

**Efficient Bioethanol Production by Overexpression of Endogenous *Saccharomyces cerevisiae*
Xylulokinase and NADPH-Dependent Aldose Reductase with Mutated Strictly NADP⁺-
Dependent *Pichia stipitis* Xylitol Dehydrogenase**

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ABSTRACT

Development of efficient xylose-fermenting *Saccharomyces cerevisiae* strain has involved a large number of trials that focus on improving ethanol production from glucose and xylose present in lignocellulosic hydrolysates. In this study, a recombinant *S. cerevisiae* strain (SK-NY) overexpressing *GRE3*-encoded NADPH-dependent aldose reductase and xylulokinase with a mutated strictly NADP⁺-dependent *Pichia stipitis* xylitol dehydrogenase was constructed and its fermentation efficiency was compared with that of an isogenic constructed reference strain expressing *P. stipitis* xylose reductase instead of the *GRE3* gene. Strain SK-NY efficiently fermented xylose and glucose mixture and the ethanol production by SK-NY was 21.4% higher than that of the reference strain. Interestingly, the yield of ethanol production by SK-NY strain increased from 0.395 g ethanol/g sugar to 0.435 g ethanol/g sugar after glucose depletion. Furthermore, xylitol accumulation by SK-NY strain (0.6% of total sugar) was considerably lower than that of the reference strain (4.8% of total sugar). These improvements may be influenced by

the effective regeneration of NADPH/NADP⁺ cofactors by *GRE3* gene and the mutated strictly NADP⁺-dependent *P. stipitis* xylitol dehydrogenase.

Keywords: Bioethanol, Xylose fermentation, Cofactor requirement, Xylose reductase, Xylitol dehydrogenase

1. Introduction

Although naturally occurring *Saccharomyces cerevisiae* strains have been used for industrial scale bioethanol production from hexose sugars, there is considerable research interest in the development of recombinant strains that can efficiently ferment both hexose and pentose sugars from lignocellulosic hydrolysates for effective bioethanol production. Lignocellulosic biomass is one of the major renewable substrates that can be used for ethanol production. Complete conversion and fermentation of cellulose and hemicellulose is essential for producing high ethanol yields. Since glucose and xylose are the two predominant sugars in the lignocellulosic hydrolysates, many previous studies have focused on developing recombinant strains that maximize ethanol production from xylose and glucose by eliminating byproducts through metabolic conversion [1, 2, 3-5].

Oxidative metabolism of xylose can be performed by using recombinant yeast *S. cerevisiae* strains expressing xylose-metabolizing enzymes. However, xylose metabolism by recombinant yeast strains can be affected by cofactor availability. Previous studies indicate that effective regeneration of cofactors by the introduced enzyme may increase the cofactor availability and prevent xylitol accumulation as a byproduct of the fermentation process. Some of these were introduced by native *XYL1* and *XYL2* coding for xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, from *Pichia stipitis* (also known as *Scheffersomyces stipitis*) [6, 7, 8]. Recombinant yeasts expressing native or mutated *P. stipitis* XR (*PsXR*) and XDH (*PsXDH*) have also been constructed [9, 10, 11-13]. The redox imbalance between the wild type XR that uses both

NADPH and NADH and the wild type XDH that uses only NAD⁺ may influence xylitol accumulation. Our recent study [5] showed that a novel recombinant strain expressing both mutated strictly NADPH-dependent *PsXR* [14] and mutated strictly NADP⁺-dependent *PsXDH* (named ARSdR) [15], and also overexpressing *XKS1*, which encodes endogenous *S. cerevisiae* xylulokinase (XK), reduced xylitol accumulation by 34.4–54.7% and increased ethanol production by 10–20%.

