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Production of xylitol from D-xylose by recombinant *Lactococcus lactis*

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Abstract

The D-xylose reductase from *Pichia stipitis* CBS 5773 and the xylose transporter from *Lactobacillus brevis* ATCC 8287 were expressed in active form in *Lactococcus lactis* NZ9800. Xylitol production was investigated using non-growing recombinant cells in high cell-density under microaerobic conditions in the presence of xylose and glucose. Besides xylose, the recombinant strain with xylose reductase activity reduced L-arabinose and D-ribose in significant extent to the corresponding pentitols.

The ratio of xylitol produced per glucose consumed was almost 10-fold higher under glucose limitation than the ratio in the presence of excess initial glucose. The co-expression of the xylose transporter with the xylose reductase did not increase the efficiency of xylitol production appreciably when compared to the strain in which only the xylose reductase gene was expressed. A fed-batch experiment with high initial xylose concentration (160 g l^{-1}) under glucose limitation was carried out using the strain co-expressing xylose reductase and xylose transporter genes. The xylitol yield from xylose was 1.0 mol mol^{-1} and the ratio of xylitol produced per glucose consumed was 2.5 mol mol^{-1}. The volumetric productivity was 2.72 g l^{-1} h^{-1} at 20 h. Of the xylose initially present, 34% was consumed. Analysis of the fermentation metabolites revealed a shift from homolactic to mixed acid fermentation at early stages of the experiment.

Keywords: *Lactococcus lactis*; Xylitol; Xylose; Metabolic engineering; Xylose reductase; Xylose transport

1. Introduction

Xylitol is a sugar-alcohol used as a natural sweetener in food and confectionary industry. The metabolism of xylitol in humans is independent of insulin, which makes it a suitable sugar substitute for use by diabetics

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(Emodi, 1978). Xylitol is also well known for its anticariogenic properties (Scheinin and Mäkinen, 1975) and it is therefore added in dental products.

Xylitol is presently produced by chemical hydrogenation of the five-carbon sugar, D-xylose, with Raney-nickel as the catalyst (Albert et al., 1980; Melaja and Hämäläinen, 1977). In order to avoid poisoning of the nickel catalyst, the xylose that is obtained from hardwood hydrolysates has to be extensively purified (Ojamo, 1994). In comparison to chemical hydrogenation, an advantage of microbial xylitol production is the possibility of using industrial side-streams as raw material instead of expensive pure xylose. Thus, there has been a considerable interest in microbial conversion of xylose to xylitol with both recombinant and non-recombinant strains.

Yeasts are generally considered to be more efficient producers of xylitol than bacteria or fungi. The best productivities so far have been achieved using yeasts belonging to the genus *Candida* (Parajó et al., 1998a, 1998b; Winkelhausen and Kuzmanova, 1998). However, the use of *Candida* in food industry is problematic because of the well-known pathogenic nature of many *Candida* species (Fridkin and Jarvis, 1996). The construction of recombinant yeasts producing xylitol has been established by the introduction of xylose reduction into *Saccharomyces cerevisiae* (Meinander and Hahn-Hägerdal, 1997; Parajó et al., 1998b; Lee et al., 2000; Govinden et al., 2001), but the highest productivities obtained using natural yeast strains have usually not been met. There are also some examples on bacterial production of xylitol. The reported productivities and final xylitol concentrations have, however, been very low both with recombinant (Suzuki et al., 1999) and natural species (Yoshitake et al., 1971, 1973; Rangaswamy and Agblevor, 2002).

The energy metabolism of lactic acid bacteria (LAB) is generally not connected to their limited biosynthetic activity. Therefore, their sugar metabolism can be engineered without disturbing the biosynthesis of structural components of the cell, which has made LAB attractive targets for metabolic engineering (Hugenholtz and Kleerebezem, 1999). The most intensively investigated LAB for metabolic engineering purposes is *Lactococcus lactis*. It is widely used in dairy and food processes, and due to its GRAS (generally recognized as safe) status, it is a potential production host for food and feed applications.

It has been reported that fructose can be reduced to mannitol very effectively by non-growing LAB cells (von Weymarn et al., 2002). In the present study, we use a similar approach for the reduction of xylose to xylitol. This is carried out by expressing a yeast xylose reductase gene in *L. lactis*. We also study the effects of introducing a xylose transporter in *L. lactis* on the xylitol production. We demonstrate for the first time that xylitol productivities comparable to those of *Candida* can be achieved with a food-compatible bacterial production strain.

2. Materials and methods

2.1. Bacterial strains, cloning vectors, media and cultivation conditions

Unless otherwise indicated, *L. lactis* NZ9800 was grown at 30 °C in M17 broth (Difco) containing 5 g l^{-1} glucose. When required, the medium was supplemented with 8 μg ml^{-1} chloramphenicol. *L. lactis* NZ9800 and the vectors pNZ8032 and pNZ8037 were obtained from NIZO Laboratories (Netherlands) (de Ruyter et al., 1996).

2.2. Chemicals

Chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Nisin stock solution (25 μg ml^{-1}) was prepared by dissolving 0.1 g of 2.5% nisin into 2 ml of 1 M HCl for 2 h at room temperature. The volume was made up to 100 ml with distilled water and the solution was filtered through a 0.2 μm membrane. Bovine serum albumin was added to the solutions at 5 mg ml^{-1} in order to prevent the adsorption of nisin onto plastic surfaces.

2.3. Plasmid construction

Total DNAs from *Pichia stipitis* CBS 5773 and *Lactobacillus brevis* ATCC 8287 were isolated using Qiagen Genomic-tips according to the instructions by the manufacturer.

