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ORIGINAL PAPER

Enhancement of xylitol production in *Candida tropicalis* by co-expression of two genes involved in pentose phosphate pathway

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Abstract The yeast *Candida tropicalis* produces xylitol, a natural, low-calorie sweetener whose metabolism does not require insulin, by catalytic activity of NADPH-dependent xylose reductase. The oxidative pentose phosphate pathway (PPP) is a major basis for NADPH biosynthesis in *C. tropicalis*. In order to increase xylitol production rate, xylitol dehydrogenase gene (*XYL2*)disrupted *C. tropicalis* strain BSXDH-3 was engineered to co-express *zwf* and *gnd* genes which, respectively encodes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH), under the control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. NADPH-dependent xylitol production was higher in the engineered strain, termed "PP", than in BSXDH-3. In fermentation experiments using glycerol as a co-substrate with xylose, strain PP showed volumetric xylitol productivity of 1.25 g l^{-1} h^{-1}, 21% higher than the rate (1.04 g l^{-1} h^{-1}) in BSXDH-3. This is the first report of increased metabolic flux toward PPP in *C. tropicalis* for NADPH regeneration and enhanced xylitol production.

Keywords Xylitol · *Candida tropicalis* · Glucose-6-phosphate dehydrogenase · 6-Phosphogluconate dehydrogenase

Abbreviation
PPP Pentose phosphate pathway

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Introduction

Xylitol, a five-carbon sugar alcohol, is used as an alternative for sucrose, fructose and various artificial sweeteners. It is roughly as sweet as sucrose despite having only approximately two-third of its calories, and does not require insulin for its metabolic regulation. Xylitol is widely and increasingly used in the food and confectionary industries because of these benefits [15], and its dietary and chemical properties have been extensively studied [17]. The chemical method generally used for production of xylitol on an industrial scale, which is based on xylose dehydrogenation [3], is costly and energy intensive [19], and also presents environmental hazards because it uses a toxic Raney nickel catalyst, and high-pressure hydrogen gas.

Alternative biotechnological approaches for xylitol production using xylose-fermenting yeasts, which utilize NADPH-dependent xylose reductase to reduce xylose to xylitol, are safe and environmentally friendly, and give high yield and productivity. In particular, the yeast *Candida tropicalis* has been extensively studied in this regard [7, 9, 10, 14, 20, 24]. Great efforts have been made to improve or optimize biological production of xylitol by yeasts, because of high demand from the pharmaceutical and food industries [4, 6, 21, 22].

In a previous study, we achieved high yield of xylitol production in *C. tropicalis* under fully aerobic conditions by disrupting the *XYL2* gene encoding XDH [11]. We screened various carbon sources for xylitol production by this mutant strain, termed BSXDH-3, and found that glycerol was the best co-substrate for cell maintenance and NADPH regeneration [12]. In micro-organisms, NADPH is produced mainly via the pentose phosphate pathway (PPP), as a co-factor of two enzymes: (1) glucose-6-phosphate

dehydrogenase (G6PDH), encoded by the gene *zwf*, catalyzing the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone and (2) 6-phosphogluconate dehydrogenase (6-PGDH), encoded by *gnd*, catalyzing the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate [23] (Fig. 1). The oxidative part of the PPP is strongly associated with xylitol production, and xylitol yield is decreased by disrupting *zwf* or *gnd* [5]. NADPH content in *Aspergillus niger* was increased by over-expression of G6PDH, encoded by *gsdA* gene [18]. In addition to generating NADPH, the PPP contributes to synthesis of ribose 5-phosphate, which is required for biosynthesis of certain amino acids, nucleotides and coenzymes.

Manipulation of redox co-factors, which participate in >300 biochemical reactions involving oxidation and reduction, has significant effects on metabolic networks [2]. Metabolic engineering strategies are often focused on manipulating pathways for co-factor regeneration. NADPH availability is a limiting factor in the reduction of xylose to xylitol in *C. tropicalis*. The goal of the present study is to enhance metabolic flux through PPP, and thereby promote NADPH regeneration. This ultimately enhances NADPH-dependent xylitol production by co-expressing *zwf* and *gnd* genes, encoding G6PDH and 6-PGDH (Fig. 2).

