Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 43 (2006) 86–89
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Elevation of glucose 6-phosphate dehydrogenase activity increases xylitol production in recombinant *Saccharomyces cerevisiae*

Do-Hyun Kwon [a], Myoung-Dong Kim [b], Tae-Hee Lee [a], Yong-Joo Oh [a], Yeon-Woo Ryu [c], Jin-Ho Seo [a,*]

[a] *Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Republic of Korea*
[b] *School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[c] *Department of Molecular Science and Technology, Ajou University, Suwon 442-749, Republic of Korea*

Available online 1 August 2006

Abstract

To increase the NAD(P)H-dependent xylitol production in recombinant *Saccharomyces cerevisiae* harboring the xylose reductase gene from *Pichia stipitis*, the activity of glucose 6-phosphate dehydrogenase (G6PDH) encoded by the *ZWF*1 gene was amplified to increase the metabolic flux toward the pentose phosphate pathway and NADPH regeneration. Compared with the control strain, the specific G6PDH activity was enhanced approximately 6.0-fold by overexpression of the *ZWF*1 gene. Amplification in the G6PDH activity clearly improved the NAD(P)H-dependent xylitol production in the recombinant *S. cerevisiae* strain. With the aid of an elevated G6PDH level, maximum xylitol concentration of 86 g/l was achieved with productivity of 2.0 g/l h in the glucose-limited fed-batch cultivation, corresponding to 25% improvement in volumetric xylitol productivity compared with the recombinant *S. cerevisiae* strain containing the xylose reductase gene only.

Keywords: *Saccharomyces cerevisiae*; NADPH; Xylitol; Xylose reductase; Glucose 6-phosphate dehydrogenase

1. Introduction

Xylitol, a five-carbon sugar alcohol, has the same order of sweetness as sucrose and fructose [1]. Xylitol can be produced by microbial catalysts using xylose-utilizing yeasts [2,3] and recombinant *Saccharomyces cerevisiae* [4]. *S. cerevisiae* cannot utilize xylose as carbon source as this yeast does not possess a metabolic pathway for conversion of xylose to xylitol or to xylulose. Expression of the xylose reductase gene from *Pichia stipitis* in *S. cerevisiae* conferred the ability to produce xylitol from xylose with almost theoretical yield [5]. Since xylose reductase of *P. stipitis* requires NAD(P)H as cofactor for its enzymatic action, cosubstrates, such as glucose and ethanol must be supplied for regeneration of cofactor and maintenance of cellular activity.

Although NADPH can be produced via the $NADP^+$-linked isocitrate dehydrogenase reaction, the oxidative pentose phosphate pathway (PPP) is thought to be a major source of NADPH biosynthesis in yeast [6]. The metabolic flux through this pathway has been reported to increase at high NADPH requirements and to decrease when the need for NADPH synthesis is reduced [7]. The availability of NADPH within a cell might be enhanced by metabolic engineering, e.g., overproduction of enzymes involved in the PPP or deletion of genes in glycolysis in case a hexose is a carbon source. NADPH is produced in two of the steps in the PPP, namely the conversion of glucose 6-phosphate to 6-phosphoglucose-δ-lactone, catalyzed by glucose 6-phosphate dehydrogenase (G6PDH) and conversion of 6-phosphogluconate to ribulose 5-phosphate catalyzed by 6-phosphogluconate dehydrogenase (6PGDH). Overproduction of these enzymes might result in an increase in the PPP. The enhanced NADPH level has been previously reported in *Escherichia coli* and *Ralstonia eutropha* by overproduction [8,9].

This study was undertaken to elevate the enzyme level of G6PDH in order to increase the metabolic flux through PPP and thereby to enhance NADPH regeneration for xylitol production in recombinant *S. cerevisiae*. Two recombinant *S. cerevisiae* strains were characterized in batch and glucose-limited fed-batch cultivations to examine the effects of G6PDH overexpression on NADPH regeneration and concomitant conversion of xylose to xylitol.

* Corresponding author. Tel.: +82 2 880 4855; fax: +82 2 873 5095.
E-mail address: jhseo94@snu.ac.kr (J.-H. Seo).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.06.014

2. Experimental

2.1. Strains, plasmids and culture conditions

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid preparation. *S. cerevisiae* BJ3505/δXR (MATα *pep*4*::HIS*3 *his*3 *lys*2-208 *trp*1, *ura*3) harboring multiple copies of the xylose reductase gene from *P. stipitis* in the genome [10] was used as host for the overexpression of the *ZWF*1 gene. The structural gene of *ZWF*1 was amplified by the polymerase chain reaction (PCR) using the genomic DNA of BJ3505 (ATCC 208281) as template and appropriate primers. After digestion with *Bam*HI and *Eco*RI, the expected-size PCR product was cloned into p426GPD [11] to construct pKZWF1 (2μ, *URA*3, 8.1 kb). The *S. cerevisiae* BJ3505/δXR strain transformed with an empty vector p426GPD was used as control strain.

