Metabolic Engineering of the Pentose Phosphate Pathway of Xylose Fermenting *Saccharomyces cerevisiae*

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Eliasson A., Boles E., Johansson B., Österberg M., Thevelein J. M., Spencer-Martins I., Juhnke H., and Hahn-Hägerdal B. Xylulose Fermentation by Mutant and Wild-type Strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology (2000) **53**(4)**:**376-382.

Paper II

Johansson B., Christensson C., Hobley T. and Hahn-Hägerdal B. Xylulokinase Over-expression in Two Strains of *Saccharomyces cerevisiae* also Expressing Xylose Reductase and Xylitol Dehydrogenase and Its Effect on Fermentation of Xylose and Ligno-cellulosic Hydrolysate. Applied and Environmental Microbiology (2001) **67**(9):4249-4255.

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Notation

AUR1-C	Dominant selectable marker, confers resistance to Aureobasidin A
E4P	Erythrose 4-phosphate
F6P	Fructose 6-phosphate
FCC	Flux control coefficient
G3P	Glyceraldehyde 3-phosphate
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
G6PDH	Glucose 6-phosphate dehydrogenase
GND1	Gene encoding the enzyme 6-PGDH
Non-oxidative PPP	Non-oxidative pentose phosphate pathway
ORF	Open reading frame
Oxidative PPP	Oxidative pentose phosphate pathway
6-PGDH	6-phosphogluconate dehydrogenase
PGI	Phosphoglucose isomerase
PGI1	Gene encoding the enzyme PGI
PPP	Pentose phosphate pathway
Ri5P	Ribulose 5-phosphate
RKI1	Gene encoding the enzyme RKI
RKI	D-ribose 5-phosphate ketol-isomerase
RPE1	Gene encoding the enzyme RPE
RPE	D-ribulose 5-phosphate 3-epimerase
Ru5P	Ribulose 5-phosphate
S7P	Sedoheptulose 7-phosphate
TAL1	Gene encoding the enzyme TAL
TAL	Transaldolase
TF	Transcription factor
TKL1	Gene encoding the enzyme TKL
TKL	Transketolase
XDH	Xylitol dehydrogenase
X5P	Xylulose 5-phosphate
XI	Xylose isomerase
XK	Xylulokinase
XKS1	Gene encoding the enzyme XK
XR	Xylose reductase
XYL1	Gene encoding the *Pichia stipitis* enzyme XR
XYL2	Gene encoding the *Pichia stipitis* enzyme XDH
xylA	Gene encoding the *Thermus thermophilus* enzyme XI
ZEO^R	Dominant selectable marker; confers resistance to zeocin
ZWF1	Gene encoding the enzyme G6PDH

All genes are from *Saccharomyces cerevisiae* unless stated otherwise.

Abstract

The aim of the work presented in this thesis is the improvement of xylose fermentation performance of recombinant *Saccharomyces cerevisiae* strains expressing *XYL1* and *XYL2* from *Pichia stipitis*, encoding xylose reductase (XR) and xylitol dehydrogenase (XDH). A recombinant strain of *S. cerevisiae,* fermenting both xylose and glucose to ethanol could decrease the production cost of ethanol from ligno-cellulosic biomass. The xylose fermentation efficiency of recombinant *S. cerevisiae* is limited by a slow xylose fermentation rate and high xylitol by-product formation.

Xylulose fermentation was used as a model of xylose fermentation, where the effect of the expression of the heterologous *XYL1* and *XYL2* is omitted. Xylulokinase over-production by over-expression of the native *XKS1* improved xylulose consumption rate of *S. cerevisiae*, but decreased xylose consumption rate by *S. cerevisiae* expressing *XYL1* and *XYL2*. This phenomenon was suggested to result from too high over-expression of the *XKS1* gene in the strain expressing *XYL1* and *XYL2*. *XKS1* over-expression decreased xylitol yield from both xylulose and xylose.

S. cerevisiae strains with a partly or completely blocked oxidative PPP, had a higher xylulose consumption rate than the control strains. This was interpreted as limiting capacity of the non-oxidative PPP, since blocking the oxidative PPP prevents the metabolic flux from this pathway from competing with the xylulose flux for the non-oxidative PPP capacity.

Blocking the oxidative PPP had a very different effect on xylose fermentation. Recombinant *S. cerevisiae* expressing *XYL1, XYL2*, over-expressing the native *XKS1* and blocked or partly blocked oxidative PPP fermented xylose slower than the control strains. Xylitol production also ceased as a result. The lower xylitol production was interpreted as a more NADH than NADPH dependent xylose reduction by the XR enzyme, and thus a better co-factor balanced xylose metabolism. The decrease in xylose consumption rate was probably the result of the absence of NADPH-dependent xylose reduction combined with inhibition of XR by higher intracellular levels of $NADP^+$. Over-expression of *XYL1* as well as allowing a low oxidative PPP activity increased the xylose consumption rate.

S.cerevisiae TMB3026 over-expressing the entire non-oxidative PPP, including the genes *RPE1, RKI1, TAL1* and *TKL1,* was constructed. This strain, also expressing the *XYL1, XYL2* and over-expressing *XKS1,* fermented xylose at the same rate as the control, *S.cerevisiae* TMB3001. This suggests that the non-oxidative PPP does not limit the xylose consumption rate in xylose-fermenting *S. cerevisiae* TMB3001.

A new expression vector was developed to achieve the multiple over-expressions of the non-oxidative pentose phosphate pathway (PPP) genes. This vector allows marker recycling, so the number of genetic modifications to a strain is independent of the number of available markers. Dominant markers were used to construct a xylose-fermenting wild-type *S. cerevisiae* strain.

Strains used with relevant genotypes and phenotypes

Strain	Relevant genotype or phenotype	Reference
CEN.PK	Expresses *XYL1, XYL2* on a 2μ plasmid	Paper II
TMB3001	CEN.PK 113-7A (*MAT*a *his3-D1 MAL2-8c SUC2) his3::YIp XR/XDH/XK* Expresses *XYL1, XYL2* and *XKS1*	Eliasson *et al*. (2000)
TMB3006	Based on Isolate #3. Expresses *XYL1, XYL2* and *XKS1*	Lindén and Hahn-Hägerdal (1989) for Isolate #3
TMB3008	Based on CEN.HJ 5-1B (*MAT*a *leu2-3,112 his3-D1 ura3-52 trp1-289 gnd1::HIS3 MAL2-8c SUC2*) *his3::*YIploxZEO Expresses *XYL1, XYL2* and *XKS1* No 6-PGDH activity, nor G6PDH activity	Paper III
TMB3026	Based on TMB3001 Over-expressing *RPE1*, *RKI1, TAL1* and *TKL1*	Paper V
TMB3030	Based on TMB3001; *CUP1-ZWF1*	Paper IV
TMB3034	Based on TMB3001; YRP34-*ZWF1*	Paper IV
TMB3035	Based on TMB3001; YRP25-*ZWF1*	Paper IV
TMB3037	Based on TMB3001; YRP13-*ZWF1*	Paper IV
TMB3250	ENY.WA-1A *his3*::YIp XR/XDH/XK Expresses *XYL1, XYL2* and *XKS1*	Paper III
TMB3251	RBY6-1 *his3*::YIp XR/XDH/XK Expresses *XYL1, XYL2* and *XKS1* 10-fold decreased PGI activity	Paper III
TMB3255	Based on TMB3001 *zwf1*::*KanMX* Expresses *XYL1, XYL2* and *XKS1* No G6PDH activity	Paper III

1 Introduction

1.1 Ligno-cellulose as a renewable feedstock for ethanol production

The world's resources of energy in the form of fossil fuels are believed to be limited, and not replenished at the rate of consumption. A solution to these finite resources is the use of renewable energy. Energy is considered to be renewable if the cycle time for its regeneration is relatively short. Solar energy, the only widely available source of renewable energy, can yield electricity by the use of solar cells or windmills or by combustion of solid biomass. None of these energy forms are suitable for replacing liquid fuel for transportation using existing internal combustion engine technology. Another alternative is to produce renewable energy by ethanolic fermentation of biomass carbohydrates by a micro-organism. The advantage of ethanol is that it can be used directly to replace all or part of the fuel for engines designed for petrol, with little modification. The most abundant form of biomass is plant and woody biomass i.e. ligno-cellulose. Softwood, hardwood, sugar-cane bagasse and other agricultural residues have been considered as feedstock for ethanol production (Hayn *et al.*, 1993).

1.2 The sugars in biomass

Ligno-cellulose contains cellulose, hemi-cellulose and lignin. Cellulose is a linear glucose polymer where the subunits are linked through β-1,4-linkages. Hemi-cellulose is mixture of polymers consisting of the pentose sugars xylose and arabinose and the hexose sugars glucose, galactose and mannose. The sugar polymers in ligno-cellulose are liberated and hydrolysed by either acid alone (Parisi, 1989) or in combination with enzymatic hydrolysis (Ladish *et al.*, 1983). Hydrolysis also yields fermentative inhibitors (Larsson *et al.*, 1999), which are an important process consideration. A ligno-cellulosic hydrolysate contains mainly glucose, galactose, mannose, xylose and arabinose in different proportions, depending on the nature of the raw material. Softwood (e.g. pine) contains low amounts of xylose while hardwood (e.g. birch) and sugar-cane bagasse contain higher amounts.

1.3 Economy of ethanol production

The production cost of ethanol must be very low to be competitive with petrol. The cost of the raw material has the greatest impact on the cost of the production of ethanol from ligno-cellulose (von Sivers *et al.*, 1994). Ethanolic xylose fermentation can decrease the production cost of ethanol by 25% (Hinman *et al.*, 1989) and would make the process more competitive. Baker's yeast, *Saccharomyces cerevisiae*, is the preferred micro-organism for industrial ethanol production. This organism ferments the hexose sugars present in ligno-

cellulosic hydrolysate to ethanol with high yield and productivity, but cannot ferment the pentoses xylose and arabinose.

1.4 Naturally xylose-fermenting yeasts

Yeasts such as *Pichia stipitis*, *Pachysolen tannophilus* and *Candida utilis* can ferment xylose (Toivola *et al.*, 1984; Schneider *et al.*, 1981; Slininger *et al.*, 1982; Bruinenberg *et al.*, 1983a) in contrast to *S. cerevisiae*. A specific productivity of 0.20 g ethanol/g xylose per hour and a yield of 0.48 g ethanol/g xylose have been obtained with *P. stipitis* (Skoog and Hahn-Hägerdal, 1990). However, *P. stipitis* has an ethanol productivity of 0.38 g ethanol /g biomass per hour (Skoog *et al.*, 1992) which is only about one fifth of the theoretical ethanol productivity of *S. cerevisiae* from glucose (around 2 g ethanol /g biomass per hour).

P. stipitis also has a lower ethanol tolerance (du Preez *et al.*, 1987; du Preez *et al.*, 1989) than *S. cerevisiae* making it less suitable for industrial ethanol production. *P. stipitis* has been observed to perform less efficiently than *S. cerevisiae* in the harsh environment of ligno-cellulosic hydrolysates (Lindén and Hahn-Hägerdal, 1989; Hahn-Hägerdal *et al.*, 1994).

S. cerevisiae is able to grow anaerobically (Andreasen and Stier, 1953 and 1954). *P. stipitis* is unable to grow anaerobically and also requires low, well controlled amounts of oxygen for optimal performance (Skoog and Hahn-Hägerdal, 1990; Skoog *et al.*, 1992). In an industrial process, it would be difficult to control the oxygen supply at these low levels. *P. stipitis* can be engineered for anaerobic growth on glucose by expression of *S. cerevisiae URA1*, encoding dihydro-orotate dehydrogenase (Shi and Jeffries, 1998), but anaerobic growth on xylose is still absent.

1.5 Recombinant bacteria

The bacterium *Escherichia coli* has been engineered with the ethanologenic enzymes pyruvate decarboxylase and alcohol dehydrogenase from the ethanol producing bacterium *Zymomonas mobilis* resulting in an efficient xylose-fermenting organism (Ohta *et al.*, 1991). However, *E. coli* is a potential pathogen, so spent biomass cannot be used directly as for example fertilizer. *E. coli* fermentation is carried out at neutral pH, which makes the fermentation sensitive to infections.

Zymomonas mobilis has also been engineered with the xylose metabolic enzymes and the non-oxidative PPP of *E. coli* creating a very efficient xylose-fermenting organism (Zhang *et al.*, 1995; Joachimsthal and Rogers, 2000). *Z. mobilis* is similarly to *S. cerevisiae,* Generally

Regarded As Safe (GRAS), so spent biomass could be used in the same way as spent *S. cerevisiae* would. However, this organism appears to be sensitive to acetic acid (Kim *et al.*, 2000), which is always present in ligno-cellulosic hydrolysates. Zang *et al.* (1995) constructed a strain with near theoretical conversion of xylose and glucose to ethanol, but there are yet no reports on large-scale ethanol production with this strain.

1.6 Xylose fermentation by recombinant *S. cerevisiae*

S. cerevisiae has been used for long time for industrial ethanol production and the organism is well suited for large scale fermentations in harsh environment. Physiology, genetics and genetic engineering of this organism are well known and the entire genome sequence is available. *S. cerevisiae* was chosen for these reasons despite the lack of natural xylose-fermenting capability.

Bacteria ferment xylose through the expression *xylA*, encoding xylose isomerase (XI). This enzyme carries out the isomerisation of xylose to xylulose in a single step (Figure 1). *S. cerevisiae* has the ability to ferment xylulose (Wang and Schneider, 1980). Many attempts have been made to express xylose isomerase in yeast, but all have failed (Ho *et al.*, 1983; Sarthy *et al.*, 1987; Amore *et al.*, 1989; Hallborn, 1995; Moes *et al.*, 1996) except in the case of the *xylA* of the thermophilic organism *Thermus thermophilus* (Walfridsson *et al.*, 1996). This enzyme is expressed at low levels and the resulting recombinant *S. cerevisiae* ferments xylose slowly (Walfridsson *et al.*, 1996). The low rate is probably due to the fact that the enzyme retains only a fraction of its activity at the temperature of yeast fermentation (30-40ºC) and that the gene may suffer from low expression efficiency due to sub-optimal codon usage (Walfridsson *et al.*, 1996).

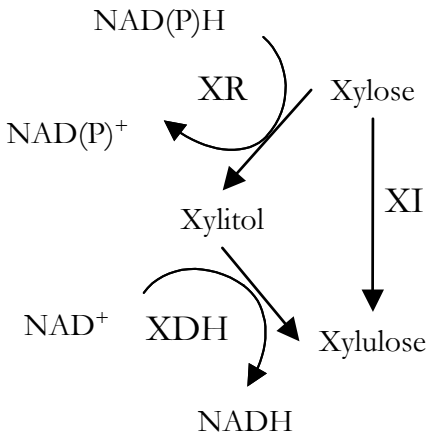


Figure 1. Initial xylose metabolism in yeast (XR and XDH) and bacteria (XI).

Faster xylose fermentation by *Saccharomyces cerevisiae* has been achieved by the expression of two genes, *XYL1* and *XYL2* (Kötter *et al.*, 1990; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1997), from the xylose-fermenting yeast *P. stipitis* encoding the enzymes xylose reductase (XR) (Kötter *et al.*, 1990; Hallborn *et al.*, 1991) and xylitol dehydrogenase (XDH) (Kötter *et al.*, 1990; Figure 1). XR reduces xylose to xylitol using NADH or preferably NADPH (Rizzi *et al.*, 1988). Xylitol is oxidised to xylulose by XDH using NAD^+ exclusively (Rizzi *et al.*, 1989; Figure 1). Xylose fermentation of such strains is still about an order of magnitude slower than glucose fermentation and a large fraction of the consumed xylose is turned into the by-product xylitol.

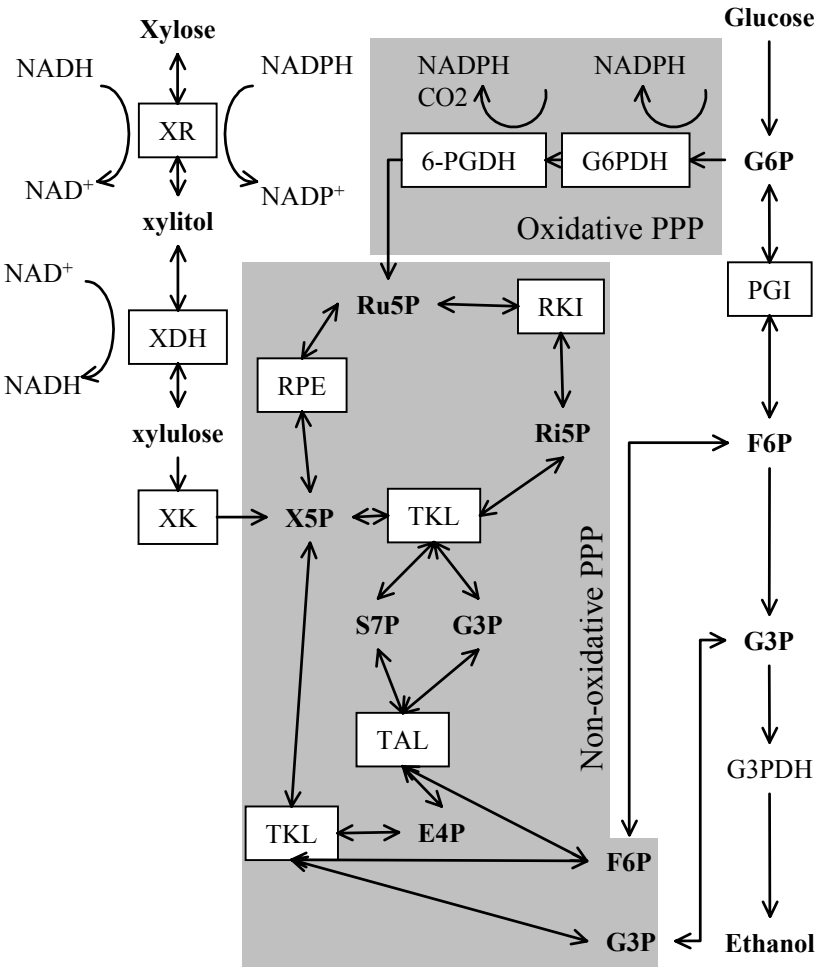


Figure 2. Model of anaerobic xylose and glucose metabolism in yeast. The enzymatic step going to and from 6-phosphogluconolactone in the oxidative PPP has been omitted for clarity, as well as some parts of glycolysis. The oxidative PPP and the non-oxidative PPP are shaded separately. Enzymes that were engineered as a part of work presented in this thesis are enclosed in boxes. Metabolites are in bold. Enzyme abbreviations are given in the Notation section.

All yeasts are assumed to metabolise xylose through the non-oxidative PPP (Figure 2). Carbon labelling experiments (Ligthelm *et al.*, 1988) and blocked pentose utilisation of non-oxidative PPP mutants (Paper I) provide evidence of that this is the main catabolic pathway for xylose. There has been a report on the presence of a xylulose 5-phosphate phosphoketolase in xylose-fermenting yeasts (Evans and Ratledge, 1984). This enzyme converts xylulose 5-phosphate to glyceraldehydes 3-phosphate and acteyl phosphate, by-passing the non-oxidative PPP. However, the presence of this enzyme has not been confirmed in other reports.

1.7 Outline of the research

The aim of the work presented in this thesis was to investigate the role of the oxidative and non-oxidative PPP in xylose fermentation by recombinant *S. cerevisiae*.

In the work described in Paper I, xylulose fermentation was used as a model system to study the effect of xylulokinase over-production and the effects of various PPP and glycolysis mutations on pentose fermentation. The effects of xylulokinase over-production on xylose fermentation in various media, including ligno-cellulosic hydrolysate are described in Paper II.

A blocked oxidative PPP was shown to decrease by-product xylitol yield, but also lower xylose fermentation rate (Paper III). In Paper IV, *XYL1* over-expression and oxidative PPP down regulation were studied as ways of increasing xylose fermentation rate of strains with blocked oxidative PPP.

A tool was developed (Paper V) and used generate a strain over-producing all non-oxidative PPP enzymes. Non-oxidative PPP over-production was found to have no effect on xylose consumption rate (Paper VI).

Additionally, the construction of a xylose fermenting ligno-cellulosic hydrolysate tolerant wild-type *S. cerevisiae* is described in Chapter 3.

2 The pentose phosphate pathway

The oxidative PPP is the major source of reducing power in the form of NADPH in *S. cerevisiae* (Nogae and Johnston, 1990) and other yeasts (Bruinenberg *et al.*, 1983a). This conclusion has been drawn partly from the finding that oxidative PPP mutants are sensitive to oxidative stress, such as exposure to hydrogen peroxide (Nogae and Johnston, 1990; Juhnke *et al.*, 1996), presumably for low capability of NADPH production. In agreement, exposure to hydrogen peroxide permitted growth on glucose of the otherwise glucose negative phosphoglucose isomerase (PGI) mutant (*pgi1*⊿). The *pgi1*⊿ mutation causes glucose to be metabolised through the oxidative PPP resulting in $NADP^+$ depletion (Boles *et al.*, 1993). Hydrogen peroxide metabolism is presumed to result in recycling of the $NADP^+$ (Boles *et al.*, 1993), and thereby restoring growth.

The non-oxidative PPP in *S. cerevisiae* produces biosynthetic precursors such as D-ribose 5-phosphate (Ri5P) and erythrose 4-phosphate (E4P). It has been estimated that between 2-30 % of the glucose is metabolised through the PPP during glucose fermentation (Bruinenberg *et al.*, 1983b; Gancedo and Lagunas, 1973).

2.1 Xylulose fermentation

Xylulose fermentation has been studied in the fermentation of xylose containing ligno-cellulosic hydrolysates with xylose isomerase and natural *S. cerevisiae*. (Lindén and Hahn-Hägerdal, 1989; Chandrakant and Bisaria, 2000). Xylulose fermentation is also useful for analytical reasons, as the physiological effect of pentose fermentation is isolated from the effect of the expression of *XYL1* and *XYL2*.

S. cerevisiae ferments xylulose at about one tenth of the rate of glucose fermentation (Yu *et al.*, 1995). This suggests that some of the reasons for slow xylose fermentation by *S. cerevisiae* are not connected to the introduced heterologous pathway consisting of XR and XDH.

2.1.1 Xylulokinase over-expression

Xylulokinase (XK) catalyses the phosphorylation of xylulose to xylulose 5-phosphate (X5P) using ATP (Figure 2). This is the first step in xylulose utilisation. Reported XK activities in *S. cerevisiae* have ranged from undetectable (Papers I & II; Jeppsson *et al.*, 1996) to about one fifth of the activity of XK in *P. stipitis* (Deng and Ho, 1990).

XK over production in CEN.PK2-1C/D increased xylulose consumption in batch-culture by approximately 50 % and reduced xylitol yield to undetectable levels (Paper I). In agreement with this, XK over-production has been reported to increase the *S. cerevisiae* growth rate on xylulose by about three times (Richard *et al.*, 2000). In contrast, XK over-production inhibited growth in xylulose medium (Rodriguez-Pena *et al.*, 1998). No inhibition was seen on glucose medium, linking the inhibition to xylulose metabolism (Rodriguez-Pena *et al.*, 1998). The reason for this discrepancy is not clear, but most available data suggest that XK activity limits the xylulose consumption rate.

2.1.2 Pentose phosphate pathway and glycolysis mutants

Disruption of *RPE1*, encoding ribulose 5-phosphate epimerase (RPE), resulted in blocked xylulose fermentation (Paper I). This adds to the evidence that the non-oxidative PPP is the main xylose metabolic pathway (Ligthelm *et al.*, 1988) and suggests that X5P phosphoketolase (Evans and Ratledge, 1984) is not present in *S. cerevisiae.*

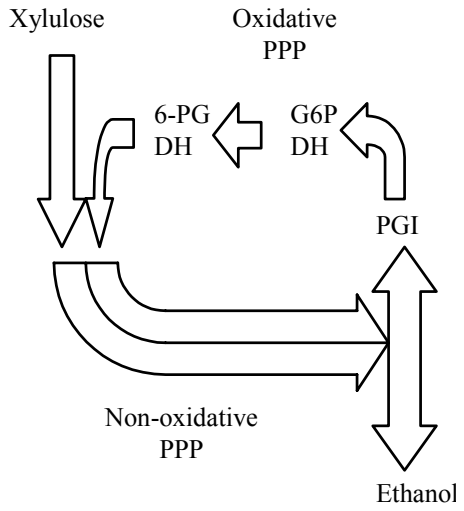


Figure 3. Putative carbon cycle explaining the increased xylulose fermentation capacity of *gnd1*⊿ and *pgi* ⊿ mutants. Oxidative PPP flux is abolished in *gnd1*⊿ and *pgi*⊿ releasing capacity in the non-oxidative PPP.

Disruption of *GND1*, encoding 6-phosphogluconate dehydrogenase (6-PGDH) and down regulation of PGI activity resulted in 20 % and 31 % higher xylulose fermentation rates (Paper I). One common denominator of these genetic alterations is that both could result in decreased flux through the oxidative PPP during xylulose fermentation (Figure 2). Lowering of the oxidative PPP flux could result directly from the abolishment of 6-PGDH activity or indirectly by lower gluconeogenetic flux as a consequence of reduced PGI activity (Figure 2).

The oxidative PPP forms a carbon cycle together with the non-oxidative PPP and gluconeogenesis (Figure 3) where glucose 6-phosphate (G6P), ribulose 5-phosphate (Ri5P), fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (G3P) are common metabolites (Figure 2). It is conceivable that cycling of PPP intermediates could compete with the xylulose entering as xylulose 5-phosphate, for non-oxidative PPP capacity and

result in the higher observed fluxes of xylulose, when this cycle is blocked (Paper I, Figure 3).

2.2 The effect of xylulokinase over-expression on xylose fermentation

Fast growing mutants of *Candida sp., P. tannophilus*, and recombinant *S. cerevisiae* showing faster xylose utilisation and higher ethanol yields, have been found to exhibit elevated levels of XK activity (McCracken and Gong, 1983; Ciriacy and Porep, 1986; Lachke and Jeffries, 1986; Tantirungkij *et al.*, 1994).

The *S. cerevisiae* gene *XKS1*, encoding XK, was first cloned by Ho and Tsao (1993) and used to construct a *S. cerevisiae* strain expressing *XYL1*, *XYL2* and over-expressing *XKS1* (Ho *et al.*, 1998). The open reading frame (ORF) published by Ho and Tsao (1993) was too short to encode an active XK (Paper II). However, the gene used to create the XK over-expressing strain was correct, since a larger piece of DNA was cloned, containing the entire gene (Ho *et al.*, 1998). The correct version of the gene now appears as the yeast ORF YGR194c/*XKS1* in the databases (Arroyo *et al.*, 1995; Rodriguez-Pena *et al.*, 1998).

XKS1 over-expression was reported to enhance the xylose consumption rate as well as the ethanol yield and to lower the xylitol yield (Ho *et al.*, 1998). However, no comparison was made of xylose fermentation capacity between a strain expressing *XYL1* and *XYL2* and a strain expressing *XYL1*, *XYL2* and *XKS1*. Instead, this conclusion was drawn from the higher apparent xylose consumption rate of the strain expressing *XYL1, XYL2* and *XKS1* (Ho *et al.*, 1998) compared with previous reports of *S. cerevisiae* expressing only *XYL1* and *XYL2* (Kötter and Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1997).

The weakness of this reasoning is that different strains were used, so the results are not directly comparable. Furthermore, the estimated value of the specific xylose consumption rate of the XK over-producing strain (Ho *et al.*, 1998) is similar to that of the *XYL1-XYL2* expressing strains studied in Paper II (Figure 3), so therefore the higher apparent xylose consumption rate may be the result of the use of high amounts of biomass rather than XK over-production.

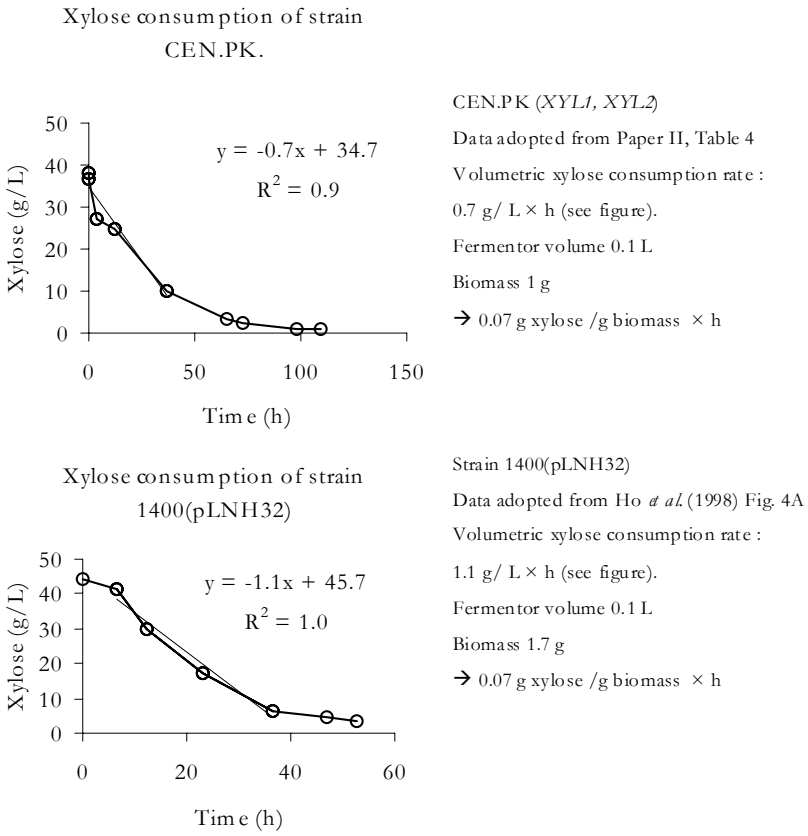


Figure 3. Comparison of specific xylose consumption rates between CEN.PK expressing *XYL1* and *XYL2* (Paper II) and strain 1400(pLNH32) (Ho *et al.* 1998). The biomass was given in OD_{600} units (Ho *et al.* 1998) and has been recalculated to biomass using the approximate relationship of 5 OD_{600} = 1 g/L biomass. Linear curve fitting was done to calculate the xylose consumption rate from the data points.

XK over-production increased ethanol yield and decreased xylitol yield but lowered xylose consumption rate in strains expressing *XYL1* and *XYL2*, regardless of media (Paper II). On the contrary, XK over-production was recently shown to increase the xylose consumption rate in strain H1691, expressing *XYL1* and *XYL2* in contrast to the results presented in Paper II (Toivari *et al.*, 2001).

The differences between CEN.PK (Paper II) and H1691 (Toivari *et al.*, 2001) are intriguing, since the same *S. cerevisiae* strain (van Dijken *et al.*, 2000) was used as host strain in both cases. One difference between the strains is the relatively low XR, XDH and XK activities of the H1691 compared to the CEN.PK (Table 1).

Table 1. Specific activities of XR, XDH, XK, the promoters used to control the respective genes and the vectors used in different recombinant *S. cerevisiae.* *) The ADH1 promoter was modified for constitutive expression. †) *XYL1* and *XYL2* were integrated into the chromosome while *XKS1* was expressed on a 2μ based plasmid.

Strain	Prom.	XR	Prom.	XDH	Prom.	XK	Vect.	Reference
CEN.PK-pXKs	*ADH1*	0.7	*PGK1*	18.0	*PGK1*	36.0	2μ	(Paper II)
H1691	*PGK1*	0.2	*ADH1**	0.1	*ADH1**	0.4	2μ/Int.†	(Toivari *et al.* 2001)
1400(pLNH32)	*ADH1*	0.4	*PYK1*	0.8	*PYK1*	0.1	2μ	(Ho *et al.* 1998)
TMB 3001	*ADH1*	0.5	*PGK1*	3.1	*PGK1*	1.6	Int.	(Eliasson *et al.* 2000)

The decreased xylose utilisation in CEN.PK upon *XKS1* over-expression may have been caused by high XK activity since *XKS1* was over expressed to a level two orders of magnitudes higher than in H1691 (Table 1). A mathematical model of glucose metabolism (Teusink *et al.*, 1998) suggested that high, uncontrolled sugar kinase activity at the beginning of a metabolic pathway could lead to abnormal accumulation of sugar phosphates and concomitant depletion of the intracellular ATP pool (Paper II).

A kinetic model of the initial xylose metabolism, disregarding any negative effects of XK over-expression, has shown XK over-production to be necessary for ethanol production with *S. cerevisiae* also expressing *XYL1* and *XYL2* (Eliasson *et al.*, 2001). This implies that the XK activity level should be over-produced at an intermediate level.

2.3 The oxidative pentose phosphate pathway

Completely or partially blocked oxidative PPP resulted in increased xylulose fermentation rate. (Paper I). In the following section, the effect of a blocked oxidative PPP on xylose fermentation will be described.

2.3.1 Inactivation of the oxidative PPP

The *GND1* mutant strain CEN.HJ5-1B (Juhnke *et al.*, 1996) was transformed with the YIploxZEO vector (Paper III, Chapter 3) to create a xylose-fermenting oxidative PPP mutant strain of *S. cerevisiae* called TMB3008 (Paper III). The *HIS3* gene had previously been used to remove *GND1*, preventing the use of the vector YIpXR/XDH/XK (Eliasson *et al.*, 2000). The construction if YIploxZEO is described in Chapter 3. A glucose positive revertant of this strain was shown to ferment xylose more slowly (Paper III) than the control strain TMB3001 (Eliasson *et al.*, 2000), but with a considerably lower xylitol yield and higher ethanol yield. This shows that the suggested competition for non-oxidative PPP capacity proposed for xylulose fermentation (Figure 3) did not occur during xylose fermentation.

The results rather suggested that the oxidative PPP is responsible for much of the xylitol formation in xylose-fermenting *S. cerevisiae* through production of NADPH and concomitant NADPH dependent xylose reduction. A *ZWF1* deletion strain of TMB3001 was constructed called TMB3255 (Paper III). This strain showed a similar phenotype to TMB3008 (*gnd1*⊿) connecting the low xylitol yield to the inactive oxidative PPP (Figure 2). TMB3255 showed even lower xylitol yield than TMB3008, but also an even lower xylose consumption rate (Paper III). This more stringent phenotype of TMB3255 could be due to the oxidative PPP being completely blocked in this strain, whereas it may be partly active in TMB3008 due to the presence of an isoenzyme of 6PGDH, encoded by *GND2* (Lobo and Maitra, 1982). However, enzyme activity measurements of 6-PGDH and G6PDH did not show that TMB3008 has a more active oxidative PPP than TMB3255, though this may be limited by the sensitivity of the assays (Paper III).

