Conversion of Waste Agriculture Biomass to Bioethanol by Recombinant *Saccharomyces cerevisiae*

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Abstract

Agricultural waste biomass has already been transferred to bioethanol and used as energy related products, although many issues such as efficiency and productivity still to be overcome. In this study, the protein engineering was applied to generate enzymes with completely reversed coenzyme specificity and developed recombinant yeasts containing those engineered enzymes for construction of an efficient biomass-ethanol conversion system. Recombinant yeasts were constructed with the genes encoding a wild type xylose reductase (XR) and the protein engineered xylitol dehydrogenase (XDH) (with NADP) of *Pichia stipitis*. These recombinant yeasts were characterized based on the enzyme activity and fermentation ability of xylose to ethanol. The protein engineered enzymes were expressed significantly in *Saccharomyces cerevisiae* as judged by the enzyme activity in vitro. Ethanol fermentation was measured in batch culture under anaerobic conditions. The significant enhancement was found in Y-ARS strain, in which NADP$^+$-dependent XDH was expressed; 85% decrease of unfavorable xylitol excretion with 26% increased ethanol production, when compared with the reference strain expressing the wild-type XDH.

Keywords: Agricultural waste biomass; Protein engineering; Xylitol dehydrogenase; Xylose-fermentation; Eethanol production.

1. Introduction

The more efficient use of biomass is demanded to solve the global crisis such as depletion of fossil fuel and global warming. Woody biomass, including agriculture residues, wood chips, municipal solid wastes, paper wastes, etc., has already been transferred to

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bioethanol and biodiesel in some cases and used as energy related products, although many issues such as efficiency and productivity still exist to be overcome. Xylose is one of the major fermentable sugars present in lignocellulosic biomass, the second most abundant carbohydrate polymer in nature to glucose. The efficient fermentation of xylose is required to develop economically viable processes for producing biofuels such as ethanol from biomass [1]. Although a few xylose fermenting yeasts are found in nature [2, 3], *Saccharomyces cerevisiae* is used universally for industrial ethanol production because of the ability to produce high concentrations of ethanol and high inherent ethanol tolerance. Although the native *S. cerevisiae* can not ferment xylose, the *S. cerevisiae* transformed with the native enzymes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis*, a most potent recombinant strain, acquires the ability to ferment xylose to ethanol [4, 5]. So engineering for xylose utilization has been focused on adapting the xylose metabolic pathway from the xylose-utilizing yeast *Pichia stipitis*. In this organism, xylose is converted into xylulose by two oxidoreductases. First, xylose is reduced to xylitol by an NADPH/NADH-linked xylose reducatase (XR) [6], and then xylitol is oxidized to xylulose by an NAD-linked xylitol dehydrogenase (XDH) [7]. Finally, xylulokinase (XK) phosphorylates xylulose into xylulose-5-phosphate, which is metabolized further via the pentose phosphate pathway (PPP) (Fig. 1). Many researchers attempt at engineering xylose metabolism expressed only native XR and XDH *from P. stipitis* in *S. cerevisiae* because *S. cerevisiae* can ferment xylulose [8]. Recombinant *S. cerevisiae* expressing native XR and XDH could grow on xylose, but ethanol production from xylose was not significant because a substantial portion of the consumed xylose was converted into xylitol [9].

The most straightforward metabolic engineering strategy is the expression of bacterial xylose isomerase (XI) gene, so that xylose can directly be converted to xylulose with no coenzyme. There have been numerous unsuccessful attemts to express XI genes from various bacteria in *S. cerevisiae* except a thermophilic enzyme [10, 11], we focused on the fungal xylose metabolic pathway in this study. XR and XDH in this fungal pathway are necessary for *S. cerevisiae* to ferment xylose to ethanol because of a lack of genes encoding these enzymes in *S. cerevisiae*. Several studies reported that *S. cerevisiae* transforming the genes encoding XR, XDH from *P. stipitis* and the endogenous gene XK, a most potent recombinant strain, has not yet been applied to the industrial bio-process due to the unfavorable excretion of xylitol [12, 13]. Because intercellular redox imbalance caused by the different coenzyme specificity of XR (with NADPH) and XDH (with NAD) has been thought to be one of the main factors of xylitol excretion. The introduction of the protein engineered XDH (with NADP) of *P. stipitis* generated in this study prevented this xylitol excretion by maintaining the intercellular redox balance.

