Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle

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Abstract

When xylose metabolism in yeasts proceeds exclusively via NADPH-specific xylose reductase and NAD-specific xylitol dehydrogenase, anaerobic conversion of the pentose to ethanol is intrinsically impossible. When xylose reductase has a dual specificity for both NADPH and NADH, anaerobic alcoholic fermentation is feasible but requires the formation of large amounts of polyols (e.g., xylitol) to maintain a closed redox balance. As a result, the ethanol yield on xylose will be sub-optimal. This paper demonstrates that anaerobic conversion of xylose to ethanol, without substantial by-product formation, is possible in *Saccharomyces cerevisiae* when a heterologous xylose isomerase (EC 5.3.1.5) is functionally expressed. Transformants expressing the XylA gene from the anaerobic fungus *Piromyces* sp. E2 (ATCC 76762) grew in synthetic medium in shake-flask cultures on xylose with a specific growth rate of 0.005 h\(^{-1}\). After prolonged cultivation on xylose, a mutant strain was obtained that grew aerobically and anaerobically on xylose, at specific growth rates of 0.18 and 0.03 h\(^{-1}\), respectively. The anaerobic ethanol yield was 0.42 g ethanol g\(^{-1}\)xylose and also by-product formation was comparable to that of glucose-grown anaerobic cultures. These results illustrate that only minimal genetic engineering is required to recruit a functional xylose metabolic pathway in *Saccharomyces cerevisiae*. Activities and/or regulatory properties of native *S. cerevisiae* gene products can subsequently be optimised via evolutionary engineering. These results provide a gateway towards commercially viable ethanol production from xylose with *S. cerevisiae*.

Keywords: Xylose isomerase; Hemicellulose; Fermentation; Pentose; Yeast; Bioethanol

1. Introduction

*Saccharomyces cerevisiae* is applied on a huge scale for the industrial production of ethanol from sugars. *S. cerevisiae* couples high fermentation rates with the ability to grow under strictly anaerobic conditions [1,2], but has a limited substrate range. This drawback is especially relevant for the alcoholic fermentation of hemicellulose-containing feedstocks, which, after hydrolysis, yield substantial amounts of the pentose sugar xylose [3]. Wild-type *S. cerevisiae* strains are capable of slow fermentation of xylulose but cannot ferment xylose itself and, moreover, cannot grow anaerobically on ei-
Fig. 1. Different metabolic options for alcoholic fermentation of xylose with a fitting redox balance. Colours indicate the central routes in carbohydrate metabolism: red: glucose-6-phosphate dehydrogenase and 6-phosphoglucose dehydrogenase (hexose–monophosphate pathway); blue: rearrangement reactions in the pentose–phosphate pathway; green: glycolysis. Abbreviations: C6P: hexose-6-phosphate, C5P: pentose-5-phosphate and C3P: triose-3-phosphate. (a) NADPH-specific xylose reductase and NAD-specific xylitol dehydrogenase (aerobic). A closed redox balance and a positive ATP balance for alcoholic fermentation of xylose can be obtained in the presence of an external electron acceptor (e.g., oxygen) for reoxidation of the NADH generated in the xylitol-dehydrogenase reaction. Note that, in this scenario, 75% of the hexose phosphate produced by the pentose–phosphate pathway has to be oxidised by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to yield the NADPH required for the xylose reductase reaction. As a result, the molar yield of CO2 on xylose exceeds the ethanol yield. (b) NADPH-specific xylose reductase and NAD-specific xylitol dehydrogenase (anaerobic). Redox balancing via the production of glycerol is not an option, as this leads to a negative ATP balance: two thirds of the triose phosphates would have to be converted to glycerol rather than entering glycolysis. (c) Xylose reductase with dual cofactor specificity (NADPH/NADH) and NAD-specific xylitol dehydrogenase (anaerobic). Redox balancing via the production of glycerol is not an option, as this leads to a negative ATP balance: two thirds of the triose phosphates would have to be converted to glycerol rather than entering glycolysis. (d) Xylose reductase and xylitol dehydrogenases with matching cofactor specificities (equivalent to xylose isomerase). No surplus NADH is generated since the reducing equivalents needed for xylose reductase are generated by the xylitol dehydrogenase. The oxidative reactions of the pentose-phosphate pathway are not needed in a redox neutral conversion of xylose into xylulose. Only this scenario allows for the absence of reduced by-products (glycerol, xylitol) and the production of equimolar amounts of ethanol and CO2.
fermentation by individual strains correlated with the presence of an NADH-linked XR activity [4]. Since yeasts lack a transhydrogenase that can interconvert NADPH and NADH [10,11] a strictly NADPH-linked XR results in a disturbed redox balance. Only reoxidation of the NADH formed in the XDH reaction with an external electron acceptor such as oxygen or acetoin (Fig. 1(a)) allows for alcoholic fermentation of xylose by yeasts with a strictly NADPH-linked XR [12].