LB medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per liter) was used for *E. coli* cultivation. A synthetic complete (SC) plate without uracil was used for selection of the transformants harboring the *URA*3-marked plasmid.

Batch and fed-batch cultures were carried out in a bench-top fermentor (KoBiotech, Incheon, Korea) with a 1.0-l working volume. Seed cultures were grown overnight in a selective medium. YEPD medium (10 g yeast extract, 20 g peptone and 20 g glucose per liter) supplemented with 40 g xylose per liter was used for batch cultures. For fed-batch cultures, initial xylose concentration of 100 g/l was used and 600 g/l glucose solution was fed at a rate of 1.8 g glucose/l h after the depletion of glucose added initially. Medium acidity of pH 5.0 and temperature of 30 °C were maintained throughout the cultivation. Agitation speed and aeration rate were set at 500 rpm and 1 vvm, respectively.

2.2. Analytical methods

Dry cell mass concentration was measured with a spectrophotometer (GE Health Care Ultrospcec 2000, Piscataway, NJ, USA) at 600 nm. Optical density was converted into dry cell mass concentration using the predetermined conversion factor. Concentrations of glucose, xylose, ethanol and xylitol were measured by HPLC (Knauer, Berlin, Germany) equipped with the HPX-87H column (Bio-Rad, Richmond, CA, USA) of which temperature was maintained at 60 °C. The mobile phase consisted of 5 mM H_2SO_4 solution and detection was carried out with a reflective index detector (Knauer).

2.3. Measurement of enzyme activities

Preparation of cell extract and measurement of xylose reductase activity were done using the method described by Walfridsson et al. [12] with some modifications. Cells were harvested by centrifugation at 8000 × *g* for 10 min, washed twice with ice-cold water and then incubated in Y-PER solution (Pierce, Rockford, IL, USA) for 20 min. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C to obtain crude extract. Xylose reductase activity was determined by measuring the oxidation of NADPH at 340 nm. One unit of xylose reductase activity was defined as the amount of enzyme that can oxidize 1 μmol of NADPH per minute at 30 °C.

G6PDH activity was determined using the method described by Deutsch [13] with some modifications. The standard assay volume of 200 μl contained 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM maleimide, 0.4 mM $NADP^+$ and 10 mM glucose 6-phosphate. One unit of G6PDH activity was defined as the amount of enzyme that can produce 1 μmol of NADPH per minute at 30 °C. Specific enzyme activity (U/mg) was estimated by dividing enzyme activity by the cellular protein concentration. Protein concentration of the crude cell extract was measured using a protein assay kit (Bio-Rad).

2.4. Determination of intracellular nucleotides

Concentrations of $NADP^+$ and NADPH were determined by enzyme cycling assays which involves 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) as the terminal electron acceptor and phenazine ethosulfate as an electron carrier [14]. Cells were harvested by rapid filtration, washed twice with ice-cold water and then resuspended in 0.5 ml HCl (0.1 M) for $NADP^+$ determination or in 0.5 ml NaOH (0.1 M) for NADPH determination. After boiling for 5 min, cell suspensions were placed on an ice bath and neutralized with an equal volume of 0.1 M NaOH or HCl. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C. Concentrations of $NADP^+$ and NADPH were determined by measuring absorbance at 570 nm.

3. Results and discussion

Batch cultures were carried out to characterize the control and engineered strains. Since the stability of plasmid pKZWF1 was considerably high (over 85% maintenance after eight generations) in our preliminary experiments, both strains were grown in YEPD medium supplemented with 40 g xylose per liter in subsequent batch cultures. Xylitol was produced with almost theoretical yield using ethanol formed from glucose utilization as cosubstrate but the two strains did not show any difference in xylose reductase activity, i.e., approximately specific xylose reductase activity of 0.8 U/mg protein were obtained for both strains. As summarized in Table 1, appreciable influences on rates of cell growth and xylitol production were observed by *ZWF*1 overexpression. An increase in G6PDH activity enhanced xylitol productivity, not so much as expected. In the xylitol-producing period, the *S. cerevisiae* BJ3505/δXR strain harboring plasmid pKZWF1 showed specific G6PDH activity of 0.10 ± 0.02 U/mg protein, which was 6.0-fold increase compared with the control strain. However, amplification in G6PDH level did not lead to a significant improvement in xylitol productivity, i.e., xylitol productivity increased from 1.0 to 1.2 g/l h. Moreover, a statistically appreciable elevation in NADPH content did not occur by *ZWF*1 overexpression (data not shown).

