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High-level production of D-mannitol with membrane cell-recycle bioreactor

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Ten heterofermentative lactic acid bacteria were compared in their ability to produce D-mannitol from D-fructose in a resting state. The best strain, *Leuconostoc mesenteroides* ATCC-9135, was examined in high cell density membrane cell-recycle cultures. High volumetric mannitol productivity (26.2 g l^{-1} h^{-1}) and mannitol yield (97 mol%) were achieved. Using the same initial biomass, a stable high-level production of mannitol was maintained for 14 successive bioconversion batches. Applying response surface methodology, the temperature and pH were studied with respect to specific mannitol productivity and yield. Moreover, increasing the initial fructose concentration from 100 to 120 and 140 g l^{-1} resulted in decreased productivities due to both substrate and end-product inhibition of the key enzyme, mannitol dehydrogenase (MDH). Nitrogen gas flushing of the bioconversion media was unnecessary, since it did not change the essential process parameters.
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Keywords: mannitol; membrane cell-recycle bioreactor; resting cells; lactic acid bacterium; *Leuconostoc mesenteroides*; response surface methodology

Introduction

D-Mannitol is a naturally occurring sugar alcohol with six carbon atoms. It is only about half as sweet as sucrose [13]. Mannitol and other sugar alcohols exhibit reduced caloric values compared to the respective value of most sugars. This makes them applicable as sweeteners in so-called "light" foods. Sugar alcohols are metabolized independently of insulin and thus are applicable in diabetic food products. Besides applications in the food industry, mannitol is also used in the pharmaceutical industry [7,19]. In medicine, mannitol is used to decrease cellular edema (excessive accumulation of fluid) and to increase urinary output [16].

At present, mannitol is produced commercially by catalytic hydrogenation of fructose-containing syrups [7,16]. The existing chemical production methods are characterized by some drawbacks, one of them being a low yield. When fructose is catalytically hydrogenated, only about 50% of it is converted into mannitol; the rest is converted into another sugar alcohol, sorbitol [16]. Typically, ultrapure (expensive) raw materials are required for efficient conversion. When more cost-effective syrups such as glucose–fructose syrups are used as starting material for the conversion, the main product is sorbitol, and mannitol is formed as a by-product [7,16]. Hence, mannitol production becomes very dependent on the market demand for sorbitol. Furthermore, mannitol is relatively difficult to purify from sorbitol, which results in even higher production costs and decreased yields [6].

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Microbial mannitol production has been studied extensively. Microbes able to produce mannitol include many yeasts, filamentous fungi, and bacteria [5]. Most studies on microbial mannitol production are based on batch cultivations and commonly only moderate production levels (yields or volumetric productivities) have been reported [4,7,19]. The yield can be improved by screening for more efficient production strains. To improve the volumetric productivity of bioprocesses, the use of membrane cell-recycle bioreactor (MCRB) systems has been suggested. Successful implementation of the MCRB has been demonstrated, e.g., for the production of lactic acid [9,17], propionic acid [2], 2,3-butanediol [11], and ethanol [1]. No reports for mannitol production with MCRBs were found.

Soetaert and coworkers have studied extensively the production of mannitol with a heterofermentative lactic acid bacterium (LAB), *Leuconostoc pseudomesenteroides* ATCC-12291. In fed-batch cultures an average volumetric mannitol productivity of about 6.3 g l^{-1} h^{-1} and a mannitol yield from fructose of 94 mol% were achieved [14]. An improved volumetric productivity (8.9 g l^{-1} h^{-1}) but a low yield (60 mol%) was reported for cells immobilized to reticulated polyurethane foam [15]. In a recent patent application, the production of mannitol by high densities of immobilized *L. pseudomesenteroides* cells is suggested [10]. In this process, the average volumetric mannitol productivity and mannitol yield from fructose were approximately 20 g l^{-1} h^{-1} and 85 mol%, respectively. It is, however, important to realize that mannitol production with LAB requires 1/3 of fructose to be used in cofactor regeneration. Hence, this fraction is typically replaced by glucose, which is significantly cheaper than fructose.

