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**ORIGINAL ARTICLE**

# Enhancing Biofuel Production from Woody Biomass

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**ABSTRACT** - Woody biomass, including agricultural wastes, wood chips, municipal solid waste, and paper waste, have been converted into bioethanol and biodiesel in certain instances, serving as energy-related products. Xylose is a significant fermentable sugar present in woody biomass and ranks as the second most abundant carbohydrate polymer in nature, following glucose. To create economically viable biofuel production processes, both glucose as well as xylose must be effectively fermented to produce ethanol from woody biomass. A recombinant strain of *Saccharomyces cerevisiae*, modified with xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes from *Pichia stipitis* (designated PsXR and PsXDH, respectively) can convert xylose to ethanol, while also excrete xylitol. The excretion of xylitol may arise from an intracellular redox imbalance due to the contrasting coenzyme preferences of the NADPH-dependent XR and the NAD+-dependent XDH. In this study, we evaluated the impact of reversing coenzyme specificity towards NADP+ and thermally stabilizing the xylitol dehydrogenase from *P. stipitis* on the fermentation of xylose into ethanol, utilizing a recombinant *S. cerevisiae* that also expressed the native xylose reductase from *P. stipitis*. The engineered enzymes demonstrated significantly enhanced activity when expressed in *Saccharomyces cerevisiae* in *vitro*. Notably, the Y-C4/ARS strain, which expressed the thermostable and NADP+-dependent XDH, exhibited an 84% reduction in undesirable xylitol excretion and a 28% increase in ethanol production relative to the reference strain with the wild-type XDH. This study focuses on optimizing metabolic flux through the pentose phosphate pathway and integrating additional cofactor-balancing strategies, with the goal of further enhancing ethanol yields and developing robust industrial strains suitable for large-scale lignocellulosic biofuel production.

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## INTRODUCTION

The efficient utilization of biomass is essential to address global challenges like fossil fuel depletion and climate change. Woody biomass, including agricultural wastes, wood chips, municipal solid waste, and paper waste, has been converted into bioethanol and biodiesel in certain cases, though significant barriers related to efficiency and productivity still limit its widespread application. Xylose is a significant fermentable sugar found in lignocellulosic biomass, ranking as the second most prevalent carbohydrate polymer in nature following glucose. Effective xylose fermentation is crucial for developing economically viable methods for biofuel production, such as ethanol, from biomass [1]. While several naturally occurring xylose-fermenting yeasts exist [2; 3], *Saccharomyces cerevisiae* is predominantly utilized in industrial ethanol production due to its ability to generate high ethanol concentrations and its strong ethanol tolerance. However, native *S. cerevisiae* is incapable of fermenting xylose. To address this

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constraint, *S. cerevisiae* has been genetically modified with the xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *Pichia stipitis*, a highly effective recombinant strain, enabling it to ferment xylose into ethanol [4; 5]. Research on xylose application have focused on integrating the xylose metabolic pathway from *Pichia stipitis*, where xylose is first reduced to xylitol by an NADPH/NADH-linked xylose reductase (XR) [6], followed by its oxidation to xylulose via a NAD-linked xylitol dehydrogenase (XDH) [7]. Subsequently, xylulose is phosphorylated by xylulokinase (XK) into xylulose-5-phosphate, which is further metabolized via the pentose phosphate pathway (PPP) (Figure 1). Numerous researchers have attempted to engineer xylose metabolism by expressing only the native XR and XDH from *P. stipitis* in *S. cerevisiae*, as *S. cerevisiae* is capable of xylulose fermentation [8]. Despite recombinant *S. cerevisiae* expressing native XR and XDH being capable of growth on xylose, ethanol generation from xylose remains limited due to a substantial fraction of the consumed xylose being converted to xylitol [9].

