

# Microbial production of xylitol, L-xylulose and L-xylose

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Anne Usvalampi



# Microbial production of xylitol, L-xylulose and L-xylose

**Anne Usvalampi**

Doctoral dissertation for the degree of Doctor of Science in  
Technology to be presented with due permission of the School of  
Chemical Technology for public examination and debate in  
Auditorium KE2 (Komppa Auditorium) at the Aalto University School  
of Chemical Technology (Espoo, Finland) on the 25th of October,  
2013, at 12 noon

**Aalto University**  
**School of Chemical Technology**  
**Department of Biotechnology and Chemical Technology**  
**Bioprocess Engineering**

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Aalto University publication series

**DOCTORAL DISSERTATIONS** 137/2013

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ISBN 978-952-60-5316-5 (printed)

ISBN 978-952-60-5317-2 (pdf)

ISSN-L 1799-4934

ISSN 1799-4934 (printed)

ISSN 1799-4942 (pdf)

<http://urn.fi/URN:ISBN:978-952-60-5317-2>

<http://lib.tkk.fi/Diss/>

Unigrafia Oy

Helsinki 2013

Finland



**Author**

Anne Usvalampi

**Name of the doctoral dissertation**

Microbial production of xylitol, L-xylulose and L-xylose

**Publisher** School of Chemical Technology

**Unit** Department of Biotechnology and Chemical Technology

**Series** Aalto University publication series DOCTORAL DISSERTATIONS 137/2013

**Field of research** Bioprocess Engineering

**Manuscript submitted** 4 June 2013

**Date of the defence** 25 October 2013

**Permission to publish granted (date)** 20 August 2013

**Language** English

**Monograph**

**Article dissertation (summary + original articles)**

**Abstract**

Rare sugars are defined as monosaccharides and their derivatives that are rare in nature. These rare sugars have many potential uses in medicine and food industry. Their derivatives can be used as antiviral and anticancer drugs. Most rare sugars taste sweet, but they are not metabolized by humans to the same level of more common sugars. Thus they can be used as low calorie sweeteners. Some rare sugars have the ability to suppress blood glucose and insulin levels and could thus be used as diabetes drugs. Also other medicinal and nutraceutical properties have been found.

Rare sugars are generally expensive, as they cannot be isolated from natural sources in significant amounts. They are often produced by chemical means, but due to several steps and poor yields, the prices have stayed high. Biotechnological production is often superior to chemical methods once suitable enzymes acting on rare sugars are found. In this thesis the microbial production of two rare sugars – L-xylulose and L-xylose – and one sugar alcohol, xylitol, was studied.

As a meso-sugar, xylitol is an intermediate between D- and L-sugars. It has several interesting characteristics, such as the ability to prevent dental caries and ear infections. In this thesis xylitol was produced from D-xylose by recombinant *Lactococcus lactis* harboring the xylose reductase gene from *Pichia stipitis*. The strain was able to produce xylitol from xylose in a quantitative yield, but it was not able to convert all the xylose initially present to xylitol. The productivity of the strain was competitive with previous reports using natural or recombinant strains. Next, the xylitol-4-dehydrogenase from *Pantoea ananatis* was produced recombinantly in *Escherichia coli* and characterized. The resting recombinant cells were used to produce L-xylulose from xylitol in quantitative yields. The volumetric productivity of this strain was one of the best reported to date. Last, the L-fucose isomerase gene from *E. coli* was overexpressed in a recombinant *E. coli* strain. The gene product was characterized and it was noticed to convert L-xylulose to L-xylose with the reaction balance strongly on the side of xylose. Contrary to earlier reports on the conversion between L-xylulose and L-xylose, practically no L-lyxose was formed with this enzyme. Resting cells of the recombinant strain were also able to convert L-xylulose to L-xylose.

In conclusion, starting from a readily available sugar, D-xylose, xylitol and two rare sugars, L-xylulose and L-xylose were produced using resting cells of recombinant bacteria. Additionally, the enzymes performing the reaction between xylitol and L-xylulose, as well as between L-xylulose and L-xylose were characterized.

**Keywords** xylitol, L-xylulose, L-xylose, rare sugars, metabolic engineering

**ISBN (printed)** 978-952-60-5316-5

**ISBN (pdf)** 978-952-60-5317-2

**ISSN-L** 1799-4934

**ISSN (printed)** 1799-4934

**ISSN (pdf)** 1799-4942

**Location of publisher** Espoo

**Location of printing** Helsinki

**Year** 2013

**Pages** 142

**urn** <http://urn.fi/URN:ISBN:978-952-60-5317-2>



**Tekijä**

Anne Usvalampi

**Väitöskirjan nimi**

Ksylitolin, L-ksyluloosin ja L-ksyloosin mikrobiologinen tuotto

**Julkaisija** Kemian tekniikan korkeakoulu**Yksikkö** Biotekniikan ja kemian tekniikan laitos**Sarja** Aalto University publication series DOCTORAL DISSERTATIONS 137/2013**Tutkimusala** Bioprosessiteknikka**Käsikirjoituksen pvm** 04.06.2013**Väitöspäivä** 25.10.2013**Julkaisuluvan myöntämispäivä** 20.08.2013**Kieli** Englanti **Monografia** **Yhdistelmäväitöskirja (yhteenveto-osa + erillisartikkelit)****Tiivistelmä**

Luonnossa harvinaiset monosakkaridit ja niiden johdannaiset on määritelty harvinaisiksi sokereiksi. Harvinaisilla sokereilla on useita käyttömahdollisuuksia lääke- ja elintarviketeollisuudessa. L-Muotoisten sokereiden johdannaisia voidaan käyttää esimerkiksi virus- ja syöpälääkkeinä. Useimmat harvinaiset sokerit maistuvat makeilta, mutta sopivien entsyymien puutteessa ruoansulatus ei kykene käyttämään niitä kuten tavallisia sokereita. Tästä syystä harvinaisia sokereita voidaan käyttää vähäkalorisina makeutusaineina. Tietyillä harvinaisilla sokereilla on myös kyky alentaa veren sokeri- ja insuliinitasoa, mistä syystä niitä voitaisiin käyttää diabetislääkkeinä.

Koska harvinaisia sokereita ei voida eristää luonnosta merkittäviä määriä, ne ovat yleensä kalliita. Harvinaisia sokereita voidaan valmistaa kemiallisesti, mutta synteesit koostuvat yleensä useista vaiheista ja saannot ovat usein huonoja. Biotekniset tuotantomenetelmät ovat monesti osoittautuneet kemiallisia paremmiksi, kunhan löydetään sopivat entsyymit, joilla on aktiivisuutta haluttuja sokereita kohtaan. Tässä väitöskirjassa tutkittiin kahden harvinaisen sokerin – L-ksyluloosin ja L-ksyloosin – sekä sokerialkoholi ksylitolin mikrobiologista tuottoa.

Ksylitoli on meso-sokeri, ja luonnollinen välimuoto muutettaessa D-sokereita L-sokereiksi. Lisäksi ksylitolilla on useita kiinnostavia ominaisuuksia, kuten kyky ehkäistä hammaskariesta ja korvatulehduksia. Tässä työssä ksylitolia tuotettiin D-ksyloosista *Lactococcus lactis* -bakteerilla, johon oli lisätty *Pichia stipitis* -hiivan ksyloosireduktaasi-geeni. Kyseinen kanta tuotti ksylitolia D-ksyloosista kvantitatiivisella saannolla, mutta se ei kyennyt käyttämään kaikkea lähtöainetta. Tuottonopeus oli kilpailukykyinen aiemmin raportoitujen tulosten kanssa. Seuraavaksi *Pantoea ananatis* -bakteerin ksylitoli-4-dehydrogenaasi-geeni kloonattiin *Escherichia coli* -bakteeriin ja sen tuottama entsyymi karakterisoitiin. Lepäävillä rekombinantti-soluilla saatiin tuotettua ksylitolista L-ksyluloosia kvantitatiivisella saannolla. Kannan tuottonopeus oli yksi parhaista tähän päivään mennessä raportoidusta. Lopulta *E. coli* -bakteerin L-fukoosi-isomeraasi-geeni yliekspressoitiin rekombinanttisessa *E. coli* -kannassa. Tuotettu entsyymi karakterisoitiin, ja sen todettiin isomeroivan L-ksyluloosia L-ksyloosiksi. Toisin kuin aiemmin raportoiduissa kokeissa, sivutuotteena ei syntynyt L-lyksoosia merkittäviä määriä. Lisäksi lepäävät solut tuottivat L-ksyloosia L-ksyluloosin toimiessa lähtöaineena.

Yhteenvetona, tässä työssä valmistettiin ksylitolia, L-ksyluloosia ja L-ksyloosia lepäävillä rekombinanttisilla bakteerisolulla. Lähtöaineena käytettiin halpaa D-ksyloosia. Lisäksi työssä karakterisoitiin entsyymit, jotka katalysoivat reaktioita ksylitolista L-ksyluloosiksi ja L-ksyluloosista L-ksyloosiksi.

**Avainsanat** Ksylitoli, L-ksyluloosi, L-ksyloosi, harvinaiset sokerit, metabolian muokkaus**ISBN (painettu)** 978-952-60-5316-5**ISBN (pdf)** 978-952-60-5317-2**ISSN-L** 1799-4934**ISSN (painettu)** 1799-4934**ISSN (pdf)** 1799-4942**Julkaisupaikka** Espoo**Painopaikka** Helsinki**Vuosi** 2013**Sivumäärä** 142**urn** http://urn.fi/URN:ISBN:978-952-60-5317-2



# Preface

The work for this thesis was conducted at the Department of Biotechnology and Chemical Technology, Aalto University School of Chemical Technology, in the research group of Bioprocess Engineering during the years 2004-2013. The work was partially funded by the Finnish Graduate School on Applied Bioscience and the Finnish Academy, which is greatly appreciated.

First and foremost I would like to express my gratitude to Professor Emeritus Matti Leisola for believing in me and allowing me to work in his laboratory. I also want to thank Professor Heikki Ojamo for giving me the opportunity to complete this thesis. Special thanks belong to my instructor Dr. Antti Nyysölä for ideas, help and guidance throughout this work, as well as for his patience in reviewing this thesis.

I would like to thank my preliminary examiners Dr. Peter Richard and Professor Per Saris for providing valuable proposals for improvement.

I would also like to express my appreciation to Dr. Ossi Turunen for sharing his vast knowledge on enzymes. Dr. Ossi Pastinen is acknowledged for helping me understand the mental life of sugars, as well as analyzing some of my difficult samples. Dr. Kristiina Kiviharju earns my thanks especially for helping me with the bioreactors and modeling.

I would also like to thank my other co-authors Dr. Johannes Aarnikunnas, Professor Airi Palva, Dr. Niklas von Weymarn and Dr. Jarkko Valjakka for their collaboration. I want to thank Noora Salonen for her friendship since the beginning of our studies. Erika Winquist is thanked for her help in teaching and encouraging me during my thesis work. Miia Helanto is especially thanked for her companionship on conference trips as well as help in the lab. I also want to thank Anna Kankaanpää for a short but precious colleagueship.

I thank Dr. Tom Granström for being a good boss and taking care of me when I was working alone. I express my sincere gratitude to Johanna Aura for always being willing to help in the lab, and Auli Murrola and Marjaana Rytelä for HPLC analyses, without which this work never could have been realized. I also want to give special thanks to Seppo Jääskeläinen for helping with all sort of equipment in the lab and to Chris Code for checking the English language of my thesis.

I want to thank the personnel of the Laboratory of Bioprocess Engineering for an enjoyable and encouraging working environment. Sasikala Anbarasan, Dr. Sandip Bankar, Dr. Tero Eerikäinen, Professor Alexander Frey, Dr. German Jurgens, Johanna



Karimäki, Essi Koskela, He Li, Galina Mai-Gisoni, Erkka Meskanen, Ulla Moilanen, Filip Mollerup, Mari Piirainen, Kirsti Pitkänen, Jorg de Ruijter, Markus Räsänen, Heidi Salo, Kalle Salonen, Pauliina Salonen, Dr. Salem Shamekh, Dr. Shrikant Survase, Tommi Timoharju, Esa Uosukainen, Tuomas Uuksulainen, Veera Virtanen, Dr. Sanni Voutilainen, Dr. Antti Vuolanto, Janne Wallenius and Dr. Svetlana Zakharova are all acknowledged for their help and good conversations along the way.

Finally, I want to express my gratitude to my parents for guiding me into university and especially to my father for sparring me in my studies. My sisters Jonna and Milla are acknowledged for their friendship especially during the years spent home taking care of children. Last but definitely not least I want to thank my husband Paul for believing in me and being my rock, and my wonderful children Nessa and Luka for showing me what is really important in life.

Espoo, August 2013  
Anne Usvalampi

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# List of Publications

- I Nyssölä, A., Pihlajaniemi, A., Palva, A., von Weymarn, N. and Leisola, M., Production of xylitol from D-xylose by recombinant *Lactococcus lactis*, *J. Biotechnol.* **118** (2005) 55-66.
- II Aarnikunnas, J.S., Pihlajaniemi, A., Palva, A., Leisola, M. and Nyssölä, A., Cloning and expression of a xylitol-4-dehydrogenase from *Pantoea ananatis*, *Appl. Environ. Microbiol.* **72** (2006) 368-377.
- III Usvlampi, A., Kiviharju, K., Leisola, M. and Nyssölä, A., Factors affecting the production of L-xylulose by resting cells of recombinant *Escherichia coli*, *J. Ind. Microbiol. Biotechnol.* **36** (2009) 1323-1330.
- IV Usvlampi, A., Turunen, O., Valjakka, J., Pastinen, O., Leisola, M. and Nyssölä, A., Production of L-xylose from L-xylulose using *Escherichia coli* L-fucose isomerase, *Enzyme Microb. Tech.* **50** (2012) 71-76.

# Author's contribution

- Publication I The author made the batch and fed batch cultivations together with Dr. Nyysölä. The author studied the effect of xylose and xylitol concentration and did the experiments with different sugars. The cloning of the strains and the transport analysis were performed by Dr. Nyysölä, as well as experiment on the effect of pH. The manuscript was written together with Dr. Nyysölä and the other authors helped in revising the manuscript.
- Publication II The author defined the research plan together with the coauthors and carried out the purification of the recombinant xylitol-4-dehydrogenase and studied the characteristics of the recombinant enzyme. The author also produced L-xylulose from xylitol and performed the polarimetric analysis of the ketose. The cloning and sequencing of the xylitol-4-dehydrogenase was done by Dr. Aarnikunnas, as well as the RNA isolation and the Northern hybridization. The purification of the native enzyme and the xylulose produced were carried out by Dr. Nyysölä. The author wrote the manuscript together with Dr. Nyysölä and Dr. Aarnikunnas. Other authors helped in revising the manuscript.
- Publication III The author defined the research plan with the help of Dr. Nyysölä. The author carried out the majority of the experiments. Dr. Kiviharju helped with the scale-up experiment and the modeling. The author wrote the manuscript together with Dr. Nyysölä and Dr. Kiviharju. Prof. Leisola helped in revising the manuscript.
- Publication IV The author defined the research plan with the help of the coauthors. The author carried out all the experiments with the exception of the modeling, which was done by Dr. Turunen and Dr. Valjakka. The author wrote the manuscript together with Dr. Nyysölä and Dr. Turunen, and the other authors helped in revising the manuscript.

# List of Abbreviations

6-PG/PK	6-phosphogluconate/phosphoketolase pathway
ADP	adenosine diphosphate
AR	adenosine receptor
araE	gene encoding for pentose transporter protein
Asp	aspartic acid
ATP	adenosine triphosphate
CCC	central composite circumscribed
CCF	central composite face-centered
CoA	coenzyme A
DE3	lysogen that encodes T7 RNA polymerase
DiP	diphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FDP	fructose-1,6-diphosphate
Fru	fructose
<i>fucI</i>	gene encoding L-fucose isomerase
FucI	L-fucose isomerase
G6PDH	Glucose-6-phosphate dehydrogenase
GC	guanine-cytosine
Glc	glucose
glpF	glycerol diffusion facilitator protein
Glu	glutamic acid
GRAS	generally recognized as safe
HBV	hepatitis B virus
HFCS	high fructose corn syrup
His	histidine
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IPTG	isopropyl $\beta$ -D-thiogalactopyranoside
$k_{cat}$	turnover number
$K_m$	Michaelis constant
LAB	lactic acid bacteria

LB	Luria Bertani cultivation broth
M17	cultivation broth for lactic acid bacteria
Man	mannose
NAD <sup>+</sup>	oxidized form of nicotinamide adenine nucleotide
NADH	reduced form of nicotinamide adenine nucleotide
NADP <sup>+</sup>	oxidized form of nicotinamide adenine nucleotide phosphate
NADPH	reduced form of nicotinamide adenine nucleotide phosphate
NRTI	nucleotide reverse transcriptase inhibitor
P value	calculated probability, estimated probability of rejecting the hypothesis
P	phosphate
PCR	polymerase chain reaction
PTS	phosphotransferase
Q <sup>2</sup>	coefficient of model prediction
R <sup>2</sup>	coefficient of determination
RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGLT	sodium-glucose transporter
T7	bacteriophage T7, a DNA virus capable of infecting bacterial cells
V <sub>max</sub>	maximal velocity
<i>xdh</i>	gene encoding the xylitol-4-dehydrogenase
XDH	xylitol-4-dehydrogenase
XI	xylose isomerase
XPDH	xylitol phosphate dehydrogenase
<i>XYL1</i>	gene encoding the xylose reductase activity
XR	xylose reductase
<i>xylA</i>	gene encoding the xylose isomerase
<i>xylT</i>	gene encoding the xylose transporter or xylose-H <sup>+</sup> symporter
XylT	xylose transporter or xylose-H <sup>+</sup> symporter
YE	yeast extract

# 1. Introduction

The International Society of Rare Sugars has defined rare sugars as monosaccharides and their derivatives that are rare in nature. In addition, disaccharides and oligosaccharides that contain rare monosaccharides are considered to be rare sugars (Anon, 2012a). Of all the possible hexoses and pentoses only seven are considered to be present in nature in significant amounts. These are glucose, galactose, mannose, fructose, xylose, ribose and L-arabinose. All other hexoses and pentoses can be classified as rare sugars (Beerens *et al.*, 2012). In addition to simple monosaccharides, there are several other types of sugar derivatives that are considered rare. These derivatives include deoxygenated, aminated and methylated monosaccharides, as well as polyols that are also called sugar alcohols. In this thesis, if no prefix is given, the D-form of the sugar is indicated.

In the literature part of this thesis different uses of rare sugars will be reviewed and enzyme classes catalyzing the reactions of sugars are covered briefly. The metabolism of xylose and xylitol in different organisms is also reviewed. The experimental part of this work focuses on three rare sugars – xylitol, L-xylulose and L-xylose – and their reactions and uses will be presented in more detail. Finally a short introduction to lactic acid bacteria is given.

## 1.1. Uses of rare sugars

Rare sugars have found use in medicine as antiviral drugs. Anticancer features have also been found. An additional feature is that most rare sugars taste sweet, but they are not metabolized by humans to the same level of natural sugars. Hence these rare sugars can be used as low calorie sweeteners. Due to their ability to suppress plasma glucose and insulin levels some rare sugars can also be used as diabetes drugs. All of these properties derive from the fact that not many enzymes can act on rare sugars.

Due to the efficient production methods of psicose, tagatose and allose, these rare monosaccharides have been available in sufficient amounts for extensive studies, and various pharmaceutical properties have been found. It is probable that if other rare monosaccharides could also be produced in bulk, several new functions would be found. In the following chapters the different uses of rare sugars are discussed in more detail.



### **1.1.1. Antiviral drugs**

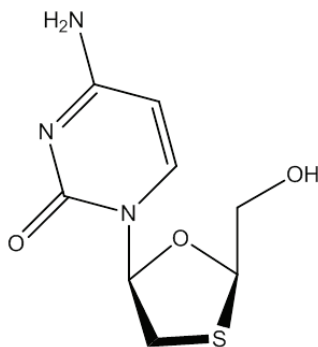
According to the World Health Organization, more than 350 million people are chronically infected with hepatitis B virus (HBV). A safe and effective vaccination is available in the developed countries, but there is still a need for medication for the millions of chronically ill individuals. Especially in eastern Asia and sub-Saharan Africa chronic liver disease and liver cancer caused by hepatitis B are major health problems (Anon, 2012b). Human immunodeficiency virus (HIV) has infected more than 60 million people since the start of the epidemic, and almost 20 million people have died of AIDS. Despite all the effort, no effective vaccine against HIV has been developed to date. Evidently there is a great need for effective antiviral drugs against HBV and HIV (Anon, 2012c).

Nucleoside analogues called nucleotide reverse transcriptase inhibitors (NRTIs) are used in medicine as antiviral drugs. Their efficacy is based on their ability to mimic natural nucleosides, as a result of which they are phosphorylated inside the cell and incorporated into growing DNA strands. Once in the DNA strand, they act as chain terminators. This way they can stop the viral DNA synthesis (Gumina *et al.*, 2001; Mathé and Gosselin, 2006).

Previously it was believed that only nucleoside analogues based on D-sugars would work due to the stereo-specificity of kinases. However, it was found that in some instances cellular kinases are able to phosphorylate nucleosides with an L-sugar moiety. The resulting nucleotides have various biological activities. Consequently, in the beginning of the 1990's L-nucleoside analogues became a new class of antiviral drugs (Mathé and Gosselin, 2006; Wang *et al.*, 1998).

Nowadays a large number of L-nucleoside analogues have been synthesized and their antiviral activities have been evaluated. It seems that in general L-nucleoside analogues are less toxic, have greater metabolic stability and similar or even greater antiviral activity than their D-counterparts (Mathé and Gosselin, 2006; Gumina *et al.*, 2001).

The first L-nucleoside analogue based antiviral drug was Lamivudine, also called 3TC, which is a cytidine analogue that has L-ribose derivative as the sugar moiety (Mathé and Gosselin, 2006). The structure of Lamivudine is presented in **Figure 1**. Lamivudine was approved by the Food and Drug Administration (FDA) in the USA in 1995 and in the EU in 1996. Today, as the patents have expired, there are also numerous generic versions available. Lamivudine is used widely against chronic HBV and HIV. However, long term use can lead to the development of resistant viral strains (Cox and Tillmann, 2011).



**Figure 1.** The molecular structure of Lamivudine or 3TC.

Emtricitabine, also called FTC, is closely related to Lamivudine. It was approved for the treatment of HIV in 2003 both in the EU and the USA. It is currently in phase III trials to be approved for HBV treatment. Emtricitabine has problems similar to Lamivudine related to the development of resistant strains (Cox and Tillmann, 2011).

Telbivudine is a thymidine nucleoside analogue based on L-ribose that is used for the treatment of chronic HBV. It was approved by the FDA in 2006 and in the EU in 2007. Telbivudine is more effective than Lamivudine and the risk of development of resistance is lower, but there have been some reports of toxicity (Cox and Tillmann, 2011).

Clevudine or L-FMAU is an analogue of uridine also with an L-ribose derivative as the sugar moiety. It is approved for anti-HBV therapy in South Korea and the Philippines. Licensing in India, Malaysia and Thailand is currently pending. Clevudine is more potent than Lamivudine, but it is unlikely to get market approval in most countries due to safety concerns. The phase III trials in the US were terminated due to myopathy cases (Cox and Tillmann, 2011; Seok *et al.*, 2009).

In addition to the above mentioned NRTIs, some L-nucleoside analogues, such as Maribavir and Elvucitabine, are currently in clinical trials (Cihlar and Ray, 2010; Anon. 2012d).

### 1.1.2. Other medicinal uses

Several types of viruses are found to have strong association with cancer. These viruses can cause chronic infections in humans. It is assumed that the inhibition of virus replication could thus help in prevention of these types of cancers. It is shown that L-nucleoside analogues that inhibit the replication of HBV can help prevent hepatocellular carcinoma (liver cancer associated with hepatitis B) (Cheng, 2001).

