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Research Article

Dual utilization of NADPH and NADH cofactors enhances xylitol production in engineered *Saccharomyces cerevisiae*

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Xylitol, a natural sweetener, can be produced by hydrogenation of xylose in hemicelluloses. In microbial processes, utilization of only NADPH cofactor limited commercialization of xylitol biosynthesis. To overcome this drawback, *Saccharomyces cerevisiae* D452-2 was engineered to express two types of xylose reductase (XR) with either NADPH-dependence or NADH-preference. Engineered *S. cerevisiae* DWM expressing both the XRs exhibited higher xylitol productivity than the yeast strain expressing NADPH-dependent XR only (DWW) in both batch and glucose-limited fed-batch cultures. Furthermore, the coexpression of *S. cerevisiae ZWF1* and *ACS1* genes in the DWM strain increased intracellular concentrations of NADPH and NADH and improved maximum xylitol productivity by 17%, relative to that for the DWM strain. Finally, the optimized fed-batch fermentation of *S. cerevisiae* DWM-ZWF1-ACS1 resulted in 196.2 g/L xylitol concentration, 4.27 g/L h productivity and almost the theoretical yield. Expression of the two types of XR utilizing both NADPH and NADH is a promising strategy to meet the industrial demands for microbial xylitol production.

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1 Introduction

Xylitol is a five-carbon sugar alcohol with a number of advantageous properties. As xylitol is as sweet as sucrose and has a 33% lower calorie, and extreme cooling and caries-preventative effects, xylitol has been commercially used as a sugar substitute in various products such as chewing gums, candies and toothpastes [1]. Xylitol was also chosen as one of the top 12 value-added chemicals from biomass and could be used as a platform chemical for polymer synthesis such as xylaric acid and glycols, directly polymerized for production of unsaturated polyester resin [2]. Ethylene glycol, an important chemical intermediate, can be produced by ruthenium-mediated hydrogenolysis of xylitol [3]. Due to these properties, demand of xylitol has increased in food and chemical industries. Currently, the annual xylitol market was estimated to be 125 000 tons of demand and priced at 4.5–5.5 US dollars per kg [4].

Xylitol is chemically synthesized by hydrogenation of xylose, one of major sugars in cellulosic biomass, in the presence of a metal catalyst and hydrogen gas at over ambient conditions such as 120°C of temperature and up to 5.5 MPa of pressure [5, 6]. Due to the sustain-

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Abbreviations: **ACS1**, acetyl-CoA synthetase; **NAD**, nicotinamide adenine dinucleotide; **NADP**, nicotinamide adenine dinucleotide phosphate; **XR**, xylose reductase; **ZWF1**, glucose-6-phosphate dehydrogenase

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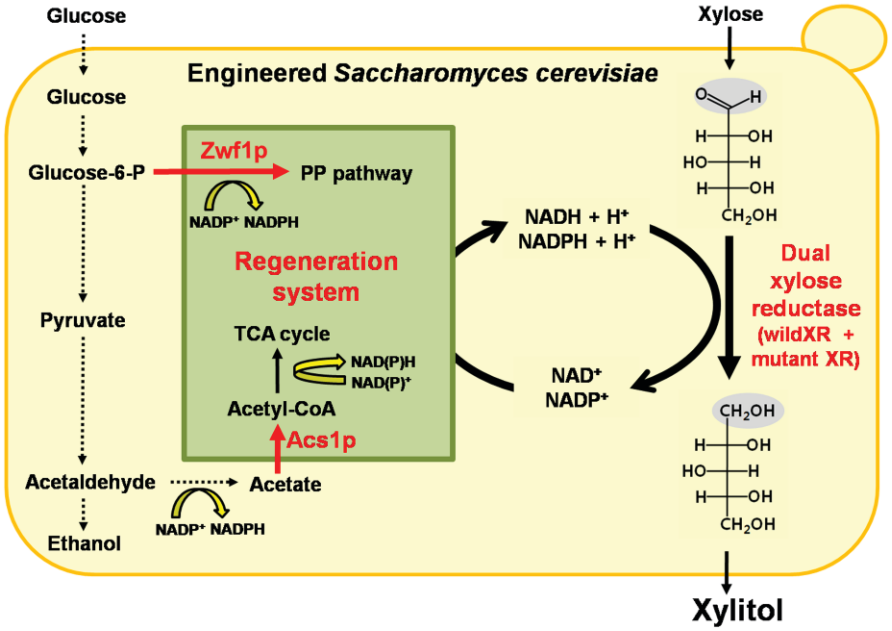

Figure 1. Metabolic pathways for xylitol biosynthesis and regeneration of NADPH and NADH cofactors in engineered *S. cerevisiae*. Zwf1p, glucose-6-phosphate dehydrogenase; Acs1p, acetyl-CoA synthetase; PP pathway, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle.

ability issue, especially biological production of xylitol has gained an interest from academia and industries [7]. Instead of metal catalysts, a biocatalyst of xylose reductase can undertake the hydrogenation reaction of xylose with aid of NADPH and NADH cofactors (Fig. 1). Because of instability and high cost of xylose reductase and the cofactors, the biological production of xylose should be devised by microbial systems. To date, robust microorganisms have been developed to express xylose reductase highly and to supply the cofactors sufficiently. Among several microorganisms, *Candida* species is well established to produce high concentration of xylitol over 237 g/L with high productivity [8]. *Candida* species is recognized as a good xylitol producer, however, it consumes xylose as an energy source so the xylitol yield does not reach a commercially applicable value over 90% [8, 9]. *Saccharomyces cerevisiae* is a GRAS (generally recognized as safe) microorganism not only used in food and alcoholic beverage industries but also applicable for production of biochemicals, biofuels and biopharmaceuticals. In spite of its outstanding properties, *S. cerevisiae* is unable to produce xylitol from xylose because it does not have an enzyme, xylose reductase (XR) endogenously. For xylitol biosynthesis in metabolically-engineered *S. cerevisiae*, various research efforts have been made including genetic introduction of the XR gene, supply of the cofactors, control of the pentose phosphate pathway [10–13]. Because engineered *S. cerevisiae* expressing XR cannot metabolize xylitol into the central carbon metabolism, it converts xylose to xylitol at almost the theoretical yield (~100%) [9].

