**Research Article**

# The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains

Marie Jeppsson[1], Björn Johansson[1#], Peter Ruhdal Jensen[2], Bärbel Hahn-Hägerdal[1] and Marie F. Gorwa-Grauslund[1]*

[1] *Department of Applied Microbiology, Lund University, PO Box 124, 221 00 Lund, Sweden*
[2] *Section of Molecular Microbiology, Biocentrum, Technical University of Denmark, Building 301, DK-2800 Lyngby, Denmark*

*\*Correspondence to:*
*Marie F. Gorwa-Grauslund,*
*Department of Applied*
*Microbiology, Lund University, PO*
*Box 124, 221 00 Lund, Sweden.*
*E-mail: Marie-*
*Francoise.Gorwa @tmb.lth.se*

*#Centro de Ciencias do Ambiente,*
*Departamento de Biologia,*
*Universidade do Minho, Campus*
*de Gualtar, 4710-057*
*Braga, Portugal.*

## Abstract

**Disruption of the *ZWF1* gene encoding glucose-6-phosphate dehydrogenase (G6PDH) has been shown to reduce the xylitol yield and the xylose consumption in the xylose-utilizing recombinant *Saccharomyces cerevisiae* strain TMB3255. In the present investigation we have studied the influence of different production levels of G6PDH on xylose fermentation. We used a synthetic promoter library and the copper-regulated *CUP1* promoter to generate G6PDH-activities between 0% and 179% of the wild-type level. G6PDH-activities of 1% and 6% of the wild-type level resulted in 2.8- and 5.1-fold increase in specific xylose consumption, respectively, compared with the *ZWF1*-disrupted strain. Both strains exhibited decreased xylitol yields (0.13 and 0.19 g/g xylose) and enhanced ethanol yields (0.36 and 0.34 g/g xylose) compared with the control strain TMB3001 (0.29 g xylitol/g xylose, 0.31 g ethanol/g xylose). Cytoplasmic transhydrogenase (TH) from *Azotobacter vinelandii* has previously been shown to transfer NADPH and NAD$^+$ into NADP$^+$ and NADH, and TH-overproduction resulted in lower xylitol yield and enhanced glycerol yield during xylose utilization. Strains with low G6PDH-activity grew slower in a lignocellulose hydrolysate than the strain with wild-type G6PDH-activity, which suggested that the availability of intracellular NADPH correlated with tolerance towards lignocellulose-derived inhibitors. Low G6PDH-activity strains were also more sensitive to $H_2O_2$ than the control strain TMB3001. Copyright $\wedge$ 2003 John Wiley & Sons, Ltd.**
**Keywords: xylose fermentation; *Saccharomyces cerevisiae*; glucose-6-phosphate dehydrogenase; xylitol; redox balance; inhibitors; synthetic promoter library; NADPH**

## Introduction

*Saccharomyces cerevisiae* efficiently converts the hexose sugars present in lignocellulosic hydrolysates to ethanol. However, it cannot utilize pentoses such as xylose. The xylose pathway has therefore been integrated into *S. cerevisiae*. The *XYL1* and *XYL2* genes from *Pichia stipitis*, encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, as well as endogeneous *XKS1* encoding xylulokinase (XK), have been chromosomally integrated into *S. cerevisiae*, resulting in the stable xylose-fermenting strain TMB3001 (Eliasson *et al*., 2000). Even though TMB3001 produces ethanol from xylose (0.21 g/g xylose), considerable amounts of xylitol are formed as well (0.2–0.4 g/g xylose). Xylitol formation has been attributed to the co-factor imbalance generated by the NAD(P)H-dependent XR and NAD$^+$-dependent XDH reactions (Bruinenberg *et al*., 1983a).

The oxidative pentose phosphate pathway (PPP) is a major source of NADPH in yeast, but NADPH is also produced via the NADP$^+$-linked isoci- trate dehydrogenase reaction (Bruinenberg *et al.*, 1983b). The *ZWF1* gene encoding glucose-6-phosphate dehydrogenase has been disrupted in strain TMB3255 to decrease the NADPH level (Jeppsson *et al.*, 2002). TMB3255 showed an enhanced ethanol yield (0.41 g/g xylose) and a decreased xylitol yield (0.05 g/g xylose), however, at the expense of a six-fold lower xylose consump- tion rate. The xylose consumption rate in TMB3255 has been enhanced by increasing the production of xylose reductase (Jeppsson *et al.*, 2003). Another strategy to maintain a high ethanol yield without losing the xylose flux would be to allow a low flux through the oxidative PPP.