The DNA isolated from *P. stipitis* was used as the template for PCR amplification of the *XYL1* gene encoding the xylose reductase (Amore et al., 1991).

Recognition sites for *Nco*I and *Hin*dIII were inserted at the 5′ and 3′ ends of the fragment, respectively. The primer pair used in the PCR was XRF 5′-TACTACC-ATGGCTTCTATTAAGTTGAACTCTG-3′ (forward) and XRR 5′-GCAACAAGCTTAGACGAAGATA-GGAATCTTGTCCCAGTCCCA**A**GGGTCG-3′ (reverse). To facilitate cloning, an authentic *Nco*I site at the end of the gene was removed by introducing a silent point mutation into its recognition sequence (shown in bold). The amplified PCR-fragment was purified using QIAquick DNA purification kit (Qiagen) and cloned into an *Nco*I and *Hin*dIII cut pNZ8032 expression vector. *L. lactis* NZ9800 was transformed with the resulting expression plasmid by electroporation as described previously by Holo and Nes (1989). The strain was named LLXR.

An operon encoding xylose reductase of *P. stipitis* and xylose-H^+ symporter XylT of *Lb. brevis* (Chaillou et al., 1998) was constructed by recombinant PCR. In the construct, *XYL1* was arranged upstream of *xylT* under the control of the *nisA* promoter present in the expression vector pNZ8032 (de Ruyter et al., 1996). The *XYL1* fragment was fused with a fragment containing the ribosomal binding site of *xylT*, the structural gene *xylT*, and the original transcription terminator of *xylT*. *Nco*I and *Hin*dIII recognition sites were inserted at the 5′ and 3′ ends of the fusion fragment, respectively. *XYL1* was amplified by PCR using primers XRF (see above) and RSDR 5′-AATCT-CGCTCCTTAGACGAAGATAGGAATCTTGTCCC-AGTCCCA**A**GGGTCG-3′ (reverse) with chromosomal *P. stipitis* DNA as the template. The point mutation which removes the *Nco*I recognition sequence is again shown in bold. The fragment containing *xylT*, its ribosomal binding site and its transcription terminator was amplified by using the primer pair TSDF 5′-TCTTCGTCTAA*GGAGC*GAGATTTATGCGAAAA-GTTTCGACC-3′ (forward) and TTERR 5′-GCGC-GAAGCTTAAAAATGGTCAGACCAGTTTTACCT-GATCCC-3′ (reverse) using chromosomal *Lb. brevis* DNA as the template. The stop codon of *XYL1* is shown underlined and the putative ribosomal binding site of *xylT* is shown in italics. The amplified PCR-fragments were purified using the Qiagen Gel Extraction kit and used as the templates in a second round of PCR with XRF and TTERR as the primers. In this PCR, the two fragments were joined by utilizing an overlap of 22 bp at the end of *XYL1* and at the beginning of *xylT* containing fragments. The amplified PCR-fragment was cloned into an *Nco*I and *Hin*dIII cut pNZ8032 expression vector and transferred into *L. lactis* NZ9800 by electroporation as described above. The strain was named LLXTXR.

2.4. Preparation of cell extracts and xylose reductase assay

Duplicate 40 ml cultures of the *L. lactis* recombinant strains were grown to an optical density (OD_{600}) of 0.5–0.7 at 600 nm and the inducer nisin was added. The cultures were induced for 2 h and the cells were harvested by centrifugation at $3000 \times g$ for 10 min at 4 °C. The cells were washed with 10 mM potassium phosphate buffer, pH 6.0 and resuspended into 1 ml of buffer. After the addition of 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, the cells were disrupted with a Labsonic 2000 U (B. Braun, Melsungen, Germany) sonicator in 1 ml batches. The cells were sonicated with maximum power in four 15 s pulses with intermittent cooling. The cell debris was removed by centrifugation at $28{,}000 \times g$ at 4 °C. The resulting extract is referred to as cell extract.

Xylose reductase activity was assayed from the cell extracts by measuring the change in absorbance at 340 nm at 30 °C (Verduyn et al., 1985). The activities were calculated as μmol NADH or NADPH oxidized per minute. Protein concentrations were determined using Bio-Rad protein assay reagent with bovine serum albumin as the protein standard.

2.5. Xylose transport analysis

Duplicate cultures (40 ml) of the recombinant *L. lactis* strains were induced with nisin and the cells were harvested as described above. Xylose transport was analyzed using a modification of the method described previously (Chaillou et al., 1998). The cells were washed once with uptake buffer (50 mM potassium phosphate buffer, pH 6.5 containing 2 mM $MgSO_4$) and resuspended. The assays were carried out at the final cell density of 6.0–7.0 OD_{600} at 30 °C. Cells were preenergized by incubation with 5 mM glucose for 5 min and [U-^{14}C]xylose (91 mCi mol^{-1}) (Amersham Pharmacia Biotech) was added at a final concentration of 0.5 mM. The cell-suspensions were incubated

for 2 min and cells from 0.5 ml samples were filtered onto 0.2 μm cellulose nitrate filters (Whatman). The filters were washed twice with 2.5 ml of ice-cold 0.1 M LiCl and their radioactivity was determined by scintillation counting as described previously (Nyyssölä and Leisola, 2001).