In spite of many attempts for modification of coenzyme specificity, only a few of them can be judged to be fully reversal, in terms of obtaining a mutant enzyme as catalytically efficient as the wild type [14, 15]. In our previous study [16], site-directed mutagenesis for complete reversal of coenzyme specificity was performed on NAD$^+$-dependent XDH from *P. stipitis* to generate NADP$^+$ dependent XDH and these mutated XDHs were

characterized *in vitro*. In the present study, we constructed recombinant yeasts that were transformed with the genes encoding the protein engineered XDH (with completely NADP-dependent) and a wild type XR of *P. stipitis* and characterized those recombinant yeasts based on the enzyme activity and fermentation ability of xylose to ethanol. The protein engineered enzymes were expressed significantly in *S. cerevisiae* as judged by the enzyme activity in vitro. Ethanol fermentation was measured in batch culture under anaerobic conditions. Introduction of NADP-dependent XDH was shown to prevent the xylitol excretion probably because of maintaining the intercellular redox balance.
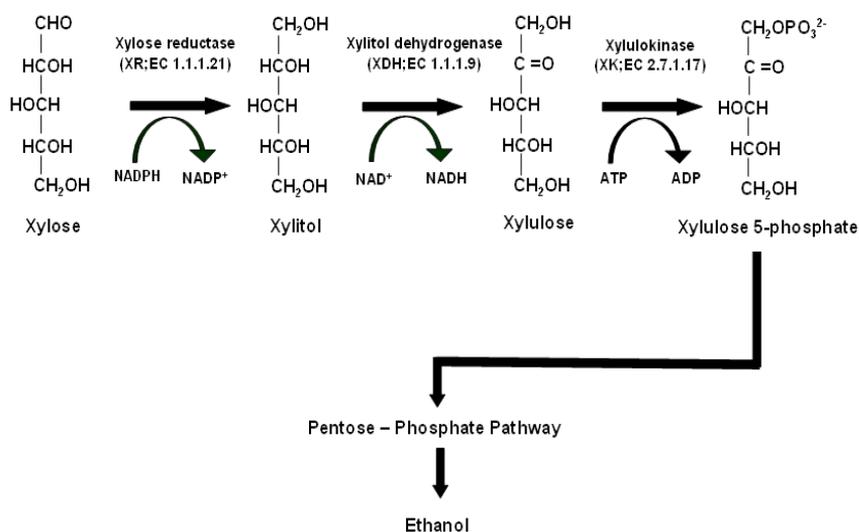

Fig. 1. Metabolic pathway from xylose to ethanol.

2. Materials and Methods

2.1. *Materials and media*

S. cerevisiae D452-2 (*MATa leu2 his3ura3can1*) [17] was used as the recipient yeast strain for the expression of *Pichia stipitis* XDH and XR. *S. cerevisiae* was grown in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) for transformation. In all other experiments, a defined minimal medium (6.7 g of yeast nitrogen base without amino acid with 5 g glucose per liter) was used. Leucine, Histidine, and Uracil were added at 20 mg/l, 20 mg/l, and 5 mg/l respectively for strain D452-2. Bacterial strains were grown in Luria-Bertani medium (yeast extract 5 g/l, peptone 10 g/l, and sodium chloride 10 g/l). Transformants were selected by adding ampicillin (50 mg/liter). For growth on solid media 20 g of agar per liter was added.

2.2. *Yeast strain transformation*

Yeast transformation was performed using the lithium acetate method [18]. Yeast cells were incubated overnight in YPD medium before being streaked on selective medium. *S. cerevisiae* transformants were selected on agar plates with minimal medium containing 20 mg/l histidine, 20 mg/l leucine and 5 mg/l uracil.

2.3. *Preparation of crude cell extract*

The recombinant yeast strains were grown in minimal medium at 30°C. The cells were harvested by centrifugation at 5000 rpm for 10 minutes. The cells were resuspended in disintegration buffer 100 mM sodium phosphate (pH 7.0) containing 1 mM MgCl$_2$, 0.5 mM EDTA and 0.5 mM dithiothreitol (DTT) and vortexed together with an equal volume of glass beads (0.5 mm diameter) twice for 5 min at 4°C. Cell debris and glass beads from the cell extract were separated by centrifugation at 15000 rpm, 10 min, 4°C and the remaining supernatant was used for enzyme determinations.

