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Original Article

Metabolic Engineering of *Saccharomyces cerevisiae* for Redox Balance of Xylose Fermentation

Soo Rin Kim[1]* and Yong-Su Jin[2]

[1]*School of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea*
[2]*Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

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Abstract The bioconversion of cellulosic biomass hydrolyzates consisting mainly of glucose and xylose requires the use of engineered *Saccharomyces cerevisiae* expressing a heterologous xylose pathway. However, there is concern that a fungal xylose pathway consisting of NADPH-specific xylose reductase (XR) and NAD^+-specific xylitol dehydrogenase (XDH) may result in a cellular redox imbalance. However, the glycerol biosynthesis and glycerol degradation pathways of *S. cerevisiae*, termed here as the glycerol cycle, has the potential to balance the cofactor requirements for xylose metabolism, as it produces NADPH by consuming NADH at the expense of one mole of ATP. Therefore, this study tested if the glycerol cycle could improve the xylose metabolism of engineered *S. cerevisiae* by cofactor balancing, as predicted by an *in-silico* analysis using elementary flux mode (EFM). When the *GPD1* gene, the first step of the glycerol cycle, was overexpressed in the XR/XDH-expressing *S. cerevisiae*, the glycerol production significantly increased, while the xylitol and ethanol yields became negligible. The reduced xylitol yield suggests that enough NAD^+ was supplied for XDH by the glycerol cycle. However, the *GPD1* overexpression completely shifted the carbon flux from ethanol to glycerol. Thus, moderate expression of *GPD1* may be necessary to achieve improved ethanol production through the cofactor balancing.

Keywords: elementary flux mode (EFM), glycerol metabolism, *GPD1*, xylitol

*Corresponding author: Soo Rin Kim
Tel: 82-53-950-7769; Fax:
E-mail: soorinkim@knu.ac.kr

Introduction

While corn- and sugarcane-based bioconversion processes can relieve the problems of using fossil fuels, their sustainability has been criticized due to the unavoidable competition with foods and their limited contribution to carbon emissions (Searchinger et al., 2008). In contrast, non-food crops, as well as crop residues and wood wastes, so called cellulosic biomass, could be promising alternatives as raw materials for current bioprocesses (Schmer et al., 2008). However, several technical difficulties still need to be overcome for the economic feasibility of cellulosic bioprocesses (Rubin, 2008).

One of the major challenges for cellulosic bioprocesses is the absence of a microbe that can efficiently ferment the mixed sugars, mainly glucose and xylose, derived from cellulosic biomass (Kim et al., 2012). *Saccharomyces cerevisiae* is an efficient host for producing bioethanol from corn and sugarcane that are mainly composed of glucose and fructose. The nature of *S. cerevisiae* that maximizes ethanol production by repressing respiration at high sugar concentrations has enabled this yeast to be the first choice for industrial bioprocesses. However, *S. cerevisiae* is unable to metabolize xylose and the other minor sugars derived from cellulosic biomass (Kim et al., 2013). Thus, many researchers have already attempted the metabolic engineering of *S. cerevisiae* to extend its substrate range, specifically to ferment xylose.

The heterologous expression of the xylose isomerase (XI) gene, as well as the overexpression of the xylulokinase (XK) gene allow *S. cerevisiae* to metabolize xylose though the native pentose phosphate pathway, followed by glycolysis (Kim, Park et al., 2013). However, the conversion of xylose to xylulose by XI is thermodynamically unfavorable. Alternatively, xylose reductase (XR) and xylitol dehydrogenase (XDH) can convert xylose to xylulose, yet these two enzymes have unbalanced cofactor preferences, NAD(P)H and NAD^+, respectively (Figure 1). Indeed, engineered *S. cerevisiae* strains expressing heterologous

