

Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054

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Abstract

The recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3399 was constructed by chromosomal integration of the genes encoding D-xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). *S. cerevisiae* TMB 3399 was subjected to chemical mutagenesis with ethyl methanesulfonate and, after enrichment, 33 mutants were selected for improved growth on D-xylose and carbon dioxide formation in Durham tubes. The best-performing mutant was called *S. cerevisiae* TMB 3400. The novel, recombinant *S. cerevisiae* strains were compared with *Pichia stipitis* CBS 6054 through cultivation under aerobic, oxygen-limited, and anaerobic conditions in a defined mineral medium using only D-xylose as carbon and energy source. The mutation led to a more than five-fold increase in maximum specific growth rate, from 0.0255 h⁻¹ for *S. cerevisiae* TMB 3399 to 0.14 h⁻¹ for *S. cerevisiae* TMB 3400, whereas *P. stipitis* grew at a maximum specific growth rate of 0.44 h⁻¹. All yeast strains formed ethanol only under oxygen-limited and anaerobic conditions. The ethanol yields and maximum specific ethanol productivities during oxygen limitation were 0.21, 0.25, and 0.30 g ethanol g xylose⁻¹ and 0.001, 0.10, and 0.16 g ethanol g biomass⁻¹ h⁻¹ for *S. cerevisiae* TMB 3399, TMB 3400, and *P. stipitis* CBS 6054, respectively. The xylitol yield under oxygen-limited and anaerobic conditions was two-fold higher for *S. cerevisiae* TMB 3399 than for TMB 3400, but the glycerol yield was higher for TMB 3400. The specific activity, in U mg protein⁻¹, was higher for XDH than for XR in both *S. cerevisiae* TMB 3399 and TMB 3400, while *P. stipitis* CBS 6054 showed the opposite relation. *S. cerevisiae* TMB 3400 displayed higher specific XR, XDH and XK activities than TMB 3399. Hence, we have demonstrated that a combination of metabolic engineering and random mutagenesis was successful to generate a superior, xylose-utilizing *S. cerevisiae*, and uncovered distinctive physiological properties of the mutant.

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1. Introduction

Unlike several bacteria, yeasts and filamentous fungi [1], wild-type *Saccharomyces cerevisiae* cannot use D-xylose for growth and ethanol production, and it has been a metabolic engineering challenge to construct a strain with these

qualities [2–7]. An efficient D-xylose-fermenting *S. cerevisiae* strain would enable the utilization of a larger fraction of hemicellulose and thus contribute to making the production of ethanol from agricultural and forestry residues economically viable [8].

Early studies of xylose fermentation with *S. cerevisiae* have shown low activities of D-xylose reductase (XR) and xylitol dehydrogenase (XDH) [9]. XR converts xylose to xylitol, using either NADPH or NADH as a cofactor [10], and XDH converts xylitol to xylulose, using NAD⁺ [11]. The genes *xyl1* and *xyl2*, encoding XR and XDH, respectively, have been cloned from *Pichia stipitis* and transformed into *S. cerevisiae* [2]. The recombinant strain was

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able to slowly use xylose for growth and ethanol production [3]. Later work has demonstrated that overexpression of xylulokinase (XK) [12], which is responsible for the phosphorylation of xylulose to xylulose 5-phosphate, improved xylose utilization [13,14]. A laboratory strain (CEN.PK) transformed with the genes for XR, XDH, and XK, *S. cerevisiae* TMB 3001, was able to produce ethanol from xylose, but could not grow anaerobically on xylose [7,15].

In the present study, we have chosen mutagenesis as a method for further improvement. This method has previously been used to improve xylose growth in a strain of *S. cerevisiae* expressing XR and XDH [16]. We describe the construction of two *S. cerevisiae* strains, *S. cerevisiae* TMB 3399 and 3400, both capable of growing on and fermenting D-xylose to ethanol. The choice of host strain in this work, the industrial, diploid *Saccharomyces* USM21 strain [17], was based on its robustness and better ethanol tolerance. *S. cerevisiae* TMB 3399 was created by chromosomal integration of the *P. stipitis* genes encoding XR and XDH, as well as the *S. cerevisiae* gene encoding XK. *S. cerevisiae* TMB 3399 was then subjected to chemical mutagenesis and mutants were selected that displayed improved growth on D-xylose. Specific growth rate, product yield, and specific productivity were determined for *S. cerevisiae* TMB 3399 and 3400 and compared with the corresponding parameter values for *P. stipitis* CBS 6054, one of the best natural D-xylose-utilizing yeasts known [1,18].