Although the wild type *S. cerevisiae* strains do not catalyze xylose as the sole carbon substrate [16], the wild type strains have a functional xylose transport system and can express xylose reductase and xylitol dehydrogenase [17] for metabolizing xylose in the presence of other sugar substrates [18]. The nonspecific aldose reductase encoded by the *GRE3* gene [19] is a monomeric NADPH-dependent aldose reductase and has significant XR activity [20]. Considering that the *GRE3* gene has significant homology with the *XYL1* gene, the *GRE3* gene is thought to be a homolog of XR. The role of the *GRE3* gene in xylose fermentation by recombinant *S. cerevisiae* has not been clearly elucidated. A previous study showed that *GRE3* gene deletion decreased xylitol and acetate accumulation during xylose fermentation by recombinant *S. cerevisiae* that expressed bacterial *Thermus thermophilus* xylose isomerase [21]. A strain overexpressing *GRE3*, *XYL2*, and *XKS1* had five-fold lower xylose consumption rate and lower ethanol yield than that of a strain overexpressing *XYL1*, *XYL2*, and *XKS1* [22]. Furthermore, xylose fermentation with yeast strains with optimized *GRE3* expression in conjunction with expression of *XYL1*, *XYL2*, and *XKS1* was better than that by strains with deleted *GRE3* gene [23, 24]. In addition, feasibility of xylose fermentation by engineered *S. cerevisiae* overexpressing *GRE3* and also expressing *XYL2* and xylulokinase (*XYL3*) from *S. stipitis* was investigated regarding the use of different express promoters and terminators of introduced genes under oxygen limited conditions [4]. In this study, a recombinant yeast strain that overexpressed *GRE3* and *XKS1* with ARSdR was constructed for elucidation of the role of *GRE3* in xylose fermentation and for investigating the effects of matched cofactors during fermentation of a mixture of xylose and glucose.

2. Material and Methods

2.1 Plasmids and Strains

All plasmids and strains used in this study are listed in Table 1. The genomic DNA of *S. cerevisiae* was isolated from D452-2 (*MATa leu2 his3 ura3 can1*) by using the DNA High Recovery Kit (TaKaRa Bio, Kyoto, Japan) and was used as template for PCR of the corresponding *GRE3* sequence. Primers were designed by using the *GRE3* gene sequence (GenBank accession number NM_001179234): forward primer sequence was 5'-acggcagaaattcatgtcttcactggttactc-3' and reverse primer sequence was 5'-cgacgaggatcctcaggcaaaagtgggg-3'. The underlined sequences in the forward and reverse primer sequences are restriction sites for EcoRI and BamHI, respectively. The target sequence was amplified by using PCR Thermal Cycler PERSONAL (TaKaRa Bio). The amplified DNA was ligated between the EcoRI and BamHI sites of pPGK plasmid with a phosphoglycerate kinase (PGK) promoter and terminator [25]. The XhoI-SalI fragment-containing cassette of PGK promoter, *GRE3*, and PGK terminator was obtained from the resulting plasmid and ligated into SalI site of YEpm4 plasmid [26] to yield YEpm4-*GRE3* plasmid. The XhoI-SalI fragment was also ligated into SalI site of pAUR-ARSdR-XK [12] to yield pAUR-*GRE3*-ARSdR-XK plasmid. In addition, XhoI-SalI fragment containing a cassette of PGK promoter, *PsXR*, and PGK terminator was obtained from pPGK-XR (WT) plasmid [10] and ligated into SalI site of YEpm4 to yield YEpm4-*PsXR* plasmid. Next, the XhoI-SalI containing a cassette with PGK promoter, XK, and PGK terminator was obtained from pPGK-XK plasmid [27] and ligated into SalI site of pHV1 plasmid [28] to yield pHV1-XK plasmid.

Transformation of yeast was carried out by using Fast Yeast Transformation KitTM (Takara Bio) and following the manufacturer's instructions. A previously constructed pPGK-ARSdR [11] was

used to transform *S. cerevisiae* D452-2, and then the transformed *S. cerevisiae* D452-2 was further transformed with pHV1-XK. Next, D452-2 containing both pPGK-ARSdR and pHV1-XK was further transformed with YEpM4-*PsXR* or YEpM4-GRE3 to obtain SK-NE or SK-NN, respectively. Transformants were selected on yeast nitrogen base (YNB) minimal medium (20 g of glucose/L and 6.7 g/L of YNB without amino acids) plates supplemented with 20 mg/L of leucine, histidine, and/or 5 mg/L of uracil as appropriate. Furthermore, D452-2 was transformed with pAUR-GRE3-ARSdR-XK after the plasmid was linearized by restriction enzyme BsiWI digestion to obtain SK-NY. Transformants were selected on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) containing 0.5 mg/L of aureobasidin A (Takara Bio).