In the present investigation we constructed strains with a range of production levels of G6PDH to generate a range of intracellular NADPH levels. The copper promoter, *CUP1* (Labbe and Thiele, 1999) was used to facilitate the regulation of the *ZWF1* gene expression by changing the Cu$^{2+}$ con-centration of the medium. Additionally, a synthetic promoter library was constructed for expression of the *ZWF1* gene at a wider span of activities. Fur-thermore, a transhydrogenase-producing strain was included to investigate the effects of the reaction converting NADPH and NAD$^+$ into NADP$^+$ and NADH (Nissen *et al.*, 2001) on xylose fermen-tation. Ethanol and xylitol yields, as well as the xylose consumption rate were monitored.

Strains with low G6PDH-activity are likely to have reduced levels of NADPH. This may influ-ence their tolerance towards inhibitors present in lignocellulose hydrolysates. For example, 5-hydroxymethyl furfural (5-HMF) requires NADPH for reduction (Wahlbom and Hahn-Hägerdal, 2002)

and this may result in reduced growth rate. In this investigation, growth rates were determined in a lignocellulose hydrolysate. Strains disrupted in the oxidative pentose phosphate pathway have also shown enhanced sensitivity to oxidative stress (Nogae and Johnston, 1990). The tolerance towards hydrogen peroxide was therefore also evaluated for strains with a range of different G6PDH activities.

## Materials and methods

### Strains

Strains of *S. cerevisiae* used in this investigation are summarized in Table 1. *Escherichia coli* DH5$\alpha$ (Life Technologies, Rockville, MD) was used for sub-cloning. All strains were stored in 20% glyc-erol at $-80$ °C. Yeast cells from freshly streaked YPD plates (Ausubel *et al.*, 1995) were used for inoculation.

### Nucleic acid manipulation

Plasmid DNA was prepared with BioRad Plas-mid Miniprep kit (Hercules, CA). Restriction and modification enzymes were obtained from Roche (Roche Diagnostics AB, Bromma, Sweden), Fer-mentas (Vilnius, Lithuania) and Life Technologies (Rockville, MD). DNA extractions from agarose gel were made by QIAGEN Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

### Transformation

Competent cells of *E. coli* DH5$\alpha$ were prepared and transformed as described elsewhere (Inoue *et al.*, 1990) and yeast transformations were made using a lithium acetate method (Gietz *et al.*, 1992),

**Table 1.** Strains used in this study

| Strain | Genotype | Reference |
|--------|----------|-----------|
| TMB3001 | CEN.PK 113-7A (*MAT***a** *his3-  1 MAL2-8c SUC2*) *his3*::YIp XR/XDH/XK | (Eliasson *et al.*, 2000) |
| TMB3255 | TMB3001 *zwf1::KanMX* | (Jeppsson *et al.*, 2002) |
| TMB3256 | TMB3001 promoterless *ZWF1* | This work |
| TMB3037 | TMB3001 YRP13 *ZWF1* | This work |
| TMB3035 | TMB3001 YRP25 *ZWF1* | This work |
| TMB3034 | TMB3001 YRP34 *ZWF1* | This work |
| TMB3030 | TMB3001 *CUP1 ZWF1* | This work |
| TMB3253 | TMB3001 YEplac112 KanR | This work |
| TMB3254 | TMB3001 YEplac112 PGK CTH KanR | This work |

which was slightly modified (Güldener *et al.*, 1996). *E. coli* transformants were selected on Luria–Bertani (LB) medium plates (Ausubel *et al.*, 1995) with 100 ∝g/ml ampicillin (IBI Shelton Scientific Inc., Shelton, CT). *S. cerevisiae* transformants were selected on Yeast Nitrogen Base without amino acids (Difco, Sparks, MD) or on YPD plates with 100 ∝g/ml zeocin (Invitrogen, Groningen, The Netherlands) or 150 ∝g/ml geneticin (Life Technologies, Rockville, MD).

## Construction of the pB3 *ZWF1* and pB3 CUP1 *ZWF1* vectors

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**AGAATGAGTGAAGGCCCCGTC-AAATTC-3 and 3*ZWF1* clon 5 -GA**GGATCC**CTGCACTCTGATGACCAGTTCG-3 , adding *Bam* - HI sites at both ends (bold face). Only the 5 part was cloned to allow simultaneous integration and disruption of the wild-type copy of the *ZWF1* gene. The flanking *Bam* HI sites were cleaved 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, resulting in the linear fragment pB3 *ZWF1* . The cohesive ends of the remaining promoterless plasmid were blunted by Klenow DNA polymerase. The resulting vector was closed by ligation with T4 DNA ligase, generating pB3 *ZWF1* vector.

