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ORIGINAL ARTICLE

Improved xylose uptake in *Saccharomyces cerevisiae* due to directed evolution of galactose permease Gal2 for sugar co-consumption

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Abstract

Aims: *Saccharomyces cerevisiae* does not express any xylose-specific transporters. To enhance the xylose uptake of *S. cerevisiae*, directed evolution of the Gal2 transporter was performed.

Methods and Results: Three rounds of error-prone PCR were used to generate mutants with improved xylose-transport characteristics. After developing a fast and reliable high-throughput screening assay based on flow cytometry, eight mutants were obtained showing an improved uptake of xylose compared to wild-type Gal2 out of 41 200 single yeast cells. Gal2 variant 2·1 harbouring five amino acid substitutions showed an increased affinity towards xylose with a faster overall sugar metabolism of glucose and xylose. Another Gal2 variant 3·1 carrying an additional amino acid substitution revealed an impaired growth on glucose but not on xylose.

Conclusions: Random mutagenesis of the *S. cerevisiae* Gal2 led to an increased xylose uptake capacity and decreased glucose affinity, allowing improved co-consumption.

Significance and Impact of the Study: Random mutagenesis is a powerful tool to evolve sugar transporters like Gal2 towards co-consumption of new substrates. Using a high-throughput screening system based on flow-through cytometry, various mutants were identified with improved xylose-transport characteristics. The Gal2 variants in this work are a promising starting point for further engineering to improve xylose uptake from mixed sugars in biomass.

Introduction

Faced with climate change and increasing global demand for transportational fuels on the one hand and decreasing natural deposits of fossil fuels on the other, there is a strong need for new and sustainable feedstocks like non-food biomass. *Saccharomyces cerevisiae* is considered one of the most promising organisms for bioconversion of lignocellulosic biomass into biofuels (van Maris *et al.* 2006). Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. Hemicellulose, the second most abundant biopolymer found in nature, is primarily composed of pentoses such as xylose and arabinose. Unfortunately, *S. cerevisiae* is unable to metabolize pentose sugars which, depending on the kind of feedstock, comprise up to 30% of lignocellulose.

Heterologous pathways have been introduced into *S. cerevisiae* to enable utilization of xylose and arabinose (Kuyper *et al.* 2004; Karhumaa *et al.* 2006; Hahn-Hägerdal *et al.* 2007; Brat *et al.* 2009; Wisselink *et al.* 2009). However, complicated by the fact that *S. cerevisiae* does not express any xylose-specific transport system, the

utilization and ethanol yield remains highly insufficient. Many efforts have been directed towards expressing heterologous xylose-specific transporters in *S. cerevisiae* (Saloheimo *et al.* 2007; Fonseca *et al.* 2011; Young *et al.* 2012), but these attempts suffer from complications such as efficient translation, posttranslational modification, correct folding and proper targeting to the plasma membrane of yeast cells. Therefore, the use of a homologous transporter system is a promising alternative to overcome these problems.

Saccharomyces cerevisiae transports glucose by means of the 18 hexose transporters (Hxt1 to Hxt17, and Gal2) which are all members of the major facilitator superfamily (MFS) (Boles and Hollenberg 1997; Pao *et al.* 1998). Depending on environmental factors like glucose concentration in the surrounding medium, the appropriate transport system is expressed that exhibits distinct glucose affinities: low (K_m of 50–100 mmol l^{-1}), moderate (K_m of about 10 mmol l^{-1}) or high (K_m of 1–2 mmol l^{-1}) affinity (Reifenberger *et al.* 1997; Diderich *et al.* 1999). To date, no xylose-specific transporter has been described for *S. cerevisiae* but it has been shown that xylose transport takes place by the same transport system as for glucose uptake, mainly by Hxt4, Hxt5, Hxt7 and Gal2 (Hamacher *et al.* 2002; Sedlak and Ho 2004). Nevertheless, the transport of xylose as a substrate via hexose transporters occurs with a two orders of magnitude lower affinity than for glucose (Kötter and Ciriacy 1993). The limitation of xylose uptake may be due to this lower affinity of the transport system towards xylose compared to glucose, and thus in the presence of glucose, xylose is a nonpreferred substrate for these transporters. Also catabolite repression by glucose is a concern (Ye *et al.* 1999). Interestingly, Gal2 is not only a galactose-specific transporter protein but when expressed in a *S. cerevisiae hxt* null strain it functions as a high-affinity glucose transporter (Reifenberger *et al.* 1997). Furthermore, the galactose permease is also known to mediate arabinose uptake in *S. cerevisiae* (Cirillo 1968). This versatility makes Gal2 an ideal candidate for directed evolution and improvement of its potential xylose-transport capacity.

Although the members of the MFS are found in all kingdoms of life and comprise secondary transporters as uniporters, antiporters and symporters that transport highly various compounds, the spatial architecture and topology is considerably conserved (Hirai *et al.* 2003; Hirai and Subramaniam 2004). The distinct functions of each transporter is based on the versatile assembly of amino acids within the substrate binding and translocation domains (Vardy *et al.* 2004). Studies on Gal2, Hxt2 and Hxt7 from *S. cerevisiae* revealed that the transmembrane (TM) segments 1, 5, 7, 8 and 10 harbour essential amino acid residues regarding substrate recognition, substrate translocation and transport affinity. Asn331 and Asp340 both in TM7 of Hxt2 and Hxt7, respectively, were shown to play a key role for recognition and transport affinity during transport of glucose. Furthermore, it was demonstrated for Gln209 and Thr213, both within TM5 of Hxt7, or Leu201 (TM5) of Hxt2 to be of high importance or located at or close to the substrate recognition site (Kasahara and Kasahara 2000a,b, 2003, 2010; Kasahara *et al.* 2004, 2006, 2011). Thus, in conjunction with its substrate promiscuity, one may speculate that the xylose relevant amino acids will be located within a similar region of the galactose permease.

The inefficient and highly unspecific transport of xylose across the plasma membrane in *S. cerevisiae* is a major bottleneck for large-scale industrial production of 2nd generation biofuels. Therefore, in this work we aimed to increase the transport of xylose in Gal2 by performing two rounds of random mutagenesis. We developed a fast and efficient screening assay based on flow cytometry to detect the desired transporter variants. Using this FACS-based method, we screened two mutant libraries with a *S. cerevisiae* hexose transporter deletion strain in which the main seven hexose and the galactose transporter genes (*Δhxt1-7, Δgal2*) were deleted. Of 28 100 single yeast cells in total, we identified five Gal2 variants with an improved xylose affinity of which Gal2 variant 2·1 showed the most significant improvement as compared to the wild-type. With this variant *2·1* we performed a third round of random mutagenesis using a *S. cerevisiae* hexokinase deletion strain, in which the hexokinase genes (*Δhxk1*, *Δhxk2*, *Δglk1*, *Δgal1*) were deleted. Out of a third mutant library of 13 100 single yeast cells in total, five further Gal2 variants with additional amino acid substitutions were identified. The variant 3·1 showed an impaired growth on glucose but not on xylose. In this article, we present *S. cerevisiae* Gal2 mutants with enhanced uptake capacity for xylose and improved sugar metabolism of glucose and xylose.

Materials and methods

Molecular biology techniques and chemicals

Restriction enzymes and T4 DNA ligase were acquired from Thermo Fisher Scientific Biosciences GmbH (St. Leon-Rot, Germany). The antibiotics hygromycin (HG), phleomycin (phleo) and geneticin (G418) were acquired from InvivoGen (Toulouse, France). pYL16 and nourseothricin (nour) were from Werner Bioagents (Jena, Germany), and ampicillin was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Oligonucleotides used for strain construction were purchased from Sigma-

Aldrich. Yeast genomic DNA was isolated using the YeaStar™ Genomic DNA Kit (ZymoResearch, Irvine, CA) following the manufacturer's instructions.

