The Expression of a *Pichia stipitis* Xylose Reductase Mutant With Higher K_M for NADPH Increases Ethanol Production From Xylose in Recombinant *Saccharomyces cerevisiae*

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Abstract: Xylose fermentation by *Saccharomyces cerevisiae* requires the introduction of a xylose pathway, either similar to that found in the natural xylose-utilizing yeasts *Pichia stipitis* and *Candida shehatae* or similar to the bacterial pathway. The use of NAD(P)H-dependent XR and NAD^+-dependent XDH from *P. stipitis* creates a cofactor imbalance resulting in xylitol formation. The effect of replacing the native *P. stipitis* XR with a mutated XR with increased K_M for NADPH (Kostrzynska et al., 1998: FEMS Microbiol Lett 159:107–112) was investigated for xylose fermentation to ethanol by recombinant *S. cerevisiae* strains. Enhanced ethanol yields accompanied by decreased xylitol yields were obtained in strains carrying the mutated XR. Flux analysis showed that strains harboring the mutated XR utilized a larger fraction of NADH for xylose reduction. The overproduction of the mutated XR resulted in an ethanol yield of 0.40 g per gram of sugar and a xylose consumption rate of 0.16 g per gram of biomass per hour in chemostat culture (0.06/h) with 10 g/L glucose and 10 g/L xylose as carbon source.

Keywords: xylose reductase; *Saccharomyces cerevisiae*; site-specific mutagenesis; xylitol; NAD(P)H

INTRODUCTION

Ethanol is efficiently produced from hexoses by *Saccharomyces cerevisiae*, but recombinant *S. cerevisiae* strains capable of xylose utilization are needed to expand the substrate range to lignocellulosic hydrolyzates. The most straightforward metabolic engineering strategy is the expression of a bacterial xylose isomerase (XI) gene, so that xylose can directly be converted to xylulose. There have been numerous unsuccessful attempts to express XI genes from various bacteria in *S. cerevisiae* (Amore et al., 1989; Briggs et al., 1984; Gardonyi and Hahn-Hägerdal, 2003; Hallborn, 1995; Ho et al., 1983; Moes et al., 1996; Sarthy et al., 1987; Schrunder et al., 1996). Only the XI gene from the thermophilic bacterium *Thermus thermophilus* has successfully been expressed in *S. cerevisiae* (Walfridsson et al., 1996), and generated xylose-fermenting recombinant strains (Karhumaa et al., 2005). Recently the cloning and successful expression of XI from the anaerobic fungus *Piromyces* sp. enabled the development of xylose-growing and fermenting strains (Kuyper et al., 2003, 2004).

The other metabolic engineering strategy, which has been developed over the last 15 years consists of expressing fungal xylose reductase (XR) and xylitol dehydrogenase (XDH) genes. Stable xylose-fermenting *S. cerevisiae* strains have been obtained by integrating the *Pichia stipitis XYL1* and *XYL2* genes encoding XR and XDH, respectively, and overexpressing the endogenous *XKS1* gene encoding xylulokinase (XK) (Eliasson et al., 2000; Ho et al., 1998; Toivari et al., 2001). However, the ethanol yield produced by these strains was far from the theoretical maximum of 0.51 g/g, because a significant fraction of the consumed xylose was secreted as its reduction product xylitol. This has been ascribed to the difference in cofactor usage between the NAD(P)H-dependent XR and the strictly NAD^+-dependent XDH steps (Kötter and Ciriacy, 1993). Xylitol formation in recombinant *S. cerevisiae* has been reduced by the addition of electron acceptors such as acetoin, furfural and acetaldehyde (Wahlbom and Hahn-Hägerdal, 2002), that re-oxidize NAD^+ which is needed in the XDH reaction. Xylitol formation has

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also been decreased by shifting the cofactor utilization in the XR step from NADPH to NADH. This was notably achieved by changing the intracellular pool of NADPH by blocking or reducing the oxidative pentose phosphate pathway (PPP) flux through modification of the glucose-6-phosphate dehydrogenase activity (Jeppsson et al., 2002, 2003a). This resulted in improved ethanol yield, however, at the expense of the growth rate on glucose (Jeppsson et al., 2003b) and the xylose consumption rate (Jeppsson et al., 2002, 2003a,b).