In addition to L-nucleosides, L-glucose (Bicher, 1997) and D-allose (Lim and Oh, 2011) have been shown to have more potential in cancer treatment. They can be used in conjunction with radiation (D-allose) or with other cancer treatments (L-glucose) to

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improve the results of the therapy. In addition, D-arabinose and D-lyxose can be used as a starting material for the synthesis of antitumor compounds (Goodwin *et al.*, 1998; Moran *et al.*, 1993; Morita *et al.*, 1996; Savage *et al.*, 2006). Furthermore, several other rare monosaccharides are extensively studied for their anticancer properties (Beerens *et al.*, 2012).

Adenosine is an important mediator in numerous biological functions. Several studies have shown that ligands (agonists or antagonists) of adenosine receptors (ARs) have potential in the treatment of several diseases. AR agonists have cardioprotective properties, whereas AR antagonists have been shown to have potential for example as anti-Alzheimer and anti-Parkinson medication. In particular, an L-nucleoside analogue of adenosine is found to work as a cardioprotective agent (Kasiganesan *et al.*, 2009).

A rare sugar, D-allose can be used to reduce blood pressure. It also functions as an anti-oxidative and an anti-inflammatory agent as well as a cryoprotectant. Due to these properties it can be used in surgery and transplantation (Lim and Oh, 2011).

### 1.1.3. Nutriceuticals

Due to the growing concern of obesity in the developed countries, the need for low-calorie sweeteners is evident. Even for non-obese people, energy restriction has been shown to increase healthy years, retard the development of many diseases and possibly also increase lifespan. Dietary energy restriction also maintains low plasma glucose and insulin levels and inhibits autoimmune diseases (Levin *et al.*, 1995).

There are several polyols that are currently used as alternative sweeteners. Compared to “high potency sweeteners” such as acesulfame K, aspartame and sucralose, the polyols can be a substitute for the bulk of sucrose as well as for the sweetness. One additional advantage is that polyols usually have no aftertaste, unlike artificial sweeteners (Kroger *et al.*, 2006).

The most used polyols in the USA are sorbitol, mannitol and xylitol. There are also disaccharide polyols permitted for use in food, such as isomalt, lactitol and maltitol. Three rare sugars are newcomers in the sweetener market: the polyol erythritol and two rare monosaccharides, D-tagatose and D-psicose (Kroger *et al.*, 2006).

Alternative sweeteners have clear advantages in the use in foods. They have fewer calories than sucrose and – unlike sucrose and starch – they do not promote tooth decay. Furthermore, they are metabolized independently of insulin, and have little or no effect on plasma glucose level. However, because most rare sugars are digested incompletely, they may have a laxative effect when used in large amounts (Kroger *et al.*, 2006).

Additionally, all polyols reduce the crystallization tendency of sugars and for this reason they are also used for increasing the shelf-life of foodstuffs and cosmetic

products (Wolever *et al.*, 2002; Saha and Nakamura, 2002). As polyols have no reducing end-groups, they all have excellent heat and acid stability (Moon *et al.*, 2010).

In the next chapters, rare monosaccharides and monosaccharide polyols used as sweeteners – with the exception of xylitol (see Chapter 1.4) – are discussed in more detail.

### *Sorbitol*

Sorbitol is a six-carbon polyol that is naturally found in apples, pears, cherries, apricots and plums. It is currently produced by chemical hydrogenation of glucose, which is derived from corn starch (Fernández-Bañares *et al.*, 2009). Sorbitol has an energy value of 2.6 kcal per gram compared to 4.0 kcal/g of sucrose, and a relative sweetness of approximately 60 % of that of sucrose. It is used as a sweetener in chewing gum, candy, mints, jam, jelly, baked goods, drinks, ice cream and chocolate. It is also used as an additive in foods (E420) and medicine due to its properties as a humectant, bulking agent, stabilizer, softener and emulsifier and its surface active properties (Fernández-Bañares *et al.*, 2009)

### *Mannitol*

Mannitol is a six-carbon sugar alcohol that is widely found in nature, for example in pumpkins, celery, onions, grasses, brown seaweeds, olives, mistletoe and lichens. It is a component of the exudates from plants, such as the manna ash tree. It is also found in the mycelium of various fungi and it is the main carbohydrate in mushrooms (Soetaert *et al.*, 1999).

Mannitol is industrially produced by the catalytic hydrogenation of a glucose/fructose mixture. The composition of the hydrogenated mixture is about 25 % mannitol and 75 % sorbitol. Mannitol is recovered by crystallization from the reaction mixture (Wisselink *et al.*, 2002).

Mannitol is about half as sweet as sucrose and has an energy value of 1.6 kcal/g. Mannitol's main application is as a food additive (E421). It is used as a sweet-tasting texturizing agent in chewing gums, often in combination with other polyols. Mannitol is also commonly used in chewable tablets and granulated powders, since it prevents moisture absorption and does not interact with the active components of pharmaceuticals. In addition, its sweet cool taste masks the unpleasant taste of many drugs (Soetaert *et al.*, 1999).

Mannitol has also use in medicine due to its osmotic effect. In surgery, parenteral mannitol solutions are applied to prevent kidney failure, and in brain surgery it is used to reduce cerebral edema. Mannitol hexanitrate is also used in the treatment of hypertension (Soetaert *et al.*, 1999).

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### *Erythritol*

Erythritol is a newcomer in the sweetener market. It is a four-carbon polyol that is widely distributed in nature, for example in seaweeds and fungi as well as in a number of common fruits. It occurs frequently in fermented foods and drinks and in processed vegetables (Moon *et al.*, 2010).

Erythritol has 60-70 % of the sweetness of sucrose (Moon *et al.*, 2010). Only 10 % of erythritol is metabolized by the human body, and the rest is excreted with the urine. Therefore, its caloric content is lower than that of other polyols, only 0.2 kcal per gram. It has no effect on plasma glucose and insulin levels, nor does it have a laxative effect (Moon *et al.*, 2010; Kroger *et al.*, 2006; Bernt *et al.*, 1996).

Erythritol is produced from glucose by fermentation. At least three different fungi can be used for this: *Moniliella pollinis*, *Trichosporonoides megachiliensis* and *Yarrowia lipolytica* (U.S. Food and Drug Administration, 2011). Erythritol is purified by ion exclusion chromatography, ultrafiltration and crystallization from the medium. Erythritol has an E-number 968 and it is used in bakery, chewing gums, candies, ice cream, beverages and yogurt (Cerestar Holding B.V., 2001).

### *D-Tagatose*

D-Tagatose is a keto-hexose and a C-4-epimer of fructose. It can be found in trace quantities in some milk products such as cheeses and yogurt as well as in some rare plants (Livesey and Brown, 1996; Levin, 2002). Tagatose has had a GRAS (Generally Regarded as Safe) status since 2001 for use in foods and beverages (U.S. Food and Drug Administration, 2001).

The taste of tagatose is virtually indistinguishable from sucrose and it is 92 % as sweet as sucrose with no cooling effect (Levin *et al.*, 1995; Levin, 2002; Kroger, 2006). Although FDA has approved 1.5 kcal/g as the caloric value for tagatose, many studies have estimated it to be lower (Levin, 2002). Tagatose is used in jams and chocolate based products as well as a table sweetener (Boudebbouze *at al.*, 2011).

Part of the tagatose ingested is absorbed in the small intestine and metabolized similarly to glucose. However, the rate of tagatose metabolism is slower than that of conventional monosaccharides, and thus the glycemic response it produces is smaller. Most of the tagatose ingested is not absorbed through the small intestine, but passes to the lower gut, where it is fermented by bacteria. Due to this fermentation, tagatose can cause gastrointestinal discomfort when consumed in large amounts (Levin, 2002; Kroger *et al.*, 2006; Oh, 2007).

Tagatose is produced enzymatically from the lactose of whey permeate. Lactose is first hydrolyzed to glucose and galactose using  $\beta$ -galactosidase. After separation, galactose is isomerized to tagatose with L-arabinose isomerase. Finally tagatose is separated from the remaining galactose using a simulated moving bed ion exclusion chromatography and crystallized (Anon, 2012e).

Due to its low glycemic index, tagatose is usable by people with diabetes, and it can also lower plasma glucose and insulin levels (Levin *et al.*, 1995). There is an ongoing phase III clinical trial investigating whether tagatose can be used as medication for diabetes (Espinosa and Fogelfeld, 2010; Boudebbouze *et al.*, 2011).

### *D-Psicose*

D-Psicose is a ketohexose and a C-3-epimer of fructose. It can be found in small quantities in agricultural products and in commercial mixtures of glucose and fructose. Psicose is formed from fructose during cooking and it is naturally present in many foodstuffs such as fruit juices and sauces (Baek *et al.*, 2010; Kim *et al.*, 2006).

Psicose has 70 % of the sweetness of sucrose (Hayashi *et al.*, 2010). Of the psicose ingested, 70 % is absorbed in the small intestine and excreted in urine. The remaining 30 % passes into feces (Hayashi *et al.*, 2010). Thus, the energy value of psicose is effectively zero. The FDA has approved a caloric value of 0.2 kcal/g for psicose (Matsuo *et al.*, 2002). Psicose was given a GRAS status in June 2012. Psicose has been proposed for use in chewing gum, baked goods, candies, ice cream, beverages, yogurt, cereals and coffee mix (U.S. Food and Drug Administration, 2012).

The commercial production of psicose is based on the use of the D-psicose-3-epimerase from *Agrobacterium tumefaciens*. Fructose solution is treated with immobilized, non-viable *Corynebacterium glutamicum* cells harboring the enzyme. In this process it takes between four and eight hours at 50 °C to convert fructose to psicose. The psicose solution is purified with active carbon, ion exclusion chromatography and crystallization (CJ Cheiljedang, Inc., 2011).

Psicose has several nutraceutical features that make it an attractive sugar substitute. It has the ability to suppress plasma glucose and insulin levels after meals (Baek *et al.*, 2010; Matsuo and Izumori, 2009; Hayashi *et al.*, 2010). Psicose has also attracted attention as an inhibitor of hepatic lipogenic enzymes and as an activator of abdominal fat loss (Baek *et al.*, 2010). These findings suggest that psicose could be an appropriate candidate for preventing and reversing diabetes (Baek *et al.*, 2010).

## **1.2. Enzymes acting on monosaccharides**

In the next chapters some general classes of enzymes acting on monosaccharides are presented as well as some relevant examples of each class. Only enzyme classes with potential in the production of rare sugars are discussed, so the list is not comprehensive.

### **1.2.1. Keto-aldol isomerases**

Enzymes that isomerize monosaccharides belong to the group EC 5.3.1 keto-aldol isomerases, also called aldose isomerases. They catalyze the redox reaction exchanging

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the carbonyl functionality between carbons C-1 and C-2. Aldose isomerases accept several different sugars as substrates and thus side products are often formed. Advantages of isomerases in bioproduction include the possibility to use unsubstituted substrates (Beerens *et al.*, 2012).

### *Xylose isomerase*

Xylose isomerase (EC 5.3.1.5) is a promiscuous enzyme that primarily catalyzes the isomerization reaction between D-xylose and D-xylulose. However, it also catalyzes the conversion of glucose to fructose and several other aldose-ketose isomerizations (Jokela *et al.*, 2002). Due to the industrially important function of producing fructose from glucose, it is also often called glucose isomerase. The isomerization of glucose to fructose is of commercial importance in the production of high-fructose corn syrup. Xylose isomerase is therefore one of the three highest tonnage value enzymes worldwide, amylase and protease being the other two. Industrially applied xylose isomerases are produced mostly by *Streptomyces* and *Bacillus* species (Bhosale *et al.*, 1996; Asbóth and Náray-Szabó, 2000).

### **1.2.2. Oxidoreductases**

Oxidoreductases belong to the group EC 1.1 and they are responsible for converting ketoses and aldoses into corresponding polyols and *vice versa*. They use pyridine nucleotides as cosubstrates, which makes them more difficult to use on an industrial scale. Generally, oxidoreductases acting on ketoses are called polyol dehydrogenases and those acting on aldoses are called aldose reductases (Beerens *et al.*, 2012).

### *Sorbitol dehydrogenase*

Ascorbic acid, which is a vitamer of vitamin C, is produced industrially from glucose by the so-called the Reichstein process. In the first step glucose is chemically hydrogenated to sorbitol and then sorbitol is oxidized to L-sorbose by the bacterium *Gluconobacter oxydans* sp. *suboxydans*. This latter step is catalyzed by the enzyme sorbitol dehydrogenase (EC 1.1.1.14). L-Sorbitol is the chiral raw material for ascorbic acid production by chemical synthesis (Hancock and Viola, 2002).

### **1.2.3. Epimerases**

Carbohydrate epimerases belong to the class EC 5.1.3. They are responsible for catalyzing the reorientation of a hydroxyl group and thereby converting sugars into one of their epimers. The advantage of epimerases is the possibility of producing a broad range of different epimers, as epimerases can, in principle, act on any of the carbons in

a sugar. However, the drawback is that side-products may be formed. Additionally, most epimerases found to date require expensive substituted substrates such as UDP-sugars (Beerens *et al.*, 2012).

#### *D-Tagatose-3-epimerase*

D-Tagatose-3-epimerase catalyzes epimerization reactions of several ketohexoses at the C-3 position. It can catalyze at least the reactions between D-fructose and D-psicose, L-fructose and L-psicose, D-tagatose and D-sorbose and L-tagatose and L-sorbose. As a consequence, D-tagatose-3-epimerase has become a key enzyme in the production of several rare monosaccharides (Izumori, 2002). In addition to the D-psicose-3-epimerase (see Chapter 1.1.3, page 7), D-tagatose-3-epimerase is, to my knowledge, the only epimerase that acts on unsubstituted monosaccharides.

### **1.3. Xylose and xylitol metabolism**

D-Xylose can be used as a growth substrate by many microorganisms, including bacteria, yeasts and molds. There are four catabolic pathways for D-xylose. In oxido-reductase pathway and isomerase pathway the substrate is converted to D-xylulose. In the Weimberg pathway and Dahms pathway D-xylose is first converted to 2-keto-3-deoxy-xylonate which can further react to 2-ketoglutarate or to pyruvate and glycolaldehyde, respectively (Zhang *et al.* 2013). In the following chapters the oxido-reductase pathway and isomerase pathway will be discussed in more detail.

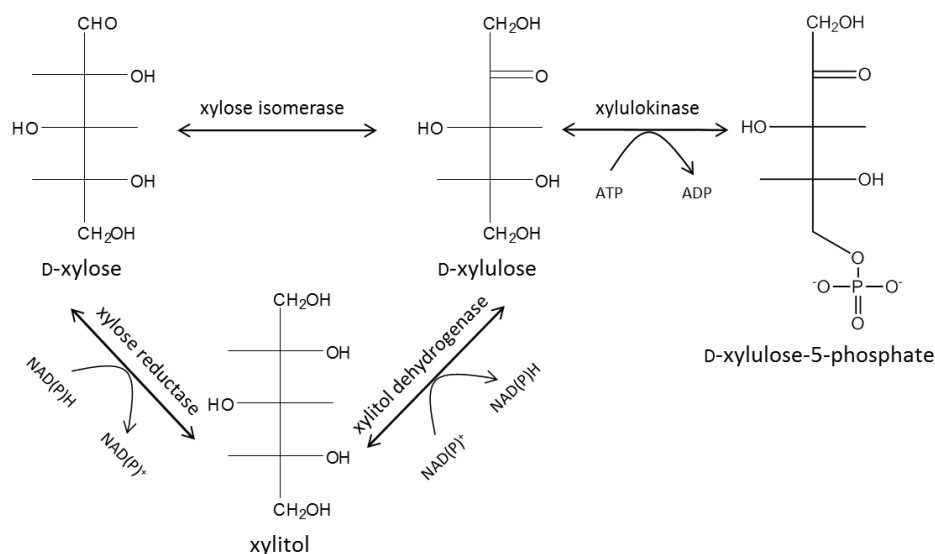
#### **1.3.1. In yeasts and molds**

Yeasts and molds generally use a two-step conversion of D-xylose to D-xylulose. In this oxido-reductase pathway xylose is first reduced to xylitol by a xylose reductase (aldose reductase, EC 1.1.1.21, abbreviated as XR). Xylitol is then oxidized to D-xylulose by a xylitol-2-dehydrogenase (EC 1.1.1.9, abbreviated as XDH). Then the D-xylulose is phosphorylated by D-xylulokinase (EC 2.7.1.17) to D-xylulose-5-phosphate, which can enter the pentose phosphate pathway (Winkelhausen and Kuzmanova, 1998; Parajó *et al.*, 1998a). These conversions are shown in **Figure 2**.

Both XR and XDH require pyridine nucleotide cofactors to function. XR and XDH can be either nicotinamide adenine dinucleotide (NAD<sup>+</sup>) or nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) dependent. NADH and NADPH are the reduced forms of these cofactors. These enzymes exhibit different cofactor specificities in different yeasts. Either an NADH or an NADPH dependent XR reduces xylose to xylitol. In most yeasts, XR has a higher or even an absolute preference for NADPH. Xylitol is usually oxidized to D-xylulose by an NAD<sup>+</sup>-dependent XDH (Winkelhausen and Kuzmanova, 1998; Parajó *et al.*, 1998a).



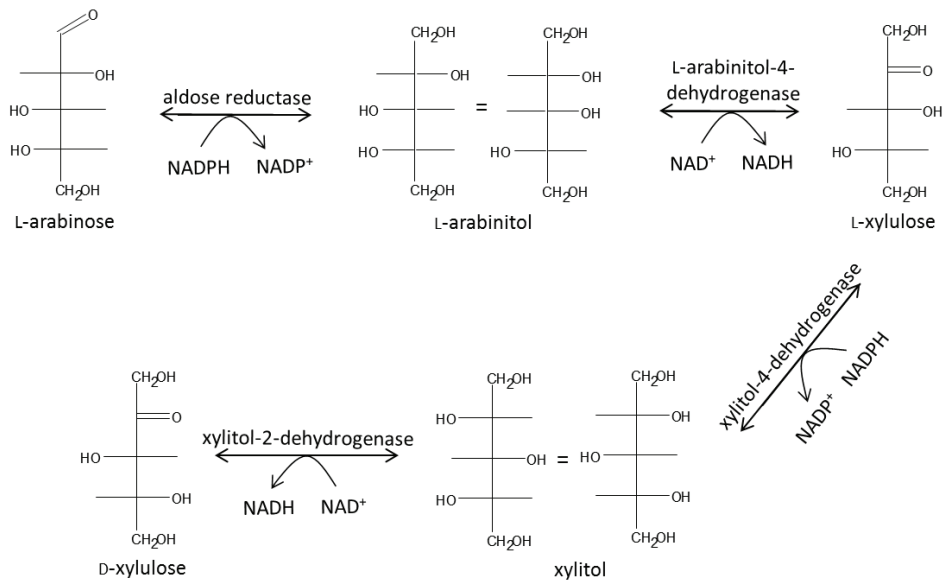
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**Figure 2.** The conversion of xylitol to D-xylulose either by a xylose isomerase in a one step reaction or *via* xylitol in a two step reaction. D-Xylulose is phosphorylated to D-xylulose-5-phosphate by a xylulokinase.

A cofactor imbalance is generated when xylose is converted to xylulose. In the first step (from xylose to xylitol) one NADPH is oxidized to NADP<sup>+</sup>, and in the second step (from xylitol to xylulose) one NAD<sup>+</sup> is reduced to NADH. As a consequence, NADPH and NAD<sup>+</sup> need to be regenerated. As yeasts lack the transhydrogenase enzyme (NADH + NADP<sup>+</sup> ↔ NAD<sup>+</sup> + NADPH), they need alternative ways to regenerate the cofactors. Under aerobic conditions NAD<sup>+</sup> can be regenerated with oxygen. Under oxygen-limited conditions, the accumulation of NADH limits the reaction catalyzed by the NAD<sup>+</sup>-linked XDH, resulting in the accumulation of xylitol (van Dijken and Scheffers, 1986). However, in some yeasts, such as in *Pichia stipitis*, the XR activity is linked to both NADH and NADPH (Verduyn *et al.*, 1985). In these cases the NAD<sup>+</sup> consumed in the second step of the xylose metabolism can be regenerated in the first step.

Although the oxido-reductase pathway appears to be common for yeasts and molds, xylose isomerases catalyzing the direct isomerization of D-xylose to D-xylulose have also been found in some yeasts – namely in *Candida boidinii* and *Candida utilis* – and in the fungus *Aspergillus oryzae* (Bhosale *et al.*, 1996; Vongsuvanlert and Tani, 1998). However, some yeasts are able to catabolize L-arabinose via L-arabitol and L-xylulose to xylitol and further to D-xylulose as shown in **Figure 3**. In this pathway L-xylulose is reduced to xylitol in a reaction catalyzed by xylitol-4-dehydrogenase (synonym L-xylulose reductase, EC 1.1.1.10). Both NAD<sup>+</sup>- and NADP<sup>+</sup>-dependent xylitol-4-dehydrogenases have been described in both, yeasts and molds (Richard *et al.*, 2002; Witteveen *et al.*, 1994; Verho *et al.*, 2004;).



**Figure 3.** The fungal pathway for L-arabinose utilization via L-xylulose and xylitol. Modified from Richard *et al.*, 2002.

### 1.3.2. In bacteria

Many bacterial species possess the enzyme xylose isomerase (EC 5.3.1.5 abbreviated as XI) that is able to convert xylose to D-xylulose in one step as presented in **Figure 2**. After the isomerization, D-xylulose is phosphorylated to D-xylulose-5-phosphate by a D-xylulokinase (Winkelhausen and Kuzmanova, 1998). As with yeasts, D-xylulose-5-phosphate enters the pentose phosphate pathway.

However, some bacteria, such as *Corynebacterium* and *Enterobacter* strains can produce xylitol. It seems that they possess the genes for XR and XDH, either in addition to or instead of the gene for the XI (Yoshitake *et al.*, 1973, Parajó *et al.*, 1998a).

Xylitol can also be used as a growth substrate by certain enteric bacteria and some lactic acid bacteria. In enteric bacteria xylitol is transported into the cell in its free form and oxidized to D-xylulose, which is then phosphorylated to D-xylulose-5-phosphate (Doten and Mortlock, 1985b). Some lactic acid bacteria, however, have a specific phosphotransferase system that imports xylitol into the cell and simultaneously converts it to xylitol-5-phosphate. Inside the cell, an NAD<sup>+</sup>-specific dehydrogenase oxidizes the xylitol-5-phosphate to D-xylulose-5-phosphate (London, 1990).

*Klebsiella pneumoniae* is an enteric bacterium that can acquire the ability to grow on xylitol by spontaneous mutations. This bacterium has a D-arabitol permease – that transports also xylitol – and a D-xylulokinase that phosphorylates D-xylulose. In addition, *K. pneumoniae* has a ribitol dehydrogenase that also has a low level of activity for the oxidation of xylitol to D-xylulose. However, this enzyme is not induced during

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growth on xylitol. A mutation in the ribitol dehydrogenase regulatory gene results in constitutive synthesis of this enzyme, which allows the utilization of xylitol. Thus the natural mutant strain can grow on xylitol by oxidizing it to D-xylulose, which is then phosphorylated to D-xylulose-5-phosphate (Doten and Mortlock, 1984; Mortlock *et al.*, 1965).