Strains and plasmids

All microbial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* XL1-blue strain was used for site-directed mutagenesis, subcloning and plasmid amplification. The *S. cerevisiae* strains DS68625 and DS69473 (Nijland *et al.* 2014) used in this study (Table 1) were provided by DSM and may be made available for academic research under a strict Material Transfer Agreement with DSM (contact: paul.waal-de@dsm.com). Supporting information on strain construction is found in the supporting information section.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or charactersistics*	Source or reference
Strains		
Saccharomyces cerevisiae		
DS68616	*Mat a, ura3-52, leu2-112, gre3::loxP, loxP-Ptpi:TAL1, loxP-Ptpi::RKI1, loxP-Ptpi-TKL1, loxP-Ptpi-RPE1, delta::Padh1XKS1Tcyc1-LEU2, delta::URA3-Ptpi-xylA-Tcyc1*	DSM, AX Delft, the Netherlands
DS68625	DS68616, *his3::loxP hxt2::loxP-kanMX-loxP, hxt367::loxP-hphMX-loxP, hxt145::loxP-natMX-loxP, gal2::loxP-zeoMX-loxP*	DSM; Nijland *et al.* (2014)
DS69473	DS68616, *his3::loxP, glk1::lox72, hxk1::loxP-hphMX-loxP, hxk2::lox72, gal1::loxP*	DSM; Nijland *et al.* (2014)
Escherichia coli		
XL1-blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*[F′ *proABlacI*qZΔ*M15* Tn*10* (Tet^r)]	Stratagene Corp., Santa Clara, CA
Plasmids		
pRS313	*E. coli*/yeast shuttle vector; *CEN6, ARSH4, HIS3,* Amp^r	Sikorski and Hieter 1989
pUC18	*E. coli* cloning vector; Amp^r	Thermo Scientific Biosciences GmbH
pRS313-GAL2	*GAL2* under control of *S. cerevisiae* truncated *HXT7* (-390) promotor and *HXT7* terminator	J. G. Nijland, Molecular Microbiology, RUG, the Netherlands

*Amp^r, ampicillin resistance; Tet^r, tetracycline resistance.

Media and culture conditions

Strains were pre-cultured in rich growth medium (YP) consisting of 10 g l^{-1} yeast extract (Sigma-Aldrich) and 20 g l^{-1} bactopeptone (BD Biosciences, Breda, the Netherlands) supplemented with either 2% w/v maltose or 2–3% w/v xylose. Yeast transformations were carried out as described by Schiestl and Gietz (1989). All growth experiments with *S. cerevisiae* were performed in 100 ml and 250 ml aerobic shake flasks using selective mineral medium according to Luttik *et al.* (2000) with minor alterations: as nitrogen source urea (2·3 g l^{-1}) was used instead of $(NH_4)_2SO_4$. To compensate for the reduced sulphate level K_2SO_4 (6·6 g l^{-1}) was added. As a carbon source maltose (2% for precultures), glucose or xylose at various concentrations were added. Cultivation was at 30°C with constant shaking at 180 rev min^{-1}.

Escherichia coli cells were grown in Luria–Bertani (LB) medium at 37°C.

Growth assays

Yeast transformants were precultured in 100 ml shake flasks containing 10 ml of mineral medium with 2% maltose. For the growth assays with xylose, 250 ml shake flasks with 50 ml of the same mineral medium supplemented with various concentrations of this sugar (2, 0·45 and 0·1%) were inoculated with the corresponding preculture at an initial optical density (OD) at 600 nm of 0·1. To shorten the typical long lag phase with xylose a small amount of maltose was given to the experiments: 0·05% maltose to growth assays with 2% xylose and 0·005% maltose to growth assays with 0·45% and with 0·1% xylose. Cells were grown at 30°C with constant shaking at 180 rev min^{-1}. All growth rates were estimated by measuring the OD at 600 nm at several time points. All measurements concerning the candidates resulting from random mutagenesis were performed in triplicates unless otherwise stated. The growth experiments for characterizing the Gal2 mutants were carried out in duplicates.

Sugar consumption analysis

The concentrations of sugars were determined by high-performance liquid chromatography (HPLC) (Agilent Technologies, Böblingen, Germany) using an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Munich, Germany). The column was eluted with 5 mmol l^{-1} sulphuric acid as mobile phase with a flow rate of 0·5 ml min^{-1} at 65°C. The run time was 15·5 min and for detection a refractive-index detector was used. All measurements were performed in triplicates.

Random mutagenesis and mutant library construction

Three rounds of error-prone PCR (epPCR) were performed to construct mutant libraries using the plasmid pRS313-GAL2 containing the *GAL2* gene. For the first two rounds of epPCR, the restriction enzyme sites *Bam*HI (between TM6/7) and *Mun*I (between TM10/11) were used to excise the template DNA. Two oligonucleotides, 8881 and 8882 (Table S1, supporting information), were used as the forward and reverse primers, respectively. The epPCR reaction with a total volume of 50 μl was performed following a standard protocol. Prior ligation, the PCR fragments and the pRS313-GAL2 vector were digested with *Bam*HI and *Mun*I, subsequently transformed into the *S. cerevisiae* strain DS68625 and screened for improved uptake of xylose.

In the first round of epPCR, plasmid pRS313-GAL2 was used as the template. The subsequently identified *GAL2* variant *1·1* served as the template for the second round of epPCR. Therefore, all further variants in the next two rounds of epPCR include the alterations in the DNA sequence from the mutant *1·1*. The identified variant *2.1* from the second round of epPCR was chosen as the template for the third round of modification and therefore all further variants of *GAL2* identified from this round include the alterations in the DNA sequence from both the variants *1·1* and *2·1*.

The silent mutation L343L in variant *2·1* which encodes for an additional *Mun*I restriction site was removed and changed back into the wild-type sequence by site-directed mutagenesis (ttg → tta) prior to creation of the third mutant library. Also to expand the range for random mutagenesis between TM3 and TM11, a silent *Eag*I restriction site located within the putative cytoplasmic loop region between TM2 and TM3 was introduced into variant *2·1* via site-directed mutagenesis. The resulting plasmid, pRS313-GAL2-2.1/EagI, was digested with *Eag*I and *Mun*I to excise the DNA for a third round of random mutagenesis using epPCR. The following oligonucleotides 8883 and 8882 were used as the forward and reverse primers respectively (Table S1). The PCR fragments were digested with the appropriate restriction enzymes and substituted for the corresponding region of pRS313-GAL2-2.1/EagI.

High-throughput screening for enhanced xylose uptake

The mutant libraries were transformed into the *S. cerevisiae* transporter deletion strain DS68625 for the first and second round of mutagenesis and into the hexokinase deletion strain DS69473 for the third round of mutagenesis, respectively. The transformed yeast cells of the first two mutant libraries were plated out in 100 μl aliquots on agar plates with mineral medium containing 2% xylose and incubated for 3 days at 30°C. All plates were washed with 5 ml of mineral medium without urea, vitamins and carbon source for 4 min. The cell suspension was then supplemented with the appropriate amount of urea, vitamins and 1% xylose and incubated overnight at 30°C at 180 rev min^{-1}. The cells were then washed twice with mineral medium without urea, vitamins and carbon source. The cells were analysed on a flow cytometer (BD FACSDiVa; Becton Dickinson, Heidelberg, Germany) and budding yeast cells were sorted out on agar plates with exactly 25 ml of mineral medium supplemented with 0·1% xylose as carbon source. Exactly 100 single cells were sorted per agar plate to guarantee the same growth conditions for every single yeast cell. The fastest growth determined by the size of each colony over a period of 2–4 days indicated an enhanced uptake of 0·1% xylose and marked the criteria for evolved uptake. The largest colonies were transferred to new agar plates to isolate the plasmid DNA. The DNA was retransformed into a fresh strain background for validation and further characterization.

The third screening round of random mutagenesis was carried out in the *S. cerevisiae* hexokinase deletion strain DS69473. The mutant library was transformed and plated on agar plates with mineral medium containing 3% xylose. The same selection procedure as described above was used, in total 131 plates (30 ml mineral medium supplemented with 2% xylose and 10% glucose) were analysed. After 12 days, eight colonies were transferred to fresh agar plates with 3% xylose for isolation of plasmid DNA. Subsequently, the plasmid DNA was transformed into the *S. cerevisiae* transporter deletion strain DS68625 and analysed for growth in liquid mineral medium under different conditions. The identified mutants were DNA sequenced and termed *1·1* (1st-round mutant), *2·1–2·4* (2nd-round mutants), and *3·1–3·7* (3rd-round mutants) respectively (Table 2).