2.6. Analysis of xylitol production with non-growing cells under microaerobic conditions

Recombinant *L. lactis* cell cultures were grown, induced with nisin and separated from the medium by centrifugation as described above. The incubation buffer contained 100 mM potassium phosphate buffer pH 7.0 supplemented with 2 mM $MgSO_4$. The harvested cells were washed once with double strength incubation buffer and resuspended. The cell suspensions were dispensed in 2 ml batches to glass test tubes. The xylitol production was started by adding 2 ml of a solution containing xylose and glucose to final concentrations of 60 g l^{-1} and 20 g l^{-1}, respectively. In order to minimize the effect of oxygen, the solutions were sparged with nitrogen before the experiment, and the cell-batches were layered with mineral oil. The cell suspensions were incubated at 30 °C and cells were separated by centrifugation for 1 min at 28,000 × *g*. The supernatants were filtered through 0.2 μm membranes and analyzed by high performance liquid chromatography (HPLC) as described below. The effect of different inducer (nisin) levels (0–0.8 ng ml^{-1}) on xylitol production was studied using non-growing cells at the cell densities of 18.4–23.7 OD_{600} and 22.1–27.1 OD_{600} with LLXR and LLXTXR, respectively.

The effect of initial xylose concentration (0–230 g l^{-1}) was studied at the cell density of 16.2 OD_{600} and the reduction of different sugars (L-arabinose, D-galactose, D-mannose, D-ribose and D-xylose) was carried out at the cell density of 7.2 g l^{-1} (cell dry weight per volume) essentially as described above.

The effect of pH on xylitol production of non-growing cells under microaerobic conditions was investigated as above with the following exceptions. The cells were suspended in 300 mM potassium phosphate buffer (pH 5.5–7.7) at OD_{600} of 11.2 or 300 mM Tris–HCl buffer (pH 7.1–8.7) at OD_{600} of 11.6. The reaction buffers were supplemented with 1 mM $MgSO_4$, 60 g l^{-1} xylose and 20 g l^{-1} glucose.

2.7. Batch and fed-batch production of xylitol with pH control using non-growing cells

Five liters of M17 growth medium containing 10 g l^{-1} glucose was inoculated with 25 ml of recombinant *L. lactis* cell culture propagated to the stationary phase. The cells were grown for 2 h and 0.8 ng ml^{-1} nisin was added. The cultures were further grown for 11 h and the cells were separated by centrifugation at 3000 × *g* for 10 min. The cells were washed with the xylitol production buffer containing 50 mM potassium phosphate buffer pH 7.0, 2 mM $MgSO_4$, 8 μg ml^{-1} chloramphenicol, 1 mM dithiothreitol and 0.8 ng ml^{-1} nisin and resuspended.

In order to compare the batch and glucose-limited fed-batch production modes, the recombinant *L. lactis* cells were suspended in the xylitol production buffer supplemented with 25 g l^{-1} xylose. One hundred and fifty milliliters of cell suspension was used in the experiment. Glucose and M17 were supplied at feed-rates of 1.0 g l^{-1} h^{-1} and 0.25 g l^{-1} h^{-1} per suspension volume, respectively. In the batch production experiment glucose and M17 were added at initial concentrations of 75 g l^{-1} and 2.0 g l^{-1}, respectively. The production experiments were performed in duplicates at 30 °C in 250 ml glass vessels with magnetic stirring. The effect of oxygen was minimized by continuously sparging the cell suspensions with nitrogen. pH was controlled at 7.0 ± 0.1 by automatic addition of 4 M KOH. The experiments were carried out at the initial cell densities of 23.5–27.4 g l^{-1} (cell dry weight per volume).

The final fed-batch production experiments at high xylose concentration were performed as described above with the exception that the initial xylose concentration was 160 g l^{-1} and glucose and M17 were fed at rates of 1.3 g l^{-1} h^{-1} and 0.1 g l^{-1} h^{-1} (per suspension volume), respectively. The experiments were carried out at the mean initial cell density of 36 g l^{-1} (cell dry weight per volume).

2.8. Other analytical procedures

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels according to the standard protocol (Laemmli, 1970). Growth rates were estimated by monitoring OD_{600} of the cultures. Glucose, xylose, xyl-

itol, formate, acetate, lactate, acetoin, diacetyl and 2,3-butanediol were analyzed from the samples at 65 °C using a HPLC system equipped with a Bio-Rad HPX-87H Aminex column and a Waters 410 RI refractive index detector. H_2SO_4 (5 mM) was used as the eluent with a flow-rate of 0.4 ml min^{-1}. L-Arabitol, galactitol, D-mannitol and ribitol were determined using a Bio-Rad HPX-87P column and a Deashing Micro-Guard pre-column (Bio-Rad) at 70 °C with deionized water as the eluent at a flow-rate of 0.6 ml min^{-1}.

3. Results

3.1. Expression of xylose reductase

In order to study the controlled expression of xylose reductase, the previously described nisin-inducible expression system NICE was used (de Ruyter et al., 1996). The xylose reductase gene *XYL1* of the yeast *P. stipitis* was translationally fused to the start codon present in the expression vector pNZ8032 under the control of the *nisA* promoter. Transformation or *L. lactis* NZ9800 with the expression plasmid resulted in the strain *L. lactis* LLXR.

Xylose reductase activities were determined with NADH as the co-substrate in LLXR cell extracts prepared from cultures induced with different concentrations of nisin (Fig. 1). The results indicate that xylose reductase was expressed in *L. lactis* in active form. The xylose reductase activity of the cell extracts increased sharply with the increasing nisin concentration until 0.2 ng ml^{-1} nisin, after which the activity reached a plateau. The maximum activity of 1.8 μmol min^{-1} mg^{-1} was obtained using 1.2 ng ml^{-1} nisin. No xylose reductase activity could be detected in the cell extract from the uninduced control cultivation. The activity ratio with NADH and NADPH as the co-factors determined at the maximal induction level was 0.64 (NADH/NADPH).

