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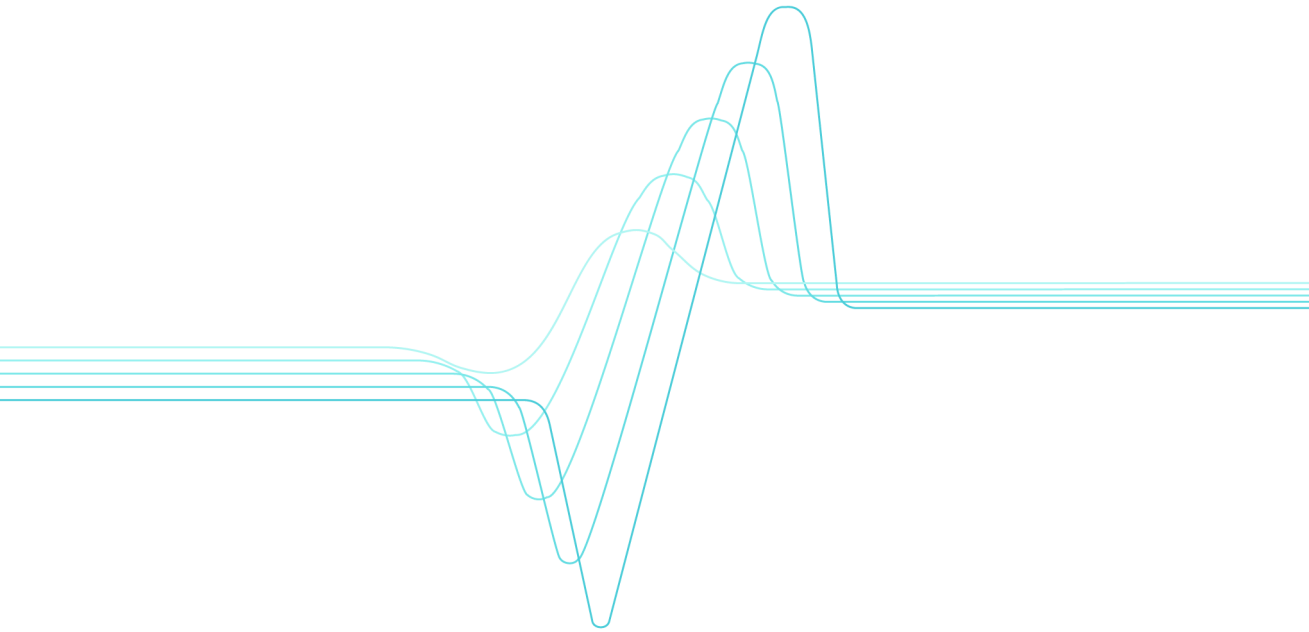

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Engineering the pentose phosphate pathway of *Saccharomyces cerevisiae* for production of ethanol and xylitol

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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

Appendix IV of this publication is 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.

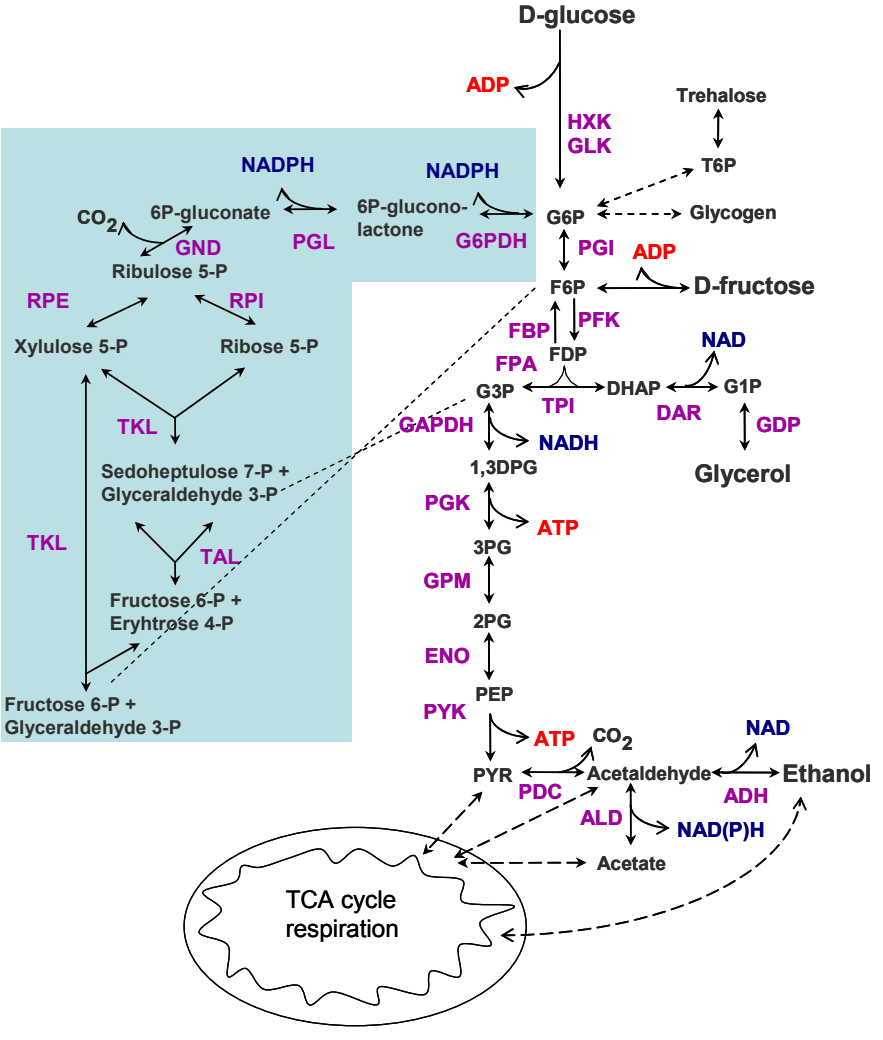

Figure 1. Schematic representation of central carbon metabolism of yeast S. cerevisiae. HXK, hexokinase; GLK, glucokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; FBP, fructose1, 6-bisphosphatase; FPA, fructose bisphosphate aldolase; TPI, triose phosphate isomerase; DAR, DHAP reductase; 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.

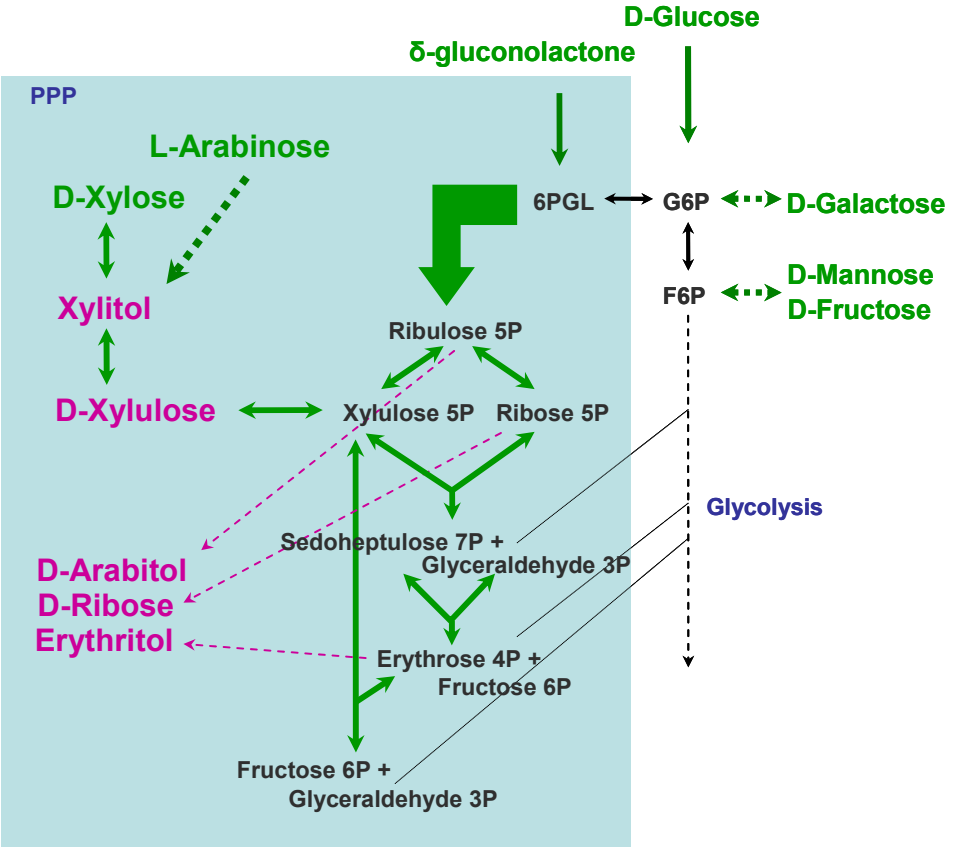

Figure 2. Role of the pentose phosphate pathway in utilization and production of sugars and sugar alcohols.

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).

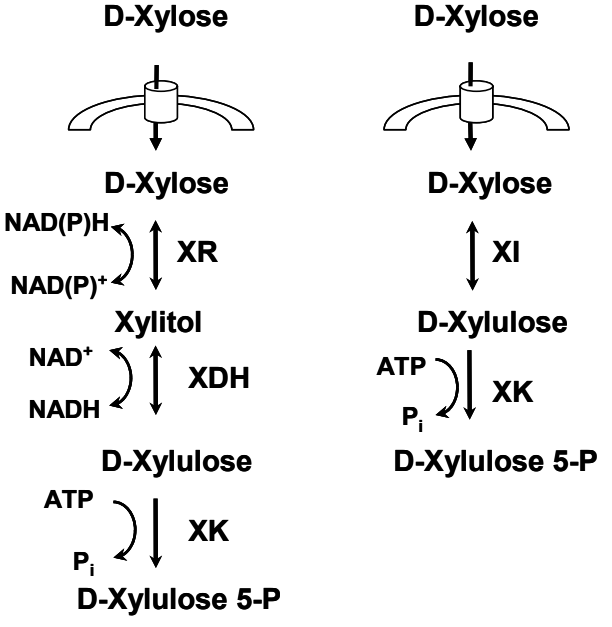

Figure 3. First steps in the metabolism of D-xylose via fungal (left) or bacterial (right) pathways.

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 $NADP^+$.

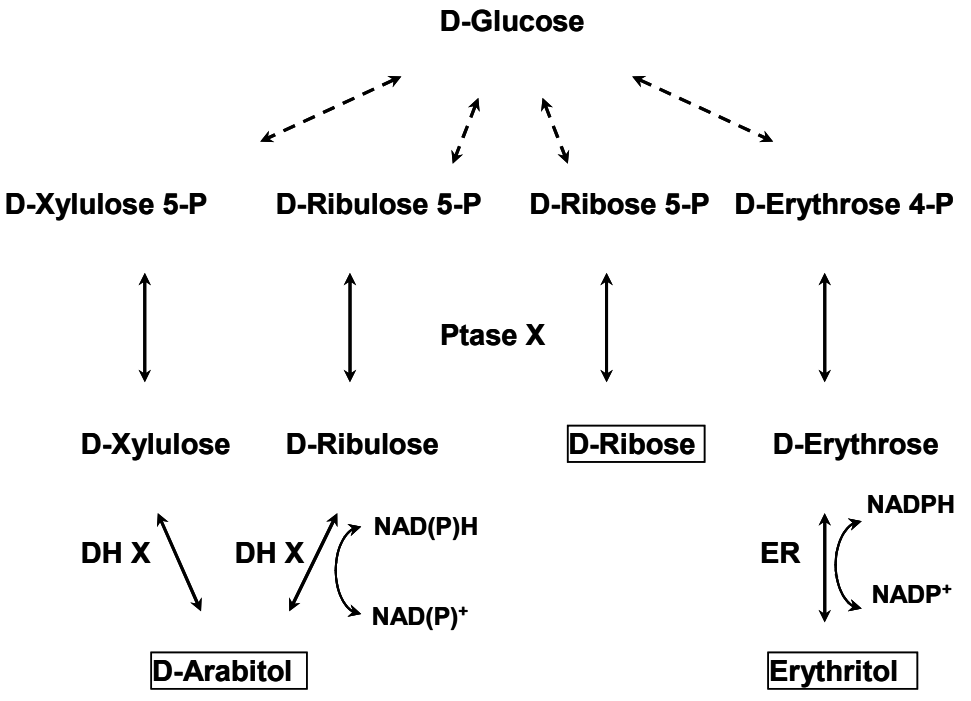

Figure 4. Schematic representation of possible pathways for formation of D-arabitol, D-ribose and erythritol from D-glucose in osmophilic yeasts. DH X; arabitol dehydrogenase, Ptase X; unknown phosphatase, ER; erythrose reductase.

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).

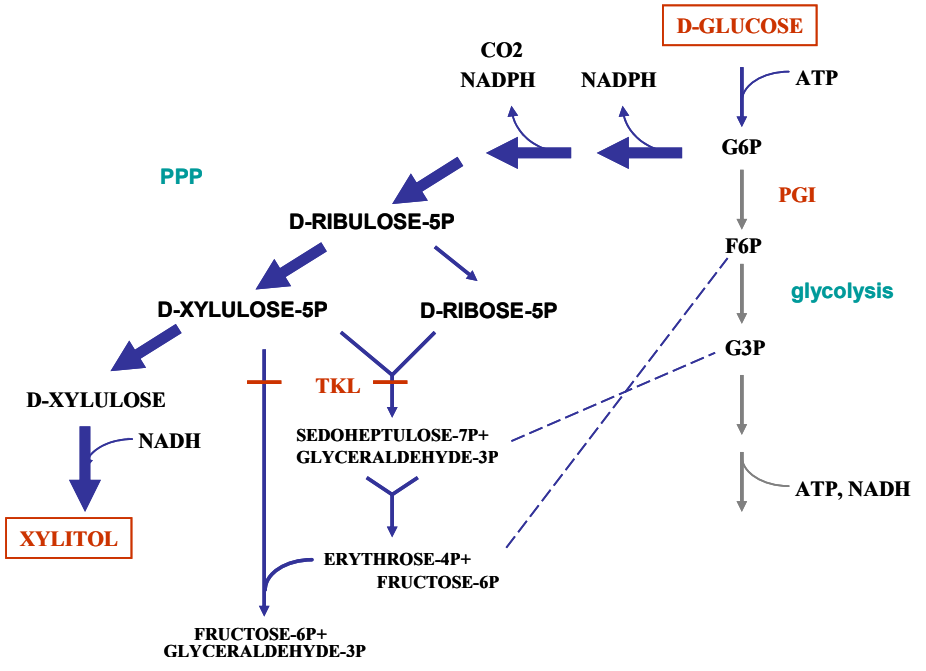

Figure 5. Hypothesized production pathway from D-glucose to xylitol in recombinant S. cerevisiae strains.

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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PUBLICATION I

Conversion of Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae*: Importance of Xylulokinase (*XKS1*) and Oxygen Availability

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Conversion of Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae*: Importance of Xylulokinase (*XKS1*) and Oxygen Availability

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The yeast *Saccharomyces cerevisiae* efficiently ferments hexose sugars to ethanol, but it is unable to utilize xylose, a pentose sugar abundant in lignocellulosic materials. Recombinant strains containing genes coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from the xylose-utilizing yeast *Pichia stipitis* have been reported; however, such strains ferment xylose to ethanol poorly. One reason for this may be the low capacity of xylulokinase, the third enzyme in the xylose pathway. To investigate the potential limitation of the xylulokinase step, we have overexpressed the endogenous gene for this enzyme (*XKS1*) in *S. cerevisiae* that also expresses the *P. stipitis* genes for XR and XDH. The metabolism of this recombinant yeast was further investigated in pure xylose bioreactor cultivation at various oxygen levels. The results clearly indicated that overexpression of *XKS1* significantly enhances the specific rate of xylose utilization. In addition, the XK-overexpressing strain can more efficiently convert xylose to ethanol under all aeration conditions studied. One of the important illustrations is the significant anaerobic and aerobic xylose conversion to ethanol by the recombinant *Saccharomyces*; moreover, this was achieved on pure xylose as a carbon. Under microaerobic conditions, 5.4 g L^{-1} ethanol was produced from 47 g L^{-1} xylose during 100 h. In fed-batch cultivations using a mixture of xylose and glucose as carbon sources, the specific ethanol production rate was highest at the highest aeration rate tested and declined by almost one order of magnitude at lower aeration levels. Intracellular metabolite analyses and *in vitro* enzyme activities suggest the following: the control of flux in a strain that overexpresses *XKS1* has shifted to the non-oxidative steps of the pentose phosphate pathway (i.e., downstream of xylose 5-phosphate), and enzymatic steps in the lower part of glycolysis and ethanol formation pathways (pyruvate kinase, pyruvate decarboxylase, and alcohol dehydrogenase) do not have a high flux control in this recombinant strain. Furthermore, the intracellular ATP levels were found to be significantly lower for the XK strain compared with either the control strain under similar conditions or glucose-grown *Saccharomyces*. The ATP:ADP ratios were also lower for the XK strain, especially under microaerobic conditions (0.9 vs 6.4). © 2001 Academic Press

Key Words: **xylose; ethanol; *Saccharomyces cerevisiae*; xylulokinase; metabolic engineering; renewable resources.**

INTRODUCTION

Cellulosic biomass is an attractive feedstock for fuel ethanol production since it is readily available, e.g., as a waste from the pulp and paper or agricultural industries, and also due to the fact that it is renewable with cycles many orders of magnitude shorter compared with those of fossil fuels. About 30–40% of lignocellulosic material is hemicellulose, which contains mostly pentoses (primarily xylose). Successful industrial production of ethanol from such raw materials depends on the quantitative conversion of carbon present in the biomass. Several industrial microorganisms can readily utilize hexose sugars; however, efficient pentose-utilizing organisms are less common. For example, the yeast *Saccharomyces cerevisiae*, one of the most prominent ethanol-producing organisms from hexose sugars, is unable to utilize pentoses.

