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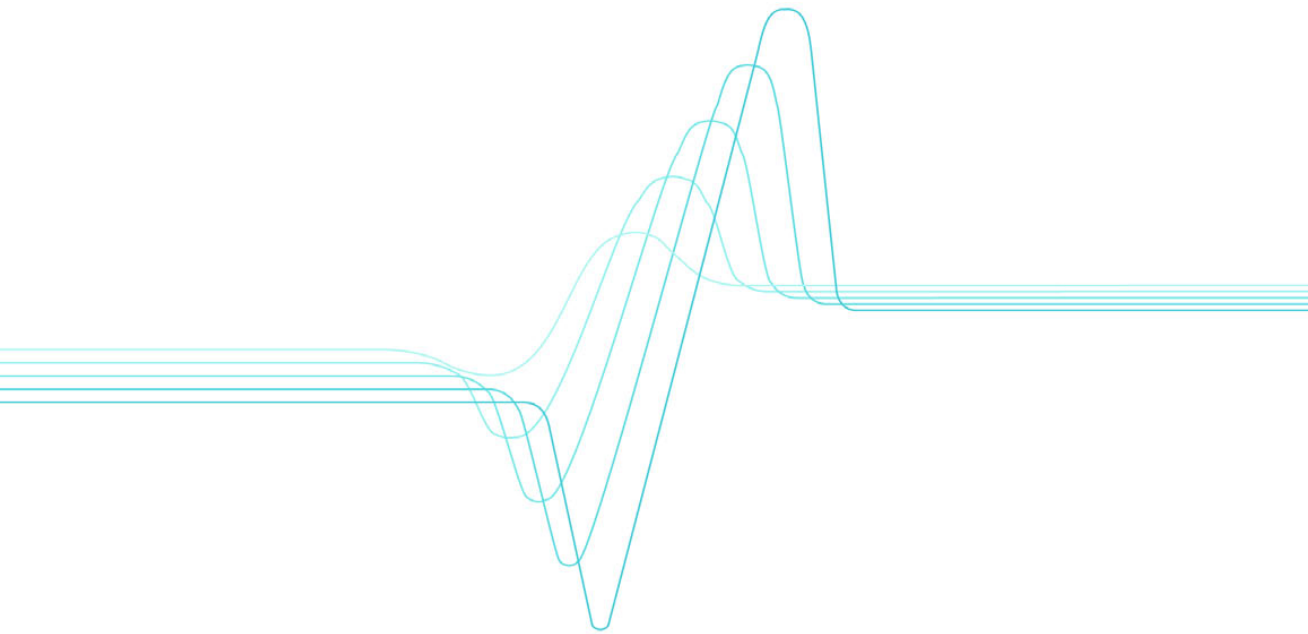

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Engineering the pentose phosphate pathway of *Saccharomyces cerevisiae* for production of ethanol and xylitol

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VTT

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Abstract

The baker's yeast *Saccharomyces cerevisiae* has a long tradition in alcohol production from D-glucose of e.g. starch. However, without genetic modifications it is unable to utilise the 5-carbon sugars D-xylose and L-arabinose present in plant biomass. In this study, one key metabolic step of the catabolic D-xylose pathway in recombinant D-xylose-utilising *S. cerevisiae* strains was studied. This step, carried out by xylulokinase (XK), was shown to be rate-limiting, because overexpression of the xylulokinase-encoding gene *XKS1* increased both the specific ethanol production rate and the yield from D-xylose. In addition, less of the unwanted side product xylitol was produced.

Recombinant D-xylose-utilizing *S. cerevisiae* strains have been constructed by expressing the genes coding for the first two enzymes of the pathway, D-xylose reductase (XR) and xylitol dehydrogenase (XDH) from the D-xylose-utilising yeast *Pichia stipitis*. In this study, the ability of endogenous genes of *S. cerevisiae* to enable D-xylose utilisation was evaluated. Overexpression of the *GRE3* gene coding for an unspecific aldose reductase and the *ScXYL2* gene coding for a xylitol dehydrogenase homologue enabled growth on D-xylose in aerobic conditions. However, the strain with *GRE3* and *ScXYL2* had a lower growth rate and accumulated more xylitol compared to the strain with the corresponding enzymes from *P. stipitis*. Use of the strictly NADPH-dependent Gre3p instead of the *P. stipitis* XR able to utilise both NADH and NADPH leads to a more severe redox imbalance. In a *S. cerevisiae* strain not engineered for D-xylose utilisation the presence of D-xylose increased xylitol dehydrogenase activity and the expression of the genes *SOR1* or *SOR2* coding for sorbitol dehydrogenase. Thus, D-xylose utilisation by *S. cerevisiae* with activities encoded by *ScXYL2* or possibly *SOR1* or *SOR2*, and *GRE3* is feasible, but requires efficient redox balance engineering.

Compared to D-xylose, D-glucose is a cheap and readily available substrate and thus an attractive alternative for xylitol manufacture. In this study, the pentose phosphate pathway (PPP) of *S. cerevisiae* was engineered for production of xylitol from D-glucose. Xylitol was formed from D-xylulose 5-phosphate in strains lacking transketolase activity and expressing the gene coding for XDH from *P. stipitis*. In addition to xylitol, ribitol, D-ribose and D-ribulose were also formed. Deletion of the xylulokinase-encoding gene increased xylitol production, whereas the expression of *DOG1* coding for sugar phosphate phosphatase increased ribitol, D-ribose and D-ribulose production. Strains lacking phosphoglucose isomerase (Pgi1p) activity were shown to produce 5-carbon compounds through PPP when *DOG1* was overexpressed. Expression of genes encoding glyceraldehyde 3-phosphate dehydrogenase of *Bacillus subtilis,* GapB, or NAD-dependent glutamate dehydrogenase Gdh2p of *S. cerevisiae*, altered the cellular redox balance and enhanced growth of *pgi1* strains on D-glucose, but co-expression with *DOG1* reduced growth on higher D-glucose concentrations. Strains lacking both transketolase and phosphoglucose isomerase activities tolerated only low D-glucose concentrations, but the yield of 5-carbon sugars and sugar alcohols on D-glucose was about 50% (w/w).

Preface

This study was carried out at VTT in the group of Metabolic Engineering. The financial support from Tekes, the Finnish Funding Agency for Technology and Innovation, Danisco Sweeteners, Academy of Finland (Finnish Centre of Excellence programme, 2000–2005, Project no. 64330) and University of Helsinki is gratefully acknowledged.

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My parents Aili and Tapsa, my brother Petri and his family and my mother in law Hiisu I would like to merit for their unquestionable help whenever needed. Without you this work would not have been possible.

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Mervi

Espoo, May 2007

List of publications

This thesis is based on the following articles referred to in text by their Roman numerals (I–IV).

I Toivari, M.H., Aristidou, A., Ruohonen, L. and Penttilä, M. 2001. Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (*XKS1*) and oxygen availability. Metab. Eng. 3, 236–249.

II Toivari, M.H., Salusjärvi, L., Ruohonen, L. and Penttilä, M. 2004. Endogenous xylose pathway in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 70, 3681–3686.

III Toivari, M.H., Ruohonen, L., Miasnikov, A.N., Richard, P. and Penttilä, M. Metabolic engineering of *Saccharomyces cerevisiae* for conversion of D-glucose to xylitol and other five-carbon sugars and sugar alcohols. Manuscript considered for publication in Applied and Environmental Microbiology.

IV Toivari, M.H., Penttilä, M. and Ruohonen, L. Enhancing flux of D-glucose to pentose phosphate pathway in *Saccharomyces cerevisiae* for production of 5-carbon sugars and sugar alcohols. Manuscript.

Contents

Appendices of this publication are not included in the PDF version.
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List of symbols

aa	amino acid
bp	base pair
DHAP	dihydroxyacetone phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gdh2p	NAD-dependent glutamate dehydrogenase of *S. cerevisiae*
GDH2	glutamate dehydrogenase-encoding gene of *S. cerevisiae*
GRE3	aldose reductase-encoding gene of *S. cerevisiae*
G6PDH	D-glucose 6-phosphate dehydrogenase
kb	kilobase
ORF	open reading frame
PCR	polymerase chain reaction
PGI1	phosphoglucose isomerase-encoding gene of *S. cerevisiae*
Pgi1p	phosphoglucose isomerase of *S. cerevisiae*
pgi1 tkl1 tkl2	strain lacking functional *TKL1*, *TKL2* and *PGI1* genes
PPP	pentose phosphate pathway
ScXYL2	xylitol dehydrogenase-encoding gene of *S. cerevisiae*
TAL1	transaldolase-encoding gene of *S. cerevisiae*

TCA	tricarboxcylic acid cycle
TKL1	transketolase-encoding gene of *S. cerevisiae*
TKL2	transketolase-encoding gene of *S. cerevisiae*
tkl1 tkl2	strain lacking functional *TKL1* and *TKL2* genes
XDH	xylitol dehydrogenase
XI	xylose isomerase
XK	xylulokinase
XKS1	xylulokinase-encoding gene of *S. cerevisiae*
XR	xylose reductase
XYL1	xylose reductase-encoding gene of *P. stipitis*
XYL2	xylitol dehydrogenase-encoding gene of *P. stipitis*
XYL3	xylulokinase-encoding gene of *P. stipitis*
wt	wild type

1. Introduction

The relatively high price, and in the long term the limited availability, of fossil fuels has increased concern about future energy and material resources. In addition, the warming of climate, accelerated by carbon dioxide emission from fossil fuels, is alarming. One solution to these challenges could be a transition from traditional oil refineries to biorefineries that would convert renewable organic matter, such as cereal or non-cereal plants and agricultural and municipal solid waste, to energy and value added products (Fernando *et al*. 2006, Kamm and Kamm 2007, Ragauskas *et al*. 2006). Biorefineries utilize "white biotechnology" (http://www.europabio.org/white_biotech.htm) aiming at more energy efficient, low cost and less polluting production processes by using enzymes and whole cell catalysts.

One of the major challenges of biorefineries is the generation of transportation fuels. Bioethanol manufactured from renewable resources by microbial fermentation is an attractive alternative because it is carbon dioxide neutral; the amount of CO_2 released in fermentation was originally absorbed from the atmosphere by the growing plants. Production of ethanol from starch of e.g. wheat, barley or maize by fermentation with the traditional baker's yeast *Saccharomyces cerevisiae* is a well known process. However, the most abundant and cheap renewable raw material for bioethanol production not used for human nutrition is lignocellulose, i.e. plant material consisting of cellulose, hemicellulose and lignin. Unfortunately, after hydrolysis only part of the sugars in lignocellulose, namely the hexose sugars D-glucose, D-galactose and D-mannose, are efficiently fermented to ethanol by the yeast *S. cerevisiae.* The hemicellulose sugars D-xylose and L-arabinose remain unutilized, thus lowering the economical feasibility of the process. Extensive strain development of *S. cerevisiae* (for a review, see Aristidou and Penttilä 2000, Hahn-Hägerdal *et al*. 2006, Hahn-Hägerdal *et al*. 2007, Jeffries 2006, van Maris *et al*. 2006) and also of bacteria such as *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* (Dien *et al*. 2003, Lindsay *et al*. 1995, Mohagheghi *et al*. 2002, Ohta *et al*. 1991) has been performed for the fermentation of pentose sugars to ethanol. The current industrial strains are being tested in pilot and demonstration scale plants in Spain, Sweden, USA and Canada (Hahn-Hägerdal *et al*. 2006). Thus, estimations on economics of bioethanol production by fermentation should emerge in the near future.

Enzymes and microbes are also increasingly utilized for production of food ingredients, one example being the relatively new sweetener erythritol that has low caloric value, anticariogenic properties and an insulin-independent metabolism similar to that of xylitol. Manufacturing of erythritol by chemical synthesis is too expensive, but microbial conversion of D-glucose present in corn syrup is feasible and therefore the method currently used for erythritol production. Xylitol production by chemical reduction of D-xylose derived from wood has a long history of process development and is thus currently more feasible compared to microbial reduction of D-xylose. However, as these processes require the extraction of D-xylose from the wood material, the more readily available and cheap D-glucose could be an attractive alternative for biotechnical production of xylitol.

Bioprocesses need tailor-made organisms in which the metabolism is redirected to utilize or produce desired compounds. Yeasts are an important group of unicellular eukaryotic microbes varying from pathogenic species to organisms used in the food industry, such as the baker's yeast *S. cerevisiae*. The ability of this yeast to grow and ferment (i.e. produce alcohol from sugar) in both aerobic and anaerobic conditions, as well as its tolerance to acids, high alcohol concentrations and low pH, make it an ideal production organism. In addition, *S. cerevisiae* is one of the most studied eukaryotic organisms. Extensive knowledge is available on the process behaviour, cell physiology and genetics of *S. cerevisiae*. *S. cerevisiae* was the first eukaryotic organism of which the genome was sequenced (Goffeau *et al*. 1996). The genome-wide methods for gene expression and global proteome and metabolome analyses enable monitoring of cell metabolism. In this study, the pentose phosphate pathway (PPP) of *S. cerevisiae* was modified to enhance utilization of D-xylose and for production of xylitol from D-glucose.