A straightforward metabolic engineering approach involves the expression of the bacterial xylose isomerase (XI) gene, allowing for the direct conversion of xylose to xylulose without coenzymes. Numerous attempts to incorporate XI genes from various bacteria into *S. cerevisiae* have proven unsuccessful, with the exception of the thermophilic enzyme [10; 11]. Consequently, this study concentrated on the fungal xylose metabolic pathway, highlighting the essential roles of XR and XDH in facilitating the fermentation of xylose into ethanol by *S. cerevisiae*, which lacks the genes encoding these enzymes. Several studies have reported that transforming *S. cerevisiae* with the genes encoding XR and XDH from *P. stipitis*, along with the endogenous XK gene, results in a highly effective recombinant strain. However, this approach has not yet been applied in industrial bioprocesses due to the problematic excretion of xylitol. The intercellular redox imbalance caused by the differing coenzyme specificities of XR (which uses NADPH) and XDH (which uses NAD) is believed to be a major factor contributing to xylitol excretion [12; 13]. The introduction of engineered XDH (which uses NADP) from *P. stipitis*, developed in this study, mitigated xylitol excretion by maintaining the intercellular redox balance.

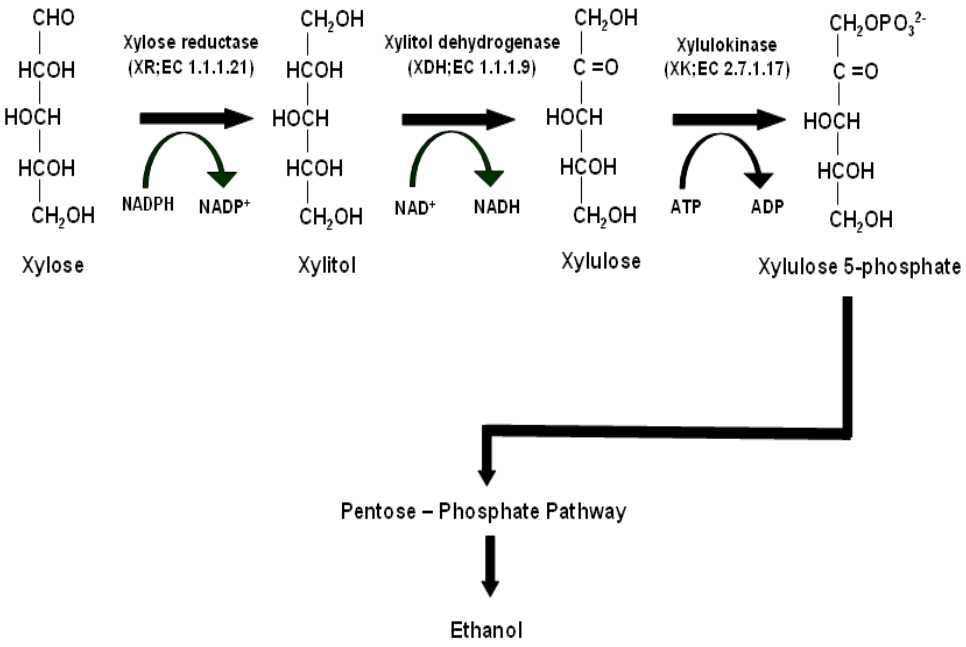


**Figure 1.** Schematic representation of xylose metabolic pathway to ethanol

Despite numerous efforts to alter coenzyme specificity, only a few modifications have successfully reverted the specificity to a level where mutant enzyme exhibits catalytic efficacy comparable to that of the wild type [14; 15]. In a prior study [16], site-directed mutagenesis was employed to fully reverse the coenzyme specificity of NAD+-dependent XDH from *Pichia stipitis*, converting it to NADP+-dependent XDH. These mutated enzymes were then analyzed *in vitro*. In the current study, we engineered recombinant yeasts through transformation with genes that encode the fully $NADP^+$- dependent XDH and a wild type XR from *P. stipitis*. The evaluation of these recombinant yeasts was conducted with respect to their enzymatic activity and capacity to ferment xylose into ethanol. The engineered enzymes demonstrated substantial expression levels in *Saccharomyces cerevisiae*, as indicated via *in vitro* enzyme activity assays. Fermentation for ethanol production was performed under anaerobic conditions using batch cultures. The presence of $NADP^+$-dependent XDH appeared to prevent xylitol formation, possibly by contributing to the stabilization of the intracellular redox state.