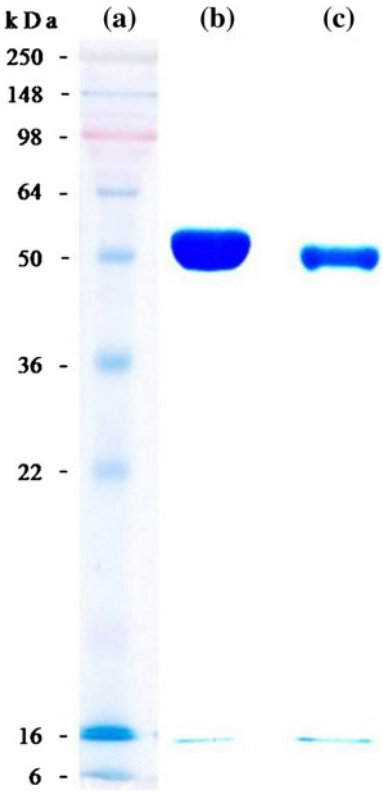

Fig. 2 SDS-PAGE analysis of recombinant G6PDH and 6-PGDH purified from *E. coli* BL21, using 12% (w/v) polyacrylamide gel stained with silver nitrate. **a** Marker, **b** purified G6PDH (58 kDa) and **c** purified 6-PGDH (57 kDa)

Materials and methods

Yeast strains and culture media

C. tropicalis L10 (ura3/ura3), a uracil auxotroph derived from *C. tropicalis* BSXDH-3, was used as a host strain for transformation. For genetic manipulation of *C. tropicalis*, YM medium (3 g yeast extract, 3 g malt extract, 5 g bactopeptone, 20 g glucose), YNB medium (6.7 g yeast–nitrogen base without amino acids, 20 g glucose) and YNB-5FOA medium (6.7 g yeast–nitrogen base without

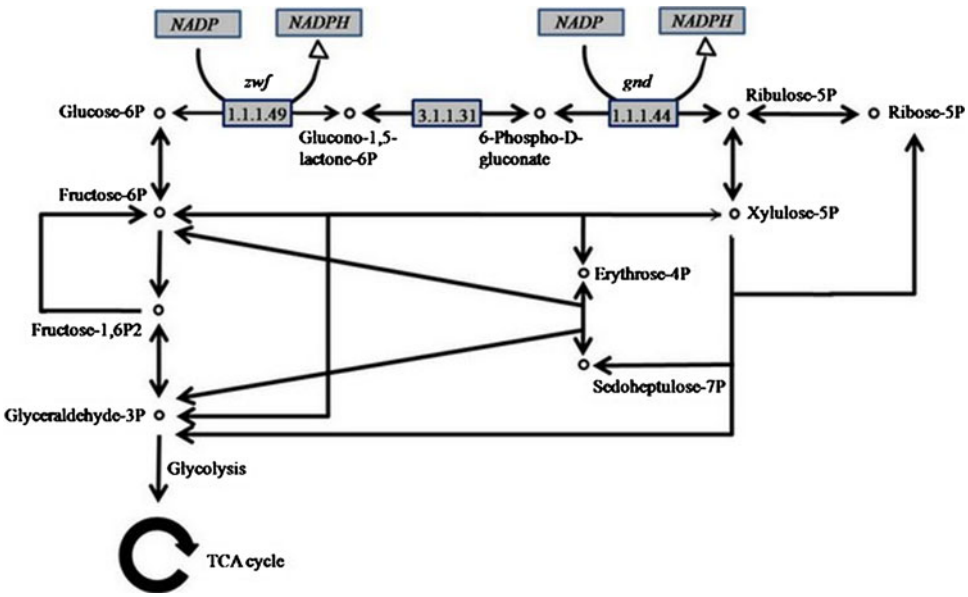

Fig. 1 Schematic summary of pentose phosphate pathway (PPP) in *C. tropicalis*

amino acids, 20 g glucose, 0.1 g uracil, 0.1 g uridine, 0.8 g 5-fluroorotic acid per liter) were used. *Escherichia coli* DH5α and BL21 (RBC Bioscience, Taiwan) were used, respectively, for plasmid preparation and for expression of recombinant enzymes, and were cultured in Luria–Bertani (LB) medium.

