

Faculty of Biosciences
Department of Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki, Helsinki, Finland

**Pentitol phosphate dehydrogenases:
Discovery, characterization and use in D-arabitol and xylitol
production by metabolically engineered *Bacillus subtilis***

Mira Povelainen

Danisco Innovation & Technology
Kantvik, Finland

ACADEMIC DISSERTATION

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Supervised by:

Doctor Andrei Miasnikov
Danisco Innovation
Genencor International, Inc.
Palo Alto, USA

Reviewed by:

Docent John Londesborough
VTT, Biotechnology
Espoo, Finland

And

Docent Heikki Ojamo
Metropolia, University of Applied Sciences
Vantaa, Finland

Opponent:

Professor Kaarina Sivonen
University of Helsinki
Helsinki, Finland

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List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

- I **Povelainen M., Eneyskaya E. V., Kulminskaya A. A., Ivanen D. R., Kalkkinen N., Neustroev K. N. and Miasnikov A. N.** (2003) Biochemical and genetic characterization of a novel enzyme of pentitol metabolism: D-arabitol-phosphate dehydrogenase. *Biochem. J.* **371**: 191-197
- II **Soroka N. V., Kulminskaya A. A., Eneyskaya E. V., Shabalin K. A., Uffimtcev A. V., Povelainen M., Miasnikov A. N., Neustroev K. N.** (2005) Synthesis of arabinitol 1-phosphate and its use for characterization of arabinitol-phosphate dehydrogenase. *Carbohydrate Res.* **340**: 539-546
- III **Povelainen M. and Miasnikov A. N.** (2006) Production of D-arabitol by metabolic engineered strain of *Bacillus subtilis*. *Biotechnol. J.* **1**: 214-219
- IV **Povelainen M. and Miasnikov A. N.** (2007) Production of xylitol by metabolically engineered strains of *Bacillus subtilis*. *J. Biotechnol.* **128**: 24-31

Additional unpublished data are also presented.

List of abbreviations

APDH	D-arabitol phosphate dehydrogenase
ATP	adenosine triphosphate
DH	dehydrogenase
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GDH	glucose dehydrogenase
GRAS	generally recognized as safe
HPLC	high performance liquid chromatography
NAD	β -nicotinamide adenine dinucleotide
NADP	β -nicotinamide adenine dinucleotide phosphate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PHMG	polyhexamethyleneguanidine hydrochloride
PPP	pentose phosphate pathway
PTS	phosphotransferase system
RPE	ribulose 5-phosphate 3-epimerase
RPI	ribose 5-phosphate isomerase
SDS	sodium dodecyl sulphate
TCA	tricarboxylic acid
TKT	transketolase
XDH	xylitol dehydrogenase
XPDH	xylitol phosphate dehydrogenase

Abstract

The ultimate goal of this study has been to construct metabolically engineered microbial strains capable of fermenting glucose into pentitols D-arabitol and, especially, xylitol. The path that was chosen to achieve this goal required discovery, isolation and sequencing of at least two pentitol phosphate dehydrogenases of different specificity, followed by cloning and expression of their genes and characterization of recombinant arabitol and xylitol phosphate dehydrogenases.

An enzyme of a previously unknown specificity, D-arabitol phosphate dehydrogenase (APDH), was discovered in *Enterococcus avium*. The enzyme was purified to homogeneity from *E. avium* strain ATCC 33665. SDS/PAGE revealed that the enzyme has a molecular mass of 41 ± 2 kDa, whereas a molecular mass of 160 ± 5 kDa was observed under non-denaturing conditions implying that the APDH may exist as a tetramer with identical subunits. Purified APDH was found to have narrow substrate specificity, converting only D-arabitol 1-phosphate and D-arabitol 5-phosphate into D-xylulose 5-phosphate and D-ribulose 5-phosphate, respectively, in the oxidative reaction. Both NAD^+ and NADP^+ were accepted as co-factors. Based on the partial protein sequences, the gene encoding APDH was cloned. Homology comparisons place APDH within the medium chain dehydrogenase family. Unlike most members of this family, APDH requires Mn^{2+} but no Zn^{2+} for enzymatic activity. The DNA sequence surrounding the gene suggests that it belongs to an operon that also contains several components of phosphotransferase system (PTS). The apparent role of the enzyme is to participate in arabitol catabolism via the "arabitol phosphate route" similar to the ribitol and xylitol catabolic routes described previously.

Xylitol phosphate dehydrogenase (XPDH) was isolated from *Lactobacillus rhamnosus* strain ATCC 15820. The enzyme was partially sequenced. Amino acid sequences were used to isolate the gene encoding the enzyme. The homology comparisons of the deduced amino acid sequence of *L. rhamnosus* XPDH revealed several similar enzymes in genomes of various species of Gram-positive bacteria. Two enzymes of *Clostridium difficile* and an enzyme of *Bacillus halodurans* were cloned and their substrate specificities together with the substrate specificity of *L. rhamnosus* XPDH were compared. It was found that one of the XPDH enzymes of *C. difficile* and the XPDH of *L. rhamnosus* had the highest selectivity towards D-xylulose 5-phosphate.

A known transketolase-deficient and D-ribose-producing mutant of *Bacillus subtilis* (ATCC 31094) was further modified by disrupting its *rpi* (D-ribose phosphate isomerase) gene to create D-ribulose- and D-xylulose-producing strain. Expression of APDH of *E. avium* and XPDH of *L. rhamnosus* and *C. difficile* in D-ribulose- and D-xylulose-producing strain of *B. subtilis* resulted in strains capable of converting D-glucose into D-arabitol and xylitol, respectively. The D-arabitol yield on D-glucose was 38 % (w/w). Xylitol production was accompanied by co-production of ribitol limiting xylitol yield to 23 %.

1 Introduction

Polyols, also called alditols or sugar alcohols, are formed by reduction of a carbonyl group of a monosaccharide into a hydroxyl group. Polyols are used in a wide range of applications. One large application of polyols is their use as sugar replacers for diabetics and low calorie sweeteners in the food industry. On the back of the health trend among consumers, the demand for polyols is expected to experience substantial growth in the near future (Moloughney, 2008). Among polyols used as sweeteners, xylitol has a number of unique properties. Xylitol has a high positive enthalpy of solution in water. This leads to a “cooling effect” – desirable in certain confectionary and chewing gum products. Perhaps, the most important property of xylitol is its cariostatic effect. Numerous studies have demonstrated that regular use of xylitol can decrease the incidence and even reverse the development of caries in humans (Scheinin et al., 1975, 1985; Isokangas et al., 1987, 1988, 1989, 1993; Kandelman et al., 1988, 1990; Mäkinen et al., 1995; Alanen et al., 2000). Recently, xylitol has found new applications also in cosmetics, which is further increasing its demand (Zacharis, 2007). Another polyol, D-arabitol, can be used as a starting material in xylitol production. However, there is certainly interest in new alternative sweeteners in the food and beverage markets as well. D-Arabitol is of interest because it is reported to be almost not metabolizable in humans (Huck et al., 2004), leading to very low calorie content of the D-arabitol molecule.

At the moment xylitol is produced industrially by chemical hydrogenation of D-xylose isolated from various hydrolysates of plant materials. Since the demand for xylitol has grown at a fast rate during the past few years, the availability of the economically viable sources of pure D-xylose has become a limiting step in xylitol manufacture. Glucose is a common and relatively a cheap material. In addition, glucose is the preferred carbon and energy source for most organisms making it an attractive starting material for microbial bioconversions. Biotechnological production of D-arabitol and xylitol using glucose as a starting material has also been studied, but no industrially viable process has yet been developed. Low yields, interfering by-product formation and multi-fermentation process architectures have prevented practical application of previously described processes.

So far the only industrially successful process wherein glucose is converted to a five-carbon sugar is the production of D-ribose by *Bacillus subtilis* (Sasajima and Yoneda, 1974; Sasajima et al., 1976; De Wulf and Vandamme, 1997). In addition, *B. subtilis* is extensively used as a host organism in many other industrial processes, such as production of different enzymes. *B. subtilis* has a number of advantages as a host for a bioconversion-based process: *B. subtilis* has the GRAS-status, its genome is sequenced and the tools for its genetic modification are available. Moreover, the PPP of *B. subtilis* has been reported to be more active in glucose metabolism than in most other bacteria (Sauer et al., 1996). D-Ribose is produced via the PPP, which proves that the fermentation products produced via PPP can reach high yields in *B. subtilis*.

Pentitols are five-carbon sugar alcohols. The best characterized pentitol catabolic pathway in bacteria starts with the uptake of a neutral pentitol by the cell followed by its oxidation to the corresponding keto-sugar catalyzed by an intracellular pentitol dehydrogenase. The keto-sugars are subsequently phosphorylated to form intermediates of the PPP. Pentitols can also be catabolized via an alternative pathway, where the pentitol is taken up and phosphorylated (typically by the PTS), followed by the oxidation of the resulting pentitol phosphates by pentitol phosphate dehydrogenases (London and Chase, 1977). However, studies of such alternative pathways have been limited to few species of lactic acid bacteria and two pentitols: xylitol and ribitol (Hausman and London, 1987). Understanding the enzymology of pentitol phosphate metabolism has considerable practical importance, since these compounds are believed to play a central role in the cariostatic activity of pentitol sweeteners (Waler et al., 1984).

In the present study, a new type of pentitol phosphate dehydrogenase has been discovered, purified and sequenced – arabitol phosphate dehydrogenase. For the first time, also the gene encoding a xylitol phosphate dehydrogenase has been cloned and sequenced. This was followed by the expression and characterization of a number of recombinant pentitol phosphate dehydrogenases with respect to the properties important for the use of these enzymes as tools for metabolic engineering. Finally, this information has been used to construct metabolically engineered *B. subtilis* strains producing significant yields of D-arabitol or xylitol in a single fermentation step using glucose as a starting material. The efficiency of these strains exceeds by a large margin that of earlier bacterial or yeast hosts engineered to produce pentitols from glucose.

2 Literature review

2.1 D-Arabitol and xylitol as alternative sweeteners

Obesity is recognized as having reached epidemic proportions in the Western world. This fact together with the continuing trend of health and wellbeing in general, has been the driver for the increased use of alternative sweeteners in food, beverage and confectionary industries. The U.S. demand alone for alternative sweeteners is projected to increase 4% a year according to a 2007 study from the Freedonia Group, Inc. (Moloughney, 2008). The sugar alcohols (polyols) are increasingly used as sweetener alternatives in the food and confectionary industries. They are regarded as “more natural” sugar substituents than the traditional synthetic, high-potency sweeteners like aspartame or acesulfame K. Therefore, the demand for polyols is expected to grow even at a faster rate in the future. Polyols are relatively poorly absorbed and metabolized by humans resulting in a low caloric content value. Their ingestion is slow, and the followed blood glucose and serum insulin responses are significantly lower than those following glucose or sucrose ingestion. These findings have clearly indicated that polyols can be regarded as suitable sweeteners for use in diabetic and carbohydrate-controlled diets (Natah et al., 1997; Nguyen et al., 1993; Tong et al., 1987).

Xylitol is a five-carbon sugar alcohol that is a natural constituent of many fruits and vegetables (Mäkinen and Söderling, 1980; Washuett et al., 1973). It has been used as a food ingredient and sweetening agent for over twenty years now and its properties have been extensively studied. Xylitol has been approved for use in food in over 50 countries and therefore it has found widespread applications in almost all sectors of the food industry like confectionary, chewing gum, hard coating applications, chocolate, dairy products and baked goods. Xylitol is the sweetest of all polyols (Moskowitz, 1971; Gutschmidt and Ordynsky, 1961; Lindley et al., 1976; Yamaguchi et al., 1970; Lee, 1977), being the only polyol to exhibit a sweetness intensity equivalent to that of sucrose (See table 1; Hyvönen et al., 1977; Munton and Birch, 1985). In addition to its sweetness, xylitol has also other advantages compared to other polyols. The dissolution of xylitol is highly endothermic reaction, meaning that xylitol absorbs energy from its environments as it dissolves, causing a drop in the temperature of the surroundings. This effect compained with xylitol's high solubility results in a significant cooling effect upon consumption. This provides xylitol with a clear taste advantage over both sugar and most other polyol sweeteners in many applications. However, the most famous advantage of xylitol as a sweetener is certainly its positive health effects, especially in dental health. The dental health effects of xylitol are discussed later in this thesis (Section 2.1.1).

Compared to xylitol little is known about the properties of D-arabitol. The literature reports concerning D-arabitol are mainly focused on D-arabitol's use in diagnosis of invasive *Candida* infections in humans (for review, see Christenssons

et al., 1999). However, in the constantly growing markets of alternative sweeteners including polyols, there is certainly space for new products as well. There are few reports describing D-arabitol being non-metabolizable in humans (Crick, 1961; McCormick and Touster, 1961; Huck et al., 2004). This non-metabolizable nature of D-arabitol results in a very low caloric content of the molecule and therefore could offer an interesting sweetener alternative especially for weight management diets.

Table 1. Summary of the characteristics of some polyols and sucrose (Lee, 1977; Mitchell, 2006)

	Relative Sweetness (Sucrose=1)	Caloric Content (USA) kcal/g	Cooling Effect ^a	Cariogenicity
D-Arabitol	0.6	~0.2 ^b	Strong	Non-cariogenic
Erythritol	0.7	0.2	Very strong	Non-cariogenic
Isomalt	0.4	2	Weak	Non-cariogenic
Lactitol	0.4	2	Moderate	Non-cariogenic
Maltitol	0.9	2.1	Weak	Non-cariogenic
Mannitol	0.5	1.6	Strong	Non-cariogenic
Sorbitol	0.6	2.6	Strong	Non-cariogenic
Xylitol	0.95	2.4	Very strong	Anti-cariogenic
Sucrose	1	4	Weak	Cariogenic

^aCooling effect (ΔH) kcal/g: very strong >35; strong 25-35; moderate 5-25; weak <5

^bEstimated value based on the reports describing that D-arabitol is not metabolized (Crick, 1961; McCormick and Touster, 1961; Huck et al., 2004)

2.1.1 Xylitol and dental health

It is generally accepted that the consumption of sugars and other fermentable carbohydrates in our diets is one of the main causative factors for dental caries (Gehring, 1978; Newburn, 1982; Shaw, 1987; Arens, 1999, Rugg-Gunn and Nunn, 1999). Xylitol and polyols in general are considered as non-cariogenic, meaning that they do not contribute to tooth decay and are therefore increasingly used as replacements for fermentable carbohydrates in foods. However, in case of xylitol it has been shown that xylitol is not only non-cariogenic but it also actually prevents tooth decay. This ability to inhibit the development of caries has been demonstrated in numerous clinical and field studies. These studies lead to a conclusion that when xylitol-containing confectionary or chewing gum is consumed as part of the normal diet, new caries incidences are typically reduced by

40 – 80% (Scheinin et al., 1975, 1985; Isokangas et al., 1987, 1988, 1989, 1993; Kandelman et al., 1988, 1990; Mäkinen et al., 1995; Alanen et al., 2000).

One particular family of bacteria associated with dental caries is the mutans streptococci group of bacteria. This group of bacteria is generally considered as among the most virulent of the cariogenic bacteria and is frequently implicated as the main cause of dental caries in the developed world (Trahan, 1995; Bagg et al., 1999). It is believed that one of the key mechanisms of xylitol's plaque-reducing, and ultimately caries-inhibitory, effect is indeed its effect against the mutans streptococci. The specific mechanism of the effect has been a subject for many studies over the last two decades and the consensus of opinion appears to be a combination of inhibitory effects, which can be divided into four main modes of action (Maguire and Rugg-Gunn, 2003; Bond and Dunning, 2006): (1) Xylitol is not fermentable and therefore does not encourage the bacterial growth. (2) When mutans streptococci are exposed to xylitol, the proportion of xylitol-tolerant strains increases. These xylitol-tolerant strains are less virulent and thus less acidogenic and produce less adhesive polysaccharides that form the plaque matrix (Söderling et al., 1997; Trahan et al., 1992). (3) The uptake and conversion of xylitol to xylitol 5-phosphate has an inhibitory effect upon metabolism in the cell (Trahan, 1995), but (4) this action also forms an energy wasting cycle, also called "futile metabolic cycle", in which the uptaken and phosphorylated xylitol is further split by sugar-phosphate phosphatases and expelled from the cell. This cycle obviously limits the metabolic efficiency of the organism (Söderling and Pihlanto-Leppälä, 1989; Pihlanto-Leppälä et al., 1990).

In addition to xylitol's effect on dental health there are also some other health benefits associated with xylitol such as prebiotic effects (Salminen et al., 1985), satiety effects (Shafer et al., 1987; King et al., 2005) and prevention of the mid-ear infections (acute otitis media) in young children (Uhari et al., 1996, 1997).

2.2 *Bacillus* as an industrial production host

Bacillus species are widely used in industry for a variety of reasons. These Gram-positive eubacteria are capable of secreting homologous proteins to the culture medium at tens of grams per liter concentrations, in particular during the post-exponential growth phase. *Bacillus* species have the ability to grow rapidly to high cell densities on low-cost carbon and nitrogen sources. In addition, the species such as *B. subtilis* and *B. licheniformis* have the GRAS (generally recognized as safe) status with the Food and Drug Administration (FDA). Much is also known about transcription and translation mechanisms, secretory machinery and genetic manipulation of *B. subtilis*. The complete genome sequence of *B. subtilis* 168 has been published, comprising 4100 protein-encoding genes (Kunst et al., 1997), which further promotes the development of products or processes.

B. subtilis has been used for decades for the bulk production of industrial enzymes, in particular for the production of native secreted enzymes such as amylases, proteases, cellulases and xylanases. These enzymes have applications in a number of industrial processes such as food, textile and fabric care, grain processing, paper and the rapidly growing bioethanol industries (Rao et al., 1989; Singh and Hayashi, 1995; Kuhad et al., 1997; Bajpai, 1997; Pandey et al., 2000). Because of the high secretion capacity of *Bacillus* species, they are great candidates as hosts for the production of heterologous proteins as well. A major problem in the production of heterologous proteins in *B. subtilis* is the large number of extracellular proteases, which attack secreted cloned foreign proteins (Doi et al., 1984; Wang et al. 1988). However, there are some examples of therapeutic human proteins that have been expressed in *B. subtilis* such as human growth hormone (Schallmey et al, 2004).

