

Changing Flux of Xylose Metabolites by Altering Expression of Xylose Reductase and Xylitol Dehydrogenase in Recombinant *Saccharomyces cerevisiae*

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Abstract

We changed the fluxes of xylose metabolites in recombinant *Saccharomyces cerevisiae* by manipulating expression of *Pichia stipitis* genes (*XYL1* and *XYL2*) coding for xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively. *XYL1* copy number was kept constant by integrating it into the chromosome. Copy numbers of *XYL2* were varied either by integrating *XYL2* into the chromosome or by transforming cells with *XYL2* in a multicopy vector. Genes in all three constructs were under control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. Enzymatic activity of XR and XDH in the recombinant strains increased with the copy number of *XYL1* and *XYL2*. XR activity was not detected in the parent but was present at a nearly constant level in all of the transformants. XDH activity increased 12-fold when *XYL2* was on a multicopy vector compared with when it was present in an integrated single copy. Product formation during xylose fermentation was affected by XDH activity and by aeration in recombinant *S. cerevisiae*. Higher XDH activity and more aeration resulted in less xylitol and more xylulose accumulation during xylose fermentation. Secretion of xylulose by strains with multicopy *XYL2* and elevated XDH supports the hypothesis that D-xylulokinase limits metabolic flux in recombinant *S. cerevisiae*.

Index Entries: Metabolic flux; metabolic engineering; xylose; xylose reductase; xylitol dehydrogenase.

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Introduction

Xylose-fermentation in lignocellulose hydrolysate is indispensable for economic conversion of biomass to fuels and chemicals (1). *Saccharomyces cerevisiae* is widely used for the industrial fermentation of glucose to ethanol because it has the capacity for high specific ethanol production rates (2,3) and will tolerate high ethanol concentrations (4). However, native strains of *S. cerevisiae* do not use xylose as a carbon source for growth (5–7). In contrast to *S. cerevisiae*, other yeasts use xylose very well. One of the best xylose-fermenting yeasts, *Pichia stipitis*, has been studied extensively for the regulatory and physiologic properties that enable it to ferment xylose (8), and it has served as the source of genes for engineering xylose metabolism in *S. cerevisiae*. The *XYL1* and *XYL2* genes coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis* have been expressed in *S. cerevisiae* (9–22). The resulting transformants can grow on xylose aerobically but only produce significant amounts of ethanol in low yield under oxygen-limited conditions (13). This results in xylitol accumulation in the medium as a byproduct, which is a major obstacle in obtaining high ethanol production from xylose. XR from *P. stipitis* has a high affinity for NADPH even though it can use NADH as a cofactor (14). However, *P. stipitis* XDH only uses NAD⁺ as a cofactor (15). This difference results in cofactor imbalance in the cytosol during xylose metabolism.

Bruinenberg et al. (16) hypothesized that xylitol accumulates during the metabolism of xylose because yeasts cannot oxidize NADH to NAD⁺ efficiently through respiration under oxygen-limited conditions, and because the cytosolic NAD⁺/NADH ratio is unfavorable to the XDH reaction. Walfridsson et al. (11) reported that the ratio of XR and XDH activity is important in reducing xylitol formation in the fermentation of glucose/xylose mixtures. They found that a strain with a lower ratio (0.06) of XR/XDH activity accumulated less xylitol and produced more ethanol than a strain with a higher ratio (17.5). However, they also obtained very low xylose consumption rates and did not report the effect of aeration (11). This prompted us to investigate whether or not increasing XDH activity can reduce xylitol accumulation during metabolism of xylose alone under aerobic and oxygen-limited conditions. To alter XDH activity, we introduced the *XYL2* gene into the *XYL1* integrated *S. cerevisiae* either by integration into chromosome or by expression from a multicopy plasmid. To determine whether we could further alter the ratio of reduced and oxidized intermediate metabolites formed by the engineered cells, we also studied the effect of aeration.

