CHAPTER 2:

INCREASED XYLOSE AFFINITY OF HXT2 THROUGH GENE SHUFFLING OF HEXOSE TRANSPORTERS IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Aims: Optimizing D-xylose transport in *Saccharomyces cerevisiae* is essential for efficient bioethanol production from cellulosic materials. We have used a gene shuffling approach of hexose (Hxt) transporters in order to increase the affinity for D-xylose.

Methods and Results: Various libraries were transformed to a hexose transporter deletion strain and shuffled genes were selected via growth on low concentrations of D-xylose. This screening yielded two homologous fusion proteins (fusion 9,4 and 9,6), both consisting of the major central part of Hxt2 and various smaller parts of other Hxt proteins. Both chimeric proteins showed the same increase in D-xylose affinity (8.1 ± 3.0 mM) compared to Hxt2 (23.7 ± 2.1 mM). The increased D-xylose affinity could be related to the C-terminus, more specifically to a cysteine to proline mutation at position 505 in Hxt2.

Conclusions: The Hxt2^{C505P} mutation increased the affinity for D-xylose for Hxt2, thus providing a way to increase D-xylose transport flux at low D-xylose concentration.

Significance and Impact of the Study: The gene shuffling protocol using the highly homologues hexose transporters family provides a powerful tool to enhance the D-xylose affinity of Hxt transporters in *S. cerevisiae*, thus providing a means to increase the D-xylose uptake flux at low D-xylose concentrations.

Keywords: Yeast, Biofuels, Xylose, Transport, Biotechnology, Fermentation, Metabolism

INTRODUCTION

The increasing awareness of the shortage of fossil fuels and the problems surrounding atmospheric CO_2 concentrations are a driving cause of innovation in the fuel industry. Sources for alternative fuels have led to many developments in the field. Frontrunners among these are microbial derived biodiesel and bioethanol. In the past, bioethanol was mainly produced by fermentation of sugars and starches from high value agricultural crops, like corn, wheat and sugar cane. This poses the problem of competition with human and animal consumption (1). The use of alternative sugars sources from agricultural waste and other by-products of industry has been a major focus in recent years. The main contender in this area is lignocellulosic biomass, an abundant by-product of agriculture and forestry. Hydrolysis of lignocellulosic biomass releases a mixture of hexose and pentose sugars and the majority of these sugars take the form of glucose and xylose, in a typical mass ratio of 2:1 (4, 5). *Saccharomyces cerevisiae*, the commonly used organism in industrial scale ethanol production, is naturally deficient in the usage of pentose sugars due to the lack of an enzyme able to transform pentose into a substrate for the pentose phosphate pathway (PPP). In some industrial strains, the first problem was overcome by inserting a fungal xylose isomerase gene from *Piromyces* species *E2* (10, 105). This enzyme allows the interconversion between D-xylose and D-xylulose, the latter of which can be phosphorylated by the xylulose kinase XKS1, which has been overexpressed in several engineered strains. The resulting D-xylulose-5-P enters the PPP and, via glyceraldehyde-3-phosphate and fructose-6-phosphate, D-xylose catabolism is connected to glycolysis and subsequent ethanol fermentation. Although this results in the desired D-xylose fermentation, the D-xylose consumption lags behind compared to D-glucose metabolism (106, 107). *S. cerevisiae* first consumes D-glucose before D-xylose and this is the direct result of the sugar affinity of hexose transporters (Hxt) (36, 94). The hexose transporters are intrinsically D-glucose transporters and their affinity for D-glucose is, in general, a 100-fold higher compared to D-xylose (7, 106) which prevents efficient D-xylose transport as long as not all D-glucose has been depleted (56). In recent years, several studies have focused on improving the affinity for D-xylose of the endogenous Hxt proteins. In these studies, two screening strategies (and strains) were

used: 1) selection for improved D-xylose metabolism in the presence of high concentrations of D-glucose by a hexokinase deletion strain which is unable to grow on D-glucose (45, 65, 71, 72, 77) and 2) selection for enhanced growth on D-xylose by a hexose transporter deletion strain in which single sugar transporters are expressed (77, 108). In particular the first strategy proved to be very effective and obtained Hxt mutants with decreased affinities for D-glucose while the D-xylose affinity remained mostly unaltered. In particular, a conserved asparagine (at position 366, 376, 370 and 376, in Hxt11, Hxt36 (72), Hxt7 and Gal2, respectively) seems highly crucial for this altered affinity for D-glucose (65, 71, 72). The method was, however, less effective when it concerned the selection for an improved D-xylose affinity.