Xylose-utilizing yeasts express the enzymes xylose reductase (XR) that reduces xylose to xylitol and xylitol dehydrogenase (XDH) that further oxidizes xylitol to xylulose. Kötter *et al.* first reported the construction of a *S. cerevisiae* strain expressing the XR- and XDH-encoding genes *XYL1* and *XYL2* derived from the xylose-utilizing yeast *Pichia stipitis* (Kötter *et al.*, 1990). We have also genetically engineered *S. cerevisiae* to utilize xylose by introducing the *XYL1* and *XYL2* genes on either multicopy plasmids or by integrating them into the chromosome (Walfridsson *et al.*, 1995). Although these strains can grow aerobically on xylose, a large fraction of the consumed xylose is excreted as xylitol. The inefficient incorporation of xylose-derived carbon into the central pathways leading to ethanol has been attributed to a number of causes. One of

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the most important is redox cofactor imbalance; XR enzymes are either specific or prefer NADPH, whereas XDH enzymes are specific for NADH. There may also be limitations that arise downstream of XDH, including the step converting xylulose to xylulose-5P catalyzed by xylulokinase (XK). To overcome the limitations in the pentose phosphate pathway, the genes coding for transketolase and transaldolase have been overexpressed in recombinant *S. cerevisiae* (Walfridsson *et al.*, 1995), but with limited success.

Although unable to utilize xylose, *S. cerevisiae* can grow on xylulose, the isomerized form of xylose. However, the activity of xylulokinase is low or undetectable (Richard *et al.*, 2000; Yang and Jeffries, 1997). Cloning of the gene coding for the *S. cerevisiae* xylulokinase (*XKS1*) was first performed in 1989 (Ho and Chang, 1989). Fermentation studies with a recombinant *S. cerevisiae* strain overexpressing the three first genes of the xylose pathway (*XYL1*, *XYL2*, and *XKS1*) followed later using corn cob pretreated by ammonia (Cao *et al.*, 1996) and complex xylose or xylose–glucose media (Ho *et al.*, 1998). Growth on xylose was observed under aerobic conditions and ethanol was produced under anaerobic conditions; however, a quantitative analysis is not possible based on these experiments. Recently, Eliasson and co-workers (2000) tested a similar strain in anaerobic chemostat cultures on glucose–xylose mixtures using mineral media. Aerobic growth on xylose and anaerobic ethanol production was reported and the authors concluded that the xylose uptake limits the xylose flux. However, more detailed physiological studies are still lacking despite the encouraging results reported thus far.

This study focuses at elucidating the effects of xylulokinase overexpression, as well as oxygen availability, on xylose metabolism in bioreactor cultivations, aimed at providing quantitative physiological data that can elucidate bottlenecks of xylose metabolism in recombinant *S. cerevisiae*. Information from metabolic rates, intracellular metabolite concentrations, and enzyme activities under various conditions is discussed in terms of further strain improvement.

MATERIALS AND METHODS

Strains and Plasmids

Genetically modified *S. cerevisiae* strains used in this work are based on the strain CEN.PK2 (H1346, *MATa*, *leu*2-3/112, *ura*3-52, *trp*1-289, *his*3*D*1, *MAL2*-8^c, *SUC*2; Boles *et al.*, 1996). Bioreactor cultivations were carried out with either strain H1693 (control) or H1691 (*XKS1* overexpressing) (see below). The *Escherichia coli* strain DH5α (Woodcock *et al.*, 1989) was used as the bacterial cloning host.

Strain construction. The *XYL1* and *XYL2* genes coding for xylose reductase and xylitol dehydrogenase, respectively, were obtained from the genomic DNA of *P. stipitis* by PCR (Walfridsson *et al.*, 1995). The *XYL1* was cloned under the *PGK*1 promoter (Mellor *et al.*, 1983) and the *XYL2* under a modified, constitutive *ADH1* promoter (Ruohonen *et al.*, 1995). An *XYL1–XYL2* integration cassette was constructed by using bacterial cloning vector B955 (J. Toikkanen and S. Keränen, submitted) containing two *URA3* gene fragments (base pairs 71–450 and 781–1135 from the encoding region of the gene) at *Sac*I–*Xba*I sites and *Xho*I–*Asp*718 sites of the Bluescript SK bacterial cloning vector (Stratagene), respectively. The *Hin*dIII fragment of the *XYL1* gene under the *PGK*1 promoter and terminator and the *Bam*HI fragment of *XYL2* under the modified *ADH*1 promoter and terminator were respectively ligated into the *Hin*dIII and *Bam*HI sites within *URA3* fragments of B955. The resulting construction containing the *XYL1* and *XYL2* expression cassettes flanked by the *URA3* regions was released from Bluescript SK by *Sac*I–*Nsi*I digestion and isolated from an agarose gel. The fragment was used to transform yeast strain H1466. Strain H1466 was constructed by integrating the functional *URA3* gene into the CEN.PK2 (H1346) (Boles *et al.*, 1996) strain to obtain a uracil prototrophic strain. The *XYL1–XYL2*-containing transformants were selected on 5-fluoro-orotic acid (5-FOA) plates for uracil auxotrophic colonies (Boeke *et al.*, 1984). The correct, functional integration of the *XYL1* and *XYL2* expression cassettes was verified by Southern blotting, *in vitro* XR and XDH activities from cell extracts, and the ability of the transformants to grow on xylose (with the CEN.PK2 strain as negative control). One of the transformants, H1469, was selected for *XKS1* overexpression as described below.

The gene coding for xylulokinase (*XKS1*) was isolated from *S. cerevisiae* by Richard *et al.* (2000). The multicopy vector YEplac195 (Gietz and Sugino, 1988) containing the *XKS1* gene under the constitutive *ADH1* promoter was transformed into strain H1469 (resulting strain H1691) and into strain H1346 (resulting strain H1695). The same plasmid without the *XKS1* expression cassette was transformed into strain H1469 (resulting strain H1693). General cloning techniques were as described in Sambrook *et al.* (1989). The yeast transformations were done by the lithium acetate transformation procedure (Gietz *et al.*, 1992; Hill *et al.*, 1991).

Cultivation Media

All cultivations took place in yeast synthetic complete media (YSC) described by Sherman *et al.* (1983),

supplemented with various xylose or glucose concentrations as described below. Uracil was omitted from these media (YSC-Ura) to minimize growth of plasmid-free cells. YSC-Ura supplemented with 20 or 50 g L^{-1} glucose was used for routine shake flask cultivations and inocula preparations. The media for the fed-batch cultivations were as follows: the initial batch phase was carried out in YSC-Ura supplemented with 20 g L^{-1} xylose and 1 g L^{-1} glucose, whereas the feed media consisted of YSC-Ura supplemented with 500 g L^{-1} xylose and 20 g L^{-1} glucose. Batch cultivations were carried out in YSC-Ura supplemented with 50 g L^{-1} xylose.

Cultivations

The inocula were prepared by growing cells overnight to an OD_{600} of 10–15 on glucose medium. The cells were then harvested and resuspended in phosphate buffer (or 0.9% NaCl) and then transferred to the bioreactor, giving an initial OD_{600} of approximately 10.

Bioreactor cultivations took place in a 1.8-L Chemap CMF bioreactor (Chemap AG, Volketswil, Switzerland) with an initial working volume of 1.2 L. Mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands) precisely regulated the air and nitrogen flow rates. For anaerobic experiments, nitrogen was passed through an Oxisorb oxygen-absorbing device (Messer Griesheim GmbH, Krefeld, Germany) to reduce the residual oxygen level to below 50 ppb. The dissolved oxygen (DO) concentration was monitored by an Ingold polarographic probe. The temperature was maintained at 30°C and the pH was controlled at 5.5 by addition of 2 M NaOH. The agitation speed was constant at 300 rpm.

The initial set of experiments consisted of a series of fed-batch cultivations with small glucose feed and decreasing aeration. For the first 24 h, the fermentation was carried out in batch mode after which a substrate feed (YSC-Ura plus 50% xylose and 2% glucose) was initiated at a flow rate of 2.5 ml h^{-1}. The aeration was maintained at 0.5 standard liters per minute (SLPM) air during the batch phase and during the initial 24 h of the fed-batch phase (operating phase 1, OP1). Subsequently, the oxygen concentration in the inlet gas was changed to various air to nitrogen mixture ratios at approximately 24-h intervals as shown in Table 1. The oxygen percentage in the previous table refers to the approximate fraction of oxygen in the gas inlet stream, e.g., 20% refers to pure air, whereas a 50:50 air:nitrogen mixture is described as 10% O_2.

The second set of experiments consisted of three separate batch cultivations carried out under fully anaerobic, microaerobic, or aerobic conditions (Table 2). The total gas flow rate in these experiments was 0.5 SLPM with oxygen inlet concentrations of 0% (anaerobic), 2% (micro aerobic), and 20% (aerobic) in the different fermentations.

TABLE 1

Summary of Culture Aeration Conditions in Xylose–Glucose Fed-Batch Experiments for the Various Operating Phases (OP)

	F_{air}	F_{N2}	Oxygen (%)	Agitation (rpm)
OP1	0.500	0	20	300
OP2	0.250	0.250	10	300
OP3	0.187	0.313	7.5	300
OP4	0.125	0.375	5	300
OP5	0.062	0.438	2.5	300
OP6	0	0.500	0	300

Note. F_{air}, airflow rate; F_{N2}, nitrogen flow rate, both in standard liters per minute (SLPM). Oxygen percentage refers to the approximate fraction of oxygen in the gas inlet stream, e.g., 20% refers to pure air, whereas a 50:50 air:nitrogen mixture is described as a 10% O_2 mixture.

Liquid samples were withdrawn from the fermentor at time intervals to measure growth, substrate consumption, and formation of extracellular products. Additional samples were also taken for measuring enzyme activities and concentrations of important intracellular metabolites.

Analytical Procedures

Growth and extracellular products. Cell growth was followed optically at 600 nm and gravimetrically by measuring the cell dry weight. For the latter, a known culture volume was first centrifuged (4500 rpm, 4°C), and the pellet was resuspended in 5 ml of water, transferred to a preweighted stainless steel dish, and dried in an oven at 105°C. Prior to measuring the final dry weight, the dish was cooled for 2–3 h in a desiccator. The culture supernatant from the above sample was filtered through a 0.2-μm syringe filter (Millipore) and stored at −20°C for further analysis by HPLC or enzymatic assays. The resin-based Aminex HPX-87H column (Bio-Rad Labs, U.S.A.) was used to separate the various compounds present in the fermentation broth. The column was maintained at 55 or 35°C. The eluent consisted of 5 mM H_2SO_4 at a constant

TABLE 2

Summary of Culture Aeration Conditions in Xylose-Only Batch Experiments (See Table 1 for Details)

	F_{air}	F_{N2}	Oxygen (%)	Agitation (rpm)
Aerobic	0.500	0.000	20	500
Microaerobic	0.125	0.375	2.0	500
Anaerobic	0	0.500	0	500

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flow rate of 0.6 ml min^{-1}. This system enabled us to quantify the following compounds: glucose, xylose, xylitol, glycerol, acetate, pyruvate, and ethanol using a combination of a refractive index (RI) and UV ($\lambda = 210$ nm) detectors connected in series.

The composition of the fermentation exhaust gas was monitored online with a QMG 421C quadrupole mass spectrometer (Balzers Pfeiffer Scandinavia AB, Sweden). The gas was first passed through a reflux condenser and then introduced to the spectrometer via a multiport valve system. Data acquisition and analysis were performed with the Balzers Quadstar 422 software, which provided the mole fractions of oxygen, carbon dioxide, argon, and nitrogen in the exhaust gas. This data provided the basis for determining the carbon evolution rates (CER, mmol L^{-1} h^{-1}) and oxygen uptake rates (OUR, mmol L^{-1} h^{-1}) for the case of aerobic or microaerobic cultivations.

Intracellular metabolite analysis. The cellular quenching and metabolite extraction methodology was based on a technique described by de Koning and van Dam (1992). Briefly, a 5- or 10-ml culture sample was rapidly quenched in 60% methanol maintained at $-40°$C in a cryostat. The intracellular samples were extracted with a chloroform solution containing 3 mM Pipes buffer, pH 7.3, and 3 mM EDTA, and then either lyophilized to dryness and subsequently suspended to 1 ml of double-distilled water or alternatively evaporated to a volume of 0.5 ml.

The levels of key intracellular metabolites (glucose 6-phosphate, fructose 6-phosphate, sedoheptulose 7-phos phate, xylulose 5-phosphate, ribulose 5-phosphate, ATP, ADP, and AMP) were measured enzymatically using an automated analyzer (Cobas MIRA Plus, Roche, Switzerland). The enzymatic assays were adapted from the manual assays described by Bergmeyer (1983).

Enzyme activities. Enzyme activities were measured from cell extracts prepared by disrupting cells using glass beads in 50 mM Hepes buffer, pH 7.0, containing 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and supplemented with protease inhibitors PMSF and pepstatin A (final concentrations of 0.17 and 0.01 mg/ml, respectively). Pyruvate kinase and pyruvate decarboxylase activities were measured as described by Bergmeyer (1983). Alcohol dehydrogenase activity was measured in the direction of ethanol consumption by using an initial ethanol concentration of 200 mM and monitoring NADH formation (Roche ethanol kit reagents). Protein amounts were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Xylose reductase, xylitol dehydrogenase, and xylulokinase activities were measured as described by Richard *et al.* (1999, 2000). To avoid interference of xylitol dehydrogenase activity in the xylulokinase assay, the activity was measured from strain H1695 containing only the plasmid expressing *XKS1* (xylulokinase). All enzymatic and protein analyses were carried out with the Cobas Mira automated analyzer (Roche).

RESULTS

Construction of a Recombinant S. cerevisiae *Strain Expressing XYL1, XYL2, and XKS1*

The *P. stipitis* genes *XYL1* and *XYL2* encoding XR and XDH were integrated into the *URA*3 locus of *S. cerevisiae*. The *XYL1* gene was expressed from the *S. cerevisiae PGK1* promoter and the *XYL2* gene from the modified *ADH1* promoter, resulting in XR activity of 3 nkat/mg and XDH activity of 2 nkat/mg of total cellular protein. The *S. cerevisiae XKS1* gene encoding XK was expressed from multicopy vector YEplac195 from the modified *ADH1* promoter, resulting in XK activity of 7 nkat/mg protein. The stability of this plasmid in strain H1695 was previously analyzed and found to be stable for several generations: about 90% of the cells contained the plasmid after 2 days of growth without selection (Richard *et al.*, 2000).

Fed-Batch Cultures on Xylose–Glucose Media with Decreasing Oxygen Concentration

The purpose of this set of experiments was twofold: to compare xylose metabolism between a strain expressing only XR and XDH (H1693, control) with that of a strain which also overexpresses XK (H1691), hence elucidating the impact of XK overexpression; and to access the importance of aeration levels on xylose metabolism by such strains. The experiments were carried out in fed-batch mode, where a solution of xylose with a small amount of glucose (xylose:glucose:500:20 g/g) was gradually fed to the culture.

The system was operated under a specific aeration rate for a time period (about 24 h), after which the oxygen inlet concentration was reduced by varying the ratio of air to nitrogen introduced into the system. The oxygen concentration was initially around 20% (pure air) and in a series of steps was gradually reduced down to 0% (pure nitrogen) as indicated in Table 1.

For each aeration state, samples were withdrawn to determine the rates of xylose utilization and types and rates of secreted by-products and also for measuring the levels of key intracellular metabolites and enzyme activities. The xylose feed rate was fixed throughout the experiment and was similar for both strains. The feed rate was chosen such that the xylose was nonlimiting under any of the conditions used. Glucose, which can have both a stimulatory and an inhibitory effect on xylose utilization, represented only a

small fraction of the total carbon substrate (4% g/g), and its residual level was zero for both strains under all studied conditions. Given all the possible effects of glucose on xylose transport and further metabolism, together with the fact that xylose assimilation to biomass is rather limited especially under oxygen-limiting conditions, this type of fed-batch operation provides a number of advantages over either batch cultivation (where the mixed sugar utilization would occur sequentially) or continuous operation (which by definition requires significant growth).

The specific rates of xylose and glucose utilization, as well as xylitol and ethanol formation, are shown in Fig. 1 for the various inlet oxygen concentrations studied. The values represent the overall rates in the 24-h period. Clearly, the presence of *XKS1* enhances the xylose uptake rate, except in the oxygen concentration of 2.5%, where it is equal for both strains. Interestingly, at this aeration level the specific ethanol production rate is higher for the control strain, although under all other aeration conditions the specific ethanol production rate is significantly higher for the XK strain. Another interesting phenomenon is that the XK strain produces ethanol under fully aerobic conditions, while no ethanol production is seen with the control under similar conditions. It is likely that part of the ethanol is derived from glucose in the feed. Nevertheless, in most cases with the XK strain, glucose consumption can account for only a small part of the ethanol. In the two lowest oxygen concentrations (0 and 2.5%), it is however possible that the ethanol is derived from glucose. The control strain does not produce very much ethanol, except in an oxygen concentration of 2.5%; there, part of it is likely produced from xylose.

