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Reduced Oxidative Pentose Phosphate Pathway Flux in Recombinant Xylose-Utilizing *Saccharomyces cerevisiae* Strains Improves the Ethanol Yield from Xylose

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In recombinant, xylose-fermenting *Saccharomyces cerevisiae*, about 30% of the consumed xylose is converted to xylitol. Xylitol production results from a cofactor imbalance, since xylose reductase uses both NADPH and NADH, while xylitol dehydrogenase uses only NAD⁺. In this study we increased the ethanol yield and decreased the xylitol yield by lowering the flux through the NADPH-producing pentose phosphate pathway. The pentose phosphate pathway was blocked either by disruption of the *GND1* gene, one of the isogenes of 6-phosphogluconate dehydrogenase, or by disruption of the *ZWF1* gene, which encodes glucose 6-phosphate dehydrogenase. Decreasing the phosphoglucone isomerase activity by 90% also lowered the pentose phosphate pathway flux. These modifications all resulted in lower xylitol yield and higher ethanol yield than in the control strains. TMB3255, carrying a disruption of *ZWF1*, gave the highest ethanol yield (0.41 g g⁻¹) and the lowest xylitol yield (0.05 g g⁻¹) reported for a xylose-fermenting recombinant *S. cerevisiae* strain, but also an 84% lower xylose consumption rate. The low xylose fermentation rate is probably due to limited NADPH-mediated xylitol reduction. Metabolic flux modeling of TMB3255 confirmed that the NADPH-producing pentose phosphate pathway was blocked and that xylitol production was mediated only by NADH, leading to a lower rate of xylose consumption. These results indicate that xylitol production is strongly connected to the flux through the oxidative part of the pentose phosphate pathway.

Fuel ethanol produced from fermentation of lignocellulosic hydrolysates is an attractive replacement for liquid fossil fuels because its production is renewable and it does not generate net carbon dioxide. Hydrolysis of lignocellulose generates mostly hexose but also some pentose sugars. The pentose sugars cannot be metabolized by *Saccharomyces cerevisiae*, the preferred ethanol-producing microorganism. In hydrolysate made from hardwood, xylose must be fermented to ethanol for the process to be economically feasible (39). The yeast *Pichia stipitis* metabolizes xylose through expression of the *XYLI* gene, encoding xylose reductase (XR), and the *XYL2* gene, encoding xylitol dehydrogenase (XDH). XR catalyzes the reduction of xylose to xylitol by using NADH or NADPH (30), whereas XDH oxidizes xylitol to xylulose exclusively with NAD⁺ (31). Unfortunately, *P. stipitis* is sensitive to ethanol (10) and requires low and carefully controlled oxygenation (34), which prevents its use for industrial ethanol production.

Recombinant *S. cerevisiae* strains expressing the *XYLI* and *XYL2* genes from *P. stipitis* have been constructed and can ferment xylose (26); however, most of the consumed xylose is secreted as xylitol (20, 26, 36, 42). Xylitol production can be lowered by overexpression of the *XKS1* gene, which encodes the native xylulokinase (XK) (23), but still about one third of the consumed xylose is converted to xylitol under anaerobic conditions (13). Xylitol formation may result from the cofactor imbalance between the NADPH-consuming XR and NADH-producing XDH reactions (26).

Eliasson et al. (12) reported improved ethanol yield from xylose in *S. cerevisiae* strains that have low phosphoglucone isomerase activity (PGI) or a deletion in the *GND1* gene, which encodes 6-phosphogluconate dehydrogenase (6-PGDH). Glucose 6-phosphate, a branch point metabolite between glycolysis and the pentose phosphate pathway (PPP), is reversibly converted to fructose-6-phosphate using PGI, while 6-PGDH converts 6-phosphogluconate to ribulose-5-phosphate in the PPP (35). This difference suggests that the increased ethanol yield from xylitol observed for these strains could be related to altered flux through the PPP.

We tested this hypothesis by lowering the PPP flux by genetic engineering. We evaluated the effects of these changes on xylose utilization and product formation in *S. cerevisiae* strains expressing XR and XDH from *P. stipitis* and overproducing native XK. The PPP flux was modified by (i) lowering PGI activity, (ii) deleting the *GND1* gene, and (iii) deleting the *ZWF1* gene, which encodes glucose-6-phosphate dehydrogenase (G6PDH). We used a flux model (40) to compare internal fluxes in the Δ*zwf1* strain and a control strain.

### MATERIALS AND METHODS

**Strains.** We used seven different *S. cerevisiae* strains in this study (Table 1). *Escherichia coli* DH5α (Life Technologies, Rockville, Md.) was used for subcloning. All strains were stored in 20% glycerol at −80°C. Yeast cells from freshly streaked YPD (4) plates were used for inoculation.

