

Boost in Bioethanol Production using Recombinant *Saccharomyces cerevisiae* with Mutated Strictly NADPH-Dependent Xylose Reductase and NADP<sup>+</sup>-Dependent Xylitol Dehydrogenase

Sadat Mohammad Reza Khattab<sup>a</sup>, Masayuki Saimura<sup>b</sup>, and Tsutomu Kodaki<sup>b,\*</sup>

<sup>a</sup>Faculty of Science, Al-Azhar University, Assiut Branch, Assiut, 71524, Egypt

<sup>b</sup>Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

The xylose-fermenting recombinant *Saccharomyces cerevisiae* and its improvement have been studied extensively. The redox balance between xylose reductase (XR) and xylitol dehydrogenase (XDH) is thought to be an important factor in effective xylose fermentation. Using protein engineering, we previously successfully reduced xylitol accumulation and improved ethanol production by reversing the dependency of XDH from NAD<sup>+</sup> to NADP<sup>+</sup>. We also constructed a set of novel strictly NADPH-dependent XR from *Pichia stipitis* by site-directed mutagenesis. In the present study, we constructed a set of recombinant *S. cerevisiae* carrying a novel set of mutated strictly NADPH-dependent XR and NADP<sup>+</sup>-dependent XDH genes with overexpression of endogenous xylulokinase (XK) to study the effects of complete NADPH/NADP<sup>+</sup> recycling on ethanol fermentation and xylitol accumulation. All mutated strains demonstrated reduced xylitol accumulation, ranging 34.4%–54.7% compared with the control strain. Moreover, compared with the control strain, the two strains showed 20% and 10% improvement in ethanol production.

**Keywords:** Xylose fermentation, Xylose reductase, Xylitol dehydrogenase, Coenzyme specificity, Ethanol production

\* Corresponding author, Tel: +81-774-38-3510, Fax: +81-774-38-3499

e-mail address: kodaki@iae.kyoto-u.ac.jp

**Abbreviations:** XR: xylose reductase; XDH: xylitol dehydrogenase; XK: xylulokinase; PGK: phosphoglycerate kinase; WT: wild type; *PsXR*: xylose reductase from *Pichia stipitis*; *PsXDH*: xylitol dehydrogenase from *Pichia stipitis*

Environmental concerns and depletion of fossil oil along with increasing demand for energy for transportation, heating, and industrial processes have prompted a search for sustainable and renewable substances for energy generation; consequently, reducing greenhouse-gas emission and subsequent the global warming. Biofuel is a biotechnology product that is attracting research attention. Use of suitable microorganisms with cellulosic biomass has the potential to meet the demand for liquid fuel (Hahn-Hägerdal et al., 2007; Rubin, 2008).

Xylose is one of the main fermentable sugars present in lignocellulosic biomass, and the second most abundant carbohydrate in nature after glucose. Complete fermentation of glucose and xylose are economically valuable. *Saccharomyces cerevisiae* is the top fermenting microorganism, although it cannot ferment xylose natively. Recombinant *S. cerevisiae* acquires the ability to ferment xylose via transformed xylose reductase (XR) and xylitol dehydrogenase (XDH) (Jeffries and Jin, 2004). However, xylitol accumulation by recombinant *S. cerevisiae* during xylose fermentation is an obstacle to industrial-scale application. The redox imbalance between XR and XDH, where the wild type (WT) XR uses both NADPH and NADH in preference to NADPH, and WT XDH uses only NAD<sup>+</sup>, is thought to be one of the main reasons for xylitol accumulation. Many attempts have been made to overcome this problem by several NADH-preferring XR mutants from *Candida tenuis* (Kavanagh et al., 2002; Kavanagh et al., 2003; Leitgeb et al., 2005; Petschacher and Nidetzky, 2005; Petschacher et al., 2005) and *Pichia stipitis* (Watanabe et al., 2007a, 2007b; Lee et al., 2012).