2.3.2 Synthetic promoters.

Disruption of the *ZWF1* gene led to low xylitol yields, but also lowered xylose consumption rate to one sixth of the reference strain (Paper III). This suggests that *ZWF1* should not be disrupted, but rather under expressed at some optimal level where low xylitol

yield could perhaps be achieved together with higher xylose consumption rate. Exchanging the wild-type promoter for a weaker one could lower the expression level of *ZWF1*.

Vectors with a wide range of promoter strengths have been developed (Mumberg *et al.*, 1995). Most of the promoters are very strong and the differences in promoter strength between them are considerable. An alternative approach is to develop a library of synthetic promoters with different strengths in much closer intervals, to facilitate expression optimisation. This strategy has been successful for *E. coli* and lactic acid bacteria (Jensen and Hammer, 1998a; 1998b)

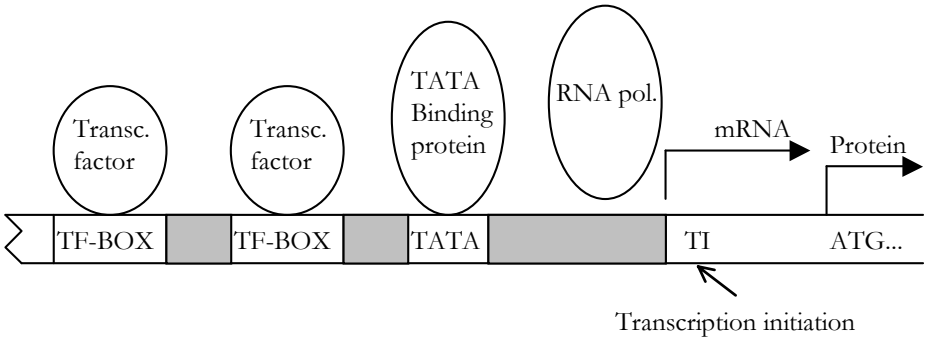


Figure 4. Schematic view of a yeast promoter. The transcription factor binding sites (TF-box and TATA) as well as the intervening sequences (grey) has impact on promoter strength.

Promoters are made up of a number of transcription factor (TF) binding sites with a more or less conserved distances between the TF binding sites and the initiation of transcription (Figure 4). Small changes in the nucleotide sequence of the TF binding site may have a profound effect on TF affinity for the promoter and thereby affect promoter strength. The intervening sequences (shown in grey in Figure 4) also controls promoter strength (Jensen and Hammer, 1998b). The idea is that different intervening spacer nucleotide sequences make the promoter wind differently and make cooperative binding of transcription factors more or less probable. The so-called helical phasing of transcription factor binding sites is

```
    EcoRI                CT        RPG              RPG          CT
ATCAGAATTCTCGAGNNNNNCTTCCNNNNNACCCATACANNNNNNNNACCCATACANNNNNCTTCCNNNNNNNNNNNNNNNN

          TATA-box
NNNNNNNNNNNNNNNTATAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTTCTTTCTTGTAACATCTCTCT

TGTAATTCCTTATTCCTTCTAGCTATTTTTCATAAAAAACCAAGCAACTGCTTATCAACACACAAACACTA
      BamHI
AATCAAAATGGATCCCGAT
```

Figure 5. The base sequence of the synthetic promoter YRP. N=25% of each base, transcription factor binding boxes has 3% error in each position.

important for promoter strength of the *S. cerevisiae ADH2* promoter (Donoviel *et al.*, 1995).

A promoter library was made from degenerate oligonucleotides as shown in Figure 5 (Paper IV). In the sequence of bases, N represents 25 % of each of the four bases. The TF binding sites, the CT-boxes and the RPG-boxes have 1% of the three other bases in each position. The oligonucleotides were designed to have two RPG-boxes and two CT-boxes. These TF binding sites bind the transcription factors RAP1p and GCR1p and are common to highly expressed genes, such as glycolytic and ribosmal protein genes (Rotenberg and Woolford, 1986; Stanway *et al.*, 1989; Santangelo and Tornow, 1990; Kuroda *et al.*, 1994).

The distance between the RPG-box and the CT-box is the same as in the glycolytic pyruvate kinase promoter, wich has been shown to be the facilitate interaction between GCR1p and RAP1p (Drazinic *et al.*, 1996). The distance between the RPG-boxes was chosen to be the same distance as in the ribosomal protein promoter RP39A (Rotenberg and Woolford, 1986).

Different promoter clones showed a variation of strengths (Paper IV, Figure 6) when used

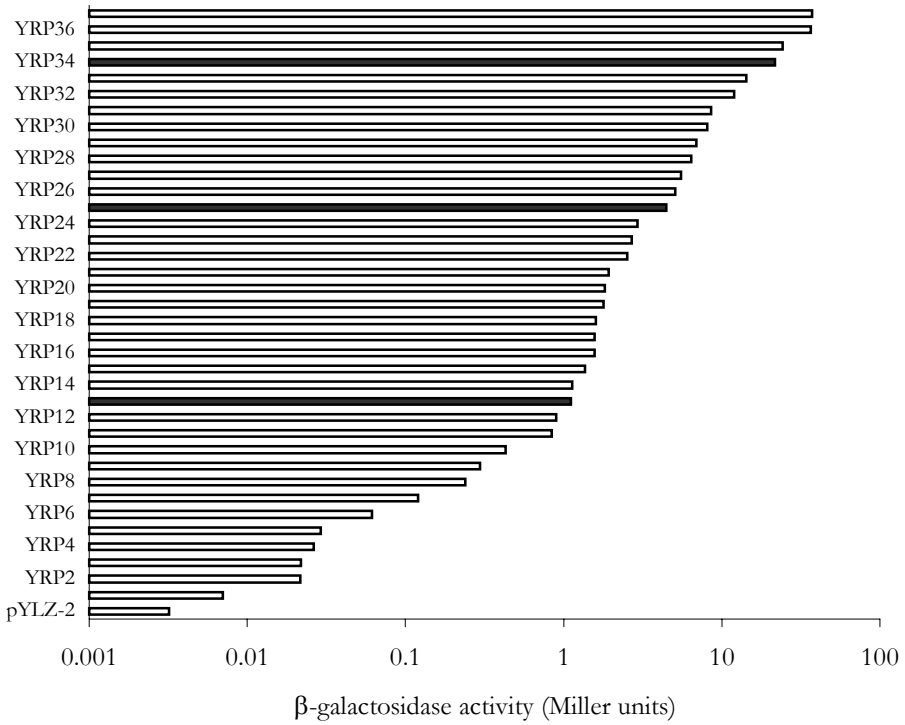


Figure 6. β-galactosidase activity in Miller units from various YRP promoter clones controlling β-galactosidase in vector pYLZ-2 (Paper IV). The shaded promoter clones were used to control *ZWF1* expression (Figure 7).

to control the β-galactosidase reporter gene in the vector pYLZ-2 (Hermann *et al.*, 1992).

2.3.3 Modulation of the *ZWF1* gene

Three promoter clones (shaded in Figure 6) was used to control the expression of the *ZWF1* gene in TMB3001. The G6PDH activity (Paper IV, Figure 7) retained the order of expression levels from the β-galactosidase construct (Paper IV, Figure 6).

TMB3037 (Figure 7), expressing *ZWF1* at 13% of the wild-type level showed about five times higher xylose consumption rate than TMB3255(*zwf1*Δ) but also 3.6 times higher xylitol yield (Paper VI). There appears to be a close relationship between xylitol yield and xylose consumption rate (Papers III and IV).

The synthetic promoters were successfully used to down-regulate *ZWF1* expression. The *CUP1* copper regulated promoter was also used in an attempt at creating down-regulation of the *ZWF1* gene (Paper IV). The lowest G6PDH activity obtainable with the *CUP1* promoter was slightly higher than the wild-type level, which made the *CUP1* promoter unsuitable for this purpose (Paper IV).

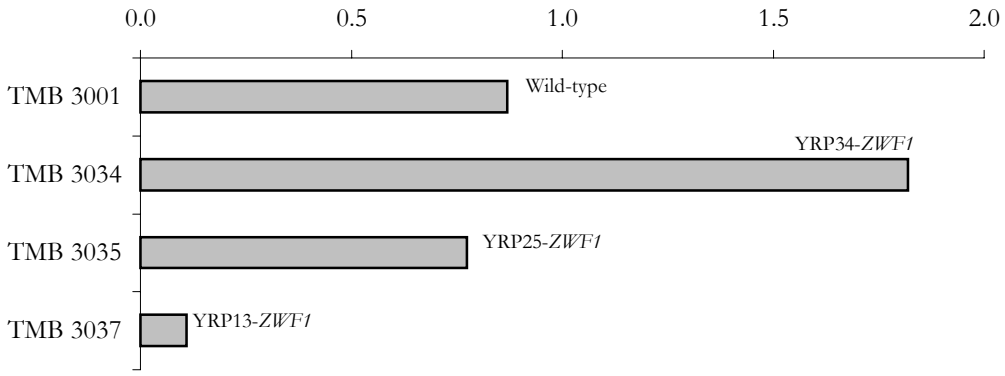


Figure 7. G6PDH activities in strains derived from TMB3001 with selected promoter clones (shaded in Figure 6) controlling the *ZWF1* expression in strains (Paper IV).

2.3.4 Over-expression of *XYL1*

The reduced xylose consumption rate of the strains with blocked oxidative PPP could be partly due to that the NADPH dependent xylose reduction is absent. Since the main NADPH producing pathway is inactive, the NADPH/$NADP^+$ ratio is higher in these

strains (Paper III). Therefore, inhibition of XR by $NADP^+$ (Verduyn *et al.*, 1985; Rizzi *et al.*, 1988) probably also contributes to the low xylose consumption rate.

Over-expression of *XYL1* increased the rate of xylose fermentation considerably in both TMB3255 (*zwf1*Δ) and the control strain TMB3001 (Paper IV). In TMB3001, the *ADH1* promoter controls the *XYL1*, which is weaker than the *PGK1* promoter (Walfridsson *et al.*, 1997). *XYL1* was over-expressed by introducing another copy of *XYL1* controlled by the *PGK1* promoter (Paper IV). Xylose consumption rate increased 70% in TMB3001 when *XYL1* was over-expressed. The increase in xylose consumption rate for TMB3255 was about 15 times upon 12 times XR over-production, suggesting that XR activity almost totally control the xylose consumption rate (see the definition of FCC, section 2.4.1).

In Paper III, I suggest that xylose utilisation proceeds with a better balance of co-factors in TMB3255 and TMB3008 than in TMB3001. The absence of NADPH results in a NADH dependent xylose conversion to xylulose. Other strategies have also been employed to achieve co-factor balanced xylose utilisation. A fusion protein of XR and XDH has been constructed in order to exploit the proximity effect to create NADH dependent xylose utilisation (Anderlund *et al.*, 2001). Xylose reductase has been engineered to lower NADPH dependent activity (Kostrzynska *et al.*, 1998). Both strategies have been hampered by reduction of the specific activity of the enzyme as a result of the protein engineering. Over-expression of *XYL1* together with disruption of *ZWF1* seems to be a better strategy (Paper IV).

2.4 The non-oxidative pentose phosphate pathway

Blocking the oxidative PPP resulted in increased xylulose fermentation rate, perhaps due to insufficient non-oxidative PPP capacity (Paper I; section 2.1.2; Figure 3). Blocking the oxidative PPP decreased xylose fermentation rate, due to decreased NADPH production (Paper III and IV). Since the oxidative PPP affected the initial xylose metabolism, the question if the non-oxidative PPP is sufficiently active for xylose fermentation remained unanswered.

Accumulation of the non-oxidative PPP intermediate sedoheptulose 7-phosphate (S7P) (Figure 2) has been observed in xylose-fermenting yeast (Kötter and Ciriacy, 1993) as well as in xylulose-fermenting yeast (Senac and Hahn-Hägerdal, 1990). This has been attributed to insufficient transaldolase (TAL) activity compared with glyceraldehyde 3-phosphate dehydrogenase (G3PDH) activity (Figure 2). G3P is a common substrate of the lower

glycolysis enzyme G3PDH and TAL (Figure 2). If G3P produced in the non-oxidative PPP is consumed through glycolysis, S7P will be trapped, since no additional G3P can be formed to take part in the TAL reaction forming E4P and F6P. Xylulose-fermenting *S. cerevisiae* was treated with iodine acetate, a specific inhibitor of G3PDH, resulting in lower S7P levels but decreased rather than increased xylulose fermentation rate was observed (Senac and Hahn-Hägerdal, 1990). This might be due to unspecific toxic effects of iodine acetate, so no reliable conclusion could be drawn as to whether the TAL activity is sufficient or not. Over-expression of the *TAL1* gene in a *S. cerevisiae* strain also expressing *XYL1* and *XYL2* improved the aerobic growth on xylose (Walfridsson *et al.*, 1995). Over-expression of *TAL1* together with *TKL1* had the same or slightly better effect than over-expressing *TAL1* alone. However, xylose fermentation rate was not improved.

A stoichometric flux model of the intracellular metabolism of the xylose-fermenting yeast strain *S. cerevisiae* TMB3001 showed that the flux through the ribulose 5-phosphate epimerase enzyme (RPE) was one order of magnitude lower than the flux through the ribose 5-phosphate isomerase enzyme (RKI) (Figure 2; Wahlbom *et al.*, 2001). This means that the ribose 5-phosphate (Ri5P) required for a balanced TKL reaction needs to be supplied through the oxidative PPP, which was also shown by the flux model (Wahlbom *et al.*, 2001). The low flux through the RPE could result from an insufficiently expressed enzyme.

It is generally accepted that *S. cerevisiae* cannot metabolise xylose without genetic engineering. Nevertheless, wild-type *S. cerevisiae* was shown to take up xylose at a low rate, provided that a co-substrate was present (van Zyl *et al.*, 1989). The major xylose fermentation product was xylitol, which may be due to the expression of aldose reductase (Kuhn *et al.*, 1995). This enzyme could function as a NADPH specific xylose reductase. However, trace experiments using radioactive xylose showed formation of radioactive carbon dioxide and biomass, proving that xylose was also assimilated (van Zyl *et al.*, 1989). The *S .cerevisiae* ORF YLR070c has been shown to encode an XDH (Richard *et al.*, 1999), which could carry out the assumed conversion of xylitol to xylulose. It is therefore theoretically possible to find a xylose utilisation pathway in *S. cerevisiae*. Among the co-substrates tested for a naturally xylose consuming *S. cerevisiae*, ribose promoted the fastest xylose utilisation (van Zyl *et al.*, 1989). C-13 NMR showed that ribitol is formed during xylose and ribose co-utilisation, suggesting that ribose is converted first to ribitol and then to to ribulose (van Zyl *et al.*, 1993). Following this route, ribose enters the non-oxidative

PPP as ribulose 5-phosphate and xylose as xylulose 5-phosphate. Recently *S. cerevisiae* XK was shown to also carry out the phosphorylation of ribulose (Richard *et al.*, 2000). Thus simultaneous xylose and ribose metabolism could by-pass the RPE (Figure 2).

Xylose-fermenting yeasts such as *C. utilis* and *P. stipitis* can utilise nitrate as the sole nitrogen source (Bruinenberg *et al.*, 1983a) whereas *S. cerevisiae* is unable to do so. Biomass growth with nitrate required expenditure of four NADPH molecules per molecule of nitrogen to convert nitrate to ammonia. The reactions are carried out by nitrate and nitrite reductase:

$NO_3^- + NAD(P)H + H^+ \rightarrow NO_{2-} + H_2O + NAD(P)^+$; Nitrate reductase

$NO_2^- + 3NADPH + 3H^+ \rightarrow NH_4^+ + 2H_2O + 3NADP^+$; Nitrite reductase

S. cerevisiae is unable to utilise nitrate, due to either a lack of suitable enzymes or possibly an insufficient capacity of the PPP. The *S. cerevisiae* genome contains the ORF YNR074c annotated as showing weak similarities with *Bacillus subtilis* nitrite reductase (Cherry *et al.* 1998).

2.4.1 The control of pathway flux

The metabolic control that a particular enzyme exerts on the flux of a metabolite can be quantitatively described as the flux control coefficient (FCC) (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). The FCC of an enzyme can be understood as the *relative* increase in the whole pathway flux divided by the *relative* increase in enzyme activity following an infinitesimal increase in the enzyme activity. Thus, the enzyme with the greatest FCC is the one where an increase of enzyme activity (over-production) has the highest impact on the metabolite flux.

The flux change resulting from the over-expression of a gene encoding a single enzyme in a pathway has been shown to be small. Over-production of single enzymes in *S. cerevisiae* tryptophan biosynthesis resulted in at most 1.3-fold increase in flux, while simultaneous over-production of five enzymes resulted in a 8.8-fold increase (Niederberger *et al.*, 1992). Over-expression of single genes in yeast glycolysis did not result in increased glycolytic flux (Schaaff *et al.*, 1989), while over-expression of seven enzymes (Hauf *et al.*, 2000) increased the capacity to ferment glucose under certain conditions (Smits *et al.*, 2000).

Over-expression of a single gene encoding an enzyme in a metabolic pathway is not likely to increase the flux very much as control is generally distributed (Fell, 1992) unless the FCC of the particular enzyme is higher than about 0.6 (Fell and Thomas, 1995). The metabolic pathway from X5P to G3P and F6P involves 5 enzymatic steps (Figure 2). Since the sum of FCCs is unity, the average FCC of the non-oxidative PPP enzymes could be no higher than 0.2. Thus it may be unlikely that a single enzyme in the non-oxidative PPP would have an FCC as high as 0.6. This suggests that multi-site modulation of gene expression is necessary to achieve increases in flux (Kacser and Acerenza, 1993). Multi-site modulation would in this case mean over-production of all non-oxidative PPP enzymes (Figure 2). The simplest way of achieving this would be to engineer a common regulator to up-regulate the whole pathway simultaneously. Unfortunately, no such regulator is known for the non-oxidative PPP so the remaining option was to create a strain over-expressing the entire non-oxidative PPP.

2.4.2 Achieving multi-site modulation

Changing the expression level of many genes at the same time by genetic engineering requires a large set of genetic markers. The over-expression of seven genes in glycolysis required the auxotrophic markers *URA*3, *LEU*2, *TRP*1 and the dominant marker *Kan*MX (Hauf *et al.*, 2000). There are more markers available in *S. cerevisiae*, but the number of markers is still limited (Cherry *et al.*, 1998). These would rapidly be used up for any multi-site modulation, since the number of genetic modifications required is not known beforehand. Furthermore, when the markers have been exhausted, it is not possible to further modify the strain; a desirable trait since metabolic engineering is an iterative process where genetic changes are performed and analysed successively (Bailey, 1991; Östergaard *et al.*, 2000).

2.4.3 The pB3 PGK / pCRE3 system

One goal of the research presented in this thesis was to develop a yeast expression system that would meet the following criteria to allow efficient multi-site modulation.

1. A recyclable marker for more efficient multi-site modulation
2. Selection for an efficient dominant marker for broad applicability
3. Wide range of exchangeable promoters

The strategy for making the expression vector pB3 PGK (Paper V), will be described in the following sections.

2.4.4 Recyclable markers

The use of recyclable markers can overcome the problem of marker exhaustion. The CRE/*lox*P system was first used to recycle auxotrophic markers in *S. cerevisiae* (Sauer, 1987; 1994). The recycling of the marker gene was accomplished by flanking *lox*P sequences. These sequences were recombined by the specific CRE recombinase, resulting in loss of the marker gene. This system was modified for the *Kan*MX marker (Güldener *et al.*, 1996) and used to delete twenty hexose transporters from a yeast strain using the same marker gene in twenty consecutive transformations (Wieczorke *et al.*, 1999). This system has been shown to be very efficient and rapid compared with the pop-in/pop-out gene replacement strategies involving spontaneously recombining sequences and counter-selectable markers (Scherer and Davis, 1979).

2.4.5 Dominant markers

There are a number of drawbacks of auxotrophic markers. They can complicate the interpretation of physiological studies as a strain with an auxotrophic mutation may not behave like its prototrophic counterpart even if the medium is supplied with the auxotrophic requirement (Baganz *et al.*, 1997). Auxotrophic markers are often not available in industrial strains, which are more interesting from an applied aspect. These points suggest that dominant markers are more efficient than auxotrophic ones.

The most commonly used marker is *Kan*MX, conferring resistance to G418, which has been used in the EUROFAN project to create knockout strains of most ORFs in the *S. cerevisiae* genome (Dujon, 1998). The drawback is that some industrial strains (such as Isolate#3, see Chapter 3; Lindén *et al.*, 1989) are insensitive to this antibiotic (Eliasson, 2001). The copper resistance selection system requires multiple copies of the marker to confer resistance (Fogel and Welch, 1982; Fogel *et al.*, 1983). The zeocin and the Aureobasidin A systems have the advantage that both are available commercially (Invitrogen, TaKaRa), where both the resistance genes (ZEO^R, *AUR1-C*) and the antibiotics is supplied by the same company. A sensitivity screen revealed that both zeocin and Aureobasidin A efficiently killed all yeast strains screened, including the tolerant Isolate#3 (Lindén *et al.*, 1989) and other industrial strains (results not shown). The toxicity mechanisms of these markers are entirely different, making simultaneous selection possible.

Zeocin induce lethal DNA double strand breaks (Mazzei, 1984; Mirabelli *et al.*, 1982), while the Aureobasidin A attacks the cytoskeleton of the yeast cell (Hashida-Okado *et al.*, 1998). The ZEO^R does not code for an enzyme, but a small polypeptide that binds the zeocin drug in a stoichometric manner. The mechanism by which *AUR-1C* confers resistance to Aureobasidin A is not known. The ZEO^R and *AUR1-C* markers were selected for the expression vector pB3 PGK and the CRE expression vector pCRE3 (Paper V).

2.4.6 Promoters

The pB3 PGK vector (Paper V) was constructed with the strong *PGK1* promoter (Mellor *et al.*, 1983) and the short *GCY1* terminator (Hermann *et al.*, 1992). The promoter was constructed so as to be excisable with restriction endonucleases *Sac*I and *Xba*I. This made the promoter easily exchangeable with the wide range of promoters summarised in Table 2. The cloning strategy of the pB3 PGK vector is described in Figure 8. The cloning strategy of the CRE recombinase vector pCRE3 is described in Figure 9a and 9b.

Table 2. Promoters excicable with *Sac*I and *Xba*I.

Promoter	Characteristics	Reference
TDH3	Strong	(Mumberg *et al.* 1995)
TEF1	Strong, constitutive	---"---
ADH1	Intermediate	---"---
CYC1	Weak	---"---
CUP1	Copper induced	(Labbé and Thiele, 1999)
CTR1	Copper repressed	---"---
CTR3	Copper repressed	---"---
GAL1	Galactose induced	(Güldener *et al.* 1996)

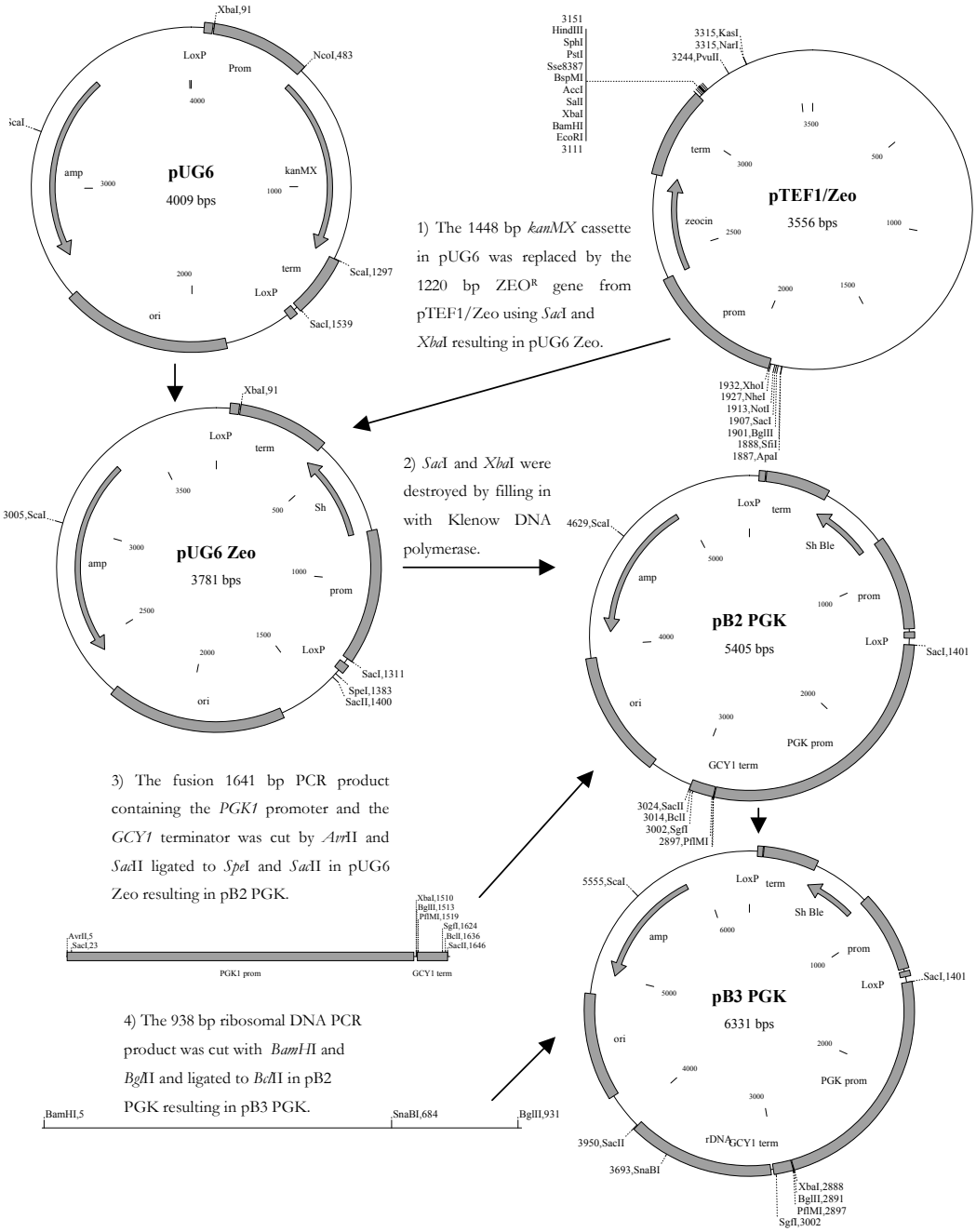
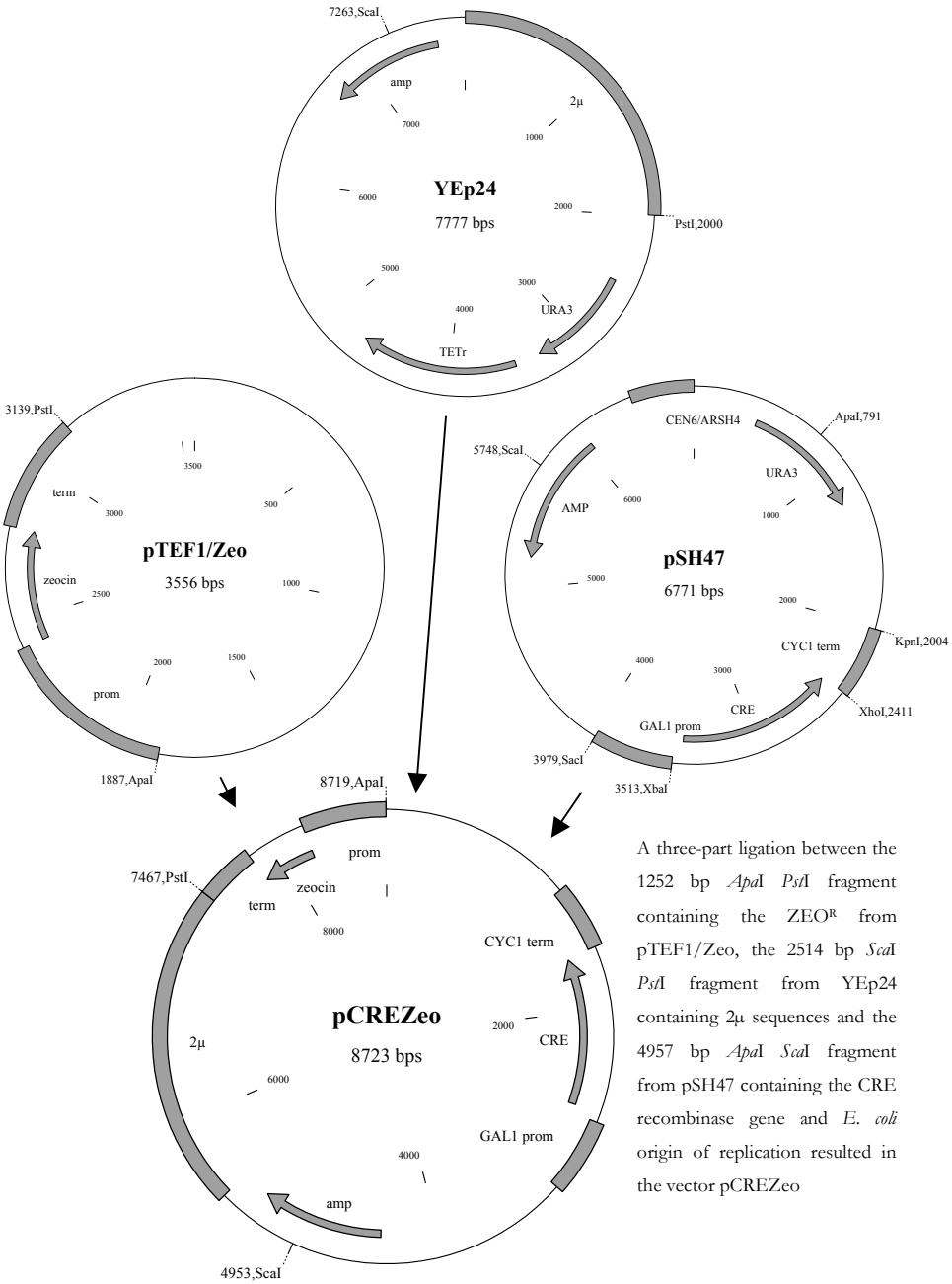
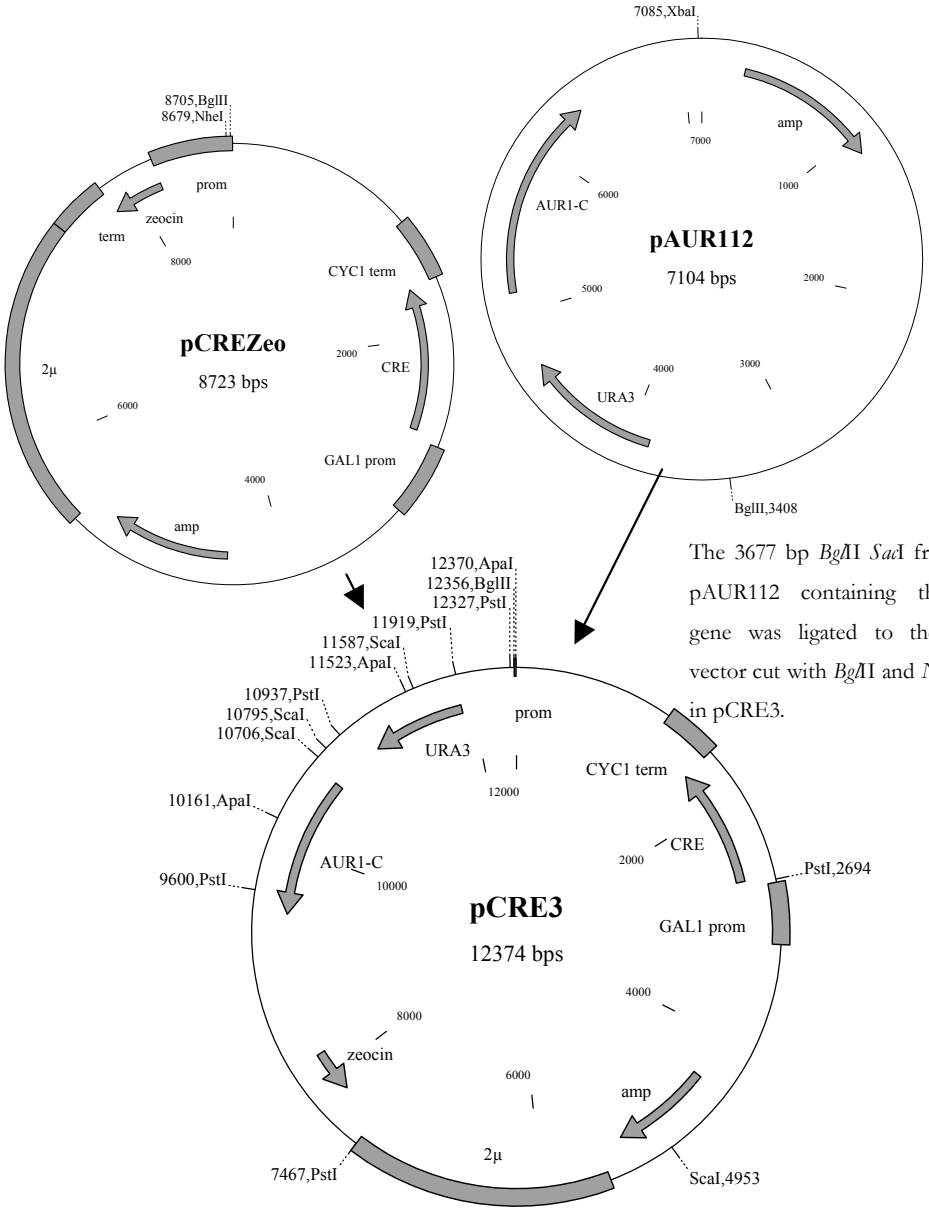


Figure 8. Construction of the pB3 PGK vector (PaperV).



A three-part ligation between the 1252 bp *Apa*I *Pst*I fragment containing the ZEO^R from pTEF1/Zeo, the 2514 bp *Sca*I *Pst*I fragment from YEp24 containing 2µ sequences and the 4957 bp *Apa*I *Sca*I fragment from pSH47 containing the CRE recombinase gene and *E. coli* origin of replication resulted in the vector pCREZeo

Figure 9a. Construction of the pCREZeo vector (Paper V).



The 3677 bp *Bgl*II *Sac*I fragment from pAUR112 containing the *AUR1-C* gene was ligated to the pCREZeo vector cut with *Bgl*II and *Nhe*I resulting in pCRE3.

Figure 9b. Construction of pCRE3 from pCREZeo (Paper V).