**Nucleic acid manipulation.** Plasmid DNA was prepared with the Qia miniprep kit (Qiagen, Valencia, Calif.) or Bio-Rad plasmid miniprep kit (Hercules, Calif.). Restriction and modification enzymes were obtained from Roche (Roche Diagnostics AB, Bromma, Sweden) and from Life Technologies (Rockville, Md.), respectively. DNA extractions from agarose gel were made using a Qiagen gel extraction kit.

**Transformation.** Competent cells of *E. coli* DH5α were prepared and transformed by the method of Inoue et al. (22), and yeast transformations were made.

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TABLE 1. S. cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Relevant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENY.WA-1A</td>
<td>MATa ura3-52 leu2-3,112 his3-D1 trp1-289 MAL2-8c MAL3 SUC1</td>
<td>1,000–2,500 mU of PGI mg of protein−1</td>
<td>24</td>
</tr>
<tr>
<td>RBY6-1a</td>
<td>ENY.WA-1A pgil-1A::URA3 PGH1::LEU2</td>
<td>190–270 mU of PGI mg of protein−1</td>
<td>7</td>
</tr>
<tr>
<td>TMB3250</td>
<td>ENY.WA-1A his3::YipXR/XDH/XK</td>
<td>Expresses XR, XDH, and XK</td>
<td>This work</td>
</tr>
<tr>
<td>TMB3251</td>
<td>RBY6-1 his3::YipXR/XDH/XK</td>
<td>Expresses XR, XDH, and XK; 10-fold-decreased PGI activity</td>
<td>This work</td>
</tr>
<tr>
<td>TMB3001</td>
<td>CEN.PK 113-7A (MATa his3-D1 MAL2-8c SUC2)</td>
<td>Expresses XR, XDH, and XK</td>
<td>13</td>
</tr>
<tr>
<td>TMB3255</td>
<td>TMB3001 zyg::KanMX</td>
<td>Expresses XR, XDH, and XK; no G6PDH activity</td>
<td>This work</td>
</tr>
<tr>
<td>TMB3008</td>
<td>CEN.HJ5-1B (MATa leu2-3,112 his3-D1 ura3-52 trp1-289 gnd1::HIS3 MAL2-8c SUC2) his3::YipLoxZEO</td>
<td>Expresses XR, XDH, and XK; no G6PDH activity</td>
<td>This work</td>
</tr>
</tbody>
</table>

a Parental strain ENY.WA-1A.

b Parental strain CEN.PK 113-7A.

c Parental strain CEN.PK2-1C.

Continuous cultivations. Cultivations were performed using defined mineral medium (38). Yeast cells were grown under oxygen-limited conditions in 200 ml of medium containing 20 g of xylose liter−1, 20 g of glucose liter−1, 10 mg of ergosterol liter−1, and 400 mg of Tween 80 liter−1 in a 250-ml baffled shake flask. The culture was incubated overnight at 30°C. Cells were centrifuged at 4,400 × g for 5 min at 4°C and used to inoculate 1.5 liters of the same medium to ca. 5 × 105 cells liter−1 in a Bioflo III fermentor (New Brunswick Scientific, Edison, N.J.). Antifoam was added at 0.05% (vol/vol) (Dow Corning Antifoam RD Emulsion; BDH Laboratory Supplies, Poole, United Kingdom). Continuous cultivation was set at dilution rates of 0.06 and 0.12 h−1 at 30°C and pH 5.5, controlled by addition of 3 M NaOH, and a stirring speed of 200 rpm. The fermentor was sparged with 0.2 liter of nitrogen min−1 (containing less than 5 ppm O2) as measured with a gas mass flowmeter (Brokhorst, Ruurlo, The Netherlands).

Analyses of substrates and products. Glucose, xylose, xylitol, sucinate, glycerol, acetate, and ethanol concentrations were determined by column liquid chromatography (CLC) using a Gilson CLC system (Gilson, Villiers-le-Bel, France). An Aminex HPX-87H column (Bio-Rad, Richmond, Calif.) and an R1D10A refractive index detector (Shimadzu, Kyoto, Japan) were used. The column temperature was 45°C, and 5 mM H2SO4 was used as the mobile phase at a flow rate of 0.6 ml min−1.

