was modeled in order to compare their growth behaviors and to find suitable values for fed-batch control parameters. The coefficient of determination (R^2) of the model fit to the batch data of *L. plantarum* BPT197 was 0.996. The kinetic constants of the growth model for the mutant strain were as follows: μ_{max}, 0.520 h^{-1}; Monod equation kinetic parameter for substrate consumption (K_S), 2.58 g $liter^{-1}$; Y_{XS}, 0.232 g g^{-1}; m_S, 0.411 h^{-1}. The corresponding values for the native strain under similar culture conditions were as follows: μ_{max}, 0.823 h^{-1}; K_S, 7.33 g $liter^{-1}$; Y_{XS}, 0.241 g g^{-1}; m_S, 0.324 h^{-1}. The results indicate that the mutant grows slower and utilizes glucose for energy production less efficiently than the native strain. The glucose concentration also appeared to have smaller effects on the growth rate of the mutant than on that of the native strain. Results of the RT-qPCR and activity assays from the batch cultivations (Fig. 1) indicated that the L-arabinose isomerase gene was not expressed when the glucose concentration was above 5 g $liter^{-1}$ in batch cultivation. Apparently, L-arabinose isomerase synthesis in the cells is strongly repressed by glucose.

TABLE 4. Comparison of growth and L-arabinose isomerase activity between *L. plantarum* BPT197 cultivations with different monosaccharides and disaccharides

Sugar	Activity of L-arabinose isomerase (U g^{-1} protein)[a]	ΔOD_{600}[b]
Glucose	160 ± 20	10.6 ± 0.2
Sucrose	396 ± 11	9.9 ± 0.1
Galactose	1,060 ± 14	8.4 ± 0.1
Lactose	2,266 ± 6	2.5 ± 0.2
Mannose	2,359 ± 4	3.8 ± 0.5
Maltose	408 ± 5	12.3 ± 0.3

[a] U, μmol min^{-1} L-ribulose formed.
[b] Change in optical density at 600 nm between 0 h and 10 h.

Comparison of *L. plantarum* BPT197 cultivations with different monosaccharides and disaccharides as the carbon sources is presented in Table 4. The results show that glucose, sucrose, maltose, and galactose were readily utilized for growth but that all of these carbohydrates had a repressive effect on L-arabinose isomerase synthesis. Although the L-arabinose isomerase activity of the cells was higher with lactose and mannose than the activity of the cells grown on other sugars, the growth was poor with these two sugars.

L-Ribulose production from L-arabinose was tested with growing cells of *L. plantarum* BPT197 in MRS medium supplemented with L-arabinose and glucose, fructose, or sucrose in different concentrations. The highest L-ribulose concentration achieved with growing cells of *L. plantarum* BPT197 was 0.9 g $liter^{-1}$ of L-ribulose after 10 h incubation in MRS medium supplemented with 10 g $liter^{-1}$ sucrose and 25 g $liter^{-1}$ L-arabinose.

The fed-batch cultivation mode was chosen to obtain high cell density with high L-arabinose isomerase activity. The K_S value determined from the batch cultivations implied that glucose concentrations below 2.5 g $liter^{-1}$ would limit growth. On the other hand, no L-arabinose isomerase activity was detected when the glucose concentration was over 5 g $liter^{-1}$. In order to maximize enzyme production, the operating point of 2.5 g $liter^{-1}$ was therefore chosen for glucose concentration. Despite some variation in the glucose concentrations the results were similar in the three fed-batch cultivation experiments that were carried out (data not shown). The results for the behavior of the mutant strain in a fed-batch cultivation presented in Fig. 2 show that the L-arabinose isomerase activity correlates with cell growth during the feed phase. The biomass growth seemed to be virtually unaffected by difficulties in glucose control in all three cultivations (data not shown). The relative L-arabinose isomerase mRNA levels before and after the beginning of the