## MATERIALS AND METHODOLOGY

### Materials and Media

*Saccharomyces cerevisiae* D452-2 (*MATa leu2 his3ura3can1*) [17] was utilized as the yeast strain for expressing *Pichia stipitis* XDH and XR. Transformation was carried out in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). For other experiments, we used a defined minimal medium (6.7 g/l yeast nitrogen base without amino acids, 5 g/l glucose). For strain D452-2, leucine, histidine, and uracil were added at 20 mg/l, 20 mg/l, and 5 mg/l, respectively. Bacterial strains were cultured in Luria-Bertani medium (5 g/l yeast extract, 10 g/l peptone, 10 g/l sodium chloride) with ampicillin (50 mg/l) for selection. For solid media, 20 g/l agar was added.

### Yeast Strain Transformation

The lithium acetate method was utilized to conduct the yeast transformation [18]. Cells were plated on selective medium after an overnight incubation in YPD. Minimal medium agar plates containing 20 mg/l histidine, 20 mg/l leucine, and 5 mg/l uracil were utilized to select the *S. cerevisiae* transformants.

### Enzyme Assays

Enzyme activity measurements were carried out using a V-550 spectrophotometer (JASCO Corporation, Japan), set at 340 nm and 35°C. For XDH assays, the reaction mixture consisted of 50 mM $MgCl_2$ and 300 mM xylitol in 50 mM Tris-HCl buffer at pH 9.0. In the case of XR, the assay mixture contained 60 mM sodium phosphate and 133 mM xylose [13]. Each reaction was initiated by adding 0.10 ml of 20 mM NAD/NADP for XDH and 0.10 ml of 1.5 mM NADPH/NADH for XR, bringing the total volume to 1.0 ml. A single unit of enzyme activity is defined as the production of 1 µmol of NAD(P)+H per minute. The Lowry method was utilized to quantify the protein concentrations [19], employing bovine serum albumin as a standard.

### Preliminary Batch Fermentation in Shake Flask

Initially, 15 ml Falcon tubes with 3 ml of minimum media were utilized to cultivate the recombinant yeast strains for a duration of 3 days. Aerobic culture of the cells was subsequently conducted in 100 ml minimum medium within 300 ml flasks, maintained at 30°C and shaken at 200 rpm for a further 3 days. Following centrifugation at 5000 rpm for 10 minutes and subsequent washing with 9 g/l NaCl, the cell pellets were inoculated into 200 ml of fermentation medium consisting of 6.7 g/l yeast nitrogen base devoid of amino acids, 5 g/l glucose, 15 g/l xylose, and necessary amino acids. The process of ethanol fermentation took place in 200 ml flasks, which were sealed using two layers of Saran wrap to maintain anaerobic conditions. Periodic samples of 1 ml were collected and preserved at -35°C for the analysis of substrates and products.

### Batch Fermentation in Bioreactor

Anaerobic batch fermentation was performed using a BioFlo 110 bioreactor (New Brunswick Scientific Co.). Recombinant yeast strains were precultured for 3 days in 5 ml of minimal medium within 15 ml Falcon tubes, followed by an additional 3 days of aerobic cultivation in 300 ml of minimal medium in a 1-liter flask. After centrifugation, the cell pellets were washed with a 9 g/l NaCl solution and then transferred to a fermentation medium containing 6.7 g/l yeast nitrogen base without amino acids, supplemented with 5 g/l glucose, 15 g/l xylose, and the necessary amino acids. Prior to autoclaving, the culture medium was supplemented with 0.5 ml/l of silicone antifoam. The fermentation process was then executed in a 1.3-liter bioreactor containing 1 liter as the initial volume. Conditions were maintained at 30°C, pH was regulated at 5.5 using 2 M NaOH and 1 M $H_2SO_4$, and agitation was kept constant at 500 rpm.