2. Materials and methods

2.1. Strains, DNA manipulations, and mutagenesis

During transformation and mutagenesis, yeast strains were grown on YPD and YPX media containing 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, and 20 g l⁻¹ D-glucose or D-xylose, respectively, or SX medium containing 6.7 g l⁻¹ Yeast Nitrogen Base (Difco, Detroit, MI, USA) and 20 g l⁻¹ D-xylose. Cultivations were carried out at 30°C unless otherwise indicated.

Plasmid YIpXR/XDH/XK [7] was linearized by digestion with *Pst*I and transformed into the *HIS3* locus of *S. cerevisiae* USM21 [17], using the lithium acetate method [19]. *S. cerevisiae* USM21 does not have any auxotrophic requirements. Therefore, the selection strategy was based on the transformants' ability to use D-xylose. DNA isolation and Southern analysis [20] confirmed correct integration and the transformed yeast was called *S. cerevisiae* XYLUSM21/TMB 3399. Mutagenesis was carried out with ethyl methanesulfonate (EMS, Sigma, St. Louis, MO, USA) [21]. The cells were incubated with EMS for 80, 100, 120, and 140 min to obtain survival rates between 60 and 10%. Samples withdrawn after these time points were pooled and added to two separate shake flasks con-

taining 400 ml YPX medium. In the first flask, the initial xylose concentration was 50 g l⁻¹ and the medium was not changed during the enrichment. In the second flask, the sugar concentration was changed every 48 h, from 2 g l⁻¹ xylose and 20 g l⁻¹ glucose in the beginning to 50 g l⁻¹ xylose and no glucose after 8 days. Between the feed changes, the cells were centrifuged and the supernatant was removed. The flasks were incubated at 30°C with stirring for 10 days. Samples were withdrawn and plated on YPX plates every 48 h. Thereafter, selected colonies from the YPX plates were inoculated into 5-ml YPX test tubes containing Durham tubes turned upside down in the medium. In this way, mutants with superior carbon dioxide evolution were selected. In the second screening, the best mutants and *S. cerevisiae* TMB 3399 were cultivated aerobically in 1-l flasks containing 100 ml of SX medium. *S. cerevisiae* TMB 3399 and the mutants with the highest growth rates were cultivated under oxygen-limited conditions in 55-ml flasks containing 50 ml SX medium. From these cultures, ethanol productivities, ethanol yields, and enzymatic activities of XR, XDH, and XK were determined. The best mutant was named *S. cerevisiae* TMB 3400.

P. stipitis CBS 6054 was obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

2.2. Medium, strain maintenance, and cultivation conditions

We used a defined mineral medium including vitamins and trace elements [22] for all fermentation experiments. During anaerobic cultivation experiments, ergosterol and unsaturated fatty acids, in the form of Tween 80 (Sigma), were added to the medium [23,24]. D-Xylose (20 g l⁻¹) was used as carbon and energy source. Antifoam (Dow Corning® Antifoam RD Emulsion, BDH Laboratory Supplies, Poole, UK) was added to a concentration of 0.5 ml l⁻¹. Both yeasts were maintained on SX plates. A loopful of yeast was inoculated in 100 ml of medium in 1000-ml baffled shake flasks, which were incubated overnight at 30°C and 140 rpm in an orbital incubator (Gallenkamp INR-200, Leicester, UK). Bioreactors were inoculated with 20 mg l⁻¹ of cells (dry weight) growing at the late-exponential phase. Batch fermentation was conducted at 30°C in 2-l Biostat® A bioreactors (B. Braun Biotech International, Melsungen, Germany). The working volume of the bioreactors was 1500 ml and the pH was automatically maintained at 5.5 by the addition of 3 M NaOH. Filter-sterilized air was supplied to the bioreactors during aerobic and oxygen-limited cultivation. During aerobic cultivation, the dissolved oxygen tension was at least 30% of the maximum. Oxygen-limited conditions were defined as 0% dissolved oxygen tension in an air-bubbled bioreactor. Anaerobic conditions were ensured by continuous flushing with nitrogen containing less than 5 ppm O₂ (ADR class2 1A, AGA, Malmö, Sweden). A gas flow rate of 1 l min⁻¹ was controlled by mass flow meters (Bronk-

horst HI-TECH, Ruurlo, The Netherlands) and the off-gas condensers were cooled to 4°C. Duplicate fermentation experiments were run for each yeast and no difference was observed in the product pattern. The different yeast strains examined were cultivated so that a substantial amount of xylose was consumed, allowing calculation of rates and yields. A strain consuming xylose slowly was therefore cultivated for a longer period of time than a strain consuming xylose rapidly.

