Identification of an important motif that controls the activity and specificity of sugar transporters†

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ABSTRACT

Efficient glucose-xylose co-utilization is critical for economical biofuel production from lignocellulosic biomass. To enable glucose-xylose co-utilization, a highly active xylose specific transporter without glucose inhibition is desirable. However, our understanding of the structure-activity/specificity relationship of sugar transporters in general is limited, which hinders our ability to engineer xylose-specific transporters. In this study, via homology modeling and analysis of hexose sugar transporter HXT14 mutants, we identified a highly conserved YYX(T/P) motif that plays an important role in controlling the activity and specificity of sugar transporters. We demonstrated that mutating the two tyrosine residues of the motif to phenylalanine respectively improved glucose transport capacity across several different sugar transporters. Furthermore, we illustrated that by engineering the fourth position in the YYX(T/P) motif, the sugar specificity of transporters was significantly altered or even reversed towards xylose. Finally, using the engineered sugar transporter, genuine glucose-xylose co-fermentation was achieved. This article is protected by copyright. All rights reserved.
Introduction

To mitigate the global warming and reduce our dependence on petroleum, efficient and economical conversion of inexpensive biomass into biofuels and industrial important chemicals presents both a great challenge and opportunity (Buijs et al. 2013; Peralta-Yahya et al. 2012; Rabinovitch-Deere et al. 2013; Stephanopoulos 2007; Zhang et al. 2011). The yeast \textit{Saccharomyces cerevisiae} is among the top choices of microorganisms due to its high efficiency in ethanol production and high tolerance to ethanol and inhibitors presented in hydrolysates (Weber et al. 2010). In addition, \textit{S. cerevisiae} is more resistant to viral infections, which is a serious problem in large scale bacterial fermentation (Los et al. 2007). Besides glucose, xylose is the most abundant sugar in cellulosic biomass. Because glucose can be efficiently utilized by native \textit{S. cerevisiae}, majority of the research have been focused on engineering \textit{S. cerevisiae} to efficiently ferment xylose. Through decades of research, significant advances have been made in xylose fermentation (Kim et al. 2013a; Laluce et al. 2012). However, due to carbon catabolite repression (CCR) (Carlson 1999; Trumbly 1992) and extremely limited xylose transport in the presence of glucose, xylose can be consumed by \textit{S. cerevisiae} only after the depletion of glucose. While xylose pathway optimization was the focus of past researches (Du et al. 2012; Kim et al. 2013b), recently, researchers started to investigate and engineer sugar transporters for efficient xylose transport (Farwick et al. 2014; Young et al. 2012; Young et al. 2014).

Sugar transporters are essential for cells from all domains of life. For example, the GLUT family of human glucose transporters plays fundamental roles in different physiological and pathophysiological processes (Mueckler 1994). Deficiency of GLUT transporters is related to a barrage of human diseases such as infantile-onset seizure (Brockmann 2009; Scheffer 2012), Fanconi-Bickel syndrome (Santer et al. 1997), and type 2 diabetes mellitus (Leney and Tavare 2009). Deeper understanding of the structure-activity/specificity relationship of a sugar transporter may provide better insight on those diseases.
the other hand, improving sugar transport activity and specificity, particularly towards xylose, is critical for efficient conversion of biomass into biofuels. The major facilitator superfamily (MFS) sugar transporters mediate monosaccharide transport in *S. cerevisiae* and related yeast strains (Boles and Hollenberg 1997; Leandro et al. 2009). Via bioprospecting, researchers have discovered novel sugar transporters with high xylose transport capacity (Leandro et al. 2009; Young et al. 2011). However, those native transporters usually confer high glucose transport and glucose inhibition as well (Young et al. 2010). A few xylose specific transporters have been discovered (Du et al. 2010). However, they usually have very low activity in xylose transport. In light of the incapability of native sugar transporters to achieve efficient xylose transport in the presence of glucose, directed evolution has been used to improve xylose transport capacity (Young et al. 2012) as well as alleviate glucose inhibition (Farwick et al. 2014) of sugar transporters. Nonetheless, our understanding of how a sugar transporter controls its activity and specificity is quite limited due to the scarcity of crystal structures of sugar transporters and the difficulty in studying protein dynamics of sugar transport process.