In this paper, we studied the production of mannitol by heterofermentative LAB in a resting state. Combining membrane cell-recycle technology and high cell density batch cultures, a very efficient mannitol production process was developed.

Materials and methods

Microbial strains and culture media

The following strains were used in the initial comparison study: *Lactobacillus brevis* ATCC-8287, *Lactobacillus buchneri* TKK-1051, *Lactobacillus fermentum* NRRL-B-1932, *Lactobacillus sanfranciscensis* ATCC-27651, *Lactobacillus* sp. B001, *Leuconostoc mesenteroides* ATCC-9135 and *L. pseudomesenteroides* ATCC-12291 [18]. *L. mesenteroides* ATCC-8086, ATCC-8293, and ATCC-10830 were also used. All microbial strains were maintained in standard MRS growth medium (Pronadisa MRS broth, Hispanlab, Madrid, Spain) supplemented with glycerol (15% [v/v]) and stored at −80°C. MRS growth medium was also used for inoculum cultures and for the production of cells for optimization studies.

The bioconversion (BC1) medium for resting cell cultures with low sugar concentration had the composition (g l^{-1} distilled water): tryptone (Pronadisa), 0.5; yeast extract (Difco Laboratories, Detroit, MI), 0.25; fructose, 20; glucose, 10; K_2HPO_4, 2; $MgSO_4$, 0.2; $MnSO_4$, 0.01. In cultures with *L. fermentum* double concentrations of $MgSO_4$ and $MnSO_4$ were used. The bioconversion (BC2) medium for resting cell cultures with high sugar concentration had the composition (g l^{-1} distilled water): tryptone (Pronadisa), 1; yeast extract (LAB M, International Diagnostics Group, Lancashire, England, UK), 0.5; K_2HPO_4, 1; $MgSO_4$, 0.2; $MnSO_4$, 0.02. If not otherwise stated, 100 g l^{-1} fructose and 50 g l^{-1} glucose were used. The cell production (CP) medium for the semicontinuous bioconversion experiment had the composition (g l^{-1} distilled water): yeast extract (LAB M, International Diagnostics Group), 15; glucose, 30; K_2HPO_4, 2; $MgSO_4$, 0.2; $MnSO_4$, 0.05. The feed solution for continuous CP had the same composition as the CP medium, except that the glucose concentration was 50 g l^{-1}. The effect of biomass, initial fructose concentration and the glucose-to-fructose ratio on mannitol production was studied in two independent experiments. Data from these experiments are given as mean values and standard deviations.

Biomass production and bioconversion

In cell production for optimization studies, cells were collected at early stationary phase from a 2-l culture by centrifugation (6000×*g*, 10 min) and suspended in 10 mM phosphate buffer (pH 6.2). The buffered cell suspension was added to either BC1 or BC2 medium in Biostat Q bioreactors for optimization studies. Cell production for the semicontinuous bioconversion experiment was performed as follows: CP medium in Biostat MD bioreactor (working volume 2 l) was inoculated (5% v/v) with a 10-h culture grown in standard MRS medium. Temperature, pH, and agitation were controlled at 30°C, 6.0, and 200 rpm, respectively. At the end of the batch biomass culture, indicated by a slowdown in base consumption rate, cell recycling was started and feed solution was pumped into the bioreactor (Figure 1). A constant 2-l volume in the bioreactor was achieved by removing cell-free permeate from the system. The dilution rate was increased stepwise from 0.3 to 1.0 h^{-1} to provide sufficient sugars and nutrients for growth. When a cell concentration of 15 g cell dry weight (cdw) l^{-1} was achieved, the cells were concentrated to about 25% (v/v). Next, the bioreactor was refilled with BC2 medium containing 220 g fructose and 121 g glucose (working volume 2 l). When the first bioconversion batch was finished, the cells were again concentrated, fresh BC2 medium containing 160 g fructose and 88 g glucose was added to the bioreactor and a second bioconversion batch was started. This latter procedure was repeated 13 times, using the same initial cell biomass. After the third bioconversion, the amount of glucose added was reduced to 80 g. When the base consumption rate in a previous batch dropped under a threshold value, the cells were revived at the beginning of the next batch with an addition of extra yeast extract (10 g) and tryptone (20 g). The permeates (approximately 1.5 l/batch) were analyzed for mannitol concentration and thereafter used for downstream processing.