Here we have used the hexose transporter deletion strain as a host for the expression of a library of Hxt genes obtained by gene shuffling to select transporters with an improved D-xylose affinity. The subfamily of expressed Hxt genes can be readily used in the gene shuffling approach since they exhibit a high homology on DNA level, which ranges from 64% to 99% (35, 94). Furthermore, previous studies on engineered Hxt chimeras already have indicated that they maintain their activity and that by this approach the affinity for D-glucose can be modulated (80, 84, 109–111). Kasahara and coworkers identified key amino acids responsibly for the affinity towards D-glucose although mainly mutations were found that had a negative effect on the affinity for D-glucose (110, 112–114) while the impact of the mutations on the D-xylose affinity was not investigated. The presented data shows that gene shuffling allows for the selection of HXT proteins with a marked improvement in the affinity for D-xylose thereby increasing D-xylose transport at low concentrations.

MATERIALS AND METHODS

YEAST STAINS, MEDIA AND CULTURE CONDITIONS

The DS68625 strain, in which the main hexose transporters Hxt1–7 and Gal2 were deleted, was used for all complementation experiments with the various shuffled Hxt genes and was described elsewhere (72) (Supplemental Table 1). They are made available for academic research under a strict Material Transfer Agreement with DSM (contact: paul.waal-de@dsm.com). All strains were inoculated in shake flasks and incubated at 30°C in mineral medium (MM) supplemented with the appropriate carbon source. Cell growth was monitored by optical density (OD) at 600 nm using an UV-visible spectrophotometer (Novaspec PLUS).

MOLECULAR BIOLOGY TECHNIQUES AND CHEMICALS.

DNA polymerase, restriction enzymes and T4 DNA ligase were acquired from ThermoFisher Scientific and used following manufacturer's instructions. Oligonucleotides used for plasmid constructions and gene shuffling were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

GENE SHUFFLING

With some minor modifications the DNA shuffling procedure was used (115). *HXT1*, *2*, *4* and *7* were amplified with a XbaI and BamHI restriction site on the C- and N-terminus respectively whereas *HXT5* and *GAL2* have a *XbaI* and *Cfr9I* restriction site at the before mentioned termini. *HXT36* was amplified with a *BcuI* and *BamHI* restriction site on the C- and N-terminus (Supplemental table 2). All *HXT* genes were digested with 0.05U/µl DNAse for 20 min at 4 °C and the DNAse was subsequently denatured by adding EDTA (5 mM) and incubated for 10 min at 65 °C. The obtained *HXT* fragments were separated on a 1.0% agarose gel and all fragments ranging from 50–300 bp were isolated from the gel. A primer-less PCR in which all Hxt fragments were mixed was for 40 cycles and an annealing temperature of 55 °C using Phire® PCR polymerase. Subsequently *HXT* fusions were amplified using all forward Hxt primers in combination with all other reverse primers except for the combination belonging to the same gene (*eg* not Forward Hxt1 xbaI and Reversed Hxt1 BamHI). The obtained full length *HXT* genes were combined and digested with the appropriate restriction enzymes and ligated into pRS313P7T7-MCS (72) and transformed to *E.coli*. Several colonies were picked and sequenced to confirm the *HXT* fusions. All colonies were subsequently combined into batches (forward *HXT1* and *HXT2* (batch 6), forward *HXT3* (batch 7), forward *HXT4* and

GAL2 (batch 8), forward *HXT5* (batch 9) and forward *HXT7* (batch 10) and transformed to DS68625 and transferred to MM supplemented with 0.1% D-xylose and grown for 144 hours.

CLONING OF THE HXT2^{C505P} MUTANT

HXT2 gene fragments were amplified from genomic DNA of the DS68616 strain using the primers listed in Supplemental Table 2 with the Phusion® High-Fidelity PCR Master Mix with HF buffer. Using F Hxt2 XbaI and a reversed primer with the F497Y and/or the C505P mutation yielded the 3' fragment of *HXT2* and the same forward primers of those mutations combined with primer R Hxt2 BamHI yielded the 5' fragment of *HXT2*. After PCR clean-up both fragments were fused using overlap-PCR (Phusion® High-Fidelity PCR Master Mix) with only the F Hxt2 XbaI and R Hxt2 BamHI primers. The full-length DNA of *HXT2*, and the mutants, was cloned into pRS313-P7T7 and subsequently sequenced.

TRANSPORT ASSAYS

To determine the kinetic parameters of sugar transport, cells were grown for 16 hours in shake flasks in minimal medium (MM) containing 2% D-maltose and standard uptake procedure was followed as shown before (72). [^{14}C]D-xylose or [^{14}C]D-glucose (ARC, USA) uptakes were analyzed at concentrations varying from 0.5–200 mM and 0.1–180 mM, respectively. In order to determine the uptake kinetics of the transporters a non-linear Michaelis-Menten least squares fit was used.