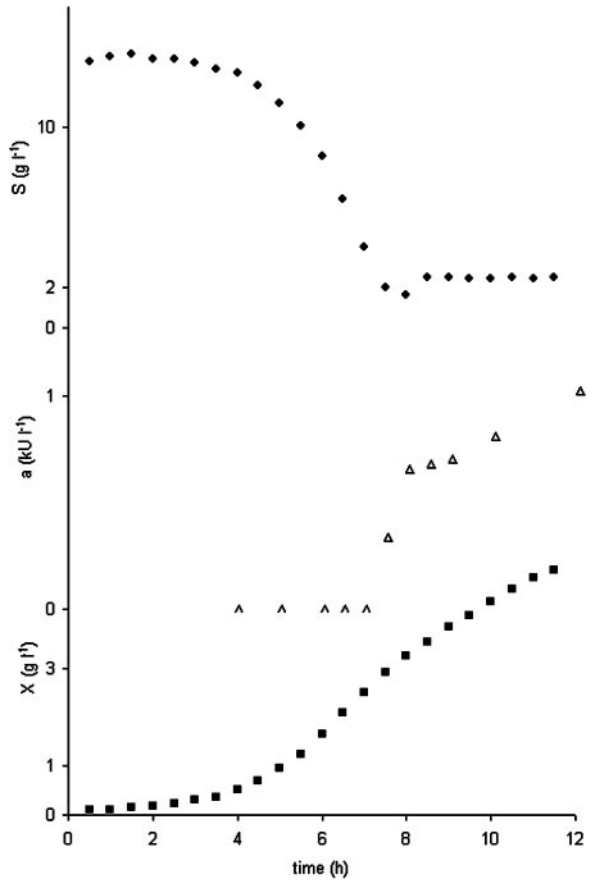

FIG. 2. Glucose concentration (S), L-arabinose isomerase activity (a), and cell density (X) in a fed-batch cultivation of *L. plantarum* BPT197. Glucose feeding started at 6 h of cultivation.

glucose feed are shown in Fig. 3 for two cultivations. Although there was a large fluctuation in the mRNA levels determined, the results show that the mRNA levels rise rapidly after the glucose concentration drops below the critical level.

Effect of borate on L-ribulose production. The effect of borate on L-ribulose production was studied by incubating resting cells of *L. plantarum* BPT197 in sodium tetraborate and in sodium phosphate buffers. We obtained 15.4 g liter^{-1} L-ribulose from 20 g liter^{-1} L-arabinose in the borate buffer, whereas in the phosphate buffer only 0.8 g liter^{-1} L-ribulose was formed. The results show that the presence of borate has a strong and favorable effect on L-ribulose production.

Optimization of L-ribulose production by resting cells. The L-ribulose production by resting cells of *L. plantarum* BPT197 was optimized with c_A, c_B, and T as the variables. The results of CCD were evaluated with statistical coefficients: R^2, coefficient of model prediction (Q^2), and probability of the null hypothesis (P value). Acceptable values for bioprocesses are R^2 over 0.8, difference between R^2 and Q^2 under 0.2, and P value under 0.01 (17). The results of the CCF experiment design are shown in Table 2. The Box-Cox analysis plot (9) of the initial linear regression fit suggested that the r_i response fit could benefit from a power transformation using a small positive value. The response x required no mathematical transformation. The model term T^2 was found to be insignificant in both models, and it was thus removed. Analysis of variance yielded the following statistical coefficients for r_i: R^2, 0.993; Q^2, 0.962; P, <0.001; P for the lack of fit, 0.062. Corresponding values for x were as follows: R^2, 0.993; Q^2, 0.960; P, <0.001; P for the lack of fit, 0.490. Figure 4 shows response surfaces of both r_i and x. A clear optimum around 500 mM can be observed for c_B. At 325 mM c_B the conversion seems to have a c_A optimum at around 30 g liter^{-1}. The results imply that the optimum c_A and T are outside the initial search area, and thus another experiment design was constructed in order to find the optimal values for these variables.

The results of the second optimization experiment design are shown in Table 3. The Box-Cox analysis plot of the initial regression fit suggested that the r_i response fit could benefit from a power transformation of 0.5. The response x required a power transformation of −1.0. The response r_{max} required a power transformation of 0.25. Analysis of variance for this experiment design yielded the following statistical coefficients for r_i: R^2, 0.997; Q^2, 0.981; $P < 0.001$; P for the lack of fit, 0.075. For x, the values are as follows: R^2, 1.000; Q^2, 0.998; P, <0.001; P for the lack of fit, 0.082. For r_{max}, the values are as follows: R^2, 0.993; Q^2, 0.952; P, <0.001; P for the lack of fit, 0.008. The response surfaces are shown in Fig. 5. The maximum L-ribulose concentrations in the experiments were around 80 g liter^{-1}. Higher temperatures yielded better productivities, and the initial rate and maximum rate gave similar results. The r_i was