During continuous cultivation, cells from the outlet were used to measure RNA (5), proteins, and polysaccharides (19). Samples for CLC and cell dry weight determination were withdrawn from the fermentor. Growth was followed by measuring the optical density at 620 nm (OD620). Cell dry weight was determined by filtering 1 volume of sample through a 0.45-μm filter and washing with 3 volumes of water. The filter was dried in a microwave oven at 350 W for 8 min, cooled in a desiccator, and weighed. The composition of the outgoing gas was monitored with a carbon dioxide and oxygen monitor (type 1308; Bruel & Kjaer, Copenhagen, Denmark).

Enzymatic measurements. Crude extracts for enzyme measurements were made using the Y-PER yeast protein extraction reagent (Pierce, Rockford, Ill.). Protein concentration was determined by the Coomassie protein assay reagent (Pierce), using bovine serum albumin as a standard. The phosphoglucone isomerase (PGI, EC 5.3.1.9) activity was measured as described previously (27). The glucose-6-phosphate dehydrogenase activity (6PGDH, EC 1.1.1.44) and 6-phosphogluconate dehydrogenase activity (6-PGDH, EC 1.1.1.44) were measured according to the method of Bergmeyer (6).

Intracellular NADPH and NADP+ were measured enzymatically (6) on a TD-700 fluorometer (Turner Designs, Sunnyvale, Calif.) after extraction with boiling ethanol (16) from samples withdrawn from anaerobic growth on glucose.

RESULTS

Effect of decreased PGI activity on xylose fermentation. The xylose pathway, consisting of XR and XDH enzymes from P. stipitis and overproduced XK from S. cerevisiae, was introduced via the YipXR/XDH/XK vector (13) into ENY.WA-1A (control) and RBY6-1 strains (10-fold decrease in PGI activity [7]), generating S. cerevisiae TMB3250 and TMB3251, respectively.
TABLE 2. Specific xylose consumption and ethanol, xylitol, acetate, and glycerol yields after 70 h of 25-ml batch fermentations with xylose (50 g/liter) as the sole carbon source

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype or genotype</th>
<th>Xylose consumed (g/g of biomass)</th>
<th>Ethanol (g/g of xylose consumed)</th>
<th>Xylitol (g/g of xylose consumed)</th>
<th>Acetate (g/g of xylose consumed)</th>
<th>Glycerol (g/g of xylose consumed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3250</td>
<td></td>
<td>7.2 ± 0.4</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.023 ± 0.001</td>
<td>0.047 ± 0.001</td>
</tr>
<tr>
<td>TMB3251</td>
<td>Low PGI</td>
<td>4.6 ± 0.3</td>
<td>0.34 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.034 ± 0.001</td>
<td>0.058 ± 0.002</td>
</tr>
<tr>
<td>TMB3008</td>
<td>Δgndl</td>
<td>5.6 ± 0.7</td>
<td>0.38 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.051 ± 0.005</td>
<td>0.052 ± 0.011</td>
</tr>
<tr>
<td>TMB3255</td>
<td>Δzwf1</td>
<td>1.5 ± 0.1</td>
<td>0.41 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.084 ± 0.005</td>
<td>0.054 ± 0.008</td>
</tr>
</tbody>
</table>

* Values are the averages of the results of two independent fermentations and deviations from the averages.

TMB3251 had about 10% of the PGI activity of its control strain, TMB3250 (data not shown).

After 70 h of xylose fermentation, TMB3250 and TMB3251 had consumed 7.2 and 4.6 g of xylose g of biomass⁻¹, respectively (Table 2). Ethanol yields were 0.30 g of xylose g⁻¹ for TMB3250 and 0.34 g of xylose g⁻¹ for TMB3251. The 11% higher ethanol yield of TMB3251 was accompanied by a lower xylitol yield (0.21 g g⁻¹) than that of TMB3250 (0.30 g g⁻¹). The acetate and glycerol yields were slightly higher in TMB3250 (0.03 g g⁻¹ and 0.06 g g⁻¹, respectively) than in TMB3250 (0.02 g g⁻¹ and 0.05 g g⁻¹, respectively).

**Xylose fermentation by a ΔGND1 strain.** GND1 encodes one of the two NADP⁺-dependent isoenzymes of 6-phosphogluconate dehydrogenase that catalyze the conversion of 6-phosphogluconate to ribulose 5-phosphate in the PPP. The xylose pathway was introduced in the CEN.HJ5-1B strain, which has an inactive GND1 gene (24), to study the effect of an altered PPP on xylose fermentation. S. cerevisiae strain TMB3008 was generated by integration of the YIpLoxZEO vector in a glucose-positive revertant of CEN.HJ5-1B. A glucose-positive revertant was used instead of the original strain to facilitate glucose metabolism (33). TMB3008 and its control, TMB3001, were used for batch fermentation of xylose. TMB3001 and TMB3008 consumed 9.2 and 5.6 g of xylose g of biomass⁻¹, respectively (Table 2). Ethanol yields were 0.31 g g⁻¹ for TMB3001 and 0.38 g g⁻¹ for TMB3008 (Table 2). TMB3008 showed 24% higher ethanol yield and a lower xylitol yield (0.13 g g⁻¹) than TMB3001 (0.29 g g⁻¹). The acetate yield was higher in TMB3008 (0.05 g g⁻¹) than in TMB3001 (0.03 g g⁻¹). The glycerol yields were similar in the two strains.