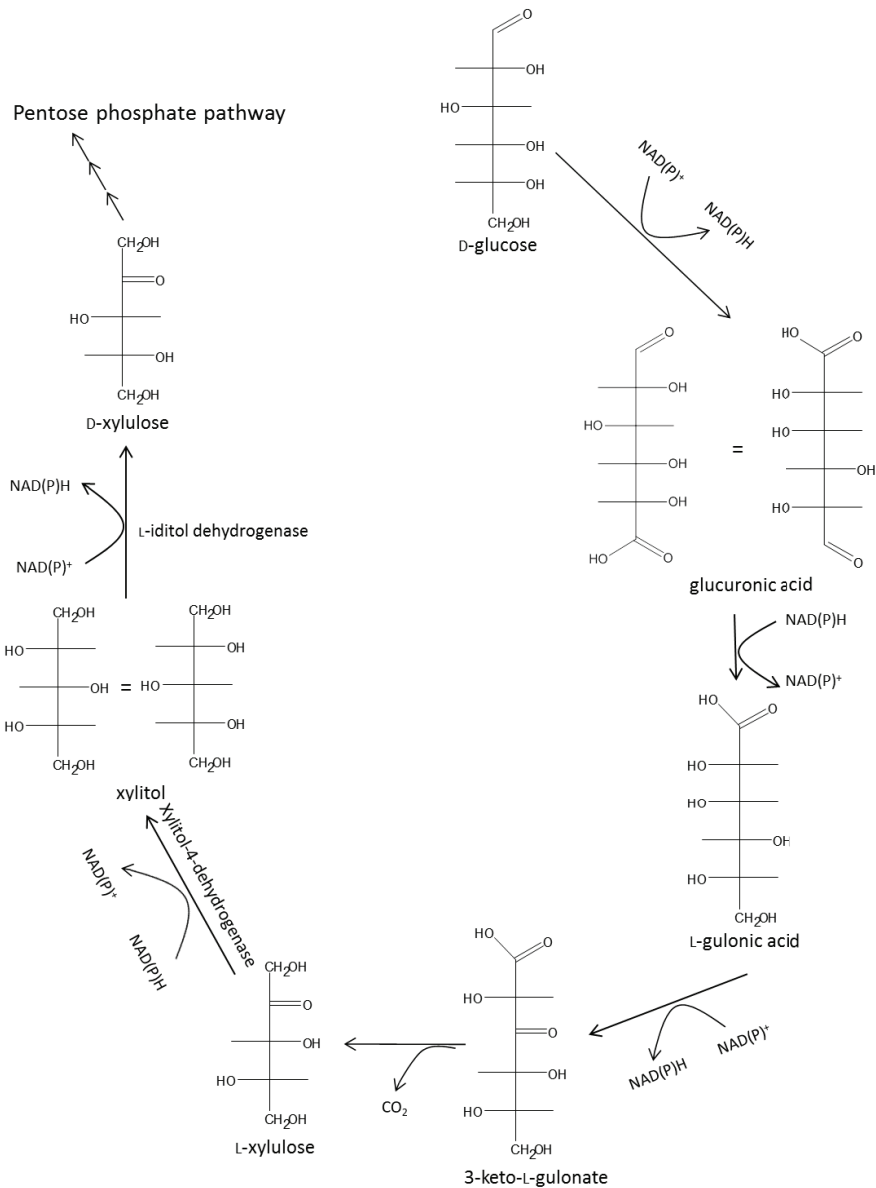
Some bacteria can also metabolize xylitol *via* L-xylulose. The bacterial plant pathogen *Pantoea ananatis* (formerly named *Erwinia uredovora*) can acquire the ability to utilize xylitol as a growth substrate by a spontaneous mutation. Xylitol utilizing mutants have been isolated and shown to constitutively synthesize xylitol-4-dehydrogenase and L-xylulokinase (EC 2.7.1.53) enzymes (Doten and Mortlock 1985a). This xylitol-4-dehydrogenase differs from most xylitol-4-dehydrogenases in being specific to NAD<sup>+</sup> as a cofactor and not showing activity with NADP<sup>+</sup> (Compte Viteri, 2004).

Additionally, *Alcaligenes* 701B strain has been shown to grow on xylitol. The growth rate is slow, as xylitol in the medium must diffuse into the cells before it can be utilized. The cells can convert xylitol either to L-xylulose by NAD<sup>+</sup>-dependent xylitol-4-dehydrogenase or to ribulose by an epimerase. Ribulose can be used by the cells, while L-xylulose is accumulated into the medium (Granström *et al.*, 2005).

### 1.3.3. In mammals

In humans, glucose is usually metabolized through glycolysis. However, about 5 % of daily glucose metabolism occurs in the glucuronic acid cycle, as shown in **Figure 4**. In this cycle glucose is converted in three steps *via* glucuronic acid and gulonic acid to L-xylulose. The L-xylulose is further converted to xylitol in a xylitol-4-dehydrogenase (also called L-xylulose reductase) catalyzed reaction. Somewhere between 5 and 15 grams of xylitol is produced daily in the glucuronic acid cycle (Carbone *et al.*, 2004; El-Kabbani *et al.*, 2002). Xylitol-4-dehydrogenase is present in e.g. humans, hamsters, rats, mice, apes and guinea pigs. Especially large amounts of the enzyme are synthesized in liver and kidneys (Hollmann and Touster 1957; Touster *et al.*, 1956).

Xylitol-4-dehydrogenase isolated from guinea pig liver is very specific to L-xylulose (Hickman and Ashwell, 1958), whereas xylitol-4-dehydrogenases from hamster or human have wide substrate specificities for several pentoses, tetroses, trioses and  $\alpha$ -dicarbonyl compounds (El-Kabbani *et al.*, 2002; Carbone *et al.*, 2004). Nearly all xylitol-4-dehydrogenases use NADP<sup>+</sup> as a cofactor (Hollmann and Touster, 1956). It has been reported that xylitol-4-dehydrogenase also acts as a detoxifying enzyme towards reactive dicarbonyl compounds, which may be formed in the tissue or ingested as components of foods and beverages (Ishikura *et al.*, 2001).



**Figure 4.** Glucuronic acid cycle in mammals with L-xylulose and xylitol as intermediates.

### *Pentosuria*

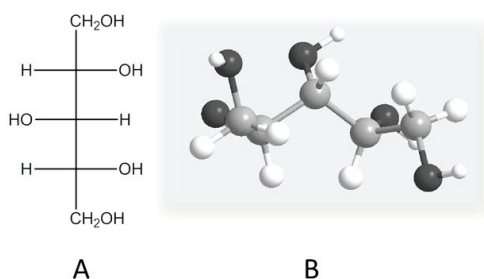
Pentosuria is a congenital metabolic disorder of the sugar metabolism that exists predominantly in Ashkenazi-Jews (Zlotogora, 2004). It leads to the excretion of L-xylulose in the urine (Levene and LaForge, 1913; Levene and LaForge, 1914; Greenwald, 1930). It is now known that those with pentosuria have a defect in the glucuronic acid cycle and that the xylitol-4-dehydrogenase activity of the erythrocytes has diminished. Pentosuria can be detected by the presence of reducing sugars in the urine. Pentosuria

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has not been found to cause any significant clinical symptoms or to lower the life expectancy of patients (Zlotogora, 2004).

### 1.4. Xylitol

Xylitol is an acyclic five-carbon sugar alcohol. The structure of xylitol is presented in **Figure 5**. Xylitol has a molecular formula of  $C_5H_{12}O_5$  and its molar mass is 152.15 g/mol. Xylitol occurs in nature in fruits and vegetables, among which the yellow plum and greengages have the highest xylitol content, nearly 1 % of dry weight (Aminoff *et al.*, 1978).



**Figure 5.** Structure of xylitol **A)** as a Fischer projection and **B)** as a ball and stick model.

#### 1.4.1. Properties

Xylitol is used as a sweetener in chewing gum, candies, and chocolates and in pharmaceutical preparations such as tablets and cough syrups. It is also used in tooth paste (Bär, 1986; Parajó *et al.*, 1998a). Xylitol dissolves well in water and is as sweet as sucrose (Bär, 1986). In theory, the caloric value of xylitol is 4 kcal/g, the same as in other dietary carbohydrates. However, in reality the caloric utilization of xylitol by the human body may be lower due to the slow and incomplete absorption of xylitol. On food labels, the FDA allows a reduced calorie claim of 2.3 kcal/g for xylitol (Peldyak and Mäkinen, 2002). As the dissolution of xylitol is an endothermic process, it gives a cool and fresh sensation. It is highly stable under heating and at acidic pH as there are no reducing end-groups. For this reason xylitol does not undergo browning reactions with amino acids (Aminoff *et al.*, 1978; Winkelhausen and Kuzmanova, 1998; Bär, 1986; Fratzke and Reilly, 1977).

### 1.4.2. Nutriceutical properties

There are two different metabolic pathways for xylitol in humans. Xylitol is slowly absorbed from the digestive tract as there is no specific transport system. Xylitol is dehydrogenated in the liver to D-xylulose by L-iditol dehydrogenase in the glucuronic acid cycle as shown in **Figure 4**. D-Xylulose is then phosphorylated and enters the pentose phosphate pathway. In the pentose phosphate pathway D-xylulose-5-phosphate is partly converted to glucose and partly metabolized to pyruvate and lactate, or it may also be completely oxidized. Glucose is produced slowly from xylitol, which causes only very low stimulation of insulin secretion. This makes xylitol a suitable sweetener for diabetic patients (Bässler, 1978; Bär 1986).

If xylitol is ingested in large amounts, a significant portion may reach the distal parts of the gut. There it is fermented by the intestinal flora (Bär, 1986). Clinical trials have shown that due to the slow absorption rate in the intestine, xylitol can cause unpleasant but harmless osmotic diarrhea. Thus the maximum allowable amount for consumption is 50 g/dose, and 70 g/day. However, adaptation to gradually increasing amounts has been observed for up to 200 g/day of xylitol (Bässler, 1978).

Xylitol is also used in parenteral nutrition since it does not react with amino acids as glucose does. It is a good energy source for patients in the post-operative state, when excessive secretion of stress hormones causes insulin resistance and hinders the use of glucose (Parajó *et al.*, 1998a).

A significant characteristic of xylitol is its ability to inhibit growth of caries causing bacteria. According to the long-lasting studies called the Turku Sugar Studies (Scheinin and Mäkinen, 1975), xylitol is clearly non-cariogenic. Several other studies have also proven xylitol to be advantageous to oral health. Dental caries is caused by bacteria that accumulate in the biofilm covering the teeth, called the dental plaque. *Streptococcus mutans* strains are the most typical caries-forming bacteria. Fermentation of common dietary carbohydrates by plaque bacteria leads to acid accumulation, which results in tooth demineralization and eventually in the formation of cavities. As a sweet substance, xylitol promotes mineralization by increasing the flow of saliva, but this effect is common for all sweeteners. However, xylitol is exceptional, because it is practically non-fermentable by oral bacteria and thus the plaque pH does not decrease after xylitol consumption. In fact, after chewing of a xylitol-sweetened gum, an elevation of plaque pH is observed. In addition, habitual xylitol consumption decreases counts of *S. mutans* as well as the amount of plaque (Franzke and Reilly, 1977; Bär, 1986; Parajó *et al.*, 1998b; Söderling, 2009).

Another significant characteristic of xylitol is its ability to inhibit the growth of *Streptococcus pneumoniae*, which is the leading bacterial cause of acute otitis media (middle ear infection). Xylitol has been shown to decrease acute otitis media incidents in day-care children by 30-40 % compared to control group (Uhari *et al.*, 2001). In addition, treating chronic otitis media with effusion with antibiotics is often not

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effective. The use of xylitol containing chewing gum in combination with antibiotics has been shown to improve the prognosis (Sezen *et al.*, 2008). It is believed that xylitol has the ability to diminish bacteria's ability to form biofilm. This makes bacteria more susceptible to antibiotics as well as immune system defenses (Kurola *et al.* 2011).

### **1.4.3. Production methods**

Xylose is the most available raw material for the production of xylitol. Xylose is a monomer component of hemicellulose, which is the third most abundant polymer in nature (Sreenivas Rao *et al.*, 2007). Currently xylitol is produced chemically, but several biotechnological methods for its production have also been developed. In the following chapters different raw materials and their treatment for xylitol production will be introduced, as well as the chemical xylitol production method and the current status of biotechnological methods.

#### *Raw materials*

Lignocelluloses are organic plant materials, which are abundant and renewable. Their major components – cellulose, hemicellulose and lignin – vary in composition and in proportion. Hemicellulose is a plant cell wall polysaccharide that is a mixture of hexosans and pentosans. It can comprise up to 40 % of the total dry material of certain plants. Hemicelluloses are easily hydrolyzed and the resulting hydrolysate will consist of a mixture of sugars such as glucose, xylose and L-arabinose. The pentose fraction, which is composed of xylose and L-arabinose, is much more abundant in hardwoods than in softwoods. High amounts of pentosans are also present in agricultural residues, such as in sugarcane bagasse, corn cobs, corn fiber as well as in wheat and rice straw (Sreenivas Rao *et al.*, 2007; Winkelhausen and Kuzmanova, 1998; Parajó *et al.*, 1998a).

Many studies have been conducted on the utilization of the hemicellulose portion of agricultural residues for xylose and xylitol production. Corn fiber represents a renewable resource that is available in significant quantities from the corn wet milling industries. Corn fiber contains about 70 % fermentable sugars, and is rich in pentoses. It has low commercial value, and there is an increasing supply of it due to fuel ethanol production from corn. Also the use of sugarcane bagasse has been studied as its hydrolysate contains xylose as the main component (Sreenivas Rao *et al.*, 2007).

Although hydrolysis can be performed enzymatically, most fermentation studies have focused on hydrolysates derived by acid hydrolysis. Because of its heterogeneous structure and relatively low degree of polymerization, hemicellulose is much easier to hydrolyze than the crystalline cellulosic components of biomass (Sreenivas Rao *et al.*, 2007).

### *Chemical production*

On an industrial scale, xylitol is currently produced by chemical reduction of xylose derived mainly from beech and other hardwoods. Corn cob is also used to some extent (Ossi Turunen and Heikki Ojamo, personal communication, May 2013). The process starts with the production of xylose from the hemicellulosic fraction of biomass by acid-catalyzed hydrolysis (Winkelhausen and Kuzmanova 1998; Parajó *et al.*, 1998a; Sreenivas Rao *et al.*, 2007). In order to prevent the contamination of the catalyst, pure xylose is needed. Thus the hydrolysate is subjected to a complicated purification process to remove contaminating substances. Ion exclusion chromatography is used for this purpose and finally xylose is crystallized. The mother liquor can be recycled into the process, and thus about 95 % of the xylose can be crystallized (Aminoff *et al.*, 1978; Heikki Ojamo, personal communication, May 2013).

After the purification, xylose is hydrogenated to xylitol. This is carried out in the presence of a Raney nickel catalyst, at about 125 °C and at hydrogen pressure of 3 bar (Albert *et al.*, 1980). Annually approximately 40,000-50,000 tonnes of xylitol are produced worldwide, and DuPont is the biggest producer (Heikki Ojamo, personal communication May 2013). The chemical process for xylitol production is rather expensive because of the need for very pure xylose as a starting material and the high temperature and pressure required for the hydrogenation step (Sreenivas Rao *et al.*, 2007).

### *Microbial production*

In comparison to chemical hydrogenation, an advantage of microbial production of xylitol is the possibility to use industrial side-streams or crude hemicellulosic hydrolysates as the raw material instead of more expensive pure xylose. In general, yeasts are considered to be the best microbial xylitol producers, and among them, the most efficient producers belong to the genus *Candida* (Sreenivas Rao *et al.*, 2007; Granström *et al.*, 2007a). However, the use of these yeasts in the food industry is problematic because of the pathogenic nature of many *Candida* species (Fridkin and Jarvis, 1996).

Recombinant strains have been constructed for the production of xylitol in a safer host strain than *Candida* by introducing the *XYL1*-gene, which encodes xylose reductase activity, into *Saccharomyces cerevisiae*. However, the highest productivities achieved with natural *Candida* strains have usually not been met (Govinden *et al.*, 2001; Meinander and Hahn-Hägerdal, 1997; Lee *et al.*, 2000; Parajó *et al.*, 1998b).

A few bacteria have been reported to produce xylitol from xylose. Both natural (Rangaswamy and Agblevor, 2002) and recombinant (Suzuki *et al.*, 1999) production strains have been investigated. However, the reported productivities and final xylitol concentrations have been significantly lower than with yeasts (Yoshitake *et al.* 1973; Parajó *et al.*, 1998a).



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Prior to the fermentation by a micro-organism, the lignocellulosic materials need to be hydrolyzed in order to release fermentable sugars. In addition to sugars, the degradation products include weak acids, furan derivatives and phenolics, which are generated during the pre-treatment process. These compounds are shown to inhibit microbial growth and therefore affect the fermentation performance. Consequently, the detoxification of the hydrolysate or the use of an inhibitor-tolerant production organism is usually required for effective hydrolysate fermentation (Huang *et al.*, 2011). Using detoxification methods such as over-liming, activated charcoal or ion exchange resin treatment or a combination of these has been reported to improve xylitol production. Over-liming has been claimed to have the best potential for use in industrial scale (Huang *et al.*, 2011).

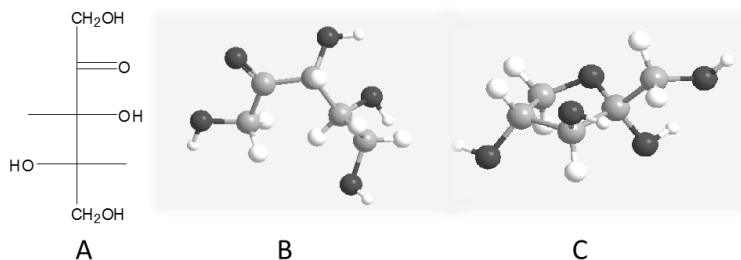
Due to the cost of purification needed for xylose and the limited availability of xylose in practice for possible bulk production, some approaches using glucose as a starting material for xylitol production have also been described. Most efficient xylitol production from glucose has been achieved using a recombinant *Bacillus subtilis* strain. A known transketolase-deficient *B. subtilis* ATCC31094 strain was used that was able to produce ribose from glucose. First the gene coding for D-ribose phosphate isomerase was disrupted and the resulting strain was hence able to produce D-ribulose and D-xylulose from glucose. Then, the xylitol phosphate dehydrogenases from *Lactobacillus rhamnosus* and *Clostridium difficile* were expressed in this strain. The strains were able to produce xylitol from glucose with a conversion of around 23 % (Povelainen and Miasnikov, 2006; Povelainen and Miasnikov, 2007).

## 1.5. L-Xylulose

### 1.5.1. Properties and occurrence

Xylulose or *threo*-2-pentulose is a ketopentose, meaning that it has five carbon atoms and a ketogroup at carbon C-2 as shown in **Figure 6**. It has a molecular formula of  $C_5H_{10}O_5$  and a molar mass of 150.13 g/mol. Xylulose is almost colorless and it forms syrup. The formation of crystals has not been reported (Budavari, 1996). Both D- and L-enantiomers of xylulose are found as intermediates in metabolic pathways of prokaryotes as well as eukaryotes. Both forms are rare in nature (Doten and Mortlock, 1985b).

The optical rotation of D-xylulose  $[\alpha]^{18}_D$  is  $-33^\circ$  and that of L-xylulose  $[\alpha]^{21}_D$   $+31^\circ$ . Xylulose cannot form pyranose rings, and in aqueous solution, depending on the source of information, 8-20 % of D-xylulose is reported to exist as a free ketose while the rest is comprised of  $\alpha$ - and  $\beta$ -furanoses (Hayward and Angyal 1977; Pastinen 2000). It would seem likely that L-xylulose behaves similarly to its enantiomer in aqueous solutions.



**Figure 6.** Structure of L-xylulose **A)** as a Fischer projection, **B)** as a ball and stick model in acyclic form and **C)** as a ball and stick model of the furanose ring form.

L-Xylulose is currently extremely expensive. Sigma-Aldrich sells 95 % pure L-xylulose at a price of 1,345 € for 25 mg, which corresponds to over 55,000 € per gram (Anon. 2012f). However, the high price reflects also the fact that markets for L-xylulose are small.

### 1.5.2. Uses

L-Xylulose has been shown to be a specific inhibitor of certain  $\alpha$ -glucosidases, while having virtually no effect on other glycosidases such as  $\beta$ -glucosidase or  $\alpha$ - and  $\beta$ -mannosidases. Furthermore, it is a specific inhibitor of the *N*-linked glycoprotein processing enzyme, glucosidase I, but does not inhibit glucosidase II or other glycoprotein processing mannosidases. Thus it could prove to be a useful inhibitor for studying glycoprotein processing, especially as it has been proven to be non-toxic and to also be effective in cell cultures (Muniruzzaman *et al.*, 1996).

$\alpha$ -Glucosidase inhibitors can also find use in the management of diabetes. Only monosaccharides can be metabolized directly, and thus starch and disaccharides such as sucrose have to be digested before they are absorbed. By inhibition of intestinal  $\alpha$ -glucosidases, carbohydrate digestion can be delayed and the overall carbohydrate digestion time prolonged. This reduces the rate of glucose absorption. L-Xylulose has been shown to have a strong inhibitory effect on the  $\alpha$ -glucosidases, sucrase and maltase, present in the small intestine. Thus, an L-xylulose containing drug preparation for reducing blood sugar levels in humans and animals has been patented (Heinz *et al.*, 1998).

### 1.5.3. Production methods

In 1933 a synthetic procedure for producing small amounts of D- and L-xylulose was introduced. In this method D- or L-xylose is epimerized to the corresponding diastereomer. The isomerization of xylose in pyridine leads to the production of xylulose (Touster, 1962). The process is, however, complicated and the conversion of xylulose is poor, approximately 15 %. In addition, several by-products are formed and the purification process would be costly. Biotechnological processes are appealing for the production of xylulose due to increased safety and the possibility of producing fewer side-products. In the following, the most promising biotechnological processes for the production of xylulose are described.

There are several biotechnological ways to produce D-xylulose. It can be produced from D-arabitol by *Gluconobacter oxydans* sp. *suboxydans*, *Acetobacter aceti* or mutant *Klebsiella pneumoniae* lacking the xylose isomerase and D-xylulokinase enzymes (Hann *et al.*, 1938; Moses and Ferrier, 1962; Ahmed *et al.*, 1999; Doten and Mortlock, 1985c). D-Xylulose can also be produced enzymatically from D-xylose by commercially available xylose isomerases (Pronk *et al.*, 1988; Olsson *et al.*, 1994) or from D-arabitol by the mannitol dehydrogenase from *Rhodobacter sphaeroides* (Schwartz *et al.*, 1994).

In *Gluconobacter oxydans* sp. *suboxydans*, two different enzymes responsible for pentitol oxidation have been found: one in the cytosolic fraction the other in the membrane fraction. The cytoplasmic enzyme is NAD<sup>+</sup> dependent and oxidizes D-arabitol or xylitol to D-xylulose, but the D-xylulose is not accumulated as it is used in the metabolism of the cell. The membrane-bound enzyme on the other hand is NADP<sup>+</sup> independent and is used in oxidative fermentation. This enzyme catalyzes the production of L-xylulose from xylitol using either PQQ (pyrroloquinoline quinone) or FAD (flavin adenine dinucleotide) as prosthetic group, and L-xylulose is accumulated in the medium (Adachi *et al.*, 1999; Adachi *et al.*, 2001).

Natural mutants of *Pantoea ananatis* that are able to use xylitol as the sole carbon source have been found. Xylitol negative mutants were achieved by transposon mutagenesis. One of these, *Pantoea ananatis* DM122 could still synthesize xylitol-4-dehydrogenase constitutively, but lacked L-xylulokinase activity. Thus, L-xylulose produced from xylitol was accumulated into the medium (Doten and Mortlock, 1985a).

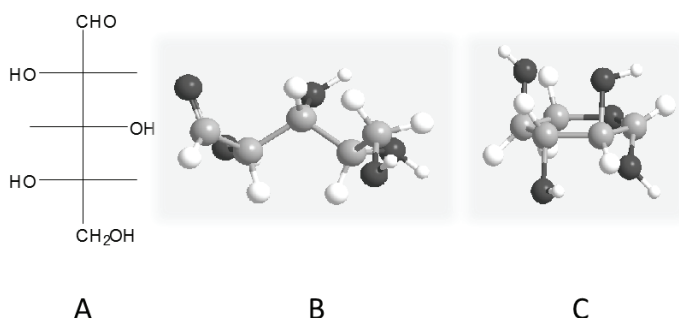
It has also been reported that the bacterium *Alcaligenes* sp. 701B can be used to produce L-xylulose from xylitol. Both washed cell suspensions grown on L-sorbose, an inducer of xylitol-4-dehydrogenase, or cells growing on D-xylose can be used for the conversion of xylitol to L-xylulose (Khan *et al.*, 1991; Granström *et al.*, 2005). Furthermore, D-tagatose-3-epimerase from *Pseudomonas cichorii* produced recombinantly in *E. coli* has been used for the production of L-xylulose from L-ribulose (Bhuiyan *et al.*, 1998).