2.3. Analyses

Soluble metabolites were analyzed by high-performance liquid chromatography as has been described previously [25]. The composition of the outgoing gas was monitored continuously by a Carbon Dioxide and Oxygen Monitor Type 1308 (Brüel and Kjør, Copenhagen, Denmark) [26]. The cell dry weight was determined by filtering a known volume of the culture broth through a 0.45-µm Supor membrane (Gelman Sciences, Ann Arbor, MI, USA). After washing with three volumes of distilled water and drying in a microwave oven for 15 min at 350 W, the filter was weighed. Cell dry weight was determined in triplicate. Cell-free extracts were obtained by treatment with a yeast protein extraction solution (Y-PER[®], Pierce, Rockford, IL, USA). Assays of XR, XDH, and XK [7] as well as pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) [27] were performed as previously described, but triethanolamine buffer at pH 7 was used instead of glycine buffer at pH 9 for the XDH and ADH assays. The high pH of the glycine buffer caused precipitation of components in the extraction solution.

3. Results

3.1. Transformation, mutagenesis, and selection

S. cerevisiae TMB XYLUSM21/3399 was constructed by transforming *Saccharomyces* USM21 [17] with the in-

tegrative plasmid YIpXR/XDH/XK [7] that carries genes for XR and XDH from *P. stipitis* and an extra copy of the endogenous gene for XK. The selection strategy was based on the ability to use xylose and transformants with the plasmid integrated into the *HIS3* locus were selected on YPX plates after 4 days at 28°C. All transformants were screened for stable xylose metabolism by passage through a non-selective medium, YPD, to assure that the plasmid was maintained in the integrated state.

Initial shake-flask experiments exhibited poor D-xylose utilization in a defined medium. Therefore, *S. cerevisiae* TMB 3399 was subjected to mutagenesis by EMS to improve D-xylose utilization. Thirty-three mutants from EMS-treated cell cultures were screened for carbon dioxide evolution in Durham tubes. Out of these 33 mutants, 18 came from the enrichment culture with an initial xylose concentration of 50 g l⁻¹ and the remaining 15 came from the culture where serial transfer to medium with increasing xylose concentration was employed. The three best mutants and *S. cerevisiae* TMB 3399 were cultivated aerobically and two mutants grew with a higher specific growth rate than *S. cerevisiae* TMB 3399 (Table 1). These two mutants (no. 125 and 145) and *S. cerevisiae* TMB 3399 were further analyzed in oxygen-limited cultures. The ethanol yield and productivity as well as the enzymatic activities were higher for mutant no. 125 than for *S. cerevisiae* TMB 3399. Mutant no. 125 was called *S. cerevisiae* TMB 3400 and was selected for further physiological characterization and comparison with *S. cerevisiae* TMB 3399 and *P. stipitis* CBS 6054. This mutant came from the enrichment culture with an initial xylose concentration of 50 g l⁻¹.

3.2. Fermentation kinetics of *S. cerevisiae* TMB 3399 and 3400 and *P. stipitis* CBS 6054

In the cultivation experiments with *S. cerevisiae* TMB 3399, *S. cerevisiae* TMB 3400 and *P. stipitis* CBS 6054 (Figs. 1–3), the stirring speed was kept constant so that the cultures initially were aerobic, but gradually became

Table 1

Maximum specific growth rates ($\mu_{\max}$), yield coefficients for ethanol ($Y_{E/S}$), specific ethanol productivities (q_{ethanol}), as well as specific enzymatic activities (U mg protein⁻¹) for D-xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) for *S. cerevisiae* TMB 3399 and the three best-performing mutants selected on the basis of CO₂ production from D-xylose

Yeast strain	$\mu_{\max}^a$	$Y_{E/S}^b$	q_{ethanol}^c	Enzymatic activity		
				XR	XDH	XK
<i>S. cerevisiae</i> TMB 3399	0.04	0.2	0.022	0.05	0.45	0.18
Mutant no. 125	0.14	0.3	0.039	0.22	1.35	0.27
Mutant no. 138	0.04	n.d. ^d	n.d.	n.d.	n.d.	n.d.
Mutant no. 145	0.07	0.24	0.024	0.18	0.35	0.2

The maximum specific growth rates were obtained in 1-l shake flasks containing 100 ml SX medium, whereas the other values were determined in 55-ml, oxygen-limited flasks containing 50 ml SX medium.