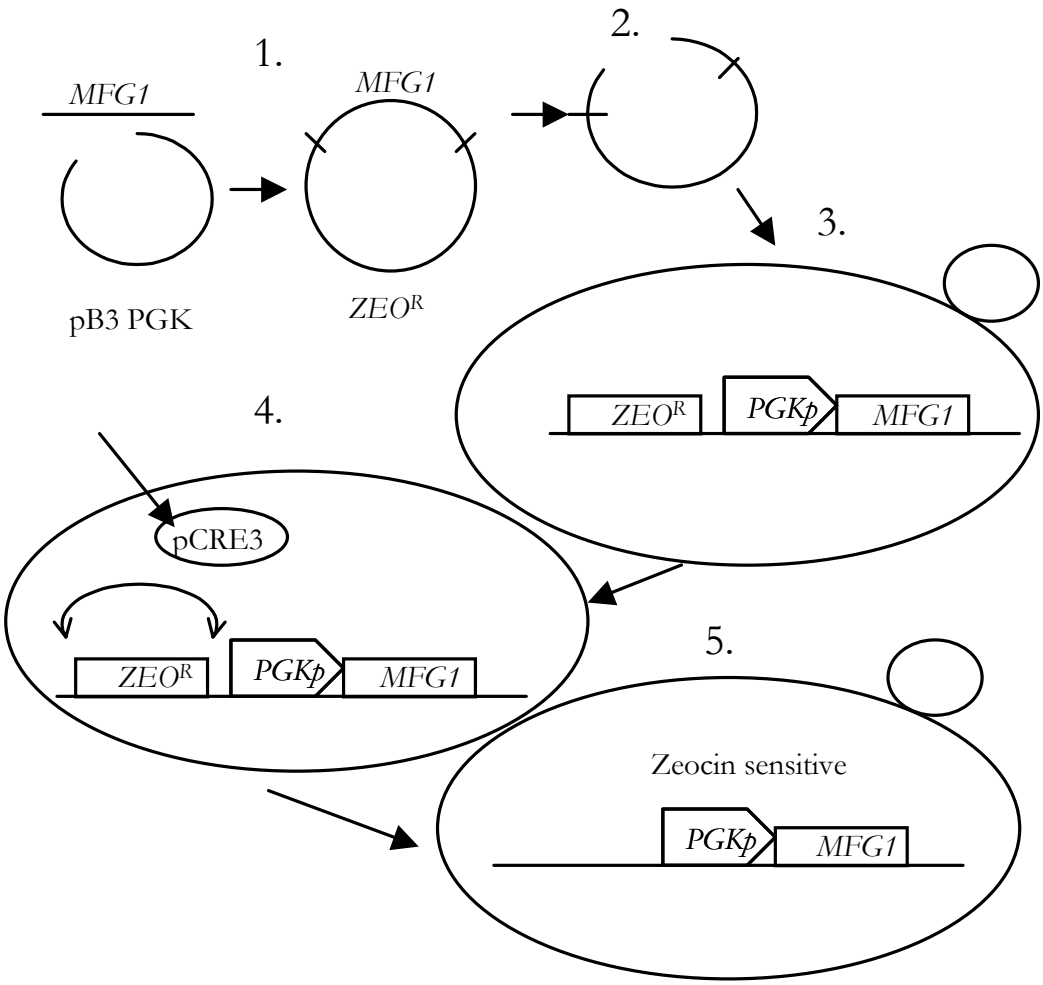


Figure 10. Genetic engineering steps using the pB3 PGK /pCRE3 system for over-expression of the gene of interest (*MFG1)* that is already present in the genome (Paper V). 1) *MFG1* is cloned in the vector pB3 PGK. 2) A single restriction cut is made within the coding region of *MFG1*. 3) pB3 PGK *MFG1* is integrated in the locus of *MFG1*. 4) The strain is transformed with pCRE3, the ZEO^R marker is looped out. 5) pCRE3 is cured, zeocin sensitivity marks both pCRE3 curing and marker loss (Paper V).

The strategy for genetic engineering using the pB3 PGK /pCRE3 vectors is outlined in Figure 10 (Paper V). The plasmids pB3 PGK and pCRE3 were used to make four chromosomal integrations in *S. cerevisiae* using the same pair of dominant markers (Paper V). The pB3 PGK and pCRE3 could probably be used for even further rounds of genetic engineering, since no increased zeocin resistance was detected in the strains where the ZEO^R had been looped out (Paper V).

Strain TMB3026 (Paper V) was constructed from TMB3001, over-expressing the genes *RPE1, RKI1, TAL1* and *TKL1*. These genes encode the enzymes ribulose 5-phosphate epimerase (RPE), ribose 5-phosphate isomerase (RKI), transaldolase (TAL) and transketolase (TKL) (Figure 2). The resulting increase in activity of the enzymes was 4

times for RPE, 23 times for RKI, 17 times for TAL, and 13.5 times for TKL. The activities of TMB3026 did not differ much from the activities of strains over expressing single genes.

Strain TMB3026 (Paper VI) did not show higher xylose consumption rate in neither chemostat nor batch-culture compared to the control strain TMB3001. The non-oxidative PPP could thus be ruled out as having any significant control of xylose consumption rate in TMB3001, and probably other strains of xylose-fermenting *S. cerevisiae*. Since XR activity level exerts a great deal of control of xylose consumption rate (Paper IV), this could be a reason why TMB3026 did not show higher xylose consumption rates than TMB3001.

A yeast strain over-expressing seven glycolytic enzymes showed a higher fermentative capacity under a transient from glucose limitation to glucose excess, suggested to result from rise in initial ATP demand (Smits *et al.*, 2000). Pentose sugars are not a natural substrate for *S. cerevisiae*, and do not induce metabolism as strongly as glucose (Müller *et al.*, 1995). This suggests that a similar transient, from xylose limitation to xylose excess is not likely to provoke a measurable difference between TMB3026 and TMB3001.

The regulation of the non-oxidative PPP is unknown, but it is conceivable that it is regulated by the demand for its products. Perhaps could it be possible to see an effect of non-oxidative PPP over-expression during growth on complex medium, since the demand for amino acid precursors should be lower than on the minimal medium used in Paper VI. The non-oxidative PPP might be less active in TMB3001 than in TMB3026 under such conditions, and a difference in xylose consumption rate might be observed between the strains.

3 Strain backgrounds

The major part of this thesis deals with the effects of various directed mutations and genetic modifications on xylulose and xylose fermentation capacity. However, the performance of the strains with respect to xylulose fermentation also depends of the strain background for reasons that are not obvious (Paper I; Jeppsson *et al.*, 1996). This is also true for xylose fermentation, since two recombinant laboratory carrying identical vectors fermented xylose at different rates with different ethanol yields (Paper II).

Tolerance of the harsh conditions in a ligno-cellulosic hydrolysate could be an important criterion for selecting the host strain. A *S. cerevisiae* strain was isolated from a spent sulphite liquor ethanol production plant (Lindén *et al.*, 1992). This strain, called Isolate#3, exhibited ethanol production in presence of higher levels of acetic acid in the medium (Lindén *et al.*, 1992).

The Isolate#3 as well as most strains described in Paper I lacks auxotrophic markers. For this reasons I created genetic tools which could engineer practically any *S. cerevisiae* strain for xylose fermentation. The YIpXR/XDH/XK vector (Eliasson *et al.*, 2000) was engineered with the *AUR1-C* marker to create YIploxAUR or the ZEO^R marker to create YIploxZEO to facilitate the construction of a xylose-fermenting yeast strain based on isolate#3 and other wild-type and industrial strains. These markers were further described in section 2.4.5. The cloning strategy to make the YIploxAUR and YIploxZEO is outlined in Figure 11. The markers of these vectors are flanked by *lox*P sequences in the same way as in the pB3 PGK, to facilitate multiple genetic engineering to the same strain.

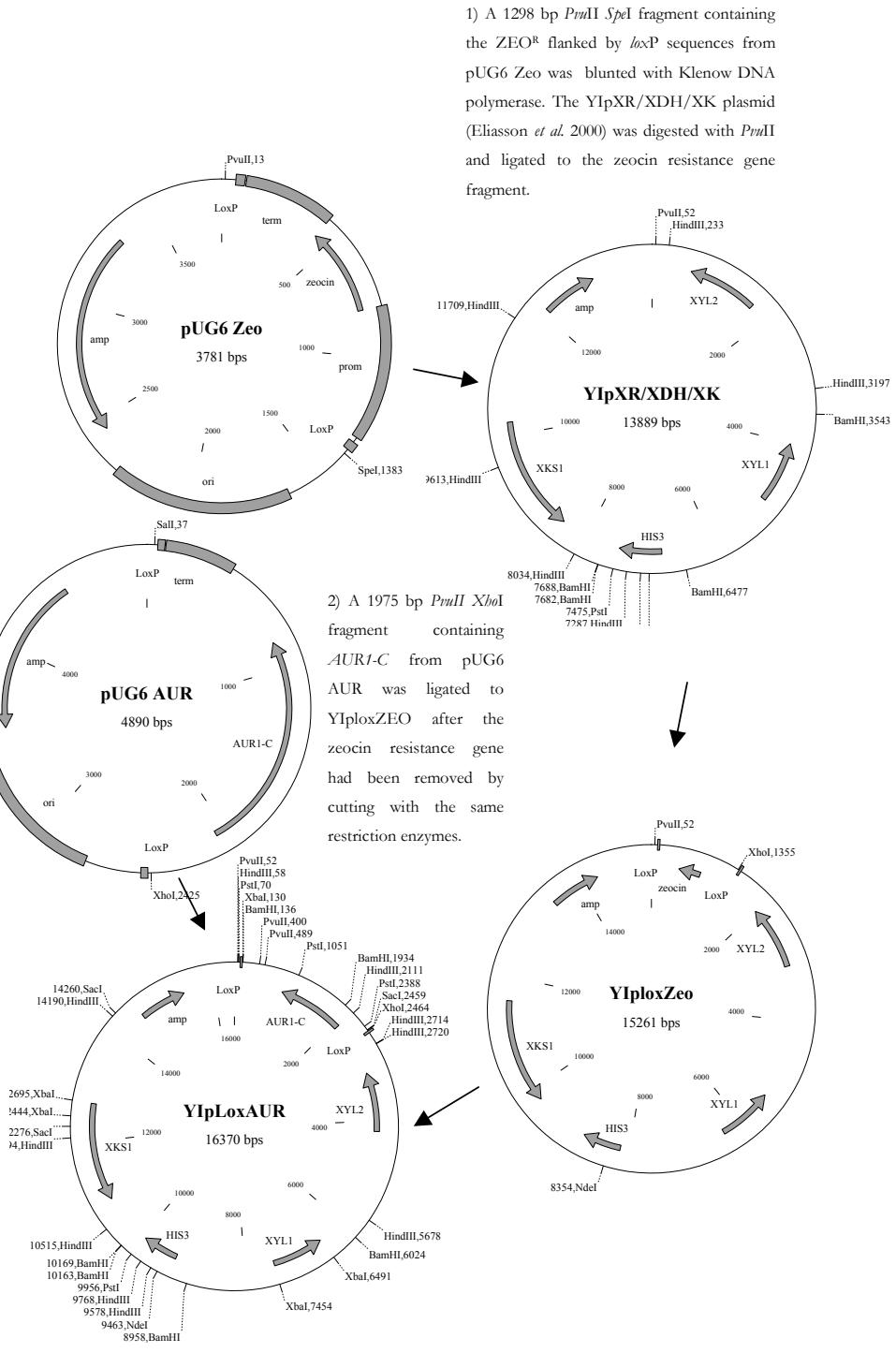


Figure 11. Cloning strategy for YIplox AUR and YIploxZEO vectors. The pUG6 AUR vector is identical to the pUG6 Zeo (Figure 8) except for that ZEO^R is exchanged for *AUR1-C*.

Isolate#3 was engineered for xylose fermentation with YIploxAUR vector, resulting in TMB3006. TMB3006 fermented xylose at the same or slightly lower rate than TMB3001 (Figure 12). This suggests that wild-type or industrial strain backgrounds are not necessarily better suited for xylose fermentation. However, TMB3006 survived and was metabolically active at 70% ligno-cellulosic hydrolysate in the medium, while TMB3001 could only survive maximum 30% (van Zyl, 2001). This could be an important trait for a strain to be used for fermentation of ligno-cellulosic hydrolysates.

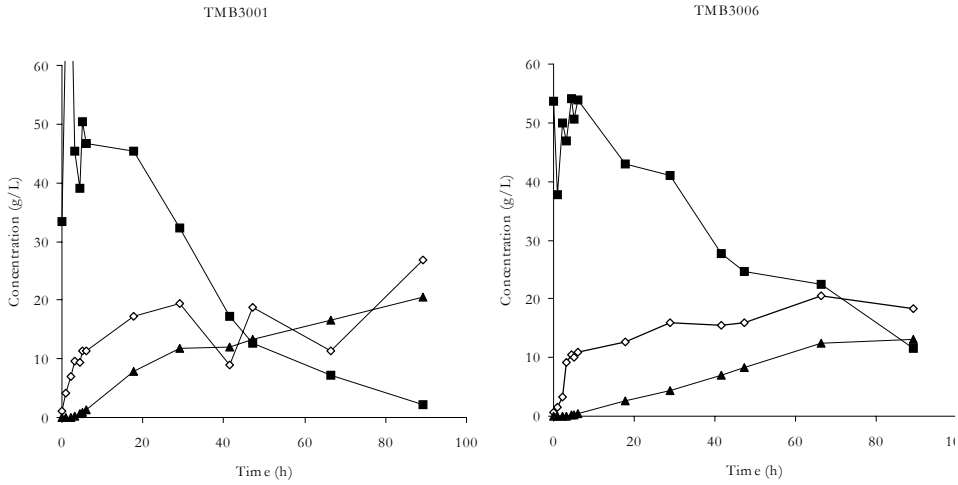


Figure 12. Fermentation of 20 g/L glucose and 50 g/L xylose by TMB3001 and TMB3006. The biomass level is 10 g/L in both cases. ■ xylose, ◊ ethanol, ▲xylitol. Glucose was omitted for clarity.

4. Conclusions

There is a strong correlation between oxidative PPP flux and xylitol formation. Xylitol formation seems to be largely NADPH dependent.

Disruption of oxidative PPP leads to low xylitol yields from xylose and an almost closed co-factor balance for xylose reduction to xylitol and xylitol oxidation to xylulose.

Allowing a reduced oxidative PPP flux or over-expression of *XYL1* can relieve the decreased xylose consumption rate associated with a blocked oxidative PPP.

XR activity rather than the non-oxidative pentose phosphate pathway capacity controls the rate of xylose fermentation in TMB3001.

The pB3 PGK /pCRE3 expression system proved to work as intended and to facilitate multiple over-expressions of many genes in the same strain. Four genes were consecutively over-expressed using the same set of markers.

Synthetic yeast promoters could be used to down-regulate gene expression.

A Blocked oxidative PPP combined with over-expression of *XYL1* was the best genetic improvement of recombinant *S. cerevisiae* xylose fermentation presented in this work.

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Paper I

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ORIGINAL PAPER

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Xylulose fermentation by mutant and wild-type strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*

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Abstract Anaerobic xylulose fermentation was compared in strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*, mutants and wild-type strains to identify host-strain background and genetic modifications beneficial to xylose fermentation. Overexpression of the gene (*XKS1*) for the pentose phosphate pathway (PPP) enzyme xylulokinase (XK) increased the ethanol yield by almost 85% and resulted in ethanol yields [0.61 C-mmol (C-mmol consumed xylulose)$^{-1}$] that were close to the theoretical yield [0.67 C-mmol (C-mmol consumed xylulose)$^{-1}$]. Likewise, deletion of gluconate 6-phosphate dehydrogenase (*gnd1*Δ) in the PPP and deletion of trehalose 6-phosphate synthase (*tps1*Δ) together with trehalose 6-phosphate phosphatase (*tps2*Δ) increased the ethanol yield by 30% and 20%, respectively. Strains deleted in the promoter of the phosphoglucose isomerase gene (*PGI1*) – resulting in reduced enzyme activities – increased the ethanol yield by 15%. Deletion of ribulose 5-phosphate (*rpe1*Δ) in the PPP abolished ethanol formation completely. Among non-transformed and parental strains *S. cerevisiae* ENY. WA-1A exhibited the highest ethanol yield, 0.47 C-mmol (C-mmol consumed xylulose)$^{-1}$. Other non-transformed strains produced mainly arabinitol or xylitol from xylulose under anaerobic conditions. Contrary to previous reports *S. cerevisiae* T23D and CBS 8066 were not isogenic with respect to pentose metabolism. Whereas, CBS 8066 has been reported to have a high ethanol yield on xylulose, 0.46 C-mmol (C-mmol consumed xylulose)$^{-1}$ (Yu et al. 1995), T23D only formed ethanol with a yield of 0.24 C-mmol (C-mmol consumed xylulose)$^{-1}$. Strains producing arabinitol did not produce xylitol and vice versa. However, overexpression of *XKS1* shifted polyol formation from xylitol to arabinitol.

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Introduction

XYL1 and *XYL2* from *Pichia stipitis* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, have been expressed in *Saccharomyces cerevisiae*, enabling the strain to metabolise xylose (Kötter and Ciriacy 1993; Tantirungkij et al. 1994; Walfridsson et al. 1995). The recombinant strains produced little ethanol from xylose and xylitol was the main product. When endogenous *XKS1* encoding xylulokinase (XK) and catalysing the phosphorylation of xylulose to xylulose 5-phosphate was overexpressed in xylose-utilising strains of *Saccharomyces* sp. harbouring *XYL1* and *XYL2*, xylose fermentation resulted in enhanced ethanol yields (Ho et al. 1998). However, xylose fermentation under anaerobic conditions was not demonstrated and xylitol remained a major by-product.

The inability of recombinant xylose-utilising strains of *S. cerevisiae* to ferment xylose efficiently under anaerobic conditions has been ascribed to the redox imbalance generated in the first two steps of the eukaryotic xylose metabolism (Bruinenberg et al. 1983), the inefficient pentose phosphate pathway (PPP) of *S. cerevisiae* (Walfridsson et al. 1995) and/or the inability of pentose

sugar metabolism to activate the lower part of glycolysis (Boles et al. 1993; Müller et al. 1995).

In the present study, strains of *S. cerevisiae* with genetically altered enzyme activities in glycolysis, trehalose and PPP metabolism were investigated (Fig. 1). The influence of these alterations on the anaerobic metabolism and fermentation of xylulose was determined. The aim was to identify metabolic modifications beneficial for anaerobic xylose fermentation in strains not exhibiting product formation related to the redox imbalance generated by the first two steps of the eukaryotic xylose metabolism. Additionally, anaerobic xylulose fermentation was investigated in a number of wild-type strains of *S. cerevisiae* and *Zygosaccharomyces* to identify genetic backgrounds potentially beneficial to anaerobic xylose fermentation.

Materials and methods

Recombinant DNA and plasmids

For recombinant DNA work, standard procedures were applied (Sambrook et al. 1989). Yeast-specific techniques were used as described by Guthrie and Fink (1991). Plasmid transformation into yeast was performed using LiAc, to yield competent cells, and single-stranded DNA as the carrier (Schiestl and Gietz 1989).

Strains

The yeast strains used are listed in Table 1. The strains were stored frozen at −80 °C. Cultures streaked from the frozen stocks on to agar plates were used to inoculate the precultures.

Construction of *PGI1* mutants

Eight different centromeric plasmids with successive promoter deletions (Rose et al. 1991) were digested with *Pst*I and *Dra*I. The fragments carrying the *PGI1* gene with promoters of different size were subcloned into the integrative plasmid YIplac128 (Gietz and Sugino 1988). The resulting plasmids (pBR1 to pBR8) were linearised with *Hpa*I and transformed separately into the *S. cerevisiae* strain EBY44 (*pgi1*Δ*-1*::*URA3*) (Boles and Zimmermann 1994). Several stable leucine prototrophic transformants were isolated and given the RBY prefix. The present study included RBY 6-1 and RBY 7-1.

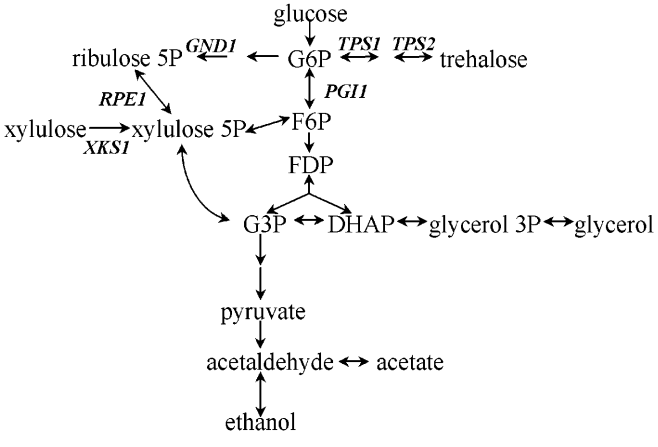

Fig. 1 Simplified scheme of xylulose metabolism in yeast. Genes encoding enzymes that have been mutated in strains used in the present study are indicated: *GND1* gluconate 6-phosphate dehydrogenase, *RPE1* ribulose 5-phosphate epimerase, *XKS1* xylulokinase, *TPS1* trehalose 6-phosphate synthase, *TPS2* trehalose 6-phosphate phosphatase, *PGI1* phosphoglucose isomerase. Intermediary metabolites: *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *FDP* fructose 1,6-diphosphate, *G3P* glyceraldehyde 3-phosphate, DHAP dihydroxyacetone phosphate

Construction of the *tps* mutant

The *TPS1* and *TPS2* genes were deleted in the *S. cerevisiae* strain W303-1A using the *TRP1* and *LEU2* marker genes, respectively, as described previously (Neves et al. 1995). The *Escherichia coli* homologue of *TPS1*, *OtsA*, was expressed on a multi-copy plasmid, pRS426 (Sikorski and Hieter 1989), using a 778-bp fragment of the *TPS1* promoter and a 296-bp fragment of the *TPS1* terminator. The construct was used to transform the above-described deletion strain, resulting in W303-1A (*tps1*Δ, *tps2*Δ, p*OtsA*).

Cloning and overexpression of the xylulokinase gene (*XKS1*)

Chromosomal DNA from *S. cerevisiae* CBS 8066 was prepared (Sambrook et al. 1989) and used as the template for amplification with the polymerase chain reaction (PCR). Two primers were constructed based on the 5′ end (5′-GCGGATCCTCTAGAA-TGGTTTGTTCAGTAATTCAG-3′) and the 3′ end (5′-AGAT-CTGGATCCTTAGATGAGAGTCTTTTCCAG-3′) of the *XKS1* gene, respectively (Rodriguez-Pena et al. 1998). The chromosomal DNA was amplified using *Pwo* DNA polymerase (Boehringer Mannheim, Germany). The amplicon was ligated between the phosphoglycerate kinase (PGK) promoter and terminator (Mellor et al. 1983) in plasmid YEp24-PGK (Walfridsson et al. 1997) using *Bam*HI, resulting in YEp24-PGK-XK. *S. cerevisiae* CEN.PK2-1D was transformed with YEp24-PGK-XK, resulting in CEN.PK (*XKS1*).

Xylulose preparation

Xylulose was prepared as described previously (Olsson et al. 1994). Enzymatic xylose isomerisation (Maxazyme; Gist Brocades, Delft, The Netherlands) yielded a mixture containing about 20% xylulose. The xylulose was then purified by ion-exchange chromatography in a water-jacketed column (XK 50/60; Pharmacia Biotech AB, Uppsala, Sweden) packed with Dowex 1-X8 ion-exchange resin (Bio-Rad Laboratories, Hercules, Calif.). The column was eluted with double-distilled water at a flow rate of 6 ml min^{-1}. In each separation, 5–8 g pure (>98%) xylulose was produced when 20–30 ml isomerisation mixture was loaded.

Inoculum

Baffled 250-ml conical flasks containing 50 ml mineral medium (Verduyn et al. 1992), supplemented for auxotrophic requirements (uracil, 0.05 g l^{-1}; leucine, 0.25 g l^{-1}; histidine, 0.05 g l^{-1}; and tryptophan, 0.05 g l^{-1}), 20 g l^{-1} xylulose and 20 g l^{-1} glucose were inoculated from plates and incubated in an INR-200 orbital incubator (Gallenkamp, Leicester, UK) at 130 rpm and 30 °C for 15–30 h depending on the strain. These cultures were used to inoculate 100 ml of the same medium in 500-ml baffled flasks that were incubated overnight under the same conditions. The cells were harvested by centrifugation (J2-21; Beckman, Geneva, Switzerland) at 6000 g for 10 min and washed twice with 0.9% NaCl. The supernatant was removed and the cells were resuspended in fresh mineral medium. The suspensions were used as inocula for the fermentations.

Fermentations

Anaerobic fermentations were performed in 25-ml flasks containing 20 ml of the same mineral medium used for inoculum preparation;

Table 1 Description of yeast strains (*Saccharomyces cerevisiae* and *Zygosaccharomyces* sp.) used in this study

Strain	Designation of strains used in this study	Genotype	Phenotype/Isolation site	Reference
Glycolytic mutants				
S. cerevisiae ENY.WA-1A		MATα *ura3-52 leu2-3, 112 his3-Δ1 trp1-289 MAL2-8*^{c} *MAL3 SUC3*	1000–2500 mU PGI (mg protein)$^{-1}$	This work
S. cerevisiae RBY6-1		*pgi1-1Δ::URA3, PGI1::LEU2*	190–270 mU PGI (mg protein)$^{-1}$	This work
S. cerevisiae RBY7-1		*pgi1-1Δ::URA3, PGI1::LEU2*	10–20 mU PGI (mg protein)$^{-1}$	This work
PPP mutants				
S. cerevisiae CEN.PK2-1D		MATα *ura3-52 leu2-3, 112 his3-Δ1 trp1-289 MAL2-8*^{c} *SUC2*	≤20 mU XK (mg protein)$^{-1}$ 48 mU GND (mg protein)$^{-1}$	This work Juhnke et al. 1996
S. cerevisiae CEN.PK(*XK*)		*XK::URA3*	2200–6000 mU XK (mg protein)$^{-1}$	This work
S. cerevisiae CEN.HJ5-1B	CEN.PK(*gnd1Δ*)	*gnd1::HIS3*	0 mU GND (mg protein)$^{-1}$	Juhnke et al. 1996
S. cerevisiae CEN.PK2-1C		MAT *ura3-52 leu2-3, 112 his3-Δ1 trp1-289 MAL2-8*^{c} *SUC2*	2100 mU RPE (mg protein)$^{-1}$	Juhnke et al. 1996
S. cerevisiae CEN.HJ1-1A	CEN.PK(*rpe1Δ*)	*rpe1::LEU2*	0 mU RPE (mg protein)$^{-1}$	Juhnke et al. 1996
Trehalose mutants				
S. cerevisiae W303-1A		MAT *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 GAL SUC2*		Thomas and Rothstein 1989
S. cerevisiae W303-1A (*tps1Δ*, *tps2Δ*, p*OtsA*)		*tps1Δ::TRP1 tps2Δ::LEU2* p*OtsA*	Overaccumulation of sugar phosphates	This work
Natural strains				
S. cerevisiae T23D		Meiotic progeny of CBS 8066		Wenzel et al. 1992, Pronk et al. 1994
S. cerevisiae IGC 2533				
S. cerevisiae IGC 2608			Beer	Rodrigues de Sousa et al. 1995
Z. bailii IGC4806^{T}			Rosé sparkling wine	
Z. rouxii IGC5276^{T}			Conc. black grape must	

as conditions were anaerobic, 0.42 g l^{-1} polyoxyethylenesorbitan mono-oleate (Tween 80; Sigma, St. Louis, Mo.), and 0.01 g l^{-1} ergosterol were added. The carbon source was 50 g l^{-1} xylulose. The flasks were sealed with rubber stoppers with one syringe needle for sampling and one for carbon dioxide outflow. The initial pH was 5.5 and the initial cell dry weight was 5 g l^{-1}. The cultures were incubated at 30 °C in a water bath with magnetic bars gently stirring the fermentation broth. Fermentations were carried out at least in duplicate and the results are reported as mean values. The standard error was < 5% for fermentations with glycolytic mutants and < 10% for fermentations with PPP and trehalose-metabolism mutants. For the wild-type strains, the standard error was < 10% except for *Zygosaccharomyces rouxii*, which gave a standard error of about 25%.

Carbon balances and yield calculations

For the calculations it was assumed that 1 mol carbon dioxide is formed for every mole ethanol or acetic acid produced. Carbon balances and yields were calculated in single carbon-unit equivalents (carbon millimoles: C-mmol) (de Jong-Gubbels et al. 1995).

Analysis

Substrates consumed and products formed were analysed by column liquid chromatography (CLC). A CLC system (Beckman Instruments, Fullerton, Calif.) was used together with an RID-6A refractive index detector (Shimadzu, Kyoto, Japan) and two cation-exchange columns (Aminex HPX-87H; Bio-Rad, Richmond, Calif.) in series. For the mobile phase, 5 mM H_2SO_4 was used. The flow rate was set to 0.5 ml min^{-1} and the separation temperature was 45 °C.

The cell dry weight was determined by filtering 5 ml culture broth through a 0.45-μm Supor membrane (Gelman Sciences, Ann Arbor, Mich.). After being washed with three volumes of double-distilled water and dried in a microwave oven for 15 min, the filter was weighed. The cell dry weight was determined in the beginning and at the end of the fermentations.

Cell extracts for enzyme-activity measurements were prepared using glass beads (0.5 mm in diameter). Cells were harvested by centrifugation and, after washing, resuspended in a disintegration buffer (0.1 M triethanolamine buffer, pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 M dithiothreitol and 0.5 mM ethylenediamine tetraacetic acid. The suspension was vortexed for 5 min at 4 °C, put on ice for 5 min and then vortexed again. In order to separate cell debris and glass beads from the cell extract, the disintegrated cell mixture was centrifuged at 20 000 g for 5 min at 4 °C. The remaining supernatant was then used for enzyme determinations. The method of Shamanna and Sanderson (1979) was used for XK-activity determination, with the following modifications. The XK reaction was coupled with the pyruvate kinase reaction as follows:

$$\text{xylulose} + \text{ATP} \xrightarrow{\text{xylulokinase}} \text{xylulose 5-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

Consumption of NADH was measured spectrophotometrically at 340 nm. The reaction mixture contained the following: 50 mM TRIS/HCl buffer (pH 7.5), 2.0 mM $MgCl_2$, 2.0 mM ATP, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 10 U pyruvate kinase (E.C. 2.7.1.40), 10 U lactate dehydrogenase (E.C. 1.1.1.27) and 8.5 mM xylulose.

PGI activity was assayed as described previously (Maitra and Lobo 1971).

The protein content was assayed using Coomassie Protein Assay Reagent (Bradford 1976) (Pierce, Rockford, Ill.) in the cell extracts used for XK determinations and by using a microbiuret method (Zamenhoff 1957) in cell extracts assayed for PGI activity. Bovine serum albumin was used as the standard.

Results and discussion

The objective of the present study was to identify genetic modifications, in *S. cerevisiae*, which enhance efficient anaerobic xylose fermentation. Additionally, parental strains of *S. cerevisiae* and wild-type strains of *S. cerevisiae* and *Zygosaccharomyces* sp. were investigated to identify genetic backgrounds suitable for metabolic engineering directed at anaerobic xylose fermentation. Anaerobic xylulose fermentation was chosen to identify metabolic modifications beneficial to anaerobic xylose fermentation in strains not exhibiting product formation related to the redox imbalance generated by the first two steps of the eukaryotic xylose metabolism.

Under the experimental conditions chosen, no new biomass was formed in any of the fermentations.

Glycolytic and trehalose mutants

The *PGI1*-promoter deletion mutants *S. cerevisiae* RBY6-1 and RBY7-1 exhibited PGI activities of 190–270 mU mg^{-1} and 10–20 mU mg^{-1}, respectively; these values are one and two orders of magnitude lower than the activity of the parental strain (Table 1). The ethanol yield from xylulose increased with decreasing PGI activity, being 0.47, 0.53 and 0.54 C-mmol (C-mmol consumed xylulose)$^{-1}$ (Table 2). *PGI1*-promoter deletion mutants have been found to accumulate higher levels of fructose 6-phosphate and fructose 1,6-diphosphate (FDP) than wild-type strains (Boles et al. 1993), and hexose phosphates were required for induction of the ethanologenic enzymes, pyruvate decarboxylase and alcohol dehydrogenase, as well as for inactivation of the gluconeogenic fructose 1,6-bisphosphatase (Boles et al. 1993; Müller et al. 1995). In xylulose-fermenting cells of *S. cerevisiae*, FDP levels were almost an order of magnitude lower relative to glucose-fermenting cells (Senac and Hahn-Hägerdal 1990). Thus, the increased anaerobic ethanol formation in the *PGI1*-promoter deletion mutants suggests that the reduction in PGI activity may enhance intracellular concentrations of FDP in cells metabolising xylulose to levels supporting ethanologenesis.

When the trehalose-pathway genes *TPS1* and *TPS2* (encoding trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase, respectively) were deleted, high levels of FDP accumulated (Hohmann et al. 1996). This was believed to cause inhibition of growth on glucose. When the *E. coli* homologue of *TPS1*, *OtsA*, was expressed in *tps1*Δ, *tps2*Δ strains, growth on glucose was restored while overaccumulation of sugar phosphates remained (data not shown). In agreement with the observation that FDP induces ethanologenic enzymes (Boles et al. 1993), 20% enhanced ethanol formation was found for *S. cerevisiae* W303-1A(*tps1*Δ, *tps2*Δ, p*OtsA*) fermenting xylulose under anaerobic conditions (Table 2). However, only 80% of the consumed carbon was accounted for in the measured products (Table 2),

Table 2 Carbon balances calculated after 70 h in fermentations of 1665 C-mmol xylulose. Yields [C-mmol (C-mmol consumed xylulose)$^{-1}$] for polyols and ethanol are in parentheses

Fermentation	Carbon in (C-mmol l^{-1})	Carbon out (C-mmol l^{-1})						Total carbon recovery (%)
	Xylulose	Ethanol	Arabinitol	Xylitol	Glycerol	Acetate	CO_2	
Glycolytic mutants								
ENY.WA-1A	495	232 (0.47)	0	20 (0.04)	72	25	129	97
RBY 6-1	631	337 (0.53)	0	41 (0.06)	70	17	177	102
RBY 7-1	650	348 (0.54)	0	48 (0.07)	69	22	185	103
PPP mutants								
CEN.PK2-1C, 1D	292	96 (0.33)	0	31 (0.11)	48	44	70	99
CEN.PK(*XKS1*)	439	268 (0.61)	22 (0.05)	0	62	46	157	126
CEN.PK(*gnd1*Δ)	352	152 (0.43)	0	22 (0.06)	41	40	96	100
CEN.PK(*rpe1*Δ)	60	0	0	12 (0.20)	1	21	11	75
Trehalose-metabolism mutants								
W303-1A	1069	262 (0.24)	141 (0.13)	0	48	45	153	61
W303-1A(*tps1*Δ, *tps2*Δ, p*OtsA*)	911	264 (0.29)	182 (0.20)	0	64	64	164	81
Wild-type strains								
T23D	584	139 (0.24)	268 (0.46)	0	29	71	105	105
IGC 2533	784	148 (0.19)	0	378 (0.48)	37	85	117	98
IGC 2608	269	43 (0.16)	102 (0.38)	0	30	39	41	95
Z. bailii IGC 4806^{T}	585	87 (0.15)	0	336 (0.57)	0	38	63	90
Z. rouxii IGC 5276^{T}	232	14 (0.06)	0	101 (0.44)	0	0	7	53

the remaining part probably having been lost as carbon dioxide in the oxidative part of the PPP.