Xylose reductase catalyzes the one-step hydrogenation of xylose to xylitol in the presence of NADPH or NADH cofactor (Fig. 1). Most XRs in nature depend on NADPH, of which the oxidized form should be reduced again inside cells. Since the NADPH regeneration is done by a few metabolic steps, insufficient supply of NADPH limits rapid production of xylitol. Meanwhile, a NADH-preferring XR mutant was developed by protein engineering and applied to conversion of xylose to ethanol in engineered *S. cerevisiae* [14, 15]. An engineered *S. cerevisiae* expressing the mutant XR showed much higher xylose consumption rate than the same yeast harboring the wild-type XR dependent on NADPH. Considering these results, we hypothesized that utilization of both NADPH and NADH as cofactors would improve xylitol biosynthesis in engineered *S. cerevisiae*. In this study, we constructed engineered *S. cerevisiae* strains expressing the NADPH-dependent wild XR (wXR) and the NADH-preferring mutant XR (mXR), which were originated from *Scheffersomyces stipitis*. Additionally, glucose-6-phosphate dehydrogenase for NADPH regeneration and acetyl-CoA synthetase for NADPH and NADH regeneration were overexpressed to improve the xylitol production performance further. Finally, fed-batch fermentation strategies were optimized to produce xylitol with a high titer and productivity.

2 Materials and methods

2.1 Strains and plasmids

Escherichia coli TOP 10 (Invitrogen, Carlsbad, CA, USA) was used for gene manipulation. LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used for *E. coli* cultivation. *S. cerevisiae* D452-2 was used as a host strain for xylitol production and a source of the

yeast genomic DNA. Plasmids p426GPD and p425GPD with the *GPD* promoter and *CYC1* terminator were used for the expression of the wild (Genbank accession No. 4839234) and mutant genes of *XYL1* encoding *S. stipitis* XRs, and the *S. cerevisiae ZWF1* (No. 855480) and *ACS1* (No. 851245) genes coding for glucose-6-phosphate dehydrogenase (Zwf1p) and acetyl-CoA synthetase (Acs1p), respectively. Plasmids pRS403, pRS405 and pITy-3 were used for chromosomal integration of the *XYL1* genes. Yeast strains and plasmids used in this study are listed in Supporting information, Table S1.

2.2 Genetic manipulation

To construct the expression cassettes of the wild XR (wXR) and mutant XR (mXR), the two XR genes were amplified by polymerase chain reaction (PCR) from previously constructed vectors of YEpM4-XR^{WT}and YEpM4-XR^{MUT} with the corresponding PCR primers: F1 and R1 (Supporting information, Table S2) [14]. Each amplified XR gene was treated with DNA restriction enzymes of *Bam*HI and *Xho*I, and inserted between the *GPD* promoter and *CYC1* terminator in plasmid p426GPD, resulting in the construction of plasmids p426GPD_wXR and p426GPD_mXR. The XR expression cassette of the *GPD* promoter, each XR gene and *CYC1* terminator in a row, was PCR-amplified with DNA primers of F2 and R2. After digestion with *Sac*I, the cassette was introduced into chromosomal integration vectors, resulting in the construction of plasmids pRS406_wXR, pRS403_mXR, pITy-3_wXR and pITy-3_mXR. Plasmids pRS406_wXR and pRS403_mXR were linearized by *Xcm*I and *Nhe*I treatment, respectively, transformed and integrated into the chromosome of *S. cerevisiae* D452-2 to construct the DWM strain. Engineered *S. cerevisiae* strains expressing multiple copies of the wXR gene (DWW) and mXR gene (DMM) were constructed by δ-integration of plasmids pITy-3_wXR and pITy-3_mXR in *S. cerevisiae* D452-2, respectively [16]. The *ZWF1* and *ACS1* genes were amplified from the *S. cerevisiae* D452-2 genomic DNA, digested with *Xma*I and *Xho*I for *ZWF1*, *Eco*RI and *Xho*I for *ACS1* and cloned into p425GPD vector to generate plasmids p425GPD_ZWF1 and p425GPD_ACS1. After amplifying the *ZWF1* expression cassette with primers of F2 and R2 and digesting with *Sac*I, the cassette was introduced into plasmid p425GPD_ACS1 to construct plasmid p425GPD_ZWF1_ACS1. Each resulting plasmid was transformed into the DWM strain, of which transformants were named DWM-ZWF1, DWM-ACS1 and DWM-ZWF1-ACS1. The schemes for plasmid construction are displayed in Supporting information, Fig. S1.

A yeast EZ-Transformation kit (BIO 101, Vista, CA, USA) was used for plasmid transformation into *S. cerevisiae*, of which transformants were selected on YNB medium containing 20 g/L glucose, appropriate amino acids and nucleotide (histidine, leucine or uracil). Yeast strains transformed with delta-integration plasmids were propagated on YP medium supplemented with 20 g/L glucose and an appropriate concentration of G418 antibiotic (Calbiochem Co., USA).