Another strategy to reduce xylitol formation consists of modifying the affinity of the XR-XDH enzymes towards more balanced co-factor utilization. The *XYL1* gene has been subjected to site-specific mutagenesis to reduce the XR affinity for NADPH (Kostrzynska et al., 1998; Zhang and Lee, 1997). As a result, a 17 times higher K_M for NADPH with xylose as substrate was achieved when the *XYL1* gene carried the K270M (lysine → methionine) mutation, while the K_M for NADH remained unchanged (Kostrzynska et al., 1998). Attempts have also been made to increase the affinity of XDH for the cofactor $NADP^+$. An $NADP^+$ recognition sequence from *Thermoanaerobium brockii* was introduced in the *XYL2* gene resulting in equal apparent K_M values for NAD^+ and $NADP^+$ (Metzger and Hollenberg, 1995). The mutated *XYL2* gene mediated xylose growth when co-expressed with the *XYL1* gene in *S. cerevisiae*, however, ethanolic fermentation of xylose was not reported (Metzger and Hollenberg, 1995). Another attempt to change the co-factor affinity of XDH using multiple site-directed mutagenesis on amino acids from the coenzyme-binding domain was recently reported (Watanabe et al., 2005), but the effect of the mutant expression in *S. cerevisiae* was not studied.

In the present investigation, we constructed xylose-utilizing *S. cerevisiae* strains expressing the K270M-mutated *P. stipitis XYL1* gene (Kostrzynska et al., 1998) at two different levels together with the native *P. stipitis XYL2* gene and the overexpressed endogenous *XKS1* gene. Xylose consumption and ethanol formation were compared with strains expressing the native *P. stipitis XYL1* gene together with *XYL2* and *XKS1* genes.

MATERIALS AND METHODS

Strains

Escherichia coli DH5α (Life Technologies, Rockville, MD) was used for sub-cloning. All strains were stored in 20% glycerol at −80°C. Yeast cells from freshly streaked YPD plates (Ausubel et al., 1995) were used for inoculation. The yeast strains used are listed in Table I.

Nucleic Acid Manipulation

Plasmid DNA was prepared with a BioRad Plasmid Miniprep Kit (Hercules, CA). Restriction and modification enzymes were obtained from Fermentas (Vilnius, Lithuania). The QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) was used for DNA extraction from agarose.

Transformation

Competent cells of *E. coli* DH5α were prepared and transformed by the method of Inoue et al. (1990). Yeast transformation was performed using the lithium acetate method (Güldener et al., 1996). Yeast cells were incubated overnight in YPD medium before being streaked on selective medium. *E. coli* transformants were selected on Luria–Bertani (LB) plates (Ausubel et al., 1995) with 100 μg/mL ampicillin (IBI Shelton Scientific, Inc., Shelton, CT). *S. cerevisiae* transformants were selected on plates with Yeast Nitrogen Base w/o amino-acids (Difco, Sparks, MD) containing 20 g/L glucose and 50 mg/L uracil, or on YPD plates with 100 μg/mL zeocin (Invitrogen, Groningen, the Netherlands).

Construction of TMB3270

The *PGK1* promoter and terminator were excised from pB3 PGK (Johansson and Hahn-Hägerdal, 2002) using *Nar*I and *Sac*I, and inserted into YIplac211 (Gietz and Sugino, 1988) using the same sites, resulting in YIplac211 PGK. Mutated *XYL1* (K270M) was amplified from pSX (K270M) (Kostrzynska et al., 1998) using primers previously described (Hallborn et al., 1991), with *Bam*HI sites added to the 5′ ends of each primer. The *XYL1* PCR product was cut with *Bam*HI and inserted after the *PGK1* promoter at the *Bgl*II site of YIplac211 PGK, resulting in YIplac211 PGK *XYL1*(K270M). The correct orientation was verified by PCR and the correctness of the vector was verified by restriction analysis.

YIplac211 PGK *XYL1*(K270M) was cleaved with *Bpu*10I within the *URA3* gene. The cleavage product was used for transformation of TMB3265 (XDH-XK) (Träff-Bjerre et al.,

Table I. Yeast strains used in the present investigation with their relevant phenotype.

Strain	Relevant genotype	Relevant phenotype	Reference
TMB3001	CEN · PK 113-7A (*MAT*α his3-*Δ*1 MAL2-8c SUC2) *his3*::YIpXR/XDH/XK	Produces XR, XDH, and XK	Eliasson et al. (2000)
TMB3260	TMB3001 *XYL1*::pB3 PGK *XYL1*	TMB3001 over-producing XR	Jeppsson et al. (2003a)
TMB3265	CEN · PK 113-11C (*MAT*α his3-*Δ*1 ura3-52 MAL2-8c SUC2) *his3*:: YIpXDH/XK	Produces XDH and XK	Träff-Bjerre et al. (2003)
TMB3270	TMB3265 *ura3*::YIplac211 PGK *XYL1*(K270M)	TMB3265 producing the mutated XR	This work
TMB3271	TMB3270 *XYL1*::pB3 PGK *XYL1* (K270M)	TMB3270 over-producing the mutated XR	This work

2003) resulting in TMB3270. Integration at the correct locus in TMB3270 was verified with PCR, using the primer 5′-CAC GGA AAT GTT GAA TAC TCA TAC TC-3′ annealing to the YIplac211 PGK *XYL1* (K270M) vector, and 5′-GTT ACT TGG TTC TGG CGA GGT A-3′ annealing downstream of the *URA3* gene in the yeast genome. The K270M mutation was verified by sequencing.

