TKK Dissertations 157
Espoo 2009

METABOLIC ENGINEERING OF LACTIC ACID BACTERIA FOR PRODUCTION OF MANNITOL, L-RIBULOSE AND L-RIBOSE

Doctoral Dissertation

Miia Helanto

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Department of Biotechnology and Chemical Technology

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Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Faculty of Chemistry and Materials Sciences for public examination and debate in Auditorium KE2 (Komppa Auditorium) at Helsinki University of Technology (Espoo, Finland) on the 24th of April, 2009, at 12 noon.

Helsinki University of Technology
Faculty of Chemistry and Materials Sciences
Department of Biotechnology and Chemical Technology

Teknillinen korkeakoulu
Kemian ja materiaalitieteiden tiedekunta
Biotekniikan ja kemian tekniikan laitos

Distribution:
Helsinki University of Technology
Faculty of Chemistry and Materials Sciences
Department of Biotechnology and Chemical Technology
P.O. Box 6100 (Kemistintie 1)
FI - 02015 TKK
FINLAND
URL: http://www.tkk.fi/Units/BioprocessEngineering/
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E-mail: miia.helanto@tkk.fi

ISBN 978-951-22-9838-9
ISBN 978-951-22-9839-6 (PDF)
ISSN 1795-2239
ISSN 1795-4584 (PDF)
URL: http://lib.tkk.fi/Diss/2009/isbn9789512298396/

TKK-DISS-2583

Yliopistopaino
Helsinki 2009

ABSTRACT OF DOCTORAL DISSERTATION	HELSINKI UNIVERSITY OF TECHNOLOGY P.O. BOX 1000, FI-02015 TKK http://www.tkk.fi

Author Miia Helanto

Name of the dissertation

Metabolic engineering of lactic acid bacteria for production of mannitol, L-ribulose and L-ribose

Manuscript submitted 12.9.2008	Manuscript revised 9.3.2009

Date of the defence 24.4.2009

☐ Monograph ☒ Article dissertation (summary + original articles)

Faculty Faculty of Chemistry and Materials Science

Department Department of Biotechnology and Chemical Tewchnology

Field of research Bioprocess Engineering

Opponent(s) PhD Pauli Kallio

Supervisor Prof. Matti Leisola

Instructor DSc (Tech) Antti Nyyssölä

Abstract

The main goal of this work was to metabolically engineer lactic acid bacteria (LAB) for the use as efficient production hosts of commercially interesting rare sugars and sugar alcohols. LAB are a very diverse group of gram positive bacteria. It is a group of bacteria that is originally identified by their ability to synthesize lactic acid. The main function of sugar metabolism in LAB is to generate the energy necessary for growth and maintenance of cell functions. Hence, their sugar metabolism is generally not connected to their limited biosynthetic activity. The possibility of modifying catabolic pathways of sugars without disturbing the biosynthesis of cell components makes the sugar metabolism of LAB attractive targets for metabolic engineering. LAB have also the GRAS (generally regarded as safe) status which makes them suitable production hosts for food and pharmaceutical applications.

In the first part of this work mannitol production of *Leuconostoc pseudomesenteroides* was improved by random mutagenesis. Mannitol is a naturally occurring six-carbon sugar alcohol, which is widely used in the food, chemical and pharmaceutical industry. The activity of fructokinase was decreased to about 10% compared to the parent strain, which resulted in a reduced leakage of fructose into the phosphoketolase (PK) pathway. The yield of mannitol from fructose was improved from 74% to 86% (mol/mol). In addition, characteristics of fructose utilization of *Lactobacillus fermentum* were studied. A novel *fruIK* operon involved in channeling fructose to the PK pathway was characterized.

In the second part of this work L-arabinose metabolism of *Lactobacillus plantarum* was modified. An L-ribulokinase deficient mutant was constructed. Resting cells of the ribulokinase deficient mutant were used for the production of L-ribulose. The isomerisation of L-arabinose to L-ribulose was very unfavourable 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 statistical software predicted initial L-ribulose production rate (r_i) of 29.1 g/(l·h), best achievable process productivity (r_{max}) of 14.8 g/(l·h) and conversion of L-arabinose to L-ribulose (x) of 0.70 mol/mol. The work was continued by introducing L-ribose isomerase activity into L-ribulokinase deficient strains of *E. coli* and *Lb. plantarum*. By adding the second reaction, isomerization of L-ribulose to L-ribose, the two-reaction sequence with L-arabinose as the starting material became favorable for L-ribose production. L-Ribose is a rare sugar that is used as a precursor for a production of antiviral drugs. The process for L-ribose production by resting cells and protein precipitates was investigated. The initial L-ribose production rates were 0.46 g/(g·h) [1.8 g/(l·h)] and 0.27 g/(g·h) [1.91g/(l·h)] for *E. coli* and for *Lb. plantarum*, respectively. Conversions were around 20% (mol/mol) at their highest in the experiments.

Keywords Lactic acid bacteria, metabolic engineering, mannitol, L-ribulose, L-ribose

ISBN (printed) 978-951-22-9838-9	ISSN (printed) 1795-2239
ISBN (pdf) 978-951-22-9839-6	ISSN (pdf) 1795-4584
Language English	Number of pages 87

Publisher HUT/Department of Biotechnology and Chemical Technology

Print distribution HUT/Department of Biotechnology and Chemical Technology

☒ The dissertation can be read at http://lib.tkk.fi/Diss/2009/isbn9789512298396

VÄITÖSKIRJAN TIIVISTELMÄ	TEKNILLINEN KORKEAKOULU PL 1000, 02015 TKK http://www.tkk.fi

Tekijä Miia Helanto

Väitöskirjan nimi

Metabolic engineering of lactic acid bacteria for production of mannitol, L-ribulose and L-ribose

Käsikirjoituksen päivämäärä 12.9.2008	Korjatun käsikirjoituksen päivämäärä 9.3.2009

Väitöstilaisuuden ajankohta 24.4.2009

☐ Monografia	☒ Yhdistelmäväitöskirja (yhteenveto + erillisartikkelit)

Tiedekunta	Kemian ja Materiaalitieteiden tiedekunta
Laitos	Biotekniikan ja Kemian tekniikan laitos
Tutkimusala	Bioprosessitekniikka
Vastaväittäjä(t)	FT Pauli Kallio
Työn valvoja	Prof. Matti Leisola
Työn ohjaaja	TkT Antti Nyyssölä

Tiivistelmä

Työn tarkoituksena oli kehittää uusia menetelmiä maitohappobakteereiden käyttämiseksi tehokkaina tuotantoisäntinä kaupallisesti kiinnostavien harvinaisten sokereiden ja sokerialkoholien tuotannossa. Maitohappobakteerit ovat laaja ryhmä gram-positiivisia bakteereita, jotka on alun perin tunnistettu kyvystä tuottaa maitohappoa. Maitohappobakteereiden sokeriaineenvaihdunnan päätarkoitus on tuottaa energiaa kasvuun ja solun toimintojen ylläpitoon eikä sokeriaineenvaihdunta normaalisti ole yhteydessä solukomponenttien synteesiin. Mahdollisuus sokerimetabolian muokkaamisen häiritsemättä solukomponenttien biosynteesiä tekee maitohappobakteereista kiinnostavan metaboliamuokkauskohteen harvinaisten sokereiden ja sokerialkoholien tuottamiseen. Maitohappobakteereilla on myös GRAS-status eli niiden käyttö on turvallista elintarvike- ja lääketieteen sovelluksissa.

Työn ensimmäisessä osassa *Leuconostoc pseudomesenteroides* -bakteerin mannitolin tuottoa parannettiin satunnaisen mutageneesin avulla. Mannitoli on kuusihiilinen sokerialkoholi, jota käytetään mm. makeutusaineena. Kannan fruktokinaasientsyymin aktiivisuus laski 10%:iin villityypin aktiivisuudesta, mikä esti osittain fruktoosin kulkeutumisen fosfoketolaasi-metaboliareitille. Mannitolin saanto fruktoosista parantui 74%:sta 86%:iin (mol/mol). Lisäksi karakterisoitiin *Lactobacillus fermentum* -bakteerin uusi *fruIK*-operoni, Tämä operoni koodaa fruktokinaasi ja fosfoglukoosi-isomeraasi entsyymejä, jotka vastaavat solun sisäisen fruktoosin kulkeutumisesta fosfoketolaasi-reitille.

Työn toisessa osassa muokattiin *Lactobacillus plantarum* -bakteerin L-arabinoosimetaboliaa. L-Ribulokinaasia koodaava geeni tuhottiin kohdistetun mutageneesin avulla. Tämän L-ribulokinaasi-negatiivisen kannan lepääviä soluja käytettiin L-ribuloosin tuotantoon. L-arabinoosin isomerointireaktion tasapaino on vahvasti lähtöaineen puolella. Kun reaktiossa syntyvä L-ribuloosi sidotaan boraattikompleksiksi, pystytään L-ribuloosin saantoa L-arabinoosista nostamaan huomattavasti. L-Ribuloosin tuotantoprosessi optimoitiin käyttäen CCD-koesuunnitelmaa. Tilastollinen mallinnus ennusti L-ribuloosin volumetriseksi tuotantonopeudeksi alussa (r_i) 29.1 g/(l·h), prosessin parhaaksi tuottavuudeksi (r_{max}) 14 g(l·h) ja konversioksi (x) 0.70 (mol/mol). Työn seuraavassa vaiheessa L-ribulokinaasi-negatiivisiin *E. coli* ja *Lb. plantarum* -kantoihin siirretiin L-riboosi-isomeraasia koodaava geeni. Näin aikaansaatu kaksivaiheinen reaktio tuotti L-arabinoosista L-ribuloosin kautta L-riboosia. L-riboosi on harvinainen sokeri, jota käytään viruslääkkeiden raaka-aineena. L-riboosin tuottoon käytettiin sekä lepääviä soluja että proteiinisakkoja. L-riboosin tuotantonopeudet lepäävillä *E. coli* -soluilla olivat 0.46 g/(g·h) [1.8 g(l·h)] ja lepäävillä *Lb. plantarum* -soluilla 0.27 g/(g·h) [1.91 g/(l·h)]. L-Arabinoosin konversio L-riboosiksi oli parhaimmillaan hieman yli 20%.

Asiasanat maitohappobakteerit, metaboliamuokkaus, mannitoli, L-ribuloosi, L-riboosi

ISBN (painettu) 978-951-22-9838-9	ISSN (painettu) 1795-2239
ISBN (pdf) 978-951-22-9839-6	ISSN (pdf) 1795-4584
Kieli englanti	Sivumäärä 87

Julkaisija TKK/Biotekniikan ja kemiantekniikan laitos

Painetun väitöskirjan jakelu TKK/Biotekniikan ja kemiantekniikan laitos

☒ Luettavissa verkossa osoitteessa http://lib.tkk.fi/Diss/2009/isbn9789512298396

Preface

The experimental work was carried out at Department of Biotechnology and Chemical Technology (in the former Laboratory of Bioprocess Engineering), Helsinki University of Technology during the years 2001-2008.

First I would like to express my warmest thanks to my supervisors Professor Matti Leisola for supervising and providing the opportunity and facilities to carry out the work and Dr. Antti Nyyssölä for supervising and sharing his expert knowledge. I would like to thank the reviewers Professor Wim Soetaert and Dr. Reetta Satokari for their constructive feedback and advisable suggestions.

I would like to thank the present and former personel of the Department of Biotechnology and Chemical Technology. Especially I want to thank my co-authors Ulla Airaksinen, Dr. Niklas von Weymarn, Dr. Kristiina Kiviharju and Dr. Tom Granström. Anne Usvalampi is particularly acknowledged for the companionship and help during several conference trips. I would like to thank Dr. Ossi Turunen and Dr. Ossi Pastinen for their help and fruitful discussions. Seppo Jääskeläinen is specially thanked for his excellent technical aid and Kalle Salonen for his computer skills. I also would like to express my gratitude to Auli Murrola and Marjaana Rytelä who have analyzed thousands of HPLC samples during this work and Johanna Aura who never said no when I needed help in the laboratory. Sasikala Anbarasan, Piia Appelqvist, Dr. Tero Eerikäinen, Dr. Fred Fenel, Mervi Hujanen, Johanna Karimäki, Anna-Kaisa Kylmä, Dr. Anja Lampio, Ulla Moilanen, Kirsti Pitkänen, Heidi Salo, Dr. Harri Santa, Dr. Salem Shamekh, Katja Skogman, Noora Sirén, Tommi Timoharju, Esa Uosukainen, Raisa Vermasvuori, Erika Winquist, Veera Virtanen, Dr. Antti Vuolanto and Antti-Jussi Zitting are acknowledged for their help and good conversations along the way.

Additionally I want to thank the co-workers at the University of Helsinki, Professor Airi Palva, Dr. Ilkka Palva and Dr. Johannes Aarnikunnas for successful collaboration.

The research was funded by Tekes (The National Technology Agency, Finland), The Finnish Academy and ABS Graduate School.

Furthermore I want express my heartfelt thanks to my husband Henri for understanding and supporting me during these years and our children Anton, Aurora, Estella and Tiffany who have fulfilled the other half of my life. Finally I want to thank my friends and family for their great support.

Espoo, March 2009
Miia Helanto

For the memory of my Father (1936-2007)
who had faith in me but never saw this thesis complete.

Contents

List of Publications

I Helanto, M., Aarnikunnas, J., von Weymarn, N., Airaksinen, U., Palva, A. and Leisola, M., Improved mannitol production by a random mutant of *Leuconostoc pseudomesenteroides* (2005) *J Biotechnol.* 116, 283-294

II Helanto, M, Aarnikunnas, J., Palva, A., Leisola, M., and Nyyssölä A., Characterization of genes involved in fructose utilization by *Lactobacillus fermentum* (2006) *Arch. Microbiol.* 186, 51-59

III Helanto, M, Kiviharju, K., Leisola, M., and Nyyssölä A., Metabolic engineering of *Lactobacillus plantarum* for production of L-ribulose (2007) *Appl. Environ. Microbiol.* 73, 7083-91.

IV Helanto, M., Kiviharju, K., Granström; T., Leisola, M., and Nyyssölä, A., Biotechnological production of L-ribose from L-arabinose, *Appl. Microbiol. Biotechnol.* In press. Published online 17th of January 2009.

The author's contribution to the appended pulications:

Publication I: The author defined the research plan together with the coauthors and carried out the cloning, sequence characterization and enzyme activity assays. RNA isolation and northern hybridizations were carried out by the author together with Dr. J. Aarnikunnas who also performed the primer extension analyses. Bioreactor cultivations and calculations were carried out by Dr. N. von Weymarn and the random mutagenesis by U. Airaksinen. The author wrote the manuscript together with Dr. J. Aarnikunnas. Other authors helped in revising the manuscript.

Publication II: The author defined the research plan with the help of the coauthors and carried out majority of the experiments. Dr. J. Aarnikunnas helped with northern hybridization. Electrophoretic mobility shift assay was carried out together with Dr. A. Nyyssölä. The author wrote the manuscript together with Dr. A. Nyyssölä. Other authors helped in revising the manuscript.

Publication III: The author defined the research plan with the help of the coauthors and carried out the cloning, enzyme activity assays, RT-qPCR, and L-ribulose production experiments. Dr. K. Kiviharju designed the optimization experiments and carried out the bioreactor cultivations and calculations. Dr. A. Nyyssölä performed the sugar purification experiments. The author wrote the manuscript together with Dr. K. Kiviharju and Dr. A. Nyyssölä. Prof. M. Leisola helped in revising the manuscript.

Publication IV: The author defined the research plan with the help of the coauthors and carried out the cloning, enzyme activity assays, and resting cell experiments. Dr. K. Kiviharju performed the bioreactor cultivations and calculations. Dr. T. Gransröm carried out the protein precipitate experiments. The author wrote the manuscript together with Dr. A. Nyyssölä and Dr. K. Kiviharju. Other authors helped in revising the manuscript.