2.3 Fermentation conditions

S. cerevisiae was grown in 5 mL of YP (10 g/L yeast extract and 20 g/L peptone) or YNB leu$^-$ medium (6.7 g/L yeast nitrogen base without amino acids and 1.6 g/L yeast synthetic drop-out medium supplement w/o leucine) containing 20 g/L glucose at 30°C and 250 rpm for 20 h. The cells were harvested and inoculated into a 500 mL baffled flask with 100 mL YP medium containing glucose and xylose for batch fermentation at 30°C and 250 rpm. For glucose-limited fed-batch fermentation, the pre-grown cells were transferred into 100 mL of YNB leu$^-$ medium with 20 g/L glucose. After 20 h of culture at 30°C and 250 rpm, the cells were harvested and suspended in sterilized water. The suspended cells were inoculated to a 3.7 L fermentor (Bioengineering AG, Wald, Switzerland) with 1 L working volume of YP medium containing 19 g/L glucose and 76–98 g/L xylose at 30°C. Acidity was controlled at pH 5.0 by addition of 2 N HCl and 2 N NaOH. An initial optical density of the cultures was adjusted at about 0.5. An agitation speed and an aeration rate was set at 500 rpm and 1 vvm, respectively. A concentrated glucose solution (600 g/L) was fed constantly at 1.8 g/L h of feed rate after depletion of glucose initially added. In addition, 600 g/L xylose solution was supplemented intermittently or continuously into the culture broth to control xylose concentration between 35 and 80 g/L or around 80 g/L.

2.4 Determination of intracellular NAD(P)H and NAD(P)$^+$ concentrations

Concentrations of NADPH, NADP$^+$, NADH and NAD$^+$ were measured using the NADP$^+$/NADPH and NAD$^+$/NADH assay kits (BioAssay Systems, Hayward, CA, USA). *S. cerevisiae* cells grown in YNB leu$^-$ medium with glucose were harvested at the mid-exponential growth phase and suspended in 125 μl of the NAD(P)$^+$ extraction buffer. The concentration measurement followed the manufacturer's instruction.

2.5 Determination of cell and metabolite concentrations

Optical density (OD) of the yeast cells was measured using a spectrometer (Ultrospec 2000, Amersham Pharmacia Biotech, Uppsala, Sweden) at 600 nm, which was converted to dry cell weight by a pre-determined conversion factor, 0.3 dry cell weight (g/L)/OD. Concentrations of metabolites in the culture broth were determined by a high performance liquid chromatography (HPLC, Agilent 1100LC, Santa Clara, CA, USA) equipped with a refractive

index detector at 35°C and a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex, Torrance, CA, USA). The column was eluted with 0.01 N of H_2SO_4 at a flow rate of 0.6 mL/min and heated at 60°C.

3 Results

3.1 Dual expression of wild XR and mutant XR improved xylitol productivity

In order to extend the availability of the two cofactors (NADPH and NADH), and examine the influence of the extended availability of reducing agents on xylitol production, engineered *S. cerevisiae* strains were constructed to express the wild XR (DWW) or mutant XR (DMM) only, and both the XRs (DWM) (Supporting information, Table S1). In vitro activity assay of the wild and mutant XRs were carried out to assess their expression and preference of cofactors in the engineered *S. cerevisiae* strains (Table 1). The DWW and DMM strains were highly specific for NADPH and NADH, respectively, whereas the DWM strain having dual XRs showed similar activities toward NADPH and NADH as expected. The DWM strain had a similar total XR activity to the DWW strain or 1.4 times higher total activity than the DMM strain.

To analyze the xylitol-producing performance, batch cultures of the DWW, DMM, and DWM strains were performed using YP medium with 21 g/L xylose and 21 g/L glucose (Supporting information, Fig. S2). All the engineered *S. cerevisiae* strains consumed glucose at the same consumption rate. After glucose added initially was utilized, the three strains started to consume xylose with different consumption rates. *S. cerevisiae* is a Crabtree positive strain able to produce ethanol from glucose even in aerobic condition [17]. Ethanol produced from glucose was consumed after glucose depletion and used for maintenance of cell viability because xylose cannot flow into the central energy metabolism in all strains. And ethanol and xylose were consumed simultaneously since ethanol was used for the regeneration of the cofactors for xylitol production. Xylitol production was linearly correlated to the consumption of xylose and ethanol (Supporting information, Fig. S2). Fermentation parameters of these batch fermentations were summarized in Table 1. The DWM strain expressing two types of XR exhibited the highest capability of xylitol production. The batch fermentation of the DWM strain resulted in improved xylitol concentration by 6.6% and 47% compared to the corresponding values for the DWW and DMM strains expressing each type of XR, respectively. As expected, the xylitol yield in all cases reached the theoretical xylitol yield (1 g-xylitol/1 g-xylose). Interestingly, the DMM and DWM strains showed 1.3 times higher final dry cell mass than the DWW strain, indicating that the partial or complete change of the wild XR to the mutant XR probably reduced a metabolic burden by expression of NADPH-consuming XR and hence elevated final cell mass.

3.2 High xylitol productivity was obtained in glucose-limited fed-batch fermentation

In batch fermentations, the DWM strain gave the best performance for xylitol production. To confirm its ability as xylitol producer, glucose-limited fed-batch fermentations of the DWW-C and DWM-C strains were performed in YP medium with 100 g/L xylose and 21 g/L glucose (Supporting information, Fig. S3). The DWW-C and DWM-C strains were constructed by introduction of plasmid p425GPD into the DWW and DWM strains, respectively. After depletion of glucose initially added, a concentrated glucose solution was fed continuously to maintain its basal level in the medium, which is called a glucose-limited feeding strategy. To relieve the glucose inhibition on xylose transport [18, 19] and to supply the cofactors and cellular energy, the glucose-limited feeding strategy was chosen in this study. By this glucose feeding strategy, xylose was linearly consumed to produce xylitol with different xylitol productivities. The glucose-limited fed-batch fermentation of the DWM strain exhibited 72 g/L xylitol concentration and 2.00 g/L h xylitol productivity which were 13% and 14% higher than those for the DWW strain. Based on the XR activity analysis and the xylitol-producing ability in batch and glucose-limited fed-batch fermentations, the balanced expression of the wild and mutant XRs under high XR expression levels could be a plausible reason for the enhanced production of xylitol by the DWM strain.

