Anaerobic Xylose Fermentation by Recombinant *Saccharomyces cerevisiae* Carrying XYL1, XYL2, and XKS1 in Mineral Medium Chemostat Cultures

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For ethanol production from lignocellulose, the fermentation of xylose is an economic necessity. *Saccharomyces cerevisiae* has been metabolically engineered with a xylose-utilizing pathway. However, the high ethanol yield and productivity seen with glucose have not yet been achieved. To quantitatively analyze metabolic fluxes in recombinant *S. cerevisiae* during metabolism of xylose-glucose mixtures, we constructed a stable xylose-utilizing recombinant strain, TMB 3001. The XYL1 and XYL2 genes from *Pichia stipitis*, encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, and the endogenous XKS1 gene, encoding xylulokinase (XK), under control of the PGK1 promoter were integrated into the chromosomal HIS3 locus of *S. cerevisiae* CEN.PK 113-7A. The strain expressed XR, XDH, and XK activities of 0.4 to 0.5, 2.7 to 3.4, and 1.5 to 1.7 U/mg, respectively, and was stable for more than 40 generations in continuous fermentations. Anaerobic ethanol formation from xylose by recombinant *S. cerevisiae* was demonstrated for the first time. However, the strain grew on xylose only in the presence of oxygen. Ethanol yields of 0.45 to 0.50 mmol of C/mmol of C (0.35 to 0.38 g/g) and productivities of 9.7 to 13.2 mmol of C h^{-1} g (dry weight) of cells^{-1} (0.24 to 0.30 g h^{-1} g [dry weight] of cells^{-1}) were obtained from xylose-glucose mixtures in anaerobic chemostat cultures, with a dilution rate of 0.06 h^{-1}. The anaerobic ethanol yield on xylose was estimated at 0.27 mol of C/(mol of C of xylose) (0.21 g/g), assuming a constant ethanol yield on glucose. The xylose uptake rate increased with increasing xylose concentration in the feed, from 3.3 mmol of C h^{-1} g (dry weight) of cells^{-1} when the xylose-to-glucose ratio in the feed was 1:3 to 6.8 mmol of C h^{-1} g (dry weight) of cells^{-1} when the feed ratio was 3:1. With a feed content of 15 g of xylose/liter and 5 g of glucose/liter, the xylose flux was 2.2 times lower than the glucose flux, indicating that transport limits the xylose flux.

To obtain an economically feasible industrial process for ethanol production from lignocellulose, it is necessary to ferment all sugars present with high yields and productivities (53). The commonly used *Saccharomyces cerevisiae* has many advantages as an ethanol producer, such as fast sugar consumption, high ethanol yield from hexoses, and high resistance to inhibitory compounds that are present in the hydrolysates. However, a major drawback is that *S. cerevisiae* cannot utilize the pentose sugar xylose, only its isomer xylulose. In xylose-utilizing yeasts, the conversion from xylose to xylulose is a two-step process catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XDH) (10), whereas bacteria perform the conversion in one step with xylulose isomerase (XI) (23).

Xylose fermentation by recombinant *S. cerevisiae* carrying heterologous XYL1 and XYL2 genes from *Pichia stipitis*, which encode XR and XDH, respectively, has resulted mainly in xylitol formation (24, 44, 48). Similarly, if xylA from *Thermus thermophilus*, which encodes XI, is introduced into *S. cerevisiae*, then only limited xylose fermentation is observed (47). Limited xylose fermentation by recombinant *S. cerevisiae* has been ascribed to poor xylose uptake (9, 24, 25), a cofactor imbalance generated by the discrepancy in cofactor usage by XR and XDH (8, 24, 49), limitations in the pentose phosphate pathway (12, 24, 38, 48), and insufficient induction or activation of ethanologenic enzymes (5, 17, 20, 29). When homologous XKS1, which encodes xylulokinase (XK), was overexpressed in a *Saccharomyces* sp. strain carrying XYL1 and XYL2, the ethanol yield and the xylose uptake rate increased under oxygen-limited conditions, but xylitol was still a major by-product (22).