Abbrevations and Symbols

ADP	adenosine diphosphate
araB	L-ribulokinase gene
ATP	adenosine triphosphate
c_A	L-arabinose concentration
c_B	borate concentration
CCC	central composite circumscribed
CCD	central composite design
CCF	central composite face centered
CcpA	catabolite control protein A
cDNA	single stranded DNA
cre	catabolite responsive element
C_T value	concentration of DNA molecules multiplied by time
DHAP	dihydroxyacetone-phosphate
DIG	digoxigenin
EMP	Embden-Meyerhof-Parnas pathway
EMSA	electrophoretic mobility shift assay
EPS	exopolysaccharide
FDP	fructose-1,6-diphosphate
FK	fructokinase
L-FMAU	1-(β-L-2-fluoro-2-deoxyarabinofuranosyl)-5-methyluracil
fruI	phosphoglucose isomerase gene
fruK	fructokinase gene
GAP	glyceraldehyde-3-phosphate
GRAS	generally regarded as safe
HTH	helix-turn-helix
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	inverted repeats
K_S	Monod equation kinetic parameter for substrate consumption
LAB	lactic acid bacteria
LDH	lactate dehydrogenase
ldh	lactate dehydrogenase gene
MDH	mannitol dehydrogenase
MNNG	1-methyl-3-nitro-1-nitrosoguanidine
m_s	maintenance coefficient (g/g/h)
NAD^+/NADH	oxidized/reduced form of nicotinamide adenine nucleotide
$NADP^+$/NADPH	oxidized/reduced form of nicotinamide adenine nucleotide phosphate
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PGI	phosphoglucose isomerase
PK	phoshoketolase
PLLA	poly-L-lactic acid

PMF	proton motive force
PTS	phosphotransferase system
Q^2	coefficient of model prediction
R^2	coefficient of determination
RBS	ribosome binding site
r_i	initial production rate
r_{max}	best achievable process productivity
RT-qPCR	real time quantitative PCR
T	temperature
unkR	putative unknown repressor gene
x	conversion
Y_{xs}	biomass yield from substrate (g/g)
μ_{max}	maximum specific growth rate (1/h)

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1. Lactic acid bacteria

1.1. Introduction

Lactic acid bacteria (LAB) are a very diverse group of gram positive bacteria. It is a group of bacteria that is originally identified by their ability to synthesize lactic acid. The classification of LAB is nowadays still greatly based on the work by Orla-Jensen (1919). According to Axelsson (1998) typical LAB is gram positive, non-sporing, catalase negative, devoid of cytochromes, facultative anaerobe, fastidious, acid-tolerant and strictly fermentative with lactic acid as the major end-product during sugar fermentation. Because LAB comprise a wide variety of microbes revisions of the classification and taxonomy still appear regularly. Gram-positivity is the only character that cannot be argued with. The present taxonomy relies on a true phylogenetic taxonomy which has been revealed by extensive work on determining rRNA sequences and whole genome sequencing (Siezen et al., 2004).

The group of LAB could be divided into about 20 genera. The 11 major genera of LAB are *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 1998). Largest and the most diverse group *Lactobacillus* can be divided into over 50 recognized species (Axelsson, 1998).

LAB are generally associated with habitats rich in nutrients, such as various food products. They have been traditionally used as starter bacteria in milk, vegetable, cereal and meat fermentations. In fermented foods LAB display numerous antimicrobial activities which increase the shelf life and microbial safety of the food. This is mainly due to the production of organic acids, but also due to other compounds produced such as bacteriocins and antifungal peptides (De Vuyst and Leroy, 2007). LAB fermentation processes also increase digestibility of the food and add desirable aroma compounds (Caplice and Fitzgerald, 1999).

LAB are members of the normal flora of the mouth, intestine and vagina of mammals (Axelsson, 1998). A number of LAB species are used as probiotic strains, for example in food supplement in order to benefit health (Ljungh and Wadström, 2006). LAB have also proved to be strong candidates for development as oral delivery vehicles for digestive enzymes and vaccine antigens (Wells et al., 1996).

LAB are aerotolerant anaerobes. They typically are unable to build catalase or cytochromes needed for oxygen breakdown and oxidative phosphorylation (Axelsson, 1998). Therefore LAB do not posses an electron transport chain and they carry out strictly fermentative metabolism of sugars both under aerobic and anaerobic conditions. The main function of their sugar metabolism is to generate the energy necessary for growth.

1.2. Primary carbohydrate metabolism of lactic acid bacteria

LAB have been traditionally classified into two metabolic sub-groups according to which pathway is active in catabolism of hexose sugars: homo- and heterofermentaive LAB.

1.2.1. Embden-Meyerhof-Parnas pathway (glycolysis)

Homofermentative pathway, Embden-Meyerhof-Parnas pathway (EMP-pathway) or glycolysis, is used by all LAB except *leuconostocs*, group III *lactobacilli*, *oenococci* and *weissellas*. It is typically characterized by the formation of fructose-1,6-diphosphate (FDP), which is split by a FDP aldolase into dihydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). GAP (and DHAP isomerized to GAP) is then converted to pyruvate in a metabolic sequence including substrate level phosphorylation at two steps (Axelsson, 1998). Under permissive conditions (anaerobic conditions and where sugars are not limiting) pyruvate is finally reduced to lactate. In theory, 1 mol glucose fermented via the EMP-pathway

results in the production of 2 moles of lactate with a net gain of 2 moles of ATP (Figure 1).

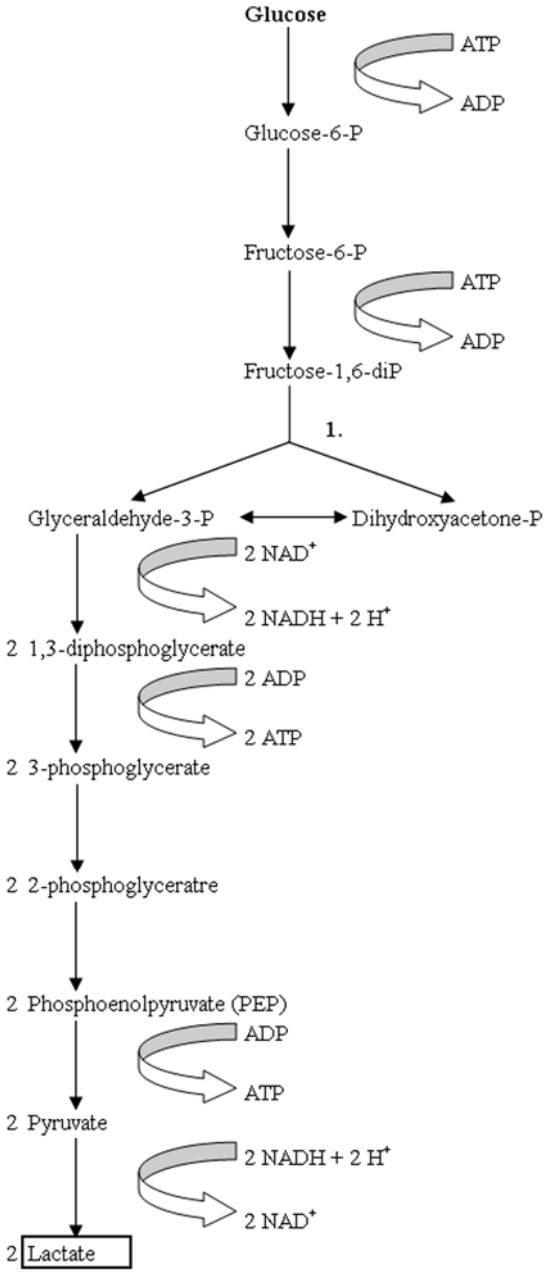

Figure 1. Embden-Meyerhof-Parnas pathway (glycolysis). The enzyme presented: 1. fructose-1,6-diphosphate aldolase.

1.2.2. 6-phosphogluconate/phosphoketolase pathway (PK pathway)

In contrast to homofermentative LAB heterofermentative LAB lack the enzyme FDP aldolase, which mediates the sugarphosphate cleavage. Instead, the pathway proceeds from glucose-6-phoshate via two oxidations by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and by one epimerization by ribulose-phosphate 3-epimerase resulting in xylulose-5-phosphate (xylulose-5-P). Heterofermentative (6-phosphogluconate/phosphoketolase) pathway is characterized by the next step, where xylulose-5-phosphate is split by a phosphoketolase to GAP and to acetyl-P. GAP is then metabolized to lactic acid via phosphorylation to pyruvate and acetyl-P is reduced to ethanol via acetyl-CoA. The latter reaction series is driven by redox imbalance in the cells (Axelsson, 1998). In theory, fermentation of 1 mole of glucose via the PK pathway results in the production of 1 mole each of lactate, carbon dioxide, ethanol and ATP (Figure 2).

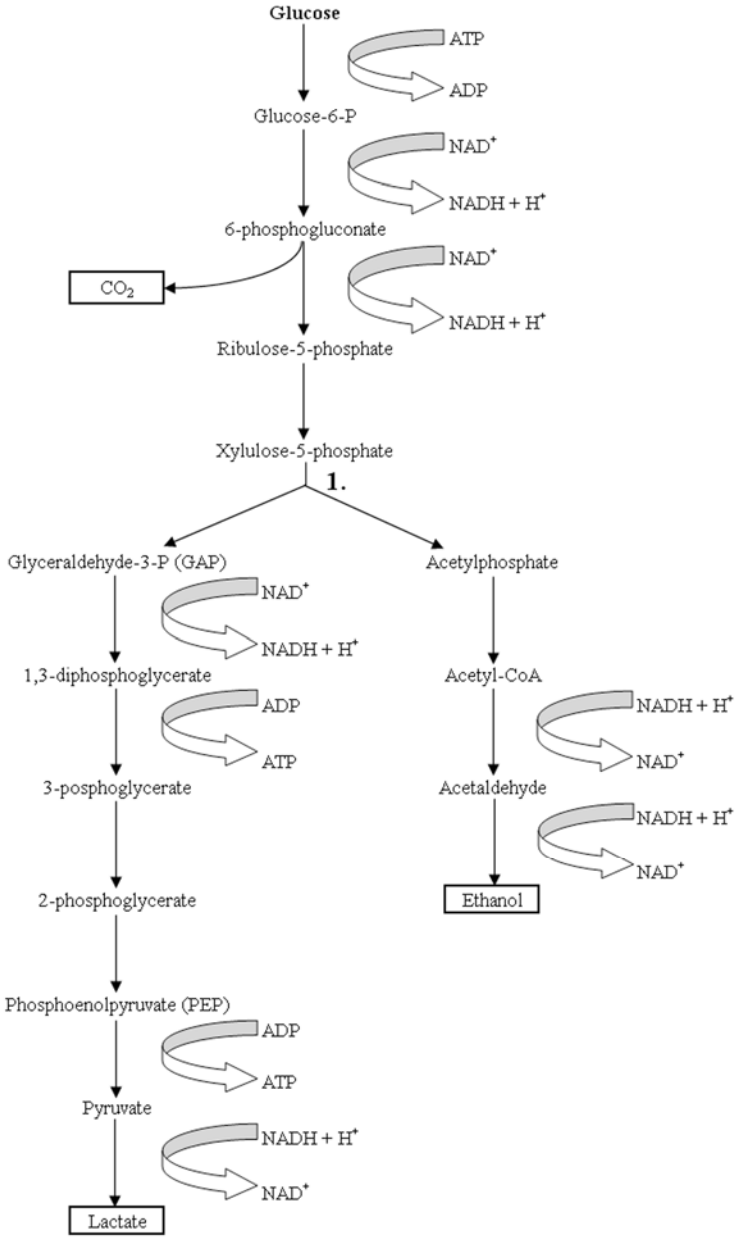

Figure 2. 6-phosphogluconate/phosphoketolase pathway (PK pathway). The enzyme presented: 1. phosphoketolase.

1.2.3. Pentose metabolism

Pentoses are readily fermented by many LAB. Specific permeases are used for sugar transport. Inside the cell, pentoses are converted to xylulose-5-P by sugar specific isomerases and epimerases (Kandler, 1983). These compounds are then metabolized by the lower half of the PK pathway. This would imply that pentoses are only fermented by heterofermentative LAB. In fact most of the LAB can ferment pentoses. In homofermentative LAB pentoses are fermented via the same PK pathway as in heterofermentative LAB. In homofermentative LAB the PK pathway is generally induced by pentoses (Kandler, 1983).

Fermentation of pentoses leads to different end-products compared to the hexose fermentation via the PK pathway. No decarboxylation steps are needed and no CO_2 is formed. Since dehydrogenation reactions are not necessary in the reactions resulting in the intermediate product xylulose-5-phosphate, the reduction of acetyl phosphate to ethanol becomes unnecessary. Instead, acetyl phosphate is converted to acetate by acetate kinase and ATP is formed (Figure 7). Fermetation of pentoses yields equimolar amounts of lactic acid and acetic acid as the end products (Axelsson, 1998; Kandler, 1983).

1.2.4. Fructose co-metabolism

In heterofermentative LAB fructose is typically fermented by the normal PK pathway with CO_2, ethanol, acetate and lactate as the end-products. Part of the fructose can simultaneously act as an electron acceptor and be reduced to mannitol by a NAD^+:mannitol dehydrogenase in order to regenerate NAD^+ (Figure 3) (Dols et al., 1997). When fructose is the sole sugar source, 2/3 mol ATP is formed per 1 mol of fructose consumed, which is less than what is produced in glucose fermentation. However, for some lactobacilli the growth rate on fructose is higher than on glucose, although fructose fermentation is energetically less efficient than glucose fermentation (Axelsson, 1998; von Weymarn, 2002). This suggests that under the conditions of excess substrate, priority is given to growth rate rather than to efficiency of substrate utilization. The reduction of fructose to mannitol could play a

role in this. The co-metabolism of fructose results in decreased ethanol production, because in this case the acetylphoshate is not reduced to ethanol in order to regenerate NAD^+. Instead, acetic acid is produced from acetyl phosphate with the formation of ATP (Axelsson, 1998).

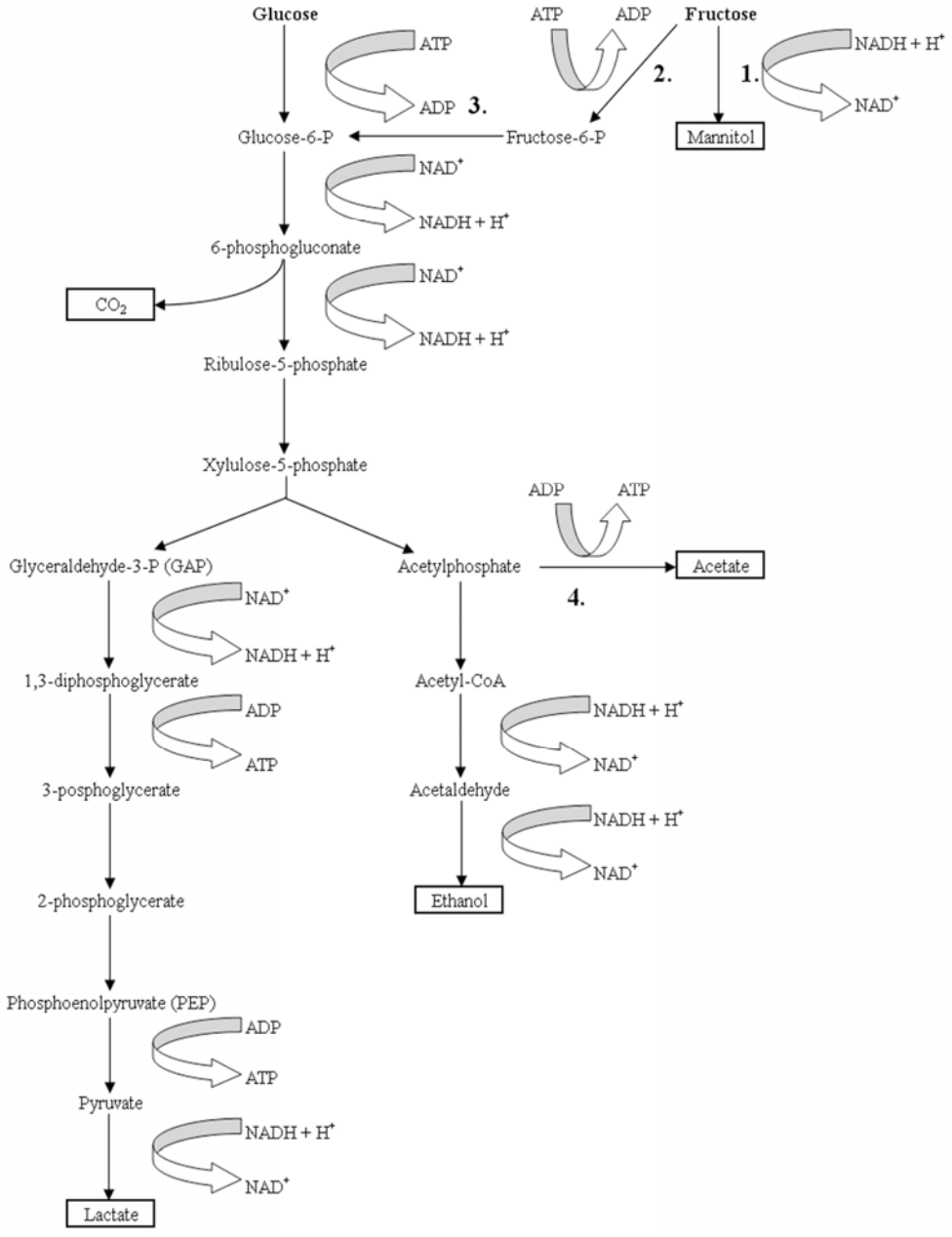

Figure 3. Co-metabolism of fructose and glucose. The enzymes presented: 1. mannitol dehydrogenase, 2. fructokinase, 3. phosphoglucose isomerase and 4. acetate kinase.