### Measurement of Intracellular Coenzyme Contents

Xylose and glucose, both at a concentration of 10 g/l, were used in minimal medium to extract intracellular coenzymes from cells grown under oxygen-limiting conditions. A 20 ml yeast culture was rapidly mixed with 80 ml of 60% methanol under temperature of -40°C to quench the reaction. Coenzymes were extracted in 50 mM potassium phosphate (pH 5.0) for NAD+ and NADP+, or 50 mM Tris/HCl (pH 9.0) for NADH and NADPH. Coenzyme concentrations were determined by measuring absorbance at 339 nm with specific coupling enzymes at room temperature [20]: alcohol dehydrogenase (Sigma) for NAD+, glucose-6-phosphate dehydrogenase (Sigma) for NADP+, glycerol-3-phosphate dehydrogenase (Wako) for NADH, and glutamate dehydrogenase (Oriental Yeast) for NADPH.

### Analysis of Fermentation Products

Gas chromatography was utilized to analyze the concentration of ethanol using a model GC-14B (Shimadzu Corporation, Japan), equipped with a flame ionization detector. The setup included a 2.0-meter glass column with a 3.2 mm internal diameter, packed with Thermon-3000, column temperature at 70°C, injector at 200°C, detector at 250°C, and nitrogen as the carrier gas at a flow rate of 25 ml/min. For glucose, xylose, xylitol, glycerol, and acetic acid, high-performance liquid chromatography (Tosoh Corporation Ltd., Japan) with an RI detector was used, employing a Bio-Rad HPX-87H ion-exclusion column and a mobile phase of 5 mM $H_2SO_4$ at 0.4 ml/min. Absorbance at 600 nm was utilized to monitor cell growth, employing a spectrophotometer (Model U-2001, Hitachi, Japan).

## RESULTS AND DISCUSSION

### Expression of *P. stipitis* XR and XDH mutants in *S. cerevisiae*

Plasmids for XDH expression in yeast pPGK-WT, pPGK-ARS, pPGK-ARSdR, pPGK-C4, pPGK-C4/ARS, and pPGK-C4/ARSdR were created by inserting either the wild-type (WT) or mutated XDH expression regions [16] into the pPGK yeast expression vector [21]. This vector, a 2 μm DNA-based shuttle vector, includes a phosphoglycerate kinase (PGK) promoter and terminator for expression, a multicloning site, and the URA3 gene for yeast selection (Figure 2). For XR expression, the XR coding region [22] was first inserted into the pPGK vector, and then this XR region with the PGK promoter and terminator was excised and transferred into the YEpM4 vector [23], another 2 μm DNA-based shuttle vector with a multicloning site and LEU2 gene for yeast selection (Figure 3), resulting in the plasmid pPGK-XR. The yeast strain S. cerevisiae D452-2 (MATa leu2 his3ura3canl) [17] was used for expressing both XDH and XR. This strain was first transformed with pPGK-XR, and then the resulting transformants were further transformed with pPGK-WT, pPGK-ARS, pPGK-ARSdR, pPGK-C4, pPGK-C4/ARS, and pPGK-C4/ARSdR to generate recombinant yeast strains Y-WT, Y-ARS, Y-ARSdR, Y-C4, Y-C4/ARS, and Y-C4/ARSdR, respectively. Additionally, pPGK and YEpM4 were transformed into D452-2 to create the control strain Y-Vector.

