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Dual modulation of glucose 6-phosphate metabolism to increase NADPH-dependent xylitol production in recombinant *Saccharomyces cerevisiae*

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Abstract

To increase the metabolic flux toward the NADPH-generating pentose phosphate pathway in a recombinant *Saccharomyces cerevisiae* strain that harbors the xylose reductase gene from *Pichia stipitis*, expression of phosphoglucose isomerase (PGI) encoded by the *PGI1* gene was modulated by a promoter replacement using the *ADH1* promoter. Although the *ADH1* promoter down-regulated PGI expression in glucose-limited condition, the decline of PGI activity did not exert a profound influence on xylitol production in a series of glucose-limited fed-batch cultivations. However, simultaneous enforcement of glucose 6-phosphate dehydrogenase (G6PDH) activity and attenuation of phosphoglucose isomerase activity worked in a cooperative manner to increase xylitol production and to reduce utilization of cosubstrate required for xylitol production in a glucose-limited fed-batch cultivation of the PGI mutant strain with an enhanced G6PDH activity. An 1.9-fold increase in specific xylitol productivity of 0.34 ± 0.03 g/g cells h was achieved compared with the control strain containing xylose reductase only.

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

1. Introduction

Microbial catalysts such as xylose-utilizing yeasts and recombinant *Saccharomyces cerevisiae* can convert xylose to xylitol, a five-carbon sugar alcohol [1–3]. Expression of the xylose reductase gene from *Pichia stipitis* in *S. cerevisiae*, one of the most widely used eukaryotic workhorses in biotechnology [3–7], conferred the ability to produce xylitol from xylose with almost theoretical yield [2,3]. Since xylose reductase of *P. stipitis* requires NAD(P)H as cofactor for its enzymatic action, cosubstrates such as glucose and ethanol are needed to regenerate cofactors, which are oxidized during the conversion of xylose to xylitol [8]. Accumulation of glucose, a preferential but repressible cosubstrate, can override xylose transport and hence, a controlled feeding strategy for glucose has been generally used for a fed-batch operation [3,9,10].

In our previous study [10], a reduction in the NADPH pool by overexpression of bacterial transhydrogenase decreased xylitol productivity in a recombinant *S. cerevisiae* strain, which was in good accordance with the fact that xylose reductase from *P. stipitis* prefers NADPH to NADH as a cofactor [11]. While several approaches have been successfully attempted to engineer the cofactor metabolism in *Escherichia coli* [12,13], there is an increased complexity in balancing the formation and consumption of cofactors in *S. cerevisiae* as this yeast does not have cytoplasmic transhydrogenase able to convert NADH directly

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into NADPH. Moreover, the metabolism of these cofactors is compartmentalized.

The oxidative pentose phosphate pathway (PPP) is thought to be a major source of NADPH biosynthesis in yeast [14]. The metabolic flux through this pathway has been reported to increase at high NADPH demand and to decrease when the need for NADPH production is reduced [15]. NADPH is produced at the two steps in PPP including the conversion of glucose 6-phosphate to 6-phosphoglucose-δ-lactone, catalyzed by glucose 6-phosphate dehydrogenase (G6PDH) and the conversion of 6-phosphogluconate to ribulose 5-phosphate catalyzed by 6-phosphogluconate dehydrogenase.

A simple metabolic engineering approach can be considered to force all carbon fluxes toward the oxidative PPP by blocking the entry of a carbon into glycolysis. This goal might be achieved by disruption of the gene *PGI1*. Phosphoglucose isomerase (PGI; E.C. 5.3.1.9) encoded by the *PGI1* gene in *S. cerevisiae* is a key enzyme in glycolysis that functions at the junction of the gluconeogenesis and catabolism. It catalyzes the reversible isomerization of D-glucose 6-phosphate and D-fructose 6-phosphate and thus its activity is tightly controlled at the pivotal point of the two metabolic pathways, glycolysis and PPP. However, disruption of the *PGI1* gene to force the carbon flux through PPP in *S. cerevisiae* is reported as lethal [16,17]. Accordingly, it is highly desirable to manipulate the expression level of PGI appropriately. It could be assumed that reduced PGI activity might lead to accumulation of glucose 6-phosphate, a substrate for G6PDH that produces NADPH.