PPP mutants

S. cerevisiae strains generally express low levels of inducible XK activity (Deng and Ho 1990; Jeppsson et al. 1996). Two different sequences for *XKS1* have been reported (Ho and Tsao 1993; Rodriguez-Peña et al. 1998). However, only one of them generates an active enzyme, as discussed by Johansson et al. (submitted for publication). In the construction of *S. cerevisiae* CEN.PK (*XKS1*), primers were designed based on the sequence published by Rodriguez-Pena et al. (1998). The overexpression resulted in an increase in XK activity from ≤0.02 U mg^{-1} protein in the host strain to 2.2–6.0 U mg^{-1} protein in the recombinant strain (Table 1). Previously, overexpression of *XKS1* has resulted in higher ethanol yields from xylulose in wild-type strains of *S. cerevisiae* (Deng and Ho 1990) and in higher ethanol yields from xylose in recombinant strains of *Saccharomyces* sp. harbouring *XYL1* and *XYL2* encoding XR and XDH, respectively (Ho et al. 1998). These results were confirmed with CEN.PK(*XKS1*), which generated the highest ethanol yield from xylulose in the present study [0.61 C-mmol (C-mmol consumed xylulose)$^{-1}$] (Table 2). Concomitantly, polyol formation shifted from xylitol to arabinitol formation with a yield reduction from 0.11 C-mmol xylitol (C-mmol consumed xylulose)$^{-1}$ in the host strain to 0.05 C-mmol arabinitol (C-mmol consumed xylulose)$^{-1}$ in CEN.PK(*XKS1*). This suggests that the redox-sink function of xylulose reduction to xylitol is essential for anaerobic xylulose metabolism in *S. cerevisiae*. When xylulose is stoichiometrically phosphorylated to xylulose 5-phosphate this function has to be fulfilled by other cellular reactions. It has previously been observed that xylose-metabolising cells of *P. stipitis* form arabinitol when the alternative oxidase is inhibited under oxygen-limited conditions (Jeppsson et al. 1995).

In *S. cerevisiae* CEN.PK(*gnd1*Δ) and CEN.PK (*rpe1*Δ) the genes for the PPP enzymes gluconate 6-phosphate dehydrogenase and ribulose 5-phosphate epimerase, respectively, have been deleted (Juhnke et al. 1996). This was expected to prevent the loss of carbon as carbon dioxide in the oxidative PPP during xylulose fermentation. In fact, CEN.PK(*gnd1*Δ) exhibited an ethanol yield 30% higher than that of the host strain (Table 2). In contrast, CEN.PK(*rpe1*Δ) consumed very little xylulose, which was mainly converted to acetate and xylitol without any ethanol formation (Table 2). It was reported previously that mutants deleted in *RPE1* were unable to grow on xylulose (Miosga and Zimmermann 1996). The present investigation shows that such mutants are not even able to ferment xylulose.

Parental and wild-type strains

In addition to the parental strains ENY.WA-1A, CEN.PK2-1C, 1D and W303-1A (Tables 1 and 2), *S. cerevisiae* strains T23D, IGC 2533 and IGC 2608 and two strains of *Zygosaccharomyces* (*Z. bailii* IGC 4806 and *Z. rouxii* IGC 5276) were also investigated for anaerobic ethanol formation from xylulose. Under the

present experimental conditions, the highest ethanol yield obtained was with ENY.WA-1A, 0.47 C-mmol (C-mmol consumed xylulose)$^{-1}$. In a previous study, *S. cerevisiae* CBS 8066 was found to be among the best xylulose-fermenting *S. cerevisiae* strains, having an ethanol yield of 0.46 C-mmol (C-mmol consumed xylulose)$^{-1}$ (Yu et al. 1995).

S. cerevisiae T23D is a homozygous, diploid strain derived from the heterozygous strain CBS 8066 (Wenzel et al. 1992; Pronk et al. 1994) and the two strains have been considered isogenic. However, under conditions used in the present study the anaerobic ethanol yield from xylulose for T23D was only half of that reported for CBS 8066 (Yu et al. 1995) and instead the arabinitol yield obtained with T23D was three times higher (Table 2). This discrepancy implies that the two supposedly isogenic strains differ significantly with respect to anaerobic PPP related redox balance. For *S. cerevisiae* strains IGC 2533 and IGC 2608, high specific growth rates on xylulose had been observed previously (I. Spencer-Martins et al., unpublished results). However, neither strain fermented xylulose to ethanol with appreciable yields (Table 2). Instead, IGC 2608 converted xylulose to arabinitol whereas IGC 2533 converted xylulose to xylitol (Table 2).

Z. bailii IGC 4806 and *Z. rouxii* IGC 5276 were included in the present investigation because of the tolerance of *Zygosaccharomyces* strains towards weak acids (Thomas and Davenport 1985), low pH and high ethanol concentrations (Sousa et al. 1996), a combination commonly encountered in fermentations of lignocellulose hydrolysates (Olsson and Hahn-Hägerdal 1996). *Zygosaccharomyces* is also known to have a better developed PPP than *Saccharomyces* (Brown 1990). However, both *Z. bailii* IGC 4806 and *Z. rouxii* IGC 5276 converted xylulose to xylitol under anaerobic conditions, with only minor amounts of ethanol being formed (Table 2). Furthermore, for *Z. rouxii* IGC 5276 47% of the assimilated carbon could not be accounted for in the products measured (Table 2), indicating that it was lost as carbon dioxide in the oxidative part of the PPP.

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Paper II

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Xylulokinase Overexpression in Two Strains of *Saccharomyces cerevisiae* Also Expressing Xylose Reductase and Xylitol Dehydrogenase and Its Effect on Fermentation of Xylose and Lignocellulosic Hydrolysate

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Fermentation of the pentose sugar xylose to ethanol in lignocellulosic biomass would make bioethanol production economically more competitive. *Saccharomyces cerevisiae*, an efficient ethanol producer, can utilize xylose only when expressing the heterologous genes *XYL1* (xylose reductase) and *XYL2* (xylitol dehydrogenase). Xylose reductase and xylitol dehydrogenase convert xylose to its isomer xylulose. The gene *XKS1* encodes the xylulose-phosphorylating enzyme xylulokinase. In this study, we determined the effect of *XKS1* overexpression on two different *S. cerevisiae* host strains, H158 and CEN.PK, also expressing *XYL1* and *XYL2*. H158 has been previously used as a host strain for the construction of recombinant xylose-utilizing *S. cerevisiae* strains. CEN.PK is a new strain specifically developed to serve as a host strain for the development of metabolic engineering strategies. Fermentation was carried out in defined and complex media containing a hexose and pentose sugar mixture or a birch wood lignocellulosic hydrolysate. *XKS1* overexpression increased the ethanol yield by a factor of 2 and reduced the xylitol yield by 70 to 100% and the final acetate concentrations by 50 to 100%. However, *XKS1* overexpression reduced the total xylose consumption by half for CEN.PK and to as little as one-fifth for H158. Yeast extract and peptone partly restored sugar consumption in hydrolysate medium. CEN.PK consumed more xylose but produced more xylitol than H158 and thus gave lower ethanol yields on consumed xylose. The results demonstrate that strain background and modulation of *XKS1* expression are important for generating an efficient xylose-fermenting recombinant strain of *S. cerevisiae*.

A yeast strain capable of fermenting xylose and glucose to ethanol with high yields would increase the economic feasibility of fuel ethanol production from lignocellulosic biomass. Xylose fermentation by natural and recombinant yeasts has recently been reviewed (14, 17, 20). *Saccharomyces cerevisiae,* which is used for industrial ethanol production, cannot ferment xylose but can ferment its isomer, xylulose (44). In yeast, xylose reductase (XR) and xylitol dehydrogenase (XDH) catalyze the conversion of xylose to xylulose via the intermediate xylitol. Xylulokinase (XK), encoded by the gene *XKS1* (32), phosphorylates xylulose to xylulose 5-phosphate, which is then metabolized through the pentose phosphate pathway and glycolysis. *S. cerevisiae* has been transformed with *XYL1* and *XYL2* from the xylose-fermenting yeast *Pichia stipitis* encoding XR and XDH, respectively (22, 37, 40, 42). Xylose fermentation by these recombinant strains of *S. cerevisiae* yields little ethanol, and xylitol is the major product (22, 37, 40, 42), perhaps due to limited XK activity in *S. cerevisiae* (6).

Saccharomyces sp. strain 1400(pLNH32), a fusion between *Saccharomyces uvarum* and *Saccharomyces diastaticus* (4), which overexpresses *XYL1, XYL2,* and *XKS1,* had an estimated ethanol yield of 0.44 carbon-millimole (c-mmol)/c-mmol in complex medium (18). Recently, *S. cerevisiae* CEN.PK overexpressing *XYL1, XYL2,* and *XKS1* was quantitatively characterized under anaerobic conditions in defined media fermenting mixtures of glucose and xylose (9). This strain, TMB3001, gave an ethanol yield of 0.27 c-mmol/c-mmol. How much of the difference in ethanol yield is due to overexpression of *XKS1*, to media composition and to strain background is not known.

Recombinant xylose utilizing *S. cerevisiae* strains have been characterized in yeast extract-peptone (YP) complex medium (18, 37) and in defined medium (9, 22, 42). The use of YP limits the interpretation of the fermentation results, since YP medium contains all of the cellular components of yeast grown on hexose sugars, including some hexose sugars. Thus, components of the YP media are cofermented with xylose and enhance product yields. Furthermore, YP is too expensive for use in industrial ethanol production (46), which makes YP unsuitable for characterizing the performance of novel xylose-fermenting recombinant yeast strains.

Strains of *S. cerevisiae* differ in their ability to ferment xylulose (8), suggesting inherent differences in their capacities to ferment pentose sugars. Recently, a majority of yeast laboratories within the European Community agreed to use *S. cerevisiae* strain CEN.PK (10) as a reference strain (43). CEN.PK is a laboratory strain specifically designed for physiological and genetic research, including the development of metabolic engineering strategies (10). CEN.PK grows well on various carbon sources, sporulates efficiently, and is available with many different markers and genotypes (10). A recombinant xylose-fermenting *S. cerevisiae* CEN.PK strain is now available (9), but *S. cerevisiae* H158 (31) has been extensively used in earlier studies (15, 16, 26, 27, 28, 40, 41, 42).

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In this study, we overexpressed *XKS1* along with *XYL1* and *XYL2* in two strains of *S. cerevisiae,* H158 (31) and CEN.PK (10), to quantitatively determine the contribution of *XKS1* overexpression and strain background, respectively, on the ethanolic fermentation of xylose. Sugar consumption and product formation in defined mineral medium, complex medium, and a birch wood hydrolysate were also monitored to quantify the contribution of YP and lignocellulose-derived fermentation inhibitors, respectively, to ethanol production in strains overexpressing *XKS1*.

MATERIALS AND METHODS

Strains and plasmids. All yeast and bacterial strains were maintained at 8°C on solid cloning medium (described below) and Luria-Bertani (LB) medium (1) with 100 mg of ampicillin liter^{-1}, respectively. The *XKS1* gene was cloned from *S. cerevisiae* CBS 8066. We obtained *S. cerevisiae* CEN.PK2-1C (MAT**α** *leu2-3 leu2-112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*) (10) from Echard Boles (University of Duesseldorf, Duesseldorf, Germany) and *S. cerevisiae* GPY55-15α (*leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir$^+$*) from Greg Payne (University of California, Berkeley) (31), transformed them with plasmid pY6 (40), and named them CEN.PK and H158, respectively. pY6 contains *XYL1* controlled by the *ADH1* promoter, *XYL2* controlled by the *PGK1* promoter, a yeast 2μm multicopy ORI, and the *URA3* marker for uracil prototrophy. The *ADH1* promoter is weaker than the *PGK1* promoter, resulting in lower XR than XDH activity (40). The yeast strains were also transformed with the integrative plasmid pDF1 (23), resulting in inactivation of the chromosomal *FUR1* gene by gene replacement. Since a Δ*fur1* strain must have an active *URA3* gene to survive, even in the presence of uracil, the *FUR1* inactivation made the strains maintain the pY6 plasmid even under nonselective conditions. *XKS1* was first subcloned in the vector YEp24PGK (40) and then transferred to the vector YEplac112 (11). *Escherichia coli* DH5α [F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1*] (Life Technologies, Rockville, Md.) was used for subcloning.

Cloning of the *XKS1*. *XKS1* was amplified from *S. cerevisiae* CBS 8066 chromosomal DNA (13) with a 5′ primer (primer 1, 5′-GC**GGATCC**TCTAGAATG GTTTGTTCAGTAATTCAG-3′) and either one of two 3′ primers (primer 2 [5′-AGATCT**GGATCC**TTAAGGGGACAATCTTGG-3′] or primer 3 [5′-AGA TCT**GGATCC**TTAGATGAGAGTCTTTTCCAG-3′]). Primer 1 was designed from published sequence information (19, 32). Primer 2 was designed to amplify a 1,776-bp open reading frame (ORF) (19), and primer 3 was designed to anneal 27 bp further downstream on the same sequence, yielding a 1,803-bp ORF (32). Both ORFs have been claimed to encode a protein with XK activity (19, 32). The complementary sequences are underlined. *Bam*HI restriction sites used for cloning are shown in boldface. Primer 1 introduced base substitutions at positions −3 (T to A) and at −2 (T to G) to maximize translational efficiency, where +1 is the A in the start codon (7, 25). Similarly, the codon of the N-terminal amino acid was altered. Protein stability in *S. cerevisiae* depends partly on the N-terminal amino acid of the protein; the half-life can range from minutes to several hours (2). Thus, we changed the *XKS1* N-terminal amino acid from a destabilizing (TTG, Leu) to a stabilizing (GTT, Val) one. The PCR product was ligated in the *Bgl*II site between the *PGK1* promoter and terminator (29) in plasmid YEp24PGK using *Bam*HI sites present on extra nucleotides added onto the primers, resulting in YEp24PGK/XK. The expression cassette containing promoter, gene, and terminator was cut out with *Bam*HI and *Sma*I and ligated into YEplac112 (11) using the same sites, resulting in pXks. CEN.PK and H158 carrying the pY6 plasmid were transformed with pXks, resulting in CEN.PK-pXks and H158-pXks.

Transformations. *S. cerevisiae* was transformed using the lithium acetate method (12), and *E. coli* DH5α was transformed with the calcium chloride method (33).

Cloning media. Yeast strains were grown in SD medium (35) supplied with 250 mg of L-leucine, 50 mg of L-tryptophan, 50 mg of L-histidine, and 50 mg of uracil per liter. Transformants were selected by omission of the appropriate amino acids or nucleotide. Bacterial strains were grown in LB medium (1), and transformants were selected by adding 100 μg of ampicillin ml^{-1}.

Lignocellulose hydrolysate. A birch wood lignocellulose hydrolysate was provided by Robert Eklund (Mid Sweden University, Örnsköldsvik, Sweden). It was prepared by mixing birch wood (10 kg) with water and concentrated sulfuric acid (5 g liter^{-1}) to a total of 30 kg and hydrolyzed for 7 min at 188°C by adding steam (36). The hydrolysate was adjusted to pH 5.5 and filter sterilized (0.45 μm [pore size]). The hydrolysate contained 39 g of xylose, 5.7 g of glucose, 3.5 g of mannose, 3.1 g of galactose, 1.7 g of arabinose, 0.16 g of hydroxy methyl furfural (HMF), and 0.7 g of furfural per liter based on high-pressure liquid chromatography (HPLC) analysis (see below).

TABLE 1. Medium composition

Medium components	Medium composition			
	Defined	Complex	Defined hydrolysate	Complex hydrolysate
Sugar mixture	+	+	−	−
Hydrolysate	−	−	+	+
Mineral medium[a]	+	−	+	−
Amino acids (50 mg liter^{-1})	+	−	+	−
Yeast extract (10 g liter^{-1})	−	+	−	+
Peptone (20 g liter^{-1})	−	+	−	+

[a] Concentrations of vitamins, mineral salts, and trace elements were as described by Verduyn et al. (45). + and −, presence or absence of a component, respectively.

Fermentation media. Defined minimal (45) or complex media were used with either birch wood hydrolysate or a mixture of sugars with the same sugar composition as the birch wood hydrolysate (Table 1). Amino acids L-histidine and L-tryptophan, each at 50 mg liter^{-1}, were added to complement amino acid auxotrophy in defined media. Complex medium contained 10 g of yeast extract and 20 g of peptone per liter in addition to the carbon source. Fermentation using xylose as sole carbon source was conducted with 80 g of xylose per liter. A high xylose concentration was used to overcome the absence of a specific xylose transport system in *S. cerevisiae* (21, 28).

Preparation of inoculum. The inoculum for batch fermentation was prepared by adding a single colony to a 500-ml shaking flask containing 200 ml of defined medium. After overnight incubation at 30°C and 120 rpm, the culture was harvested by centrifugation at 6,400 × *g* for 10 min at 4°C and used to inoculate up to eight 1-liter shaking flasks containing 500 ml of defined medium. The cells were grown to an optical density at 620 nm (OD_{620}) of 4 to 5, harvested at 4°C by centrifugation (6,400 × *g* for 10 min), and resuspended in ice-cold 0.9% (wt/vol) NaCl. An aliquot of this cell suspension was centrifuged at 6,400 × *g* for 10 min and resuspended in 25 ml of ice-cold medium of the same type to be used in fermentation.

Fermentation. Fermentation was conducted batchwise in 120-ml fermentors, with a 100-ml working volume and magnetic bar stirring (100 rpm), and 10 g (dry weight) of inoculum per liter, at 30°C by water jacket and at pH 5.5 by the addition of NaOH.

Analysis of substrates and products. Samples for quantification of substrates and products were analyzed by HPLC. Xylose, glucose, mannose, galactose, arabinose, and xylitol were separated using an HPX-87P ion-exchange column (Bio-Rad Laboratories, Hercules, Calif.) operated at 85°C using water as the mobile phase at 0.6 ml min^{-1} and determined with a refractive index detector (Shimadzu, Kyoto, Japan). Ethanol, glycerol, acetate, xylulose, HMF, and furfural were separated using an Aminex HPX-87H (Bio-Rad) ion-exchange column operated at 45°C, with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}, and detected using a refractive index detector (Shimadzu)—except for HMF and furfural which were detected using a SPD 6A UV detector (Shimadzu).

Enzyme activity analyses. Cell extracts were prepared from 50-ml batch cultures grown to an OD_{620} of 4 to 5. The cells were lysed in 100 mM triethanolamine buffer (pH 7.0), 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol by vortexing them twice with glass beads for 5 min each time at 4°C, with cooling on ice for 5 min in between vortexing. Bovine serum albumin (5 mg ml^{-1}) was added after the cells were lysed to increase protein stability. The protein content in cell extracts was measured by the method of Bradford (3). XR, XDH, and XK activities were measured as previously described (9). XK activity was determined in two steps. First, the XDH activity was determined in the absence of ATP, and then the sum of the XK and XDH activities in the presence of ATP was determined, the XK activity being the difference (9). All enzyme activity measurements were performed at 30°C. The absorbance change per minute (ΔA min^{-1}) was divided by the molar absorptivity for NADH (6.22 cm^{-1}μmol^{-1}) to calculate substrate consumption per minute. One unit of enzyme activity is defined as 1 μmol of substrate converted per min for all assays.

Calculations. Carbon balances were calculated using single carbon unit equivalents (i.e., c-mmol) (5) consumed and produced after 65 h to allow comparison

TABLE 2. Specific XR, XDH, and XK activities in cell extracts of cells grown in defined media with a sugar mixture as the carbon source

Strain	Enzyme sp act (U mg of protein^{-1})[a]		
	XR	XDH	XK
H158	0.8	14	<0.1
H158-pXks	0.8	15	28
CEN.PK	0.7	19	<0.1
CEN.PK-pXks	0.7	18	36

[a] Values represent the average of duplicate experiments with a <10% variation between samples.

of hexose and pentose sugar metabolism. Yields were expressed as c-mmol/c-mmol. The carbon balance was calculated assuming 1 c-mmol of CO_2 produced for every 2 c-mmol of ethanol and acetate produced, according to the metabolic stoichometry. No cell growth, as measured by dry weight determination, occurred during fermentation (data not shown). Biomass production was omitted from the carbon balance calculations for this reason. The specific xylose consumption rate was calculated as the c-mmol gram (cell dry weight)$^{-1}$ hour^{-1}, as based on the amount of xylose consumed after 65 h.

RESULTS

Cloning of the xylulokinase gene (*XKS1*). Two sequences have been reported to encode XK activity in *S. cerevisiae;* one with an ORF of 1,776 bp (19) and another one that is similar but slightly longer, with an ORF of 1,803 bp, originally designated YGR194c (accession no. Z72979) (38) and now called *XKS1* (32). PCR amplification of the *XKS1* gene from *S. cerevisiae* CBS 8066 using primers 1 and 2 (see Materials and Methods) generated the 1,776-bp ORF, whereas the use of primers 1 and 3 generated the 1,803-bp ORF. Both ORFs were fused to the *PGK1* promoter and terminator in the YEp24PGK vector. When transformed into either *S. cerevisiae* CEN.PK or H158, the 1,803-bp ORF resulted in more than 300 times higher XK activity (Table 2), whereas the 1,776-bp ORF did not cause any measurable increase in XK activity (results not shown). The 1,803-bp sequence was used throughout this work. The 1,776-bp sequence was considered incomplete and did not code for an active enzyme since several PCR products of the 1,776-bp ORF were cloned to rule out the possibility of PCR errors. When *Saccharomyces* sp. strain 1400(pLNH32) was constructed, a larger piece of DNA was cloned (18, 19), which yielded the complete gene, whereas the 1,776-bp ORF (19) is too short to generate an active enzyme.

XR, XDH, and XK activity. Enzyme activities were measured under conditions used for preparation of inoculum (Table 2). XR activities were 0.7 to 0.8 U mg^{-1} and were similar in all strains. XDH activities were higher in CEN.PK strains than in H158 strains (18.2 to 18.9 and 13.9 to 15.3 U mg^{-1}, respectively). A low XR/XDH ratio was deliberately chosen, since xylitol production is reduced in such strains compared to strains in which the activity ratio is high (40). The XR and XDH activities were higher than previously reported (40), which may be due to the use of different growth media. XK activities increased at least 300-fold to 28 to 36 U mg^{-1} from overexpression of *XKS1* on a multicopy plasmid under the *PGK1* promoter-terminator sequences (Table 2). When *XKS1* was chromosomally integrated, the specific XK activity was only 2 U mg^{-1} (9). The XK activity cannot be directly compared to that seen in other reports (6, 18), since an assay (34) determining the sum of XK and XDH activity was used (see Materials and Methods).

Xylose fermentation in defined and complex media. CEN.PK-pXks consumed more xylose than H158-pXks and generated slightly higher ethanol concentrations and considerably higher final xylitol concentrations (Table 3). Both strains consumed more xylose in complex medium than in the defined medium. H158-pXks had a higher ethanol yield and a lower xylitol yield than CEN.PK-pXks.

Sugar mixture fermentation in defined and complex media. A sugar mixture reflecting the sugar composition of a birch wood hydrolysate (see Materials and Methods) was fermented by H158, H158-pXks, CEN.PK, and CEN.PK-pXks in both defined and complex media (Table 4). The xylose consumption rate was initially much higher for H158 than for H158-pXks, whereas H158-pXks consumed xylose at a constant rate throughout the fermentation (Fig. 1). Ethanol, xylitol, and

TABLE 3. Product yields of *XKS1* overexpressing strains H158-pXks and CEN.PK-pXks in defined and complex media containing xylose as the sole carbon source[a]

Strain	Medium[h]	Consumed xylose[b] (c-mmol)		Products[c] (c-mmol)			C-bal (%)[d]	Sp Xyl Cons[e]	Y (Et/Xyl)[f]	Y (Xol/Xyl)[g]
		Xyl	Et	Xol	Ac	Gly				
H158-pXks	D	30	6	0	1	2	46	0.5	0.22	0
	C	35	9	1	1	2		0.5	0.27	0.03
CEN.PK-pXks	D	49	8	12	2	2	59	0.8	0.16	0.25
	C	51	11	18	1	3		0.8	0.21	0.36

[a] Both strains express *XYL1* and *XYL2*. Displayed values are the average of duplicate experiments with a <10% difference. The values represent c-mmol consumed or produced after 65 h of fermentation.
[b] Xyl, xylose; Et, ethanol.
[c] Xol, xylitol; Ac, acetate; Gly, glycerol.
[d] The carbon balance (C-bal) is calculated for defined medium only and is the sum of the produced carbon divided by the sum of consumed carbon (c-mmol/c-mmol). A total of 1 c-mmol of CO_2 is assumed to be formed for every 2 c-mmol of ethanol and acetate formed.
[e] Sp Xyl Cons, specific xylose consumption (c-mmol of xylose g [cell dry weight]$^{-1}$ h^{-1}). This value is the xylose consumed within 65 h divided by the biomass (in grams) and time (in hours).
[f] Y (Et/Xyl), c-mmol of ethanol per c-mmol of consumed xylose.
[g] Y (Xol/Xyl), c-mmol of xylitol per c-mmol of xylose consumed.
[h] D, defined; C, complex.

TABLE 4. Product yields of H158 and CEN.PK expressing *XYL1* and *XYL2* and H158-pXks and CEN.PK-pXks also overexpressing *XKS1* in defined and complex media containing a sugar mixture[a]

Strain	Medium	Consumed sugars (c-mmol)		Products (c-mmol)				C-bal (%)	Sp Xyl Cons	Y [Et/(Xyl+Hex)][b]	Y (Xol/Xyl)
		Hex	Xyl	Et	Xol	Ac	Gly				
H158	D	40	114	36	67	17	6	101	1.8	0.24	0.59
	C	41	107	19	79	6	2		1.6	0.13	0.74
H158-pXks	D	39	24	24	1	1	4	68	0.4	0.38	0.04
	C	32	21	27	1	2	3		0.3	0.51	0.04
CEN.PK	D	33	132	22	103	13	3	97	2.0	0.13	0.78
	C	39	133	19	91	10	2		2.0	0.11	0.69
CEN.PK-pXks	D	41	64	27	27	2	6	73	1.0	0.26	0.42
	C	43	77	31	32	5	4		1.2	0.25	0.42

[a] Displayed values are the average of duplicate experiments with a <10% difference. Values represent the c-mmol consumed or produced as calculated after 65 h of fermentation. Column headings and abbreviations are as defined in Table 3.
[b] Y [Et/(Xyl + Hex)], ethanol per c-mmol of consumed xylose and hexoses.

acetic acid were produced when xylose was consumed, whereas glycerol production was not related to xylose consumption.

For both strains, *XKS1* overexpression lowered the total amount of xylose consumed and final xylitol concentrations (Table 4). Final acetate concentrations were lowered by *XKS1* overexpression. In the presence of hexose sugars, complex medium had little effect on xylose consumption. Lower xylitol yields in *XKS1*-overexpressing strains translated into higher ethanol yields on consumed sugars. CEN.PK-pXks consumed more xylose in fermentation of the sugar mixture than in fermentation of xylose alone (Tables 3 and 4), whereas H158-pXks consumed less xylose in the sugar mixture. H158-pXks produced almost no xylitol, while CEN.PK-pXks produced a third of the xylitol produced by CEN.PK. CEN.PK-pXks gave a lower ethanol yield on consumed sugars than H158-pXks. H158-pXks showed the highest ethanol yield from consumed sugars, 0.38 c-mmol/c-mmol in defined medium, equivalent to 57% of the theoretical yield. However, H158-pXks consumed only about a third as much xylose as did CEN.PK-pXks.

Hydrolysate fermentation in defined and complex media. Sugar consumption was reduced in fermentation of birch wood hydrolysate compared to a medium without hydrolysate, a finding possibly due to inhibitory components in the birch wood hydrolysate (24, 36) (Tables 4 and 5). Xylitol production was

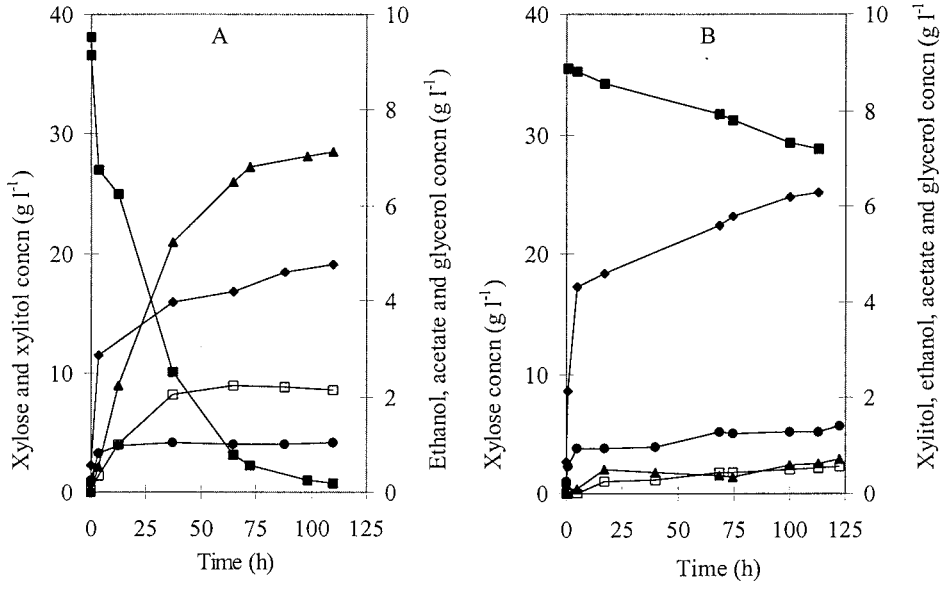

FIG. 1. Fermentation of a sugar mixture (see Materials and Methods) by *S. cerevisiae* H158 expressing *XYL1* and *XYL2* (A) and by *S. cerevisiae* H158-pXks also overexpressing *XKS1* (B) in complex medium. The concentrations of xylose (■) are indicated on the left axis, and the concentrations of ethanol (◆), acetate (□), and glycerol (●) are indicated on the right axis in both panels. The concentration of xylitol (▲) is on the left axis in panel A and in the right axis in panel B. Glucose, mannose, and galactose were omitted for clarity since they were consumed within 5 h. Duplicate fermentation experiments differed less than 10%.

TABLE 5. Product yields of H158 and CEN.PK expressing *XYL1* and *XYL2* and H158-pXks and CEN.PK-pXks also overexpressing *XKS1* in defined and complex media containing a birch wood hydrolysate[a]

Strain	Medium	Consumed sugars (c-mmol)		Products (c-mmol)				C-bal (%)	Sp Xyl Cons	Y [Et/(Xyl+Hex)]	Y (Xol/Xyl)
		Hex	Xyl	Et	Xol	Ac	Gly				
H158	D	33	89	17	59	2	1	72	1.4	0.14	0.67
	C	38	114	22	71	4	2		1.8	0.14	0.62
H158-pXks	D	29	22	15	0	2	0	51	0.3	0.30	0
	C	26	22	23	0	0	1		0.3	0.48	0
CEN.PK	D	31	16	11	6	3	1	60	0.3	0.23	0.38
	C	27	38	8	23	6	1		0.6	0.13	0.62
CEN.PK-pXks	D	26	26	19	5	4	0	75	0.4	0.36	0.17
	C	29	31	24	6	1	15		0.5	0.41	0.20

[a] Displayed values are the average of duplicate experiments with a <10% difference. Values represent c-mmol consumed or produced calculated after 65 h of fermentation. Column headings and abbreviations are as defined in Tables 3 and 4.

reduced in hydrolysate media, more so for CEN.PK than for H158. Complex medium promoted xylose consumption in birch wood hydrolysate. CEN.PK-pXks consumed slightly more xylose than H158-pXks in hydrolysate media but also produced more xylitol, as was the case in the sugar mixture medium. CEN.PK and CEN.PK-pXks showed higher ethanol yields on consumed sugars with hydrolysate than without hydrolysate (Tables 4 and 5), whereas the ethanol yields were lower for H158-pXks.

DISCUSSION

XKS1 overexpression in recombinant *S. cerevisiae* improved ethanolic fermentation of xylose because the xylitol yield decreased in all media we examined. However, *XKS1* overexpression also reduced xylose consumption considerably. The final ethanol concentration was only marginally affected, since reduced xylose consumption was balanced by reduced xylitol production. The decreased xylose utilization in H158-pXks and CEN.PK-pXks may have been caused by uncontrolled XK activity since *XKS1* was overexpressed under the control of the strong *PGK1* promoter, which overrules possible feedback control of xylulose phosphorylation. Teusink et al. (39) suggested that uncontrolled sugar kinase activity in the beginning of a metabolic pathway could lead to abnormal accumulation of sugar phosphates and concomitant depletion of the intracellular ATP pool. A mathematical model (39) has been developed in which glucose utilization is reduced by lack of feedback control of hexokinase and phosphofructokinase activity. This lack of control also led to glucose-6-phosphate and fructose-1,6-bisphosphate accumulation and ATP depletion. The model showed that sugar phosphate accumulation and ATP depletion could be relieved by lower hexokinase activity. By analogy with the effect of uncontrolled hexokinase activity, *XKS1* overexpression may retard cellular metabolism by xylulose-5-phosphate accumulation and/or ATP depletion.

The level of XK activity may be crucial for the xylose uptake rate and the subsequent ethanolic fermentation. When *XYL1*, *XYL2,* and *XKS1* were integrated into the *his3* locus of a CEN.PK strain, yielding strain TMB3001 (9), the XK activity was only about 2 U mg of protein^{-1} compared to ca. 30 U mg of protein^{-1} in H158-pXks and CEN.PK-pXks. Strain TMB3001 had a maximum xylose consumption rate of 6.8 c-mmol g (cell dry weight)$^{-1}$ h^{-1} compared to 1 c-mmol g (cell dry weight)$^{-1}$ h^{-1} for CEN.PK-pXks (Table 4). For *Saccharomyces* sp. strain 1400(pLNH32), with an XK activity of 0.1 U mg^{-1}, the xylose consumption rate has been estimated to be 14.3 c-mmol g (cell dry weight)$^{-1}$ h^{-1} (9, 18). However, in this strain the XK activity is not directly comparable to our results, since an assay also measuring XDH activity was used (34). When *XKS1* was overexpressed in *S. cerevisiae* FY1679 and W303, the XK activity was not reported (32). However, whereas aerobic growth on xylulose was reduced, growth on glucose was unaffected. These results suggest that it is necessary to carefully modulate the XK activity to achieve efficient xylose fermentation by recombinant *S. cerevisiae*.