2.2. Enzyme Activity

The recombinant *S. cerevisiae* strains were cultivated in flasks with 100 mL growth media (YNB or YPD) and incubated at 30°C on a shaker rotating at 150 rpm. For cultivation, YNB medium was used for SK-NE and SK-NN strains, whereas YPD medium was used for SK-N1 and SK-NY strains. The cell pellets were harvested by centrifugation at $6000 \times g$ for 5 min at 4°C and the cell-free extracts were prepared as described previously [5]. Enzyme activities of XR, XDH, and XK were determined by spectrophotometric assessment of freshly prepared cell-free extracts as described previously [11, 12]. Protein concentrations were determined using the Bio-Rad Quick Start Bradford 1X dye reagent (Bio-Rad Laboratories, CA), and the absorbance was measured at 595 nm wavelength with γ -globin as a standard.

2.3. Fermentation

SK-NE and SK-NN strains were precultured in YNB minimal medium. The cells were harvested by centrifugation and suspended in 100 mL of YNB medium containing 20 g/L xylose instead of glucose (YNB-X20). Fermentation was carried out in a 250-mL Erlenmeyer flask incubated on a shaker rotating at 150 rpm and temperature maintained at 30°C. SK-N1 and SK-NY were precultured in YPD rich medium. The cells were harvested by centrifugation and suspended in 1000 mL of YPD medium containing 45 g/L of xylose and 15 g/L of glucose instead of 20 g/L of glucose (YPD15X45). Fermentation was carried out in a high-performance bioreactor (BioFlo 110, New Brunswick Scientific Co.). The initial cell density in the fermentation culture was approximately adjusted to an optical density of 1.0 measured at 600 nm wavelength. All growth rates were monitored by using a spectrophotometer (U-2001, Hitachi, Japan). Concentrations of ethanol, glucose, xylose, xylitol, glycerol, and acetic acid were determined using high performance liquid chromatography as described previously [5].

3. Results and discussion

In the first part of this study, we transformed three extrachromosomal plasmids into *S. cerevisiae* D452-2 to construct SK-NE and SK-NN strains and investigated whether the *GRE3* gene located on an extrachromosomal plasmid has better performance on fermentation than the *XYL1* gene with the same plasmids of pPGK-ARSdR and pHV1-XK (Table 1). All introduced genes were subjected to a strong PGK promoter and terminator (Table 1). Although XR activity of SK-NN strains with *GRE3* expression was 2.37 fold lower than that of the reference strain SK-NE with *XYL1* expression (Table 2), SK-NN demonstrated higher xylose consumption rate (2.5 g per day) than that of the reference strain (2.3 g per day; Fig. 1). Nevertheless, both strains showed relatively slower consumption rate, which may be due to the use of minimum medium as the growth medium (Table 3). Both XDH and XK in SK-NE and SK-NN strains showed similar activities (Table 2).

Xylitol accumulation by SK-NN was 21.5% lower than that by SK-NE (Fig. 1). The lower xylitol accumulation by SK-NN may be due to the efficient recycling of NADPH/NADP⁺ between GRE3 and ARSdR. Notably, the enzyme activities ratios for ARSdR/GRE3 and ARSdR/*Ps*XR were 13.6 and 5.55, respectively. A previous report showed that the enzyme ratio of XR/XDH/XK influenced xylitol accumulation and ethanol production where 1:≥10:≥4 of the XR/XDH/XK ratio was optimal in minimizing xylitol formation during xylose utilization by yeast [29]. The reduction in xylitol accumulation in SK-NN resulted in 25% higher ethanol yield than that of the reference SK-NE (Fig. 1). The cell growth was monitored for both strains and no significant difference in growth characteristics was observed (data not shown).