1.2.5. Co-metabolism and the role of oxygen

Lactic acid bacteria can adapt their metabolism in response to varying conditions, resulting in different end-products under different conditions. Pyruvate has a vital role serving as an electron acceptor in the regeneration of NAD^+. Depending on conditions LAB can convert pyruvate to other end products than reduce it to lactate.

LAB are able to grow in the presence of air, but they lack the typical oxygen removal mechanisms (e.g. catalase, cytochromes, and hemes). When exposed to air, oxygen can act as an external electron acceptor in a reaction catalyzed by NADH oxidases. The NADH regeneration reaction results in the formation of NAD^+ and either H_2O or H_2O_2, depending on whether four or two electrons are transferred to the oxygen molecule. Induction of NADH oxidases leads to pyruvate accumulation and the cells are forced to change their metabolism. The modified metabolite composition is strongly depended on external conditions and the pathways of pyruvate metabolism are strain specific (Axelsson, 1998).

1.2.6. Carbohydrate transport systems

The transport of solutes and the bioenergetics of the cell are in general tightly connected. Cellular metabolism leads to an electrochemical proton gradient across the cytoplasmic membrane. The most commonly known system for creating a proton gradient is the membrane-linked electron transport chain, present in respiring organisms. Protons are excreted outside the cell by specific transport proteins situated in the cytoplasmic membrane. This creates an inwardly directed force named the proton motive force (PMF). In respiring cells the PMF and the action of ATP synthase result in the phosphorylation of ADP to ATP. LAB do not posses an electron transport chain and hence, are not able to form ATP *via* oxidative phosphorylation. In LAB, ATP is formed by substrate level phosphorylation, which is characteristic to all strictly fermentative organisms. LAB do have an enzyme very similar to ATP synthase. However, this enzyme catalyses the reverse reaction, pumping protons out of the cell by hydrolyzing ATP. This ATP consuming reaction generates PMF, which can drive transport of metabolites and ions.

In LAB substrates are taken up by primary, secondary or group translocation systems. Primary transporters are driven by ATP, whereas secondary transporters utilize the free energy stored in the electrochemical gradient of the translocated solute across the membrane (Poolman and Konings, 1993).

The ABC transport systems (ATP-binding cassette transporters) are the largest class of primary transport systems. Translocation of substrates is coupled to ATP hydrolysis. This mechanism is not only used to accumulate substrates and different solutes but also to excrete unwanted products (Poolman, 2002). Sugars are transported by carbohydrate transport ATPases (Neves et al., 2005).

Secondary transporters, aka permeases, are divided into three groups, symporters (cotransport of two or more solutes), uniporters (transport of one molecule) and antiporters (countertransport of two or more solutes). Symporters usually translocate the solute across the membrane with a proton or sodium ion. Presumably, many sugars are transported in by PMF-driven symport as free sugars and phosphorylated inside the cell by kinases (Axelsson, 1998). Antiporters use the electrochemical ion gradient to excrete substrates and drive protons in, whereas uniporters do not use a coupling ion (Poolman, 2002).

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a complex enzyme machinery whose main function is to translocate a sugar across the membrane with simultaneous phosphorylation (Reizer et al., 1988; Postma et al., 1993). The energy for the process is provided by the high-energy phosphate bond of phosphoenolpyruvate (PEP). The energy of the phosphoryl group is transferred in a series of reactions, *via* the energy coupling proteins Enzyme I and HPr, and the substrate specific phosphoryl transfer protein/domain EIIA to a membrane-located enzyme EIIBC that mediates transport and phosphorylation of the sugar (Figure 4) (Axelsson, 1998). PTS is tightly coupled with the EMP-pathway (glycolysis) and is therefore not common in heterofermentative bacteria (Romano et al., 1979; Saier et al., 1996).

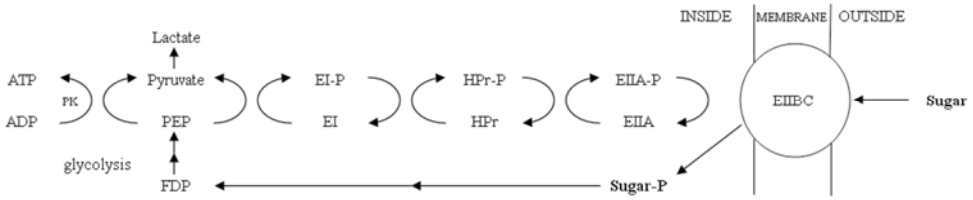

Figure 4. Sugar transport mediated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and relation to glycolysis. PK represents pyruvate kinase. Adapted from Axelsson (1998).

1.2.7. Metabolic engineering of carbohydrate metabolism in lactic acid bacteria

LAB are industrially important microbes that are used worldwide in a variety of food fermentations. Their contribution primarly consist of the rapid formation of lactic acid from the available carbon source resulting in acidification of the food raw material. Besides their lactic acid forming capacity, LAB also have the ability to contribute to other product characteristics like flavour, texture and nutrition. Metabolic engineering of LAB have focused on re-routing pyruvate metabolism to produce commercially important end products like sweeteners, flavour and aroma compounds (Kleerebezem and Hugenholtz, 2003). Metabolic engineering has been successfully utilized in enhancing the production of exopolysaccharides (EPSs) and sugaralcohols in LAB (Kleerebezem and Hugenholtz, 2003; Nyyssölä and Leisola, 2005). Recently, LAB have also been used for the engineering of complex biosynthetic pathways leading to the production of valuable metabolites with health benefits for the consumers (Smid et al., 2005).

1.2.7.1. Metabolic engineering of lactic acid production

Lactic acid is widely used in food, pharmaceutical, textile, leather, and chemical industries. L-Lactic acid is preferred for food and pharmaceutical applications because it is a normal intermediate in mammalian metabolism. L-Lactic acid is also used industrially as a starting material in the production of valuable synthetic biopolymers, such as poly-L-lactic acid (PLLA) (Jahno et al., 2007). Since many LAB are naturally quite optimal lactic acid producers, metabolic engineering in lactic acid production has focused on the production of pure L-lactic acid. Some LAB use two isomer specific enzymes D- and L-lactate dehydrogenases (LDH) to produce respective lactic acid isomers. In *Lactobacillus helveticus* the D-lactate dehydrogenase gene has been disrupted by plasmid integration which resulted in the production of pure L-lactic acid (Bhowmik and Steele, 1994). Production of L-lactic acid at low pH was increased 20% when the disrupted *ldhD* gene was replaced by an additional copy of *ldhL* gene (Kylä-Nikkilä et al., 2000).

1.2.7.2. Metabolic engineering of pyruvate metabolism

Pyruvate has an essential role in sugar catabolism of LAB as the main molecule generated from all metabolic pathways (de Vos, 1996). By metabolic engineering it has been possible to use pyruvate as a source for new products such as ethanol, diacetyl and L-alanine. Ethanol is a common fermentation product in heterofermentative LAB and it's produced by a reduction of acetaldehyde. Homofermentative LAB do not usually produce ethanol in significant amounts because all reducing equivivalents generated during glycolysis are used for reduction of pyruvate to lactic acid. Metabolic engineering has been used to increase the production of ethanol in a lactate dehydrogenase deficient *L. lactis* by expressing the *pet* operon from the high alcohol producing bacterium *Zymomonas mobilis* (de Vos and Hugenholtz, 2004). The other product that can be generated from pyruvate is diacetyl, which provides the typical butter aroma in dairy products. This compound is naturally produced by some LAB from citrate in cometabolic fermentation of lactose (Hugenholtz, 1993). Sugarmetabolism of *L. lactis* was diverted towards the production of α-acetolactate, the precursor of diacetyl either by disruption of lactate dehydrogenase gene or overproduction of NADH oxidase. By combining the latter strategy with disruption of

the gene encoding α-acetolactate decarboxylase, high diacetyl production from glucose and lactose was achieved (Hugenholtz et al., 2000). Hols et al. (1999) expressed the alanine dehydrogenase from *Bacillus megaterium* in lactate dehydrogenase deficient *L. lactis*, resulting in production of alanine instead of lactic acid. More than 99.5% (mol/mol) of glucose was converted to alanine. By deletion of the alanine racemase activity in these bacteria, complete stereo-specific production of L-alanine was conceived. L-alanine is used as a sweetener and in pharmaceutical applications.

2. D-Mannitol

2.1. Properties and applications

D-Mannitol is a naturally occurring six-carbon sugar alcohol (polyol), which is found in many plants, such as pumpkins, celery, onions, grasses, olives, mistletoe and lichens. Mannitol is also found in manna, the dried exudate of manna ash tree (*Fraxinus ornus*), which has been for many years the commercial source of mannitol in Sicily, Italy (Soetaert et al., 1999). Mannitol is also a common component of brown marine algae and mushrooms.

Mannitol forms white orthorhombic needles and the crystals have a melting point of 165-168ºC (Schwartz, 1994) At 25ºC mannitol has the solubility of 18% (w/v) in water (Soetaert et al., 1999).

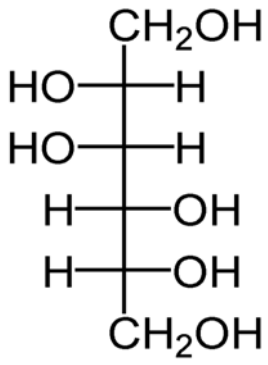

Figure 5. Structure of D-mannitol

Mannitol is widely used in the food, chemical and pharmaceutical industry, as well as in medicine (Soetaert et al., 1995). It has a sweet cool taste and a relative sweetness compared to sucrose around 50-70% (Anon., 2008a). Mannitol is only partially metabolized by humans and does not induce hyperglycemia, which makes it a suitable component of diabetic food products (Griffin and Lynch, 1972). Mannitol has also reduced physiological caloric value, which makes it ideal for low calorie (light) products. Mannitol and other sugar alcohols reduce the crystallization tendency of sugars, and for this reason they are also used for increasing the self-life of foodstuffs and cosmetic products. In medicine, mannitol is used as a powerful osmotic diuretic. Mannitol is used in many types of surgery for the prevention of kidney failure and for reduction of dye and brain edema (Saha et al., 2002).

2.2. Chemical hydrogenation

Mannitol is currently produced industrially by catalytic hydrogenation of glucose/fructose (50:50) mixtures using Raney nickel as the catalyst (Makkee et al., 1985). The hydrogenation reaction typically results in a 25:75 mixture of mannitol and sorbitol. This means that when fructose is catalytically hydrogenated, only about

50% of it is converted into mannitol; the rest is converted to sorbitol (Soetaert et al., 1999). In addition, all glucose present is reduced to sorbitol. Mannitol is also relatively difficult to separate from sorbitol and ultrapure raw materials are required for efficient conversion, which results in even higher production costs (Wisselink et al., 2002).

2.3. Enzymatic hydrogenation

Mannitol can be produced enzymatically. D-Fructose is reduced to D-mannitol by NAD(P)H-dependent mannitol dehydrogenase (MDH). An NADH-dependent MDH (EC 1.1.1.67) has been purified from e.g. *Pseudomonas fluorescens* (Kavanagh et al., 2002), *Leuconostoc mesenteroides* (Yamanaka, 1975; Aarnikunnas et al., 2002), *Leuconostoc pseudomesenteroides* (Hahn et al., 2003), *Lactobacillus brevis* (Martinez et al., 1963; Liu et al., 2005), *Lactobacillus sanfrancesciscensis* (Korakli and Vogel, 2003), and *Rhodobacter sphaeroides* (Schneider and Giffhorn, 1989). An NADPH-dependent MDH (EC 1.1.1.138) has been purified from e.g. head blight fungus *Gibberella zeae* (Trail and Xu, 2002), *Candida mangoliae* (Lee et al., 2003a), *Lactobacillus intermedius* (Saha, 2003) and *Cladosporium herbarum* (Simon-Nobbe et al., 2006). The major disadvantage of the enzymatic processes is the co-factor dependency of MDH. NADH and NADPH are very expensive which makes the reaction unfeasible for industrial processes.

2.4. Microbial production

Microbial production of mannitol using food grade microorganisms is an interesting alternative to the chemical processes. Microbes that are able to produce mannitol include many yeasts (Oinishi and Suzuki, 1970; Lee et al., 2003b), filamentous fungi (Smiley et al., 1967; Domelsmith et al., 1988) and lactic acid bacteria (LAB) (Soetaert, 1990).

2.4.1. Mannitol production by heterofermentative LAB

Certain heterofermentative LAB have been reported to convert fructose into mannitol with yields up to 100% (mol/mol) when fructose and glucose are present in the medium in a ratio of 2:1 (Korakli et al., 2000). When fructose is the single sugar source, heterofermentative LAB ferment it *via* the PK pathway to produce a mixture of CO_2, ethanol, acetate and lactate. A significant part of fructose is simultaneously reduced to mannitol by a NAD^+:mannitol dehydrogenase (MDH) (Dols et al., 1997). Co-metabolism of glucose and fructose in heterofermentative LAB is presented in Figure 3.

The heterofermentative strains that have been studied for mannitol production belong mainly to *Lactobacillus* and *Leuconostoc* (Nyyssölä and Leisola, 2005). Mannitol can be accumulated in concentrations close to the solubility limit of mannitol (180 g/l at 25ºC) in the medium of these LAB (Soetaert et al., 1995). Up to 7.6 g/(l·h) volumetric productivities have been reached using simple batch cultivations (von Weymarn et al., 2002a). Increasing the volumetric productivities has been attempted by immobilizing the cells or by recycling the cells in high density in a membrane bioreactor. A volumetric productivity of 8 g/(l·h) and a 59% (mol/mol) yield from fructose have been achieved using *L. pseudomesenteroides* cells immobilized in polyurethane foam (Soetaert and Vandamme, 1991). A far higher volumetric production rate of 30 g/(l·h) and a yield of 84% (mol/mol) have been achieved using fructose-glucose feed in a ratio of 3:1 and by immobilizing the cells on an anion exchanger (Ojamo et al., 2003). Efficient mannitol production has also been reported using resting cells of *L. mesenteroides* ATCC 9135 in a membrane reactor at high cell density (16 g/l cell dry weight per volume). With initial fructose and glucose concentrations of 100 g/l and 50 g/l, respectively, a volumetric productivity of 26 g/(l·h) and a yield of 97% (mol/mol) were reached. This process was also scaled up to pilot plant scale (100 l) (von Weymarn et al., 2002b, 2003).

There are only few reports on improving the production of mannitol by genetic engineering of heterofermentative LAB. Soetaert (1992) used UV-irritation to construct a *L. pseudomesenteroides* mutant that was unable to ferment fructose *via*

the phosphoketolase pathway. This mutant was characterized to lack the enzyme mannofructokinase. It produced mannitol from fructose in the presence of glucose in both batch and fed-batch processes with a conversion efficiency of 107%. Some of the mannitol may possibly be formed from some components in the complex nitrogen source, since no glucose was converted to mannitol.