During anaerobic growth of S. cerevisiae on hexoses, glycolysis, a closed redox balance via glycerol formation can only be obtained in the presence of another source of ATP (Fig. 1(b)).

In the few yeast species that, in addition to the common NADPH-linked XR activity, also contain NADH-linked XR activity, anaerobic fermentation of xylose becomes possible since excess NADH can be shuttled into xylitol formation (Fig. 1(c)). Obviously, xylitol formation goes at the expense of the ethanol yield on xylose. Anaerobic alcoholic fermentation of xylose without xylitol formation is only possible when XR and XDH would have matching coenzyme specificities (Fig. 1(d)). This is equivalent to the one-step interconversion of xylose to xylulose by xylose isomerase (XI, EC 5.3.1.5) that occurs in xylose-metabolising bacteria and Archaea (Fig. 1(d)).

Many studies have focussed on the combined functional expression of the XR and XDH genes from the xylose-fermenting yeast Pichia stipitis in S. cerevisiae [7,8]. Consistent with the biochemical constraints discussed above, these studies were confronted with problems related to the intracellular redox balance. For example, a recent study in which an XR/XDH-expressing S. cerevisiae strain was subjected to random mutagenesis and selection yielded a mutant that was capable of slow anaerobic growth on xylose [15]. In accordance with the scenario depicted in Fig. 1(c), this strain still produced large amounts of xylitol.

In yeasts that metabolise xylose via XR and XDH, the resulting excess NADH can, in principle, be effectively removed via aeration. Studies on the effects of oxygen on xylose fermentation by yeasts [16,17] have revealed that excessive xylitol formation can only be prevented when sufficient oxygen is provided. However, too intensive aeration would lead to a competition for glycolytic NADH between mitochondrial respiration and alcoholic fermentation, thus reducing the ethanol yield.

Genetic engineering of S. cerevisiae for efficient anaerobic xylose fermentation via the XR/XDH pathway requires protein engineering to exactly match the coenzyme specificities of the two oxidoreductases. Either the NADPH-linked XR should be made NADH-specific or the NAD-linked XDH should be converted into an NADP-linked enzyme, resulting in the situation depicted in Fig. 1(d). If at all possible, such protein engineering will require a substantial research effort.

Functional expression of a heterologous XI in S. cerevisiae seems a logical approach to engineer this yeast as a cell factory for efficient alcoholic fermentation of xylose. Various groups have attempted this but, until recently, found negligible or no XI activities under conditions that permit growth of S. cerevisiae [18] and references cited therein). We have recently identified a xylose isomerase (encoded by the XylA gene of the anaerobic fungus Piromyces sp E2) that can be functionally expressed in S. cerevisiae at high levels [19–21]. However, the rate of xylose consumption in a XylA expressing S. cerevisiae strain was barely sufficient to meet the maintenance energy requirement of the cells [20].

The aim of the present study was to test the hypothesis that expression of a xylose isomerase eliminates the redox problems inherent to the XR/XDH approach. To this end, a XylA-expressing strain was subjected to directed evolution. Subsequently, a mutant that had acquired the ability to grow anaerobically on xylose was analysed for growth, ethanol production and by-product formation in anaerobic fermenter cultures.