As an alternative strategy, we reversed *P. stipitis* XDH (*PsXDH*) specificity from NAD<sup>+</sup>-dependent to NADP<sup>+</sup>-dependent (Watanabe et al., 2005). In addition to an 86% reduction in unfavorable xylitol accumulation, a recombinant *S. cerevisiae* harboring mutated *PsXDH* combined with WT XR showed a 41% improvement in bioethanol production (Watanabe et al., 2007c). Furthermore, a recombinant *S. cerevisiae* was constructed by chromosomally integrating WT *PsXR* gene and mutated NADP<sup>+</sup>-dependent *PsXDH* gene with endogenous xylulokinase (XK) gene overexpression (Matsushika and Sawayama, 2008; Matsushika et al., 2008). This recombinant strain demonstrated a 28% reduction in xylitol accumulation and a 15% increase in

ethanol production compared with the control strain. Nevertheless, these strains may retain some redox imbalance because of the use of both NADH and NADPH cofactors of *PsXR*, with preference for the latter. Therefore, strictly NADPH-dependent XR with mutated strict NADP<sup>+</sup>-dependent XDH may be more effective in increasing bioethanol production and decreasing xylitol accumulation.

Site-directed mutagenesis was applied to construct a novel strictly NADPH-dependent XR from *P. stipitis* (Khattab et al., 2011a, 2011b). Three constructed double mutants E223A/S271A (AA), E223D/S271A (DA), and E223G/S271A (GA) demonstrated strict NADPH dependency without any significant loss of enzyme activity.

In this study, we constructed chromosomally integrated recombinant *S. cerevisiae* strains harboring WT *PsXR*, or mutated strictly NADPH-dependent *PsXR*, AA, DA, and GA (Khattab et al., 2011a, 2011b) with a strictly NADP<sup>+</sup>-dependent *PsXDH* (Watanabe et al., 2005, ARSdR) and overexpression of endogenous XK. Subsequently, we examined the effects of efficient NADPH/NADP<sup>+</sup> recycling between XR and XDH for xylose fermentation and bioethanol production. We constructed a set of chromosomally integrated recombinant *S. cerevisiae* of xylose-fermenting yeast strains (SK-N2, SK-N3, and SK-N4) containing mutated NADPH-dependent *PsXR* and mutated NADP<sup>+</sup>-dependent *PsXDH*, and a control strain SK-N1 containing WT *PsXR* and mutated NADP<sup>+</sup>-dependent *PsXDH* (Table 1).

Expression of XR, XDH, and XK activities in these cells was determined (Table 2). XR in SK-N2, SK-N3, and SK-N4 showed much higher activities with NADPH compared with NADH, while XR in SK-N1 showed activities with both NADPH and NADH preferred to NADPH. These characteristics of XR activities were consistent with those of mutated XR and WT XR expressed in *E. coli* (Khattab et al., 2011a, 2011b) with little increase in DA XR activity of the recombinant strain. XDH activities in all four strains exhibited almost the same activity showing NADP<sup>+</sup> dependency; furthermore, XK activities were almost identical in the four strains.

Xylose and glucose fermentation abilities of these strains were measured in a medium with 20 g/l glucose and 55 g/l xylose at initial fermentation time. Because a practical sugar

solution obtained from biomass usually contains both glucose and xylose, we focused on a fermentation process employing a sugar mixture of glucose and xylose. Glucose was consumed within the first 6 h at almost the same rate in all strains (Fig. 1a). Because *S. cerevisiae* has a native mechanism for glucose consumption and fermentation, all four strains attained a glucose consumption rate of 3.6 g/h.