Metabolic engineering of purine metabolism of *B. subtilis* has been used to produce purine nucleosides and nucleotides. Mutants of *B. subtilis* K produce inosine with the yield greater than 20 g/l (Shiio, 1989) and guanosine-producing *B. subtilis* strains have been derived by mutation of inosine producers, with reported yields of >8 g/l (Momose and Shiio, 1969). In *B. subtilis*, riboflavin biosynthesis is associated with purine metabolism as well. Recombinant DNA techniques have been used together with fermentation strategies to develop processes with commercially attractive levels of riboflavin by *B. subtilis* (Perkins et al., 1999). *B. subtilis* has been developed to produce yields up to 30 g/l riboflavin in 3-day fermentation and hence it is becoming the most competitive microbial riboflavin producer. Other industrially interesting products from *B. subtilis* are poly- γ -glutamic acid and D-ribose (Ogawa et al., 1997; De Wulf and Vandamme, 1997).

2.2.1 Production of D-ribose

D-Ribose is used in food (as a flavor enhancer), pharmaceuticals, cosmetics, health food, and animal feed, as well as for the treatment of myocardial ischemia and muscular pain. D-Ribose can be produced by fermentation. The microorganisms that secrete D-ribose are deficient in the enzyme transketolase and (or) D-ribulose 5-phosphate 3-epimerase. Several strains of *B. subtilis* and its mutants have been reported to produce significant amounts of D-ribose (Sasajima and Yoneda, 1974; Sasajima et al., 1976; De Wulf and Vandamme, 1997). Developments in genetic engineering and fermentation technology have contributed to improvements in D-ribose productivity in *Bacillus* fermentations. D-Ribose yields exceeding 90 g/l from 200 g/l D-glucose, with significant reduction in fermentation time and the concentration of undesirable by-products, have been achieved (Kishimoto et al., 1990; Miyagawa et al., 1992; De Wulf and Vandamme, 1997). This D-ribose production by *B. subtilis* in context with this thesis is especially of

importance because it is so far the only industrially successful process for bioconversion of D-glucose into a five-carbon sugar.

2.3 Carbohydrate uptake and metabolism in *Bacillus subtilis*

2.3.1 Carbohydrates utilized by *Bacillus subtilis*

B. subtilis is a soil bacterium. This natural habitat of *B. subtilis* contains a wide variety of carbohydrates including a large number of polysaccharides derived from plants, animals and microbes. By using extracellular polysaccharide-degrading enzymes *B. subtilis* is capable of degrading these complex nutrients to oligo-, di- and monosaccharides which are then transported into the cells, phosphorylated and subsequently catabolized via glycolysis or the PPP. *B. subtilis* produces enzymes such as: α -amylase (encoded by *amyE*) (Yang et al., 1983), pullulanase (*amyX*) (Kunst et al., 1997), endo- β -1,4-mannanase (*ydhT*) (Araujo and Ward, 1990), levanase (*sacC*) (Martin et al., 1987), glucan-1,4- α -maltohydrolase (*yvdF*) (Kunst et al., 1997), pectate lyases (*pel*, *pelB*) (Nasser et al. 1993), β -1,3-1,4-endoglucanase (*bgIS*) (Murphy et al, 1984), and endo-1,4- β -xylanases (*xynA*, *xynD*) (Wolf et al. 1995). The genes encoding these polysaccharide-hydrolyzing enzymes are often organized in an operon or a regulon together with genes that encode the enzymes catalyzing the uptake of the extracellular hydrolytic products and the first intracellular steps in their catabolism. The following carbohydrates seem to be utilized by *B. subtilis*: mono-, di-, and oligosaccharides, amino sugars and their N-acetyl derivatives, sugar-containing opines, glyconic, glycaric, and glycuronic acids, and sugar-derived polyalcohols (linear or cyclic).

2.3.2 The PEP:carbohydrate phosphotransferase system

The phosphotransferase system (PTS) is a complex carbohydrate transport system found in many Gram-positive and Gram-negative bacteria as well as in *B. subtilis*. It catalyzes the simultaneous transport and phosphorylation of its substrates using a phosphorylation cascade composed of enzyme I (EI) and HPr (shared by PTS systems of different substrate specificities) and the sugar-specific proteins, enzymes IIA (EIIA) and EIIB (Fig. 1). Phosphoenolpyruvate (PEP) is the source of energy for the transport step and it also provides the phosphoryl group for the phosphorylation of carbohydrates via the PTS. PTS proteins are intermediately phosphorylated at a histidyl residue or at a cysteyle residue. P~EIIB transfers the phosphoryl group to the substrate bound to the corresponding membrane-spanning

EIIC, and the phosphorylated carbohydrate is then released to the cell (for review, see Postma et al. 1993).

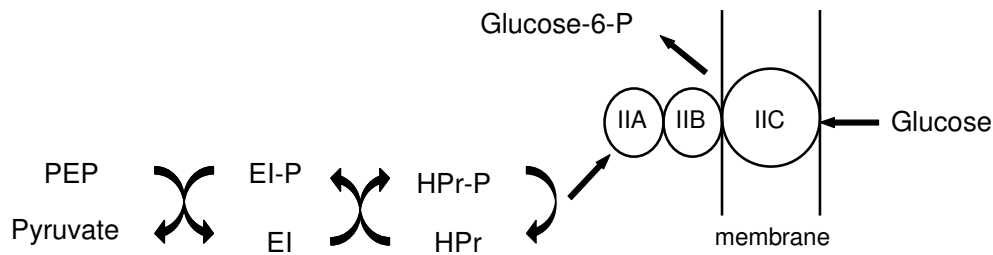


Figure 1. The PEP:glucose phosphotransferase system of *B. subtilis*

2.3.3 Glucose transport

Glucose is the preferred carbon and energy source of *B. subtilis*. Most glucose uptake and phosphorylation are achieved by a specific EII of the PTS that is encoded by *ptsG* (EIICBA in Fig. 2; Gonzy-Tréboul et al., 1991), but there are at least two other glucose transporters, one of which is also PTS dependent. The *ptsG* mutants of *B. subtilis* exhibit impaired glucose utilization (Bachem et al., 1997). Moreover *ptsH* mutants, where the HPr is inactivated, are unable to use fructose or maltose, but they grow very slowly on glucose (Stülke et al., 1995). Thus, the second EII besides the *ptsG* gene product must be capable of transporting and phosphorylating glucose, and there must be a PTS-independent glucose transport system. There are two candidates for PTS-independent glucose uptake (Fiegler et al., 1999; Paulsen et al., 1998). The glucose permease (GLP in Fig. 2) encoded by *GlcP* is a glucose:H⁺ symporter, i.e. a proton is transported together with each glucose molecule. Intracellular glucose originating from non-PTS glucose transport or the degradation of disaccharides is phosphorylated by glucose kinase (Skarlatos and Dahl, 1998). The other is assumed to be a glucose facilitator (GDF in Fig. 2). It was identified by homology with glucose uptake protein of *Staphylococcus xylosus*. In *B. subtilis* it is encoded by the *ycxE* gene and it is part of the *ycxE-gdh* operon, in which the *gdh* gene encodes glucose dehydrogenase (GDH). The coexpression of GDF and GDH suggests that GDF recruits glucose for GDH which further transforms it into gluconate. After phosphorylation by gluconate kinase (GLAK), 6-phosphate gluconate enters the PPP.

2.3.4 The general pathways of carbon metabolism

2.3.4.1 Glycolysis

Metabolic flux analyses revealed that glucose is catabolized in *B. subtilis* via the PPP and glycolysis (Sauer et al., 1996 and 1997).

The first reaction in glycolysis, the conversion of glucose 6-phosphate to fructose 6-phosphate, is specific for glucose and disaccharides that are cleaved to yield glucose 6-phosphate. The genes required for the glycolytic reaction sequence from glucose 6-phosphate to pyruvate in *B. subtilis* have been identified. The *pfk* and *pykA* genes encode phosphofructokinase and pyruvate kinase, respectively, and form an operon (Kunst et al., 1997). The expression of the *pfk-pykA* operon and of the *fbaA* gene, which encodes fructose 1,6-bisphosphate aldolase, is constitutive (Mitchell et al., 1992; Tobisch et al., 1999). The genes that encode the enzymes catalyzing the interconversion of triose-phosphates are clustered in *B. subtilis*, whereas *gap*, which encodes glyceraldehyde 3-phosphate dehydrogenase, is the distal gene of the bicistronic *yvbQ-gap* operon. The genes coding for phosphoglycerate kinase, triose phosphate isomerase, phosphoglycerate mutase and enolase form the *pgk-tpi-pgm-eno* operon (Leyva-Vazquez and Setlow, 1994; Tobisch et al., 1999). The expression of the latter two operons is induced by glucose (Tobisch et al., 1999).

Pyruvate dehydrogenase, which provides the link between glycolysis and the tricarboxylic acid (TCA) cycle or overflow metabolism, is encoded by *phdABCD* (Hederstedt, 1993). Synthesis of pyruvate dehydrogenase subunits is constitutive in *B. subtilis*, as judged from a proteomic analysis (Tobisch et al., 1999). The acetyl-coenzyme A (CoA) formed by the action of the pyruvate dehydrogenase complex can be further metabolized in the TCA cycle to generate energy and intermediates for anabolic reactions. Alternatively, it can be converted into acetate and excreted. The latter reaction is a part of the so-called overflow metabolism which eliminates superfluous carbon from the cell (Nakano and Hulett, 1997). This notion is in agreement with the observation that genes involved in overflow metabolism (such as *ackA*, *pta* and *alsS*, which encode acetate kinase, phosphotransacetylase and α -acetolactate synthase, respectively) are induced in the presence of glucose (Grundt et al., 1994; Presecan-Siedel et al., 1999; Renna et al., 1993; Shin et al., 1999). By contrast, the genes that encode enzymes of TCA cycle are repressed by glucose (Hanson and Cox, 1967).

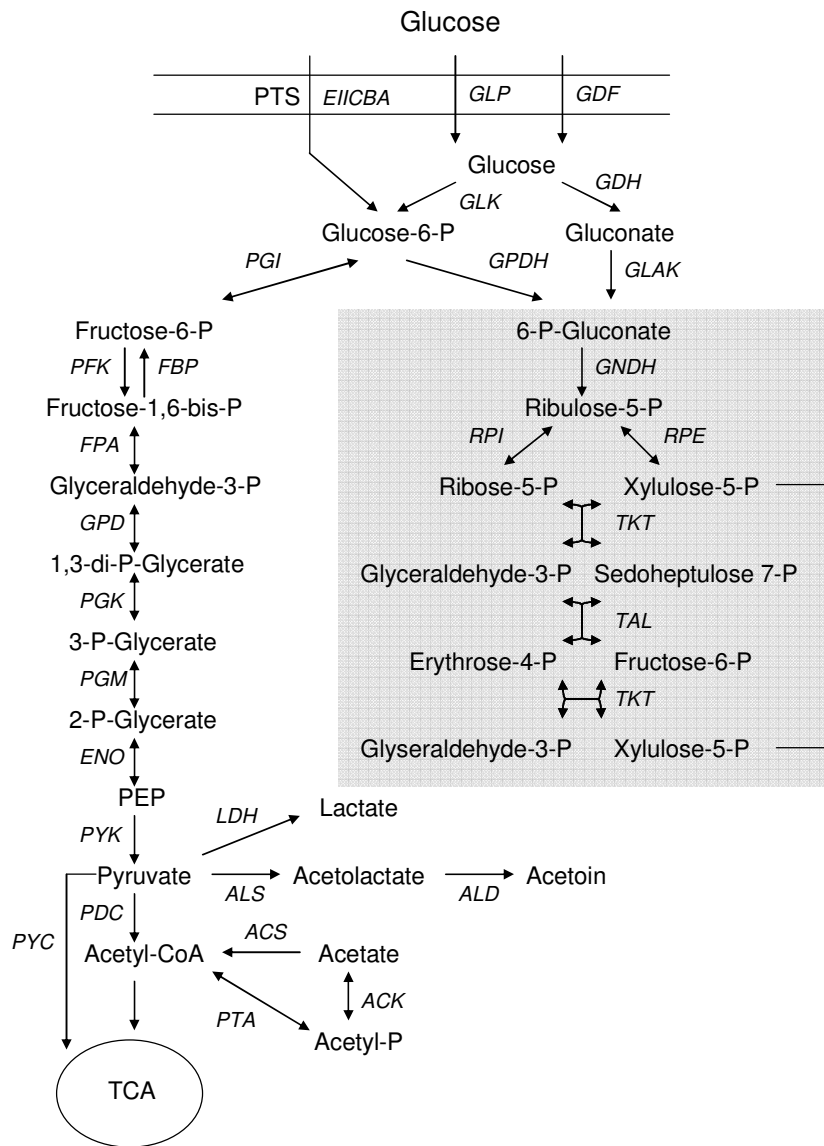


Figure 2. Schematic representation of central carbohydrate metabolism via glycolysis and PPP of *Bacillus subtilis*.

GLP, glucose permease; *GLK*, glucose kinase; *GDF*, glucose facilitator; *GDH*, glucose dehydrogenase; *GLAK*, gluconate kinase. Glycolytic enzymes: *PGI*, phospho glucose isomerase; *PFK*, 6-phosphofructokinase; *FBP*, fructose-1,6-bisphosphatase; *FPA*, fructose-1,6-bisphosphate aldolase; *GPD*, Glyceraldehyde-3-phosphate dehydrogenase; *PGK*, 3-phosphoglycerate kinase; *PGM*, phosphoglycerate mutase; *ENO*, enolase; *PYK*, pyruvate kinase; *PDC*, pyruvate dehydrogenase complex. PPP enzymes: *GPDH*, glucose-6-phosphate dehydrogenase; *GNDH*, gluconate-6-phosphate dehydrogenase; *TKT*, transketolase; *RPE*, ribulose-5-phosphate epimerase; *RPI*, ribose-5-phosphate isomerase; *TAL*, transaldolase. Other enzymes: *PYC*, pyruvate carboxylase; *LDH*, lactate dehydrogenase; *ALS*, α -acetolactate synthase; *ALD*, α -acetolactate decarboxylase; *ACS*, Acetyl-CoA synthetase; *PTA*, phosphotransacetylase; *ACK*, Acetate kinase

2.3.4.2 PPP

Knowledge about the regulation of the genes and enzymes of the PPP in *B. subtilis* is rather limited. PPP serves two major functions: it provides the NADPH needed for anabolic reactions and generates precursors for biosynthesis of a number of essential molecules such as aromatic amino acids and nucleotides. These roles of PPP may explain why the genes encoding the enzymes of this pathway are constitutively expressed and showing little dependence on presence or absence of glucose. A notable exception is the *gntZ* gene. Its expression is weakly induced in the presence of glucose (Blencke et al., 2003). However, it is not totally clear, what is the role of the enzyme encoded by *gntZ* gene in the *B. subtilis* metabolism. The *gntZ* gene was generally assumed to encode the 6-phosphogluconate dehydrogenase, but more recently it was demonstrated that the major 6-phosphogluconate dehydrogenase is encoded by the *yqjI* gene in *B. subtilis* (Zamboni et al., 2004).

Schilling et al. have studied the transcriptional and metabolic responses of *B. subtilis* to the availability of organic acids. In the presence of glutamate and succinate, significantly less glucose 6-phosphate is converted into fructose 6-phosphate by the phospho glucose isomerase (PGI, Fig. 2) than in the absence of the organic acids. In contrast, the portion of glucose 6-phosphate that is oxidized by glucose 6-phosphate dehydrogenase is significantly increased. This regulation of glucose 6-phosphate partition is not reflected by the transcriptional regulation of the genes encoding the two major enzymes (PGI and GPDH) that use glucose 6-phosphate as a substrate. Schilling et al. hypothesize that the candidate causing the increased flux through the PPP in the presence of succinate and glutamate could be the *ywIF* gene, tentatively believed to encode ribose 5-phosphate isomerase. The expression of this gene was weakly but significantly increased upon the addition of organic acids (Schilling et al., 2007). Moreover, both the transketolase (TKT) and transaldolase (TAL) were recently shown to be phosphorylated in vivo in *B. subtilis* (Lévine et al., 2006). It is, however, unknown under which conditions these phosphorylations occur and how they affect the activities of the two enzymes.

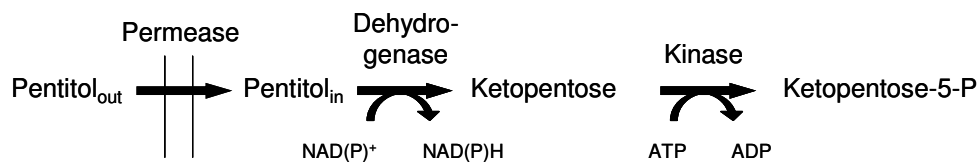
The PPP flux is generally reported to be in the range of 20 to 30 % of the total glucose in bacteria. However, the analysis of metabolic flux of glucose in wild type and riboflavin producing *B. subtilis* strains revealed that at a higher growth rate there is a substantial flux (30 to 50 %) through the PPP (Sauer et al., 1996). This estimated high flux through the oxidative branch of the PPP challenges the classical hypothesis of the two functions of the PPP: to provide NADPH and to generate precursors for tryptophan and nucleotide synthesis. In *B. subtilis* the PPP must be considered as a major pathway of glucose metabolism.

2.4 Pentitol metabolism in bacteria

Five-carbon sugar alcohols (pentitols) do not occur abundantly in nature, so it is not surprising that the ability of both prokaryotic and eukaryotic microorganisms to metabolize this class of organic compounds is rather variable although not at all unusual. Mortlock (Mortlock, 1976) identified the following examples of bacteria as being able to utilize or grow at the expense of pentitols: *Klebsiella aerogenes*, *Pseudomonas* species, *Acetobacter suboxydans*, *Gluconobacter oxydans*, *Bacillus subtilis* and *Azotobacter agilis*. The list can be expanded to include also *Erwinia uredovora* (Doten and Mortlock, 1985a), *Morganella morganii*, *Providencia stuartii*, *Serratia marcescens* (Doten and Mortlock, 1985b), *Escherichia fergusonii* (Farmer et al., 1985), *Klebsiella pneumoniae* (Doten and Mortlock, 1984), *Staphylococcus saprophyticus* (Tselenis-Kotsowilis et al., 1982), *Leminorella* species (Hickmann-Brenner et al., 1985), and several species of lactic acid bacteria (Nowlan and Deibel, 1967; London and Chase, 1977).