To confirm the fermentation results obtained using SK-NE and SK-NN grown on minimal medium and to investigate whether similar data can be obtained under more practical conditions by using rich medium, we transformed D452-2 by chromosomal integration of plasmid for constructing SK-NY strain. Both SK-NY and SK-N1 [5] reference strain were used for measuring enzyme activities and performing fermentation by using a rich medium containing both glucose and xylose. The XR activity of SK-NY was 6.2 fold lower than that of the SK-N1 reference strain. Justification of co-expressing of *XKS1* and ARSdR were previously studied [12] and our data also indicated no significant difference in enzyme activities of XK and ARSdR (Table 2). Thus, xylose consumption rate of SK-NY was slightly lower than that of SK-N1; SK-NY consumed 24.8 g (4.1 g/h) of mixed sugars (41.3% of total sugars) and SK-N1 consumed 27.7 g (4.61 g/h) of mixed sugars after 6 hours. However, the consumption rate of SK-NY recovered within 48 h and more than 98% of mixed sugars were consumed at the rate of 1.2 g/h by both strains (Table 3 and Fig. 2A). Although the glucose concentration is generally higher than the xylose concentration in lignocellulosic biomass, xylose concentration used in this experiment was three fold higher than the glucose concentration to assess the effects of NADPH/NADP⁺ recycling between GRE3 and ARSdR at higher xylose concentrations, considering glucose helps transporting xylose and

influences the fermentation rate [30]. Similar ethanol conversion rates were obtained using 15 g/L glucose and 5 g/L xylose mixture (Data not shown). Although both SK-NY and SK-N1 had similar consumption rates for glucose and xylose, total ethanol yield of SK-NY was 21.4% higher than that of SK-N1. Thus, SK-NY produced 25 g/L ethanol, which was equivalent to 0.42 g ethanol/g sugar (85.7% of the theoretical yield), whereas SK-N1 produced 20.6 g/L ethanol, which was equivalent to 0.346 g ethanol/g sugar (70.5% of the theoretical yield) after complete consumption of glucose and xylose (Table 3 and Fig. 2A). The yield of ethanol production by SK-NY strain increased from 0.395 g ethanol/g sugar to 0.435 g ethanol/g sugar after glucose depletion. On the other hand, the ethanol production by SK-N1 decreased from 0.364 g ethanol/g sugar to 0.329 g ethanol/g sugar after glucose consumption. Since both SK-N1 and SK-NY strains had similar ARSdR and XK activities (Table 2), the increased ethanol production may be influenced by the strict NADPH/NADP⁺-dependence of GRE3/ARSdR combination that regenerates the cofactors for each other. These results are consistent with that of our previous report, which showed that strains SK-N2 with double mutated *PsXR*, ARSdR and XK, showed 20% increase in ethanol production [5], although GRE3 activity was lower than that of strictly NADPH-dependent double mutated *PsXR*. The ratio of total activities of ARSdR/GRE3 in SK-NY was 63.7, whereas the ratio of total activities of ARSdR/*PsXR* in SK-N1 was 10.1 (Table 2). Optimizing the expression levels of XR, XDH, and XK may be another important factor for generating an efficient xylose-fermenting recombinant *S. cerevisiae* strain [4]. Xylitol accumulation in fermentation with SK-NY was lowered by 87.5% and 0.6% of the total sugar was accumulated as xylitol. In contrast, 4.8% of the total sugar was accumulated as xylitol in fermentation reactions with the reference strain (Fig. 2B). These data are compatible with our previous data that showed that xylitol accumulation by strains expressing mutated strictly NADPH-dependent *PsXR* was 34.4% to 54.7% lower than that of the reference strains [5].

Although the feasibility of xylose fermentation by engineered *S. cerevisiae* overexpressing NADPH dependent endogenous aldose reductase (*GRE3*) and expressing NAD⁺ dependent xylitol dehydrogenase (*XYL2*) from *S. stipitis* under oxygen-limited conditions was previously assessed and the expression levels were optimized using different promoters, the findings indicated that the DGX23 strain could not efficiently ferment xylose under strict anaerobic conditions [4]. Our study indicated that SK-NY strain having matched cofactor (NADPH/NADP⁺) of overexpressed *GRE3* and mutated *PsXDH* (ARSdR) and with overexpressed XK produced ethanol at a conversion rate that was 85.7% of the theoretical yield and the xylitol accumulation yield of the recombinant SK-NY strain was 87.5% lower than that of the reference strain (Table 3). Considering the data of DGX23 strain [4] and SK-NY stain, the cofactor regeneration between XR and XDH appears to play a crucial role in fermentation reactions conducted under strict anaerobic conditions or with un-optimized enzyme ratios.