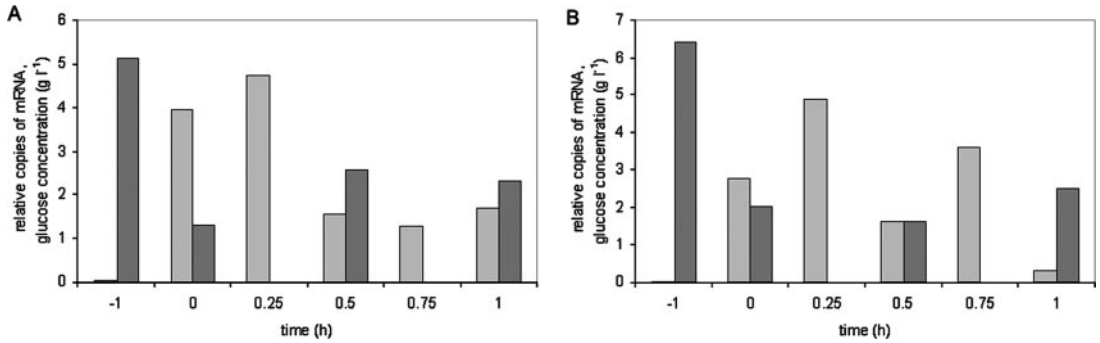

FIG. 3. Relative mRNA levels of *araA* during two different fed-batch cultivations of *L. plantarum* BPT197. The time is relative to the start of glucose feeding to the reactor. Glucose concentrations are presented as dark gray bars, and relative mRNA levels as light gray bars. Glucose concentrations are presented at 30-min intervals.

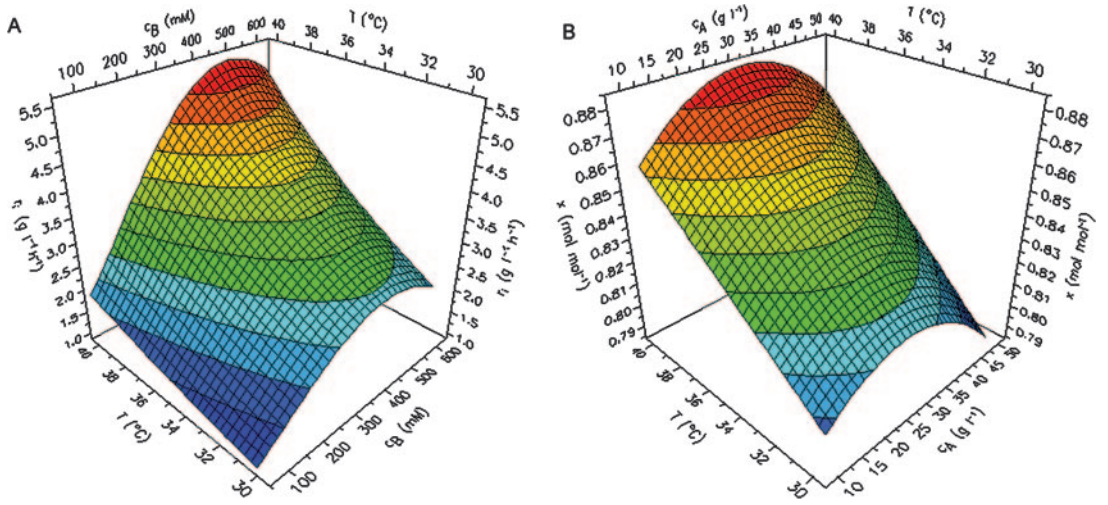

FIG. 4. Response surface plots of the optimization of L-ribulose production using resting cells of *L. plantarum* BPT197. (A) r_i at 50 g liter^{-1} L-arabinose concentration (standard deviation, 0.06 g liter^{-1} h^{-1}). (B) x at 325 mM borate concentration (standard deviation, 0.03 mol mol^{-1}).

highest at c_A values from 250 to 320 g liter^{-1}. The r_{max} favored even higher c_A values. The x, on the other hand, was highest at a smaller c_A value, the optimal value being around 70 g liter^{-1}. Although the temperature effects on x were small, the results suggest a temperature optimum between 42 and 46°C.