Although the shortcomings of xylose fermentation by recombinant *S. cerevisiae* have been investigated in several studies, data from anaerobic fermentations do not exist and quantitative data are sparse. Chemostat cultivations in which growth rate and concentrations of substrates and products are constant enable quantitative determinations of metabolic fluxes. Analysis of xylose fluxes is the first step towards identifying causes for by-product formation and low productivity during xylose fermentation. A stable recombinant strain was constructed by integration of the XYL1 and XYL2 genes from *P. stipitis* and the homologous XKS1 gene under control of the PGK1 promoter into the chromosomal HIS3 locus of *S. cerevisiae*. Metabolic fluxes were determined for different xylose-to-glucose ratios in chemostat cultivations under anaerobic conditions. Glucose was used as a cosubstrate since data from anaerobic fermentations do not exist.

**MATERIALS AND METHODS**

*Strains and plasmids.* *Escherichia coli* DH5α [F− ΔlacZAM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(k88- λ- strR188 thy-1 gyrA96 relA1)] (GIBCO BRL, Gaithersburg, Md.) was used for subcloning. *Saccharomyces cerevisiae* CEN.PK113-7A (MATα his3Δ1 MAL2-8Δ MAL2-8Δ SUC2) (18) was used as the recipient yeast strain for the integrating plasmid YIpXR/XDH/XK. All strains were stored...
Frozen at -80°C. Agar plates streaked from the frozen stocks were used to inoculate the precultures.

Plasmids used for cloning of the YXL1 and YXL2, XKS1, and HIS3 genes were pY7 (46), pXa (B. Johansson, C. Christenson, T. Hобley, and B. Hahn-Hагrdal, submitted for publication), and YDp-H (3), respectively. YDp-H was obtained from Jorg Haaf (Scientific Research and Development GmbH, Oberschleuse, Germany).

Media. *S. cerevisiae* CEN.PK PK113-7A was grown in YPD medium (40) for transformation. In all other experiments, a defined medium including vitamins and trace elements was used (50). 1-Histidine was added at 50 mg per liter for strain CEN.PK PK113-7A. For the continuous cultivations, the medium was also supplemented with ergosterol and unsaturated fatty acids in the form of Tween 80 (Sigma, St. Louis, Mo.) (1, 2). Ergosterol and Tween 80 were dissolved in boiling 96% (vol/vol) ethanol to final concentrations of 0.01 and 0.42 g/liter, respectively. Bacterial strains were grown in Luria-Bertani medium (35). Transformants were selected by adding ampicillin (50 mg/liter). For growth on solid media, 20 g of agar per liter was added.

Nucleic acid manipulations. Standard techniques for nucleic acid manipulations were used (35). Plasmids were prepared using the QIAdraw Maxi Plasmid Purification Kit (Qiagen GmbH, Hilden, Germany). Restriction enzymes and other modifying enzymes were purchased from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). DNA fragments separated by agarose gel electrophoresis were purified with the QiAquick Gel extraction Kit (Qiagen GmbH).

Construction of integrating vector expressing YXL1, YXL2, and XKS1. The 2μ origin of replication and the P-ribosyl-antranilate isomerase (TRP1) gene were removed by partial digestion with *Xmn*I and *Pvu*II from plasmid pXa, a yeast episomal plasmid carrying the *XKS1* gene under the control of the phosphoglycerate kinase (PGK) promoter and terminator (Johansson et al., submitted) (Fig. 1a). This construct has two base substitutions introduced in the *HIS3* gene upstream from the start codon to maximize translational efficiency; furthermore, the codon for the N-terminal amino acid was altered to increase the protein stability of the gene product. The remaining fragment of 6,465 bp was recircularized by self-ligation and then partially digested with *Hind*III and BamHI, creating a fragment of 6,435 bp (Fig. 1b). The genes *YXL1* and *YXL2* under the control of alcohol dehydrogenase (ADH) and PGK promoters, respectively, were excised from pY7 (46) by partial digestion with *Hind*III and BamHI, yielding a fragment of 6,153 bp (Fig. 1c). The 1,150-bp *HIS3* cassette was excised from YDp-H (3) by BamHI (Fig. 1d). Finally, the plasmid carrying *XKS1* was ligated to the *HIS3* cassette and the *YXL1-YXL2* fragment, yielding YpXp/XK in an integrating vector carrying *YXL1*, *YXL2*, and *XKS1* and with *HIS3* as a selection marker (Fig. 1e). Restriction enzyme digests verified the map of the constructed vector.

Yeast strain transformation. The lithium acetate method was used for transformation (37).