2.4. *Enzyme assays*

Enzyme activities were measured by using a V-550 model spectrophotometer (JASCO Corporation, Japan) operating at 340 nm and 35°C. The standard assay mixture for XDH contained 50 mM MgCl$_2$ and 300 mM xylitol in 50 mM Tris-HCl (pH 9.0); for XR contained 60 mM sodium phosphate and 133 mM xylose [13]. All reactions were started by addition of 0.10 ml of a 20 mM NAD/NADP solution for XDH and 0.10 ml of a 1.5 mM NADPH/NADH solution for XR to a final volume of 1.0 ml (standard assay condition). One unit of enzyme activity refers to 1 μmol of NAD(P)$^+$H produced/min. Protein concentrations were determined by the method of Lowry *et al*. [19] with bovine serum albumin as a standard.

2.5. *Preliminary batch fermentation in shake flask*

After precultivation of recombinant yeast strains in 3 ml minimal medium in 15 ml falcon tube for 3 days, yeast cells were aerobically cultivated for 3 days at 30°C in 100 ml minimal medium in 300 ml flask in incubator at 200 rpm. The cell pellets were collected by centrifugation for 10 min at 5000 rpm and 4°C, and were washed once with a solution containing 9 g of NaCl/liter. The cell pellets were then inoculated into 200 ml fermented medium (6.7 g of yeast nitrogen base without amino acid per liter with 5 g of glucose/liter plus 15 g of xylose/liter and appropriate amino acid). Ethanol fermentation was carried out as batch cultures in 200 ml flask with anaerobic condition in incubator at 150 rpm. The flasks were sealed with two layers of Saran wrapping paper to allow fermentation to occur anaerobically. Samples (1 ml) of the fermentation broth were removed at intervals and stocked at -35°C for analyses of substrates and fermentation products.

2.6. *Batch fermentation in bioreactor*

A high performance bioreactor (BioFlo 110, New Brunswick Scientific Co.) was used for anaerobic batch fermentation. After precultivation of recombinant yeast strains in 5 ml minimal medium in 15 ml falcon tube for 3 days, yeast cells were aerobically cultivated for 3 days at 30°C in 300 ml minimal medium in 1 liter flask in incubator at 200 rpm. The cell pellets were collected by centrifugation for 10 min at 5000 rpm and 4°C, and were washed once with a solution containing 9 g of NaCl/liter. The cell pellets were then inoculated into fermented medium (6.7 g of yeast nitrogen base without amino acid per liter with 5 g of glucose/liter plus 15 g of xylose/liter and appropriate amino acid). Silicone antifoam 0.5 ml/liter was added to the media before autoclaving. Anaerobic cultivation took place in a 1.3 liter bioreactor with an initial working volume 1 liter. The temperature was maintained at 30°C. The pH was controlled at 5.5 by addition of 2 M NaOH and 1 M H_2S0_4. The agitation speed was constant at 500 rpm.

2.7. *Analysis of fermentation products*

Ethanol concentration was measured using gas chromatography. The gas chromatograph (model GC -14B, Shimadzu Corporation, Kyoto, Japan, fitted with a flame ionization detector) was operated under the following conditions: glass column (2.0 m by 3.2 mm) packed with Thermon-3000 (Shimadzu); temperature of column 70°C, injector 200°C, and detector 250°C; and nitrogen carrier gas flow rate 25 ml/min. A high-performance liquid chromatograph (Tosoh Corporation Ltd., Japan) equipped with an RI detector was used to analyze the concentrations of glucose, xylose, xylitol, glycerol, and acetic acid. A Bio-Rad type HPX-87H ion-exclusion column was used. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.4 ml/min. Cell growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer (model U-2001, Hitachi, Japan).

3. Results and Discussion

3.1. *Expression of p. stipitis XR and XDH mutants in s. cerevisiae*

Plasmids pPGK-WT and pPGK-ARS for XDH expression in yeast were constructed by inserting the wild-type (WT) or corresponding mutated XDH expression region [16] into a yeast expression vector, pPGK [21], which is a 2 μm DNA-based shuttle vector containing a phosphoglycerate kinase (PGK) promoter and terminator for expression, a multicloning site, and the URA3 gene as a yeast selectable marker (Fig. 2). To construct a plasmid for XR expression in yeast, the XR coding region [22] was first inserted into pPGK vector. Then the XR coding region with PGK promoter and terminator was excised