Alcohol dehydrogenase I encoded by the *ADH1* gene in yeast reduces acetaldehyde to ethanol during glucose fermentation. Although originally thought to be constitutive, *ADH1* transcription is repressed when cells are grown on a non-fermentable carbon source such as glycerol and ethanol [18]. On glucose, activity of the *ADH1* promoter decreases during the late exponential phase where glucose is almost exhausted [19].

This study was undertaken in order to increase the metabolic flux through PPP, to accelerate NADPH regeneration and thus to enhance the NADPH-dependent xylitol production in the recombinant *S. cerevisiae* strain. A 'push and pull' strategy to increase NADPH regeneration was evaluated, i.e., G6PDH activity was fortified by overexpression of the *ZWF1* gene on plasmid and expression of the genomic *PGI1* gene was modulated by the *ADH1* promoter. Performances of the engineered strains for xylitol production were compared in a series of glucose-limited fed-batch cultivations.

2. Experimental

2.1. Strains and DNA manipulations

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid preparation. *S. cerevisiae* BJ3505/δXR harboring multiple copies of the *P. stipitis* xylose reductase gene [9] was used as host strain for promoter replacement and transformation of plasmid pKZWF1 (2 μm, *URA3*, 8.1 kb, lab stock) which constitutively overexpresses the *ZWF1* gene [20]. Recombinant *S. cerevisiae* strains used in this study were listed in Table 1. Empty vectors p426GPD (ATCC 87361) and pMK103 (2 μm, *TRP1*, 8.0 kb, lab stock) were used as control.

The truncated structural *PGI1* gene was obtained by the polymerase chain reaction (PCR) using the genomic DNA of *S. cerevisiae* BJ3505 (ATCC 208281) as template and two primers YJ1 [5′-CGGGATCCCTAAAAATGTCCAATAACTCATTCA-3′] and YJ2 [5′-CCGCTCGAGGTCAACAACCTTCAAGGTTT-3′]. After digestion with *Bam*HI and *Xho*I, the expected-size PCR product was cloned into plasmid pMK103 that contains the *ADH1* promoter and *CYC1* terminator up- and downstream of the cloning site, respectively, to construct pYJ103. Plasmid pYJ103 was linearized with *Sal*I restriction enzyme before transformation into the yeast. Control strains were also constructed by transformation with an empty vector pMK103 or p426GPD. Plasmid pMK103 was linearized by *Bsp*MI of which recognition site is located in the *TRP1* open reading frame. Promoter replacement was confirmed by diagnostic PCR using primers DIAG5 [5′-ATCTTAAAAAGGTCCTTTCTTCATAA-3′] and DIAG3 [5′-AAATATAAATAACGTTCTTAATACTAACATAACTA-3′]. All recombinant DNA techniques were based on the methods described by Sambrook and Russell [21]. Schematic representation of the promoter replacement is shown in Fig. 1.

2.2. Growth conditions

LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) was used for *E. coli* cultivation. Synthetic complete (SC) plates without appropriate nutrients were used for selection of the yeast transformants. Fed-batch cultures were carried out in a bench-top fermentor (Bioengineering AG, Wald, Switzerland) with 1.0-l working volume. Seed cultures were grown overnight in a selective SC medium. YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 10% xylose was used for fed-batch cultures. After the depletion of glucose added initially, glucose solution (60%) was fed at a rate of 0.6 g

Table 1
Recombinant *S. cerevisiae* strains used in this study

Strain	Genotype	PGI activity[a] (unit/mg)	Ref.
BJ3505/δXR	*MATα his3 lys2-208 trp1 ura3 pep4::HIS3 Ty-δ*::P_{GPD1}-*XYL1*-T_{GPD1}-neo^r	0.40 ± 0.02	[9]
YJO-4	BJ3505/δXR, *trp1*::*TRP1*, p426GPD	0.40 ± 0.02	This study
YJO-11	BJ3505/δXR, *PGI1*::P_{ADH1}-*PGI1*-T_{CYC1}-*TRP1*, p426GPD	0.12 ± 0.02	This study
YDK-5	BJ3505/δXR, *trp1*::*TRP1*, pKZWF1	0.42 ± 0.02	This study
YJO-12	BJ3505/δXR, *PGI1*::P_{ADH1}-*PGI1*-T_{CYC1}-*TRP1*, pKZWF1	0.12 ± 0.02	This study

[a] For enzyme assay, cells growing exponentially in YEPD were shifted to yeast extract–peptone medium supplemented with 0.05% glucose for 1 h.