## 1.6. L-Xylose

### 1.6.1. Properties and occurrence

L-Xylose is an aldopentose that has a molecular formula  $C_5H_{10}O_5$  and a molar mass of 150.13 g/mol. The structure of L-Xylose is presented in **Figure 7**. The optical rotations of D- and L-xylose are  $[\alpha]^{20}_D = -18.6^\circ$  and  $[\alpha]^{20}_D = +18.6^\circ$ , respectively (Fischer and Ruff, 1900). L-Xylose can exist in open chain form, as a pyranose or as a furanose. The relative concentrations of different stereoisomers have not been specified for L-xylose. However, for D-xylose the distribution of different forms in water are: 36.5 % as  $\alpha$ -pyranose, 63 % as  $\beta$ -pyranose and less than 1 % in open chain and furanose form (Pastinen, 2000). Most likely L-xylose acts similarly to its enantiomer, and thus pyranose rings are predominant.

L-Xylose is very rare in nature, and an L-xylose moiety has only been found in two natural compounds: aseric acid (3-C-carboxy-5-deoxy-L-xylose) and 3-O-methyl-L-xylose, which have been found in the cell walls of some plants and bacteria, respectively (Shin *et al.*, 1997; Brown *et al.*, 1977). L-Xylose costs 163 € per 25 grams, which corresponds to a theoretical price of 6500 € per kilogram. Thus there is a need for a more economic production process (Anon, 2012g).



**Figure 7.** Structure of L-xylose **A)** as a Fischer projection, **B)** as a ball and stick model in acyclic form and **C)** as a ball and stick model in pyranose ring form.

### 1.6.2. Uses

L-Xylose is a suitable starting material for the production of L-ribonucleosides. L-Xylose can be converted into an L-ribose derivative *via* oxidation and reduction steps. This product is then glycosylated to give L-ribonucleosides: L-uridine, L-cytidine, L-adenosine and L-guanosine (Moyroud and Strazewski, 1999; Chelain *et al.*, 1995).

L-Xylose can also be used as a starting material for the production of polyhydroxypyrrolidines and related analogues. These compounds have many biological activities. They are shown to have anti-HIV effects, inhibit tumor growth, and

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also act as  $\alpha$ - and  $\beta$ -glucosidase inhibitors, which is of relevance for the development of diabetes drugs (Behr and Guillermin, 2007).

Sodium-glucose transporter (SGLT) inhibitors form a novel class of diabetes drugs. As plasma glucose is filtered in the kidneys, it is normally reabsorbed by the SGLTs. Inhibition of SGLTs leads to urinary glucose excretion, a mechanism that does not require insulin secretion. L-Xylose derived SGLT inhibitors are highly potent and stable (Goodwin *et al.*, 2009).

### 1.6.3. Production methods

Several methods for the production of L-xylose have been reported, though most of them are not economically feasible. L-Xylose can be produced from L-xylulose by isomerization. This can be achieved either enzymatically or chemically under alkaline conditions. The equilibrium of the reaction between D-xylose and D-xylulose in an aqueous solution has been determined at pH 6.8-7.4 and at 25 °C to be 85:15 in favor of D-xylose (Tewari *et al.*, 1985).

In the chemical isomerization of L-xylulose to L-xylose, aqueous solution of L-xylulose is incubated at 40 °C for 40 hours in the presence of 0.05 M NaOH. The final reaction mixture contains 18 mol-% L-xylose and 64 mol-% L-xylulose. In other words, 18 %, of the starting material is converted to side products. The solution can be purified by ion exchange chromatography, after which L-xylose and L-xylulose can be separated in a chromatographic separation column (Heikkilä *et al.*, 2002).

Xylose isomerase from *Streptomyces rubiginosus* can also be used for the production of L-xylose. Jokela *et al.* (2002) used a column packed with XI crystals to produce L-xylose from L-lyxose through the intermediate L-xylulose. Hence, it would also be possible to produce L-xylose from L-xylulose with this enzyme. However, L-lyxose would probably be formed as a side product. Another problem with this method was that unlike the isomerization of the D-forms by XI, the reaction between the L-forms is very slow.

Granström *et al.* (2005) used immobilized L-rhamnose isomerase from *Pseudomonas stutzeri* for the production of L-xylose from L-xylulose. L-Lyxose was formed as a side product and the final concentrations were 53:26:21 for L-xylulose, L-xylose and L-lyxose, respectively.

L-Xylose can also be produced from other starting materials than L-xylulose. For example glucose (Reichstein *et al.*, 1933), sorbitol (Vargha, 1935; Dimant and Banay, 1960), D-gluconolactone (Yang *et al.*, 2002), 2-keto-L-gulonic acid (Heikkilä *et al.*, 2002; Heikkilä *et al.*, 2005) or D-glucuronic acid (Heikkilä *et al.*, 2002; Heikkilä *et al.*, 2005; Fischer and Ruff, 1900) have been used as the starting materials. However, the synthesis methods with these starting materials require several steps and the yields are generally low.

### *L-Fucose isomerase*

L-Fucose is a deoxy-hexose, namely 6-deoxy-L-galactose. The enzyme L-fucose isomerase (FucI, EC 5.3.1.25) catalyzes the reaction between L-fucose and L-fuculose (6-deoxy-L-tagatose). L-Fucose isomerase is also called D-arabinose isomerase due to its ability to isomerize D-arabinose to D-ribulose (Seemann and Schulz, 1997). It has been reported that L-fucose isomerase from *Klebsiella (Aerobacter) aerogenes* can convert L-xylose to L-xylulose, although the  $K_m$  was over 10 times higher and the  $V_{max}$  more than hundred times lower than the values for L-fucose. Additionally, L-xylose became inhibitory already at low concentrations (0.25 M) (Oliver and Mortlock, 1971a; Oliver and Mortlock, 1971b). Later L-fucose isomerase activity has also been found in *Escherichia coli* (Lu and Lin, 1989).

## **1.7. Lactic acid bacteria**

Lactic acid bacteria (LAB) are industrially important microbes that are used all over the world in a large variety of industrial food fermentations. LAB are primarily used for generating lactic acid into food products from the available carbon sources. This results in fast acidification of the food material and aids in the preservation of these products. In addition to the lactic acid formation, LAB also contribute to the texture and flavor of the product. LAB are mostly used in the dairy industry, but other raw materials such as cellulose or wood are also fermented by LAB (Kleerebezem *et al.*, 2002; Wee *et al.*, 2006).

### **1.7.1. Metabolic pathways**

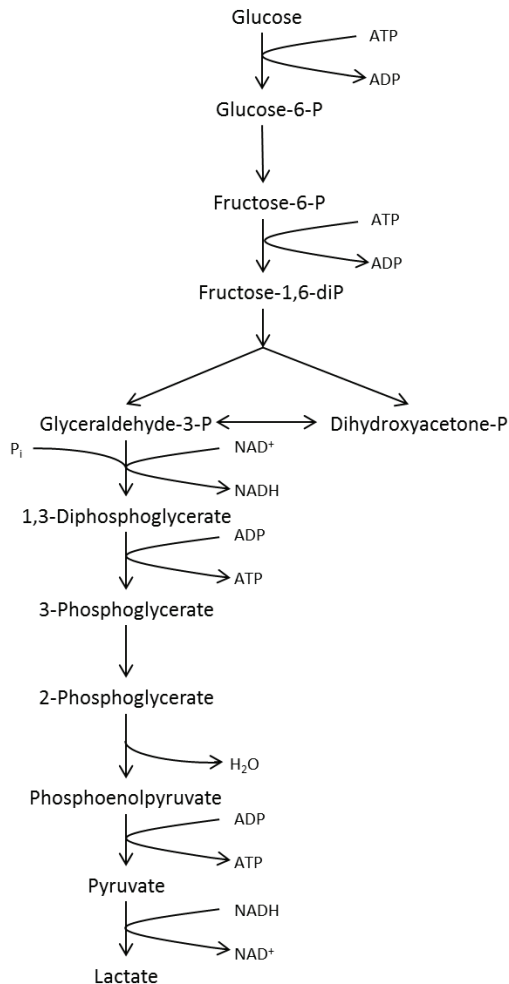
Two main sugar fermentation pathways can be distinguished among LAB. These are glycolysis - also called Embden-Meyerhof pathway - and the 6-phosphogluconate/phosphoketolase pathway (6-PG/PK). In glycolysis lactic acid is formed as the only end-product under standard conditions and this form of metabolism is therefore called homolactic fermentation. Standard growth conditions are defined as non-limiting concentrations of glucose and growth factors (amino acids, vitamins and nucleic acid precursors) available and an oxygen limitation. The 6-PG/PK pathway results in the formation of several end-products including lactic acid, ethanol, acetate and carbon dioxide. This form of metabolism is referred to as heterolactic fermentation (Kandler, 1983; Axelsson, 1998).

Growth conditions differing from the standard conditions may significantly change the end-product distribution. These changes may be caused e.g. by an altered pyruvate metabolism and the possibility to use external electron acceptors, such as oxygen (Kandler, 1983; Axelsson, 1998).

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### Glycolysis

The glycolytic pathway of glucose is presented in **Figure 8**. It starts with the formation of fructose 1,6-diphosphate, which is split by fructose-1,6-diphosphate (FDP-) aldolase into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate that are interconvertible. Glyceraldehyde-3-phosphate is then converted to pyruvate in a sequence of reactions in which ATP is formed in two of the reaction steps.



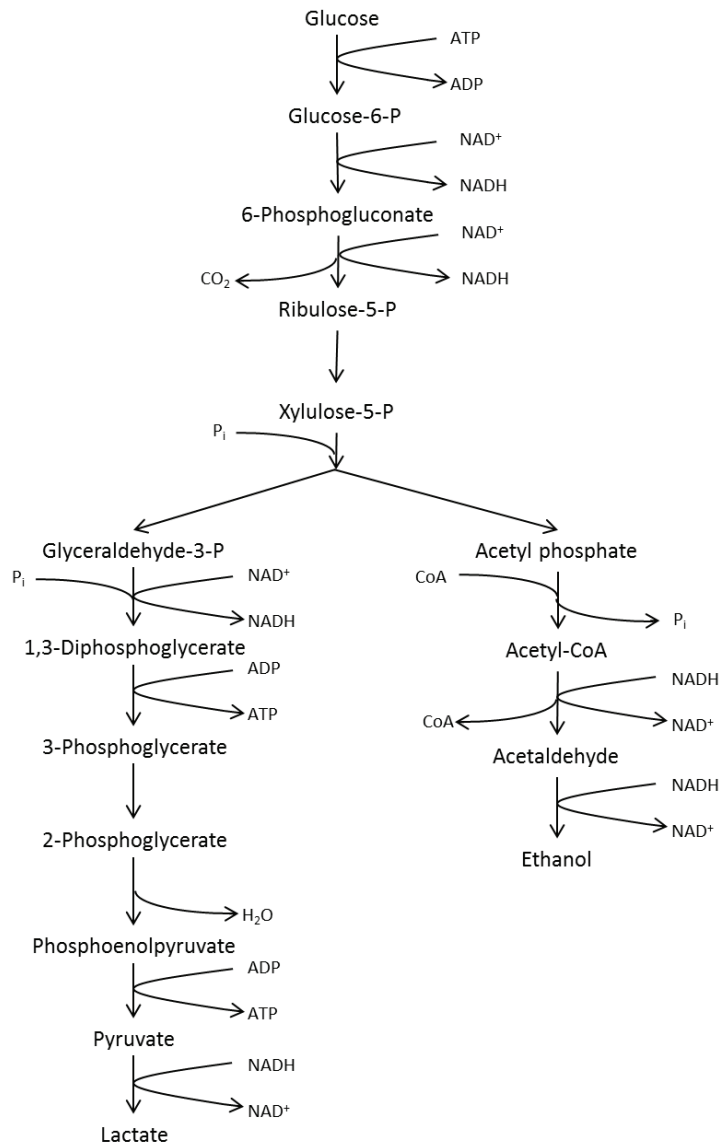
**Figure 8.** Glycolysis or homofermentative pathway of glucose metabolism in lactic acid bacteria. Modified from Axelsson, 1998.

Under standard conditions, pyruvate is reduced to lactic acid by a lactate dehydrogenase. In this step the NADH formed during the earlier steps of the pathway is re-oxidized. Thus, the redox balance is preserved and lactic acid is virtually the only end-product (Kandler, 1983; Axelsson, 1998). In theory the homolactic fermentation of

glucose results in two moles of lactic acid and a net gain of two moles of ATP per one mole of glucose consumed (Axelsson, 1998).

### 6-PG/PK pathway

The fermentation of glucose through the 6-PG/PK pathway is presented in **Figure 9**. In this pathway glucose is first phosphorylated and dehydrogenated and 6-phosphogluconate is formed. This is followed by reductive decarboxylation and



**Figure 9.** 6-Phosphogluconate/phosphoketolase or heterofermentative pathway for glucose metabolism in lactic acid bacteria. Modified from Axelsson, 1998.



## *Introduction*

ribulose-5-phosphate is formed. Ribulose-5-phosphate is converted to xylulose-5-phosphate, which is split into glyceraldehyde-3-phosphate and acetyl phosphate by the enzyme phosphoketolase. Glyceraldehyde-3-phosphate is metabolized in the same way as in glycolysis, resulting in lactic acid formation. Under standard conditions acetyl phosphate is converted to acetyl-CoA, acetaldehyde and finally to ethanol (Kandler, 1983; Axelsson, 1998). The heterolactic fermentation of one mole of glucose through pentose phosphate pathway yields one mole of lactate, ethanol, CO<sub>2</sub> and ATP (Axelsson, 1998).

### *Fermentation of pentoses*

The heterolactic fermentation of pentoses leads to different end-products compared to hexose fermentation. Since no decarboxylation steps are needed to produce xylulose-5-phosphate, no carbon dioxide is formed. Additionally, as no dehydrogenation occurs in the reactions resulting in xylulose-5-phosphate formation, the reduction of acetyl phosphate to ethanol becomes unnecessary. Instead, the acetyl phosphate is converted to acetate and ATP is formed. The fermentation of pentoses thus yields equimolar amounts of lactic acid and acetic acid (Kandler, 1983; Axelsson, 1998).

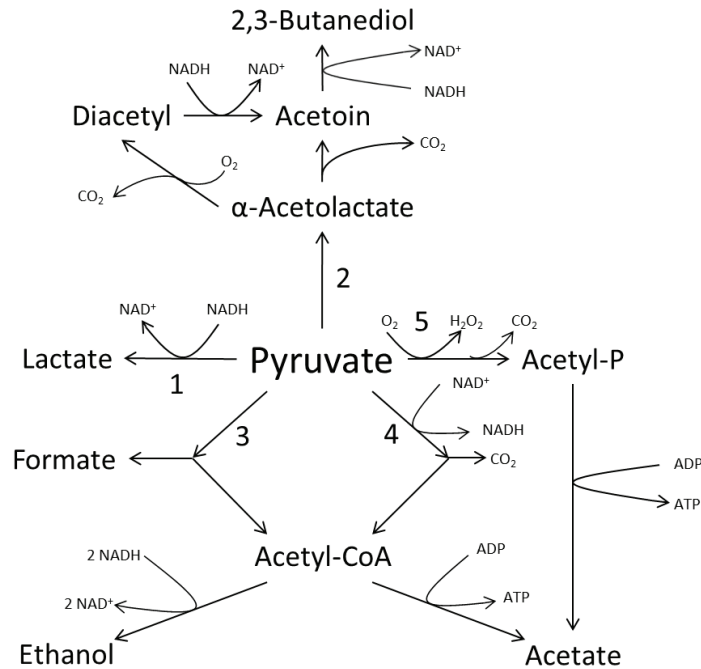
## **1.7.2. Categories of lactic acid bacteria**

LAB can be divided into three metabolic categories. The LAB belonging to the first category are obligately homofermentative. This means that sugars can only be fermented by glycolysis due to the lack of phosphoketolase activity that is needed to convert xylulose-5-phosphate to glyceraldehyde-3-phosphate and acetyl phosphate. This category includes some lactobacilli and some individual species from other genera. The second category comprises of obligately heterofermentative LAB, meaning that all sugars are fermented through the 6-PG/PK pathway. These bacteria lack FDP aldolase needed in glycolysis. This category includes leuconostocs, some lactobacilli, oenococci and weisellas. The third category consists of facultatively heterofermentative LAB that possess a constitutive FDP aldolase, and thus they are homofermentative regarding hexoses. However, pentoses induce the synthesis of phosphoketolase, and as a consequence these LAB are heterofermentative for pentoses. All the remaining lactic acid bacteria, including *Lactococcus lactis*, belong to this category (Axelsson, 1998).

## **1.7.3. Different fates of pyruvate**

Lactic acid bacteria can alter their metabolism in response to varying conditions, resulting in different end-product patterns in different conditions. Most often the change is due to an altered pyruvate metabolism or to the availability of an external

electron acceptor. In homolactic and heterolactic fermentation, pyruvate holds a key position in serving as an electron acceptor in the regeneration of  $\text{NAD}^+$ . However, under certain conditions LAB can use other ways to utilize pyruvate than reducing it to lactic acid. In the following, some possible reactions for pyruvate are represented. The different fates of pyruvate are also presented in **Figure 10** (Axelsson, 1998).



**Figure 10.** Different fates of pyruvate in lactic acid bacteria. 1. Homolactic fermentation. 2. Diacetyl/acetoin pathway. 3. Pyruvate-formate lyase system. 4. Pyruvate dehydrogenase pathway. 5. Pyruvate oxidase pathway. Modified from Axelsson, 1998.

#### *Diacetyl/acetoin pathway*

Diacetyl, acetoin and 2,3-butanediol are the end products of diacetyl/acetoin pathway. This pathway is used only when there is a surplus of pyruvate relative to the need for  $\text{NAD}^+$  regeneration in the cell. This can be obtained in two ways: an alternative source of pyruvate than the fermented carbohydrate exists or another compound acts as an electron acceptor. Oxygen can function as an alternative electron acceptor under aerobic conditions. A group of enzymes called NADH oxidases are common among different species of LAB and they are often induced by oxygen. Under aerated conditions the induction of NADH oxidases may lead to a situation where pyruvate excess is available (Kandler, 1983; Axelsson, 1998).

In general, low sugar concentrations and low pH favor the production of diacetyl and acetoin. Diacetyl is formed by chemical decomposition of  $\alpha$ -acetolactate and this

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reaction is favored by aeration and low pH. Acetoin and/or 2,3-butanediol are produced in much larger amounts than diacetyl. This pathway is rather common among different LAB (Axelsson, 1998).

### *The pyruvate-formate lyase pathway*

The enzyme complex pyruvate-formate lyase catalyzes the reaction in which formate and acetyl-CoA are produced from pyruvate and CoA. Acetyl-CoA can play two different roles: it can be used as an electron acceptor, leading to ethanol formation or as a precursor for ATP formation with acetate as the end product (Axelsson, 1998).

This pathway has been shown to be common among many species of LAB. It is used by *Lactococcus lactis* under anaerobic conditions under substrate limitation. Under these conditions the metabolism switches from homolactic to heterolactic fermentation and the end-products formed are lactate, acetate, formate and ethanol. The change happens due to the reduction in the rate of glycolysis, which leads to changed levels in glycolytic intermediates and subsequently different activities of the enzymes (Thomas *et al.*, 1979). In general, lower levels of intracellular fructose 1,6-diphosphate are found in cells carrying out heterolactic fermentation than in cells performing homolactic fermentation. It can be concluded, that under partial starvation caused by substrate limitation, the cells respond by regulating their enzyme activities so as to partly prevent pyruvate from being reduced to lactic acid. This leads to the production of more ATP per glucose than in normal homolactic fermentation. However, this pathway is only active under anaerobic conditions (Axelsson, 1998).

### *The pyruvate dehydrogenase pathway*

Under aerobic conditions the pyruvate formate lyase pathway is inactivated and its role is played by the pyruvate dehydrogenase pathway. When exposed to air, the cells depend on the pyruvate dehydrogenase for the acetyl CoA production. The excess NADH produced can be reoxidized by NADH oxidases (Axelsson, 1998).

Non-growing *Lactococcus lactis* cells under substrate limitation and aeration can perform homoacetic fermentation, in which all the pyruvate is routed through the pyruvate dehydrogenase pathway with acetic acid as the sole end product (Smart and Thomas, 1987).

### *The pyruvate oxidase pathway*

Under aerated conditions, acetate can also be produced by the pyruvate oxidase pathway. In this pathway pyruvate reacts with oxygen to form acetyl phosphate, CO<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. Acetyl phosphate is further converted to acetate while one ATP is formed (Axelsson, 1998).

## 2. Aims of the study

As discussed in the literary review, using biotechnological methods is an interesting approach to the production of rare sugars, as their chemical synthesis is often difficult and expensive. There are already several such applications for rare L-sugars. New applications will also be found as new production methods are developed and rare sugars can be produced in sufficient quantities for application studies. In this study, the aim was to produce two L-sugars – L-xylulose and L-xylose - which are both very rare and expensive. The sugar alcohol, xylitol, was also chosen for production experiments for two reasons: as a meso-sugar it is a natural and cheap intermediate in the production of L-sugars from D-xylose and as a nutraceutical it has many interesting uses.

The aims of this study were to:

1. Produce xylitol from D-xylose with recombinant lactic acid bacteria.
2. Purify and characterize *Pantoea ananatis* xylitol-4-dehydrogenase from recombinant *Escherichia coli*.
3. Produce L-xylulose from xylitol with recombinant *E. coli* and optimize the production parameters.
4. Construct an expression host able to produce L-xylulose from D-xylose.
5. Overexpress and characterize the enzyme L-fucose isomerase from *Escherichia coli*.

### 3. Materials and methods

In this section only a brief summary of the materials and methods used will be given. The more detailed descriptions can be found in the appended publications.

#### 3.1. Strains and culture conditions

A list of strains and basic culture conditions used in this thesis are presented in **Table 1**.

#### 3.2. DNA-techniques

The *Pichia stipitis* xylose reductase gene *XYL1* was amplified by polymerase chain reaction (PCR) and cloned into the *NcoI* and *HindIII* sites of the pNZ8032 expression vector (de Ruyter *et al.*, 1996). *L. lactis* NZ9800 was transformed with the resulting expression plasmid by electroporation as described previously (Holo and Nes, 1989). The resulting strain was named LLXR. For co-expression of *XYL1* with the xylose permease gene *xyfT* from *Lactobacillus brevis*, a fragment containing *xyfT*, its ribosomal binding site and its transcription terminator was fused directly after the stop-codon of *XYL1* by PCR. The fragment was cloned to pNZ8032 and *L. lactis* NZ9800 was transformed with the construct. The resulting strain was named LLXTXR.

The purified xylitol-4-dehydrogenase from *Pantoea ananatis* was subjected to N-terminal sequencing. The whole reading frame of *xdh* was sequenced using the Vectorette (Sigma Genosys Ltd) system and a degenerate primer was designed on the basis of the N-terminal sequence obtained. The xylitol-4-dehydrogenase gene (abbreviated *xdh*) was amplified by PCR and cloned into the *EcoRI* and *HindIII* sites of the pKK223-3 expression vector (PL-Pharmacia). *E. coli* M15 was transformed with the resulting plasmid and the strain was named ERF2157.

*XYL1* and *xdh* were multiplied by PCR using the genome of *Pichia stipitis* as the template for *XYL1* and the genome of *Pantoea ananatis* as the template for *xdh*. The forward primer for *xdh* had the end of *XYL1* gene at its 5'-end followed by the putative ribosomal binding site of *xdh*. The reverse primer for *XYL1* had the ribosomal binding site and the beginning of *xdh* at its 5'-end. The resulting polymerization products were used as templates for an additional PCR-reaction with splicing by overlap extension technique. The fusion gene had the putative ribosomal binding site of

Table 1. Strains used in this study.