Xylose consumption rates varied in the recombinant strains. SK-N1 (WT XR) consumed 74.1% and 97.5% of xylose after 48 h and 96 h, respectively, with 0.55 g/h xylose consumption rate (Fig. 1a). SK-N2 (AA XR) consumed 64.2% and 90.5% of xylose after 48 h and 96 h, respectively, with 0.52 g/h xylose consumption rate (Fig. 1a). SK-N3 (DA XR) consumed 74.7% and 96.3% of xylose after 48 h and 96 h, respectively, with 0.55 g/h xylose consumption rate (Fig. 1a). SK-N4 (GA XR) showed the lowest xylose consumption rate of 0.38 g/h, where 40% and 66.1% xylose consumption occurred after 48 h and 96 h, respectively (Fig. 1a). These consumption rates in SK-N1, SK-N2, SK-N3, and SK-N4 may reflect in both the  $K_m$  of WT XR, AA XR, DA XR, and GA XR for xylose and the total enzyme activities with NADPH and NADH. These enzymes showed  $K_m$  of 97.1, 226, 108, and 270 mM for xylose (Khattab et al., 2011a, 2011b), and 2.07, 1.59, 2.79, and 1.17 U/mg protein for the total enzyme activities (Table 2). The effects of lower  $K_m$  of WT XR in SK-N1 for xylose and higher total enzyme activity of DA XR in SK-N3 probably resulted in almost the same xylose consumption rate between them. The slow consumption rate of SK-N4 probably resulted from the three-fold higher  $K_m$  of GA XR for xylose compared with WT XR (Khattab et al., 2011b), in addition to the lower total enzyme activity of GA XR expressed in SK-N4 (57% of WT XR in SK-N1) as shown in Table 2.

This study aims to assess the effects of a mutated pair of strictly NADPH-dependent XR and NADP<sup>+</sup>-dependent XDH to reduce xylitol excretion and consequently improve ethanol production. At 13.3% of xylose, xylitol was accumulated in SK-N1, whereas SK-N2, SK-N3, and SK-N4 showed 46.1%, 34.4%, and 54.7% reductions in xylitol accumulation, respectively, compared with SK-N1 (Fig. 1b). Although SK-N1 and SK-N3 consumed xylose at almost the same rate, SK-N3 exhibited lower xylitol accumulation, probably reflecting the effective

NADPH/NADP<sup>+</sup> recycling. The mutated XR and XDH in SK-N3 use only NADPH and NADP<sup>+</sup>, whereas WT XR in SK-N1 uses both NADPH and NADH, and mutated XDH (ARSdR) in SK-N1 uses only NADP<sup>+</sup>. For ethanol production, SK-N1 produced 34.2% of total consumed mixed glucose and xylose sugars, representing 67% of the theoretical yield with 0.15 g/h ethanol production rate (Fig. 1c). SK-N2 showed a 20% improvement in ethanol production where 30.1 g/l were produced with 0.41 g/g ethanol of total consumed sugars (80% of the theoretical yield). In addition, this improvement can be seen in 0.18 g/h production rate (Fig. 1c). SK-N3 produced ethanol at 25.8 g/l with a yield of 35% of total consumed sugars (glucose and xylose), representing 68.2% of the theoretical yield (Fig.1c). SK-N4 showed 10% improvement in ethanol production with a relatively slow xylose consumption rate, producing 27.9 g/l, representing 0.41 g/g of the total consumed sugars (80% of the theoretical yield) (Fig. 1c). This improvement in ethanol production by 20% and 10% for SK-N2 and SK-N4, respectively, may have resulted from efficient NADPH/NADP<sup>+</sup> recycling and diminished xylitol excretion. Contrary to our expectation, SK-N3 showed only a 1.8% improvement in ethanol production compared with SK-N1. Considering that NADPH-dependent XR activity in SK-N3 was approximately two times higher compared with SK-N2 and SK-N4, the shortage of cofactors may be a factor affecting the metabolic activity for ethanol production. Further investigation is needed to clarify this point, for example by increasing the NADPH pool (Verho et al., 2003; Bera et al., 2011).