**Xylose fermentation by a ΔZWF1 strain.** The strain with low PGI activity and the ΔGND1 strain had phenotypes similar to those of their respective wild-type strains with respect to xylose fermentation (Table 2). Both of these genetic alterations decreased the flux through the PPP during xylose fermentation. Lowering the PPP flux could result from abolishing the 6-PGDH activity or from lower gluconeogenic flux as a consequence of low PGI activity. Therefore, the disruption of ZWF1 also should result in lower xylitol and higher ethanol yields. Δzwf1 mutations have no negative side effects with respect to glucose utilization (24), whereas both the GND1 deletion and the reduction in PGI activity resulted in defective glucose metabolism (7, 33). The ZWF1 gene was deleted in TMB3001, resulting in TMB3255, and the strains were compared for xylose fermentation.

TMB3255 had the highest ethanol yield (0.41 g g⁻¹), the lowest xylitol yield (0.05 g g⁻¹), and the highest acetate yield (0.08 g g⁻¹) of the three strains (Table 2). However, the specific xylose consumption after 70 h (1.5 g per g of biomass) was also the lowest of the three strains (Table 2). This qualitatively similar product pattern suggests that the phenotypes of Δgnd1, low-PGI, and Δzwf1 strains are all related to lower PPP flux.

The Δzwf1 genotype has been reported to cause a requirement for organic sulfur (37). We did not observe this requirement, and xylose uptake was not affected by the addition of methionine.

**Enzyme activities.** The ΔZWF1 strain (TMB3255) showed a more pronounced effect on xylose fermentation than that observed in the ΔGND1 strain (TMB3008) (Table 2). These results suggest that the oxidative part of the PPP might not be totally blocked in TMB3008, since we inactivated only one of the two 6-PGDH isoenzymes in S. cerevisiae.

We measured the enzyme activities for G6PDH and 6-PGDH of TMB3001, TMB3008, and TMB3255 in cultures grown on glucose and xylose (Table 3). The enzyme activities of G6PDH and 6-PGDH in TMB3001 (control strain) were comparable with previously published values for S. cerevisiae (27). The 6-PGDH enzyme activity increased in TMB3001 when cultivated on xylose instead of glucose. In TMB3008 (Δgnd1), 6-PGDH and G6PDH activities were undetectable. This might be an effect of the reversion to a glucose-positive phenotype of this strain, since ΔGND1 mutants often lose 6-PGDH activity when exposed to high concentrations of glucose (33). As expected, G6PDH activity was not detectable in TMB3255 (Δzwf1). A fourfold decrease in 6-PGDH activity was measured in TMB3255 compared with the control strain (TMB3001). The hypothesis of a partly active PPP in TMB3008 was therefore not confirmed by higher activities of G6PDH or 6-PGDH in TMB3008 relative to those in TMB3255.

**Table 3. Specific G6PDH and 6-PGDH activities measured after growth on glucose or incubation on xylose**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Sp act* (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G6PDH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>TMB3001</td>
<td></td>
<td>0.90</td>
</tr>
<tr>
<td>TMB3008</td>
<td>Δgnd1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TMB3255</td>
<td>Δzwf1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Displayed values are the averages of the results of duplicate experiments with less than a 10% difference.
Continuous cultivations and flux analyses. A flux analysis was performed to compare intracellular fluxes in TMB3001 and TMB3255 using a stoichiometric model (40). Chemostat cultivations on a mixture of 20 g of xylose liter$^{-1}$ and 20 g of glucose liter$^{-1}$ were performed at dilution rates of 0.06 and 0.12 h$^{-1}$. The flux values for TMB3001 and TMB3255 (Fig. 1) were normalized to 100 mmol per g (dry weight) of cells per h. Substances shown inside boxes are substrates or products excreted into the medium. P, phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate.