2.4.2. Mannitol production by homofermentative LAB

In contrast to heterofermentative LAB mannitol production in homofermentative LAB is not very common. In homofermentative LAB mannitol is synthesized from the glycolytic intermediate fructose-6-phosphate (Figure 6). Under normal conditions mannitol biosynthesis is virtually nonexistent in *L. lactis*, presumably because of catabolic repression. Mannitol synthesis has been successfully activated by genetic engineering. *Lb. plantarum* and *L. lactis* strains deficient in lactate dehydrogenase activity have been reported to produce small amounts of mannitol (Ferain et al., 1996; Neves et al., 2000). Since NAD^+ was no longer regenerated in the reaction in which pyruvate is reduced to lactate, the mannitol-1-phosphate dehydrogenase reaction was used for this purpose instead. Gaspar et al. (2004) succeeded in enhancing the mannitol production by disrupting parts of the mannitol-PTS operon, *mtlA* or *mtlF* genes, of the lactate dehydrogenase deficient *L. lactis* strain. The mannitol yield from glucose was significantly improved to 33% (mol/mol) by using resting cells of the double mutant**.**

Mannitol production has been enhanced by overexpression of the genes encoding enzymes involved in mannitol synthesis. However, overexpression of a mannitol-1-phosphate dehydrogenase gene in *L. lactis* alone does not guarantee efficient mannitol production in this strain. Low intracellular concentration of fructose-6-phosphate due to its fast flux to glycolysis has been suggested to be the reason for limited mannitol formation (Wisselink et al., 2002). The mannitol-1-phosphate dehydrogenase from *Lb. plantarum* has been expressed in a lactate dehydrogenese deficient *L. lactis* strain to enhance mannitol production. The mannitol yield from glucose was only 1% (mol/mol) with growing cells. However, with resting cells the yield of mannitol from glucose was 25% (mol/mol) (Wisselink et al., 2004). Even

more efficient mannitol production was achieved by co-expressing of both of the genes involved in converting fructose-6-phosphate to mannitol. The mannitol-1-phosphatase gene from *Eimeria tenella* and the mannitol-1-phosphate dehydrogenase gene from *Lb. plantarum* were expressed in a lactate dehydrogenase deficient *L. lactis* strain. A mannitol yield of 50% (mol/mol) from glucose was achieved by using growing cells of this strain. This is close to the theoretical maximum of 67% (mol/mol) (Wisselink et al., 2005).

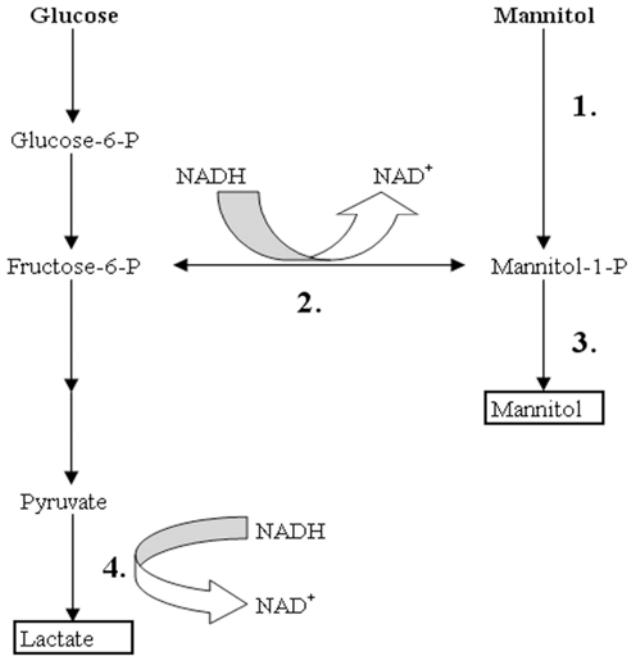

Figure 6. Mannitol biosynthesis in homofermentative LAB. Enzymes presented: 1. mannitol specific phosphoenolpyruvate (PEP)-dependent sugartransferase system (PTS), 2. mannitol-1-phosphate dehydrogenase, 3. mannitol-1-phosphatase and 4. lactate dehydrogenase.

2.5. Summary of the mannitol production methods and the hypothesis of this work

The chemical dehydrogenation of mannitol has some drawbacks. The need for metallic catalyst, purification of mannitol from sorbitol, relatively high price of raw material, chromatographic purification of metal catalyst from the final product, and also high temperature and pressure in operating conditions makes this method a mediocre performer from an economical standpoint.

Alternative mannitol production methods such as enzymatic and microbial processes have been studied extensively. The major disadvantage of the enzymatic processes is the co-factor dependency of MDH. NADH and NADPH are very expensive which makes the reaction unfeasible for industrial processes. Microbial production of mannitol has been reported with bacteria, yeasts, and filamentous fungi. Some of these processes have very low volumetric productivities, prolonged cultivation time, and a risk of contamination. LAB have proved to be very promising candidates for biotechnological mannitol production. The reports show that a nearly quantitative yield from fructose and very high volumetric productivities can be achieved by using heterofermentative LAB.

In heterofermentative LAB fructose is fermented *via* the phosphoketolase (PK) pathway and a significant part of fructose is simultaneously reduced to mannitol by a mannitol dehydrogenase. To be channeled into the PK pathway, intracellular fructose is first phosphorylated into fructose-6-phosphate by fructokinase (FK) and then isomerized to glucose-6-phosphate by phosphoglucose isomerase (PGI). The hypothesis of this thesis was that inactivation of a gene encoding for one of these enzymes would prevent the leakage of fructose into the PK pathway and improve the yield of mannitol from fructose.

3. Rare sugars

3.1. Introduction

The International Society of Rare Sugars (ISRS) has defined rare sugars as monosaccharides and their derivatives that rarely exist in nature (Anon., 2008b). In the past few years the use of L-carbohydrates and their nucleoside derivatives have greatly increased in medicine. Several nucleoside analogues derived from from L-sugars, such as L-FMAU [1-(β-L-2-fluoro-2-deoxyarabinofuranosyl)-5-methyluracil], have been shown to be potent antiviral agents against hepatitis-B, HIV and Eppstein-Barr viruses (Mathé and Gosselin, 2006). L-Nucleosides have also shown their potential in cancer treatments as chemoterapeutic agents against viral associated cancers (Gumina et al., 2001; Cheng, 2001). Thanks to their 'unnatural' stereochemistry L-nucleosides have in most cases lower toxicity and higher metabolic stability compared to their natural counterparts (Gumina et al., 2001).

Rare sugars, such as D-tagatose, are also used in the food industry. They have the same sweetness as the natural sugars, but they are either metabolized in lesser degree than the natural sugars or not metabolized at all. This will make their use suitable as a low-calorie sweeteners. The other advantage of rare sugars is the absence of an objectionable aftertaste typical for many artificial sweeteners (Levin et al., 1995).

Rare sugars cannot be isolated from natural sources in large quantities. The price of rare sugars has been high and the production volumes small. However the markets and interest for rare and unnatural sugars have been growing recently. It would be advantageous to utilize cheap natural resources such as starch, wood, whey or crude plant hydrolysates for the production of rare sugars.

3.2. L-Ribulose

3.2.1. Properties and applications

L-Ribulose is a ketopentose, in other words it is a five carbon monosaccharide containing a ketogroup. It has a molecular formula of $C_5H_{10}O_5$ and molecular weight of 150.13. Despite being a common metabolic intermediate in different organisms, L-ribulose is scarce in nature and classified as a rare sugar (Ahmed, 2001).

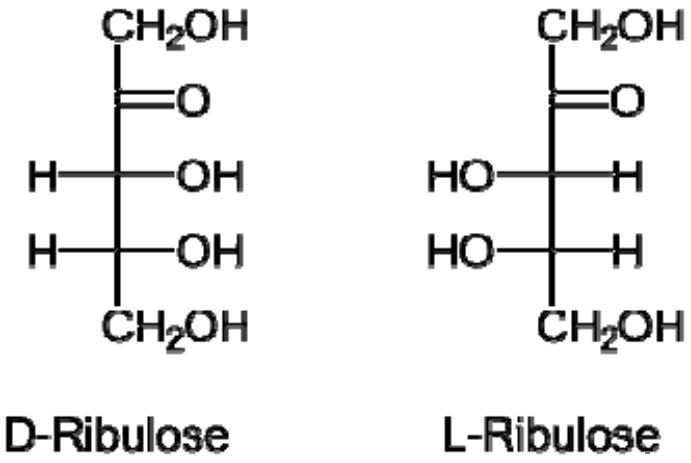

Figure 7. Structures of D- and L-ribulose.

The L-form of ribulose is an intermediate in the pathway for L-arabinose utilization in many bacteria. 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 (Figure 8) (Heath et al., 1957; Burma and Horecker, 1957a, 1957b; Lee et al., 1986; Lin et al., 1985a, 1985b, 1985c; Kandler, 1983). The genes for the enzymes of the reaction sequence, L-arabinose isomerase (*araA*), L-ribulokinase (*araB*) and L-ribulose 5-phosphate 4-epimerase (*araD*), have been reported to be arranged as an operon in

bacteria (Lee et al., 1986; Lin et al., 1985a, 1985b, 1985c, Sá-Nogueira and de Lencastre, 1989).

L-Ribulose market is currently small and there is no bulk production. The only available price at the moment is 995 $ per 2 g (ZuCarb). L-Ribulose has no industrial use at present but it is an important precursor for the synthesis of L-ribose, a high value rare sugar used in preparation of new antiviral drugs (Ahmed, 2001).

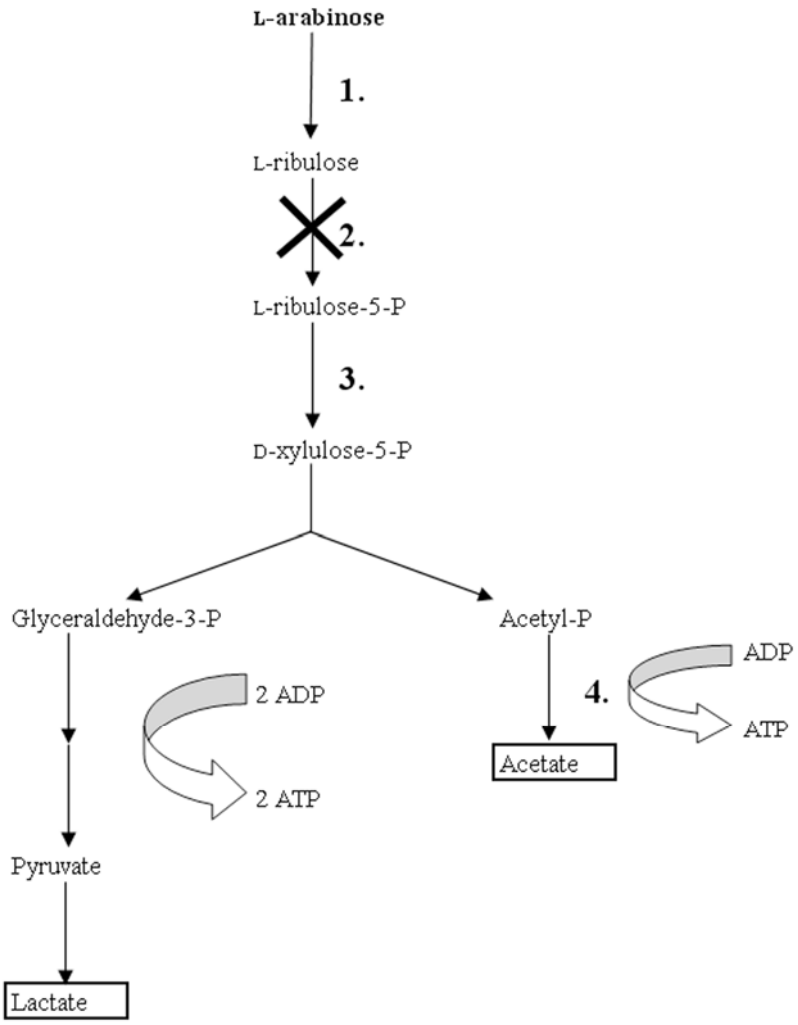

Figure 8. L-arabinose metabolism in LAB. The enzymes presented: 1. L-arabinose isomerase, 2. L-ribulokinase, 3. L-ribulose 5-phosphate 4-epimerase and 4. acetate kinase. Symbol X represents the place for the knockout mutation in L-ribulose producing strains.

3.2.2. Production methods

L-Ribulose production has been studied almost exclusively by using acetic acid bacteria for the dehydrogenation of ribitol to L-ribulose (Moses and Ferrier, 1961; Ahmed et al, 1999; Kylmä et al., 2004; De Muynck et al., 2006). 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 (Adachi et al., 2001). The L-ribulose yield of 98% (mol/mol) from ribitol was reached by using washed cells of *Acetobacter aceti* IFO 3281 (Ahmed et al., 1999). With an initial ribitol concentration of 50 g/l Kylmä et al. (2003) attained specific production rate of 1.2 g/(g·h) and a yield of 96% (mol/mol) using resting cells of the same strain. The productivity of 5.9 g/(g·h) [15.7 g/(l·h)] achieved using resting cells of *Gluconobacter oxydans* MC14 is the highest achieved so far using this approach (De Muynck et al., 2006).

A problem with using ribitol as the raw material is that it is presently very expensive and that it cannot be isolated from any natural sources. As most of the other sugar alcohols ribitol would therefore have to be produced from the corresponding pentose, D-ribose, by chemical or microbial hydrogenation (Albert et al., 1980). Furthermore, the precursor of ribitol, D-ribose, is currently produced from glucose by fermentation (Ahmed, 2001), which makes the entire production chain to L-ribulose very complicated. The production of ribitol from glucose by fermentation using the fungus *Trichosporonoides megachillensis* has also been reported (Kawaguchi et al., 2001). However, the yield of ribitol from glucose is low in this process (less than 30%), since a large fraction of the glucose is lost to other polyols.

Unlike other L-sugars, L-arabinose is fairly abundant in nature. It is a common component of polymers of lignocellulosic materials. 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% (w/w) of the deproteinated mass). L-arabinose can easily be isolated from the pulp by acid or enzymatic hydrolysis, filtration and acetone fractionation (Spagnuolo et al., 1999).

L-Ribulokinase catalyzes the phosphorylation of L-ribulose in reaction 2. as shown on Figure 8. L-Ribulokinase deficient mutant of *E. coli* has been previously constructed by chemical mutagenesis (Englesberg, 1961). The mutant has been shown to convert L-arabinose to L-ribulose in the presence of borate ions. The thermodynamic equilibrium in the isomerization reaction between L-arabinose and L-ribulose is very unfavourable for L-ribulose formation (Heath et al., 1958). Borate ions form a complex with L-ribulose, which increases the conversion efficiency of L-arabinose to L-ribulose. With the initial L-arabinose concentration of 18.2 g/l, a volumetric productivity of 0.66 g/(l·h) and the yield of 84% (mol/mol) was reached using resting cells of the *E. coli AraB* mutant (Englesberg, 1961).

3.3. L-Ribose

3.3.1. Properties and applications

L-Ribose is an aldopentose and has a molecular formula of $C_5H_{10}O_5$. It forms solid white crystals with a melting point of 87ºC (Budavari, 1996). The molecular weight of L-ribose is 150.13.

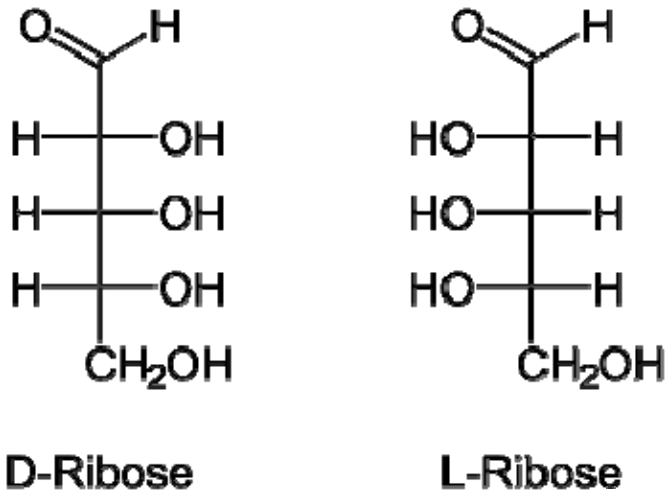

Figure 9. Structures of D- and L-ribose.