^ah⁻¹.

^bg ethanol g D-xylose⁻¹.

^cg ethanol g biomass⁻¹ h⁻¹.

^dNot determined.

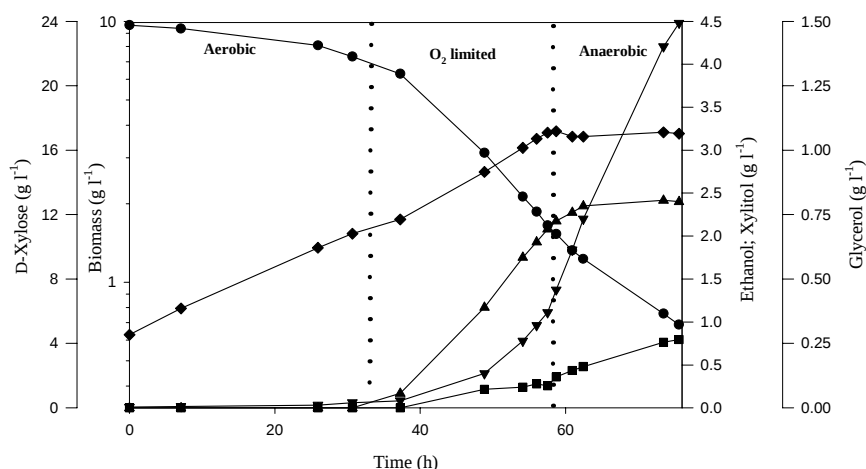


Fig. 1. D-Xylose consumption (●) and the formation of ethanol (▲), biomass (◆), xylitol (▼), and glycerol (■) during aerobic, oxygen-limited, and anaerobic cultivation of *S. cerevisiae* TMB 3399. Oxygen limitation was defined as 0% dissolved oxygen in an air-bubbled bioreactor. Anaerobic conditions were ensured by continuous flushing of nitrogen through the bioreactor. A defined mineral medium, supplemented with 20 g l⁻¹ D-xylose, was used and the pH was kept at 5.5. The graph is a representative example of fermentation experiments performed in duplicate.

more oxygen-limited as cell growth proceeded. Towards the end of the fermentation, replacing air with nitrogen imposed anaerobic conditions.

During the aerobic cultivation of *S. cerevisiae* TMB 3399 and 3400, only biomass was formed from D-xylose (Figs. 1 and 2). Ethanol, glycerol and xylitol were formed as the availability of oxygen decreased. Biomass formation still occurred in *S. cerevisiae* TMB 3399 at the same specific growth rate as under aerobic conditions, whereas oxygen limitation caused slower growth for *S. cerevisiae* TMB 3400. Once anaerobiosis began, biomass growth ceased and the rate of xylitol excretion increased, especially for *S. cerevisiae* TMB 3399. At the end of the fermentation, concentrations of ethanol, xylitol and glycerol reached 2.4 g l⁻¹, 4.5 g l⁻¹ and 0.26 g l⁻¹ for *S. cerevisiae* TMB 3399 and 3.4 g l⁻¹, 2.5 g l⁻¹ and 1.1 g l⁻¹ for *S. cerevisiae* TMB 3400, respectively. Acetate and succinate accumulated to less than 0.25 g l⁻¹ and 0.060 g l⁻¹, respectively. Similarly,

P. stipitis CBS 6054 excreted metabolites only when oxygen was limited (Fig. 3), but unlike *S. cerevisiae* TMB 3399 and 3400, *P. stipitis* CBS 6054 produced ethanol almost exclusively. The final concentrations of ethanol, xylitol, glycerol, and acetate were 3.7 g l⁻¹, 0.1 g l⁻¹, 0.04 g l⁻¹, and 0.03 g l⁻¹, respectively. No succinate was detected.