Saccharomyces strains that overexpress *XYL1* and *XYL2* have been shown in the past to convert xylose primarily to xylitol. This appears to be the case here as well for the control strain, especially in the absence of oxygen (Fig. 1C). However, by overexpressing *XKS1* we have succeeded in significantly lowering xylitol excretion, especially at the two extremes of oxygen availability: fully anaerobic or highly aerobic. The attenuation of the xylose to xylitol flux by *XKS1* overexpression is apparently less significant under microaerobic conditions (2.5 or 5% O_2).

The nonlinearity of this response with respect to oxygen availability, and in extension to what happens downstream of xylitol, illustrates the complexity of the system and the fact that oxygen is likely to play a variety of physiological roles in xylose metabolism. Results for oxygen concentration of 2.5% appear to differ from all other aeration levels. At this unique point, xylose utilization rates appear to converge for the two strains, whereas ethanol production rates and yields for the XK strain are below that of the control. Off-gas analysis data indicate that at this particular aeration

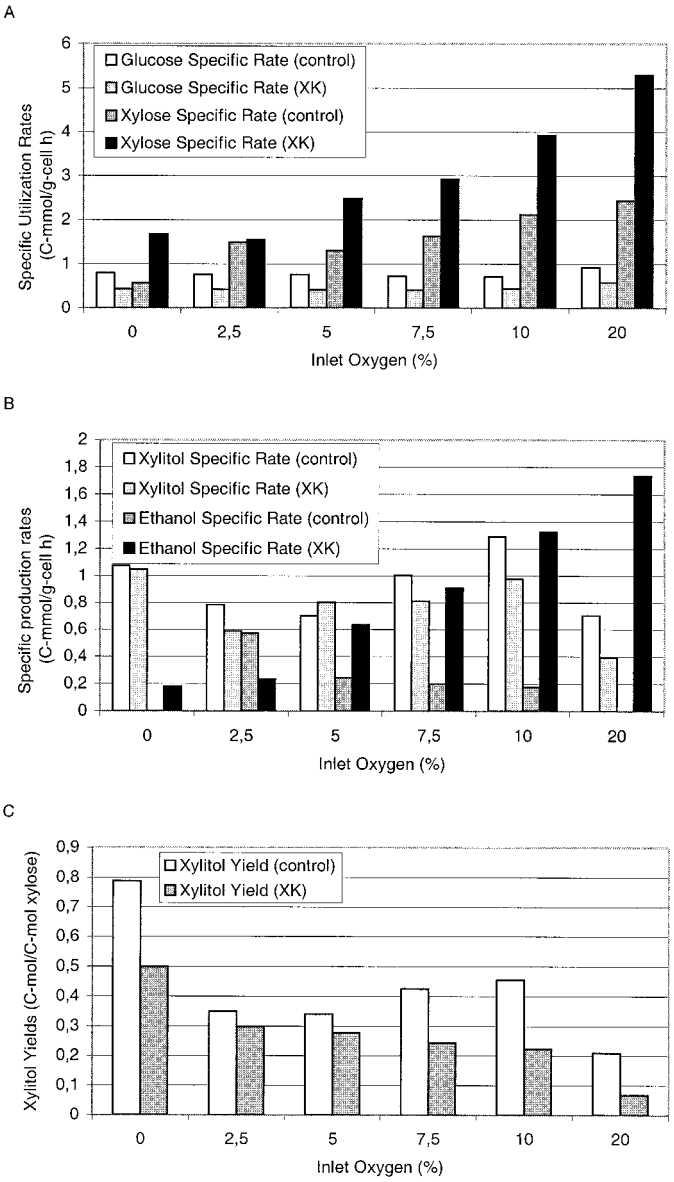

FIG. 1. Specific rates (C-mmol/g cell dry wt h) of xylose and glucose consumption (A) and ethanol and xylitol formation (B) for the fed-batch cultivations of the control and XK-overexpressing strains plotted vs the inlet oxygen concentration. (C) Xylitol yields on xylose (C-mol/C-mol xylose) for the same experiments.

the XK strain directs more carbon to CO_2 and none to acetate. Higher CO_2 production may be linked to higher futile cycling through the pentose phosphate pathway as explained in the Discussion. Possibly at an aeration level of 2.5% oxygen, the *XKS1* expression level is optimal for ethanol production in the control strain, but not in the *XKS1* strain. It may be that a high XK amount leads to too high xylulose 5-phosphate levels which results in futile cycling.

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TABLE 3

Comparison of Intracellular Metabolite Levels for Xylulose 5-Phosphate (Xu5P), Ribulose 5-Phosphate (Ru5P), and Adenosine Triphosphate (ATP), for the XK and Control Strains in the Fed-Batch Cultivations at Various Aeration Levels

	Xu5P		Ru5P		ATP	
O_2%	XK	control	XK	control	XK	control
20	3.67	1.58	2.24	0.51	0.92	1.17
10	2.98	1.31	1.59	0.14	0.80	2.35
7.5	3.52	2.20	1.96	1.11	2.00	4.00
5.0	4.43	2.71	2.58	1.62	1.68	4.46
2.5	4.66	1.95	2.71	0.93	1.94	4.16
0	3.59	1.45	1.00	0.30	1.78	4.41

Note. The metabolite levels were determined enzymatically as described under Materials and Methods and reported below as intracellular millimolar concentrations.

As shown in Table 3, irrespective of the aeration conditions, the XK strain accumulated higher levels of the pentose phosphate pathway metabolic intermediates xylulose 5-phosphate and ribulose 5-phosphate. Furthermore, the intracellular ATP levels were significantly lower for the XK strain under all aeration conditions, except for the aerobic conditions where the difference was not significant. Another interesting difference between the XK and control strain was with respect to the *in vitro* measured levels of alcohol dehydrogenase (ADH). The ADH activities for the XK strain were about 20 to 25% lower compared with the control strain in the cultures with 20, 7.5, 5, and 2.5% oxygen concentrations (data not shown).

Batch Cultures under Aerobic, Microaerobic, and Anaerobic Conditions on Pure Xylose

A second set of experiments was carried out to investigate xylose metabolism in the absence of glucose at various oxygen levels. The existing literature describing recombinant *S. cerevisiae* for xylose utilization indicates that cosubstrates, usually in the form of a hexose sugar, or oxygen, are essential components for xylose utilization. The experiments described below were carried out in minimal media using xylose as the only carbon source, and it consisted of a series of independent batch cultivations each operating at a certain inlet oxygen concentration as summarized in Table 2.

The volumetric xylose consumption and main product formation rates of the XK and control strains under the aerobic, microaerobic, and anaerobic conditions as a function of time are presented in Fig. 2. As a confirmation of the fed-batch cultivations, we observed that under all aeration conditions xylose consumption was much more efficient with the XK strain. Under anaerobic conditions the control strain utilized only a small fraction of the xylose available. Ethanol production was observed with the XK strain also under fully aerobic conditions. This aerobic xylose fermentation continued until the residual xylose concentration approached 10 g L^{-1}, after which cells began consuming the ethanol formed in the first phase. The inability of this strain to convert xylose to ethanol at residual xylose levels below 10 g L^{-1} (65 mM) is likely to be due to a limitation in xylose transport, which presumably has a rather high apparent K_m value for xylose (150–1500 mM; see Kötter *et al.*, 1993). No net ethanol production was observed with the control strain under aerobic or microaerobic conditions. Glycerol production was only seen with the XK strain. Another important and reproducible finding is the fact that under oxygen-limited conditions the XK strain can better sustain the measurable biomass concentration, as well as its viability and metabolic activity vs time, compared to the control strain. The XK strain was found to sustain its fermentation capacity for several days in a row. Furthermore, microscopic comparisons of fermentation samples showed that a large population from the control strain appeared as "ghost cells," and this fraction increased rapidly over the 4- to 5-day cultivation period (contrary to the XK strain).

Overexpression of xylulokinase leads to higher production rates and ethanol yields. Table 4 summarizes the specific rates of xylose utilization and product formation for the two strains at the various aeration levels in the logarithmic production phase (as marked with arrows in Fig. 2). The xylose uptake rate was increased by a factor of 2–2.4 when overexpressing *XKS1* at all aeration levels. As in the fed-batch cultures, the most striking result is the significantly higher ethanol production rate: the rate is eightfold higher compared to the control under anaerobic conditions. Under microaerobic and aerobic conditions no ethanol production was seen with the control strain, in contrast to the significant ethanol production observed with the XK strain under similar conditions. The highest specific ethanol production rate with the XK strain was observed under microaerobic conditions.

As in the previous set of experiments (Fig. 1B or 1C), *XKS1* overexpression resulted in significant reduction of the specific xylitol excretion rate under aerobic conditions. However, under anaerobic and microaerobic conditions specific xylitol production by the XK strain was 1.5- to 2-fold higher vs the control. Nevertheless, the fraction of xylose carbon excreted as xylitol is always less for the XK strain, being only around 5% (mol/mol) under fully aerobic conditions. The strong correlation of xylitol production on aeration for both strains may be indicative of the metabolic

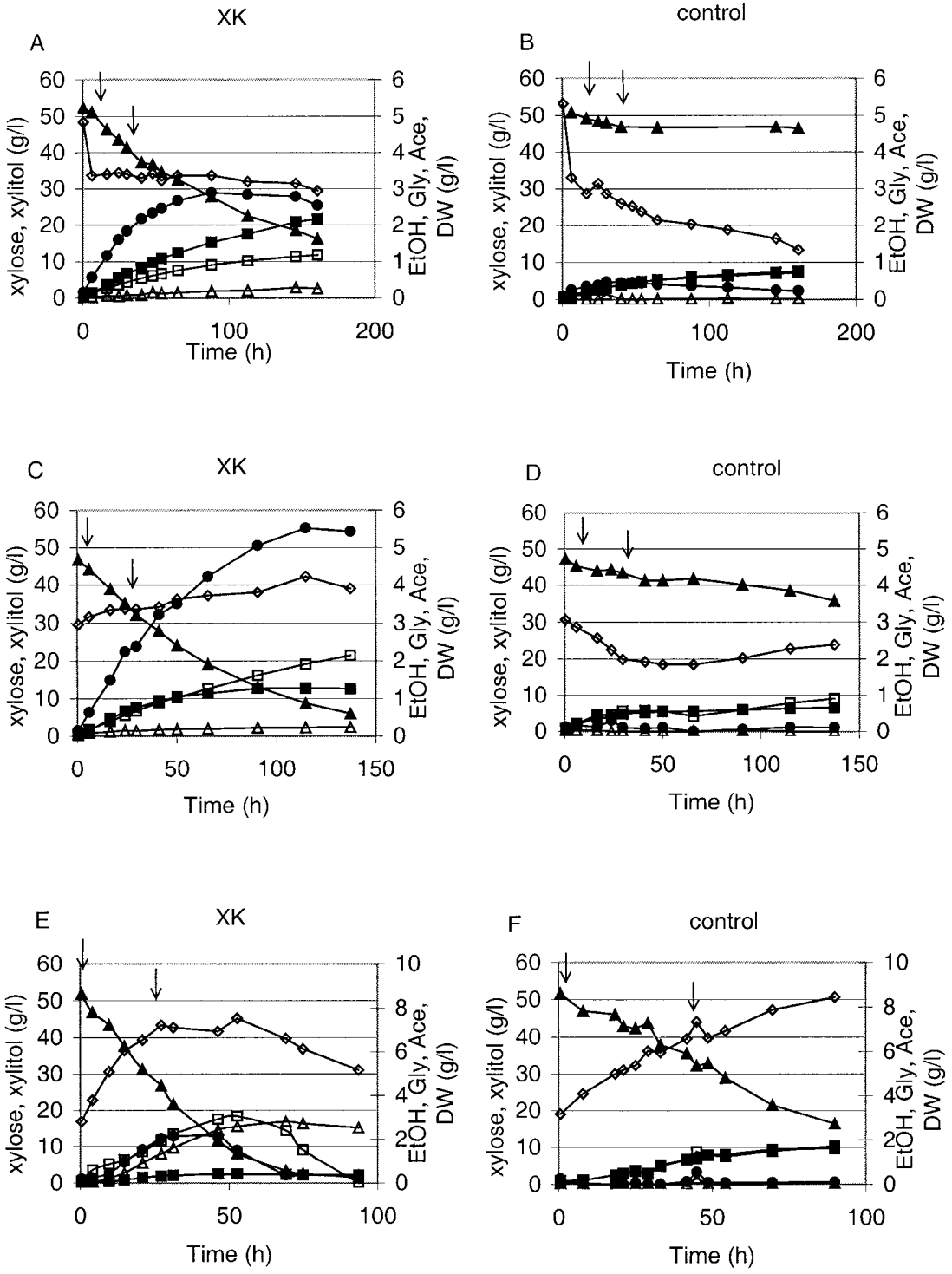

FIG. 2. Substrate consumption and product formation (g L^{-1}) for the XK and the control strain in anaerobic (0%, A, B), microaerobic (2%, C, D), and aerobic (20%, E, F) batch cultures on pure xylose. Xylose (▲), xylitol (■), dry weight (DW, ◇), ethanol (EtOH, ●), glycerol (Gly, △), and acetate (Ace, □). The logarithmic production/consumption phase used in the calculations is marked with arrows.

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TABLE 4

Specific Production/Consumption Rates of the Xylulokinase Overexpressing Strain (XK) and the Control Strain (C-mmol/g-cell dry wt h) at the Various Aeration Levels in Batch Cultivations

	Anaerobic		Microaerobic		Aerobic	
	XK	Control	XK	Control	XK	Control
Xylose	6.64	2.95	6.08	2.49	7.67	3.74
Xylitol	2.73	1.40	2.55	1.70	0.47	1.05
Glycerol	0.02	0	0.03	0	0.34	0
Acetate	0.16	0.11	0.20	0.20	0.47	0.15
Ethanol	0.83	0.10	0.94	0	0.62	0
CER	0.52	0.19	1.18	0.84	2.33	1.18
OUR	—	—	—	—	3.34	2.02

redox imbalance linked with the conversion of xylitol to xylulose (XDH reaction) which results in raising the cellular $NADH:NAD^+$ ratio.

The acetate production rate is three times higher for the XK strain under aerobic conditions. The specific carbon dioxide production rate for the XK strain is higher by a factor of 1.5–2.7 compared with the control. The glycerol production rate is highest under the aerobic conditions for the XK strain; in all other cases, it is very low or no glycerol is produced.

Table 5 compares the yields on total xylose consumed (C-mol/C-mol). A significant increase is seen in ethanol yields with the XK strain compared to the control. Ethanol yield is highest under microaerobic and anaerobic conditions. Under aerobic conditions, the ethanol yield is about half that of the lower oxygen concentrations, most likely because ethanol reconsumption is rather high under these conditions. Xylitol yields are slightly lower under anaerobic conditions, but only 60 and 20% under microaerobic and aerobic conditions of those of the control strain. The yields of CO_2 are comparable for the two strains except for the microaerobic conditions where the control strain has a 1.7-fold higher yield.

TABLE 5

Yields of Product Formation of the Xylulokinase Overexpressing Strain and the Control Strain (C-mol/C-mol)

	Anaerobic (0%)		Microaerobic (2%)		Aerobic (20%)	
O_2%	XK	Control	XK	Control	XK	Control
Biomass	—	—	—	—	0.16	0.15
Xylitol	0.41	0.47	0.42	0.68	0.06	0.28
Glycerol	0	0	0	0	0.04	0
Acetate	0.02	0.04	0.03	0.08	0.06	0.04
Ethanol	0.12	0.04	0.15	0	0.08	0
CER	0.08	0.07	0.20	0.34	0.30	0.32

Intracellular metabolite patterns vary with XK expression level and oxygen concentration. Figure 3 shows the intracellular levels of xylulose 5-phosphate (Xu5P), sedoheptulose 7-phosphate (S7P), glucose 6-phosphate (G6P), and fructose 6-phosphate (F6P) in the logarithmic consumption/metabolite production phase. The metabolites were measured from two to four time points in the logarithmic phase and from later time points. All points gave very similar results, showing a clear trend of intracellular metabolite concentrations. Clearly, aeration does not influence the intracellular levels of these metabolites, for the control strain, a direct result perhaps of the low rate of xylose utilization of this strain under any of the aeration conditions tested. However, the pattern is very different for the XK strain: the levels of Xu5P, the product of the xylulokinase reaction, are significantly higher in the XK strain under all aeration conditions, and the levels appear to be inversely related to aeration. Aeration is, hence, likely to enhance the rate of metabolism downstream of Xu5P. It has previously been shown that sedoheptulose

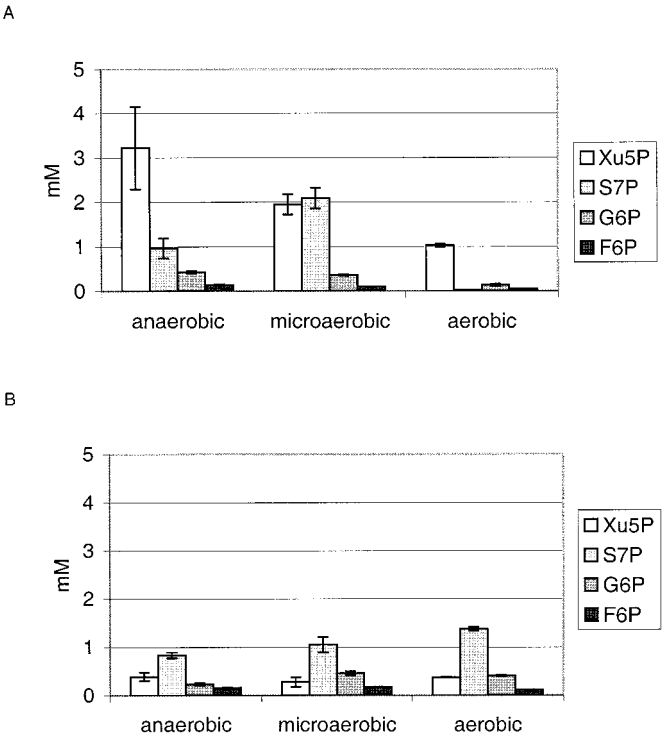

FIG. 3. Intracellular metabolite levels (mM) in the XK strain (A) and control strains (B). Xylulose 5-phosphate (Xu5P), sedoheptulose 7-phosphate (S7P), glucose 6-phosphate (G6P), and fructose 6-phosphate (F6P).