L-ribose is used as a starting material in the synthesis of many L-nucleoside based pharmaceutical compounds, such as Clevudine, Valtorcitabine, Elvucitabine and Epivir (Lamivudine) (Woodyer et al., 2008). Epivir has been approved for the treatment of hepatitis-B infection, and Clevudine and Valtorcitabine are in clinical trials (Mathé and Gosselin, 2006). Elvucitabine is currently in clinical trials against HIV (Mathé and Gosselin, 2006).

3.3.2. Chemical production of L-ribose

L-Ribose can be obtained chemically by molybdate catalyzed epimerization of L-arabinose (Jumppanen et al., 2001; Angyal, 2005). However, the L-ribose yield is low (10-35% (mol/mol)) and a significant proportion of the consumed L-arabinose is lost to by-products. In addition, the L-arabinose used in this process requires extensive purifications before the chemical conversion (Jumppanen et al., 2000). The conversion of D-mannono-1,4-lactone into L-ribose in eight steps has been described. The key step of this reaction sequence is the cyclization of a γ-hydroxyalkoxamate (Takahashi et al., 2002). Also the treatment of D-mannono-1,4-lactone with piperidine, followed by mesylation-induced SN2-type O-alkylation has been described to result in L-ribose formation (Seo et al., 2003). Additionally, D-ribose has been converted chemically to L-ribose with the interconversion of the ends of the D-ribose molecule in six steps with an overall yield of 45% (mol/mol) (Jung and Xu, 1997). Also D-galactose has been used as the starting material in a ten step chemical synthesis resulting in a 57% (mol/mol) overall yield (Shi et al., 2001). A method of synthesizing L-ribose from an L-arabinose derivative, which is an intermediate of the deoxyribose synthesis with overall yield of 77% (mol/mol) has also been reported (Akagi at al., 2002).

3.3.3. Biotechnological production of L-ribose

Biotechnological production of L-ribose has focused on converting L-ribulose to L-ribose. An L-ribose isomerase catalyzing the reaction from L-ribulose to L-ribose has been isolated from the bacterium *Acinetobacter* sp. DL-28 and the corresponding gene

sequenced (Shimonishi and Izumori, 1995; Mizanur et al., 2001). The use of toluene permeabilized cells of *Acinetobacter* sp. DL-28 for L-ribose production has also been described (Ahmed et al., 1999). A novel enzyme, D-lyxose isomerase, which also isomerizes L-ribulose to L-ribose as a side-activity, has been isolated and sequenced recently (Cho et al., 2007). Furthermore, an isomerase having L-ribose isomerizing activity has been created by directed evolution. This was achieved by random mutagenesis of *Escherichia coli* L-arabinose isomerase gene to construct a mutant library and by screening for transformants having activity towards L-ribose (De Myunck et al., 2007). A one step conversion of ribitol to L-ribose has also been studied. Woodyer et al. (2008) reported a production rate of 0.73 g/(l·h) and a conversion of over 70% using an *E. coli* strain expressing a mannitol-1-dehydrogenase from *Apium graveolens*.

4. Aims of this Study

The aim of this study was metabolic engineering of lactic acid bacteria for the production of industrially interesting compounds such as mannitol and rare sugars. LAB are very promising targets for metabolic engineering since their carbon metabolism is relatively simple and not connected to the biosynthetic activity. Therefore their carbohydrate metabolism can be engineered without disturbing the synthesis of cell components. LAB have also the GRAS (generally regarded as safe) status, which makes them suitable production hosts for food and pharmaceutical applications.

The specific aims of this study were:

1. to improve the mannitol production process by altering the fructose metabolism of *Leuconostoc pseudomesenteroides* by random mutagenesis.
2. to characterize genes involved in fructose metabolism in *Lactobacillus fermentum* to find ways to enhance the mannitol production.
3. to construct an L-ribulose producing strain of *Lactobacillus plantarum* and to develop and optimize the L-ribulose production process.
4. to construct L-ribose producing strains of *Escherichia coli* and *Lactobacillus plantarum*.

5. Materials and Methods

Only a brief summary of materials and methods of this study is presented in here. A more detailed description can be found in publications I-IV.

5.1. Bacterial strains and growth conditions

All *Leuconostoc* and *Lactobacillus* strains were cultivated at 30°C in standard MRS growth medium (Difco, LabM). Erythromycin was used as a selective marker for plasmids at the final concentration of 5 μg/l. Bioreactor cultivations were performed using simplified MRS medium or SP medium (von Weymarn et al., 2002a). The simplified MRS medium comprised 0 or 10 g/l glucose, 10 g/l bacto peptone (Difco), 10 g/l yeast extract (Lab M Limited), 2 g/l K_2HPO_4, 0.2 g/l $MgSO_4$ and 0.05 g/l $MnSO_4$, and 5-30 g/l various sugars.

All *Escherichia coli* strains were cultivated aerobically in standard LB medium (Difco, Pronadisa) at 37°C. Ampicillin was used as a selective marker for plasmids in the final concentration of 100 μg/l. *Lactococcus lactis* NZ9000, obtained from NIZO Laboratories (Netherlands) (Kuipers et al., 1998), was grown in M17 medium (Difco) containing 5 g/l sucrose at 30°C.

5.2. DNA techniques and transformation methods

Basic molecular biology techniques were used as described by Sambrook and Russel, (2001). Isolation of chromosomal DNA is described in studies I-IV. Restriction enzymes and other DNA modifying enzymes were used as described by the manufacturer (New England Biolabs). Polymerase chain reaction (PCR) was performed in the reaction conditions recommended by the manufacturers of the polymerases (Finnzymes, Invitrogen, or Roche).

The Vectorette II system (Sigma Genosys, Ltd., UK) was used for creating a genomic library of *L. pseudomesenteroides* and *Lb. fermentum.* Chromosomal DNA of *L. pseudomesenteroides* and *Lb. fermentum* was digested either with *Bam*HI, *Cla*I, *Eco*RI or *Hind*III (New England Biolabs) and ligated with the respective Vectorette units. Vectorette amplicons were amplified with PCR using sequence specific and Vectorette unit specific primers, subcloned into the cloning vector pGM-T Easy and sequenced.

Lb. plantarum was transformed by electroporation as previously described by Aarnikunnas et al. (2001). Transformation mehod for *L. lactis* and *E. coli* were performed as described by Holo and Nes (1989) and Sambrook and Russel (2001), respectively.

5.3. RNA techniques

For isolation of total RNA the cells were treated with the RNAprotect® Bacteria Reagent (Qiagen). The cells were incubated in the presence of lysozyme (Sigma) and mutanolysin (Sigma) and the RNA was isolated using the RNAeasy® Mini Kit (Qiagen). The RNA for cDNA synthesis was treated with deoxyribonuclease I (Sigma).

Northern blotting was carried out as described in Studies I-II. The gel was blotted on Zeta-probe membrane (Bio-Rad) and hybridized with a [α-^{33}P]dATP-labeled probe, generated by PCR amplification. The membranes were visualized using a molecular imaging system (Model GS-525, Bio-Rad). Quantification of RNA on the membrane was performed by hybridization with 16S RNA specific PCR probes.

The 5'-end determination of *fruK* transcripts was performed at the Institute of Biotechnology, University of Helsinki by using 5 pmol Cy5-labelled oligonucleotide Cy5LPFRK and an ALF Express DNA Sequencer as described previously (Vesanto et al., 1995).

The relative amount of *araBAD* mRNA from *Lb. plantarum* cells was determined by reverse transcription quantitative PCR (RT-qPCR) using comparative C_T method (Study III). cDNA for the RT-qPCR assays was synthesized with StrataScript® cDNA Synthesis Kit (Stratagene) using random hexamer primers and an RNA template. PCR amplification was performed in final volumes of 20 µl using 2 × Brilliant® SYBR® Green Master Mix (Stratagene) and ROX reference dye according to the instructions by the manufacturer. All the amplifications were carried out in optical grade 8 well strips using an Mx3000P real-time PCR system (Stratagene).

5.4. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was used for studying interactions between the unkR repressor protein and the promoter region of the *fruIK* operon of *Lb. fermentum* (Study II). The *unkR* gene was amplified with PCR from *L. fermentum* NRRL-B-1932 genomic DNA and the resulting fragment was cloned into the expression vector pQE-60 (Qiagen). *E. coli* XL1-Blue was transformed with the plasmid. The UnkR repressor protein was produced by inducing the cells with 0.5 mM IPTG. Cells were disrupted by sonication and the lysate from the cells was used as the source of UnkR repressor protein in the gel shift reaction. The gel shift reaction was performed using the DIG Gel Shift Kit, 2nd Generation according to the instructions by the manufacturer (Roche). Lysate of *E. coli* XL1-blue cells containing pQE-60 plasmid without an insert was used as a negative control.

5.5. Enzyme activity assays

5.4.1. Fructokinase and phosphoglucose isomerase assays

Cell extracts were prepared by sonication and the fructokinase and phosphoglucose isomerase activities were determined from the cell extracts as described in studies I-II. Enzyme activities were assayed at 30°C by measuring the change in absorbance at 340

nm. Protein concentrations were determined by the Bradford method using Bio-Rad protein assay reagent with bovine serum albumin as a protein standard (Bradford, 1976).

5.4.2. PTS activity assay

Fructose PTS activity of *Lb. fermentum* was determined at 30°C using toluene treated cells as described by Nagasaki et al. (1992). The method is described in more detail in Study II. The PTS activity was assayed by measuring the change in absorbance at 340 nm. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a protein standard.

5.4.3. L-arabinose isomerase and L-ribose isomerase assays

Cell extracts were prepared by sonication and the L-arabinose isomerase and L-ribose isomerase activities determined at 30°C from the cell extracts as described in more detail in Studies III-IV. Protein concentrations were determined using the Qubit™ fluorometer (Invitrogen) according to the instructions by the manufacturer.

5.5. Bioreactor cultivations

All *Leuconostoc* and *Lactobacillus* cultivations were carried out in a Biostat MD reactor (total volume 2 l, B. Braun Biotech International) at 1.5 l with a stirring rate of 200 rpm (two 6-blade Rushton type impellers) at 30 ˚C. pH was controlled at a minimum of 6.2 with 3 M NaOH. The cultivations were inoculated with 5% (v/v) of culture in late exponential phase.

E. coli BPT234 cultivations were carried out at 1.5 l of LB medium at 37°C. The culture was aerated at a constant rate of 0.5 vvm. Dissolved oxygen was controlled at 30% by varying the stirring rate (minimum 500 rpm).

Sugar, ethanol, organic acid and mannitol concentrations were determined from the cultivation samples by HPLC. 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.

5.6. Optimizations

5.6.1. Optimization of L-ribulose production

A central composite face centered (CCF) experiment design for three variables was used in the initial investigation of L-ribulose production with resting cells of *Lb. plantarum* BPT197. The variables were temperature (T), and L-arabinose (c_A) and borate (c_B) concentrations at ranges from 30 to 40°C, from 10 to 50 g/l and from 50 to 600 mM, respectively. Initial L-ribulose production rate (r_i) and conversion of L-arabinose to L-ribulose (x) were used as the responses. A further two variable central composite circumscribed (CCC) experiment design with a star distance of 1.5 was constructed in order to optimize temperature (from 38 to 46°C) and L-arabinose concentration (from 100 to 300 g/l). 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 in all optimizations.

5.7. Purifications

5.7.1. Purification of L-ribulose

The cell suspensions from the optimization experiment 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 (200-400 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 (Englesberg, 1961). Fractions containing 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 freeze dried and the dried samples were dissolved into methanol. The boric acid was removed as methyl borate under vacuum from the sample. L-ribulose was dissolved into distilled water and analyzed with HPLC.

6. Results and Discussion

6.1. Characterization of *Leuconostoc pseudomesenteroides* random mutants unable to grow on fructose (Study I)

In heterofermentative LAB fructose is fermented *via* the phosphoketolase (PK) pathway to produce a mixture of CO_2, ethanol, acetate and lactate. A significant part of fructose is simultaneously reduced to mannitol by a NAD^+:mannitol dehydrogenase (MDH) (Dols et al., 1997). To be channeled into the PK pathway, intracellular fructose is first phosphorylated into fructose-6-phosphate by a fructokinase (FK) (EC 2.7.2.4) and then isomerized to glucose-6-phosphate by a phosphoglucose isomerase (PGI) (EC 5.3.1.9). The hypothesis of this work was that inactivation of a gene encoding for one of these enzymes would prevent the leakage of fructose into the PK pathway and give an improved yield of mannitol from fructose.

6.1.1. Isolation of Leuconostoc pseudomesenteroides mutants unable to grow on fructose

The fructokinase of the mannitol producer *L. pseudomesenteroides* was inactivated by chemical mutagenesis in order to prevent the loss of fructose to the PK pathway. After mutagenesis the mutants unable to grow on fructose were screened. Conversion of fructose to mannitol by these mutants was tested to ensure that the fructose permease was not affected by the mutagen. Three colonies out of 1000 screened were found to possess lowered fructokinase activity in the range of 10 to 60% of that of the wild type. The mutant with the lowest fructokinase activity (10% of that of the parent strain), named BPT143, was selected for further studies. There was no significant difference in the mannitol dehydrogenase activities of the mutant and of the wild type strain.

6.1.2. Sequence analysis of the fruK gene

To characterize the fructokinase gene *fruK* of the wild type *L. pseudomesenteroides* ATCC12291, sequence data of the corresponding genes from other bacteria were utilized. A fragment of the *L. pseudomesenteroides* fructokinase gene (*fruK*) was isolated by PCR amplification using degenerate oligonucleotides which were designed according to the conserved regions of fructokinase genes from various LAB and *B. subtilis*. Sequence analysis of the 0.5 kb PCR fragment revealed high similarity with other bacterial fructokinases. The unknown upstream (0.5 kb) and downstream (0.8 kb) regions of the *fruK* gene were isolated using the Vectorette II system (Sigma) and specific inverse primers, followed by sequencing and by assembling the sequences of the three *fruK* fragments.

The coding sequence of the *L. pseudomesenteroides fruK* gene was found to be 862 bp in size and preceded by a putative -35 and -10 promoter region starting 94 base pairs upstream of the translation initiation site. A *cre*-like consensus sequence (TGWAARCGYTWNCW) (Inancio et al., 2003) was found in the promoter region. A putative ribosome binding site was located six base pairs upstream of the translation initiation codon. A putative stem-loop transcription terminator with ΔG =

-84 kJ was located 290 - 325 bp downstream of the translation stop codon (Figure 10). In addition, an upstream stem-loop terminator with ΔG = -84 kJ was found 490 - 453 bp upstream of the translation initiation codon of *fruK*. No additional open reading frames were found 450 bp upstream or 460 bp downstream of the *fruK* coding sequence by the sequence analyses performed. The sequence analyses further showed that the *fruK* gene region is flanked by approximately 350 bp long inverted repeats (IR). This suggests that the ability of *L. pseudomesenteroides* to use unphosphorylated intracellular fructose has evolved *via* lateral Tn-transposition during evolution.

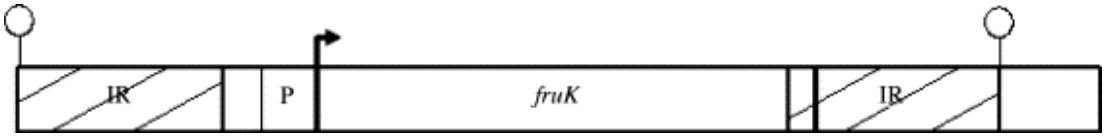

Figure 10. Map of the *L. pseudomesenteroides* ATCC12291 *fruK* gene and its flanking areas. The coding sequence is marked with *fruK*, promoter area with P and 350 bp long inverted repeat sequences with IR. Putative transcription terminators are marked with hairpinloops.