Purification of L-ribulose. The purification of L-ribulose was investigated using the procedure described in Materials and Methods. The complexes of borate with L-ribulose and L-arabinose were efficiently separated by the chromatographic method. In the sample applied to the column L-ribulose comprised 61% (wt/wt) of the total amount of the two isomers. In the fractions of the eluent containing over 90% of the L-ribulose the proportion of L-ribulose was 96% (wt/wt). There was a small unidentified peak eluting before L-ribulose in the HPLC chromatogram of the L-ribulose fraction (data not shown). However, this impurity was clearly separated to some extent from L-ribulose during the chromatographic separation, which suggests that it could be fully removed by optimization of the chromatographic conditions and by recycling. The L-ribulose–borate complex was broken by ion exchange and the borate removed as methyl borate. Eighty-five percent of the L-ribulose moiety of the complex was recovered using this procedure.

DISCUSSION

In this work we have studied the possibility of developing a new and efficient process for L-ribulose production from L-arabinose. The pathway of L-arabinose utilization in *L. plantarum* NCIMB8826 was blocked by inactivating the ribulokinase gene. The effects of this modification on L-ribulose production using resting cells were investigated.

In order to obtain information on growth behavior of the ribulokinase-deficient mutant *L. plantarum* BPT197, the growth was modeled using batch cultivation data. Several possibilities exist for evaluating microbial growth rate, but the traditional Monod model was used in this work. Kinetic cultivation parameters for different *Lactobacillus* species reported

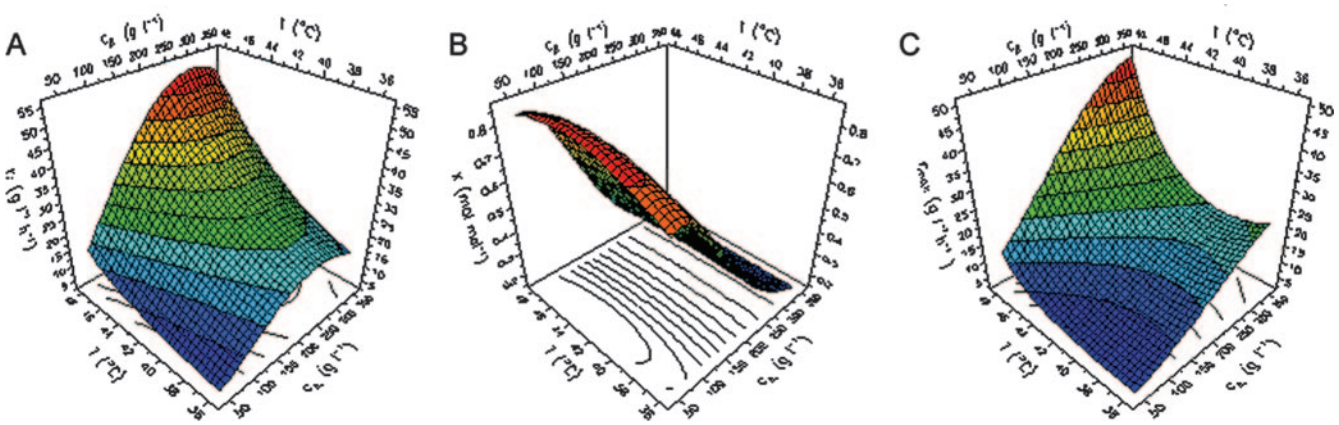

FIG. 5. Response surface plots of the temperature and L-arabinose concentration optimization of L-ribulose production using resting cells of *L. plantarum* BPT197. r_i (standard deviation, 0.35 g liter^{-1} h^{-1}) (A), conversion (x) of L-arabinose to L-ribulose (standard deviation, 0.003 mol mol^{-1}) (B), and r_{max} (standard deviation, 0.13 g liter^{-1} h^{-1}) (C) values are presented as a function of T and c_A.

in the literature were compared to our results. The μ_{max} determined in the present study is within the wide range of μ_{max} values reported. Also, our result for the K_S value (2.58 g liter^{-1}) is in agreement with the K_s values for lactic acid bacteria, ranging from 0.3 to 4.5 g liter^{-1} (7, 36). The literature values of Y_{XS} with different lactic acid bacteria vary from 0.25 to 1.38 g g^{-1} (27, 39). The results for *L. plantarum* BPT197 obtained in this study were slightly below the reported values, presumably due to differences in the cultivation conditions. No literature values were found for m_S.

The growth and L-arabinose isomerase activities were investigated with different sugars as the substrate for growth. In general, cell growth was poor on sugars which gave high L-arabinose isomerase activities. The *araA* gene of *L. plantarum* has been reported to be induced by L-arabinose and strongly repressed by glucose, fructose, and mannose (12). Contrary to this, our results showed that the activity of L-arabinose isomerase was high in cells grown on mannose, whereas 100% repression in the presence of mannose has been reported previously (12). When *L. plantarum* BPT197 was grown in batch culture in the presence of different sugars and L-arabinose, only minor amounts of L-ribulose were formed. These results prompted us to look for alternatives for batch production of L-ribulose by growing cells.