The *CUP1* promoter was released from the plasmid pCu413 CUP1 (Labbe and Thiele 1999) by restriction cleavage with *Sac* I and *Spe* I. The *CUP1* promoter was ligated to pB3 *ZWF1* , resulting in pB3 *CUP1 ZWF1*.

## Oligonucleotides for synthetic promoter design

Two synthetic oligonucleotides (DNA Technology A/S, Aarhus, Denmark) were used to generate synthetic promoters: oligonucleotide 1: 5 ATCAGAATTCTCGAGNNNNNCTTCCNNNNNACCCATACANNNNNNNNACCCATACANNNNNCTTCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTATAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTTCTTTCTTGTAACATC3 (159-mer) and oligonucleotide 2: 5 ATCGGGATCCATTTTGATTTAGTGTTTGTGTGTTGATAAGCAGTTGCTTGGT-

TTTTTA TGAAAAATAGCTAGAAGGAATAA-GGAATTACAAGAGAGATGTTACAAGAAAG-AAG3 (113-mer). 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 2 h at 37 °C with 23.6 units Klenow DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) in a total volume of 200 ∝l. The double-stranded DNA was purified by agarose gel electrophoresis and cleaved with *Eco*RI and *Bam*HI.

## Construction of the pYLZ-2 YRP vectors

The pYLZ-2 vector (Hermann *et al.*, 1992) containing the *E. coli lacZ* gene was 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 were identified by PCR using the primers 5YRPclon and 3YRPclon, as described below, and *E. coli* colonies as template. Plasmid DNA was prepared from 37 positive clones, designated pYLZ-2 YRP 1–37, and used for transformation of *S. cerevisiae* CEN.PK 113-5D (Entian and Kötter, 1998). Transformants were selected by uracil prototrophy.

## Construction of the pB3 YRP *ZWF1* vectors

Three clones of the synthetic promoter library (YRP13, YRP25 and YRP34) were used for PCR-amplification with the primers 5YRPclon 5 -GATC**GAGCTC**TGGCCGATTCATTAATCCAG-CTGAA-3 and 3YRPclon 5 -GATC**TCTAGA**TT-TGATTTAGTGTTTGTGTGTTGAT-3 . The synthetic promoters were inserted into the promoterless pB3 *ZWF1* vector after cleavage with *Sac* I and *Xba* I (bold face), generating pB3 YRP13 *ZWF1*, pB3 YRP25 *ZWF1* and pB3 YRP34 *ZWF1*.

## Construction of TMB3256, TMB3030, TMB3037, TMB3035 and TMB3034

The vectors pB3 *ZWF1*, pB3 CUP1 *ZWF1*, pB3 YRP13 *ZWF1*, pB3 YRP25 *ZWF1* and pB3 YRP34 *ZWF1* were cleaved with *Bgl* II within the *ZWF1* gene. The cleavage products were

used for transformation of TMB3001, generating TMB3256, TMB3030, TMB3037, TMB3035 and TMB3034 after selection for zeocin resistance. Integration was confirmed by PCR amplification with the pB3 PGK-specific primer 5 - GAAGTTATTAGGTGATATCAGATCC-3 and the *ZWF1*-specific primer 3*ZWF1* clon.

## Construction of TMB3253 and TMB3254

The *c*th gene encoding the *Azotobacter vinelandii* cytoplasmic transhydrogenase was removed from YEp24-pPGK-CTH (Nissen *et al.*, 2001) using *Hin* dIII and inserted into YEplac112 (Gietz and Sugino, 1988) using the same site, resulting in YEplac112 PGK CTH. The *KanMX* gene was removed from pFA6 – KanMX4 (Wach *et al.*, 1994) using *Bam*HI and *Sac*I, and thereafter inserted in YEplac112 and YEplac112 PGK CTH using the same sites, resulting in YEplac112 KanR and YEplac112 PGK CTH KanR. TMB3001 was transformed with YEplac112 KanR and YEplac112 PGK CTH KanR, resulting in TMB3253 and TMB3254, respectively, after selection for geneticin resistance.

## Batch fermentation

Batch fermentation under oxygen-limited conditions in a defined mineral medium (Verduyn *et al.*, 1992) with xylose as sole carbon source was performed as previously described (Jeppsson *et al.*, 2002). Sampling occurred before xylose was totally consumed. Geneticin (150 $\propto$g/ml) was added in the batch-fermentation with TMB3253 and TMB3254. Maximal specific growth rates in the presence of 2% lignocellulose hydrolysate (Nilvebrant *et al.*, 2003), 20 g/l glucose, and 0.1 mM methionine were determined using a defined mineral medium (Verduyn *et al.*, 1992) and in-house-built fermentors. Cultivation was conducted anaerobically by sparging with 0.6 l/min $N_2$ gas at 30 °C, 250 rpm stirring, pH 5.5 controlled by autoaddition of 3 M NaOH and a total volume of 750 ml. For TMB3030, 10 $\propto$M $Cu^{2+}$ was added to generate a G6PDH-activity above the one of the wild-type.