Complex medium overcame the inhibitory effects of lignocellulose-derived inhibitors so that the specific xylose consumption and the ethanol yield increased when YP medium was added to birch wood hydrolysate. Complex medium had little effect on xylose fermentation in the absence of inhibitory hydrolysate. However, yeast extract and peptone are too expensive for industrial ethanol production from lignocellulosic hydrolysate (46). Xylitol production was lower in hydrolysate media than in sugar mixture media, possibly due to the reduction of furfural to furfuryl alcohol, which may provide the XDH reaction with reduced cofactors (30).

The host strain influenced the efficiency of the xylose fermentation by the resulting recombinant strain. The highest ethanol yield, 0.51 c-mmol/c-mmol, was obtained with H158-pXks in complex medium (Table 4). H158 is a laboratory strain used extensively as a host for genetic engineering for xylose fermentation (15, 16, 26, 27, 28, 40, 41, 42). CEN.PK is a laboratory strain, specifically designed for physiological and genetic research, including the development of metabolic engineering strategies (10). CEN.PK grows well on various carbon sources, sporulates efficiently, and is available with many different markers and genotypes (10). CEN.PK has been chosen as a standard strain for laboratories within the European Community (43). CEN.PK-pXks consumed more xylose than H158-pXks in the fermentation of xylose only and of the sugar

mixture. CEN.PK-pXks also performed better in hydrolysate media than did H158-pXks, with higher sugar consumption and higher ethanol production. Furthermore, when H158-pXks fermented hydrolysate media, the ethanol yield decreased compared with the level of fermentation in other media.

Our study shows that the results of a metabolic engineering strategy aimed at introducing a new metabolic pathway is highly dependent on the choice of host strain and the modulation of overexpressed genes. In particular, our results on the deleterious effect of uncontrolled *XKS1* overexpression showed that it is necessary to quantify the effect of individual genetic modifications introduced in a metabolic pathway. Medium composition influenced the results to a lower extent. Even so, any quantitative characterization of new metabolically engineered strains must be considered incomplete if not also performed in defined mineral medium.

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Paper III

Reduced oxidative pentose phosphate pathway flux in recombinant xylose utilizing *Saccharomyces cerevisiae* strains improves the ethanol formation from xylose

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Running title: Improved xylose fermentation by lower oxidative PPP flux.

Keywords: yeast, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, NADPH, xylitol, redox balance.

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ABSTRACT

In recombinant xylose fermenting *Saccharomyces cerevisiae*, about thirty percent of the consumed xylose is converted to xylitol, which is a major by-product. Xylitol production results from a co-factor imbalance since xylose reductase uses both NADPH and NADH while xylitol dehydrogenase only uses NAD^+. In this study we increased the ethanol yield and decreased the xylitol yield by lowering the flux through the NADPH producing oxidative pentose phosphate pathway (PPP). The oxidative PPP was blocked either by disruption of the *GND1* gene, one of the isogenes of 6-phosphogluconate dehydrogenase or by disruption of the *ZWF1* gene encoding glucose 6-phosphate dehydrogenase. Decreasing the phosphoglucose isomerase activity by 90% also lowered the oxidative PPP flux. These modifications all resulted in lower xylitol yield and higher ethanol yield than in the control strains. TMB3255, carrying a disruption of the *ZWF1* gene, gave the highest ethanol yield (0.41 g g^{-1}) and the lowest xylitol yield (0.05 g g^{-1}) ever reported for a xylose-fermenting recombinant *S. cerevisiae*, but also a lower xylose fermentation rate. The low xylose fermentation rate may be partly due to a very low NADPH mediated xylose reduction. Metabolic flux modeling of TMB3255 confirmed that the NADPH producing oxidative PPP was blocked and that xylose reduction was mediated only by NADH. These results prove that xylitol production is strongly connected to the flux through the oxidative PPP.

INTRODUCTION

Fuel ethanol produced from fermentation of lignocellulosic hydrolysates is an attractive replacement of liquid fossil fuels, since its production is renewable and does not generate net carbon dioxide. Hydrolysis of lignocellulose generates mainly hexose but also pentose sugars, which the preferred ethanol producing microorganism *Saccharomyces cerevisiae* cannot metabolize (39). In hydrolysate made from hardwood, it has been demonstrated that xylose must be fermented to ethanol for the process to be economically feasible (42). The yeast *Pichia stipitis* has the ability to metabolize xylose through the expression of the *XYL1* gene encoding xylose reductase (XR) and the *XYL2* gene encoding xylitol dehydrogenase (XDH). XR catalyzes the reduction of xylose to xylitol using NADH or NADPH (26), whereas XDH oxidizes xylitol to xylulose exclusively using NAD^+ (27). However, *P. stipitis* is sensitive to ethanol (9) and requires low and carefully controlled oxygenation (30), which prevents its use for industrial ethanol production.

Recombinant *S. cerevisiae* strains expressing *XYL1* and *XYL2* genes from *P. stipitis* have been constructed and the ability to ferment xylose has been demonstrated (23). However most of the consumed xylose is secreted as xylitol (23, 32, 37). Xylitol production can be lowered by over-expression of the *XKS1* gene encoding native xylulokinase (XK) (20), but still about one third of the consumed xylose is converted to xylitol under anaerobic conditions (12). Xylitol formation has been proposed to result from the co-factor imbalance between the NADPH-consuming XR and the NADH-producing XDH reactions (23).

In a previous investigation, we reported improved ethanol yield from xylulose in *S. cerevisiae* strains having a low phosphoglucose isomerase activity (PGI) or a deletion in the *GND1* gene that encodes 6-phosphogluconate dehydrogenase (6-PGDH) (11). Glucose 6-phosphate, a branch point metabolite between glycolysis and the oxidative pentose phosphate pathway (PPP), is

reversibly converted to fructose 6-phosphate using PGI, whereas 6-PGDH converts 6-phosphogluconate to ribulose 5-phosphate in the oxidative PPP (31). This suggests that the increased ethanol yield from xylulose observed for these strains could be related to an altered flux through the oxidative PPP.

In this investigation we lowered the oxidative PPP flux by genetic engineering and studied the effects on xylose utilization and product formation in *S. cerevisiae* strains expressing XR and XDH from *P. stipitis* and overproducing native XK. The oxidative PPP flux was modified by (i) lowering PGI activity, (ii) deleting the *GND1* gene and (iii) deleting the *ZWF1* gene encoding glucose 6-phosphate dehydrogenase (G6PDH). A flux model (35) was used to compare internal fluxes in the *zwf1D* strain and a control strain.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this investigation are summarized in Table 1. *Escherichia coli* DH5α (Life Technologies, Rockville, MD) was used for sub-cloning. All strains were stored in 20% glycerol at –80ºC. Yeast cells from freshly streaked YPD (3) plates were used for inoculation.

Nucleic acid manipulation. Plasmid DNA was prepared with QIA Miniprep kit (QIAGEN, Valencia, CA) or BioRad Plasmid Miniprep kit (Hercules, CA). Restriction and modification enzymes were obtained from Roche (Roche Diagnostics AB, Bromma, Sweden) and from Life Technologies (Rockville, MD) respectively. DNA extractions from agarose gel were made by QIAGEN Gel Extraction Kit.

Transformation. Competent cells of *E. coli* DH5α were prepared and transformed by the method of Inoue *et al.* (1990) (19), and yeast transformations were made using the modified lithium

acetate method (14). *E. coli* transformants were selected on Luria-Bertani (LB) medium (3) plates with 30 µg ml^{-1} kanamycin (ICN Biochemical Inc., Aurora, OH) and or 100 µg ml^{-1} ampicillin (IBI Shelton Scientific Inc., Shelton, CT). *S. cerevisiae* transformants were selected on YPD-plates with 100 µg ml^{-1} zeocin (Invitrogen, Groningen, The Netherlands) or 200 µg ml^{-1} geneticin (Life Technologies, Rockville, MD) or on Yeast Nitrogen Base w/o amino-acids (Difco, Sparks, MD) supplemented for auxotrophic requirements.

Construction of TMB3250 and TMB3251. The YIpXR/XDH/XK vector (12) was digested with *Pst*I within the *HIS3* gene and used to transform strains ENY.WA-1A and RBY6-1 generating *S. cerevisiae* TMB3250 and TMB3251, respectively, after selection for histidine prototrophy.

Construction of TMB3008. The plasmid pUG6 (16) was digested with *Sac*I and *Xba*I and the *KanMX* gene was replaced with the zeocin resistance gene from pTEF1/Zeo (Invitrogen, Groningen, The Netherlands) using the same restriction sites, resulting in pUG6 Zeo. The zeocin resistance gene flanked by *lox*P sequences was removed from pUG6 Zeo using *Pvu*II and *Spe*I and blunted with Klenow DNA polymerase. The YIpXR/XDH/XK plasmid (12) was digested with *Pvu*II and ligated to the zeocin resistance gene fragment. The resulting plasmid, YIpLoxZEO, was digested with *Nde*I within the *HIS3* gene and used to transform CEN.HJ5-1B generating *S. cerevisiae* TMB3008 after selection for zeocin resistance.

Construction of TMB3255. The *ZWF1* gene was PCR-amplified from *S. cerevisiae* TMB3001 chromosomal DNA, using the oligonucleotides 5´-CG**GGATCC**AAAATGTC ACTGACCGCGGC-3´ adding a *Bam*HI-restriction site at 3' end (bold) and 5´-GTTTCG GCTCGGCCGGAGGAGG-3´. The *ZWF1* PCR-product was inserted in the pUC19 vector (43) after restriction cleavage with *Eco*RI and *Bam*HI. The *KanMX* gene with *loxP* sequences was

PCR-amplified from pUG6 with the oligonucleotides 5´-TCC**CCC** **GGG**AGCTTCGTACGCTGCAG-3´ adding a *Sma*I restriction site (bold) and 5´-GG**GGT** **ACC**ATAGGGAGACCGGCAGATCC-3´ adding a *Kpn*I restriction site (bold). The KanMX PCR product was inserted into the *ZWF1* gene using restriction sites *Msc*I-*Kpn*I within the *ZWF1* gene and *Sma*I-*Kpn*I flanking the *KanMX* gene. The plasmid was digested with *Bam*HI and *Eco*RI, and the linear product was used for transformation of TMB3001 (12), generating TMB3255 strain.

Small-flask cultivations. Defined medium (50 ml) (40) with 40 g l^{1} glucose in a 250 ml baffled shake-flask was inoculated and incubated overnight at 30°C on an orbital incubator (Gallenkamp INR-200, Leicester, UK). TMB3250 and TMB3251 were grown on 40 g l^{-1} fructose instead of glucose since the low PGI activity causes a growth defect on glucose (6). These precultures were used to inoculate a second culture of 200 ml in a 1000 ml baffled shake-flask, which was incubated with the same conditions. The cells were harvested in exponential phase by centrifugation at 4400×g, 10 min, 4°C (Avanti^{TM} J-251, Beckman Instruments, Palo Alto, CA) and washed twice with 0.9% NaCl.

Stirred 25ml vials containing 20 ml of defined medium (40) with 50 g l^{1} xylose as sole carbon source were inoculated with ca. 5 g l^{1} cells. The medium was supplemented with 100 mM citrate buffer (pH 5.5), the required amino acids (50 µg ml^{-1} L-tryptophan and/or 250 mg l^{1} L-leucine) and/or 50 mg l^{1} uracil. Ergosterol and Tween 80 were added to final concentrations of 0.01 and 0.4 g l^{1}, respectively (1, 2). Fermentation was conducted at 30ºC in duplicates. Samples were withdrawn using a 2 mm hypodermic needle with a syringe and fermentation gasses were expelled through a 0.8 mm needle.

Continuous cultivations. Yeast cells were grown in 200 ml defined medium (40) containing 20 g l^{1} xylose and 20 g l^{1} glucose, 10 mg l^{1} ergosterol and 0.4 g l^{1} Tween 80 in a 250 ml baffled

shake-flask. The culture was incubated overnight at 30°C. Cells were centrifuged at 4400×g for 5 min and 4°C, and used to inoculate 1.5 l of the same medium to OD_{620} 0.5 in a Bioflo III fermentor (New Brunswick Scientific, Edison, NJ). Antifoam was added at 0.5 % (v/v) (Dow Corning® Antifoam RD Emulsion, BDH Laboratory Supplies, Poole, England). Continuous cultivation was set up at dilution rates of 0.06 and 0.12 h^{-1} at 30°C, pH 5.5 controlled by addition of 3M NaOH, and a stirring speed of 200 rpm. The fermentor was sparged with 0.2 l min^{-1} nitrogen (containing less than 5 ppm O_2) as measured with a gas mass flowmeter (Bronkhorst, Ruurlo, The Netherlands).

Analyses of substrates and products. Glucose, xylose, xylitol, succinate, glycerol, acetate and ethanol concentrations were determined by column liquid chromatography (CLC) using a Gilson CLC system (Gilson, Villiers-le-bel, France). An Aminex HPX-87H column (BioRad, Richmond, CA) and a R1D-10A refractive index detector (Shimadzu, Kyoto, Japan) were used. The column temperature was 45°C and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml min^{-1}.

In continuous cultivation, cells from the outlet were used for measuring the biomass content of RNA (4), proteins and polysaccharides (17). Samples for CLC and cell dry weight determination were withdrawn from the fermentor. Growth was followed by measurement of OD_{620}. The cell dry weight was determined by filtering one volume of sample through a 0.45 µm filter and washing with three volumes of water. The filter was dried in a microwave oven at 350 W for 8 min, cooled in a desiccator and weighed. The composition of outgoing gas was monitored with a Carbon Dioxide and Oxygen Monitor Type 1308 (Brüel&Kjœr, Copenhagen, Denmark).

Enzymatic measurements. Crude extracts for enzyme measurements were made using the Y-PER reagent (Pierce, Rockford, IL). Protein concentration was determined by Coomassie Protein Assay Reagent (Pierce), using bovine serum albumin as a standard. The phosphoglucose isomerase (PGI, E.C 5.3.1.9) activity was measured as described by Maitra and Lobo (1971) (24). The

glucose-6-phosphate dehydrogenase activity (G6PDH, EC 1.1.1.49) and the 6-phosphogluconate dehydrogenase activity (6-PGDH, EC 1.1.1.44) were measured according to Bergmeyer (5).

Intracellular NADPH and $NADP^+$ were measured enzymatically (5) on a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) after extraction with boiling ethanol (15) from samples withdrawn from anaerobic growth on glucose.

RESULTS

Effect of decreased PGI activity on xylose fermentation. The xylose pathway, consisting of XR and XDH enzymes from *P. stipitis* and overproduced XK from *S. cerevisiae* was introduced in a strain with reduced PGI activity to study the effect of the flux distribution between glycolysis and PPP on xylose fermentation. For this purpose, the YIpXR/XDH/XK vector (12) was introduced in the ENY.WA-1A strain (control) and the derived RBY6-1 strain (10-fold decrease in PGI activity, (6)) generating *S. cerevisiae* TMB3250 and TMB3251 respectively. Enzymatic measurements confirmed that TMB3251 had about 10% of the PGI activity of its control strain TMB3250 (data not shown).

After 70 hours of xylose fermentation, TMB3250 and TMB3251 had consumed 7.2 and 4.6 g xylose g^{-1} biomass, respectively (Table 2). Ethanol yields were 0.30 g g^{-1} xylose for TMB3250 and 0.34 g g^{-1} xylose for TMB3251. The 11% higher ethanol yield of TMB3251 was accompanied by a lower xylitol yield (0.21 g g^{-1}) compared to TMB3250 (0.30 g g^{-1}). The acetate yield was slightly higher in TMB3251 (0.03 g g^{-1}) than in TMB3250 (0.02 g g^{-1}). Glycerol yield was also higher for TMB3251 (0.05 g g^{-1}) than for TMB3250 (0.06 g g^{-1}).

Xylose fermentation by a *GND1* deleted strain. The *GND1* gene encodes one of the two $NADP^+$-dependent isoenzymes of 6-phosphogluconate dehydrogenase that catalyze the

conversion of 6-phosphogluconate to ribulose 5-phosphate in the oxidative PPP. The xylose pathway was introduced in the CEN.HJ5-1B strain that has an inactive *GND1* gene (21) to study the effect of an altered oxidative PPP on xylose fermentation. *S. cerevisiae* strain TMB3008 was generated by integration of the YIpLoxZEO vector in a glucose positive revertant of CEN.HJ5-1B. A glucose positive revertant was used instead of the original strain to facilitate glucose metabolism (28).

TMB3008 and its control TMB3001 were used for batch fermentation of xylose. TMB3001 and TMB3008 consumed 9.2 and 5.6 g xylose g^{-1} biomass, respectively (Table 2). Ethanol yields were 0.31 g g^{-1} for TMB3001 and 0.38 g g^{-1} for TMB3008 (Table 2). TMB3008 showed 24% higher ethanol yield and a lower xylitol yield (0.13 g g^{-1}) compared to TMB3001 (0.29 g g^{-1}). The acetate yield was higher in TMB3008 (0.05 g g^{-1}) than in TMB3001 (0.03 g g^{-1}). The glycerol yields were similar in the two strains.

Xylose fermentation by a *ZWF1* deleted strain. The strain with low PGI activity and the *GND1* deleted strain showed similar phenotypes compared to their respective wild-type strain with respect to xylose fermentation (Table 2). One common denominator of these two strains is that both these genetic alterations could decrease the flux through the oxidative PPP during xylose fermentation. Lowering of the oxidative PPP flux could result directly from abolishment of 6-PGDH activity or indirectly by lower gluconeogenetic flux as a consequence of low PGI activity. Therefore, the disruption of the *ZWF1* gene encoding glucose 6-phosphate dehydrogenase should also result in lower xylitol and higher ethanol yields. The *zwf1D* genotype has not been reported to have any negative side effects regarding glucose utilization (21), whereas both the *GND1* deletion and the lowering of PGI activity cause a defective glucose metabolism (6, 28). The *ZWF1* gene was deleted

in TMB3001 resulting in TMB3255 and the strains were compared with respect to xylose fermentation.

TMB3255 showed the highest ethanol yield (0.41 g g^{-1}), the lowest xylitol yield (0.05 g g^{-1}) and the highest acetate yield (0.08 g g^{-1}) of the three strains (Table 2). However, the specific xylose consumption after 70 hours (1.5 g g^{-1} biomass) was also the lowest of the three strains (Table 2). This qualitative similar product pattern suggests that the phenotypes of *gnd1D*, low PGI and *zwf1D* are all related to lower oxidative PPP flux.

The *zwf1D* genotype has been reported to cause a requirement for organic sulfur (33). This was not observed in our hands and xylose uptake was not affected by addition of metionine.

Enzyme activities. The deletion of the *ZWF1* gene (TMB3255) led to a more pronunced effect on xylose fermentation than the deletion of the *GND1* gene (TMB3008), i.e. lower xylitol yield, enhanced ethanol yield and 3.7-fold decrease in specific xylose consumption (Table 2). This suggested that the oxidative PPP might not be totally blocked in TMB3008. In *S. cerevisiae* there are two isoenzymes of 6-PGDH, encoded by *GND1* and *GND2* (28). Since only *GND1* gene was deleted in TMB3008, the oxidative PPP might still be partly active.

We measured the enzyme activities for G6PDH and 6-PGDH of TMB3001, TMB3008 and TMB3255 in cultures grown on glucose and xylose (Table 3). The enzyme activities of G6PDH and 6-PGDH in TMB3001 (control strain) were comparable with previously published values for *S. cerevisiae* (24). The 6-PGDH enzyme activity increased in TMB3001 when cultivated on xylose instead of glucose. In TMB3008 (*gnd1D*), 6-PGDH but also G6PDH activities were undetectable. This might be an effect of the reversion to a glucose positive phenotype of this strain, since *GND1* deletion mutants often loose G6PDH activity when exposed to high concentrations of glucose (28). As expected, G6PDH activity was not detectable in TMB3255 (*zwf1D*). A 4-fold decreased 6-

PGDH activity was measured in TMB3255 compared with the control strain (TMB3001). The hypothesis of a partly active oxidative PPP in TMB3008 was therefore not confirmed by higher activities of G6PDH or 6-PGDH compared to TMB3255.

Continuous cultivations and flux analyses. A flux analysis was performed to compare intracellular fluxes in TMB3001 and TMB3255 using a stoichometric model (35). Chemostat cultivations on a mixture of 20 g l^{1} xylose and 20 g l^{1} glucose were performed at dilution rates of 0.06 and 0.12 h^{-1}. The flux values for TMB3001 (Fig. 1A) and TMB3255 (Fig. 1B) were normalized to 100 mmol consumed glucose and xylose per g biomass and hour.

The xylose consumption rate and the xylitol production rate were lower in TMB3255 than in TMB3001 (Table 4), confirming the results from batch fermentation. However, the difference in xylose utilization between the two strains were not as pronounced in chemostat cultivation with glucose and xylose as substrate (Table 4) as it was in batch fermentation with xylose as sole carbon source (Table 2). At higher dilution rates the xylose consumption decreased, and the glycerol production increased as previously found (35). In TMB3001, the xylose consumption was 37% lower at 0.12 h^{-1} than at 0.06 h^{-1}, whereas it was only 12% lower for TMB3255 at 0.12 h^{-1}.

The flux analyses confirmed that there was no flux through the oxidative PPP in TMB3255 deleted for the *ZWF1* gene (Fig. 1B). In contrast, 14-18% of the consumed glucose was channeled through the oxidative PPP of the control strain TMB3001 (Fig. 1A). The flux from ribulose-5-phosphate to xylulose-5-phosphate was reversed in TMB3255, compared to TMB3001. The model also predicted that XR only used NADH in TMB3255, whereas XR used 53 and 57% NADPH in TMB3001 at 0.06 h^{-1} and 0.12 h^{1}, respectively. Intracellular concentrations of NADPH and $NADP^{+}$ in TMB3001 and TMB3255 were analyzed using enzymatic assays with fluorimetric

detection. Average values indicated that the $NADPH/NADP^+$ ratio was 3-5 times lower in TMB3255 than in TMB3001 (data not shown).

DISCUSSION

The design of an efficient recombinant *S. cerevisiae* strain for xylose fermentation has been a major challenge for many years. Up to now, xylitol remained a major fermentation by-product from xylose in *S. cerevisiae* strains expressing *XYL1* and *XYL2* genes (23, 32, 37). It has been proposed that xylitol formation results from the apparent co-factor imbalance between the XR and XDH enzymes (7). The XR enzyme has been engineered for lower affinity for NADPH, however lower enzymatic activity and substantially higher Km for xylose were obtained (22, 44). Expression of bacterial xylose isomerase, which converts xylose to xylulose without co-factors involvement was also attempted. So far, only xylose isomerase from *Thermus thermophilus* has been actively expressed in *S. cerevisiae*, however with very low activity (38).

It has been demonstrated that both the XK level and the XR/XDH ratio had an effect on xylitol formation in *S. cerevisiae*. Strains with low XR/XDH ratio showed lower xylitol formation than strains with a high ratio (37). Overexpression of *XKS1* increased the ethanol yield (18, 20). However, strains overexpressing *XKS1* still secret about one third of the consumed xylose as xylitol (35) under anaerobic conditions.

In this work, improved recombinant xylose-fermenting *S. cerevisiae* strains have been constructed by combining parameters that have previously been shown to improve xylose fermentation, low XR/XDH ratio and *XKS1* overexpression, with the inactivation of the oxidative PPP. To the best of our knowledge, TMB3255 has the highest ethanol yield (0.41 g g^{-1}) and the lowest xylitol yield (0.05 g g^{-1}) ever reported for a xylose-fermenting recombinant *S. cerevisiae*.

The inactivation or lowering of the oxidative PPP activity was achieved by deletion of either the *GND1* gene or the *ZWF1* gene, or by lowering the PGI activity. Deletion of *ZWF1* and *GND1* genes blocks the oxidative PPP flux directly, while low PGI activity decreases the gluconeogenetic flux towards glucose 6-phosphate which is the substrate of G6PDH, the first enzyme of the oxidative PPP. Since the oxidative PPP is the main source of NADPH in the cell (8), we suggest that the low xylitol yield is directly linked to depletion of NADPH in strains with defective oxidative PPP. The higher acetate yield is an indication of NADPH depletion, since oxidation of acetaldehyde to acetate requires a $NADP^+$-dependent acetaldehyde dehydrogenase (25, 36). Furthermore, a lower NADPH/$NADP^+$ ratio was measured in TMB3255 compared to TMB3001.

The XR enzyme uses both NADPH and NADH (26), whereas the XDH enzyme exclusively uses NAD^+, which leads us to propose the following model. In strains with a low flux through oxidative PPP, the level of NADPH is low and a greater fraction of xylose is reduced with NADH. Since NADH is consumed in the XR step and produced in the XDH step, the xylose conversion to xylulose is balanced with respect to co-factors, which would explain why only minor amounts of xylitol are formed (Fig. 2). This model is further strengthened by previous results (35) and present results with the control strain TMB3001 at different dilution rates; NADH usage by XR increased and xylitol yield decreased at higher dilution rate, where faster anabolism results in reduced NADPH availability.

Our results in *S. cerevisiae* agree with the decreased sorbitol accumulation in rats deficient in the G6PDH enzyme (34). In this metabolic model, glucose is reduced to sorbitol with NADPH as co-factor by aldose reductase in analogy with the reduction of xylose to xylitol by XR. The fact that this connection is found in different model systems further strengthens that there is a connection between NADPH production capacity and xylitol formation.

Lowering the oxidative PPP activity resulted in reduced xylose consumption rate. One reason is that the NADPH-dependent reduction of xylose to xylitol is reduced, resulting in a reduced overall xylose fermentation rate. However the xylose uptake of TMB3255 was even lower than could be explained by the lack of the $NADP^+$-dependent reaction. An explanation for this observation could be the inhibition of XR by $NADP^+$. The lower NADPH producing capacity in a strain with low oxidative PPP leads to increased accumulation of $NADP^+$ and it has been shown that $NADP^+$ inhibits XR *in vitro* (26, 41).

A low XR/XDH activity ratio (0.06) has been shown to result in decreased xylitol production by xylose fermenting *S. cerevisiae* compared to higher ratios (5 and 17.5) (37). The XR/XDH ratio in *P. stipitis* has been reported to be much higher, 0.7-4 (10, 29). Despite this difference, *P. stipitis* produce less xylitol than recombinant *S. cerevisiae* (23). A mathematical core model of the ratio of the XR, XDH and XK enzymes showed that co-factor concentrations had a greater impacton xylitol by-product formation than the activity ratios of the enzymes (13). This suggests that levels and ratios of NADPH and/or NADH are differently regulated in *P. stipitis* than in *S. cerevisiae*.

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LEGENDS TO FIGURES

Figure 1. Internal metabolic fluxes of TMB3001 (*ZWF1)* (**A**) and of TMB3255 (*zwf1D*) (**B**) in chemostat cultures at a dilution rate of 0.06 h^{-1} (upper value) and 0.12 h^{-1} (lower value) with a feed containing 20 g l^{1} xylose and 20 g l^{1} glucose. TMB3001 has wild-type level of G6PDH (gray arrow). TMB3255 is deficient in G6PDH (open arrow). All fluxes are normalized to a total specific sugar consumption of 100 mmol g^{1} cell dw h^{1}). Substances showed in italics are substrates or products secreted into the medium.

Figure 2. Proposed model of the connection between NADPH level, xylitol production and xylose consumption rate. **A)** In a strain with wild-type level of the oxidative PPP, xylose is reduced with both NADH and NADPH. Xylose reduced with NADPH is mainly secreted as xylitol. **B)** In a strain with lower oxidative PPP activity, the fraction of xylose reduced by NADPH is smaller due to NADPH depletion. This results in lower xylitol production and a lower xylose consumption.

TABLE 1. Strains used with relevant genotypes and phenotypes.[a]Parental strain ENY.WA-1A.[b]Parental strain CEN.PK 113-7A.
[c]Parental strain CEN.PK2-1C

Strain	Relevant genotype	Relevant phenotype	Reference
ENY.WA-1A	*MAT* α *ura3-52 leu2-3, 112 his3-D 1 trp1-289 MAL2-8c MAL3 SUC3*	1000-2500mU PGI (mg protein)$^{-1}$	Juhnke *et al.* 1996
RBY6-1[a]	ENY.WA-1A *pgi1-1 D ::URA3, PGI1::LEU2*	190-270mU PGI (mg protein)$^{-1}$	Boles and Zimmermann, 1994
TMB3250 [a]	ENY.WA-1A *his3* ::YIp XR/XDH/XK	Expresses XR, XDH and XK	This work
TMB3251[a]	RBY6-1 *his3* ::YIp XR/XDH/XK	Expresses XR, XDH and XK 10-fold decreased PGI activity	This work
TMB3001[b]	CEN.PK 113-7A (*MAT* a *his3-D 1 MAL2-8c SUC2*) *his3::* YIp XR/XDH/XK	Expresses XR, XDH and XK	Eliasson *et al* . 2000
TMB3255[b]	TMB3001 *zwf1::Kan* MX	Expresses XR, XDH and XK No G6PDH activity	This work
TMB3008[c]	CEN.HJ 5-1B (*MAT* α *leu2-3,112 his3-D 1 ura3-52 trp1-289 gnd1::HIS3 MAL2-8c SUC2*) *his3::* YIploxZEO	Expresses XR, XDH and XK No 6-PGDH activity	This work

TABLE 2. Specific xylose consumption (g g biomass^{-1}) and ethanol, xylitol, acetate and glycerol yields (g g consumed xylose^{-1}) after 70 hours of 25 ml batch fermentations with 50 g l^{-1} xylose as sole cabon source. Values are the average of two independent fermentations and deviation from the average.

Strains	Relevant phenotype	Consumed xylose	Yields			
			ethanol	xylitol	acetate	glycerol
TMB3250		7.2 ± 0.37	0.30 ± 0.01	0.30 ± 0.01	0.02 ± 0.01	0.05 ± 0.01
TMB3251	low PGI	4.6 ± 0.26	0.34 ± 0.01	0.21 ± 0.01	0.03 ± 0.01	0.06 ± 0.01
TMB 3001		9.2 ± 0.1	0.31 ± 0.01	0.29 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
TMB 3008	*gnd1 D*	5.6 ± 0.72	0.38 ± 0.01	0.13 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
TMB 3255	*zwf1 D*	1.5 ± 0.1	0.41 ± 0.02	0.05 ± 0.01	0.08 ± 0.01	0.05 ± 0.01

TABLE 3. Specific glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) activities measured after growth on glucose or incubation in xylose.

		Specific activity (U/mg protein)			
		G6PDH		6-PGDH	
Strains	Relevant genotype	Glucose	Xylose	Glucose	Xylose
TMB 3001		0.90	0.95	0.54	0.83
TMB 3008	*gnd1 D*	<0.01	<0.01	<0.01	<0.01
TMB 3255	*zwf1 D*	<0.01	<0.01	0.21	0.21

TABLE 4. Specific uptake rates (negative values) and production rates (positive values) (mmol g^{-1} $biomass^{-1}$ h^{-1}) of substrates and products at dilution rate 0.06 h^{-1} and 0.12 h^{-1} for TMB 3001 and TMB 3255 (*zwf1D*). The carbon and degree of reduction balances were obtained using measured values for ethanol and carbon dioxide. [a] calculated from carbon balance and degree of reduction balance.

	TMB 3001		TMB 3255	
	D = 0.06 h^{-1}	D = 0.12 h^{-1}	D = 0.06 h^{-1}	D = 0.12 h^{-1}
Xylose	-1.50	-1.93	-0.80	-1.29
Glucose	-3.39	-6.88	-4.00	-7.32
$CO2_{measured}$	6.59	11.69	7.56	11.85
$CO2_{calculated}$ [a]	7.21	13.59	7.78	13.42
$Ethanol_{measured}$	5.44	10.28	7.10	12.00
$Ethanol_{calculated}$ [a]	6.47	12.42	7.33	12.39
Xylitol	0.64	0.71	0.10	0.17
Glycerol	0.81	1.58	0.75	1.80
Acetate	0.04	0.07	0.36	0.78
Succinate	0.02	0.04	0.00	0.00

FIGURE 1A.

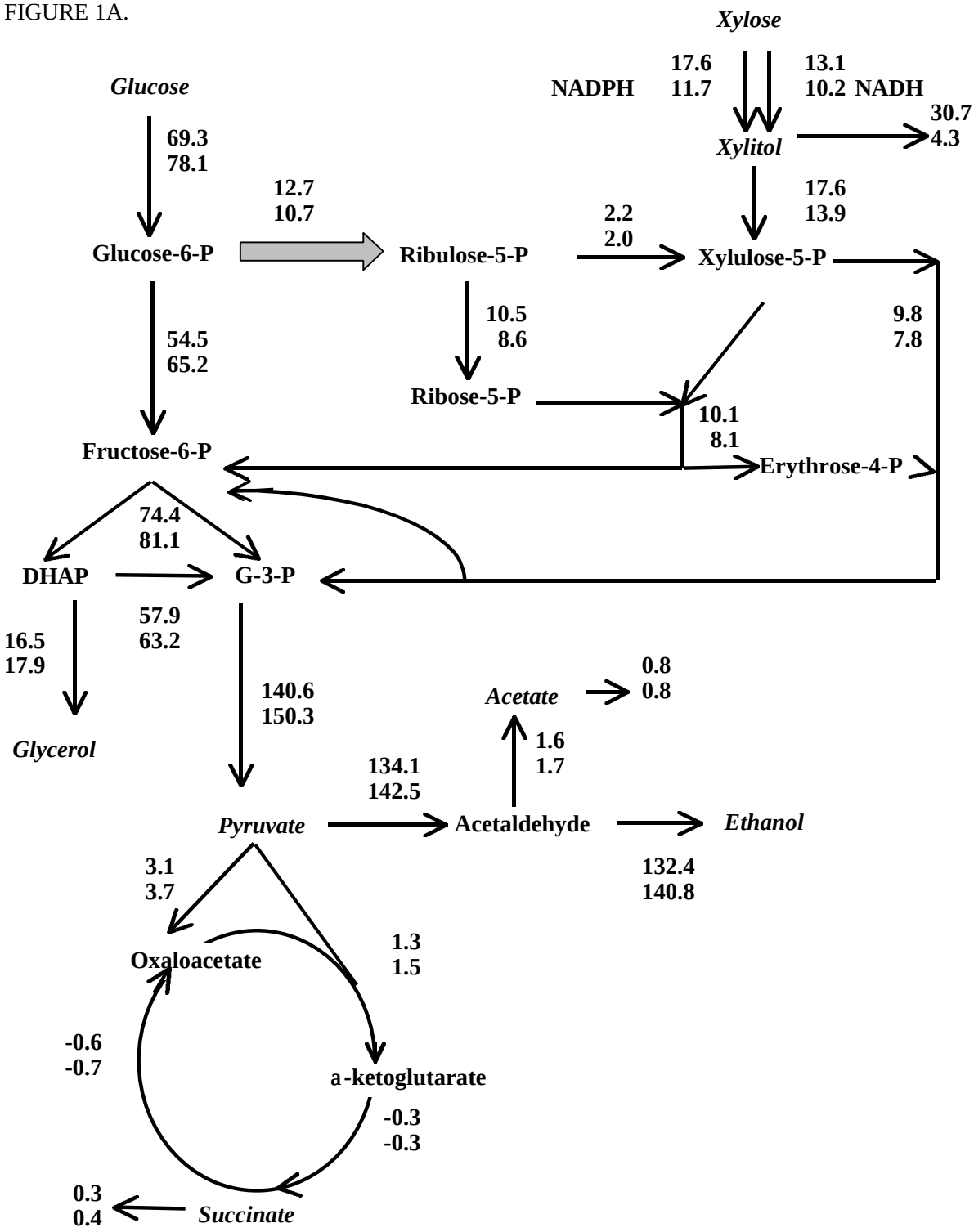

Xylose
17.6
11.7
NADPH
13.1
10.2
NADH
30.7
4.3
Xylitol
Glucose
69.3
78.1
12.7
10.7
17.6
13.9
2.2
2.0
Glucose-6-P
Ribulose-5-P
Xylulose-5-P
10.5
8.6
9.8
7.8
54.5
65.2
Ribose-5-P
10.1
8.1
Fructose-6-P
Erythrose-4-P
74.4
81.1
DHAP
G-3-P
57.9
63.2
16.5
17.9
0.8
0.8
Acetate
140.6
150.3
Glycerol
1.6
1.7
134.1
142.5
Pyruvate
Acetaldehyde
Ethanol
3.1
3.7
132.4
140.8
1.3
1.5
Oxaloacetate
-0.6
-0.7
a-ketoglutarate
-0.3
-0.3
0.3
0.4
Succinate

FIGURE 1B.