Rare sugars and sugar alcohols have been successfully produced using resting cells (14, 15, 37). It has been reported that a benefit of this production mode, especially in the case of lactic acid bacteria, is the possibility of reusing the cells in several sequential batches without significant loss of productivity (43). The fed-batch strategy for producing *L. plantarum* cells with high L-arabinose isomerase activity for resting-cell experiments was chosen on the basis of the results from the batch cultivations. Controlling the glucose concentration was necessary in order to avoid repression of *araB* expression without limiting the growth rate. The cells were cultivated for 12 h since longer cultivation times resulted in decreased enzyme activity (data not shown). The large variation in relative mRNA levels determined during glucose feeding is possibly a result of the glucose control point (2.5 g liter^{-1}) being close to the glucose repression limit.

Only minor amounts of L-ribulose were produced from L-arabinose by resting cells in the phosphate buffer used. It has been reported that at equilibrium in an L-arabinose isomerase-catalyzed reaction about 90% of the total pentose is present as L-arabinose (21), which would explain the poor conversion. L-Ribulose is known to form a more stable complex with borate than with L-arabinose (13). By complexing the formed L-ribulose with borate, the equilibrium of L-arabinose isomerization can therefore be shifted towards L-ribulose. The production of L-ribulose from L-arabinose with resting cells of the *E. coli araB* mutant has been described previously. In the presence of borate 90% conversion of L-ribulose from L-arabinose was achieved with the *E. coli* mutant (16). In our studies with resting cells of *L. plantarum* BPT197 we achieved almost 90% conversion of L-ribulose from L-arabinose in low L-arabinose concentrations ($\leq$50 g liter^{-1}) in borate buffer. At high L-arabinose concentrations ($>$100 g liter^{-1}) in the presence of 0.5 M potassium tetraborate a precipitate of unknown composition was formed. In this case the conversion was below 50%.

Rational experiment design is a beneficial tool for deriving knowledge of process interdependencies. It allows statistical evaluation of results in a modeling environment, where both model and result validity can be estimated. CCD are typically used in optimization problems of two or three variables (18, 35). Some experiment designs have been used in studies with *Lactobacillus* species. A bioreactor study on temperature, pH, and Casitone (tryptic digest of casein) concentration effects on the production of an exopolysaccharide with *L. delbrueckii* subsp. *bulgaricus* RR has been conducted using a quadratic optimization scheme (24). *L. casei* cultivation in shake flasks has been optimized using a CCD in two blocks with temperature, yeast extract, glucose, and tryptone concentrations as the variables (38). The kinetic growth constants in pH-controlled batch cultures of this species have been studied with a CCF design (19). Experiment designs are more widely and systematically used in medical experiments concerning lactic acid bacteria and their role in the digestive tracts of mammals.

A combination of the two optimization experiment designs constructed in this study was used for obtaining a wider view of L-ribulose production. The statistical coefficients were at acceptable levels, but, as the *P* for the lack of fit was too significant, the resulting response surfaces are not presented here. The combination of these two experiment designs suggested that the process has an optimal operation point around a c_A of 100 g liter^{-1}, a c_B of 500 mM, and *T* of 48°C, where the Modde software predicted an r_i of 29.1 g liter^{-1} h^{-1}, an r_{max} of 14.8 g liter^{-1} h^{-1}, and an *x* of 0.70 mol mol^{-1}. The reusability of *L. plantarum* BPT197 cells in 500 mM borate buffer was tested, and there was no loss of productivity in two sequential batches (results not shown). L-Ribulose production from L-arabinose has been only rarely studied. A significantly lower volumetric productivity of 0.66 g liter^{-1} h^{-1} was reported for resting cells of the *E. coli araB* mutant (16). In addition, *L. plantarum* has the status of generally recognized as safe, which makes it a more suitable production host for pharmaceutical applications than the endotoxin-producing *E. coli*. Furthermore, from the process point of view a benefit of using of lactic acid bacteria for production is that no instrumentation for aeration is required.