Cloning of zwf and gnd genes from *C. tropicalis*

zwf and *gnd* were isolated from genomic DNA of *C. tropicalis* (ATCC20336) by XHL PCR kit (Bioneer, Daejeon, Korea) using synthesized primers complementary to the coding regions of the genes. Amplified PCR products were purified from agarose gel after electrophoresis with a gel extraction kit (Promega, WI, USA). Sequence data of *zwf* and *gnd* were submitted to GenBank database under accession numbers FJ380923 and FJ380925.

Enzyme activity assays

G6PDH and 6-PGDH activities were measured as described previously [8], with conversion of NADP to NADPH determined by change in absorbance at 340 nm (A_{340}) at 25°C. Enzyme activities are expressed in terms of specific activity [U (mg of protein)$^{-1}$], where one unit corresponds to conversion of 1 μmol $NADP^+$ per minute.

Gene transformation for over-production of G6PDH and 6-PGDH

zwf and *gnd* genes were cloned into pET-21a(+), an expression vector containing *C*-terminal *His*-tag sequence (Invitrogen). The resulting plasmids were transformed into *E. coli* BL21 for over-production of G6PDH and 6-PGDH. The recombinant enzymes were induced by 0.5 mM IPTG, and purified by nickel nitrilotriacetic acid agarose affinity chromatography (Qiagen).

Construction of *zwf* and *gnd* gene expression cassettes, and yeast transformation

In our previous study, two copies of the *XYL2* gene encoding xylitol dehydrogenase were disrupted by inserting the *hisG* fragment and *URA*3 gene at two *XYL2* gene positions in *C. tropicalis*. The resulting strain, BSXDH-3, was incubated on a YNB-5FOA plate for 3 days to detect deletion of *URA*3 marker gene. The resulting uracil auxotroph strain was designated L10. L10 has two *hisG* sequences that permit integration of two expression cassettes including a homologous region of *hisG*. The genes *glu* and *leu*, which encode glutamic acid and leucine, were cloned from genomic DNA of *Bacillus subtilis*. The first constructed cassette was composed of 5′ *HisF1,* glutamic acid (*glu*), uracil marker (*URA3*), glutamic acid (*glu*), GAPDH promoter (P_{GAPDH}), *zwf*, GAPDH terminator (T_{GAPDH}), and 3′ *HisR1*. The cassette contained two DNA fragments 5′ *HisF1* and 3′ *HisR1* flanking the first target *hisG* region, and was transformed into *C. tropicalis* L10 by homologous recombination system using lithium acetate method [12]. A *gnd* expression cassette was constructed by similar procedure.

Fermentation and analytical methods

The fermentation medium for xylitol production consisted of 50 g D-xylose, 10 g yeast extract, 5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, and 20 g glycerol per liter as a co-substrate for cell growth. Xylitol production was performed in 250 ml Erlenmeyer flask with 50 ml xylitol fermentation medium in a shaking incubator, 200 rpm at 30°C. Concentrations of D-xylose, xylitol, and glycerol were determined by high-pressure liquid chromatography (Waters, MA, USA), equipped with a Sugar-Pak 1 column and a refractive-index detector (Waters). Distilled water was used as the mobile phase, at column temperature 90°C and flow rate 0.5 ml min^{-1}. Cell growth was monitored spectrophotometrically at 600 nm. One A_{600} was equivalent to 0.474 g cells l^{-1}.

Results and discussion

Purification and analysis of recombinant G6PDH and 6-PGDH

Recombinant G6PDH and 6-PGDH were purified from *E. coli* BL21 using nickel nitrilotriacetic acid agarose affinity chromatography. G6PDH generated a much higher level of NADPH than 6-PGDH. Enzymatic activities of G6PDH and 6-PGDH are shown in Table 1. Molecular weights of recombinant G6PDH and 6-PGDH were identified, respectively as 58 and 57 kDa.

Construction of *C. tropicalis* strain co-expressing *zwf* and *gnd* genes

Most xylose-assimilating yeasts, including *C. tropicalis*, have a xylose metabolic pathway, in which xylose reductase converts D-xylose to xylitol using NADPH as a co-factor. Regeneration of NADPH is therefore crucial for increasing reducing power of the cell. The oxidative PPP is the major source of NADPH biosynthesis in yeast [1].