Characteristics of the control and engineered strains were further investigated in glucose-limited fed-batch cultivations. After depletion of glucose added initially, the fermentation mode was switched to a fed-batch phase by feeding glucose solution (60%) as sole carbon source. The *S. cerevisiae* BJ3505/δXR strain with

Table 1
Batch and fed-batch fermentation results of recombinant *S. cerevisiae* BJ3505/δXR strains

Strain	Plasmid	Fermentation mode	Final cell mass (g/l)	Xylitol productivity (g/l h)[a]	Final xylitol concentration (g/l)
S. cerevisiae BJ3505/δXR	p426GPD	Batch[b]	7.5	1.0	19
		Fed-batch	14.5	1.6	71
S. cerevisiae BJ3505/δXR	pKZWF1	Batch	10.0	1.2	24
		Fed-batch	18.0	2.0	86

[a] Values were estimated during the xylitol-producing period of corresponding fermentation mode and averaged from two separate experiments.
[b] For batch fermentation, all values were measured after 20 h of cultivation and averaged from two separate experiments.

an elevated G6PDH activity resulted in 18.0 g/l of final dry cell mass and 86.0 g/l of final xylitol concentration. The engineered strain maintained specific G6PDH activity of 0.10 ± 0.02 U/mg protein throughout the fed-batch period (Fig. 1). Accordingly, amplification of G6PDH activity improved xylitol productivity from 1.6 to 2.0 g/l h, 25% increase in volumetric xylitol productivity. Since such an improvement in xylitol productivity was attributed to an elevated NADPH level, intracellular concentrations of $NADP^+$ and NADPH in the control and engineered strains were measured using the enzyme cycling assays. As shown in Fig. 1, a significant change in NADPH concentration was not observed by G6PDH overexpression in the glucose-limited fed-batch cultures. It was speculated that G6PDH played a role for balancing the redox state in the xylitol-producing strain rather than for increasing the NADPH pool itself. It appeared that NADPH was more rapidly regenerated by an elevated G6PDH activity, which in turn improved the NADPH-dependent xylose conversion to xylitol.

It was interesting to note that amplification in G6PDH activity improved the overall xylitol production in a recombinant *S. cerevisiae* strain although specific xylitol productivity was not improved as much as an elevation of the G6PDH activity. The rapid regeneration of cofactors by an elevation of the G6PDH activity might lead to an increase in the carbon flux through PPP where the metabolites would subsequently be transformed to biomass [15,16].

The catabolism of pentose sugars, such as xylose and arabinose requires NADPH and NADH. Xylose is reduced to xylitol by a reaction that requires NADPH preferentially or exclusively. The xylose reductase from *P. stipitis* prefers NADPH but can use NADH as well [4]. All other fungal xylose reductases described in the literature can use NADPH only as a cofactor.

NADPH is mainly regenerated from the oxidative part of PPP. The oxidative PPP generates 2 mol of NADPH and 1 mol of CO_2 by enzymatic actions of G6PDH and 6PGDH. G6PDH encoded by the *ZWF*1 gene in yeast, the rate-limiting enzyme in PPP, produces NADPH, which is a critical modulator of the cellular redox state. In our previous study, a reduction in NADPH pool by overexpression of bacterial transhydrogenase decreased the xylitol productivity in recombinant *S. cerevisiae* [17]. It was recently reported that NADPH-dependent xylose accumulation was responsible for most of xylitol production in recombinant *S. cerevisiae* [18]. Thus, overexpression of enzymes in PPP, such as G6PDH and 6PGDH might increase intracellular NADPH availability for xylose conversion.