In the majority of bacteria and eukaryotic microorganisms, the general sequence of enzymatic steps for pentitol catabolism is the same, although the dehydrogenases and kinases that participate in the enzymatic reactions differ from species to species in their specificity for substrate or substrate and co-factor (Mortlock, 1976). In that so called ketopentose route (Fig. 3) pentitols are transported into the cells by a permease and oxidized to their corresponding ketopentoses by an NAD- or NADP-dependent dehydrogenase. An appropriate kinase subsequently phosphorylates the ketopentose, producing ketopentose 5-phosphate which then enters the PPP.

Ketopentose route



Pentitol phosphate route

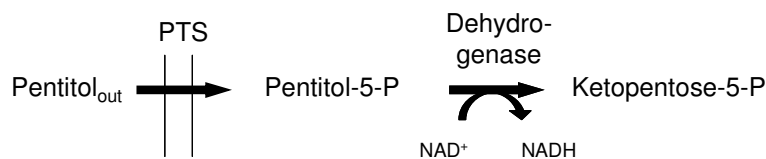


Figure 3. The routes of pentitol metabolism in bacteria

In the late 1970's London and Chase noticed that *Streptococcus avium* and *Lactobacillus casei* assimilate pentitols by a mechanism that is different from the ketopentose route described above (London and Chase, 1977). Resting cell and cell extract studies revealed that ribitol and xylitol were transported into the cell by substrate-specific PEP-dependent phosphotransferases. Ribitol 5-phosphate and xylitol 5-phosphate formed in these reactions were then oxidized to ribulose 5-phosphate and xylulose 5-phosphate by NAD-dependent pentitol phosphate dehydrogenases (Fig. 3). Later, Hausman and London purified and characterized two types of pentitol phosphate dehydrogenases – xylitol phosphate dehydrogenase (XPDH) and ribitol phosphate dehydrogenase - from the cell extracts of *L. casei* (Hausman and London, 1987).

2.4.1 Pentitol phosphate dehydrogenases of *L. casei*

The physical properties of the two purified pentitol phosphate dehydrogenases were analysed by Hausman and London (Hausman and London, 1987). It was found that the molecular weights of the native forms of ribitol phosphate dehydrogenase and XPDH of *L. casei* were 115 kDa and 180 kDa, respectively, by molecular size exclusion chromatography. Denaturing PAGE resolved the ribitol phosphate dehydrogenase into a single polypeptide band with an estimated molecular weight of 49 kDa, while XPDH migrated as a single band with a molecular weight of approximately 42 kDa. Thus, in their native states, ribitol phosphate dehydrogenase appeared to be a dimer, while the XPDH existed as a tetramer. The pI of XPDH was estimated to be between 5.3 and 5.5 by isoelectric focusing.

In the analysis of kinetic parameters of the enzymes, both enzymes exhibited an absolute specificity for NAD⁺ as a co-factor. The ribitol phosphate dehydrogenase utilized only D-ribitol 5-phosphate as a substrate, while XPDH was found to oxidize both D-xylitol 5-phosphate and D-arabitol 5-phosphate. The latter finding appears to be rather strange. Assuming C-2 hydroxyl is oxidized in both D-arabitol 5-phosphate and D-xylitol 5-phosphate, it means that the enzyme accepts two opposing stereochemical configurations at the reactive center of the substrate – a highly unusual property for any enzyme. This would also mean that both substrates are oxidized into the same product - D-xylulose 5-phosphate. Thus, in the reverse reaction D-xylulose 5-phosphate should be reduced to a mixture of D-arabitol 5-phosphate and D-xylitol 5-phosphate – which, again, seems to be rather improbable (see figure 6 on page 35 for structures). The authors provide no explanation or discussion of this apparent controversy. Fructose 6-phosphate, fructose 1-phosphate, mannitol 1-phosphate and sorbitol 6-phosphate did not serve as substrates for either enzyme. Some intermediate products of glycolysis were tested as potential inhibitors of the two dehydrogenases. The compounds included fructose 6-phosphate, glucose 6-phosphate, fructose 1,6-bisphosphate, ribose 5-

phosphate, glyceraldehyde 3-phosphate, 3-phosphoglycerate, PEP, pyruvate, acetylphosphate as well as mono-, di-, and triphosphate esters of guanosine and adenosine. With the exception of ATP, none of the compounds tested inhibited either oxidative or reductive activity at 10 mM. At 10 mM, ATP produced a 45% inhibition in both the forward and reverse directions of the XPDH. However, the authors concluded that the inhibition might be due to the ability of ATP to chelate the divalent cation Mn^{2+} which was required by the enzyme for optimal activity (Dixon and Webb, 1964).

2.5 Bioconversion of D-glucose into D-arabitol and xylitol

D-Arabitol can be produced by catalytic hydrogenation of D-arabinose and by chemical reduction of lactones of arabinonic and lyxonic acids (Kirk-Othmer, 1978; Lemmens et al., 1998; Flèche et al., 2000; Fabre et al., 2002). It is also well known that many osmophilic yeast species such as *Zygosaccharomyces*, *Debaryomyces*, *Metschnikowia*, *Candida*, *Pichia*, *Hansenula* and others (Onishi et al., 1961; Ingram and Wood, 1965; Hanssens et al., 1972; Bernard et al., 1981; Nobre and da Costa, 1985; Van Eck, 1989 and 1993; Jovall et al., 1990; Groleau et al., 1995; Bisping et al., 1996; Nozaki et al., 2003) produce D-arabitol when grown on D-glucose. Most of these organisms have in common tolerance to high concentrations of sugars and salts and therefore it has been suggested that the accumulation of polyols in yeasts may be related to osmotic stress (Van Eck et al., 1989). Two biosynthetic pathways for D-arabitol in fungi have been proposed (Ingram and Wood, 1965; Jovall et al., 1990). Both of these pathways are extensions of the PPP, in which either D-xylulose 5-phosphate or D-ribulose 5-phosphate is de-phosphorylated and reduced with a corresponding polyol dehydrogenase to D-arabitol. Since D-arabitol is a byproduct of yeast cell mass production, the typical yield is between 20-30% of consumed D-glucose. However, regardless of the growth conditions, the production of D-arabitol by yeast and fungi are always accompanied by the production of other polyols, most commonly, glycerol.

Currently, xylitol is industrially produced by chemical reduction of D-xylose isolated from hydrolysates of plant materials, but D-xylose can also be reduced to xylitol with high yields by various yeast species (Hallborn et al., 1991; Winkelhausen and Kuzmanova, 1998). D-Xylose is abundant and renewable; however, it is difficult to obtain as a pure and defined chemical from the hemicellulose fraction of plant biomass. To overcome this problem, a process for xylitol production that starts from D-glucose has become an attractive alternative to the D-xylose based processes.

It has been known for almost 40 years that D-glucose can be converted into xylitol by fermentation via D-arabitol and D-xylulose. Onishi and Suzuki described a three-step process wherein D-glucose is fermented into D-arabitol by *Debaryomyces hansenii* followed by the oxidation of D-arabitol into D-xylulose with *Acetobacter*

suboxydans and the reduction of D-xylulose into xylitol by *Candida guilliermondii* (Onishi and Suzuki, 1969). Later, Mayer et al. used a similar approach but replaced the last fermentation step with an enzymatic *in vitro* reaction using recombinant XDH of *Galactocandida mastotermitis* (Mayer et al., 2002).

During 1990's, a metabolically engineered xylitol-producing yeast strain has been constructed in our laboratory. This strain converted D-glucose into xylitol via an artificial pathway composed of the same intermediates as in the process of Onishi and Suzuki. The genes encoding arabitol dehydrogenase from *Klebsiella terrigena* and XDH from *Pichia stipitis* were expressed in osmophilic yeast *Zygosaccharomyces rouxii* that naturally produces D-arabitol from D-glucose. The resulting strain was capable of directly converting D-glucose to xylitol, although in a low yield (see table 2) (Harkki et al., 1997).

Mihara et al. have extensively screened osmophilic microorganisms from soil collected from the bank of Tama River, Kawasaki-shi, Kanagawa-ken (Mihara et al., 2002). As a result they found microorganisms having ability to produce xylitol or D-xylulose from glucose. By molecular taxonomic analysis, the two isolated bacterial strains were identified as new species and designated as *Asaia ethanolifaciens* and *Zucharibacter floricola*.

Recently, Toivari et al. have demonstrated a procedure by which D-glucose can be converted to xylitol by a metabolically engineered yeast *Saccharomyces cerevisiae* (Toivari et al., 2007). They have shown that a transketolase-deficient *S. cerevisiae* strain accumulated D-xylulose 5-phosphate intracellularly and released ribitol and pentose sugars (D-ribose, D-ribulose and D-xylulose) into the growth medium. When the XDH encoding gene *XYL2* of *Pichia stipitis* was expressed in the transketolase-deficient strain, the strain resulted in an 8.5-fold enhancement of the total amount of the excreted sugar alcohols ribitol and xylitol. The deletion of the endogenous xylulokinase encoding gene *XKS1* further increased the amount of xylitol excreted by the strain.

Table 2. Summary of xylitol production from D-glucose using microbiological methods

Initial D-glucose (g/l)	Time (h)	Xylitol production (g/l)	Xylitol yield from initial D-glucose (%)	Xylitol yield from D-glucose consumed (%)	Reference
155	211	18	12	13	Onishi et al. 1969
250	144	7.5	3	n.k.	Harkki et al. 1997
200	120	1.9-6.4	1-3.2	n.k.	Mihara et al. 2002
20	100	0.3	1.5	52	Toivari et al. 2007

n.k. = not known

Considerable effort has been spent in an attempt to develop a technology for the production of xylitol from D-glucose. However, low yields and/or complicated

multi-fermentation process architectures have prevented the practical application of any of these processes described above. Table 2 summarizes the xylitol production from D-glucose by microbial methods.

In addition to D-arabitol's potential as an alternative sweetener, D-arabitol can also be used as a starting material for xylitol production. Suzuki et al. have reported the discovery of microorganisms that possess two enzymes enabling the production of xylitol from D-arabitol. *Gluconobacter oxydans* was found to have a membrane-bound D-arabitol dehydrogenase as well as a soluble XDH, which made the strain able to produce xylitol directly from D-arabitol (Suzuki et al., 2002; Sugiyama et al., 2003). Therefore there has been a growing interest in developing an effective method for D-arabitol production as well.

3 Aims of the study

The main goal of this study was to design improved metabolically engineered microbial hosts that can produce xylitol from glucose in a single fermentation step. The target was to achieve xylitol yield on glucose about two times higher than in any of the previously known processes. A secondary goal of the work was to use the same approach for constructing pathway-engineered microbial hosts producing D-arabitol. At the beginning of this work we made a strategic choice of using *B. subtilis* as a fermentation host and focusing on artificial pathways for pentitol production that involve a block in PPP and the use of pentitol phosphate dehydrogenases to direct the carbon flow towards production of pentitols.

In order to achieve these goals and according to our plan we needed to achieve the following intermediate milestones:

- To find bacterial strains - sources of APDH and XPDH
- To purify the two above-mentioned enzymes and partially sequence them
- To clone the genes encoding APDH and XPDH and express them in a suitable strain of *B. subtilis*.
- To expand our enzyme toolbox by cloning and expressing homologues of the first characterized pentitol phosphate dehydrogenases
- To characterize the recombinant pentitol phosphate dehydrogenases with respect to properties important for their application as pathway engineering tools (especially, substrate specificity)
- To construct pentulose (D-ribulose and D-xylulose) overproducing strains of *B. subtilis*
- To use the genes of selected pentitol phosphate dehydrogenases for construction of *B. subtilis* strains producing D-arabitol and xylitol.

4 Materials and methods

The most essential and previously unpublished materials and methods are presented here. The previously unpublished material is additionally pointed out with an asterisk (*) within the text. For more detailed descriptions the reader is referred to the supplemented original papers I-IV.

4.1 Microbial strains and growth media (I, II, III, IV, *unpublished)

The host strains of isolated enzymes and the strains used for the expression and cloning are listed in Table 3. Table 4 provides information about sources and genotypes of *Bacillus* strains used in this study.

Table 3. Strains used in this study

ATCC – American Type Culture Collection, JCM – Japan Collection of Microorganism, BGSC – Bacillus Genetic Stock Centre

Strain	Code	Source	Use in the study
* <i>Morganella morganii</i>	25829	ATCC	Source of XDH gene
<i>Lactobacillus rhamnosus</i>	15820	ATCC	Source of XPDH gene
<i>Bacillus halodurans</i>	9153	JCM	Source of XPDH and APDH genes
<i>Clostridium difficile</i>	43603	ATCC	Source of XPDH genes
<i>Enterococcus avium</i>	33665	ATCC	Source of APDH gene
<i>Escherichia coli</i>	XL1-Blue MRF ⁺	Stratagene	Expression host, cloning
<i>Escherichia coli</i>	XL0LR	Stratagene	Cloning
<i>Bacillus subtilis</i>	BD170	BGSC	Expression host
<i>Bacillus subtilis</i>	6051	ATCC	Source of RPE, TKT and RPI genes
<i>Bacillus subtilis</i>	31097	ATCC	Expression host

E. avium and *L. rhamnosus* were cultivated at 30°C in M.R.S. broth (Lab M, Bury, UK). For the induction of XPDH, a modified M.R.S. medium containing xylitol (20g/l) instead of glucose was used. *B. halodurans* was grown at 37°C in Horikoshi-I media containing glucose, 10 g; polypeptone 5 g; yeast extract, 5 g; K₂HPO₄, 1 g; MgSO₄·7H₂O, 0.2 g per 900 ml. After autoclaving 100 ml of 10% Na₂CO₃ solution

was added. *C. difficile* was grown in ATCC Culture medium 2107 Reinforced clostrial broth containing tryptone, 10 g; beef extract, 10 g; yeast extract, 3 g; dextrose, 5 g; NaCl, 5 g; soluble starch, 1 g; cysteine HCl, 0.5 g; sodium acetate, 3 g per 1000 ml at 37°C under anaerobic conditions. Other bacteria were grown in the LB medium (Sambrook et al. 1989) at 37°C using rotary shakers (200 rpm).

Table 4. The strains of *B. subtilis* used in this study

Strain	Source	Known genotype
6051	Obtained from ATCC	Wild type
BD170	Obtained from BGSC	<i>trpC2, trh-5</i>
31094	Obtained from ATCC	<i>tktA</i>
GX1	D-ribose phosphate isomerase disrupted in BD170	<i>trpC2, thr-5, Δ rpi, Cm</i>
GX2	D-ribose phosphate isomerase disrupted in ATCC 31094	<i>tktA, Δ rpi, Cm</i>
GX4	Transketolase gene disrupted in BD170	<i>trpC2, trh-5, Δ tktA, Ery</i>
GX5	Transketolase gene disrupted in GX1	<i>trpC2, trh-5, Δ rpi, Δ tktA, Ery, Cm</i>
GX7	Xylose resistant mutant of GX2	<i>tktA, Δ rpi, Cm</i>

4.2 Enzyme activity assays (I, II, III, IV)

APDH and XPDH activities were analyzed in a reaction mixture containing 50 mM Tris HCl, pH 7.0/8.5, 0.2 – 0.5 mM NADH/NAD⁺, 5 mM substrate (e.g. D-xylulose 5-phosphate) and about 1 mU of the enzyme. Reactions were carried out at 30°C in microtitre plate wells and the decrease in absorbance at 340 nm was followed kinetically for 10-20 minutes using a Spectramax 250 plate reader (Molecular Devices, Sunnyvale, USA). When measuring the kinetic parameters of enzymes, at least five different substrate concentrations ranging from 0.05 to 1 mM, were used. One unit of enzyme activity was defined as an amount catalyzing the oxidation of 1 μmol of NADH in 1 min.

The products of D-xylulose 5-phosphate reduction were analysed after the incubation of different pentitol phosphate dehydrogenases (10-100 mU) with D-xylulose 5-phosphate under the reaction conditions described above. The reaction mixture was treated with a phosphatase (Sigma catalogue no. P4252) and the resulting polyols were analysed with HPLC as described in the Analytical methods section.

The XDH activity was measured as XPDH and APDH activities, but using D-xylulose as a substrate.

Transketolase and D-ribose 5-phosphate isomerase were assayed by the methods described by Bublitz and Steavenson (Bublitz and Steavenson, 1988) adapted to microtitre plate format. D-Xylulokinase was assayed by the method of Simpson (Simpson, 1966).

4.3 Analytical methods (I, II, III, IV)

Sugars were analysed by HPLC on a Shimadzu LC-10AD chromatograph equipped with the RID-10A refractive index detector using an Aminex HPX87P column (Bio-Rad, Hercules USA) equilibrated at 70°C and H₂O as a mobile phase.

4.4 Purification of *E. avium* APDH (I)

All steps were carried out at 4°C. Cells from 5 liters of culture medium were separated by centrifugation (3000xg, 20 min), washed with water and re-suspended in 20 mM Tris-HCl buffer, pH 7.2, 3 mM DTT (buffer A). Cells were incubated with 0.3 % (w/v) lysozyme (Sigma Chemical Co., USA) at 20°C for 60 min, sonicated (3 × 20 sec) and centrifuged (12000xg, 20 min). The supernatant was dialysed against buffer A, applied to a DEAE 5PW (Pharmacia Biotech, Uppsala, Sweden) column (21.5×159 mm) equilibrated with the same buffer and eluted with a linear gradient (0 - 1 M) of NaCl (total volume 100 ml) at a flow rate of 3 ml/min. Fractions containing APDH activity were pooled, concentrated using an Amicon PM-30 membrane to 10 ml, and dialysed against 20 mM Tris-HCl buffer, pH 7.8, supplemented with 3 mM DTT. This material was further fractionated by chromatography on a Mono Q HR(5/5) (Pharmacia, Sweden) with a linear gradient (0 - 1 M) of NaCl in the same buffer (total volume 24 ml). The elution flow rate of 0.6 ml/min was used. The fractions with APDH activity were pooled and applied to a Sepharose Blue CL 6B (Pharmacia, Sweden) column (10 × 5 mm) equilibrated with 50 mM Tris HCl buffer, pH 8.0, 100 mM NaCl, 3 mM DTT. Fractions containing APDH activity were eluted with 3 mM NADH in the equilibration buffer (total volume 32 ml, elution flow rate 0.8 ml/min). Finally, the enzyme solution was brought to 1.7 M (NH₄)₂SO₄/30 mM Tris-HCl, pH 7.4, loaded into a Phenyl Superose HR 5/5 (Pharmacia) column equilibrated with 30 mM Tris-HCl, pH 7.4 containing 1.7 M (NH₄)₂SO₄ and eluted with a linear gradient to 30 mM Tris-HCl buffer, pH 7.4 (total volume 40 ml, flow rate 0.6 ml/min). Purified APDH was dialysed against 30 mM Tris-HCl buffer, pH 7.2.