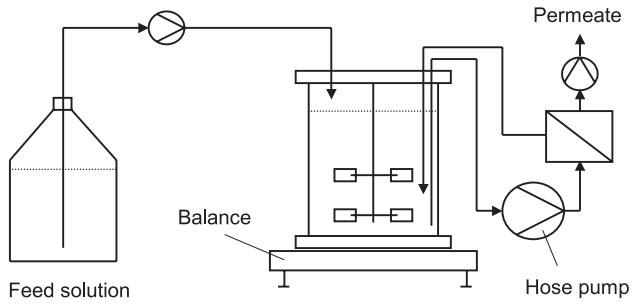

Figure 1 Schematic diagram of the MCRB.

Bioreactor equipment and crystallization

A Biostat Q multiple bioreactor unit (B. Braun Biotech International, Melsungen, Germany) with four culture vessels was used in the optimization studies (working volume 400–600 ml). The Biostat Q system was equipped with automatic probes for the measurement and control of temperature and pH. Magnetic bars and a magnetic drive unit were used for mixing (400 rpm). When strict anaerobic conditions were required, nitrogen gas was added to the bioreactor through a sparger pipe equipped with a frit.

A Biostat MD bioreactor unit (B. Braun Biotech International) equipped with an M2 culture vessel (working volume 2.0 l) was used for the production of biomass and in the semicontinuous bioconversion experiment. The Biostat MD system was equipped with automatic probes for the measurement and control of temperature and pH. Two disc impellers were used for mixing (200 rpm). The culture vessel was placed on a balance connected to the control unit of the MD system.

The MCRB consisted of the MD bioreactor system attached to a tangential flow filtration module, Pellicon 2 Biomax 1000 (1000 k, 0.1 m^2 filtration area, V channel; Millipore, Bedford, MA), and a hose pump, Masterflex model no. 754944 (Cole-Parmer, Barrington, IL) for cell recycle with a flow rate of 1.5–2.0 l min^{-1} (Figure 1). The inlet and retentate pressures were 1.2–1.4 and 0.7–0.9 bar, respectively, and the average permeate flux was about 0.06 l min^{-1}. Feed and harvest pumps used were of type 101U/R from Watson Marlow, Cornwall, UK. The filtration unit of the MCRB was disinfected with 0.5 M NaOH and washed thoroughly with sterile water before use. All data from the cultures were collected by a PC with MFCS/win software (B. Braun Biotech International).

Purification of mannitol was performed using a rotary evaporator, a 400-ml decanter glass equipped with external mixing placed in a temperature-controlled water bath; a Büchner funnel with suction for crystal separation from the mother liquor; and a drying oven (60°C). The cell-free permeate was evaporated at 35°C to 250 g mannitol L^{-1}. The supersaturated concentrate was poured into a 400-ml decanter glass, which was cooled to 5°C (cooling rate 2°C/h) with slow mixing. The crystals were separated

by filtration, dried overnight at 60°C, and finally homogenized in a porcelain mortar.

Analytical methods

The optical density of the bioconversion media was measured at 600 nm against distilled water. The samples were diluted in such a manner that the absorbance values were in the range of 0.1 to 0.6. The cdw were measured as follows: a sample of cultivation broth was pipetted into a preweighed centrifuge tube followed by centrifugation at 6000×*g* for 5 min. The cell pellet was washed with sterile 0.9% (w/v) NaCl, the centrifugation was repeated and the centrifuge tube was dried at 80°C until a constant cdw was achieved. Frequently, a predetermined linear correlation factor was used to convert OD values to cdw. Viable cell counts were measured by diluting a sample of cultivation broth in sterile 0.9% (w/v) NaCl, and 100 μL of diluted suspension was spread on MRS agar (pH 6.2). Plates were incubated at 30°C for 24 h, and then subsequently, the colonies were counted.