The genomic DNA from the fructose-negative strain BPT143 was isolated and possible mutations in the *fruK* gene sequence were analyzed by sequencing the *fruK* gene region after PCR amplification. A silent point mutation in Thr104 was identified in the *fruK* sequence of the mutant. This was not, however, likely to be the reason for the reduced fructokinase activity. No mutations could be found in the 450 bp upstream region of *fruK*, suggesting that the putative *fruK* promoter sequence was not directly affected by the mutagen.

6.1.3. Analyses of L. pseudomesenteroides fruK transcripts

No detectable amounts of *fruK* transcripts were synthesized in the BPT143 mutant. This was in accordance with the reduced fructokinase activity of the mutant. The size of the hybridized *fruK* transcript was determined to be approximately 1.3 kb, which is

in good agreement with the size of the putative *fruK* mRNA deduced from the gene sequence. Determination of the 5′-end of the 1.3 -kb transcript, using primer extension with an *fruK*-specific oligonucleotide, located the transcription start site seven nucleotides downstream of the -10 region of the *fruK* promoter and 59 nucleotides upstream of the initiation codon. According to these results and the transcription terminator analysis the actual size of the *fruK* transcript was verified to be 1257 bp.

However, no mutations directly affecting the expression of *fruK* could be found in the *fruK* gene sequence or in its upstream region of 450 bp. It would therefore seem likely that some more distant sequences involved in the regulation of *fruK* transcription were mutated. For example, a regulatory protein may have been affected by the mutagen. In an activator based system, inactivation of this protein or its synthesis would have directly resulted in the phenotype observed. On the other hand, if the *fruK* gene is controlled by a repressor, e.g. a constitutive repressor mutation or a non-inducible repressor mutation could then result in the phenotype found. It is neither possible to completely rule out DNA conformation rearrangement effects as the cause of the change in the BPT143 phenotype.

6.1.4. Bioreactor cultivations

The effect of the random mutation on mannitol production was studied in bioreactors under process conditions. Comparison of wild type *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant BPT143 revealed that the mutant grew faster than the parent strain. The maximum specific growth rate was improved from 0.59±0.04 1/h to 0.69±0.01 1/h. In addition, the fructose consumption rate was slightly increased in BPT143. More importantly, the specific fructokinase activity was significantly reduced resulting in an improved mannitol yield [from 74% to 86% (mol/mol)]. A faster fructose consumption rate and an improved yield subsequently resulted in a slightly better volumetric mannitol productivity by the mutant [from 2.1 g/(l·h) to 2.8 g/(l·h)]. This was expected because both the mannitol yield and the fructose consumption rate were improved in the mutant.

Both the parent strain and the mutant consumed approximately the same amount of glucose in relation to fructose (Figure 11). Due to a greater leakage of fructose into the PK pathway, the parent cells produced clearly more carbon dioxide, lactic acid and ethanol than the mutant cells did. To balance the increased NADH oxidation due to increased mannitol production, the mutant cells produced less ethanol and more acetic acid. However, the yield of ATP per mole of fructose consumed was still approximately the same with both strains: 112 mol/mol for the parent strain and 110 mol/mol for the mutant. Hence, the yields of ATP correlated well with the final cell dry weights observed with both strains.

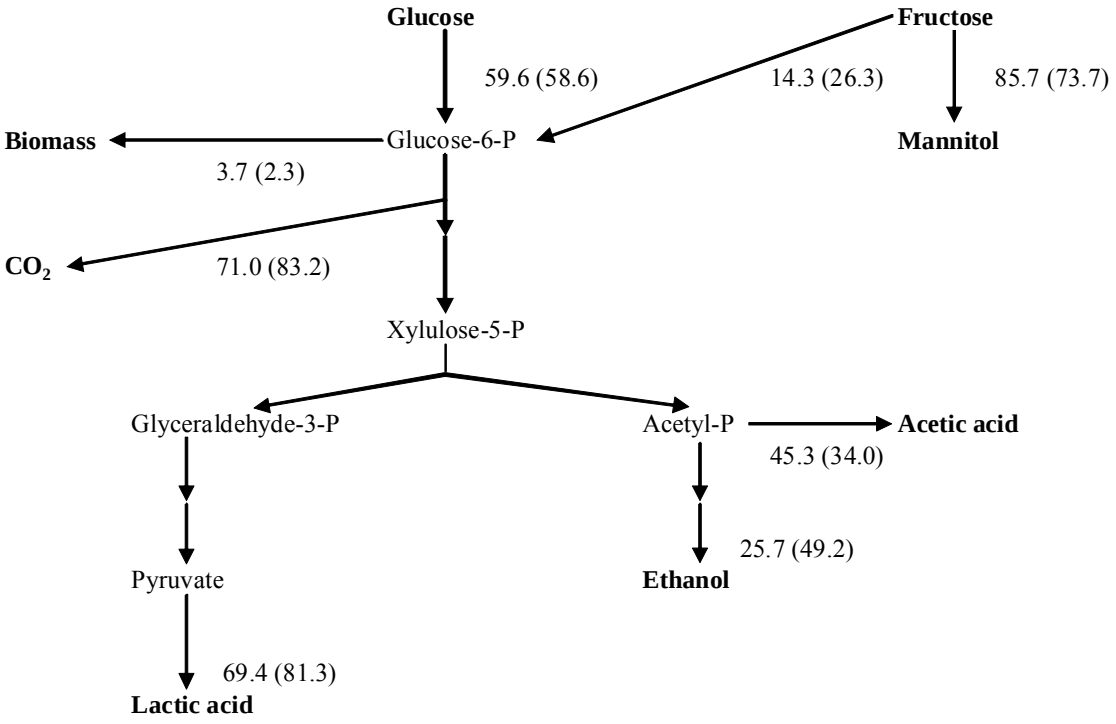

Figure 11. The primary metabolism of *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant (BPT143) at 30°C and pH 5.0. The values represent yields on fructose [(mol/mol)×100]. Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were analyzed by HPLC, whilst the amount of biomass and carbon dioxide were calculated. The respective values for the parent strain are shown in brackets.

It has been reported for some native LAB strains that only negligible amounts of fructose leak into the PK pathway when the cells are grown in the presence of a 2:1 mixture of fructose and glucose (e.g. Korakli et al., 2000; Saha and Nakamura, 2003). This is usually achieved at low pH. However, acidic process conditions result in decreased cell metabolism, which subsequently cause decreased volumetric mannitol productivity. A fructokinase-negative mutant could enable higher pH to be used in the production process without lowering the yield (von Weymarn, 2002). With such a mutant both yield and productivity could be maximized.

Despite the 90% decrease in fructokinase activity a 100% (mol/mol) yield of mannitol from fructose was not achieved. The results suggest that the fructose phoshorylation pathway is more efficient than the fructose reduction reaction, which makes it difficult to direct the flux to mannitol. Nevertheless, our results indicate that the yield can be improved by lowering the fructokinase activity. It is possible that a quantitative yield would be achieved, if the fructokinase activity was completely disrupted. In addition, the absence of a PTS transporter in the mutant strain would be a prerequisite for a quantitative yield.

Targeted mutagenesis to disrupt fruktokinase gene of *Leuconostoc pseudomesenteroides* ATCC12291 was tested using thermosensitive pGHOST system. The temperature maximum of this strain was too low for successful integration of the plasmid and the deletion of the fructokinase gene failed. A good mannitol producer, *Lactobacillus fermentum* NRRL-B-1932 (von Weymarn et al., 2002a), that tolerates high temperatures, was selected for the targeted fructokinse disruption instead.

6.2. Characterization of genes involved in fructose utilization in *Lactobacillus fermentum* (Study II)

In this work we characterized the genes involved in fructose metabolism in *Lactobacillus fermentum* to find new ways to enhance the mannitol production by genetic engineering.

6.2.1. Sequence analysis of the fruIK operon of Lb. fermentum

A fragment of the *Lb. fermentum* fructokinase gene (*fruK*) was isolated by PCR amplification using degenerated oligonucleotides, which were designed according to the conserved regions of the fructokinase genes from various LAB and *B. subtilis*. Sequence analysis of the 0.7 kb PCR fragment revealed a high similarity with the other bacterial fructokinases. The unknown upstream (2700 bp) and downstream (1400 bp) regions of the *fruK* gene were isolated using the Vectorette II system (Sigma) and specific inverse primers. Amplified PCR fragments were sequenced and contiguous sequences were joined together and analyzed. Two additional open reading frames (ORF:s), *unkR* and *fruI* upstream of *fruK*, were found. Sequence analysis of *fruI* revealed that it shared a high similarity with other bacterial phosphoglucose isomerases.

The sequence analysis suggests that *Lb. fermentum fruI* and *fruK* form a single transcript, which is expressed from a promoter located upstream of *fruI* and terminated with a stem-loop structure downstream of *fruK*. The coding sequence of the *L. fermentum fruI* gene was found to be 1352 bp in size and preceded by the putative -35 and -10 promoter region starting 107 bp upstream of the translation initiation site. Putative ribosome binding sites were located 9 bp upstream of the translation initiation codon of *fruI* and 16 bp upstream of *fruK* translation initiation codon, respectively. A putative stem-loop terminator with $\Delta G = -95$ kJ was located 204-244 bp downstream of the translation stop codon of the 871 bp-sized *fruK*. In addition, two catabolite responsive elements (*cre*) upstream of *fruIK* were found: a complete, inverted *cre*-like consensus sequence (WTGNAANCGNWNNCW) (Miwa et al., 2000) and a partial consensus sequence in the 5´-3´ direction. This suggests that the *fruIK* operon is under the transcriptional control of a CcpA protein. Organization of the genes, their putative ribosome binding sites and transcription terminators are presented in Figure 12.

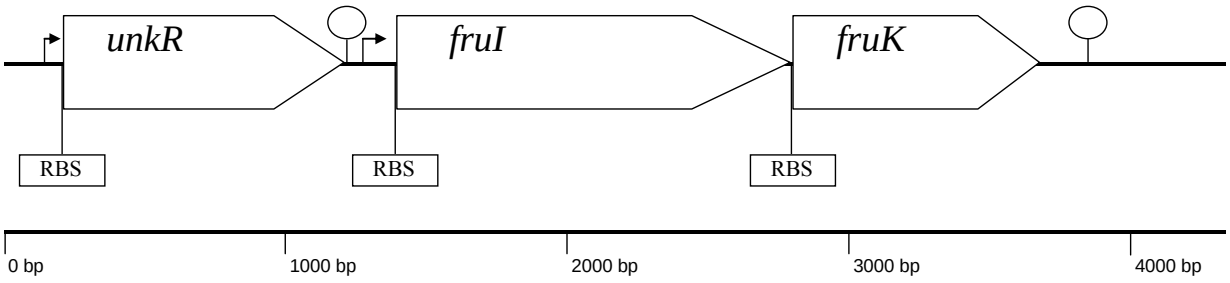

Figure 12. Organization of the *L. fermentum* NRRL-B-1932 *fruIK* operon, the *unkR* gene and their flanking regions. Putative promoter regions are marked with arrows, putative transcription terminators with hairpin loops and putative riboseme binding sites by "RBS".

6.2.2. Sequence analysis and EMSA of repressor unkR

The coding sequence of the *Lb. fermentum unkR* gene was found to be 989 bp in size. It was preceded by a putative -35 and -10 promoter region starting 68 bp upstream of translation initiation codon TTG. A putative ribosome binding site was located 6 bp upstream of the translation intiation codon (Figure 12). A putative stem loop terminator with ΔG = -102.5 kJ was located 9-35 bp downstream of the translation stop codon.

Sequence analysis suggested that UnkR belongs to the LacI/GalR family of repressors. UnkR contains the DNA binding HTH (helix-turn-helix) -motif, which is typical for these repressors. Purification of the His-tagged UnkR was not successful. Therefore the electrophoretic mobility shift assay (EMSA) was performed using cell lysate of the transformant overproducing UnkR to determine whether the *fruIK* operon is under the transcriptional control of an UnkR repressor protein. Despite many repetitive analyses we were not able to show that UnkR repressor protein binds to the putative promoter region of the *fruIK* operon. Thus, the target of the repressor protein UnkR remains to be elucidated.

6.2.3. Activities of fructokinase and phosphoglucose isomerase in cells grown on various sugars

L. fermentum NRRL-B-1932 was able to grow on glucose, fructose, sucrose, ribose, maltose, lactose and galactose. The results show that of the ones tested, fructose was the most readily utilized substrate for growth.

Assays of fructokinase and phosphoglucose isomerase activity were performed from cells grown on these different sugar substrates. The results show that the expression of the *fruIK* operon is stimulated when fructose or sucrose is the sole source of sugar (Table 1). With ribose and maltose no significant fructokinase or phosphoglucose isomerase activity could be detected.

Table 1. Volumetric sugar consumption rates and activities of fructokinase and phosphoglucose isomerase in *Lb. fermentum* cells grown anaerobically with various sugars as the substrates.

Sugar	Volumetric consumption rate (g/(l·h)[a]	Activity of fructokinase (U/mg protein)[b]	Activity of phosphoglucose isomerase (U/mg protein)[b]
fructose	2.01	0.25 ± 0.07	2.22 ± 0.56
maltose	1.49	0.03 ± 0.00	-
sucrose	1.37	0.14 ± 0.09	1.17 ± 0.06
lactose	0.98	0.08 ± 0.01	-
galactose	0.97	0.11 ± 0.08	1.18 ± 0.32
glucose	0.95	0.08 ± 0.02	0.91 ± 0.03
ribose	0.84	0.01 ± 0.01	-

[a]Between t = 0 and 15 h
[b]Cells used in activity assays were grown 5 h

6.2.4. mRNA analysis of the putative fruIK operon

To investigate the expression of the putative *fruIK* operon, northern analyses were performed on *Lb. fermentum* cells (Figure 13). No transcript hybridizing with a *fruK* specific probe and only a very weak signal with a *fruI* specific probe could be detected when the cells were incubated in the presence glucose. However, in the presence of

fructose or glucose + fructose a clear signal could be distinguished with both probes. According to the analyses with the Quantity One program (Bio-Rad) the intensity of the signal was approximately two times stronger with both probes when incubated in the presence of only fructose as compared to the signal obtained with a glucose-fructose mixture. The size of the hybridized *fruI* and *fruK* transcript was approximately 2.5 kbp which corresponds well with the size of the putative *fruIK* operon transcribed from a promoter upstream of *fruI* and terminated at the stem-loop structure downstream of *fruK*. These results are compatible with the conclusion drawn from sequence analysis that *fruI* and *fruK* form an operon and that the *fruIK* operon is under the control of catabolite repression.

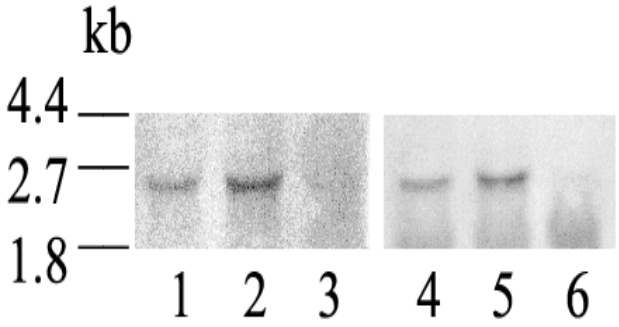

Figure 13. Northern hybridizations of the *Lb. fermentum* NRRL-B-1932. Total RNA of *L. fermentum* was hybridized with 0.5 kbp *fruK* (lanes 1-3) and 0.65 kbp *fruI* (lanes 4-6) specific probes. Cells were induced for 30 minutes with 15 g/l fructose + 15 g/l glucose (lanes 1 and 4), 30 g/l fructose (lanes 2 and 5) and 30 g/l glucose (lanes 3 and 6). Positions of the fragments of the RNA-ladder are shown on the left hand side.