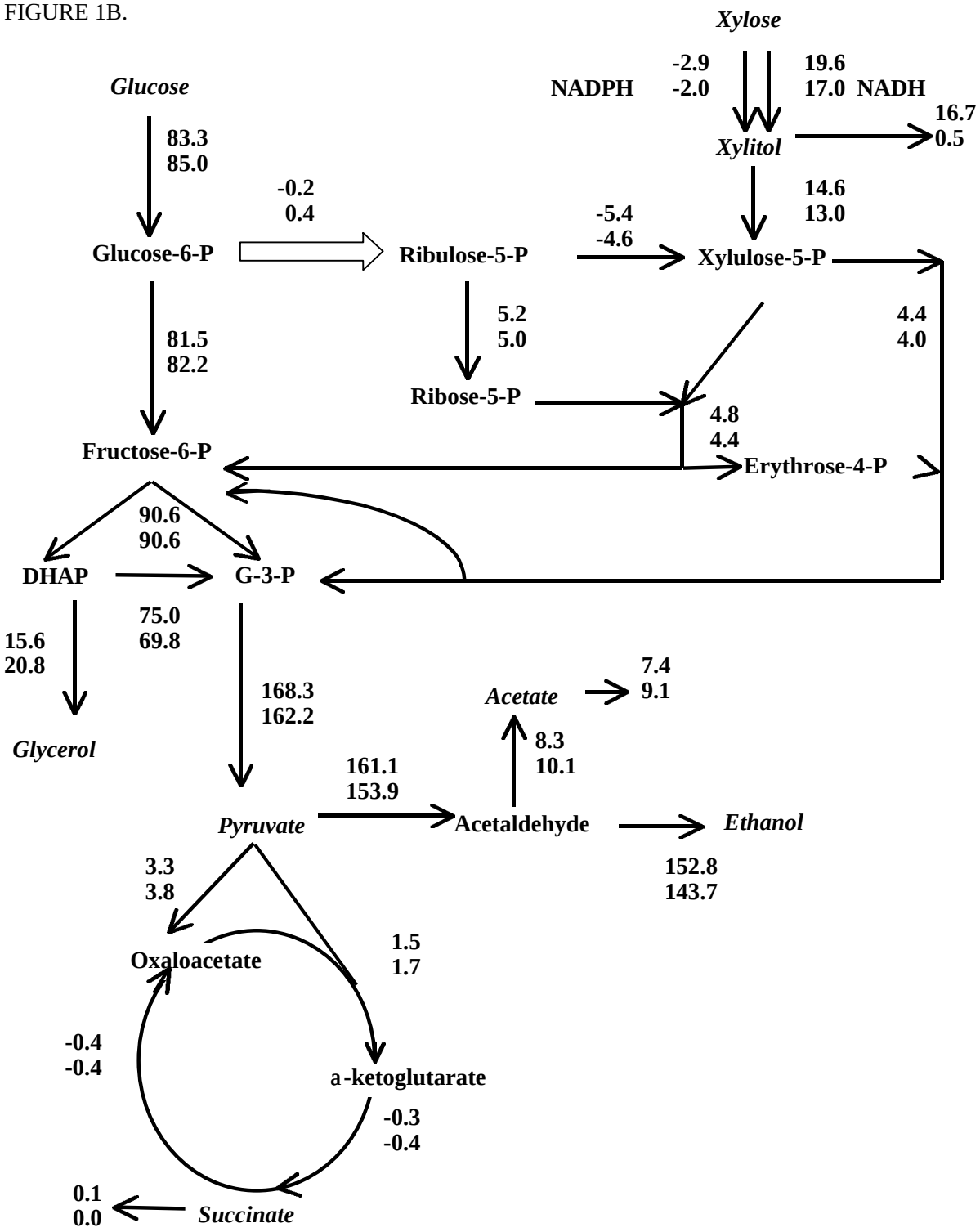
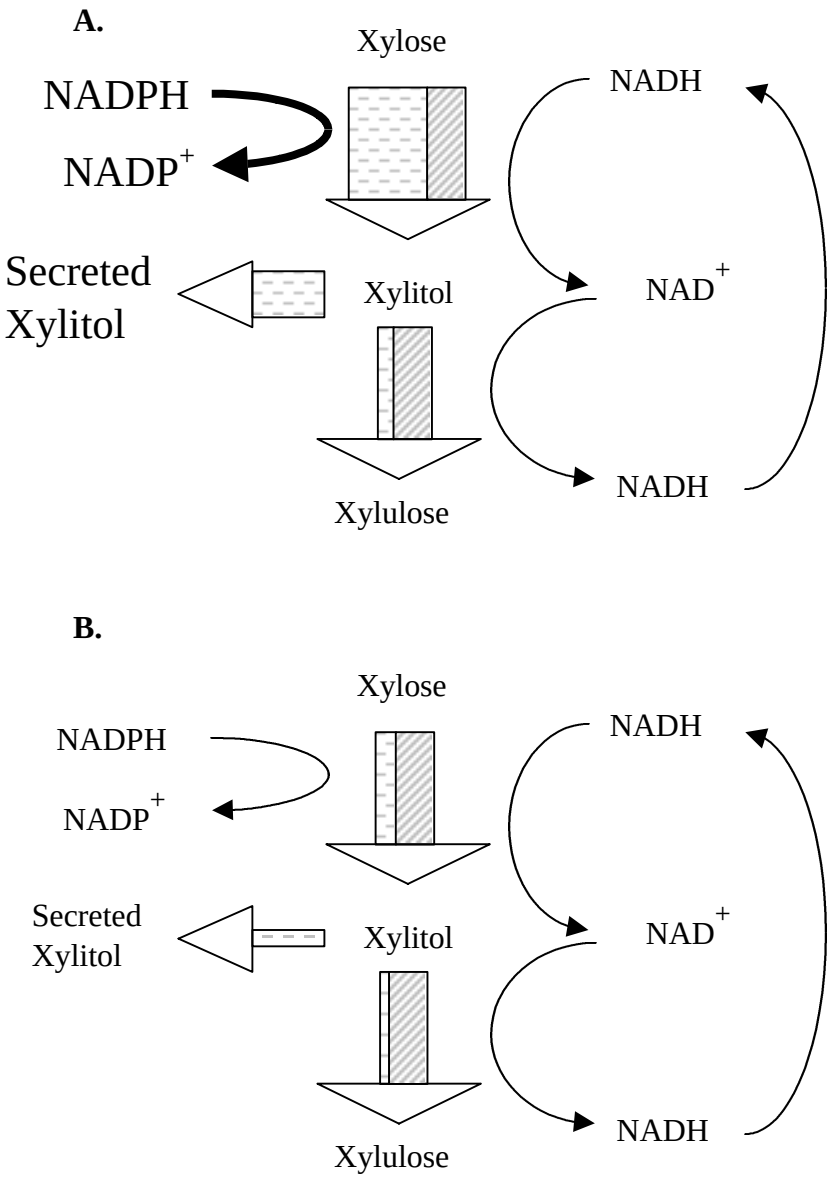

Xylose
-2.9
19.6
NADPH
-2.0
17.0
NADH
16.7
0.5
Xylitol
Glucose
83.3
85.0
-0.2
0.4
14.6
13.0
-5.4
-4.6
Glucose-6-P
Ribulose-5-P
Xylulose-5-P
5.2
5.0
4.4
4.0
81.5
82.2
Ribose-5-P
4.8
4.4
Fructose-6-P
Erythrose-4-P
90.6
90.6
DHAP
G-3-P
75.0
69.8
15.6
20.8
168.3
162.2
7.4
9.1
Acetate
Glycerol
8.3
10.1
161.1
153.9
Pyruvate
Acetaldehyde
Ethanol
3.3
3.8
152.8
143.7
1.5
1.7
Oxaloacetate
-0.4
-0.4
a-ketoglutarate
-0.3
-0.4
0.1
0.0
Succinate

FIGURE 2.


A.
Xylose
NADPH
NADP+
NADH
Secreted
Xylitol
Xylitol
NAD+
NADH
Xylulose
B.
Xylose
NADPH
NADP+
NADH
Secreted
Xylitol
Xylitol
NAD+
NADH
Xylulose

Paper IV

Improved Xylose Fermentation by Modulation of Xylose Reductase and Glucose 6-Phosphate Dehydrogenase Activities in Xylose Fermenting Recombinant *Saccharomyces cerevisiae*

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Running title: Improved Xylose Fermentation by Modulation of XR and G6PDH Activities

Keywords: yeast, xylose reductase, glucose 6-phosphate dehydrogenase, NADPH, xylitol, ethanol, redox balance, synthetic promoters

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ABSTRACT

In anaerobic xylose fermentation, about 30% of the consumed xylose is secreted as xylitol, which is a major by-product. In a previous study, we showed that disruption of the *ZWF1* gene, encoding glucose 6-phosphate dehydrogenase in the oxidative pentose phosphate pathway (PPP), resulted in decreased xylitol yields accompanied by increased ethanol yields from xylose. However, a 6-fold decrease in specific xylose consumption rate was observed, which was interpreted as being due to the absence of NADPH-dependent xylose reduction and/or $NADP^+$-dependent inhibition of xylose reductase (XR). In the present work, the xylose consumption rate was enhanced by over-expression of XR and by allowing a small flux through the oxidative PPP by down-regulation of *ZWF1* rather than disruption. Over-expression of XR from 0.42 to 3.36 U $(mg\ protein)^{-1}$ resulted in a 1.7-fold increase in xylose fermentation rate compared with the control strain TMB3001. XR over-expression in a *zwf1*-deleted background resulted in a 15-fold increase in the specific xylose consumption rate.

A novel library of synthetic promoters was developed to facilitate down-regulation of *ZWF1*. Glucose 6-phosphate dehydrogenase activities of 1% and 13% of the wild-type level resulted in 2.8-fold and 5.1-fold increases in specific xylose consumption, respectively, compared with the *zwf1*-deleted strain. Both strains exhibited decreased xylitol yields (0.132 and 0.186 g $(g\ xylose)^{-1}$) and enhanced ethanol yields (0.355 and 0.336 g $(g\ xylose)^{-1}$) compared with the control strain TMB3001 (0.290 g xylitol $(g\ xylose)^{-1}$, 0.307 g ethanol $(g\ xylose)^{-1}$). *S. cerevisiae* TMB3261, which combines deletion of *ZWF1* and over-expression of *XYL1*, showed a specific ethanol productivity of 0.11 g ethanol $(g\ biomass \times h)^{-1}$, which is 2.4-fold higher than that of the reference strain TMB3001, and corresponds to 55% of the maximal ethanol productivity of the natural xylose-fermenting yeast *P. stipitis*.

INTRODUCTION

Lignocellulosic biomass is a potential inexpensive feedstock for the production of renewable energy in the form of fuel ethanol. Fermentation of the xylose fraction in addition to the hexose sugars, has been found to decrease the ethanol production cost by 25% (15). However, the industrial ethanol-producing microorganism *Saccharomyces cerevisiae* cannot metabolise the xylose fraction in the biomass. *Pichia stipitis* has the ability to metabolise xylose through the expression of the *XYL1* gene encoding xylose reductase (XR) and the *XYL2* gene encoding xylitol dehydrogenase (XDH). XR catalyses the reduction of xylose to xylitol using NADH and preferably NADPH (28), whereas XDH oxidizes xylitol to xylulose, which *S. cerevisiae* can ferment (37) exclusively using NAD^+ (29).

Recombinant *S. cerevisiae* strains expressing *XYL1* and *XYL2* genes from *P. stipitis* have been constructed and the ability to ferment xylose has been demonstrated (23). However, most of the consumed xylose was secreted as xylitol (23, 34, 36), which has been proposed to be the result of co-factor imbalance between the NADPH-consuming XR and the NADH-producing XDH reactions (23).

We recently showed that NADPH-dependent xylose reduction was responsible for most of the xylitol secretion in recombinant *S. cerevisiae* and that blocking the oxidative pentose phosphate pathway lowered the xylitol yield to very low levels (20). Blocking of the PPP was achieved by disruption of either *GND1*, encoding 6-phosphogluconate dehydrogenase, or *ZWF1*, encoding glucose 6-phosphate dehydrogenase. Disruption of *ZWF1* resulted in the lowest xylitol yields, but also lowered the specific xylose consumption rate to about one sixth of that in the control strain. We suggested that the decrease in xylose consumption rate with a lower PPP activity was due to the absence of NADPH-dependent xylose reduction and/or the inhibition of XR by $NADP^+$ (28, 39).

In this investigation we used two different strategies to restore a high xylose consumption rate in a strain with a blocked oxidative PPP. In the first, the *XYL1* gene was over-expressed with the strong glycolytic *PGK1* promoter (25), while in the second, the *ZWF1* gene was down-regulated using a new library of synthetic promoters.

MATERIALS AND METHODS

Strains. The strains of *S. cerevisiae* used in this investigation are summarized in Table 1. The *ZWF1* gene was cloned from *S. cerevisiae* CBS 8066 chromosomal DNA and the *XYL1* gene from *P. stipitis* CBS 6054 chromosomal DNA. *Escherichia coli* DH5α (Life Technologies, Rockville, MD, USA) was used for sub-cloning. All strains were stored in 20% glycerol at –80ºC. Yeast cells from freshly streaked YPD plates (3) were used for inoculation.

Nucleic acid manipulation. Plasmid DNA was prepared with a BioRad Plasmid Miniprep Kit (Hercules, CA, USA). Restriction and modification enzymes were obtained from Roche (Roche Diagnostics AB, Bromma, Sweden) and from Life Technologies (Rockville, MD, USA), respectively. A QIAGEN Gel Extraction Kit was used for DNA extractions from agarose.

Transformation. Competent cells of *E. coli* DH5α were prepared and transformed by the method of Inoue *et al.* (1990) (16), and yeast transformation was performed using the modified lithium acetate method (13). *E. coli* transformants were selected on Luria-Bertani (LB) plates (3) with 100 μg ml^{-1} ampicillin (IBI Shelton Scientific Inc., Shelton, CT, USA). *S. cerevisiae* transformants were selected on YPD plates with 100 μg ml^{-1} zeocin (Invitrogen, Groningen, The Netherlands).

Construction of the pB3 PGK *XYL1* vector. The *XYL1* gene encoding XR was PCR-amplified from *P. stipitis* CBS 6054 chromosomal DNA, using the primers 5*XYL1*clon `5'-GC`**`GGATCC`**`TCTAGAATGCCTTCTATTAAGTTGAACTCTGG-3'` and 3*XYL1*clon `5'TT`**`GGA`**

TCCTCTAGATTAGACGAAGATAGGAATCTTGTCCC-3′, adding *Bam*HI sites to both ends (denoted in bold face). The *XYL1* PCR product was cut with *Bam*HI and inserted after the *PGK1* promoter at the *Bgl*II site of pB3 PGK (21), resulting in pB3 PGK *XYL1*.

Construction of the pB3 CUP1 *ZWF1* vector. A fragment of approximately 75% of the 5′ end of *ZWF1* was cloned by PCR from *S. cerevisiae* CBS 8066 chromosomal DNA using the primers 5*ZWF1*clon 5′-GA**GGATCC**AGAATGAGTGAAGGCCCCGTCAAATTC-3′ and 3*ZWF1*clon 5′-GA**GGATCC**CTGCACTCTGATGACCAGTTCG-3′, adding *Bam*HI sites at both ends (bold face). The flanking *Bam*HI sites were cut at the 1150 bp PCR product. The partial *ZWF1* ORF was ligated to the pB3 PGK, cut with *Bgl*II, resulting in pB3 PGK *ZWF1*. The *PGK1* promoter was removed by restriction cleavage with *Sac*I and *Xba*I. The *CUP1* promoter was released from the plasmid pCu413 CUP1 (24) by a restriction cleavage with *Sac*I and *Spe*I. The *CUP1* promoter was ligated to the promoterless pB3 PGK *ZWF1* vector, resulting in pB3 CUP1 *ZWF1*.

Construction of the promoterless pB3 *ZWF1* vector. The *PGK1* promoter was removed from pB3 PGK *ZWF1* by restriction cleavage with *Sac*I and *Xba*I. The cohesive ends of the remaining promoterless plasmid were blunted by Klenow DNA polymerase. The resulting pB3 *ZWF1* vector was closed by ligation with T4 DNA ligase.

Oligonucleotides for synthetic promoter design. Two synthetic oligonucleotides were used to generate synthetic promoters: oligonucleotide 1: 5′ATCAGAATTCTCGAG NNNNNCTTCCNNNNNACCCATACANNNNNNNNACCCATACANNNNNCTTCCNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNTATAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNCTTCTTTCTTGTAACATC3′(159-mer) and oligonucleotide 2: 5′ATCGGGATCCA TTTTGATTTAGTGTTTGTGTGTTGATAAGCAGTTGCTTGGTTTTTTATGAAAAATAGCTAGA AGGAATAAGGAATTACAAGAGAGATGTTACAAGAAAGAAG3′ (113-mer). The oligo-nucleotides were manufactured by DNA Technology A/S, Aarhus, Denmark. The underlined

sequences are complementary, facilitating annealing of the downstream part of oligonucleotides 1 and 2.

Synthesis of synthetic promoters. To create double-stranded DNA, 0.6 nmol oligonucleotide 1 and 1.04 nmol oligonucleotide 2 were mixed with 100 nmol of each dNTP and incubated for two hours at 37°C with 23.6 units Klenow DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) in a total volume of 200 µl. The double-stranded DNA was purified by agarose gel electrophoresis and cut with the restriction enzymes *Eco*RI and *Bam*HI.

Construction of the pYLZ-2 YRP vectors. Plasmid pYLZ-2 reporter gene vectors (14) containing the *E. coli lacZ* gene were cut with *Eco*RI and *Bam*HI and the synthetic double-stranded promoters were ligated to the vector resulting in the pYLZ-2 YRP vectors. Positive clones of pYLZ-2 vectors containing synthetic promoters were identified by analytical PCR using the primers 5YRPclon and 3YRPclon, as described below, and *E. coli* colonies as template. Plasmid DNA was prepared from thirty-seven positive clones, designated pYLZ-2 YRP 1 to 37.

Construction of the pB3 YRP *ZWF1* vectors. Three selected clones of the synthetic promoter library (YRP13, YRP25 and YRP34) were amplified by PCR with the primers 5YRPclon `5′-GATC`**`GAGCTC`**`TGGCCGATTCATTAATCCAGCTGAA-3′` and 3YRPclon `5′-GATC`**`TCTAGA`**`TTTGATTTAGTGTTTGTGTGTTGAT-3′`. The primers introduced *Sac*I and *Xba*I restriction sites on the 5′ side and 3′ side of the promoter for compatibility with the pB3 PGK promoter (denoted in bold face). The *PGK1* promoter was removed from pB3 PGK *ZWF1* vector by restriction cleavage with *Sac*I and *Xba*I. The synthetic promoters were inserted into the promoterless pB3 *ZWF1* vector using the same sites.

Construction of TMB3260 and TMB3261. pB3 PGK *XYL1* was cleaved within the *XYL1* gene using *Sna*BI. The cleavage product was used for transformation of TMB3001 and

TMB3255, generating TMB3260 and TMB3261 after selection for zeocin resistance. Integration at the correct locus was confirmed using PCR by amplification of the PGK promoter together with the *XYL1* gene as described earlier (21).

Construction of TMB3030, TMB3034, TMB3035, TMB3037 and TMB3256. The vectors pB3 CUP1 *ZWF1*, pB3 YRP13 *ZWF1*, pB3 YRP25 *ZWF1,* pB3 YRP34 *ZWF1* and pB3 *ZWF1* were cleaved with *Bgl*II within the *ZWF1* gene. The cleavage products were used for transformation of TMB3001, generating TMB3030, TMB3037, TMB3035, TMB3034 and TMB3256, after selection for zeocin resistance. Integration at the correct locus was confirmed by PCR amplification with the pB3 PGK-specific primer BJ 7729 5´-GAAGTTATTAGGTG ATATCAGATCC-3´ and the *ZWF1*-specific primer 3*ZWF1*clon.

Small-flask cultivation. Defined medium (50 ml) (38) with 40 g l^{-1} glucose in a 250 ml baffled shake-flask was inoculated and incubated overnight at 30°C on an orbital incubator (Gallenkamp INR-200, Leicester, UK). These pre-cultures were used to inoculate a second culture of 200 ml in a 1000 ml baffled shake-flask, which was incubated under the same conditions. Cells were harvested in the exponential phase by centrifugation at 4400×g, 10 min, 4°C (Avanti^{TM} J-251, Beckman Instruments, Palo Alto, CA, USA) and washed twice with 0.9% NaCl. The dry cell weight was determined by filtering one volume of sample through a 0.45 µm filter and washing with three volumes of water. The filter was dried in a microwave oven at 350 W for 8 min, cooled in a desiccator and weighed.

Stirred 25 ml vials containing 20 ml defined medium (38) with 50 g l^{-1} xylose as sole carbon source were inoculated with approximately 5 g l^{-1} cells. The medium was supplemented with 100 mM citrate buffer (pH 5.5). Ergosterol and Tween 80 were added to final concentrations of 0.01 and 0.4 g l^{-1}, respectively (1, 2). Fermentation was conducted in duplicates at 30ºC. Samples were withdrawn using a 2 mm hypodermic needle with a syringe and fermentation gases were expelled through a 0.8 mm needle.

Analysis of substrates and products. Xylose, xylitol, glycerol, acetate and ethanol concentrations were determined by column liquid chromatography (CLC) using a Gilson CLC system (Gilson, Villiers-le-bel, France). An Aminex HPX-87H column (BioRad, Richmond, CA, USA) and a R1D-10A refractive index detector (Shimadzu, Kyoto, Japan) were used. The column temperature was 45°C and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml min^{-1}.

Enzymatic measurements. Crude extracts for enzyme measurements were made using the Y-PER reagent (Pierce, Rockford, IL, USA). The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce), with bovine serum albumin as standard. The xylose reductase activity (EC 1.1.1.21) and the glucose-6-phosphate dehydrogenase activity (G6PDH, EC 1.1.1.49) were measured according to Bergmeyer (7). Thirty-seven *S. cerevisiae* strains CEN.PK 113-5D (12) harbouring YEpYRP1 to YEpYRP37 were grown in liquid culture to the late log phase. Beta-galactosidase activities were measured according to Miller (1972) (26). Each measurement was performed on duplicate cultures.

RESULTS

Effect of increased XR activity on xylose fermentation. We recently showed that the strain TMB3255, in which the *ZWF1* gene encoding glucose 6-phosphate dehydrogenase (G6PDH) in the oxidative PPP had been deleted, had a higher ethanol yield (0.409 g g^{-1}) than its control strain TMB3001 (0.307 g g^{-1}) in batch fermentation of xylose (Table 2) (17). However, TMB3255 consumed xylose at one sixth of the rate of TMB3001. The lower rate could be the result of low or absent NADPH-mediated xylose reduction and/or the inhibition of XR by $NADP^+$ (28, 39). The effect of increased XR activity on xylose fermentation was therefore assessed in TMB3001 (control) and TMB3255 (*zwf1Δ*) by introduction of the pB3

PGK *XYL1* vector, generating TMB3260 and TMB3261, respectively. The NADPH-dependent XR activity in TMB3001 was 0.42 U (mg protein)$^{-1}$ and the NADH-dependent activity was 0.26 U (mg protein)$^{-1}$, which corresponds well with the earlier reported values for this strain (10). The TMB3255 strain was directly derived from this strain, so the XR activity was not measured. The NADPH-dependant XR activities in TMB3260 and TMB3261 were 3.36 and 4.99 U (mg protein)$^{-1}$, and the NADH-dependent XR activities were 2.36 and 3.10 U (mg protein)$^{-1}$ (Table 2).

Batch fermentation with 50 g/l xylose was carried out with the two XR over-expressing strains TMB3260 (*PGK1-XYL1*) and TMB3261 (*PGK1-XYL1*, *zwf1Δ*), and their control strains, TMB3001 and TMB3255 (*zwf1Δ*). After 60 hours, TMB3260 (*PGK1-XYL1*) and TMB3261 (*PGK1-XYL1*, *zwf1Δ*) had consumed 14.7 and 19.0 g xylose (g biomass)$^{-1}$ (Table 4), which corresponds to increases of 1.7 fold and 15 fold compared with TMB3001 and TMB3255 (*zwf1Δ*), respectively.

As a result of increased XR activity the xylitol yield was 55% lower for TMB3260 (*PGK1-XYL1*) than for the control strain TMB3001 (Table 2). However, because of increased acetate and glycerol yields in TMB3260 (0.046 g g^{-1} and 0.161 g g^{-1}) compared with TMB3001 (0.025 g g^{-1} and 0.052 g g^{-1}), the ethanol yield was unchanged. The glycerol yield was also increased in the other high-XR-activity strain TMB3261 (*PGK-XYL1*, *zwf1Δ*) (0.128 g g^{-1}) compared with its control TMB3255 (*zwf1Δ*) (0.054 g g^{-1}). TMB3261 exhibited a lower ethanol yield (0.344 g g^{-1}) than TMB3255 (0.409 g g^{-1}), accompanied by a 12-fold increase in specific ethanol production after 60 hours (6.5 g (g biomass)$^{-1}$) compared with TMB3255 (0.5 g (g biomass)$^{-1}$).

A comparison between the two high-XR-activity strains revealed that the *zwf1*-deleted strain TMB3261 consumed xylose 29% faster than TMB3260 with wild-type G6PDH activity.

The ethanol yield was 13% higher for TMB3261 (0.344 g g^{-1}) than for TMB3260 (0.304g g^{-1}). TMB3261 also had a lower xylitol yield (0.076 g g^{-1}) than TMB3260 (0.130 g g^{-1}). The acetate yield was slightly higher in TMB3261 (0.056 g g^{-1}) than in TMB3260 (0.046 g g^{-1}), whereas the glycerol yield was lower in TMB3261 (0.128 g g^{-1}) than in TMB3260 (0.161 g g^{-1}).

Effect of modulation of G6PDH activity on xylose fermentation. The direct correlation between the availability of NADPH and the level of xylitol formation in xylose fermentation was assessed by expressing the *ZWF1* gene under control of the Cu^{2+}-regulated *CUP1* promoter (24). The *ZWF1* gene was placed under regulation of the *CUP1* promoter in TMB3030 after integration of pB3 *CUP1 ZWF1* into TMB3001. The addition of 0, 1 and 10 µM Cu^{2+} gave G6PDH activities of 1.00, 1.81 and 2.90 U (mg protein)$^{-1}$, respectively (Table 3), which resulted in xylitol yields of 0.319 g g^{-1}, 0.328 g g^{-1} and 0.383 g g^{-1}, respectively (results not shown). This further supports the hypothesis that there is a strong connection between G6PDH activity and xylitol formation (20). The lowest G6PDH activity obtainable with the *CUP1* promoter was 1.00 U (mg protein)$^{-1}$ (Table 3), which is slightly higher than the activity of the control strain TMB3001 (0.87 U (mg protein)$^{-1}$), and which makes the *CUP1* promoter unsuitable for down-regulation of *ZWF1* expression.

Construction of a promoter library for down-regulation of *ZWF1*. In order to facilitate the regulation and optimisation of *ZWF1* expression, a library of synthetic promoters was designed. A library of synthetic promoters has already been reported for *S. cerevisiae* (17). However, this library was designed from the *ARG8* promoter, which is regulated by amino acids in the medium. Since a promoter independent of external effectors was desired, a new promoter was designed as outlined in Figure 1. The promoter, called YRP, contains two regulatory structures, so-called RPG boxes (27) and CT boxes (5, 6). These elements have been well characterized and are known to promote transcription of constitutive genes such as

glycolytic and ribosomal protein genes (22, 30, 31, 33). The CT boxes and RPG boxes were designed to be degenerated; at each position, 1% each of the three other bases is present, instead of the one that is indicated (Figure 1). The concept on which the promoter design is based is that completely degenerated intervening sequences combined with less degenerated transcription factor binding sites results in a wide range of different promoter strengths (17-19). Prokaryotic promoters have a relatively compact and constant consensus sequence, upon which a synthetic promoter can be modelled (18, 19). However, *S. cerevisiae* does not appear to have a strict consensus sequence for promoters, instead the synthetic promoter was pieced together forming a combination of structures from several *S. cerevisiae* promoters. The distance between the RPG box and the CT box was the same as in the glycolytic *PYK1* promoter (8), since it had been shown to be the best for interaction between the transcription factors GCR1p and RAP1p (8), which bind to the RPG box and CT box, respectively. The distance between the RPG boxes was chosen to be the same as in the ribosomal protein promoter RP39A (30) due to its compact size. The sequence between the TATA box and the 3´ end of the promoters was the same as in the *ENO1* promoter (35). The structure of the *ENO1* promoter and the distance from the TATA box to the CT box were chosen based on size considerations. Since the quality of synthetic oligonucleotides rapidly decreases with increasing size, the synthetic promoters were limited to about 250 bp. The synthetic double-stranded promoters were ligated to the vector pYLZ-2 (14) containing the *E. coli lacZ* reporter gene, resulting in pYLZ-2 YRP vectors. Thirty-seven different clones of the promoter were used to control the *lacZ* reporter gene in vectors pYLZ-2 YRP1 to 37. The resulting beta-galactosidase activities ranged from 0.007 to 37 Miller units (Figure 2), covering about three orders of magnitude between the lowest and the highest activity. The range of promoter activities was covered in small steps, facilitating fine-tuning of gene expression.

Three selected promoter clones YRP13 (1 Miller unit (MU)), YRP25 (4 MU), and YRP34 (22 MU) (shaded in Figure 2), were used to control the expression of the *ZWF1* gene in TMB3001, resulting in TMB3037, TMB3035 and TMB3034, respectively. The strains showed different levels of G6PDH activity (Figure 3), ranging from 0.11 for TMB3037 to 1.82 U mg^{-1} for TMB3034. The G6PDH activities in the different strains (Figure 3) followed the expected order of the strength of the promoters (Figure 2).

Xylose fermentation with strains down-regulated for *ZWF1*. The synthetic promoter YRP13 produced the lowest G6PDH activity in TMB3037, so this strain was selected for batch fermentation with 50 g/l xylose. TMB3037 consumed 6.7 g xylose (g biomass)$^{-1}$, which is 5 times more than TMB3255 (*zwf1Δ*), and which represents 80% of the specific consumption observed in the control strain TMB3001 (Table 2). As expected, TMB3037 had a higher ethanol yield (0.336 g g^{-1}) than TMB3001 (0.307 g g^{-1}), accompanied by a lower xylitol yield (0.186 g g^{-1} vs. 0.290 g g^{-1}).

In an attempt to obtain even lower expression levels of *ZWF1*, a strain with a promoterless *ZWF1* gene was constructed by integration of pB3 *ZWF1* into TMB3001. The resulting strain, TMB3256, had a G6PDH activity of 0.01 U (mg protein)$^{-1}$, which is about 1% of the wild-type activity (Table 3). Batch fermentation conducted with 50 g/l xylose showed that TMB3256 had a specific xylose consumption of 3.7 g (g biomass)$^{-1}$, which corresponds to a 2.8-fold increase compared with TMB3255 (*zwf1Δ*). At the same time, TMB3256 showed an ethanol yield of 0.355 g g^{-1} and a xylitol yield of 0.132 g g^{-1}, which are close to the values for TMB3255 (*zwf1*) (Table 4).

The connection between G6PDH activity, xylitol yields, ethanol yields and xylose consumption have been illustrated in strains with G6PDH activities spanning from 0 U (mg protein)$^{-1}$ to 2.9 U (mg protein)$^{-1}$ (Figure 4). The *zwf1*-deleted strain TMB3255 exhibited the highest ethanol yield (0.407 g g^{-1}) and lowest xylitol yield (0.051 g g^{-1}), whereas the

TMB3030 strain at 10 μM Cu^{2+}, had the lowest ethanol yield (0.260 g g^{-1}) and the highest xylitol yield (0.383 g g^{-1}) of the strains in Table 4. Between 1 and 2 U G6PDH (mg protein)$^{-1}$, there is a plateau where yields are constant compared with lower G6PDH activities. The rate of xylose consumption (g (biomass h)$^{-1}$) increased when G6PDH activities increased, but only up to the wild-type level of G6PDH (0.87 U (mg protein)$^{-1}$), after which no further increase was observed.

DISCUSSION

In a previous investigation (20), we demonstrated the strong connection between NADPH production and xylitol formation during xylose fermentation by recombinant *S. cerevisiae* with a disrupted *ZWF1* gene. However, the specific xylose consumption in the *zwf1*-deleted strain (TMB3255) was 6 times lower than in the control strain TMB3001.