Continuous fermentations. Continuous fermentations were conducted anaerobi- cally in computer-controlled glass bioreactors (Belach Biotechnik AB, Stockholm, Sweden) at 30°C with a stirring speed of 200 rpm. The working volume of the bioreactors was 600 ml, and the pH was adjusted to 5.5 with 3 M KOH. Anaerobic conditions were maintained by sparging with nitrogen containing less than 5 ppm of O2 (ADR class2 1A; AGA, Sundbyberg, Sweden) at a constant gas flow rate. The dry weight of the cells was determined by filtering a known volume of the culture broth through a 0.45-μm-pore-size Supor membrane (Gelman Sciences, Ann Arbor, Mich.). After being washed with 3 volumes of double-distilled water and dried in a microwave oven for 15 min, the filter was weighed. The dry weight of the cells was determined in triplicate for each steady state.

Enzymatic assays. Cell extracts for enzyme assays were prepared using glass beads (0.5 mm in diameter). Cells were harvested by centrifugation and, after washing, were resuspended in a disintegration buffer, 0.1 M triethanolamine buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithio-
A plasmid was cut with PstI within the TMB 3001, grew in the absence of histidine and "cerevisiae" gous ends to initiate integration. The resulting recombinant, HindIII deletion in the recipient strain, to generate homolo-

HindIII is deleted between the XYL2, XYL1, XKS1.

on a xylose-glucose mixture in shaken-flask cultures were 0.03, CEN.PK 113-7A and the recombinant strain TMB 3001 grown expressed XR, XDH, and XK activities. The specific activities was constructed (Fig. 1).

HIS3 was deleted between the

PGK (EC 2.7.2.3) Tris-HCl (100 mM, pH 8.0), EDTA (0.9 mM), MgCl2 (2 mM)

ADH (EC 1.1.1.1) Glycine (100 mM, pH 9.0) NAD+ (5.0 mM) Ethanol (1.7 M)

"All concentrations refer to the final assay mixture. The final assay volume was 1 ml.

c, d Ethanol yield was corrected by assuming that the missing percentage in the degree of reduction balance was due to evaporated ethanol.

b Ethanol yield was calculated based on consumed glucose only.

c Ethanol yield was calculated based on consumed carbohydrate.

The protein content was assayed using Coomassie protein assay reagent (6)

buffers and reagents according to Table 1. Assays were adapted from previously reported assays (4, 7, 32, 33, 39, 51). Specific activities are expressed as units per milligram of protein. Units are defined as micromoles of NADH reduced or oxidized per minute. For the XK assay, the reaction occurring before the addition of ATP (XDH in the reverse direction) was subtracted from the reaction observed in the presence of ATP. No reaction was observed in the absence of xylulose. Xylulose was produced as previously described (31).

The protein content was assayed using Coomassie protein assay reagent (6) (Pierce, Rockford, Ill.) with bovine serum albumin as a standard.

Calculations. Carbon balances and yields were calculated using single carbon unit equivalents (expressed as moles of carbon) (13) to allow comparison of hexose and pentose sugar metabolism. The elemental formula CH1.745O0.627N0.129S0.0025 was used for calculation of assimilated carbon converted to biomass (16).

RESULTS

Construction of a recombinant strain expressing XYL1, XYL2, and XKS1. An integrating vector, YipXR/XDH/XK, carrying XYL1, XYL2, and XKS1 and with H3S3 as selectable marker, was constructed (Fig. 1). HIS3 was in S. cerevisiae CEN.PK 113-7A is deleted between the HindIII sites (37). The constructed plasmid was cut with PstI within the HIS3 gene, but outside the HindIII deletion in the recipient strain, to generate homologous ends to initiate integration. The resulting recombinant, S. cerevisiae TMB 3001, grew in the absence of histidine and expressed XR, XDH, and XK activities. The specific activities of XR, XDH, and XK in cell lysates of the parental strain CEN.PK 113-7A and the recombinant strain TMB 3001 grown on a xylose-glucose mixture in shaken-flask cultures were 0.03, 0.01, and 0.02 U/mg of protein and 0.21, 1.78, and 0.93 U/mg of protein, respectively.