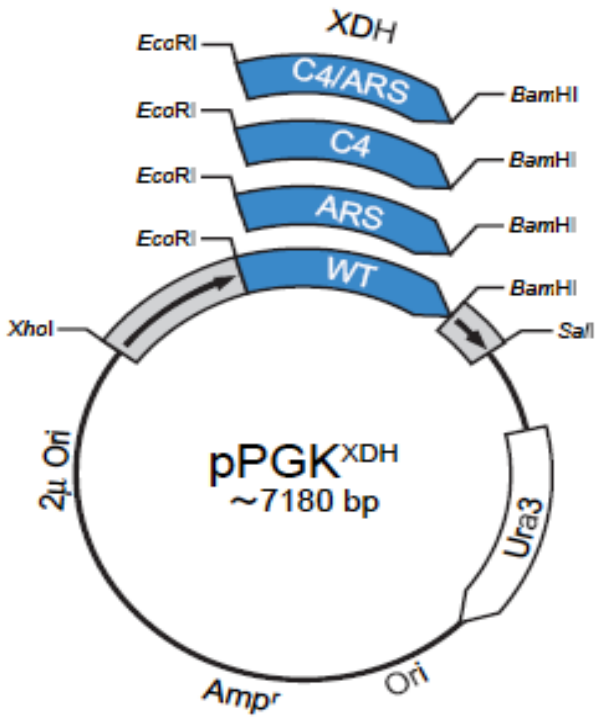


**Figure 2.** Schematic diagram of the yeast expression vector pPGK

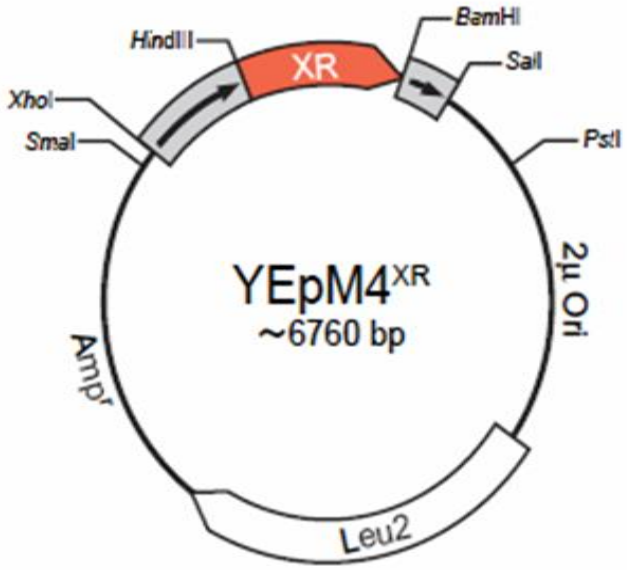


**Figure 3.** Schematic diagram of the yeast expression vector YEpM4

## Effect of Psxdh Mutants in *S. Cerevisiae*

Normal growth was observed for each recombinant *S. cerevisiae* strain developed in this study when cultured in minimal medium (data not shown). Following cultivation in minimal medium, the strains were harvested and disrupted with glass beads to obtain crude extracts, as detailed in the Materials and Methods section. Spectrophotometric methods were employed to quantify the activities of XDH and XR in the prepared crude extracts. Figure 4 illustrates that the Y-ARS strain demonstrated a significant increase in enzyme activity with NADP+, exceeding that of Y-WT by more than eightfold. The Y-ARSdR strain, which contains a quadruple mutant XDH, demonstrated 13 times more enzyme activity with NADP+ relative to Y-WT. The Y-C4 strain, which includes an additional zinc-introduced XDH, showed a 5-fold increase in enzyme activity with NAD+ compared to Y-WT. The Y-C4/ARS and Y-C4/ARSdR strains displayed, respectively, 22-fold and 11-fold higher XDH enzyme activity with NADP+. XR activities in these recombinant yeasts were nearly identical (Table 1). The XDH and XR activities observed in the control strain were likely due to endogenous XDH and XR activities.