L-Ribulose production has been studied almost exclusively by using acetic acid bacteria for the dehydrogenation of ribitol to L-ribulose (4, 14, 29). The volumetric productivity of 15.7 g liter^{-1} h^{-1} achieved using resting cells of *Gluconobacter oxydans* MC14 is the highest reported using this approach (14). However, ribitol is presently very expensive and, unlike L-arabinose, is not available from natural sources in any significant amounts. Like most of the other sugar alcohols, ribitol would therefore have to be produced from the corresponding pentose, D-ribose, by chemical or microbial hydrogenation (5). Furthermore, the precursor of ribitol, D-ribose, is currently produced from glucose by fermentation (3), which makes the entire production chain to L-ribulose very complicated.

The advantages of using resting cells for the production of rare sugars include the relatively simple purification of the product, since no major by-products are formed and complex medium components are omitted during the production phase. An HPLC method for the analysis of ribulose, arabinose, and ribose mixtures has been reported previously (13). The method is based on complexing these sugars with borate and separating the negatively charged complexes by ion exclusion chromatog-

raphy. Our results suggest that a similar method can be developed for purifying L-ribulose in preparative scale. After the L-ribulose–borate complex is purified, it will have to be broken in order to separate the L-ribulose moiety of the complex. The recovery of L-ribulose has previously been achieved by exchanging the counterion of borate for H^+ and by removing the boric acid as methyl borate by evaporation (16). We are currently investigating different methods that could be used in the downstream process of L-ribulose purification.

L-Ribose is a rare sugar of high market value that is used in the pharmaceutical industry as a precursor of antiviral drugs. The rationale for research on L-ribulose production has mainly been the possibility of isomerizing it to L-ribose. L-Ribose can also be obtained by molybdate-catalyzed epimerization of L-arabinose, the raw material of the present study. However, the reported yield of L-ribose is low and a significant proportion of it is lost to by-products in this process (6). Although L-ribulose is currently very expensive, the markets for it are small. However, an efficient production method for L-ribulose would enable new applications of commercial value for this rare sugar to be found.

The bulk price for pure crystalline L-arabinose is around $50 per kg. However, this process could utilize a much cheaper material, liquid L-arabinose made from corn fiber. The price of this liquid L-arabinose has been estimated to be around $0.1 per kg (42). The L-ribulose market is currently small, and there is no bulk production. The only available price at the moment was $995 per 2 g (ZuCarb), and it is very hard to predict at which level the bulk price for L-ribulose will settle. The process seems very profitable when the material prices are compared. Furthermore, the estimated 75% overall yield of L-ribulose from L-arabinose is excellent process efficiency.

ACKNOWLEDGMENTS

We thank Ossi Pastinen for excellent advice. Esa Huusela and AH Diagnostics are thanked for assistance in RT-QPCR experiments. Kalle Salonen, Marjaana Rytelä, and Auli Murrola are acknowledged for technical support.

The research was funded by the Academy of Finland (210778).