RESULTS

GENE SHUFFLING AND IMPROVED HXT SELECTION

The quest for a specific D-xylose transporter with high affinity for this pentose sugar has been a main focus in the bioethanol field in recent years (33, 65, 71, 72, 116). D-xylose uptake is inhibited by the high D-glucose concentration present in lignocellulosic biomass causing a delayed fermentation of D-xylose. To enhance the affinity for D-xylose uptake by *S. cerevisiae*, we applied gene shuffling on the highly homologues family of *HXTs*. *HXT1–7* and *GAL2* were all amplified and used in gene shuffling. In the last PCR amplification step only non-matching forward and reverse primers were used in order to obtain an increased number of fusions compared to the original Hxt DNA fragments. The complete library of Hxt fusions, divided in to 5 batches with different forward primers, were cloned into the pRS313P7T7 (72) expression vector and was subsequently transformed to the DS68625 strain, which lack the main Hxt proteins Hxt1–7 and Gal2. This strain cannot grow on D-xylose due to the lack of efficient D-xylose transporters. Immediately after transformation, cells were transferred to minimal medium supplemented with 0.1% D-xylose. As a control for the growth experiments, plasmid pRS313P7T7-mcs and pRS313P7T7 carrying the genes of *HXT1*, *HXT36*, *HXT4* or *GAL2* were used. After 144 hours in minimal medium with 0.1% D-xylose only batch 9, obtained from the amplification with primer F Hxt5 XbaI and all reverse primers, and batch 10, amplified with F Hxt7 XbaI and all reverse primers, were able to reach a higher OD than the control transformants (Supplemental Figure 1).

VALIDATION OF HXT FUSION PROTEINS

Cells from batch 9 and 10 were plated on 0.1% D-xylose and plasmid isolation was done on 6 single colonies of each batch with subsequent DNA sequencing. Out of the 6 isolated *S. cerevisiae* strains from batch 9, four were identical and contained a Hxt fusion named 9,4 whereas 2 others contained fusion 9,6. DNA sequencing revealed that fusion 9,4 consists of Hxt5 (102 amino acids), Hxt2 (388 amino acids), Hxt4 (49 amino acids) and Hxt2 (32 amino acids). Fusion 9,6 is very homologous to fusion 9,4 and consists of Hxt5 (102 amino acids), Hxt2 (254 amino acids), Hxt3 (37 amino acids), Hxt2 (120 amino acids) and Hxt4 (78 amino acids). All 6 colonies isolated from batch 10 yielded the same fusion (10,1) which consists of Hxt7 (65 amino acids), Hxt4 (33 amino acids), Hxt7 (53 amino acids), Hxt4 (42 amino acids), Hxt7 (83 amino acids), Hxt4 (97 amino acids), Hxt7 (67 amino acids), Hxt1 (21 amino acids), Hxt7 (35 amino acids), Hxt4 (14 amino acids) and Hxt1 (59 amino acids) (Figure 1).

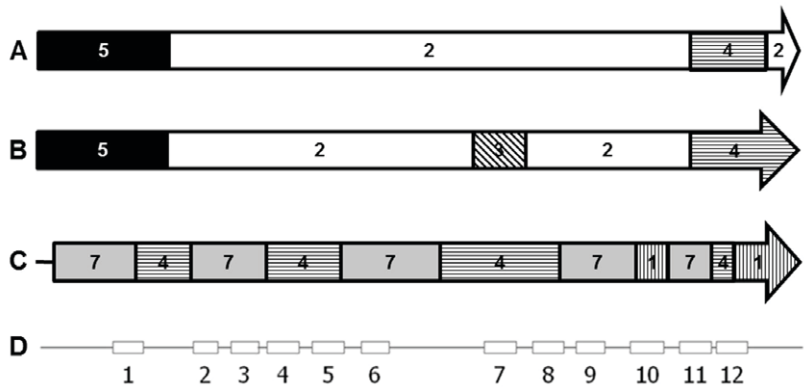

Figure 1. Schematic representation of hexose transporter fusions 9,4 (A), 9,6 (B) and 10,1 (C). The colors and stripes indicate the various parts of each of Hxt1 (vertically striped), Hxt2 (white), Hxt3 (slanted striped), Hxt4 (horizontally striped), Hxt5 (black) and Hxt7 (grey). The matching 12 transmembrane domains (TMDs) are depicted in D.