Construction of TMB3271

The *PGK1* promoter and terminator together with the *XYL1* (K270M) gene were excised from YIplac211 PGK *XYL1*(K270M) using *Nar*I and *Sac*I. The fragment was inserted into pB3 PGK after removal of the *PGK1* promoter and terminator with *Nar*I and *Sac*I, resulting in pB3 PGK *XYL1*(K270M). The correctness of the vector was verified by restriction analysis and the K270M mutation was verified by sequencing. The plasmid was cleaved with *Bsh*TI within the *XYL1* gene. The cleaved plasmid was used for integration in the *XYL1* K270M gene of TMB3270. The transformant TMB3271 was selected on YPD plates with zeocin.

Batch-Fermentation

Oxygen-limited batch fermentation of xylose was conducted as follows. For the pre-culture, defined mineral medium consisting of 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$, 0.3 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L $MnCl_2 \cdot 4H_2O$, 0.3 mg/L $CuSO_4 \cdot 5H_2O$, 4.5 mg/L $CaCl_2 \cdot 2H_2O$, 3 mg/L $FeSO_4 \cdot 7H_2O$, 0.4 mg/L $Na_2MoO_4 \cdot 2H_2O$, 1 mg/L H_3BO_3, 0.1 mg/L KI, 15 mg/L EDTA, 0.05 mg/L biotin, 1 mg/L calcium pantothenate, 1 mg/L nicotinic acid, 25 mg/L inositol, 1 mg/L thiamine · HCl, 1 mg/L pyridoxine · HCl, 0.2 mg/L para-amino benzoic acid, 0.2 mg/L riboflavin, 0.002 mg/L folic acid was used. Fifty milliliters of the mineral medium containing 40 g/L glucose as carbon source 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 1,000 mL baffled shake-flask, which was incubated under the same conditions. Cells were harvested in exponential phase by centrifugation at 4,400*g*, 10 min, 4°C (Avanti™ J-25I, Beckman Instruments, Palo Alto, CA) and washed twice with 0.9% NaCl. 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. Stirred 25 mL vials containing 20 mL of the same defined medium with 50 g/L xylose as sole carbon source were inoculated with approximately 5 g/L cells. The medium was supplemented with 100 mM citrate buffer (pH 5.5), 10 mg/L ergosterol, and 0.42 g/L Tween-80. The fermentation was conducted at 30°C in duplicates. Samples were withdrawn using a 2 mm hypodermic needle with a syringe and fermentation gases were expelled through a 0.8 mm needle.

Continuous Cultivation

Cells from an overnight culture were used for inoculation of 1.5 L defined mineral medium described above with 10 g/L glucose and 10 g/L xylose as carbon sources. Tween-80 (0.42 g/L) and ergosterol (10 mg/L) were added to the medium to ensure growth under anaerobic conditions. The cultivations were performed in a Bioflo-III bioreactor (New Brunswick Scientific, Edison, NJ). Antifoam was added at 0.05% (v/v) (Dow Corning® Antifoam RD Emulsion, BDH Laboratory Supplies, Poole, UK). After depletion of glucose, continuous cultivation with 10 g/L glucose and 10 g/L xylose was set up at a dilution rate of 0.06/h at 30°C and pH 5.5 controlled by 3 M KOH. Anaerobic conditions were obtained by sparging the reactor with 0.2 L/min nitrogen (containing less than 5 ppm O_2) as measured with a gas mass flowmeter (Bronkhorst, Ruurlo, the Netherlands), and a stirring speed of 200 rpm was used.

Analyses

Substrate and product concentrations, cell dry weight, RNA, proteins and carbohydrates were measured as earlier described (Jeppsson et al., 2002). The previously developed stoichiometric flux model (Wahlbom et al., 2001) was used to evaluate the in vivo cofactor usage by XR.

Enzyme Activities

Enzyme activities were determined in cells from shake-flask cultures growing exponentially in the defined mineral medium described above with 20 g/L glucose as carbon source. Pre-cultures were prepared under the same conditions. Crude extracts were obtained using the Y-PER reagent (Pierce, Rockford, IL). The protein concentration was determined using the Coomassie protein assay reagent (Pierce) with bovine serum albumin as a standard. A U-2000 model spectrophotometer (Hitachi Ltd., Tokyo, Japan) operating at 340 nm and 30°C was used for XR assay. XR activities were measured with two different sample concentrations (each in triplicate). Due to the low affinity of the mutated XR for both NADPH and xylose, the standard assay (Eliasson et al., 2000) was modified by using 2 mM NADPH or NADH and 1.05 M xylose in the final mixture. Samples were diluted ten times in ice-cold ion free water to be in the linear range of the spectrophotometer. Activities were measured over an extended time period (4 vs. 2 min for the original assay).