from the plasmid and reinserted into YEpM4 [23] vector, which is a 2 μm DNA-based shuttle vector containing a multicloning site LEU2 gene as a yeast selectable marker (Fig. 3) to yield pPGK-XR. *S. cerevisiae* D452-2 (*MATa leu2 his3ura3canl*) [17] was used as the recipient yeast strain for the expression of XDH and XR. D452-2 was transformed with pPGK-XR and then D452-2 containing pPGK-XR was further transformed with pPGK-WT and pPGK-ARS to construct recombinant yeast strains Y-WT and Y-ARS respectively. pPGK and YEpM4 were transformed into D452-2 to construct Y-vector as a control strain.
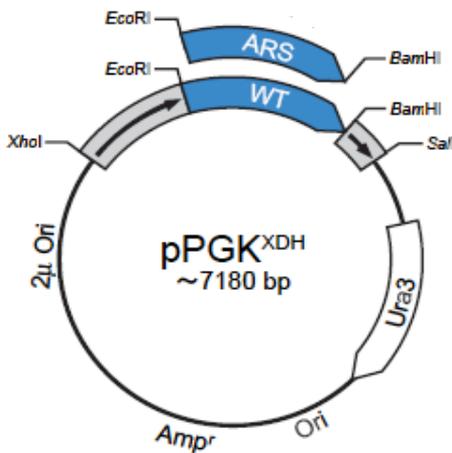
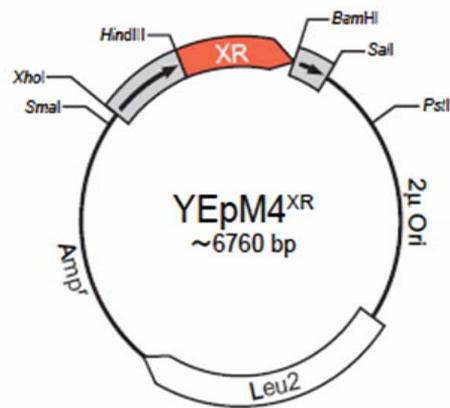Fig. 2. Schematic diagram of yeast expression vector pPGK.

Fig. 3. Schematic diagram of yeast expression vector YEpM4.

3.2. *Effect of xylitol dehydrogenase from pichia stipitis (PsXDH) mutants in s. cerevisiae*

All the recombinant S. cerevisiae strains constructed in this study grew normally in the minimal medium (data not shown). The recombinant strains were cultured in the minimal medium, harvested and then the crude extracts were prepared using glass beads as described in materials and methods. XDH and XR activities in the crude extracts were measured spectrophotometrically. As shown in Fig. 4, Y-ARS showed higher enzyme activity with $NADP^+$ compared with Y-WT (more than 8-fold higher values of Y-WT). The XR activities in these recombinant yeast were observed at al most the same levels (Table 1). The XDH and XR activities observed in control strain were probably due to endogenous XDH and XR activities.
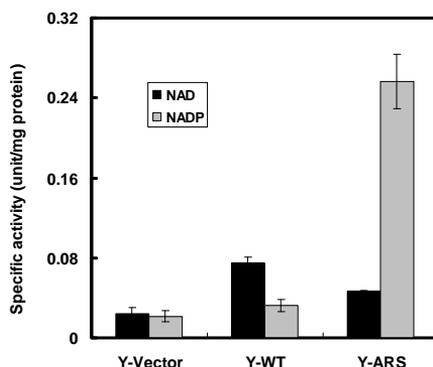
Fig. 4. Specific enzyme activities of xylitol dehydrogenase (protein engineered XDH) in the recombinant *S. cerevisiae* were assayed in the direction of polyol oxidation by measuring the reduction of NAD^+ or $NADP^+$. Values are the mean ± S.D., $n = 3$.

Table 1. Activities of XR and XDH in recombinant yeast strains.

Strain	XR (unit/mg protein)	XDH (unit/mg protein)	
		NAD^+	$NADP^+$
Y-vector	0.010 ± 0.001	0.024 ± 0.006	0.022 ± 0.005
Y-WT	0.041 ± 0.002	0.074 ± 0.006	0.032 ± 0.006
Y-ARS	0.051 ± 0.005	0.046 ± 0.002	0.256 ± 0.022

3.3. *Effect of NADP-preferring XDH mutant in ethanol fermentation*

Ethanol production and xylitol excretion of the recombinant yeasts grown in an incubator with shaker at 30°C were measured. One set of fermentation (100 ml) was started with 5 g/l glucose and 15 g/l xylose (Fig. 5). In case of Y-Vector, an increase of ethanol concentration was found in initial phase (3–25 h), which may be due to glucose consumption. Y-WT produced ethanol at 2.22 g/l and excluded xylitol at 1.42 g/l. Y-ARS produced the highest amount of ethanol at 2.79 g/l and excreted the lowest amount of xylitol at 0.66 g/l. In all these recombinant yeast strains, glucose was completely fermented in 25 to 40 h. Of the xylose, 25–58% was fermented in 68 h.

