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

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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.

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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