Results

Screening assay

To enhance the transport of xylose in *S. cerevisiae* we aimed to improve the xylose affinity of Gal2 by random mutagenesis. For screening such a random mutant library, a reliable, fast and efficient screening assay using agar plates was developed to detect the transporter variants showing enhanced affinity or uptake capacity of xylose. The wild-type Gal2 transporter was transformed into the *S. cerevisiae* strain DS68625, in which the main hexose transporters (Hxt1-7, Gal2) are deleted. Based on this genetic background, this strain is not able to grow

Table 2 Generated *GAL2* variants from three rounds of random mutagenesis

Designation	Mutations	Round
1·1	L311R (L362L, D363D, K469K)	1st round
Variant *1·1* was used as the template for 2nd round of random mutagenesis (further variants include these mutations)		
2·1	L301R, K310R, N314D, M435T (L343L)	2nd round
2·2	M435T, S468T (K394K)	2nd round
2·3	L301R, K310R, N314D, Q425R, S427P, M435T (L343L, I356I)	2nd round
2·4	M435T (Q329Q, Q338Q, C434C)	2nd round
Variant *2·1* was used as template for 3rd round of random mutagenesis (further variants include these mutations except L343L)		
3·1	T386A	3rd round
3·2	F444L (G151G, I256I)	3rd round
3·3	(S285S, E367E)	3rd round
3·4	(D364D)	3rd round
3·5	Y176H	3rd round
3·6	Y226C, L280P (I165I, I178I, A250A)	3rd round
3·7	M339V, L464Q (Y446Y)	3rd round

[0]Silent mutations.

on xylose and growth on glucose is substantially hampered (Nijland *et al.* 2014). After plating the transformants on agar plates with 2% xylose, the plates were incubated for 3 days to enable xylose sugar metabolism. By flooding the plates with mineral medium, a cell suspension was prepared and incubated with 1% xylose overnight. Subsequently, the cell suspension was analysed on a flow cytometer (FACS) to identify the desired yeast cells: since growth includes sugar uptake as well as sugar metabolism, we analysed for budding yeast cells only. On the one hand, these cells are able to grow and multiply themselves and therefore transport xylose. On the other hand, by choosing budding yeast cells only, all yeast cells are in the same growth state. Based on these criteria, the budding yeast cells were sorted on agar plates with either 2, 1 and 0·1% xylose to test for the lowest xylose concentration possible at which growth could be still determined by colony size. Exactly 100 single cells were sorted per agar plate (Fig. 1a) with exactly the same volume of mineral medium to guarantee the exact same selection pressure and growth conditions for every yeast cell. Over a period of up to 3 days, we observed growth for each of the tested xylose concentrations. At the lowest concentration of 0·1% xylose the yeast colonies showed the smallest colonies as compared to 2 and 1% xylose, but nevertheless significant growth. Using this FACS-based screening assay the xylose concentration of 0·1% was chosen to screen a Gal2 random mutant library for transporter variants with improved affinity towards xylose.

Directed evolution of Gal2 to increase its affinity for xylose

First round of random mutagenesis

A distinct part of the transporter protein Gal2 was selected for mutagenesis, between amino acid positions Asp292 and Ser477. This segment, which contains 185 amino acids, includes a large part of the proposed central cytoplasmic loop between the transmembrane (TM) segments 6 and 7, TM7 to TM10, and the corresponding cytoplasmic and extracellular loops (see Fig. 1b). This part of the Gal2 transport protein was chosen based on relevant amino acids concerning hexose sugar transport identified in related hexose transporters in *S. cerevisiae* (Kasahara and Kasahara 2000a,b, 2003, 2010; Kasahara *et al.* 2004, 2007, 2011).

After creating a random mutant library with mutagenic parameters to obtain approximately up to four point mutations within the selected segment, which was confirmed by sequencing 10 randomly selected PCR fragments, the library was transformed into the *S. cerevisiae* transporter deletion strain DS68625 (Δ*hxt1-7*, Δ*gal2*). In addition, this strain has been engineered for xylose utilization by introduction of the xylose isomerase gene from the anaerobic fungus *Piromyces* sp. E2, overexpression of *XKS1* and four genes involved in the nonoxidative pentose phosphate pathway (Kuyper *et al.* 2005) and deletion of *GRE3*. Applying the developed FACS-based high-throughput screening assay as described in the method section budding yeast cells were sorted in total on 137 agar plates with exactly 25 ml of mineral medium containing 0·1% xylose as carbon source. In this 1st screening round, after 3 days of incubation the 80 biggest colonies were picked and transferred to fresh agar plates containing the exact same medium with 0·1% xylose. After another 4 days at 30°C, nine colonies which were significantly larger than the background colonies were tested in liquid mineral medium with 2% xylose as sole carbon source. For validation of the growth results the plasmid DNA from promising mutants was isolated and retransformed into the *S. cerevisiae* deletion strain DS68625. Of these nine candidates, one candidate, designated as variant 1·1, showed a faster growth as compared to the wild-type Gal2 permease (Fig. 2). DNA sequencing of this variant revealed a single amino acid substitution at position 311 where a leucine, located within the cytoplasmic loop region between TM6/7, was changed into an arginine. Additionally, three silent mutations were identified (L362L, D363D, K469K) (Table 2).

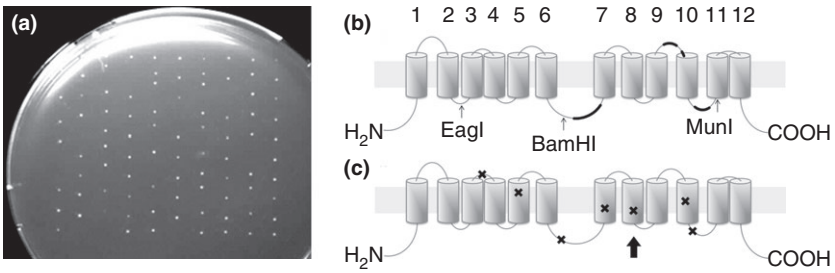

Figure 1 *Saccharomyces cerevisiae* mutant library screening agar plate after 3 days of incubation with mineral medium containing 0·1% xylose (a). After flow cytometry analysis, budding yeast cells were sorted 10 by 10 on each agar plate. Bigger colonies show faster growth according to quicker xylose transport and consumption as compared to the surrounding smaller colonies. Empty spaces in-between show yeast cells which did not grow during the time of 3 days. (b) and (c): Gal2 wild-type transporter protein topology model predicted by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/; 6/2013). The cytoplasm is located at the bottom of each model, the extracytoplasm is on top and the numbers depict the transmembrane segments. For the first two rounds of epPCR, the restriction enzyme sites *Bam*HI and *Mun*I were used to excise the template DNA. *Eag*I and *Mun*I were used to excise the template DNA for the third round of epPCR. The highlighted black areas indicate the identified amino acid substitutions after two rounds of random mutagenesis (b). The black crosses indicate the identified alterations in the amino acid sequence after the third round of random mutagenesis. The black arrow shows the mutation in TM8 in candidate 3·1 that led to a significantly impaired uptake of glucose but not of xylose (c).

Second round of random mutagenesis

Using this variant 1·1 as a template, a 2nd round of random mutagenesis was performed using the same parameters on a similar segment of the transporter protein. Here, 14 400 yeast cells harbouring the new mutant library were sorted out on agar plates containing mineral medium with 0·1% xylose as sole carbon source. Four second round mutants were identified (*2·1–2·4*) and sequenced. DNA sequencing of the mutant *2·1* revealed beside the mutations in variant *1·1*, three new amino acid substitutions (L301R, K310R, N314D) within the central cytoplasmic loop between TM6/7 and an additional substitution at position 435 where a methionine was changed to a threonine. This M435T mutation was also identified in all the other 2nd round mutants (*2·2*, *2·3*, and *2·4*) (Table 2). After retransformation into a fresh strain background all the candidates were tested in liquid mineral medium at two different xylose concentrations for detailed characterization of the mutants: 0·45% for improved affinity and 0·1% for high affinity (Fig. 3a and b). Using HPLC analysis sugar utilization was measured over time (Fig. 3c and d).