1.1 The pentose phosphate pathway in yeast metabolism

1.1.1 Source of NADPH and metabolites

The role of the pentose phosphate pathway (PPP) in yeasts and other eukaryotic organisms is to produce reducing power in the form of NADPH for the cellular reactions and also to produce precursors such as D-ribose 5-phosphate and

D-erythrose 4-phosphate for nucleotide and amino acid biosynthesis. The pentose phosphate pathway in its current form was first described by Horecker and Mehler in 1955 (Fig. 1) (Horecker and Mehler 1955).

The phosphorylated D-glucose molecule, D-glucose 6-phosphate, is channelled either to storage carbohydrates via D-glucose 1-phosphate, to glycolysis or to the PPP. In PPP, D-glucose 6-phosphate is oxidized to 6-phospho-gluconolactone by D-glucose 6-phosphate dehydrogenase (G6PDH) with the concomitant reduction of $NADP^+$ to NADPH. Subsequently, the 6-phospho-gluconolactone is converted to 6-phosphogluconate by 6-phosphogluconolactonase. This reaction (hydrolysis of the lactone ring) also occurs spontaneously, but at a slow rate. 6-phosphogluconolactone is oxidised to D-ribulose 5-phosphate and CO_2 by 6-phosphogluconate dehydrogenase with $NADP^+$ as electron acceptor. Thus, in this so-called oxidative pentose phosphate pathway, two NADPH molecules and one CO_2 molecule are formed when D-glucose 6-phosphate is sequentially oxidized to D-ribulose 5-phosphate. D-Ribulose 5-phosphate can be isomerised to D-ribose 5-phosphate by D-ribose 5-phosphate ketol-isomerase or epimerized to D-xylulose 5-phosphate by D-ribulose 5-phosphate 3-epimerase. Both of these reactions, contrary to the oxidative pathway enzymes, are reversible. The following, also reversible steps of transketolase and transaldolase, convert the two 5-carbon sugar phosphates D-xylulose 5-phosphate and D-ribose 5-phosphate through sedoheptulose 7-phosphate and erythrose 4-phosphate to the glycolytic intermediates glyceraldehyde 3-phosphate and D-fructose 6-phosphate. Of these intermediates, D-ribose 5-phosphate serves as a precursor for purine and pyrimidine ring and histidine biosynthesis, whereas erythrose 4-phosphate is needed for synthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine.

Because PPP produces both reducing power and precursors for biosynthesis, it has a central role in biomass generation and its regulation has been thought to be modulated by the need for NADPH and D-ribose 5-phosphate (Stryer 1989). $NADP^+$ as a substrate and NADPH as a competitive inhibitor determine the activity of D-glucose 6-phosphate dehydrogenase, the first enzymatic reaction in the pathway (for a review see, Levy 1979). In addition, ATP may inhibit the G6PDH activity (Levy 1979, Vaseghi *et al*. 1999). However, the ratio of $NADP^+$ to NADPH is considered as the most important factor modulating the G6PDH activity.

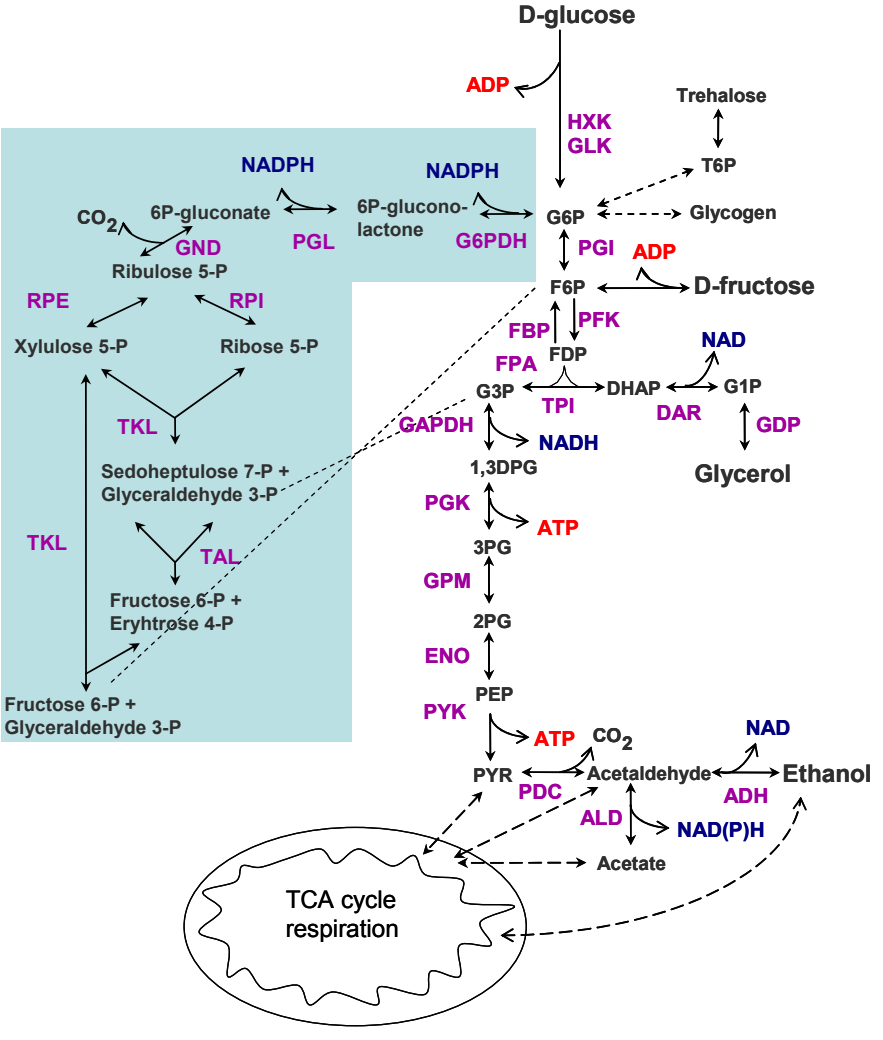

Figure 1. Schematic representation of central carbon metabolism of yeast S. cerevisiae. HXK, hexokinase; GLK, glucokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; FBP, fructose1, 6-bisphosphatase; FPA, fructose bisphosphate aldolase; TPI, triose phosphate isomerase; DAR, DHAP reductase; GDP, glycerol phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; GPM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; PDC, pyruvate decarboxylate; ADH, alcohol dehydrogenase; ALD, acetaldehyde dehydrogenase; G6PDH, D-glucose 6-phosphate dehydrogenase; PGL, 6-phospho-gluconolactonase; GND,6-phosphogluconate dehydrogenase; RPE, D-ribulose 5-phosphate 3-epimerase; RPI, D-ribulose 5-phosphate ketol-isomerase; TKL, transketolase; TAL, transaldolase.

1.1.2 Regulation of gene expression and carbon flux of PPP

Although the regulation of the flux to PPP follows the need for reducing power and precursors for biosynthesis, the regulation in reality may be more complex than modulation of the D-glucose 6-phosphate dehydrogenase (G6PDH) activity. For example, it is not always known how the environmental conditions in different yeast species affect the $NADP^+$ to NADPH ratio, and thus the G6PDH activity. Furthermore, the induction of the other pathway enzymes, in addition to G6PDH, also influences the carbon flow through this pathway. The molecular mechanisms regulating the expression of these genes are only beginning to be unravelled.

In general, kinetic data on yeast G6PDH is scarce. True K_m values of 0.015, 0.051 and 0.076 mM for D-glucose 6-phosphate and 0.003, 0.008 and 0.006 mM for $NADP^+$, for *S. cerevisiae*, *Saccharomyces carlsbergensis* and *Candida utilis* respectively, have been reported (Levy 1979). The K_i value for NADPH for *S. carlsbergensis* G6PDH, 0.07 μM, is much lower compared to K_i of 32 μM reported for *C. utilis*. Apparent K_m values are 0.23 and 0.66 mM for D-glucose 6-phosphate and 0.067 and 0.044 mM for $NADP^+$, for *C. utilis* and *Schizosaccharomyces pombe*, respectively. The K_m values for D-glucose 6-phosphate of *S. cerevisiae* G6PDH and phosphoglucose isomerase, the two main enzymes active on this compound, are similar or 3- to 6-fold higher for the phosphoglucose isomerase depending on the study [(Noltmann 1972) and references therein].

The PPP pathway is known to be important in protection against oxidative stress (Krems *et al*. 1995, Nogae and Johnston 1990). In *S. cerevisiae* D-glucose 6-phosphate dehydrogenase, gluconate 6-phosphate dehydrogenase, D-ribulose 5-phosphate epimerase, transketolase and transaldolase mutants were all sensitive to hydrogen peroxide (Juhnke *et al*. 1996). Similarly, *zwf1* mutants lacking G6PDH activity were found to be sensitive to oxygen and auxotrophic for methionine (Slekar *et al*. 1996, Thomas *et al*. 1991). In addition, overexpression of the transketolase-encoding gene was able to rescue the oxygen sensitivity and methionine auxotrophy of the cytosolic superoxide dismutase (*SOD1*) mutant (Slekar *et al*. 1996). The methionine biosynthesis pathway requires NADPH possibly explaining the auxotrophy. Defence mechanisms of oxidative stress, such as glutathione, glutathione reductase and peroxidase and thioredoxin, thioredoxin reductase and peroxidase, eliminate reactive oxygen

species via reduced sulfhydryl groups. The reductase and peroxidase enzymes restore the reduced state of glutathione and thioredoxin either directly or indirectly by NADPH (for a review see, Grant 2001, Jamieson 1998). The importance of PPP in oxidative stress response has been also observed in genome-wide studies of *S. cerevisiae*. On the proteome level H_2O_2 elevated the amount of Zwf1, Tkl2 and Tal1 proteins (Godon *et al*. 1998). In addition, by using a complete set of viable deletion strains, agents such as H_2O_2, linoleic acid 13-hydroperoxide (LoaOOH) and menadione, generating different reactive oxygen species, all affected growth of the *gnd1*, *rpe1* and *tkl1* mutants of the PPP pathway (Fig. 1) (Thorpe *et al*. 2004). Similarly, the *zwf1*, *gnd1*, *rpe1* and *tkl1* mutant strains were found to be sensitive to furfural and 5-hydroxymethylfurfural (Gorsich *et al*. 2006), inhibitory compounds often present in lignocellulose hydrolysates.

The expression of *ZWF1* and *TAL1* genes is regulated by the Yap1p transcription factor during oxidative stress (Lee *et al*. 1999). Oxidative stress caused e.g. by H_2O_2 catalyses, with the aid of Gpx3 protein, the disulphide bond formation of Yap1p leading to conformational changes that guide the protein to the nucleus for activation of antioxidant stress genes (for a review, see Fedoroff 2006, Liu *et al*. 2005). Recently another transcription factor, Stb5p, was shown to regulate many PPP genes and also other genes coding for NADPH-dependent enzymes (Larochelle *et al*. 2006). Stb5p was required for growth in the presence of oxidative stress generated by H_2O_2 or diamine. It binds to promoter regions of the PPP genes *ZWF1*, *SOL3*, *GND1* and *TKL1*. It also binds to promoters of *ALD6* and *IDP2* coding for NADPH dependent acetaldehyde dehydrogenase and isocitrate dehydrogenase, respectively (Grabowska and Chelstowska 2003, Minard *et al*. 1998). Stb5p is able to repress the *PGI1* gene coding for phosphoglucose isomerase. Pgi1p converts D-glucose 6-phosphate to D-fructose 6-phosphate in glycolysis, and thus regulation of *PGI1* expression could direct D-glucose 6-phosphate to PPP or to storage carbohydrates instead of glycolysis. The activation of the Stb5b was suggested to be different from that of Yap1p (Larochelle *et al*. 2006).

Of the PPP genes only the deletion of *RKI1* coding for D-ribose-5-phosphate ketol-isomerase (RPI) was found detrimental, first by Miosga and Zimmermann (Miosga and Zimmermann 1996) and subsequently in the systematic study of *S. cerevisiae* gene-deletion mutants (Giaever *et al*. 2002). Previously, a mutant

without 6-phosphogluconate dehydrogenase activity was also reported to be unable to grow on D-glucose. This was possibly due to toxic accumulation of 6-phosphogluconate, because growth was restored when the D-glucose 6-phosphate dehydrogenase activity was also lost (Lobo and Maitra 1982). It may be that in the systematic deletion study the presence of a second isoenzyme for 6-phosphogluconate dehydrogenase (Gnd1p or Gnd2p) was able to perform the reaction, whereas in the previous study by Lobo and Maitra both isoenzymes could have been defected.