In this study, via homology modeling and analysis of sugar transporter HXT14 mutants, we identified a highly conserved YYX(T/P) motif that plays an important role in determining the activity and specificity of sugar transporters. We demonstrated that mutating two tyrosine residues of the motif to phenylalanine could improve glucose transport across several different sugar transporters. And these two residues may act as a “gate” to restrict the diffusion of glucose molecule to its binding site via interactions through hydroxyl groups of tyrosine residues. Furthermore, we also illustrated that by engineering the fourth position in the YYX(T/P) motif, the specificity of a sugar transporter was significantly altered or even reversed towards xylose. Finally, using the engineered sugar transporter, genuine glucose-xylose co-fermentation was achieved in a sugar transporter knockout strain. Our study
shed some light on the structure-activity/specificity relationship of sugar transporters and made strides towards the goal of efficient co-utilization of glucose and xylose in *S. cerevisiae* for biofuel production.

**Materials and methods**

**Strains, media, and cultivation conditions**

*S. cerevisiae* HZ848 (MATa, ade2-1, *Aura3*, his3-11, 15, *trp1-1*, *leu2-3, 112*, and *can1-100*) and *Escherichia coli* WM1788 was used for recombinant DNA manipulation. Hexose transporter knockout strain MW01 (CEN.PK2-1C Δ*hxt1-17 Δstl1 Δagt1 Δydl247w Δyjr160c Δgal2*, aka VW4000, a gift from Dr. Eckhard Boles in Goethe University Frankfurt) was used as the host for sugar transporter characterization and engineering. See supplementary material for detail.

**Plasmid construction**

Single mutations of HXT14 are cloned into helper plasmid pRS414-HXT7p-SacII-HXT7t using Gibson assembly method (Gibson et al. 2009). In order to simplify library screening, we cloned the xylose utilization pathway and sugar transporters (*HXT14*, *HXT10*, *GAL2* and *HXT2*) on the same plasmid instead of two plasmids system in HXT14 mutants. See supplementary material for detailed plasmids construction.

**Saturation mutagenesis and mutant screening**

Here we describe saturation mutagenesis and screening of HXT2 as an example. The *HXT2* gene was separated into two fragments with overlapping region. The first fragment was amplified using primers HXT7p-HXT2-F and HXT2-T339-degererate-R. The second fragment (with degenerate codon) was amplified using primers HXT7p-HXT2-R and HXT2-T339-degererate-R. The saturation mutagenesis library was created *in situ* in *S. cerevisiae* MW01 strain by assembling two *HXT2* gene fragments with
linearized pRS416-xylose pathway-HXT7p-SacII-HXT7t via the DNA assembler method. Single colonies were inoculated into 3 mL of SC-Ura liquid medium supplemented with 20 g/L xylose or 20 g/L glucose. Clones with significant growth in xylose and limited growth in glucose were selected for further analysis. Plasmids from positive clones were isolated and re-transformed to S. cerevisiae MW01 strain to confirm their phenotypes. See supplementary material for detailed description of cell growth assay, high cell-density fermentation assay, HPLC analysis method, and $^{14}$C-sugar uptake assay.

**Results**

**Analysis of HXT14 mutants revealed a highly conserved YYX(T/P) motif that controls the activity and specificity of sugar transporters**

Previously, we obtained several HXT14 mutants with improved xylose transport capacity by directed evolution (Wang et al. 2016). Compared with wild type HXT14 (HXT14WT), we found that mutants HXT14-T365A, HXT14-#14(Y13N, Y363F, I506V), and HXT14-T365S (Table S2) improved glucose transport significantly, while mutant HXT14-#38(F300Y, N387H, N435D) had a dramatic 13.7-fold specificity switch towards xylose. HXT14-#38 contained a key mutation N387H, which aligned with N376 of GAL2 in the multiple sequence alignment (Figure 1). It may support the conclusion made by Farwick et al. that asparagine in this position plays an important role in controlling sugar specificity (Farwick et al. 2014). Mutations Y362F and Y363F from mutants HXT14-#9(C129G, I240L, Y362F, M419L) and HXT14-#14 (Table S2) respectively are extremely close to residue T365 of HXT14 and may contribute significantly to the improved activity of HXT14-#9 and HXT14-#14. Multiple sequence alignment of sugar transporters (Figures 1 and Figure S1) revealed that Y362 and Y363 of HXT14 were highly conserved throughout all MFS sugar transporters, and position 365 of HXT14 was occupied
mostly by either threonine or proline in sugar transporters (Figure S1). Therefore, we hypothesized that the highly conserved YYX(T/P) motif might play an important role in MFS sugar transporters.