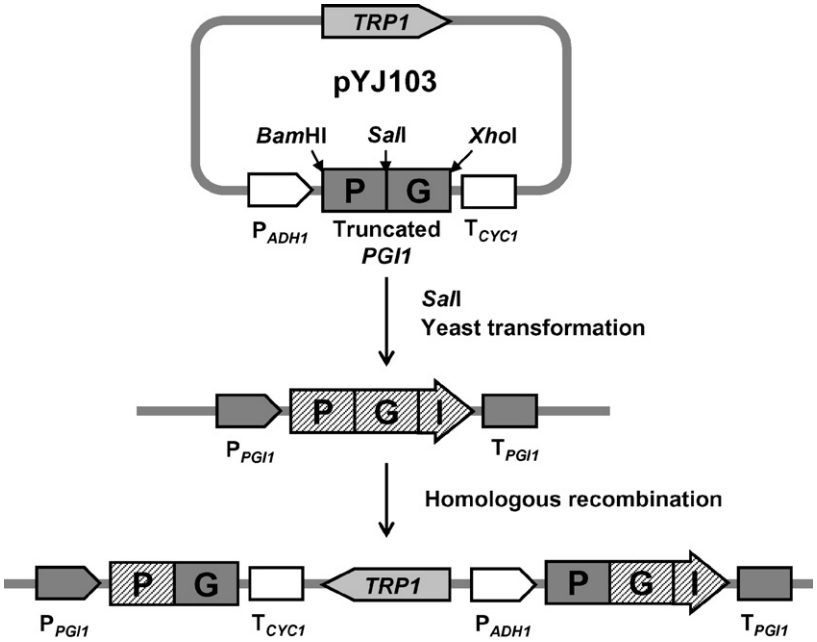

Fig. 1. Schematic representation of promoter replacement through homologous recombination. Plasmid pYJ103 that harbors truncated *PGI1* gene under the control of the *ADH1* promoter was linearized by restriction enzyme *Sal*I of which restriction site is located in the structural gene of *PGI1*.

glucose/h. Medium acidity and temperature were maintained at pH 5.0 and 30 °C throughout the cultivation. Agitation speed and aeration rate were set at 500 rpm and 1 vvm, respectively. To measure the expression levels of PGI and G6PDH, the recombinant *S. cerevisiae* strains grown in the respective cultivation mode were harvested by rapid filtration and stored at −80 °C until further analysis.

2.3. Analytical methods

Dry cell mass concentration was measured with a spectrophotometer at 600 nm. Optical density was converted into dry cell mass concentration using the predetermined conversion factors for the respective strains. Concentrations of glucose, xylose, ethanol, and xylitol were measured using HPLC (Knauer, Berlin, Germany) equipped with the HPX-87H column (Bio-Rad, Richmond, CA, USA). The mobile phase was 5 mM H_2SO_4 solution. For enzyme assays, cells corresponding to an optical density of 20 at 600 nm were harvested by centrifugation, washed twice with ice-cold water and incubated in an appropriate volume of Y-PER solution (Pierce, Rockford, IL, USA) for 20 min. Cell debris was removed by centrifugation at 8000 × *g* for 10 min and the supernatant was used for enzyme assays. PGI activity was determined using the method described by Deutsch [22] with some modifications. The standard assay (200 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM maleimide, 0.4 mM $NADP^+$, 10 mM fructose 6-phosphate and 3 units of glucose 6-phosphate dehydrogenase (G6PDH). One unit of PGI activity was defined as the amount of an enzyme that produces one micromole of NADPH per minute at 30 °C. For G6PDH assay, the same procedures as mentioned for PGI were used except that glucose 6-phosphate was used as substrate. One unit of G6PDH activity was defined as the amount of an enzyme that produces one micromole of NADPH per minute at 30 °C.