Catabolite repression is a system in which a rapidly metabolizable sugar, such as glucose, is preferentially used instead of other available carbon sources. In low-G+C gram-positive bacteria catabolite repression has been reported to be mediated by a CcpA protein, which can bind to a so-called catabolite responsive element, *cre*, upstream of genes involved in the utilization of carbon sources other than the preferred

one. The binding of CcpA to *cre* is believed to block transcription of the genes being down-regulated in catabolite repression (Hueck et al., 1995; Monedero et al., 1997). Although fructose appeared to be more efficiently consumed by *Lb. fermentum* NRRL-B-1932 than glucose, the two *cre*-consensus sequences found in the promoter region of *fruIK* suggest that fructose metabolism is under the control of catabolite repression in this strain. In addition, enzyme activity data and results from the northern blot hybridization experiments presented are in accordance with this assumption.

6.2.5. PTS activity of Lb. fermentum NRRL-B-1932

In order to investigate PTS activity, *Lb. fermentum* cells were grown in a medium containing fructose and treated with toluene. No PTS activity for the transport of glucose, fructose, sucrose or mannose could be detected in *Lb. fermentum* cells. *L. lactis* cells grown with sucrose as the substrate were used as the positive control and showed a PTS activity of 0.2 U/mg.

The transport of hexoses in LAB can be coupled to simultaneous phosphorylation by a phosphoenolpyruvate-sugar phosphotransferase system (PTS), or the hexoses can be transported into the cell in free form by a permease. Fructose transport by PTSs appears to be more common among bacteria than fructose uptake by non-PTS-systems (Barrière et al., 2005). However, fructose transport by permeases is known to be functional in some cases (Chiou et al., 2002). It has been reported that the heterofermentative *Lactobacillus* species, *Lb. brevis*, has both a functional PTS and the ability to transport fructose by a sugar:cation symport (Saier et al., 1996). However, the results presented in the current work show no evidence for the presence of a PTS-system in *Lb. fermentum* NRRL-B-1932. This and the evidence for the presence of a *fruIK* operon involved in channeling unphosphorylated fructose to the PK pathway are compatible with the assumption that *Lb. fermentum* NRRL-B-1932 uses a permease for fructose transport.

In addition to being phosphorylated to fructose-6-phosphate and metabolized further by the pentose PK pathway, free intracellular fructose can be reduced to mannitol with the concomitant regeneration of NAD^+ in heterofermentative LAB. When both glucose and fructose are present in the growth medium, catabolite repression most likely leads to

more efficient channeling of fructose to mannitol production than in the absence of glucose. *L. fermentum* strains have been reported to be able to reduce fructose to mannitol in high yield. When fructose is co-fermented with glucose, over 90% (mol/mol) of the fructose present can be converted to mannitol (von Weymarn et al., 2002).

Efficient biotechnological mannitol production by LAB appears to be a trade-off between efficiency of conversion and rate of product formation. At high pH a high volumetric productivity can be achieved but the yield will be low. At low pH, the situation is the reverse (Soetaert, 1990). If only one mechanism for fructose uptake is indeed present in *Lb. fermentum* NRRL-B-1932 strain, the metabolic engineering of this strain for mannitol production would be simplified, since the loss of fructose to central sugar metabolism could be prevented by disrupting the fructokinase gene. With the resulting fructose-negative mutant it could be possible to adjust the process conditions so that both high yield and high volumetric productivity would be achieved.

Disruption of the fructokinase gene of *Lb. fermentum* NRRL-B-1092 by pGHOST system failed. Integration of the deletion plasmid was confirmed but the second recombination step was unsuccessful. The reason for the failure was not found.

6.3. Metabolic engineering of *Lactobacillus plantarum* for production of L-ribulose (Study III)

In this work we studied the possibility of developing a new and efficient process for L-ribulose production from L-arabinose. The pathway of L-arabinose utilization in *Lb. plantarum* NCIMB 8826 was blocked by inactivating the ribulokinase gene. The effects of this modification on L-ribulose production using resting cells were investigated.

6.3.1. Inactivation of Lb. 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 of pRKDEL was obtained by 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. The second homologous recombination was achieved by growing the cells for 100 generations at 30°C without selection. The clones were analyzed by PCR with *araB* specific oligonucleotides and the clones having the deletion of the right size were isolated. No amplification products could be detected by PCR amplification with oligonucleotides specific to the integration vector. 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 confirmed that the deletion had occurred. Furthermore the *araB* specific RT-qPCR product was detected from arabinose induced wild type *Lb. plantarum* strain but not from the mutant *Lb. plantarum* BPT197.

6.3.2. Lb. plantarum BPT197 growth

The growth of the L-ribulokinase deficient mutant and the native *Lb. plantarum* strain were modeled in order to compare their growth behavior and to find suitable values for control parameters. The coefficient of determination (R^2) of the model fit to the batch data of *Lb. plantarum* BPT197 was 0.996. The kinetic constants of the growth model were maximum specific growth rate (μ_{max}) 0.520 1/h, the Monod equation kinetic parameter for substrate consumption (K_S) 2.58 g/l, biomass yield from substrate (Y_{XS}) 0.232 g/g and maintenance coefficient (m_S) 0.411 1/h for the mutant strain. The corresponding values for the native strain under similar culture conditions were μ_{max} 0.823 1/h, K_S 7.33 g/l, Y_{XS} 0.241 g/g, and m_S 0.324 1/h. 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 indicated that the L-arabinose isomerase gene was not expressed when the glucose concentration was above 5 g/l in batch cultivation. Apparently L-arabinose isomerase synthesis in the cells is strongly repressed by glucose.

In comparison of *Lb. plantarum* BPT197 cultivations with different monosaccharides and disaccharides as the carbons sources the results (Table 2) showed 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.

Table 2. Comparison of growth and L-arabinose isomerase activity between *Lb. plantarum* BPT197 cultivations with different monosaccharides and disaccharides.

Sugar	Activity of L-arabinose isomerase (U/g protein)	ΔOD_{600}[a]
Glucose	160±20	10.6±0.2
Sucrose	396±11	9.9±0.1
Galactose	1060±14	8.4±0.1
Lactose	2266±6	2.5±0.2
Mannose	2359±4	3.8±0.5
Maltose	408±5	12.3±0.3

U = µmol/min L-ribulose formed
[a] Change in OD_{600} between t = 0 h and 10 h

L-Ribulose production from L-arabinose was tested with growing cells of *Lb. 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 *Lb. plantarum* BPT197 was 0.9 g/l of L-ribulose after 10 h incubation in MRS medium supplemented with 10 g/l sucrose and 25 g/l L-

arabinose. These results prompted us to look for alternatives for batch production of L-ribulose by growing cells.

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/l would limit growth. On the other hand, no L-arabinose isomerase activity was detected, when the glucose concentration was over 5 g/ l. In order to maximize enzyme production the operating point of 2.5 g/l 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. The biomass growth in all three cultivations seemed to be virtually unaffected by the technical difficulties in the glucose control. The relative L-arabinose isomerase mRNA levels before and after the beginning of the glucose feed are shown in Figure 14 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. The cells were cultivated for 12 h since longer cultivation times resulted in decreased enzyme activity.

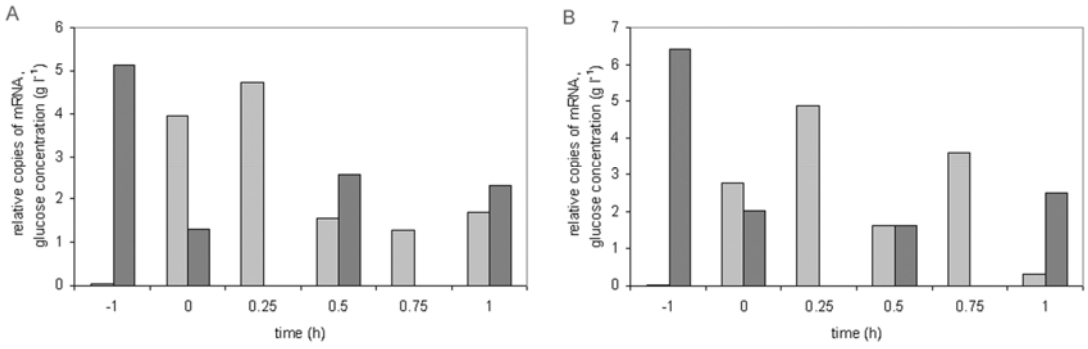

Figure 14. Relative mRNA levels of *araA* during two different fed-batch cultivations of *Lb. plantarum* BPT197. The time is relative to the start of glucose feeding to the reactor. Glucose concentrations are presented as dark grey bars and relative mRNA levels as light grey bars. Glucose concentrations are presented at 30 minute intervals.

6.3.3. Effect of borate on L-ribulose production

Borate is known to form complexes with carbohydrates having two adjacent diol groups. Since the stabilities of different complexes vary, this phenomenon has been utilized in changing the balances of isomerization reactions between sugars (Takasaki, 1971).

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

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 (Heath et al., 1958), which would explain the poor conversion. L-Ribulose is known to form a more stable complex with borate than with L-arabinose (De Muynck et al., 2006). By complexing the formed L-ribulose with borate, more efficient conversion of L-arabinose to L-ribulose was achieved.

6.3.4. Optimization of L-ribulose production by resting cells and recycling of cells

The L-ribulose production by resting cells of *Lb. plantarum* BPT197 was optimized with L-arabinose and borate concentrations and temperature as the variables. The results of CCDs were evaluated with statistical coefficients: coefficient of determination (R^2), coefficient of model prediction (Q^2) and probability to 0-hypothesis (P value). Acceptable values for bioprocesses are R^2 over 0.8, difference of R^2 and Q^2 under 0.2, and P value under 0.01 (Eriksson et al., 2000). The Box-Cox analysis plot (Box and Cox, 1964) 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. Anova

analysis yielded the statistical coefficients R^2 0.993, Q^2 0.962, $P < 0.001$ and P for the lack of fit 0.062 for r_i, and R^2 0.993, Q^2 0.960, $P < 0.001$ and P for the lack of fit 0.490 for x. Figure 15 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/l. 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.

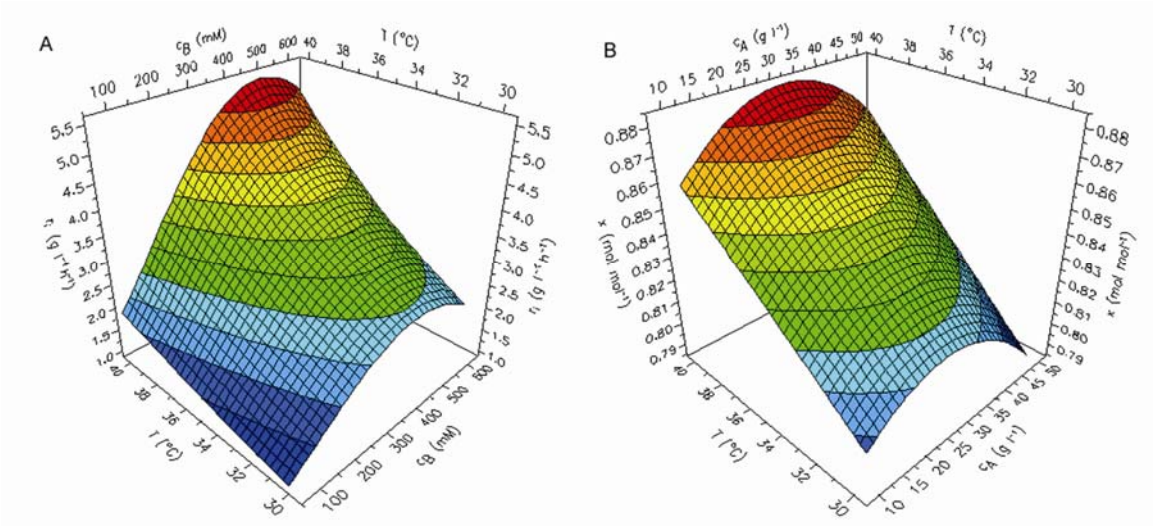

Figure 15. Response surface plots of the optimization of L-ribulose production using resting cells of *Lb. plantarum* BPT197. A) Initial ribulose production rate (r_i) at 50 g/l arabinose concentration (standard deviation 0.06 g/(l·h). B) Conversion (x) at 325 mM borate concentration (standard deviation 0.03 mol/mol).

A further two variable CCC experiment design with a star distance of 1.5 was constructed in order to optimize temperature (from 38 to 46°C) and L-arabinose concentration (from 100 to 300 g/l). In addition to the responses used in the previous experiment design, the best achievable process productivity (r_{max}) was used as the response. 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. Anova analysis of this experiment design yielded the

statistical coefficients R^2 0.997, Q^2 0.981, $P < 0.001$ and P for the lack of fit 0.075 for r_i, R^2 1.000, Q^2 0.998, $P < 0.001$ and P for the lack of fit 0.082 for x, and R^2 0.993, Q^2 0.952, $P < 0.001$ and P for the lack of fit 0.008 for r_{max}. The response surfaces are shown in Figure 16. The maximum L-ribulose concentrations in the experiment were around 80 g/l. Higher temperatures yielded better productivities, both the initial rate and maximum rate gave similar results. The r_i was highest at c_A values from 250 to 320 g/l. 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 range being around 70 g/l. Although the temperature effects on x were small, the results suggest a temperature optimum between 42 and 46°C.

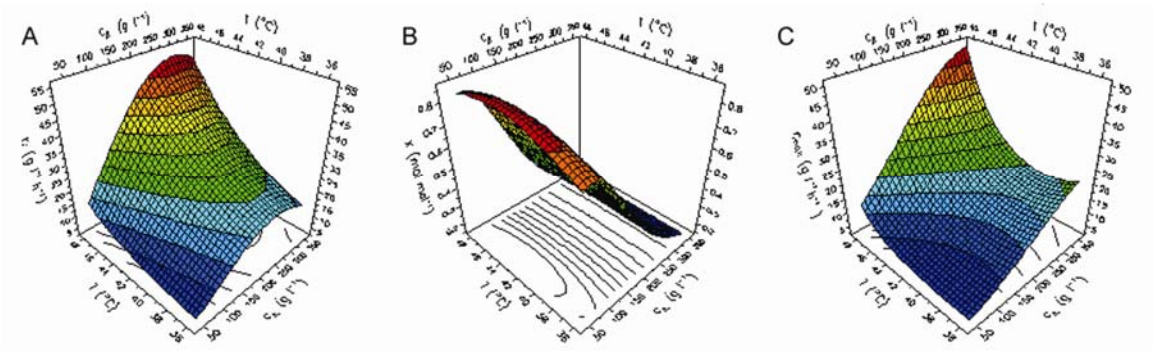

Figure 16. Response surface plots of the temperature and L-arabinose concentration optimization of L-ribulose production using resting cells of *Lb. plantarum* BPT197. A) Initial ribulose production rate (r_i) [standard deviation 0.35 g/(l·h)], B) conversion (x) of arabinose to ribulose (standard deviation 0.003 mol/mol) and C) maximum ribulose production rate (r_{max}) [standard deviation 0.13 g/(l·h)] are presented as a function of temperature (T) and arabinose concentration (c_A).

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 the P for the lack of fit was too significant. The combination of these two experiment designs suggested that the process has an optimal operation point around c_A 100 g/l, c_B 500 mM and T 48°C, where the Modde software predicted r_i 29.1 g/(l·h), r_{max} 14.8 g/(l·h) and x 0.70 mol/mol.

Rare sugars and sugar alcohols have been successfully produced using resting cells (De Myunck et al., 2006; Doten and Mortlock, 1985; Nyyssölä et al., 2005). 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 (von Weymarn et al., 2002b). The reusability of *Lb. plantarum* BPT197 cells was tested in 500 mM borate buffer and there was no loss of productivity in two sequential batches.

6.3.5. Purification of L-ribulose

The purification of L-ribulose was investigated using the procedure described in Study III. The borate complexes of L-ribulose and L-arabinose were efficiently separated by the chromatographic method used. In the sample applied to the column L-ribulose comprised 61% (w/w) 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% (w/w). There was a small unidentified peak eluting before L-ribulose in the HPLC-chromatogram of the L-ribulose fraction. 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. 85% of the L-ribulose moiety of the complex was recovered using this procedure.

It has been suggested that an advantage 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 media components are omitted during the production phase. The results of the present study suggest that L-ribulose would be fairly easily purified from the medium of the resting cells. However, since complexing L-ribulose with borate appears to be necessary in order to achieve efficient L-ribulose production, the method for recycling borate would be an economically important part of this process.