To verify our hypothesis, we first generated Y362F, Y363F, and N387H single mutants of HXT14 and determined their exponential growth rates in glucose or xylose containing medium as a surrogate for their sugar transport capacities (Young et al. 2011; Young et al. 2012; Young et al. 2014). As we predicted, single mutant HXT14-N387H reduced glucose transport by 3.5-fold while increased xylose transport by 30% (from 0.00310±0.00026 to 0.00403±0.00042 hr⁻¹), which resulted in a 4.6-fold decrease in terms of specificity towards glucose (Figure 2A). Single mutants HXT14-Y362F and HXT14-Y363F only provided limited improvements in terms of xylose transport capacity compared with HXT14WT respectively. However, surprisingly, Y362F and Y363F increased glucose transport capacity by 3-fold and 7.7-fold respectively, which indicated that these two tyrosine residues might have specific interactions with glucose molecule.

The YYX(T/P) motif is part of the solvent-accessible channel of sugar transporters

Although the YYX(T/P) motif is highly conserved, its spatial occupation and detailed molecular mechanism for controlling activity and specificity remains unclear. To gain some insight, we constructed a homology model of HXT14 using the crystal structure of XylE (PDB: 4GBZ) from *Escherichia coli* (Sun et al. 2012) as the template using the modeling program Molecular Operating Environment (Chemical Computing Group, Montreal, Canada) (Figure 3A and 3B). XylE is a xylose/H⁺ symporter, which can be inhibited by glucose (Henderson 1990; Henderson and Maiden 1990; Lam et al. 1980). Unlike other sugar transporters, the co-crystal structures of XylE with xylose or glucose were obtained (Sun et al. 2012), which would provide valuable insight on the activity and specificity of HXT14 and its mutants. Despite the relatively low sequence identity (28.2%) between
HXT14 and XylE, the homology model of HXT14 had good resemblance to XylE. Most interestingly, the putative YYX(T/P) motif is part of the transmembrane helix 7 (TM7), which is critical for forming the solvent-accessible channel of sugar transporters (Sun et al. 2012). In particular, in the co-crystal structure of XylE with xylose, the kinked TM7 hosts three polar residues (Q288, Q289, and N294) and an aromatic residue Y298 that contributed to the recognition of xylose (Sun et al. 2012). In addition, TM7 is a discontinuous helix which was proposed to play an important role in conformational change during substrate transport (Sun et al. 2012). Figure 3B showed the top view of the molecular surface of the homology model of HXT14, the YYX(T/P) motif (highlighted as red in Figure 3B) occupied the extracellular end of TM7 and formed part of the entrance of the solvent-accessible channel. Through the solvent-accessible channel, we can clearly see the glucose molecule (highlighted as green) at its binding site (Figure 3B). Although no direct contact between the YYX(T/P) motif and the glucose molecule was observed in our model, the co-crystal structure only captured one instance of the transport process. It is quite possible that the YYX(T/P) motif acts as a “gate” and has specific interactions with the sugar molecule during its diffusion into the channel to its binding site.

To characterize the HXT14WT and its mutants in detail, we performed [14C]-sugar uptake assay to determine their kinetic parameters (Table 1). Compared to HXT14WT, HXT14-Y362F had a similar \(K_m\) value, but its \(V_{\text{max}}\) value was 35-fold higher than that of HXT14WT. In the case of HXT14-Y363F, its affinity for glucose was 73% higher than wild type HXT14. In addition, the \(V_{\text{max}}\) value of HXT14-Y363F was 24-fold higher than HXT14WT. The significantly increased \(V_{\text{max}}\) values of both HXT14-Y362F and HXT14-Y363F were consistent with their significantly improved growth rates in glucose, which supported our hypothesis that the two tyrosine residues may act as a “gate” to restrict transport of glucose. On the other hand, we also determined the kinetic parameters of HXT14-T365A towards D-
xylose. It had 3.3-fold higher affinity towards xylose and 3.6-fold higher $V_{\text{max}}$ value than wild type HXT14, which may explain its improved growth rate in xylose.