Concentrations of organic acids, sugars, and mannitol were determined by high-performance liquid chromatography (HPLC). Mannitol concentrations were analyzed using an Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA) at 70°C with distilled water as the mobile phase. Glucose and fructose concentrations were measured using an Aminex HPX-87C column at 70°C with distilled water as the mobile phase. Acetic and lactic acid concentrations were measured using Aminex HPX-87H ion exclusion column at 60°C with 5 mM H_2SO_4 as the mobile phase. All components were analyzed with a refractive index detector. Deashing Micro-Guard precolumns (Bio-Rad Laboratories) were used in analysis of mannitol and sugars, while a Cation H Micro-Guard precolumn (Bio-Rad Laboratories) was used in analysis of organic acids. The elution rate in all systems was 0.6 ml min^{-1}.

The cell-free extracts for mannitol dehydrogenase (MDH) activity measurements were prepared as follows: Cells at late exponential growth phase grown in standard MRS medium containing 0.75 g l^{-1} glycine were harvested, washed twice in cold 25 mM phosphate buffer (pH 7.0) and resuspended in 4 ml cold sonication buffer, which contained in a total volume of 50 ml: 2 M Tris–HCl, pH 7.0, 1.25 ml; 1 M $MgCl_2$, 0.5 ml; 0.5 M EDTA, 0.1 ml; 50% (v/v) glycerol, 30 ml; Complete Mini EDTA-free protease inhibitor, two tablets (Roche Diagnostics, Indianapolis, IN); 100 mM DTT, 0.5 ml; distilled water, 17.65 ml. The suspension was sonicated in the presence of glass beads for 8× 15 s with 30 s cooling on ice between the treatments. Cell debris was separated by centrifugation and supernatants were assayed immediately for MDH activity at 30°C in 20 mM acetate buffer (pH 5.35) by following the oxidation of 0.15 mM NADH at 340 nm. Kinetic parameters (K_m and K_i) were determined by DynaFit software performing a nonlinear least-squares regression of enzymatic data [8]. One MDH activity unit (U) was defined as the formation of 1 μmol NAD^+ per minute at 30°C.

Concentrations of Mn^{2+} and Mg^{2+} in the permeates were analyzed with atomic absorption spectrometry (AAS). Mn^{2+} was measured by means of graphite furnace AAS (Varian 400 P, Varian, Walnut Creek, CA) with GTA 96 graphite furnace and deuterium background correction. Mg^{2+} measurements were performed using flame AAS (Varian 600) with an N_2O–acetylene flame. Wavelengths used were 279.5 nm (Mn^{2+}) and 285.2 nm (Mg^{2+}).

The combined effect of temperature and pH on mannitol production by resting cells was studied using response surface methodology (multiple linear regression). A quadratic polynomial model with four center point runs was applied and the results were analyzed and plotted using Modde Version 4.0 software (Umetri, Umeå, Sweden). The levels of the variables were: temperature 28, 33, 38°C; and pH 4.8, 5.4, and 6.0. The experiments (altogether 12) were conducted in random order in Biostat Q bioreactors.

The purity of crystalline mannitol was analyzed by HPLC and by spectrophotometry following the spectrum from 220 to 820 nm.

Results and discussion

Initial comparison of the strains

Seven strains of heterofermentative LAB were tested for mannitol production in a resting state using BC1 medium. Based on these preliminary test tube level experiments (data not shown), *L. mesenteroides*, *L. pseudomesenteroides* and *Lb. fermentum* were selected for a comparison study using bioreactors (Table 1). The specific mannitol productivity and mannitol yield from fructose by *L. mesenteroides* were higher than those of the two other strains. In order to study the *L. mesenteroides* species in more detail, three additional commercial strains (ATCC-8086, -8293, and -10830) were acquired. Only strain 8293 showed mannitol productivities comparable to 9135, whereas 8086 and 10830 were poor producers (data not shown). Strain 9135 was chosen for further process development studies.