7-phosphate accumulates when *S. cerevisiae* is grown on xylulose (Senac and Hahn-Hägerdal, 1990). As shown in Fig. 3, the S7P levels are around 1 mM for both strains at all aeration levels, except for the case of the XK strain under microaerobic conditions where S7P is around 2 mM.

The levels of the glycolytic intermediates G6P and F6P are for the most part significantly lower compared with those of Xu5P and S7P. The levels of G6P and F6P remain again unaffected by aeration for the control strain. However, while G6P and F6P levels are comparable between the control and XK strain under anaerobic and microaerobic conditions, these levels fall to almost zero for the XK strain under fully aerobic conditions. During growth on xylose, glucose 6-phosphate is expected to be derived by reversal of the glycolytic steps from fructose 6-phosphate or glyceraldehyde 3-phosphate [resulting from Xu5P by the transketolase (TKL)/transaldolase (TAL) reactions]. The lower G6P/F6P levels for this particular case may reflect an enhanced flux of G6P into the oxidative PPP pathway, which would generate NADPH for the XR reaction.

The intracellular ATP levels (Table 6) are significantly lower for the XK strain compared with either the control strain under similar conditions or glucose-grown *Saccharomyces* (on glucose, [ATP]: 2–5 mM; [ATP]:[ADP] ratios: 4–7). Furthermore, under aerobic and microaerobic conditions the ATP, ADP, and AMP levels are about equal in the XK strain, whereas the ATP level is clearly higher in the control strain (Table 6). Under anaerobic conditions the ATP level is higher than ADP and AMP also in the XK strain; however, the variation is more pronounced for the control strain.

It has been suggested that when cells utilize xylose as the primary carbon source the enzymes of the lower part of glycolysis may not be sufficiently induced (Boles *et al.*, 1993; Muller *et al.*, 1995). To access this potential limitation, especially with the XK strain, the enzyme activities of

TABLE 6

ATP, ADP, and AMP Levels in the Batch Cultivation

	Anaerobic (0%)		Microaerobic (2%)		Aerobic (20%)	
O_2%	XK	Control	XK	Control	XK	Control
ATP	1.73	2.04	0.97	1.66	0.67	1.68
ADP	0.83	0.76	1.04	0.26	1.33	1.00
AMP	0.53	0.46	0.70	0.26	1.17	0.60
ATP:ADP	2.1	2.7	0.9	6.4	0.5	1.7

Note. Values are from time point 30 h, but represent the trend of all the five to nine time points measured.

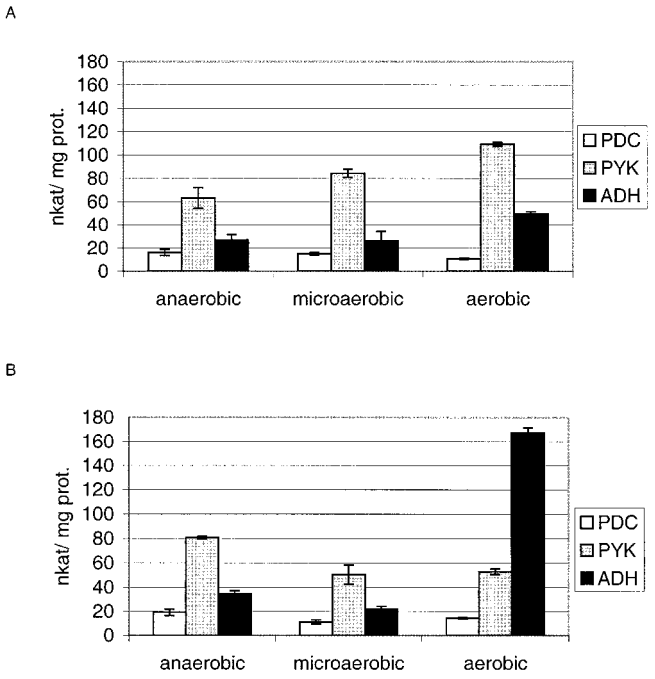

FIG. 4. Activities of pyruvate kinase (PYK), pyruvate decarboxylase (PDC), and alcohol dehydrogenase (ADH) in the XK strain (A) and in the control strain (B) in the batch cultures on pure xylose in logarithmic production/consumption phase.

pyruvate kinase (PYK), pyruvate decarboxylase (PDC), and ADH were measured and the results are presented in Fig. 4.

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate simultaneously producing ATP. The pyruvate kinase activities seem to increase with aeration in the XK strain, but not in the control strain. The activities in the XK strain under aerobic and microaerobic conditions were about twice the activity of the control strain.

Pyruvate decarboxylase catalyzes the conversion of pyruvate to acetaldehyde. Acetaldehyde is converted to either ethanol or alternatively to acetyl-CoA, which will primarily enter the TCA cycle when the cycle is active or be converted to other biosynthetic intermediates. In our studies, the *in vitro* PDC activity remained relatively unaffected by either the aeration level or the overexpression of *XKS1*.

Alcohol dehydrogenase catalyzes the reversible reaction that converts acetaldehyde and NADH to ethanol and NAD^+. Four genes have been found in *Saccharomyces* that encode isoenzymes of alcohol dehydrogenases (see Discussion). The ADH activity was clearly higher in the control strain under aerobic conditions compared with the XK strain. The ADH activity is lowest under microaerobic and anaerobic conditions. Notice that the enzyme activity measurement detects all ADH isozymes.

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DISCUSSION

Carbon Distribution

While the preparation of this article was in progress, Eliasson *et al.* (2000) described the fermentation properties of a similar recombinant *Saccharomyces* strain, expressing XR, XDH, and XK, and the illustration of anaerobic xylose conversion to ethanol by this recombinant *S. cerevisiae* on mixtures of glucose and xylose. One of the important additional contributions of our results is the fact that not only have we illustrated significant anaerobic xylose conversion to ethanol by recombinant *Saccharomyces*, but also, for the first time, we have achieved this on minimal salt media with pure xylose as a carbon source. In the work of Eliasson *et al*., mixtures of glucose and xylose were used because their recombinant strain could not grow anaerobically on xylose only.

In addition, we report ethanol production from pure xylose in fully aerated bioreactor cultivations for the first time. Generally, in aerobic xylose cultivations, ethanol, if formed, has been believed to be simultaneously consumed and used as a cosubstrate possibly producing NADPH. However, with the *XKS1*-overexpressing strain we observed significant ethanol accumulation, and this was maintained for as long as ethanol is formed and only subsequently consumed when xylose concentration drops below 10 g L^{-1}. This patterns resembles aerobic glucose fermentation, suggesting repression and derepression of gluconeogenic enzymes. On the other hand, ethanol accumulation under fully aerobic conditions by the XK strain may just be a reflection of enhanced xylose flux by XK overexpression. To the best of our knowledge, ethanol formation from xylose under fully aerobic conditions has not been reported before for any of the recombinant *Saccharomyces* strains studied so far.

The overexpression of xylulokinase was shown to drastically increase the xylose uptake rates as well as ethanol production rates, compared to strain with basal endogenous XK activity. The ethanol yields are higher, but xylitol yields lower. The increased flux is also seen as elevated carbon evolution rate and in glycerol and acetate formation. Most likely, the more efficient conversion of xylulose to xylulose 5-phosphate leads to enhanced oxidation of xylitol and therefore enhanced flux of xylose to ethanol. Also, the increased phosphorylation of xylulose may change the intracellular metabolite concentration in a way that enhances the xylose flux, for example by inducing or repressing various enzymes by yet unknown mechanisms.

Although *XKS1* overexpression enhances the specific xylose utilization rates, as well as ethanol yields on xylose, a significant fraction of the utilized xylose is still excreted as xylitol. Even though the *P. stipitis* XR can utilize NADH, NADPH is the preferred cofactor. The accumulation of xylitol suggests a high $NADH:NAD^+$ ratio and hence NAD^+ limitation for the xylitol dehydrogenase reaction. As indicated in Table 5, fully aerobic conditions can drastically reduce the yield of xylitol on xylose, in agreement with the above hypothesis (since in the presence of oxygen NADH can easily be oxidized to NAD^+). Under oxygen limitation, on the other hand, we would expect at least part of the excess NADH to be regenerated via the glycerol pathway. This is believed to be at least one of the primary mechanisms of redox balancing in glucose-grown *Saccharomyces* under oxygen limitation. Surprisingly, however, very little glycerol was formed under anaerobic or microaerobic conditions for either strain. It appears as if the glycerol pathway is not significantly activated under growth on xylose only (although some glycerol was observed under fully aerobic conditions). If this is indeed the case, this phenomenon has again not been well documented or discussed. As a corollary, it may be postulated that alternative redox balancing cycles may actually play a more dominant role instead, when cells are grown on xylose. One possibility is that glycerol is indeed being produced, hence acting as an NADH sink; however, new pathways may be activated that reconsume glycerol. If such glycerol reconsumption were to take place via an $NADP^+$ oxidoreductase, this would in essence create a futile cycle whereupon NADH is converted to NADPH (transhydrogenase equivalent). Besides such a glycerol cycle, other cycles may be activated under such nonphysiological circumstances, one possibility being the malate–aspartate cycle (Small and McAlister-Henn, 1998), which could in essence operate under microaerobic (or aerobic) conditions transferring NADH to the mitochondria for ATP generation.

The two different set ups (fed-batch and batch) gave very similar results, as summarized above; only a few differences were detected. In the fed-batch experiment, the xylitol production rate was higher for the control strain under all aeration conditions, in contrast to the batch experiments. This is probably due to the higher xylose concentration in the media in the fed-batch experiment and therefore faster xylose uptake and conversion to xylitol. Another difference was the higher ethanol production rate and yield for the control strain under microaerobic conditions in the fed-batch experiment. This may reflect the slightly different aeration level (2.5 and 2%) in the two experiments or possibly the small amount of glucose present in the fed-batch experiments. The ethanol production rate of the XK strain varied also under other aeration conditions, being highest under aerobic conditions in fed-batch and under microaerobic conditions in the batch cultivation. This is probably due to the different experimental set ups: unlike

the fed-batch cultivations, no glucose was present in the batch experiments, and also the residual xylose level was significantly higher during the later stages of the fed-batch cultivations compared to the batch experiments. The carbon balances could not be closed in either of the experiments, and the carbon recovery varied from about 60 to 80%. The inability to close the carbon balance has been reported in the literature for both xylulose and xylose fermentations (Yu *et al.*, 1995; Eliasson *et al.*, 2000). For our case, at least, this discrepancy cannot be due to ethanol evaporation since with an identical set up using glucose only as the carbon source, we can account for almost 100% of the carbon. A more likely possibility is production of yet unidentified products, and according to our observations small unidentified HPLC peaks were detected, especially under aerobic conditions.

The Intracellular Metabolite Concentrations Suggest Limitations in the Nonoxidative Pentose Phosphate Pathway

The overexpression of xylulokinase drastically changes the amounts and ratios of the two important pentose phosphate pathway metabolites xylulose 5-phosphate and sedoheptulose 7-phosphate. The concentrations of these metabolites are relatively unaltered by oxygenation in the control strain, whereas in the XK strain the metabolites vary significantly in different oxygen concentrations. This can be interpreted as an inability of the XK strain to maintain the homeostasis of the cell. In the XK strain xylulose 5-phosphate accumulated, suggesting a limitation in enzymatic steps downstream of this metabolite, such as transketolase. The accumulation decreased with increasing aeration. This is of interest as more ATP should be available under aerobic conditions; possibly the regulation of transketolase and transaldolase or the activity of glycolysis varies under different aeration conditions. Under anaerobic and microaerobic conditions the accumulation of sedoheptulose 7-phosphate was also significant with the XK strain, pointing to a limitation in the transaldolase reaction or to competition of glyceraldehyde 3-phosphate between the pentose phosphate pathway and glycolysis as suggested previously to be the case in xylulose fermentations (Senac and Hahn-Hägerdal, 1990). Also, the accumulation of these sugar phosphates under oxygen-limited conditions may reflect the lack of NAD^+, which would limit the downstream reaction of glyceraldehyde-3-phosphate dehydrogenase that also uses NAD^+ as substrate. In the control strain only sedoheptulose 7-phosphate accumulated.

Our results with the *XKS1*-overexpressing strain indicate that the steps immediately downstream of the XK reaction can have a high flux control coefficient in xylose utilization. The regulation of the nonoxidative pentose phosphate pathway enzymes for cells grown on xylose is still elusive. For *Saccharomyces* yeast grown on glucose, only a small fraction (1–2.5%) of the utilized carbon is believed to be processed via the pentose phosphate pathway (Gancedo, 1973), indicating that this yeast has a low-capacity pentose phosphate pathway.

The limitations can be envisioned at various levels. The steps catalyzed by the TKL and TAL reactions are likely to have high control on the xylose flux. Kötter *et al.* studied a strain that had been mutated and selected for higher xylulokinase activity and which accumulated xylulose 5-phosphate (Kötter, 1993). Metzger and Hollenberg (1994) overexpressed the transketolase-encoding gene of *P. stipitis* in this strain; however, this led to a reduction in the specific growth rate. Subsequent work with a *XYL1*-and *XYL2*-containing *S. cerevisiae* strain showed that overexpressing *TAL1* considerably enhanced growth on xylose compared with a strain containing *XYL1* and *XYL2* only but had no effect on ethanol formation (Walfridsson *et al.*, 1995). For the XK strain used in this study, we are likely to be even more limited by the TKL and TAL reactions due to the significantly higher Xu5P formation rates.

Furthermore, the competition for ribose 5-phosphate between enzymes of the nonoxidative pentose phosphate pathway enzymes TKL and TAL on one hand and biosynthetic reactions on the other (since this molecule and its derivatives are components of the important metabolites ATP, CoA, NAD+, FAD, RNA, and DNA) cannot be overlooked, especially under significant growth rates. Also, aeration levels may influence the coupling of PPP intermediates and glycolysis, as well as glycolytic fluxes.

Cellular Energetics

Since *Saccharomyces* has been evolved to utilize hexoses, growth and metabolism of this yeast on pentose sugars will inevitably influence its cellular energetics. Earlier studies with *Saccharomyces* overexpressing *XKS1* (only) showed growth inhibition on xylulose (Rodriguez-Pena *et al.*, 1998). ATP is a substrate of XK, and any imbalances in terms of ATP utilization (by XK) and ATP regeneration (lower glycolysis, respiration) will gradually reduce metabolic fluxes and eventually shut down the pathway. Similar metabolic schemes, such as the hexokinase (HK, equivalent to XK) reaction together with glycolysis, typically require complex regulation, such that the rate of ATP production matches that of ATP utilization for sugar phosphorylation. In the case of glycolysis, it has been shown that Tps1p (trehalose-6-phosphate synthase) is an essential regulatory enzyme that inhibits (one of) the first steps in glycolysis and thereby limits the flux of glucose to pyruvate. A *tps1*

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disruptant accumulates hexose phosphates and consumes ATP and inorganic phosphate rapidly. Growth on glucose can be restored in such a mutant by attenuating HK or transporter activity.

The well-established cariostatic effect of xylitol (Cutress *et al*., 1992) has also been linked with a dramatic decrease in glycolytic rates and ATP levels (Forbord *et al*., 1992). Xylitol or xylulose is also known to cause hepatic ATP catabolism by inducing the trapping of Pi in the form of glycerol 3-P as a consequence of an increase in the $NADH:NAD^+$ ratio (Vincent *et al*., 1989a). In isolated rat hepatocytes, 5 mM D-xylulose or xylitol drastically decreased the ATP levels, while increasing the concentrations of Xu5P, S7P, ribose 5-P, and phosphoribosyl pyrophosphate (Vincent *et al*., 1989b). Our XK strain follows a similar pattern for at least ATP, Xu5P, and S7P.

As anticipated, the intracellular ATP levels for the XK strain were found to be indeed significantly lower compared with either the control strain under similar conditions or glucose-grown *Saccharomyces*. This can be an important limitation in xylose metabolism in more ways than one: ATP is a substrate for the xylulokinase reaction, and ATP levels or the ATP:ADP ratio act as physiological signals, both as allosteric enzyme regulators and for controlling the rate of protein synthesis. Significant deviations from physiological levels will eventually slow down metabolism and result in cell death. ATP limitations are likely to arise under anaerobic conditions, where ATP synthesis is restricted to oxidative phosphorylation, a rather inefficient means of ATP generation vs respiration. Nevertheless, according to the results of Table 4, the specific xylose utilization rates for the XK strain are comparable under anaerobic and microaerobic conditions and only marginally higher under fully aerobic conditions, which may indicate that other factors, besides energetics, may have higher xylose flux control. An important difference between glucose and xylose metabolism in this yeast is the fact that the apparent xylose uptake rate is about an order of magnitude less than glucose uptake. This by itself may provide a safety mechanism against the rapid depletion of the ATP pool below physiologically acceptable thresholds. Note also that the previous hypothesis can still agree with the results observed earlier with xylulose and XK overexpression (Rodriguez-Pena *et al*., 1998), since the rate of xylulose uptake can be significantly higher compared with that of xylose. Accumulation of pentose phosphate intermediates as well as lowering of the ATP levels can indeed be detrimental; however, their impact is likely to be attenuated if the xylose uptake rate were to have significant control on the flux.