There are two isoenzymes, not only for the 6-phosphogluconate dehydrogenase, but also for the PPP enzymes 6-phosphoglucono lactonase, transketolase and possibly also for transaldolase, for which the open reading frame YGR043C shows about 80% similarity on the amino acid level. The putative transaldolase activity has not, however, been verified and the protein is reported to localize in the nucleus (supplemental material (Huh *et al*. 2003)). Recently this open reading frame (ORF) was named *NQM1* for Non-Quiescent Mutant by the *Saccharomyces* Genome Database (SGD), and it may be linked to decreased capacity of non-quiescent *nqm1* strains to reproduce (SGD Werner-Washburne, M., (Allen *et al*. 2006)). The *S. cerevisiae* genome has undergone a duplication, subsequent rearrangement and loss of genes. However, the remaining paralogous genes may have diverged functionally as well as in regulation (Dujon 2006, Wagner 2002). The *TKL1*- and *GND1*-encoded isoenzymes have been reported to be responsible for the majority of the transketolase and 6-phosphogluconate dehydrogenase activities on D-glucose (Schaaff-Gerstenschläger *et al*. 1993, Sinha and Maitra 1992), whereas for *SOL3*- and *SOL4*-encoded 6-phosphoglucono lactonase such data is not available. The genes encoding the "second isoenzymes" *GND2*, *TKL2* and also *SOL4* and *NQM1* (YGR043C), are similarly induced after diauxic shift (DeRisi *et al*. 1997). They also respond similarly in several other conditions, for example to histone depletion (Wyrick *et al*. 1999), heat shock and nitrogen depletion (Gasch *et al*. 2000) and to inhibitors of the drug rapamycin (Huang *et al*. 2004). However, the actual physiological role of the "second" isoenzymes has not been established.

The values for the proportion of D-glucose 6-phosphate that enters PPP reported for *S. cerevisiae* vary considerably. Gancedo and Lagunas and Maaheimo and coworkers found values from 1 to 4% (Gancedo and Lagunas 1973, Maaheimo *et al*. 2001), but Gombert and coworkers reported a flux of up to 44% (Gombert

et al. 2001) and several others have reported values intermediate to those (Blank *et al*. 2005, Fiaux *et al*. 2003, van Winden *et al*. 2005). These differences probably reflect the culture conditions, particularly the oxygen availability, since higher fluxes were obtained with more aerobic conditions and in D-glucose-limited chemostat cultures i.e. with respiratory metabolism.

Compared to other Hemiascomycetous (subdivision of ascomycetes) yeast species studied in the Genolevoures project (Souciet *et al*. 2000) such as *Zygosaccharomyces rouxii*, *Kluyveromyces thermotolerans*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Pichia angusta*, *Debaryomyces hansenii*, *Pichia sorbitophila*, *Candida tropicalis* and *Yarrowia lipolytica*, the relative flux or flux ratio to PPP in *Saccharomyces* species was significantly lower (Blank *et al*. 2005). In *S. cerevisiae* only about 10% of the carbon was channelled through PPP, whereas in the non-*Saccharomyces* species about 40% of the D-glucose entered PPP (Blank *et al*. 2005). The higher flux correlated with higher biomass formation in all yeasts studied, except for *P. angusta* in which additional NADPH-consuming reactions may also be present. *S. cerevisiae* probably exhibited fermentative or respiro-fermentative metabolism in the applied experimental setup (deep-well microtitre plates containing 5 g l^{-1} D-glucose as a carbon source), whereas the metabolism of the other yeast species was most probably more respiratory. When the flux ratios of *S. cerevisiae* and *Pichia stipitis* were compared in aerobic D-glucose-limited chemostats, where both species showed respiratory metabolism, more similar PPP flux ratios of 40% and 61%, respectively, were obtained (Fiaux *et al*. 2003).

The carbohydrate metabolism of *S. cerevisiae* and its close relatives differs from that of the more distant members of hemiascomycetes. *S. cerevisiae*, a Crabtree positive yeast, is able to ferment hexoses even in the presence of oxygen, whereas other yeast species, e.g. *K. lactis* exhibit respiratory metabolism in the presence of oxygen. The different mode of metabolism may be due to variations in respiratory capacity or in sugar transport activities. Indeed, in *S. cerevisiae* mutants having lower D-glucose transport capacity, the glycolytic flux was also lower and metabolism shifted from fermentation to respiration (Elbing *et al*. 2004a). Accordingly, increase in transport capacity in *K. lactis* enabled fermentation of D-galactose and raffinose in the presence of oxygen (Fukuhara 2003, Goffrini *et al*. 2002). Because respiratory metabolism produces more ATP per D-glucose consumed compared to ethanol production and thus enables

formation of a higher amount of biomass, and because NADPH may also donate electrons for oxidative phosphorylation in some yeast species (see below), the flux to PPP is also linked to respiratory metabolism and ATP formation.

Interestingly, the yeast species with higher PPP flux may have additional NADPH consuming reactions. Two mechanisms have been suggested for oxidation of cytosolic NADPH in *K. lactis*, by mitochondrial dehydrogenases and a cycle of $NADP^+$- and NAD^+-dependent alcohol dehydrogenases (Gonzalez-Siso *et al*. 1996, Overkamp *et al*. 2002). Recently, external NADPH-utilizing dehydrogenases, Nde1p and Nde2p, located in the inner mitochondrial membrane were described in *K. lactis* (Tarrio *et al*. 2005, Tarrio *et al*. 2006). In addition, a NADPH-consuming ethanol-acetaldehyde shuttle could indeed be formed by the NADPH-utilizing mitochondrial alcohol dehydrogenase Ald3p and a possible cytosolic acetaldehyde reductase of *K. lactis* (Saliola *et al*. 2006a, Tarrio *et al*. 2006). Redox shuttles, for example glycerol 3-phosphate shuttle and ethanol-acetaldehyde shuttle, are described for oxidation of NADH, (Bakker *et al*. 2000, Bakker *et al*. 2001, Overkamp *et al*. 2000), but less is known about NADPH shuttles. In addition to various shuttles, the metabolic reactions of for example the NAD- and NADPH-dependent glutamate dehydrogenases may form transhydrogenase cycles that oxidise NADPH and reduce NAD^+ (Boles *et al*. 1993, Dickinson *et al*. 1995). Tarrio and coworkers have discussed the possible NADPH reoxidating reactions in *K. lactis* (Tarrio *et al*. 2006). It is possible that the biomass formation and the various NADPH-consuming reactions present in *K. lactis* and other Crabtree-negative yeasts maintain the $NADP^+$ to NADPH ratio high enough to allow the carbon flux to PPP in these yeast species.

When the gene encoding D-glucose 6-phosphate dehydrogenase was deleted in *K. lactis*, the biomass formation was significantly lower in the mutant strain compared to the wild type (wt) strain (Saliola *et al*. 2006b). In *S. cerevisiae* the deletion of the *ZWF1* gene resulted in methionine auxotrophy and in sensitivity to oxidative stress, but did not affect the formation of biomass (Slekar *et al*. 1996, Thomas *et al*. 1991). Possibly in *S. cerevisiae*, the NADPH-producing function of PPP can be overcome by other reactions such as the cytoplasmic acetaldehyde dehydrogenase (Ald6p) encoded by *ALD6*, the malic enzyme (Mae1p), encoded by *MAE1*, or isocitrate dehydrogenase (Idp2p) encoded by *IDP2*. Overexpression of *ALD6* restored growth in the absence of methionine of a *zwf1* mutant strain and *zwf1 ald6* double mutants were not viable (Grabowska

and Chelstowska 2003). The malic enzyme contributed to the NADPH level in the *zwf1* mutant but not in wt cells grown on D-glucose (Blank *et al*. 2005, Minard *et al*. 1998). The NADPH-forming role of the isocitrate dehydrogenase was evident during growth on fatty acids (Minard *et al*. 1998, Minard and McAlister-Henn 1999). Acetaldehyde dehydrogenase also contributed to NADPH production of the other hemiascomycetous yeasts although to lower extent compared to *S. cerevisiae* (Blank *et al*. 2005). Shuttle reactions of malate and pyruvate may be needed, because malic enzyme is located in mitochondria. In what proportion the PPP and the other NADPH-producing and consuming reactions contribute to the overall NADPH pool in different yeast species is difficult to estimate precisely, due to the still limited knowledge of all the reactions involved.

In strains deficient in the phosphoglucose isomerase activity, that converts D-glucose 6-phosphate to D-fructose 6-phosphate, D-glucose must be channelled via the PPP. The *pgi1* strain of *S. cerevisiae*, first described by Maitra (Maitra 1971), was unable to grow on D-glucose, and on D-fructose required small amounts of D-glucose but concentrations over 2 g l^{-1} inhibited the growth. This was possibly due to accumulation of D-glucose 6-phosphate and ATP depletion (Ciriacy and Breitenbach 1979, Maitra 1971, Ugarova *et al*. 1986). It may also be that the accumulating D-glucose 6-phosphate was converted to trehalose 6-phosphate by trehalose 6-phophate synthase (Tps1p) (Stambuk *et al*. 1993), which in turn could lead to the postulated feedback inhibition of hexokinases (Blazquez *et al*. 1993, Gancedo and Flores 2004). Alternatively, other toxic intermediates, for example gluconate 6-phosphate, accumulate and inhibit growth. The phosphoglucose isomerase mutants of the yeast *K. lactis* and of *E. coli* are able to grow on D-glucose by channelling it through PPP (Goffrini *et al*. 1991, Vinopal *et al*. 1975). The inability of a *S. cerevisiae pgi1* mutant to grow on D-glucose can be overcome by increasing the activities of the PPP enzymes (Dickinson *et al*. 1995) or by introducing NADPH-consuming reactions such as the transhydrogenase cycle of Gdh1p and Gdh2p (Boles *et al*. 1993), the *E. coli* transhydrogenase UdhA (Fiaux *et al*. 2003), *K. lactis* NADPH-dependent GAPDH (Verho *et al*. 2002), *K. lactis* thioredoxin reductase or *K. lactis* NADPH-dependent external dehydrogenase (Tarrio *et al*. 2006).

1.1.3 Role of PPP in substrate entry and product formation

In addition to producing NADPH and precursors for biosynthesis, the PPP is also an entry point for various carbon sources into the metabolism (Fig. 2). Several yeast species of for example the genera *Candida*, *Pichia*, *Pachysolen*, *Kluyveromyces* and *Debaryomyces* are able to utilize D-xylose and some species also L-arabinose, the pentose sugars present in the hemicellulose fraction of plant material. These sugars enter the metabolism through the PPP and thus require a good interplay between PPP and glycolysis. In D-xylose and probably also in L-arabinose metabolism, part of the carbon channelled to glyceraldehyde 3-phosphate and D-fructose 6-phosphate is directed to D-glucose 6-phosphate which may enter the PPP to produce NADPH. D-Xylose utilization is further described in section 1.2. Xylitol and D-xylulose are also assimilated via the PPP sugar phosphate D-xylulose 5-phosphate. Sugar alcohols D-arabitol and erythritol are probably channelled to glycolysis via D-ribulose 5-phophate and D-erythritol 4-phosphate, although these pathways have not been confirmed (Wong *et al*. 1995, Nishimura *et al*. 2006). Inversely, D-arabitol, erythritol and D-ribose produced from D-glucose by osmotolerant yeast species are derived from PPP intermediates (Fig. 2). The baker's yeast *S. cerevisiae* is not able to produce sugar alcohols from D-glucose or to use them as a carbon source.