Effect of temperature and pH

Using response surface methodology, the effects of temperature and pH on mannitol production were studied. The responses (mannitol yield and specific mannitol productivity) are shown in Table 2 and Figure 2. Mannitol yield was strongly influenced by pH, but not by temperature. The best yields were achieved at low pH (4.8) and low temperature (28°C) values. Unfortunately, the specific mannitol productivities were negatively influenced by these conditions. Hence, an optimum response area for the specific mannitol productivity was identified within a pH and temperature range of 5.4–5.8 and 33–35.5°C, respectively. As a compromise between the two responses, pH and temperature values of 5.2 and 32°C, respectively, were chosen for further studies. Using these values, the model predicted a mannitol yield and a specific mannitol productivity of 92.8±1.9 mol% and 1.49±0.06 g (g cdw)$^{-1}$ h^{-1}, respectively.

Table 1 Volumetric (r_{mtol}) and specific (q_{mtol}) mannitol productivities and yield of mannitol from fructose consumed (Y_{mtol}) in batch bioconversions with resting cells of three heterofermentative LAB at 30°C and pH 5.0

Strain	r_{mtol} (g $l^{-1}h^{-1}$)	q_{mtol} (g (cdw g)$^{-1}h^{-1}$)	Y_{mtol} (mol/mol)
L. mesenteroides ATCC-9135	2.3	2.6	0.98
L. pseudomesenteroides ATCC-12291	1.6	1.5	0.80
Lb. fermentum NRRL-B-1932	0.8	1.0	0.86

BC1 medium was used.

Table 2 Yields of mannitol from fructose (Y_{mtol}) and specific mannitol productivities (q_{mtol}) by resting cells of *L. mesenteroides* ATCC-9135 using response surface methodology

Real values		Coded values		Y_{mtol}	q_{mtol}
pH	Temp (°C)	pH	Temp (°C)	(mol%)	(g (cdw g)$^{-1}$ h^{-1})
4.8	28	−1	−1	99.88	1.22
6.0	28	+1	−1	85.44	1.20
4.8	38	−1	+1	91.55	1.30
6.0	38	+1	+1	77.65	1.46
4.8	33	−1	0	97.42	1.30
6.0	33	+1	0	87.80	1.48
5.4	28	0	−1	90.60	1.28
5.4	38	0	+1	84.63	1.39
5.4	33	0	0	87.23	1.52
5.4	33	0	0	89.63	1.54
5.4	33	0	0	90.73	1.59
5.4	33	0	0	90.47	1.58

The R^2 value for the model was 0.949.

Effect of biomass concentration and initial fructose concentration

Increasing the biomass concentration in a resting cell bioconversion did not alter the specific mannitol productivities (Table 3). When the biomass concentration was raised to about 16 g cdw l^{-1}, a volumetric mannitol productivity of 26.2±0.1 g l^{-1} h^{-1} was achieved. In addition, increasing the biomass concentration also improved the yield and conversion of fructose to mannitol. It seems tempting to further increase the biomass concentration, but this would result in difficulties with overall control of the process. Correct timing of the concentration phase is essential because the cells consume mannitol after sugar depletion. At cell concentrations over 15 g cdw l^{-1}, the batch time was reduced to about 4 h. Hence, further reduction of the batch time would most likely decrease the yield and productivity due to incorrect timing of the concentration phase.

Increasing the initial fructose concentration of the bioconversion medium had a negative effect on the productivities (Table 3). Volumetric productivities decreased when the initial fructose concentration was increased from 100 to 120 and 140 g l^{-1}. However, both mannitol yield and conversion were benefited by a high initial fructose concentration. In an additional experiment, the K_m and K_i (product) values for MDH were measured from *L. mesenteroides* ATCC-9135 cell lysates. When assayed with fructose as the substrate, MDH showed Michaelis–Menten kinetics up to 100 g fructose l^{-1} (K_m=13.1±0.9 g l^{-1}). With 200 g fructose l^{-1} a small decrease in total activity was seen (from 8.4 to 7.8 U). This indicates some level of substrate inhibition. On the other hand, a high osmotic pressure can also be the cause of productivity decline at higher initial fructose concentrations. The K_m measured for this enzyme was higher than reported previously for pure enzyme, 6.3 g l^{-1} [12]. The K_i value, measured with 5 g l^{-1} initial mannitol, was 66.4 g l^{-1}, indicating a strong end-product inhibition at high mannitol concentrations.