2. Materials and methods

2.1. Strains and maintenance

Saccharomyces cerevisiae strain used in this study is RWB202. This strain was obtained by transforming CEN.PK113-5D (ura3), an isogenic member of the CEN.PK strain family [22], with the 2-micron-based vector pAKX002 [20]. This expression vector carries the XylA gene from the anaerobic fungus Piromyces sp E2 (ATCC 76762) under the control of the constitutive TPII promoter, as well as the URA3 marker gene. Stock cultures were grown at 30 °C in shake flasks on synthetic medium [23] supplemented with 20 g of glucose l−1. When stationary phase was reached, sterile glycerol was added to 30% (vol/vol), and 2 ml aliquots were stored in sterile vials at −80 °C.

2.2. Cultivation and media

Shake-flask cultivation was performed at 30 °C in a synthetic medium [23]. The pH of the medium was adjusted to 6.0 with 2 M KOH prior to sterilisation.
Precultures were prepared by inoculating 100 ml medium containing 20 g\textsuperscript{-1} xylose in a 500-ml shake flask with a frozen stock culture. After 24-48 h incubation at 30 °C in an orbital shaker (200 rpm), this culture was used to inoculate either shake-flask cultures or fermenter cultures. The synthetic medium for anaerobic cultivation was supplemented with 0.01 g l\textsuperscript{-1} ergosterol and 0.42 g l\textsuperscript{-1} Tween 80 dissolved in ethanol [24,25], this resulted in 11–13 mM ethanol in the medium.

2.3. Selection of fast-growing mutants

Mutants of RWB202 with a higher specific growth rate on xylose were selected by serial transfer in shake flasks and by sequencing-batch cultivation in fermenters. For serial transfer experiments, a 500-ml shake flask containing 100 ml synthetic medium with 2% xylose was inoculated with RWB202. When the optical density at 660 nm had reached 1.5, the culture was used to inoculate a new shake flask and the procedure was repeated. Anaerobic sequencing-batch cultivation was carried out at 30 °C in 2-l laboratory fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1 l. The culture pH was kept at pH 5.0 by automatic addition of 2 M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 l min\textsuperscript{-1} nitrogen (<10 ppm oxygen) per minute. To minimise diffusion of oxygen, fermenters were equipped with Norprene tubing (Cole Palmer Instrument company, Vernon Hills, USA). Dissolved oxygen was monitored with an autocleavable oxygen electrode (Applisens, Schiedam, The Netherlands). New cycles of batch cultivation were initiated manually by removing 90% of the culture volume and replacing it with fresh medium. Severely oxygen-limited conditions were achieved in the same experimental set-up by discontinuing the nitrogen sparging through the culture in above-mentioned set-up and thus allowing for a small influx of oxygen into the cultures via diffusion.

2.4. Anaerobic batch cultivation in fermenters

Anaerobic batch cultures were carried out in two-litre laboratory fermenters (Applikon, Schiedam, The Netherlands) equipped with Norprene tubing, with a working volume of 1.5 l, at 30 °C and at pH 5.0. Cultures were stirred at 800 rpm and sparged with 0.5 l min\textsuperscript{-1} nitrogen (<5 ppm oxygen). The synthetic medium was supplemented with the anaerobic growth factors ergosterol and Tween 80 (0.01 and 0.42 g l\textsuperscript{-1}, respectively) as well as 100 μl l\textsuperscript{-1} of silicone antifoam (BDH, Poole, UK).

2.5. Determination of culture dry weight

Culture samples (10.0 ml) were filtered over pre-weighed nitrocellulose filters (pore size 0.45 μm; Gelman laboratory, Ann Arbor, USA). After removal of medium the filters were washed with demineralised water and dried in a microwave oven (Bosch, Stuttgart, Germany) for 20 min at 360 W and weighed. Duplicate determinations varied by less than 1%.

2.6. Gas analysis

Exhaust gas was cooled in a condensor (2 °C) and dried with a Permapure dryer type MD-110-48P-4 (Permapure, Toms River, USA). O\textsubscript{2} and CO\textsubscript{2} concentrations were determined with a NGA 2000 analyser (Rosemount Analytical, Orrville, USA). Exhaust gas-flow rate and specific oxygen-consumption and carbon-dioxide production rates were determined as described previously [26,27]. In calculating these biomass-specific rates, a correction was made for volume changes caused by withdrawing culture samples.