4.5 Cloning of the APDH gene of *E. avium* and the XPDH gene of *L. rhamnosus* (I, IV)

The purified *E. avium* APDH and *L. rhamnosus* XPDH enzymes were partially sequenced. Based on the partial amino acid sequences, oligonucleotide primers were designated for both the enzymes. These primers were used to amplify a 0.6 kb and 0.85 kb fragments of the chromosomal DNA of *E. avium* and *L. rhamnosus*, respectively. Genomic libraries of *E. avium* and *L. rhamnosus* were constructed by partially digesting the chromosomal DNA with *Sau3A* and cloning the 3-10 kb fragment fractions in λ -ZAP Express phage vector (Stratagene). The libraries were subsequently screened by DNA-DNA hybridization using the PCR products as probes. Library construction and screening were performed according to the methods recommended by Stratagene.

4.6 Construction of expression vectors (III, IV, *unpublished)

The expression vectors used in this study were derivatives of pGT23 (Kerovuo et al., 2000). *B. subtilis* *degQ36* promoter (Yang et al. 1986) modified to inactivate a presumptive CRE site (Hueck et al. 1994) was amplified from *B. subtilis* ATCC 6051 genomic DNA. The PCR product was digested with *SalI* and *EcoRI* and ligated to *SalI/EcoRI* digested pGT23 to generate pGT74. The kanamycin resistance gene was amplified from pDG738 (*Bacillus* Genetic Stock Centre). The *SalI/BamHI* digested PCR product was further ligated with *SnaBI/BclI* digested pGT74 generating pGTK74.

For construction of the expression vectors, the presumptive coding areas of the XDH, APDH and XPDH genes were amplified by the PCR introducing the *EcoRI* or *MfeI* restriction site immediately upstream of the initiation codon and the second restriction site (*XbaI* or *BamHI*) downstream of the coding region. These PCR fragments were cloned into the poly-linker of pGTK74 digested with *EcoRI* and either *XbaI* or *BamHI*. *The vector for the expression of XDH gene of *M. morgani* was named as pGTK74(MXD2). The vector for the expression of APDH gene of *E. avium* was named as pGTK74(APDH3). For the expression of the APDH of *B. halodurans* the derivative of pTAC (named as pTAC(APDH)) expression vector was used. The vectors for the expression of the XPDH genes of *L. rhamnosus*, *B. halodurans*, *C. difficile* 1 and *C. difficile* 2 were named as pGTK74(LRXPDH), pGTK74(BHDH), pGTK74(CDDH980) and pGTK74(CDDH1043), respectively. The expression vectors constructed in this study are listed in table 5.

Table 5. Expression vectors constructed in this study

Vector	Expressed enzyme
*pGTK74(MXD2)	XDH of <i>Morganella morganii</i>
pGTK74(APDH3)	APDH of <i>Enterococcus avium</i>
pTAC(APDH)	APDH of <i>Bacillus halodurans</i>
pGTK74(LRXPDH)	XPDH of <i>Lactobacillus rhamnosus</i>
pGTK74(BHDH)	XPDH of <i>Bacillus halodurans</i>
pGTK74(CDDH980)	XPDH 1 of <i>Clostridium difficile</i>
pGTK74(CDDH1043)	XPDH 2 of <i>Clostridium difficile</i>

4.6 Construction of *B. subtilis* strains disrupted in *rpi* and *tktA* genes (III)

B. subtilis rpi gene encoding D-ribose 5-phosphate isomerase was identified in the genomic sequence by homology to the *rpiA* gene of *E. coli*. It was amplified by PCR using the chromosomal DNA of *B. subtilis* ATCC 6051 as a template. The 1.25 kb PCR product was digested with *EcoRI* and *HindIII* and ligated with pUC19 digested with the same restriction enzymes to form plasmid pUC(BSRPIB). The pUC(BSRPIB) was further modified by inserting the filled-in 0.83 kb *Sau3A-DraI* fragment (carrying chloramphenicol resistance gene) of pMK4 into a unique *SfuI* site within the *rpi* coding area to form plasmid pUC(BSRPIB)-cm. Similarly, the *tktA* gene was amplified from chromosomal DNA of *B. subtilis* ATCC 6051. The 2.48 kb PCR product was digested with *BamHI* and *SalI* and ligated with pUC19 to form pUC19(TKT). The *tktA* gene was disrupted by inserting into its unique *MluI* site the filled-in 1.6 kb *BamHI* fragment from pDG647 (Guérout-Fleury et al. 1995) carrying erythromycin resistance gene. The whole of the *rpi* and *tktA* genes disrupted with antibiotic-resistance cassettes were excised from the plasmids pUC(BSRPIB)-cm and pUC(TKT)-ery by digesting with restriction enzyme pairs *EcoRI/HindIII* and *XmaI/SalI*, respectively and the resulting linear fragments were used to transform *B. subtilis* strain BD170 to chloramphenicol or erythromycin resistance using the natural competence-based transformation method. In each experiment, a few hundred transformants were pooled, grown in liquid culture and re-plated on antibiotic-containing medium. Resulting clones were screened by PCR using pairs of primers annealing to the *rpi* (RPI5 and RPI3) and *tktA* (TKT5 and TKT3) genes. Clones generating PCR products of expected size were confirmed by enzymatic assays to have lost the activity of transketolase or D-ribose 5-phosphate isomerase. Disruption of the *rpi* gene was also confirmed by a rather leaky but detectable D-ribose auxotrophy of the strain GX1 (Table 4). Chromosomal DNA was isolated from the strain GX1 and used to transform *B. subtilis* strains ATCC 31094. Screening of transformants by PCR was done in the same way as during the isolation of BD170 mutants. A transformant of ATCC 31094 carrying only the

disrupted *rpi* gene and retaining the five-carbon sugar producing phenotype was named GX2.

A literature report describes the absence of D-glucose repression in the D-ribose-producing strain of *B. subtilis* ATCC 21951 (De Wulf et al., 1996). The strain ATCC 21951 is similar to the strain ATCC 31094 which is the parent of the strain GX2. Therefore we wanted to measure the D-xylulokinase activity of the GX2. In order to create positive controls to the D-xylulokinase activity measurement, the strain GX2 was plated on a plate containing LB-agar and D-xylose. D-Xylose completely arrested the growth of the GX2, but eventually spontaneous D-xylose-resistant mutants appeared as noticed also earlier with *B. subtilis* 168 (Schmiedel and Hillen, 1996). The analysis of these mutants revealed that some of them had dramatically changed profiles of D-glucose fermentation products. Out of about two hundred D-xylose-resistant mutants of GX2, seven (about 3% of all mutants) produced about 5 times more D-xylulose than GX2. The rest of the mutants produced D-ribulose and D-xylulose in the same ratio as the parent strains. To confirm that the high D-xylulose phenotype was indeed caused by a chromosomal mutation, the isolates were subjected to several rounds of sub-cloning on non-selective medium (LB). No instability was observed. One of the mutants (GX2-1) was re-named as GX7 as used in all subsequent studies.

4.7 Construction of D-arabitol and xylitol producing *B. subtilis* strains (III, IV)

B. subtilis BD170 strain was transformed with the expression vectors pGTK74(MXD2), pGTK74(LRXPDH), pGTK74(BHDH), pGTK74(CDDH980), pGTK74(CDDH1043) and pGTK74(APDH3). The chromosomal DNA from these transformed BD170 strains was isolated and used to transform GX7 strain.

4.8 Conversion of D-glucose into D-arabitol and xylitol by fermentation of *B. subtilis* (III, IV)

The fermentations were carried out in LB containing 10% D-glucose and appropriate antibiotics at 37°C in shaking flasks. The production of sugar alcohols was monitored throughout the first 300 hours. All cultures have been weighed both at the start of fermentation and then prior to taking aliquots for analysis. Sterile water was added when necessary to compensate for medium evaporation during prolonged cultivation.

5 Results

5.1 APDH

5.1.1 Purification of *E. avium* APDH (I)

The APDH of *E. avium* was purified 240-fold from crude cellular extract of *E. avium* in four chromatographic steps with a yield of 8% of the initial activity and a specific activity of 12 U/mg protein. The resulting preparation appeared homogeneous on SDS/PAGE, revealing a single polypeptide band with an estimated molecular mass of 41 ± 1 kDa. Analytical gel filtration on a Superose 12 column revealed a single symmetrical APDH peak corresponding to 160 ± 5 kDa. Essentially identical estimates were obtained using native PAGE. These observations led us to the conclusion that in its native state APDH is a tetramer.

5.1.2 Properties of *E. avium* APDH (I)

The isoelectric point of the *E. avium* APDH was 6.4. The maximal catalytic activity of APDH in the reductive reaction with NADH was found at pH 6.8 - 7.3 and in the oxidative reaction with NAD^+ at pH 8.3 - 8.6 (Fig. 4). The same pH optima in both reductive and oxidative reactions were observed using NADP^+ and NADPH as cofactors.

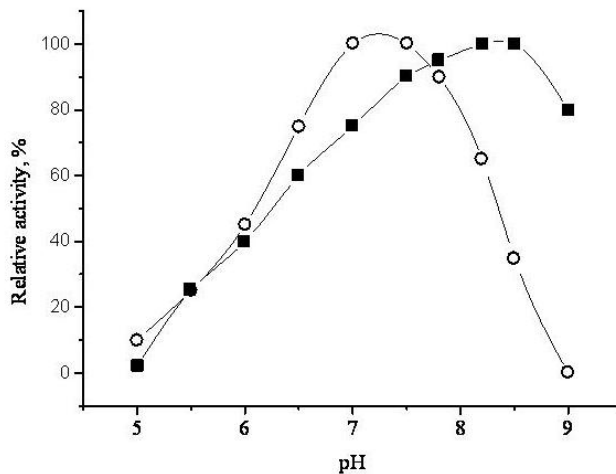


Figure 4. pH profiles for the oxidative(■) and reductive(O) reactions of the APDH

The metal content of APDH purified from *E. avium* was analysed by atomic absorption spectrometry. Pure APDH contains 4.05 ± 0.02 ions of Mn^{2+} and less than 0.1 mol of Zn^{2+} , Mg^{2+} , Ca^{2+} and other bivalent ions (Cu^{2+} , Cd^{2+} , Co^{2+} and Fe^{2+}) per tetramer. Essentially identical results were obtained using the recombinant enzyme purified from *B. subtilis*. Recombinant APDH was also used in the metal ion reconstruction experiments described below. The purified APDH was found to be inactivated by EDTA in a time- and concentration-dependent manner. The second-order rate constant for EDTA inactivation at 20 °C was $12.2 M^{-1} \cdot s^{-1}$. Inactive EDTA-modified enzyme contained less than 0.1 mol of Mn^{2+} per tetramer. Analytical gel filtration on a Superose 12 column in the presence of 1 mM EDTA and native gel electrophoresis showed that APDH treated with EDTA retained its tetrameric structure. D-Xylulose 5-phosphate did not protect the enzyme from EDTA inactivation.

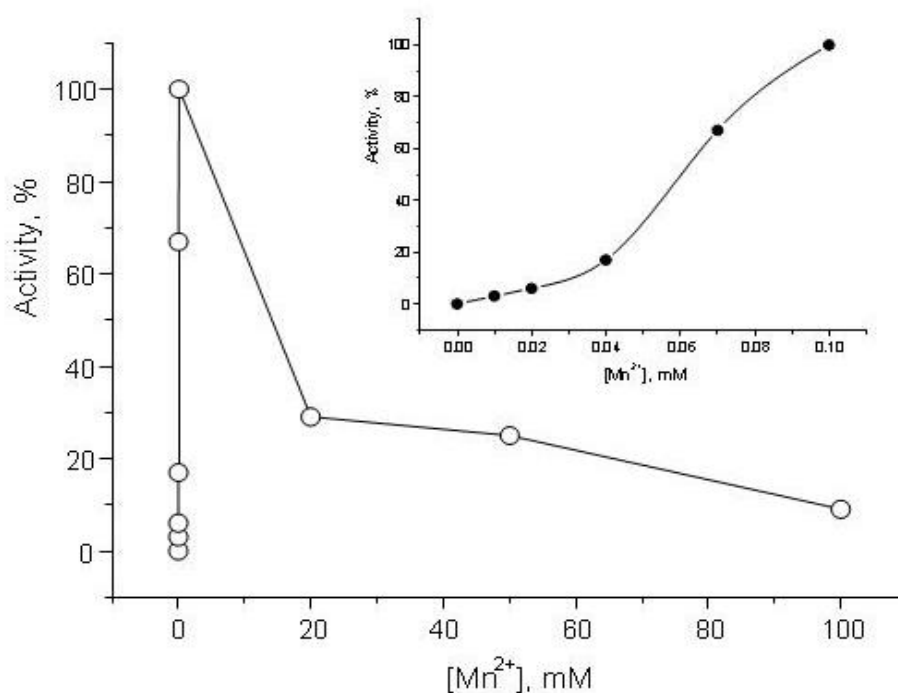


Figure 5. Effect of Mn^{2+} concentration on the EDTA-inhibited APDH
 Inset: Reconstitution of the EDTA-inhibited APDH at lower Mn^{2+} concentrations (0 – 100 μM)

The EDTA-inactivated APDH was treated with several bivalent cations (Zn^{2+} , Mg^{2+} , Ca^{2+} , Mn^{2+} and Fe^{2+}) in a concentration range of 0.1-20 mM. Only the

addition on Mn^{2+} at concentrations up to 2 mM resulted in complete re-activation of APDH. The specific activity of the re-activated enzyme was 12 ± 1 U/mg, similar to the non-treated APDH. The pH optimum of the Mn^{2+} -dependent restoration of the enzyme was studied by steady-state kinetics using D-xylulose 5-phosphate as a substrate and was about 7.0 ± 0.5 . The enzyme restored after Mn^{2+} treatment also contained 4.05 ± 0.05 ions/tetramer. Mn^{2+} ions at concentrations above 10 mM inhibited APDH activity (Fig. 5). Similarly to the yeast Mn^{2+} -dependent enolase (Lee and Nowak 1992), this may be explained by the presence of an additional Mn^{2+} -binding site in the enzyme molecule. Indeed, increasing the concentration of Mn^{2+} up to 20 mM led to the incorporation of 6.05 ions/tetramer, and up to 30 mM resulted in 8.0 ± 1 ions/tetramer. PHMG, Hg^{2+} and Zn^{2+} ions at a concentration of 2 mM completely inactivated APDH.

5.1.3 Substrate specificity of *E. avium* APDH and kinetic parameters (I)

D-Xylulose 5-phosphate was incubated with the either native or recombinant APDH in the presence of NADH followed by the treatment of the reaction products with alkaline phosphatase. When the reaction products were analysed by HPLC, only arabitol and small amounts of xylulose were observed (see Fig. 6 for illustration of the structural relationships between D-arabitol phosphates and pentulose phosphates). A very similar product profile was obtained when the experiment was conducted using D-ribulose 5-phosphate rather than D-xylulose 5-phosphate as a substrate. The initial velocity of the APDH-catalyzed reduction of D-ribulose 5-phosphate was estimated at 2-3% of the D-xylulose 5-phosphate reduction rate. Products of the APDH-catalyzed oxidation of D-arabitol 1-phosphate by NAD^+ were analysed by HPLC and after dephosphorylation of the reaction products. In addition to arabitol, only xylulose was detected in these analyses. D-Arabitol 5-phosphate was also observed to be a substrate for APDH resulting in D-ribulose 5-phosphate production. The initial rate of the APDH-catalyzed oxidation of D-arabitol 5-phosphate was 8 times lower than for D-arabitol 1-phosphate. No oxidation by APDH of xylitol 5-phosphate, D-sorbitol, D-mannitol and xylitol with NAD^+ could be detected. Likewise, D-erythrose 4-phosphate and D-ribose 5-phosphate were not reduced by APDH. Thus, the substrate specificity of APDH from *E. avium* seems to be limited to oxidation of D-arabitol 1-phosphate to D-xylulose 5-phosphate and D-arabitol 5-phosphate to D-ribulose 5-phosphate using either NAD^+ or $NADP^+$ as a co-factor.

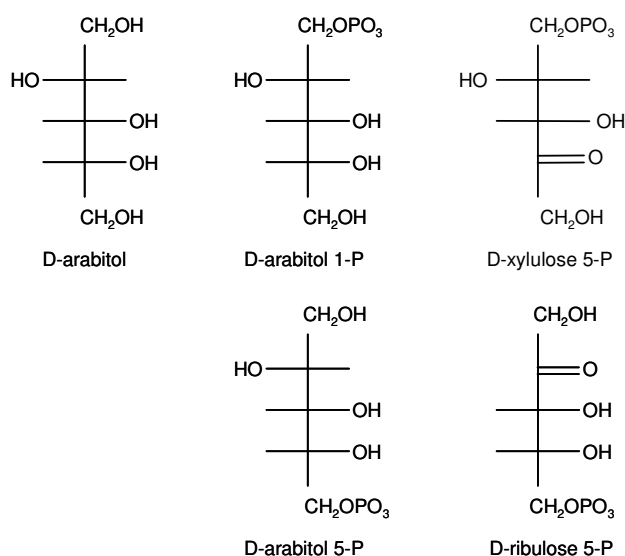


Figure 6. Structural formula of the two D-arabitol phosphates and some closely related compounds. Note that the formula for D-xylulose 5-phosphate is drawn “upside-down” to emphasize its structural relatedness to D-arabitol 1-phosphate.

Table 6 summarizes kinetic parameters obtained for the oxidative and reductive reactions of APDH catalysis. As it is seen in Table 6, the V_{\max} of the D-xylulose 5-phosphate reduction reaction is about 12-fold higher than for the oxidative reaction. Kinetic parameters of the oxidative and reductive reactions using NADP^+ and NADPH as co-factors are also listed in Table 6. The rates of both reductive and oxidative reactions with NAD^+ and NADH as cofactors were about 14 times higher than with NADP^+ and NADPH .