**Tyrosine to phenylalanine mutations of the YYX(T/P) motif can improve glucose transport in sugar transporters**

We sought to investigate whether the YYX(T/P) motif of other sugar transporters is also involved in controlling the activity and specificity. We first tested the function of tyrosine to phenylalanine mutations of the YYX(T/P) motif in sugar transporter HXT10, GAL2, and HXT2 from *S. cerevisiae*. Based on the multiple sequence alignment (Figure 1), we generated mutants HXT10-Y329F, HXT10-Y330F, GAL2-Y351F, GAL2-Y352F, HXT2-336F, and HXT2-337F and determined their exponential growth rates in glucose or xylose containing medium. Summarized in Figure 2B, all mutants except for HXT10-Y329F showed increased glucose transport capacity. Combined with similar or even decreased xylose transport, their specificities towards glucose were increased. The amount of improvement seems to be negatively correlated with the glucose transport capacities of the wild type transporters. In the case of sugar transporters with very low glucose transport capacity (such as HXT14), mutating tyrosine to phenylalanine significantly improved their activities (300%-770%). The wild type HXT10 and GAL2 (HXT10WT and GAL2WT) are moderate glucose transporters, and only 60%-123% improvement of glucose transport capacity were seen. When the best glucose transporter HXT2 was used as the template (Young et al. 2014), only ~19% increase was achieved. The two tyrosine residues of the YYX(T/P) motif are highly conserved across all MFS sugar transporters, and combined with the fact that tyrosine to phenylalanine mutations have universal effect of improving glucose transport for several different glucose transporters, our results strongly supported our hypothesis that these two tyrosine residues may act as a “gate” for sugar transporters by having specific interactions between hydroxyl groups and the glucose molecule.
Rewiring sugar specificity of sugar transporters via engineering the fourth position of the YYX(T/P) motif

Unlike the two tyrosine residues in the YYX(T/P) motif, the fourth position is not highly conserved, and is mostly occupied by either threonine or proline (Figure 1 and Figure S1). The T365A and T365S mutations of HXT14 significantly increased both glucose and xylose transport capacity and steered the specificity towards xylose (Figure 2A). Therefore, we predicted that the fourth position of the YYX(T/P) motif may be involved in controlling specificity in sugar transporters. To test our hypothesis, we generated threonine to alanine or serine mutations in both HXT10 (HXT10-T332A and HXT10-T332S) and GAL2 (GAL2-T354A and GAL2-T354S). Surprisingly, all mutants were non-active in terms of glucose or xylose transport (Figure 2C). It seems that this particular conclusion from HXT14 engineering study cannot be readily applied to other sugar transporters by simply mutating threonine to alanine or serine. However, the disruptive nature of these mutations indicated that the fourth position of the YYX(T/P) motif is critical for sugar transporters. We argued that, because of the difference between sugar transporters, the specific mutation required in this position to change the sugar specificity is different in various sugar transporters. Therefore, we first performed saturation mutagenesis on residue T354 of GAL2. Because high xylose specificity is very hard to achieve and highly favored in sugar transporter engineering, in our screening, only mutants with comparable growth rate in glucose and xylose were selected. As a result, mutant GAL2-T354N reversed sugar specificity of WT GAL2 and had a 1.8-fold preference towards xylose (Figure 2C).