Anaerobic fermentations of xylose and glucose. Product formation by S. cerevisiae TMB 3001 was investigated in a high-performance bioreactor under anaerobic conditions. The recombinant strain carrying YipXR/XDH/XK grew aerobically on xylose, but not anaerobically. We included glucose in all fermentations to enable anaerobic chemostat cultivation. The maximum growth rate anaerobically on glucose was 0.35 h−1 (data not shown). Four different steady states were established, three with mixtures of xylose and glucose, the xylose-to-glucose ratios in the feed being 3:1, 1:1, and 1:3, respectively, and one with glucose as the sole carbon source (Table 2). For one set of fermentations, the first steady state was established with the medium containing the highest xylose concentration; the following three steady states were conducted in the order of decreasing xylose concentration in the feed. A second set was conducted in the opposite mode, i.e., started with a feed containing glucose only. The total amount of carbon source in the feed was 667 mmol of C, equivalent to 20 g/liter, and the D was 0.06 h−1. A post hoc analysis (Scheffe, α = 0.05) showed no significant difference between replicates. Steady-state concentrations were independent of the order in which steady states were reached. The standard deviation was less than 5% of the consumption and production for mean values of all substances (Table 2).

Xylose was coutilized with glucose under anaerobic conditions. The xylose uptake rate increased with increasing xylose concentration and decreasing glucose concentration in the feed, from 3.3 mmol of C h−1 g (dry weight) of cells−1 when the xylose-to-glucose ratio in the feed was 1:3 to 6.8 mmol of C h−1 g (dry weight) of cells−1 when the xylose-to-glucose ratio

<p>| TABLE 1. Buffer and reagent concentrations for enzyme assays a |
|---------------------------------|-----------------|-----------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th>Enzyme (EC no.)</th>
<th>Buffer</th>
<th>Cofactor</th>
<th>Reagents</th>
<th>Start reagent (conc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XR (EC 1.1.1.21)</td>
<td>Triethanolamine (100 mM, pH 7.0)</td>
<td>NADPH (0.2 mM)</td>
<td>Xylose (350 mM)</td>
<td></td>
</tr>
<tr>
<td>XDH (EC 1.1.1.9)</td>
<td>Glycine (100 mM, pH 9.0), MgCl2 (50 mM)</td>
<td>NAD+ (3.0 mM)</td>
<td>Xylitol (300 mM)</td>
<td></td>
</tr>
<tr>
<td>XK (EC 2.7.1.17)</td>
<td>Tris-HCl (50 mM, pH 7.5), MgCl2 (2.0 mM)</td>
<td>NADH (0.2 mM)</td>
<td>Xylose (8.5 mM), phosphoenol pyruvate (0.2 mM), pyruvate kinase (10 U), lactate dehydrogenase (10 U), ATP (2.0 mM)</td>
<td></td>
</tr>
<tr>
<td>PGK (EC 2.7.2.3)</td>
<td>Tris-HCl (100 mM, pH 8.0), EDTA (0.9 mM), MgCl2 (2 mM)</td>
<td>NADH (0.2 mM)</td>
<td>3-Phosphoglycerate (20 mM)</td>
<td></td>
</tr>
<tr>
<td>ADH (EC 1.1.1.1)</td>
<td>Glycine (100 mM, pH 9.0)</td>
<td>NAD+ (5.0 mM)</td>
<td>Ethanol (1.7 M)</td>
<td></td>
</tr>
</tbody>
</table>

a All concentrations refer to the final assay mixture. The final assay volume was 1 ml.

<p>| TABLE 2. Specific substrate consumption rates and product yields for xylose and glucose fermentation at different steady states a |
|-----------------|-----------------|-----------------|-----------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th>Feed denomination (g/liter)</th>
<th>Substrate consumption rate (mmol of C h−1 g [dry wt] of cells−1)</th>
<th>Product yield (mmol of C of product/mmol of C of total carbohydrates)</th>
<th>% C recovery a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose</td>
<td>Glucose</td>
<td>EtOH</td>
<td>EtOH (corrected)</td>
</tr>
<tr>
<td>15 xylose + 5 glucose</td>
<td>−6.8</td>
<td>−15.1</td>
<td>0.34</td>
</tr>
<tr>
<td>10 xylose + 10 glucose</td>
<td>−4.7</td>
<td>−18.0</td>
<td>0.38</td>
</tr>
<tr>
<td>5 xylose + 15 glucose</td>
<td>−3.3</td>
<td>−22.7</td>
<td>0.37</td>
</tr>
<tr>
<td>0 xylose + 20 glucose</td>
<td>0.0</td>
<td>−23.8</td>
<td>0.39</td>
</tr>
</tbody>
</table>

a The total carbon concentration in the feed was 667 mmol of C/liter, or 20 g/liter. EtOH, ethanol; XOH, xylitol; Acet, acetate; Glyc, glycerol.