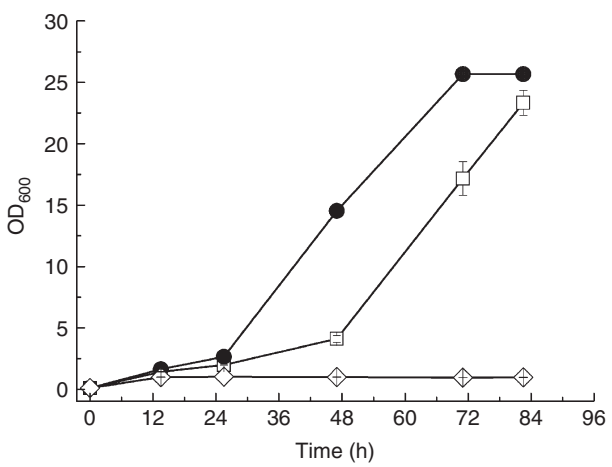

Figure 2 Comparison of the Δ*hxt1-7, Δgal2* strain DS68625 expressing wild-type *GAL2* (□) and variant *1·1* (●) from the first round of mutagenesis in mineral medium with 2% xylose as carbon source. DS68625 (◊) expressing an empty vector was included as control. The error bars represent standard deviations calculated from three independent experiments.

Compared to the wild-type and the mutant 1·1 identified from the first round of mutagenesis all mutants from the second round of mutagenesis (2·1–2·4) showed an improved growth on 0·45% xylose (Fig. 3a). After 62 h no xylose was detectable by HPLC for the mutant 2·1 and <0·03% for the variants 2·2 and 2·3 (Fig. 3c). At the same time point, 0·35% xylose and 0·42% xylose for the variant 1·1 and the wild-type Gal2 was present in the remaining medium respectively. Experiments with 0·1% xylose revealed variant 2·1 as a potential high-affinity transporter variant (Fig. 3b). 2·1 showed the fastest growth among all the tested variants: only 0·02% xylose was present in the remaining medium after 114 h of incubation, whereas variant 1·1 and the wild-type transporter showed no growth or consumption at this xylose concentration (Fig. 3d). The *S. cerevisiae* deletion strain DS68625 bearing the empty vector (pRS313) was included as a control in the experiments and showed neither growth nor consumption of xylose.

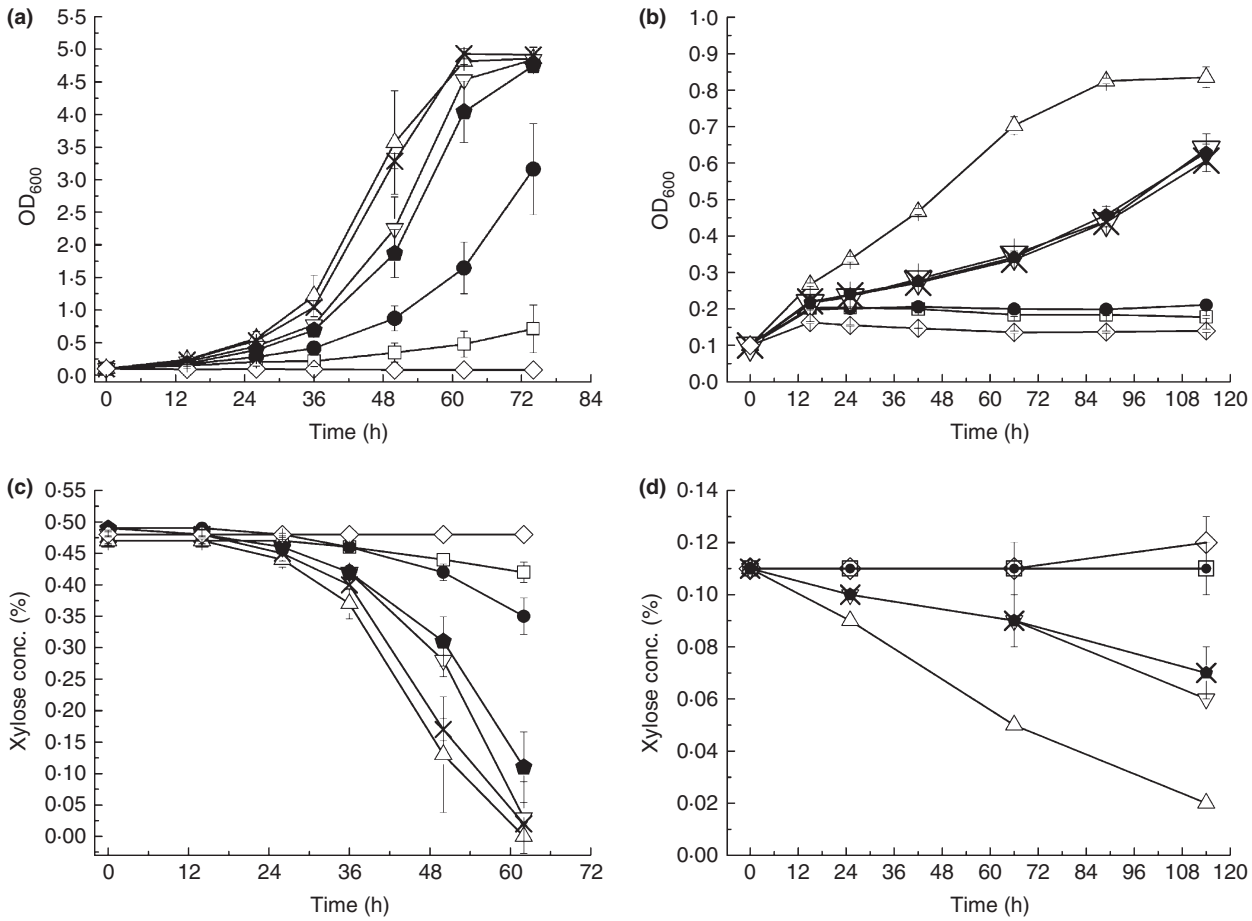

Figure 3 Comparison of growth (a and b) and consumption (c and d) of Gal2 (wild-type) and identified mutants after two rounds of random mutagenesis at low xylose concentrations. Strain DS68625 expressing wild-type *GAL2* (□) and the variants *1·1* (●), *2·1* (△), *2·2* (▽), *2·3* (×) and *2·4* (⬟) in mineral medium with 0·45% (a and c) or 0·1% (b and d) xylose as carbon source. DS68625 (◇) expressing an empty vector was included as a control. The error bars represent standard deviations calculated from three independent experiments.

Directed evolution of Gal2 to improve the xylose uptake in the presence of glucose

Third round of random mutagenesis

The screening conditions with concentrations of 0·1% xylose led to significantly increased affinity towards xylose due to alterations within the different loop regions of Gal2 (Fig. 1b). In the subsequent screening of the mutant library, the conditions were changed to improve the uptake of xylose in the presence of glucose. Due to the diauxic growth behaviour of DS68625, in which glucose is first consumed, the hexokinase deletion strain DS69473 (Δ*hxk1*, Δ *hxk2*, Δ*glk1*, Δ*gal1*) was used for transformation of the mutant library (Nijland *et al.* 2014). Growth of glucose as the sole carbon source is completely abolished in this strain. However, glucose transport is intact. Therefore, the strain DS69473 is an ideal host for screening of a xylose-transport enhanced Gal2-variant competitive to glucose transport.

To create a new mutant library to screen for improved xylose uptake in the presence of glucose the best candidate of the 2nd round of mutagenesis, *2·1*, was chosen as the template.

The new mutant library was transformed into the hexokinase deletion strain DS69473. In total 131 agar plates containing 2% xylose and 10% glucose as carbon sources with single budding yeast cells were incubated at 30°C. After 12 days of incubation at 30°C eight colonies could be identified on all the plates. Out of these eight candidates, DNA sequencing revealed five new *GAL2* mutants with amino acid substitutions. Two candidates were also found with silent mutations and one candidate was found twice (Table 2). From the 3rd round mutants the plasmid DNA harbouring the amino acid substitutions from the five *GAL2* mutants *3·1*, *3·2*, *3·5*, *3·6* and

3·7 was isolated and transformed into the transporter deletion strain DS68625 to characterize each mutant more in detail concerning growth on 2% glucose and 2% xylose (Fig. 4a and b).