RESULTS

Effect of the *XYL1* K270M Mutation on Xylose Fermentation

P. stipitis XR has been subjected to site-specific mutagenesis to modify its affinity for NADPH (Kostrzynska et al., 1998). The exchange of lysine into methionine at amino-acid position 270 resulted in an apparent K_M of 185 μM for

NADPH using xylose as a substrate, which is 17 times higher than the apparent K_M for NADPH of the native XR (11 μM) (Kostrzynska et al., 1998). However, the K270M mutation in XR also enhanced the apparent K_M for xylose 14-fold (625 vs. 44 mM), whereas the apparent K_M for NADH was not affected when glyceraldehyde was used as the substrate (31 vs. 28 μM) (Kostrzynska et al., 1998).

The *XYL1* K270M gene was introduced into *S. cerevisiae* strain TMB3265 already harboring the *P. stipitis XYL2* (encoding XDH) and the endogenously overexpressed *XKS1* (encoding XK) genes (Träff-Bjerre et al., 2003), resulting in strain TMB3270. The NADPH- and NADH-dependent in vitro XR activities in TMB3270 (mutated XR) were 1.04 and 0.54 U/mg, respectively, whereas the control strain TMB3001, that harbors the native *XYL1* gene together with the *XYL2* and *XKS1* genes (Eliasson et al., 2000) had NADPH- and NADH-dependent activities of 0.25 and 0.13 U/mg, respectively (Table II). Despite the lower affinity of K270M mutant for NADPH (Kostrzynska et al., 1998), similar in vitro NADPH/NADH activity ratios (around 1.9) were obtained with both strains.

Product formation from xylose was analyzed in batch fermentation with glucose-grown resting cells under oxygen-limited conditions (Table III). A negligible amount of biomass was formed under these conditions. The xylose consumption rate of TMB3270 (*XYL1* K270M) (0.155 g per biomass/h) was similar to that of TMB3001 (0.145 g per biomass/h). The ethanol yield was enhanced (0.36 g/g) and the xylitol yield was reduced (0.17 g/g) in TMB3270 compared with the control strain TMB3001 (0.31 g ethanol per gram of xylose, 0.29 g xylitol per gram of xylose). Acetate and glycerol yields were low, but were higher in TMB3270 (0.035, 0.072 g/g) than in TMB3001 (0.025, 0.052 g/g).

The effect of the mutation on product formation during co-fermentation of glucose and xylose was also assayed in anaerobic continuous cultivation with TMB3001 and TMB3270 at 10 g/L glucose and 10 g/L xylose (Table IV). TMB3270 (*XYL1* K270M) exhibited a 58% lower xylose consumption rate than the control strain TMB3001. Ethanol and CO_2 yields were about 10% higher in TMB3270 (0.40 and 0.48 g per gram of substrate) than in TMB3001 (0.37 and 0.43 g per gram of substrate). TMB3270 showed a reduced xylitol yield per gram consumed xylose (0.31 g per gram of xylose) compared with TMB3001 (0.52 g per gram of xylose), which agreed with the results from the batch fermentation (Table III). TMB3270 also had a lower glycerol yield (0.084 g per gram of substrate) than TMB3001 (0.095 g per gram of substrate), while the acetate yield was slightly higher in TMB3270 (0.002 g per gram of substrate) compared with TMB3001 (0.001 g per gram of substrate). The biomass formation was 1.1 and 1.0 g/L for TMB3001 and TMB3270, respectively, resulting in a similar biomass yield for the two strains (0.094 and 0.095 g per gram of substrate).

Table II. Specific XR activity (U per mg protein) for strains TMB3001, TMB3260, TMB3270, and TMB3271.

	XR activity (U per mg)	
Strain	NADPH	NADH
TMB3001 (one copy of *XYL1*)	0.25 ± 0.05	0.13 ± 0.04
TMB3260 (two copies of *XYL1*)	1.41 ± 0.25	1.23 ± 0.46
TMB3270 (one copy of *XYL1* (K270M))	1.04 ± 0.24	0.54 ± 0.14
TMB3271 (two copies of *XYL1* (K270M))	11.03 ± 1.51	4.72 ± 0.99

The cells were grown aerobically in defined mineral medium with 20 g/L glucose as carbon source and were harvested during the exponential phase.

Effect of Enhanced Expression of *XYL1* K270M on Xylose Fermentation

Product formation in batch (Table III) and continuous cultivation (Table IV) supported the hypothesis that the increased K_M value for NADPH of the mutated XR reduced xylitol formation. However, the lower xylose consumption rate of strain TMB3270 (Table IV) suggested that the low NADH-dependent XR activity limited xylose metabolism.

XR activity has previously been shown to limit xylose consumption in TMB3001 (Jeppsson et al., 2003b). In order to check whether this statement also hold for the mutated XR, another copy of the mutated *XYL1* (K270M) gene was integrated into TMB3270, resulting in TMB3271. The high-XR-activity strain TMB3260 which harbors two copies of the native *XYL1* gene together with the *XYL2* and *XKS1* genes (Jeppsson et al., 2003b) was used as a control strain for TMB3271.