Glycerol accumulation in the fermentation medium amounted to 4.8, 6.24, 5.68, and 5.99 g/l for SK-N1, SK-N2, SK-N3, and SK-N4, respectively (Fig. 1d). Acetic acid also accumulated to 1.37, 0.91, 1.35, and 0.77 g/l for SK-N1, SK-N2, SK-N3, and SK-N4, respectively (Fig. 1d). Glycerol slightly increased in these strains, while acetic acid slightly decreased in SK-N2 and SK-N4. A combination of the previous strategies, such as reducing glycerol accumulation by deletion of the glycerol 3-phosphate dehydrogenase gene (Michnick et al., 1997), may be needed to improve ethanol production by preventing glycerol accumulation.

In our previous work, we reported the development of a xylose-utilizing *S. cerevisiae* strain by reversing the coenzyme specificity of WT *Ps*XDH from NAD<sup>+</sup> to NADP<sup>+</sup> for compatibility with WT *Ps*XR (Watanabe et al., 2007b, 2007c; Matsushika and Sawayama, 2008; Matsushika et al., 2008). In this study, using the complete NADPH-dependent *Ps*XR, xylitol accumulation was further reduced in SK-N2, SK-N3, and SK-N4 by 46.1%, 34.4%, and 54.7% over SK-N1, respectively. In addition, SK-N2 and SK-N4 showed improvements in ethanol production by 20% and 10%, respectively. To our knowledge, this is the first report describing the exclusive use of NADPH/NADP<sup>+</sup> cofactor between a matched pair of mutated *Ps*XR and *Ps*XDH with overexpression of XK for xylose fermentation.

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Table 1

Characteristics of recombinant *S. cerevisiae* strains and plasmids used in this study. A plasmid pPGK harboring WT *PsXR* (Watanabe et al., 2007b) was used as a template for constructing three double mutants E223A/S271A (AA), E223D/S271A (DA), and E223G/S271A (GA) with phosphoglycerate kinase (PGK) expression promoter and terminator by site-directed mutagenesis. Details of the codons used for mutations, methods, electroporation, and DNA sequencing are previously described (Khattab et al., 2011a, 2011b). Two *SalI* restriction sites in the *PsXR* gene were eliminated by single-round PCR with the following two primers:  $\Delta$ SalI-S (5'-GTTGGAAAGTTGACGTGACACCTGTTC-3') and  $\Delta$ SalI-AS (5'-GAACAGGTGTCACGTCACTTTCCAAC-3') (underlining indicates mutated regions). The substitutions of *SalI* sites in the *PsXR* gene produced the same amino acid residues. Subsequently, approximately 2.0 kbp DNA fragment comprising *SalI* site-eliminated WT *PsXR* or *SalI* site-eliminated double mutants, AA, DA, or GA with the PGK expression promoter and terminator were excised from the plasmids using restriction enzymes *XhoI* and *SalI*. These *SalI*-*XhoI* fragments composed of the WT XR or mutated, AA XR, DA XR, and GA XR coding region with PGK promoter and terminator were introduced to the *SalI* site of a plasmid, which was a shuttle vector based on pAUR101 (Takara Bio) and harbored mutated *PsXDH* (ARSdR) and endogenous XK (Matsushika et al., 2008). The constructed plasmids were named pAUR-WTXR-ARSdR-XK, pAUR-AAXR-ARSdR-XK, pAUR-DAXR-ARSdR-XK, and pAUR-GAXR-ARSdR-XK. pAUR101 shuttle vector (Takara Bio) is a chromosomal integration vector for *S. cerevisiae* containing an aureobasidin resistance gene (*AUR1-C*). Yeast transformation with plasmids pAUR-WTXR-ARSdR-XK, pAUR-AAXR-ARSdR-XK, pAUR-DAXR-ARSdR-XK, and pAUR-GAXR-ARSdR-XK was carried out with the Fast Yeast Transformation™ kit (Takara Bio). Those plasmids, linearized with restriction enzyme *BsiWI*, were chromosomally integrated into the *aur1* locus of D452-2 to construct SK-N1, SK-N2, SK-N3, and SK-N4, respectively.