All 3 unique fusions (9,4, 9,6 and 10,1) were re-transformed into the original DS68625 strain and a similar growth experiment (in minimal medium supplemented with 0.1% D-xylose and 0.025% D-maltose) was done to confirm the previous result. The 0.025% D-maltose was added to shorten the lag-phase. Fusion 9,4 and 9,6 showed increased growth rates and reached the highest OD_{600} levels after 40 hours. They outperformed the DS68616 strain containing all Hxt proteins. The controls Hxt1, Hxt36, and Gal2 showed no growth on the low D-xylose concentrations while Hxt2 showed limited growth rates. Fusion 10,1 did perform better compared to the low affinity transporters Hxt 1 and Hxt36, but it showed lower growth rates compared to Hxt2 and was not further investigated (Figure 2).

SUGAR UPTAKE KINETICS OF HXT CHIMERAS

To assess if the improved D-xylose fermentation characteristics on low D-xylose concentrations of the cells bearing fusion 9,4 and 9,6 were caused by improved D-xylose uptake, affinity transport assays were performed with D-xylose and D-glucose. Since a major part of fusions 9,4 and 9,6 consists of Hxt2, a Hxt with natively already an high affinity for D-xylose (70, 71), Hxt2 was used as control (Supplemental figure 2). The K_m for D-xylose by Hxt2 is 23.7 ± 2.1 mM. Both chimeras 9,4 and 9,6 showed increased affinities for D-xylose uptake, *i.e.*, 9.4 ± 3.9 mM and 6.9 ± 2.3 mM, respectively (Table 1). The V_{max} of D-xylose uptake by 9,4, 9,6 and Hxt2 were very similar. *i.e.*, 35.2 ± 3.1 nmol/mgDW.min, 31.6 ± 4.1 nmol/mgDW.min and 33.0 ± 2.1 nmol/mgDW.min respectively. In contrast, the affinity for D-glucose uptake hardly changed (Table 1), although this might be due to the fact that D-glucose uptake rates via Hxt2 are extremely fast and any further improvement might not be readily detected. These data show that the chimeric transporters obtained by gene shuffling exhibit an improved D-xylose uptake affinity.

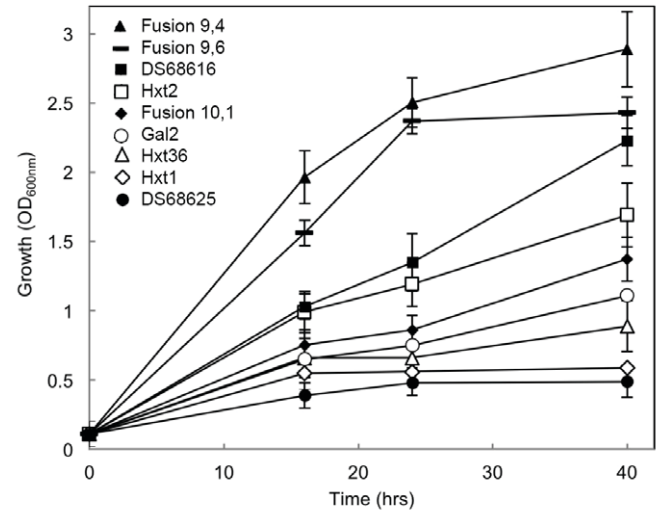

Figure 2. Growth (OD_{600}) of strain DS68625 expressing individual hexose transporters on minimal medium supplemented with 0.1% D-xylose and 0.025% D-maltose. Strains tested contained Hxt1 (◇), Hxt2 (□), Hxt36 (△), Gal2 (○), the gene-shuffled hexose transporters (fusion 9,4 (▲), fusion 9,6 (—) and fusion 10,1 (◆)) or the empty control plasmid pRS313-P7T7mcs (●). As control, the DS68616 strain, with the full Hxt landscape, was used complemented with the empty control plasmid pRS313-P7T7mcs (■). Error bars were obtained from biological duplicates.

Table 1. K_m and V_{max} values for D-glucose and D-xylose uptake by Hxt2 and the fusion transporters 9,4 and 9,6 and Hxt2^{C505P} expressed in strain DS68625.

	K_m (mM)		V_{max} (nmol mg/DW.min)	
	D-glucose	**D-xylose**	**D-glucose**	**D-xylose**
Hxt2	0.95 ± 0.07	23.7 ± 2.10	91.45 ± 0.60	32.98 ± 2.09
Fusion 9,4	0.76 ± 0.01	9.37 ± 3.91	82,90 ± 3.68	35.18 ± 3.05
Fusion 9,6	0.78 ± 0.02	6.86 ± 2.30	73.70 ± 2.05	31.55 ± 4.10
Hxt2 C505P	1.22 ± 0.05	8.30 ± 1.0	67.30 ± 4.88	27.70 ± 1.70

Errors are the standard of the mean of 2 (D-glucose) or 4 (D-xylose) independent experiments.