Materials and Methods

Microbial Strains and Plasmids

The microbial strains and plasmids used are listed in Table 1 (12,17,18). We routinely used *Escherichia coli* DH5a (*F⁻ recA1 endA2 hsdR17 [r_K⁻ m_K⁺]*)

Table 1
Plasmids and Microbial Strains Used

Name	Description	Reference
Plasmids		
pY2XR	<i>TRP1</i> , 2 μ m origin, <i>GAPDH_p-PSXYL1-GAPDH_t</i>	12
pY2XDH	<i>TRP1</i> , 2 μ m origin, <i>GAPDH_p-PsXYL2-GAPDH_t</i>	12
pRS305	Yeast integration vector, <i>URA3</i>	20
pRS306	Yeast integration vector, <i>LEU2</i>	20
pYS10	<i>URA3-GAPDH_p-PsXYL1-GAPDH_t</i>	This study
pYS20	<i>LEU2-GAPDH_p-PsXYL2-GAPDH_t</i>	This study
Strain		
<i>S. cerevisiae</i> L2612	<i>MATa leu2-3 leu2-112 ura3-52</i> <i>trp1-298 can1 cyn1 gal+</i>	17
<i>S. cerevisiae</i> FPL-YS10	Isogenic of L2612 except for <i>leu2::LEU2-PsXYL1</i>	This study
<i>S. cerevisiae</i> FPL-YS1020	Isogenic of L2612 except for <i>leu2::LEU2-PsXYL1</i> , <i>ura3::URA3-PsXYL2</i>	This study
<i>S. cerevisiae</i> FPL-YS1022	FPL-YS10 (pYXDH)	This study
<i>P. stipitis</i> FPL-UC7	<i>ura3-3</i> , NRRL Y-21448	18

supE44 thi-1 gyrA relA1)(Gibco-BRL, Gaithersburg, MD) for gene cloning and manipulation.

Enzymes, Primers and Chemicals

Restriction enzymes, DNA-modifying enzymes, and other molecular reagents were obtained from New England Biolabs (Beverly, MA), Promega (Madison, WI), Stratagene (La Jolla, CA), and Roche (Indianapolis, IN). Reaction conditions were as recommended by the suppliers. All general chemicals were purchased from Sigma (St. Louis, MO).

Media and Culture Conditions

Yeast and bacterial strains were stored in 15% glycerol at -70°C . Yeast strains were routinely cultivated at 30°C in YP medium (10 g/L of yeast extract, 20 g/L of Bacto peptone) with either 20 g/L of glucose (YPD), 20 g/L of xylose (YPX-2%), or 40 g/L of xylose (YPX-4%). YPD or YPX plus 20 g/L of agar was used for plates. To select for yeast transformants using the *URA3*, *TRP1*, or *LEU2* selectable markers, we used

6.7 g/L of yeast nitrogen base (YNB) without amino acids plus 20 g/L of glucose, 20 g/L of agar, and a mixture of appropriate nucleotides and amino acids. To prevent loss of pY2XDH during inoculum development on glucose, we used yeast synthetic complete (YSC) medium, consisting of 6.7 g/L of YNB plus 20 g/L of glucose supplemented with all necessary amino acids and nucleotides except tryptophan. For fermentation, recombinant strains were cultivated in YPX-2% or YPX-4% with xylose as the sole sugar source, which maintained the xylose metabolism genes. Aeration conditions were modulated by the choice of flask type or agitation speeds in a shaker. We cultivated cells in 50 mL of medium in a 125-mL Erlenmeyer flask shaken at 100 or 200 rpm or in a 125-mL baffled flask shaken at 200 rpm. The oxygen transfer rates were determined by the sulfite method (19). All cultures were incubated at 30°C.

Plasmid Construction

The plasmids used are summarized in Table 1. To construct an integration vector, pY2XR (12) was digested with *Hind* III. The 2.3-kbp *Hind* III-*Hind* III fragment, which contains *PsXYL1* between the *S. cerevisiae* GAPDH promoter and terminator, was inserted into the *Hind* III site of pRS305 (20). The resulting plasmid was named pYS10. In a similar manner, the 2.8-kbp *Hind* III-*Hind* III fragment, having *PsXYL2* between the GAPDH promoter and terminator, was isolated from pY2XDH (12). The vector pYS20 was constructed by inserting the 2.8-kbp *Hind* III-*Hind* III fragment into the *Hind* III site of pRS306 (20).

Yeast Transformation

A yeast EZ-Transformation kit (BIO 101, Vista, CA) or Alkali-Cation Yeast Kit (BIO 101) was used for yeast transformations. Integration vectors were linearized with an appropriate enzyme prior to transformation. Transformants were selected on YSC medium (21) containing 2% glucose with 20 g/L of agar plus appropriate nutritional supplements.