Strain	Parent strain	Cloned genes	Plasmid	Used for	Medium	Conditions	Induction
<i>L. lactis</i> LLXR	<i>L. lactis</i> NZ9800	XYL1 <i>P. stipitis</i> CBS5773	pNZ8032	Production of xylitol	M17 + 5 g/l glu 8 mg/l Cm	30 °C no stirring	0.8 µg/l nisin
<i>L. lactis</i> LLXTXR	<i>L. lactis</i> NZ9800	XYL1 <i>P. stipitis</i> CBS5773 <i>xyIT Lb. brevis</i> ATCC8287	pNZ8032	Production of xylitol	M17 + 5 g/l glu 8 mg/l Cm	30 °C no stirring	0.8 µg/l nisin
<i>E. coli</i> ERF2157	<i>E. coli</i> M15	<i>xdh P. ananatis</i> ATCC43072	pKK223-3	Characterization of XDH	LB Broth 100 mg/l Amp 25 mg/l Km	37 °C 200 rpm	1 mM IPTG
<i>E. coli</i> BPT228	<i>E. coli</i> HB101 (DE3)	<i>xdh P. ananatis</i> ATCC43072	pACYC-Duet	Production of L-xylulose	LB Broth 34 mg/l Cm	37 °C 200 rpm	1 mM IPTG
<i>E. coli</i> BPT244	<i>E. coli</i> XL1-Blue	<i>fucI E. coli</i> K12	pQE-60	Characterization of FucI Production of L-xylulose	LB Broth 100 mg/l Amp	37 °C 200 rpm	1 mM IPTG

IPTG = Isopropyl-β-D-thiogalactopyranoside

## Materials and methods

*xdh* between the two genes and it was inserted into plasmid pQE-60 (Qiagen) for transformation into *E. coli* XL1Blue.

Because the desired results were not achieved using the *XYL1* and *xdh* fusion, pACYC-Duet vector containing two separate cloning sites was used in an attempt to obtain an *E. coli* strain capable of converting D-xylose to L-xylulose via xylitol. *E. coli* HB101 cells were used as this strain lacks xylose isomerase activity but the xylose transporter is still active (Sambrook *et al.* 1989; Boyer *et al.*, 1969). Since pACYC-Duet requires a strain containing a copy of the gene for T7 RNA-polymerase for expression, HB101 was subjected to DE3-lysogenisation using a DE3-lysogenisation kit (Novagen). The resulting HB101(DE3) cells were transformed with a pACYC-Duet plasmid containing *NcoI* and *HindIII* cut *XYL1* gene in the first cloning site and *NdeI* and *XhoI* cut *xdh* gene in the second cloning site. HB101(DE3) strain containing a construct with the *xdh* cloned into the *NdeI* and *XhoI* sites of pACYC-Duet vector was named BPT228.

*FucI* was amplified by PCR using the genomic DNA of *E. coli* K-12 as the template. A His<sub>6</sub>-tag was inserted into the C-terminus of the gene. The PCR-product was cloned into the expression vector pQE-60 and the resulting plasmid was transformed into *E. coli* XL1-Blue cells. The resulting strain was named BPT244.

### 3.3. Enzyme activity assays

Xylose reductase and xylitol-4-dehydrogenase activities were assayed from the cell extracts by incubating with xylose and NADH or xylitol and NAD<sup>+</sup>, respectively, and measuring the change in absorbance at 340 nm at 30 °C. The enzyme activities are calculated as nanokatals (nkat), corresponding to nmoles of NADH or NAD<sup>+</sup> regenerated per second (Verduyn *et al.*, 1985).

Xylose transport was analyzed using [U-<sup>14</sup>C]xylose by a modification of the method by Chaillou *et al.* (1998).

L-Fucose isomerase activities were determined from cell extracts by incubating L-xylulose or L-xylose solution with the enzyme sample. The reaction was stopped by heating at 100 °C for 2 min. Sugar concentrations were determined from the reaction mixtures by high performance liquid chromatography (HPLC).

Protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin as the protein standard. Alternatively Qubit (Invitrogen) quantification system, based on fluorescent dyes that emit signals when bound to specific target molecules, was used.

### 3.4. Calculations

As there are different definitions for conversion and yield, the following are used in this thesis:

$$\text{conversion} = \frac{P_f - P_i}{S_i} \quad (1)$$

$$\text{yield} = \frac{P_f - P_i}{S_i - S_f} \quad (2)$$

where

$S_i$  = initial amount of substrate (mol)

$S_f$  = final amount of substrate (mol)

$P_i$  = initial amount of product (mol)

$P_f$  = final amount of product (mol)

### 3.5. Effect of reaction conditions on the production of L-xylulose

#### 3.5.1. CCC-design for pH, shaking speed and initial xylitol concentration

The effects of pH, shaking speed, and xylitol concentration and their interactions on L-xylulose production were studied using a central composite circumscribed (CCC) design. pH was varied between 5.0 and 9.0 with star points at 3.6 and 10.4, and the shaking speed was varied between 100 rpm and 250 rpm with 49 rpm and 300 rpm as the star points. The initial xylitol concentration was varied logarithmically between 10 g/l and 100 g/l, with star points at 4.6 g/l and 219 g/l. This resulted in an experiment design comprising 17 experiments with three replicates in the center point.

The experiments were performed in 250 ml Erlenmeyer flasks in 25 ml with resting cells of *E. coli* BPT228 as the catalyst. Citrate (50 mM) – Tris (50 mM) buffer was supplemented with 10 mM MgCl<sub>2</sub> and 34 mg/l chloramphenicol. The reaction was continued for 48 h at 37 °C, and samples were analyzed by HPLC. pH values of the samples were estimated using pH-indicator strips.

#### 3.5.2. CCF-design for temperature, pH and initial xylitol concentration

The effects of temperature, pH and initial xylitol concentration and their interactions on L-xylulose production were further studied using a central composite face-centered (CCF) design. pH was varied between 7.0 and 8.5. Temperatures ranged from 36 °C to 44 °C in the experiments. The initial xylitol concentration was varied between 150 g/l and 450 g/l. In addition, four extra experiments were performed at the star points of pH and xylitol concentration (star distance 1.5). This resulted in an experiment design of 21 experiments.



## Materials and methods

The experiments were performed in 250 ml Erlenmeyer flasks in 25 ml in the presence of resting cells of BPT228, at a shaking speed of 300 rpm. K-phosphate (50 mM) – Tris (50 mM) buffer was supplemented with 5 mM MgCl<sub>2</sub> and 17 mg/l chloramphenicol. Samples were analyzed by HPLC. The pH-values of the samples were measured at the reaction temperature with a pH electrode.

### 3.5.3. Experiment designs

The results were evaluated using statistical coefficients: coefficient of determination (R<sup>2</sup>), coefficient of model prediction (Q<sup>2</sup>), P value, and P for the lack of fit. These values indicate how well the model corresponds to the observations (R<sup>2</sup>), how well the model is able to predict experimental results (Q<sup>2</sup>), and how valid the model is (P values). The experiment designs were made and evaluated using Modde 5.0 (Umetri, Sweden). The optimum values were predicted with a prediction tool provided by Modde.

## 3.6. Bioreactor experiments

### 3.6.1. *Lactococcus lactis* producing xylitol

The *Lactococcus lactis* LLXR and LLXTXR strains were used to produce xylitol from xylose. Batch and fed batch experiments were performed with resting cells at high cell density. Glass vessels of 250 ml were used at a working volume of 150 ml with magnetic stirring and nitrogen sparging to minimize the effect of oxygen. pH was controlled at 7.0 and the temperature was 30 °C. The experiments were performed at a xylose concentration of 25 g/l. In the batch production experiments glucose and M17 were used at initial concentrations of 75 g/l and 2 g/l, respectively. In fed batch experiments glucose and M17 were supplied at feed rates of 1.0 g/(l·h) and 0.25 g/(l·h) per suspension volume, respectively. The fed batch experiments at high xylose concentration were performed at 160 g/l xylose. Glucose and M17 were added at feed rates 1.3 g/(l·h) and 0.1 g/(l·h), respectively. All experiments were performed in duplicates.

### 3.6.2. *Escherichia coli* producing L-xylulose

Resting cells of *E. coli* BPT228 were used to produce L-xylulose from xylitol in a Biostat MD reactor (total volume 2 l, B. Braun Biotech International) equipped with two six-blade Rushton-type impellers. The cells were grown in the reactor at 1.8 liter and induced with IPTG. The cells were cultivated for an additional 3.5 h after induction and concentrated by tangential flow filtration using a Pellicon Mini (Millipore) holder and two Biomax 1,000 K membranes (total filtration area 0.2 m<sup>2</sup>, Millipore). The cells

were resuspended into a solution (1 l) containing K-phosphate (50 mM) – Tris (50 mM) – HCl buffer pH 8.0, 5 mM MgCl<sub>2</sub> and 250 g/l xylitol. Production of L-xylulose with resting cells was carried out in the bioreactor at 40 °C for 24 h. The pH was maintained at 8.0 by the addition of 3 M NaOH. Dissolved oxygen was controlled at a minimum of 30 % air saturation.

### 3.7. Protein purifications

#### 3.7.1. Native xylitol-4-dehydrogenase

*Pantoea ananatis* ATCC 43072 cells were grown and separated from the culture by centrifugation. All buffers used in the purification were supplemented with 10 mM MgCl<sub>2</sub>. The cells were suspended in buffer and disrupted by sonication. Ammonium sulfate was added to the cell extract to achieve 20 % saturation. The resulting precipitate was removed and the supernatant was adjusted to 60 % ammonium sulfate saturation. The precipitate formed was dissolved in 20 % saturation ammonium sulfate and applied to a phenyl Sepharose (Amersham Biosciences) column. The column was washed with 20 % saturated ammonium sulfate and eluted with a gradient of ammonium sulfate from 20 % to 0 % saturation. The selected active fractions were pooled and ammonium sulfate was added at 75 % saturation to the pool. The precipitate was dissolved in gel filtration buffer containing Tris (25 mM) – HCl at pH 8.0 and 150 mM NaCl. The solution was applied to a Sephacryl S-300 (Amersham Biosciences) gel filtration column and eluted. The buffer of the pooled active fractions was changed by ultrafiltration followed by dilution with potassium phosphate (10 mM) buffer, pH 7.0. The sample was applied to a hydroxyapatite Bio-Gel HTP (Bio-Rad) column and eluted with a gradient of 10 mM to 400 mM potassium phosphate buffer pH 7.0. The pooled active fractions were concentrated by ultrafiltration and the buffer was changed to Tris (10 mM) – HCl, pH 8.0. The sample was applied to a DEAE-Memsep 1000 HP (Millipore) column and eluted with a linear gradient of 0 to 0.6 M NaCl. The selected fractions were concentrated and subjected to native polyacrylamide gel electrophoresis in a 7.5 % gel (Bio-Rad). The gel was stained for xylitol-4-dehydrogenase activity using a previously described Nitro Blue Tetrazolium-phenazine methosulfate system (Neuberger *et al.*, 1979). The band showing xylitol-4-dehydrogenase activity was cut from the gel and incubated in Laemmli sample buffer. The sample was loaded into 12.5 % SDS-PAGE gel and electrophoresed. The proteins were electroblotted from the SDS-PAGE gel onto a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). The N-terminal amino acid sequence of the protein sample was determined at the Protein Chemistry Unit of the Biomedicum Center (Helsinki, Finland).

### 3.7.2. Recombinant xylitol-4-dehydrogenase

*E. coli* ERF2157 cells were grown and induced with 0.25 mM IPTG to produce xylitol-4-dehydrogenase. All buffers used in the purification were supplemented with 15 mM MgCl<sub>2</sub>. The cells were suspended in buffer and disrupted by sonication. The resulting cell extract was subjected to ammonium sulfate fractioning at 20 % saturation. The precipitate was removed by centrifugation and the supernatant was adjusted to 60 % ammonium sulfate saturation. The precipitate formed was dissolved in 20 % saturation ammonium sulfate and applied to a hydrophobic interaction column (butyl sepharose 4 Fast Flow, Amersham Biosciences). The column was washed with 20 % saturated ammonium sulfate and eluted with a gradient of ammonium sulfate from 20 % to 0 % saturation. The fractions with activity were combined and concentrated by ultrafiltration. The concentrated sample was applied to a gel filtration column (HiLoad 26/60 Superdex 200 preparation grade, Amersham Biosciences) and eluted with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. The selected active fractions were combined, and the buffer was changed by ultrafiltration and dilution to 10 mM Tris-HCl, pH 8.0. The sample was applied to an ion-exchange column (Biosepra Q Ceramic HyperD 20, Ciphergen, Cergy-Saint-Christophe, France) and eluted with a linear gradient of 0 to 0.7 M NaCl. The active fractions were pooled and concentrated by ultrafiltration.

### 3.7.3. Recombinant L-fucose isomerase

*E. coli* BPT244 cells were grown and induced with 1 mM IPTG to produce L-fucose isomerase. The cells were disrupted by sonication and the His-tagged protein was purified from the cell extract by Ni-affinity chromatography (His-Trap HP column, Amersham Biosciences) according to the instructions of the manufacturer. The selected fractions were pooled and concentrated by ultrafiltration.

## 3.8. Purification of L-xylulose

L-Xylulose was produced by resting cells of recombinant *E. coli* in 1 liter of potassium phosphate (20 mM) buffer, pH 7.0 supplemented with MgCl<sub>2</sub> (5 mM), appropriate antibiotics and xylitol (10 g/l). The cells were removed by centrifugation and the supernatant was filtered through a layer of activated carbon, followed by filtration through a 0.2 µm membrane. The filtered solution was evaporated to 50 ml, and 100 ml of ethanol was added. The solution was centrifuged and the supernatant was concentrated by evaporation to 50 ml and ionic compounds were removed by anion exchange (Amberlite IRA-958) followed by cation exchange (Finex CS 16 G PE, Finex, Finland). The remaining solution was concentrated by evaporation to 10 ml and

subjected to strong acid cation exchange chromatography (Dowex 50WX4-400) in  $\text{Ca}^{2+}$  form. The sample was eluted with deionized water and xylulose was analyzed from the collected fractions by HPLC. The fractions containing xylulose were combined and concentrated.

### 3.9. Stability of L-xylulose

The stability of L-xylulose was studied at different pH-values at 37 °C. L-Xylulose was produced from xylitol using resting cells of *E. coli* BPT228. After the production, cells were separated by centrifugation and the reaction mixture containing L-xylulose was divided into 7 batches. These batches were buffered to different pH-values in citrate (100 mM) – Tris (100 mM). The final pHs varied between 7.0 and 10.0. The reaction mixtures were incubated at 37 °C. Samples (1 ml) were taken at different time points and the pH of the samples was adjusted to 7.0 with 1 M HCl. The sugar concentrations of the samples were measured using HPLC.

### 3.10. Levels of intracellular metabolites

Resting cells of *E. coli* BPT228 were incubated in the presence of 10 g/l xylitol and D-[1-<sup>3</sup>H]xylitol (American Radiolabeled Chemicals, Ltd) at  $1.7 \times 10^5$  Bq/l for 22 hours for the determination of intracellular ratio of xylitol and L-xylulose as well as the ratio of  $\text{NAD}^+$  to NADH. L-Xylulose and xylitol concentrations of the culture supernatant were determined by HPLC. The cells were suspended in 2 ml of  $\text{NAD}^+$ /NADH extraction buffer ( $\text{NAD}^+$ /NADH Quantification kit, BioVision) and disrupted. An aliquot of 100  $\mu\text{l}$  of the resulting cell extract was used for the quantification of intracellular pyridine nucleotides using the  $\text{NAD}^+$ /NADH quantification kit.

The rest of the cell extract was neutralized with HCl to prevent L-xylulose degradation, and subjected to cation exchange (Dowex 50WX4, 200-400 mesh) chromatography in order to separate L-xylulose from xylitol. The fractions containing L-xylulose and xylitol were collected and concentrated by evaporation under reduced pressure. The amount of radioactivity in the fractions was determined by scintillation counting.

### 3.11. Analytical methods

Glucose, xylose, xylitol, formate, acetate, lactate, acetoin, diacetyl and 2,3-butanediol were analyzed from the reactions with *L. lactis* LLXR and LLXTXR cells. An HPLC system equipped with a Bio-Rad HPX-87H Aminex column with 5 mM  $\text{H}_2\text{SO}_4$  as the eluent at 65 °C with a flow-rate of 0.4 ml/min was used in the analyses.

### *Materials and methods*

Xylitol and xylulose were determined using a Bio-Rad HPX-87P column and a Deashing Micro-Guard pre-column (Bio-Rad) in XDH characterization and in the experiments with resting cells of *E. coli* ERF2157. Deionized water was used as the eluent at 70 °C and at a flow-rate of 0.6 ml/min.

From the reaction mixtures with *E. coli* BPT228 as the catalyst, xylulose and xylitol were analyzed using an HPX-87K column (Bio-Rad), a K<sup>+</sup> pre-column and a Waters 410 refractive index detector. Water was used as the eluent at 85 °C at a flow rate of 0.4 ml/min.

Lyxose, xylose and xylulose in the reactions with L-fucose isomerase as the catalyst were analyzed using Rezex RCM monosaccharide Ca<sup>2+</sup> column with a Phenomenex Inc. pre-column. Distilled water was used as the eluent at 75 °C and at an elution rate of 0.5 ml/min. The detection was carried out using a RID-10A refractive index detector.

Because the HPLC system used cannot separate D- and L- forms of sugars, the xylulose produced was subjected to polarimetric analysis. Perkin-Elmer Polarimeter 343 was used and the solutions were evaporated to a sugar concentration of 10 g/l before analysis. The specific optical rotation was determined at 589 nm and at 20 °C. D- And L-xylulose (Sigma-Aldrich) were used as the standards under the same conditions.

The molecular mass of recombinant xylitol-4-dehydrogenase was estimated by analytical gel filtration using a HiLoad 26/60 Superdex 200 preparation-grade (Amersham Biosciences) column according to the manufacturer's instructions.

## 4. Results and Discussion

### 4.1. Production of xylitol using recombinant *Lactococcus lactis* (I)

#### 4.1.1. Genetic engineering of the strain

The xylose reductase gene *XYL1* from *Pichia stipitis* was chosen for this study as it has a reasonably high specific activity with NADH in addition to the activity with NADPH (Verduyn *et al.*, 1985). *XYL1* has also previously been expressed in a prokaryotic host (Amore *et al.*, 1991). The gene was expressed in *Lactococcus lactis* NZ9800 using the nisin inducible expression vector pNZ8032. The strain was named LLXR. The results show that XR can be produced in *L. lactis* in an active form. The NADH to NADPH –linked activity ratio of the recombinant enzyme was determined to be 0.64. This is in agreement with the previously reported ratio 0.72 for the native enzyme (Verduyn *et al.*, 1985).

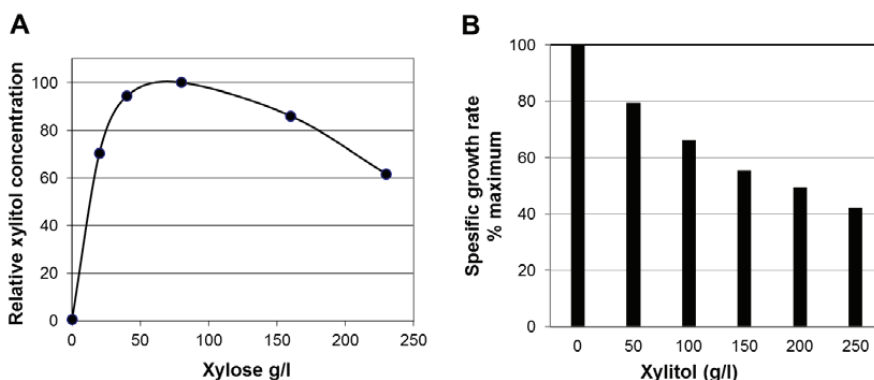
The *L. lactis* strain used was unable to utilize xylose, neither in the presence or absence of glucose (data not shown). It is known that majority of *L. lactis* strains used in dairy fermentations cannot utilize xylitol due to the accumulation of mutations in genes encoding for enzymes of xylose metabolism (Erlandson *et al.*, 2000). The fact that the yield of xylitol from xylose was nearly quantitative may have been a consequence of this. Since it was possible that xylose transport has also been affected by such mutations, an attempt was made to enhance the influx of xylose by co-expression of the xylose-H<sup>+</sup>-symporter (XylT) of *Lactobacillus brevis* together with the xylose reductase. The resulting strain, LLXTXR, produced both proteins in active form. The activities of both the enzyme and the transporter protein were determined from the coexpression strain as well as from strains expressing the genes singly. The results showed that the activities of the coexpressed proteins were 61 % and 60 % for XR and XylT respectively, of the activities of the singly expressed proteins. However, the coexpression of XylT did not improve the xylitol production by resting cells to any significant extent, possibly due to the lowered activity of XR or because xylose transport was originally not limiting. Hence it seems that some form of xylose transport takes place in *L. lactis* NZ9800.

A likely explanation to this would be the use of EII<sup>man/glc</sup> complex responsible for glucose transport in lactic acid bacteria, including *L. lactis* NZ9000 (Pool *et al.*, 2006). This complex has been shown to be responsible of facilitated diffusion of D-xylose in *Lactobacillus pentosus* (Chaillou *et al.*, 1999).

#### 4.1.2. Effects of environmental factors

The effect of pH on xylitol production by resting cells LLXR was investigated and the maximal productivity of xylitol was obtained at pH 8.3. This suggests that slightly alkaline conditions favor xylitol production, which is compatible with the previously reported observation that higher pH can result in considerably higher intracellular NADH/NAD<sup>+</sup> ratio (Snoep *et al.*, 1991). pH 7.0 was chosen for further production experiments in order to not risk cell viability.

The effect of different initial xylose concentrations on the production of xylitol was studied with resting cells of LLXTXR. The results are shown in **Figure 11A**, and it can be seen that the cells produce more xylitol as initial xylose concentration is increased from 0 g/l to 100 g/l. However, at initial xylose concentrations higher than this, the xylitol production was decreased. The addition of the well-known osmoprotectant (Galinski, 1993; Molenaar *et al.*, 1993) glycine betaine (4 mM) into the cell suspensions did not significantly improve the xylitol productivity at high xylose concentrations (data not shown).



**Figure 11.** **A)** The effect of initial xylose concentration on the production of xylitol by LLXTXR cells. The values are presented as final concentrations of xylitol obtained relative to the maximum. **B)** The effect of xylitol concentration on the growth of LLXR cells. The specific growth rates are presented relative to the value with no added xylitol.

The effect of xylitol on growth of LLXTXR cells is shown in **Figure 11B**. It was shown that at high concentrations of xylitol the growth of *L. lactis* was clearly inhibited. According to Verduyn *et al.* (1985), no product inhibition could be detected with xylitol concentrations up to 75 g/l with xylose reductase from *Pichia stipitis*. Possibly the osmotic stress caused by xylitol in the medium is inhibiting the growth of the cells.

### 4.1.3. Batch and fed batch production of xylitol

The production of xylitol from xylose under micro-aerobic conditions was studied. Glucose was added as a carbon source for continuous regeneration of NADH. Batch and glucose-limited fed batch modes were studied. A summary of the results is presented in **Table 2**. According to the results, the specific productivity of xylitol in the fed batch mode was 70 % of that in the batch. However, the ratio of xylitol produced per glucose consumed was 10 times higher in the fed batch fermentation. The yield of xylitol from xylose was in effect quantitative in all production experiments.

**Table 2.** The production of xylitol by *L. lactis* cells: comparison between strains and production modes. The initial xylose concentration was 25 g/l. The experiments were performed in duplicates.

Strain	Mode	Incubation time h	Initial cell dry weight g/l	Specific productivity g/(g·h)	Yield xylitol/xylose mol/mol	Xylitol production/ glucose consumption mol/mol
LLXR	Batch <sup>a</sup>	5	23.5 ± 0.1	0.12	0.98 ± 0.05	0.22 ± 0.03
	Fed batch <sup>b</sup>	8	24.7 ± 0.2	0.086	0.98 ± 0.05	2.3 ± 0.04
LLXTXR	Fed batch <sup>b</sup>	8	27.4 ± 0.6	0.092 ± 0.015	1.00 ± 0.01	2.7 ± 0.3

<sup>a</sup> Initial glucose and M17 concentrations were 75 g/l and 2.0 g/l, respectively.

<sup>b</sup> Glucose and M17 were supplied at a feed-rate of 1.0 g/(l·h) and 0.25 g/(l·h) per suspension volume, respectively.