We attempted to co-express two PPP genes of *C. tropicalis* to enhance the rate of xylitol production from xylose using glycerol as co-substrate in the medium. For this

Table 1 Enzymatic activity of recombinant G6PDH and 6-PGDH in *E. coli* BL21

Expressed enzyme	Specific activity* [U (mg of protein)$^{-1}$]
G6PDH	0.20
6-PGDH	0.06

Specific activity was determined in cells grown in 50 ml LB medium in a 250 ml flask for 12 h. Values represent means of three independent experiments

purpose, two expression cassettes were prepared as shown in Fig. 3, with over-expression of G6PDH and 6-PGDH driven by the constitutive GAPDH promoter.

The first expression cassette was successfully transformed into strain L10, and uracil prototrophs were selected from YNB plate. The cassette was integrated into *hisG* fragment region in the L10 genome, and site-directed insertion of the cassette into transformants was confirmed by PCR (Fig. 3a1).

To pop-out *URA3* marker gene, the first cassette transformant was incubated on YNB-5FOA plate for 3 days, and the *URA*3 pop-out mutant obtained from selected colonies was then used for transformation of the second cassette in the remaining *hisG* fragment region. Site-directed insertion of the second cassette was also confirmed by PCR (Fig. 3b1). The engineered *C. tropicalis* strain over-expressing G6PDH and 6-PGDH, was designated as PP.

Comparison of xylitol production in parent strain BSXDH-3 versus recombinant PP

To evaluate and compare rates of xylitol production in BSXDH-3 and PP, fermentation experiments were