Xylose reductase activity was determined by measuring the oxidation of NADPH at 340 nm as described in our previous report [2]. One unit of xylose reductase activity was defined as the amount of an enzyme that oxidizes one micromole of NADPH per minute at 30 °C. Specific enzyme activity (unit/mg cellular protein) of an enzyme was determined by dividing enzyme activity by the cellular protein concentration. Protein concentration was measured using a protein assay kit (Bio-Rad).

3. Results and discussion

To assess the *PGI1* promoter replacement with *ADH1*, PGI activities were assayed in recombinant *S. cerevisiae* strains grown to the exponential phase in YEPD (2% glucose). For comparison, cells grown in YEPD were shifted to yeast extract–peptone medium supplemented with 0.05% glucose for 1 h. At low glucose condition (0.05%), PGI activities in the strains YJO-11 and YJO-12 were notably reduced to give specific PGI activities of 0.12 ± 0.02 (unit/mg protein), which corresponded to 72% reduction compared with the control strains YJO-4 and YDK-5. On the other hand, at high (2%) glucose condition, the PGI expression level controlled by the *ADH1* promoter was comparable with that of the native promoter to give similar specific PGI activity of 0.42 ± 0.02 units/mg, indicating that the *ADH1* promoter strength was attenuated to some extent at low glucose condition. Promoter replacement was also confirmed by a diagnostic PCR. Accordingly, it was concluded that the mutant *S. cerevisiae* strains (YJO-11, YJO-12) in which the *ADH1* promoter controls the *PGI1* gene expression were successfully constructed.

Table 2
Summary of glucose-limited fed-batch cultivations of recombinant *S. cerevisiae* strains

Strain	Dry cell mass[a] (g/l)	Xylitol yield[a] (g/g)	Specific xylitol productivity[a] (g/g cells h)	Glucose consumed per xylitol produced[b] (g/g)
YJO-4 (control)	12.1 ± 0.3	0.98	0.18 ± 0.03	0.38 ± 0.01
YJO-11 (low PGI)	13.2 ± 0.3	1	0.16 ± 0.02	0.39 ± 0.01
YDK-5 (high G6PDH)	10.2 ± 0.2	0.99	0.28 ± 0.03	0.33 ± 0.01
YJO-12 (high G6PDH plus low PGI)	9.5 ± 0.2	1	0.34 ± 0.03	0.28 ± 0.01

Initial xylose concentration of 100 g/l was supplemented to YEPD medium and glucose was fed at a constant rate of 0.6 g/h after glucose depletion. All measurements were performed in triplicate and averages and standard errors are shown.

[a] Values were obtained after 69 h of cultivation.

[b] Values are averages obtained based on the volumetric changes during the glucose-limited fed-batch mode.

Glucose-limited fed-batch cultivations were carried out to compare xylitol productivities for the YJO-4 (control) and YJO-11 (low PGI) strains. As shown in Table 2, an altered PGI expression context did not make a significant change in final concentrations of dry cell mass and xylitol productivities. It indicated that a change in PGI activity alone did not profoundly affect xylitol production in the recombinant *S. cerevisiae* strain.

Another series of glucose-limited fed-batch cultivations were performed further to examine whether the dual control over the glucose 6-phosphate metabolism would increase NADPH-dependent xylitol production. Recombinant *S. cerevisiae* YDK-5 and YJO-12 strains were constructed to increase G6PDH activity. Such a 'push and pull' strategy over glucose 6-phosphate was expected to increase the metabolic flux toward PPP for NADPH generation by increasing the supply of glucose 6-phosphate, the substrate for G6PDH, and concomitantly by enhancing the G6PDH activity for NADPH generation from glucose 6-phosphate and $NADP^+$. PGI activities for the YJO-12 strain were 0.28 ± 0.03 units/mg protein during the fed-batch mode while those of the YDK-5 strain were 0.44 ± 0.02 units/mg protein. By overexpression of the *ZWF1* gene on pKZWF1 plasmid, G6PDH activities of 0.13 ± 0.03 units/mg protein were maintained for both strains, YDK-5 (high G6PDH) and YJO-12 (high G6PDH plus low PGI), which was in good accordance with our previous study [18]. Specific xylitol productivity in glucose-limited fed-batch cultivation of the YDK-5 strain increased from 0.18 ± 0.03 to 0.28 ± 0.03 g/g cells h by overexpression of G6PDH. In addition, effects of G6PDH amplification became more evident for the PGI mutant. Specific xylitol productivity for YJO-12 (high G6PDH plus low PGI) strain was 0.34 ± 0.03 g/g cells h, which was a 1.9-fold enhancement in the specific xylitol productivity compared with the parental strain, YJO-4 containing xylose reductase only (Table 2 and Fig. 2). Enzyme assays supported that these results were not caused by changes in xylose reductase activity (data not shown).