Strategies to increase a cellular NADPH level include metabolic engineering of ammonium assimilation pathways in yeast [19]. Poulsen et al. [20] increased the NADPH content in

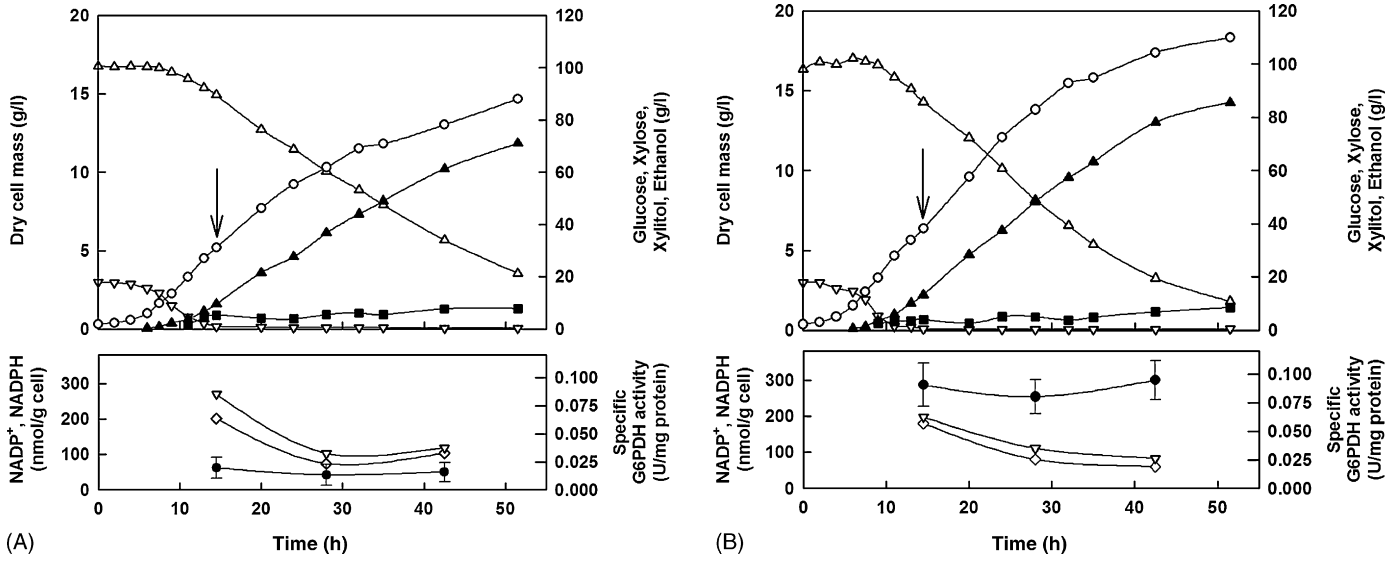

Fig. 1. Fed-batch fermentation profiles of *S. cerevisiae* BJ3505/δXR strains harboring plasmid p426GPD (A) and pKZWF1 (B) at 30 °C and pH 5.0. Dry cell mass (◯), glucose (▽), xylose (△), xylitol (▲), ethanol (■), $NADP^+$ (□), NADPH (▽) and specific G6PDH activity (●). Glucose solution (60%) was fed at a rate of 1.8 glucose/l h in the fed-batch phase. Arrows indicate initiation of the fed-batch mode. Xylitol productivities shown in Table 1 were estimated during the xylitol-producing period.

Aspergillus niger by overexpression of G6PDH encoded by the *gsdA* gene. Nissen et al. [21] constructed a transhydrogenase-producing strain to investigate the effects of the reaction converting NADPH and NAD^+ into $NADP^+$ and NADH on xylose fermentation. Overexpression of cytoplasmic transhydrogenase from *Azotobacter vinelandii* resulted in lower xylitol productivity, which is in good accordance with our previous report [17]. These facts supported the influence of NADPH on xylose conversion to xylitol. It was also suggested that the availability of intracellular NADPH produced from PPP correlated with tolerance against lignocellulose-derived inhibitors, such as 5-hydroxymethyl furfural [22].

Although an enhancement in G6PDH activity improved the cell growth, such an amplification in G6PDH level did not lead to corresponding elevations in NADPH content and specific xylitol productivity, indicating that *S. cerevisiae* has a robust redox control apparatus.

It should be noted from a metabolic engineering approach that overexpression of single enzyme, such as G6PDH may not be sufficient to meet NADPH requirement in a redox-perturbed recombinant *S. cerevisiae* strain.

Since NADPH content in the cell was not profoundly affected by G6PDH overexpression, deletion or modulated expression of the *PGI*1 gene, encoding phosphoglucoisomerase, could be a plausible strategy to reduce the glycolytic flux and thereby, to increase availability of glucose 6-phosphate, a substrate for G6PDH. Through a genome-wide analysis, Bro et al. [23] found that a gene subset consisting of genes encoding metabolic enzymes requiring NAD(H)/NADP(H) as cofactors indicated possible connection of NADPH-requirement with expression of NADPH-regenerating enzymes. It would be very interesting to overproduce all NADPH-producing enzymes or combinations of thereof for improving xylitol conversion in recombinant yeast.