b Ethanol yield was corrected by assuming that the missing percentage in the degree of reduction balance was due to evaporated ethanol.

c Ethanol yield was calculated based on consumed glucose only.

d Xylitol yield was calculated based on xylose only.

e C recovery includes the product yields calculated for total carbohydrates.
in the feed was 3:1 (Fig. 2; Table 2). However, even at the highest xylose concentration, only 12% of the xylose was consumed (Table 3).

Carbon balances at four steady states with increasing xylose concentration showed that the measured products accounted for 86.9, 84.8, 94.4, and 88.1% of consumed carbon (Table 2). Using a balance of the degree of reduction (34) of substrates and products and assuming that ethanol accounted for the missing percentage in the degree of reduction balances, the carbon balances were recalculated and closed to within 2% (Table 2). The resulting rates of ethanol evaporation calculated from measured values and degree of reduction balances were 0.04, 0.08, 0.11, and 0.14 g liter\(^{-1}\) h\(^{-1}\) for steady states with increasing glucose concentration. At an initial concentration of 6 g of ethanol/liter, the experimentally determined evaporation rate was 0.06 g liter\(^{-1}\) h\(^{-1}\), which is in agreement with the calculated values. The ethanol concentration differed at the four steady states, which may have influenced the evaporation rate. Furthermore, ethanol is highly volatile and the experimental error is large. To obtain as accurate a determination of the ethanol concentrations as possible, degree of reduction balances were used throughout the study. The corrected ethanol yield on total carbohydrates decreased with increasing xylose in the feed, from 0.53 to 0.45 mol of C/(mol of C of consumed carbohydrates) (0.41 to 0.35 g/g) (Table 2). However, the corrected ethanol yield calculated on consumed glucose increased, showing that xylose is converted to ethanol under anaerobic conditions. The corrected ethanol yield for the steady state with only glucose in the feed was 0.53 mol of C/(mol of C of glucose) (0.41 g/g), and with a xylose-to-glucose ratio in the ratio of 3:1, the ethanol yield increased to 0.65 mol of C/(mol of C of glucose) [(0.65 – 0.53) \times 15.1/6.8 (Table 2)] (0.21 g/g), assuming the ethanol yield on glucose to be constant for the four different steady states.

The xylitol yield, calculated with total consumed carbohydrates, increased with increasing xylose uptake rate, from 0.03 to 0.12 mol of C/(mol of C of carbohydrates) (0.03 to 0.12 g/g), when the xylose concentration in the feed increased from 5 to 15 g/liter (Table 2) so that the xylose fraction excreted as xylitol increased from 24 to 40%. The glycerol yield increased slightly at the lowest xylose concentration. At the highest xylose concentration, the lowest glycerol yield and highest acetate yield were observed.

The biomass yield on consumed sugar was rather constant (Table 2), but the concentration of biomass increased from 0.64 to 1.78 g/liter with increasing glucose concentration (Table 3).

**Enzyme activities and strain stability.** The specific activities of the enzymes XR, XDH, XK, PGK, and ADH were measured throughout the fermentations (Table 4). The recombinant *S. cerevisiae* strain TMB 3001 expressed XR, XDH, and XK with activities of 0.4 to 0.5, 2.7 to 3.4, and 1.5 to 1.7 U/mg of protein, respectively, corresponding to an approximate XR-to-XDH-to-XK ratio of 1:6:4. Furthermore, the strain exhibited stable recombinant enzyme activities throughout more than 4 weeks of continuous cultivation, equivalent to more than 40 generations. The PGK and ADH activities were 15 to 23 and 21 to 28 U/mg of protein, respectively (Table 4).