2.7. Metabolite analysis

Glucose, xylose, xylitol, organic acids, glycerol and ethanol were detected by HPLC analysis on a Waters Alliance 2690 HPLC (Waters, Milford, USA) containing a BioRad HPX 87H column (BioRad, Hercules, USA). The column was eluted at 60 °C with 0.5 g l\textsuperscript{-1} H\textsubscript{2}SO\textsubscript{4} at a flow rate of 0.6 ml min\textsuperscript{-1}. Detection was by means of a Waters 2410 refractive-index detector and a Waters 2487 UV detector. Xylulose was determined enzymatically in the following manner. The reaction mixture consisted of 100 mM Tris–HCl buffer (pH 7.5) with 10 mM MgCl\textsubscript{2}, 0.30 mM NADH and an adequate amount of sample (1 ml total volume). The assay was started by the addition of 0.2 U sorbitol dehydrogenase (Sigma, St. Louis, USA). The xylulose concentration was calculated using an absorption coefficient of 6.3 mM\textsuperscript{-1} cm\textsuperscript{-1} for NADH.

2.8. Carbon recoveries and ethanol evaporation

Carbon recoveries were calculated as carbon in products formed, divided by the total amount of sugar carbon consumed, and were based on a carbon content of biomass of 48%. To correct for ethanol evaporation during the fermentations, the amount of ethanol produced was assumed to be equal to the measured cumulative production of CO\textsubscript{2} minus the CO\textsubscript{2} production that occurred due to biomass synthesis (5.85 mmol CO\textsubscript{2} per gram biomass [28]) and the CO\textsubscript{2} associated with acetate formation. This assumption was verified by determining the ethanol evaporation rates in a sterile fermenter set-up under the conditions used for the anaerobic batch experiments. Rates of evaporation were determined at ethanol concentrations of 25, 75 and 175 mM, in liquid volumes that corresponded to those present at these ethanol concentrations during the xylose fermentation.
experiments. From these evaporation rates a linear relationship was found of 0.006 l−1 h−1/C0. The ethanol concentrations in fermentation experiments, as directly measured by HPLC, were then corrected for evaporation by applying this evaporation rate on the average ethanol concentration between two time points, and adding the sum of all previous evaporation values to the measured ethanol value. These corrected ethanol values fitted well with the ethanol values calculated from CO2 production.

The three different ethanol graphs corresponding to the three different methods of ethanol determination based on the data from the anaerobic batch of RWB202-AFX on xylose are depicted in Fig. 2.

3. Results

3.1. Selection of fast-growing mutants on xylose medium

The initial specific growth rate of S. cerevisiae RWB202 on xylose was 0.005 h−1, corresponding to a doubling time of ca. 140 h [20]. To select for spontaneous mutants with an improved specific growth rate on xylose, the strain was subjected to serial transfer in shake flasks containing a synthetic medium with xylose as the sole carbon source. After 30 transfers, covering a period of 79 days, the specific growth rate had increased 24-fold to 0.12 h−1 (Fig. 3). At this stage, the serial-transfer culture was used to inoculate a fermenter for strictly anaerobic batch cultivation. However, even after two weeks of incubation, no growth or xylose consumption was observed (data not shown), suggesting that fast aerobic xylose metabolism was not sufficient to allow for anaerobic growth as well.

To further select for anaerobic growth, the fermenter culture was subjected to a severe oxygen limitation. This was accomplished by terminating the nitrogen flushing, thus allowing slow diffusion of air into the reactor (see Section 2). Under these conditions the culture was able to grow, albeit very slowly, and gradually consumed all xylose present in the medium. When xylose was depleted, a new batch was initiated by removing approximately 90% of the culture volume and replacing it with fresh medium. After 10 cycles of this severely oxygen-limited sequencing batch cultivation, covering 46 days, a new batch was started under fully anaerobic conditions by resuming the sparging with nitrogen. This time, anaerobic growth and xylose consumption as well as ethanol production was observed. The culture was then subjected to a further 10 cycles of anaerobic sequencing batch cultivation, covering 33 days. From the final cycle, a sample was streaked on mineral medium xylose plates and after 48 hours incubation at 30 °C, single colonies were restreaked on identical plates. From these plates, two colonies were picked (A and B) and used to inoculate anaerobic batch fermenters for characterisation. As single-cell isolate A exhibited a slightly higher anaerobic growth rate on xylose (data not shown), it was designated RWB202-AFX (for anaerobic fermentation of xylose) and used for further experiments.