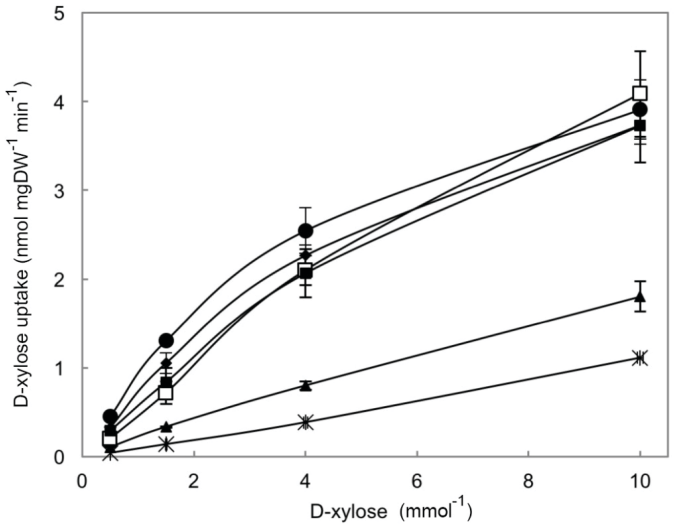

Figure 3. D-xylose uptake of the DS68625 hexose transporter deletion strain expressing Hxt2 (▲), the gene fusions 9,4 (♦) and 9,6 (●) and the N-terminal fusion (✳), C-terminal fusion 9,4 (□) and C-terminal fusion 9,6 (■) in plasmid pRS313-P7T7. Errors are the standard deviation of two independent experiments

UNRAVELING HXT CHIMERAS

To determine what brings about this increased affinity for D-xylose uptake, variants of the fusions 9,4 and 9,6 were made. This concerned a N-terminal fusion protein which consists of the first 102 amino acids of Hxt5 fused to Hxt2 to analyze if the N-terminus of Hxt5 causes the increase in D-xylose affinity. The other 2 variants concerned the C-terminus of fusion 9,4 or 9,6 in which the first 102 amino acids of Hxt5 are replaced for the first 102 amino acids of Hxt2. The three variants were tested for D-xylose uptake using the lower D-xylose concentration range (0.5–10 mM). The N-terminal fusion protein showed a similar uptake compared as Hxt2 whereas the C-terminal fusions 9,4 and 9,6 showed significantly improved D-xylose uptake compared to Hxt2 and similar uptake compared to the original fusions 9,4 and 9,6 (Figure 3). This suggests that the C-terminus of both chimera contains the determinants that increased the D-xylose affinity of Hxt2. Since the C-terminal fusion 9,4 and 9,6 only have 49 amino acids of Hxt4 in the C-terminus in common, the increase in D-xylose affinity must come from amino acids present in this region. Alignment of the first 49 amino acids of Hxt4 with the corresponding region of Hxt2 revealed two amino acids (F497 and C505) that vastly differ in this region. Both mutations (F497Y, C505P), and the combination were generated in Hxt2 and tested for D-xylose uptake. Both variants containing the C505P mutation show a marked increased uptake of D-xylose compared to Hxt2. D-xylose uptake, measured at 5 mM D-xylose, in Hxt2 F497Y+C505P, Hxt2 C505P and Hxt2 is 2.63 ± 0.10, 2.62 ± 0.06 and 1.62 ± 0.09 nmol/mgDW.min respectively and D-xylose uptake compares to fusions 9,4 and 9,6 (Figure 4). These data suggest that the C505P mutation of Hxt2 is responsible for the improved D-xylose affinity. Sugar transport assays of the Hxt2^{C505P} mutant showed similar K_m values (8.3 ± 1.0 mM for D-xylose and 1.22 ± 0.05 mM for D-glucose) compared to the chimeras 9,4 and 9,6 (Table 1). To further verify the improved affinity for D-xylose, the Hxt deletion strain DS68625 expressing the Hxt2^{C505P} mutant was aerobically grown in 0.1 % D-xylose and 0.025 % D-maltose (Figure 5). Indeed, the Hxt2^{C505P} mutant grows at similar growth rate on D-xylose as compared to fusion 9,4 and reaches the levels of biomass, whereas the strain with the wild-type Hxt2 lags behind. To explore the sequence space of Hxt2^{C505} all amino acid substitutions at position 505 were tested, but none showed the same improvement in D-xylose affinity as compared to the proline (data not shown).