Another interesting observation was the fact that under aerobic and microaerobic conditions the ATP, ADP, and AMP levels are about equal in the XK strain, whereas the ATP level is clearly higher in the control strain (Table 6). Under anaerobic conditions, the ATP level is higher than that of ADP and AMP in the XK strain, but the differences are more pronounced for the control strain. However, the different distribution of ATP, ADP, and AMP in the two strains may indicate novel pathways of ATP generation and, thus, faster metabolism in the XK strain. Different kinases, such as adenylate kinase ($ATP + AMP = ADP + ADP$), may change the distribution of nucleotides and result in ATP generation. Since this observation was more pronounced in the presence of oxygen, it is likely to be mitochondria linked.

Pyruvate Kinase, Pyruvate Decarboxylase, and Alcohol Dehydrogenase Are Active on Xylose

Metabolism of an unnatural substrate, such as xylose, is likely to have very different effects on enzyme function and gene expression compared with growth on glucose. For example, the level of intracellular metabolites or ATP and free phosphate levels will influence the activity of many enzymes. We measured the activities of enzymes converting phosphoenolpyruvate to ethanol, PYK, PDC, and ADH.

PYK is one of the most highly expressed glycolytic enzymes on glucose. Full induction of the *PYK1* gene requires an increase in glucose 6-phosphate and fructose 6-phosphate concentrations (Boles *et al*., 1993; Boles and Zimmermann, 1993). Similarly to phosphofructokinase, the expression of this enzyme is likely to be linked to the cellular energetics. We saw an increase in the PYK activity as a function of increasing oxygen in the XK strain, but not in the control strain. The increase in PYK expression is likely to be a response to the declining levels or ATP:ADP ratio (see Table 6); this enzyme is believed to be an extremely sensitive sensor of the cytosolic ATP/ADP ratio (Larsson *et al*., 2000). The activities under microaerobic and aerobic conditions were higher compared to those of the control. However, the activity does not correlate with measured glucose 6-phosphate and fructose 6-phosphate levels indicating a more complex mode of induction. Interestingly, higher pyruvate kinase activities were detected under conditions where ethanol was produced by the XK strain only and where lower ATP levels were also detected. However, considering its capacity with respect to the observed xylose flux, pyruvate kinase is unlikely to be rate limiting on pure xylose.

The structural protein of pyruvate decarboxylase of *S. cerevisiae* is coded by genes *PDC1* (Schmitt *et al*., 1983) and *PDC5* (Seeboth *et al*., 1990). The PDC enzyme is activated by its substrate pyruvate, while the expression of *PDC1* and *PDC5* is induced by glucose. The activity has been found to correlate well with the intracellular levels of

triose phosphates (Boles *et al*., 1993). Overall, the regulation of PDC expression is complex and the real mechanism of glucose induction is yet unclear. Compared to pyruvate kinase and alcohol dehydrogenase activities, the PDC activities we measured were low, about 20 nkat/mg protein. In literature, the values reported for glucose-grown cells vary from 11 to 50 nkat/mg protein (0.7–3 U/mg protein), whereas they are about three times lower in cells grown on ethanol (Boles and Zimmermann, 1993; Flikweert *et al*., 1996; Liesen *et al*., 1996; van Hoek *et al*., 1998).

Four genes have been found so far in *Saccharomyces* that encode isoenzymes of alcohol dehydrogenases. *ADH1* encodes the glucose-inducible isoenzyme prominent during glucose fermentation (Denis *et al*., 1983), while *ADH2* is repressed by glucose and is implicated in the utilization of ethanol (Denis *et al*., 1981; Vallari *et al*., 1992). Under aerobic conditions the control strain had about three times higher ADH activity compared to the XK strain. A high ADH(2) activity in the control strain can presumably lead to rapid ethanol reassimilation and may explain the observed low ethanol accumulation levels. These results combined with mRNA or protein electrophoresis analyses will help to further elucidate this phenomenon.

Overexpression of xylulokinase clearly increases ethanol production from xylose, both aerobically and under oxygen-limited conditions. Despite the XK amplification, the ethanol production rates and yields can still be further improved. Based on our measurements the enzyme activities of the ethanologenic pathway are not limiting in the xylose metabolism. Moreover, the steps that link xylulose 5-phos phate with the glycolytic pathway are likely to have high flux control coefficients as discussed above. The exact effects on the xylose fluxes as a result of altering the levels of either the endogenous transketolase or transaldolase reactions still remain to be seen.

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PUBLICATION II

Endogenous Xylose Pathway in *Saccharomyces cerevisiae*

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Endogenous Xylose Pathway in *Saccharomyces cerevisiae*

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The baker's yeast *Saccharomyces cerevisiae* is generally classified as a non-xylose-utilizing organism. We found that *S. cerevisiae* can grow on D-xylose when only the endogenous genes *GRE3* (*YHR104w*), coding for a nonspecific aldose reductase, and *XYL2* (*YLR070c*, *ScXYL2*), coding for a xylitol dehydrogenase (XDH), are overexpressed under endogenous promoters. In nontransformed *S. cerevisiae* strains, XDH activity was significantly higher in the presence of xylose, but xylose reductase (XR) activity was not affected by the choice of carbon source. The expression of *SOR1*, encoding a sorbitol dehydrogenase, was elevated in the presence of xylose as were the genes encoding transketolase and transaldolase. An *S. cerevisiae* strain carrying the XR and XDH enzymes from the xylose-utilizing yeast *Pichia stipitis* grew more quickly and accumulated less xylitol than did the strain overexpressing the endogenous enzymes. Overexpression of the *GRE3* and *ScXYL2* genes in the *S. cerevisiae* CEN.PK2 strain resulted in a growth rate of 0.01 g of cell dry mass liter^{-1} h^{-1} and a xylitol yield of 55% when xylose was the main carbon source.

The pentose sugar xylose is a major constituent of lignocellulose. *Saccharomyces cerevisiae* cannot use xylose, instead converting it primarily to xylitol with only a small fraction going into biomass or ethanol (44, 45). Recombinant xylose-metabolizing *S. cerevisiae* strains contain genes from the xylose-utilizing yeast *Pichia stipitis* coding enzymes for the first two steps in xylose conversion (23, 38, 46). However, the potential of *S. cerevisiae*'s own enzymes, if they are overexpressed, has not been evaluated.

In xylose-utilizing fungi, xylose reductase (XR) reduces xylose to xylitol, which is oxidized to xylulose by xylitol dehydrogenase (XDH). Xylulose is subsequently phosphorylated to xylulose 5-phosphate by xylulokinase and metabolized through the pentose phosphate pathway. *S. cerevisiae* cannot utilize xylose but can grow on xylulose (15, 43). Thus, the inability of *S. cerevisiae* to utilize xylose was attributed to its inability to convert xylose to xylulose (15), even though low XR and XDH activities are known in *S. cerevisiae* (4). A nonspecific aldose reductase, converting xylose to xylitol, was purified and characterized from *S. cerevisiae* (24); however, the genes coding for the putative XR and XDH enzymes remained unknown. The third enzyme in the xylose pathway, xylulokinase, is encoded by *XKS1*, a gene that has been cloned from, and probably is functional in, *S. cerevisiae* (19). Moderate increases in xylulokinase activity are beneficial in recombinant xylose-metabolizing *S. cerevisiae* strains (9, 18, 20, 21, 40).

Based on the *S. cerevisiae* genome sequence (14), the N-terminal amino acid sequence of the previously purified aldo-keto reductase corresponds to the open reading frame *YHR104w* (*GRE3*), which has 72% amino acid similarity to the XR enzyme of *P. stipitis*. This enzyme can reduce a wide variety of ketose substrates and requires a NADPH cofactor (24). The XR of *P. stipitis* can use either NADH or NADPH in the reduction reaction. *GRE3* is induced under various stress conditions and may act on the toxic intermediates generated (2, 11, 30, 31). Under some conditions, deletion of *GRE3* can decrease xylitol production (42). Two other aldose reductase gene homologs, *YPR1* and *YJR096w*, also encode enzymes requiring NADPH and can utilize xylose as a substrate, but they have much higher K_m values for xylose than does Gre3p (29, 41). Thus, several genes encoding aldose reductases that could utilize xylose are expressed in *S. cerevisiae*.

There are three genes in the *S. cerevisiae* genome that are similar to the gene encoding XDH, *XYL2* of *P. stipitis*. Open reading frame *YLR070c* encodes an enzyme with XDH activity (referred to here as *ScXYL2* and ScXDH, respectively) (32). The other two genes are *SOR1*, which encodes sorbitol dehydrogenase (SDH), and an open reading frame, *YDL246c*, that is almost identical to *SOR1*. The SDH enzyme also can use xylitol as a substrate (35).

The aim of this work was to study the endogenous xylose pathway in *S. cerevisiae*. The gene homologs needed for xylose metabolism exist in the *S. cerevisiae* genome, but it is not known how they are expressed or if the activities they encode form a functional pathway, collectively capable of metabolizing xylose. This study is the first attempt to use only endogenous genes for generating a xylose-metabolizing *S. cerevisiae* strain. The use of endogenous genes as alternatives in constructing recombinant xylose-utilizing strains is evaluated.

MATERIALS AND METHODS

Strains. The yeast strain W303-1B (MATα *leu2-3/112 his3-11/15 trp1-1 can1-100 ade2-1 ura31*) (39) was used for cloning of the *GRE3* and *ScXYL2* genes. The yeast strains S150-2B (H308) (MAT**a** *his3Δ-1 leu2-3/112 trp1-289 ura3-52 cir*$^+$ *gal*$^+$) (26) and CEN.PK2 (H1346) (*MAT***a** *leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8*^{c} *SUC2*) (6) were used as host strains for expressing the *XYL1* and *XYL2* genes of *P. stipitis* and the *GRE3* and *XYL2* genes of *S. cerevisiae*. The strains CEN.PK2 and ENY.WA-1A (*MAT*α *ura3-52 leu2-3/112 trp1-289 his3Δ1 MAL2-8*^{c} *MAL3 SUC3*) (7) were used for gene expression analysis. The previously described CEN.PK2-derived strains expressing the *XKS1* gene on a multicopy vector (strain H1695) and the corresponding control strain with an empty vector (strain H1697) were used as control strains in the *XKS1* expression analyses (33). The genomic DNAs of strains S288C (34) or CEN.PK2 were used as the template for PCR probes. The *Escherichia coli* DH5α strain was used for the bacterial cloning steps.

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TABLE 1. Primers used for gene cloning and probe generation

Gene	Primers
GRE3 (*YHR104w*)	fwd, AAATTGGATCCAGATGTCTTCACTGGTTA; rev, CATACGGATCCTGAGTATGGATTTTACTG
ScXYL2 (*YLR070c*) probe	fwd, GCCTTGAGGATAGAGAACACC; rev, GATTCATGTCCCAGCACCATT
SOR1 probe	fwd, ATGTCTCAAAATAGTAACCC; rev, TCATTCAGGACCAAAGATAATAGT
GRE3 (*YHR104w*) probe	fwd, AACCATCCAGGCAGTACCAC; rev, AGCATCGGAATGAGGGAAAT
TKL1 probe	fwd, TTTGAGTGTTGAAGCTGCTA; rev, CAAATCTGATGATCTACGATC
TAL1 probe	fwd, CGCTTAAGGAAGTATCTCGGA; rev, GCTTTGCTGCAAGGATTCAT
IPP1 probe	fwd, CCGATTGGAAAGTTATTGCC; rev, AGAACCGGAGATGAAGAACCA
ACT1 probe	fwd, AAGAAATGCAAACCGCTGCT; rev, TGGTGAACGATAGATGGACCA

Northern analysis. Total RNA was isolated with a Trizol reagent kit (Invitrogen, Carlsbad, Calif.). The samples taken at 70 h were treated with 10 mg of Zymolyase (Seikagaku Corporation, Tokyo, Japan) per ml for 10 min at room temperature (22 to 24°C) prior to the RNA isolation. Probes for the Northern analysis (Table 1) were prepared by PCR for all genes except for *XKS1* from the genomic DNA of strains S288C or CEN.PK2. To avoid cross-reactions with homologous genes, the probes for *ScXYL2*, *TKL1*, and *TAL1* were chosen partly from the 3′ or 5′ noncoding regions. The probe for *SOR1* also detects *YDL246c*. The PCR products were cloned into pCR2.1-TOPO (Invitrogen), excised from the vector by digestion with EcoRI, and verified by sequencing. *XKS1* was amplified by PCR as described previously (33). The fragments were purified from agarose gels, and labeled with a random primed DNA labeling kit (Roche, Basel, Switzerland) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden). Six identical gels were run and blotted to Hybond-N nylon filters (Amersham Pharmacia Biotech) and fixed with UV light (UV Stratalinker 2400; Stratagene, La Jolla, Calif.). Hybridized mRNAs were scanned with a Typhoon Phosphoimager and quantified with ImageQuant software (Amersham Pharmacia Biotech). The measured values were normalized by using the specified amount of pyrophosphate phosphohydrolase (*IPP1*) mRNA, coding for inorganic diphosphatase or actin (*ACT1*) mRNA, which codes for structural protein actin as a control for each sample.

Strain construction. *GRE3* (*YHR104w*) was amplified from the genomic DNA of strain W303-1B (Table 1). The resulting fragment was cloned into the BglII site between the *PGK* promoter and terminator of the pMA91 vector (27), resulting in plasmid B1165. The *ScXYL2* (*YLR070c*) gene was cloned into the pMA91 vector as described previously (32). The *ScXYL2* gene with the *PGK* promoter and terminator was released from pMA91 by digestion with HindIII and the fragment ligated into the HindIII site of the YEp24H (1) vector (plasmid B1180). The PCR fragment containing *ScXYL2* also was inserted into the BglII site of plasmid B1181, which contains the *PGK* promoter and terminator from the pMA91 vector as a HindIII fragment cloned into the corresponding site in the YEplac195 vector (13) (plasmid B1523). The *XYL1* gene of *P. stipitis* was previously amplified by PCR and cloned between the *PGK* promoter and terminator in the pMA91 vector (plasmid B383) (16). The *XYL2* gene of *P. stipitis* also was cloned into pMA91 (plasmid B731) (46). The *XYL2* expression cassette was ligated into the HindIII site of YEplac195 (plasmid B1530) or blunt ended and cloned into the PvuII site of YEp24 vector (17) (plasmid B733).

To give a control strain, the yeast strain H308 (S150-2B) was transformed with the empty pMA91 and YEp24 vectors. The strain H308 was also transformed with corresponding vectors containing the *P. stipitis XYL1* and *XYL2* genes (B383 and B733) to give strain H1356 and with vectors containing the *S. cerevisiae* genes *GRE3* and *ScXYL2* (B1165 and B1180) to give strain MTen. The CEN.PK2 strain was transformed with *GRE3*- and *ScXYL2*-containing vectors (B1165 and B1523) or with *GRE3*- and *P. stipitis XYL2* (B1165 and B1530)-expressing vectors, resulting in strains H2558 and H2560, respectively. Standard recombinant DNA methods were used (25). Yeast transformations were done as described by Gietz et al. (12).

Enzyme activities. XR and XDH activities were measured in cell extracts made by disrupting cells with glass beads either in 100 mM sodium phosphate buffer (pH 7.0) or in 50 mM HEPES buffer (pH 7.0), containing 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol. Both buffers were supplemented with the protease inhibitors phenylmethylsulfonyl fluoride and pepstatin A (final concentrations of 0.17 mg ml^{-1} and 0.01 mg ml^{-1}, respectively). XDH activity was measured as described earlier (32). XR activity was measured in 100 mM sodium phosphate buffer (pH 7.0) containing 1 M xylose and 0.2 mM NADPH as a decrease in A_{340}. Protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). All enzymatic and protein analyses were carried out with the Cobas Mira automated analyzer (Roche).

Yeast cultures and metabolite analysis. The recombinant strains were cultured in shake flasks containing synthetic complete (YSC) medium (modified from that of Sherman et al. [37]) either with or without glucose and without either leucine or uracil for plasmid selection. The ratio of xylose to glucose was 19:1 or 20:1, with either 19 or 20 g of xylose liter^{-1} and 1 g of glucose liter^{-1}. Cultures were started from an initial optical density at 600 nm (OD_{600}) of 0.2. Alternatively, no glucose was added and the cultures were started with a high biomass (OD_{600} of approximately 2). Growth was measured as OD_{600}. Cells also were cultivated on 20 g of glucose liter^{-1} in YSC medium without leucine and uracil. For growth on xylulose, a mixture of xylose and xylulose (30 g of xylose liter^{-1} and 10 g of xylulose liter^{-1}) was prepared by isomerization of D-xylose (28). Cultures were made in 50 ml of media and carried out in 250-ml Erlenmeyer flasks at 30°C and 250 rpm on an orbital shaker. Each strain was cultured in duplicate.

Anaerobic growth experiments were performed in a 1.8-liter Chemap CMF bioreactor (Chemap AG, Volketswil, Switzerland) with an initial working volume of 1.2 liters. Mass flow controllers (Bronkhorst High-Tech BV, Ruurlo, The Netherlands) regulated the air and nitrogen flow rates. Nitrogen was passed through an Oxisorb oxygen-absorbing device (Messer Griesheim GmbH, Krefeld, Germany) to reduce the residual oxygen level to >50 ppb. The dissolved oxygen concentration was monitored by an Ingold polarographic probe (Mettler-Toledo, Columbus, Ohio). The temperature was maintained at 30°C, and the pH was controlled at 5.5 by the addition of 2 M NaOH. The medium was YSC without leucine and uracil supplemented with 47.5 g of xylose liter^{-1} and 2.5 g of glucose liter^{-1}. Cultures were started from an initial OD of 8 to 9. The total gas flow rate in this experiment was 0.5 standard liters per minute. Cell growth was measured as OD_{600} and cell dry mass (DM) as described previously (40).