In this investigation we showed that xylose uptake could be restored to a level 15 times greater than that in TMB3255 (*zwf1Δ)* by over-expression of XR. This result confirms that xylose reductase was present in a limiting amount in TMB3255, probably as a result of reduced NADPH-usage by XR (20), and inhibition of XR by $NADP^+$ (28, 39). A 70% enhancement of the xylose uptake also resulted from increased XR expression in the control strain TMB3001, indicating that XR activity also limited the xylose fermentation rate in this strain.

In this work, the XR over-expression was increased ten-fold, from an XR/XDH ratio of 0.1 in TMB3001 (10) to a ratio of 1.1 in TMB3260 (Table 2), resulting in a 55% lower xylitol yield, but an unchanged ethanol yield (Table 4). This ratio is in the vicinity of the XR/XDH ratio of 0.7-4 observed in *Pichia stipitis* (9, 32). However, this contradicts earlier observations that a low XR/XDH ratio results in decreased xylitol production in recombinant *S. cerevisiae* (11, 36). However, the observations of Walfridsson *et al.* (1997) were made with

strains expressing XR and XDH but not over-expressing XK. In the strains compared by Eliasson *et al.* (2001), absolute levels of XR, XDH and XK activities differed, and it is therefore not clear that the low xylitol yield depended solely on the XR/XDH ratio.

The construction of a synthetic YRP promoter library was shown to be useful for metabolic optimisation of the *ZWF1* gene expression in *S. cerevisiae*. The promoters covered three orders of magnitude in expression level when used to control the *lacZ* gene. The span of beta-galactosidase activities of the promoters selected for *ZWF1* expression was similar to the G6PDH activity span obtained with the same promoters. The library of synthetic promoters has the advantage of facilitating gene expression optimisation without the need for external regulators, which are necessary for regulated promoters, such as *CUP1*.

This investigation showed that xylose uptake in a G6PDH-deficient strain could be greatly enhanced by allowing a low G6PDH activity, at the expense, however, of a slightly higher xylitol yield. In the strains TMB3256 and TMB3037 with 1% and 13% of the wild-type level of G6PDH activity, respectively, the xylose consumption rate was 2.8 times and 5 times higher than for TMB3255 (*zwf1Δ*). The xylitol yields were 2.6 times higher for TMB3256 and 3.6 times higher for TMB3037 than for TMB3255 (*zwf1Δ*). Thus, there is a clear trade-off between high xylose consumption rate and low xylitol yield when the *ZWF1* expression level is modulated.

The results obtained in this study support the view that metabolic engineering is an iterative process in which genetic modifications must be implemented and analysed before proceeding further (4, 40). TMB3261 (*PGK1-XYL1*, *zwf1Δ*) is the best performing strain found in this work. It combines an increased ethanol yield of 0.344 g (g xylose)$^{-1}$) with a 2.4-fold higher ethanol productivity (0.11 g ethanol (g biomass × h)$^{-1}$) from xylose compared with TMB3001. This productivity corresponds to 55% of the maximal ethanol productivity from xylose of the efficient xylose-fermenting yeast *P. stipitis* (32).

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FIGURE LEGENDS

Figure 1. Base sequence of the synthetic promoter YRP. N=25% of each base, transcription factor binding sites (RPG box and CT box) have 1% each of the bases other than indicated at each position. The *Eco*RI and *Bam*HI sites were used to clone the promoters in pYLZ-2.

Figure 2. Specific beta-galactosidase activity (Miller units) from YRP1 to YRP37 controlling *lacZ* in vector pYLZ-2 in *S. cerevisiae*. The empty vector pYLZ-2 produced 0.0032 Miller units. Shaded clones (YRP13, YRP25, YRP34, YRP37) were used to regulate the expression of *ZWF1* (Figure 3).

Figure 3. Specific glucose 6-phosphate dehydrogenase activities (U (mg protein)$^{-1}$)) in strains with synthetic promoters controlling the *ZWF1* gene (TMB3034, TMB3035 and TMB3037) and TMB3001 representing the wild-type level.

Figure 4. Ethanol (□) and xylitol (■) yields (g g^{-1}) and xylose consumption (▲) (g (g biomass h)$^{-1}$) versus measured G6PDH activity. Yields calculated after 60-70 hours of batch fermentation with TMB3255, TMB3256, TMB3037, TMB3001 and TMB3030 in 50 g l^{-1} xylose. The dashed line indicates the G6PDH activity in the control strain TMB3001 (0.87 U (mg protein)$^{-1}$, see Table 3).

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TABLE 1. Strains used with relevant genotypes and phenotypes.

Strain	Relevant genotype	Relevant phenotype	Reference
CEN.PK 113-5D	CEN.PK 113-5D (*MAT* a *ura3-52 MAL2-8c SUC2*)	Uracil auxotrophy	Entian and Kötter, 1998
CEN.PK 113-7A	CEN.PK 113-7A (*MAT* a *his3- D 1 MAL2-8c SUC2*)	Histidine auxotrophy	Entian and Kötter, 1998
TMB3001	CEN.PK 113-7A his3::YIp XR/XDH/XK	Expresses XR, XDH and XK 0.42 U XR (mg protein)$^{-1}$ 0.87 U G6PDH (mg protein)$^{-1}$	Eliasson *et al* ., 2000 This work This work
TMB3030	TMB3001 *CUP1 -ZWF1*	Specific G6PDH activity depends on Cu^{2+} levels	This work
TMB3034	TMB3001 YRP34-*ZWF1*	1.82 U G6PDH (mg protein)$^{-1}$	This work
TMB3035	TMB3001 YRP25-*ZWF1*	0.77 U G6PDH (mg protein)$^{-1}$	This work
TMB3037	TMB3001 YRP13-*ZWF1*	0.11 U G6PDH (mg protein)$^{-1}$	This work
TMB3255	TMB3001 *zwf1::Kan* MX	Expresses XR, XDH and XK No G6PDH activity	Jeppsson *et al* ., 2001
TMB3256	TMB3001 promoterless *ZWF1*	0.01 U G6PDH (mg protein)$^{-1}$	This work
TMB3260	TMB3001 *PGK1-XYL1*	3.36 U XR (mg protein)$^{-1}$	This work
TMB3261	TMB3255 *PGK1-XYL1*	4.99 U XR (mg protein)$^{-1}$	This work

TABLE 2. NADPH- and NADH-dependent XR activities and XR/XDH ratio in the control strain, TMB3001, and in the strains with increased XR activities, TMB3260 and TMB3261.

Strain	Relevant genotype	U XR (mg protein)$^{-1}$		XR / XDH*
		NADPH	NADH	
TMB3001		0.42	0.26	0.1
TMB3260	TMB3001 *PGK1-XYL1*	3.36	2.36	1.1
TMB3261	TMB3255 *PGK1-XYL1*	4.99	3.10	1.7

* 3 U XDH (mg protein)$^{-1}$ (Eliasson *et al.*, 2000)

TABLE 3. G6PDH activities in TMB3001 (control), TMB3255 (*zwf1D*), Cu^{2+}-regulated TMB3030, and in the strains with decreased G6PDH activity, TMB3037 and TMB3256.

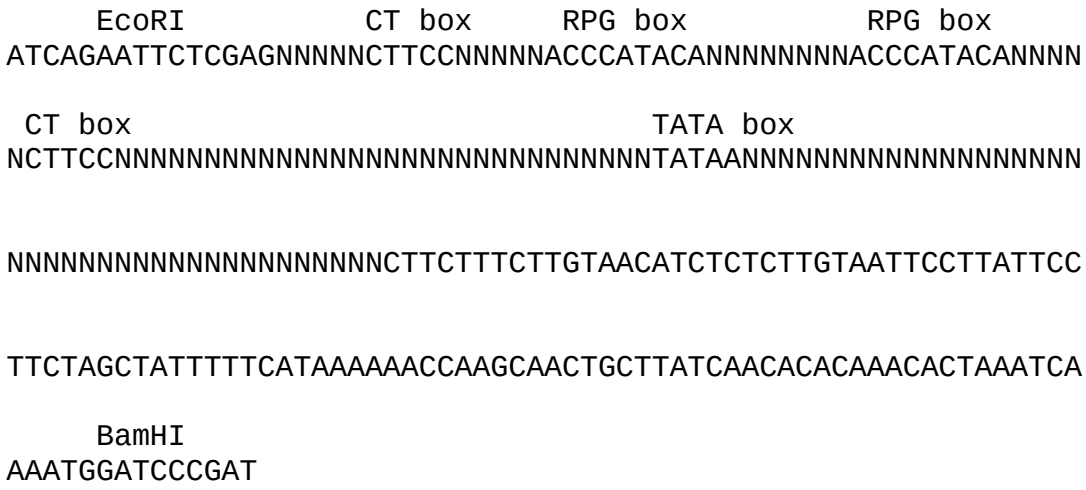
Strain	Relevant genotype		U G6PDH (mg protein)$^{-1}$
TMB3001			0.87
TMB3255	*zwf1 D*		<0.01
TMB3030	TMB3001 *CUP1-ZWF1*	0 µM Cu^{2+}	1.00
TMB3030	TMB3001 *CUP1-ZWF1*	1 µM Cu^{2+}	1.81
TMB3030	TMB3001 *CUP1-ZWF1*	10 µM Cu^{2+}	2.90
TMB3037	TMB3001 YRP13-*ZWF1*		0.11
TMB3256	TMB3001 promoterless *ZWF1*		0.01

TABLE 4. Effect of different expression levels of *XYL1* and *ZWF1*. Specific xylose consumption (g (g biomass)$^{-1}$) and ethanol, xylitol, acetate and glycerol yields (g (g consumed xylose)$^{-1}$) after 60-70 hours of 25 ml batch fermentation with 50 g l^{-1} xylose as sole carbon source. The presented values are the average of two independent fermentations plus/minus the deviation of the samples from the average.

Strains	Relevant genotype	Consumed Xylose	Yields			
			Ethanol	Xylitol	Acetate	Glycerol
TMB3001*		8.7 ± 0.1	0.307 ± 0.001	0.290 ± 0.006	0.025 ± 0.001	0.052 ± 0.004
TMB3255*	*zwf1 D*	1.3 ± 0.1	0.409 ± 0.015	0.051 ± 0.014	0.084 ± 0.005	0.054 ± 0.008
TMB3260	*PGK-XYL1*	14.7 ± 0.3	0.304 ± 0.007	0.130 ± 0.009	0.046 ± 0.007	0.161 ± 0.001
TMB3261	*PGK-XYL1* *zwf1 D*	19.0 ± 0.6	0.344 ± 0.014	0.076 ± 0.016	0.056 ± 0.006	0.128 ± 0.004
TMB3256	*ZWF1* w/o promoter	3.7 ± 0.1	0.355 ± 0.019	0.132 ± 0.017	0.054 ± 0.003	0.044 ± 0.001
TMB3037	YRP13-*ZWF1*	6.7 ± 0.2	0.336 ± 0.008	0.186 ± 0.003	0.039 ± 0.001	0.037 ± 0.001

* Data adopted from Jeppsson *et al.* (2001)

Figure 1


EcoRI
CT box
RPG box
RPG box
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BamHI
AAATGGATCCCGAT

Figure 2

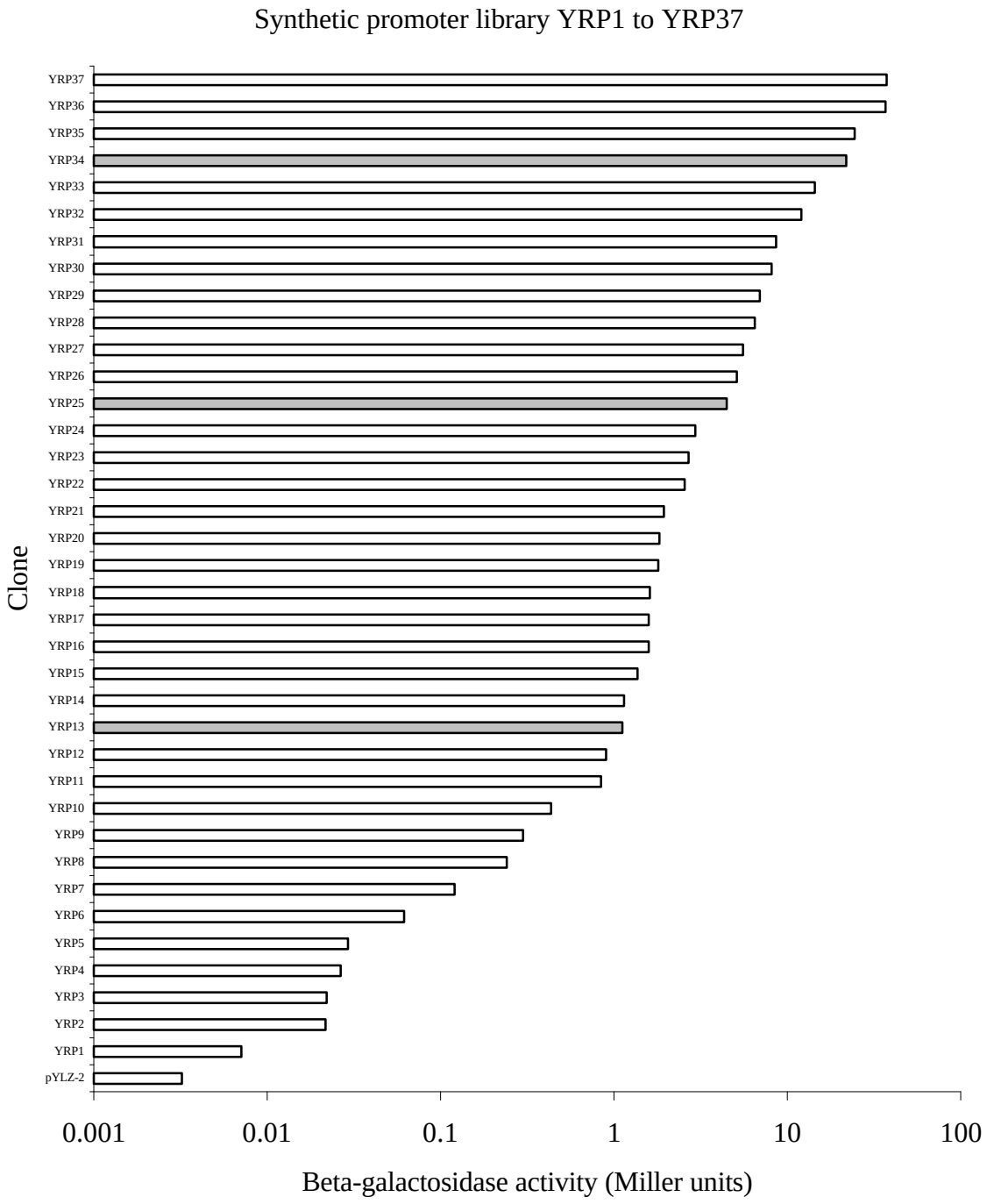

Synthetic promoter library YRP1 to YRP37
Clone
YRP37
YRP36
YRP35
YRP34
YRP33
YRP32
YRP31
YRP30
YRP29
YRP28
YRP27
YRP26
YRP25
YRP24
YRP23
YRP22
YRP21
YRP20
YRP19
YRP18
YRP17
YRP16
YRP15
YRP14
YRP13
YRP12
YRP11
YRP10
YRP9
YRP8
YRP7
YRP6
YRP5
YRP4
YRP3
YRP2
YRP1
pYLZ-2
0.001
0.01
0.1
1
10
100
Beta-galactosidase activity (Miller units)

Figure 3

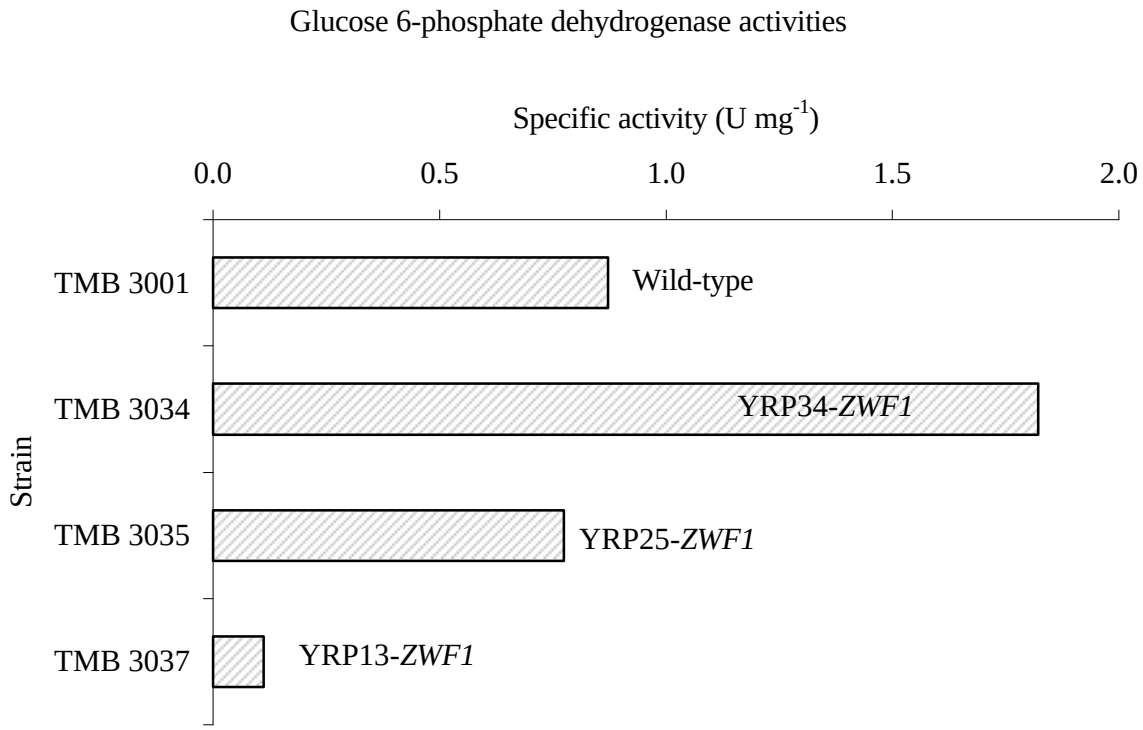
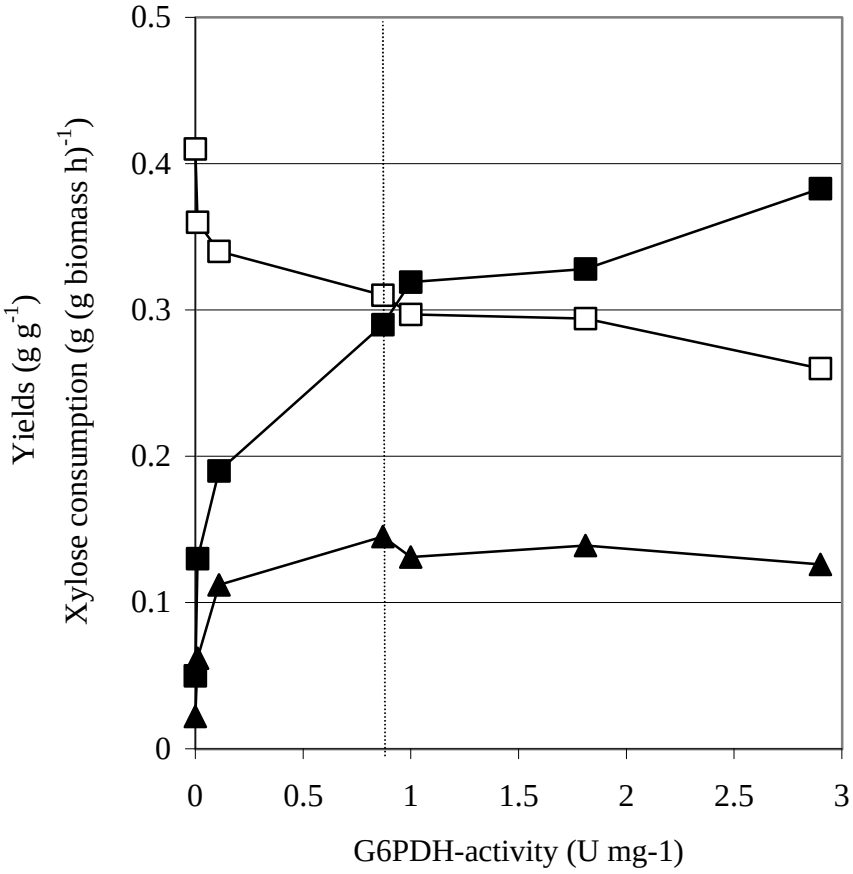

Glucose 6-phosphate dehydrogenase activities
Specific activity (U mg-1)
0.0
0.5
1.0
1.5
2.0
Strain
TMB 3001
Wild-type
TMB 3034
YRP34-ZWF1
TMB 3035
YRP25-ZWF1
TMB 3037
YRP13-ZWF1

Figure 4


0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
Yields (g g-1)
Xylose consumption (g (g biomass h)-1)
G6PDH-activity (U mg-1)

Paper V

Over-production of pentose phosphate pathway enzymes using a new CRE/*lox*P expression vector for repeated genomic integration in *Saccharomyces cerevisiae*

Short title: *Saccharomyces cerevisiae* vector for repeated genomic over-expression

Keywords: *Saccharomyces cerevisiae*, *lox*P, CRE, marker recycling, integrative vector, pentose phosphate pathway

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Abstract

Two new vectors are described, the expression vector pB3 PGK and the CRE recombinase vector pCRE3. The pB3 PGK has a zeocin selectable marker flanked by *lox*P sequences and an expression cassette consisting of the strong *PGK1* promoter and the *GCY1* terminator. The *S. cerevisiae* genes *RKI1, RPE1, TAL1* and *TKL1* were cloned in pB3 PGK and integrated in the locus of the respective gene, resulting in over-expression of the genes. *S. cerevisiae* TMB 3026, simultaneously over-expressing the *RKI1, RPE1, TAL1* and *TKL1* genes, was created by successive integrations and removal of the *lox*P-zeocin-*lox*P cassette using pCRE3. The 2µ based pCRE3 carries the Aureobasidin A, zeocin and *URA3* markers. pCRE3 proved to be easily cured without active counter selection. The zeocin marker is present on both the pB3 PGK and on pCRE3, so that screening for zeocin sensitivity indicates both chromosomal marker loss and loss of the pCRE3 vector. This feature saves time, since only one screening step is needed between successive chromosomal integrations. Marker recycling did not lead to increased zeocin resistance, indicating that the zeocin marker could be used for more than four rounds of transformation. The use of the CRE/*lox*P system proved to be a practical strategy to over-express multiple genes without exhausting available markers.

Introduction

Metabolic engineering, the directed genetic improvement of productivity, substrate and product range of a micro-organism (Bailey, 1991), commonly involves manipulation at transcriptional level of multiple genes. Metabolic engineering aimed at increasing glycolytic flux in *Saccharomyces cerevisiae* by over-expressing single glycolytic enzymes did not succeed (Schaaff *et al.*, 1989) while simultaneous over-expression of seven genes (Hauf *et al.*, 2000) in lower glycolysis gave the yeast a higher fermentative capacity under certain conditions (Smits *et al.*, 2000). The theory of metabolic control analysis predicts that all enzymes along a metabolic pathway share the control of the metabolite flow through that pathway to various degrees (Heinrich and Rapoport, 1974; Kacser and Burns, 1973). Simultaneous alteration of many genes by genetic engineering requires a large set of genetic markers. The simultaneous over-expression of the enzymes of lower glycolysis required the auxotrophic markers *URA*3, *LEU*2, *TRP*1 and the dominant *Kan*MX (Hauf *et al.,* 2000). The drawback of such a strategy is that the number of genetic modifications is limited by the number of available markers. Metabolic engineering is an iterative process where genetic changes are performed and analysed successively (Bailey, 1991; Stephanopoulos *et al.*, 1998; Östergaard *et al.*, 2000), and the number of required genetic alterations to attain a certain goal is not known beforehand. Furthermore, auxotrophic markers are not available in industrial strains.

A different approach was taken when twenty hexose transporters (Wieczorke *et al.*, 1999) were deleted consecutively using the dominant *Kan*MX marker flanked by *lox*P repeats (Güldener *et al.*, 1996). The CRE recombinase induces a specific recombination

between the sites, removing the marker gene. The CRE/*lox*P system has proved to be very effective and a range of removable markers has been developed (Delneri *et al.*, 2000).

To generate an integrative expression system that overcomes marker exhaustion and makes use of different promoters we aimed to construct an expression vector system that would use the CRE/*lox*P system for marker recycling of an efficient dominant marker. The plasmid should have an expression cassette where the promoter is excisable by *Sac*I and *Xba*I. Thus the promoter would be exchangeable for a large set of previously constructed promoters, both strong and weak promoters (Mumberg *et al.*, 1995), copper regulated promoters (Labbé and Thiele, 1999) and a galactose regulated promoter (Güldener *et al.,* 1996). Further, the CRE vector should have a dominant marker for increased host range as well as a 2μ ORI. The lower stability of 2μ vectors compared to CEN-ARS vectors (Gietz and Sugino, 1988; Rose and Broach, 1991) should make plasmid curing easier without counter selection of the *URA*3 gene. Counter selection of *URA*3 is not useful for prototrophic strains carrying a functional *URA*3 gene.

In this investigation we constructed the dominant marker integrative expression vector pB3 PGK, carrying the zeocin marker flanked by *lox*P sequences. The vector also contains a *PGK1* promoter / *GCY1* terminator expression cassette. The vector was used to simultaneously over-express *RKI1, RPE1, TAL1* and *TKL1* in the prototrophic xylose fermenting *S. cerevisiae* TMB 3001 (Eliasson *et al.*, 2000) with CRE mediated marker recycling. These genes code for the enzymes of the lower pentose phosphate pathway;

ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, transaldolase and transketolase, respectively. We also constructed a new CRE expression plasmid, pCRE3, carrying three markers, *URA*3, Aureobasidin A and zeocin, and having 2μ ORI instead of CEN-ARS. A recently reported CRE expression vector also carried a dominant marker, but retained the CEN-ARS ORI (Steensma and Linde, 2001).

Materials and Methods

Methods

Standard molecular biology techniques were used unless otherwise stated (Ausubel *et al.*, 1995; Sambrook *et al.*, 1989). The LiAc method was used for yeast transformations (Agatep *et al.*, 1998). After transformation, the yeast was incubated for six hours in YPD medium before transfer to selective media to allow for the zeocin or Aureobasidin A marker to be expressed. *E. coli* was transformed by SEM (Inoue *et al.*, 1990). *E. coli* plasmid DNA was prepared with Quantum prep from Bio-Rad (Hercules, CA, USA).

Strains and media

S. cerevisiae TMB 3001 (Eliasson *et al.,* 2000) was used as host for transformations. *E. coli* DH5α (Life Technologies, Rockville, MD, USA) was used for sub cloning. Yeast was grown on 20 g/l Difco peptone, 10 g/l yeast extract, 20 g/l agar for solid medium and 20 g/l glucose (YPD) or galactose (YPGal). The pH of the medium was set to 5.5 except for zeocin selection where pH was set to 7.5. Zeocin (Invitrogen, Groningen, The Netherlands) was added at 50 mg/l or Aureobasidin A (Takara Biomedical Europe S.A., Gennevilliers, France) at 0.15 mg/l for selection of transformants. *E. coli* was grown in LB (Sambrook *et al.,* 1989) with 200 mg/l ampicillin for selection.

Plasmid construction

pB3 PGK

The 1220 bp zeocin gene from pTEF1/Zeo (Invitrogen) replaced the 1448 bp *Kan*MX gene in pUG6 (Güldener *et al.,* 1996) using restriction enzymes *Sac*I and *Xba*I, resulting in pUG6 Zeo. Filling in with klenow DNA polymerase destroyed the *Sac*I and the *Xba*I sites. A 1641 bp PCR fragment (described below) containing the *PGK1* promoter and the *GCY1* terminator was cut by *Avr*II and *Sac*II and ligated to the *Spe*I and *Sac*II sites of pUG6 Zeo resulting in pB2 PGK. A 938 bp ribosomal DNA PCR product (Nieto *et al.*, 1999) was cut with *BamH*I and *Bgl*II and cloned into the *Bcl*II site of pB2 PGK resulting in pB3 PGK (Figure 1). PCR products of the ORFs of *RKI1*, *RPE1*, *TAL1* and *TKL1* were cut with *BamH*I and ligated to the *Bgl*II site between *PGK1* promoter and *GCY1* terminator in pB3 PGK, resulting in pB3 PGK *RKI1*, pB3 PGK *RPE1*, pB3 PGK *TAL1* and pB3 PGK *TKL1*, respectively.

pCRE3

A three-part ligation between the 1252 bp *Apa*I *Pst*I fragment containing the zeocin marker from pTEF1/Zeo, the 2514 bp *Sca*I *Pst*I fragment from YEp24 (Parent *et al.*, 1985) containing 2µ sequences, and the 4957 bp *Apa*I *Sca*I fragment from pSH47 (Güldener *et al.,* 1996) containing the CRE recombinase gene and *E. coli* ORI resulted in the vector pCREZeo. The 3677 bp *Bgl*II *Sac*I fragment from pAUR112 containing the Aureobasidin A resistance marker AUR1-C gene was ligated to the pCREZeo vector cut with *Bgl*II and *Nhe*I resulting in pCRE3 (Figure 2).

Primers

Primer sequences are summarized in Table 1. The *PGK1* promoter was amplified from pMA91 (Mellor *et al.*, 1983) using Primers A and B. The *GCY1* terminator was

amplified from pYLZ-2 (Hermann *et al.*, 1992) using primers C and D. The fragments were mixed and fused by PCR using Primers A and D. A ribosomal DNA sequence (Nieto *et al.,* 1999) was amplified with primers 5rDNA and 3rDNA. *RKI1, RPE1*, *TAL1* and *TKL1* ORFs (Cherry *et al.*, 1998) were amplified from *S. cerevisiae* CBS 8066 chromosomal DNA with the primers indicated in Table 1 and cut with *Bam*HI on flanking sites introduced by the PCR primers.

Chromosomal integration and diagnostic PCR

Plasmids pB3 PGK- *RKI1, RPE1*, *TAL1* and *TKL1* were linearised within the coding region of their respective cloned genes with restriction endonuclases *Spe*I, *Xcm*I, *Bgl*II and *Pin*AI, respectively (Figure 3A). This directed the integration to the chromosomal locus of the cloned gene in the vector (Figure 3B). Yeast colony PCR was performed on cells washed with water using Lyse-n-go (PIERCE, Rockford, IL, USA) according to the manufacturers specifications. Amplification of the *PGK1* promoter - *MFG1* gene fragment using primers BJ5756 and the downstream cloning primer of the respective gene verified the correct integrations of the vectors (Table 1, Figure 3) except for pB3 PGK *TKL1*. The correct pB3 PGK *TKL1* integration was verified by amplification of a 826 bp fragment containing the PGK promoter and a part of the TKL1 gene using the primer 3TKL1cont (Table 1). This was done since it was hard to obtain a PCR product with the 3TKL1clon primer, perhaps because of the increased length compared to the other genes. The primer 3TKL1cont anneals downstream of the restriction cut in the vector, so the presence of the PCR product proves correct integration of the vector.

Enzyme activity measurements

The activities of ribulose 5-phosphate epimerase activity and ribose 5-phosphate isomerase were determined by the method of Miosga and Zimmermann (1996).

Transaldolase and transketolase activity were determined as described by Bergmeyer (1974).

Results

Over-expression of the *RKI1, RPE1*, *TAL1* and *TKL1* genes

The pB3 PGK- *RKI1, RPE1, TAL1* and *TKL1* plasmids were linearised within the respective coding region of the cloned gene and used to transform TMB 3001. Zeocin positive clones appeared two days after plating transformants on selective medium. Diagnostic PCR showed that an average of 60% of the clones were positive for the presence of the *PGK1* promoter - *MFG1* gene cassette (Figure 3, 4). Enzyme activity measurements confirmed four to 24 times higher enzymatic activity of the over-expressed gene (Table 2). These results showed that the integrative vector pB3 PGK worked as intended and that correct integrations were obtained at a high frequency with all the cloned genes.

Consecutive over-expression of *RKI1, RPE1*, *TAL1* and *TKL1*

Consecutive over-expression of more than one gene with marker recycling was demonstrated by simultaneous over-expression of *RKI1, RPE1, TAL1* and *TKL1*. We transformed TMB3014 (over-expressing *TAL1*, Table 2) with pCRE3 selecting for Aureobasidin A resistance. The transformants were selected on YPGal medium for induction of the *GAL1* promoter controlling the CRE protein expression. Three resistant colonies were picked and grown in 100 mL of YPD to saturation (24h) for plasmid curing. One hundred and fifty colonies were screened for sensitivity to 50 mg/l zeocin. Since the pCRE3 vector also carries the zeocin marker, zeocin sensitivity marks both loss of the chromosomal marker and curing of the pCRE3 plasmid. About 20% of the

colonies were sensitive, corresponding to a plasmid loss of about 3% per generation, given that the chromosomal zeocin gene was lost in all cases. This corresponds well to earlier reports on the stability of 2μ based vectors (Rose and Broach, 1991) and the high efficiency of the CRE recombinase (Güldener *et al.,* 1996). The resulting zeocin sensitive strain was transformed with the pB3 PGK *RPE1* plasmid selecting for the same zeocin concentration as before. The background from transformation without DNA was as low as in previous transformations (2-8 colonies). The resulting strain TMB 3026a showed over-expression of both *TAL1* and *RPE1* as measured by enzyme activities (Table 2) as well as correct products from diagnostic PCR (Figure 4). pB3 PGK *RKI1* and *TKL1* was inserted into TMB 3026a in the same manner resulting in TMB 3026b (*RPE1, RKI1, TAL1*) and TMB 3026 (*RPE1, RKI1, TAL1* and *TKL1*) (Table 2). TMB 3026 was positive in diagnostic PCR for all four genes (Figure 4) and had about the same activity of the over-expressed enzymes as the strains over-expressing single genes (Table 2). No increased zeocin resistance could be detected in TMB 3026b compared to the TMB 3001, indicating efficient marker removal by pCRE3.

Discussion

The pB3 PGK expression vector proved to work as intended. Previous reports where marker recycling using CRE/*lox*P was successful (Delneri *et al.,* 2000; Güldener *et al.,* 1996; Wieczorke *et al.,* 1999), there was only the short *lox*P sequence (34 bp) left on the chromosome after marker recycling. The pB3 PGK is 6.3 kb, and most of it is left on the chromosome after the marker has been looped out (Figure 3C). This could have caused problems since pB3 PGK sequences already present in the chromosome may

lead to incorrect integration when the same vector is used again. Nevertheless, four different genes could be over-expressed simultaneously using the same marker by recycling with pCRE3. The presence of the zeocin marker in both pCRE3 and pB3 PGK makes the CRE/*lox*P system more practical, since only one screening step is required to verify marker loss and pCRE3 curing. The zeocin marker and the Aureobasidin A marker have been found to be efficient for a number of industrial strains in contrast to G418 (results not shown). In the TMB 3026 all genes encoding enzymes in the lower PPP were simultaneously over-expressed and the zeocin marker can be used to further modify this strain, which may be more difficult using earlier strategies (Hauf *et al.,* 2000; Smits *et al.,* 2000).