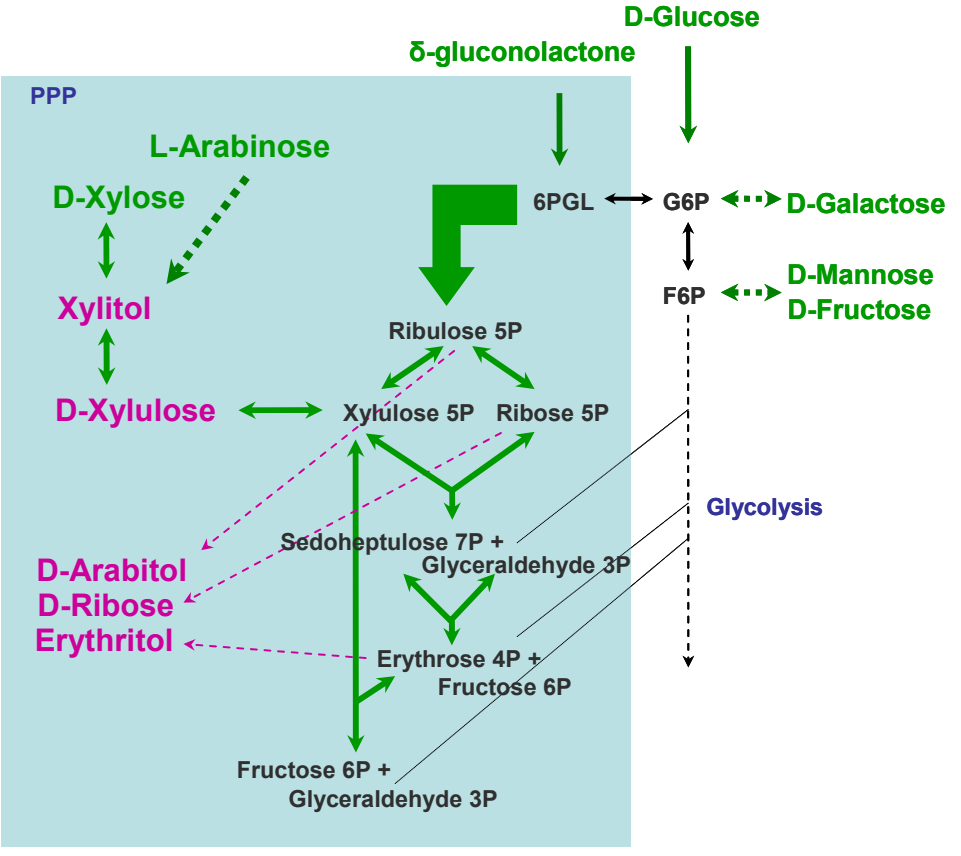

Figure 2. Role of the pentose phosphate pathway in utilization and production of sugars and sugar alcohols.

PPP may also be an entry point for δ-gluconolactone, which in *S. cerevisiae* induces the enzyme activities of 6-phosphogluconate dehydrogenase, 6-phosphogluconolactonase and gluconokinase (Sinha and Maitra 1992). In *Schizosaccharomyces pombe* the PPP enzymes are induced by δ-gluconolactone and are repressed by D-glucose (Mehta *et al*. 1998). The δ-gluconolactone can enter the PPP either via hydrolysis to gluconic acid and subsequent phosphorylation to 6-phosphogluconate or by phosphorylation to 6-phosphogluconolactone. Alternatively, δ-gluconolactone or gluconate can be converted to D-glucose by NADPH-dependent D-glucose dehydrogenase, as reported for *Saccharomyces bulderi*, thus entering the metabolism via D-glucose 6-phosphate (van Dijken *et al*. 2002).

1.2 Modifications of *S. cerevisiae* PPP for conversion of D-xylose to ethanol

1.2.1 Conversion of D-xylose to D-xylulose 5-phosphate

Transport of D-xylose into *S. cerevisiae* occurs by facilitated diffusion via some of the many hexose transporters that transport D-glucose. These transporters have higher K_m for D-xylose (0.1-1.5 M) compared to D-glucose (1-100mM) and D-glucose inhibits the uptake of D-xylose (Kötter and Ciriacy 1993, Boles and Hollenberg 1997, Saloheimo *et al*. 2007). Hxt1p, Hxt2p, Hxt4p, Hxt5p, Hxt7p and Gal2p have all been shown to transport D-xylose (Lee *et al*. 2002a, Hamacher *et al*. 2002, Saloheimo *et al*. 2007). The naturally D-xylose-utilizing yeasts may transport D-xylose either via facilitated diffusion or by proton gradient-linked transporters, which also have lower affinity for D-xylose compared with D-glucose. Proton symporters able to transport both D-glucose and D-xylose (with lower affinity) have been reported for example for *P. stipitis*, *Pichia heedii* and *Debaryomyces hansenii* (Does and Bisson 1989, Nobre *et al*. 1999) and cloned from *P. stipitis* and *Candida intermedia* (Weierstall *et al*. 1999, Leandro *et al*. 2006). A D-xylose specific transporter from *Trichoderma reesei* TrXlt1p was recently described (Saloheimo *et al*. 2007). However, prolonged culture and possibly adaptive mutation(s) were needed for growth on D-xylose of a recombinant *S. cerevisiae* strain expressing *TrXLT1* and deficient in major endogenous hexose transporters. In general, overexpression of genes encoding the facilitated diffusion transporters of either *S. cerevisiae* or *C. intermedia*, or of the *C. intermedia* proton symporter did not enhance D-xylose utilisation in recombinant *S. cerevisiae* strains (Hamacher *et al*. 2002, Leandro *et al*. 2006, Saloheimo *et al*. 2007). Accessory proteins are known to be needed for the activity of some transporters such as ion carriers and monocarboxylate transporters (Lichtenberg *et al*. 1999, Makuc *et al*. 2004). Possibly D-xylose transporters also need still unidentified auxiliary factor(s) for functional expression.

In naturally D-xylose-utilising yeasts and filamentous fungi, D-xylose is reduced to xylitol by xylose reductase (XR) after entering the cell and subsequently oxidized to D-xylulose by xylitol dehydrogenase (XDH; Fig. 3). D-Xylulose is then phosphorylated to yield D-xylulose 5-phosphate, a PPP intermediate. *S. cerevisiae* is able to grow on D-xylulose, but not on D-xylose (Chiang *et al*.

1981, Richard *et al*. 2000, Ueng *et al*. 1981, Wang and Schneider 1980). The genes encoding the activities of the first two reactions, namely *XYL1* for xylose reductase and *XYL2* for xylitol dehydrogenase were cloned from the D-xylose-utilising yeast *P. stipitis* and expressed in *S. cerevisiae* under constitutive promoters (Kötter *et al*. 1990, Kötter and Ciriacy 1993, Walfridsson *et al*. 1995). This resulted in biomass and xylitol formation from D-xylose, but the ethanol amounts produced were low and anaerobic D-xylose utilisation was slow. This was proposed to be due to insufficient capacity of the pentose phosphate pathway and to cofactor imbalance caused by the different cofactors preferred by the xylose reductase (NADPH) and xylitol dehydrogenase (NAD^+) (Kötter and Ciriacy 1993, Walfridsson *et al*. 1995). XDH enzymes (EC 1.1.1.9, D-xylulose reductase) have been cloned and characterized from several yeast species, most recently from *C. tropicalis* by Ko and coworkers (Ko *et al*. 2006) and by Lima and coworkers (Lima *et al*. 2006). Properties of various yeast D-xylose (aldose) reductases (EC 1.1.1.21, aldehyde reductase) have been reviewed by Ellis (Ellis 2002) and the nonspecific aldose reductases of *S. cerevisiae* described by Träff and coworkers (Träff *et al*. 2002).

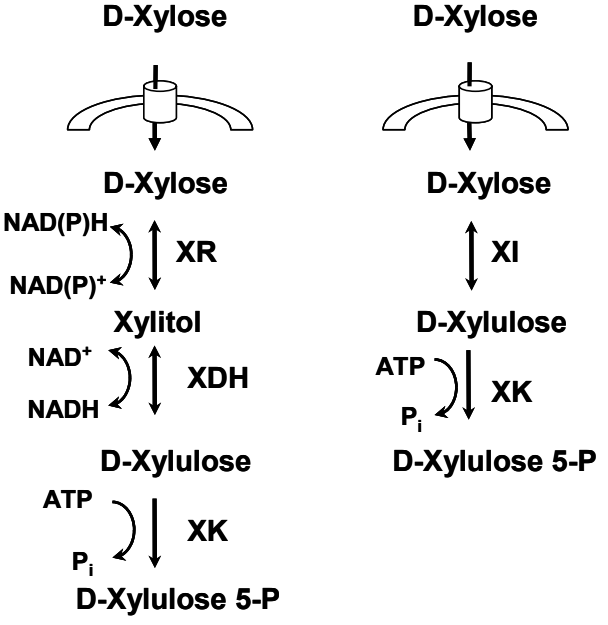

Figure 3. First steps in the metabolism of D-xylose via fungal (left) or bacterial (right) pathways.

In most bacteria, D-xylose is converted to D-xylulose in one step by xylose isomerase (XI, EC 5.3.1.5, also referred to as glucose isomerase) (Fig. 3). Cofactors are not needed in the isomerisation and thus the redox balance is not an issue. The bacterial xylose isomerases have been expressed in *S. cerevisiae*, but with little success (Amore *et al*. 1989, Ho *et al*. 1983, Moes *et al*. 1996, Sarthy *et al*. 1987, Walfridsson *et al*. 1996). Xylose isomerase from *Thermus thermophilus* was expressed in an active form in *S. cerevisiae* generating small amounts of ethanol from D-xylose, but the temperature optimum of this enzyme was 85ºC and it had low activity at 30ºC (Walfridsson *et al*. 1996). Later this enzyme has been mutagenised to decrease the inhibition by xylitol and to obtain a higher activity also at low temperatures (Lönn *et al*. 2002). Recently, a fungal xylose isomerase from an anaerobic fungus *Piromyces* sp. E2 was expressed successfully in *S. cerevisiae* showing XI activity from 0.03 to 1.1 μmol min^{-1} mg $protein^{-1}$ and ability to grow on D-xylose (Harhangi *et al*. 2003, Kuyper *et al*. 2003, Kuyper *et al*. 2004). However, xylitol inhibits the activity of xylose isomerases (Yamanaka 1969, Yamanaka 1975), and D-xylose may be reduced to xylitol by some of the several non-specific aldose reductases present in *S. cerevisiae* (Träff *et al*. 2002). Therefore the *GRE3* gene coding for the major aldose reductase activity in *S. cerevisiae* has been deleted from strains expressing xylose isomerases (Karhumaa *et al*. 2005, Kuyper *et al*. 2005a, Träff *et al*. 2001). When the performance of the isomerase and oxido-reductive pathways were compared in a same strain background on mineral medium containing D-xylose, the strain with xylose isomerase pathway had higher ethanol yield by 30% and it produced less xylitol (Karhumaa *et al*. 2007). However, the strain with the oxido-reductive pathway had higher D-xylose consumption rate and specific ethanol productivity. Results of D-xylose fermentation by various recombinant *S. cerevisiae* strains constructed with either the oxido-reductive or xylose isomerase pathways were compared by van Maris and coworkers (van Maris *et al*. 2006). The highest ethanol yields on D-xylose were 0.43 g g^{-1} for both types of strains, but the yield of xylitol on D-xylose was remarkably lower for the strains with XI.

The phosphorylation step after conversion of D-xylose to D-xylulose catalyzed by the xylulokinase (XK, EC 2.7.1.17) enzyme is known to occur in *S. cerevisiae*, since it can grow on D-xylulose. Xylulokinase is encoded by the *XKS1* gene in *S. cerevisiae* (Ho *et al*. 1990, Rodriguez-Pena *et al*. 1998), and xylulokinase-encoding genes (named *XYL3*) have also been characterised from *P. stipitis* and *Candida maltosa* (Guo *et al*. 2006, Jin *et al*. 2002).

The interplay of the first three reactions of the oxido-reductive pathway converting D-xylose to the metabolic intermediate D-xylulose 5-phosphate has attracted considerable interest in the context of optimal expression levels of the XR, XDH and XK enzymes. A kinetic model suggested a ratio of 1 : >10 :> 4 for xylose reductase, xylitol dehydrogenase and xylulokinase, respectively, which was also shown experimentally to be valid (Eliasson *et al*. 2001). In a strain overexpressing the non-oxidative PPP genes, high level expression of XR and XDH enzymes enhanced D-xylose fermentation (Karhumaa *et al*. 2006). Similarly, a recombinant D-xylose-utilising *S. cerevisiae* strain C1 that evolved in an anaerobic chemostat culture on D-xylose showed increased expression of XR and XDH encoding genes (Sonderegger *et al*. 2004).