**DISCUSSION**

For the first time, anaerobic ethanol formation from xylose has been demonstrated for a recombinant xylose-utilizing strain of *S. cerevisiae*. The corrected ethanol yields (0.45 to 0.50 mmol of C/mmol of C; 0.35 to 0.38 g/g) and productivities (9.7 to 13.2 mmol of C h\(^{-1}\) g [dry weight] of cells\(^{-1}\); 0.24 to 0.30 g h\(^{-1}\) [dry weight] of cells\(^{-1}\)) obtained for cofermentation of xylose and glucose by TMB 3001 were slightly lower than those previously reported for *Saccharomyces* sp. strain 1400(LNH-ST) in oxygen-limited batch fermentation, 0.56 mmol of C/mmol of C and 14.3 mmol of C h\(^{-1}\) g [dry weight] of cells\(^{-1}\), respectively (21). The discrepancy could arise from differences in strain background as well as in the fermentation setup, i.e., absence or presence of oxygen and continuous or batch mode. For *P. stipitis*, the most efficient natural xylose-fermenting yeast, the highest ethanol yield on xylose, 0.63 mmol of C/mmol of C (0.48 g/g), was obtained in oxygen-limited continuous culture with a D of 0.06 h\(^{-1}\) (42). The productivity obtained under the same conditions was 8.7 mmol of C h\(^{-1}\) g (dry weight) of cells\(^{-1}\) (0.20 g h\(^{-1}\) g [dry weight] of cells\(^{-1}\)). In contrast, both ethanol yield and productivity under anaerobic conditions were considerably lower (42). One reason for choosing *S. cerevisiae* as the host strain for development of a xylose-fermenting yeast is its rapid anaerobic growth on glucose (30, 52). However, anaerobic growth on xylose has not yet been demonstrated for recombinant xylose-utilizing *S. cerevisiae*. This lack of growth has been attributed to slow xylose metabolism (24), resulting in too little ATP formation to maintain growth. *P. stipitis* has recently been metabolically engineered for anaerobic growth (41). When *S. cerevisiae URA1* was expressed in *P. stipitis*, the yeast grew anaerobically on glucose, but not on xylose, supporting the suggestion that anaerobic growth on xylose is limited by slow xylose metabolism.

**TABLE 3. Concentrations of xylose and glucose in the different feeds and in the fermentor and biomass concentrations for the different steady states**

<table>
<thead>
<tr>
<th>Feed denomination (g/liter)</th>
<th>Feed concn (g/liter)</th>
<th>Residual concn (g/liter)</th>
<th>Biomass concn (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylose</td>
<td>Glucose</td>
<td>Xylose</td>
</tr>
<tr>
<td>15 xylose + 5 glucose</td>
<td>15.04</td>
<td>5.08</td>
<td>12.80</td>
</tr>
<tr>
<td>10 xylose + 10 glucose</td>
<td>9.56</td>
<td>9.67</td>
<td>7.05</td>
</tr>
<tr>
<td>5 xylose + 15 glucose</td>
<td>5.23</td>
<td>15.27</td>
<td>3.01</td>
</tr>
<tr>
<td>0 xylose + 20 glucose</td>
<td>21.02</td>
<td>0.032</td>
<td>1.78</td>
</tr>
</tbody>
</table>
The strategy for chromosomal integration of the genes encoding XR, XDH, and XK resulted in a stable recombinant strain which retained its physiological characteristics through more than 4 weeks of continuous cultivation without selection pressure. Translated to generation time, strain TMB 3001 was stable for more than 40 generations, which is considerably longer than the four to five generations of stability reported for strain 1400 (pLNH32), which carries the same genes on a 2µm-derived vector (22). Recombinant xylose-utilizing *Saccharomyces* strains carrying 2µm-based vectors have been stably maintained in batch cultivation (22, 24, 27, 44, 48), but in continuous cultivation they tend to be unstable (27, 28). The instability of strains carrying 2µm-based vectors may result from genetic instability at the plasmid level, i.e., spontaneous loss of the transformed phenotype and the plasmid (19, 26, 28), or high frequency of recombination, resulting in cells that still carry the selectable marker but have lost the cloned gene (15, 28). Consequently, strains carrying less foreign DNA usually dominate the culture since the high-copy-number plasmids and the high expression of the heterologous genes can reduce the growth rate and glycolytic flux (26–28, 43). For the construction of TMB 3001, a single target sequence rather than multiple integrations in ribosomal DNA or transposons was chosen to diminish the burden on the cells caused by expression of foreign DNA. The resulting XR, XDH, and XK activities for TMB 3001 were lower than those for a CEN.PK strain carrying the same genes on two multicopy plasmids (pY7 [46] and pXks [Johansson et al., submitted]) cultivated in batch, 0.4, 3, and 1.6 U/mg compared to 0.7, 18, and 36 U/mg, respectively (Johansson et al., submitted). Still, the ethanol yields of the two strains were similar, 0.45 and 0.42 mol of C/mol of C (0.35 and 0.37 mol of C/mol of C, respectively) (Johansson et al., submitted). The ethanol yields of the two strains were similar, 0.45 and 0.42 mol of C/mol of C (0.35 and 0.37 mol of C/mol of C, respectively) (Johansson et al., submitted).