The NADPH- and NADH-dependent XR activities in TMB3271 were 11.03 and 4.72 U/mg, respectively, which is 9–11 times higher than for TMB3270 (Table II). TMB3260 exhibited NADPH- and NADH-dependent activities of 1.41 and 1.23 U/mg, respectively, which is six to nine times higher than for TMB3001 (Table II). The NADPH/NADH ratio was higher for TMB3271 carrying the mutated XR than for TMB3260 (2.3 vs. 1.1), however, these activity ratios were calculated from values with a large standard deviation (Table II).

Xylose consumption and product formation with TMB3271 (two copies of *XYL1* (K270M)) were compared with TMB3270 (one copy of *XYL1* (K270M)), TMB3001 (one copy of *XYL1*), and TMB3260 (two copies of *XYL1*) in oxygen-limited batch fermentation using resting cells (Table III). Similar results were obtained for strains TMB3260 and TMB3271, expressing high levels of *XYL1* and *XYL1* (K270M), respectively. Compared with TMB3001 both strains had higher xylose consumption rate, decreased xylitol yield, enhanced glycerol and acetate yields and similar ethanol yield.

Product formation was also compared in anaerobic continuous culture with 10 g/L glucose and 10 g/L xylose as carbon sources (Table IV). The enhanced XR activity resulted in 58% and 220% higher xylose uptake in TMB3260 and TMB3271 (*XYL1* K270M), respectively, compared with their respective low-XR-activity strains TMB3001 and TMB3270 (*XYL1* K270M). The K270M mutation in the high XR-expressing strain TMB3271 resulted in higher

Table III. Effect of reduced XR affinity for NADPH on xylose fermentation by recombinant *S. cerevisiae* in oxygen-limited batch cultivation with 50 g/L xylose as carbon source.

Strain	Xylose consumption rate	$Y_{ethanol}$	$Y_{xylitol}$	$Y_{acetate}$	$Y_{glycerol}$	$C_{recovery}$
TMB3001[a] (one copy of *XYL1*)	0.145 ± 0.002	0.31 ± 0.01	0.29 ± 0.01	0.025 ± 0.001	0.052 ± 0.004	0.98
TMB3260[b] (two copies of *XYL1*)	0.245 ± 0.005	0.30 ± 0.01	0.13 ± 0.01	0.046 ± 0.007	0.161 ± 0.001	0.95
TMB3270 (one copy of *XYL1* (K270M))	0.155 ± 0.005	0.36 ± 0.01	0.17 ± 0.01	0.035 ± 0.001	0.072 ± 0.005	0.99
TMB3271 (two copies of *XYL1* (K270M))	0.226 ± 0.020	0.31 ± 0.01	0.09 ± 0.01	0.060 ± 0.008	0.181 ± 0.014	0.97

Specific xylose consumption rate (gram per gram of biomass per hour), ethanol, xylitol, acetate, and glycerol yields (gram per gram of consumed xylose) and carbon recoveries (C_{moles} out per (C_{moles} in)) were determined after 60–70 h with 2–3 g per L cells. The values given are the average of two independent fermentation experiments ± the deviation from the average. In the calculation of carbon recovery, 0.5 moles of CO_2 were assumed to be formed for each C_{mole} of ethanol or acetate produced.

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

[b]Fermentation data from Jeppsson et al. (2003b).

ethanol and CO_2 yields (0.40 and 0.40 g/g) than with the control high XR-expressing strain TMB3260 (0.36 and 0.39 g/g), which agreed with the enhanced ethanol and CO_2 yields found at low XR activity in TMB3270 compared to TMB3001 (Table IV). The xylitol yield was lower in TMB3271 (0.44 g xylitol per gram of xylose) than in TMB3260 (0.58 g xylitol per gram of xylose). The biomass formation was similar in TMB3260 and TMB3271 (1.1 and 1.2 g/L, respectively). However, the biomass yield was higher in TMB3271 (0.098 g per gram of substrate) than in TMB3260 (0.085 g per gram of substrate) as a result of a reduced glucose consumption rate. TMB3271 showed a twofold decrease in glycerol yield and a slight decrease in acetate yield compared with TMB3260, different to what was observed in non-growing conditions (Table III).

Flux Analysis

Since no obvious relationship could be made between the in vitro XR activity measurement and the physiology of the recombinant yeast under fermentation conditions, a stoichiometric flux model (Wahlbom et al., 2001) was used to elucidate the cofactor utilization by XR in strains harboring native *XYL1* (TMB3001 and TMB3260) and mutated *XYL1* K270M (TMB3270 and TMB3271) during anaerobic chemostat cultivation. Strains expressing mutated XR utilized a greater fraction of NADH in the XR reaction than strains expressing native XR (Fig. 1). According to the model, the XR reaction in TMB3001 utilized 47% NADH, while the XR reaction in TMB3270 (K270M) used only NADH for the reduction. In the strains with higher XR activity, TMB3260 and TMB3271 (*XYL1* K270M), 36 and 86% NADH, respectively, was used in the XR step. The oxidative pentose phosphate pathway is a major source of NADPH in yeast (Lagunas and Gancedo, 1973), and more glucose was channeled through this pathway in TMB3001 (15%) and TMB3260 (22%) than in the mutant-carrying TMB3270 (7%) and TMB3271 (12%). As a result less carbon dioxide was formed in the mutant-carrying strains.