The use of borate ions complicates the process considerably and L-ribulose is only considered as an intermediate product in the process of producing L-ribose. These concerns in this process led to the idea of changing the equilibrium of the two isomerization reaction sequence from L-arabinose to L-ribose so that the reaction becomes favorable for L-ribose production. This could be done by adding the second isomerization reaction of L-ribulose to L-ribose in L-ribulokinase deficient strain. In this way the whole process from L-arabinose to L-ribose could be simplified by getting rid of the use of the borate ions and producing L-ribose from L-arabinose in one bioconversion step.

6.4. Biotecnological production of L-ribose from L-arabinose by resting cells of *Lactobacillus plantarum* and *Escherichia coli* (Study IV)

In this work we studied the possibility of producing L-ribose from L-arabinose in one bioconversion step using whole cells and protein precipitates. The conversion of L-arabinose to L-ribose was achieved by the introduction of L-ribulose isomerizing activity into the L-ribulokinase deficient strains of *E. coli* and *Lb. plantarum.*

6.4.1. Expression of the L-ribose isomerase gene

A synthetic L-ribose isomerase gene (NCBI sequence AB062121) was used as a template for the PCR amplification of the L-ribose isomerase gene with the insertion of an *Nco*I site at the 5´ end and an *Xho*I site at the 3´ end. It was cloned into pTrcHis 2B expression vector (Invitrogen) and pSIP401 expression vector (Sorvig et al., 2005), which then were transformed into the ribulokinase deficient strains *E. coli* UP1110 and *Lb. plantarum* BPT197, respectively. The growth medium of *E. coli* UP1110 was supplemented with L-arabinose to induce the expression of the endogenous L-arabinose isomerase gene of the strain. L-Ribose isomerase gene expression was induced by the addition of IPTG. The specific L-ribose isomerase

activity determined from the cell extracts of *E. coli* UP1110 was 103±1 U/g protein 3 h after the onset of the IPTG induction. The specific L-arabinose isomerase activity determined from the cell extracts was 610±6 U/g protein at this point. Only negligible L-arabinose isomerase and L-ribose isomerase activity could be detected in non-induced *E. coli* cells.

The heterogenous expression of the L-ribose isomerase gene in *Lb. plantarum* BPT232 was studied using the peptide SPPIP as an inducer at concentrations ranging from 0 to 200 µg/l. A specific L-ribose isomerase activity of 183±28 U/g protein was determined from the cell extracts 1 h after the addition of 25 µg/l SPPIP, whereas no activity could be detected in the non-induced cells. The specific L-ribose isomerase activity reached a plateau above the SPPIP concentration of 25 µg/l. At 100 µg/l SPPIP the specific L-ribose isomerase activity determined was 155±25 U/g protein, and this concentration was chosen for further studies. No L-arabinose isomerase activity was detected in the *Lb. plantarum* BPT232 cells during the experiment, most likely because the glucose concentration was above the repression limit (Study III).

6.4.2. Production of L-ribose using resting cells

We investigated the use of resting cells for this purpose. The method is described in more detail in study IV. The results are shown in Figure 17. The initial L-ribose production rates (r_i) determined between 0 and 3 h at 39°C and pH 8 were 0.46±0.01 g/(g·h) [1.84±0.03 g/(l·h)] and 0.27±0.01 g/(g·h) [(1.91±0.1 g/(l·h)] for *E. coli* and for *Lb. plantarum*, respectively. Conversions of L-arabinose to L-ribose (x) were 19.7±0.1% (mol/mol) and 20±1% (mol/ mol) for *E. coli* and for *Lb. plantarum*, respectively.

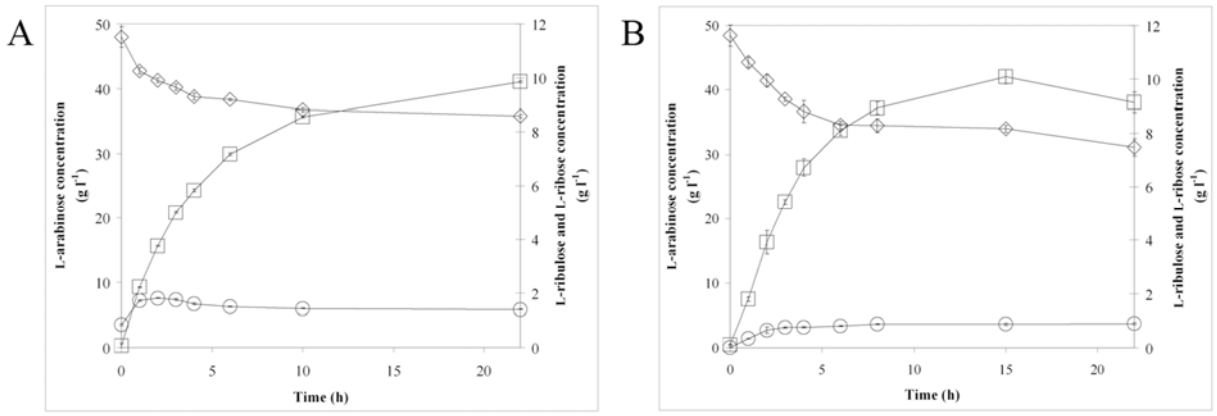

Figure 17. L-Ribose production by resting cells of *E. coli* BPT 234 (A) and *Lb. plantarum* BPT232 (B). L-Arabinose concentration ◊, L-ribose concentration □, and L-ribulose concentration ○.

At the equilibrium of the isomerization reaction the ratio of L-arabinose to L-ribulose has previously been determined to be 90:10 (Heath et al, 1958) and the ratio of L-ribulose to L-ribose 30:70 (Shimonishi and Izumori, 1996). This would suggest that in a reaction mixture containing the substrate and the two isomerases the maximum L-ribose yield would not exceed 26% (mol/mol). However, with whole cells the situation is more complicated since the reaction takes place in the cytoplasm and the produced L-ribose is excreted and/or transported into the medium. It may therefore be possible to reach even higher yields using whole cells.

6.4.3. Repetitive batch experiments with Lb. plantarum BPT232

Growing up the lactic acid bacteria cells for production can be costly because of their complex nutritional requirements. Therefore, an important consideration in using resting cells of these bacteria is the recyclability of the cells. The reusability of *Lb. plantarum* BPT232 cells for L-ribose production was studied using three parallel samples of the first bioconversion cycle. The cells were washed and used for another batch under the same conditions. An initial L-ribose production rate (r_i) of 0.22±0.01 g/(g·h) [1.54±0.02 g/(l·h)] and a conversion of L-arabinose to L-ribose (x) of 21±0.1% (mol/mol) were achieved in this second production cycle. The L-ribose

isomerase and L-arabinose isomerase activities determined from the cell lysates were 34% lower and 12% higher at the end of the second cycle than at the end of the first cycle, respectively. The results suggest that the cells can be used for several successive batches, even without addition of nutrients to the media.

6.4.4. Production of L-ribose by polyethylglycol protein precipitates

It has been previosly reported that the L-ribose isomerase of *Acinetobacter* sp. DL-28 is stable only for 10 min at 30°C (Shimonishi and Izumori, 1996). In our studies we were able to increase the stability of the enzyme by preparing a cell lysate and precipitating it with PEG. The method is described in more detail in study IV. The L-ribose isomerase and L-arabinose isomerase containing cell extract was fractionated by PEG precipitation. The protein precipitates with the highest activity were pooled and used for catalyzing the direct conversion of L-arabinose to L-ribose. The production of L-ribose and L-ribulose from L-arabinose by the protein precipitates is shown in Figure 18. The results indicate that L-arabinose isomerase and L-ribose isomerase enzymes were both active in the PEG precipitate.

The equilibrium between L-arabinose, L-ribulose and L-ribose was not completely reached during the 20 h experiments, but after 4 hours the reaction rates decreased significantly and continuation of the reaction was not reasonable. The conversions of L-arabinose to L-ribose were around 24% (mol/mol), which corresponds to the level achieved by the resting cells. The production rates between 0 and 3 h were 18.1±1.1 g/g protein/h [2.09±0.13 g/(l·h)] and 34.8±4.4 g/(g protein·h) [2.71±0.03 g/(l·h], for *E. coli* and *Lb. plantarum*, respectively.

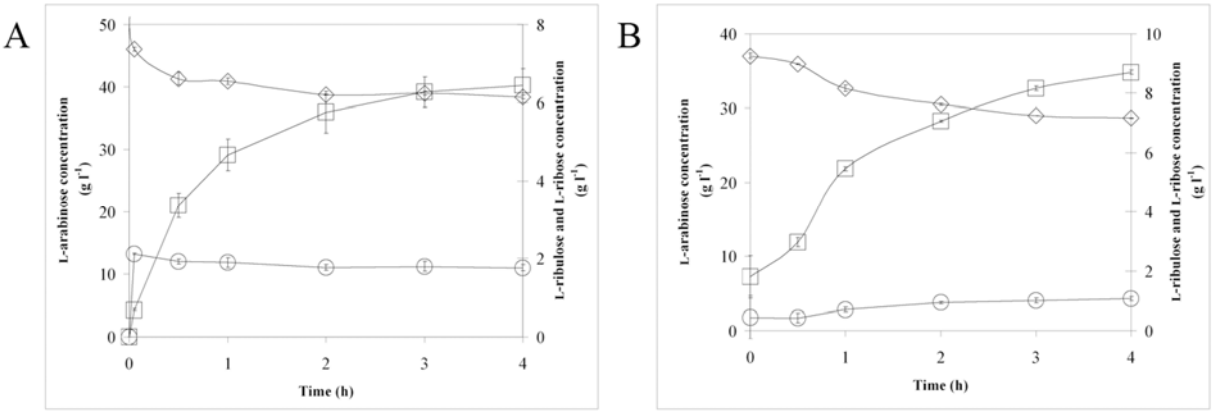

Figure 18. L-Ribose production with protein precipitates from *E. coli* BPT 234 (A) and *Lb. plantarum* BPT232 (B). L-Arabinose concentration ◊, L-ribose concentration □, and L-ribulose concentration ○.

The results of the present study suggest that the biotechnological L-ribose production from L-arabinose by resting cells or by protein precipitates containing L-arabinose isomerase and L-ribose isomerase activities holds promise of becoming an alternative for chemical L-ribose production from L-arabinose. Woodyer et al. (2008) have reported a one step conversion of ribitol to L-ribose with a production rate of 0.73 g/(l·h) and a conversion of over 70% using *E. coli* strain expressing a mannitol-1-dehydrogenase from *Apium graveolens*. A problem with using ribitol as the raw material is that it is presently very expensive and that it cannot be isolated from any natural sources.

It has been shown previously that L-ribose can be easily and efficiently separated from L-arabinose and L-ribulose by ion exclusion chromatography (Jumppanen et al., 2000). This would enable the recycling of the reagents back to the bioconversion. Unlike in the chemical process also low purity grade L-arabinose or even crude or fractionated plant material can be utilized as a raw material in the current bioprocess. However, the production conditions should be studied further and a downstream process developed in order to fully evaluate the feasibility of the current approach.

7. Conclusions

The aim of this thesis was to study whether efficient production of commercially interesting rare sugars and sugar alcohols can be achieved by metabolic engineering of LAB. The main function of sugar metabolism in LAB is to generate the energy necessary for growth and maintenance of cell functions (Axelsson, 1998). Hence, their sugar metabolism is generally not connected to their limited biosynthetic activity. The possibility of modifying catabolic pathways of sugars without disturbing the biosynthesis of cell components makes LAB attractive targets for metabolic engineering for production of rare sugars and sugar alcohols. LAB have also the GRAS status which makes them suitable production hosts for food and pharmaceutical applications.

Most of the rare sugars and sugar alcohols are currently produced by chemical reactions, even though they require high purity starting materials. There could also be problems with environmentally hazardous components, undesired side products and lack of the right stereochemistry associated to chemical processes. The biochemical production of rare sugars and sugar alcohols has become an attractive alternative to chemical processes. Advancements in genetic engineering have enabled the development of novel pathways by re-routing the existing metabolic pathways and combining enzymes from different origins. Progress in metabolic modeling has given the tools to predict and simulate metabolic pathways *in silico*.

In the first part of the study mannitol production of *L. pseudomesenteroides* was improved by random mutagenesis. Despite the 90% decrease in fructokinase activity a 100% yield (mol/mol) of mannitol from fructose was not achieved. A possible explanation for this is that the fructose phosphorylation pathway is simply more efficient than the fructose reduction reaction which makes it difficult to direct the flux to mannitol. It is possible that a quantitative yield would be achieved, if the fructokinase was completely disrupted and the mutant strain did not have PTS transporter for fructose.

In addition characteristics of fructose utilization of *Lb. fermentum* was studied. A novel *fruIK* operon involved in channeling fructose to the PK pathway was characterized. Due to the lack fructose-PTS of this strain, the metabolic engineering would be simplified, since the loss of fructose to central sugar metabolism could be prevented by disrupting either of the genes from the *fruIK* operon. Attemps to disrupt the fructokinase gene by pGHOST system failed. The reason for the failure was not found. The use of novel commercial kits for gene knockout could solve the problems concerning the gene disruption.

The commercial chemical dehydrogenation process for mannitol production has several drawbacks. The need for catalyst, difficult and expensive purification and the high price of the pure raw material are just few of them. Due to these shortcomings extensive studies have been undertaken to provide more advanced techniques for mannitol production. Biotechnological production of mannitol has become an attractive alternative to the chemical process. Some heterofermentative LAB have been reported to produce a quantitative yield of mannitol from fructose, but these are not optimal strains for industrial production of mannitol due to their slow growth or fructose consumption rate (von Weymarn *et al.*, 2002b). Metabolic engineering of a strain, that is an efficient mannitol producer and lacks the fructose-PTS, would be the easiest way to have an optimal strain for commercial mannitol production.

In the second part of this work L-arabinose metabolism of *Lb. plantarum* was engineered in order to produce the rare sugars L-ribulose and L-ribose. An L-ribulokinase deficient mutant was constructed. Fed-batch cultivation strategy was used to avoid catabolite repression and maximize L-arabinose isomerase production during growth. Resting cells of the ribulokinase deficient mutant were used for the production of L-ribulose. The isomerisation of L-arabinose to L-ribulose was very unfavourable for L-ribulose formation. However, high L-ribulose yields were obtained by complexing the produced L-ribulose with borate. Unfortunately the use of borate ions complicates the process considerably, since free L-ribulose can only be recovered after breaking the L-ribulose-borate complex. Since L-ribulose is an intermediate product towards L-ribose, the work was continued by introducing L-ribose isomerase activity into L-ribulokinase deficient strains of *E. coli* and *Lb.*

plantarum. By adding the second reaction, isomerization of L-ribulose to L-ribose, the two-reaction sequence with L-arabinose as the starting material became favorable for L-ribose production. The process for L-ribose production by resting cells and protein precipitates was investigated.

In this part of the thesis, a novel and efficient way of producing L-ribose from the readily available raw material L-arabinose in one bioconversion step was described. The results of the present study suggest that biotechnological L-ribose production from L-arabinose by resting cells or by protein precipitates containing L-arabinose isomerase and L-ribose isomerase activities holds promise of becoming an alternative for chemical L-ribose production from L-arabinose. Unlike in the chemical process also low purity grade L-arabinose or even crude or fractionated plant material can be utilized as a raw material in the current bioprocess. However, the production conditions should be studied further and a downstream process developed in order to fully evaluate the feasibility of the current approach.

The future of the metabolic engineering of LAB holds a great promise. As GRAS organisms they are attractive production hosts for different compounds for food and pharmaceutical applications. Recent developments in LAB metabolic engineering include the re-routing of complex, biosynthetic pathways leading to the production of metabolites with a health benefit for the consumer. Genomics, proteomics and metabolomics have become important tools to understand the LAB metabolic network. Global understanding of the pathways that can be manipulated and the genes involved will be the key for the design of the future metabolic engineering strategies.

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ISBN 978-951-22-9838-9
ISBN 978-951-22-9839-6 (PDF)
ISSN 1795-2239
ISSN 1795-4584 (PDF)