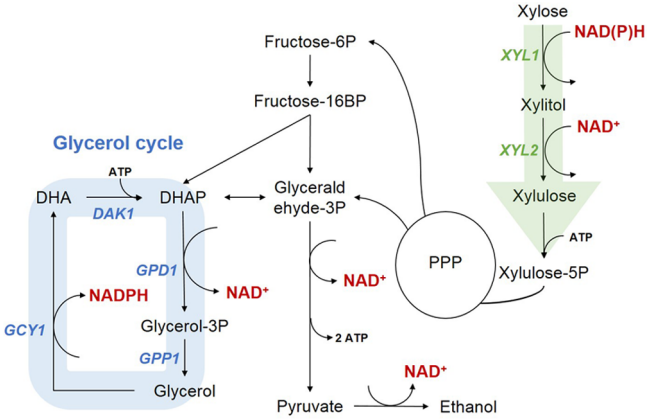

Figure 1. Cofactor recycling between xylose-metabolizing pathway and glycerol cycle. The heterologous xylose-catabolizing pathway, consisting of *XYL1*, *XYL2*, and *XYL3*, followed by the pentose phosphate pathway (PPP) and glycolysis, allowed *S. cerevisiae* to metabolize xylose. The glycerol cycle consists of NADH-consuming glyceraldehydes-3-phosphate dehydrogenase (*GPD1*), glycerol-3-phosphatase (*GPP1*), NADPH-producing glycerol dehydrogenase (*GCY1*), and ATP consuming dihydroxyacetone kinase (*DAK1*).

XR/XDH genes have shown limited xylose fermentation capability (Kim et al., 2013). Specifically, XR/XDH-expressing strains consumed xylose very slowly, accumulated byproducts, such as xylitol, and produced a low amount of ethanol. Thus, it has been hypothesized that the unbalanced cofactor preferences of the XR/XDH pathway may lead to a cellular redox imbalance, resulting in inefficient xylose fermentation.

As glycerol biosynthesis and degradation pathways, termed here as the glycerol cycle, also employ different types of cofactor, it was hypothesized that activating glycerol metabolism could serve as a redox balancing mechanism of xylose metabolism. Specifically, the glycerol cycle consisting of four enzymes (NADH-consuming glyceraldehydes-3-phosphate dehydrogenase, glycerol-3-phosphatase, NADPH-producing glycerol dehydrogenase, and ATP consuming dihydroxyacetone kinase in Figure 1) can convert NADH into NADPH at the expense of one ATP. If these reactions are operational, xylose fermentation could become redox neutral. To test this hypothesis, an elementary flux mode (EFM) analysis was performed to mathematically calculate the feasibility of redox balancing between xylose metabolism and the glycerol cycle. This prediction was also experimentally validated by up-regulating the glycerol cycle.

Materials and Methods

Elementary flux mode analysis

To computationally predict the positive effects of the glycerol cycle on xylose metabolism, an EFM analysis was performed as described previously (Quarterman et al., 2014). The product (xylitol, glycerol, and ethanol) yields (g/g xylose) were calculated from each EFM.

Strains and plasmids

To test the effect of *GPD1* overexpression on xylose metabolism, the *S. cerevisiae* SR1u strain was used. The SR1u strain is a uracil auxotrophic mutant of the prototrophic YSX3-pX2 strain (Kim et al., 2012), which expresses three heterologous genes (*XYL1*, *XYL2,* and *XYL3*, encoding XR, XDH, and XK, respectively) derived from *Sheffersomyces stipites*. The uracil auxotrophy was induced by introducing a Ty1 insertional mutation at the *URA3* gene (*ura3-52*), as described previously (Ha et al., 2013). An integrating plasmid (pRS406 $TEF1_P$-*GPD1*-$CYC1_T$) was constructed to overexpress the *GPD1* gene under the control of the *TEF1* promoter. The plasmid was linearized by *Xma*I treatment and integrated into the genome of the SR1u strain, resulting in the SR1u-*GPD1* strain.