It has previously been shown that XR reduces dihydroxyacetone phosphate (DHAP) with NADPH (Jeppsson et al., 2003b). The flux model, however, assumes strictly NADH-dependent DHAP reduction. When the model reaction was changed to 50% NADH and 50% NADPH for the reduction of DHAP, the NADH usage in the XR reaction increased for all strains, and it was higher in the strains carrying the mutated XR (data not shown). Strains carrying the mutated XR (TMB3270 and TMB3271) also showed a lower oxidative PPP flux in the modified model.

DISCUSSION

Among natural xylose-utilizing yeasts, only those harboring an NADH-linked XR activity display significant alcoholic

Table IV. Continuous cultivation of TMB3001, TMB3270, TMB3260, and TMB3271 under anaerobic conditions at a dilution rate of 0.06/h with 10 g/L glucose and 10 g/L xylose as carbon-source.

Strains	Glucose consumption rate	Xylose consumption rate	$Y_{ethanol}$	$Y_{xylitol}$	$Y_{xylitol}$[a]	$Y_{acetate}$	$Y_{glycerol}$	Y_{CO_2}	$Y_{biomass}$	$C_{recovery}$
TMB3001 (one copy of *XYL1*)	0.52	0.12	0.37	0.10	0.52	0.001	0.095	0.43	0.094	1.08
TMB3260 (two copies of *XYL1*)	0.51	0.19	0.36	0.16	0.58	0.004	0.107	0.39	0.085	1.09
TMB3270 (one copy of *XYL1* (K270M))	0.58	0.05	0.40	0.02	0.31	0.002	0.084	0.48	0.095	1.07
TMB3271 (two copies of *XYL1* (K270M))	0.45	0.16	0.40	0.12	0.44	0.003	0.053	0.40	0.098	1.07

Consumption rates (gram per gram of biomass per hour), yields Y (gram per gram of consumed substrate), and $C_{recovery}$ are displayed. Steady state was assumed after at least six bioreactor volumes had passed. The presented data are the average values from two steady-states with less than 5% deviation.

[a]Xylitol yield on xylose.

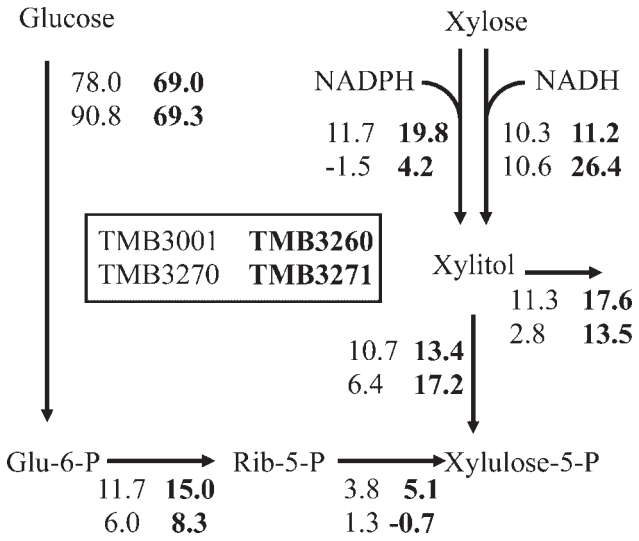

Figure 1. Simplified scheme showing the fluxes in the xylose pathway (mmol per g biomass/h) for TMB3001 (upper value, lean-faced type), TMB3270 (lower value, lean-faced type), TMB3260 (upper value, bold), and TMB3271 (lower value, bold) cultivated in continuous culture with 10 g/L glucose and 10 g/L xylose. Sugar uptake (glucose + xylose) was normalized to 100 mmol per g biomass/h.

fermentation (Bruinenberg et al., 1984). However, so far no strictly NADH-dependent XR has been reported. *Candida utilis* XR utilizes exclusively NADPH, while *C. shehatae*, *P. segobiensis*, *P. stipitis*, and *Pachysolen tannophilus* XRs utilize both NADPH and NADH in the reduction of xylose to xylitol (Bruinenberg et al., 1984). Because of its dual cofactor utilization, XR from *P. stipitis* has been preferred for the construction of xylose-fermenting recombinant *S. cerevisiae* strains (Eliasson et al., 2000; Ho et al., 1998; Kötter and Ciriacy, 1993; Tantirungkij et al., 1993), although the recently cloned XR gene from *C. parapsilosis* could be a better choice due to its putative preference for NADH (Lee et al., 2003).