Preparation of Crude Extract and Enzyme Assays

Yeast cells were grown to midlog phase at 30°C in YSC with glucose supplemented with appropriate nucleotides and amino acids. Cells were harvested by centrifuging at 3000g for 5 min. The cell pellet was washed and suspended in the buffer (50 mM phosphate buffer, pH 6.5). The suspended cells were mixed with glass beads (Sigma) and vortexed at maximum speed for 5 min at 4°C. Vortexing was repeated two or three times with intermittent cooling on ice. Cell debris was removed by centrifuging at 13,000g for 10 min. XR (EC 1.1.1.21) activity was measured in a reaction mixture with the following composition: 50 mM phosphate buffer, pH 6.0; 100 mM xylose; and 0.4 mM NADPH (24). XDH (EC 1.1.1.9) activity was measured in a reaction mixture containing 50 mM Tris-HCl buffer, pH 8.5; 4 mM NAD⁺, 5 mM MgCl₂, and 100 mM xylitol (15). We used a photodiode array spectrophotometer (Hewlett Packard, Wilmington, DE)

to monitor the rate of NADPH oxidation and NAD⁺ reduction in the reaction by absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes 1 μ mol of substrate/min at 30°C. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

Analytical Methods

Glucose, xylose, xylitol, xylulose, and ethanol concentrations were determined by high-performance liquid chromatography (Hewlett Packard) with an ION 300 column (Interaction Chromatography, San Jose, CA). Cell growth was monitored by optical density at 600 nm (OD₆₀₀). One unit at 600 nm was equivalent to 0.167 g of dry cell/L.

Results and Discussion

*Construction of Recombinant *S. cerevisiae* Strains*

Having Different Levels of XDH Activity

The vector pYS10 containing *PsXYL1* under control of *S. cerevisiae* GAPDH_p was cut with *StuI* within *URA3* and integrated into the chromosome of *S. cerevisiae* L2612 by homologous recombination. We observed a significant increase in XR activity after transformation of pYS10. Crude extract from FPL-YS10 showed XR activity of 0.367 U/mg, whereas cell homogenate from L2612 did not show measurable XR activity (Table 2). We then introduced *PsXYL2* under the control of GAPDH_p into FPL-YS10 by two methods. First, we integrated the linearized pYS20 containing *PsXYL2* into the *S. cerevisiae* chromosome at the *LEU2* locus to construct FPL-YS1020. Second, we transformed FPL-YS10 with the multi-copy vector pY2XDH, which resulted in FPL-YS1022. As expected, specific activity of XR was almost the same in both strains, but XDH activity increased 12-fold (0.625 vs 0.051 U/mg) in FPL-YS1022 compared with FPL-YS1020 (Table 2). Even though we used GAPDH_p for both the *XYL1* and *XYL2* expression cassettes, XR activity was seven times higher than XDH. XR activity remained essentially constant even when *XYL2* was highly expressed from the multicopy (2 μ) vector in FPL-YS1022.

Xylose Fermentation by Recombinant YS1020 and YS1022

Growth on xylose provides selective pressure to maintain genes coding for xylose assimilation (12,22). The parental strain L2612 and FPL-YS10 could not grow on YPX-4%. Therefore, we used YP medium with 40 g/L of xylose for xylose fermentation with the recombinant strains FPL-YS1020 and FPL-YS1022. However, in order to prevent loss of the pY2XDH plasmid from FPL-YS1022 during preculture with glucose, YNB medium supplemented with appropriate amino acids and nucleotides was used for inoculum preparation. To change the oxygen transfer rate, fermentation was performed with 50 mL of medium in a 125-mL Erlenmeyer flask or a 125-mL baffled flask, each of which was shaken at 200 rpm. Both

Table 2
In Vitro Enzyme Activities in Crude Extract
from Cell Cultures of Yeast Strains

Strains	Specific enzyme activity (U/mg) ^a		
	XR	XDH	Xylulokinase
L2612	ND	ND	ND
YS10	0.367	ND	ND
YS1020	0.354	0.051	ND
YS1022	0.357	0.625	ND
<i>P. stipitis</i> UC7	0.881	0.129	0.529

^aData are the average of at least two experiments and SDs were <10% for all assays. ND, not detected.

recombinant strains consumed and grew on xylose under both conditions. Xylitol and xylulose accumulated in the media as byproducts. An insignificant amount of ethanol (<1 g/L) was detected at the end of fermentation in both cultures (Fig. 1). Interestingly, the pattern of byproduct formation differed with the copy number of *PsXYL2* gene and with the oxygen transfer rate (Fig. 2). The FPL-YS1022 strain, containing *PsXYL2* in a multicopy vector, produced more xylulose and less xylitol as compared to the FPL-YS1020 strain that has *PsXYL2* integrated into the chromosome. The changes in XDH activity clearly affected metabolic fluxes and resulted in altered byproduct formation patterns. The higher XDH activity resulted in more xylulose and less xylitol accumulation in recombinant *S. cerevisiae* (Fig. 1).