Table 6. Kinetic data obtained for the purified APDH from *E. avium*

Substrate and reaction direction	Co-factor	Substrate K_m (mM)	V_{\max} ($\mu\text{mol}/\text{min per mg}$)
D-xylulose 5-P, reductive	NADH	0.23 ± 0.01	14.0 ± 0.2
D-arabitol 1-P, oxidative	NAD^+	2.9 ± 0.1	1.2 ± 0.02
D-arabitol 5-P, oxidative	NAD^+	0.63 ± 0.03	0.15 ± 0.003
D-xylulose 5-P, reductive	NADPH	0.65 ± 0.03	1.2 ± 0.02
D-arabitol 1-P, oxidative	NADP^+	3.6 ± 0.2	0.09 ± 0.002

5.1.4 Cloning and expression of *E. avium* APDH gene (I)

The purified APDH was partially sequenced. Using degenerate oligonucleotides derived from 5 of these amino acid sequences (Pep1: QYNLCPHR, Pep2: EIEYIGSR, Pep3: KQGQFIQVGLFANK, Pep4: GAINIDEMITK, Pep5: VVNEITDGYGVDK) numerous PCR products were generated. The largest product (about 650 bp) was cloned and used to screen the *E. avium* genomic library. One hybridisation-positive clone containing the smallest insert was sequenced (the sequence has been deposited with GenBank under accession number AY078980). The deduced functional map of the sequenced DNA fragment is shown in Fig. 7. Four open reading frames can be identified therein (the start of frame 1 is outside of the sequenced area). The amino acid sequences of the products of these open reading frames were compared to the GenBank database using the BLASTP service of NCBI. Open reading frames 1 and 3 show high homology to components of the galactitol-PTS of *E. coli* and *Salmonella typhimurium*. The highest scoring homologue of Orf2 is a component of the PTS system in *L. monocytogenes*. The sequence of Orf4 encodes the APDH. All the sequenced peptides were found within the translated sequence of Orf4 (Fig. 8).



Figure 7. The chromosomal area of *E. avium* around the APDH gene

The whole coding sequence of Orf4 was amplified by PCR and placed under control of a *degQ36M* promoter from *B. subtilis* in a plasmid named pGTK74(APDH3). *B. subtilis* transformed with this plasmid expressed APDH at a level of about one third of the total soluble protein. The enzyme was purified from the cell extract of *B. subtilis* to near homogeneity using Sepharose-Blue chromatography. The kinetic characteristics of the recombinant enzyme were measured in both oxidative and reductive reactions. The results were essentially identical to those obtained with the enzyme isolated from *E. avium*.

MSKTMKGVSKQAPGYDQMAFIDLSVPEATDDKVLIKVAYTGICGSDIHTF
KGEYKNPTTPVVLGHEFSGQVVEVGANVTKVKVGDRVTSETTFYVCGECD
YCKEKQYNLCPHRKGIGTQQNGSMANYVLAREESIHLDPDHLSEYEGAAMS
EPLACCVHAMYQKSHLELKDTIIIMGPGPIGLYLLQIAKEIGAFVIMTGI
TKDAHRLALAKKLGADVIVDTMKEDLAKVVNEITDGYGVDKVYDASGAVP
AVNASLPLIRKQGQFIQVGLFANKMVDLDTESIQRREIEYIGSRSQNPYD
WPPIAHLLAKGAINIDEMITKKYPLTEWREAFDKVMEGNEIKVMIESNPE
EF

Figure 8. Deduced amino acid sequence of APDH from *E. avium*. The sequences of the peptides identified in the native APDH are underlined.

5.1.5 Substrate specificity of *B. halodurans* APDH (II)

The enzyme used in this study was a recombinant APDH from *B. halodurans* produced in *E. coli* and partially purified. The specificity of the enzyme and its enzymatic properties were investigated using the oxidative reaction of D-arabitol 1-phosphate. The products of the D-arabitol 1-phosphate oxidation by *B. halodurans* APDH with NAD^+ were dephosphorylated by phosphatase digestion and analyzed by HPLC. Only xylulose and trace quantities of arabitol were detected. Since D-xylulose 5-phosphate is the only (xylulose) stereoisomer that can be obtained by oxidation of D-arabitol 1-phosphate, the product of the enzymatic reaction was obviously D-xylulose 5-phosphate. The analysis of dephosphorylated products of the APDH-mediated D-xylulose 5-phosphate reduction using NADH as a co-factor revealed only arabitol as a product. As expected, the only product of the APDH-catalyzed oxidation of D-arabitol 5-phosphate was ribulose 5-phosphate. Furthermore D-erythrose 4-phosphate, D-Ribose 5-phosphate and D-fructose 6-phosphate were tested as substrates. Unlike the APDH of *E. avium* the *B. halodurans* enzyme had significant activity with D-fructose 6-phosphate as a substrate as well. Table 7 summarizes kinetic parameters obtained for the forward and reversed directions of *B. halodurans* ADPH catalysis.

Table 7. Kinetic data obtained for the purified APDH from *B. halodurans*

Substrate and reaction direction	Co-factor	Substrate K_m (mM)	V_{max} ($\mu\text{mol}/\text{min per mg}$)
D-xylulose 5-P, reductive	NADH	0.22 ± 0.004	27.0 ± 0.5
D-arabitol 1-P, oxidative	NAD ⁺	4.0 ± 0.1	5.9 ± 0.15
D-ribulose 5-P, reductive	NADH	4.0 ± 0.051	3.6 ± 0.1
D-fructose 6-P, reductive	NADH	0.27 ± 0.005	5.0 ± 0.15

5.2 XPDH

5.2.1 Cloning of XPDH gene of *L. rhamnosus* (IV)

The only XPDH that has been previously described in literature is the enzyme from *Lactobacillus casei* CI83 (Hausman and London 1987). However, this strain has never been deposited into a public strain collection and no DNA or protein sequence information related to XPDH has yet been published. A private communication from J. London (NIDR, Bethesda, USA) suggested the use of a strain of *L. rhamnosus* ATCC 15820 as a precursor of the XPDH-producing strain of *L. casei* CI83. Based on this information; the enzyme was isolated and partially sequenced. Using the protein sequence data, the gene encoding XPDH was cloned. The sequence has been deposited with GenBank under accession number AY533148.

In an attempt to find an enzyme with the most suitable substrate specificity for the xylitol production process, several candidate XPDH genes were identified based on their amino acid sequence homology to *L. rhamnosus* enzyme and the genes encoding the candidate XPDH enzymes were cloned.

5.2.2 Substrate specificity of the XPDH enzymes (IV)

The substrate specificity of different recombinant XPDH enzymes was investigated with a number of substrates. The recombinant enzymes of *L. rhamnosus*, *B. halodurans* and *C. difficile* were all found to catalyze the reduction of both D-xylulose 5-phosphate and D-ribulose 5-phosphate, but none of the neutral keto-sugars. Xylitol was the only polyol found among the dephosphorylated reaction products of D-xylulose 5-phosphate. It was also found (Table 8) that there is a considerable variation in substrate specificity among the XPDH from different sources. The preferred substrate for XPDH of *B. halodurans* and XPDH 2 of *C.*

difficile seemed to be D-ribulose 5-phosphate suggesting that these two enzymes should be referred to as ribitol phosphate dehydrogenases rather than XPDH. In addition, the XPDH of *B. halodurans* also catalyzed the reduction of D-fructose 6-phosphate. D-Erythrose 4-phosphate and D-xylulose were also tested as substrates, but none of the enzymes catalyzed the reduction of either of them. XPDH of *L. rhamnosus* and XPDH 1 of *C. difficile* demonstrated narrower substrate specificity. XPDH of *L. rhamnosus* and XPDH 1 of *C. difficile* had also similar K_M values for D-xylulose 5-phosphate (0.75 and 0.52 mM respectively). These results are in agreement with the data on XPDH from *L. casei* (Hausman and London, 1987).

Table 8. Substrate specificity of different XPDH enzymes.

The substrates are abbreviated as follows: Xlu5P – D-xylulose 5-phosphate; Rlu5P – D-ribulose 5-phosphate; Glu6P – D-glucose 6-phosphate; Fru6P – D-fructose 6-phosphate

Enzyme	Relative activity at 5 mM substrate concentration (%)			
	Xlu5P	Rlu5P	Glu6P	Fru6P
<i>L. rhamnosus</i> XPDH	100	45.6	2.2	1
<i>B. halodurans</i> XPDH	100	115.2	2.8	27.1
<i>C. difficile</i> XPDH 1	100	38.7	< 1	8.6
<i>C. difficile</i> XPDH 2	100	120.2	< 1	< 1

Both XPDH of *L. rhamnosus* and XPDH 1 of *C. difficile* displayed high degree of selectivity towards D-xylulose 5-phosphate even when their selectivity towards D-xylulose 5-phosphate and D-ribulose 5-phosphate were tested by a procedure using increasing D-ribulose 5-phosphate 3-epimerase concentrations. At the highest D-ribulose 5-phosphate 3-epimerase concentration, the two pentulose phosphates were continuously in equilibrium and the ratio of xylitol phosphate to ribitol phosphate was approximately 100:1 (Fig. 9).

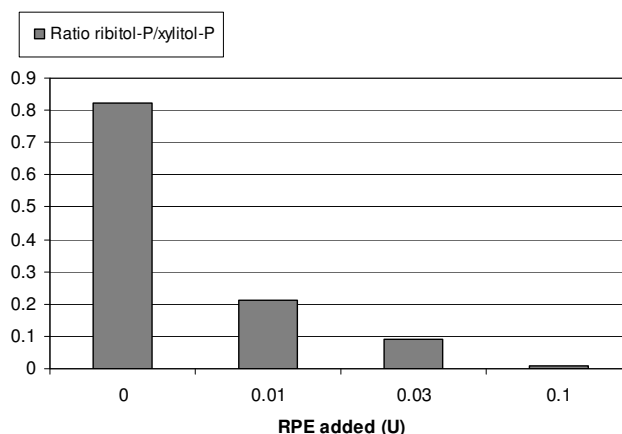


Figure 9. Products of the reaction between D-ribulose 5-phosphate and excess NADH in the presence of XPDH from *L. rhamnosus* (0.1 U) and varying amounts of D-ribulose 5-phosphate 3-epimerase (RPE). The crude preparation of recombinant XPDH derived from *E. coli* used in this experiment contained a small amount of D-ribulose 5-phosphate 3-epimerase. This and a significant amount of D-xylulose 5-phosphate present in the commercial preparation of D-ribulose 5-phosphate account for the production of xylitol phosphate even in the absence of added epimerase.

5.3 Engineering of PPP of *B. subtilis* (III)

A plasmid containing *B. subtilis tktA* disrupted with erythromycin resistance gene was constructed and used to transform *B. subtilis* strain BD170. The disruption of the chromosomal *tktA* gene in transformants was confirmed by PCR. The resulting strain (GX4, Table 4) was cultivated in a medium containing 10% glucose and fermentation products were analysed by HPLC. Indeed, the strain GX4 produced ribose. However, the ribose production levels were approximately 5-10 times lower than by *B. subtilis* strain ATCC 31094 when cultivated under the same conditions. With a strain where both *tktA* and *rpi* genes were disrupted (GX5, Table 4) no five-carbon sugar production could be detected. The genetically engineered *tktA* mutants also grew slower than D-ribose-producing strains obtained by random mutagenesis (ATCC 31094), particularly on high-glucose media. Based on these results, *B. subtilis* ATCC 31094 was used as a starting point for construction of strains producing other five-carbon sugars.

In order to study the effect of *rpi* gene disruption in D-ribose-producing *B. subtilis* strains, the *rpi* gene from *B. subtilis* strain ATCC 6051 was cloned by PCR. The cloned *rpi* gene was disrupted with chloramphenicol resistance gene and used to transform *B. subtilis* BD170 to chloramphenicol resistance. Chromosomal DNA of

the resulting strain GX1 was subsequently used to transform the strain ATCC 31094 thereby leading to strain GX2 (Table 4). This “indirect” transformation procedure was necessary because the transformability of the D-ribose-producing strain ATCC 31094 was very low. D-Ribulose was the main product of D-glucose fermentation by GX2 (obtained in about 35% yield on consumed D-glucose, Table 9). D-Xylulose was also detected but in a much lower amount (about 2% on consumed D-glucose). No D-ribose was detected. Strain GX7 is a derivative of GX2, carrying a spontaneous mutation that confers resistance to D-xylose. The total amount of pentuloses produced by GX7 in glucose fermentations is similar to that of GX2; however, the D-xylulose : D-ribulose ratio is much higher (about 1:2).

Table 9. D-Xylulose and D-ribulose yields in fermentation of *B. subtilis* strains

<i>B. subtilis</i> strain	D-xylulose, (g/l) ^{a)}	D-ribulose, (g/l)	ribose (g/l)
ATCC 31094	0.6 ± 0.2	4.8 ± 0.8	7.4 ± 1.9
GX2	0.9 ± 0.3	14.2 ± 1.8	0
GX7	4.3 ± 0.7	8.1 ± 0.9	0

a) The figures in the table are averages from three to five independent fermentation experiments. 95% confidence intervals are provided.

5.4 Production of D-arabitol and xylitol by *B. subtilis* strains (III, IV, *unpublished)

In order to study the xylitol and D-arabitol productivity in *Bacillus*, the XDH of *M. morgani*, XPDH of *L. rhamnosus* and *C. difficile* as well as APDH of *E. avium* were expressed in pentulose producing *B. subtilis* GX7 strain. The expression levels of the enzymes in *B. subtilis* BD170 cell extracts grown in LB were 10.0 U/mg protein for XDH of *M. morgani*, 0.5 and 1.1 U/mg for XPDH of *L. rhamnosus* and *C. difficile*, respectively, and 4.1 U/mg for APDH of *E. avium*. However, the expression levels dropped 5-10 times when the genes were transferred into the *B. subtilis* GX7 strain and grown on LB-glucose. Several other promoters (all derived from *B. subtilis*) were also tested: *cdd* (also known as p43 promoter) (Wang and Doi, 1984), *fba1* (*tsr*) and *pst* (Qi et al., 1997), but none of the alternative expression vectors provided an improvement in the activity of recombinant XDH, XPDH or APDH in GX7 cultivated under high-glucose conditions (data not shown).

In GX7[pGTK74(MXD2)] strain relatively high levels of XDH activity (over 2 U/mg protein) were measured. When grown on LB-glucose, this strain, as expected, did produce xylitol; however, the yield remained rather low and D-xylulose was not completely converted into xylitol under any conditions tested. Although D-xylulose – xylitol conversion improved somewhat when the cells were cultivated with low

aeration, the total five-carbon sugar yield under these micro-aerobic conditions dropped significantly (data not shown).

In GX7[pGTK74(LRXPDH)], GX7[pGTK74(CDDH980)] and GX7[pGTK74(APDH3)] strains, even though the levels of the pentitol phosphate dehydrogenase enzyme activities were modest, pentitols were the major D-glucose fermentation product of these strains (see Table 10).

Table 10. Production of five carbon sugars and pentitols by *B. subtilis* strain GX7 expressing XDH, XPDH or APDH

GX7 and enzyme expressed	Products in fermentation broth, % of consumed glucose ^(a)				
	xylitol	ribitol	D-arabitol	D-xylulose	D-ribulose
None	1.2± 0.1	1.4± 0.1	< 1	9.5± 1.4	20.0± 1.7
<i>M. morgani</i> XDH	3.4± 0.3	n.d.	< 1	3.5± 0.5	14.5± 1.5
<i>L. rhamnosus</i> XPDH	22± 1.9	5.4± 0.8	< 1	4.1± 0.6	3.8± 1.3
<i>C. difficile</i> XPDH 1	23± 1.8	2.0± 0.1	< 1	2.7± 0.3	4.5± 0.6
<i>E. avium</i> APDH	< 1	< 1	38± 4.3	< 1	2.6± 0.5

(a) The values in the table are averages from three to five independent fermentation experiments. 95% confidence intervals are provided.

n.d. = not detected

6 Discussion

6.1 APDH – a novel enzyme of pentitol metabolism

As described in the literature review (section 2.4) of this thesis, it has been shown that pentitols can be catabolized by bacteria via pentitol phosphate intermediates (London and Chase, 1977). Studies of such pathways have, however, been limited to a few species of *Lactobacillus* and only two pentitols: xylitol and ribitol (Hausman and London, 1987). Two types of dehydrogenases involved in arabitol assimilation via pentulose intermediates have been characterized (Scangos and Reiner, 1978; Neuberger et al., 1979; Wong et al., 1995). Route 1 (Fig. 10) is common in bacteria and route 2 occurs typically in yeast. Hausman and London concluded (Hausman and London, 1987) that in *Lactobacillus casei* XPDH can also oxidize D-arabitol 5-phosphate and is probably involved in arabitol assimilation by such strains. This hypothesis implies that the reduction of D-xylulose 5-phosphate and D-ribulose 5-phosphate catalyzed by this enzyme leads to products with different stereochemical configurations around C-2. Such specificity would be very unusual for an NAD⁺-dependent dehydrogenase. In this thesis a dehydrogenase

with specificity towards D-arabitol phosphate is isolated from a pentitol-utilizing *Enterococcus avium* and an initial structural and functional characterization of the enzyme is presented.

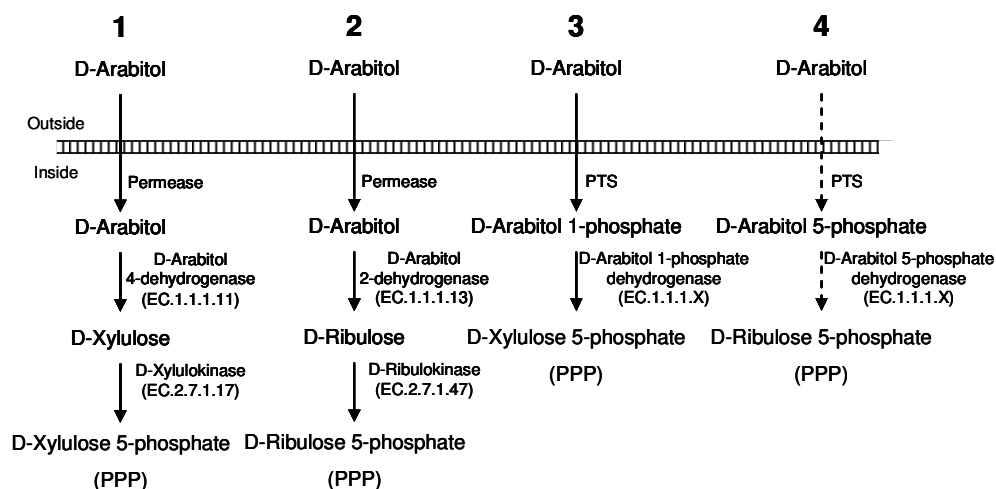


Figure 10. Different catabolic routes for D-arabitol

The analysis of the *E. avium* APDH sequence places it within the large family of medium chain polyol/alcohol dehydrogenases (Jörnvall et al. 1993). It is well known that in most medium chain dehydrogenases Zn^{2+} plays the role of metal co-factor, although in some enzymes, for example bovine lens sorbitol dehydrogenase, either Zn^{2+} or Mn^{2+} can function interchangeably (Marini et al. 1997). The metal content analysis of the purified APDH from *E. avium* revealed that Mn^{2+} appears to be the only acceptable co-factor ion for the enzyme activity. Thus, the metal-ion dependency of *E. avium* APDH is different from that of threonine dehydrogenase, where both Zn^{2+} and Mn^{2+} are required for maximal activity (Epperly and Dekker 1991).