In the present study, we investigated the effect of expressing a mutated *P. stipitis* XR with an enhanced K_M for NADPH, with the hypothesis that this would shift product formation from xylitol to ethanol in recombinant *S. cerevisiae*. The overproduction of the mutated XR in recombinant *S. cerevisiae* is, to the best of our knowledge, the first successful construction using a mutated enzyme to shift the anaerobic product formation from xylitol to ethanol. Indeed, strains harboring the mutated XR showed increased ethanol and CO_2 yields at the expense of reduced xylitol and glycerol yields, compared with strains harboring native *P. stipitis* XR.

Although only one extra copy of the native and mutated XR gene should have been integrated in TMB3260 and TMB3271, respectively, a 6- to 11-fold increase in activity was observed in both cases. The native *XYL1* gene was expressed with the *ADH1* promoter and the second copy was expressed with the strong and constitutive *PGK*1 promoter, which could explain the more than twofold increase in activity. However, a similar trend was found for the mutated XR gene for which both copies were expressed under the same promoter (*PGK1*). This suggests that more than one extra-copy has been integrated and that strains with multiple integration have been selected after transformation due to the high selective pressure from the antibiotic.

The introduction of the K270M mutation in the XR encoding gene induced a small but significant change in product distribution in favor of ethanol, despite the fact that the mutated XR displayed a higher in vitro NADPH- to NADH-activity ratio than the native XR. Previous characterization of the K270M mutant focused on the affinity change for the cofactors and xylose (Kostrzynska et al., 1998), however, no information on the change in catalytic efficiency was available. A thorough characterization of the kinetics of a *C. tenuis* XR mutant carrying the same mutation indicated that the catalytic constant (k_{cat}) was increased in the mutant with both NADH (19 vs. 11/s in the control) and NADPH (36 vs. 13/s in the control) as cofactors, with the fold change being significantly higher for NADPH (2.8) than for NADH (1.7) (Petschacher et al., 2004). This result may suggest that the K270M mutation has not only reduced the in vitro affinity for NADPH but may also have enhanced the catalytic rate of the *P. stipitis* mutated XR since product formation and flux analysis demonstrates increased utilization of NADH by the K270M mutant. The metabolic flux model (Wahlbom et al., 2001) that was used to estimate the relative in vivo NADPH- and NADH-fluxes for the native and the mutated XR, respectively, clearly showed that strains harboring the mutated XR used a larger fraction of NADH for the reduction of xylose. When grown anaerobically on a mixture of glucose and xylose, the strain overproducing the mutated XR yielded more biomass per consumed sugar and significantly less glycerol. Biomass formation is accompanied by NADH formation which, in the absence of oxygen, is oxidized by glycerol formation (Albers et al., 1996). In the recombinant strain carrying an additional copy of the mutated XR, the flux model showed that more NAD^+ was produced during xylose reduction, which could be translated into decreased glycerol formation and increased biomass formation.

Some discrepancies were observed between the xylose batch cultivation with resting cells and the glucose–xylose continuous cultivations, notably for the ethanol yield with the strains having high level of XR. A more or less pronounced effect of XR mutation on product distribution may be resulting from altered intracellular levels of NADH and NADPH related to the cultivation conditions. Indeed the presence of glucose should enable increased production of NADPH via the PPP whereas cell growth should lead to the generation of excess NADH and to increased NADPH consumption.

Xylitol production has been ascribed to the difference in cofactor usage in the NAD(P)H-dependent XR and the NAD^+-dependent XDH reactions (Kötter and Ciriacy, 1993). Instead of engineering the XR enzyme, previous metabolic engineering strategies have targeted the production and consumption of NADPH/$NADP^+$ and NADH/NAD^+. In Table V, the results of these investigations have been summarized also taking into account strain background, mode of

Table V. Xylose consumption rate (gram per gram of biomass per hour), ethanol yield (gram per gram of sugar) and xylitol yield (gram per gram of xylose) in fermentation with *S. cerevisiae* strains engineered to enhance ethanol yield from xylose.