Table 1. Summarized results of batch cultures of engineered *S. cerevisiae* DWW, DMM and DWM strains using 21 g/L xylose and 21 g/L glucose

Strain	Final dry cell mass[a] (g/L)	Xylitol concentration[a] (g/L-h)	Xylitol productivity[a] (g/L)	Xylitol yield (g xylitol /g xylose)	Specific XR activity[b] (U/mgprotein)			XR activity ratio[c]
					NADPH	NADH	Total	
DWW	5.97	16.5	0.827	~1	2.11 ± 0.02	1.53 ± 0.03	3.64	1.39
DMM	7.41	12.0	0.599	~1	0.19 ± 0.01	2.47 ± 0.03	2.66	0.08
DWM	8.14	17.6	0.881	~1	1.89 ± 0.10	1.77 ± 0.03	3.66	1.07

[a] These values are the mean of triplicate experiments.
[b] Specific XR activity was measured three times independently.
[c] This value was calculated by the division of XR activities on NADPH to NADH.

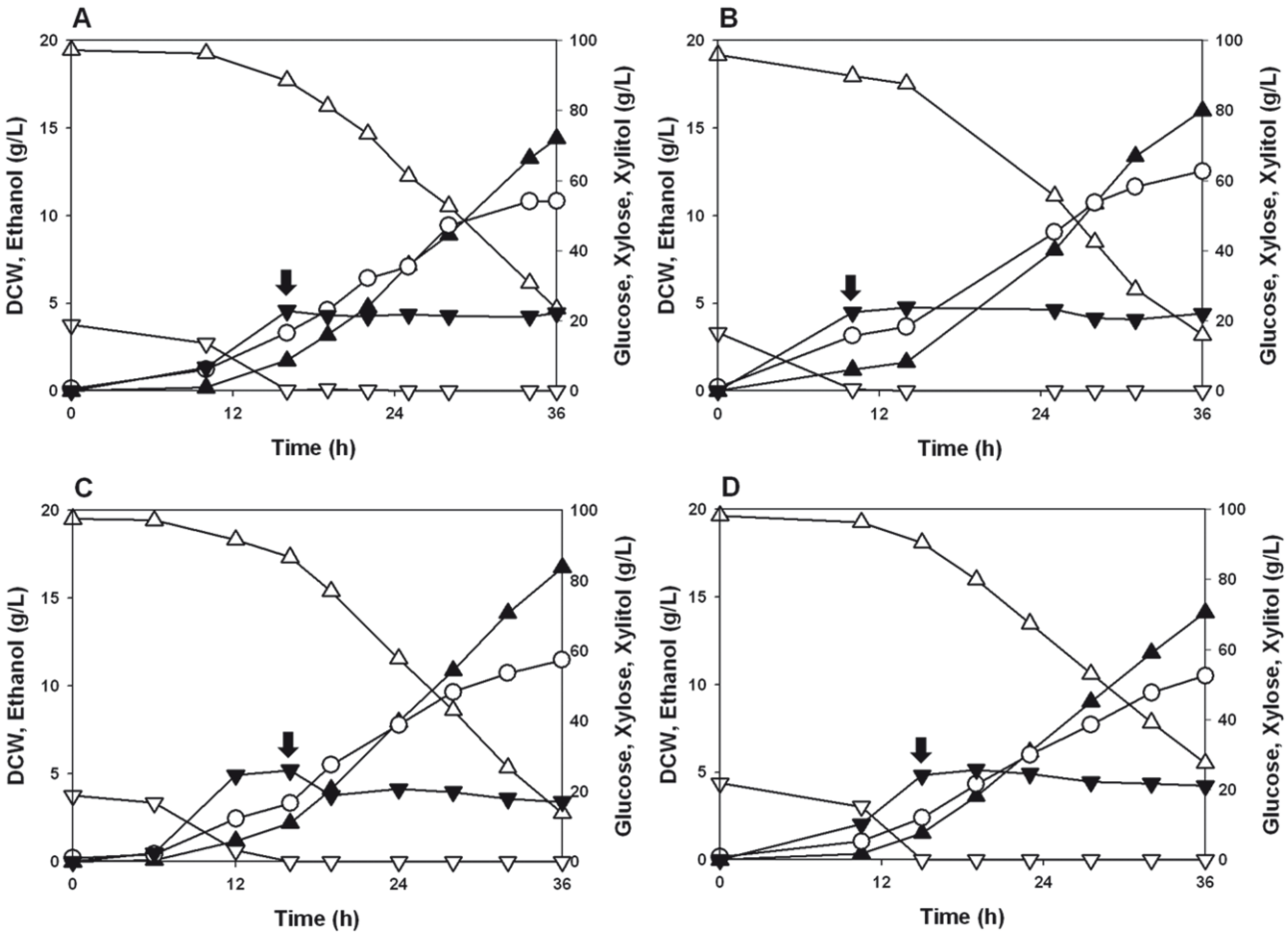

Figure 2. Effect of Zwf1p and Acs1p overexpression on xylitol production in glucose-limited fed-batch fermentations of engineered *S. cerevisiae* DWM-ZWF1 (**A**), DWM-ACS1 (**B**), DWM-ZWF1-ACS1 (**C**) and DWW-ZWF1-ACS1 (**D**) at 30°C, 500 rpm, 1 vvm and pH 5.0. A mixture of 18 g/L glucose and 98 g/L xylose was added initially. The arrow indicates the start point of the glucose feeding at a rate of 1.8 g/L h. Symbols are denoted as follows: ○, dry cell weight (DCW); ▽, glucose; △, xylose; ▲, xylitol; ▼, ethanol.

3.3 Overexpression of *ZWF1* and *ACS1* increased intracellular pools of NADPH and NADH

As shown in Fig. 1, biological production of xylitol is controlled by the activity and cofactor dependency of XR, and the supply of NADPH and NADH. As harmonized expression of the wild and mutant XRs significantly improved the xylitol-producing performance as presented above, sufficient supply of NADPH and NADH is expected to enhance the xylitol production further. In microbial systems, NADPH and NADH are regenerated by many metabolic enzymes. Among them, glucose-6-phosphate dehydrogenase encoded by the *ZWF1* gene and acetyl-CoA synthetase by the *ACS1* gene were selected as the NADPH and NADH regenerating enzymes, because of their high efficiency.