3.2. Physiological characterisation of strain RWB 202-AFX

The selected strain RWB202-AFX was grown under strict anaerobic conditions on xylose or glucose in pH-controlled fermenters. In synthetic medium containing 20 g l−1 xylose and the anaerobic-growth factors Tween.
and ergosterol, the selected strain grew anaerobically with a specific growth rate of 0.03 h\(^{-1}\) and virtually completely consumed the xylose (Table 1 and Fig. 4(a)). During cultivation the strain displayed normal anaerobic growth behaviour and no unexpected products could be observed. The overall by-product formation was low, succinate and lactate did not exceed 1 mM each and the concentration of acetate remained below 2.5 mM. Also xylitol, which is usually formed in large amounts during anaerobic fermentation of xylose by yeasts, was only formed in minor amounts (Table 1 and Fig. 4) of the by-products glycerol reached the highest concentration.

Anaerobic cultivation of RWB202-AFX and the ‘wild-type’ strain CEN.PK 113-7D on glucose-containing medium yielded similar results. The main differences during growth on glucose were a reduced specific growth rate, a lower biomass yield and a 2-fold higher acetate production of the selected strain RWB202-AFX as compared to that of the wild-type strain (Table 1). Despite these differences, glucose consumption as well as ethanol, glycerol and carbon dioxide production showed the same trend lines (Fig. 5) and reached near-identical values.

The carbon balances calculated from the initial data based on HPLC measurements of ethanol revealed that on xylose 11% and on glucose 3% of the carbon was unaccounted for. Based on CO\(_2\) measurements, the evaporation of ethanol was assumed to be the cause of the missing carbon. The fact that the xylose growth experiments lasted 10-fold longer than the glucose batches supported this assumption. To compensate for ethanol evaporation, the assumption was made that the amount of ethanol produced was equal to the amount of CO\(_2\) produced minus the amount of CO\(_2\) produced during biomass formation and acetate production (see

### Table 1

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>RWB202-AFX</th>
<th>RWB202-AFX</th>
<th>CEN.PK 113-7D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific growth rate (h(^{-1}))</td>
<td>0.03 ± 0.00</td>
<td>0.24 ± 0.00</td>
<td>0.34 ± 0.00</td>
</tr>
<tr>
<td>Biomass yield (g g(^{-1}))</td>
<td>0.088 ± 0.004</td>
<td>0.079 ± 0.000</td>
<td>0.099 ± 0.003</td>
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<tr>
<td>Ethanol yield(^a) (g g(^{-1}))</td>
<td>0.42 ± 0.00</td>
<td>0.40 ± 0.00</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>Carbon recovery(^a) (%)</td>
<td>105.5 ± 0.0</td>
<td>103.7 ± 0.8</td>
<td>104.0 ± 1.1</td>
</tr>
<tr>
<td>Sugar consumed (mM)</td>
<td>137.4 ± 0.2</td>
<td>114.9 ± 0.4</td>
<td>116.1 ± 0.3</td>
</tr>
</tbody>
</table>