Table 2 summarizes the growth parameters for the three yeast strains under aerobic, oxygen-limited, and anaerobic conditions. The maximum specific growth rates, biomass yields, and the specific D-xylose uptake rates were determined from a second, completely aerobic culture (graphs not shown), whereas the other values are from the cultures with varying oxygen availability (Figs. 1–3). During aerobic growth, *S. cerevisiae* TMB 3399 grew at a specific growth rate of 0.0255 h⁻¹. *S. cerevisiae* TMB 3400 grew more than five times faster, at a specific growth rate of 0.14 h⁻¹. Both *S. cerevisiae* strains had a biomass yield of

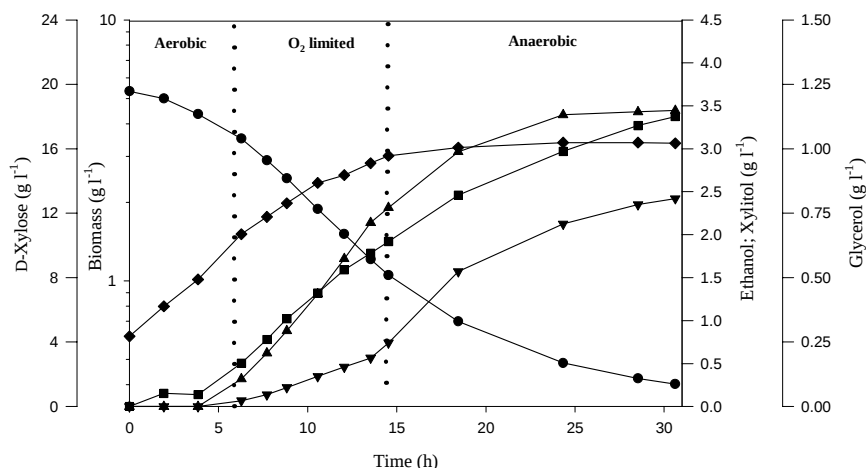


Fig. 2. Aerobic, oxygen-limited, and anaerobic cultivation of *S. cerevisiae* TMB 3400. Same symbols and conditions as in Fig. 1.

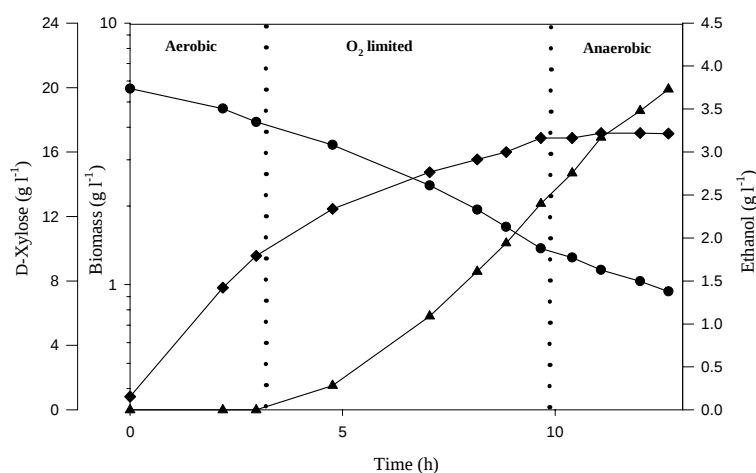


Fig. 3. Aerobic, oxygen-limited, and anaerobic cultivation of *P. stipitis* CBS 6054. Same symbols and conditions as in Fig. 1.

around 0.4 g biomass g xylose⁻¹. *P. stipitis* CBS 6054 grew faster than both *S. cerevisiae* strains at 0.44 h⁻¹, and also had a higher biomass yield – around 0.57 g biomass g D-xylose⁻¹. The carbon and degree-of-reduction balances closed within 95–105% for all yeast strains in the separate, completely aerobic cultivation experiment. Ethanol yields and specific ethanol productivities were higher for *S. cerevisiae* TMB 3400 than for *S. cerevisiae* TMB 3399. *S. cerevisiae* TMB 3399 had a xylitol yield about twice as high as *S. cerevisiae* TMB 3400 during both oxygen-limited conditions and anaerobiosis – 0.14 compared to 0.07 g xylitol g D-xylose⁻¹ and 0.59 compared to 0.25 g xylitol g D-xylose⁻¹, respectively. Anaerobic ethanol yield and ethanol productivity for *P. stipitis* CBS 6054 were 2–5 times higher than that of *S. cerevisiae* TMB 3400.