Effect of glucose-to-fructose ratio

Increasing the initial fructose concentration was disadvantageous with respect to mannitol productivity, but advantageous with respect to conversion (Table 3). Therefore, a bioconversion protocol with an initial fructose concentration of 120 g l^{-1} was used. The biomass concentration was about 15 g cdw l^{-1}. Based on previous experiments we concluded that the glucose-to-fructose ratio should be increased from 0.5:1, due to a severe slowdown of fructose consumption and reduced conversion, when glucose was depleted before fructose. Consequently, a bioconversion experiment with a 0.6:1 glucose-to-fructose ratio was performed. Applying this ratio, a total and rapid consumption of fructose was observed. Furthermore, extending the bioconversion by a further 0.5 h resulted in over 0.95 mol/mol conversion. At the same time, the volumetric productivity decreased only slightly and was still above 22 g l^{-1} h^{-1}. No other important parameter changes were seen and a 0.55:1 ratio was chosen for further studies. A typical plot of such a bioconversion is shown in Figure 3.

Effect of nitrogen gas flushing

The importance of nitrogen gas flushing of the bioconversion medium on mannitol production was assessed with two parallel experiments: one with constant nitrogen gas flushing and one where no gases were added to the bioreactor. The changes in essential production parameters were not significant. Therefore, as a technical implication, it was concluded that nitrogen gas flushing of the bioconversion medium was unnecessary.

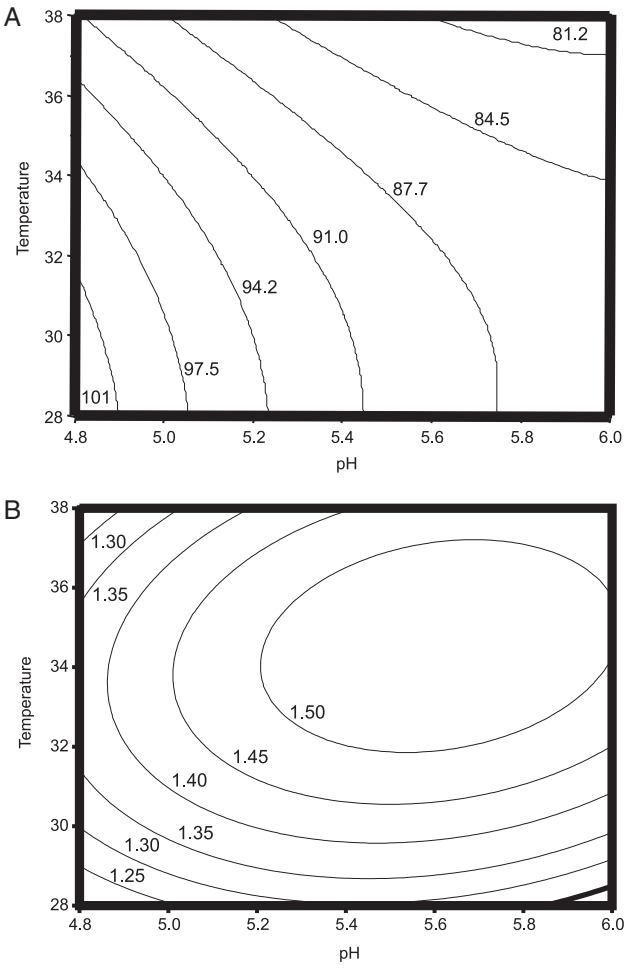

Figure 2 Contour plots showing the dependence of (A) the yield of mannitol from fructose consumed (mol%) and (B) the specific mannitol productivity (g (g cdw)$^{-1}$ h^{-1}) by resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (100 g fructose l^{-1} and 50 g glucose l^{-1}) on temperature and pH.