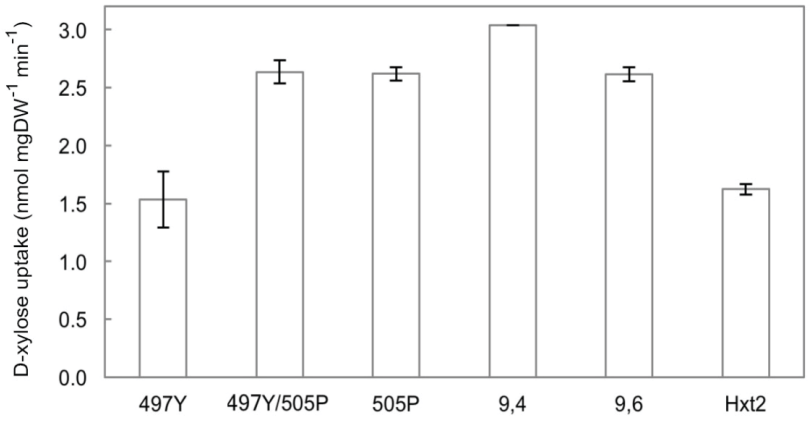

Figure 4. Uptake experiment with 5 mM of D-xylose in the DS68625 hexose transporter deletion strain expressing Hxt2 with the point mutations F497Y, F497Y/C505P and C505P and the controls fusion 9,4, 9,6 and Hxt2 in plasmid pRS313-P7T7. Errors are the standard deviation of two independent experiments

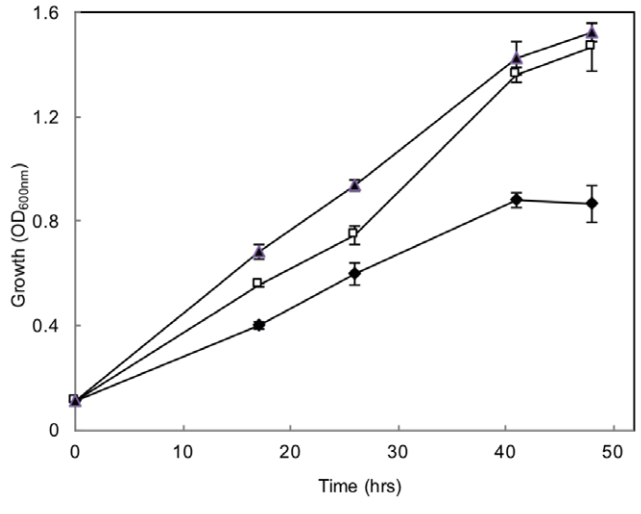

Figure 5. Growth (OD_{600}) on MM supplemented with 0.1% D-xylose and 0.025% D-maltose of the DS68625 strain expressing Hxt2 (◆), fusion 9,4 (■) and $Hxt2^{C505P}$ (▲) in plasmid pRS313-P7T7. Error bars were obtained from biological duplicates.

DISCUSSION

The use of lignocellulosic biomass for ethanol production with *S. cerevisiae* is a promising technology for the additional supply of energy from renewable and non-food resources. The first and main hurdle to overcome is the efficient co-fermentation of hexoses and pentoses since transport rates for pentoses in general, and D-xylose in particular, are insufficient. To increase the transport activity for D-xylose it is important to also improve on the transport affinity which is orders of magnitude poorer than for glucose (70). Without any structural bias, a generic gene shuffling method (115) was used to generate shuffled libraries of the main Hxt genes. Using the transporter deletion strain DS68625, in which the main hexose transporters Hxt1–7 and Gal2 are deleted, we screened 5 shuffling libraries at a low D-xylose concentration of 0.1% for 40 hours and obtained 2 different variants with improved affinities towards D-xylose. In this screen, only limited growth was observed with the wild-type Hxt2 transporter while growth was even poorer when Gal2, Hxt36 and Hxt1 were tested. Both shuffling variants (fusions 9,4 and 9,6) show a remarkable homology as they both share the identical N-terminus of Hxt5 and a similar C-terminus of Hxt4. The main, and central part, of both chimeras consists of Hxt2, the hexose transporter which shows the highest affinity for D-glucose (94, 117) and D-xylose (70) amongst the Hxt transporter family (Table1). However, fusion 9,6 also contains a small part of Hxt3, but since this is missing from fusion 9,4, it cannot be responsible for the D-xylose affinity change. Rather, the change in affinity is due to altered amino acids within the C-terminus of both fusions and this could be pinpointed at the cysteine at position 505. When this cysteine residue in Hxt2 was mutated into the proline found in the chimeras 9,4 and 9,6, similar improved affinity (Table 1) and growth on 0.1% D-xylose was observed (Figure 5). This proline residue is conserved in Hxt1,3–7 and Gal2 with the only exception a cysteine in Hxt2. The C505P mutation is located in the C-terminal tail of Hxt2, just downstream the last transmembrane domain 12. Possibly, conformational rigidity introduced by the proline causes a conformational change in Hxt2 that results in an improved D-xylose affinity without affecting the D-glucose affinity. Interestingly, the cysteine (TGT) to proline (CCA/T) mutation required at least two base pair changes which is

more readily obtained in the gene shuffling approach as compared to classical error prone mutagenesis.