The enzymatic activities in the cell extracts were generally higher for *S. cerevisiae* TMB 3400 than for *S. cerevisiae* TMB 3399 (Table 3). When the specific activity (in U mg protein⁻¹) was compared, both *S. cerevisiae* strains displayed higher XDH than XR activity. In contrast, *P. stipitis* CBS 6054 showed higher XR than XDH activity.

PDC and ADH activity were found under all cultivation conditions for *S. cerevisiae* TMB 3399 and 3400, whereas these activities were not detected during the aerobic cultivation of *P. stipitis* CBS 6054.

4. Discussion

The construction of an *S. cerevisiae* strain growing on D-xylose has been the object of intense research efforts [2–7]. Most of the work has focused on overexpression of the genes involved in the metabolism of xylose. The introduction of genes encoding XR, XDH, and XK was considered necessary due to insufficient activity of these enzymes in wild-type *S. cerevisiae* [2,14]. However, using only metabolic engineering had not proved sufficient for efficient xylose utilization. Random mutagenesis combined with subsequent selection has previously been used to increase the maximum specific growth rate from 0.03 h⁻¹ to 0.08 h⁻¹ in a strain containing a plasmid carrying the genes for XR and XDH [16].

Table 2

Maximum specific growth rates ($\mu_{\max}$), yield coefficients (Y_X for biomass, $Y_{\text{Xyli/S}}$ for xylitol and $Y_{\text{E/S}}$ for ethanol), specific xylose uptake rates (q_{xylose}), and specific ethanol productivities (q_{ethanol}) from aerobic, oxygen-limited, and anaerobic batch cultivation of *S. cerevisiae* TMB 3399 and 3400 and *P. stipitis* CBS 6054

Yeast	Aerobic			O ₂ limited			Anaerobic		
	$\mu_{\max}^a$	$Y_{X/S}^b$	q_{xylose}^c	$Y_{\text{E/S}}^d$	q_{ethanol}^e	$Y_{\text{Xyli/S}}^f$	$Y_{\text{E/S}}^d$	q_{ethanol}^e	$Y_{\text{Xyli/S}}^f$
<i>S. cerevisiae</i> TMB 3399	0.0255 ± 0.002	0.39 ± 0.02	0.065 ± 0.01	0.21 ± 0.01	0.001 ± 0.0	0.14 ± 0.03	0.05 ± 0.02	0.0006 ± 0.0	0.59 ± 0.04
<i>S. cerevisiae</i> TMB 3400	0.14 ± 0.004	0.41 ± 0.02	0.35 ± 0.024	0.25 ± 0.01	0.10 ± 0.0	0.07 ± 0.0	0.18 ± 0.007	0.024 ± 0.001	0.25 ± 0.0
<i>P. stipitis</i> CBS 6054	0.44 ± 0.02	0.57 ± 0.025	0.78 ± 0.068	0.31 ± 0.02	0.16 ± 0.01	< 0.01	0.40 ± 0.064	0.11 ± 0.001	< 0.01

The average biomass concentration was used in the calculation of the specific productivities of ethanol during oxygen-limited and anaerobic cultivations, respectively. The numbers represent mean values and deviations from the mean of duplicate fermentation experiments.

^ah⁻¹.

^bg biomass g D-xylose⁻¹.

^cg xylose g biomass⁻¹ h⁻¹.

^dg ethanol g D-xylose⁻¹.

^eg ethanol g biomass⁻¹ h⁻¹.

^fg xylitol g D-xylose⁻¹.

Table 3

Specific enzymatic activities (U mg protein⁻¹) for xylose reductase (XR), xylitol dehydrogenase (XDH), xylulokinase (XK), pyruvate decarboxylase (PDC), and alcohol dehydrogenase (ADH) from cells of *S. cerevisiae* TMB 3399, *S. cerevisiae* TMB 3400 and *P. stipitis* CBS 6054 cultivated under aerobic, oxygen-limited, and anaerobic conditions