Strain	Relevant genotype/phenotype	O_2	Mode	D	Glu	Xyl	Xylose consumption rate	$Y_{ethanol}$	$Y_{ethanol}$ (% increase)	$Y_{xylitol}$	Reference strain	Reference ferm. data
TMB3001	1 × *XYL1*	–	C	0.06	10	10	0.12	0.37		0.52	Eliasson et al. (2000)	This work
TMB3260	2 × *XYL1*	–	C	0.06	10	10	0.19	0.36		0.58	Jeppsson et al. (2003b)	This work
TMB3270	1 × *XYL1* (K270M)	–	C	0.06	10	10	0.05	0.40	8	0.31	This work	This work
TMB3271	2 × *XYL1* (K270M)	–	C	0.06	10	10	0.16	0.40	11	0.44	This work	This work
TMB3001		–	C	0.06	20	20	0.23	0.30		0.43	Eliasson et al. (2000)	Jeppsson et al. (2002)
TMB3255	*Δzwf1*	–	C	0.06	20	20	0.12	0.39	30	0.13	Jeppsson et al. (2002)	Jeppsson et al. (2002)
TMB3001		+/–	B	—	-	50	0.15	0.31		0.29	Eliasson et al. (2000)	Jeppsson et al. (2002)
TMB3255	*Δzwf1*	+/–	B	—	-	50	0.02	0.41	32	0.05	Jeppsson et al. (2002)	Jeppsson et al. (2002)
TMB3253	Control	+/–	B	—	-	50	0.16	0.28		0.34	Jeppsson et al. (2003a)	Jeppsson et al. (2003a)
TMB3254	TH	+/–	B	—	-	50	0.16	0.28		0.30	Jeppsson et al. (2003a)	Jeppsson et al. (2003a)
H2674	Control	–	B	—	-	50	0.07[a]	0.14		0.56	Verho et al. (2003)	Verho et al. (2003)
H2673	*GDP1*	–	B	—	-	50	0.06[a]	0.17	25	0.49	Verho et al. (2003)	Verho et al. (2003)
H2684	*GDP1 Δzwf1*	–	B	—	-	50	0.06[a]	0.31	127	0.35	Verho et al. (2003)	Verho et al. (2003)
TMB3001		–	C	0.05	20	50	0.31	0.28		0.56	Eliasson et al. (2000)	Roca et al. (2003)
CBP·CR4	*Δgdh1* GDH2	–	C	0.05	20	50	0.31	0.34	19	0.47	Roca et al. (2003)	Roca et al. (2003)
CBP·CR5	*Δgdh1* GS-GOGAT	–	C	0.05	20	50	0.34	0.33	16	0.44	Roca et al. (2003)	Roca et al. (2003)

The relevant genotype and phenotype are given together with oxygenation, O_2 (anaerobic (–), oxygen limited (+/–)), fermentation mode (continuous cultivation (C), batch cultivation (B)), dilution rate, D in per hour, and initial glucose (Glu) and xylose (Xyl) concentrations in g/L.

[a]Calculated using 120 h as cultivation time (only approximate cultivation time was reported by Verho et al. (2003) and the initial biomass.

cultivation including co-substrate and oxygenation. When the *ZWF1* gene encoding the oxidative PPP enzyme glucose 6-phosphate dehydrogenase (G6PDH) was disrupted (TMB3255) to reduce the intracellular NADPH pool, the ethanol yield increased by 32% (Jeppsson et al., 2002). On the other hand, when a transhydrogenase from *Azotobacter vinelandii*, which in *S. cerevisiae* converts NAD^+ and NADPH into NADH and $NADP^+$ (Nissen et al., 2001), was overexpressed (TMB3254) xylitol formation decreased by 12%, however, not for the benefit of improved ethanol formation (Jeppsson et al., 2003a).

The overproduction of a $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in strain H2673 increased ethanol formation by 25% (Verho et al., 2003). When used in combination with the deletion of *G6PDH* gene (strain H2684), the ethanol formation increased by 127%, resulting in an ethanol yield of 0.31 g per gram of sugar (Verho et al., 2003). The ammonium assimilation has been metabolically engineered to reduce NADPH consumption by deleting the *GDH1* gene, encoding an NADPH-dependent glutamate dehydrogenase (Roca et al., 2003). In the same strain, CBP · CR4, the *GDH2* gene, encoding a NADH-dependent glutamate dehydrogenase was overexpressed, which resulted in 19% increased ethanol yield (Table V) (Roca et al., 2003). The overexpression of *GLT1* and *GLN1* genes, encoding the NADH and ATP requiring GS-GOGAT complex, in a *GDH1*-deleted strain CBP.CR5 increased the ethanol yield by 16% (Roca et al., 2003).

The *ZWF1* deletion and the expression of the mutated XR gene enhanced the ethanol yield at the expense of a significantly reduced xylose consumption rate (Table V). However, the xylose consumption rate could be restored by fine-tuning the G6PDH activity (Jeppsson et al., 2003a) and by producing the mutated XR at a higher level (strain TMB3271), respectively (Table V).

High ethanol yields (0.40 g per gram of sugar) from a glucose–xylose mixture were obtained with the newly developed strains, TMB3270 and TMB3271, in which the mutated XR was produced (Table V). Thus the overproduction of the mutated XR with reduced affinity for NADPH is one of the hitherto most successful examples of strain engineering for ethanol production from xylose. The strain comparison in Table V summarizes the difficulty in manipulating the redox metabolism to enhance the ethanol yield and the ethanol productivity, and it raises the question of the flexibility limit of the central metabolic pathways in *S. cerevisiae*.

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