1.2.2 Non-oxidative PPP

The non-oxidative pentose phosphate pathway converts D-xylulose 5-phosphate to glyceraldehyde 3-phosphate and D-fructose 6-phosphate by the reversible reactions of D-ribose 5-phosphate ketol-isomerase, D-ribulose 5-phosphate 3-epimerase, transketolase and transaldolase enzymes. In the first recombinant *S. cerevisiae* strains constructed that expressed the *P. stipitis XYL1* and *XYL2* genes, accumulation of sedoheptulose 7-phosphate occurred (Kötter and Ciriacy 1993). Increase in transaldolase activity enhanced the D-xylose consumption rate, whereas overexpression of either *S. cerevisiae* or *P. stipitis* transketolase-encoding genes either had no effect or decreased the growth (Metzger and Hollenberg 1994, Walfridsson *et al*. 1995). Similarly, a *P. stipitis TAL1* gene was found to increase growth on D-xylose and also to improve ethanol production in a strain containing the *P. stipitis XYL1*, *XYL2* and *XYL3* genes overexpressed (Jin *et al*. 2005). When all the non-oxidative PPP genes were overexpressed in *S. cerevisiae* they increased the growth rate on D-xylulose but not on D-xylose (Johansson and Hahn-Hägerdal 2002) in recombinant D-xylose-utilising strains. Recently, in a strain with high level expression of genes encoding xylose reductase and xylitol dehydrogenase, overexpression of the non-oxidative PPP genes also increased the D-xylose utilization rate (Karhumaa *et al*. 2006). The importance of the non-oxidative PPP enzymes in the D-xylose metabolism of recombinant *S. cerevisiae* strains is also evident from mutants with improved D-xylose metabolism in which the transketolase and transaldolase activities (Pitkänen *et al*. 2005) or expression of *TKL1* and *TAL1*

were elevated (Sonderegger *et al*. 2004, Wahlbom *et al*. 2003). In addition, overexpression of the *XKS1, RKI1*, *RPE1*, *TKL1* and *TAL1* genes in strains harbouring the *Piromyces* sp. E2 xylose isomerase increased the specific growth rate on D-xylose in anaerobic conditions (Kuyper *et al*. 2005a).

1.3 Pentose phosphate pathway-derived sugars and sugar alcohols

1.3.1 Formation of D-arabitol, erythritol and D-ribose via PPP

Yeast species of e.g. genera *Zygosaccharomyces*, *Debaryomyces*, *Hansenula* and *Pichia* are able to grow in environments with low water activity i.e. in the presence of high sugar or salt concentrations. The baker's yeast *S. cerevisiae* does not, however, tolerate such conditions. The so-called osmotolerant yeasts accumulate compatible solutes when encountering salt or osmotic stress. Compatible solutes protect and stabilize enzymes, enabling the cellular functions in osmotic conditions. Glycerol is the most common osmolyte in yeasts, but sugar alcohols such as D-arabitol, erythritol and mannitol may also serve as osmolytes. The sugar alcohols produced may also have role in redox balancing or as storage compounds (for a review, see Brown 1978).

D-arabitol production is best described for *Z. rouxii*, a yeast used in soy sauce and miso paste production, and for the opportunistic human pathogen *Candida albicans*. *Z. rouxii* strains produce both glycerol and D-arabitol as a compatible solute. The precursor for D-arabitol in *Z. rouxii* is thought to be D-ribulose 5-phosphate (Ingram and Wood 1965, Jovall *et al*. 1990), although D-xylulose 5-phosphate has also been suggested as a precursor (Blakley and Spencer 1962). It is postulated that D-ribulose 5-phosphate is dephosphorylated and the subsequently formed D-ribulose is reduced to D-arabitol by NADPH-dependent D-arabitol dehydrogenase (Fig. 4.) (Moran and Witter 1979, Weimberg 1962). Alternatively, dephosphorylated D-xylulose could be reduced to D-arabitol. Many aspects of D-arabitol production by *Z. rouxii* and other osmotolerant yeasts, such as the effect of sugar concentration, salts, medium composition and oxygen availability, have been studied (for a review, see Spencer 1968, Spencer and Spencer 1978). The D-arabitol yield can be up to 60% of the D-glucose consumed.

Relatively little is known about the enzymes and the corresponding genes involved in D-arabitol production in yeasts. Neither the phosphatase nor the dehydrogenases have been characterized from *Z. rouxii*. Acid and alkaline phosphatases are known to be active on D-ribulose 5-phosphate and D-xylulose 5-phosphate (Ingram and Wood 1965), but their extracellular or vacuolar localization, the inability of phosphorylated compounds to cross biological membranes and their wide substrate specificity do not support their role in intracellular dephosphorylation. NADH-dependent D-arabitol dehydrogenases (D-arabinitol 4-dehydrogenase, EC 1.1.1.11) have been cloned from *P. stipitis, C. albicans* and *C. tropicalis* (Hallborn *et al*. 1995, Murray *et al*. 1995, Wong *et al*. 1993). Interestingly, deletion of the NADH-dependent D-arabitol dehydrogenase from *C. albicans* did not abolish D-arabitol production, but led to inability to grow on D-arabitol (Wong *et al*. 1995). This suggests that there may be other, perhaps NADPH-dependent, D-arabitol dehydrogenases active in D-arabitol production as suggested by Weimberger and by Moran and Witter (Moran and Witter 1979, Weimberg 1962), and that the D-arabitol formation and utilization proceed via different enzymes. The phosphatase dephosphorylating D-ribulose 5-phosphate is not known in *Pichia* or *Candida* species either. The D-arabitol dehydrogenases are not active on L-arabitol and are thus separate from L-arabinitol 4-dehydrogenase (EC 1.1.1.12) that is active on L-arabinose converting L-arabitol to L-xylulose.

D-arabitol is probably transported by passive diffusion. However, in *Z. rouxii* it may be also transported by the recently identified Fps1p channel protein (Tang *et al*. 2005). In *S. cerevisiae* Fps1p releases glycerol from inside the cell in hypo-osmotic conditions (Tamas *et al*. 1999). In *C. albicans* glycerol production increased in osmotic stress conditions, whereas temperature and oxidative stresses increased D-arabitol production (Kayingo and Wong 2005). The D-arabitol production is probably regulated differently to that of glycerol in *C. albicans* (Kayingo and Wong 2005). When D-arabitol is formed from D-glucose the effect on redox balance is different compared to that of glycerol production. Two NADPH molecules are formed in the PPP per D-arabitol formed and the subsequent reaction with D-arabitol dehydrogenase may yield either NAD^+ or $NADP^+$.

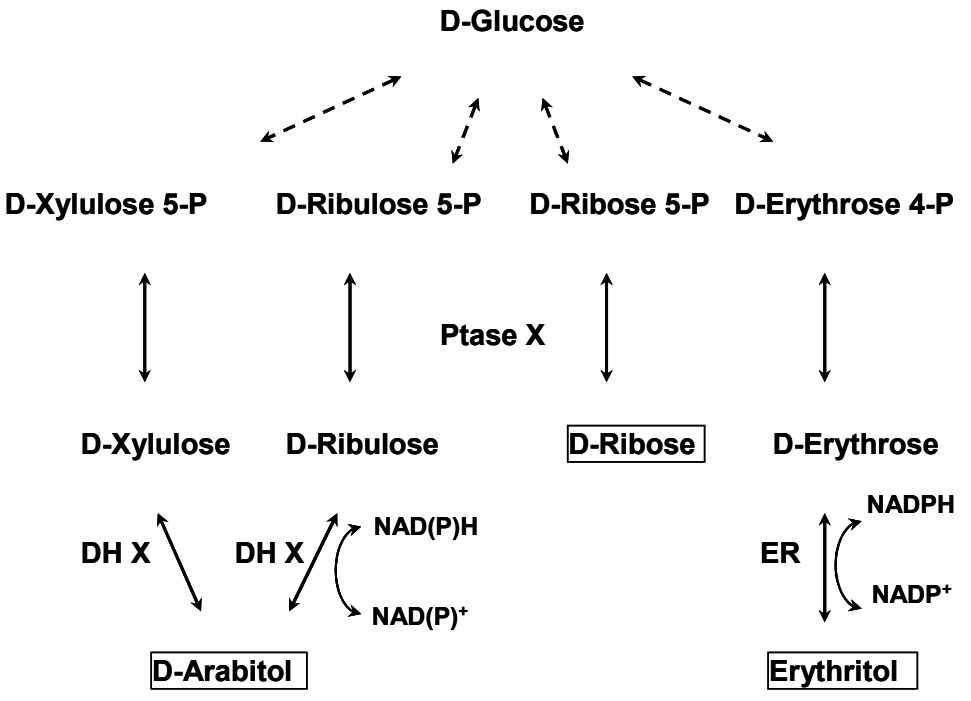

Figure 4. Schematic representation of possible pathways for formation of D-arabitol, D-ribose and erythritol from D-glucose in osmophilic yeasts. DH X; arabitol dehydrogenase, Ptase X; unknown phosphatase, ER; erythrose reductase.

Erythritol, another sugar alcohol possibly acting as an osmosolute, is synthesized from D-erythrose 4-phosphate after dephosphorylation and reduction reactions. Several yeast species such as *Aureobasidium* sp., *Torula coralline*, *Candida magnoliae* and *Ustilaginomycetes* are known to produce erythritol and the strains or their mutagenised derivatives reach over 40% yields of erythritol on D-glucose (w/w) (Ishizuka *et al*. 1989, Kim *et al*. 2000). These strains have mainly been studied in the context of process engineering to increase the yield of erythritol on D-glucose. As in the case of D-arabitol, the phosphatase enzyme dephosphorylating D-erythrose 4-phosphate is not known. However, an erythrose reductase catalyzing the D-erythrose reduction to erythritol has been characterized from *Aureobasidium* sp. and *Torula corallina* (Tokuoka *et al*. 1992, Lee *et al*. 2002b). Recently, proteome analysis of *C. magnoliae* and its mutant derivative having enhanced growth and erythritol production capacity from D-glucose, showed altered expression levels of some of the genes encoding TCA and glycolytic proteins. The levels of the TCA enzymes citrate synthase,

succinyl-CoA ligase and fumarase and the glycolytic pyruvate decarboxylase were increased in the mutant strain, whereas the level of enolase was decreased compared to the wild type strain (Lee *et al*. 2003). The higher level of fumarase may lead to lower fumarate levels and thus decrease the inhibition of the erythrose reductase by fumarate (Lee *et al*. 2002b). Low expression of enolase on the other hand may prevent the flux of D-erythrose 4-phosphate towards shikimate, an aromatic amino acid precursor, formed from D-erythrose 4-phosphate and phosphoenolpyruvate, the product of enolase reaction.

When screening for yeast species producing D-ribose *Candida pelliculosa* (*Pichia anomala*) was found to produce D-arabitol and small amounts of D-ribose derived from D-ribose 5-phosphate (De Wulf *et al*. 1996). A strain producing only D-ribose with yields of 15% of the consumed D-glucose was obtained by mutagenesis. However, no further studies on D-ribose production with these yeast strains have been reported.

1.3.2 Potential of PPP metabolites as precursors in xylitol production

Xylitol is a five-carbon sugar alcohol widely used as a non-cariogenic sweetener. It also prevents acute middle ear infection (otitis media) in children and is an ideal sweetener for persons with diabetes, because its metabolism is insulin-independent. Currently, xylitol is produced from D-xylose by chemical reduction (Melaja and Hämäläinen 1977), but biotechnical conversion of D-xylose to xylitol by various yeast species is also well characterized (Hallborn *et al*. 1991, Granström *et al*. 2007a, Granström *et al*. 2007b). Compared to D-xylose, D-glucose, that is a common substrate in the food industry, is a cheaper and more readily available raw material, making it an attractive substrate for xylitol production. However, studies on natural micro-organism producing xylitol from D-glucose are either lacking completely or reported in patent application(s) (e.g. patent US6335177). Processes combining various microbes or microbes and an enzyme catalyst have generated xylitol from D-glucose via D-arabitol (Mayer *et al*. 2002, Onishi and Suzuki 1969). Recently, a *Bacillus subtilis* strain was engineered to produce xylitol from D-glucose with yield of 23% (w/w) (Povelainen and Miasnikov 2006). In the *B. subtilis* strains the precursor of xylitol is D-xylulose 5-phophate, which is converted to xylitol 5-phosphate by a

xylitol-phosphate dehydrogenase and then dephosphorylated to xylitol by an unknown phosphatase. Miersch and coworkers have reported activity of ribitol 5-phosphate dehydrogenase converting ribitol 5-phosphate to D-ribulose 5-phosphate in *Pichia guilliermondii* (Miersch *et al*. 1980). However, the current view of D-arabitol or erythritol production in yeast is that dephosphorylation produces a keto sugar which is then reduced to the corresponding sugar alcohol by a dehydrogenase enzyme (Fig. 4). In *S. cerevisiae* xylitol could be formed via D-xylulose 5-phosphate by a pathway similar to that of D-arabitol and erythritol production.

1.4 Aims of the study

This work aimed to improve the performance of recombinant D-xylose-utilising *S. cerevisiae* strains by studying the effect of the expression level of the xylulokinase-encoding gene in various oxygen concentrations and thus in various energy and redox states.

In addition, the functioning of endogenous xylose reductase- and xylitol dehydrogenase-encoding genes of *S. cerevisiae* was studied with relation to D-xylose metabolism in order to evaluate whether strain construction without heterologous expression could be feasible.

Furthermore, this study explored the potential of the yeast *S. cerevisiae* for production of xylitol from D-glucose via PPP in a single fermentation step.