Glycerol accumulation of SK-NY was slightly higher than that of the SK-N1 reference strain, whereas acetic acid accumulation of SK-NY was slightly lower than that of the SK-N1 reference strain (Fig. 2C). Furthermore, deletion of glycerol 3-phosphate dehydrogenase gene [31] may be effective for decreasing glycerol accumulation.

Based on these results and our previous study using strictly NADPH-dependent mutated *PsXR* [5], we concluded that cofactor (NADPH/NADP⁺) regeneration between XR and XDH was highly effective for improving fermentation of xylose and glucose mixtures for increased ethanol production. Furthermore, constructing recombinant strains by using the strain development approach of this study with those of other studies may yield strains with better performance and production capabilities. For example, recombinant strains that overexpress the enzymes used in this study along with enzymes involved in the pentose phosphate pathway, such as transaldolase encoded by the *TAL* gene and transketolase encoded by the *TKL* gene [3, 32], may be used to produce synergistic effects that enable efficient ethanol production.

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Figure legends

Fig. 1. Fermentation of xylose by SK-NE and SK-NN strains in a minimal medium

Fermentation by SK-NE (open symbols) and SK-NN (closed symbols) strains for xylose consumption (triangular symbol), ethanol production (square symbol), and xylitol accumulation (rhomboid symbol) are shown. The values are the average of three independent experiments.

Fig. 2. Fermentation of xylose and glucose mixture in a rich medium by SK-N1 and SK-NY strains

Fermentation by SK-N1 (open symbols) and SK-NY (closed symbols) strains that leads to **(A)** xylose consumption (triangular symbols), glucose consumption (rhomboid symbols), and ethanol production (square symbols); **(B)** xylitol accumulation; **(C)** glycerol (triangular symbols) and acetic acid (square symbols) accumulation. The values are the average of three independent experiments.

Table 1Characteristics of *S. cerevisiae* strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Source or reference
Plasmids		
YEpm4	<i>LEU2</i>	[26]
pPGK	<i>URA3</i> , PGK promoter and terminator	[25]
pHV1	<i>HIS3</i>	[28]
YEpm4- <i>GRE3</i>	<i>LEU2</i> , expression of <i>GRE3</i>	This study
pPGK-XR(WT)	<i>URA3</i> , expression of <i>PsXR</i>	[10]
YEpm4- <i>PsXR</i>	<i>LEU2</i> , expression of <i>PsXR</i>	This study
pPGK-ARSdR	<i>URA3</i> , expression of mutated <i>PsXDH</i> (ARSdR)	[11]
pPGK-XK	<i>AUR1-C</i> , expression of XK	[27]
pHV1-XK	<i>HIS3</i> , expression of XK	This study
pAUR-ARSdR-XK	<i>AUR1-C</i> , expression of mutated <i>PsXDH</i> (ARSdR) and XK	[12]
pAUR- <i>GRE3</i> -ARSdR-XK	<i>AUR1-C</i> , expression of <i>GRE3</i> , mutated <i>PsXDH</i> (ARSdR) and XK	This study
<i>S. cerevisiae</i> strains		
D452-2	<i>MATa leu2 his3 ura3 can1</i>	[33]
SK-NE	D452-2, YEpm4- <i>PsXR</i> , pPGK-ARSdR, pHV1-XK	This study
SK-NN	D452-2, YEpm4- <i>GRE3</i> , pPGK-ARSdR, pHV1-XK	This study
SK-N1	D452-2, <i>AUR1</i> : [PGKp- <i>PsXR</i> -PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]	[5]
SK-NY	D452-2, <i>AUR1</i> : [PGKp- <i>GRE3</i> -PGKt, PGKp-ARSdR-PGKt, PGKp-XK-PGKt]	This study

Table 2

Activities of xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) in cell-free extracts of recombinant yeast strains