The cell extracts from samples induced with 0.8 ng ml^{-1}, 0.4 ng ml^{-1}, 0.2 ng ml^{-1}, and without nisin were further analyzed by SDS-PAGE (Fig. 2). A clearly visible band was present in the samples from the induced cultures, but not in the sample from the culture without added nisin. The position of the band corresponded well with the calculated molecular weight of the *P. stipitis* xylose reductase (35.9 kDa).

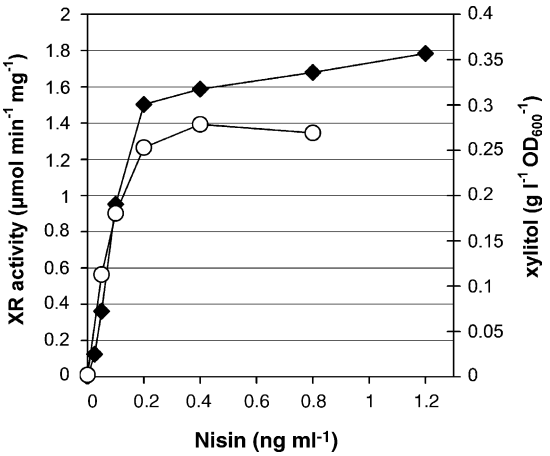

Fig. 1. Analysis of *XYL1* expression in *L. lactis* LLXR cells grown in the presence of different concentrations of the inducer nisin. Xylose reductase activity of cell extracts (♦) and production of xylitol by non-growing cells (◯). Non-growing cells were incubated for 3 h in a cell suspension supplemented with 60 g l^{-1} xylose and 20 g l^{-1} glucose as described in Section 2. The errors were less than 10%.

In a separate experiment, LLXR batch cultures were induced with different concentrations of nisin, separated from the medium and incubated under microaerobic conditions in the presence of 60 g l^{-1} xylose and

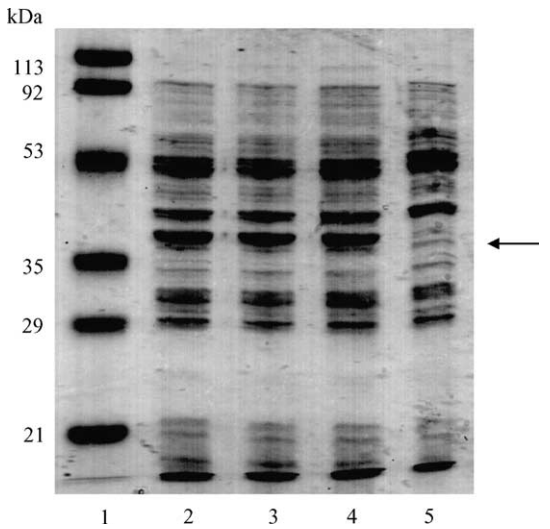

Fig. 2. Coomassie stained SDS-PAGE gel of cell extracts of *L. lactis* LLXR after induction with different concentrations of nisin. Lane 1, molecular weight marker; lane 2, 0.8 ng ml^{-1} nisin; lane 3, 0.4 ng ml^{-1} nisin; lane 4, 0.2 ng ml^{-1} nisin; and lane 5, no nisin added. The position of the putative xylose reductase band is shown by an arrow.

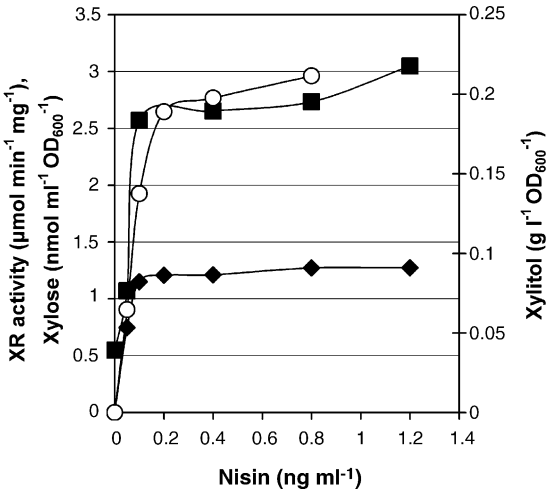

Fig. 3. Analysis of *XYL1* and *xylT* co-expression in *L. lactis* LLXTXR cells grown in the presence of different concentrations of the inducer nisin. Xylose reductase activity of cell extracts (♦) (errors less than 11%), accumulation of [U-^{14}C]xylose in cells during 2 min (■) (errors less than 22%) and production of xylitol by non-growing cells (○) (errors less than 15%). Xylitol production was analyzed by incubating the cells for 3 h in a cell suspension supplemented with 60 g l^{-1} xylose and 20 g l^{-1} glucose as described in Section 2.

20 g l^{-1} glucose for 3 h as described in Section 2. As illustrated in Fig. 1, the concentrations of the xylitol produced correlated with the expression levels of xylose reductase.