2. Materials and methods

Materials and methods are described in the original articles (I–II), in the submitted manuscript (III) and in the manuscript (IV).

Method	Used in
Bioreactor cultures, batch, fed batch	I, II
Batch cultures, shake flask	II–IV
Cell dry weight measurement	I, II
Metabolite analysis, HPLC	I–IV
Extraction of intracellular metabolites	I
Enzymatic assays for metabolites	I
Enzyme activity measurements	I, II, III
RNA extraction and Northern hybridization	II
Strain construction, DNA methods	I–IV

3. Results

3.1 Role of xylulokinase in D-xylose metabolism in recombinant *S. cerevisiae* strains (I)

S. cerevisiae strains can grow on D-xylulose which is the isomerised form of D-xylose. When strains overexpressing the xylulokinase-encoding gene were grown on D-xylulose medium Rodrigues-Pena and coworkers found the overexpression of *XKS1* detrimental, whereas Richard and coworkers observed an increase in the growth rate on D-xylose (Richard *et al*. 2000, Rodriguez-Pena *et al*. 1998). We wanted to study whether the reaction catalysed by xylulokinase is a rate-limiting step in recombinant D-xylose-utilising *S. cerevisiae* strains. Thus we cloned and overexpressed the *XKS1* gene in a multicopy plasmid under the *PGK1* promoter in *S. cerevisiae* also harbouring the *P. stipitis XYL1* and *XYL2* genes integrated into the genome. Because oxygen affects the NAD^+ and ATP availability in the cell, cultures were performed in various oxygen concentrations.

3.1.1 Increase in xylulokinase activity enhances D-xylose metabolism in various oxygen concentrations

The effect of the overexpression of the xylulokinase-encoding gene was studied in fed-batch and batch cultures, because the slow growth rate of the parental strain on D-xylose alone would have caused difficulties in setting up chemostat experiments. The fed-batch culture medium contained a small amount of D-glucose in addition to D-xylose, whereas the batch cultures were performed on D-xylose alone to exclude the possible effects of D-glucose. In previous studies by both Ho and coworkers (Ho *et al*. 1998) and Eliasson and coworkers (Eliasson *et al*. 2000) D-glucose was used as a cosubstrate.

In the fed-batch experiment oxygen concentration was stepwise changed from anaerobic to aerobic conditions and the substrates and products were measured in conditions of 0; 2.5; 5; 7.5; 10 and 20% oxygen enrichment of the inlet gas. Overexpression of the xylulokinase-encoding gene clearly improved the specific D-xylose uptake rate and ethanol production rate and decreased the xylitol yield

in all aeration conditions studied except for 2.5% oxygen enrichment, where there was no difference in specific D-xylose consumption rate or xylitol yield and the specific ethanol production rate was higher for the control strain (I, Fig. 1).

In batch cultures the two strains were compared using only D-xylose as a carbon source in three different oxygen concentrations; anaerobic (0% inlet oxygen), microaerobic (2% inlet oxygen) and aerobic (20% inlet oxygen). The xylulokinase-overexpressing strain had a higher specific D-xylose consumption rate (2-2.4-fold) and ethanol production rate (8-fold) in all oxygen concentrations studied (I, Table 4). The xylitol yield was lower especially in microaerobic and aerobic conditions (I, Table 5). In both studies xylitol production rate and yield decreased when aeration increased, showing the importance of regeneration of the NADH cofactor back to NAD^+ by respiration (I, Figs. 1 and 2, Tables 4 and 5). The observed variation between the ethanol production rates and yields of fed-batch culture at low aeration (2.5%) and the microaerobic batch culture (2%) may reflect the different set-ups, presence of D-glucose in the fed-batch experiment or the small variation in the aeration level.

3.1.2 Intracellular metabolite levels and enzyme activities suggest limitations in pentose fermentation

When intracellular metabolites of the above described cultures were measured, significant accumulation of D-xylulose 5-phosphate in the XK-overexpressing strain compared to the control strain was observed in all aeration conditions (I, Table 3 and Fig. 3). The level of accumulation of D-xylulose 5-phosphate decreases with increasing aeration, suggesting that increase in the overall D-xylose flux releases the accumulation. Interestingly, sedoheptulose 7-phosphate, previously reported to accumulate in recombinant D-xylose-utilising *S. cerevisiae* strains (Kötter and Ciriacy 1993), only accumulated to a higher level in microaerobic conditions (batch culture) compared to the control. In aerobic conditions it was below the detection limit in the strain overexpressing the XK-encoding gene. The intracellular ATP levels were lower for the XK-strain compared to the control strain which is in agreement with the D-xylulose 5-phosphate accumulation (I, Table 6). The ADH activity was significantly lower in aerobic conditions (I, Fig. 4) in the *XKS1*-overexpressing strain compared to the control strain.

3.2 The endogenous D-xylose pathway in *S. cerevisiae* (II)

The yeast *S. cerevisiae* is not able to grow on D-xylose without modifications. This inability was generally concluded to be due to the absence of the xylose reductase and xylitol dehydrogenase activities needed for conversion of D-xylose to D-xylulose in *S. cerevisiae*. Low XR and XDH activities have been reported to exist in *S. cerevisiae* strains, although not consistently (Batt *et al*. 1986, van Zyl *et al*. 1989).

3.2.1 Endogenous genes are sufficient for growth of *S. cerevisiae* on D-xylose

The *S. cerevisiae* genome has several genes or open reading frames homologous to the *P. stipitis* XR and XDH-encoding genes *XYL1* and *XYL2*, respectively. The amino acid sequences of *P. stipitis* XR and XDH were compared to the translated *S. cerevisiae* genome ORF sequences in order to identify similar genes. The gene with highest similarity (72%) towards the *P. stipitis* XR was *GRE3* coding for NADPH-specific aldose reductase. In addition, also *YPR1*, *GCY1*, YJR096w, *ARA1* and YDL124w sequences showed 50 to 60% sequence similarity on amino acid level with the *P. stipitis* XR. We showed previously that the open reading frame YLR070c (*ScXYL2*), having 59% sequence similarity with the *P. stipitis* XDH on amino acid level, codes for XDH activity (Richard *et al*. 1999). In addition, *SOR1*, encoding sorbitol dehydrogenase and *SOR2* (YDL246c) almost identical to *SOR1*, showed sequence similarity with the *P. stipitis* XDH. In order to study the possible endogenous pathway formed by these candidates we overexpressed the *GRE3* and *ScXYL2* genes under the *PGK1* promoter from multicopy plasmids in the *S. cerevisiae* strain S150-2B. The strain with the endogenous genes overexpressed was compared with a strain expressing the corresponding genes from *P. stipitis*. Overexpression of the endogenous genes enabled growth on D-xylose, although at a lower rate than the strain expressing the *XYL1* and *XYL2* genes from *P. stipitis* (II, Fig. 1). In addition, more xylitol was formed with the strain carrying the *S. cerevisiae* homologues.

The XDH activity of the strain harbouring the ScXDH was about ten times lower than that of the strain with *P. stipitis* XDH. When the *P. stipitis* XDH was

expressed in combination with *GRE3* in strain the CEN.PK2, higher XDH activity was achieved but xylitol accumulation was comparable to that observed in the strain with the ScXDH. Thus the difference in growth and xylitol production rates was concluded to be due to the Gre3 protein.

3.2.2 Genes encoding sorbitol dehydrogenase, transketolase and transaldolase are induced in the presence of D-xylose

In order to determine whether D-xylose, if present in the medium, affects the expression pattern of the endogenous genes *GRE3* and *ScXYL2* as well as the genes encoding sorbitol dehydrogenase (*SOR1, SOR2*), transketolase (*TKL1*) and transaldolase (*TAL1*), the mRNA levels were measured in the *S. cerevisiae* strains CEN.PK2 and ENY.WA-1A grown on D-glucose in the presence or absence of D-xylose. The transcription levels were analyzed from two time points (II, Fig 3.) from which XR and XDH activities were also measured. The XDH activity was 30 to 125-fold higher in the presence of D-xylose for the strains ENY.WA-1A and CEN.PK2, respectively, but the *SOR1* or *SOR2* gene rather than the *ScXYL2* gene was induced. In addition, the *TKL1* and *TAL1* genes showed increased expression (10- and 2 to 8-fold, respectively) in both strains in the presence of D-xylose. Only 9 and 6 g l^{-1} of D-xylose was used, resulting mainly in 3 and 5.5 g l^{-1} xylitol with the strains CEN.PK2 and ENY.WA-1A, respectively.

3.3 Production of xylitol and other five-carbon sugars and sugar alcohols from D-glucose with *S. cerevisia*e (III, IV)

Production of xylitol from D-glucose, a readily available substrate, in a single fermentation step has been accomplished in genetically modified bacteria (Povelainen and Miasnikov 2006). The common baker's yeast *S. cerevisiae* although a widely used production organism has not hitherto been engineered to produce xylitol from D-glucose. *B. subtilis* strains, obtained by classical mutagenesis for high-yield production of D-ribose from D-glucose, were deficient in transketolase activity and possibly also in D-ribulose 5-phosphate 3-epimerase activity (De Wulf *et al*. 1997, Sasajima and Yoneda 1989). Thus, a straight forward approach to engineer *S. cerevisiae* for D-glucose conversion to

xylitol would be to delete the transketolase-encoding genes in order to accumulate the PPP sugar phosphates, especially D-xylulose 5-phosphate, and subsequently to apply a sugar phosphatase and then reduce the formed D-xylulose to xylitol by a xylitol dehydrogenase (Fig. 5).

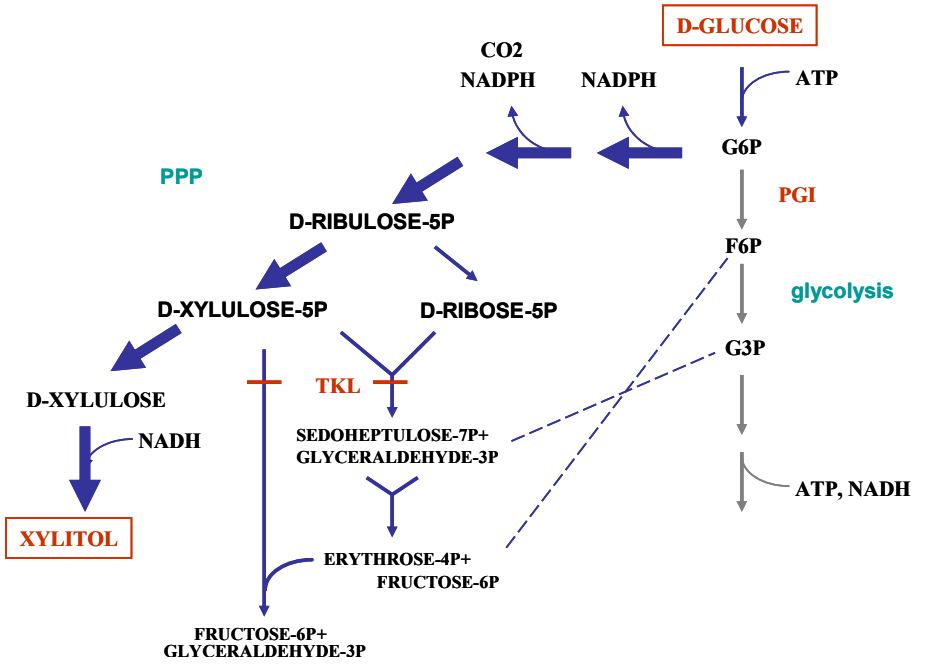

Figure 5. Hypothesized production pathway from D-glucose to xylitol in recombinant S. cerevisiae strains.

3.3.1 Production of sugar alcohols and five-carbon sugars by modified transketolase-deficient strains (III)

In order to study the production of pentose sugars and sugar alcohols in *S. cerevisiae*, the strain W303-1B and its transketolase-deficient derivative were tested for production of the pentose sugars and the sugar alcohols. Small amounts of D-ribose plus D-ribulose (which coeluted in HPLC) were detected in culture supernatants of the *tkl1tkl2* strains but not with the wt strain (III, Fig. 2a). Our previous studies on intracellular accumulation of sugar phosphates suggested that the amount of D-xylulose 5-phosphate was increased compared to the host strain in the transketolase-deficient strain (Teleman *et al*. 1999). However, D-xylulose was not observed in the culture supernatants.

Since *S. cerevisiae* does not produce significant xylitol dehydrogenase activity on D-glucose (Richard *et al*. 1999, II), also verified by the fact that only keto sugars were detected in the culture supernatants, xylitol dehydrogenase-encoding gene *XYL2* from *P. stipitis* was expressed in the transketolase-deficient strain. Subsequently, the pattern of sugars produced changed from keto sugars to the corresponding sugar alcohols (III, Fig. 2b). Only about one third of the sugar alcohols produced was xylitol and the rest was ribitol (D-ribose), suggesting that an unknown phosphatase acting in the cells may favour D-ribulose 5-phosphate or D-ribose 5-phosphate as a substrate.