The homology comparison of the amino acid sequence of *E. avium* APDH using the BLAST service pointed out that the strongest matches are found to the proteins deduced from the genomic DNA sequences of several Gram-positive species, particularly from *Lactobacillus plantarum* (GenBank accession number NP_786748), *Listeria monocytogenes* (ZP_01928588.1), *Listeria innocua* (NP_472141.1), *Clostridium difficile* (ZP_02745459.1), *Staphylococcus aureus* (YP_492956.1) and *Bacillus halodurans* (NP_241055.1). None of these proteins had been characterized biochemically. In comparison to these homologues, all the enzymes with known substrate specificity are more distantly related to APDH. Among such enzymes, bacterial sorbitol dehydrogenases (also referred to as polyol

or xylitol dehydrogenases) show the closest match. In order to verify that the high-scoring homologues of APDH share the substrate specificity with the enzyme from *E. avium*, the ORF from the genomic sequence of *B. halodurans* was amplified, expressed in *E. coli* and purified. Indeed, these two enzymes have rather similar kinetic characteristics in the reactions with D-arabitol 1-phosphate and D-xylulose 5-phosphate. Both enzymes also accept NADP⁺ and NADPH as co-factors. The only difference between the two enzymes is the fact that the APDH of *B. halodurans* has wider substrate specificity than the *Enterococcus* enzyme and can use D-fructose 6-phosphate in the reductive reaction. However, the similarity in the enzyme kinetics of these two enzymes strongly suggests that the other uncharacterized open reading frames with even higher homology than that of *B. halodurans* enzyme (in the genomes of *L. monocytogenes*, *L. innocua* and *S. aureus*) also encode APDH. In the case of *Listeria*, the conclusion that this bacterium assimilates D-arabitol via the D-arabitol phosphate route is supported by the findings of Saklani-Justforgues et al. (Saklani-Justforgues et al., 2001). These authors mapped a mutation that leads to the inability of *L. monocytogenes* to assimilate D-arabitol to a component of the PTS system. The APDH homologue identified in our searches is located in the same operon.

The strong preference of APDH for D-arabitol 1-phosphate argues in favor of a hypothesis that D-arabitol is assimilated in *E. avium* and *B. halodurans* via route 3 in Figure 10. However, theoretically, it may also be possible that both routes 3 and 4 work in parallel. Biochemical studies of the D-arabitol-PTS would be needed to resolve this question.

6.1.1 Pentitol phosphate route is common among Gram-positive bacteria

When London and Chase discovered the new route for pentitol metabolism in *L. casei* and identified the two enzymes – XPDH and ribitol phosphate dehydrogenase – catalyzing the reactions, they came to a conclusion that the pentitol phosphate route is characteristic for lactic acid bacteria (London and Chase, 1977). In this thesis the XPDH of *L. rhamnosus* has been cloned and sequenced. When the amino acid sequence of *L. rhamnosus* XPDH was used to search homologies using a BLAST service, the result was very similar to the homology comparisons of *E. avium* APDH. The closest matches for *L. rhamnosus* XPDH were uncharacterized proteins from species like *Listeria monocytogenes* (NP_466186), *Listeria innocua* (NP_472142), *Bacillus halodurans* (NP_241053), *Clostridium difficile* (YP_001088839, YP_001086964) and *Staphylococcus aureus* (YP_039708). When the results obtained from homology searches made by APDH and XPDH enzymes as well as the biochemical evidence from the cloned and characterized enzymes are taken together, it can be concluded that the pentitol phosphate route does not

exist just in lactic acid bacteria, but seems to be much more widely distributed among Gram-positive bacteria than was previously concluded.

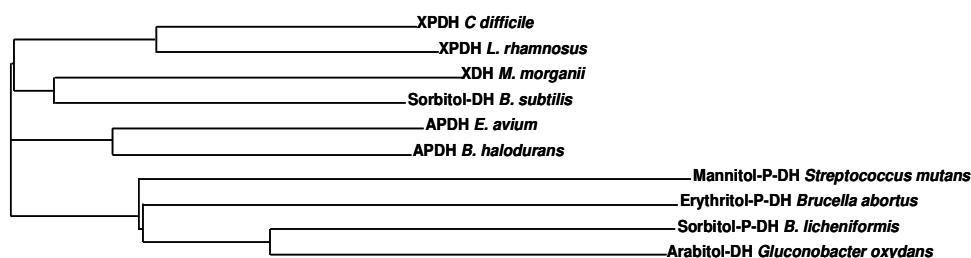


Figure 11. Phylogenetic relationships between several polyol and polyol phosphate dehydrogenases. NCBI accession numbers of the sequences used in phylogenetic comparison: YP_001088839 *C. difficile* XPDH, AAT02414 *L. rhamnosus* XPDH, Q59545 *M. organii* XDH, NP_388496 *B. subtilis* sorbitol dehydrogenase, AAL78068 *E. avium* APDH, NP_241055 *B. halodurans* APDH, AAA26942 *Streptococcus mutans* mannitol phosphate dehydrogenase, AAD11520 *Brucella abortus* erythritol phosphate dehydrogenase, YP_093371 *B. licheniformis* sorbitol phosphate dehydrogenase, AAX35768 *Gluconobacter oxydans* arabitol dehydrogenase

The phylogenetic tree presented in Figure 11 includes the sequences of several polyol and polyol phosphate dehydrogenases. The homology comparisons suggest that APDH and XPDH enzymes are derived from (or closely related to) medium chain polyol dehydrogenases like XDH and sorbitol dehydrogenase. It can also be noted that the sequences of hexitol and tetritol phosphate dehydrogenases show much weaker homology to APDH and XPDH enzymes indicating that polyol phosphate dehydrogenases do not form a common evolutionary branch.

6.2 Modifying the PPP of *B. subtilis* for D-arabitol and xylitol production

6.2.1 Mutation in transketolase gene alone is insufficient for high D-ribose production in *B. subtilis*.

D-Ribose-producing strains of *B. subtilis* obtained by random mutagenesis are known since the 1970's and available from public strain collections (e.g. ATCC 31094). In literature, production of five-carbon sugars by these strains has always been explained only by mutations in transketolase or D-ribulose 5-phosphate

epimerase genes (De Wulf et al., 1997; Sasajima and Yoneda, 1971 and 1974). In our hands, the transformability of the chemically mutagenised D-ribose-producing strains of *B. subtilis* was very poor. Therefore, the study was started by checking whether a disruption of transketolase gene by targeted mutagenesis would be sufficient to create a similar D-ribose-producing phenotype in a well transformable strain of *B. subtilis*. Towards this end, a *B. subtilis* strain disrupted in its transketolase gene was constructed and its production of D-ribose analysed. It was observed, however, that this strain produced less D-ribose and grew slower than the strain ATCC 31094, which strongly suggests that the D-ribose-producing strains obtained by Sasajima and Yoneda by random mutagenesis (Sasajima and Yoneda, 1971 and 1974) contain additional mutations partly compensating for the metabolic defects caused by the inactivation of transketolase gene.

6.2.2 Expression of XDH in pentulose producing *B. subtilis*

Figure 12 presents the most relevant metabolic pathways to bioconversion of D-glucose to five-carbon sugars. As it can be seen in Figure 12 the first logical step towards conversion of a D-ribose-producing strain into a D-ribulose and D-xylulose-producing strain is the disruption of the D-ribose 5-phosphate isomerase gene (E2, Fig 12). This mutation blocks D-ribose 5-phosphate formation and leads to intracellular accumulation of D-ribulose 5-phosphate and D-xylulose 5-phosphate.

When this project was started, the production process for xylitol was set to be the ultimate target of the project. At that time no sequence information of the polyol phosphate dehydrogenases was available, so it was decided to overexpress XDH (E6, Fig. 12.) gene within GX7 cells. It is known that most polyol dehydrogenases (including xylitol/sorbitol dehydrogenases) have quite a broad substrate specificity. However, the substrate specificity of XDH from *M. morgani* has been reported to be rather narrow. It accepts xylitol but not ribitol or D-arabitol as a substrate (Doten and Mortlock, 1985). Therefore, *M. morgani* XDH was selected as a candidate for xylitol production. The results in fermentations of GX7[pGTK74(MXD2)] were however disappointing, since xylitol yield remained very low. In addition, the observed D-xylulose:xylitol ratios did not reflect the thermodynamic equilibrium between these two compounds under normal (reducing) intracellular conditions. For example, in a strain of *Zygosaccharomyces rouxii* producing xylitol from D-arabitol via D-xylulose, no keto-sugars were detected in the fermentation broth (Harkki et al., 1997). The reasons for poor D-xylulose – xylitol conversion despite high levels of XDH activity are not quite clear. One possible explanation could be that sugar-phosphate dephosphorylation and sugar excretion may be somehow linked in *B. subtilis* making dephosphorylated sugar (D-xylulose) inaccessible to the intracellular dehydrogenase.

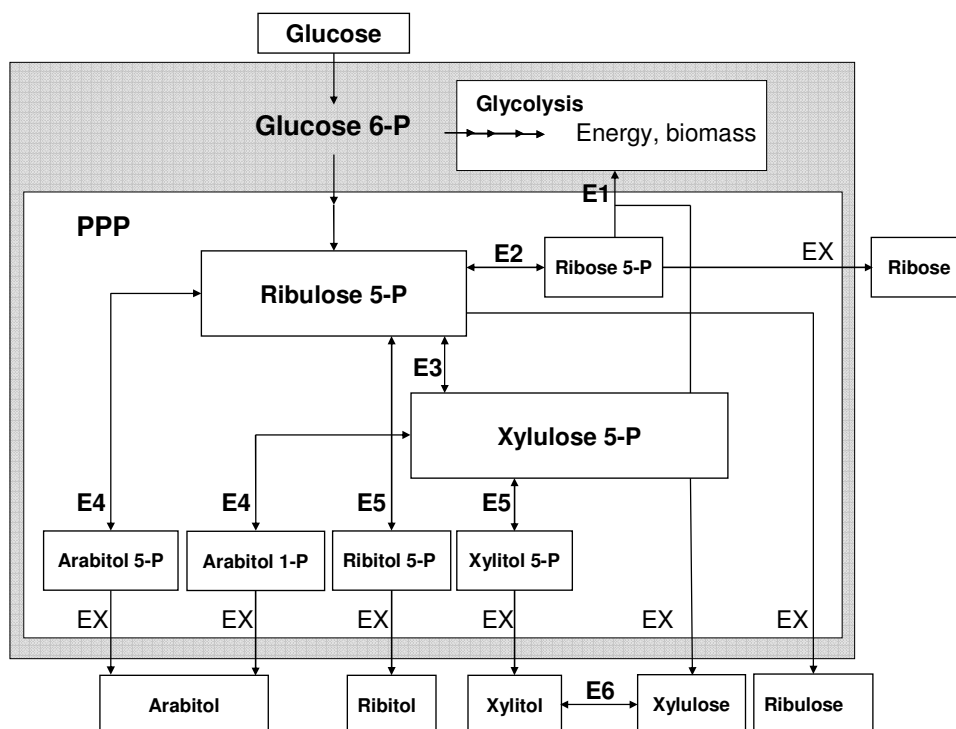


Figure 12. The metabolic pathways most relevant to the bioconversion of D-glucose into five-carbon sugars in *Bacillus*. All the sugars are presumed to be in D-configuration, “P” – denotes phosphoester moiety. The enzymes are designated as follows: E1 – transketolase; E2 – D-ribose 5-phosphate isomerase; E3 – D-ribulose 5-phosphate 3-epimerase; E4 – APDH; E5 – XPDH; E6 – XDH; EX – a presumptive intracellular (or membrane-associated) sugar-phosphate phosphatase.

6.2.3 Using APDH and XPDH for D-arabitol and xylitol production

We hypothesized that the problems occurring in the xylitol production using XDH could be solved by using specific pentitol phosphate dehydrogenases. Because of the favorable equilibrium position in a NADH-dependent reaction such an enzyme could convert the total pentulose phosphate pool into pentitol phosphates in high yield. Subsequent dephosphorylation and excretion could be mediated by the same non-specific mechanism that operates during the production of keto-sugars.

Indeed, in *B. subtilis* GX7[pGTK74(APDH3)] D-arabitol was produced from D-glucose with a yield of 38% in shake-flask cultivations. The only fermentation processes for D-arabitol production from D-glucose in significantly higher yields are

described for yeast species such as *Metschnikowia reukaufii* (Nozaki et al., 2003) and *Zygosaccharomyces rouxii* (Saha et al., 2007). The D-arabitol yield in *M. reukaufii* is reported to be 52% and that from *Z. rouxii* 48%. However, these yields can not be directly compared with the *B. subtilis* yields of the current study, since the yeast data is obtained under an optimized fermentation conditions.

It is known that certain osmophilic yeasts when grown in the presence of high concentrations of D-glucose produce a variety of polyols like D-arabitol, erythritol, mannitol or glycerol (Blaklet and Spencer, 1962; Onishi and Suzuki, 1969). In contrast to these D-arabitol producing yeasts, no other polyols were produced by metabolically engineered *B. subtilis*. Complete absence of other pentitols in the culture medium can be explained by the fact that the *E. avium* APDH (E4 in Fig. 12) accepts both D-xylulose 5-phosphate and D-ribulose 5-phosphate as substrates reducing them to D-arabitol 1-phosphate and D-arabitol 5-phosphate, respectively. After dephosphorylation both pentitol phosphates yield the same product, D-arabitol. A certain amount of D-ribulose can be detected as a by product in *B. subtilis* GX7[pGTK74(APDH3)] fermentations. That is probably due to the relatively low expression level of APDH in high-glucose medium.

In xylitol production the substrate specificity of the XPDH enzyme is clearly essential for its use as a metabolic engineering tool. In particular, the high ratio of “D-xylulose 5-phosphate reductase” to “D-ribulose 5-phosphate reductase” activity is crucial to the efficient production of xylitol by microbial strains blocked in the PPP. The two reactions catalyzed by XPDH (E5) are illustrated in Figure 12. In an attempt to find an enzyme with the highest specificity towards the D-xylulose 5-phosphate, several candidate XPDH genes were cloned and expressed. The substrate specificities of the XPDH enzymes were studied and the results led us to a conclusion that XPDH of *L. rhamnosus* and XPDH 1 of *C. difficile* are both suitable candidates for the construction of artificial D-glucose-xylitol pathway in *B. subtilis*.

Xylitol was produced from D-glucose by *B. subtilis* GX7[pGTK74(LRXPDH)] and GX7[pGTK74(CDDH980)] strains with a yield of 22-23% in the shake-flask cultivations. The yield is markedly lower than the D-arabitol yield in D-arabitol producing GX7[pGTK74(APDH3)] strain. This can be explained by two factors. Firstly, the expression levels of both XPDH genes of *L. rhamnosus* and *C. difficile* are lower compared to the expression level of *E. avium* APDH resulting in the higher residual D-xylulose and D-ribulose production by GX7[pGTK74(LRXPDH)] and GX7[pGTK74(CDDH980)]. Secondly, the substrate specificity of XPDH enzymes differs from APDH. While *E. avium* APDH reduces both D-xylulose phosphate and D-ribulose phosphate to D-arabitol phosphate, XPDH of *L. rhamnosus* and *C. difficile* reduce D-xylulose phosphate to xylitol phosphate and D-ribulose phosphate to ribitol phosphate (see Fig. 12). This leads to the co-production of ribitol during fermentation.

A study on xylitol production by metabolically engineered *Saccharomyces cerevisiae* has been conducted in parallel to the one presented in this thesis. The results of this study have recently been published by Toivari et al (2007). The strategy of the yeast study had a number of similarities with ours – using a transketolase mutation to introduce a metabolic block into PPP and a specific

dehydrogenase to convert pentuloses into pentitols. However, Toivari et al. used only polyol dehydrogenases to convert neutral pentuloses into pentitols. Pentitol phosphate dehydrogenases were not tested in the yeast study. In our opinion, this may be one of the major reasons for a much lower xylitol yield obtained with the xylitol-producing yeast strains compared to the *B. subtilis* strains of the present study. Another likely reason for low xylitol yield in yeast is the low capacity of the PPP of *S. cerevisiae*; 4-10 % of D-glucose is channeled through PPP during respirofermentative growth (Gancedo and Lagunas, 1973; Maaheimo et al., 2001). In *B. subtilis*, the PPP is a major pathway in D-glucose metabolism. The flux of D-glucose through PPP can reach 50% of the total D-glucose (Sauer et al., 1996).

6.2.4 Improving the D-arabitol and xylitol productivity

The observations made with D-arabitol producing and xylitol producing strains provide a clear guidance for further improvement of D-arabitol and xylitol production by metabolically engineered *B. subtilis* strains. D-xylulose and D-ribulose production is expected to become negligible, if APDH and XPDH genes were expressed from a promoter that remains highly active under the conditions of prolonged cultivation in high-glucose media. Furthermore, ribitol production may be reduced in xylitol-producing strains. In experiments simulating the transformation of D-ribulose 5-phosphate by mixtures of D-ribulose 5-phosphate 3-epimerase and XPDH, ribitol phosphate : xylitol phosphate ratio is about 1:100 (Fig. 9). When this result is compared to ribitol : xylitol ratio in culture broth of GX7[pGTK74(LRXPDH)] (1:10), it leads to a conclusion that the rather high level of ribitol formation during fermentation of xylitol-producing *B. subtilis* strains is caused by a limitation in activity of D-ribulose 5-phosphate 3-epimerase.