Growth rates (Table 2) were calculated from time periods in which growth, xylose consumption, and xylitol production were linear, i.e., 63 to 121 h (Fig. 1) and 69 to 147 h (Fig. 2A). The growth rate was calculated as the change in DM per hour (gram liter^{-1} hour^{-1}). The specific production and consumption rates

TABLE 2. Growth rates and specific xylose consumption and xylitol production rates for the experiments of Fig. 1 and 2

Genotype[a]	Growth (g of DM liter^{-1} h^{-1})	Xylose consumption (C-mmol g of DM^{-1} h^{-1})	Xylitol production (C-mmol g of DM^{-1} h^{-1})
PsXYL1 PsXYL2[b]	0.014 ± 0.001	2.7 ± 0.1	0.44 ± 0.04
ScGRE3 ScXYL2[b]	0.008 ± 0.001	2.5 ± 0.2	0.89 ± 0.13
ScGRE3 ScXYL2[c]	0.010 ± 0.001	3.5 ± 0.1	2.0 ± 0.1
ScGRE3 PsXYL2[c]	0.011 ± 0.000	3.4 ± 0.0	1.9 ± 0.2

[a] *Ps*, gene originating from *P. stipitis; Sc*, gene originating from *S. cerevisiae*.
[b] Parental *S. cerevisiae* strain S150-2B. The rates were calculated between time points 63 and 121h (Fig. 1).
[c] Parental *S. cerevisiae* strain CEN.PK2. The rates were calculated between time points 69 and 147 h (Fig. 2A).

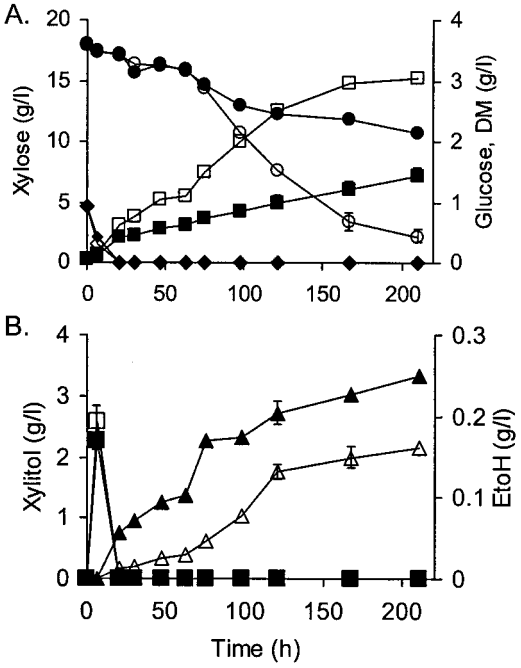

FIG. 1. Comparison of *S. cerevisiae* S150-2B strains harboring XR and XDH either from *P. stipitis* or from *S. cerevisiae* in shake flask cultures. Growth and product formation on 20 g of xylose plus 1 g of glucose liter^{-1} as a carbon source. Filled symbols represent the strain overexpressing *GRE3* and Sc*XYL2* genes, and open symbols represent the strain with *P. stipitis XYL1* and *XYL2* genes. (A) Shown are data for DM (■), glucose (♦), and xylose (●). (B) Shown are data for xylitol (▲) and ethanol (EtOH) (■). Results are based on two replications and are given in grams liter^{-1}. Points without error bars have an associated error that is <10% of the value of the point.

were calculated as C-mmol of substrate consumed or product formed by 1 g of DM per h. The DM values for shake flask cultures were obtained by using a conversion of 0.3 g of DM liter^{-1} for an OD_{600} value of 1 (unpublished data).

The nontransformed strains CEN.PK2 and ENY.WA-1A were cultured in YP medium (10 g of yeast extract liter^{-1}, 20 g of peptone liter^{-1}) with either 20 g of glucose liter^{-1} or 20 g of glucose liter^{-1} and 100 g of xylose liter^{-1}. Cells were harvested by centrifugation (3400 × *g*, 2 min at 20°C) and either frozen in liquid nitrogen (RNA samples) or moved directly to −70°C (samples for enzyme activities).

Metabolite production was analyzed from growth media by high-pressure liquid chromatography with an Aminex HPX-87H column (Bio-Rad Laboratories). The column was maintained at 35°C with an eluant of 5 mM H_2SO_4 at a constant flow rate of 0.6 ml min^{-1}. Glucose, xylose, xylitol, glycerol, acetate, and ethanol were quantified by using a combination of a refractive index and UV (λ = 210 nm) detectors connected in series.

Plasmid stability was measured by plating cells from different time points on plates without selection, growing them for 2 days, and then replicating them on plates of selection media. All of the recombinant strains contained two expression vectors, so we studied possible recombination between the plasmids. Samples were taken when growth was complete, and plasmids were extracted and transformed into *E. coli*. Plasmid DNA was extracted from bacterial transformants and analyzed by restriction enzyme digestion.

RESULTS

Recombinant strains. The XR activities of strains expressing *XYL1*, *XYL2*, *GRE3*, and *ScXYL2* in glucose-grown cultures were similar, 15 to 22 nkat (per mg of total protein), for both *GRE3*- and *XYL1*-encoded enzymes. The XDH activity varied between about 10 nkat (per mg of total protein) for ScXDH and about 90 nkat (per mg of total protein) for the XDH of *P. stipitis*. Over 90% of the cells harbored both plasmids after cultivation for 2 days without auxotrophic selection, and no recombination between the plasmids was detected.

Growth on D-xylose. When the S150-2B-derived strains containing XR and XDH either from *P. stipitis* or from *S. cerevisiae* were cultivated in shake flasks with 20 g of xylose liter^{-1} and 1 g of glucose liter^{-1} as the carbon source, glucose was consumed during the first 10 h, and then xylose consumption began. The strain with the *S. cerevisiae* XR and XDH consumed xylose slower and formed less biomass than did the strain containing the enzymes from *P. stipitis* (Fig. 1A). The control strain (data not shown) used practically no xylose, and growth on glucose and on ethanol derived from glucose resulted in 0.3 g of DM liter^{-1}, compared to 1.5 and 3.0 g liter^{-1} with strains containing *S. cerevisiae* and *P. stipitis* enzymes, respectively. The biomass yield on xylose was 20% for both overexpressing strains.

Significantly more xylitol was formed by the strain with the *S. cerevisiae* enzymes (Fig. 1B). Equal amounts of glycerol were formed (0.1 g liter^{-1}) and subsequently consumed by both strains (data not shown). Acetate (maximally 0.2 g liter^{-1}) was detected only with the strain overexpressing the *S. cerevisiae* enzymes (data not shown).

The cultures grew in a biphasic manner with a period of slow xylose consumption (up to 60 h), followed by a faster metabolic phase (from 63 to 121 h) from which the specific rates (Table 2) were calculated. The strain overexpressing the *S. cerevisiae* genes grew much slower than did the strain with the *P. stipitis* genes. The specific xylose consumption rate differed by 15%, but the specific xylitol production rate was twice as high in the strain overexpressing the *S. cerevisiae* enzymes. The xylitol yield on xylose was about 45% for the strain overexpressing the *S. cerevisiae* enzymes and about 10% for the strain expressing the *P. stipitis* enzymes.

Effect of XDH activity on xylose metabolism of strains expressing *GRE3*. XDH activity in the strains overexpressing the ScXDH-encoding gene was about 10 times lower than in the strains overexpressing the gene for *P. stipitis* XDH. Significantly higher amounts of xylitol were accumulated by the strain carrying the *S. cerevisiae* ScXDH. A CEN.PK2 strain overexpressing the *S. cerevisiae GRE3* and *P. stipitis XYL2* genes grew (Fig. 2A) and consumed xylose at the same rate as a strain carrying the *GRE3* and *ScXYL2* genes (Table 2). The strain expressing ScXDH initially produced xylitol slightly faster than did the strain with the *P. stipitis* XDH, but the final xylitol yield did not differ much, being 55 and 49%, respectively. Also, these strains showed a biphasic growth curve (Fig. 2A). Compared to the S150-2B-based strain, the growth rate of CEN.PK2-based strains was about 1.3 times higher, the specific xylose consumption rate was nearly 1.4-fold higher, and the specific xylitol production rate was over twofold higher, demonstrating the effects of different parental strains on the efficiency of xylose metabolism. Ethanol was produced only from glucose in the beginning of the cultivation, no glycerol was detected, and acetate accumulated in low quantities, maximally 0.6 g liter^{-1}.

In shake flask cultures with xylose (20 g liter^{-1}) as the sole carbon source, the biomass of the strain carrying the *P. stipitis* XDH increased more rapidly than did the biomass of the strain

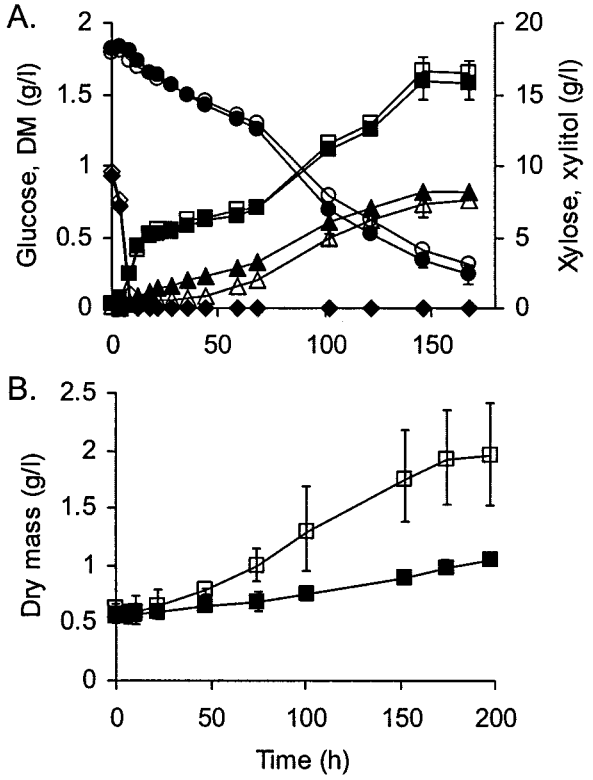

FIG. 2. Xylose metabolism with the CEN.PK2 strains overexpressing *GRE3* and *ScXYL2* (filled symbols) or *GRE3* and the *P. stipitis XYL2* (open symbols) in shake flask cultures. Points without error bars have an associated error that is <10% of the value of the point. Results are based on two replications. (A) Growth and xylose consumption and xylitol formation on 19 g of xylose plus 1 g of glucose liter^{-1} as a carbon source. DM (■), glucose (♦), xylose (●), and xylitol (▲), all in grams liter^{-1}. (B) Growth on 20 g of xylose liter^{-1} as a carbon source.

expressing ScXDH (Fig. 2B). With the latter, strain growth was almost negligible. The strain with *P. stipitis* XDH consumed more xylose than did the strain with *S. cerevisiae* XDH (16.2 versus 9.5 g in 200 h). The xylitol yield was 50% for the strain with *P. stipitis* XDH and 60% for the strain expressing the *S. cerevisiae* XDH-encoding gene. Thus, even 10-fold-higher XDH activity levels did not decrease the high xylitol yield with Gre3p. In bioreactor culture under anaerobic conditions, a small amount of xylitol (about 3.0 g liter^{-1}) was formed from 47.5 g of xylose liter^{-1} and 2.5 g of glucose liter^{-1} in 140 h by both strains, and the xylose was not consumed further. The strain with *P. stipitis* XDH maintained more biomass under anaerobic conditions than did the strain with the *S. cerevisiae* XDH (data not shown).

Transcript pattern of the endogenous xylose pathway genes. The glucose consumption and ethanol formation and subsequent consumption in cultures of nontransformed strains, CEN.PK2 and ENY.WA-1A, with and without xylose were similar (Fig. 3). Xylose metabolism and ethanol consumption began after glucose was exhausted. With the CEN.PK2 strain, biomass of the culture containing xylose and glucose continued to increase even after glucose, ethanol, and acetate consumption had stopped. The CEN.PK2 and ENY.WA-1A strains

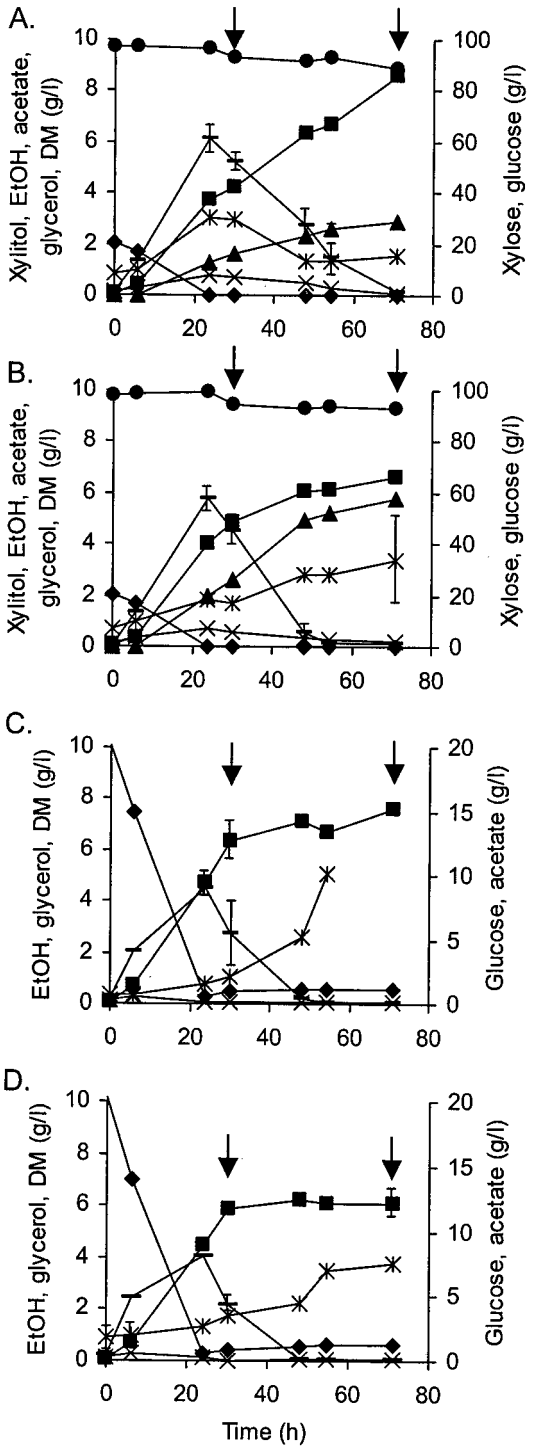

FIG. 3. Metabolism of nontransformed *S. cerevisiae* strains on glucose with and without xylose in shake flask cultures. Substrate consumption and main product formation in 100 g of xylose plus 20 g of glucose liter^{-1} (A, B) and 20 g of glucose liter^{-1} (C, D) media. Points without error bars have an associated error that is <10% of the value of the point. Results are based on two replications. Panels A and C, the CEN.PK2 strain; panels B and D, the ENY.WA-1A strain. DM (■), xylitol (▲), xylose (●), glucose (♦), ethanol (EtOH) (-), glycerol (×), and acetic acid (*), all in grams liter^{-1}. Time points for gene expression analysis are indicated by arrows.

used about 9.0 and 6.0 g of xylose liter^{-1} and produced 3.0 and 5.5 g of xylitol liter^{-1}, respectively. The ENY.WA-1A strain converted almost all of the xylose to xylitol, but the CEN.PK2 strain excreted only one-third of the xylose consumed as xylitol. This shows that *S. cerevisiae* strains differ in their ability to metabolize xylose.

RNA samples were taken at 29 h, after glucose depletion but while ethanol consumption and xylose metabolism were in progress, and at 70 h, when xylose alone was being slowly consumed. XR and XDH activities were measured at the same time.

ScXYL2 was not induced in the presence of xylose, but the expression in the presence of xylose remained two- to threefold higher than in the control culture at 70 h. The expression of *SOR1* increased 12- and 220-fold (time point, 29 h) and 21- and 3-fold (70 h) compared to that of the nontransformed control cultures (data not shown). The XDH activity was 30- and 125-fold higher at both 29 and 70 h for the ENY.WA-1A and CEN.PK2 strains, respectively (data not shown). Therefore, the presence of xylose resulted in higher XDH activity and induced the expression of the *SOR1* gene coding for SDH, which also has XDH activity.

The expression of *GRE3* was not affected by xylose in the ENY.WA-1A strain but was 2.6 times higher in the CEN.PK2 strain than in the control at 29 h (data not shown). XR activity was very similar in both cultures and was 4 and 20 times lower than the XDH activity for ENY.WA-1A and CEN.PK2, respectively (data not shown).

XKS1 was not induced in the presence of xylose, and its expression was 2 to 10 times lower than that in the control culture (data not shown). Furthermore, in the CEN.PK2 strain, the *XKS1* expression on xylulose was 1.6 times lower than in glucose-grown cells at the early growth phase (0.7 g of DM liter^{-1}, 5 h) and showed no increase during the later growth phase (2.1 g of DM liter^{-1}, 21 h; data not shown). A multicopy expression construct in which *XKS1* was controlled by an *ADH1* promoter had over 60 times higher expression than cells grown on xylulose. Thus, neither xylulose nor xylose induced *XKS1* expression.