Media preparation

A synthetic complete medium (6.7 g/L yeast nitrogen base with ammonium sulfite and 0.77 g/L CSM-Ura; MP Biomedicals, Solon,OH) containing 20 g/L D-glucose and 20 g/L agar was used to select the SR1u-*GPD1* transformants. For the xylose fermentation, a YP medium (10 g/L yeast extract and 20 g/L peptone) containing 40 g/L D-xylose (YPX) was used.

Fermentation and analysis

The preculture was made by inoculating a single colony into 5 mL YP medium containing 20 g/L glucose (YPD) and incubating it at 200 rpm for 36 h. The amount of cells equivalent to OD (optical density at 600 nm) 50 were resuspended in 50 mL YPX prepared in a 250 mL Erlenmeyer flask, yielding an initial cell concentration of OD 1. The flask was covered with foil and incubated at 100 rpm for 72 h. Samplings were performed every 24 h to analyze the cell density and extracellular metabolites, as described previously (Kim et al., 2012a).

Results and Discussion

EFM analysis predicted that glycerol cycle increases maximum ethanol yield from xylose

An EFM is defined as a minimal set of metabolic reactions that allow a steady state of the network. Thus, an EFM analysis allows a mathematical calculation of the feasible product profiles with a given substrate. Therefore, this approach was used to predict the metabolic outcomes caused by activating the glycerol cycle. The EFM analysis in this study used a yeast core metabolic network consisting of 55 reactions, 47 internal metabolites, and 7 external metabolites with xylose as the only substrate. As a result, the

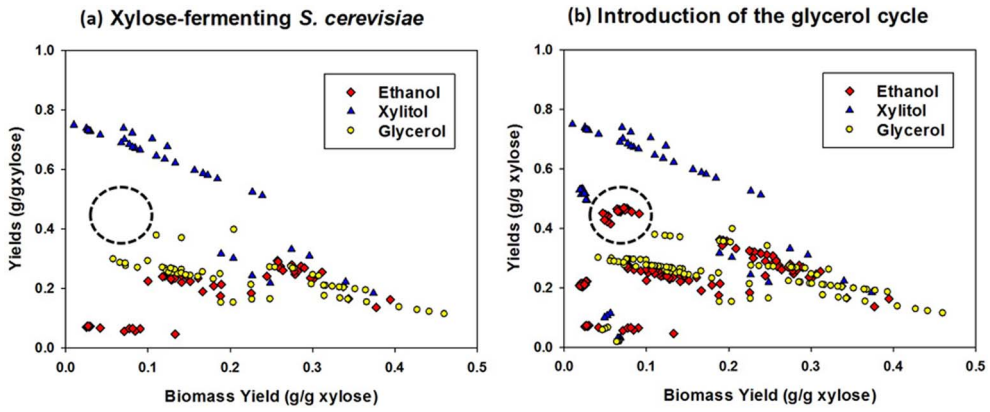

Figure 2. *In-silico* analysis of feasible product yields from xylose (g/g xylose) without (a) and with (b) glycerol cycle. Dotted lines represent increased maximum ethanol yields with the glycerol cycle.