At only 2% glucose, the 2nd round mutant 2·1 which was included as a control showed a significant decreased growth compared to that of the wild-type Gal2 (Fig. 4a). Interestingly, the 3rd round mutant 3·7 which was developed from mutant 2·1 displayed an almost identical growth as the wild-type Gal2. Also mutant 3·2 showed an increased growth at this glucose concentration as compared to 2·1 but not as fast as 3·7 or the wild-type. Furthermore, the mutants 3·1, 3·5 and 3·6 revealed a strong impaired growth on 2% glucose up to 48 h. At 2% xylose the mutant 2·1 displayed the fastest growth among all the tested candidates and the wild-type (Fig. 4b). The mutants 3·2 and 3·7 showed a slightly reduced growth than 2·1, but a clearly faster growth than the wild-type. Mutant 3·1 which showed no growth at 2% glucose for the first 48 h grew almost as fast as 3·2 and 3·7 at 2% xylose. Surprisingly, the variants 3·5 and 3·6 showed no growth at 2% xylose over the tested period of time.

Improved xylose uptake in the presence of glucose

To facilitate simultaneous consumption of glucose and xylose the two mutants 2·1 and 3·1 were evaluated. After retransformation into DS68625 these two evolved mutants were analysed for growth at 2% xylose in the presence of 2% glucose (ratio 1 : 1) to test for improved xylose consumption at higher sugar concentrations. In parallel, the mutants were also tested at a lower xylose and glucose concentration, i.e. 0·45% xylose/glucose. Besides measuring the optical density at 600 nm the consumption of both sugars was analysed by HPLC by measuring the remaining sugar concentration in the medium over time (Fig. 5a-d).

The diauxic sugar consumption profile, in which glucose is consumed first, was detectable for both the Gal2 wild-type and mutant 2·1 under the growth conditions of 2% xylose with 2% glucose. The variant 2·1 showed a faster growth than the Gal2 wild-type. This was confirmed by HPLC analysis which revealed a faster consumption of both sugars for this mutant (compare Fig. 5a and c). In medium containing 0·45% xylose and 0·45% glucose, mutant 2·1 showed also a faster growth than the wild-type (Fig. 5b). In Fig. 5d mutant 2·1 showed an almost simultaneous consumption of xylose and glucose, as compared to the wild-type Gal2. After 35 h of incubation there was no glucose and only 0·07% xylose left for the mutant 2·1 but still 0·04% glucose and 0·38% xylose present with the wild-type Gal2. The variant 3·1 showed neither consumption nor growth over the tested period of time.

Relevant amino acid for sugar transport in Gal2

We further investigated mutant 3·1 in more detail, whether the mutation T386A might be involved in the transport of glucose in Gal2, as this mutant showed an impaired growth at 2% glucose but not at 2% xylose (Fig. 4a and b). Threonine at amino acid position 386 was substituted by alanine in the wild-type protein by site-directed mutagenesis. Subsequently, after transformation into the transporter deletion strain DS68625 and growth at 2% glucose this new Gal2 variant Gal2_T386A showed a slower lag phase than the wild-type but a

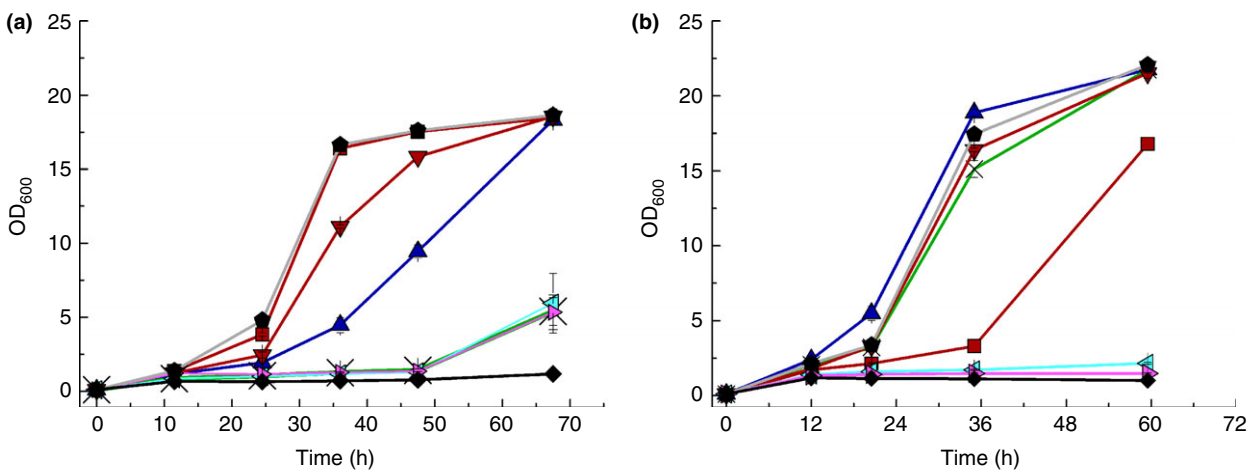

Figure 4 Comparison of growth of Gal2 variants after three rounds of directed evolution. DS68625 expressing wild-type *GAL2* (□) and the variants with amino acid substitutions *2·1* (△), *3·1* (×), *3·2* (▽), *3·5* (◁), *3·6* (▷) and *3·7* (⬟) in mineral medium with 2% glucose (a) or 2% xylose (b) as sole carbon source. DS68625 (♦) expressing the empty vector was included as control. The error bars represent standard deviations calculated from two independent experiments.