Y-ARS was further investigated in a high performance bioreactor under anaerobic conditions and compared with Y-WT. Y-ARS produced ethanol at 6.88 g/l with a yield of 0.45 g of ethanol/g of total consumed sugars (Fig. 6). The yield of ethanol from consumed sugars reached 88% of the theoretical yield and xylitol excretion was 0.41 g/l. Glucose was completely fermented in 15 h. Of the xylose, 68% was consumed in 72 h. A small amount of glycerol (0.29 g/l) and acetic acid (0.28 g/l) was produced mainly during the

glucose consumption phase. Y-WT produced ethanol at 5.45 g/l with a yield of 0.30 g of ethanol/g of total consumed sugars. The yield of ethanol from consumed sugars reached 60% of the theoretical yield. The concentration of xylitol excreted 2.66 g/l. Glycerol (0.42 g/l) and acetic acid (0.38 g/l) were produced during fermentation. The significant enhancement was found in Y-ARS strain. There was 85% decrease of unfavorable xylitol excretion and 26% increase of ethanol production, when compared with the reference strain expressing the wild–type XDH.
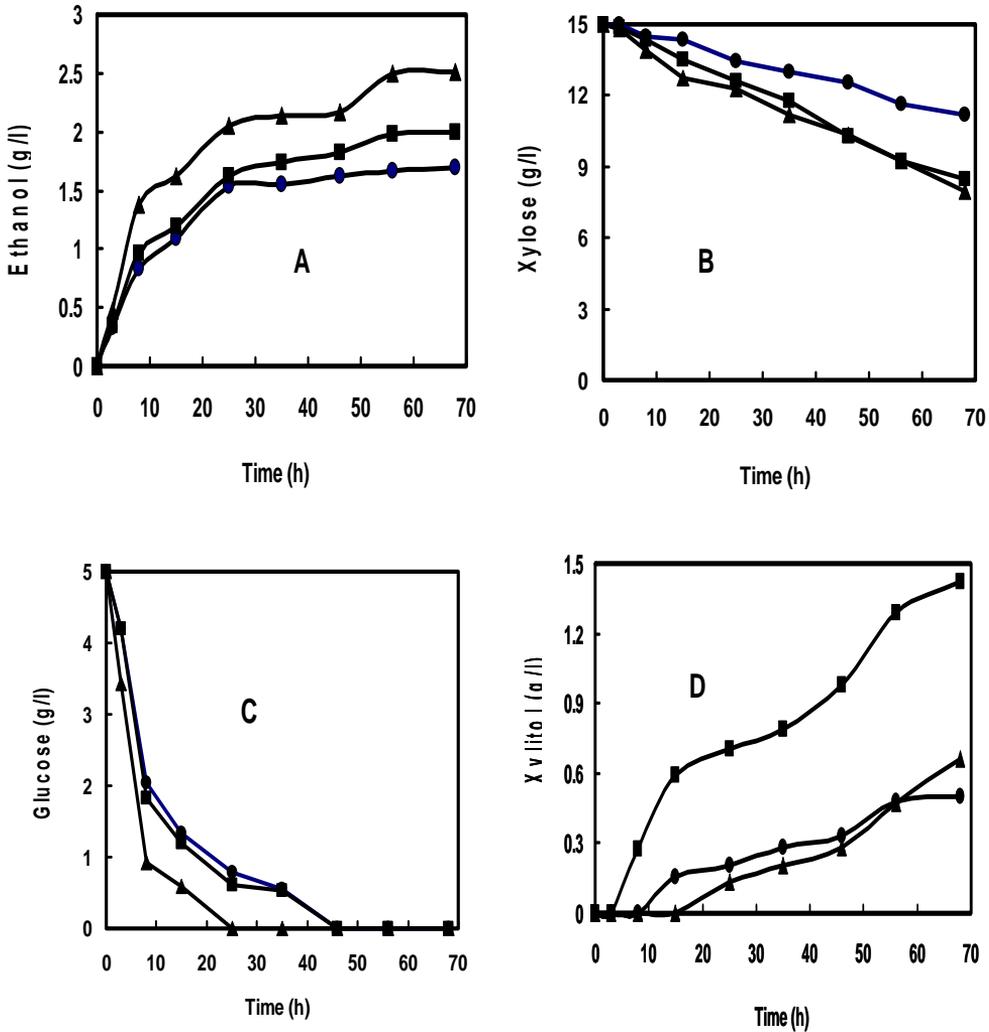