## Enzymatic measurements

Crude extracts for G6PDH-activity determination were made using the Y-PER reagent (Pierce,

Rockford, IL). Crude extracts for TH-activity determination were made with 0.5 mm glass beads in disintegration buffer (100 mM triethanolamine, 0.5 mM EDTA, 0.5 mM DTT and 1 mM PMSF) through 3 × 5 min of vigorous vortexing. Protein concentration was determined using Coomassie Protein Assay Reagent (Pierce), with bovine serum albumin as standard. The glucose-6-phosphate dehydrogenase activity (G6PDH; EC 1.1.1.49) was measured according to Bergmeyer (1974). $\beta$-Galactosidase activities were measured in whole cells (Miller, 1972) and the transhydrogenase activity was measured according Voordouw *et al.* (1979).

Enzyme activities for strains used in batch fermentation were measured in precultures, since the cells are in a better physiological condition before than after fermentation with xylose as sole carbon source.

## Analysis of substrates and products

Xylose, xylitol, glycerol, acetate and ethanol concentrations were determined by column liquid chromatography (CLC) as previously described (Jeppsson *et al.*, 2002). Cell dry weight was determined as previously described (Jeppsson *et al.*, 2002).

## Sensitivity towards $H_2O_2$

Minimal inhibitory concentrations (MIC) of $H_2O_2$ were determined in 2 ml YPD medium kept overnight in test tubes at 30 °C and 140 rpm. MIC was defined as the lowest $H_2O_2$ concentration for which no growth was found.

## Results

### Effect of modulation of G6PDH activity using the *CUP1* promoter

The connection between the availability of NADPH and xylitol formation during xylose fermentation was investigated by expressing the *ZWF1* gene under control of the $Cu^{2+}$-regulated *CUP1* pro- moter (Labbe and Thiele, 1999). Integration of pB3 *CUP1 ZWF1* into TMB3001 resulted in TMB3030. gave G6PDThe addition of 0 and 10 $\propto$M $Cu^{2+}$ activities of 1.040 and 1.567 U/mg protein, respectively. In the control strain TMB3001 the activity was 1.015 U/mg protein, which is about the same

as the lowest activity feasible with the copper promoter.

A batch fermentation of xylose was performed under oxygen-limited conditions with the control strain TMB3001 and with TMB3030 at 10 $\propto$M $Cu^{2+}$. The cells did not grow under these conditions, and the xylose was therefore converted only to xylitol, glycerol, acetate, ethanol and carbon dioxide. The higher G6PDH-activity in TMB3030 resulted in a higher xylitol yield (0.33 g/g) than in TMB3001 (0.29 g/g) (Table 2). The xylose consumption rate was the same for the two strains, indicating that the xylose consumption rate can not be enhanced by increasing the G6PDH activity above the wild-type level. Addition of 10 $\propto$M $Cu^{2+}$ to TMB3001 did not affect xylose consumption and product yields (data not shown). Since the *CUP1* promoter turned out to be unsuitable for downregulation of *ZWF1* expression, a synthetic promoter library was constructed.

## Construction of a promoter library for downregulation of *ZWF1*

In order to modulate the *ZWF1* expression, a library of synthetic promoters was designed. The promoter (Figure 1), called YRP, contains two regulatory structures, RPG boxes (Nieuwint *et al.*, 1989) and CT boxes (Baker, 1986, 1991). These elements promote transcription of ribosomal protein genes and glycolytic genes (Rotenberg and

Woolford, 1986; Baker, 1991). The CT boxes and RPG boxes were designed to be degenerated; at each position, 1% each of the three other bases was incorporated during synthesis (Figure 1). Completely degenerated intervening sequences combined with less degenerated transcription factor binding sites results in a wide range of different promoter strengths (Jensen, 1997; Jensen and Hammer, 1998a, 1998b).