Yeast	Cultivation condition	Enzyme				
		XR	XDH	XK	PDC	ADH
<i>S. cerevisiae</i> TMB 3399	Aerobic	0.03 ± 0.0034	0.34 ± 0.1	0.22 ± 0.09	0.06 ± 0.001	0.06 ± 0.04
	O ₂ limited	0.10 ± 0.01	0.42 ± 0.05	0.16 ± 0	0.05 ± 0.002	0.15 ± 0.03
	Anaerobic	0.024 ± 0.002	0.09 ± 0.001	0.11 ± 0.001	0.011 ± 0	0.20 ± 0.03
<i>S. cerevisiae</i> TMB 3400	Aerobic	1.1 ± 0.08	1.7 ± 0.26	2.4 ± 0.8	0.3 ± 0.07	0.7 ± 0.34
	O ₂ limited	0.7 ± 0.02	1.2 ± 0.39	1.3 ± 0.34	0.2 ± 0.04	0.5 ± 0.25
	Anaerobic	0.5 ± 0.05	1.2 ± 0.27	1.45 ± 0.07	0.2 ± 0.02	0.9 ± 0.15
<i>P. stipitis</i> CBS 6054	Aerobic	2.1 ± 0.20	0.4 ± 0.11	0.14 ± 0.07	0	0
	O ₂ limited	3.0 ± 0.09	0.8 ± 0.22	0.8 ± 0.57	0.02 ± 0.01	0.09 ± 0.04
	Anaerobic	2.2 ± 0.15	0.5 ± 0.13	0.52 ± 0.22	0.01 ± 0.001	0.04 ± 0.01

The numbers represent mean values and deviations from the mean in duplicate fermentation experiments.

Here, we demonstrate that the combination of metabolic engineering, that is the integration of the genes encoding XR, XDH and XK, and random mutagenesis proved successful to generate an *S. cerevisiae* strain capable of good growth on D-xylose in a defined mineral medium. The mutation in *S. cerevisiae* TMB 3400 led to a more than five-fold increase in specific growth rate, from 0.0255 h⁻¹ to 0.14 h⁻¹. A maximum specific growth rate of 0.14 h⁻¹ is higher than what is commonly reported for aerobic growth on ethanol (0.08–0.13 h⁻¹), but lower than growth on preferred substrates, such as glucose and sucrose (0.34–0.44 h⁻¹) [28]. Table 4 compares the strains developed in this study with previously constructed recombinant, xylose-utilizing *S. cerevisiae* strains. The use of different media (complex versus mineral media) and different cultivation conditions (shake flasks versus bioreactors) prevents a fair comparison. Furthermore, some investigators do not report the biomass concentration [6] and therefore neither the specific growth rate nor the specific ethanol productivity can be calculated. In our opinion, the specific ethanol productivity, g ethanol g biomass⁻¹ h⁻¹, is the most relevant parameter to evaluate the ethanol production of recombinant *S. cerevisiae* strains. The volumetric productivity, g ethanol l⁻¹ h⁻¹, is highly dependent on the biomass concentration in the fermentor. The data in Table 4 have been estimated from published diagrams. The specific growth rate and specific ethanol productivity of *S. cerevi-*

siae TMB 3400 is among the highest reported. Furthermore, we are the first to report a D-xylose-utilizing, recombinant *S. cerevisiae* strain cultivated aerobically in a bioreactor using a defined mineral medium. The rigorous choice of experimental conditions unequivocally proves that *S. cerevisiae* TMB 3400 is capable of recovering energy and producing biomass building blocks from D-xylose as the only carbon source.

The level of oxygenation differently influenced the enzymes involved in ethanol production, namely, PDC and ADH. In *S. cerevisiae* TMB 3399 and 3400, these two enzymes were expressed under all aeration conditions, but in *P. stipitis* CBS 6054 they were expressed only during oxygen-limited and anaerobic conditions, which is consistent with previous reports on the regulation of the corresponding genes [29–32]. For *S. cerevisiae*, PDC activity is required under all aeration conditions to generate cytosolic acetyl coenzyme A for the synthesis of lipids [33]. In spite of PDC and ADH activities, no ethanol was produced by *S. cerevisiae* TMB 3399 and 3400 during aerobic growth; only biomass and CO₂ were formed. The most probable explanation for the lack of ethanol secretion is that the flux over the pyruvate branch point is not high enough. The ability to divert carbon to ethanol production under aerobic conditions, the Crabtree effect, is thought to occur only when the specific growth rates are greater than 0.25–0.30 h⁻¹ [27].