3.2. Co-expression of xylose reductase and xylose transporter

In order to study the co-expression of the *P. stipitis* xylose reductase and *Lb. brevis* xylose transporter genes in *L. lactis* NZ9800, an operon structure was constructed under the control of *nisA* promoter in pNZ8032. The *Lb. brevis* xylose transporter gene region containing the ribosomal binding site, structural gene *xylT* and transcription terminator was positioned downstream of *XYL1*. As shown in Fig. 3, both the xylose transporter and the xylose reductase were active at variable concentrations of the inducer nisin in LLXTXR cells expressing the *XYL1–xylT* operon. The accumulation level of [U-^{14}C]xylose in LLXTXR cells during 2 min correlated with the activity of the xylose reductase in the cell extracts, and with the concentration of xylitol in the xylitol production experiment with non-growing cells. [U-^{14}C]xylose accumulation, xylose reductase activity and xylitol production increased steeply with increasing nisin concentration until 0.1–0.2 ng ml^{-1} nisin. At the higher nisin concentrations the values increased only slightly. The maximum xylose reductase activity of 1.3 µmol min^{-1} mg^{-1} and the maximum [U-^{14}C]xylose accumulation of 3.0 nmol OD$_{600}$ $^{-1}$ ml^{-1} were reached at 1.2 ng ml^{-1} nisin. The maximum value for the xylose accumulation was estimated to correspond to 11 nmol mg^{-1} (per cell dry weight) using the correlation 1.00 OD$_{600}$ = 0.265 mg ml^{-1} (cell dry weight per volume). [U-^{14}C]xylose was accumulated in LLXTXR at least fivefold at the maximum accumulation level as compared to the uninduced control or to the control strain transformed with plasmid pNZ8037 without the xylose reductase gene. However, only 3.9% of the total amount of [U-^{14}C]xylose in the reaction mixture was accumulated in the LLXTXR cells at this induction level.

Under the conditions used, both [U-^{14}C]xylose accumulation level and xylose reductase activity appeared to be lower when *XYL1* and *xylT* were co-expressed than when these genes were expressed singly. The maximum xylose reductase activity determined from cell extracts of LLXTXR was 61% of the activity in the cell extracts of LLXR which carries only *XYL1*. The maximum accumulation of [U-^{14}C]xylose in LLXTXR cells was 60% of the accumulation in *L. lactis* cells expressing only *xylT* (data not shown).

3.3. Xylose utilization and effect of xylitol on cell growth

The host strain *L. lactis* NZ9800 was not able to use xylose as a carbon source when grown in M17 medium at 30 °C. No growth on xylose (5 g l^{-1}) above the growth of the control cultivation without added sugars was detected in 24 h. In addition, according to the HPLC-analysis of the culture medium no xylose was metabolized. Similar results were obtained when the cells were grown in the presence of 5 g l^{-1} xylose and 5 g l^{-1} glucose: no co-metabolization of xylose with glucose could be detected.

High concentrations of xylitol clearly inhibited the growth of *L. lactis* NZ9800. Specific growth rates were estimated from the optical densities measured at 600 nm. At 50 g l^{-1}, 100 g l^{-1} and 200 g l^{-1} of xylitol

present in the M17 growth medium the specific growth rates were approximately 80%, 66% and 49% of the growth rate without added xylitol, respectively (data not shown).

3.4. Effect of pH on xylitol production

The effect of pH on xylitol production by non-growing LLXR cells expressing xylose reductase are presented in Fig. 4. The maximal xylitol production rate with the Tris–HCl buffer used was obtained around pH 8.3. The results suggest that neutral or slightly alkaline conditions are favorable for xylose reduction, which is compatible with the previously reported observation that higher pH can result in considerably higher intracellular NADH/NAD^+ ratio (Snoep et al., 1991). The pH 7.0 was chosen for further production experiments.

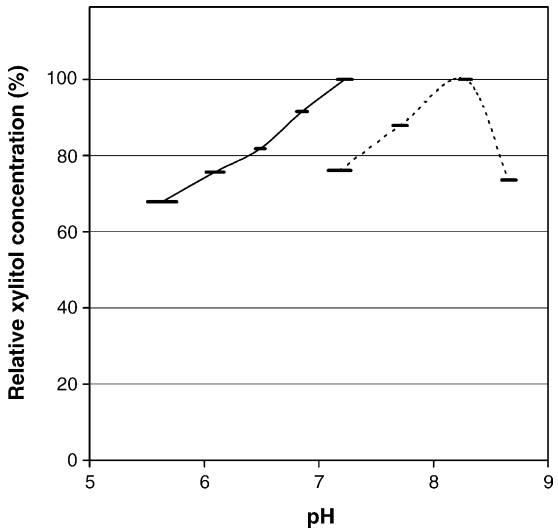

Fig. 4. Effect of pH on xylitol production by non-growing *L. lactis* LLXR cells under microaerobic conditions. The cells were suspended into 300 mM potassium phosphate buffer (—) at OD_{600} of 11.2 or 300 mM Tris–HCl buffer (----) at OD_{600} of 11.6. Cultures were induced with 0.8 ng ml^{-1} nisin. The buffers were supplemented with 60 g l^{-1} xylose and 20 g l^{-1} glucose. The values presented are final concentrations of xylitol relative to the maximum obtained with each buffer system. The changes in pH during 1 h of incubation are shown by horizontal bars.

3.5. Comparison of batch and fed-batch xylitol production modes

The batch and glucose-limited fed-batch production modes were compared using non-growing LLXR cells in high cell-density under microaerobic conditions in the presence of xylose and glucose (Table 1). The results indicate that the specific productivity of xylitol in the fed-batch fermentation was 70% of that in the batch. However, the ratio of xylitol produced per glucose consumed was 10-fold higher in the fed-batch experiment.

Comparison of LLXR and LLXTXR strains in the fed-batch mode shows that expression of the xylose transporter gene *xylT* did not significantly increase the efficiency of xylitol production. However, the results suggest that the transporter could be slightly beneficial in terms of higher ratio of xylitol produced per glucose consumed. Thus, the strain LLXTXR was chosen for further production experiments.

The yield of xylitol from xylose was in effect quantitative in all production experiments. No glucose could be detected in the medium in the fed-batch experiment. In addition, no appreciable cell growth could be observed in any of the cell suspensions used.