Strain	(U/mg protein)*				XK
	XR		XDH		
	NADPH	NADH	NADP ⁺	NAD ⁺	
SK-NE	2.63 ± 0.02	1.20 ± 0.01	14.59 ± 0.06	0.34 ± 0.01	2.61 ± 0.02
SK-NN	1.11 ± 0.01	0.01 ± 0.00	15.12 ± 0.04	0.43 ± 0.01	3.15 ± 0.03
SK-N1	1.49 ± 0.01	0.67 ± 0.01	15.03 ± 0.04	0.97 ± 0.01	2.72 ± 0.02
SK-NY	0.24 ± 0.01	0.02 ± 0.00	15.29 ± 0.05	0.74 ± 0.01	3.13 ± 0.03

The given values are the average of three independent experiments.

* 1U = 1μmol of NADH or NADPH was produced or consumed in 1 min/mg protein

Table 3

Fermentation parameters of strains SK-NE and SK-NN using YNB-X20 medium; strains SK-N1 and SK-NY using YPD15X45.

Strains	Media	Ratio of sugar consumed to total sugar [% (w/w)] ^a	Ethanol (g/l) ^a	Ethanol yield (g/g) ^a	Ethanol productivity (g/l/h) ^a
SK-NE	YNB-X20	70.0± 0.1	3.01 ± 0.01	0.22 ± 0.01	0.02 ± 0.00
SK-NN	YNB-X20	75.0± 0.1	4.02 ± 0.01	0.27 ± 0.01	0.03 ± 0.00
SK-N1	YPD15X45	99.3± 0.1	20.6 ± 0.1	0.34 ± 0.01	0.29 ± 0.01
SK-NY	YPD15X45	99.4± 0.1	25.0 ± 0.1	0.42 ± 0.01	0.35 ± 0.01

Values are averages of at least three independent experiments.

^a Ethanol titer, yield, productivity and ratio of sugar consumption calculated after 144 h with strains SK-NE and SK-NN and after 72h with strains SK-N1 and SK-NY.

Figure 1

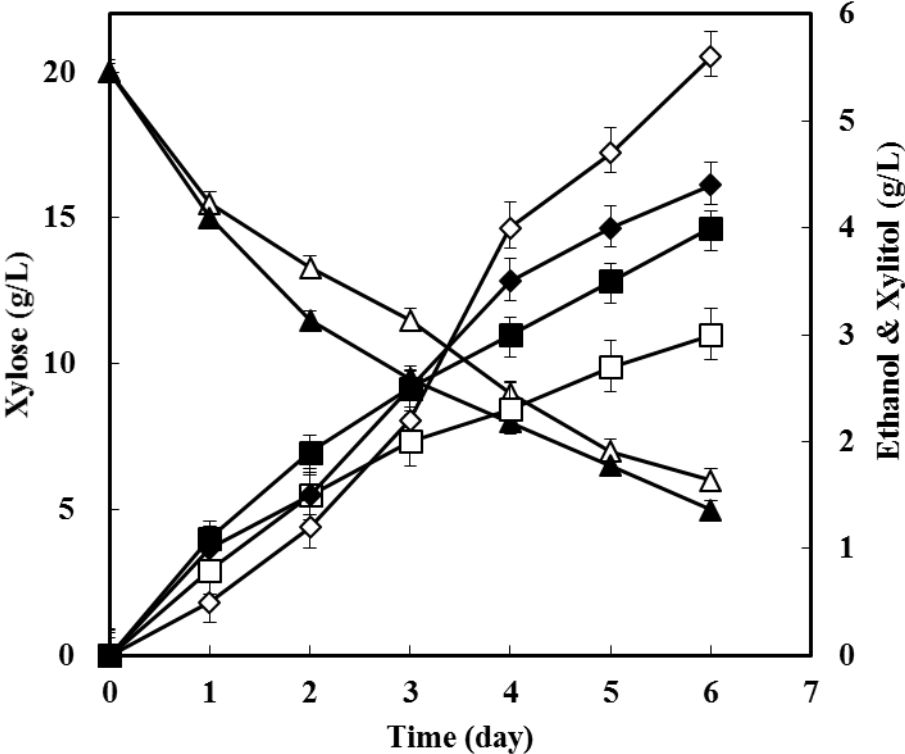


Figure 2