To investigate the effects of the intracellular levels of NADPH and NADH on xylitol production, Zwf1p and Acs1p were overexpressed in the DWM strain. Plasmids p425GPD_ZWF1 and p425GPD_ACS1 were constructed to harbor each coding gene and transformed into the DWM strain to make the DWM-ZWF1 and DWM-ACS1 strains, respectively. When intracellular concentrations of $NADP^+$, NADPH, NAD^+ and NADH in the DWM-C, DWM-ZWF1 and DWM-ACS1 strains were measured (Supporting information, Table S3), overexpression of Zwf1p and Acs1p changed the pools of the hydrogen donors and increased the intracellular concentrations of both NADPH and NADH with different extents. The *ZWF1* overexpression increased the ratios of NADPH to $NADP^+$ and of NADH to NAD^+ by 19%, compared to the DWM-C control strain. The *ACS1* overexpression led to 2.26- and 1.27-fold improvements in NADPH and NADH concentrations, respectively, relative to those of the DWM-C strain. In addition, the DWM-ACS1 strain showed 4.15 and 1.25 times higher NADPH/$NADP^+$ and NADH/NAD^+ ratios, respectively, than those of the DWM-C strain. As a result, intracellular pools of NADPH and NADH in the

engineered strain were increased by overexpression of the NADPH and NADH-regenerating enzymes in the DWM strain.

To investigate the effects of the overexpression of *ZWF1* or *ACS1* on xylitol production, glucose-limited fed-batch fermentations of DWM-ZWF1 and DWM-ACS1 were performed using 18 g/L glucose and 98 g/L xylose (Fig. 2). The DWM-ZWF1 and DWM-ACS1 strains showed 2.04 and 2.15 g/L h maximum xylitol productivity, which were 4–15% higher than that of the DWM-C strain (Supporting information, Fig. S3B). It indicated that either *ZWF1* or *ACS1* overexpression enhanced the xylitol concentration and productivity. As expected, the increments of NADPH and NADH concentrations, and the NADPH/$NADP^+$and NADH/NAD^+ ratios elevated the xylitol producing performances of the DWM-ZWF1 and DWM-ACS1 strains. Irrespective of the *ZWF1* and *ACS1* overexpression, xylitol yields in each case were close to the theoretical value.

3.4 Coexpression of *ZWF1* (for NADPH regeneration) and *ACS1* (for NADH regeneration) enhanced xylitol productivity synergistically

Individual expression of the *ZWF1* and *ACS1* genes conferred the improved ability of xylitol production to the DWM strains. To maximize the merit of the DWM strain with the broad specificity of the cofactors, plasmid p425GPD_ZWF1_ACS1 was constructed to express both the *ZWF1* and *ACS1* genes, and transformed into the DWM strain. In the analysis of the intracellular cofactor contents (Supporting information, Table S3), overexpression of both Zwf1p and Acs1p in the resulting strain of DWM-ZWF1-ACS1 highly elevated the pools of both NADPH and NADH, and the ratios of the reduced cofactors (NADPH and NADH) to the oxidized cofactors ($NADP^+$ and NAD^+) by a four-fold maximally, relative to those for the DWM-C strain. The coexpression also led to higher ratios of NAD(P)H to $NAD(P)^+$ than the single expression of Zwf1p and Acs1p.

To evaluate the xylitol-producing performance, the DWM-ZWF1-ACS1 strain was cultured using 18 g/L glucose and 98 g/L xylose, and by the same fermentation strategy as the two fed-batch cultivations performed above (Supporting information, Fig. S3). As a control, the DWW-ZWF1-ACS1 strain was cultured by the same fermentation strategy. As shown in Fig. 2C and 2D, the patterns of xylose consumption and xylitol production were similar to other glucose-limited fed-batch fermentations in this study. The DWM-ZWF1-ACS1 strain produced 83.7 g/L xylitol with the final xylitol productivity of 2.33 g/L h, while the DWW-ZWF1-ACS1 strain did 70.6 g/L xylitol with 1.96 g/L h productivity. Especially, the DWM-ZWF1-ACS1 strain showed 17% higher xylitol productivity than that of the DWM-C strain (Fig. 2C). In comparison to the sole expression of either *ZWF1* or *ACS1*, the DWM-ZWF1-ACS1 strain exhibited 8–14% improvement in xylitol productivity. Considering these fermentation results, it was certain that the balanced expression of the wild and mutant XRs with an aid of the NADPH and NADH regeneration systems exhibited a synergistic effect on xylitol production. To ensure functional expression of Zwf1p and Acs1p in the DWM-ZWF1-ACS1 strain, crude extracts of the yeast cells were collected in the fed-batch culture and their enzyme activities were measured. As shown in Supporting information, Fig. S4, the DWM-ZWF1-ACS1 strain had at least five-fold higher specific activity of glucose-6-phosphate dehydrogenase than the control DWM-C strainat all times. Specific acetyl-CoA synthetase activity of the DWM-ZWF1-ACS1 strain was also five times higher than that of the control strain.