The pB3 PGK vector could also be used for functional genomic investigations, where an altered expression rather than a knockout of the gene is desired (Delneri *et al.,* 2000; Güldener *et al.,* 1996). In pB3 PGK, the *PGK1* promoter is excisable by *Sac*I and *Xba*I. This makes the plasmid compatible with a wide range of promoters (Güldener *et al.,* 1996; Labbé and Thiele, 1999; Mumberg *et al.,* 1995). We have used this feature to exchange the *PGK1* promoter for the regulated *CUP1* promoter (Labbé and Thiele, 1999) to obtain regulated expression of the cloned genes (data to be published). The pCRE3 extends the usefulness of the CRE/*lox*P system since it has selection markers not previously used for CRE recombination vectors.

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Legends to figures

Figure 1. The pB3 PGK expression vector. Relevant restriction sites are marked. The *RPE1, RKI1, TAL1* and *TKL1* was inserted in the *Bgl*II site.

Figure 2. The pCRE3 CRE recombinase vector with Aureobasidin A, zeocin and *URA3* markers. The pCRE3 has 2µ sequences for replication in yeast.

Figure 3. Integration and marker loss strategy for the pB3 PGK *MFG1* vector. *MFG1* (*My Favourite Gene 1*) represents *RPE1, RKI1, TAL1* or *TKL1*. The amp gene as well as *E. coli* ORI were left out for clarity. *PGK1*, phosphoglycerate kinase promoter; *GCYt*, *GCY1* terminator. **A)** The yeast strain is transformed with the pB3 PGK *MFG1*. **B)** Integration occurs at the locus of *MFG1* where the expression is controlled by the wild-type promoter (*WT P*). **C)** The zeocin marker between the *lox*P sites is lost by specific CRE mediated recombination.

Figure 4. Diagnostic PCR on whole yeast cells. The PGK1 promoter - *MFG1* was amplified as described in Figure 3. The expected band sizes are given next to the positive lanes. The band sizes of the marker are 1031, 900, 800, 700, 600, 500**,** 400, 300, 200, 100 and 80 bp.

Table 1. Primers used in this study.

Name	Sequence (5´-3´)
Primer A	CAG TCC TAG GTA CCG GCG CGC CGA GCT CTA ACT GAT CTA TCC
Primer B	GCC CGG GCT GTA CAC CAA TCT TTG GAG ATC TAG ATT TGG TTT TAT ATT TGT TG
Primer C	GGT GTA CAG CCC GGG CGG CCG GCC TAA TTG TTT TTG CGT G
Primer D	CAG TCC GCG GCG CCT GAT CAG CAT GCG ATC GCT CGA CAT TTG ATA TAC
5rDNA	GAT CGG ATC CCA TGA GAG TAG CAA ACG TAA GTC
3rDNA	GAT CGG CGC CTC AGG TTC CAC CAA ACA GAT AC
BJ5756	CAT CAA GGA AGT AAT TAT CTA CT
5RKI1clon	GAT GGA TCC AGA ATG GCT GCC GGT GTC C
3RKI1clon	GAT GGA TCC TCA CTT TTC GGT AAC TTC AAC ACT AC
5RPE1clon	GAT GGA TCC AGA ATG GTC AAA CCA ATT ATA GCTC
3RPE1clon	GAT GGA TCC CTA ATC TAG CAA ATC TCT AGA AC
5TAL1clon	GAT GGA TCC AGA ATG TCT GAA CCA GCT CA
3TAL1clon	GAT GGA TCC TTA AGC GGT AAC TTT CTT TTC AAT C
5TKL1clon	GAT GGA TCC AGA ATG ACT CAA TTC ACT GAC ATT G
3TKL1clon	GAT GGA TCC CAA ATA ATA TCA TAT CAA ATC TGA TG
5TKL1cont	TTG CTT TGT CCA GAC AAA ACT TGC

Table 2. Specific activities (U/mg protein) in cell extracts of glucose grown cells. Values are means from two different cell extracts with less than 10 % difference. -, not measured.

Strains	Overexpressed gene(s)	Specific activity (U/mg)			
		RPE	RKI	TAL	TKL
TMB 3001		8.1	0.26	2.0	0.2
TMB 3013	***RPE1***	36	-	1.7	-
TMB 3017	***RKI1***	-	6.3	-	-
TMB 3014	***TAL1***	-	-	35	-
TMB 3016	***TKL1***	-	-	-	2.8
TMB 3026	***RPE1, RKI1, TAL1,TKL1***	33	6.0	34	2.7

Figure 1.

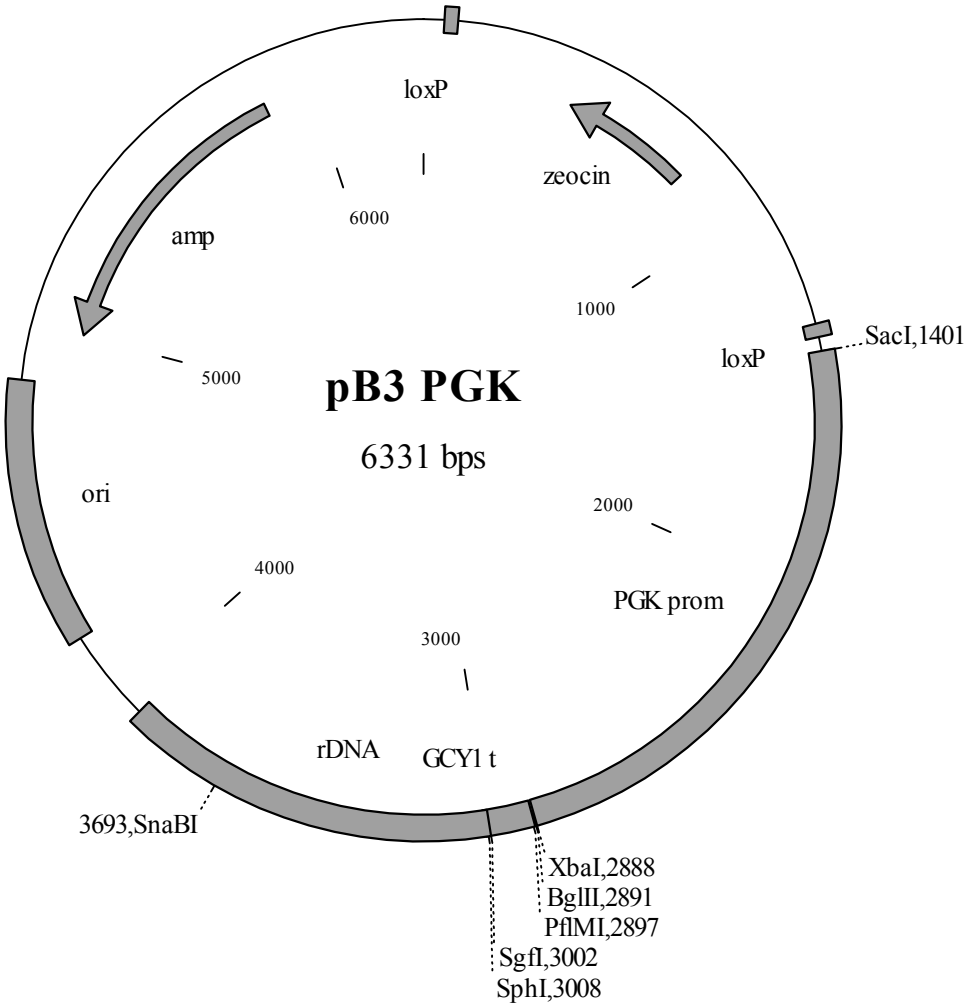

loxP
zeocin
6000
amp
1000
SacI,1401
loxP
5000
pB3 PGK
6331 bps
ori
2000
4000
PGK prom
3000
rDNA
GCY1 t
3693,SnaBI
XbaI,2888
BglII,2891
PflMI,2897
SgfI,3002
SphI,3008

Figure 2.

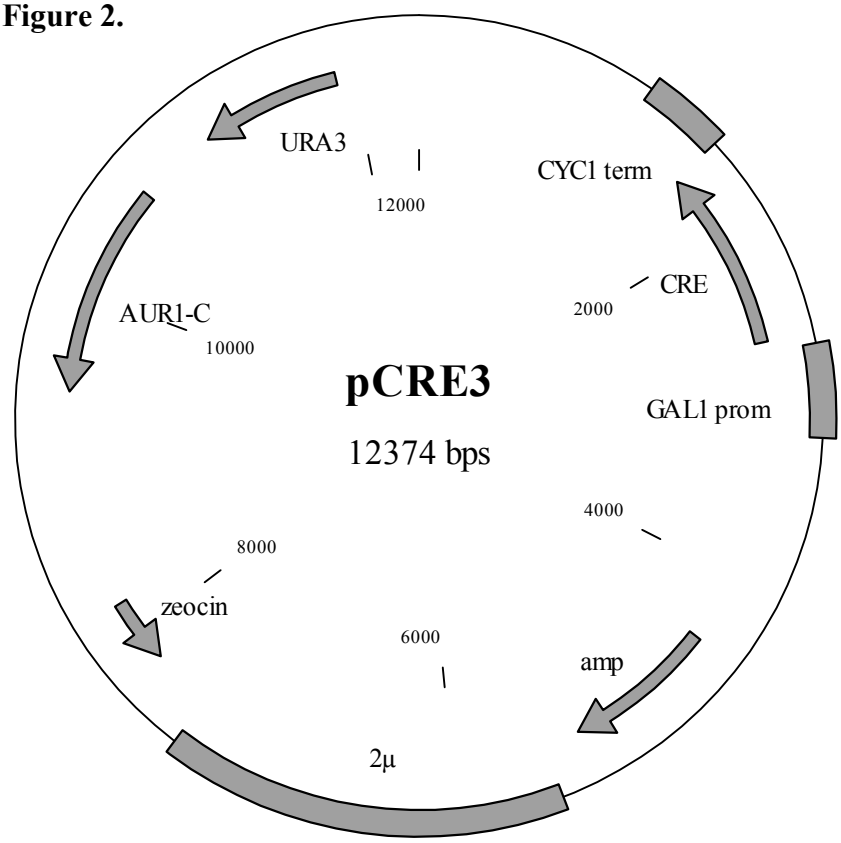
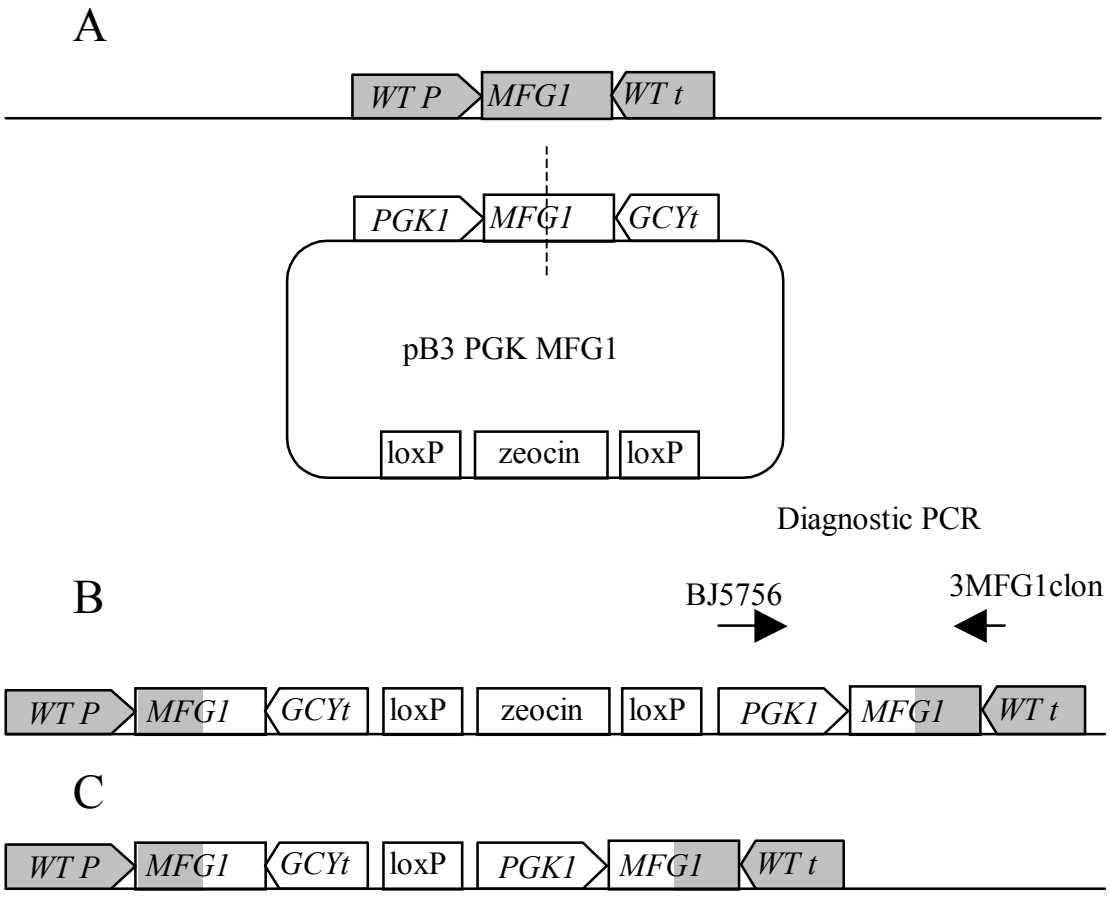

URA3
12000
CYC1 term
CRE
2000
AUR1-C
10000
pCRE3
12374 bps
GAL1 prom
4000
8000
zeocin
6000
amp
2μ

Figure 3.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WT P
MFG1
WT t
PGK1
MFG1
GCYt
pB3 PGK MFG1
loxP
zeocin
loxP
Diagnostic PCR
B
BJ5756
3MFG1clon
WT P
MFG1
GCYt
loxP
zeocin
loxP
PGK1
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WT t
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WT P
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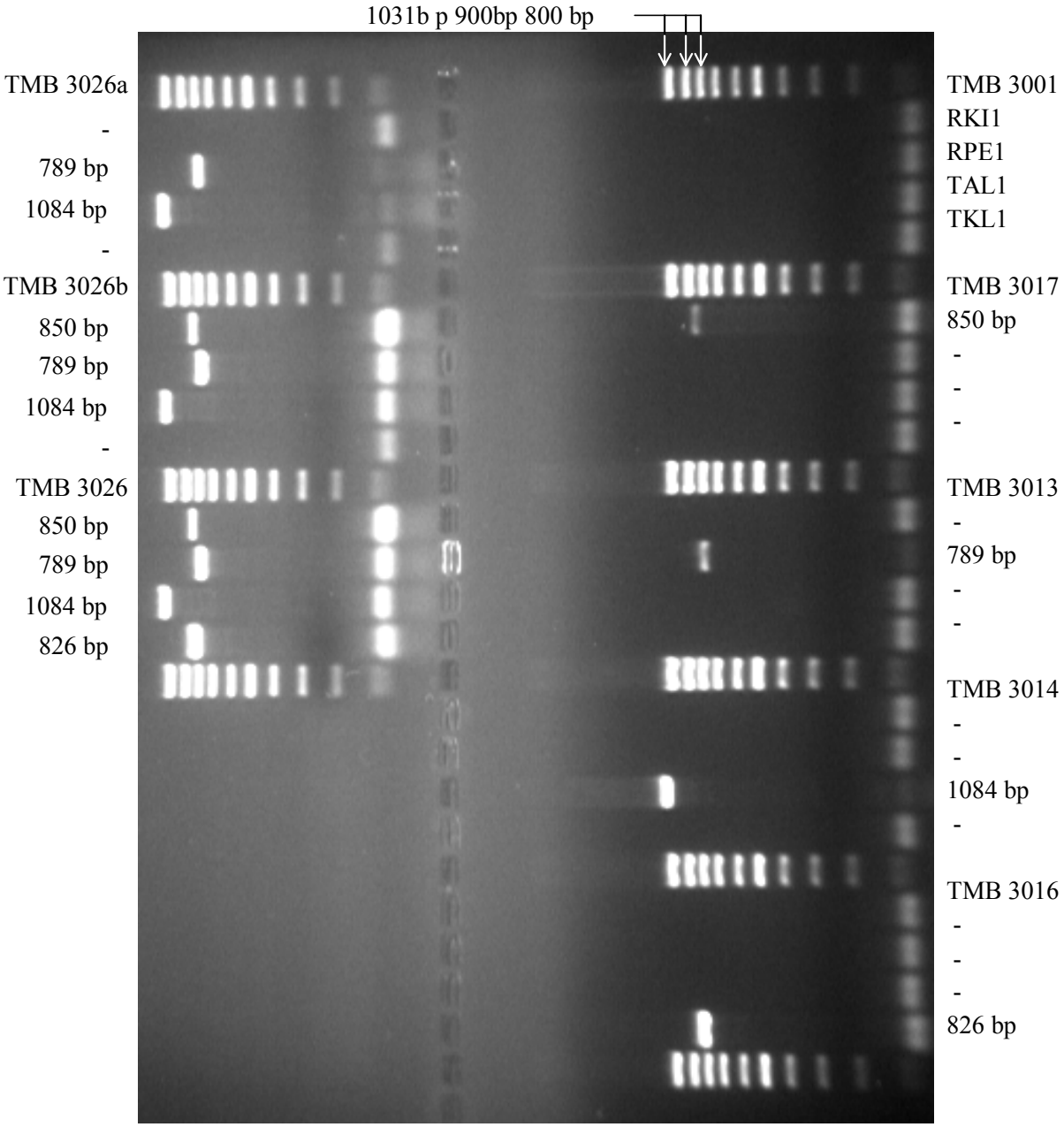
GCYt
loxP
PGK1
MFG1
WT t

Figure 4.


1031b p 900bp 800 bp
TMB 3026a
-
789 bp
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TMB 3026b
850 bp
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TMB 3026
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TMB 3001
RKI1
RPE1
TAL1
TKL1
TMB 3017
850 bp
-
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-
TMB 3013
-
789 bp
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-
TMB 3014
-
-
1084 bp
-
TMB 3016
-
-
-
826 bp

Paper VI

The Lower Pentose Phosphate Pathway Does Not Control the Xylose Fermentation Rate in Xylose Fermenting *Saccharomyces cerevisiae* TMB3001 expressing *XYL1, XYL2* and Over-expressing *XKS1.*

Running title: Over-expression of non-oxidative PPP in *S. cerevisiae*

Keywords: Xylose, *Saccharomyces cerevisiae*, xylose reductase, xylitol dehydrogenase, xylulokinase, pentose phosphate pathway, ribulose 5-phosphate epimerase, ribose 5-phosphate ketol-isomerase, transaldolase, transketolase.

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ABSTRACT

Fast and efficient xylose fermentation by recombinant *S. cerevisiae* is of economical interest for ethanol production from ligno-cellulosic biomass. *S. cerevisiae* is able to ferment xylose, when engineered with the enzymes xylose reductase and xylitol dehydrogenase. However xylose fermentation is one or two orders of magnitude slower than glucose fermentation. It has been proposed that the non-oxidative pentose phosphate pathway has an insufficient capacity of for xylose fermentation in *S. cerevisiae*. In this investigation, non-oxidative pentose phosphate pathway (PPP) genes *RPE1, RKI1, TAL1* and *TKL1* were simultaneously over-expressed in the xylose-fermenting *S. cerevisiae* TMB3001, already expressing *XYL1* (xylose reductase), *XYL2* (xylitol dehydrogenase) and over-expressing *XKS1* (xylulokinase). The level of over-expression ranges between 4 and 23 times the wild-type level of the non-oxidative PPP enzymes. The level of over-expression did not depend significantly on the number of over-expressed genes in the same strain.

Over-expression of *RPE1, RKI1, TAL1* and *TKL1* did not influence the xylose fermentation rate in batch cultures of 50 g/l xylose or chemostat cultures of 20 g/l glucose and 20 g/l xylose. This result suggests that the non-oxidative PPP enzymes does not control the xylose fermentation rate in *S. cerevisiae* TMB 3001.

INTRODUCTION

Ethanol produced from fermentation of ligno-cellulosic biomass could be a renewable alternative to fossil fuels. Ligno-cellulosic biomass contains the pentose sugar xylose, which cannot be metabolised by the preferred ethanol production organism *Saccharomyces cerevisiae*. *S. cerevisiae* can be engineered to utilise xylose by expression of the genes *XYL1* encoding xylose reductase (XR) and *XYL2* encoding xylitol dehydrogenase (XDH) from *Pichia Stipitis* (Kötter *et al.*, 1990). Xylose fermentation of such recombinant yeast strains is slow and xylitol is a major by-product (Kötter and Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1997). We showed in a previous study, that xylitol production could be almost completely abolished by blocking the oxidative pentose phosphate pathway (PPP) (Jeppsson *et al.*, 2001b), but resulted in even slower xylose fermentation rate. In contrast, blocking the oxidative PPP enhanced xylulose fermentation rate (Eliasson *et al.*, 2000a). Xylose is metabolised through the non-oxidative PPP in *P. stipitis* (Ligthelm *et al.*, 1988) and probably also in *S. cerevisiae* (Eliasson *et al.*, 2000a).

The non-oxidative PPP of *S. cerevisae* supplies the cell with erythrose 4-phosphate for amino acids (Braus, 1991), ribose 5-phosphate and NADPH for biosynthesis (Bruinenberg *et al.*, 1983; Nogae and Johnston, 1990). The fact that the non-oxidative PPP is not a catabolic pathway in *S. cerevisiae* suggests that it may not have adequate capacity for rapid xylose fermentation. *S. cerevisiae* has been shown to metabolise xylose together with ribose, but neither of the sugars alone (van Zyl *et al.*, 1993; 1999). Then non-oxidative PPP enzymes ribulose 5-phosphate isomerase (RPE) and ribose 5-phosphate isomerase (RKI) could be by-passed by xylose and ribose co-metabolisation. Further, flux modelling showed that the reaction catalysed by RPE was very slow in anaerobic xylose-fermenting *S. cerevisiae* (Wahlbom *et al.*, 2001). The non-oxidative PPP intermediate sedoheptulose 7-phosphate has

been shown to accumulate in xylose-fermenting *S. cerevisiae*, suggesting insufficient transaldolase activity (Kötter and Ciriacy, 1993; Senac and Hahn-Hägerdal, 1990). Over-expression of transaldolase led to faster aerobic growth on xylose (Walfridsson *et al.*, 1995), but did not lead to faster xylose fermentation.

Engineering of single genes to increase metabolic flux is generally not successful. Over-production of single enzymes in glycolysis did not improve the rate of glucose fermentation (Schaaff *et al.*, 1989). However, simultaneous over production of seven enzymes in the lower glycolysis (Hauf *et al.*, 2000) increased the acceleration of fermentative capacity under a certain conditions (Smits *et al.*, 2000). Tryptophane biosynthesis could also be enhanced by multiple over production of five enzymes; where over production of the single enzymes gave little effect (Niederberger *et al.*, 1992). These experimental results are supported by the theory of metabolic control analysis (Heinrich and Rapoport, 1974; Kacser and Burns, 1973) which suggests that overproduction of individual metabolic enzymes will not produce large increases in flux unless the control coefficient is larger than 0.6 (Fell and Thomas, 1995). It was therefore suggested that simultaneous alteration of multiple genes was suggested to be necessary (Fell and Thomas, 1995; Kacser and Acerenza, 1993).

The *S. cerevisiae* genes *RPE1, RKI1 TAL1* and *TKL1* encode the non-oxidative PPP enzymes ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, transaldolase and transketolase, respectively. In this investigation we explored the effect of simultaneous over-expression of the non-oxidative PPP genes *RPE1, RKI1 TAL1* and *TKL1* on xylose fermentation by *S. cerevisiae* TMB3001 (Eliasson *et al.*, 2000b). Over production of the single enzymes was also investigated.

MATERIALS AND METHODS

Strains *S. cerevisiae* strains TMB3013, TMB3014, TMB3016, and TMB3023 over-expresses the genes *RPE1*, *TAL1*, *TKL1*, and *RKI1*, respectively (Table 1) (Johansson and Hahn-Hägerdal, 2001). The *S. cerevisiae* genes *RPE1, RKI1 TAL1* and *TKL1* encode the non-oxidative PPP enzymes ribulose 5-phosphate epimerase, ribose 5-phosphate ketol isomerase, transaldolase and transketolase, respectively. TMB3026 over-expresses all four genes simultaneously (Table1) (Johansson and Hahn-Hägerdal, 2001). The over-expressing strains were constructed by chromosomal integration with a new vector with a recyclable dominant marker (Johansson and Hahn-Hägerdal, 2001). *Saccharomyces cerevisiae* TMB3001 (Eliasson *et al.*, 2000b) was used as control strain.

Inoculum and batch fermentations Defined minimal medium (Verduyn *et al.*, 1992) was supplied with 50 g/l glucose for growth of inoculum or 50 g/l xylose for batch fermentation. The yeast was grown in 500 ml baffled shake flasks in glucose medium. The cells were harvested at a dry weight of about 3 g/l, well within exponential phase. The cells were washed twice in ice-cold water and re-suspended in ice-cold xylose medium. For each strain, two flasks with rubber stoppers and 20 ml working volume were inoculated to 5 g/l cell dry weight. The flasks were incubated at 30ºC in a water bath with magnetic stirring. Samples were withdrawn through a 2 mm hypodermic needle with a syringe and fermentation gases were expelled through a 0.8 mm needle.

Continuous cultivations. Yeast cells were grown overnight in 200 ml defined minimal medium (Verduyn *et al.*, 1992) containing 50 g l^{-1} glucose, 10 mg l^{-1} ergosterol and 0.4 g l^{-1} Tween 80 in a 250 ml baffled shake-flask. Cells were centrifuged at 5000×g for 5 min and 4°C, and used to inoculate 1.5 l of the same medium to an optical density of 0.5 at 620 nm in a Bioflo III fermentor (New Brunswick Scientific, Edison, NJ, USA). Antifoam was added at 0.5 % (v/v) (Dow Corning® Antifoam RD Emulsion, BDH Laboratory Supplies, Poole,

England). Continuous cultivations were set up at dilution rates of 0.06 and 0.12 h^{-1} at 30°C, pH 5.5 controlled by addition of 5M NaOH, and a stirring speed of 200 rpm. The fermentor was sparged with 0.2 l min^{-1} nitrogen (containing less than 5 ppm O_2) as measured with a gas mass flow meter (Bronkhorst, Ruurlo, The Netherlands).

Analysis of substrates and products. Fermentation samples were analysed by HPLC. Glucose, xylose, xylitol, succinate, glycerol, acetate and ethanol were separated with an Aminex HPX-87H (Bio-Rad) ion exchange column operated at 45°C, with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} and detected using a refractive index detector (Shimadzu, Kyoto, Japan). Cell dry weight was determined by drying to constant weight in a microwave oven.

RESULTS AND DISCUSSION

Gene over-expression The hypothesis that xylose fermentation is hampered in *S. cerevisiae* by a weak PPP has been discussed in many previous reports (Ciriacy and Porep, 1986; Kötter and Ciriacy, 1993; Senac and Hahn-Hägerdal, 1990; Walfridsson *et al.*, 1995). Since no common regulator is known for the non-oxidative PPP by which the entire pathway could be up-regulated, the remaining option was to create a strain over-expressing the entire non-oxidative PPP. We have reported previously on the construction of strains over-expressing the genes *RPE1, RKI1 TAL1* and *TKL1* one-by-one and simultaneously (Johansson and Hahn-Hägerdal, 2001). The genes *RPE1, RKI1 TAL1* and *TKL1* encode the non-oxidative pentose phosphate pathway (PPP) enzymes ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, transaldolase and transketolase. The levels of over-expression over wild-type levels were 4×, 23×, 17×, and 13× for the *RPE1, RKI1 TAL1* and *TKL1*, respectively. Different levels of over-expression of the enzymes was also obtained when seven glycolytic genes were over-expressed (Hauf *et al.*, 2000) in a range from 1.4-fold to 20-

fold. The difference in enzyme activities of over produced enzymes between strains over-expressing single genes compared to TMB3026, over-expressing all four is small (Table 1). The level of over-expression remained at the same level even with increased number of integrated constructs. Hauf *et al.* (2000) found up to 1.8 times difference in expression between single over-expressing strains and a strain over-expressing seven genes. This difference could be due to the fact that the TMB3026 contains a lower number of simultaneously integrated constructs. Additionally, the *PGK1* promoter (Mellor *et al.*, 1983) used in this work is strong enough to create the desired over-expression of the genes reported here, but only about one tenth of the strength of the truncated *HXT7* promoter used by Hauf *et al.* (2000), possibly creating less strain on the cell.

Batch fermentations The xylose consumption rates in batch culture are summarized in Table 2. The xylose consumption rate was calculated as the amount of xylose (g) consumed after 40-50 hours, divided by time (h) and biomass (g). The xylose consumption rates range from 0.11 g/g×h for the TMB3014, over-expressing *TAL1* to 0.14 g/g×h for the TMB3017, over-expressing *RKI1*. Production of xylitol, ethanol, acetate, and glycerol did not vary significantly between the strains (results not shown). The TMB3026, over-expressing all four non-oxidative PPP genes (Johansson and Hahn-Hägerdal, 2001) showed an intermediate xylose consumption rate of 0.12 g/g×h (Table 2).

Over-expression of *TKL1* has been reported to inhibit growth on fermentative carbon sources (Sundström *et al.*, 1993). Expression of the *P. stipitis TKT1*, encoding transketolase, in *S. cerevisiae* expressing *XYL1* and *XYL2* resulted in increased generation times of aerobic xylose growth (Metzger and Hollenberg, 1994). Neither of the strains over-expressing *TKL1* (TMB3016 and TMB 3026) appeared to have impaired xylose metabolism (Table 2). Walfridsson et al. (1995) found no negative effects of *TKL1* over-expression in a *XYL1/XYL2* expressing *S. cerevisiae*. The reason for these differences could be lower specific

activity in TMB3016 and TMB 3026 (2.7-2.8 U/mg protein) and reported by Walfridsson *et al.* (1995) (Walfridsson *et al.*, 1995) (0.92-0.97 U/mg protein) compared with 6.7 U/mg reported by Metzger and Hollenberg (1994).

Continuous cultivations Since only small differences in xylose consumption rate between strains over-expressing various non-oxidative PPP enzymes were detected in batch cultivation, TMB3026 was also compared with the TMB3001 in anaerobic chemostat cultivation with 20 g/l glucose and 20 g/l xylose in the feed. Two steady states were obtained with TMB3026 and TMB3001 at dilution rate 0.06 and 0.012 (Table 3). The two strains consumed nearly identical amounts of glucose and xylose. There were small differences in the product formation for the two strains. TMB3001 produced small amounts of acetate and succinate at both dilution rates. The acetate production of TMB3026 was below detection limit and succinate production was lower than in TMB3001. These differences have no obvious connection to the over-expression of non-oxidative PPP genes, so it is not clear whether these differences are due to the over-expressed genes or if they are side-effects of the genetic engineering. Since TMB3026 did not show significantly higher xylose fermentation rates in neither chemostat nor batch-culture compared with the control strain TMB3001, the non-oxidative PPP could thus be ruled out as having any significant control of xylose fermentation rate in TMB3001.

Since flux control coefficients are additive, the individual control coefficients of the non-oxidative PPP enzymes must be even lower than for the whole pathway. The classical view is that reactions carried out by the non-oxidative PPP are so called “close to equilibrium” reactions that the size and direction of the fluxes are be determined by enzymes and metabolite pools outside the non-oxidative PPP. However, it has been suggested that few reactions are sufficiently close to equilibrium for this kind of regulation (Fell and Thomas, 1995).

We recently showed that some of the control of xylose consumption rate is in the XR step, since eight times over-production of XR resulted in 70% increased xylose fermentation rate of TMB3001 (Jeppsson *et al.*, 2001a). Enzymes of the lower glycolysis are expressed at lower levels during metabolism of xylulose than during glucose metabolism (Müller *et al.*, 1995), so some of the control of fermentation rate might be due to inadequate induction of glycolysis by pentoses.

The TMB3026 generated by the work presented in this investigation will be interesting for future research, once the part of metabolism where most of the metabolic flux control of xylose fermentation is identified. The integrative vector system by which the TMB3026 was created (Johansson and Hahn-Hägerdal, 2001), facilitates virtually unlimited rounds of genetic engineering so future genetic modifications are easily carried out.

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TABLE 1. Specific activities (U/mg protein) in cell extracts of glucose grown cells. Values are means are means from two cultures with less than 10 % difference. -, not measured. The data is adopted from Johansson and Hahn-Hägerdal (2001).

Strains	Overexpressed gene(s)	Specific activity (U/mg)			
		RPE	RKI	TAL	TKL
TMB 3001		8.1	0.26	2.0	0.2
TMB 3013	***RPE1***	36	-	1.7	-
TMB 3017	***RKI1***	-	6.3	-	-
TMB 3014	***TAL1***	-	-	35	-
TMB 3016	***TKL1***	-	-	-	2.8
TMB 3026	***RPE1, RKI1, TAL1,TKL1***	33	6.0	34	2.7

TABLE 2. Specific xylose consumption rates, g xylose / g biomass × hour calculated after 40-50 hours of batch cultivation. Values are means from two cultures with less than 10 % difference.

Strains	Overexpressed gene(s)	Xylose consumption rate g xylose / g biomass × hour
TMB 3001		0.13
TMB 3013	*RPE1*	0.13
TMB 3017	*RKI1*	0.14
TMB 3014	*TAL1*	0.11
TMB 3016	*TKL1*	0.12
TMB 3026	*RPE1, RKI1, TAL1,TKL1*	0.12

TABLE 3. Specific uptake rates (negative values) and production rates (positive values) (mmol g^{-1} $biomass^{-1}$ h^{-1}) of substrates and products at dilution rate 0.06 h^{-1} and 0.12 h^{-1} for TMB 3001 and TMB 3026 (overexpressing *RKI1*, *RPE1*, *TAL1* and *TKL1*) balance.

	TMB 3001		TMB 3026	
	D = 0.06 h^{-1}	D = 0.12 h^{-1}	D = 0.06 h^{-1}	D = 0.12 h^{-1}
Xylose	-1.50	-1.93	-1.53	-1.96
Glucose	-3.39	-6.88	-3.47	-6.61
$CO2_{measured}$	6.59	11.69	6.73	11.89
$Ethanol_{measured}$	5.44	10.28	6.13	11.17
Xylitol	0.64	0.71	0.65	0.72
Glycerol	0.81	1.58	0.89	1.43
Acetate	0.04	0.07	0.00	0.00
Succinate	0.02	0.04	0.01	0.01