**Products**

<table>
<thead>
<tr>
<th>Product</th>
<th>RWB202-AFX</th>
<th>RWB202-AFX</th>
<th>CEN.PK 113-7D</th>
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</thead>
<tbody>
<tr>
<td>Biomass (g l(^{-1}))</td>
<td>1.81 ± 0.08</td>
<td>1.64 ± 0.01</td>
<td>2.07 ± 0.06</td>
</tr>
<tr>
<td>CO(_2) (mmol l(^{-1}))</td>
<td>199.7 ± 1.5</td>
<td>196.9 ± 1.3</td>
<td>197.1 ± 3.4</td>
</tr>
<tr>
<td>Ethanol (mM)</td>
<td>186.8 ± 2.2</td>
<td>180.3 ± 1.4</td>
<td>181.6 ± 3.9</td>
</tr>
<tr>
<td>Xyritol (mM)</td>
<td>2.76 ± 0.03</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glycerol (mM)</td>
<td>18.3 ± 0.3</td>
<td>24.2 ± 0.1</td>
<td>22.9 ± 0.2</td>
</tr>
<tr>
<td>Acetate (mM)</td>
<td>2.26 ± 0.16</td>
<td>6.93 ± 0.02</td>
<td>3.42 ± 0.11</td>
</tr>
<tr>
<td>Succinate (mM)</td>
<td>0.75 ± 0.00</td>
<td>0.27 ± 0.02</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>0.95 ± 0.02</td>
<td>1.49 ± 0.02</td>
<td>1.70 ± 0.02</td>
</tr>
</tbody>
</table>

Values are presented as the average and experimental deviation of two independent batch cultivations.

\(^a\) Calculated based on the ethanol concentrations deduced from the CO\(_2\) production, see Section 2.

\(^b\) Deduced from the CO\(_2\) production, see Section 2.

Fig. 4. Anaerobic growth of strain RWB202-AFX in fermenters on synthetic medium with 2% (w/v) xylose as the carbon source. Data points represent the average values per litre culture and experimental deviation of two independent experiments. (a) Xylose (•), ethanol (○), glycerol (●), xylitol (□) and cumulative CO\(_2\) produced per litre as deduced from gas analysis (–). (b) (Deviation from average not shown): dry weight (●), acetate (○), xylulose (■), xylitol (□), succinate (▲), lactate (∆).
Section 2). Based on this assumption, the carbon recoveries of all batches were on average 104% (Table 1). This overestimation of products is probably due to inevitable experimental and measurement errors combined with the dynamics of growing batch cultures. The validity of this assumption was further supported by the fact that redox balances calculated for all batch experiments closed to within 3%. To verify the assumed impact of ethanol evaporation, the relationship between ethanol concentration and evaporation rate was determined in a sterile fermenter set-up under conditions used for batch cultivation. The ethanol loss corresponded to an evaporation constant of 0.006 l / h . When ethanol concentrations measured by HPLC were corrected based on this experimentally determined ethanol-evaporation constant, the corrected values were ca. 90% of the values calculated from the CO2 measurements (Fig. 2). The difference between these two methods depicted in Fig. 2 seemingly is small due to the initial ethanol present which results from the addition of Tween 80 and ergosterol. Furthermore, it should be noted that the sterile set-up used for measuring ethanol evaporation could only approximate the actual loss of ethanol in ‘real’ cultures. Relevant factors such as gas hold-up, bubble size and therefore gas transfer will be different in cultures that contain biomass.

4. Discussion

4.1. By-product formation during anaerobic xylose fermentation by yeasts

A mutant of RWB202 selected for anaerobic growth on xylose was cultivated under strictly anaerobic conditions on glucose and xylose. In both these conditions the mutant, designated RWB202-AFX, displayed stoichiometries of biomass and (by-)product formation that were comparable to those of the isogenic wild-type strain CEN.PK 113-7D cultivated on glucose. In particular, ethanol and biomass yield were nearly identical, proving that the selected strain could ferment xylose with the same efficiency as glucose.

The ethanol yields of RWB202-AFX on glucose and on xylose were 0.40 and 0.42 g g⁻¹, respectively, corresponding to around 80% of the theoretical maximum. These values correspond favourably to published data on anaerobic xylose fermentation by engineered S. cerevisiae strains expressing xylose reductase and xylitol dehydrogenase, which produce large amounts of xylitol (Table 2). As outlined in Fig. 1(a,c), anaerobic fermentation of xylose must involve polyol production when the xylose reductase is not specific for NADH. Engineered S. cerevisiae strains based on xylose reductase will exhibit an anaerobic fermentation stoichiometry that is a combination of Fig. 1(b)–(d).