3.5 Control of xylose concentration in fed-batch fermentation improved xylitol productivity

The final strain (DWM-ZWF1-ACS1) showed the highest productivity than other *S. cerevisiae* strains engineered for xylitol production. To extent the period for the highest xylitol productivity and hence elevate final xylitol titer, fed-batch cultivation strategies were designed using intermittent addition or continuous feeding of a concentrated xylose solution to maintain an optimized concentration of xylose in the culture medium [9]. In the glucose-limited fed-batch fermentation of the DWM-ZWF1-ACS1 strain (Fig. 2C), the highest xylitol productivity was achieved when xylose concentration in the medium ranged from 40 g/L to 80 g/L. So, this range of xylose concentration was adopted to keep the highest xylitol-producing ability. As shown in Fig. 3A and 3B, glucose-limited fed-batch cultures of the DWM-C and DWM-ZWF1-ACS1 strains were carried out by intermittent addition of 600 g/L xylose solution to a bioreactor when xylose concentration in the medium dropped below 40 g/L. The concentrated xylose solution was added four times to control the xylose concentration range between 40 and 80 g/L. Finally, 185.3 g/L xylitol concentration and 3.71 g/L hr productivity were obtained in 50 h fed-batch culture of the DWM-ZWF1-ACS1 strain. On the other hand, the DWM-C strain cultured by the same fermentation strategy produced 150.2 g/L xylitol. Generally, sugar uptake rate depends on extracellular sugar concentration [20]. Xylose consumption rate decreased gradually whenever xylose concentration fluctuated. For maximizing xylitol production, a glucose-limited fed-batch fermentation was performed by continuous feeding of 600 g/L xylose solution at a constant rate of 10 mL/h (Fig. 3C). The feed rate was decided from the stage showing the highest xylose consumption rate in Fig. 2C. When initial glucose was depleted, the continuous xylose feeding started at 6 h culture time. During 24 h of the feeding, xylose concentration in the medium was maintained well at 80 g/L as

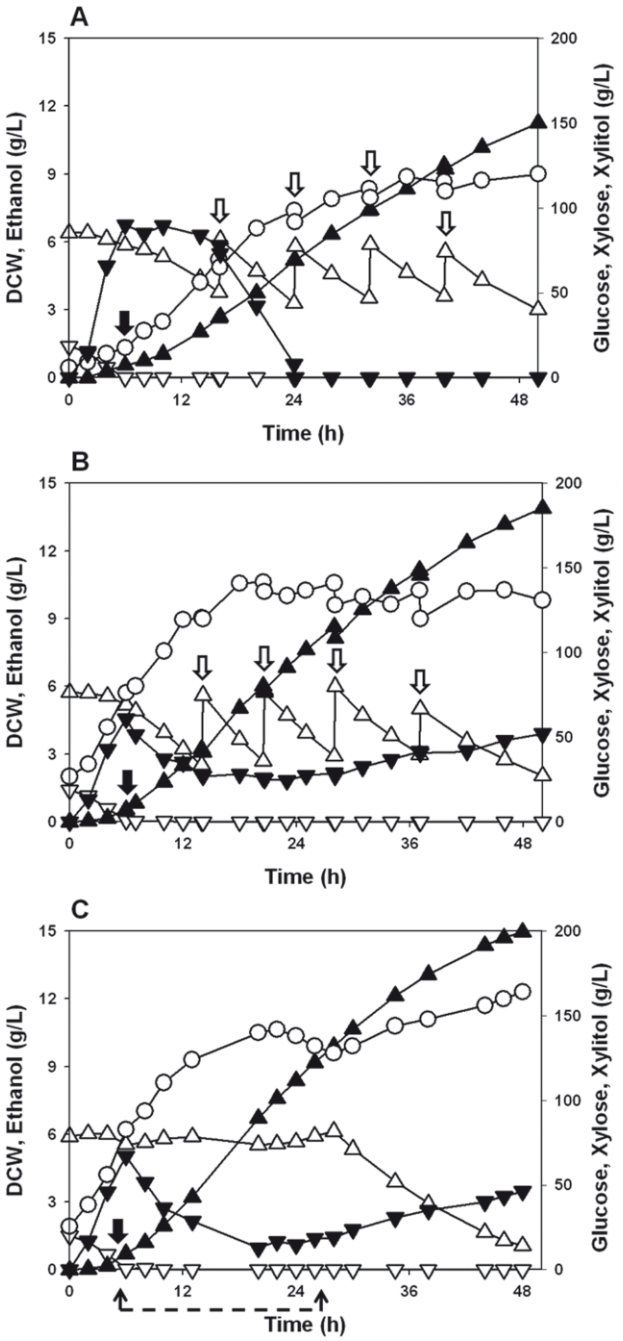

Figure 3. Fed-batch fermentations of engineered *S. cerevisiae* DWM-C (**A**), DWM-ZWF1-ACS1 (**B**, **C**). The closed arrow indicates the initiation of the glucose feeding at a rate of 1.8 g/L h. In the panels (**A**) and (**B**), a concentrated xylose solution of 600 g/L was added intermittently (open arrow). In the panel (**C**), the concentrated xylose solution was fed continuously at a rate of 10 mL/h and the dotted line indicates the period of the continuous feeding. Symbols are denoted as follows: ○, dry cell weight (DCW); ▽, glucose; △, xylose; ▲, xylitol; ▼, ethanol.

planned and most xylose added was converted into xylitol with the theoretical yield (~1 g/g). As a result, the final xylitol concentration of 196.2 g/L and xylitol productivity of 4.26 g/L h were obtained in the fed-batch culture by the continuous xylose-feeding strategy.

4 Discussion

Xylose is biologically reduced to xylitol by xylose reductase (XR). The wild XR from *S. stipitis* used in this study is known to be able to utilize both NADPH and NADH as cofactors. But the activity of wild XR on NADPH was 30% higher than that on NADH [21], so most engineered *S. cerevisiae* strains expressing the wild XR gene as well as conventional xylitol-producing yeasts convert xylose to xylitol using NADPH primarily. Because the intracellular NADH level is much higher than the NADPH level [22], balanced utilization of both NADPH and NADH is suggested to avoid the depletion of cofactors and hence improve xylitol production in engineered *S. cerevisiae*. Previously, a mutant XR preferring NADH was developed by protein engineering [15], and its expression reduced a metabolic disorder of redox-imbalance in the xylose metabolism of engineered *S. cerevisiae* [14]. As shown in the batch and glucose-limited fed-batch cultures (Supporting information, Fig. S2, Fig. S3; Table 1), the DWM strain expressing both the wild and mutant XRs showed higher xylitol producing performances than the DWW and DMM strains, indicating that the harmonized expression of two XRs with different cofactor specificities is a certain reason for the enhanced xylitol production by the DWM strain.