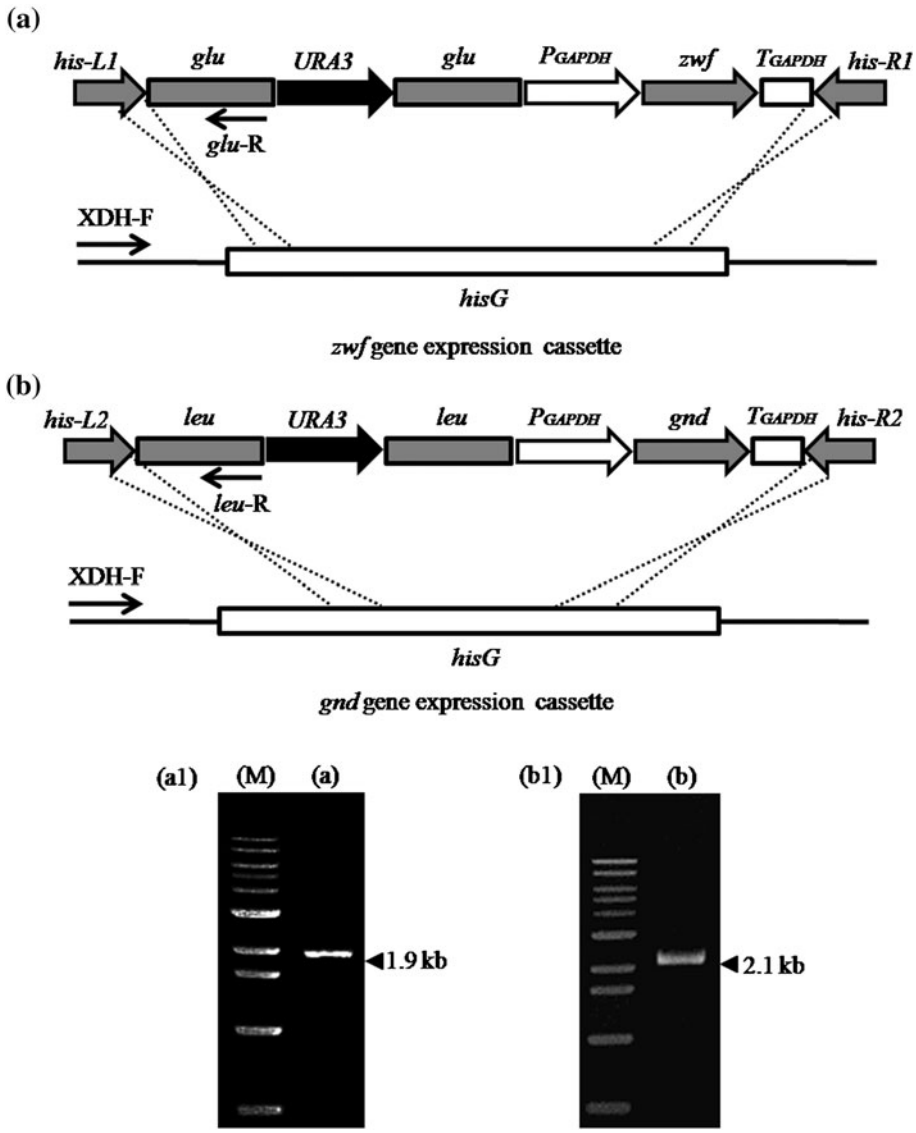

Fig. 3 *Top* Construction of *C. tropicalis* strain co-expressing *zwf* and *gnd* genes. *zwf* expression cassette (**a**) and *gnd* expression cassette (**b**) were integrated into *hisG* region of *C. tropicalis* L10. *Bottom* PCR confirmation of the specific integration of (*left*) *zwf* expression cassette (**a1**), where **a** indicates PCR with primers XDH-F and *glu*-R for amplification of 1.9 kb product; (*right*) *gnd* expression cassette (**b1**), where **b** indicates PCR with primers XDH-F and *leu*-R for amplification of 2.1 kb product

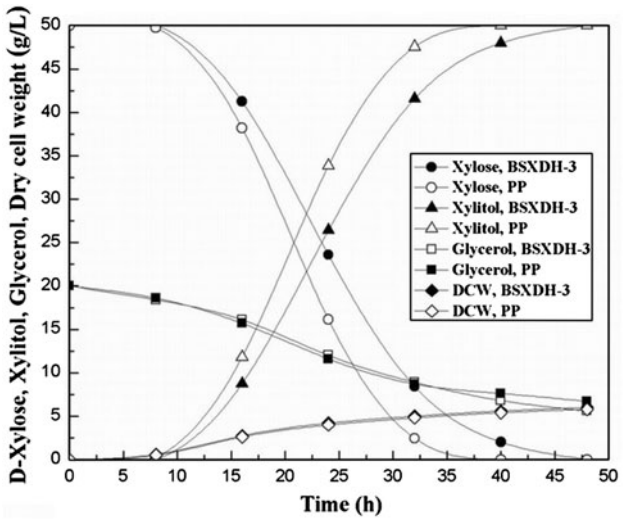

Fig. 4 Comparison of fermentation profiles of *C. tropicalis* strains BSXDH-3 (parent) and PP (over-expressing G6PDH and 6-PGDH). *Black* and *white* symbols are used, respectively, for BSXDH-3 and PP, for D-xylose consumption (*filled circle*, *open circle*), xylitol production (*filled triangle*, *open triangle*), glycerol consumption (*filled square*, *open square*) and dry cell weight (*closed diamond*, *open diamond*)

performed in 250 ml Erlenmeyer flasks with 50 ml xylitol fermentation medium in a shaking incubator, 200 rpm at 30°C. Glycerol consumption rate showed no significant difference between BSXDH-3 and PP. Rates of xylitol production at 16 and 24 h for PP were 0.74 g l^{-1} h^{-1} and 1.41 g l^{-1} h^{-1}, respectively; these values are 19 and 31% higher than corresponding rates for BSXDH-3. Volumetric productivity at 48 h was 1.25 g l^{-1} h^{-1} for PP, compared to 1.04 g l^{-1} h^{-1} for BSXDH-3 (Fig. 4).

The co-expression of *zwf* and *gnd* genes in PP clearly increased NADPH regeneration, and thereby enhanced xylitol production rate. Results were reasonably consistent with those of the previous studies [13, 16].

Conclusion

In engineered strain PP, co-expressing *zwf* and *gnd* genes, enzymatic activities of G6PDH and 6-PGDH were increased, NADPH regeneration was enhanced, and volumetric xylitol productivity at 48 h was 1.25 g l^{-1} h^{-1}, which was 21% higher than the rate (1.04 g l^{-1} h^{-1}) in parent strain BSXDH-3.

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