In *Lactococcus lactis*, nucleotides at a number of positions are well conserved between different promoters, and upon these sequences the synthetic promoter can be modelled (Jensen and Hammer, 1998a, 1998b). However, *S. cerevisiae* does not appear to have a strict sequence for promoters; instead the synthetic promoter was pieced together with 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 (Drazinic *et al.*, 1996), since it had been shown to be the best for interaction between the transcription factors GCR1p and RAP1p (Drazinic *et al.*, 1996), 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 (Rotenberg and Woolford, 1986). The constant sequence between the TATA box and the 3 end of the promoters was the same as in the *ENO1* promoter (Uemura *et al.*, 1997). The synthetic

**Table 2.** Effect of different expression levels of *ZWF1* in recombinant *S. cerevisiae* in oxygen-limited batch fermentation with 50 g/l xylose as sole carbon source. Specific xylose consumption rate (g/g biomass/h) and ethanol, xylitol, acetate and glycerol yields (g/g consumed xylose) and carbon recoveries (cmoles out/cmoles in) were determined after 60–70 h. The presented values are the mean of two independent fermentation experiments plus/minus the deviation from the mean. In calculation of carbon recovery, 0.5 moles $CO_2$ are assumed to be formed for each cmole of ethanol or acetate produced. Maximal specific growth rates, $\propto$max ($h^{-1}$) were measured under anaerobic conditions with 2% lignocellulose hydrolysate and 20 g/l glucose. For minimal inhibitory concentration, MIC, of $H_2O_2$ (%) steps of 0.75% $H_2O_2$ were investigated and double samples were made. NM, not measured. G6PDH activities were measured in precultures

| Strains | G6PDH activity | Xylose consumption rate | Y(Ethanol) | Y(Xylitol) | Y(Acetate) | Y(Glycerol) | C-recovery | $\propto$max ($h^{-1}$) | MIC (% $H_2O_2$) |
|---|---|---|---|---|---|---|---|---|---|
| TMB3255[*] | <0.01 | 0.022 ± 0.002 | 0.41 ± 0.02 | 0.05 ± 0.01 | 0.084 ± 0.005 | 0.054 ± 0.008 | 0.95 | 0.19 | 6.75 |
| TMB3256 | 0.014 | 0.062 ± 0.002 | 0.36 ± 0.02 | 0.13 ± 0.02 | 0.054 ± 0.003 | 0.044 ± 0.001 | 0.96 | 0.21 | NM |
| TMB3037 | 0.056 | 0.112 ± 0.003 | 0.34 ± 0.01 | 0.19 ± 0.01 | 0.039 ± 0.001 | 0.037 ± 0.001 | 0.99 | 0.26 | 6.75 |
| TMB3001[*] | 1.015 | 0.145 ± 0.002 | 0.31 ± 0.01 | 0.29 ± 0.01 | 0.025 ± 0.001 | 0.052 ± 0.004 | 0.98 | 0.28 | 9.75 |
| TMB3030 (0 $\propto$M $Cu^{2+}$) | 1.040 | NM | NM | NM | NM | NM | NM | NM | NM |
| TMB3030 (10 $\propto$M $Cu^{2+}$) | 1.567 | 0.150 ± 0.001 | 0.28 ± 0.01 | 0.33 ± 0.01 | 0.025 ± 0.001 | 0.051 ± 0.003 | 0.97 | 0.21 | NM |

[*] Fermentation data from Jeppsson *et al.* (2002).

| EcoRI | CT-box | RPG-box | RPG-box |
|---|---|---|---|

ATCAGAATTCTCGAGNNNNNCTTCCNNNNNACCCATACANNNNNNNNACCCA

| CT-box | | | TATA-box |
|---|---|---|---|

TACANNNNNCTTCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTATAANN

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTTCTTTCTTGTA

ACATCTCTCTTGTAATTCCTTATTCCTTCTAGCTATTTTTCATAAAAAACCAAG

| | BamHI | |
|---|---|---|

CAACTGCTTATCAACACACAAACACTAAATCAAAATGGATCCCGAT

**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

double-stranded promoters were ligated to the vector pYLZ-2 (Hermann *et al.*, 1992) 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 YRP 1–37. 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.

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. When harvested in exponential phase on YPD medium TMB3037, TMB3035 and TMB3034 had G6PDH-activities of 0.11, 0.77 and 1.82 U/mg protein, respectively, which corresponded with the expected order of the strength of the promoters.

## Xylose fermentation with strains with downregulated *ZWF1*

The synthetic promoter YRP13 produced the lowest G6PDH activity in TMB3037 (0.056 U/mg protein in defined medium), so this strain was selected for batch fermentation with xylose. TMB3037 consumed 0.112 g xylose g biomass/h, which is five times faster than in TMB3255 (*zwf1*), and represents 77% of the specific consumption observed in the control strain TMB3001 (Table 2). As expected, TMB3037 had a higher ethanol yield (0.34 g/g) than TMB3001 (0.31 g/g), accompanied by a lower xylitol yield (0.19 g/g vs. 0.29 g/g).