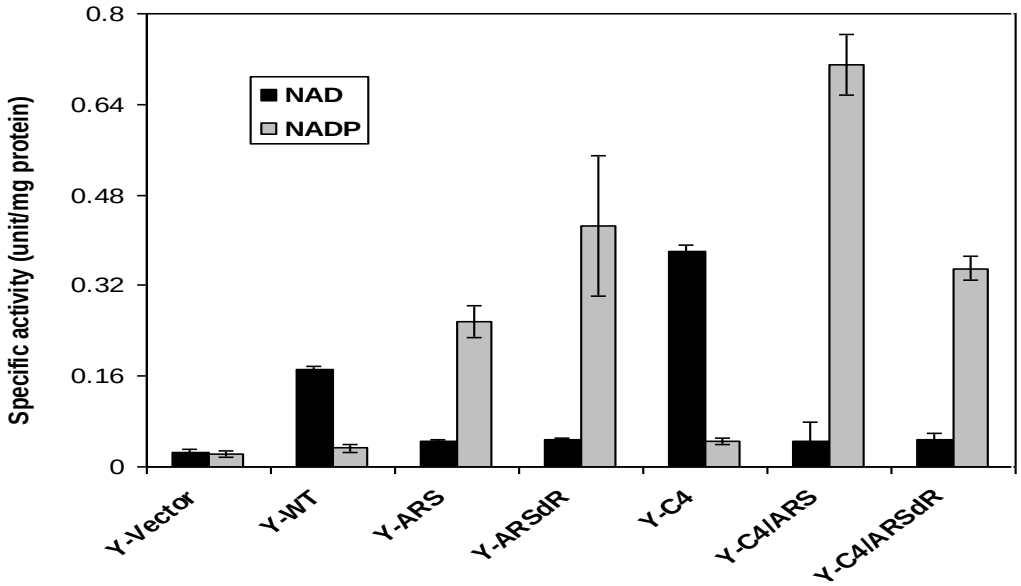


**Figure 4.** Specific enzyme activities of xylitol dehydrogenase (protein engineered XDH) in the recombinant *S. cerevisiae* were assayed in the direction of polyol oxidation by measuring the reduction of $NAD^+$ or $NADP^+$. Values are the means ± S.D., n = 3.

**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 |
| Y-C4 | 0.050 ± 0.003 | 0.381 ± 0.029 | 0.045 ± 0.002 |
| Y-C4/ARS | 0.045 ± 0.008 | 0.046 ± 0.032 | 0.709 ± 0.054 |

## Effect of NADP-Preferring XDH Mutant in Ethanol Fermentation

The recombinant yeasts were cultivated at 30°C in an incubator, containing a shaker followed by the evaluation of their ethanol production and xylitol excretion. Single fermentation batch (100 ml) was initiated using 5 g/l glucose and 15 g/l xylose (Figure 5). Ethanol concentration increased only during the initial phase (3–25 hours) in the Y-Vector strain, likely as a result of glucose consumption. Y-WT produced ethanol at 2.22 g/l and excreted xylitol at 1.42 g/l. Y-C4 yielded ethanol at 2.58 g/l, which was

higher than Y-WT, but still excreted a relatively high amount of xylitol (1.24 g/l). Y-ARSdR produced the highest ethanol concentration at 2.97 g/l and the lowest xylitol excretion at 0.16 g/l. Y-ARS, Y-C4/ARS, and Y-C4/ARSdR produced ethanol at 2.79, 2.82, and 2.91 g/l, respectively. All recombinant yeast strains completely fermented glucose within 25 to 40 hours, while 25–58% of xylose was fermented within 68 hours.

Given that Y-ARSdR demonstrated the highest ethanol production and lowest xylitol excretion, it was further examined in a high-performance bioreactor under anaerobic conditions and compared with Y-WT. Y-ARSdR achieved an ethanol production of 7.02 g/l with a yield of 0.46 g of ethanol per gram of total consumed sugars (Figure 6). The ethanol yield from consumed sugars reached 90% of the theoretical maximum, and xylitol excretion was below 0.35 g/l. The xylitol yield was 0.2 g/g of consumed sugars. Glucose was completely fermented within 15 hours, and 68% of xylose was consumed within 72 hours. A minor amount of glycerol (0.28 g/l) and acetic acid (0.27 g/l) was produced mainly during glucose consumption.