When comparing the theoretical stoichiometry of xylose fermentation via a redox neutral conversion of xylose into xylulose (Fig. 1(d)) with our experimental data (Table 1), a few apparent discrepancies are

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of RWB202-AFX to the earlier anaerobic xylose-fermenting yeast strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sonderegger et al.*</td>
</tr>
<tr>
<td>Selection time (days)</td>
<td>266</td>
</tr>
<tr>
<td>Chemical mutagenesis</td>
<td>3 times</td>
</tr>
<tr>
<td>μ glucose anaerobic (h⁻¹)</td>
<td>0.11</td>
</tr>
<tr>
<td>μ xylose aerobic (h⁻¹)</td>
<td>0.12</td>
</tr>
<tr>
<td>μ xylose anaerobic (h⁻¹)</td>
<td>0.012</td>
</tr>
<tr>
<td>Biomass yield (g g⁻¹)</td>
<td>0.021</td>
</tr>
<tr>
<td>Ethanol yield (g g⁻¹)</td>
<td>0.24</td>
</tr>
<tr>
<td>Xylitol yield (g g⁻¹)</td>
<td>0.32</td>
</tr>
<tr>
<td>Glycerol yield (g g⁻¹)</td>
<td>0.044</td>
</tr>
</tbody>
</table>

*Data obtained from Sonderegger et al. [15].
observed. Firstly, our xylose-isomerase-based strain also produced small, but significant amounts of xylitol. This, however, probably results from the activity of the non-specific aldose reductase encoded by the S. cerevisiae GRE3 gene product [29]. If so, it should be eliminated upon deletion of this gene. Secondly, the ethanol yield of strain RWB202-AFX was only 82% of the theoretical maximum depicted in Fig. 1(d). This is not unexpected. In the batch experiments (Figs. 4 and 5) with relatively low sugar concentrations and therefore low ethanol concentrations, biomass formation and the production of glycerol that is obligately associated with it [13,14] has a significant impact on the carbon balance. In the industrial practice of bioethanol production, yields on (hexose) sugars are as high as 90–95% of the theoretical maximum. This maximum is 0.51 g g⁻¹ for both glucose and xylose. The high industrial alcohol yields are due to reduced biomass formation in such processes caused by high maintenance-energy requirements of cells, resulting among others from ethanol stress at concentrations of around 100 g l⁻¹ ethanol (W.de Laat, personal communication).

The present study underlines that ethanol evaporation during batch cultivation in laboratory experiments cannot be ignored; carbon balances calculated for the batches on xylose were missing more than 10% of the carbon. With the assumption of the ethanol produced being equal to the CO₂ produced minus the amount produced in biomass and acetate formation, the carbon recoveries were acceptable (Table 1) and also the redox balances for all batches closed within a 3% margin, thus confirming the validity of the assumption.

As stressed above, anaerobic xylose fermentation without considerable xylitol production can only take place when the conversion of xylose to xylulose is a redox neutral process (Fig. 2). The results presented in this work provide a proof of principle for this claim. Functional expression of a heterologous xylose-isomerase gene in S. cerevisiae, followed by selection, yielded a strain capable of anaerobic growth and ethanolic fermentation on xylose as the sole carbon source (Fig. 4). This paper is the second report of an S. cerevisiae strain capable of anaerobic growth on xylose [15] and the first report of anaerobic xylose fermentation by a yeast with a satisfactory ethanol yield. It should be noted that theoretically high ethanol yield (i.e. absence of extensive polyol production) is not obligately connected to the conversion of xylose to xylulose via an isomerase reaction. Also with the combination of xylitol reductase and xylitol dehydrogenase a theoretical ethanol yield of 0.46 is possible when (just) enough oxygen is supplied to close the redox balance (Fig. 1(a)). In this case the oxygen supply must be substantial and by far exceeds the oxygen requirement for sterol and unsaturated-fatty-acid synthesis. This strategy for alcoholic fermentation of xylose therefore harbours the risk of oxygen ‘oversupply’, resulting in a competition between respiration and fermentation. Therefore, relieving the redox bottleneck by extensive aeration is in our opinion not a realistic option for large-scale industrial ethanol production.