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 0.014 U/mg protein, which is about 1% of the wild-type activity (Table 2). Batch fermentation conducted with 50 g/l xylose showed that TMB3256 had a specific xylose consumption of 0.062 g/g biomass/h, which corresponds to a 2.8-fold increase compared with TMB3255 (*zwf1*). At the same time, TMB3256 showed an ethanol yield of 0.36 g/g and a xylitol yield of 0.13 g/g, which are close to the values of TMB3255 (*zwf1*) (Table 2).

The connection between G6PDH activity, xylitol yields, ethanol yields and xylose consumption are illustrated in strains with G6PDH activities spanning from 0 U/mg protein to 1.6 U/mg protein (Table 2). The *ZWF1*-disrupted strain TMB3255 exhibited the highest ethanol yield (0.41 g/g) and lowest xylitol yield (0.05 g/g), whereas the TMB3030 strain at 10 $\propto$M $Cu^{2+}$ had the lowest ethanol yield (0.28 g/g) and the highest xylitol yield (0.33 g/g) of the strains in Table 2. The xylose consumption rate was not further enhanced at G6PDH-activity above wild-type level.
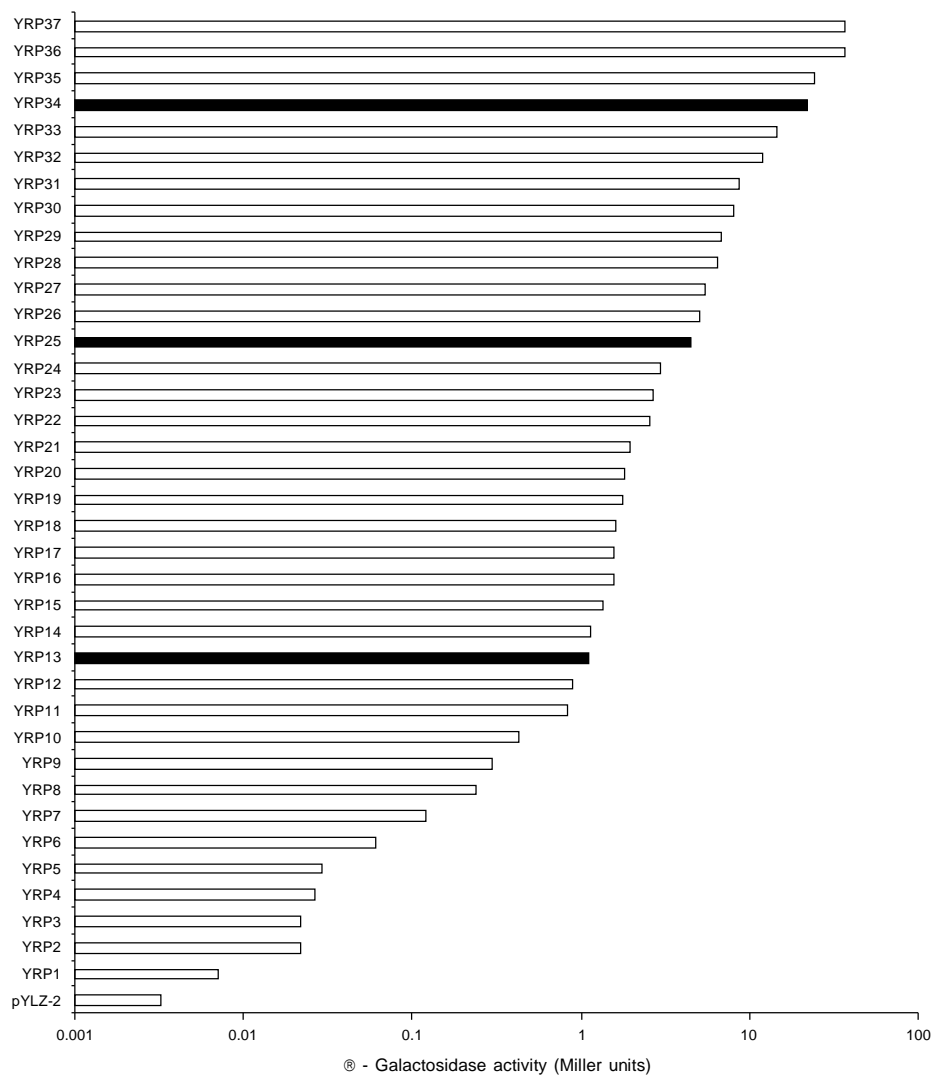
**Figure 2.** Specific $\beta$-galactosidase activity (Miller units) from YRP1 to YRP37 controlling *lacZ* in pYLZ-2 in *S. cerevisiae*. Shaded clones (YRP13, YRP25, YRP34) were used to regulate the expression of *ZWF1*

## Overproduction of transhydrogenase

If the fermentation pattern seen in low G6PDH-activity strains is a result of low intracellular levels of NADPH, a similar phenotype should be observed at overproduction of the cytoplasmic transhydrogenase (TH) from *Azotobacter vinelandii*. In *S. cerevisiae*, this enzyme has been shown to catalyse the conversion of NADPH and $NAD^+$ to $NADP^+$ and NADH, so that the ratio $NADPH : NADP^+$ shifted from 5.1 to 3.0 at TH-overproduction (Nissen *et al*., 2001).