## Intracellular Coenzyme Levels

The concentrations of NADH, NAD+, NADPH, and NADP+ were measured in cells of the recombinant strains during the late exponential growth phase (Table 2). There were no significant differences in the levels of these coenzymes between early, middle, and late exponential growth phases.

Intracellular redox states were assessed using the ratios of NADH/NAD+ and NADPH/NADP+ (designated as $R_{NAD(H)}$ and $R_{NADP(H)}$, respectively). Compared to Y-Vector, the $R_{NADP(H)}$ in Y-WT was three times higher, while $R_{NAD(H)}$ was reduced to half. Notably, Y-ARSdR exhibited $R_{NAD(H)}$ and $R_{NADP(H)}$ values that were closer to those of Y-Vector than Y-WT, suggesting that the NADP-preferring mutation in PsXDH helped maintain redox balance in the yeast cells

**Table 2.** Intracellular concentration of coenzymes in recombinant yeast cells

| Strain | Intracellular concentration (μmol/g dry weight of biomass) | | | | $R_{NADH}$ * | $R_{NADPH}$¶ |
|---|---|---|---|---|---|---|
| | $NAD^+$ | NADH | $NADP^+$ | NADPH | | |
| Y-Vector | 6.58 ±0.11 | 3.19 ± 0.29 | 3.41 ± 0.18 | 5.24 ± 0.01 | 0.49 ± 0.01 | 1.54 ± 0.08 |
| Y-WT | 8.26 ± 0.16 | 2.69 ± 0.08 | 1.68 ± 0.04 | 8.24 ± 0.14 | 0.33 ± 0.01 | 4.92 ± 0.18 |
| Y-ARSdR | 6 .45 ±0.13 | 3.02 ± 0.01 | 5.55 ± 0.04 | 7.53 ± 0.19 | 0.47 ± 0.01 | 1.36 ± 0.04 |

*Ratio of NADH/$NAD^+$
Ratio of NADPH/$NADP^+$

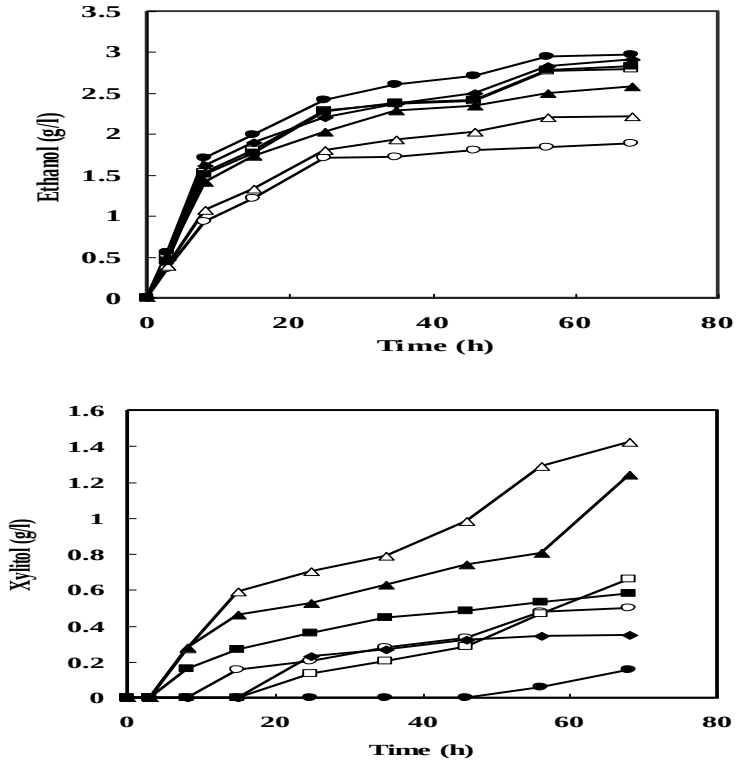


**Figure 5.** Time dependent ethanol production (upper panel) and xylitol excretion (bottom panel) profiles of Y-Vector (open circle), Y-WT (open triangle), Y-ARS (open square), Y-ARSdR (closed circle), Y-C4 (closed triangle), Y-C4/ARS (closed square), and Y-C4/ARSdR (closed rhombus) grown in shaker flasks.