Production of xylitol at the initial xylose concentration of 160 g/l under glucose limitation was performed in two parallel experiments. The results are shown in **Figure 12A**. The volumetric productivity decreased as the experiment progressed. Also the ratio of xylitol produced per glucose consumed decreased. The volumetric productivity was 2.72 g/(l·h) after 20 hour incubation. At this point the xylitol yield from xylose was quantitative, 34 % of the initial xylose amount was consumed, and xylitol produced per glucose consumed was 2.51 mol/mol. At 40.5 h the volumetric productivity was 1.83 g/(l·h) and the ratio of xylitol produced per glucose consumed was 1.02 mol/mol. The yield from xylose was still quantitative and a xylose conversion of 50 % was achieved.

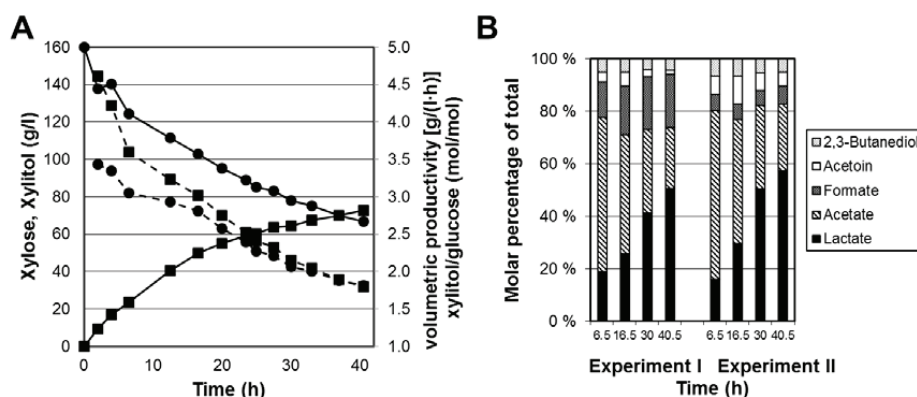
The medium was also analyzed for common fermentation metabolites. The distribution of metabolites is shown in **Figure 12B**. For an unknown reason there was a clear difference between the results in the two production experiments. However, an apparent trend in the behavior of the cells can be seen. At early stages of the experiment, acetate was the main product (61 % at 6.5 h) and lactate accounted for 20 % of the fermentation metabolites. However, at 40.5 h lactate accounted for 50 % and acetate only for one fifth of the metabolites.

It is known that in *L. lactis* pyruvate is reduced to lactate and one NADH is oxidized to NAD<sup>+</sup> during normal homolactic fermentation. When changes in the fermentation conditions lead to the depletion of intracellular NADH, pyruvate can be



## Results and Discussion

rerouted to other metabolites. In a reaction catalyzed by pyruvate formate lyase one pyruvate molecule reacts with coenzyme A forming acetyl-coenzyme A and formate. The acetyl-coA can then be converted to acetate and one equivalent of ATP is formed. Thus the formation of acetate also leads to higher energy yield from glucose (Axelsson, 1998). The shift to this mixed acid fermentation has previously been shown for non-growing cells under anaerobic conditions and glucose limitation (Fordyce *et al.*, 1984; Melchiorson *et al.*, 2002).



**Figure 12.** Behavior of *L. lactis* LLXTXR cells in a glucose limited fed batch fermentation under micro-aerobic conditions. **A)** Xylose consumption —●—, xylitol production —■—, volumetric productivity of xylitol —■— and ratio of xylitol produced per glucose consumed —●—. **B)** Distribution of fermentation metabolites relative to the total amount determined in the two experiments. Ethanol and carbon dioxide were not analyzed.

In the fed batch experiments the reduction of xylose to xylitol caused the accumulation of  $\text{NAD}^+$  inside the cell. The pyruvate formate lyase pathway of *L. lactis* was activated. Acetate was formed from acetyl-CoA and simultaneously ATP was formed. This led to a higher energy yield from glucose, which can be seen as a high ratio of xylitol produced per glucose consumed. Also the diacetyl/acetoin pathway was activated, and both acetoin and 2,3-butanediol were formed. As there was an excess of  $\text{NAD}^+$ , only a small amount of lactate was formed. As the experiment progressed, the pyruvate formate lyase pathway became less active. In the end of the experiment acetoin was no longer produced, but it was still reduced to 2,3-butanediol. The main product from pyruvate was lactate, and the product distribution approached that of homolactic fermentation.

The equilibrium of the reaction between xylitol and xylose with  $\text{NAD}^+$  and  $\text{NADH}$  as the cofactor has been shown to be strongly on the side of xylitol under physiological conditions ( $K_{\text{eq}} = 5.5 \cdot 10^9$ , Rizzi *et al.*, 1988). Thus the reaction should proceed despite the accumulation of xylitol. Although no product inhibition of the *P. stipitis* xylose reductase has been reported for up to 76 g/l (0.5 M) xylitol (Verduyn *et al.*, 1985), according to our results (**Figure 11B**) already at 50 g/l xylitol clearly inhibits the

growth of *L. lactis*. This could lead to slower metabolism as xylitol accumulates in the medium, and a slower regeneration rate of NADH. Hence the unavailability of NADH for the reduction of xylose could be a limiting step.

#### 4.1.4. Comparison with earlier results

Currently xylitol is produced by chemical reduction of D-xylose. The nickel catalyst used is susceptible to poisoning and thus the xylose needs to be extremely pure (Aminoff *et al.*, 1978). Among microbes, yeasts are generally considered to be the most efficient xylitol producers. As shown in **Table 3** the highest productivities using native strains have been achieved with *Candida tropicalis* or *Debaryomyces hansenii* (also called *Candida famata*). However, the use of *Candida* in food industry is problematic due to the well-known pathogenic nature of some *Candida* strains (Fridkin and Jarvis, 1996). In addition, the highest productivities have been achieved using complicated processes (Granström *et al.*, 2007b).

Different recombinant strains have also been constructed in order to produce xylitol. Most efficient strains are summarized in **Table 4**. *Saccharomyces cerevisiae* has been used as a production host for recombinant production of xylitol due to its GRAS status and the availability of genetic tools for its manipulation. The highest productivity of 5.80 g/(l·h) with *S. cerevisiae* has been achieved using a continuous reactor. However, the authors reported, that the strain was genetically quite unstable (Roca *et al.*, 1996).

Prior to the current work on *L. lactis*, only very low productivities had been obtained using natural or recombinant bacterial strains. The highest productivity of 1.19 g/(l·h) had been reported for *Gluconobacter oxydans*, but in this work the xylitol yield from the substrate, arabitol, was only 0.25 g/g (Sugiyama *et al.*, 2002). Only recently, a study has been made with *Corynebacterium glutamicum*, where a productivity of 7.90 g/(l·h) was achieved. In this study, the strain was heavily engineered. To improve the xylose uptake of the strain, the *araE* gene encoding a pentose transporter derived from another strain of *C. glutamicum* was integrated into the genome. In order to eliminate consumption of NADH when pyruvate is converted to lactate, the lactate dehydrogenase gene was disrupted. A xylose reductase from *Candida tenuis* with a single site mutation in order to improve coenzyme specificity was expressed in the strain. It has been shown that xylitol-phosphate can inhibit the growth of micro-organisms, and the xylulokinase gene was deleted in order to eliminate the conversion of xylitol to xylitol-phosphate. Additionally, the phosphoenolpyruvate-dependent fructose phospho-transferase (PTS<sup>fru</sup>) system is in charge of transporting and probably also phosphorylating xylitol. In order to further improve xylitol productivity, the PTS<sup>fru</sup> system was deleted (Sasaki *et al.*, 2010). These two deletions

**Table 3.** Xylitol production by natural yeast strains.

Strain	Xylose added g/l	Other substrates	Productivity g/(l·h)	Yield g/g	Conversion g/g	Final xylitol concentration g/l	Mode	Reference
<i>Candida parapsilosis</i>	300	-	3.18	0.70	0.70	210	batch	Kim <i>et al.</i> , 1997
<i>Debaryomyces hansenii</i>	279	glucose	4.60	0.79	0.79	221	batch	Dominguez <i>et al.</i> , 1997
<i>Candida tropicalis</i>	300	-	3.79	0.83	0.83	250	fed batch	Kim <i>et al.</i> , 1998
<i>Candida tropicalis</i>	270	glucose	4.56	0.93	0.93	251	fed batch	Oh and Kim, 1998
<i>Candida tropicalis</i>	n.r.	glucose + YE <sup>a</sup>	4.94 <sup>b</sup>	0.82	n.r.	189	cell recycling	Choi <i>et al.</i> , 2000
<i>Candida tropicalis</i>	200	glucose	3.66	0.87	0.86	172	batch	Kwon <i>et al.</i> , 2006a
<i>Candida tropicalis</i>	260	glucose	4.88	0.90	0.90	234	fed batch	Kwon <i>et al.</i> , 2006a
<i>Candida tropicalis</i>	214	-	12.0	0.85	0.85	182	cell recycling <sup>c</sup>	Kwon <i>et al.</i> , 2006a

n.r. Not reported

<sup>a</sup> YE = Yeast extract<sup>b</sup> Calculated from xylitol producing period of the fermentation.<sup>c</sup> Submerged membrane bioreactor with suction pressure and air sparging. Values are averages of the recycle rounds.

**Table 4.** Xylitol production by recombinant yeasts and bacterial strains.

Yeast strains	Inserted activity	Xylose added g/l	Other starting materials	Productivity g/(l·h)	Yield g/g	Conversion g/g	Final xylitol concentration g/l	Mode	Reference
<i>Saccharomyces cerevisiae</i>	<i>P. stipitis</i> XR	100	ethanol	1.00	n.r.	0.93	95	two step batch	Meinander <i>et al.</i> , 1994
<i>Saccharomyces cerevisiae</i>	XR	50 <sup>a</sup>	glucose	5.80	1.03	0.42	15 <sup>a</sup>	continuous	Roca <i>et al.</i> , 1996
<i>Saccharomyces cerevisiae</i>	<i>P. stipitis</i> XR	n.r.	glucose	1.69	n.r.	n.r.	105	fed batch	Lee <i>et al.</i> , 2000
<i>Saccharomyces cerevisiae</i>	<i>P. stipitis</i> XR	141 <sup>b</sup>	glucose+YE <sup>c</sup>	2.34	0.97	0.82	116	cell recycle	Bae <i>et al.</i> , 2004
<i>Saccharomyces cerevisiae</i>	<i>P. stipitis</i> XR + <i>S. cerevisiae</i> G6PDH <sup>d</sup>	100	glucose	2.00 <sup>e</sup>	0.97	0.86	86	fed batch	Kwon <i>et al.</i> , 2006b
<i>Candida tropicalis</i>	chemical mutagenesis	237	glycerol + glucose	3.28	0.93	n.r.	220	fed batch	Jeon <i>et al.</i> , 2011
<b>Bacterial strains</b>									
<i>Gluconobacter oxydans</i>	<i>G. oxydans</i> XDH	-	arabitol + ethanol	1.19	0.26	0.25	57	batch	Sugiyama <i>et al.</i> , 2003
<i>Lactococcus lactis</i>	<i>P. stipitis</i> XR	160	glucose + M17	2.72	1.04	0.34	55	fed batch	Study I
<i>Bacillus subtilis</i>	<i>C. difficile</i> XPDH <sup>f</sup>	-	glucose	n.r.	0.23	n.r.	23	batch	Povelainen and Miasnikov, 2007
<i>Corynebacterium glutamicum</i>	<i>P. stipitis</i> XR	60	glucose	0.90	0.97	0.57	34	fed batch	Kim <i>et al.</i> , 2010
<i>Corynebacterium glutamicum</i>	<i>C. tenuis</i> XR + <i>C. glutamicum</i> <i>araE</i> <sup>g</sup>	250	glucose	7.90	0.72	0.66	166	fed batch	Sasaki <i>et al.</i> , 2010

n.r. Not reported

<sup>a</sup> Continuous feed/output<sup>b</sup> Estimated from figures<sup>c</sup> YE = Yeast extract<sup>d</sup> G6PDH = Glucose 6-phosphate dehydrogenase<sup>e</sup> Calculated from xylitol producing period of the fermentation<sup>f</sup> XPDH = xylitol phosphate dehydrogenase<sup>g</sup> *araE* = gene encoding for pentose transporter gene, additionally three genes were disrupted as explained in Chapter 4.1.4.

presumably inhibited the majority of xylitol phosphorylation as the productivity of xylitol improved with each deletion.

In most reports with *Candida* yeasts, the xylose has been almost completely consumed, whereas in the fed batch fermentation of the current work only 34 % of the initial xylose was consumed during 20 hours. After this the production rate slowed down, as the sugar metabolism shifted towards homolactic fermentation. With yeasts the yield from xylose consumed range from 70 to 90 mol-%, whereas with the *L. lactis* strains the yields were equimolar, because glucose was used as the cosubstrate. Additionally the results in several reports indicate that after the xylose is consumed, naturally xylitol utilizing yeasts start to degrade xylitol. *L. lactis* on the other hand is not able to metabolize the xylitol it produces, making the process easier to control.

## **4.2. Purification and characterization of recombinant xylitol-4-dehydrogenase (II)**

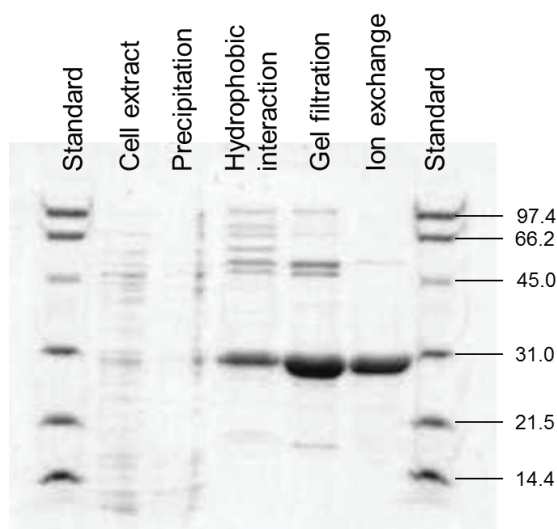
### **4.2.1. Isolation of xylitol-4-dehydrogenase from *Pantoea ananatis* and cloning the corresponding gene**

Xylitol-4-dehydrogenase was isolated from *P. ananatis* by ammonium sulfate fractioning, four chromatographic steps, native gel electrophoresis and SDS-PAGE as described in Materials and Methods. The molecular mass was estimated to be 30 kDa by SDS-PAGE. The results from the N-terminal sequencing of the intact protein were used to design a degenerate oligonucleotide. Using *P. ananatis* ATCC 43073 *Hind*III Vectorette amplicons as a template, three PCR products were obtained with the degenerate primer and Vectorette primers. The PCR products ligated to pCR2.1-TOPO vector and cloned in *E. coli* TOP10. After sequencing with vector-specific primers, one of the products appeared to contain part of the putative xylitol dehydrogenase gene. To isolate the rest of the *xdh* gene encoding the 30-kDa protein, *Eco*RI and *Hind*III digested *P. ananatis* ATCC 43072 Vectorette amplicons and PCR primers specific for the putative *xdh* gene and the Vectorette linkers were used to amplify and sequence the *xdh* gene region.

### **4.2.2. Production of xylitol-4-dehydrogenase in *Escherichia coli* and purification of the recombinant enzyme**

The *E. coli* strain ERF2157 expressing the xylitol-4-dehydrogenase gene from *Pantoea ananatis* was used to produce recombinant xylitol-4-dehydrogenase. The enzyme was purified approximately 16-fold by ammonium sulfate fractionation and three chromatographic steps. As seen in **Figure 13**, practically no impurities could be detected in the SDS-PAGE of the purified enzyme. The molecular mass of the enzyme estimated from the gel was 29 kDa, which corresponds well with the molecular mass of

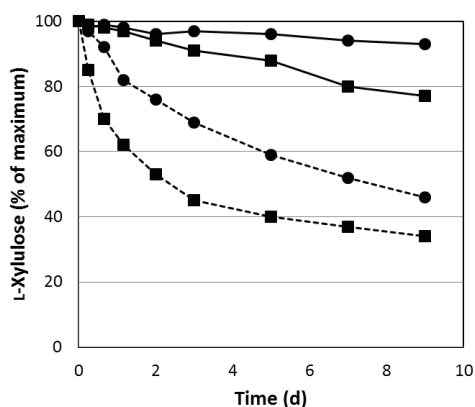
28.0 kDa calculated on the basis of the amino acid sequence. A molecular mass of 100 kDa was determined for the native enzyme by analytical gel filtration, which suggests that the enzyme is either a trimer or a tetramer.



**Figure 13.** SDS-PAGE from the purification of recombinant xylitol-4-dehydrogenase produced by *E. coli* ERF2157 cells.

#### 4.2.3. Stability of L-xylulose (unpublished data)

The stability of L-xylulose at different pHs at 37 °C was studied as described in Materials and Methods and the results are shown in **Figure 14**.



**Figure 14.** pH stability of L-xylulose. The incubations were carried out at 30 °C in citrate (100 mM) – Tris (100 mM) buffer at different pH-values. —●— pH 7.0; —■— pH 8.0; - -●- - pH 9.0; - -■- - pH 10.0.

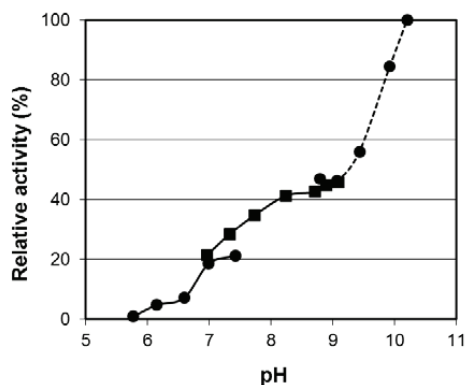
## Results and Discussion

It seems that L-xylulose is stable at pH 7.0 and 97 % of the sugar can be detected still after 48 hours. At pH 9.0, L-xylulose is somewhat less stable, and after 48 h incubation 76 % of the sugar originally present can be detected. Higher pH decreases the stability considerably.

### 4.2.4. Characterization of xylitol-4-dehydrogenase

To determine the metal dependence of recombinant xylitol-4-dehydrogenase, the enzyme was incubated with 10 mM EDTA (Ethylenediaminetetraacetic acid) to deplete  $Mg^{2+}$  and the buffer was changed by ultrafiltration and dilution to Tris-HCl (100 mM) buffer, pH 9.0. The metal-depleted apoenzyme was incubated in the presence of different divalent cations (only 10 mM  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$  or  $Ni^{2+}$  were used, other divalent cations formed precipitates under the conditions of the assay). It seems that  $Mg^{2+}$  and  $Ca^{2+}$  (70 % of maximal activity) act as cofactors of XDH. Without added metal cations the activity was only 10 % of the maximal level.

The effect of pH on the activity of XDH was also determined. As shown in **Figure 15**, maximal activity was achieved under alkaline conditions. However,  $NAD^+$  appeared to be unstable at very high pH values (data not shown), and reliable measurements could not be made above pH 10.5. In addition, according to our results xylulose was unstable at highly alkaline conditions. For these reasons the enzymatic activities in this study were routinely determined at pH 9.0.



**Figure 15.** The effect of pH on the activity of xylitol-4-dehydrogenase in different buffer systems. ●— 50 mM Potassium phosphate; ■— 50 mM Tris-HCl and ●--- 50 mM Tris-50 mM glycine-NaOH.

The substrate specificity of the enzyme was tested with different polyols and D- and L-xylulose as the substrates. The highest activity in the oxidizing direction was obtained with xylitol. Significant activity was also found towards D-threitol (62 % of the activity towards xylitol) and D-arabitol (27 %). The activities with D-sorbitol and L-threitol as

the substrates were low and no activity was detected towards other polyols. In the reducing direction the enzyme also had significant activity towards D-xylulose, 20 % of that on L-xylulose. The enzyme had a clear preference for NAD<sup>+</sup> over NADP<sup>+</sup>. The activity with NADP<sup>+</sup> as the cosubstrate was only 4 % of that with NAD<sup>+</sup>.

#### 4.2.5. Kinetic parameters

The purified recombinant XDH displayed Michaelis-Menten kinetics for xylitol and NAD<sup>+</sup> under the conditions used. The apparent  $K_m$  and  $V_{max}$  values determined are presented in **Table 5**. For comparison of the native enzyme with the recombinant one, the apparent  $K_m$  values for the partially purified native enzyme were also determined. These values were nearly identical for both substrates when error margins are taken into account. Xylitol showed distinct substrate inhibition at concentrations of over 100 mM (15 g/l). At 500 mM (76 g/l) xylitol concentration the initial velocity of the reaction was 85 % of that with 50 mM xylitol as the substrate.

**Table 5.** Apparent  $K_m$  and  $V_{max}$  values for the purified recombinant XDH and a partially purified native XDH.

Enzyme	Substrate with varied concentration	Substrate with fixed concentration	Apparent $K_m$ mM	$V_{max}$ nkat/mg
Purified recombinant XDH	Xylitol	NAD <sup>+</sup>	9.4 ± 0.7	700 ± 20
	NAD <sup>+</sup>	Xylitol	0.37 ± 0.05	790 ± 30
Partially purified native XDH	Xylitol	NAD <sup>+</sup>	11.0 ± 0.8	ND <sup>a</sup>
	NAD <sup>+</sup>	Xylitol	0.35 ± 0.02	ND <sup>a</sup>

<sup>a</sup> ND Not determined

#### 4.2.6. Comparison with earlier results

Several xylitol-4-dehydrogenases have been characterized previously. NADP<sup>+</sup>-linked xylitol-4-dehydrogenases have been identified from mammalian tissues (Arsenis and Touster, 1969; Ishikura *et al.*, 2001; Nakagawa *et al.*, 2002). An NAD<sup>+</sup>-specific xylitol-4-dehydrogenase has been reported for the yeast *Ambrosiozyma monospora* by Verho *et al* (2004) and for *Aeribacillus (Bacillus) pallidus* Y25 by Poonperm *et al.* (2007).

Doten and Mortlock (1985a) have reported the partial purification and characterization of the native xylitol-4-dehydrogenase from *Pantoea ananatis* ATCC43072. The results obtained with the recombinant XDH in the current study and with the native enzyme described in the previous report differ to some extent. Firstly, the use of metal cations in the buffers or activity assays was not reported for the native enzyme. However, in the current work, the attempts to purify the recombinant enzyme without added Mg<sup>2+</sup> led to total loss of activity (data not shown). Secondly, the



## Results and Discussion

molecular mass determined previously for the native enzyme by nondenaturing polyacrylamide gel electrophoresis was 136 kDa whereas the molecular mass of 100 kDa was obtained for the recombinant enzyme by analytical gel filtration in the present study. The results obtained for the recombinant XDH suggest that the enzyme had significant side activity towards D-xylulose, which was not reported by Doten and Mortlock (1985a).

The apparent  $K_m$  value for the native xylitol-4-dehydrogenase from *P. ananatis* presented in the previous report was five-fold higher than what was determined for the recombinant enzyme in the current work. However, in the previous report the cosubstrate  $NAD^+$  was used at a lower concentration, which may partly explain the difference in the values obtained. However, in general, the many differences between the results for the native and the recombinant enzyme cannot be explained at the moment.

The  $NADP^+$ -linked xylitol-4-dehydrogenases from mammalian tissues have apparent  $K_m$  values for xylitol ranging from 10 to 40 mM (Arsenis and Touster, 1969; Ishikura et al., 2001; Nakagawa et al., 2002). These are of the same order of magnitude as the current result. The kinetic parameters reported for the XDH from *A. monospora* are also similar to the XDH from *P. ananatis*. The XDH from *A. pallidus* Y25 has been previously produced in *E. coli*. The  $V_{max}$  value for the enzyme from *A. pallidus* is significantly lower than the  $V_{max}$  value for XDH from *P. ananatis*, even though the reaction with the *A. pallidus* enzyme was carried out at a 10 °C higher temperature. However, the  $K_m$  values for the enzyme from *A. pallidus* are significantly smaller.

The molecular masses of the different XDH monomers are very similar. The oligomeric molecular masses have not been reported for the enzymes from other sources than *P. ananatis*. The optimum for activity of *A. pallidus* Xhd has been determined, and the pH optimum is very similar to that of the XDH from *P. ananatis* (over pH 10). The cofactor preferences of the two enzymes show slight differences, as shown in **Table 6**. However, the activity with  $Mn^{2+}$  was not determined in the present work, as precipitates were formed under the assay conditions.