It was interesting to note that an enhanced G6PDH activity alone increased xylitol production, i.e., a 1.6-fold enhancement in specific xylitol productivity for the YDK-5 strain was achieved by an elevation of G6PDH activity compared with the YJO-4 strain in glucose-limited fed-batch cultivation. A similar result was also obtained in our previous study [20]. This indicated that NADPH was efficiently regenerated by an elevation of G6PDH activity. Redirection of the carbon flux to PPP by reducing PGI activity synergistically assisted xylitol production. Based on the amount of glucose consumed per xylitol produced for YJO-4 and YJO-12 strains, the cosubstrate requirement was reduced by 26% via dual modulation of PGI and G6PDH activities during the glucose-limited fed-batch cultivation. Therefore, it was suggested that amplification of G6PDH activity along with efficient supply of its substrate, glucose 6-phosphate, resulted in a synergistic enhancement of NADPH generation for xylose conversion to xylitol.

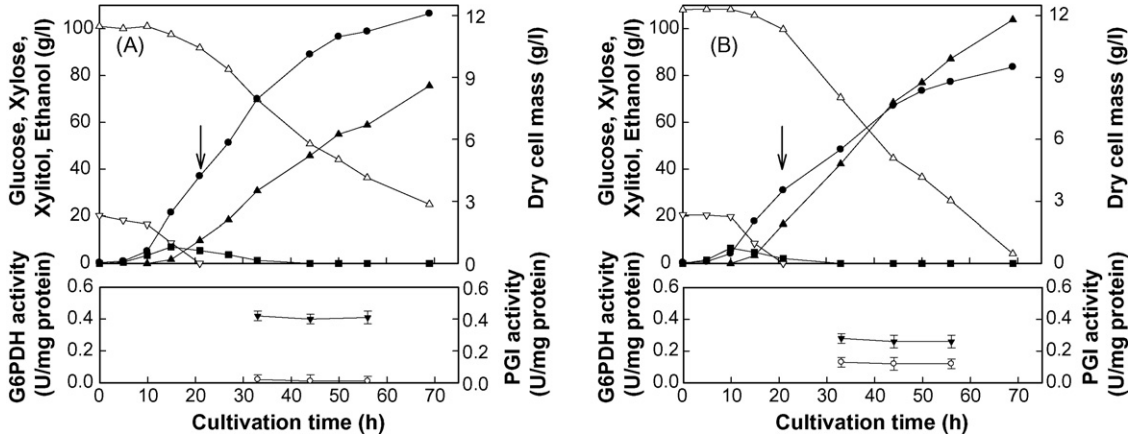

Fig. 2. Profiles of glucose (▽), xylose (□), cell growth (●), xylitol (▲), ethanol (■), G6PDH activity (○) and PGI activity (▼) in glucose-limited fed-batch cultivations of recombinant *S. cerevisiae* strains YJO-4 (A) and YJO-12 (B). Arrows indicate the initiation of the fed-batch mode in which glucose was fed at a constant feed rate of 0.6 g/h after the exhaustion of the glucose added initially. Medium acidity and temperature were maintained at pH 5.0 and 30 °C throughout the cultivation.

Inactivation of PGI might be particularly useful for exploring the metabolic network behavior since PGI is located at the first junction of two different metabolic pathways for glucose catabolism. Then, a large portion of the carbon flux from glucose should be proceeded to PPP in the PGI inactivated cells. Such a redirection in the metabolic flux can result in a profound influence on the balance of 'reducing power budget' because NADPH is formed in excess when catabolism proceeds exclusively through these pathways. The use of *ADH1* promoter enables to down-regulate PGI at low glucose condition such as a glucose-limited fed-batch mode and to grow efficiently the cells when a high level of glucose is present, however, it also limits the strain usage to an operation set-up with low glucose feeding.