The sugar phosphate phosphatase dephosphorylating the D-xylulose 5-phosphate is a major determinant of the conversion of D-glucose to xylitol. However, no protein specifically dephosphorylating D-xylulose 5-phosphate is known. The only cytosolic proteins thus far reported to dephosphorylate D-ribose 5-phosphate or D-ribulose 5-phosphate are the 2-deoxy-6-phosphate phosphatases, Dog1p and Dog2p, found in cells resistant to 2-deoxy D-glucose (Randez-Gil *et al*. 1995). Of the two similar enzymes, the Dog1p had greater activity towards 5-carbon sugar phosphates. Recently, the *DOG2* gene was reported to be expressed in conditions with oxidative stress (Tsujimoto *et al*. 2000). When expressed in a strain lacking transketolase activity also expressing xylitol dehydrogenase, the expression of Dog1p increased the amounts of D-ribose and ribitol produced (III, Table 2), but not that of xylitol, suggesting that it is indeed more specific to D-ribose 5-phosphate than to D-xylulose 5-phosphate.

Another consideration in xylitol production is the endogenous xylulokinase. Although it is known that the activity in the strain W303-1B used in this study is negligible (Richard *et al*. 2000), it still grows slowly on D-xylulose. Thus, it appeared that the D-xylulose possibly formed by dephosphorylation was phosphorylated again and subsequently not observed as xylitol. This was indeed the case since the amount of xylitol increased 1.9 fold after deletion of the xylulokinase-encoding gene (III, Table 2). When the aforementioned Dog1p was expressed in the transketolase-deficient strain harbouring *XYL2* and also deficient in xylulokinase, the best yield of ribitol and D-ribose as well as of xylitol, 3.6% of D-glucose consumed, was achieved (III, Table 2). In this strain 40% of the sugar alcohols produced was xylitol.

3.3.2 Directing D-glucose flux to the pentose phosphate pathway (IV)

In the context of xylitol production from D-glucose, channelling of all the D-glucose through the PPP in *S. cerevisiae* strain deficient in phosphoglucose isomerase activity is of interest. We studied the *S. cerevisiae pgi1* mutant strains in order to produce five-carbon sugars and sugar alcohols from D-glucose by increasing the flux to PPP with two different NADPH-consuming reactions: the *GDH2*-cycle previously reported by Boles and coworkers (Boles *et al*. 1993) and a NADPH-utilizing glyceraldehyde 3-phosphate dehydrogenase gapB of *B. subtilis* (Fillinger *et al*. 2000). To enhance the dephosphorylation of the sugar phosphates the Dog1p phosphatase was also expressed in the strains.

Both the Gdh2p, as reported previously, and the gapB enabled growth of the *pgi1* mutant strain on D-glucose. The sugar phosphate phosphatase Dog1p was needed for five-carbon sugars and sugar alcohols to be produced (IV, Fig. 2C). However, when the *DOG1* gene was expressed together with *gapB* or *GDH2* in the *pgi1* strain the growth was impaired: the *GDH2+DOG1* strain did not grow on D-glucose at all and the strain *gapB+DOG1* only very slowly (data not shown). In addition, the D-glucose tolerance in the presence of D-fructose of the strains overexpressing the *DOG1* gene was lower than without the phosphatase (IV, Fig. 2A and B). At most about 0.4 g l^{-1} of ribitol plus D-ribose and D-ribulose was produced from 4 g l^{-1} of D-glucose and 20 g l^{-1} of D-fructose with the strain expressing both *gapB* and *DOG1* genes (IV, Fig. 3).

In order to increase the accumulation of the five-carbon sugar phosphates the *PGI1* gene was deleted from a transketolase mutant strain to obtain a *pgi1 tkl1 tkl2* triple mutant. Deletion of both phosphoglucose isomerase and transketolase activities created a closed pathway from D-glucose to 5-carbon sugars and sugar alcohols. Thus D-glucose no longer served as a carbon source, but another sugar such as D-fructose was needed to maintain metabolism. Although this pathway is unable to work as such, it provides an interesting model for studying energy and redox demands. When expressed in the *pgi1 tkl1 tkl2* strain, the resulting strains with *gapB*, *gapB+DOG1*, *GDH2*, *GDH2+DOG1* and *DOG1* strains all grew in the presence of 0.5 g l^{-1} D-glucose on 20 g l^{-1} D-fructose. The strains expressing *gapB* did not grow in the presence of higher D-glucose concentrations, whereas the other strains were able to grow to some extent (IV,

Fig. 4A). The strains expressing *GDH2*, *DOG1* or both and the control strain produced ribitol, D-ribose and D-ribulose, at most about 1 g l^{-1} from 2 g l^{-1} of D-glucose in the presence of 20 g l^{-1} D-fructose (IV, Fig. 4B). However, the *GDH2* or the *DOG1* or their combination did not have a clear effect on the yield of 5-carbon sugars and sugar alcohols.

4. Discussion

4.1 Role of the xylulokinase enzyme in D-xylose metabolism

The higher D-xylose consumption and ethanol production rates by the strain overexpressing *XKS1* clearly show that the xylulokinase step was rate-limiting. In addition, the fact that ethanol was produced in both set-ups in aerobic conditions by the XK-overexpressing strain but not by the control strain, possibly by shifting the metabolism from respirative to fermentative mode, shows the importance of this modification. Moreover, to best of our knowledge, this was the first time when ethanol was produced in anaerobic conditions D-xylose only as a carbon source.

The accumulation of D-xylulose 5-phosphate suggests that the activity of the transketolase reaction is not optimal. Indeed, mutant strains with improved ability to utilize D-xylose showed increased transketolase activity (Pitkänen *et al*. 2005, Wahlbom *et al*. 2003). However, overexpression of the *TKL1* gene and the other non-oxidative PPP genes enhanced D-xylose metabolism only if XR and XDH activities were also high (Johansson and Hahn-Hägerdal 2002, Karhumaa *et al*. 2006). Overexpression of *TAL1* of either *S. cerevisiae* (Walfridsson *et al*. 1995) or *P. stipitis* (Jin *et al*. 2005) increased growth, however, further implying that these reactions should be optimized.

The significantly lower ADH activity and ethanol production by the *XKS1*-overexpressing strain in aerobic conditions may reflect the higher D-xylose flux. Possibly this higher flux affects the expression of *ADH2* gene, Adh2p being the enzyme enabling ethanol utilization. Consistent with this hypothesis, on D glucose *ADH2* was expressed in strains with low glycolytic rate, but in strains having higher glycolytic rates the expression of *ADH2* ceased rapidly after D-glucose addition (Elbing *et al*. 2004b).

While our studies were in progress, the xylulokinase of *S. cerevisiae* was also studied in other laboratories. Ho and coworkers (Ho *et al*. 1998) reported aerobic growth and ethanol formation from D-xylose on rich medium also containing D-glucose. Eliasson and coworkers (Eliasson *et al*. 2000), on the other hand,

observed improvement in ethanol production in anaerobic chemostat cultures in the presence of D-glucose when the *XKS1* was overexpressed. On defined and complex media containing a D-xylose-D-glucose mixture or birch wood hydrolysate as a carbon source the xylitol yield decreased when the *XKS1* gene was overexpressed, but the total D-xylose consumption rate was reduced (Johansson *et al*. 2001). This was suggested to be due to too high xylulokinase activity (30 U mg^{-1}) possibly leading to accumulation of D-xylulose 5-phosphate and ATP depletion (Johansson *et al*. 2001) similar to that occurring in uncontrolled D-glucose phosphorylation (Teusink *et al*. 1998, Thevelein and Hohmann 1995). The toxic effect was also observed when a *XKS1* overexpressing strain was unable to grow on D-xylulose (Rodriguez-Pena *et al*. 1998). Recently it was shown that the xylulokinase activity should be optimal and that too high expression levels lead to growth inhibition (Jin *et al*. 2003). XK activities of about 10 U mg^{-1} protein inhibited growth whereas activities in the range of 0.3 to 3.5 U mg^{-1} protein increased growth on D-xylose. Thus the xylulokinase activity of 0.4 U mg^{-1} protein in our recombinant strain was rather optimal.

The ultimate goal of anaerobic D-xylose fermentation faces the same challenges as the metabolism of D-glucose to maintain high carbon flux in order to obtain sufficient ATP for cell maintenance and/or growth in anaerobic conditions. In general, the control of sugar metabolism is thought to be distributed over several reactions (Fell and Thomas 1995, Jensen *et al*. 1995). However, the uptake rate may be a crucial determinant of whether the sugar is fermented or respired (Elbing *et al*. 2004a, Fukuhara 2003, Goffrini *et al*. 2002, Ye *et al*. 1999). In D-glucose metabolism the following step of D-glucose phosphorylation by hexokinase Hxk2p is also an important regulatory step, since the Hxk2p itself participates in D-glucose repression (Ahuatzi *et al*. 2007, Moreno *et al*. 2005) and uncontrolled phosphorylation leads to growth inhibition (Blazquez *et al*. 1993, Gancedo and Flores 2004, Teusink *et al*. 1998, Thevelein and Hohmann 1995). Xylulokinase does not have the same regulatory role on D-xylose metabolism that the Hxk2p has on the metabolism of D-glucose. D-Xylose has been shown to provoke some of the regulatory cascades of D-glucose metabolism (Belinchon and Gancedo 2003, Roca *et al*. 2004, Salusjärvi *et al*. 2007). Genes linked to respiratory metabolism were expressed and the cells were not completely D-glucose repressed or derepressed on D-xylose (Jin *et al*. 2004, Salusjärvi *et al*. 2006).

4.2 The endogenous D-xylose pathway in *S. cerevisiae*

We showed that an alternative approach of using the endogenous genes *ScXYL1* and *GRE3* of *S. cerevisiae* is feasible, although not as efficient as the pathway with *P. stipitis* enzymes. The *P. stipitis* XR can utilize both NADPH and NADH as cofactors, although favouring the former. Thus part of the NADH generated in oxidation of xylitol to D-xylulose by the XDH enzyme can be utilized by the *P. stipitis* XR. The Gre3 protein, however, is strictly NADPH-dependent. Thus the NADH formed in the XDH reaction must solely be oxidised in other reactions, such as respiration or glycerol production. The strains harbouring *GRE3* were unable to utilize D-xylose anaerobically. This is in accordance with previous studies on the D-xylose metabolizing yeast *C. utilis* harbouring an NADPH-specific xylose reductase, demonstrating that the inability to utilize D-xylose anaerobically is due to lack of NADH oxidation (Bruinenberg *et al*. 1983, Bruinenberg *et al*. 1984).

The use of endogenous genes enables simple cloning by for example with promoter replacement. In order to construct a *S. cerevisiae* strain efficiently converting D-xylose to ethanol by using only the endogenous genes, the *SOR1/SOR2* are probably the best candidates for replacing the *P. stipitis XYL2* gene. The lower K_m for D-xylose of the Gre3p could be beneficial [17 mM for Gre3p vs. 42 mM for *P. stipitis* XR (Ellis 2002)], but still the cofactor imbalance caused by the strict NADPH-dependence of the Gre3p is a problem especially under anaerobic conditions. Thus, engineering of the redox balance would be needed before the NADPH-dependent Gre3p could replace the XRs also able to utilize NADH. The recently reported approaches of NADPH-utilizing GAPDH of *K. lactis* (Verho *et al*. 2003) or the change of cofactor need of nitrogen metabolism from that of NADPH to NADH (Roca *et al*. 2003, Grotkjær *et al*. 2005) could possibly enhance D-xylose metabolism in strains with Gre3p.

In a comparable study, Träff-Bjerre and coworkers observed growth on D-xylose with a strain overexpressing *GRE3*, *XKS1* and with *P. stipitis XYL2* (Träff-Bjerre *et al*. 2004). In addition, they noticed that when *GRE3* was deleted less biomass was produced, showing that the Gre3p participates in D-xylose metabolism in the recombinant D-xylose-utilising strains. In the same context, a *S. cerevisiae* strain was recently generated by repeated selection and breeding on a minimal D-xylose medium, resulting in a strain able to grow aerobically on D-xylose

(Attfield and Bell 2006). Possibly, the genes that we overexpressed by genetic engineering, or the genes *SOR1* or *SOR2*, were induced in this strain during the prolonged selection period. Sorbitol dehydrogenase-encoding gene(s), *SOR1* and/or *SOR2,* are known to be induced by sorbitol (Sarthy *et al*. 1994), but probably D-xylose or xylitol or D-xylulose formed from D-xylose intracellularly also induce these genes. These studies demonstrate the flexibility of the *S. cerevisiae* genome for developing new (or refreshing old) capabilities. It may be that *S. cerevisiae* has evolved from an ancestral strain that was able to use D-xylose aerobically. Many of the so called D-xylose-utilizing yeasts also have an NADPH specific XR enzyme. In addition to *GRE3*, *S. cerevisiae* genome contains several genes coding for non-specific aldose reductases also able to reduce D-xylose (Ellis 2002, Träff *et al*. 2002). These enzymes, or the Gre3p, are not, however, necessarily linked to D-xylose metabolism, but may for example act in detoxification of harmful metabolites such as methyl glyoxal (Aguilera and Prieto 2001).