(Continued on the next page)

(Table 1 continued)

Strain/plasmid	Relevant genotype	Source or reference
<i>S. cerevisiae</i> strains		
D452-2	<i>MATa leu2 his3 ura3 can1</i>	(Hosaka et al., 1992)
SK-N1	D452-2, <i>AUR1: [PGKp-WTXR-PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]</i>	This study
SK-N2	D452-2, <i>AUR1: [PGKp-AAXR-PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]</i>	This study
SK-N3	D452-2, <i>AUR1: [PGKp-DAXR-PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]</i>	This study
SK-N4	D452-2, <i>AUR1: [PGKp-GAXR-PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]</i>	This study
Plasmids		
pAUR-ARSdR-XK	AUR1-C expression of mutated XDH and XK genes	(Matsushika et al., 2008)
pAUR-WTXR-ARSdR-XK	AUR1-C expression of WTXR, mutated XDH, and XK genes	This study
pAUR-AAXR-ARSdR-XK	AUR1-C expression of mutated AAXR, mutated XDH, and XK genes	This study
pAUR-DAXR-ARSdR-XK	AUR1-C expression of mutated DAXR, mutated XDH, and XK genes	This study
pAUR-GAXR-ARSdR-XK	AUR1-C expression of mutated GAXR, mutated XDH, and XK genes	This study

Table 2

Specific enzyme activities of XR, XDH, and XK in cell-free extracts of recombinant yeast strains. The recombinant *S. cerevisiae* strains were cultivated at 30°C with shaking at 240 rpm for 24 h in 10-ml YPD medium (10 g yeast extract, 20 g peptone, and 20 g glucose in 1 L) in a closed 50-ml Falcon tube. The cell pellets were harvested by centrifugation at  $6,000 \times g$  for 5 min at 4°C. Subsequently, the cell pellets were resuspended in 100 mM sodium phosphate (pH 7.5), containing 1 mM MgCl<sub>2</sub> and 10 mM 2-mercaptoethanol and vortexed with glass beads. Cell-free extracts were separated from the beads by centrifugation and used for enzyme measurements. Protein concentrations were determined using the Bio-RAD Quick Start Bradford 1× dye reagent (Bio-Rad Laboratories) by measuring absorbance at 595 nm with  $\gamma$ -globulin as a standard. Enzyme activities of XR, XDH, and XK were determined with freshly prepared cell-free extracts as previously described (Watanabe et al., 2007c; Matsushika et al., 2008).

Strain	Enzyme activity (U/mg protein)				
	XR		XDH		XK
	NADPH	NADH	NADP <sup>+</sup>	NAD <sup>+</sup>	
SK-N1	1.41	0.66	14.11	0.97	2.42
SK-N2	1.50	0.09	14.88	1.04	2.45
SK-N3	2.52	0.27	14.81	0.65	2.63
SK-N4	1.11	0.06	15.29	0.63	2.54

The given values are the averages of three independent experiments.

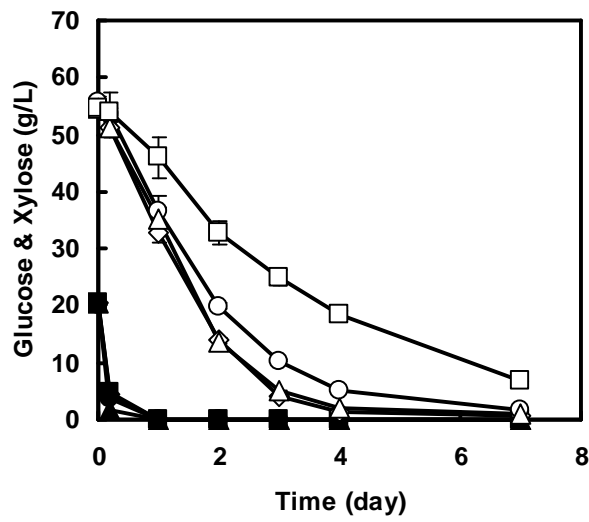
1 U = 1  $\mu$ mol of NADH or NADPH was produced or consumed in 1 min.