Table 3 Cell dry weights (cdw), initial fructose concentrations (C_{fru}), volumetric (r_{mtol}) and specific (q_{mtol}) mannitol productivities, mannitol yields (Y_{mtol}) and conversions (x_{mtol}) in batch bioconversions with resting cells of *L. mesenteroides* ATCC-9135 at 32°C and pH 5.2

cdw (g l^{-1})	C_{fru} (g l^{-1})	r_{mtol} (g l^{-1} h^{-1})	q_{mtol} (g (cdw g)$^{-1}$ h^{-1})	Y_{mtol} (mol%)	x_{mtol} (mol/mol)
5.9±0.2	100	8.3±0.1	1.4±0.0	87.1±2.8	0.84±0.04
8.6±0.5	100	13.4±0.6	1.6±0.0	94.1±2.3	0.91±0.02
12.1±0.3	100	16.4±0.1	1.4±0.0	97.7±0.8	0.95±0.01
16.0±1.4	100	26.2±0.1	1.6±0.1	96.6±3.2	0.91±0.02
15.7±0.4	120	24.1±1.0	1.5±0.0	98.4±0.1	0.95±0.01
15.8±0.2	140	21.0±0.1	1.4±0.0	98.9±0.1	0.96±0.00

BC2 medium was used. Values in the table are averages and standard deviations of two independent experiments.

Semicontinuous production

In order to study the viability of the microbial cells and to identify any changes brought to the essential process parameters in an ongoing industrial process, the bioconversion was run semi-continuously. The MCRB equipment with a working volume of 2 l was used. This resulted in stable production of mannitol for 14 batches (Figure 4). The average volumetric mannitol productivity of the 14 batches was lower than seen in a single batch bioconversion (17.1±1.1 compared to 23.5 g l^{-1} h^{-1}). The decrease was primarily due to dead volumes of the existing process equipment and some technical mistakes in specific batches. Consequently, a corresponding decrease was also seen in total mannitol conversion (from 0.93 to 0.85 mol%). Proper process design would most likely result in even smaller dead volumes and, hence, reduce the losses in volumetric productivity and conversion. We also believe that correct timing of the filtration is important because of the slow consumption of mannitol in the absence of fructose.

The optical density measured at 3 h in each batch was constantly increasing. This was first assumed to be a consequence of cell debris corrupting the spectrophotometric analysis. However, very similar viable cell count-to-optical density ratios were observed throughout the batches. For example, the viable cell counts in batches 1 and 10 were 4.02×10^9 and 5.33×10^9 ml^{-1}, respectively. Hence, the viable cell count-to-optical density ratios were 12.0×10^7 (batch 1) and 12.4×10^7 (batch 10).

In another set of experiments with successive bioconversions, both the mannitol yield and a possible accumulation of organic acids and metals were studied. The yield of the seventh batch was 98.7 mol%, which is comparable to results gained in single batch bioconversions. Thus, the performance of the microbial cells observed in single batch bioconversions was not altered in a semicontinuous mode. In the same experiment, lactate and acetate concentrations remained fairly stable for the duration of the experiment. Their concentrations were approximately 22 and 19 g l^{-1}, respectively. Stable final base consumption levels confirmed this observation. Also, the permeates from each batch were analyzed with AAS to determine the concentrations of Mg^{2+} and Mn^{2+}. Moreover, when triple amounts of both metals were added to batches 8 (Mn) and 9 (Mg) no significant accumulation of the metal ions was observed. The Mn^{2+} and Mg^{2+} concentrations in the permeates varied in the range of approximately 2–10 and 40–50 mg l^{-1}, respectively. No effect on productivity was seen when the initial amounts were tripled.