Table 1
Comparison of xylitol production in batch and fed-batch modes using *L. lactis* LLXR and comparison of *L. lactis* LLXR and LLXTXR in fed-batch mode[a]

Strain	Mode	Incubation time (h)	Initial cell density (g l^{-1})[b]	Specific productivity (g l^{-1} g^{-1})	Yield xylitol/xylose (mol mol^{-1})	Xylitol production/glucose consumption (mol mol^{-1})
LLXR	Batch[c]	5	23.5 ± 0.1	0.12	0.98 ± 0.05	0.22 ± 0.03
	Fed-batch[d]	8	24.7 ± 0.2	0.086	0.98 ± 0.05	2.3 ± 0.04
LLXTXR	Fed-batch[d]	8	27.4 ± 0.6	0.092 ± 0.015	1.00 ± 0.01	2.7 ± 0.3

[a] The cells were induced with 0.8 ng ml^{-1} nisin. The initial xylose concentration was 25 g l^{-1}. The experiments were performed in duplicates.
[b] Cell dry weight per volume.
[c] Initial glucose and M17 concentrations were 75 g l^{-1} and 2.0 g l^{-1}, respectively.
[d] Glucose and M17 were supplied at a feed-rate of 1.0 g l^{-1} h^{-1} and 0.25 g l^{-1} h^{-1}, respectively (per suspension volume).

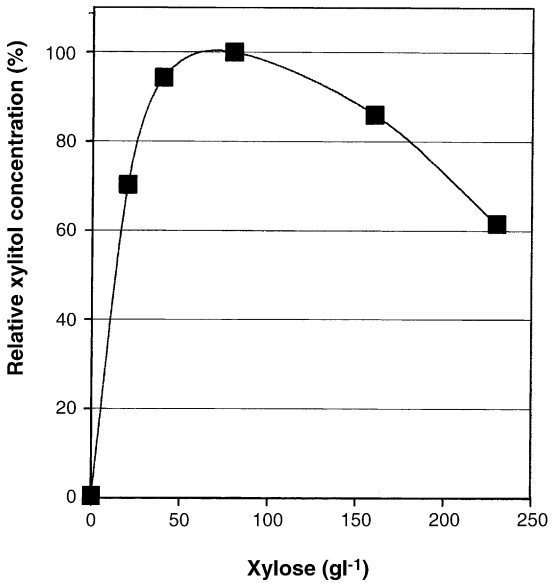

Fig. 5. Xylitol production by non-growing *L. lactis* LLXTXR cells at different initial xylose concentrations. The values presented are final concentrations of xylitol relative to the maximum obtained. The cell cultures were induced with 0.8 ng ml^{-1} nisin. The cells were incubated at 20 g l^{-1} initial concentration of glucose under microaerobic conditions for 3 h. The cells were suspended at OD_{600} of 16.2 into incubation buffer as described in Section 2.

3.6. Xylitol production at different initial xylose concentrations

The effect of initial xylose concentration on xylitol production under microaerobic conditions with non-growing LLXTXR cells is presented in Fig. 5. The results show that the cells produced more xylitol when the initial concentration of xylose was increased from 0 g l^{-1} to 100 g l^{-1}. At initial xylose concentrations higher than 100 g l^{-1} the xylitol production decreased.

The organic osmolyte glycine betaine is well known for its osmoprotective effect in many different bacterial strains, including *L. lactis* (Galinski, 1993; Molenaar et al., 1993). It has been reported that the osmotic stress resulting from high xylose concentrations can inhibit xylitol production in yeast (da Silva and Afschar, 1994). However, in our study, the addition of 4 mM glycine betaine into the cell suspensions did not significantly stimulate xylitol production by the cells (data not shown).

3.7. Production of other sugar alcohols

The production of other sugar alcohols by non-growing LLXR cells in the presence of glucose and L-arabinose, D-galactose, D-mannose, D-ribose or D-xylose is presented in Table 2. The results indicate that D-ribose and L-arabinose were reduced to the corresponding sugar alcohols (ribitol and L-arabitol) at least as efficiently as D-xylose. D-Mannitol and galactitol were produced only in minor amounts from D-mannose and D-galactose, respectively. No sugar alcohols were produced by *L. lactis* NZ9800 transformed with the plasmid pNZ8037 without the xylose reductase gene.

3.8. Fed-batch production of xylitol at high xylose concentration

Non-growing cells of LLXTXR were incubated in the presence of 160 g l^{-1} xylose in glucose-limited fed-batch in high cell-density. Fig. 6A illustrates data from a production experiment. The results indicate that both the volumetric productivity and the ratio of xylitol produced per glucose consumed decreased as the production experiment progressed. The volumetric xylitol productivity calculated as a mean of two experi-

Table 2
Reduction of different sugars to corresponding sugar alcohols by *L. lactis* LLXR[a]

	Sugar alcohol				
	L-Arabitol	Galactitol	D-Mannitol	Ribitol	Xylitol
Final concentration (mM)	34	3.0	0.8	33	28

[a] The cell cultures were induced with 0.8 ng ml^{-1} nisin. Cell suspensions (cell density 7.2 g l^{-1} cell dry weight per volume) were incubated for 3 h. Initial concentrations of the sugars were 400 mM and initial concentration of glucose was 110 mM. The errors were within 10%.