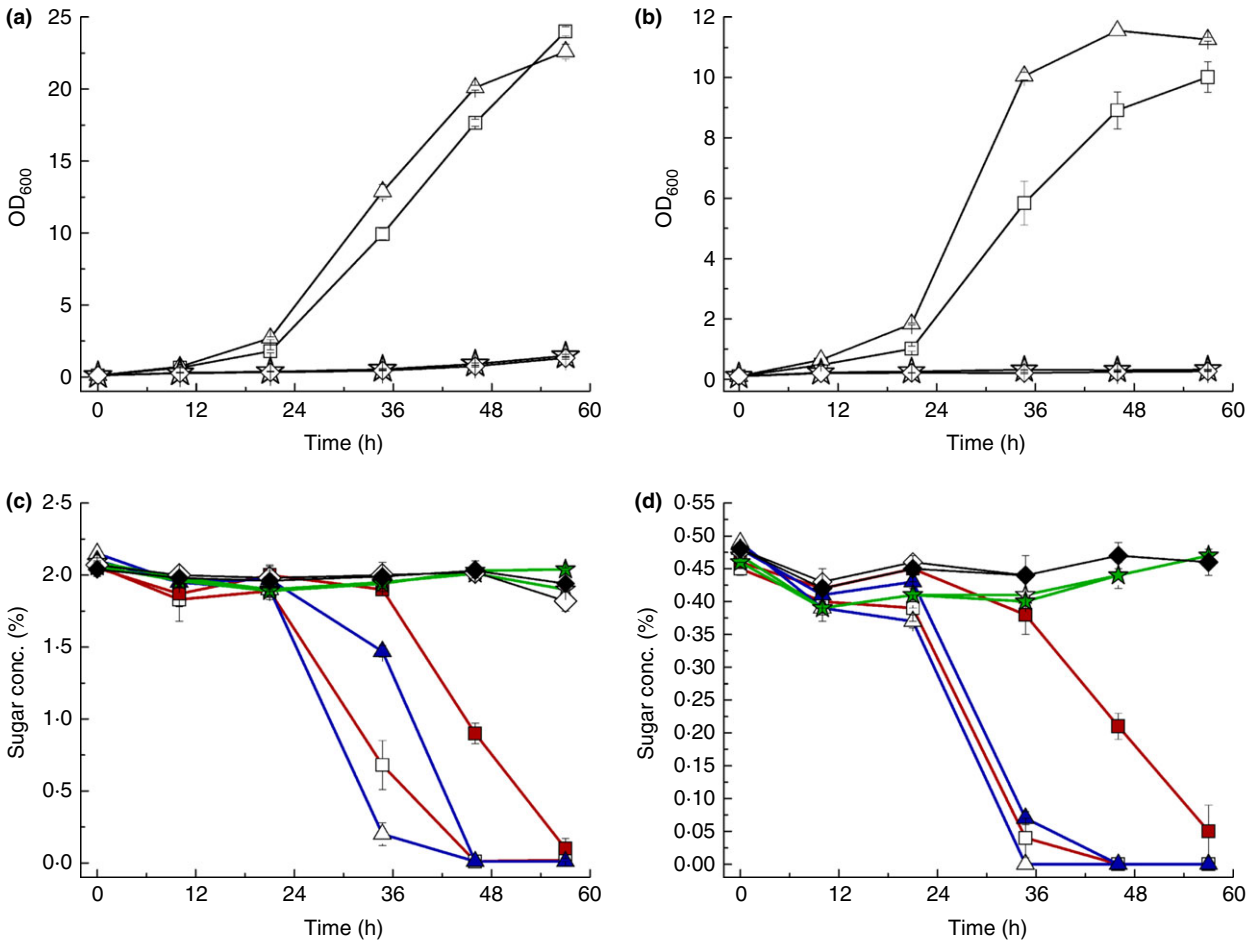

Figure 5 Growth and consumption measurements with xylose in the presence of glucose. (a) shows growth in mineral medium with 2% xylose and 2% glucose, (b) shows growth in mineral medium with 0·45% xylose and 0·45% glucose: wild-type Gal2 (□), mutants 2·1 (△) and 3·1 (☆), DS68625 (◇). (c) shows consumption measurements for 2% xylose with 2% glucose and (d) for 0·45% xylose with 0·45% glucose respectively: wild-type Gal2 with glucose (empty □), wild-type Gal2 with xylose (filled ■), mutants 2·1 with glucose (empty △), 2·1 with xylose (filled ▲), 3·1 with glucose (empty ☆), 3·1 with xylose (filled ★), DS68625 with glucose (empty ◇), DS68625 with xylose (filled ♦). Gal2 and mutants were expressed in the deletion strain DS68625. DS68625 expressing the empty vector was included as control. The error bars represent standard deviations calculated from three independent experiments. Empty symbols: glucose; filled symbols: xylose.

clearly faster growth than mutant 2·1 (Fig. 6a). The results were similar for growth at 0·1% glucose (Fig. 6c). Mutant 3·1 which was again included as a control in these experiments, showed no growth on either 2% glucose or 0·1% glucose and confirmed our previous results of decreased growth on glucose (Fig. 6a and c). At 2% xylose the new variant Gal2_T386A showed a faster growth than the wild-type Gal2 but significantly slower growth than the mutants 2·1 and 3·1 (Fig. 6b). At 0·1% xylose, both Gal2_T386A and wild-type Gal2 did not grow (Fig. 6d), whereas the mutants 3·1 and in particular 2·1 showed growth. These results suggests that the mutation T386A alone is not crucial but in combination with the mutations identified in variant 2·1, it plays a critical role in glucose transport.

Discussion

Ethanol production from lignocellulosic biomass with *S. cerevisiae* is a promising technology for the additional supply of energy from renewable and nonfood resources. Competitive inhibition by glucose during co-fermentation and highly insufficient transport rates for pentoses, with xylose in particular, still remain major hurdles. In order to increase the transport activity for xylose it is important to increase the transport affinity which is orders of magnitude lower than for glucose.

Lacking structural and specific functional information on Gal2 we generated various *GAL2* variants by error-prone PCR, a well-established random mutation procedure. In our experiments, we observed no growth for the

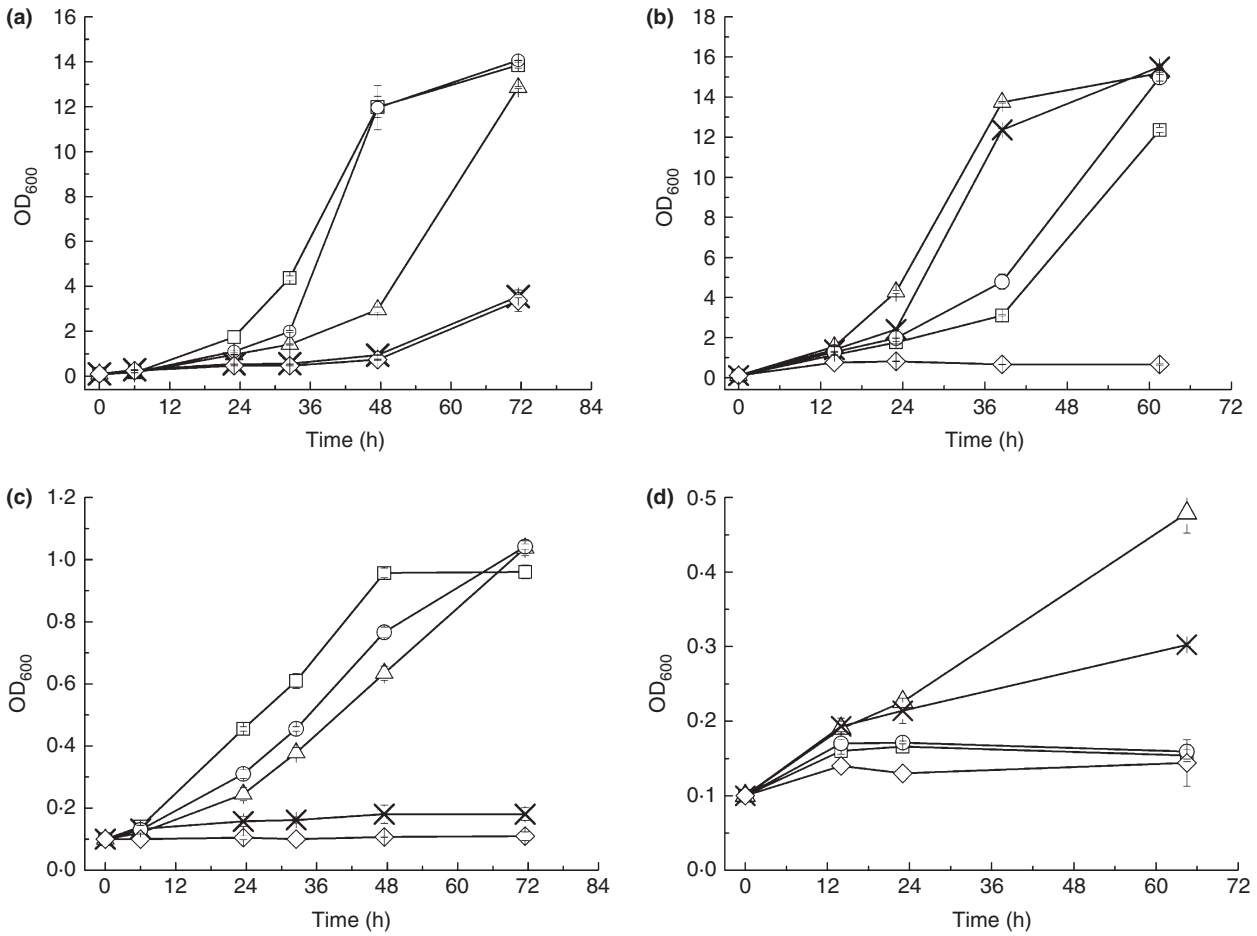

Figure 6 Growth of wild-type Gal2 in comparison to mutants 2·1, 3·1 and Gal2_T386A. DS68625 expressing wild-type *GAL2* (□) and mutants *2·1* (△), *3·1* (×) and *GAL2_T386A* (○) in mineral medium with 2% glucose (a), 2% xylose (b), 0·1% glucose (c) and 0·1% xylose (d) as carbon sources. DS68625 (◊) expressing the empty vector was included as control. The error bars represent standard deviations calculated from two independent experiments.

wild-type Gal2 transporter in the deletion strain DS68625 at xylose concentrations of 0·1% for at least 2 days. Therefore, using the transporter deletion strain DS68625 in which the eight main hexose transporters Hxt1-7 and Gal2 are deleted we screened two mutant libraries at this low xylose concentration and obtained different Gal2 variants with improved affinities towards xylose. The increased xylose affinity was found to be due to alterations of amino acids within diverse loop regions of Gal2. Of all the identified Gal2 variants from the first two mutant libraries, variant 2·1 showed the most significant improvement (Fig. 3). Given that wild-type Gal2 is a high-affinity glucose transporter, after characterization of variant 2·1, several properties became apparent. First, similar to the other variants from the first two screening rounds the variant 2·1 showed no mutations within the TM segments but within the large loop region 6/7 (L301R, K310R, N314D) and one mutation at the interface to loop 9/10 (M435T) (Fig. 1b). Considering that substrate binding is likely to be located in the central cavity of the transporter protein as shown recently from the crystal structure of XylE (Sun *et al.* 2012) or for LacY (Abramson *et al.* 2003), the mutations from 2·1 are probably not directly involved in substrate translocation. Although XylE and LacY are from *E. coli* they both are, as Gal2, members of the sugar porter (SP) family, representing the biggest subfamily within the MFS. Second, besides an improved xylose acceptance by variant 2·1, the same mutations cause the glucose transport activity to decrease at high (2%) and low (0·1%) concentrations of glucose indicating that these mutations decrease the glucose specificity in Gal2 (Fig. 4a and 6a,c). Third and most striking, when exposed to xylose and glucose simultaneously variant 2·1 showed an improved sugar metabolism of both sugars at a concentration of 0·45 and 2% each, resulting in faster growth than the wild-type

(Fig. 5). These observations of variant 2·1 indicate that the loop regions in Gal2, if not necessarily directly involved in sugar transport, to be of relevance for substrate acceptance. Furthermore, the mutations from variant 2·1 are of benefit for the co-transport of xylose with glucose as sole carbon sources.