An increase in the carbon flux through the PPP would also improve the pentitol production in metabolically engineered *B. subtilis* strains. Zhu et al. have shown that the overexpression of glucose dehydrogenase (*gdh*) gene in riboflavin-producing *B. subtilis* resulted in increased riboflavin production and an increased rate of growth while D-glucose consumption remained unchanged (Zhu et al., 2006). The precursor for riboflavin biosynthesis in *B. subtilis* is D-ribulose 5-phosphate. Since D-ribulose 5-phosphate is also a precursor for formation of xylitol and D-arabitol in the *B. subtilis* strains of the present study, over-expression of *gdh* gene can be expected to have a similar effect on pentitol yield.

Another approach towards higher yields of D-arabitol and xylitol production could be the enhancement of the D-glucose uptake by the cells. Our preliminary results suggest that overexpression of the gene encoding glucose permease (GLP) as well as the *ycxE-gdh* operon encoding glucose facilitator (GLF) and GDH indeed lead to increased utilization rate of glucose by *B. subtilis* (data not shown).

Acetoin is a product of fermentative metabolism in many prokaryotic and eukaryotic microorganisms and also in *Bacillus* spp. In *B. subtilis* grown on D-

glucose acetoin is a major extracellular product and the production of acetoin was also observed in our experiments with D-arabitol and xylitol-producing *B. subtilis* strains. Acetoin acts as an external carbon storage material and it is synthesized and excreted during exponential growth (Renna et al., 1993). Even though acetoin is not formed via the PPP, the inactivation of the α -acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD*) genes in the acetoin synthesis pathway might increase the yields of D-arabitol and xylitol. Another reason for acetoin synthesis pathway (see Fig. 2) inactivation is the organoleptic properties of acetoin, which are highly undesirable for D-arabitol and xylitol intended to be used as a food ingredient.

Finally, all the fermentation experiments of this study were done using shake flasks. There is little doubt that by running the bioconversion process in a fermentor under optimized conditions both productivity and product yield could be substantially improved.

6.3 Future perspectives

In addition to D-arabitol and xylitol production, the enzymes involved in the pentitol and pentose metabolism in bacteria are of interest because of their potential in production of other products as well. Especially the interest in production of rare sugars, which are used in the pharmaceutical industry, has increased in the last few years. One example is L-ribose, which is a potential starting material for many pharmaceutical compounds targeting for treatment of hepatitis B virus infection (Doong et al., 1991; Casey et al, 2003; Tam et al., 2003). Recently, Woodyer et al. have described an enzyme, mannitol-1-dehydrogenase of *Apium graveolens*, which is able to catalyse the conversion of ribitol to L-ribose. They have expressed the mannitol-1-dehydrogenase of *A. graveolens* in *E. coli* and thereby constructed an efficient method for L-ribose production using ribitol as a starting material (Woodyer et al., 2008). With minor changes the metabolically engineered *B. subtilis* strains described in this thesis could be modified to produce ribitol. By expression of an enzyme with the same substrate specificity as the *A. graveolens* enzyme even a *B. subtilis* strain producing L-ribose from D-glucose could be created, but there are certainly rare sugars that could be enzymatically converted from D-arabitol and xylitol as well.

7 Summary and conclusions

The ultimate target of the project described in this thesis was to develop a new, single host fermentation process for D-arabitol and xylitol production using D-glucose as a starting material. *B. subtilis* was selected as a production host because of its GRAS-status and its highly active PPP compared to other bacteria, and because it is easily amenable for genetic modifications.

When the study was started, little was known about the bacterial pentitol phosphate dehydrogenases. However, the very fact that such enzymes were known to exist, allowed us to draft a research plan wherein these enzymes would be a key for development of an efficient D-arabitol and xylitol production process. Therefore, a search for pentitol phosphate dehydrogenase producing Gram-positive bacteria was initiated. This search has eventually led us to a discovery of a novel pathway for D-arabitol metabolism. The key enzyme of the pathway, APDH, was purified from *E. avium*, characterized and the gene encoding APDH was cloned and sequenced. Although some information about XPDH from *L. rhamnosus* has been available for a long time (Hausman and London, 1987) no sequence information making this enzyme available as a tool for pathway engineering has ever been published. We were first to clone and sequence an XPDH gene, the gene from *L. rhamnosus*. Based on the sequence homology a number of other XPDH enzyme candidates were identified. Several genes encoding the presumptive pentitol phosphate dehydrogenases were cloned from various species of Gram-positive bacteria. Biochemical characterization of the products of these genes confirmed that they do indeed encode enzymes with pentitol phosphate dehydrogenase activity. The analysis of the genomic sequences of the bacteria from which the pentitol phosphate genes were cloned revealed that these genes invariably occur as parts of operons that also include substrate-specific components of the PTS system. These observations were considered as a strong evidence that both D-arabitol and xylitol catabolism via a metabolic route involving the corresponding pentitol phosphate is widely distributed among Gram-positive bacteria. This conclusion significantly expands the earlier findings of London and Chase (1977), which were limited to only two pentitols (xylitol and ribitol) and only one bacterial family (*Lactobacillus*).

The work on construction of pentitol-producing *B. subtilis* strains started by demonstration that the inactivation of the D-ribose 5-phosphate isomerase gene in the D-ribose-producing *B. subtilis* strain ATCC 31094 results in a strain producing elevated levels of ketopentuloses, in particular D-ribulose and D-xylulose. Furthermore it was found that the expression of APDH or XPDH genes in D-ribulose and D-xylulose-producing *B. subtilis* strain results in strains producing high yields of D-arabitol or xylitol, respectively. D-Arabitol was produced with the yield of 38% of consumed D-glucose. Xylitol production was accompanied by co-production of ribitol, which was due to the substrate specificity of XPDH and therefore the xylitol yield (23%) remained lower than that of D-arabitol.

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Mira Povelainen

9 References

- Alanen P, Isokangas P, Gutmann K (2000) Xylitol candies in caries prevention: results of a field study in Estonian children. *Community Dent Oral Epidemiol* 28: 218-24
- Araujo A, Ward OP (1990) Hemicellulases of *Bacillus* species: preliminary comparative studies on production and properties of mannanases and galactanases. *J Appl Bacteriol* 68: 253-261
- Arens A (1999) *Oral Health – Diet and Other Factors*. Elsevier Science B.V., Amsterdam
- Bachem S, Faires N, Stülke J (1997) Characterization of the presumptive phosphorylation sites of *Bacillus subtilis* glucose permease by site-directed mutagenesis: implication in glucose transport and catabolite repression. *FEMS Microbiol Lett* 156: 233-238
- Bagg J, MacFarlane TW, Poxton IR, Miller CH, Smith AJ (1999) Dental caries. In *Essentials of Microbiology for Dental Students*. Oxford University Press, New York, pp. 247-258
- Bajpai P (1997) Microbial xylanolytic enzyme system: properties and applications. *Adv Appl Microbiol* 43: 141-194
- Bernard EM, Christiansen KJ, Tsang SF, Kiehn TE, Armstrong D (1981) Rate of arabinitol production by pathogenic yeast species. *J Clin Microbiol* 14: 189-194
- Bisping B, Baumann U, Simmering R (1996) Effect of immobilization on polyol production by *Pichia farinosa*. *Prog Biotechnol* 11: 395-401
- Blaklet ER, Spencer JFT (1962) Studies on the formation of D-arabitol by osmophilic yeasts. *Can J Biochem Physiol* 40: 1737-1748
- Blencke H-M, Homuth G, Ludwig H, Mäder U, Hecker M, Stülcke J (2003) Transcriptional profiling of gene expression in response to glucose in *Bacillus subtilis*: regulation of the central metabolic pathways. *Metabolic Engineering* 5: 133-149
- Bond M, Dunning N (2006) Xylitol. In Mitchell H (ed.) *Sweeteners and Sugar Alternatives in Food Technology*. Blackwell Publishing Ltd, Oxford, UK, pp. 295-324
- Bublitz C, Steavenson S (1988) The pentose phosphate pathway in the endoplasmic reticulum. *J Biol Chem* 263: 12849-12853

- Casey J, Korba B, Cote P, Gerin J, Tennant B, Chu C (2003) Method of treating hepatitis delta virus infection. Patent US6670342
- Christensson B, Sigmundsdottir G, Larsson L (1999) D-Arabinitol – a marker for invasive candidiasis. *Med Mycol* 37: 391-396
- Crick RGD (1961) Improvements in or relating to sweetening agents for food. Patent Specification 884,961
- De Wulf P, Soetaert W, Schwengers D, Vandamme EJ (1996) D-Glucose does not catabolite repress a transketolase-deficient D-ribose-producing *Bacillus subtilis* mutant strain. *J Ind Microbiol* 17: 104-109
- De Wulf P, Soetaert W, Schwengers D, Vandamme EJ (1997) Optimisation of D-ribose production with transketolase-affected *Bacillus subtilis* mutant strain in glucose and gluconic acid-based media. *J Applied Microbiol* 83, 25-30.
- De Wulf P, Vandamme EJ (1997) Production of D-ribose by fermentation. *Appl Microbiol Biotechnol* 48: 141-148
- Dixon ME, Webb EC (1964) In *Enzymes*. Academic Press, Inc., New York, pp. 346-347
- Doi RH, Wong S, Kawamura F (1984) Potential use of *Bacillus subtilis* for secretion and production of foreign proteins. *Trens Biotechnol* 4: 232-235
- Doong S, Tsai C, Schinazi R, Liotta D, Cheng Y (1991) Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci USA* 88: 8495-8499
- Doten RC, Mortlock RP (1984) Directed evolution of a second xylitol catabolic pathway in *Klebsiella pneumoniae*. *J Bacteriol* 159:730-735
- Doten RC, Mortlock RP (1985a) Characterization of xylitol-utilizing mutants of *Erwinia uredovora*. *J Bacteriol* 161: 529-533
- Doten RC, Mortlock RP (1985b) Inducible xylitol dehydrogenases in enteric bacteria. *J Bacteriol* 162 (2): 845-848
- Epperly BR, Dekker EE (1991) L-threonine dehydrogenase from *Escherichia coli*. Identification of an active site cysteine residue and metal ion studies. *J Biol Chem* 266: 6086-6092

- Fabre L, Gallezot P, Perrard A (2002) Catalytic hydrogenation of arabinonic acid and lactones to arabitol. *J Catalys* 208: 247-254
- Farmer JJ 3rd, Fanning GR, Davis BR, O'Hara CM, Riddle C, Hickmann-Brenner FW, Ashbury MA et al. (1985) *Escherichia fergusonii* and *Enterobacter tayloreae*, two new species of *Enterobacteriaceae* isolated from clinical specimens. *J Clin Microbiol* 21: 77-81
- Fiegler H, Bassias J, Jankovic I, Brückner R (1999) Identification of a gene in *Staphylococcus xylosus* encoding a novel glucose uptake protein. *J Bacteriol* 181: 4929-4936
- Flèche G, Fuertes P, Tamion R (2000) Process for the conversion of oxidised sugars to hydrogenated sugars by catalytic hydrogenation. Patent EP0999197
- Gancedo JM, Lagunas R (1973) Contribution of the pentose-phosphate pathway to glucose metabolism in *Saccharomyces cerevisiae*: a critical analysis on the use of labeled glucose. *Plant Sci Lett* 1: 193-200
- Gonzy-Tréboul G, de Waard JH, Zagorec M, Postma PW (1991) The glucose permease of the phosphotransferase system of *Bacillus subtilis*: evidence for IIGlc and IIIGlc domains. *Mol Microbiol* 5: 1241-1249
- Gehring F (1978) Cariogenic bacteria. In Counsell JN (ed) *Xylitol*. Applied Science Publishers Ltd., London, pp. 111-132
- Groleau D, Chevalier P, Yuen TH (1995) Production of polyols and ethanol by the osmophilic yeast *Zygosaccharomyces rouxii*. *Biotechnol Lett* 17: 315-320
- Grundy FJ, Waters DA, Allen SH, Henkin TM (1994) Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. *J Bacteriol* 175: 7348-7355
- Guérout-Fleury A-M, Shazand K, Frandzen N, Stagier P (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* 167: 335-336
- Gutschmidt J, Ordynsky G (1961) Bestimmung des Süßungsgrades von Xylit. *Deutsche Lebensmittel Rundschau* 57: 321-324
- Hallborn J, Walfridsson M, Airaksinen U, Ojamo H, Hahn-Hägerdal B, Penttilä M, Keränen S (1991) Xylitol production by recombinant *Saccharomyces cerevisiae*. *Biotechnology* 9: 1090-1095
- Hanson RS, Cox DP (1967) Effect of different nutritional conditions on the synthesis of tricarboxylic cycle enzymes. *J Bacteriol* 93: 1777-1787

- Hanssens L, Van Regenmortel A, Verachtert H (1972) pH-dependent polyol production in *Moniliella tomentosa*. *Appl Microbiol* 24: 831-833
- Harkki AM, Myasnikov AN, Apajalahti JHA, Pastinen OA (1997) Manufacture of xylitol using recombinant microbial hosts. US patent 5631150
- Hausman SZ, London J (1987) Purification and characterization of ribitol-5-phosphate and xylitol-5-phosphate dehydrogenases from strains of *Lactobacillus casei*. *J Bacteriol* 169: 1651-1655
- Hederstedt L (1993) The Krebs citric acid cycle. In Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis* and other gram-positive bacteria: Biochemistry, physiology, and molecular genetics. *Am Soc Microbiol*, Washington DC, pp. 181-197.
- Hickmann-Brenner FW, Vohra MP, Huntley-Carter GP, Fanning GR, Lowery VA, Brenner DJ, Farmer JJ 3rd (1985) *Leminorella*, a new genus of *Enterobacteriaceae*: identification of *Leminorella grimontii* sp nov. and *Leminorella richardii* sp nov. found in clinical specimens. *J Clin Microbiol* 21: 234-239
- Huck JHJ, Roos B, Jakobs C, van der Knaap MS, Verhoeven NM (2004) Evaluation of pentitol metabolism in mammalian tissues provides new insight into disorders of human sugar metabolism. *Mol Gen Metab* 82: 231-237
- Hueck CJ, Hillen W, Saier MH, Jr. (1994) Analysis of a cis-active sequence mediating catabolite repression in gram-positive bacteria. *Res Microbiol* 145: 503-518
- Hyvönen L, Kurkela R, Koivistoinen P, Merimaa P (1977) Effects of temperature and concentration on the relative sweetness of fructose, glucose and xylitol. *Lebensmittel Wissenschaft und Technologie* 10: 316-320
- Ingram JM, Wood WA (1965) Enzymatic basis for D-arabitol production by *Saccharomyces rouxii*. *J Bacteriol* 89: 1186-1194
- Isokangas P. (1987) Xylitol chewing gum in caries prevention. A longitudinal study on Finnish school children. *Proc Finn Dent Soc* 83(Suppl 1): 1-117
- Isokangas P, Alanen P, Tiekso J, Mäkinen KK (1988) Xylitol chewing gum in caries prevention: a field study in children. *J Am Dent Assoc* 117: 315-320
- Isokangas P, Tiekso J, Alanen P, Mäkinen KK (1989) Long-term effect of xylitol chewing gum on dental caries. *Community Dent Oral Epidemiol* 17: 200-203
- Isokangas P, Mäkinen KK, Tiekso J, Alanen P (1993) Long-term effect of xylitol chewing gum in the prevention of dental caries: a follow-up 5 years after termination of a prevention program. *Caries Res* 27: 495-498

- Jovall P-A, Tunblad-Johansson I, Adler L (1990) ^{13}C NMR analysis of production and accumulation of osmoregulatory metabolites in the salt-tolerant yeast *Debaryomyces hansenii*. Arch Microbiol 154: 209-214
- Jörnvall H, Danielson O, Ecklund H, Heimqvist L, Höög J-O, Pares X, Shafcat J (1993). Enzyme and isoenzyme developments within the medium-chain alcohol dehydrogenase family. In Weiner H, Crabb D, Flynn TG (eds) Enzymology and Molecular Biology of Carbonyl Metabolism. Plenum, New York, pp. 533-544
- Kandelman D, Bar A, Hefti A (1988) Collaborative WHO xylitol field study in French Polynesia. I. Baseline prevalence and 32-month caries increment. Caries Res 22: 55-62
- Kandelman D, Gagnon G (1990) A 24-month clinical study of the incidence and progression of dental caries in relation to consumption of chewing gum containing xylitol in school preventive programs. J Dent Res 69: 1771-1775
- Kerovuo J, von Weymarn N, Povelainen M, Auer S, Miasnikov A (2000) A new efficient expression system for *Bacillus* and its application to production of recombinant phytase. Biotechnol Lett 22: 1311-1317
- King NA, Craig SAS, Pepper T, Blundell JE (2005) Evaluation of the independent and combined effects of xylitol and polydextrose consumed as a snack on hunger and food intake over 10 days. Br J Nutr 93: 911-915
- Kirk-Othmer (1978) Encyclopedia of Chemical Technology, 3rd edn., John Wiley & Sons, New York
- Kishimoto K, Kinataka K, Ochiyama N (1990) Production of D-ribose. Patent US4904587
- Kuhad RC, Singh A, Eriksson K-EL (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. Adv Biochem Eng Biotechnol 57: 45-125
- Kunst F, Ogasawara N, Moszner I, Albertini AM, Alloni G, Azevedo V, Bertero MG et al. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390: 249-256
- Lee BH, Nowak T (1992) Influence of pH on the Mn²⁺ activation of and binding to yeast enolase: a functional study. Biochemistry 31: 2165-2171
- Lee CK (1977) Structural functions of taste in the sugar series: taste properties of sugar alcohols and related compounds. Food Chem 2: 95-105
- Lemmens H, Coomans S, Röper H (1998) Process for the production of arabinitol. Patent EP0820979