The K_m value of Hxt2 for D-xylose measured is 2 fold lower compared to what we measured previously (71) and significantly lower than reported before (70). The conditions used in these three studies are not comparable as Saloheimo *et.al.* used a different promotor (pTPI1) and high copy plasmid to express Hxt2, and incubated cells on-ice before the uptake experiment. Also, variations in growth phase and pre-growth carbon source may lead to variations in uptake. It should be noted that in this study, the K_m values for Hxt2 (C505P) and both fusions were obtained using a lower cell density (OD_{600} of 10 instead of 100) and longer uptake times (10 instead of 1 minute) compared our previous study (80). This modification resulted in more reliable uptake data. D-xylose uptake was linear up to 15 minutes (data not shown). Importantly, D-xylose uptake via Hxt2 at high D-xylose concentrations (and thus low specific radioactivity) shows greater variability (70, 71) which impacts the exact calculation of the K_m value more significantly at the shorter uptake times. However, analyzing the uptake at a higher cell density and shorter uptake times, a similar trend in the affinity improvement was observed for fusion 9,4, fusion 9,6 and $Hxt2^{C505P}$ of 53.7 ± 6.14, 51.1 ± 4.78 and 58.4 ± 5.43 mM respectively, compared to Hxt2 of 70.1 ±8.87 mM (data not shown). Furthermore, Saloheimo *et.al.* used the Direct Linear Plot method (118, 119) to calculate the kinetic values (K_m 260 ± 130 mM) with large error whereas we use least squares fitting employing non-linear Michaelis-Menten kinetics. Extracting the data from the 2 most reliable datasets (70) and analysis by non-linear Michaelis-Menten least squares fitting revealed a K_m of 94.4 ± 6.8 mM. Therefore, it appears that the low affinity for D-xylose of Hxt2 claimed does not follow from the kinetic data and also does not explain why Hxt2 allows growth on low concentrations of D-xylose whereas other HXT proteins do not (Figure 2, Supplemental Figure 1). Thus, we conclude that Hxt2 exhibits a better affinity for D-xylose than previously suggested.

We observe improved growth and uptake of xylose with the $Hxt2^{C505P}$ mutant and conclude that gene shuffling (115) of the highly homologues hexose transporters family provides a powerful tool to enhance D-xylose transport in *S. cerevisiae*. The $Hxt2^{C505P}$ mutation increased the affinity for D-xylose for Hxt2, thus providing a way to increase the D-xylose transport flux at low D-xylose concentration.

ACKNOWLEDGEMENTS

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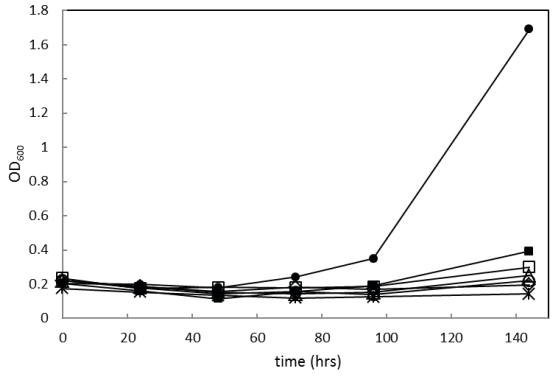
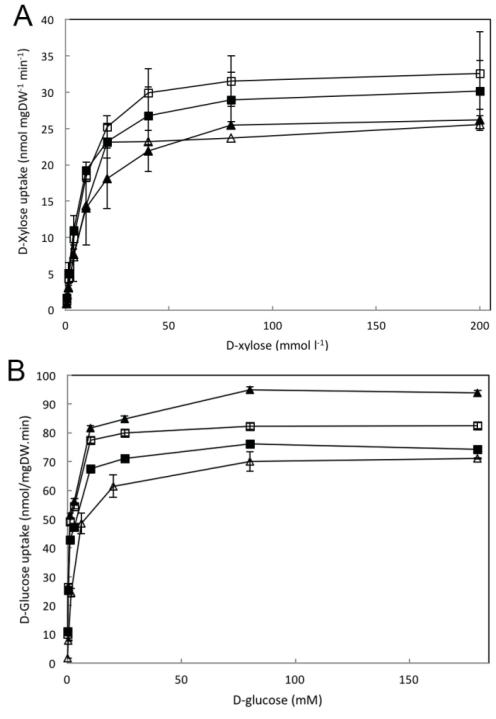
CONFLICT OF INTEREST

No conflict of interest declared.