REFERENCES

1. **Aarnikunnas, J., N. von Weymarn, K. Rönnholm, M. Leisola, and A. Palva.** 2003. Metabolic engineering of *Lactobacillus fermentum* for production of mannitol and pure L-lactic acid and pyruvate. Biotechnol. Bioeng. **82:**653–663.
2. **Adachi, O., Y. Fujii, Y. Ano, D. Moonmangmee, H. Toyama, E. Shinagawa, G. Theeragool, N. Lotong, and K. Matsushita.** 2001. Membrane-bound sugar alcohol dehydrogenase in acetic acid bacteria catalyzes L-ribulose formation and NAD-dependent ribitol dehydrogenase is independent of the oxidative fermentation. Biosci. Biotechnol. Biochem. **65:**115–125.
3. **Ahmed, Z.** 15 August 2001, posting date. Production of natural and rare pentoses using microorganisms and their enzymes. Electron. J. Biotechnol. doi:10.2225/vol4-issue2-fulltext-7.
4. **Ahmed, Z., T. Shimonishi, S. H. Bhuiyan, M. Utamura, G. Takada, and K. Izumori.** 1999. Biochemical preparation of L-ribose and L-arabinose from ribitol: a new approach. J. Biosci. Bioeng. **88:**444–448.
5. **Albert, R., A. G. Strätz, and G. Vollheim.** 1980. Die katalytische Herstellung von Zuckeralkoholen und deren Verwendung. Chem.-Ing.-Tech. **52:**582–587.
6. **Angyal, S. J.** 2005. L-Ribose: an easily prepared rare sugar. Aust. J. Chem. **58:**58–59.
7. **Berry, A., C. Franco, W. Zhang, and A. Middelberg.** 1999. Growth and lactic acid production in batch culture of *Lactobacillus rhamnosus* in a defined medium. Biotechnol. Lett. **21:**163–167.
8. **Bhowmik, T., L. Fernández, and J. L. Steele.** 1993. Gene replacement in *Lactobacillus helveticus*. J. Bacteriol. **175:**6341–6344.
9. **Box, G., and D. Cox.** 1964. An analysis of transformations. J. R. Stat. Soc. B **26:**211–252.
10. **Burma, D. P., and B. L. Horecker.** 1958. Pentose fermentation by *Lactobacillus plantarum*. III. Ribulokinase. J. Biol. Chem. **231:**1039–1051.
11. **Burma, D. P., and B. L. Horecker.** 1958. Pentose fermentation by *Lactobacillus plantarum*. IV. L-Ribulose-5-phosphate 4-epimerase. J. Biol. Chem. **231:**1053–1064.
12. **Chakravorty, M.** 1964. Induction and repression of L-arabinose isomerase in *Lactobacillus plantarum*. Biochim. Biophys. Acta **85:**152–161.
13. **De Muynck, C., J. Beauprez, W. Soetaert, and E. J. Vandamme.** 2006. Boric acid as a mobile phase additive for high performance liquid chromatography separation of ribose, arabinose and ribulose. J. Chromatogr. **1101:**115–121.
14. **De Muynck, C., C. Pereira, W. Soetaert, and E. Vandamme.** 2006. Dehydrogenation of ribitol with *Gluconobacter oxydans*: production and stability of L-ribulose. J. Biotechnol. **125:**408–415.
15. **Doten, R. C., and R. P. Mortlock.** 1985. Production of D- and L-xylulose by mutants of *Klebsiella pneumoniae* and *Erwinia uredovora*. Appl. Environ. Microbiol. **49:**158–162.
16. **Englesberg, E.** 1961. Enzymatic characterization of 17 L-arabinose negative mutants of *Escherichia coli*. J. Bacteriol. **81:**996–1006.
17. **Eriksson, L., E. Johansson, N. Kettaneh-Wold, S. Wikström, and S. Wold.** 2000. Design of experiments—principles and applications. Umetrics, Umeå, Sweden.
18. **Gabrielsson, J., N.-O. Lindberg, and T. Lundstedt.** 2002. Multivariate methods in pharmaceutical applications. J. Chemometr. **16:**141–160.
19. **Ha, M. Y., S. W. Kim, Y. W. Lee, M. J. Kim, and S. J. Kim.** 2003. Kinetics analysis of growth and lactic acid production in pH-controlled batch cultures of *Lactobacillus casei* KH-1 using yeast extract/corn steep liquor/glucose medium. J. Biosci. Bioeng. **96:**134–140.
20. **Hayn, M., W. Steiner, R. Klinger, H. Steinmüller, M. Sinner, and H. Esterbauer.** 1993. Basic research and pilot studies on the enzymatic conversion of lignocellulosics, p. 33–72. *In* J. N. Saddler (ed.), Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, United Kingdom.
21. **Heath, E. C., B. L. Horecker, P. Z. Smyrniotis, and Y. Takagi.** 1958. Pentose fermentation by *Lactobacillus plantarum*. II. L-Arabinose isomerase. J. Biol. Chem. **231:**1031–1037.
22. **Helanto, M., J. Aarnikunnas, A. Palva, M. Leisola, and A. Nyyssölä.** 2006. Characterization of genes involved in fructose utilization of *Lactobacillus fermentum*. Arch. Microbiol. **186:**51–59.
23. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
24. **Kimmel, S., R. Roberts, and G. Ziegler.** 1998. Optimization of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* RR grown in a semidefined medium. Appl. Environ. Microbiol. **64:**659–664.
25. **Kiviharju, K., K. Salonen, M. Leisola, and T. Eerikäinen.** 2006. Modeling and simulation of *Streptomyces peucetius* var. *caesius* N47 cultivation and ε-rhodomycinone production with kinetic equations and neural networks. J. Biotechnol. **126:**365–373.
26. **Kuipers, O. P., P. G. de Ruyter, M. Kleerebezen, and W. M. de Vos.** 1998. Quorum sensing controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
27. **Kwon, S., I.-K. Yoo, W. G. Lee, H. N. Chang, and Y. K. Chang.** 2001. High-rate continuous production of lactic acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor. Biotechnol. Bioeng. **73:**25–34.
28. **Kylä-Nikkilä, K., M. Hujanen, M. Leisola, and A. Palva.** 2000. Metabolic engineering of *Lactobacillus helveticus* CNRZ32 for production of pure L-(+)-lactic acid. Appl. Environ. Microbiol. **66:**3835–3841.
29. **Kylmä, A. K., T. Granström, and M. Leisola.** 2004. Growth characteristics and oxidative capacity of *Acetobacter aceti* IFO 3281: implications for L-ribulose production. Appl. Microbiol. Biotechnol. **63:**584–591.
30. **Lee, N., W. Gielow, R. Martin, E. Hamilton, and A. Fowler.** 1986. The organization of the *araBAD* operon of *Escherichia coli*. Gene **47:**231–244.
31. **Lin, H.-C., S.-P. Lei, and G. Wilcox.** 1985. The *araBAD* operon of *Salmonella typhimurium* LT2. I. Nucleotide sequence of *araB* and primary structure of its product, ribulokinase. Gene **34:**111–122.
32. **Lin, H.-C., S.-P. Lei, and G. Wilcox.** 1985. The *araBAD* operon of *Salmonella typhimurium* LT2. II. Nucleotide sequence of *araA* and primary structure of its product, L-arabinose isomerase. Gene **34:**123–128.
33. **Lin, H.-C., S.-P. Lei, G. Studnicka, and G. Wilcox.** 1985. The *araBAD* operon of *Salmonella typhimurium* LT2. III. Nucleotide sequence of *araD* and its flanking regions, and primary structure of its product, L-ribulose-5-phosphate 4-epimerase Gene **34:**129–134.
34. **Mizanur, R. M., G. Takada, and K. Izumori.** 2001. Cloning and characterization of a novel gene encoding L-ribose isomerase from *Acinetobacter* sp. strain DL-28 in *Escherichia coli*. Biochim. Biophys. Acta **1521:**141–145.
35. **Montgomery, D. C.** 1997. Design and analysis of experiments, 4th ed. John Wiley and Sons, New York, NY.
36. **Moonchai, S., W. Madlhoo, K. Jariyachavalit, H. Shimizu, S. Shioya, and S. Chauvatcharin.** 2005. Application of a mathematical model and differential evolution algorithm approach to optimization of bacteriocin production by *Lactococcus lactis* C7. Bioprocess Biosyst. Eng. **28:**15–26.