Canonaco et al. [23] analyzed the metabolic flux response to the PGI knockout in *E. coli* and found that the primary response in the metabolic pathway was the flux rerouting via PPP. Since *S. cerevisiae* does not contain a transhydrogenase that could transfer electrons from NADPH to NAD^+, the null mutant of PGI in the yeast cannot grow on glucose as sole carbon source. In this study, a significant improvement in xylitol production was not achieved in the fed-batch cultivation via the decline of PGI activity. It was conceived that the diminution in PGI activity was not enough to enforce the flux toward PPP. Accordingly, an optimum PGI level should be determined to maximize the carbon flux toward PPP, an NADPH-generating pathway in yeast.

Redox cofactors participate in more than 300 biochemical reactions involving oxidation and reduction, and their manipulation is expected to have significant effects on metabolic networks [24]. Cofactor engineering has therefore a potential as a tool both for studying metabolism and for manipulating metabolic pathways. Most current metabolic engineering strategies have focused on manipulating enzyme levels through amplification, interruption or addition of a given metabolic pathway. NADH is preferentially used in assimilatory pathways whereas NADPH functions mainly as a reducing power in anabolism. *S. cerevisiae* does not have transhydrogenase activity and the redox pairs NADH/NAD^+ and NADPH/$NADP^+$ are not able to cross the mitochondrial inner membrane. Therefore, the reduced coenzymes should be reoxidized via utilization of a carbon source to avoid depletion of these pyridine nucleotides, which may result in reduction or termination of cell growth.

From this standpoint, maintaining the cellular redox balance is a basic requirement for living cells to sustain growth. The intracellular redox state is to a large extent dependent upon the intracellular concentration ratios of the two pyridine nucleotide systems NADH/NAD^+ and NADPH/$NADP^+$. In the case of xylitol-producing recombinant *S. cerevisiae* which harbors xylose reductase from *P. stipitis*, regeneration of NAD(P)H is crucial for the enzymatic action of xylose reductase that prefers NADPH but can use NADH as well. Since the enzymatic reaction of xylose reductase occurs at cytoplasm, manipulation of PPP is conceived as one of the most plausible ways for efficient regeneration of NADPH. Poulsen et al. [25] increased the NADPH content in *Aspergillus niger* by overexpression of G6PDH encoded by the *gsdA* gene. In our previous study [10], effects of NADPH on xylitol production were investigated by introducing a transhydrogenase converting NADPH and NAD^+ into $NADP^+$ and NADH into a recombinant *S. cerevisiae* strain.

Although PPP represents the main source of NADPH biosynthesis in *S. cerevisiae*, the other strategies to increase intracellular NADPH availability can include engineering of the ammonium pathway or the malic enzyme shunt in *S. cerevisiae*. In addition, $NADP^+$-dependent isocitrate dehydrogenase and acetaldehyde dehydrogenase can be considered. It would be also very interesting to overproduce all NADPH-producing enzymes or combinations of thereof. In this study, amplification of G6PDH enzyme activity and concomitant attenuation of PGI activity worked in a cooperative manner to enhance xylitol production in the recombinant *S. cerevisiae* strain.

In conclusion, this study demonstrated that the tuning of NADPH metabolism could be achieved by manipulating two metabolic enzymes located at the glycolytic and the PPP metabolic nodes. This study also suggested experimentally the quantitative relation among PPP, NADPH generation and xylitol production in a recombinant *S. cerevisiae* strain. A metabolic engineering approach used in this study could find applications to other cell-based biotransformation reactions that require NADPH as reducing equivalent.

4. Conclusions

Simultaneous enforcement of G6PDH activity and attenuation of phosphoglucose isomerase activity in the recombinant *S. cerevisiae* strain worked in a cooperative manner to achieve maximum specific xylitol productivity of 0.34 ± 0.03 g/g cells h in the glucose-limited fed-batch cultivation, which corresponded to a 1.9-fold enhancement in specific xylitol productivity compared with the parental strain containing xylose reductase only.

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