SUPPLEMENTAL DATA



Supplemental Figure 1. Growth (OD_{600}) of the DS68625 strain expressing individual hexose transporters on minimal medium supplemented with 0.1% D-xylose. Traces are pRS313P7T7-mcs (✳), combined Hxt1, Hxt36, Hxt4 and Gal2 (□); gene-shuffled library #6 (○), #7 (◇), #8 (△), #9 (●) and #10 (■).



Supplemental Figure 2. D-xylose (Panel A) and D-glucose (panel B) uptake by the DS68625 strain expressing plasmid pRS313-P7T7 born Hxt2 (▲), fusion 9,4 (□), fusion 9,6 (■) and $Hxt2^{C505P}$ (△). Errors are the standard deviation of four independent experiments for D-xylose and two independent experiments for D-glucose.

Supplemental Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype and/or characteristics	Source or reference
Strains		
S. cerevisiae		
DS68616	*Mat a. ura3–52. leu2–112. gre3::loxP. loxP-Pt-pi:TAL1. loxP-Ptpi::RKI1. loxP-Ptpi-TKL1. loxP-Ptpi-RPE1. delta::Padh1XKS1Tcyc1-LEU2. delta::URA3-Ptpi-xylA-Tcyc1. His3::LoxP*	DSM, The Netherlands
DS68625	DS68616. *his3::loxP. hxt2::loxP-kanMX-loxP. hxt367::loxP-hphMX-loxP. hxt145::loxP-natMX-loxP. gal2::loxP-zeoMX-loxP*	(71, 72)
Plasmids		
pRS313	*E. coli*/yeast shuttle vector; *CEN6*, *ARSH4*, *HIS3*, Amp^r	(120)
pRS313P7T7	pRS313 with promoter and terminator of Hxt7	(72)

Supplemental Table 2. Oligonucleotides used in cloning.

Name	Sequence (5' ✳ 3')
F HXT1 Xbai	GCATTCTAGAATGAATTCAACTCCCGATCTAATATC
R HXT1 BamHi	TGCATGGATCCTTATTTCCTGCTAAACAAACTCTTGTA
F HXT2 Xbai	GTCCTCTAGAATGTCTGAATTCGCTACTAGCCG
R HXT2 BamHi	CATCGGGATCCTTATTCCTCGGAAACTCTTTTTTCTTTTG
F HXT36 Bcui	GCATACTAGTATGAATTCAACTCCAGATTTAATATCTC
R HXT36 BamHi	ACGTGGATCCTTATTTGGTGCTGAACATTCTCTTGT
F HXT4 Xbai	GTCCTCTAGAATGTCTGAAGAAGCTGCCTATCAAG
R HXT4 BamHi	TATCGGGATCCTTAATTAACTGACCTACTTTTTTCCGA
F HXT5 Xbai	GTCCTCTAGAATGTCGGAACTTGAAAACGCTCATC
R HXT5 Cfr9i	GCATCCCGGGTTATTTTTCTTTAGTGAACATCCTTTTATA
F HXT7 Xbai	GTCCTCTAGAATGTCACAAGACGCTGCTATTGCA
R HXT7 BamHi	CATCGGGATCCTTATTTGGTGCTGAACATTCTCTTG
F Gal2 Xbai	ACTCGTCTAGAATGGCAGTTGAGGAGAACAATATG
R Gal2 Cfr9i	GCATCCCCGGGTTATTCTAGCATGGCCTTGTACC
F 497 Hxt2	GTTTGGTATTT**TAC**TACTTCTACGTGT
R 497 Hxt2	ACACGTAGAAGTA**GTA**AAATACCAAAC
F 505 Hxt2	GTGTTTTTCTTTGTC**CCT**GAAACCAAGG
R 505 Hxt2	CCTTGGTTTC**AGG**GACAAAGAAAAACAC
F 497 505 Hxt2	GGTATTT**TAC**TACTTCTACGTGTTTTTCTTTGTC**CCT**GAAACCAAG
R 497 505 Hxt2	CTTGGTTTC**AGG**GACAAAGAAAAACACGTAGAAGTA**GTA**AAATACC

Underlined are restriction sites used. In **bold** are the codons for mutated amino acids.