Accumulation of xylulose in the medium has not been reported previously even though there have been many studies of *XYL1* and *XYL2* expression in *S. cerevisiae* (9-12). This could be owing to product analysis methods used in previous studies or to a difference among host strains. Xylitol and xylulose accumulation were also affected by aeration. The FPL-YS1022 strain accumulated more xylulose when cultivated in a baffled flask than when cultivated in an Erlenmeyer flask (3.4 vs 2.6 g/L of xylulose), whereas the FPL-YS1020 strain accumulated almost the same amount of xylulose in both flasks (Fig. 2). This suggested that XDH activity was limiting in FPL-YS1020 because increased aeration did not enhance xylulose accumulation. However, FPL-YS1022, which showed higher XDH activity, accumulated less xylitol with increased aeration. The increase in xylulose accumulation with increased expression of *XYL2* in FPL-YS1022 provided additional evidence that D-xylulokinase limits xylose metabolism in *S. cerevisiae* (22). The most likely limitation is low expression of endogenous *S. cerevisiae* xylulokinase (*ScXKS1*) (23). Alternatively, *S. cerevisiae* could be limited by other enzymes in the pentose phosphate pathway (24).

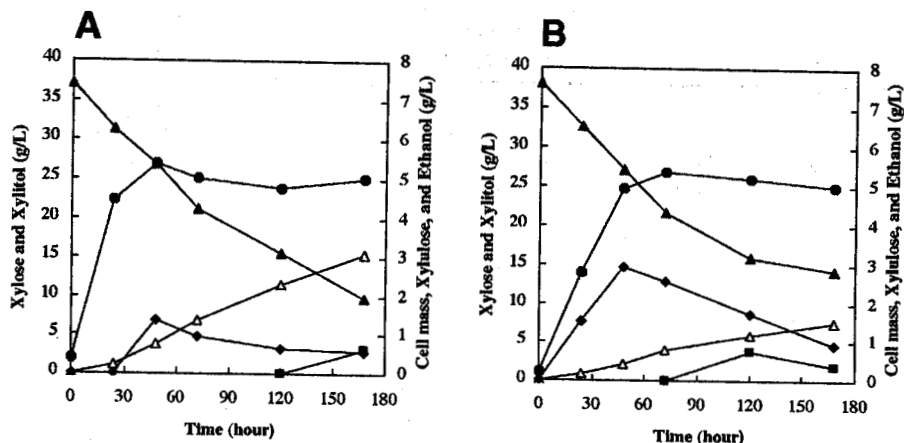


Fig. 1. Xylose-fermentation profiles by recombinant *S. cerevisiae* expressing *PsXYL1* and *PsXYL2*: (A) FPL-YS1020; (B) FPL-YS1022. Cells were cultured in YP medium with 40 g/L of xylose at an agitation rate of 200 rpm. (p) xylose; (n) cell mass; (r) xylitol; (®) xylulose; (¢) ethanol