4. Conclusions

The enzyme activity of G6PDH was elevated to enhance NADPH regeneration and concomitantly to improve the NAD(P)H-dependent xylose conversion to xylitol in the recombinant *S. cerevisiae* strain. Overexpression of G6PDH resulted in 25% enhancement in overall xylitol productivity and hence, metabolic engineering of redox metabolism appeared to be an attractive strategy for improving the conversion of xylose to xylitol.

Acknowledgements

This work was supported by Ministry of Education through the BK21 program and Korea Research Foundation Grant (KRF-2005-206-F00006).

References

[1] L. Hyvõnen, P. Koivistoinen, F. Voirol, Adv. Food Res. 28 (1982) 373.
[2] S.H. Jang, H.Y. Kang, G.J. Kim, J.H. Seo, Y.W. Ryu, J. Microbiol. Biotechnol. 13 (2005) 501.
[3] H. Jeppsson, K. Holmgren, B. Hahn-Hägerdal, Appl. Microbiol. Biotechnol. 53 (1999) 92.
[4] M.D. Kim, Y.S. Jeun, S.G. Kim, Y.W. Ryu, J.H. Seo, Enzyme Microbial. Technol. 31 (2002) 862.
[5] W.J. Lee, Y.W. Ryu, J.H. Seo, Proc. Biochem. 35 (2000) 1199.
[6] P. Bruinenberg, P. de Bot, J. van Dijken, A. Scheffers, Eur. J. Appl. Microbiol. Biotechnol. 18 (1983) 287.
[7] M. dos Santos, G. Thygesen, P. Kotter, L. Olsson, J. Nielsen, FEMS Yeast Res. 4 (2003) 59.
[8] S.J. Lim, Y.M. Jung, H.Y. Shin, Y.H. Lee, J. Biosci. Bioeng. 93 (2002) 543.
[9] J.C. Choi, H.D. Shin, Y.H. Lee, Enzyme Microbial. Technol. 32 (2003) 178.
[10] Y.S. Chung, M.D. Kim, W.J. Lee, Y.W. Ryu, J.H. Kim, J.H. Seo, Enzyme Microbial. Technol. 30 (2002) 809.
[11] D. Mumberg, R. Müller, M. Funk, Gene 156 (1995) 119.
[12] M. Walfridsson, M. Anderlund, X. Bao, B. Hahn-Hägerdal, Appl. Microbiol. Biotechnol. 48 (1997) 218.
[13] J. Deutsch, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. 3, Deerfield Beach, Verlag Chemie, 1983, p. 190.
[14] Y. Gibon, F. Larher, Anal. Biochem. 251 (1997) 153.
[15] J. Fiaux, Z.P. Çakar, M. Sonderegger, K. Wüthrich, T. Szyperski, U. Sauer, Eur. Cell. 2 (2003) 170.
[16] H. Maaheimo, J. Fiaux, Z.P. Çakar, J.E. Bailey, U. Sauer, T. Szyperski, Eur. J. Biochem. 268 (2001) 2464.
[17] Y.S. Jeun, M.D. Kim, Y.C. Park, T.H. Lee, M.S. Yoo, Y.W. Ryu, J.H. Seo, J. Mol. Catal. B: Enzyme 26 (2003) 251.
[18] M. Jeppsson, K. Träff, B. Johansson, B. Hahn-Hägerdal, M.F. Gorwa-Grauslund, Appl. Environ. Microbiol. 68 (2002) 1604.
[19] C. Roca, J. Nielsen, L. Olsson, Appl. Environ. Microbiol. 69 (2003) 4732.
[20] B.R. Poulsen, J. Nóhr, S. Douthwaite, L.V. Hansen, J.J.L. Iversen, J. Visser, G.J.G. Ruijter, FEBS J. 272 (2005) 1313.
[21] T.L. Nissen, M. Anderlund, J. Nielsen, J. Villadsen, M.C. Kielland-Brandt, Yeast 18 (2001) 19.
[22] C.F. Wahlbom, B. Hahn-Hägerdal, Biotechnol. Bioeng. 78 (2002) 172.
[23] C. Bro, B. Regenberg, J. Nielsen, Biotechnol. Bioeng. 85 (2004) 269.