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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.