The *XYL1* gene, which codes XR, that was expressed in *P. stipitis* uses either NADPH or NADH as a cofactor, while XDH exclusively uses NAD⁺. Under anaerobic conditions, the fraction of xylose converted to xylitol by NADPH-dependent XR activity is not further converted to xylitol. This was demonstrated by increased xylitol excretion following the addition of the respiratory inhibitor antimycin A to an oxygen-limited culture of a recombinant xylose-utilizing strain of *S. cerevisiae* (24). Under the conditions applied in the present study, less than 50% of the consumed xylose was excreted as xylitol. The difference could result from the overexpression of XK, which increases ethanol production and lowers xylitol excretion (14, 17, 44), or from the simultaneous utilization of glucose, which may supply NAD⁺ by glycolysis. The latter hypothesis is supported by the high glycolylic yield at the steady state with the lowest xylose-to-glucose ratio, 1.3 (Table 2). The supplementary NAD⁺ might be used by XDH and reduce xylitol excretion. ATP is used in glycerol formation, but not in xylitol formation, and this result indicates that only when a surplus of glucose is available can the yeast use carbon for glycerol formation to supply XDH with NAD⁺.

In addition to redox constraints, xylose transport also may limit xylose utilization by recombinant *S. cerevisiae*. Xylose is transported by the facilitated glucose transport system in *S. cerevisiae* cells, which have a 200-fold lower affinity for xylose than for glucose (9, 24, 25). Increasing the xylose concentration in the feed enhanced the xylose flux. However, the xylose flux was still 2.2 times lower than the glucose flux when a feed consisting of 15 g of xylose/liter and 5 g of glucose/liter was utilized, suggesting that xylose transport to a large extent determines the xylose flux in recombinant *S. cerevisiae* TMB 3001.

**ACKNOWLEDGMENTS**

We thank Dace Leveika for technical assistance and Jörg Haufler for supplying the vector YDp-H.

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**REFERENCES**


**TABLE 4.** Specific enzyme activities in cell lysates of samples (four replicates) from different steady states of strain *S. cerevisiae* TMB 3001 in anaerobic, glucose-limited continuous cultures.

<table>
<thead>
<tr>
<th>Feed denomination (g/liter)</th>
<th>XR (U/mg of protein ± SD)</th>
<th>XDH (U/mg of protein ± SD)</th>
<th>XK (U/mg of protein ± SD)</th>
<th>PGK (U/mg of protein ± SD)</th>
<th>ADH (U/mg of protein ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 xylose + 5 glucose</td>
<td>0.42 ± 0.05</td>
<td>2.74 ± 0.10</td>
<td>1.54 ± 0.07</td>
<td>21.9 ± 1.2</td>
<td>23.1 ± 1.2</td>
</tr>
<tr>
<td>10 xylose + 10 glucose</td>
<td>0.52 ± 0.04</td>
<td>3.37 ± 0.09</td>
<td>1.71 ± 0.07</td>
<td>23.1 ± 1.30</td>
<td>28.4 ± 0.75</td>
</tr>
<tr>
<td>5 xylose + 15 glucose</td>
<td>0.52 ± 0.05</td>
<td>3.14 ± 0.06</td>
<td>1.61 ± 0.03</td>
<td>15.4 ± 0.16</td>
<td>27.7 ± 0.54</td>
</tr>
<tr>
<td>0 xylose + 20 glucose</td>
<td>0.43 ± 0.03</td>
<td>2.75 ± 0.11</td>
<td>1.56 ± 0.07</td>
<td>17.3 ± 1.21</td>
<td>21.2 ± 0.81</td>
</tr>
</tbody>
</table>