37. **Nyyssölä, A., A. Pihlajaniemi, A. Palva, N. von Weymarn, and M. Leisola.** 2005. Production of xylitol from D-xylose by recombinant *Lactococcus lactis*. J. Biotechnol. **118:**55–66.
38. **Oh, S., S. Rheem, J. Sim, S. Kim, and Y. Baek.** 1995. Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology. Appl. Environ. Microbiol. **61:**3809–3814.
39. **Rodrigues, L., A. Moldes, J. Teixeira, and R. Oliveira.** 2006. Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. Biochem. Eng. J. **28:**109–116.
40. **Scott, A.** 2002. Danisco aims to ramp up sales of "pharmaceutical sugars." Chem. Week **164:**43.
41. **Spagnuolo, M., C. Crecchio, M. D. R. Pizzigallo, and P. Ruggiero.** 1999. Fractionation of sugar beet pulp into pectin, cellulose, and arabinose by arabinases combined with ultrafiltration. Biotechnol. Bioeng. **64:**685–691.
42. **U.S. Department of Energy.** 1999. Chemicals project fact sheet. Fractionation of corn fiber for production of polyols. Office of Industrial Technologies, Energy Efficiency and Renewable Energy, U.S. Department of Energy, Washington, DC.
43. **von Weymarn, N., K. Kiviharju, and M. Leisola.** 2002. High-level production of D-mannitol with membrane cell-recycle bioreactor. J. Ind. Microbiol. Biotechnol. **29:**44–49.
44. **Wang, P., J. H. Hong, J. S. Cooperwood, and C. K. Chu.** 1998. Recent advances in L-nucleosides: chemistry and biology. Antivir. Res. **40:**19–44.
45. **Yun, M., H. R. Moon, H. O. Kim, W. J. Choi, Y.-C. Kim, C.-S. Park, and L. S. Jeong.** 2005. A highly efficient synthesis of unnatural L-sugars from D-ribose. Tetrahedron Lett. **46:**5903–5905.