## Legends to figures

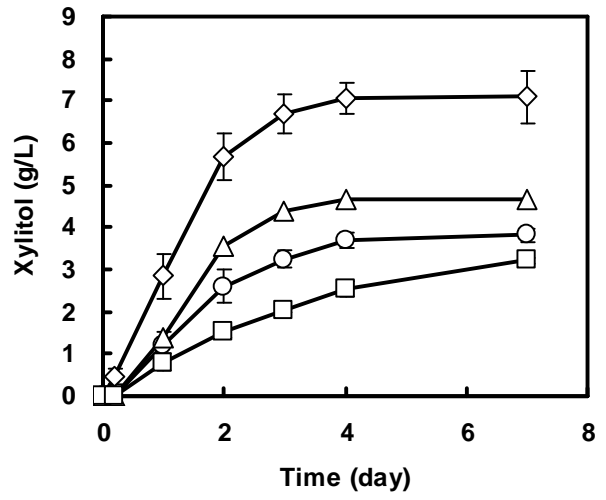
Fig. 1. Time course of fermentation by recombinant SK-N1, SK-N2, SK-N3, and SK-N4 strains. The recombinant *S. cerevisiae* strains were first cultivated at 30°C with shaking at 240 rpm for 65 h in two baffle flasks each containing 500 ml YPD medium. The cells were harvested by centrifugation at  $6,000 \times g$  for 5 min at 4°C and washed with sterile water. The collected cells were cultivated at 30°C in 1,000-ml YPD medium supplemented with 55 g/l xylose with a high-performance bioreactor (BioFlo 110, New Brunswick Scientific Co.). The cell growth was monitored spectrophotometrically (U-2001, Hitachi, Japan) at the absorbance of 600 nm revealing almost the same growth rate (data not shown). Samples of the fermentation culture were withdrawn at regular intervals, and concentration of glucose, xylose, xylitol, glycerol, acetic acid, and ethanol were determined using high performance liquid chromatography as described previously (Matsushika et al., 2008). (A) glucose (closed symbols) and xylose (open symbols) consumption; (B) xylitol production; (C) ethanol production; (D) glycerol (open symbols) and acetic acid (closed symbols) production. Symbols: rhomboid (SK-N1), circle (SK-N2), triangle (SK-N3), and square (SK-N4). Values represent means  $\pm$  SD,  $n = 3$ .

Figure 1

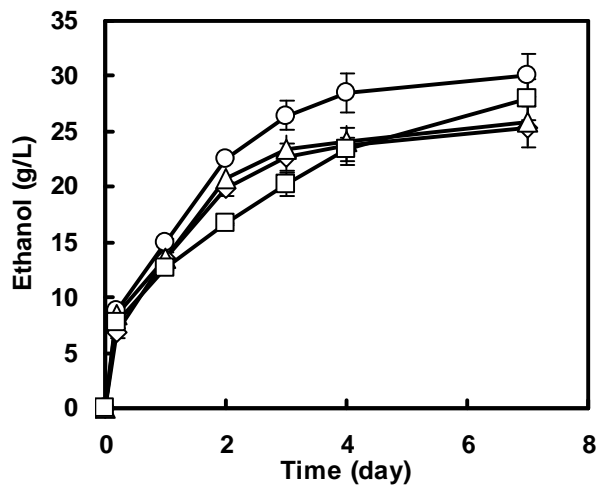
(A)



(B)



(C)



(D)