Inspired by our success with GAL2 engineering, we sought to apply the same strategy to HXT2, which is the most efficient native glucose and xylose transporter (Young et al. 2014). We screened the saturation mutagenesis library of T339 residue of HXT2 and identified a few mutants with dramatically
reversed sugar specificity. When T339 was mutated to glycine or phenylalanine, the glucose transport capacities of the mutant transporters reduced by 41-fold and 46-fold respectively. Single mutants HXT2-T339G and HXT2-T339F decreased their glucose specificity by 14-fold and 18-fold respectively, and now had ~2-fold preference towards xylose. Interestingly, another HXT2 mutant was selected in our screening effort, which later was determined to have two mutations, T339P and M420I (HXT2-T339P-M420I). The second mutation M420I was likely introduced via PCR or spontaneous mutagenesis. We also determined the kinetic parameters of HXT2WT and HXT2-T339P-M420I via sugar uptake assay (Table 1). As a result, compared with HXT2WT, HXT2-T339P-M420I had similar K_m and ~75% V_max value towards D-xylose, which was consistent with its slightly decreased growth rate in xylose. On the other hand, the K_m value of HXT2-T339P-M420I towards glucose was significantly increased (6.4-fold) to a similar level as D-xylose, and its V_max value was also decreased by 4.5-fold, which supported our observation that HXT2-T339P-M420I had a higher growth rate in xylose than glucose. Because HXT2-T339P-M420I had similar affinity towards glucose and xylose, and it had the highest growth rate in xylose among selected HXT2 mutants, it became the best candidate for further application in glucose-xylose co-fermentation.

To analyze the double mutant, we generated single mutants HXT2-T339P and HXT2-M420I, and determined their growth rates in glucose or xylose. Unexpectedly, both single mutants had similar glucose transport capacity and slightly lower xylose transport capacity compared to wild type HXT2. It was an interesting example of synergistic effect of single mutations that led to dramatic phenotype change in the double mutant (Reetz 2013). T339P mutation is of particular interest. Based on sequence alignment, at the fourth position of the YYX(T/P) motif, proline is tolerated and frequently appeared (Figure 1 and Figure S1). Actually, a few xylose specific transporters (Ec.XylE and Nc.AN25) (Du et al. 2010; Sun et al. 2012), and sugar transporters with high xylose transport activity (Ss.XUT3) (Young
et al. 2014) have proline at the fourth position (Figure 1). However, proline is not exclusively required for high xylose specificity and transport capacity. Residue M420 of HXT2 is not near the sugar binding site or within the solvent-accessible channel. However, it is part of another discontinuous helix TM10, which was proposed to facilitate protein dynamics during sugar transport (Sun et al. 2012). It may explain the synergistic effect of mutation M420I and mutation T339P.

**Engineered HXT2 mutant enabled glucose-xylose co-fermentation in *S. cerevisiae***

Glucose-xylose co-fermentation is extremely hard to achieve. The major challenge is that sugar transporters usually have high affinity towards glucose than xylose (Young et al. 2011; Young et al. 2012). In addition, even engineered sugar transporters with high xylose specificity still have significant glucose inhibition (Young et al. 2014), which impaired xylose transport in the presence of glucose. Because our engineered sugar transporter had higher xylose transport capacity than glucose, we sought to evaluate whether our engineered transporter can achieve xylose-glucose co-fermentation. We performed high cell-density xylose-glucose co-fermentation with MW01 strain carrying either HXT2WT or mutant HXT2-T339P-M420I. As shown in Figure 2D, the HXT2WT transporter could transport glucose very efficiently such that all glucose was transported inside the cell and consumed within 12 hours. During that time period, only 2.2 g/L xylose was consumed, which was due to the extreme low concentration of glucose at the end of glucose utilization phase. It would not be surprising that under continuous co-fermentation condition, almost no xylose would be utilized. On the other hand, the engineered mutant HXT2-T339P-M420I consumed glucose and xylose simultaneously with very similar rates (0.062 g/L/h for glucose and 0.052 g/L/h for xylose). Compared with previously reported sugar transporters with high xylose specificities, which showed either significant diauxic shift or no glucose-xylose co-utilization (Young et al. 2012; Young et al. 2014), our engineered HXT2 transporter demonstrated genuine co-fermentation under a high sugar concentration condition.
Admittedly, the co-consumption rate was very low due to the low activity of the HXT2 mutant, which seems to be a universal trade-off of high xylose specificity for engineered sugar transporters (Farwick et al. 2014; Young et al. 2014). It is likely because the reversal of specificity usually only happens at the expense of overall activity. However, such disadvantage can be compensated via further engineering through directed evolution (Farwick et al. 2014; Wang et al. 2016). Taken together, our engineering effort provided encouraging evidences that not only high xylose specificity can be obtained via rewiring the YYX(T/P) motif of sugar transporters, glucose-xylose co-fermentation is also achievable, which is another significant step towards obtaining a highly active xylose specific sugar transporter that would enable glucose-xylose co-utilization in a *S. cerevisiae* strain with a full set of native sugar transporters.