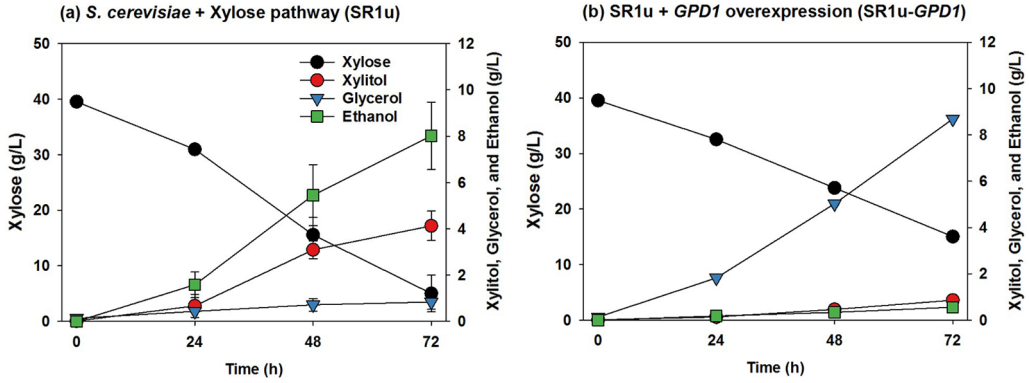

Figure 3. Xylose fermentation profiles of SR1u (*S. cerevisiae* expressing xylose pathway) and its *GPD1*-overexpressing mutant (SR1u-*GPD1*).

original model yielded 1249 EFMs, while the model with a complete glycerol cycle (glycerol dehydrogenase and DHA kinase in Figure 1) yielded 2044 EFMs. The xylitol, glycerol, and ethanol yields (mol/mol xylose) of each EFM were also calculated to determine the feasible product profiles for the original and glycerol models. As highlighted by the dotted lines in Figure 2, EFMs in the glycerol model had higher ethanol yields (0.46 g ethanol/g xylose) than that of the original model. This result supports the hypothesis that the glycerol cycle may have a positive impact on xylose fermentation by facilitating cofactor regeneration.

GPD1 overexpression led to glycerol production from xylose with no other products

To confirm the prediction experimentally, a xylose-fermenting strain, SR1u, was engineered by overexpressing the *GPD1* gene, the first step of glycerol biosynthesis. The resulting strain, SR1u-*GPD1*, was compared to the SR1u strain during the fermentation of 40 g/L xylose for 72 h. The original SR1u strain mainly produced ethanol (8.0 g/L) and some xylitol (4.1 g/L), with negligible glycerol (0.6 g/L). In contrast, the SR1u-*GPD1* strain produced significant glycerol (8.7 g/L), while the other products, such as xylitol and ethanol, became negligible (Figure 3). To better characterize the product profiles of the two strains, the carbon distribution was calculated from xylose to xylitol, glycerol, ethanol, biomass, and CO_2 (Table 1). Based on the assumption of 100% carbon recovery, an amount of CO_2 production was estimated. Apparently, the *GPD1* overexpression shifted the carbon distribution from xylitol and ethanol (42%) to glycerol (35%) with increased level of CO_2 production. The result suggests that the glycerol cycle induced the oxidative pentose phosphate pathway and respiratory metabolism, primarily for regenerating NADPH.

Table 1. Product yields (C-mol of product/C-mol of consumed xylose) for xylose fermentation

	Xylitol	Glycerol	Ethanol	Cell mass	CO_2*
SR1u	0.12±0.03	0.02±0.01	0.30±0.03	0.21±0.00	0.35±0.00
SR1u-*GPD1*	0.03±0.01	0.35±0.01	0.03±0.01	0.19±0.01	0.40±0.00

*CO_2 production was estimated by assuming 100% carbon recovery from consumed xylose.

Xylitol production during xylose fermentation is due to limited xylitol dehydrogenase (XDH) activity, resulting from either low expression levels of the enzyme or a shortage of the required cofactor, NAD^+. In this study, the SR1u-*GPD1* strain showed a significant reduction in xylitol accumulation compared to the original strain, indicating that the *GPD1* overexpression led to an

adequate supply of NAD^+ for XDH. However, the xylose consumption rate, the primary parameter for xylose reductase activity, was not improved by the *GPD1* overexpression, suggesting that the NADPH was limiting. Thus, overexpression of the *GCY1* gene encoding NADPH-producing glycerol dehydrogenase might be necessary to activate the glycerol cycle. In conclusion, this study confirmed that perturbation of glycerol metabolism can lead to significant changes in xylose fermentation.

Conflict of Interest

The authors declare no competing financial interests.

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