Meanwhile, supply of NADPH or NADH cofactor is a rate-limiting factor for xylitol production as well as the XR expression. Among several metabolic enzymes regenerating NADPH or NADH in the substrate level, glucose-6-phosphate dehydrogenase (Zwf1p) catalyzes the main metabolic pathway for NADPH regeneration in *S. cerevisiae* [11, 23]. Acetyl-CoA synthetase (Acs1p) converts acetate to acetyl-CoA, which is metabolized further into the tricarboxylic acid cycle with the NADH and NADPH regeneration metabolism. Since acetate is produced from acetaldehyde by NADPH regeneration, overexpression of acetyl-CoA synthetase is expected to modulate both NADH and NADPH levels simultaneously and indirectly [24]. Overexpression of Zwf1p and Acs1p enhanced the intracellular pools of NADPH and NADH (Supporting information, Table S3), resulting in maximum 17% increases in xylitol productivity than the control strain (Supporting information, Fig. S3B; Fig. 2C and Table 2). The similar effects of improved production of target molecules by the regeneration of the cofactors could be found in some reports. The overexpression of Zwf1p triggered the production of xylitol, GDP-L-fucose, ε-caprolactone by enhanced NADPH levels [12, 25, 26].

Table 2. Summarized results of glucose-limited fed-batch fermentations using 76–98 g/L initial xylose in this study

S. cerevisiae strain	Xylose feeding strategy	Final dry cell mass (g/L)	Final xylitol concentration (g/L)	Final xylitol Productivity (g/L-h)	Xylitol yield (g/g xylose)
DWW-C	No xylose feeding	8.8	63.5	1.76	~1
DWW-ZWF1-ACS1	No xylose feeding	10.5	70.6	1.96	~1
DWM-C	No xylose feeding	10.9	72.0	2.00	~1
DWM-ZWF1-ACS1	No xylose feeding	11.5	83.7	2.33	~1
DWM-C	Intermittent xylose addition[a)]	9.1	150.2	3.00	~1
DWM-ZWF1-ACS1	Intermittent xylose addition[a)]	10.4	185.3	3.71	~1
DWM-ZWF1-ACS1	Continuous xylose feeding[b)]	12.2	196.2	4.27	~1
BJ3505/δXR[c)]	Intermittent xylose addition	22.0	116	2.34	~1
C. tropicalis ATCC 13803[d)]	Continuous xylose feeding	10.4	187	3.90	0.75

[a)] A concentrated xylose solution of 600 g/L was added four times to control 40–80 g/L xylose concentration in the medium.
[b)] A concentrated xylose solution of 600 g/L was fed continuously to maintain 80 g/L xylose concentration in the medium.
[c)] These data were obtained from cell-recycle fermentation [10].
[d)] These data were obtained by optimizing initial xylose concentration and feeding rate [9].

The Acs1p overexpression influenced the production of many biochemicals such as hydrogen, isopropanol, butanol by increasing the carbon flux into a desired product and decreasing acetate accumulation, which was toxic to cell growth [27, 28]. When cellobiose and xylose were consumed simultaneously, overexpression of *S. cerevisiae* Idp2p (isocitrate dehydrogenase) increased the NADPH availability and allowed a 63% improvement in xylitol productivity [29]. As a result, the modulation of the pools and ratios of NADPH and NADH was one of the effective ways to improve xylitol production.

Dual expression of NAD(P)H regenerating enzymes would be an easy way to increase target molecules by changing the pools of NADPH and NADH cofactors. In case of microbial xylitol production, however, this strategy was not always effective. Coexpression of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two main NADPH-regenerating enzymes, increased xylitol productivity by engineered *Candida tropicalis* PP [30]. Coexpression of Acs1p and $NADP^+$-dependent aldehyde dehydrogenase 6 (Ald6p) in engineered *S. cerevisiae* BJ3505:δXR exerted a negative effect on xylitol production, compared to their single expression [24]. As shown in Fig. 2C and 2D, simultaneous expression of Zwf1p and Acs1p in the DWM strain exhibited 19% higher enhancement of xylitol productivity compared to the coexpression of Zwf1p and Acs1p in the DWW strain. It was certain that the coexpression of Zwf1p and Acs1p with different mechanisms of cofactor regeneration was beneficial for xylitol production mediated by both the wild and mutant XRs in the DWM-ZWF1-ACS1 strain.

The results of the glucose-limited fed-batch fermentations in this study are summarized in Table 2. The control of xylose concentration at an optimized range improved both xylitol concentration and productivity considerably. The continuous xylose-feeding strategy gave the best performances of xylitol production which were 2.3- and 1.8-fold increases in xylitol concentration and xylitol productivity, respectively, relative to the culture of the same strain without controlling xylose concentration. In comparison to the previous results done by other microorganisms, the DWM-ZWF1-ACS1 strain showed 82% and 9% higher xylitol productivity than a engineered *S. cerevisiae* BJ3505 strain expressing the NADPH-dependent XR from *S. stipitis* [10] and a wild type of *C. tropicalis* ATCC13803 [9], respectively, which were cultured using a similar fed-batch fermentation strategy. All cultures of the *S. cerevisiae* strains expressing foreign XRs in this study resulted in almost the theoretical yield (~1 g/g), whereas a traditional xylitol producer of *C. tropicalis* produced xylitol below the theoretical yield [9, 31]. Expression of two XRs highly specific for each cofactor along with two metabolic enzymes for increasing the pools of the reduced cofactors could clearly overcome both the process limitation of engineered *S. cerevisiae* (low productivity) and *C. tropicalis* (low yield) reported previously.