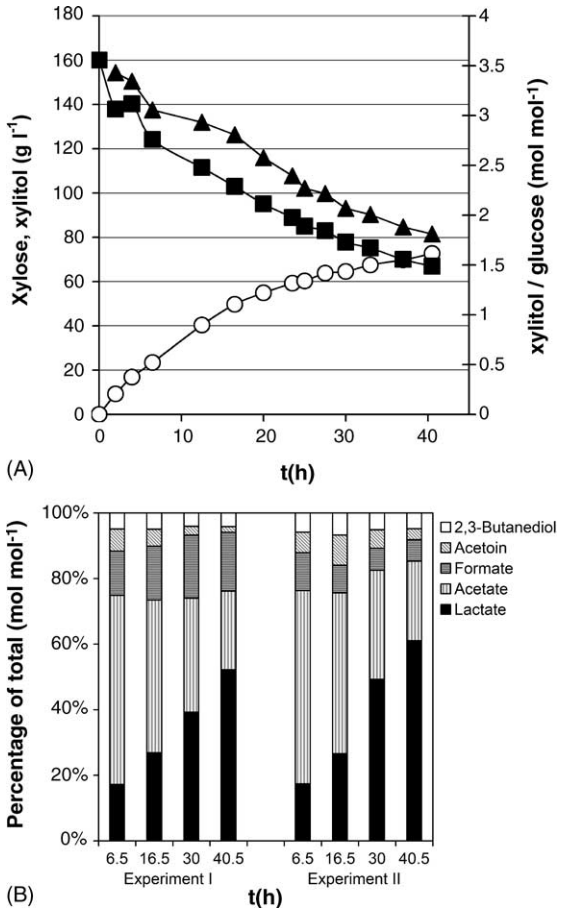

Fig. 6. Fermentation behavior of non-growing *L. lactis* LLXTXR cells under microaerobic conditions and under glucose limitation in fed-batch. The cell cultures were induced with 0.8 ng ml^{-1} nisin. The cells were supplied with glucose and M17 at feed-rates of 1.3 g l^{-1} h^{-1} and 0.1 g l^{-1} h^{-1}, respectively. The initial xylose concentration was 160 g l^{-1} and initial cell density 36 g l^{-1} (cell dry weight per volume). (A) Xylose utilization (■), xylitol production (◯) and ratio of xylitol produced per glucose consumed (▲). (B) Distribution of fermentation metabolites relative to the total amount determined in the two experiments.

ments was 2.72 ± 0.03 g l^{-1} h^{-1} after 20 h of incubation. At this point the xylitol yield from xylose and the ratio of xylitol produced per glucose consumed were 1.04 ± 0.08 mol mol^{-1} and 2.51 ± 0.09 mol mol^{-1}, respectively. Of the xylose initially present, 34% was consumed. At 40.5 h the volumetric productivity had decreased to 1.83 ± 0.06 g l^{-1} h^{-1}. The yield of xylitol from xylose and the ratio of xylitol produced per glucose consumed were 1.02 ± 0.006 mol mol^{-1} and 1.78 ± 0.05 mol mol^{-1} at 40.5 h, respectively, and of the xylose initially present, 50% was consumed. No significant growth of the cell mass could be observed during the experiment and no glucose could be detected in the medium.

In order to study the primary sugar metabolism of LLXTXR at different time points, the medium was analyzed for common fermentation metabolites. The distribution of the metabolites is shown in Fig. 6B. At 6.5 h the compounds determined accounted for approximately 87–88% of glucose consumed. No diacetyl could be detected in the media. For unknown reason there was a large variation between the two production experiments. Nevertheless, a clear trend can be observed. At early stages of the experiment, the overall product distribution showed a deviation from the normal homolactic sugar metabolism. As the experiment progressed, the product distribution approached that of homolactic fermentation. At 6.5 h, lactate accounted for approximately 20% of the total fermentation metabolites determined, but at 40.5 h its fraction had increased to approximately 50%. As shown in Fig. 6B, acetate was the main product at early stages of the fermentation (61% at 6.5 h) but its proportion decreased in time.

4. Discussion

Because of their relatively limited biosynthetic capabilities, LAB generally require complex culture media. This can be problematic for their use in industrial cultivations because of the high cost of such media. However, production costs can be lowered by the reuse of non-growing LAB cells. For example, in our laboratory *Leuconostoc mesenteroides* cells have been used in 14 sequential batches without loss of productivity in mannitol production from fructose (von Weymarn et al., 2002).

The xylose reductase from *P. stipitis* was chosen for the current study because it has a reasonably high specific activity for NADH in addition to NADPH (Verduyn et al., 1985). Furthermore, it has been reported that the *P. stipitis XYL1* can be expressed in *Escherichia coli* (Amore et al., 1991). However, there is no information available on the enzymatic activity of this xylose reductase in any bacterial host. Our results

show that the recombinant xylose reductase is active in *L. lactis*. The NADH/NADPH-linked activity ratio of 0.64 determined in the present study is in agreement with the ratio of 0.72 reported previously for the native enzyme (Verduyn et al., 1985).

We attempted to enhance the influx of xylose by co-expression of the xylose transporter of *Lb. brevis* with the xylose reductase. The heterologous expression of the xylose transporter gene in a heterofermentative host, *Lactobacillus plantarum*, has been described previously (Chaillou et al., 1998). In that study, xylose was accumulated to a final intracellular concentration of 3.8 nmol mg^{-1} (per cell dry weight) in 2 min under similar conditions as were used in the present study, with the exception that the initial xylose concentration was lower in the previous study (100 mM) than that used here (500 mM). In the present study, the maximum xylose accumulation was estimated to be 11 nmol mg^{-1}. Thus, the results suggest that *xylT* can most likely be expressed as efficiently in *L. lactis* as in *Lb. plantarum*. Surprisingly, however, co-expression of the xylose transporter gene with the xylose reductase gene did not improve the xylitol production in any significant extent.

Majority of *L. lactis* strains used in dairy fermentations are not able to utilize xylose. This has been reported to be a consequence of accumulation of mutations in genes encoding for enzymes of xylose metabolism (Erlandson et al., 2000). According to our results *L. lactis* NZ9800 did not use xylose neither in the presence nor absence of glucose when grown in complex medium. The lack of a metabolic pathway for xylose utilization was also demonstrated by the nearly quantitative yield of xylitol from xylose in the fermentation experiments. These observations suggest that despite the inability of the strain to metabolize xylose, xylose transport is functional in *L. lactis* NZ9800.