Purification

Permeates from three successive batches were crystallized separately. The average mannitol yield of the first crystallization was as high as 72 mass%. However, the crystals were not pure enough and were thus recrystallized. After recrystallization crystal purity according to HPLC was over 99%, although a minimal impurity absorbance peak was detected at about 260 nm. The mother liquors were combined and crystallized twice. The purity of these secondary crystals after the second crystallization was slightly

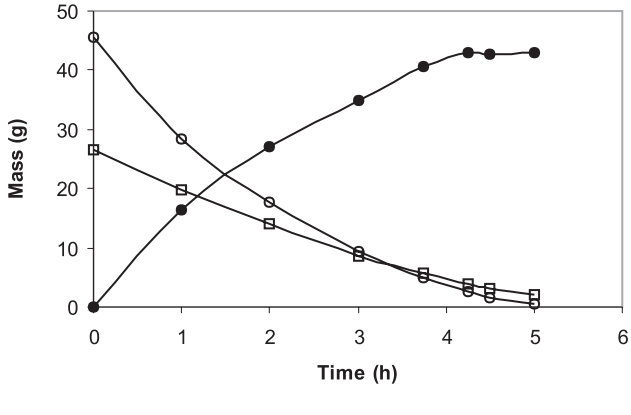

Figure 3 Masses of fructose (open circles), glucose (open rectangles), and mannitol (closed circles) in a 400-ml bioconversion experiment with resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (32°C, pH 5.2, 15.2 g cdw l^{-1}, initial fructose and glucose concentrations 120 and 66 g l^{-1}, respectively).

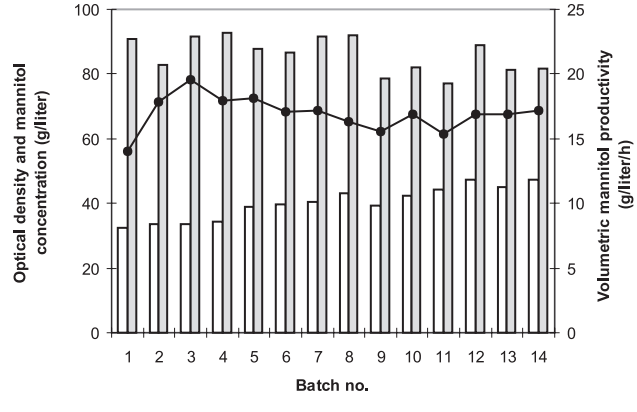

Figure 4 Optical densities (white columns), mannitol concentrations in permeates (g l^{-1}; gray columns), and volumetric mannitol productivities (g l^{-1} h^{-1}; closed circles) in a semicontinuous bioconversion experiment with resting cells of *L. mesenteroides* ATCC-9135 in BC2 medium (T=32°C, pH 5.2, and about 15 g cdw l^{-1}). Bleeding (100 ml) was performed in batches 9 and 13. Cells were revived in batches 5, 9, and 13.

lower (>97.5%) than that of the primary crystals. In addition, the impurity peak was now somewhat bigger. Combining the primary and secondary crystals a total crystallization yield of 56.4% (w/w) was achieved. When the mannitol present in the mother liquor of the second crystallization (washing) of primary crystals was added to the calculations, a total crystallization yield of 85.5% was achieved. This solution can, e.g., be used to wash the next batch of crystals, thus keeping the mannitol in the system. Moreover, using regular downstream processing equipment the purities and perhaps the yields can be improved further.

Conclusions

In this paper, a new bioprocess concept for production of mannitol is described. In preliminary tests, an efficient heterofermentative strain was identified. It was used in a semicontinuous process utilizing an MCRB. The volumetric productivity was around 20 g l^{-1} h^{-1} and the conversion was 0.9 mol/mol. Using a simple purification protocol the crystallization yield was 85 mol%. Hence, the total yield for the process was 0.77 g crystalline mannitol per gram of fructose and 0.52 g crystalline mannitol per gram initial sugar (fructose and glucose). Furthermore, in the novel bioprocess, only 0.67 g by-products are formed for each gram of crystalline mannitol obtained. According to Devos [3] the respective values for a typical chemical mannitol production process are 0.39 g crystalline mannitol per gram initial sugar and 1.58 g by-products for each gram of crystalline mannitol. Hence, we now report a clear enhancement to these key values.

The by-products of the bioprocess are mainly acetate and lactate. Mannitol and some residual sugars are also present. Hence, to improve the economy of the process, applications for these compounds should be found. Using tools of genetic engineering or by screening new strains, cells producing pure D- or L-lactate could be identified. Such a system would have two products, resulting in a more cost-effective process.

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