In conclusion, insufficient supply of NADPH cofactor and xylose utilization to cellular energy prevent the commercialization of microbial xylitol production. To overcome these hurdles, *S. cerevisiae* unable to metabolize xylose was engineered to express two types of XR using both NADPH and NADH cofactors. Additionally, Zwf1p and Acs1p were coexpressed to facilitate the NADPH and NADH regeneration. Finally, the best engineered *S. cerevisiae* DWM-ZWF1-ACS1 coexpressing NADPH-dependent and NADH-preferring XRs, and Zwf1p and Acs1p produced almost 196.2 g/L xylitol with over 4.27 g/L-h productivity and almost theoretical yield, which might meet the industrial demands for commercialization of microbial xylitol production.

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The authors declare no financial or commercial conflict of interest.

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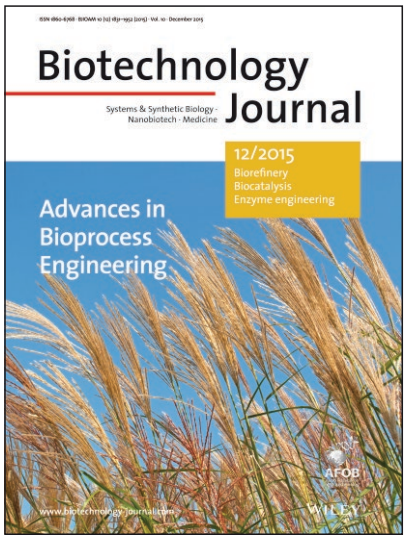

Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Yoon-Mo Koo and Penjit Srinophakun , covers advances in bioprocess engineering. Several articles in this issue discuss different aspects of lignocellulose-based biorefining. Miscanthus (shown on the cover) is one example of a plant whose lignocellulosic biomass can be used for bioethanol production. © M. Schuppich, Fotolia.com

Biotechnology Journal – list of articles published in the December 2015 issue.

Editorial

Bioprocess beyond the large scale production

Yoon-Mo Koo and Penjit Srinophakun

http://dx.doi.org/10.1002/biot.201500618

Editorial

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Jing Zhu

http://dx.doi.org/10.1002/biot.201500624

BiotecVisions

Beyond Asian Biotechnology: Collaboration between Asia and Europe

Hyun Jung Kim

http://dx.doi.org/10.1002/biot.201500623

BiotecVisions

European's Biochemical Engineering to face the megatrends and associated challenges beyond 2020

Guilherme Ferreira

http://dx.doi.org/10.1002/biot.201500627

Review

Bacterial cellulose composites: Synthetic strategies and multiple applications in bio-medical and electro-conductive fields

Mazhar Ul-Islam, Shaukat Khan, Muhammad Wajid Ullah and Joong Kon Park

http://dx.doi.org/10.1002/biot.201500106

Review

Incorporating unnatural amino acids to engineer biocatalysts for industrial bioprocess applications

Yuvaraj Ravikumar, Saravanan Prabhu Nadarajan, Tae Hyeon Yoo, Chong-Soon Lee and Hyungdon Yun

http://dx.doi.org/10.1002/biot.201500153

Review

Aggregating tags for column-free protein purification

Zhanglin Lin, Qing Zhao, Lei Xing, Bihong Zhou and Xu Wang

http://dx.doi.org/10.1002/biot.201500299

Research Article

Fatty acid hydration activity of a recombinant *Escherichia coli*-based biocatalyst is improved through targeting the oleate hydratase into the periplasm

Sang-Min Jung, Joo-Hyun Seo, Jung-Hoo Lee, Jin-Byung Park and Jin-Ho Seo

http://dx.doi.org/10.1002/biot.201500141

Research Article

Understanding β-mannanase from *Streptomyces* sp. CS147 and its potential application in lignocellulose based biorefining

Hah Y. Yoo, G. C. Pradeep, Soo K. Lee, Don H. Park, Seung S. Cho, Yun H. Choi, Jin C. Yoo and Seung W. Kim

http://dx.doi.org/10.1002/biot.201500150

Research Article

Improved growth and ethanol fermentation of *Saccharomyces cerevisiae* in the presence of acetic acid by overexpression of *SET5* and *PPR1*

Ming-Ming Zhang, Xin-Qing Zhao, Cheng Cheng and Feng-Wu Bai

http://dx.doi.org/10.1002/biot.201500508

Research Article

Enhanced hydrolysis of lignocellulosic biomass: Bi-functional enzyme complexes expressed in *Pichia pastoris* improve bioethanol production from *Miscanthus sinensis*

Sang Kyu Shin, Jeong Eun Hyeon, Young In Kim, Dea Hee Kang, Seung Wook Kim, Chulhwan Park and Sung Ok Han

http://dx.doi.org/10.1002/biot.201500081

Research Article

Phenolic compounds: Strong inhibitors derived from lignocellulosic hydrolysate for 2,3-butanediol production by *Enterobacter aerogenes*

Sang Jun Lee, Ju Hun Lee, Xiaoguang Yang, Sung Bong Kim, Ja Hyun Lee, Hah Young Yoo, Chulhwan Park and Seung Wook Kim

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Research Article

Salt tolerant chromatography provides salt tolerance and a better selectivity for protein monomer separations

Noriko Yoshimoto, Daisuke Itoh, Yu Isakari, Ales Podgornik and Shuichi Yamamoto

http://dx.doi.org/10.1002/biot.201400550

Research Article

Dual utilization of NADPH and NADH cofactors enhances xylitol production in engineered *Saccharomyces cerevisiae*

Jung-Hyun Jo, Sun-Young Oh, Hyeun-Soo Lee, Yong-Cheol Park and Jin-Ho Seo

http://dx.doi.org/10.1002/biot.201500068

Biotech Method

Phosphonium alkyl PEG sulfate ionic liquids as coating materials for activation of *Burkholderia cepacia* lipase

Yui Matsubara, Shiho Kadotani, Takashi Nishihara, Yoshichika Hikino, Yukinobu Fukaya, Toshiki Nokami and Toshiyuki Itoh

http://dx.doi.org/10.1002/biot.201500413