**Table 4.** Measured specific uptake rates (negative values) and production rates (positive values) of substrates and products at dilution rates of 0.06 and 0.12 h$^{-1}$ for TMB3001 and TMB3255 (Δzwf1)$^*$

<table>
<thead>
<tr>
<th>Substrate or product</th>
<th>Uptake or production (mmol/g of biomass/h)</th>
<th>TMB3001</th>
<th>TMB3255</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D = 0.06$ h$^{-1}$</td>
<td>$D = 0.12$ h$^{-1}$</td>
<td>$D = 0.06$ h$^{-1}$</td>
</tr>
<tr>
<td>Xylose</td>
<td>$-1.5$</td>
<td>$-1.9$</td>
<td>$-0.80$</td>
</tr>
<tr>
<td>Glucose</td>
<td>$-3.4$</td>
<td>$-6.9$</td>
<td>$-4.0$</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>$6.6$</td>
<td>$11.7$</td>
<td>$7.6$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>$5.4$</td>
<td>$10.3$</td>
<td>$7.1$</td>
</tr>
<tr>
<td>Xylitol</td>
<td>$0.64$</td>
<td>$0.71$</td>
<td>$0.10$</td>
</tr>
<tr>
<td>Glycerol</td>
<td>$0.81$</td>
<td>$1.6$</td>
<td>$0.75$</td>
</tr>
<tr>
<td>Acetate</td>
<td>$0.04$</td>
<td>$0.07$</td>
<td>$0.36$</td>
</tr>
<tr>
<td>Succinate</td>
<td>$0.02$</td>
<td>$0.04$</td>
<td>$0.00$</td>
</tr>
</tbody>
</table>

$^*$ Displayed values are the averages of the results of duplicate experiments with less than a 5% difference.
NADPH and/or NADH are regulated differently in *P. stipitis* than in *S. cerevisiae*.

We constructed a recombinant xylose-fermenting *S. cerevisiae* strain with a low XR/XDH ratio, *XKS1* overexpression, and an inactivated oxidative PPP. To the best of our knowledge, TMB3255 has the highest ethanol yield (0.41 g g⁻¹) and the lowest xyitol yield (0.05 g g⁻¹) reported for any xylose-fermenting recombinant *S. cerevisiae*. PPP activity was lowered or inactivated by deleting either *GND1* or *ZWF1* or by lowering PGI activity. A low PGI activity decreases the flux from fructose 6-phosphate to glucose 6-phosphate, which is the substrate of G6PDH, the first enzyme of the PPP. Since PPP is the main source of NADPH (9), we suggest that the low xyitol yield is directly linked to depletion of NADPH in strains with defective PPP. The higher acetate yield is an indication of NADPH depletion, since oxidation of acetaldehyde to acetate requires an NADP⁺-dependent acetaldehyde dehydrogenase (29, 41). Furthermore, the NADPH/NADP⁺ ratio was lower in TMB3255 than in TMB3001.

The XR enzyme uses both NADPH and NADH (30), whereas the XDH enzyme uses NAD⁺ exclusively. We therefore propose the following model. In strains with a low flux through the PPP, the level of NADPH is low and a greater fraction of xylose is reduced with NADH. Since NADH is consumed in the XR step and produced in the XDH step, the xylose conversion to xylitol is balanced with respect to cofactors, which would explain why only minor amounts of xyitol are formed. This model is also supported by chemostat results with the control strain TMB3001. At higher dilution rates, the faster anabolism leads to reduced NADPH availability and results in increased use of NADH versus NADPH by XR, concomitant with a decreased xyitol yield (40; this study).

Lowering the oxidative PPP activity resulted in a reduced rate of xylose consumption. One reason for this decrease is that the NADPH-dependent reduction of xylose to xylitol is reduced, resulting in a reduced overall xylose fermentation rate. This was observed for TMB3255 cultivated in a chemostat on a mixture of glucose and xylose. The NADPH formed, which occurred mainly via acetate formation, was sufficient only for biomass synthesis. A strictly NADH-dependent xylose reduction was observed, and the xylose consumption rate was reduced accordingly. In xylose fermentation by nongrowing cells, the rate of xylose consumption by TMB3255 was 84% lower than that by TMB3001, which could not be explained only by the lack of the NADP⁺-dependent reaction. We suggest that the difference is connected to the nongrowing conditions and to the absence of glucose as a cosubstrate to ensure maintenance.

This work has demonstrated the connection between the level of the oxidative PPP and xyitol by-product formation in recombinant xylose-utilizing *S. cerevisiae*. By removing the NADPH-dependent xylose reduction, we obtained the highest ethanol yield from xylose ever reported for *S. cerevisiae*. However, the xylose consumption rate decreased accordingly, suggesting a limitation of XR activity. Strains with enhanced XR activity may overcome this limitation.

ACKNOWLEDGMENTS

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