Table 4

Specific growth rate ($\mu_{\max}$) and specific ethanol productivity (q_{ethanol}) of recombinant xylose-utilizing *Saccharomyces* strains

Strain	Medium	Maximum specific growth rate, $\mu_{\max}^a$	Maximum ethanol productivity, q_{ethoh}^b	Reference
<i>S. cerevisiae</i> TMB 3399	Mineral medium	0.03	0.001	this work
<i>S. cerevisiae</i> TMB 3400	Mineral medium	0.14	0.11	this work
<i>S. cerevisiae</i> TMB 3001	Mineral medium	n.d. ^c	0.04	[15]
<i>S. cerevisiae</i> pRD1	SX medium	0.11	0.03	[2]
<i>S. cerevisiae</i> IM2	YPX	0.08	n.a. ^d	[16]
<i>Saccharomyces</i> 1400(pLNH33)	YPX	0.19	n.a.	[40]

^ah⁻¹.

^bg ethanol g biomass⁻¹ h⁻¹.

^cNot determined.

^dNot available.

The enzymatic activities of XR, XDH, and XK agree well with activities reported from *S. cerevisiae* TMB 3001 [7] that carries the same integrated plasmid as *S. cerevisiae* TMB 3399 and 3400. Although the levels vary between the strains, the relationship between the activities is the same: XDH had the highest and XR the lowest. For *P. stipitis*, the relationship was the reverse: XR displayed the highest activity and XDH the lowest.

In *S. cerevisiae* TMB 3399 and 3400, the construct for expression of XR and XDH was chosen to achieve a high level of XDH activity and a low XR activity, since kinetic calculations [14] have shown that this favors low xylitol formation. Nevertheless, both xylitol and glycerol were produced in addition to ethanol under oxygen-limited or anaerobic conditions. The mutant strain, *S. cerevisiae* TMB 3400 displayed a shift in product formation. In *S. cerevisiae* TMB 3399, considerable amounts of xylitol were produced in response to oxygen limitation or anaerobiosis, whereas little glycerol was produced. In *S. cerevisiae* TMB 3400, the xylitol yield was lower, but this strain produced more glycerol than *S. cerevisiae* TMB 3399. Both xylitol [34] and glycerol are formed to oxidize excess NADH. In both cases, the exogenous genes for XR and XDH in *S. cerevisiae* TMB 3399 and 3400 were cloned from *P. stipitis* and, thus, the enzymes synthesized in both yeasts should be the same. Evidently, mechanisms other than the level of the two enzymes regulate by-product formation in *S. cerevisiae* TMB 3399 and 3400. In addition to enzyme activity levels, the intracellular NADH/NAD⁺ ratio has been suggested to influence xylitol formation [14].

P. stipitis CBS 6054 produces only ethanol when exposed to oxygen limitation or anaerobiosis. Experiments using ¹³C-labelled D-xylose have indicated that, under anaerobic conditions, *P. stipitis* used only NADH in the first step of D-xylose utilization [35]. Thus, the first two steps of D-xylose utilization under anaerobiosis should be redox-neutral, with no xylitol excretion. Another possible explanation for the absence of xylitol excretion in *P. stipitis* CBS 6054 could be the presence of a transhydrogenase-like enzymatic reaction cycle. Such a cycle would catalyze the interconversion of NADPH and NADH. Recently, *S. cerevisiae* was genetically engineered to include an artificial transhydrogenase cycle, resulting in reduced glycerol formation during anaerobic glucose fermentation [36].

Our results in the present study demonstrate that an *S. cerevisiae* strain capable of good growth and ethanol production from D-xylose in a defined mineral medium can be generated through a combination of metabolic engineering and random mutagenesis. *P. stipitis* CBS 6054 still grows faster with a higher ethanol yield and the performance of this yeast serves as a benchmark for further improvement of recombinant strains of *S. cerevisiae*. However, it has been shown that *P. stipitis* is sensitive to inhibitors present in lignocellulosic hydrolysates [37,38] and requires a low, well-controlled oxygenation for optimal ethanol productivity [39], which taken together severely

limit its industrial exploitation. The established industrial fermentation technology for *S. cerevisiae* and this yeast's tolerance to high ethanol concentrations motivate the continued development of *S. cerevisiae* for the efficient bio-conversion of lignocellulosic material to ethanol.

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