The genes encoding transketolase (*TKL1*) and transaldolase (*TAL1*) were expressed at significantly higher levels in the presence of xylose than in the control culture. *TKL1* expression increased about 10-fold in both strains at 29 h, while *TAL1* expression increased by two- to eightfold (data not shown). At 72 h, *TKL1* expression was not much higher than in the control, but *TAL1* expression was still six- to eightfold higher than in the control.

DISCUSSION

Overexpression of the endogenous genes *GRE3* and *ScXYL2* enabled *S. cerevisiae* to grow on xylose in the presence of glucose in aerobic shake flask cultures. Relative to an *S. cerevisiae* strain expressing XR and XDH from *P. stipitis*, however, strains expressing the endogenous genes grew slower and accumulated more xylitol.

The accumulation of xylitol by the strain overexpressing *ScXYL2* could be due to low XDH activity, but the xylitol yield decreased by <10% when *ScXYL2* was replaced with the *P. stipitis XYL2*. A more likely explanation for the xylitol accumulation in the strain overexpressing the *S. cerevisiae GRE3* and *ScXYL2* is the strict NADPH specificity of Gre3p, since XRs accepting only NADPH as a cofactor cannot supply the NAD^+ needed by the XDH reaction (8). In anaerobic conditions, where NAD^+ regeneration is even lower due to lack of respiration, the strain overexpressing the endogenous enzymes was unable to utilize xylose. The NADPH specificity of Gre3p must create a severe redox imbalance, resulting in xylitol accumulation and the inability to metabolize xylose anaerobically.

The XR and XDH activities in xylose-utilizing yeasts (3, 5, 22, 36) and the XR activity in *S. cerevisiae* (4) increased when cells were grown on xylose. We detected an increase in only the XDH activity of *S. cerevisiae*. At the mRNA level, the *SOR1* gene encoding SDH was induced on xylose. Thus, the increased *SOR1* expression may lead to the higher SDH/XDH activity detected. The reason van Zyl and coworkers (44) did not detect XDH activity may be because they measured XDH activity in glucose-grown cells. Contradictory to Batt and coworkers (4) but in agreement with van Zyl and coworkers (44), we did not see an increase in the XR activity when *S. cerevisiae* was grown in the presence of xylose but detected a low level of activity with and without xylose. The differences between our results and the results of Batt and coworkers may be strain dependent.

Several putative aldose reductase (*GRE3*, *YPR1*, and *YJR096w*)- and polyol dehydrogenase (*ScXYL2*, *SOR1*, and *YDL246c*)-encoding genes exist in the *S. cerevisiae* genome, but no clear physiological functions have been attributed to the corresponding enzymes. It has been postulated that Ypr1p is involved in isoleucine catabolism and fusel alcohol formation, as it has activity with 2-methylbutyraldehyde (10). The *GRE3* gene is up-regulated in stress conditions, such as osmotic and oxidative stress, high temperature, and carbon starvation (2, 11, 30, 31). It may have a role in detoxification of methylglyoxal synthesized in response to stress (2). The transcriptional analysis of xylose and glucose cultures performed in our laboratory did not show any xylose-specific responses for these genes, except for *SOR1* (L. Salusjärvi, unpublished results).

The observation that the XDH activity and the *TKL1* and *TAL1* expression were induced in the presence of xylose, in addition to *S. cerevisiae* being able to grow on xylulose, suggests that *S. cerevisiae* has in its evolutionary past consumed xylose. On the other hand, the absence of induction on xylose and a wide substrate specificity of Gre3p indicate that the enzyme also has other roles. In the construction of recombinant, xylose-utilizing *S. cerevisiae* strains, the endogenous Gre3p and ScXDH cannot replace the *P. stipitis* XR and XDH enzymes before the redox constraints of the pathway are solved.

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PUBLICATION III

Metabolic engineering of *Saccharomyces cerevisiae* for conversion of D-glucose to xylitol and other five-carbon sugars and sugar alcohols

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PUBLICATION III

Metabolic engineering of Saccharomyces cerevisiae for conversion of D-glucose to xylitol and other five-carbon sugars and sugar alcohols

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Abstract

Recombinant *Saccharomyces cerevisiae* strains that produce sugar alcohols xylitol and ribitol and the pentose sugar D-ribose, from D-glucose, in a single fermentation step are described. A transketolase-deficient *S. cerevisiae* strain accumulated D-xylulose 5-phosphate intracellularly and released ribitol and pentose sugars (D-ribose, D-ribulose, D-xylulose) into the growth medium. Expression of the xylitol dehydrogenase encoding gene *XYL2* from *Pichia stipitis* in the transketolase-deficient strain resulted in 8.5-fold enhancement in the total amount of the excreted sugar alcohols ribitol and xylitol. The additional introduction of the 2-deoxy-glucose 6-phosphate phosphatase encoding gene, *DOG1*, into the transketolase-deficient strain expressing *XYL2* resulted in a further 1.6-fold increase in ribitol production. Finally, the deletion of the endogenous xylulokinase encoding gene, *XKS1*, was necessary to increase the amount of xylitol to 50% of the 5-carbon sugar alcohols excreted.

Keywords: *sugar alcohol, pentose sugar, S. cerevisiae, pentose phosphate pathway (PPP), transketolases, xylitol dehydrogenase, xylulokinase, 2-deoxy glucose 6-phosphate phosphatase*

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INTRODUCTION

Xylitol is a naturally-occurring 5-carbon sugar alcohol present in fruits and vegetables. Its sweetening power is comparable to that of sucrose, while the negative heat solution value gives a cool taste for this sugar alcohol. Xylitol inhibits dental caries and acute otitis media (18, 42), and is an ideal sweetener for diabetics because its metabolism is insulin-independent. Xylitol is used in products such as chewing gums, sweets and toothpaste. It is currently produced by chemical reduction, with a nickel catalyst, of the five-carbon sugar D-xylose from birch wood hydrolysates. D-xylose can also be reduced to xylitol with high yields by various yeast species (9, 43). Both processes rely on the hydrolysis and/or purification of D-xylose from lignocellulosic materials.

D-glucose, on the other hand, is a common substrate in the food industry and is, compared to D-xylose, cheap and readily available. Thus it would be attractive if xylitol could be produced from D-glucose. However, no natural micro-organisms are known that produce xylitol from D-glucose. *Bacillus*, *Zygosaccharomyces*, *Aureobasidium*, *Torula* and *Candida* species convert D-glucose to other sugars and sugar alcohols such as D-ribose, D-arabitol and erythritol (1, 4, 5, 12, 36). In D-ribose-producing *Bacillus* and *Candida* strains, the precursor is D-ribose 5-phosphate, a pentose phosphate pathway (PPP) intermediate. In these strains transketolase activity, catalyzing the conversion of D-xylulose 5-phosphate, and D-ribose 5-phosphate or erythrose 4-phosphate to C7 and C3, and C6 and C3 products, respectively, in the non-oxidative branch of the pentose phosphate pathway (PPP), is missing or defective (5, 13, 33). It was suggested that this results in the accumulation of D-ribose 5-phosphate and its dephosphorylation and excretion as D-ribose. Similarly, the PPP intermediates D-ribulose 5-phosphate or possibly xylulose 5-phosphate and erythrose 4-phosphate act as precursors for D-arabitol and erythritol, which are formed from the sugar phosphates by dephosphorylation and reduction reactions (1, 3, 11, 37). D-xylulose 5-phosphate and D-ribulose 5-phosphate also serve as precursors for D-arabitol when the D-arabitol phosphate dehydrogenase of *Enterococcus avium* is applied (22, 24). D-ribose, a drug precursor, and erythritol, a sweetener, are commercially produced from D-glucose by *Bacillus*, *Aureobasidium* and *Torula* species with a yield of almost 50% (w/w) of consumed D-glucose (5, 12, 14, 16).

Xylitol has been produced from D-glucose via D-arabitol and D-xylulose by Onishi and Suzuki in a three step fermentation process (21). D-glucose was converted to D-arabitol by *Debaryomyces hansenii*, then oxidized to D-xylulose by *Acetobacter suboxydans* and subsequently reduced to xylitol by *Candida guilliermondii*. Mayer and coworkers used a similar approach, but replaced the last fermentation step by an enzymatic *in vitro* reaction using xylitol dehydrogenase (19). These multi-step processes are, however, complex and less applicable to industrial scale. In addition, they require a high yield at each individual step to make the overall process feasible. Recently, a pentulose-producing *B. subtilis* was modified to produce xylitol from D-glucose with a yield of around 20% (w/w) by expressing a xylitol-phosphate dehydrogenase encoding gene in the host strain (23).

In the present study, we were able to demonstrate that D-glucose can be converted to xylitol by the yeast *S. cerevisiae* in a single fermentation step. We have shown earlier that a transketolase-deficient *S. cerevisiae* strain accumulated D-xylulose 5-phosphate (39). We now investigated whether the accumulation of this 5-carbon sugar phosphate could be redirected to xylitol by dephosphorylation of D-xylulose 5-phosphate and further reduction of D-xylulose to xylitol (Fig. 1a). Transketolase-deficient *S. cerevisiae* strains overexpressing xylitol dehydrogenase and a sugar phosphate phosphatase encoding genes were constructed. The phosphorylation of D-xylulose back to D-xylulose 5-phosphate was prevented by additionally deleting the endogenous xylulokinase encoding gene.

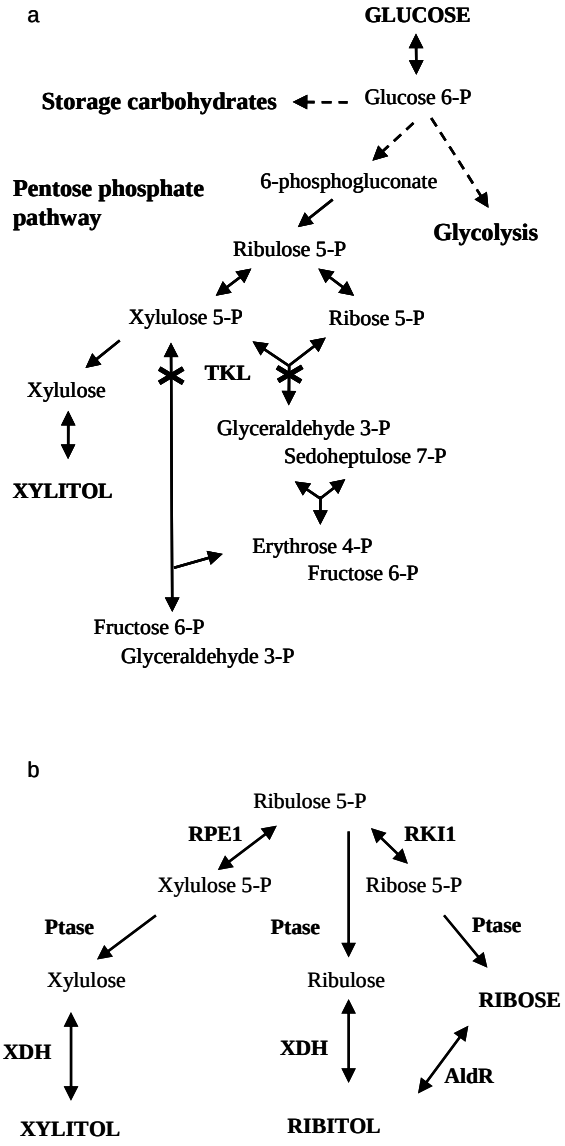

Figure 1. a. The pentose phosphate pathway of S. cerevisiae showing how xylitol could be formed from D-glucose. TKL; transketolase isoenzymes 1 and 2. b. The suggested routes to xylitol, ribitol and D-ribose in the metabolically engineered S. cerevisiae strains created in this study. RPE1; D-ribulose-phosphate 3-epimerase, RKI1; D-ribose-5-phosphate ketol-isomerase, Ptase; sugar phosphate phosphatase (e.g. Dog1p), XDH; xylitol dehydrogenase; AldR; nonspecific aldose reductase (e.g. Gre3p). All sugars presented are in D-configuration.

MATERIALS AND METHODS

Strains and strain constructions

Two yeast strains were used, W303-1B (40) and the transketolase-deficient strain (i.e. strain lacking transketolase activity) W303/tkl1 tkl2/2C, referred here as *tkl1,2* or H1055 (34). All strains constructed and used in this study are listed in Table 1. *E. coli* strain DH5α was used for bacterial cloning steps.

Table 1. Yeast strains used in the study. PsXYL2, XYL2 gene from P. stipitis; mc, gene expressed on a multicopy vector; sc, gene integrated as a single copy in the genome.

Denotation	Genotype (W303-1B) and modifications thereof	Source
W303-1B	MATα leu2-3/112 his3-11/15 trp1-1	(40)
(parental strain)	can1-100 ade2-1 ura3-1	
H1055	tkl1 tkl2	(34)
(W303/tkl1 tkl2/2C)		
H1506	tkl1 tkl2 PsXYL2sc	this work
H1520	tkl1 tkl2 PsXYL2sc DOG1mc	this work
H1514	DOG1mc	this work
H1518	YEplac195mc	this work
H1524	tkl1 tkl2 PsXYL2sc YEplac195mc	this work
H1852	tkl1,tkl2 xks1	this work
H1854	tkl1 tkl2 PsXYL2sc xks1	this work
H2282	tkl1 tkl2 PsXYL2sc xks1 DOG1mc	this work
H2284	tkl1 tkl2 PsXYL2sc xks1 YEplac195mc	this work

The plasmid pAOS66 containing the *XYL2* gene, encoding xylitol dehydrogenase (XDH) of *P. stipitis* under a modified *ADH1* promoter (30) in the BamHI site of pMA91 vector (20) was used as the source of the *XYL2* expression cassette for integration. The expression cassette was released from the plasmid pAOS66 as a 2.2 kbp BamHI fragment. To create an integration cassette targeted to *URA3* locus, a 1.2 kbp *URA3* fragment was cloned into the HindIII site of the bacterial cloning vector Bluescript SK (-) (Stratagene, CA, USA). The *XYL2* fragment was blunt ended by the Klenow enzyme and ligated into the *URA3* gene at NcoI site also made blunt ended with the Klenow enzyme. The resulting *XYL2* integration cassette was released with PvuII and HindIII

enzymes. The HindIII-fragment, containing the *XYL2* expression cassette flanked by *URA3* sequences, was purified from an agarose gel and used to transform the yeast strain H1055. 5-fluoroorotic acid (FOA) was used to select transformants which did not have a functional *URA3* gene (29). The correct integration was confirmed by measuring XDH activity from the crude cell extracts and by Southern blots. The resulting strain was named as H1506.

The 2-deoxy-glucose 6-phosphate (2-deoxy-glucose 6-P) phosphatase (Dog1p) encoding gene *DOG1* was amplified by PCR with the oligonucleotide pair 5'TGAGTAAGCTTATGGCAGAATTTTCAGCT3' and

5'TTGTCAAGCTTTTGTTTACTCAGGCCCTT3', using the vector YEp11HP as a template (31). The PCR fragment was digested with HindIII and ligated into the HindIII site located between the modified *ADH1* promoter (30) and *ADH1* terminator in the Bluescribe M13 vector. The resulting *ADH1* promoter – *DOG1* – *ADH1* terminator -cassette was released with BamHI and PvuII enzymes and the BamHI fragment was cloned into the BamHI site of the YEplac195 vector (8). The new plasmid was transformed into strain H1506, resulting in the strain H1520, and into the parental strain W303-1B, resulting in strain H1514. The respective control strains with the empty YEplac195 vector, but no expression cassette, were named as H1524 and H1518, respectively. The *S. cerevisiae* gene coding for xylulokinase was deleted as described previously (26). In short, the deletion cassette was transformed to the transketolase-deficient strain containing the integrated *XYL2* gene (H1506), resulting in strain H1854, and to the transketolase-deficient strain alone, resulting in the strain H1852. The YEplac195 vector alone or carrying the *DOG1* expression cassette was also transformed into the H1854 strain, resulting in strains H2284 and H2282, respectively. All yeast transformations were carried out using the lithium acetate method (7, 10).

Culture conditions

The recombinant and non-transformed strains were cultured in 50 ml growth medium in 250 ml Erlenmeyer flasks, with 250 rpm shaking at 30° C. D-glucose (20 g l^{-1}) was used as carbon source in modified Yeast Synthetic Complete (YSC) medium (35). For the selection of plasmid and integration transformants uracil and/or leucine were omitted from the growth medium.

Growth of yeast was monitored as increase in the optical density of the cultures at 600 nm (OD_{600}) at regular intervals, and supernatant samples were stored at -20º C. Ratio of one OD_{600} unit equals 0.3 g l^{-1} cell dry weight was used for biomass calculations.

Analyses of enzyme activities and metabolites

Enzyme activities were measured from cell extracts prepared by disrupting the cells with glass beads (∅ 425–600 μm, Sigma-Aldrich Corporation, MO) in 100 mM sodium phosphate buffer pH 7.0 for XDH activity and in 50 mM imidazole-HCl, 10 mM $MgCl_2$ pH 6.0 buffer for Dog1p activity. Phenylmethylsulfonyl fluoride (PMSF) and Pepstatin A were added as protease inhibitors in final concentrations of 0.17 mg ml^{-1} and 0.01 mg ml^{-1}, respectively.

The XDH activity was essentially determined as described earlier (27), with a Cobas Mira Plus automated analyzer (Roche, Switzerland). The specificity of Dog1p towards D-xylulose-5-P, D-ribulose-5-P, D-ribose-5-P and 2-deoxy-glucose 6-P was determined at substrate concentrations of 20 mM. Yeast cell extracts were prepared as described above. 10 μl cell extract and 210 μl substrate (20 mM) in the same buffer were mixed and incubated for 30 minutes at 30°C. The reaction was terminated with trichloro acetic acid (final concentration 2% (v/v)) and the phosphate released was measured with ammonium molybdate (15 mM) and zinc acetate (100 mM), pH 5.0. The formation of molybdenum blue was measured at 350 nm, and quantified against phosphate standards (2, 31). One unit (U) refers to amount of enzyme required to convert one μmole of substrate in one minute.