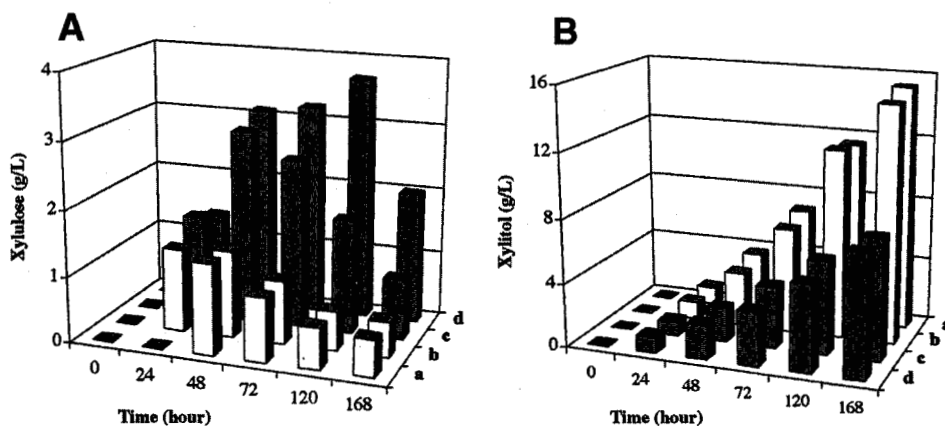


Fig. 2. Comparison of (A) xylulose and (B) xylitol production during xylose fermentation by recombinant *S. cerevisiae*. Strains and culture conditions: (a) FPL-YS1020 in an Erlenmeyer flask at 200 rpm; (b) FPL-YS1020 in a baffled flask at 200 rpm; (c) FPL-YS1022 in an Erlenmeyer flask at 200 rpm; (d) FPL-YS1022 in a baffled flask at 200 rpm.

Metabolic Flux Distributions in FPL-YS1020 and FPL-YS1022 Strains

Metabolic fluxes of the xylose assimilation steps were calculated from the xylose consumption rates, and the xylitol and xylulose accumulation rates (Table 3), during xylose fermentation by two of the recombinant *S. cerevisiae* strains (FPL-YS1020 and FPL-YS1022). As shown in Fig. 3, altering levels of XDH activity significantly altered metabolic flux distributions in xylose assimilation. Higher XDH activity in FPL-YS1022 decreased xylitol accumulation 20% and increased xylulose accumulation 54% com-

Table 3
Specific Rates During Xylose Fermentation by Recombinant *S. cerevisiae*

Strain	Oxygen transfer rate (mM O ₂ /h)	Specific rate (mM [g cell h] ⁻¹) ^a		
		Xylose uptake	Xylulose production	Xylitol production
YS1020	4.3	1.24 ± 0.27	0.22 ± 0.03	0.31 ± 0.02
YS1022	4.3	1.78 ± 0.26	0.34 ± 0.03	0.24 ± 0.01
YS1020	5.3	1.09 ± 0.06	0.22 ± 0.03	0.26 ± 0.02
YS1022	5.3	1.70 ± 0.28	0.37 ± 0.03	0.21 ± 0.01

aData represent the average ± SD of three replicates. Specific rates shown here were initial rates (from 0 to 24 h) of batch fermentations in YP medium with 40 g/L of xylose.

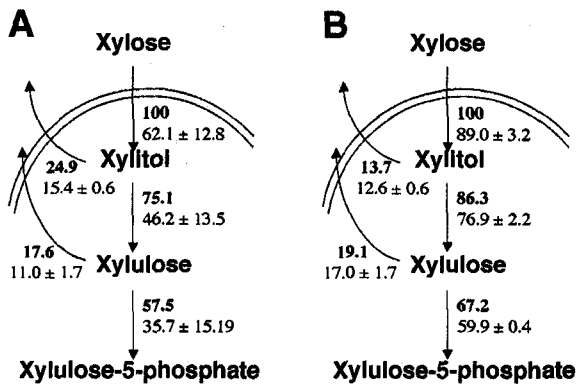


Fig. 3. Metabolic flux distributions in recombinant (A) *S. cerevisiae* FPL-YS1020 and (B) FPL-YS1022 during xylose fermentation. Fluxes were calculated from initial xylose consumption rates, and xylitol and xylulose accumulation rates of three independent batch fermentations in YP medium with 40 g/L of xylose at an oxygen transfer rate of 4.3 mM O₂/h. Fluxes are represented as the average ± SD in micromoles per gram of cell times hour. Bold figures correspond to the normalized fluxes with respect to xylose uptake flux.

pared with FPL-YS1020. Although there was more than a 12-fold increase in XDH activity in FPL-YS1022 compared with FPL-YS1020, the change in metabolic flux was not proportional to the increase in enzymatic activity. This clearly shows that not only enzyme activities but also physiologic states of cell are responsible for the control of metabolic flux in the xylose assimilation pathway.

Conclusion

During xylose fermentation by recombinant *S. cerevisiae* expressing *XYL1* and *XYL2* from *P. stipitis*, metabolic flux partitioning from xylitol

to xylulose depends on aeration and XDH activity. Increased aeration resulted in less xylitol accumulation and more xylulose accumulation. We hypothesize that the cytosolic NAD⁺/NADH ratio becomes more favorable for the XDH reaction under aerobic conditions. An increase in XDH activity could reduce xylitol formation. However, the metabolic flux from xylitol to xylulose does not increase proportionately with XDH activity.

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