In the co-consumption experiments, we observed the diauxic growth phenomenon for wild-type Gal2 (Fig. 5c, d), where glucose as preferred carbon source is consumed first and impairs the uptake of the alternative carbon source. In the same experiments at 4% sugar concentration (2% xylose with 2% glucose) variant 2·1 displayed both a faster glucose and xylose consumption as compared to the wild-type resulting in a compressed diauxic shift. Although this variant showed a slower growth on glucose only than the wild-type (Fig. 4a), this confirms the mutations of variant 2·1 to be beneficial for growth on glucose and xylose simultaneously.

During co-consumption with 0·9% sugar (0·45% xylose with 0·45% glucose), we observed with variant 2·1 an almost simultaneous consumption of glucose and xylose resulting in a clearly faster growth as compared to the wild-type Gal2. The reduced glucose specificity and improved xylose affinity based on the mutations in variant 2·1 lead to less inhibited xylose uptake in the presence of glucose. These findings correspond to published data from Subtil and Boles (2012), where the inhibitory effect by glucose on the simultaneous uptake of xylose was shown to be the main competitive step in co-consumption.

Furthermore, three mutations from variant 2·1 (L301R, K310R, N314D) were found to be located within the large central loop 6/7 of Gal2. Regarding a proper folding of the TMs within the membrane, a structural relevance of the central loop 6/7 could be demonstrated for GLUT1, a related mammalian glucose transporter and SP family member (Monden *et al.* 2001). The crystal structure of this glucose transporter was published very recently (Deng *et al.* 2014). Evident from this structure, the large central loop 6/7 in GLUT1 is forming an intracellular helical bundle of four short α-helices. Deng and co-workers showed this helical bundle to function as a latch to secure closure of the intracellular gate in the outward-facing conformation. Noteworthy, this helical bundle is present also in the crystal structures of the bacterial transporter proteins XylE from *E. coli* and GlcP from *Staphylococcus epidermidis* (Iancu *et al.* 2013) suggesting its presence also in many other SP-members such as Gal2, within the MFS superfamily. To date, the role or arrangement of the large central loop 6/7 in Gal2 or in the different Hxts from *S. cerevisiae* has not yet been described.

Using the *GAL2* variant *2·1* as a template to create a third mutant library, we focused to enhance the xylose uptake of the Gal2 transporter in the presence of glucose. Therefore, we screened for variants able to grow on 2% xylose in the presence of 10% glucose. By using a hexokinase deletion strain which is unable to metabolize hexose sugar but has wild-type transport abilities, five further Gal2 variants were identified exhibiting seven new amino acid substitutions (Table 2). Of these seven mutations, four (Y226C, M339V, T386A, F444L) were located within the TM segments 5, 7, 8 and 10 (Fig. 1c). These correspond to the TM segments shown by Kasahara *et al.* (2011) to surround the substrate pathway in Hxt7. Furthermore, in Hxt2 these TMs were shown to be important for glucose high-affinity transport (Kasahara and Kasahara 2003). Considering the high amino acid sequence identity between Gal2 and Hxt7 of approx. 71% and Gal2 and Hxt2 of approx. 61% (*Saccharomyces* Genome Database, http://www.yeastgenome.org) it is quite possible that similar TMs are also important for substrate transport in Gal2.

From the third round mutants, Gal2 variant 3·1 revealed a strong impaired growth on glucose, whereas growth on xylose was not affected (Fig. 4). Since variant 3·1 is directly developed from variant 2·1 and the mutation at position 386 (TM8) is the only difference on the protein level between these two Gal2 variants, this suggests that the mutation T386A from variant 3·1 may be involved in glucose transport but not in xylose transport. Moreover, in co-consumption experiments involving stoichiometric mixtures of xylose and glucose at high (4%) and low (0·9%) concentrations, the growth of variant 3·1 was strongly impaired and no growth or sugar metabolism was detectable (Fig. 5). Correlating with the observed inhibited growth on glucose only (Fig. 4), the mutations in variant 3·1 also inhibited xylose transport when both sugars were present.

In Gal2, threonine 386 corresponds to threonine 371 of Hxt2, both located in TM8. Investigations of TM8 in Hxt2 showed that this TM segment harbours several amino acids which are important or supportive for high-affinity glucose uptake (Kasahara *et al.* 2004, 2006). This was also shown recently by Boles and co-workers in Gal2. Different mutations at arginine 376 within TM8 of Gal2 were found to diminish the inhibition by glucose (Farwick *et al.* 2014). The authors showed that among their mutants N376F had the highest affinity for xylose with a moderate transport velocity.

Despite the significant effect of T386A in variant 3·1 in glucose transport during co-consumption with xylose, only a minor effect on the growth behaviour could be observed for glucose and xylose when this position was changed to alanine in the wild-type Gal2 transporter. Possibly, the blocked glucose transport is due to a combination of more than one amino acid alteration in 3·1 indicating a necessity to change multiple amino acids in

Gal2 in order to modify its substrate spectrum to accept xylose for co-consumption of both sugars.

Summarizing, our data show that random mutagenesis is a powerful tool to evolve sugar transporters like Gal2 towards co-consumption of new substrates. Using a high-throughput screening system based on flow-through cytometry we identified various mutants with improved xylose-transport characteristics pointing at a critical role of the loop regions in Gal2 in substrate recognition as well as an important role of threonine 386 and the TM8 in transport. The investigated Gal2 variants in this work are a promising starting point for further engineering to improve xylose uptake from mixed sugars in biomass.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers used in this study for mutagenesis and *S. cerevisiae* strain construction.

Supporting information

Materials and Methods

Hexose transporter deletion strain construction

The eight major *HXT* (*HXT1-7* and *GAL2;* Reifenberger *et al*. 1997) genes were deleted to generate a xylose transport-negative model strain for screening of putative xylose transport properties of *GAL2* variants. *S. cerevisiae* strain DS68616 equipped with DSM proprietary xylose fermentation technology (based on Kuyper *et al.* 2004; 2005) was used for the following genetic modifications. Genes were deleted using standard methods to disrupt the target locus by integrating a floxed dominant resistance marker; markers were surrounded by *loxP* sites to allow for CRE-meditated marker removal and recycling (Güldener *et al*. 1996). Locus-specific flanks were 400-700 bp large and PCR-amplified using the primers listed in Table S1. Up- and downstream flanks were fused to the floxed dominant resistance markers by standard cloning methods, and re-amplified by using the forward primer of the upstream flank and the reverse primer of the downstream flank.

First, the *HIS3* gene was deleted to serve as a marker for the introduction of putative enhanced xylose transporter(s) (libraries) as described below. The histidine auxotroph DS68616 was generated by the insertion of *loxP-kanMX-loxP* at the *HIS3* locus. Subsequently, the marker was removed by CRE-mediated marker removal (Güldener *et al.* 1996). For the deletion of the eight main hexose transporters (*HXT1-7* and *GAL2*) in *S. cerevisiae,* four deletion constructs were generated bearing either the *kanMX*, *zeoMX*, *hphMX* or *natMX* markers (Steensma and Ter Linde 2001; Güldener *et al.* 2002; Hansen *et al.* 2003). The hexose transporters were deleted in the following order: 1) *HXT3-HXT6-HXT7* cluster, 2) *HXT5-HXT1-HXT4* cluster, 3) *GAL2* and 4) *HXT2*. All transformations were plated on yeast extract (10 g l^{-1}), peptone (20 g l^{-1}) agar (15 g l^{-1}) medium supplemented with 20 g l^{-1} maltose. Maltose was added to the medium, because the uptake of this disaccharide uses an alternative

transport system other than the glucose transport systems (Wieczorke *et al.* 1999). With each deletion of a (cluster of) *HXT* gene(s), an additional marker was inserted in the order: 1) *hphMX*, 2) *natMX*, 3) *zeoMX* and 4) *kanMX*. With each inserted additional marker the respective antibiotic was supplemented to the medium in the following order: 1) HG, 2) HG and nour, 3) HG, nour and phleo, 4) HG, nour, phleo and G418. After integration of all four deletion constructs, a single colony was isolated under selection of all four antibiotics. The resulting strain, DS68625, did not grow on mineral medium (Luttik *et al.* 2000) with 2% xylose, and showed delayed growth with 2% glucose (data not shown). Correct integrations were verified by PCR analysis on genomic DNA isolates.