TH was overproduced at 1.0 U/mg in TMB3254 (Table 3). No TH activity was detected in the control strain. The xylose consumption was similar in the TH-overexpressing strain compared to its control. TH-overproduction resulted in 12% lower xylitol yield and 29% higher glycerol yield. Ethanol and acetate yields were similar with and without TH overproduction.

## Maximal specific growth rates in lignocellulose hydrolysate

Strains in which the G6PDH activity is reduced are impaired in their ability to produce NADPH. This may influence the strains' tolerance towards inhibitors present in lignocellulosic hydrolysates,

**Table 3.** Effect of transhydrogenase overproduction in recombinant *S. cerevisiae* in oxygen-limited batch fermentation with 50 g/l xylose as sole carbon source. Specific xylose consumption rate (g/g biomass/h) and ethanol, xylitol, acetate and glycerol yields (g/g consumed xylose) and carbon recoveries (cmoles out/cmoles in) were determined after 40 h. The presented values are the mean of two independent fermentation experiments ± the deviation from the mean. In calculation of carbon recovery, 0.5 moles $CO_2$ are assumed to be formed for each cmole ethanol or acetate produced. Specific TH activities were measured in cells harvested in exponential phase after growth on YPD medium

| Strains | TH activity | Xylose consumption rate | Y(Ethanol) | Y(Xylitol) | Y(Acetate) | Y(Glycerol) | C-recovery |
|---|---|---|---|---|---|---|---|
| TMB3253 | <0.01 | 0.157 ± 0.004 | 0.28 ± 0.007 | 0.34 ± 0.001 | 0.036 ± 0.001 | 0.059 ± 0.002 | 0.98 |
| TMB3254 | 1.0 | 0.162 ± 0.002 | 0.28 ± 0.003 | 0.30 ± 0.002 | 0.036 ± 0.001 | 0.076 ± 0.001 | 0.97 |

since for example, 5-HMF is reduced with NADPH (Wahlbom and Hahn-Hägerdal, 2002).

In previous work, we showed that the disrupted strain TMB3255 (*zwf1*) and TMB3001 had similar anaerobic maximal specific growth rates on glucose in defined mineral medium (Verduyn *et al.*, 1992) supplemented with 0.1 mM methionine (Jeppsson *et al.*, 2003). TMB3255 (*zwf1*) grows 54% slower than TMB3001 when methionine is left out (Jeppsson *et al.*, 2003). Under aerobic conditions, the growth rate of TMB3255 was not fully restored by methionine-addition (Jeppsson *et al.*, 2003). Anaerobic growth rates were measured for TMB3030, TMB3001, TMB3037, TMB3256 and TMB3255 in 2% lignocellulosic hydrolysate and 20 g/l glucose, supplemented with 0.1 mM methionine. Since we supplemented the medium with methionine, and the cultures were run anaerobically, differences in growth rate between strains result solely from the presence of hydrolysate. For TMB3030, 10 ∝M $Cu^{2+}$ was also added.

Maximal specific growth rates decreased with decreasing G6PDH-activity (Table 2). TMB3001, TMB3037, TMB3256 and TMB3255 had maximal specific growth rates of 0.28, 0.26, 0.21 and 0.19/h, respectively. TMB3030 had the highest G6PDH activity but it only grew at 0.21/h, which is probably due to inhibition by $Cu^{2+}$.

### Minimal inhibitory concentrations (MIC) of $H_2O_2$

Strains disrupted in the oxidative pentose phosphate pathway display enhanced sensitivity to oxidative stress (Nogae and Johnston, 1990), which has been ascribed to reduced levels of NADPH. This was also observed in our hands, where TMB3001 had a MIC of 9.75 vol% $H_2O_2$, whereas

TMB3037 and TMB3255 both had a MIC of 6.75 vol% $H_2O_2$ (Table 2).