In *L. lactis*, the glycolytic intermediate pyruvate is reduced to lactate with the concomitant formation of NAD^+ during normal homolactic fermentation. When changes in the fermentation conditions lead to the depletion of intracellular NADH, pyruvate can be rerouted in order to fulfill the requirement for the reducing form of the coenzyme. Pyruvate is split to acetyl-coenzyme A and formate in a pyruvate formate lyase catalyzed reaction. The acetyl-coenzyme A can then be further converted to acetate with an equivalent of ATP being formed in the reaction by substrate level phosphorylation. Hence, the formation of acetate would also lead to higher energy yield from glucose (Axelsson, 1998). This shift to mixed acid metabolism by *L. lactis* has been shown for non-growing cells under glucose limitation and anaerobic conditions. The glucose limitation presumably leads to the activation of pyruvate formate lyase and inhibition of lactate dehydrogenase. The main metabolic products in this case are acetate, formate and ethanol (Fordyce et al., 1984; Melchiorsen et al., 2002).

The batch production of xylitol with non-growing cells resulted in a low ratio of xylitol produced per glucose consumed. However, by glucose limited fed-batch production mode a 10-fold enhancement in the ratio was achieved. A possible explanation for the improved ratio is that the sugar metabolism of the cells was changed from homolactic to mixed-acid fermentation as described above. In agreement with this assumption is that the analysis of the final fermentation products of the fed-batch experiment showed a deviation from the normal homolactic product formation pattern of *L. lactis* with acetate and formate as the main products. Hence, the product distribution observed suggests the activation of the pyruvate formate lyase pathway.

The majority of xylitol productivities reported for yeasts fall well below the volumetric productivity of 2.72 g l^{-1} h^{-1} obtained in this study during 20 h (for references see Parajó et al., 1998a, 1998b; Winkelhausen and Kuzmanova, 1998). Among the few reports in which higher productivities have been achieved using high initial concentrations of xylose are the following: *C. tropicalis* produced 4.94 g l^{-1} h^{-1} xylitol from xylose in a cell recycling process with yeast extract, glucose and xylose feed. The yield from xylose was 0.81 mol mol^{-1} and the final xylitol concentration was 189 g l^{-1} (Choi et al., 2000). At initial xylose concentration of 279 g l^{-1} in complex medium *Debaryomyces hansenii* (*Candida famata*) produced 4.6 g l^{-1} h^{-1} xylitol with a xylitol yield of 0.78 mol mol^{-1} from xylose (Dominguez et al., 1997). The final xylitol concentration was 221 g l^{-1}. In these reports, the xylose was almost completely consumed whereas in the fed-batch fermentation reported in the present study only 34% of the xylose was used for xylitol production during 20 h. The production rate clearly slowed down after 20 h. In addition, the ratio of xylitol produced per glucose consumed decreased, which was

accompanied by reversal of the sugar metabolism back towards homolactic fermentation.

A possible explanation for the decreasing productivity is that the accumulated xylitol inhibits the reduction reaction. However, no product inhibition by xylitol (up to 0.5 M) has been observed for the xylose reductase of *P. stipitis* (Verduyn et al., 1985). In addition, in the reaction where xylose is reduced to xylitol with NADH as the cofactor the equilibrium has been reported to be strongly on the side of xylitol under physiological conditions ($K_{eq} = 5.5^9$ at pH 7.0, Rizzi et al., 1988). Alternatively, the poor utilization of xylose at the later stages of the fermentation could be connected to the unavailability of NADH for xylose reduction. The results presented in this study indicate that xylitol inhibits the growth of *L. lactis* significantly already at 50 g l^{-1}. The slower metabolism could decrease the regeneration rate of NADH.

Plant material hydrolysates contain a mixture of various sugars such as D-xylose, D-galactose, L-arabinose and D-mannose (Converti et al., 1999). This can be problematic if plant hydrolysates are considered as raw materials for xylose production, since reduction of xylose could be accompanied with the reduction of other sugars present in the medium. Yeast xylose reductases have been reported to reduce also other pentoses effectively (Verduyn et al., 1985; Granström, 2002). Purification of xylitol from a mixture of polyols is difficult to achieve under industrial conditions. The present results with *L. lactis* suggest that xylose should be separated from other sugars before its reduction.

Very pure xylose is used in the chemical hydrogenation process. For example ions, phenolic compounds and sugar degradation products have to be removed before the hydrogenation (Ojamo, 1994). It would, however, seem possible that such an extensive purification of xylose would not be required in microbial reduction. On the other hand, the results raise the question whether ribitol and L-arabitol could be produced from plant hydrolysates by the current approach.

In conclusion, the present results show that using LAB as production hosts holds promise for efficient reduction of xylose to xylitol. The benefits of the production strategy with *L. lactis* in comparison to the use of *Candida* are: a nearly quantitative yield of xylitol from xylose, competitive volumetric productivity, no requirement for aeration, and a non-pathogenic production strain. However, the disadvantage is that the conversion does not proceed to completion at high initial xylose concentrations. Therefore, the xylose remaining in the medium would have to be separated and recycled.

The efficient production of mannitol by heterofermentative LAB suggests the potential of using these bacteria for polyol production (von Weymarn et al., 2002). In addition, we have observed that xylitol exhibits only minor inhibition of growth of some heterofermentative LAB (Pihlajaniemi, unpublished results). Therefore, work is in progress to enhance xylitol production by using a heterofermentative LAB production host.

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