- Lévine A, Vannier F, Absalon C, Kuhn L, Jackson P, Scrivener E, Labas V et al. (2006) Analysis of the dynamic *Bacillus subtilis* Ser/Thr/Tyr phosphoproteome implicated in a wide variety of cellular processes. *Proteomics* 6: 2157-2173
- Leyva-Vazquez MA, Setlow P (1994) Cloning and nucleotide sequence of the genes encoding triose phosphate isomerase, phosphoglycerate mutase, and enolase from *Bacillus subtilis*. *J Bacteriol* 176: 3903-3910
- Lindley MG, Birch GG, Khan R (1976) Sweetness of sucrose and xylitol, structural considerations. *J Sci Food Agric* 27: 140-144
- London J, Chace NM (1977) New pathway for the metabolism of pentitols. *Proc Natl Acad Sci* 74(10):4296-4300
- Maaheimo H, Fiaux J, Cakar ZP, Bailey JE, Sauer U, Szyperski T (2001) Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional ¹³C labeling of common amino acids. *Eur J Biochem* 268: 2464-2479
- Maguire A, Rugg-Gunn AJ (2003) Xylitol and caries prevention – is it a magic bullet? *Brit Dent J* 194: 429-436
- Marini I, Bucchioni L, Borella P, Del Corso A, Mura U (1997) Sorbitol dehydrogenase from bovine lens: purification and properties. *Arch Biochem Biophys* 340: 383-391
- Martin I, Débarbouillé M, Ferrari E, Klier A, Rapoport G (1987) Characterization of the levanase gene of *Bacillus subtilis* which shows homology to yeast invertase. *Mol Gen Genet* 208: 177-184
- Mayer G, Kulbe KD, Nidetzky B (2002) Utilization of xylitol dehydrogenase in a combined microbial/enzymatic process for production of xylitol from D-glucose. *Appl Biochem Biotechnol* 98-100: 577-589
- McCormick DB, Touster O (1961) Conversion of D-[1-¹⁴C]arabitol, L-[1-¹⁴C]arabitol, and D-[1-¹⁴C]ribitol to liver glycogen in the rat and guinea-pig. *Biochim Biophys Acta* 54: 598-600
- Mihara Y, Sonoko T, Jojima Y, Tonouchi N, Fudou R, Yokozeki K (2002) Microorganisms and method for producing xylitol or D-xylulose. US patent 6335177
- Mitchell C, Morris PW, Lum L, Spiegelman G, Vary JC (1992) The amino acid sequence of a *Bacillus subtilis* phosphoprotein that matches an *orfY-tsr* coding sequence. *Mol Microbiol* 6: 1345-1349
- Mitchell H (2006) In Mitchell H (ed.) *Sweeteners and Sugar Alternatives in Food Technology*. Blackwell Publishing, Oxford, UK

- Miyagawa K, Miyazaki J, Kanazaki N (1992) Method of producing D-ribose. Patent EP0501765
- Moloughney S (2008) Natural ingredients sweeten the market for sugar substitutes: growing public concern about overall health has opened the door for natural alternatives to sucrose and chemical substitutes. *Nutraceuticals World* 11: 68
- Momose H, Shio I (1969) Genetic and biochemical studies on 5'-nucleotide fermentation. IV. Effect of GMP reductase and purine analogue resistance on purine nucleoside accumulation patterns in adenine auxotrophs of *Bacillus subtilis*. *J Gen Appl Microbiol* 15: 399-411
- Mortlock RP (1976) Catabolism of unnatural carbohydrates by microorganisms. In Rose AH, Tempest DW (eds.). *Advances in Microbial Physiology*, Vol 13, Academic Press, London, pp. 1-53
- Moskowitz HR (1971) The sweetness and pleasantness of sugars. *Am J Psychology* 84: 387-405
- Munton SL, Birch GG (1985) Accession of sweet stimuli to receptors. I. Absolute dominance of one molecular species in binary mixtures. *J Theoretical Biol* 112: 539-551
- Myrphy ND, McConnel DJ, Cantwell BA (1984) The DNA sequence of the gene and genetic control sites for the excreted *Bacillus subtilis* enzyme β -glucanase. *Nucleic acids res* 12: 5355-5367
- Mäkinen KK, Söderling A (1980) A quantitative study of mannitol, sorbitol, xylitol and xylose in wild berries and commercial fruits. *J Food Sci* 45: 367-374
- Mäkinen KK, Bennett CA, Hujoel PP, Isokangas PJ, Isotupa KP, Pape HR Jr, Mäkinen PL (1995) Xylitol chewing gums and caries rates: a 40-month cohort study. *J Dent Res* 74: 1904-1913
- Nakano MM, Hulett FM (1997) Adaptation of *Bacillus subtilis* to oxygen limitation. *FEMS Microbiol Lett* 157: 1-7
- Nasser W, Awadé AC, Reverchon S, Robert-Baudouy J (1993) Pectate lyase from *Bacillus subtilis*: molecular characterization of the gene, and properties of the cloned enzyme. *FEBS Lett* 335(13):319-326
- Natah SS, Hussien KH, Tuominen JA, Koivisto VA (1997) Metabolic response to lactitol and xylitol in healthy men. *Am J Clin Nutr* 65: 947-950
- Neuberger MS, Patterson ARA, Hartley BS (1979) Purification and properties of *Klebsiella aerogenes* arabinol dehydrogenase. *Biochem J* 183: 31-42

- Nguyen NU, Dumoulin G, Henriet MT, Berthelay S, Regnard J (1993) Carbohydrate metabolism and urinary excretion of calcium and oxalate after ingestion of polyol sweeteners. *J Clin Endocrinol Metab* 77: 388-392
- Nobre MF, da Costa MS (1985) Factors favouring the accumulation of arabinitol in the yeast *Debaryomyces hansenii*. *Can J Microbiol* 31: 467-471
- Nowlan SS, Deibel RH (1967) Group Q streptococci. 1. Ecology, serology, physiology and relationship to established enterococci. *J Bacteriol* 94: 291-296
- Nozaki H, Suzuki S, Tsuyoshi N, Yokozeki K (2003) Production of D-arabitol by *Metschnikowia reukaufii* AJ14787. *Biosci Biotechnol Biochem* 67: 1923-1929
- Ogawa Y, Yamaguchi F, Yuasa K, Tahara Y (1997) Efficient production of poly- γ -glutamic acid by *Bacillus subtilis* (natto) in jar fermenters. *Biosci Biotechnol Biochem* 61: 1684-1687
- Onishi H, Saito N, Koshiyama I (1961) Studies on osmophilic yeasts part XI. Various factors affecting on polyalcohol production by *Pichia miso*. *Agric Biol Chem* 26: 124-130
- Onishi H, Suzuki T (1969) Microbial production of xylitol from glucose. *Appl Microbiol* 18: 1031-1035
- Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000) Advances in microbial amylases. *Biotechnol Appl Biochem* 31: 135-152
- Paulsen IT, Chauwax S, Choi P, Saier MH Jr. (1998) Characterization of glucose-specific catabolite repression-resistant mutants of *Bacillus subtilis*: identification of a novel hexose:H⁺ symporter. *J Bacteriol* 180: 498-504
- Perkins JB, Sloma A, Hermann T, Theriault K, Zachgo E, Erdenberger T, Hannett N et al. (1999) Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin. *J Ind Microbiol Biotechnol* 22: 8-18
- Pihlanto-Leppälä A, Söderling E, Mäkinen KK (1990) Expulsion mechanism of xylitol 5-phosphate in *Streptococcus mutans*. *Scand J Dent Res* 98: 112-119
- Postma PW, Lengeler JW, Jacobson GR (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems in bacteria. *Microbiol Rev* 57: 543-594
- Presecan-Siedel E, Galinier A, Longin R, Deutscher J, Danchin A, Glaser P, Martin-Verstraete I (1999) Catabolite regulation of the *pta* gene as part of carbon flow pathways in *Bacillus subtilis*. *J Bacteriol* 181: 6889-6897

- Qi Y, Kobayashi Y, Hulett FM (1997) The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the *pho* regulon. *J Bacteriol* 179: 2534-2539
- Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev* 62: 597-635
- Renna MC, Najimudin N, Winik LR, Zahler SA (1993) Regulation of the *Bacillus subtilis alsS*, *alsD* and *alsR* genes involved in post-exponential-phase production of acetoin. *J Bacteriol* 175: 3863-3875
- Rugg-Gunn AJ, Nunn JH (1999) *Nutrition, Diet and Oral Health*. Oxford University Press, Oxford
- Saha BC, Sakakibara Y, Cotta MA (2007) Production of D-arabitol by newly isolated *Zygosaccharomyces rouxii*. *J Ind Microbiol Biotechnol* 34: 519-523
- Saklani-Justforgues H, Fontal E, Goossens PL (2001) Characterisation of a *Listeria monocytogenes* mutant deficient in D-arabitol fermentation. *Res Microbiol* 152: 175-177
- Salminen S, Salminen E, Koivistoinen P, Bridges J, Marks V (1985) Gut microflora interactions with xylitol in the mouse, rat and man. *Food Chem Toxicol* 23: 985-990
- Sambrook J, Fritsch EF, Maniatis T (1989). In: Ford N, Nolan C, Ferguson M (eds.) *Molecular Cloning: A laboratory manual*. 2nd edn. Cold Spring Harbour Laboratory Press, New York
- Sasajima K, Yoneda M (1971) Carbohydrate metabolism in *Bacillus* species. Part II. D-ribose accumulation by pentose phosphate pathway mutants. *Agric Biol Chem* 35(4): 509-517
- Sasajima K, Yoneda M (1974) D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase and D-ribulose-5-phosphate 3-epimerase mutants of *Bacillus* species. *Agric Biol Chem* 38(7): 1305-1310.
- Sasajima K, Doi M, Yokota K, Nakao Y, Yoneda M (1976) Method for producing D-ribose, Patent US3970522
- Sauer U, Hatzimanikatis V, Hohmann HP, Manneberg M, van Loon APGM, Bailey JE (1996) Physiology and metabolic fluxes of wild-type and riboflavin-producing *Bacillus subtilis*. *Appl Environ Microbiol* 62: 3687-3696
- Sauer U, Hatzimanikatis V, Bailey JE, Hochuli M, Szyperski T, Wütrich K (1997) Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. *Nat Biotechnol* 15: 448-452

- Scangos GA, Reiner AM (1978) Ribitol and D-arabitol catabolism in *Escherichia coli*. J Bacteriol 134: 492-500
- Schafer RB, Levine A, Marlette JM, Morley JE (1987) Effects of xylitol in gastric emptying and food intake. Am J Clin Nutr 45: 744-747
- Schallmeyer M, Singh A, Ward OP (2004) Developments in the use of *Bacillus* species for industrial production. Can J Microbiol 50: 1-17
- Scheinin A, Mäkinen KK, Ylitalo K (1975) Turku sugar studies V. Final report on the effect of sucrose, fructose and xylitol on the caries incidence in man. Acta Odontol Scand 33(Suppl 70): 67-104
- Scheinin A, Mäkinen KK, Tammissalo E, Rekola M (1975) Turku sugar studies XVIII. Incidence of dental caries in relation to a 1-year consumption of xylitol chewing gum. Acta Odontol Scand 33(Suppl 70): 307-316
- Scheinin A, Bánóczy J, Szöke J, Esztári I, Pienihäkkinen K, Scheinin U, Tiekso J et al. (1985) Collaborative WHO xylitol field studies in Hungary. I. Three-year caries activity in institutionalized children. Acta Odontol Scand 43: 327-347
- Schilling O, Frick O, Herzberg C, Ehrenreich A, Heinzle E, Wittmann C, Stülke J (2007) Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: transcription regulation is important but not sufficient to account for metabolic adaptation. Appl Environ Microbiol 73(2): 499-507
- Schmiedel D, Hillen W (1996) A *Bacillus subtilis* 168 mutant with increased xylose uptake can utilize xylose as sole carbon source. FEMS Microbiol Lett 135: 175-178
- Shin BS, Choi SK, Park SH (1999) Regulation of the *Bacillus subtilis* phosphotransacetylase gene. J Biochem 126: 333-339
- Shio I (1989) Industrial applications of *Bacillus subtilis*. Production of primary metabolites. In Maruo B, Yoshikawa H (eds.) *Bacillus subtilis: molecular biology and industrial applications*. Elsevier Science B. V., Amsterdam, Netherlands, pp. 191-211
- Simpson FJ (1966). D-Xylulokinase. In Wood WA (ed) *Methods in Enzymology. Carbohydrate Metabolism*. Academic Press, New York, pp. 454-458
- Singh A, Hayashi K (1995) Microbial cellulases: protein architecture, molecular properties and biosynthesis. Adv Appl Microbiol 40: 1-44
- Skarlatos P, Dahl MK (1998) The glucose kinase of *Bacillus subtilis*. J Bacteriol 180: 3222-3226

- Stülke J, Martin-Verstraete I, Charrier V, Klier A, Deutscher J, Rapoport G (1995) The HPr protein of the phosphotransferase system links induction and catabolite repression of the *Bacillus subtilis* levanase operon. *J Bacteriol* 177: 6928-6936
- Sugiyama M, Suzuki S, Tonouchi N, Yokozeki K (2003) Cloning of the xylitol dehydrogenase gene from *Gluconobacter oxydans* and improved production of xylitol from D-arabitol. *Biosci Biotechnol Biochem* 67: 584-591
- Suzuki S, Sugiyama M, Mihara Y, Hashiguchi K, Yokozeki K (2002) Novel enzymatic method for the production of xylitol from D-arabitol by *Gluconobacter oxydans*. *Biosci Biotechnol Biochem* 66: 2614-2620
- Söderling E, Pihlanto-Leppälä A (1989) Uptake and expulsion of 14C-xylitol by xylitol-cultured *Streptococcus mutans* ATCC 25175. *Scand J Dent Res* 97: 511-519
- Söderling E, Trahan L, Tammiala-Salonen T, Häkkinen L (1997) Effects of xylitol, xylitol-sorbitol, and placebo chewing gums on the plaque of habitual xylitol consumers. *Eur J Oral Sci* 105: 170-177
- Tam R, Averett D, Ramasamy H (2003) Monocyclic L-nucleosides, analogs and uses thereof. Patent US6642206
- Trahan L, Söderling E, Dréan MF, Chevrier MC, Isokangas P (1992) Effect of xylitol consumption on the plaque-saliva distribution of mutans streptococci and the occurrence and long-term survival of xylitol-resistant strains. [Erratum in *J Dent Res* (1993) 72: 87-88] *J Dent Res* 71: 1785-1791
- Tharan L (1995) Xylitol: a review of its actions on mutans streptococci and dental plaque – its clinical significance. *Int Dent J* 45: 77-92
- Tobisch S, Zühlke D, Bernhardt J, Stülke J, Hecker M (1997) Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*. *J Bacteriol* 181: 6996-7004
- Toivari MH, Ruohonen L, Miasnikov AN, Richard P, Penttilä M (2007) Metabolic engineering of *Saccharomyces cerevisiae* for conversion of d-glucose to xylitol and other five-carbon sugars and sugar alcohols. *Appl Environ Microbiol* 73: 5471-5476
- Tong Z-H, Gu W-Z, Gen Z (1987) Effect on plasma glucose and insulin after xylitol loading in 30 normal adults. *Zhonghau Nei Ke Za Zhi* 26: 420-422
- Tselenis-Kotsowilis AD, Koliomichalis MP, Papavassiliou JT (1982) Acute pyelonephritis caused by *Staphylococcus xylosum*. *J Clin Microbiol* 16: 593-594

Uhari M, Kontiokari T, Koskela M, Niemelä M (1996) Xylitol chewing gum in the prevention of acute otitis media: double blind randomized trial. *Br Med J* 313: 1180-1184

Uhari M, Kontiokari T, Niemelä M (1998) A novel use of xylitol sugar in preventing acute otitis media. *Pediatrics* 102: 879-884

Van Eck JH, Prior BA, Brandt EV (1989) Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. *J Gen microbiol* 135: 3505-3513

Van Eck JH, Prior BA, Brandt EV (1993) The water relations of growth and polyhydroxy alcohol production by ascomycetous yeasts. *J Gen Microbiol* 139: 1047-1054

Waler SM, Rolla G, Assev S, Ciardi JE (1984) The effect of xylitol on plaque metabolism. *Swed Dent J* 8: 155-161

Wang L-F, Wong S, Lee S-G, Kalyan NK, Hung PP, Hilliker S, Doi RH (1988) Expression and secretion of human atrial natriuretic α -factor in *Bacillus subtilis* using subtilisin signal peptide. *Gene* 69: 39-47

Wang PZ, Doi RH (1984) Overlapping promoters transcribed by *Bacillus subtilis* sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. *J Biol Chem* 259: 8619-8625

Washuett J, Riederer P, Bancher E (1973) A qualitative and quantitative study of sugar-alcohols in several foods. 38: 1262-1263

Winkelhausen E, Kuzmanova S (1998) Microbial conversion of D-xylose to xylitol. *J Ferment Bioeng* 86: 1-14

Wolf M, Geczi A, Simon O, Borriss R (1995) Genes encoding xylan and β -glucan hydrolising enzymes in *Bacillus subtilis*: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase. *Microbiology* 140: 1829-1838

Wong B, Leeson S, Grindle S, Magee P, Brooks E, Magee PT (1995) D-Arabitol metabolism in *Candida albicans*: construction and analysis of mutants lacking D-arabitol dehydrogenase. *J Bacteriol* 177: 2971-2976

Woodyer RD, Wymer NJ, Racine M, Khan SN, Saha BC (2008) Efficient production of L-ribose with recombinant *Escherichia coli* biocatalyst. *Appl Environ Microbiol* 74: 2967-2975

Yamaguchi S, Yoshikawa T, Ikeda S, Ninomiya T (1970) Studies of the taste of some sweet substances. *Agric Biol Chem* 34: 181-197

Yang M, Galizzi A, Henner D (1983) Nucleotide sequence of the amylase gene from *Bacillus subtilis*. *Nucleic Acids Res* 11: 237-249

Yang M, Ferrari E, Chen E, Henner DJ (1986) Identification of the pleiotropic sacQ gene of *Bacillus subtilis*. *J Bacteriol* 166: 113-119

Zacharis C (2007) Expanding the use of xylitol in cosmetics. *NutraCos*, March/April: 18-19

Zamboni N, Fischer E, Laudert D, Aymerich S, Hohmann H-P, Sauer U (2004) The *Bacillus subtilis* *yqjI* gene encodes the NADP⁺-dependent 6-P-gluconate dehydrogenase in the pentose phosphate pathway. *J Bacteriol* 186: 4528-4534

Zhu Y, Chen X, Chen T, Shi S, Zhao X (2006) Over-expression of glucose dehydrogenase improves cell growth and riboflavin production in *Bacillus subtilis*. *Biotechnol Lett* 28: 1667-1672

10 Appendices