4.3 Production of xylitol from D-glucose in yeast

The approach of deleting the transketolase encoding genes showed that the production of PPP-derived 5-carbon sugars and sugar alcohols in *S. cerevisiae* is possible, although the process is not yet commercially feasible. At most 730 mg l^{-1} of sugar alcohols was produced from D-glucose, 40% of which was xylitol. The formation of several sugar alcohols and 5-carbon sugars instead of only one is a major problem, since separation of these chemically similar compounds is difficult and also increases costs. In addition, the yield of product over substrate is low, only about 4%. This is clearly lower compared to results with an engineered *B. subtilis* strain, where yield of about 23% (w/w) was obtained (Povelainen and Miasnikov 2006). Our yields were also lower compared with the approaches using multiple steps with different organisms and/or enzymes (Mayer *et al*. 2002, Onishi and Suzuki 1969), however, the robust organism and single fermentations step support our approach.

To increase the proportion of xylitol from D-glucose, the activities of ribulokinase and D-ribulose 5-phosphate 3-epimerase enzymes could be increased in the cell, and by protein engineering with random mutagenesis together with a suitable selection system, the specificity of the sugar phosphatase

could possibly be increased. In addition, new candidate genes for phosphatases may appear with the increasing number of microbial genomes sequenced. The major difficulty with the approach utilized in this study is most probably the low flux to PPP, which should be increased in order to enhance the product formation. However, the production conditions were not optimized in any way in this study. The erythritol production for example has been significantly improved by process optimization. Recent studies suggest that respiratory metabolism increases the PPP flux of *S. cerevisiae*. Thus it would be interesting to see how oxygen availability affects the 5-carbon sugar and sugar alcohol yields in our strains lacking transketolase activity. Alternatively, the bacterial pathway of reducing the xylulose 5-phosphate to xylitol 5-phosphate and subsequently dephosphorylating it could be studied in yeast. However, since the phosphatase(s) responsible for the dephosphorylation in for example *B. subtilis* are currently unknown. Moreover, the bacterial phosphatases may be membrane bound which makes their application in yeast more difficult.

In the other approach of this study the Dog1 sugarphosphate phosphatase enabled formation of 5-carbon compounds and sugar alcohols from D-glucose via PPP in *S. cerevisiae* strain lacking phosphoglucose isomerase activity. Maximally about 0.4 g l^{-1} of 5-carbon sugars and sugar alcohols from 4 g l^{-1} of D-glucose and 20 g l^{-1} of D-fructose was produced. The NADPH-consuming reactions of glutamate dehydrogenase and glyceraldehyde 3-phosphate altered redox balance and enabled growth on D-glucose of the strains lacking phosphoglucose isomerase activity. However, overexpression of the *DOG1* gene encoding sugar phosphate phosphatase decreased growth on higher D-glucose concentrations. This may be explained by the ability of the Dog1 protein also to dephosphorylate D-glucose 6-phosphate, although with 2 times lower affinity compared to D-ribose 5-phosphate (Randez-Gil *et al*. 1995). Most probably, when D-glucose 6-phosphate accumulates the sequential phosphorylation and dephosphorylation leads to ATP depletion even when D-fructose serves as a carbon source. The accumulation probably follows the rate of D-glucose consumption mediated by the capacity of the PPP route itself and by the rate of NADPH oxidation. Thus when the capacity of the pathway is exceeded, D-glucose 6-phosphate accumulates to a level at which the futile cycle catalyzed by Dog1p starts. Thus, in this approach the rate of carbon flow through PPP should be optimal. The five-carbon sugar phosphates should accumulate in suitable concentration, but D-glucose 6-phoshate level should be low to avoid the

sequential dephosphorylation and phosphorylation by Dog1p and hexokinases, respectively. Thus, as with the transketolase approach, there is a strong need for a more specific sugarphosphate phosphatase. Alternatively, to circumvent the growth defect caused by *DOG1* overexpression, a production process with regulated feed of D-glucose could be designed to increase the yield of five-carbon sugars and sugar alcohols.

In the strain lacking phosphoglucose isomerase activity carbon is channelled exclusively through the PPP. Applying reactions that consume NADPH first of all enable such channelling in *S. cerevisiae* but also may remarkably increase the yield of 5-carbon sugars and sugar alcohols from D-glucose. If the flux to PPP could be increased by oxygen availability, as suggested above for strains lacking transketolase activity, biomass is a likely by-product. However, consumption of NADPH would in addition to guiding the flux to PPP, prevent, or at least decrease, the biomass formation and thus increase the yield of desired compounds. Other NADPH-consuming reactions, such as the soluble transhydrogenase UdhA of *E. coli* or the external dehydrogenase Nde1p of *K. lactis* would also be interesting alternatives as NADPH-consuming reactions.

In the triple mutant strains lacking both phosphoglucose isomerase and transketolase activities, at most about 1 g l^{-1} of 5-carbon sugars and sugar alcohols were produced from 2 g l^{-1} D-glucose, but the D-glucose tolerance of these strains was low. An unknown endogenous phosphatase appeared to be active, since D-ribose and ribitol were also formed in strains without *DOG1*. Thus most probably D-glucose 6-phosphate or 5-carbon sugar phosphates accumulated and induced the phosphatase, similar to the transketolase-deficient strains. The NADPH-consuming reactions did not have clear enhancing effect on the production of 5-carbon sugars and sugar alcohols. The yield of these compounds in the *pgi1 tkl1 tkl2* strains, about 50%, was, however, promising and similar to the *pgi1* mutant strains, a process of regulated feed of D-glucose might enhance the productivity of the triple mutant strains.

5. Conclusions and future perspectives

This study focused on two metabolic engineering cases; D-xylose utilization and conversion to ethanol by recombinant *S. cerevisiae* strains and production of xylitol from D-glucose with recombinant strains of *S. cerevisiae*. With the current interest in sustainable development and conversion of biomass to bio-fuels and other value added compounds in biorefineries, the development of new engineered production strains is even more important.

In the case of improving the ability of recombinant *S. cerevisiae* strains to metabolize D-xylose, we observed that overexpression of the xylulokinase-encoding gene *XKS1* in a D-xylose-metabolizing recombinant *S. cerevisiae* strain enhanced the D-xylose consumption rate and decreased the xylitol yield in different oxygen concentrations, and enhanced both the ethanol yield and the production rate. Later it was shown that the expression level of *XKS1* should be optimal (Jin *et al*. 2003) and that both the strain background and production conditions, particularly oxygen availability, should be considered when designing strains.

An alternative way of constructing a D-xylose-metabolizing strain is to use only endogenous *S. cerevisiae* genes. In addition to the xylulokinase-encoding gene, genes coding for XDH and XR activities can also be obtained from *S. cerevisiae* itself, thus enabling strain construction with e.g. promoter replacement. However, a major drawback of this approach is the inability of such strains to utilize D-xylose anaerobically, due to the fact that all *S. cerevisiae* aldose/xylose reductases are NADPH-specific. This and the low D-xylose metabolic capacity of recombinant *S. cerevisiae* strains using the XR XDH pathway particularly under anaerobic conditions, demand solutions to maintain the redox balance and particularly that of NAD^+ regeneration.

Production of xylitol from D-glucose in a single fermentation step was studied with three different approaches: the *S. cerevisiae* transketolase-deficient strains, *S. cerevisiae* strains deficient in phosphoglucose isomerase activity and harboring NADPH-consuming reaction(s) and with strains deficient in both phosphoglucose isomerase and transketolase activities. These approaches showed that production of the five-carbon sugars D-ribose plus D-ribulose and

the sugar alcohols xylitol and ribitol with recombinant *S. cerevisiae* strains is possible. However, further engineering of e.g. the phosphatase specificity and side product formation by introducing specific kinases is needed in order to obtain strains with commercial interest. In addition, reactions like export of xylitol should perhaps be enhanced.

In this study various redox problems were encountered. The NADPH specificity of the *S. cerevisiae* aldose reductase did not allow anaerobic D-xylose metabolism. In the *pgi1* mutant, the rate of NADPH consumption reflected the rate of flux to PPP and to 5-carbon sugar and sugar alcohol production. Another common factor of the two cases was the delicate balance of cellular ATP levels. In our studies the overexpression level of the xylulokinase-encoding gene appeared rather optimal, whereas in other cases too high expression level possibly led to substrate accelerated death (Teusink *et al*. 1998). Similarly, the possible futile cycle between D-glucose and D-glucose 6-phosphate in the *pgi1* mutant of *S. cerevisiae* expressing the *DOG1* gene probably led to ATP depletion. Both of the metabolic engineering cases studied here show that in a eukaryotic organism such as yeast, the redox and energy balances are rather rigid and careful optimization of the engineering is needed.

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Title

Engineering the pentose phosphate pathway of *Saccharomyces cerevisiae* for production of ethanol and xylitol

Abstract

The baker's yeast *Saccharomyces cerevisiae* has a long tradition in alcohol production from D-glucose of e.g. starch. However, without genetic modifications it is unable to utilize the 5-carbon sugars D-xylose and L-arabinose present in plant iomass. In this study, one key metabolic step of the catabolic D-xylose pathway in recombinant D-xylose-utilizing *S. cerevisiae* strains was studied. This step, carried out by xylulokinase (XK), was shown to be rate-limiting, because overexpression of the xylulokinase-encoding gene *XKS1* increased both the specific ethanol production rate and the yield from D-xylose. In addition, less of the unwanted side product xylitol was produced.

Recombinant D-xylose-utilizing *S. cerevisiae* strains have been constructed by expressing the genes coding for the first two enzymes of the pathway, D-xylose reductase (XR) and xylitol dehydrogenase (XDH) from the D-xylose-utilizing yeast *Pichia stipitis*. In this study, the ability of endogenous genes of *S. cerevisiae* to enable D-xylose utilization was evaluated. Overexpression of the *GRE3* gene coding for an unspecific aldose reductase and the *ScXYL2* gene coding for a xylitol dehydrogenase homologue enabled growth on D-xylose in aerobic conditions. However, the strain with *GRE3* and *ScXYL2* had a lower growth rate and accumulated more xylitol compared to the strain with the corresponding enzymes from *P. stipitis*. Use of the strictly NADPH-dependent Gre3p instead of the *P. stipitis* XR able to utilize both NADH and NADPH leads to a more severe redox imbalance. In a *S. cerevisiae* strain not engineered for D-xylose utilization the presence of D-xylose increased xylitol dehydrogenase activity and the expression of the genes *SOR1* or *SOR2* coding for sorbitol dehydrogenase. Thus, D-xylose utilization by *S. cerevisiae* with activities encoded by *ScXYL2* or possibly *SOR1* or *SOR2*, and *GRE3* is feasible, but requires efficient redox balance engineering.

Compared to D-xylose, D-glucose is a cheap and readily available substrate and thus an attractive alternative for xylitol manufacture. In this study, the pentose phosphate pathway (PPP) of *S. cerevisiae* was engineered for production of xylitol from D-glucose. Xylitol was formed from D-xylulose 5-phosphate in strains lacking transketolase activity and expressing the gene coding for XDH from *P. stipitis*. In addition to xylitol, ribitol, D-ribose and D-ribulose were also formed. Deletion of the xylulokinase-encoding gene increased xylitol production, whereas the expression of *DOG1* coding for sugar phosphate phosphatase increased ribitol, D-ribose and D-ribulose production. Strains lacking phosphoglucose isomerase (Pgi1p) activity were shown to produce 5-carbon compounds through PPP when *DOG1* was overexpressed. Expression of genes encoding glyceraldehyde 3-phosphate dehydrogenase of *Bacillus subtilis,* GapB, or NAD-dependent glutamate dehydrogenase Gdh2p of *S. cerevisiae*, altered the cellular redox balance and enhanced growth of *pgi1* strains on D-glucose, but co-expression with *DOG1* reduced growth on higher D-glucose concentrations. Strains lacking both transketolase and phosphoglucose isomerase activities tolerated only low D-glucose concentrations, but the yield of 5-carbon sugars and sugar alcohols on D-glucose was about 50% (w/w).

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