### Discussion

In a previous investigation (Jeppsson *et al.*, 2002), we demonstrated the connection between a low flux through the oxidative PPP and a low xylitol yield. However, the specific xylose consumption in the *ZWF1*-disrupted strain (TMB3255) was sixfold lower than for the control strain TMB3001. In the present investigation we looked into the effect of different expression levels of *ZWF1* on xylose fermentation. We showed that xylose consumption in a G6PDH-deficient strain could be enhanced by allowing a low G6PDH activity, although at the expense of a higher xylitol yield. TMB3256 and TMB3037 had 1% and 6% of the wild-type level of G6PDH activity, respectively. The xylose consumption rate was 2.8 times higher for TMB3256 and 5.1 times higher for TMB3037 than in the *ZWF1*-disrupted strain TMB3255 (*zwf1*). The xylitol yields increased 2.6 times for TMB3256 and 3.8 times for TMB3037. Thus, there is a trade-off between high xylose consumption rate and low xylitol yield when *ZWF1* expression level is modulated.

The synthetic promoter library was useful for downregulation of *ZWF1*. The *β*-galactosidase activities covered a range of about three orders of magnitude between the lowest and the highest activity, and since the maximal strength appears to be in the same range as the *CUP1* promoter, this library should be useful for both up- and downregulation of many genes in *S. cerevisiae*. Synthetic promoter libraries have earlier been described for *Lactococcus lactis* (Jensen and Hammer, 1998a), *E.*

*coli* (Jensen and Hammer, 1998b) and mammalian cells (Tornoe *et al*., 2002). The synthetic promoters did not require an inducing agent, in contrast to, for example, the *CUP1* promoter which requires supplementation of $Cu^{2+}$.

The cytoplasmic transhydrogenase from *A. vinelandii* has been shown to transfer $NAD^+$ and NADPH into NADH and $NADP^+$ (Nissen *et al*., 2001). The overproduction of transhydrogenase in *S. cerevisiae* resulted in a lower xylitol yield. Since this was also observed in strains with decreased G6PDH activity, it further strengthens the hypothesis that the decreased xylitol yield is a result of lower NADPH level. We observed enhanced glycerol yield from xylose at TH overproduction. This has also been observed on glucose (Nissen *et al*., 2001) and was then ascribed to the necessary reoxidation of NADH formed in the TH reaction.

If the transhydrogenase had been working in the opposite direction we might have been able to remove excess $NADP^+$ and NADH formed in the XR and XDH reactions, respectively. A strategy to overcome the redox imbalance between the XR and XDH reactions has recently been initiated by Verho *et al*. (2002). The *Kluyveromyces lactis* gene encoding a $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase has been introduced in xylose-fermenting *S. cerevisiae* to reduce accumulation of $NADP^+$ and NADH, and thereby enhance the ethanol yield.

Another strategy for creating a co-factor balanced breakdown of xylose was to subject xylose reductase to site-specific mutagenesis to shift the co-factor preference towards NADH (Zhang and Lee, 1997; Kostrzynska *et al*., 1998). However, when the $K_m$ of NADPH was enhanced, $K_m$ for xylose increased concomitantly.

The growth rates in lignocellulose hydrolysate were significantly lower in the strains with 0% and 1% G6PDH activity, whereas no significant decrease was observed for TMB3037 with 6% G6PDH activity compared to the control strain. Lignocellulose-derived inhibitors fall in three groups: low-molecular-weight aliphatic acids (predominantly acetic acid, formic acid and levulinic acid), furan derivatives (furfural and 5-HMF) and phenolic compounds (Larsson *et al*., 1999). 5-HMF requires NADPH for reduction (Wahlbom and Hahn-Hägerdal, 2002). This co-factor is also needed for biomass formation, which may explain the lower growth rates found for the low G6PDH activity strains.

Another mechanism of growth inhibition is the interference of aliphatic acids with ATP requirements. Acetic acid has been shown to lower the maximal specific growth rate of *S. cerevisiae* (Pampulha and Loureiro-Dias, 2000) and at high external acetic acid concentrations the intracellular pH is decreased (Pampulha and Loureiro-Dias, 1989). When the plasma membrane ATPase pumps out protons it uses ATP, which results in less ATP being available for biomass formation. We showed in the present investigation that the acetate yield is enhanced in strains with low G6PDH activity. These strains may therefore encounter difficulties with providing the ATP required for maintaining the intracellular pH.

In this investigation we have illustrated the effect of modulated G6PDH activities on xylose fermentation. The best ethanol yield was found in the *ZWF1*-disrupted strain TMB3255, but this strain also had the lowest xylose consumption rate and a significantly reduced growth rate in lignocellulose hydrolysate. TMB3037 had only 6% of wild-type G6PDH activity but the xylose consumption rate was 77% of the consumption rate found in the control strain. This strain also had almost the same maximal specific growth rate in a lignocellulose hydrolysate as TMB3001.

## Acknowledgements

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