In the reducing direction the activity of the XDH from *A. monospora* was 100 times higher for L-xylulose than for D-xylulose, whereas for the recombinant *P. ananatis* XDH described in the current study the activity was determined as 5 times higher for L-xylulose. The ratio of activities towards the L- and D-forms of xylulose of the enzyme from *A. pallidus* Y25 has not been studied.

**Table 6.** Comparison of characteristics between different xylitol-4-dehydrogenases.

Source organism	Kinetic parameters					Molecular weight	pH and temperature optimum	Cofactors	Polyol substrates	Reference
	Substrate with varied concentration	Substrate with fixed concentration	Apparent $K_m$ mM	$V_{max}$ nkat/mg	T and pH used					
<i>P. ananatis</i>	xylitol	NAD <sup>+</sup> (1.8 mM)	9.4	700	30 °C pH 9.0	100 kDa monomer 28 kDa	pH > 10	Mg <sup>2+</sup> >Ca <sup>2+</sup>	xylitol > D-threitol > D-arabitol	this study
	NAD <sup>+</sup>	xylitol (94 mM)	0.37	790						
<i>P. ananatis</i>	xylitol	NAD <sup>+</sup> (0.83 mM)	48		25 °C pH 8.9	136 kDa				Doten and Mortlock, 1985a
<i>A. monospora</i>	xylitol	NAD <sup>+</sup> (2 mM)	7.2	630	30 °C pH 9.0	monomer 30 kDa			xylitol > D-arabitol	Verho <i>et al.</i> , 2004
	L-xylulose	NADH (0.2 mM)	9.6	1700	30 °C pH 7.0					
<i>A. pallidus</i> Y25	xylitol	NAD <sup>+</sup> (25 mM)	2.1	98						
	NAD <sup>+</sup>	xylitol (100 mM)	0.034		40 °C pH 10	monomer 28 kDa	55 °C pH 11.0	Mn <sup>2+</sup> >Ca <sup>2+</sup> >Mg <sup>2+</sup>	xylitol > D-threitol > D-Iditol	Takata <i>et al.</i> , 2010
	L-xylulose	NADH (25 mM)	1.3	40						

It seems that all these three enzymes have very similar substrate specificities, as all of them prefer a polyol substrate that has a hydroxyl groups in the L- and D-configurations in C-2 and C-3, respectively. However, no activity towards hexitols was reported for the enzyme from *A. monospora*, whereas in the present work with the *P. ananatis* XDH slight activity towards D-sorbitol could be detected. In the reducing direction, the *P. ananatis* also seems to be a bit more robust than the other two enzymes, as it also reduces D-xylulose in addition to L-xylulose.

### 4.3. Co-expression of *XYL1* and *xdh*

As described above, xylitol can be produced from D-xylose efficiently in *L. lactis* containing the xylose reductase encoded by *XYL1* gene from *P. stipitis*. L-Xylulose on the other hand can be produced from xylitol by *E. coli* harboring the xylitol-4-dehydrogenase from *P. ananatis*.

In order to produce L-xylulose from D-xylose inside the cell, an attempt was made to express *XYL1* and *xdh* in the same operon with the ribosomal binding site of the latter between the genes. Thus two separate proteins – xylose reductase and xylitol-4-dehydrogenase – would be synthesized and L-xylulose would be produced from D-xylose via xylitol. The coenzyme NADH/NAD<sup>+</sup> would also be circulated in the reaction between the reduced and oxidized forms.

According to the sequencing analysis, the *XYL1+xdh* operon was successfully constructed. In addition, *E. coli* XL1-Blue containing pQE-60 plasmid with the *XYL1+xdh* fragment showed both XR and XDH activity. The cell extract of the most active clone had specific activities of 10.8 nmol/(s·mg) and 10.2 nmol/(s·mg) towards XR and XDH, respectively. This results in specific activities corresponding to 68 % of XR activity and 18 % of XDH activity compared to singly expressed enzymes.

The ability of resting cells of this strain to convert D-xylose or xylitol was tested in an overnight reaction. Starting from 10 g/l D-xylose, the cells produced 0.42 g/l xylitol and 0.40 g/l L-xylulose. When 10 g/l xylitol was used as a starting material the cells produced 3.2 g/l L-xylulose and 0.31 g/l xylose in an overnight reaction. Of added D-xylose, 31 % and of added xylitol, 29 % was used in other metabolic pathways.

Since only very small amount of L-xylulose was produced by the cells with the *XYL1+xdh* operon, the genes were cloned into a vector under the control of separate promoters. As the pACYC-Duet vector used needs a host with a genomic copy of the gene encoding the T7 RNA-polymerase, HB101 cells were  $\lambda$ DE3-lysogenized to construct HB101(DE3). The *XYL1* and *xdh* genes were cloned into pACYC-Duet and the resulting plasmid was transformed into HB101(DE3)-cells. The resting cells of this strain were used for an overnight conversion reaction. With 10 g/l D-xylose as a substrate, 0.25 g/l xylitol and 0.34 g/l L-xylulose was formed. 75 % of the xylose added was lost during the reaction, so it seems that the strain can still utilize xylose to some

extent. When 10 g/l xylitol was used as the substrate, 8.0 g/l L-xylulose was formed in an overnight reaction.

To conclude, the attempt to produce L-xylulose from D-xylose by expressing the two genes, *XYL1* and *xdh* into the same cell, was not successful. The activities of the enzymes expressed together remained much lower than those of singly expressed enzymes. This might be based on the burden from overexpressing several enzymes. Even though both enzymes showed some activity in the recombinant strains constructed, none of the strains could convert D-xylose either to xylitol or L-xylulose.

As the recombinant *E. coli* HB101(DE3) strain containing the construct with pACYC-Duet and the two genes was able to produce xylulose from xylitol with a good yield, an HB101(DE3) strain was constructed containing the pACYC-Duet plasmid and *xdh* in the second cloning site. The strain was named BPT228. This strain showed only minimal XDH-activity, 0.22 nmol/(s·mg), but in an overnight reaction it was able to produce 8.1 g/l L-xylulose from 10 g/l xylitol using resting cells. Since BPT228 was significantly more effective in producing L-xylulose than *E. coli* ERF2157, it was chosen for the production experiments.

#### 4.4. Production of L-xylulose (II, III)

##### 4.4.1. Purification of L-xylulose

L-Xylulose was produced from xylitol by resting recombinant *E. coli* ERF2157 cells. The sugar was purified from the production medium by filtration through activated carbon, anion and cation exchange to remove ionic compounds. A cation exchange column in Ca<sup>2+</sup> form was used to separate xylitol and L-xylulose. No peaks corresponding to significant impurities or by-products were present in the HPLC chromatogram of the purified sugar. The specific optical rotation of the purified xylulose in H<sub>2</sub>O at 20 °C was +32.6°, and those of pure, commercially available D- and L-xylulose were -29.4 ° and +26.5 °, respectively, under the same conditions.

Although our results suggest that the XDH enzyme has activity for D-xylulose in the reducing direction, no evidence for the presence of D-xylulose was found in the sugar produced from xylitol by the recombinant *E. coli* cells. A similar production experiment has also been carried out previously using resting cells of *Pantoea ananatis* ATCC 43074 mutant as the catalyst (Doten and Mortlock, 1985c). In this experiment the analysis also suggested that no D-xylulose was produced. If some D-xylulose would be formed, it is possible that *E. coli* could phosphorylate it to xylulose-5-phosphate, which can be utilized in the metabolic pathways of the cell. However, as the yields of xylulose from xylitol were quantitative, it seems unlikely that any D-xylulose would be produced.

#### 4.4.2. Intracellular metabolites

The equilibrium constant of the XDH-catalyzed reaction can be presented by the equation:

$$K_{eq} = \frac{[\text{L-xylulose}] [\text{NADH}] [\text{H}^+]}{[\text{xylitol}] [\text{NAD}^+]} \quad (3)$$

According to previous results, the value for  $K_{eq}$  of the reaction is  $6.9 \cdot 10^{-11}$  mol/l (Rizzi *et al.*, 1989), meaning that the thermodynamic equilibrium between xylulose and xylitol is strongly on the side of xylitol. However, in the current experiments, the yield of L-xylulose from xylitol was very high when resting cells were used. As  $\text{NAD}^+$ , NADH and  $\text{H}^+$  are also substrates in the reaction; low intracellular NADH/ $\text{NAD}^+$ -ratio might contribute to the efficient production of L-xylulose. To study this, the intracellular molar ratios of xylitol to L-xylulose and NADH to  $\text{NAD}^+$  were analyzed.

As shown in **Table 7**, the results suggest that the intracellular ratio of xylulose to xylitol is about one third of the extracellular ratio throughout the experiment. The presence of xylitol had very little effect on the intracellular ratio of NADH to  $\text{NAD}^+$  during the first hours of the reaction. At 22 hours the relative concentration of NADH is 1.5 times higher in the presence of xylitol.

**Table 7.** Molar ratios of intracellular and extracellular metabolites in an experiment with an initial xylitol concentration of 10 g/l using *E. coli* BPT228. Control samples without added xylitol.

Time h	Intracellular metabolites			Extracellular metabolites	Extracellular pH	Intracellular pH
	Samples		Control			
	xylulose/xylitol	NADH/ $\text{NAD}^+$	NADH/ $\text{NAD}^+$	xylulose/xylitol		
0	0.041	0.155	0.154	0.21	6.7	7.51
1	0.070	0.097	0.098	0.21	5.1	7.35
3	0.076	0.044	0.040	0.24	6.0	7.44
22	0.527	0.077	0.053	1.66	7.0	7.54

According to the relationship reported previously for resting cells of *E. coli* the intracellular pH can be estimated from the extracellular pH (Slonczewski *et al.*, 1981). The estimated intracellular pHs are presented in. Substituting the right hand side of equation 3 with the estimated intracellular ratios of the reactants and the hydrogen ion concentration corresponding to the estimated intracellular pH gives values  $3.0 \cdot 10^{-10}$  mol/l,  $1.2 \cdot 10^{-10}$  mol/l and  $1.2 \cdot 10^{-9}$  mol/l at 1 h, 3 h and 22 h, respectively. These are of the same magnitude as the equilibrium constant and the productivity of L-xylulose seems to occur near the equilibrium.

The total amount of intracellular xylitol and xylulose was constant throughout the experiment. The ratio of NADH to NAD<sup>+</sup> decreased over threefold in the first three hours both in the absence and presence of xylitol. Possibly the efficient aeration resulting from high shaking speed led to a quick decrease in the ratio.

Aeration is required for the dehydrogenation of xylitol to L-xylulose by resting *E. coli* BPT228 cells. It has been reported, that under aerobic conditions *E. coli* uses NADH dehydrogenases from their respiratory chains for NAD<sup>+</sup> regeneration, with oxygen being the final electron acceptor (Melo *et al.*, 2004; Yagi *et al.*, 1998). It is apparent that the NAD<sup>+</sup> used in the dehydrogenation reaction is efficiently regenerated by aeration.

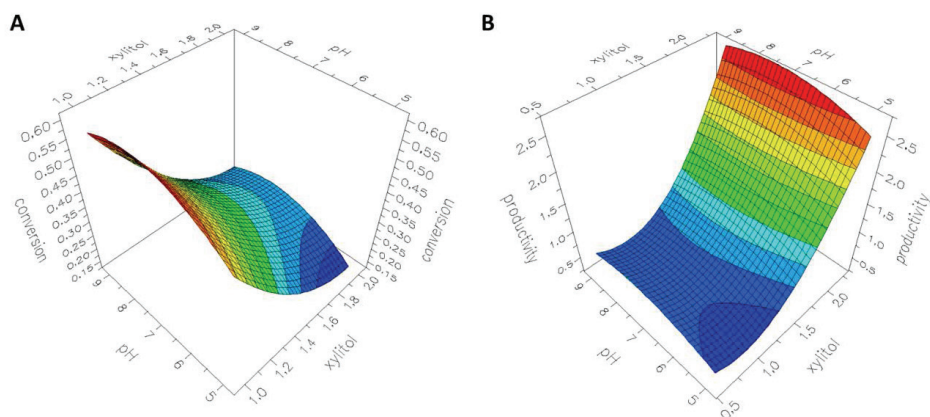
#### 4.4.3. Effects of reaction conditions on the production of L-xylulose

A CCC experiment design was used to investigate L-xylulose production by resting cells of *E. coli* BPT228 at a high cell density after 12 h of incubation. Temperature was held constant at 37 °C, and pH, shaking speed and initial xylitol concentration were used as the variables. The statistical coefficients, presented in **Table 8**, indicate that the models for conversion and volumetric productivity were highly significant and able to predict conversion and productivity at certainty levels of 72 % and 83 %, respectively. In both models the effect of shaking speed was positive but very small.

**Table 8.** Statistical coefficients for the conversion and volumetric productivity of L-xylulose from xylitol by resting cells of *E. coli* BPT228. pH, shaking speed and initial xylitol concentration were used as the variables in the CCC model and temperature, pH and initial xylitol concentration in the CCF model.

Coefficient	CCC model		CCF model	
	Conversion	Productivity	Conversion	Productivity
R <sup>2</sup>	0.933	0.941	0.995	0.977
Q <sup>2</sup>	0.716	0.828	0.990	0.942
P	< 0.001	< 0.001	< 0.001	< 0.001
P <sub>lack of fit</sub>	0.145	0.103	0.238	0.337

The response surface models for conversion and productivity are presented in **Figure 16**. According to the models, a nearly neutral pH (7.7) was optimal for both conversion and productivity. As expected, a low initial xylitol concentration (10 g/l) was optimal for achieving high L-xylulose conversion. On the other hand, productivity was higher at high initial xylitol concentrations. Since the optimum for productivity was outside the investigated region of xylitol concentrations, we used another experiment design with a different range of xylitol concentrations.

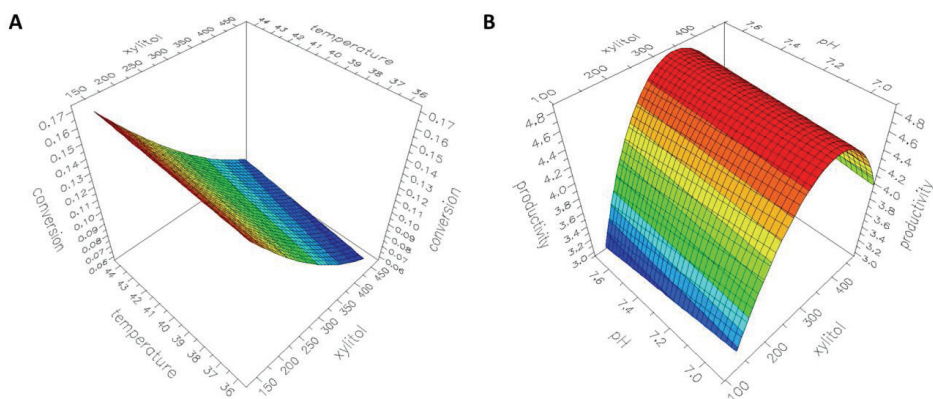


**Figure 16.** Response surface models of the effects of pH and xylitol concentration on **A**) conversion (mol/mol) and **B**) productivity [g/(l·h)] with resting cells of *E. coli* BPT228. Xylitol concentration is presented on a logarithmic scale. The shaking speed was 250 rpm in both models.

In the second experiment design the effect of temperature was also investigated, and shaking speed was not taken as a variable as it had only a small effect. A CCF experiment design with temperature, pH and initial xylitol concentration was made with additional experiments performed for the star points of pH and initial xylitol concentration. For each experiment, a time point was determined at which the concentration of L-xylulose increased by 7 % per hour. Conversion and volumetric productivity of L-xylulose were determined at these time points.

The coefficients for the models for productivity and conversion are presented in **Table 8** and the response surface models are presented in **Figure 17**. These statistical coefficients indicate that the models were highly significant and able to predict conversion and volumetric productivity at certainty levels of 99 % and 94 %, respectively. The effect of pH was very small or negligible in these models. The results suggest that the optimum temperature for volumetric productivity would be outside the investigated area (over 44 °C). Although the temperature affected the productivity significantly, it had very little effect on the conversion. The optimum of xylitol concentration for productivity was predicted to be at around 350 g/l.

It is surprising that although xylitol clearly exhibits substrate inhibition on xylitol-4-dehydrogenase activity at concentrations above 15 g/l, the optimal initial xylitol concentration for productivity was 350 g/l. This was near the highest xylitol concentration that did not affect cell viability (400 g/l, data not shown). According to previous reports, xylitol can be transported to *E. coli* cells via the glycerol diffusion facilitator protein (glpF) and the rate of transport is diffusion-limited (Heller *et al.*, 1980). If there is no specific transport mechanism for xylitol, it would seem possible that the intracellular xylitol concentration does not rise to an inhibitory level even when



**Figure 17.** Response surface models of the effects of temperature, pH and xylitol concentration on **A)** conversion (the effect of pH was negligible) and **B)** productivity (temperature 44 °C) with resting cells of *E. coli* BPT228.

extracellular xylitol level is high, and the optimal xylitol concentration for productivity is dictated by the cell viability.

As expected, better conversions can be obtained at lower xylitol concentrations. As a compromise between volumetric productivity and conversion, the scale-up experiment in the 2 liter bioreactor was conducted at 250 g/l of initial xylitol. Temperature of 40 °C was chosen for the scale-up experiment because of the poor stability of L-xylulose at alkaline pH and high temperatures (data not shown). Additionally the viability of *E. coli* cells declines rapidly at 44 °C. Already at 42 °C the viability of the cells starts to diminish (data not shown).

The productivity at the 7 % hourly L-xylulose increase was  $1.50 \pm 0.14$  g/(l·h). The model presented above predicted a total productivity of  $3.76 \pm 0.19$  g/(l·h). However, the cell density was significantly different in the bioreactor compared to the modeling experiments. The corresponding specific productivity of  $1.09 \pm 0.10$  g/(g·h) obtained in the bioreactor corresponds reasonably well with the specific productivity of  $0.77 \pm 0.04$  g/(g·h) predicted by the model. The results suggest that scaling up this process would most likely be straightforward.

#### 4.4.4. Comparison with earlier results

There are several strains that have been used for the bioconversion of xylitol to L-xylulose. Data on these is summarized in **Table 9**. It is difficult to properly compare different production strains reported, since data on the specific productivities is only rarely available. However, the specific productivity of resting cells of *Alcaligenes* sp. 701B (Khan *et al.*, 1991) has been reported, and this was significantly lower, only one fourth of the specific productivity of BPT228.



**Table 9.** Strains used for the bioconversion of xylitol to L-xylulose.

Strain (host of recombinant gene)	Initial xylitol concentration g/l	pH, T, time	Final L-xylulose concentration g/l	Conversion mol/mol	Volumetric productivity g/(l·h)	Specific productivity g/(g·h)	Side products	Reference
<i>Pantoea ananatis</i>	5	pH 7; 30 °C; 18 h	3.2	0.65	0.18	n.r. <sup>c</sup>	no	Doten and Mortlock, 1985c
<i>Alcaligenes</i> sp. 701B	5	pH 7.0; 30 °C; 24 h	4.0	0.81	0.17	0.26	no	Khan <i>et al.</i> , 1991
Recombinant <i>E. coli</i> ( <i>P. ananatis</i> ) <sup>a</sup>	250	pH 8.0; 40 °C; 10 h	15.0	0.061	1.5	1.09	no	This study
Recombinant <i>E. coli</i> ( <i>P. ananatis</i> ) <sup>b</sup>	440	pH 7.0; 44 °C; 10 h	37.3	0.086	3.7	n.r.	no	This study
<i>Aeribacillus pallidus</i> Y25	100	pH 9.0; 50 °C; 12 h	27.6	0.27	2.3	n.r.	L-xylulose, L-arabitol	Poonperm <i>et al.</i> , 2007
Recombinant <i>E. coli</i> ( <i>A. pallidus</i> )	100	pH 10; 37 °C; 24 h	24.0	0.24	1.0	n.r.	L-xylulose, L-ribulose	Takata <i>et al.</i> , 2010

<sup>a</sup> BPT228, scale-up experiment in a 2 liter bioreactor<sup>b</sup> BPT228, the optimization reaction with the best volumetric productivity<sup>c</sup> n.r. = not reported

*Aeribacillus pallidus* Y25 cells have also been used for the production of L-xylulose. This reaction was carried out at pH 9.0, 50 °C. It was reported, that when the incubation time was prolonged, the concentration of L-xylulose started to decline. Additionally, L-xylose and L-arabitol were produced as side products, presumably because of the presence of L-rhamnose isomerase and L-fucose isomerase activities in the cells (Poonperm *et al.*, 2007). In comparison, in the optimization experiment the best volumetric productivity obtained was 3.7 g/(l·h) whereas with *A. pallidus* Y25 a volumetric productivity of 2.3 g/(l·h) was achieved.

Recombinant *E. coli* cells harboring the *xdh* gene from *A. pallidus* Y25 have also been used in the conversion reaction (Takata *et al.*, 2010). However, the volumetric productivity was less than half of what was obtained with the natural *A. pallidus* Y25. In addition, L-xylose and L-ribulose were produced as side products.

In conclusion, the best productivities have been achieved with *E. coli* BPT228 and *A. pallidus* Y25. However, the high production rate using *A. pallidus* Y25 cells was reached at high temperature and alkaline pH. The results of the current study suggest that L-xylulose is unstable under alkaline conditions as shown in **Figure 14**. L-xylulose is also sensitive to elevated temperatures (data not shown). It is likely, that at least part of the decline in the L-xylulose concentration seen in the study with *A. pallidus* Y25 is due to the degradation of the product. In addition, the formation of side-products (L-xylose and L-arabitol) makes the purification of L-xylulose more difficult. No side products were detected in the reaction with *E. coli* BPT228.

## 4.5. Cloning and characterization of L-fucose isomerase (IV)

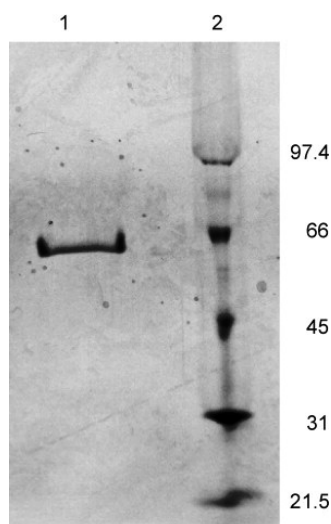
### 4.5.1. Production of recombinant L-fucose isomerase

It has been reported that the L-fucose isomerase from *Klebsiella aerogenes* is able to convert L-xylose to L-xylulose. However, the  $K_m$  of the enzyme towards L-xylose is only one tenth and the  $V_{max}$  only 1 % of that with L-fucose as the substrate. Additionally, L-xylose is inhibitory already at low concentrations (Oliver and Mortlock, 1971a; Oliver and Mortlock, 1971b). However, as reports on enzymes acting on L-xylose are rare, and as L-fucose isomerase is found in *Escherichia coli* (Lu and Lin, 1989), the *E. coli* fucose isomerase was cloned and overexpressed in order to study its activity towards L-xylulose.

The *E. coli* K12 L-fucose isomerase gene containing a His<sub>6</sub>-tag was overexpressed in *E. coli* XL1-Blue cells and the strain harboring the gene was named BPT244. The *fucI* gene was expressed at high levels in the cells. As estimated by SDS-PAGE the L-fucose isomerase represented roughly 20–40 % of the total protein in the cell extracts. The protein was purified by Ni-affinity chromatography, and no significant impurities could be detected in the SDS-PAGE of the purified protein, as shown in **Figure 18**. The

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molecular mass of the recombinant protein estimated from the gel was approximately 65 kDa, which corresponds to the value of 65 kDa calculated from the amino acid sequence of a monomer.



**Figure 18.** SDS-PAGE of His-tagged L-fucose isomerase purified by Ni-affinity chromatography. Lane 1: Purified FucI. Lane2: molecular weight marker (kDa).

Resting cells of *E. coli* BPT244 were able to convert L-xylulose to L-xylose. In an overnight reaction, starting with 10.6 g/l L-xylulose, the cells were able to produce 6.1 g/l L-xylose. Therefore, in these conditions, 58 % of L-xylulose was converted to L-xylose.