Another theoretical option to circumvent the redox imbalance due to NADPH-linked XR would be the simultaneous conversion of glucose to glycerol during alcoholic fermentation of xylose in the absence of oxygen as follows:

\[ 6 \text{xylose} \rightarrow 9 \text{ethanol} + 12\text{CO}_2 + 9\text{ATP} + 6\text{NADH} \]
\[ 3 \text{glucose} + 6\text{NADH} + 6\text{ATP} \rightarrow 6 \text{glycerol} \]

\[ 6 \text{xylose} + 3 \text{glucose} \rightarrow 9 \text{ethanol} + 12\text{CO}_2 + 6 \text{glycerol} + 3\text{ATP} \]

However, such a metabolic scenario will also lead to unacceptably low ethanol yields of only 0.29 g ethanol per g sugar.

Simple metabolic flux analysis, as outlined above, can only give the upper limits of alcohol and polyol formation. In addition to stoichiometry, kinetics can be decisive for metabolic fluxes in vivo. Recently an elegant approach has been used to circumvent the redox imbalance caused by xylose reductase and xylitol dehydrogenase. Verho et al. [30] have engineered a dual coenzyme specificity for glyceraldehyde-3-phosphate dehydrogenase by introducing a gene encoding NADP-linked glyceraldehyde-3-phosphate dehydrogenase. However, the engineered strain still exhibited substantial xylitol formation, suggesting that the heterologous enzyme had insufficient in vivo activity.

4.2. Selection for anaerobic growth of strains transformed with xylose isomerase

Currently a diversity of examples exists in which the substrate range and/or product range of microorganisms has been altered by means of extensive genetic modifications such as the deletion and/or overexpression of endogenous genes or the introduction of heterologous genes. Zang et al. [31] have successfully introduced a pathway for xylose metabolism in Zymomonas mobilis. Similarly, Becker and Boles [32] have constructed an arabinose-fermenting S. cerevisiae strain by using three heterologous genes encoding the bacterial pathway and overexpressing an endogenous fourth gene. Richard et al. [33] had already done the same with the more elaborate five-gene fungal pathway. Examples of altered product formation include homolactic acid fermentation with Kluyveromyces lactis [34], propane-1,3-diol production with Escherichia coli [35,36], as well as homoeathanolic xylose fermentation by E. coli [37,38]. In all these cases an assortment of genetic modifications was necessary to obtain the required result. The alternative,
altering the substrate spectrum of a microorganism without genetic engineering, via directed evolution in chemostat cultures, has also been reported [39].

The strain described in this work was obtained through a combination of only one genetic modification and selective pressure. Because *S. cerevisiae* is unlikely to acquire the ability to metabolise xylose through natural selection, genetic engineering had to be employed to introduce the initial step of xylose metabolism. After the xylose isomerase had been functionally expressed, a more efficient strain could be rapidly selected by applying the correct selective pressure (Fig. 3). In this manner a yeast capable of anaerobic growth on xylose was attained in a relatively short period of time without the need to search for genetic targets for modification. The fact that the aerobic growth rate on xylose could be increased 25-fold in only 80 days (Fig. 3) is evidence of the efficiency of this method. A high aerobic growth rate, however, was not connected to anaerobic growth on xylose. A different selective pressure had to be applied to successfully overcome this bottleneck.

Sonderegger and Sauer [15] have used a combination of three genetic modifications (functional expression of heterologous XR and XDH as well as overexpression of the native XKS1-encoded xylulokinase), chemical mutagenesis and directed evolution to obtain a *S. cerevisiae* strain capable of anaerobic growth on xylose. The overexpression of XKS1 encoding xylulokinase seems unnecessary in view of our results. The growth characteristics of our strain indicate that the endogenous xylulokinase activity can be changed as part of the selection process. In this way an optimal activity level is ensured without the risk of harmful levels of the kinase [40]. However, the observation of minor but significant xylose excretion during the batch cultivation on xylose (Fig. 4(b)) indicates that the capacity of xylulokinase or that of enzymes further downstream is barely sufficient.

The next logical steps in our approach is to perform a detailed investigation into the genetic changes that have taken place during the directed-evolution experiments and that enable *S. cerevisiae* to grow anaerobically on xylose. Such studies may be used for engineering strains with even higher fermentation rates.

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**References**