Pentose and hexose sugars were analyzed with HPLC from culture supernatant. The HPLC analyses were carried out with the Aminex HPX-87H Ion Exclusion Column (300 mm x 7.8 mm, Bio-Rad Laboratories, CA, USA) with 2.5 mM H_2SO_4 in water as the mobile phase, a flow rate of 0.3 ml min^{-1} at 55ºC. In order to see whether D-arabitol, which co-eluted with D-ribose and D-ribulose in the Aminex HPX-87H column, was produced, the DIONEX DX-500 device with CarboPac PA-10 column (Dionex Corporation, CA, USA) was used to analyze some of the culture supernatants. Analysis conditions were the following: column temperature 30°C, flow rate 1 ml min^{-1}; eluents A=water, B=100 mM NaOH, C=300 mM Na-acetate, 100 mM NaOH, D=300 mM NaOH. The first 21

minutes were run with 100% of A, from 21 to 40 minutes linear gradient up to 100% of B, followed by linear gradient of up to 50% B and 50% C in 20 minutes. The column was washed for four minutes with 100% of C and for three minutes with 100% of D. After washing steps, column was equilibrated for 15 minutes before next injection. Analyses not stated otherwise were performed with Aminex HPX-87H column, where the detection limit was 25 mg l^{-1}.

The Aminex HPX-87H column enabled measurement of D-glucose, ethanol, glycerol, D-xylulose, ribitol and xylitol and the co-eluted D-ribose, D-ribulose and D-arabitol. The D-ribose, D-ribulose and D-arabitol peak was manually integrated to separate it from the neighboring xylitol and ribitol peaks. The CarboPac PA-10 column enabled separation of D-arabitol from D-ribose and D-ribulose, but D-ribulose and D-xylulose co-eluted and their amounts were not quantitative due to degradation in alkaline pH.

RESULTS

Excretion of ribitol and pentose sugars by the transketolase-deficient yeast strain

Deletion of transketolase encoding genes results in accumulation of D-xylulose 5-phosphate (39), which could serve as a precursor for xylitol. To study whether xylitol and/or D-xylulose are excreted by the parental or transketolase-deficient strain, the strains were cultured on YSC medium containing 20 g D-glucose l^{-1} as the carbon source. The parental strain W303-1B produced essentially no extracellular 5-carbon sugars and sugar alcohols (maximally 20 mg l^{-1}, data not shown). The transketolase-deficient strain H1055, on the other hand, produced approximately 260 mg l^{-1} extracellular D-ribulose plus D-ribose which co-eluted in the HPLC, and approximately 60 mg l^{-1} ribitol (Fig. 2 a and Table 2). The concentration of the compounds in the culture medium increased with cultivation time. However, only a few milligrams of D-xylulose and no xylitol were observed in the culture medium (data not shown). When analyzed with the DIONEX CarboPac PA-10 column, the concentration of D-arabitol was found negligible.

Overexpression of the xylitol dehydrogenase encoding gene *XYL2* increases the amount of excreted ribitol, xylitol and D-ribose in the transketolase-deficient strain

Xylitol dehydrogenase catalyses the reduction of D-xylulose to xylitol but it also converts D-ribulose to ribitol, the equilibrium of the reaction being on the sugar alcohol side. *S. cerevisiae* does not have measurable XDH activity on D-glucose although it possesses genes coding for xylitol and sorbitol dehydrogenases (27, 32, 41). Therefore the *XYL2* gene of *P. stipitis*, encoding xylitol dehydrogenase, was introduced into *S. cerevisiae*; in a *XYL2* integrant strain H1506, the XDH activity was 1.3 U [mg total soluble proteins]$^{-1}$. The strain H1506 produced higher amounts of extracellular ribitol compared to the transketolase-deficient strain H1055 when cultured on YSC medium containing 20 g D-glucose l^{-1} as the carbon source (Fig. 2). In addition, xylitol was formed; approximately 30% of the total excreted xylitol plus ribitol in the medium was xylitol (Fig. 2b), in contrast to absence of xylitol in the transketolase-deficient strain (Fig. 2a). The amount of total xylitol plus ribitol was 8.5-fold higher in the H1506 strain than in the H1055 strain, but the total amount of 5-carbon sugars and sugar alcohols in the medium increased only 1.6-fold, as the amount of D-ribulose plus D-ribose concomitantly decreased in the H1506 strain.

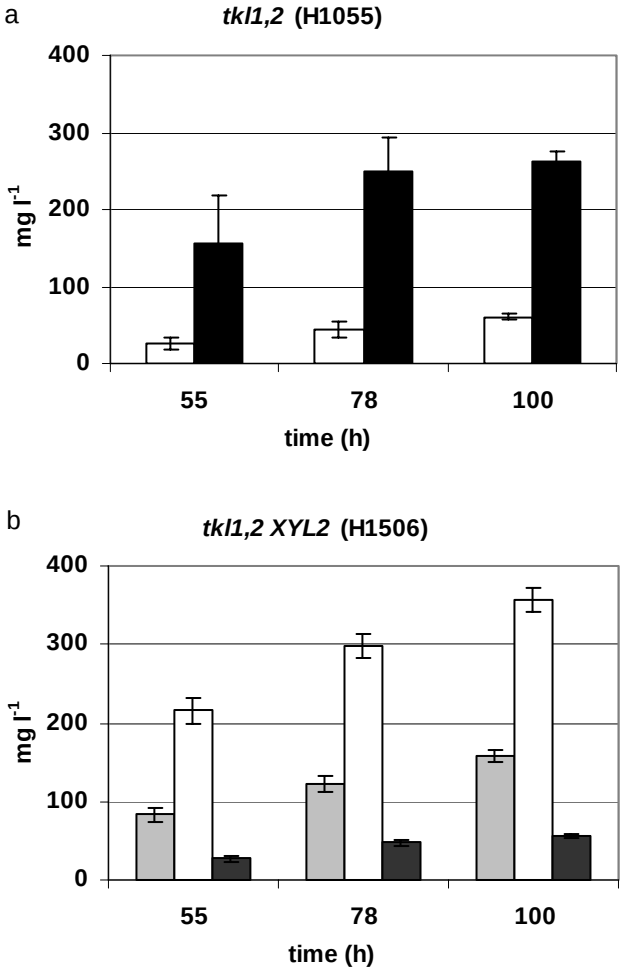

Figure 2. Volumetric production (mg l^{-1}) of extracellular pentose sugars and sugar alcohols by the transketolase-deficient strain H1055 (a) and the transketolase-deficient strain H1506 containing the XYL2 gene integrated to the genome (b), grown on YSC-medium with 20 g l^{-1} D-glucose as a carbon source in aerobic shake flask cultures. Production of sugar alcohols ribitol (white) and xylitol (grey), D-ribulose plus D-ribose (black) at 55, 78 and 100 h of culture. The 5-carbon sugars were analyzed by HPLC (see Materials and methods).

In a batch culture the strain H1506 exhibited a clear respiro-fermentative mode of growth; approximately 6 g l^{-1} ethanol was formed in 60 hours (Fig. 3). However, the strain ceased producing biomass, when still around 10 g l^{-1} D-

glucose was present in the growth medium, whereas production of ethanol, ribitol and xylitol continued until D-glucose was completely consumed. The final biomass produced by the transketolase-deficient strain was lower compared to the parental strain (approximately 0.9 g l^{-1} and 1.5 g l^{-1}, respectively), although the approximate specific growth rates of the strains did not vary much (0.11 and 0.13 g of cell dry weight h^{-1} for the transketolase-deficient and parental strain, respectively). Reduced accumulation of biomass of a transketolase-deficient strain compared to a parental strain was also noticed by Sundström and co-workers (38). Possibly, lack of transketolase activity provokes accumulation of toxic intermediates in the PPP or leads to insufficient production of NADPH and D-ribose 5-phosphate needed for anabolic reactions. This would explain the early cessation of biomass production as well as the relatively large variation in the final biomass accumulated by the H1506 strain (Fig. 3)

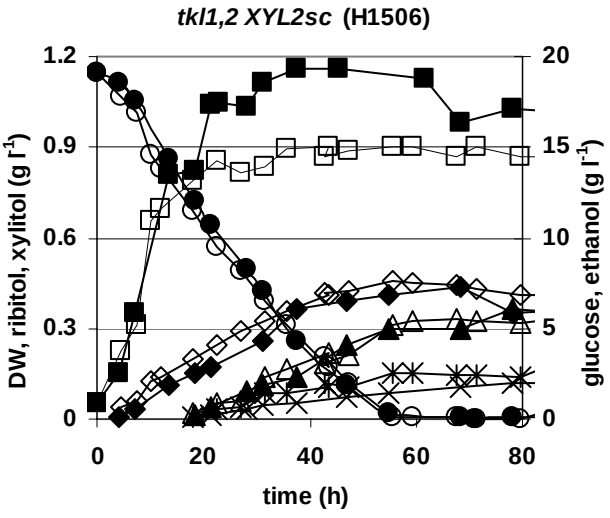

Figure 3. Volumetric consumption of D-glucose (g l^{-1}; circles) and production of biomass (dry weight, DW; squares), ethanol (g l^{-1}; diamonds), ribitol (g l^{-1}; triangles) and xylitol (g l^{-1}; cross and star) by the transketolase-deficient strain H1506, expressing the XYL2 gene integrated to the genome, in aerobic shake flask cultures on YSC-medium with 20 g l^{-1} D-glucose as a carbon source. Open and closed symbols indicate two identical, independent experiments.

Overexpression of the sugar phosphate phosphatase encoding gene *DOG1* increases the excretion of ribitol and D-ribose by the transketolase-deficient strain containing the integrated *XYL2* gene

A possible limiting step in the production of extracellular pentose sugars is the dephosphorylation of the corresponding sugar phosphates. The only cytoplasmic phosphatases known to be active on pentose phosphates are the 2-deoxy-glucose 6-P phosphatases Dog1p and Dog2p (25). The Dog1p was chosen for this study since it has higher activity towards D-ribose 5-phosphate and D-ribulose 5-phosphate compared to Dog2p (25). Our preliminary analyses indicated that, in addition to activity on 2-deoxy-glucose-6-P (100%), D-ribulose 5-phosphate (7%) and D-ribose 5-phosphate (42%), Dog1p also accepts D-xylulose 5-phosphate as a substrate, with ca. 15% activity compared to its major substrate 2-deoxy-glucose-6-P (data not shown).

When the *DOG1* gene was overexpressed in the transketolase-deficient strain H1520 with the *XYL2* gene integrated into its genome, the accumulation of extracellular ribitol was 1.6-fold higher than in the strain lacking *DOG1* (Table 2), whereas the fraction of D-ribulose plus D-ribose was higher by 3.8-fold (data not shown). The amount of xylitol, however, did not increase in the *DOG1* overexpressing strain, suggesting that this enzyme is indeed more efficient with D-ribose 5-phosphate as a substrate. The overexpression of *DOG1* had no effect on growth of the transketolase-deficient strains cultured on YSC medium, or to that of the parental strain (data not shown). The strain H1514, a parental strain overexpressing *DOG1*, had phosphatase activity of 0.06 U [mg total soluble proteins]$^{-1}$ compared to activity of 0.001 U [mg total soluble proteins]$^{-1}$ in the control strain with 2-deoxy-glucose 6-phosphate as a substrate.

Table 2. Production of the sugar alcohols xylitol and ribitol in different transketolase-deficient strains after 100 h incubation with 20 g l^{-1} glucose as a carbon source. Xol, xylitol; Rol, ribitol; DW, dry weight.

Genotype/denotation	Xol [a] mg l^{-1}	Rol [a] mg l^{-1}	DW g l^{-1}	Xol[b] (%)	Xol+Rol yield[c] (%)
tkl1 tkl2 (H1055)	-	60±10	0.49±0.05	-	0.5
tkl1 tkl2 XYL2 (H1506)	150±20	350±20	0.66±0.20	31	2.8
tkl1 tkl2 XYL2 DOG1 (H1520)	130±20	560±10	0.72±0.03	19	3.5
tkl1 tkl2 xks1 (H1852)	-	30±05	0.57±0.02	-	0.2
tkl1 tkl2 XYL2 xks1 (H1854)	300±10	270±10	0.67±0.03	52	2.9
tkl1 tkl2 XYL2 xks1 control[d] (H2284)	290±20	270±20	0.69±0.08	51	2.8
tkl1 tkl2 XYL2 xks1 DOG1 (H2282)	290±10	440±20	0.79±0.07	40	3.6

[a]Results are mean±STD, n=2-4.
[b] of total sugar alcohols xylitol and ribitol (w/w)
[c] on D-glucose (w/w)
[d] empty vector

Deletion of the xylulokinase encoding gene (*XKS1*) increases the proportion of xylitol excreted in the transketolase-deficient *XYL2* expressing strain

Xylulokinase activity in the parental strain (W303-1B) is known to be low and the strain grows only slowly on D-xylulose (26). In the transketolase-deficient strain containing the *XYL2* gene, deletion of the xylulokinase encoding gene, however, increased the amount of xylitol produced by 1.9-fold (Table 2), and concomitantly decreased ribitol production by 23%. D-ribulose plus D-ribose concentration was under 50 mg l^{-1} (data not shown). Once the Dog1p phosphatase was introduced into the xylulokinase-deficient strain, the amount of D-ribulose plus D-ribose excreted increased by a factor of 4 (data not shown) and the amount of ribitol 1.6-fold, whereas the amount of xylitol was not affected (Table 2).

At most, 730 mg l^{-1} or 3.6% of the D-glucose consumed (20 g l^{-1}) was converted to sugar alcohols (Table 2). The highest volumetric concentration was obtained

with the strain deficient in transketolase and xylulokinase activities and expressing the *XYL2* and *DOG1* genes.

DISCUSSION

A *S. cerevisiae* strain that is able to convert D-glucose to xylitol in a single fermentation step was constructed. The strain, deficient in both transketolase and xylulokinase activities, presumably accumulated D-xylulose 5-phosphate and redirected it to xylitol with the action of the introduced xylitol dehydrogenase. In addition, ribitol was formed.

The observation that ribitol, xylitol and D-ribulose plus D-ribose (the latter two not separated with the methods used in this study) were synthesized simultaneously may be explained by D-xylulose 5-phosphate, D-ribulose 5-phosphate and D-ribose 5-phosphate being present at similar concentrations in the cell (see Fig. 1). However, the need of NADPH and D-ribose 5-phosphate in the anabolic reactions as well as the lack of transketolase activity itself are likely to affect the equilibrium. Earlier studies (39) suggest that the concentration of D-xylulose 5-phosphate was about twice as high as that of D-ribulose 5-phosphate or D-ribose 5-phosphate in the transketolase-deficient strain. This is not, however, reflected by increased production of D-xylulose or xylitol. The fact that similar amounts of ribitol and xylitol were produced by the strain deficient in transketolase and xylulokinase activities and expressing the *XYL2* (strains H1854 and H2284) suggests that an unknown phosphatase in the transketolase-deficient strains dephosphorylated D-ribose 5-phosphate and D-ribulose 5-phosphate more efficiently than D-xylulose 5-phosphate. The Dog1p also favored ribitol and D-ribulose plus D-ribose production but whether the phosphatase activity in the transketolase-deficient strain is indeed Dog1p or possibly Dog2p is currently unknown.

Dephosphorylation of the 5-carbon sugar phosphates D-ribose 5-phosphate, D-xylulose 5-phosphate and D-ribulose 5-phosphate, result in D-ribose, D-xylulose and D-ribulose, respectively (Fig. 1b). The xylitol dehydrogenase of *P. stipitis* is able to reduce D-xylulose to xylitol as well as D-ribulose to ribitol (Fig. 1b) (28), and in addition, endogenous aldose reductases, Gre3p for example (15), are able to reduce D-ribose to ribitol. Thus, the ribitol observed may be derived

either from D-ribulose or D-ribose. Interestingly, while the xylulokinase activity in the parental W303-1B strain is low (26), it still phosphorylates D-xylulose, since deletion of the endogenous xylulokinase encoding gene resulted in higher amounts of xylitol produced. No D-xylulose was, however, detected in the medium in the transketolase-deficient strain deficient also in the xylulokinase activity.

In comparison with organisms that naturally produce 5-carbon sugars or those improved by classical mutagenesis (1, 4, 5, 12, 36), or the previously reported processes for xylitol production from D-glucose (19, 21, 23), the amount of 5-carbon sugars and sugar alcohols produced by the recombinant *S. cerevisiae* strains of this study were low. One very likely reason is the low capacity of the PPP in *S. cerevisiae*; less than 4% of D-glucose is channeled through this pathway during respiro-fermentative metabolism (6, 17)**.** On the other hand, in this study, production conditions were not optimized and parameters like aeration and biomass concentration are likely to affect the product yield. However, the pathway for xylitol production could be further engineered by forcing all glucose through the PPP by deleting the phosphoglucose isomerase encoding gene and simultaneously modifying the NADPH demand of the cell by introducing a transhydrogenase reaction. Moreover, through engineering of the epimerase and isomerase reactions, the balance of PPP sugar phosphates could possibly be altered. In addition, protein engineering could provide a sugar phosphate phosphatase specific for D-xylulose 5-phosphate.

The present study demonstrates the feasibility of engineering *S. cerevisiae* for the production of xylitol from D-glucose. Further studies are, however, needed to improve this method before xylitol production from D-glucose by *S. cerevisiae* would have commercial potential.

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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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