Fig. 5. Ethanol fermentation in minimal medium by recombinant *S. cerevisiae*, Y-Vector (circle), Y-WT (square), and Y-ARS (triangle). Concentration of ethanol (A), xylose (B), glucose (C), and xylitol (D) are shown.
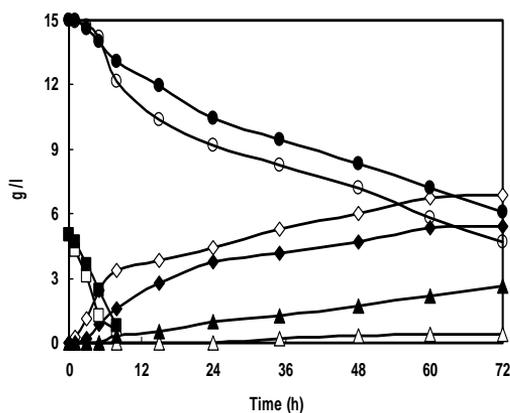
Fig. 6. Time dependent xylose (circle) and glucose (square) utilization and ethanol (rhomboid), and xylitol (triangle) concentration profiles of Y-WT (open symbol) and Y-ARS (closed symbol) grown in a bioreactor. Values are the mean of the results of duplicate experiments which differed by less than 10%. Small amount of glycerol and acetic acid were not shown (see text).

Most interesting insight in this study is to estimate the effect modifications of PsXDH (*in vitro*) [16] on fermentation of xylose to ethanol (*in vivo*). A functional modification of PsXDH in this study is to shift coenzyme specificity toward $NADP^+$. ARS mutant shows reversal coenzyme specificity toward $NADP^+$ (under coenzyme-saturated condition in vitro) [16]. The k_{cat}/K_m^{NADP} of ARS dropped 15-fold compared with WT by an increase of K_m and decrease of k_{cat}. On the other hand, k_{cat}/K_m^{NADP} increased dramatically (up to 4100-fold), caused by a large decrease of K_m value and increase of k_{cat}, which seems to be due to more efficient fermentation ability in Y-ARS (in particular, decrease of xylitol excretion), compared with Y-WT.

As described in the Introduction, an alternative strategy for maintaining intracellular redox balance is to utilize the xylose-fermenting pathway via XI. Recently, it was first reported that XI from the fungus *Piromyces sp*. strain E3 resulted in significant activity in *S. cerevisiae* cells [11]. However, comparison of xylose-fermenting ability by XR-XDH- and XI-carrying recombinant *S. cerevisiae* strains revealed that the XR-XDH xylose utilization pathway is much better than the XI pathway [24]. In this study, we introduced only exotic *XYL1* and *XYL2* genes into *S. cerevisiae*. On the other hand, in addition to the metabolic engineering described above, the introduction and overexpression of other endogenous genes, including xylulokinase (*XK*), transketolase (*TKL1*), transaldolase (*TAL1*) and several hexose-transporter (*HXT1*–7) genes, have been attempted to enhance the pentosephosphate pathway and/or xylose uptake [1]. Combined approaches of these strategies and our strategy using the protein-engineered enzyme should be considered to achieve more effective ethanol production from xylose by recombinant *S. cerevisiae*.

4. Conclusions

We applied the protein engineering to generate enzymes with completely reversed coenzyme specificity and developed recombinant yeasts containing those engineered enzymes for construction of an efficient biomass-ethanol conversion system. Most interesting insight in this study is to estimate the effect modifications of PsXDH (*in vitro*) [16] on fermentation of xylose to ethanol (*in vivo*). A functional modification of PsXDH in this study is to shift coenzyme specificity toward $NADP^+$. ARS mutant shows reversal coenzyme specificity toward $NADP^+$ (under coenzyme-saturated condition in vitro) [16]. The recombinant yeast Y-ARS containing NADP-dependent mutant ARS was expressed much more highly than each parent enzyme in *S. cerevisiae* cells, similar to in *E. coli* cells. The significant enhancement was found in Y-ARS strain, in which $NADP^+$-dependent XDH was expressed; 85% decrease of unfavorable xylitol excretion with 26% increased ethanol production, when compared with the reference strain expressing the wild-type XDH.

Acknowledgement

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