Hexokinase deletion strain construction

A hexokinase deletion strain was constructed to allow screening for Gal2 variants which transport xylose in the presence of glucose. For deletion of hexokinase genes, PCR primers were designed (647-654, listed in Table S1) that comprised of 60 nucleotide flanking sequences homologous to a hexokinase gene locus, and 20 nucleotides homologous to a floxed dominant resistance marker cassette (similar as described above except that *kanMX* was surrounded by *lox66* and *lox71*). PCR products were column filter-purified (GeneJet Kit, Thermo Fisher Scientific, Landsmeer, The Netherlands) prior to transformation experiments.

Firstly, to enable selection for transporter constructs the *HIS3* marker was used similar as in the Δ*hxt*1-7; Δ*gal2* strain. The histidine-auxotroph strain (DS68616-*his3*::*loxP*) was the same strain, as was constructed in the DS68625 lineage. For the generation of a strain incapable of hexose metabolism but capable of hexose transport, four hexokinase gene deletions were made in the xylose-fermenting strain DS68616. The hexokinase genes were deleted in the following order: 1) *GLK1*, 2) *HXK1*, 3) *HXK2*, 4) *GAL1*, integrating floxed *kanMX*, *natMX*, *kanMX* and *hphMX*, respectively. After the deletion of *GLK1*, the *kanMX* marker was recycled by CRE-mediated marker removal. After deletion of *HXK2*, the intermediate strain

was maintained on xylose-containing rich medium with 10 g l^{-1} yeast extract, 20 g l^{-1} peptone (YP) and 20 g l^{-1} xylose. After *GAL1* deletion, again, the integrated markers were removed by galactose-induced CRE-*lox* recombination. To ensure growth of the strain (since *GAL1* coding for galactokinase was deleted), 2% xylose was added to YP medium supplemented with 2% galactose and hygromycin. Selection on hygromycin ensured maintenance of the *hphMX* marker at the *GAL1* locus leaving a selection trait to be used possibly later on. After single colony isolation, the strain was verified for its deletions and by colony PCR, and designated as DS69473. The final quadruple hexokinase mutant DS69473 was screened for growth on mineral medium supplemented with 2% glucose or xylose. DS69473 did not grow on glucose, but did grow on xylose (data not shown). DS69473 was further maintained on YPX for storage and handling.

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93 **Table S1.** Primers used in this study for mutagenesis and *S. cerevisiae* strain construction

Number	Primer	Sequence (5′- 3′)	Gene(s)
28	H3f	TGTACATCCGGAATTCTAGATTGGTGAGCGCTAGGAGTCACTGCC	*HIS3*
29	H3r	CTCGAGTATTTCACACCGCATATGATCCGTCG	*HIS3*
115	Natf	ACATGTAAAATGACCACTCTTGACGACACGGC	*NAT1*
116	Natr	CAGTACTAGGGGCCAGGGCATGCTC	*NAT1*
201	Hx2uf	GACTAGTACCGGTGTTTTCAAAACCTAGCAACCCC	*HXT2*
202	Hx2ur	CTCGAGTATTTCACACCGCATATGATCCGTCG	*HXT2*
203	Hx2df	ACATGTAAAATGACCACTCTTGACGACACGGC	*HXT2*
204	Hx2dr	GTCGACGGGCCCTTATGTTGGTCTTGTTTAGTATGGCCG	*HXT2*
205	Hx3uf	AAGCGGCCGCACTAGTACCGGTGAAACAACTCAATAACGATGTGGGAC	*HXT3*
206	Hx3ur	ATCCGGACGTCTTCCTCAAGAAATCAGTTTGGGCGACG	*HXT3*
210	Hx4df	AGAAGACGCTCGAGACGTCCCTTATGGGAAGAAGGTGTTTTGCC	*HXT4*
211	Hx4dr	ATGGATCCTAGGGGTTCTTGCAGAGTAAACTGCG	*HXT4*
212	Hx5uf	AAGCGGCCGCACTAGTACATGTGAACTTGAAAACGCTCATCAAGGC	*HXT5*
213	Hx5ur	TTCGTACGCGTCTTCCGGAGTAACATGAAACCAGAGTACCACG	*HXT5*
229	Hx7df	AGAAGACCCTCGAGACGTCCGACGCTGAAGAAATGACTCACG	*HXT7*
230	Hx7dr	AGTCGACGGATCCGTAATTTTTCTTCTTTTAAGTGACGGGCG	*HXT7*
233	Gal2df	AGAAGACCCTCGAGACGTCTTACCTTGGAAATCTGAAGGCTGG	*GAL2*
234	Gal2dr	GTGGATCCTAGGTAAAACGGTACGAGAAAAGCTCCG	*GAL2*
243	Gal2ufn	AAGCGGCCGCACTAGTACCGGTGATCTATATTCGAAAGGGGCGG	*GAL2*
244	Gal2urn	AACGTACGTCCGGATCATTAGAATACTTTTGAGATTGTGCGCT	*GAL2*
647	Hxk1loxf	TCGGTTTCACTTCCTTGGGAATATTCTACCGTTCCTTCATCTTGTATTCCGG ATCCACTAGCATAACTTCG	*HXK1*
648	Hxk1loxr	GACAATGCAGCAATAACAGCAGCACCTGCACCTGAACCATCCTCAGCTTTG GGCCGCCAGTGTGATGG	*HXK1*
649	Gal1loxf	TGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCG GATCCACTAGCATAACTTCG	*GAL1*
650	Gal1loxr	AGGTATCCAAAACGCAGCGGTTGAAAGCATATCAAGAATTTTGTCCCTGTT TGGGCCGCCAGTGTGATGG	*GAL1*
651	Hxk2loxf	CCACGAAATTACCTCCTGCTGAGGCGAGCTTGCAAATATCGTGTCCAATTC CGTGATGTCTCGACCTCGAGTACCGTTCG	*HXK2*
652	Hxk2loxr	TACAAAAGAAAGTACGCAAGCTATCTAGAGGAAGTGTAGAGAGGGTTAAA ATTGGCGTGCCGGATCCTACCGTTCGTATAG	*HXK2*
653	Glk1loxf	TATCACGTGCAGCCCAGGATAATTTTCAGGACACGTGTTTCGAAAGGTTTG TCGCTCCGATCGACCTCGAGTACCGTTCG	*GLK1*
654	Glk1loxr	ATTTAGTGAGCTGTTTCTTGTCAAAACAACCAACGGAAGAGGGCGAGGCT GTTTCCTCCGCGGATCCTACCGTTCGTATAG	*GLK1*
5034	Kanf	AAGCTTGCCTCGTCCCCGCC	*kanMX*
535	Kanr	GTCGACACTGGATGGCGGCG	*kanMX*
5115	Lf1	ATCCGGACGTACGTATAACTTCGTATAGCATACATTATACGAAGTTATTCT AGTAACGGCCGCCA	*loxP*
516	Lf2	ATTCTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTAAGCTTGCCTCG TCCCCGCCG	*loxP*
517	Lr1	TCATGACGTCTCGAGGCCTATAACTTCGTATAGCATACATTATACGAAGTT ATGCGCGCT	*loxP*
518	Lr2	CATACATTATACGAAGTTATGCGCGCTCTAGATATCGTCGACACTGGATGG CG	*loxP*
8881	Gal2Bamf	CAGGGATCCTGCCGTCCAGG	*GAL2*
8882	Gal2Munr	CTGCAATTGGAAGCAGAGGCC	*GAL2*
8883	Gal2Eagf	GTACGGCCGTAAAAAGGGTCTTTCG	*GAL2*

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