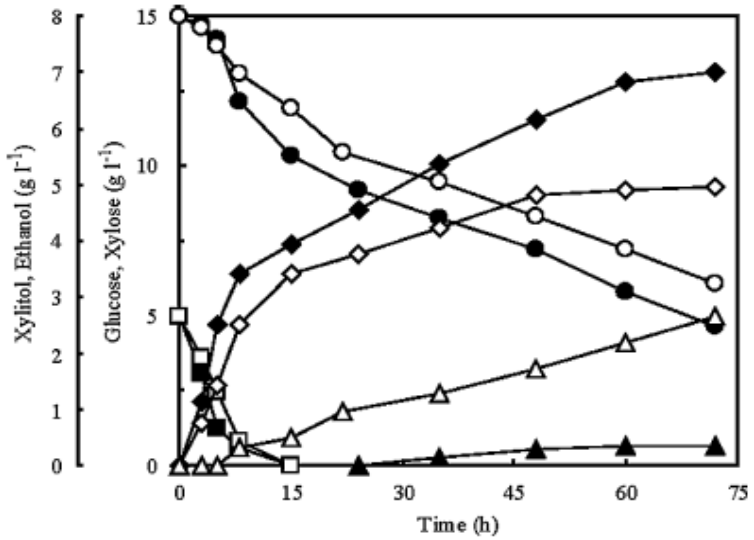


**Figure 6.** Time dependent xylose (circle) and glucose (square) utilization and ethanol (rhomboid), and xylitol (triangle) concentration profiles of Y-WT (open symbol) and Y-ARSdR (closed symbol) grown in a bioreactor.

## CONCLUSION

In this study protein engineering was utilized to create enzymes with completely reversed coenzyme specificity and developed recombinant yeast strains carrying these engineered enzymes to establish an efficient biomass-to-ethanol conversion system. A key finding from this study is the impact of PsXDH modifications (*in vitro*) on the fermentation of xylose to ethanol (*in vivo*). One notable modification to PsXDH is its thermostabilization through the incorporation of a structural zinc ion. Three thermostable mutants with this C4 mutation (C4, C4/ARS, and C4/ARSdR) were expressed at significantly higher levels than their parent enzymes in *S. cerevisiae* cells, similar to the expression levels in *E. coli* cells. Overexpressing XDH in *S. cerevisiae* using a promoter-modification approach could prevent xylitol formation, though it only slightly enhanced ethanol yield. A similar trend was observed between Y-WT and Y-C4, but no positive effects were seen with Y-C4/ARS and Y-C4/ARSdR compared to Y-ARS and Y-ARSdR. Another functional modification of PsXDH in this study involved shifting its coenzyme specificity toward NADP+. Both Y-ARS and Y-ARSdR mutants showed similar reversal of coenzyme specificity toward NADP+ (under coenzyme-saturated conditions *in vitro*), with Y-ARSdR demonstrating better fermentation efficiency, notably reduced xylitol excretion, compared to Y-ARS. The intracellular coenzyme concentration in Y-ARSdR may be optimized for NADP+, with a 22% decrease in NAD+ and a 230% increase in NADP+ relative to Y-WT. The Y-C4/ARS strain, which expressed the thermostable and NADP+-dependent XDH, exhibited a 84% reduction in undesired xylitol excretion and a 28% increase in ethanol production compared to the wild-type XDH strain. In summary, shifting coenzyme dependency proves more effective than merely increasing enzyme activity through stabilization. Combining these strategies using protein-engineered enzymes should be considered to enhance ethanol production from xylose using recombinant S. cerevisiae.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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