### 4.5.2. Kinetic properties

The recombinant L-fucose isomerase displayed Michaelis-Menten kinetics for L-xylulose with a  $K_m$  of 41 mM and a  $V_{max}$  of 3.9 nkat/mg. The turnover number ( $k_{cat}$ ) of 0.76 1/s was calculated taking into account that the hexameric L-fucose isomerase contains two active sites.<sup>1</sup>

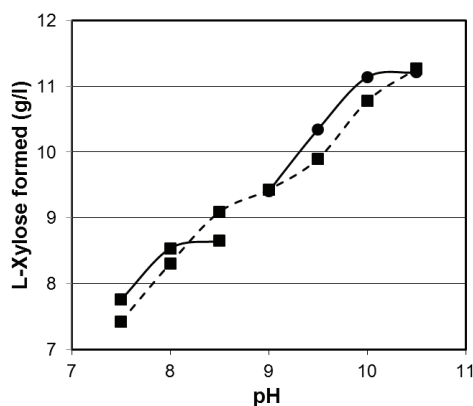
The  $K_m$  value for *E. coli* K-12 L-fucose isomerase has been reported to be 45 mM for L-fucose and 280 mM for D-arabinose (Boulter and Gielow, 1973). The  $K_m$  determined in the current study for L-xylulose is very close to the value for L-fucose. This suggests that the affinity of the enzyme towards the two C-6 sugar compounds is very similar.

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<sup>1</sup> There was a calculation error in the  $k_{cat}$  value in article (IV).

### 4.5.3. Effect of environmental factors

The effect of pH on the activity of L-fucose isomerase was determined. The results presented in **Figure 19** indicate that with L-xylulose as the substrate the pH-optimum is very alkaline (higher than pH 10.5). It has been reported that sugars can isomerize spontaneously under highly alkaline conditions (de Wit *et al.*, 1979). However, according to our studies, no spontaneous isomerization occurred under the conditions used (data not shown). In order to study the enzyme reaction under conditions at which the risk of side reactions of L-xylulose would be negligible, pH 9.0 was chosen for the rest of the experiments. For the isomerization between L-fucose and L-fuculose, the maximal activity has been determined to be between pH 8 and 10 (Boulter and Gielow, 1973). In this study the reactions were carried out in glycylglycine (125 mM) buffer supplemented with 0,025 mM MnCl<sub>2</sub> and 150 mM L-fucose.



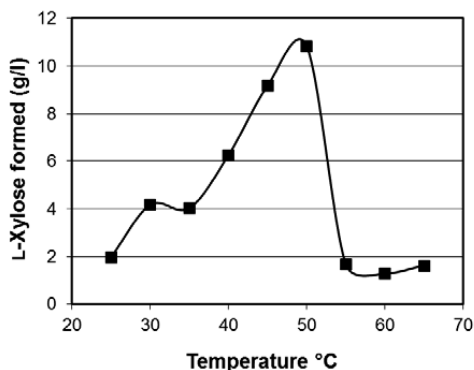
**Figure 19.** The effect of pH on the activity of L-fucose isomerase with L-xylulose as the substrate. Buffers used: Tris-HCl —■—, glycine-NaOH —●— and Tris-glycine - ■ -. The reaction mixtures were incubated for 1 hour.

The effect of temperature on L-xylulose isomerization was examined, and the results are shown in **Figure 20** for 30-min reactions. The rate of isomerization increased strongly with rising temperature up to 50 °C, after which the rate decreased sharply, apparently due to the instability of the enzyme. Very little activity was left at 55 °C or above. The half-lives determined for the enzyme at 35 °C and at 45 °C were 6 h 50 min and 1 h 31 min, respectively.

To my knowledge, the effects of temperature on activity and enzyme stability of L-fucose isomerase have not been reported previously. The L-rhamnose isomerase from *Pseudomonas stutzeri* that can also be used for L-xylose production from L-xylulose has previously been shown to have an optimum temperature for activity of 60 °C with a reaction time of 10 minutes (Leang *et al.*, 2004). It seems that this L-rhamnose

## Results and Discussion

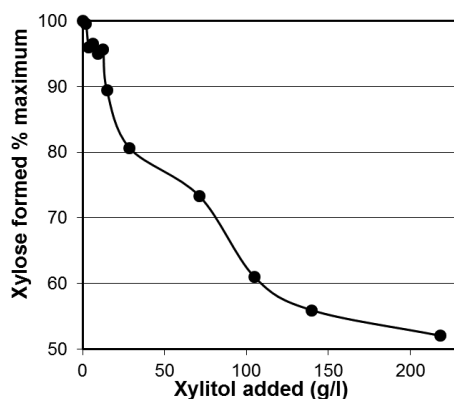
isomerase is clearly more thermostable than FucI from *E. coli*, as the present results indicate that FucI activity was completely lost at 55 °C in 30 minutes.



**Figure 20.** The effect of temperature on the activity of L-fucose isomerase. The reaction mixture was incubated for 30 minutes at each temperature.

### 4.5.4. Inhibition by xylitol

As the inhibition of sugar isomerases with polyols has been previously reported (van Bastelaere *et al.*, 1991, Pastinen 2000), the inhibition of L-fucose isomerase activity by xylitol was studied. If the inhibition by xylitol would be low enough, the oxidization of xylitol to L-xylulose and the isomerization of L-xylulose to L-xylose could be performed in one pot. As shown in **Figure 21**, the L-fucose isomerase catalyzed isomerization of L-xylulose to L-xylose was inhibited by xylitol. However, even at a high ratio of xylitol to xylulose (over 3.5) the inhibition was less than 50 %.

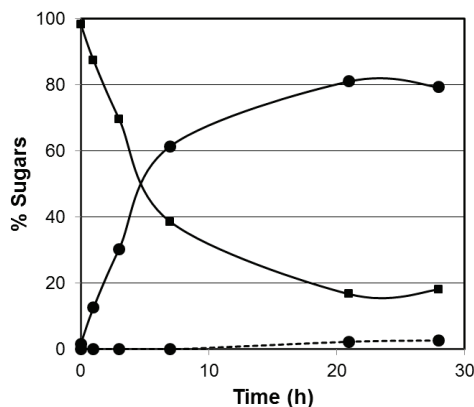


**Figure 21.** Inhibition of L-fucose isomerase catalyzed isomerization of L-xylulose to L-xylose by xylitol.

#### 4.5.5. Equilibrium ratio

It has previously been reported that in the reaction between D-xylose and D-xylulose, higher temperatures favor the production of xylulose (Tewari *et al.*, 1985). The equilibrium ratios between L-xylulose and L-xylose were determined at 25 °C and 35 °C. The time course of the formation of L-xylose from L-xylulose at 35 °C is presented in **Figure 22**.

At 25 °C the equilibrium was reached at 50 h or somewhat after it, and the equilibrium ratio determined between L-xylulose, L-xylose and L-lyxose was 13:85:2. At 35 °C the equilibrium was reached before 21 h and the corresponding equilibrium ratio was 17:81:2 (**Figure 22**). These results correspond quite well to the previously reported results, according to which the ratios of xylulose to xylose are 15:85 at 25 °C and 17:83 at 35 °C (Tewari *et al.*, 1985). As expected on the basis of previous reports, the balance was strongly on the side of L-xylose. Lower temperatures favored the formation of aldose even more compared to higher temperatures.



**Figure 22.** The reaction balance of L-fucose isomerase catalyzed reaction between L-xylulose (—■—), L-xylose (—●—) and L-lyxose (---●---) at 35 °C.

The same phenomenon is well known in the formation of HFCS (High fructose corn syrup), where higher temperatures favor the formation of fructose (Tewari and Goldberg, 1984; Tewari, 1990). The current results suggest that no side products were formed during the first seven hours of the reaction. However, after 21 hours small amounts of lyxose had formed in the reaction mixture. One possibility is that the enzyme is able to isomerize L-xylulose also to L-lyxose. However, a more likely explanation is that as cell lysate was used in the reaction instead of pure protein, other enzymes in the lysate perform this reaction. At least L-rhamnose isomerase from *E. coli* has been reported to catalyze the isomerization between L-xylulose and L-lyxose (Badia *et al.*, 1991). When L-lyxose was used as a starting material, no L-xylulose was detected

## Results and Discussion

in the reaction mixture. However, L-xylose was formed very slowly (after 7 hours 0.82 g/l xylose from 44 g/l lyxose).

The stabilities of L-xylose and L-xylulose were determined under the conditions used for the analysis of the equilibrium ratio. At 25 °C all sugars were detectable at their initial concentrations after 50 hours incubation. At 35 °C there was no loss of any sugars after 29 hours under the reaction conditions. Additionally, no spontaneous chemical isomerization was detected.

The reaction catalyzed by the L-rhamnose isomerase from *P. stutzeri* has been reported to have an equilibrium ratio between L-xylulose, L-xylose and L-lyxose of 53:26:21 (Granström *et al.* 2005). Also D-xylose isomerase from *Streptomyces rubiginosus* has been shown to catalyze the reaction between the aldoses L-lyxose and L-xylose through the ketose intermediate L-xylulose (Jokela *et al.*, 2002). Furthermore, L-rhamnose isomerase from *Thermotoga maritima* produces L-lyxose from L-xylulose with an equilibrium of 45:55. Surprisingly, no L-xylose production was reported for this enzyme (Park *et al.*, 2010).

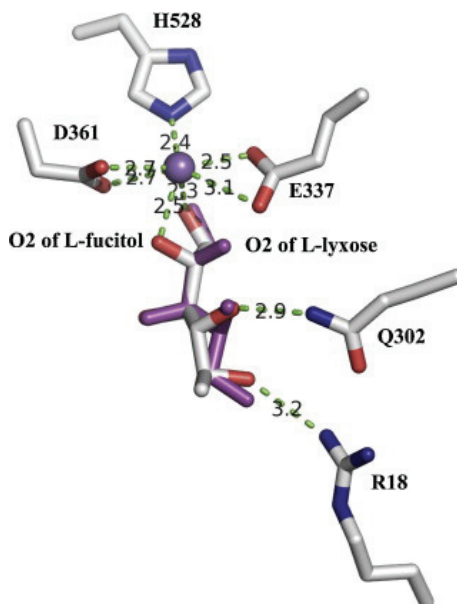
### 4.5.6. Structure and mechanism

The three-dimensional structure of L-fucose isomerase from *E. coli* has been previously determined by X-ray crystallography with L-fucitol in the catalytic center, as shown in **Figure 23**. The structure of the enzyme shows that it is a hexamer with two active sites. The enzyme has no structural similarity with other known ketol isomerases (Seemann and Schulz, 1997).

An ene-diol mechanism has been suggested for the reaction catalyzed by L-fucose isomerase. In the isomerization of L-xylulose, the double bond shifts from carbon C-2 to carbon C-1. The suggested mechanism starts with the opening of the sugar ring. After this a proton is shifted from carbon C-1 to C-2 and another proton from O-1 to O-2 (Seemann and Schulz, 1997).

The isomerization of L-xylulose could theoretically produce either L-xylose or L-lyxose. In L-xylose and L-lyxose the hydroxyl groups at C-2 are on the opposite sides of the carbon backbone whereas the C-2 and O-2 of xylulose are planar. The experimental results indicated that practically no lyxose was formed in the reaction. It seems that in the active site of L-fucose isomerase, the hydrogen bonding network between the sugar, the metal cofactor and the two catalytic residues is tight and may restrict the conformations that can be adopted during the catalysis (**Figure 23**). It is possible that when the proton is shifted from O-1 to O-2 *via* the catalytic residue Asp361 (aspartic acid) (Seemann and Schulz, 1997), the approaching proton attracts the O-2 towards Asp361. Hence it could be forced to the same side in the Fischer projection resulting in L-xylose formation. In order for the L-lyxose to form, the O-2 would have to be placed on the opposite side of C-2, which according to this catalytic mechanism may not be

possible. Glu337 (glutamic acid) may also stereochemically prevent the shifting of O-2 to the L-lyxose side.



**Figure 23.** The orientation of L-fucitol and the modeled orientation of L-lyxose in the active site of L-fucose isomerase. L-Fucitol is shown in gray and red (oxygen in red) and L-lyxose is shown in magenta. Coordination of  $Mn^{2+}$  to nearby atoms is shown by dotted green lines (bond lengths in Å).

The difference in the product formation of different isomerases can be explained by different reaction mechanisms. The proposed mechanism of action for L-rhamnose isomerase and D-xylose isomerase is a hydride shift (Korndörfer *et al.*, 2000; Yoshida *et al.*, 2010), and L-fucose isomerase uses ene-diol mechanism (Seeman and Schulz, 1997). In hydride shift enzymes there are two metals in the active site. Catalytic water mediates the transfer of a proton between O-1 and O-2 and the hydrogen atom between C-1 and C-2 is transferred as a hydride (Pastinen, 2000; Yoshida *et al.*, 2010).

The ene-diol enzyme contains only one metal and the reaction is mediated by a differing set of side chains around the substrate as in the hydride shift enzyme. It is likely that the active site configurations determine the fine tuning of the isomerization reactions. Still, a surprising finding was that the same enzyme, rhamnose isomerase, from two different species (*P. stutzeri* and *T. maritima*) show a differing reaction balance. Further studies are needed to find out what is the structural basis for these amazing results, providing that there are no confusing technical issues in the reaction conditions.



## **5. Conclusions and future prospects**

Rare and unnatural sugars have many potential uses in both medicine and food industry. L-Sugars are generally as sweet as the corresponding D-sugars, but are not fully absorbed by the human body. Additionally, several rare D-sugars are not substrates of the digestive enzymes, making them ideal low-calorie sweeteners. Rare sugar sweeteners generally also lack the typical aftertaste of artificial sweeteners. Rare sugars and sugar alcohols often possess nutraceutical properties, for example xylitol inhibits caries and D-tagatose lowers blood sugar levels. Furthermore, rare sugars have many possible uses in medicine. Some rare sugars and their derivatives have antiviral and anticancer properties, some act as cardioprotectants or as anti-inflammatory agents.

Rare sugars are generally expensive, since they cannot be isolated from natural sources in significant amounts. As new economic production methods are discovered, the sugars can be studied more extensively, and various new pharmaceutical, nutritional and other applications will most likely be found.

Potentially the biotechnological production methods of rare sugars are superior to chemical ones. A major problem in producing rare sugars biotechnologically is finding specific enzymes that can act as catalysts. In this thesis, the biotechnological production of two rare L-sugars, L-xylulose and L-xylose, and one sugar alcohol, xylitol, were studied. In the following paragraphs the aims of the study and the major results from each are summarized. Additionally, future targets for research and development are suggested.

Resting state cells were used in this thesis. The advantages of using resting cells are the ease of purification of the product and the low cost of the reaction medium as no complex and expensive media components are present. However, the cells should be recyclable from batch to batch to make the process economically feasible. In our laboratory the production of mannitol from fructose has been studied using *Leuconostoc mesenteroides*. In this study, the cells were used in 14 sequential batches without loss of productivity (von Weymarn *et al.*, 2002).

The first aim of this thesis was the production of xylitol by recombinant lactic acid bacteria. Xylitol is currently produced by chemical reduction of D-xylose. The raw material is derived from hemicellulose hydrolysates. The catalyst is susceptible to poisoning and thus the xylose needs to be extremely pure (Aminoff *et al.*, 1978). It is likely that in microbial production, less pure xylose could be used. Microbial

production of xylitol has been studied widely, and several reports using *Candida* yeasts have been published.

In this thesis, the production of xylitol by recombinant *Lactococcus lactis* harboring the *XYL1* gene encoding a xylose reductase from *Pichia stipitis* and the *xyIT* gene from *Lactobacillus brevis* encoding a xylose transporter was studied. *L. lactis* has several benefits over *Candida* yeasts. It has a GRAS status for use in food industry and it has been used in dairy industry for a long time, whereas several *Candida* strains have a pathogenic nature (Fridkin and Jarvis, 1996). Additionally *L. lactis* does not require aeration for the production of xylitol, which makes the process more economical. On the other hand, when using *L. lactis*, glucose needs to be added for the continuous regeneration of NADH. The yields of xylitol from xylose in this study were quantitative, whereas with yeasts the yields have generally been under 90 %.

All the xylose initially present could not be converted to xylitol, as the production slowed down and the fermentation mode was switched from mixed acid fermentation to homolactic fermentation. This could be caused by the accumulation of xylitol into the medium. As the optimal xylose concentration was under 100 g/l, the productivity could probably be improved by using a continuous process with cell recycling. This way fresh xylose would be added while xylitol would be removed from the process. Thus the high productivity could possibly be maintained throughout the process as the metabolism of the cells would stay in a mixed acid fermentation mode.

The second aim of the thesis was to purify and characterize *Pantoea ananatis* xylitol-4-dehydrogenase produced by recombinant *Escherichia coli*. The affinity of the xylitol-4-dehydrogenase from *P. ananatis* towards xylitol seems to be in the range of the affinities of NADP<sup>+</sup> dependent xylitol dehydrogenases from mammalian tissues. The only NAD<sup>+</sup> dependent xylitol-4-dehydrogenase found from yeast to date, the XDH from *Ambrosiozyma monospora*, also has quite similar  $K_m$  and  $V_{max}$  values for xylitol as the *P. ananatis* enzyme. However, the xylitol-4-dehydrogenase found in *Aeribacillus pallidus* Y25 has 4-5 times higher  $K_m$  towards xylitol, and the  $V_{max}$  value is 7 times smaller.

The substrate specificity of the enzyme was also studied, and the enzyme appears to have a preference for polyol substrates that have a hydroxyl group of C-2 in the left side and hydroxyl group of C-3 in the right side in the Fischer projection. This seems also to be the case with other NAD<sup>+</sup> dependent xylitol-4-dehydrogenases reported. Additionally, the reaction in the reducing direction was studied and the enzyme had activity towards D-xylulose, about 20 % of that towards L-xylulose. Despite this activity towards the D-form, the product from xylitol oxidation was pure L-xylulose as indicated by polarimetric analysis.

The next aim of this thesis was to produce L-xylulose from xylitol using resting *E. coli* cells expressing the *xdh* gene and to optimize the production parameters. For

### *Conclusions and future prospects*

this, DE3-lysogenized *E. coli* HB101 cells were used as the host because of the good productivity it had shown.

Optimal conditions for the production of L-xylulose from xylitol were found to be at pH 7.7, at low initial xylitol concentration (10 g/l) and at high temperature (44 °C). The highest volumetric productivity was also found at pH 7.7, at a high xylitol concentration (350 g/l) and at high temperature (44 °C). The final production experiment at 40 °C and pH 8.0 with 250 g/l initial xylitol concentration gave a specific productivity of 1.09 g/(g·h). This value was higher than the value predicted by the model, which was 0.77 g/(g·h).

The best L-xylulose producers reported seem to be *E. coli* harboring the xylitol-4-dehydrogenase gene from *P. ananatis* and *A. pallidus* Y25 (Poonperm *et al.*, 2007). However, the recombinant *E. coli* produces L-xylulose with an equimolar yield from xylitol whereas with *A. pallidus* side products are formed. Additionally, such reaction conditions were used with *A. pallidus* that L-xylulose is most likely not stable under them. When *xdh* from *A. pallidus* was expressed recombinantly in *E. coli* the productivities were not nearly as high as with the natural strain. Side products were also produced, which decreased the overall yield.

The microbial production methods of L-xylulose seem to be superior to the chemical methods known. The chemical methods typically require multiple steps, are expensive and the yields are low. Additionally the purification of the product from a reaction catalyzed with resting cells is quite simple. Higher volumetric productivities can also be achieved using higher cell densities. According to the current results, the scale-up of this process would be straightforward.

The next aim of this thesis was to construct a host able to produce L-xylulose from D-xylose. A recombinant strain was constructed that would express the genes of the intracellular enzymes: xylose reductase and xylitol-4-dehydrogenase. Resting cells of this strain could then be used for the production of L-xylulose from D-xylose through the intermediate xylitol. The coenzymes NAD<sup>+</sup> and NADH would be recycled in the reaction. Additionally, the accumulation of xylitol would not slow down the reaction catalyzed by xylose reductase as xylitol would be further converted.

Even though several attempts were made in constructing an *E. coli* strain containing both of these genes, the production of L-xylulose from D-xylose was not achieved at an acceptable level. Even though both genes were separately expressed in their active forms, the activities decreased when they were expressed in parallel.

The last aim of this study was to produce L-xylose from L-xylulose. No L-xylose isomerases have been reported to date, but some enzymes have been found to have slight isomerizing activity towards L-xylulose and L-xylose. One of these enzymes is the L-fucose isomerase from *Aerobacter aerogenes* (Oliver and Mortlock, 1971a; Oliver and Mortlock, 1971b). In this thesis the production of L-xylose from L-xylulose with L-fucose isomerase from *E. coli* was studied for the first time. Recombinant *E. coli* cells

overexpressing the *fucI* gene were used in an overnight reaction and 58 % of the L-xylulose was converted to L-xylose.

L-Fucose isomerase enzyme was characterized in the production of L-xylose. According to the results, the optimum pH for activity was very alkaline, above 10. Activity was highest at a temperature of 50 °C during 30 minutes but the stability was very poor above this temperature. The balance of the L-fucose isomerase catalyzed reaction was strongly on the side of L-xylose. At 35 °C and at pH 9.0 using cell lysate the L-xylose – L-xylulose – L-lyxose ratio was 81:17:2. Lyxose was only formed in prolonged incubations. The most probable explanation for the formation of lyxose is the activity of other enzymes in the *E. coli* cell lysate.

The balance of the reaction between L-xylulose, L-xylose and L-lyxose using L-rhamnose isomerase from *Pseudomonas stutzeri* has formerly been reported to be 53:26:21 (Granström *et al.* 2005). On the other hand the L-rhamnose isomerase from *Thermotoga maritima* catalyzes the reaction between L-xylulose and L-lyxose (55:45) and no L-xylose is formed. The differences in the product distributions are most likely a result of different reaction mechanisms and the structures of the catalytic centers of the enzymes. Apparently L-fucose isomerase uses an ene-diol mechanism, whereas L-rhamnose isomerases follow the hydride shift mechanism (Seemann and Shulz, 1997; Korndörfer *et al.*, 2000; Yoshida *et al.*, 2010).

As there are still some unanswered questions about the reaction balance of the L-fucose isomerase catalyzed reaction between L-xylulose and L-xylose, it would be interesting to purify the enzyme to homogeneity and use it for the conversion reaction until equilibrium is reached. This way it could be determined, whether there actually is a slight activity also towards lyxose, or whether this reaction was catalyzed by other enzymes in the cell lysate.

As L-fucose isomerase was quite unstable especially at elevated temperatures, the enzyme reaction should be performed by resting whole cells. When resting cells of *E. coli* BPT244 were used to convert L-xylulose to L-xylose, no lyxose was produced in an overnight reaction. Thus the purification of L-xylose should be quite straightforward. The inhibition of the L-fucose isomerase by xylitol was also studied. Although there was clear inhibition, even at high ratios of xylitol to L-xylulose, the inhibition was less than 50 %. This opens up a possibility for the production of L-xylose from xylitol in one pot with two different production strains. Additionally, this way the poor stability of L-xylulose would not be an issue, as it would be quickly converted to L-xylose. Furthermore, one possibility would be to coexpress the *xdh* and *fucI* in the same recombinant strain for the production of L-xylose from xylitol through the intermediate L-xylulose.

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Rare sugars have many potential uses in medicine and food industry. Their derivatives can be used as antiviral and anticancer drugs. Most rare sugars taste sweet but they are not metabolized by humans to the same level of more common sugars. Thus they can be used as low calorie sweeteners and they might also be used as diabetes drugs.

Rare sugars, as their name implies, cannot be isolated from natural sources in large quantities. Due to new biotechnological production methods, the prices of some rare sugars, such as tagatose and psicose, have decreased significantly. In this study, the biotechnological production of two rare sugars – L-xylulose and L-xylose – and one sugar alcohol, xylitol, was studied. D-Xylose, which is a common sugar in organic plant materials, was used as a starting material. The results achieved using metabolically engineered bacteria were promising, and the methods have the potential to be used in industrial processes.



ISBN 978-952-60-5316-5  
ISBN 978-952-60-5317-2 (pdf)  
ISSN-L 1799-4934  
ISSN 1799-4934  
ISSN 1799-4942 (pdf)

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