**Discussion**

On the road of pursuing economical biorefinery, the simultaneous and efficient co-utilization of glucose and xylose is a major challenge (Kim et al. 2012). Efficient xylose transporters without glucose inhibition would be a major part of the solution. However, our understanding of the structure-activity/specificity relationship of sugar transporters are very limited. In this study, we identified a YYX(T/P) motif for controlling the activity and specificity in sugar transporters. This motif is highly conserved in MFS family of monosaccharide transporters. By manipulating residues of this motif, either improved glucose transport or high xylose specificity can be achieved across different sugar transporters. In addition, our engineered mutant transporter enabled a genuine glucose-xylose co-fermentation in *S. cerevisiae*.

Because glucose utilization is already extremely efficient in *S. cerevisiae*, all previous studies on sugar transporter engineering were focused on xylose transport, which led to general overlook of occasional
mutant transporters with improved glucose transport capacity (Young et al. 2012; Young et al. 2014). However, glucose transport is the rate-limiting step in glucose utilization (Ozcan and Johnston 1999). Therefore, whether over-expressing a mutant transporter with improved glucose transport capacity would also improve glucose fermentation is still largely an open question. We have demonstrated that mutating tyrosine to phenylalanine in the YYX(T/P) motif provided almost universal improvement of glucose transport in several different sugar transporters, among which HXT2 is the best known glucose transporter in *S. cerevisiae* (Young et al. 2014). Therefore, over-expressing HXT2 mutant with improved glucose transport in *S. cerevisiae* to further improve glucose fermentation would be an interesting direction for future research. The exclusively conserved tyrosine residue at the second position of the YYX(T/P) motif had been proposed to prevent the escape of xylose to the extracellular side in the xylose transporter XylE from *E. coli*. However, the impaired function of XylE-Y298A mutant is not sufficient to support such conclusion (Sun et al. 2012). Instead, our study of the tyrosine to phenylalanine mutation indicated that their *V*<sub>max</sub> values were significantly increased, which suggested that the hydroxyl groups of the two tyrosine residues may interact with the glucose molecule during sugar transport so that the two tyrosine residues may act as a “gate” to restrict the diffusion of glucose to its binding site. Interestingly, during the preparation of this manuscript, Wang et al. discovered that an aromatic-residue rich motif YFFYY (position 332-336) of Mgt05196p from *Meyerozyma guilliermondii* plays an important role in xylose transport activity (Wang et al. 2015). Based on sequence alignment, the last two tyrosine residues of YFFYY motif take the same positions as the first two tyrosine residues in our YYX(T/P) motif. These two complementary studies demonstrated that the TM7 is critical for sugar transport activity.

Engineering sugar transporter towards higher xylose transport capacity and low glucose inhibition was the focus of several past studies. Young *et al.* demonstrated that the sugar specificity of transporters can
be altered via modifying a conserved motif (G-G/F-XXX-G) on TM1 (Young et al. 2014). This motif, including a sugar-binding residue F24 (position according to XylE), is also part of the solvent-accessible channel. Large and nonpolar residue substitutions in this motif were proposed to attenuate glucose transport via steric exclusion (Young et al. 2014). On the other hand, our study on the YYX(T/P) motif focused on a different transmembrane segment (TM7). In previous studies, mutations of N376 from TM7 of GAL2 can also eliminate glucose transport (Farwick et al. 2014). In our homology model, the YYX(T/P) motif formed part of the entrance of solvent-accessible channel. Unfortunately, no universal mutation that can reverse sugar specificity towards xylose was obtained. However, we demonstrated that the fourth position of YYX(T/P) motif was a “hot-spot”, and when subjected to saturation mutagenesis, mutants with reversed sugar specificity towards xylose can be obtained. It is likely due to the difference between sugar transporters so that different mutations are required at the fourth position to confer high xylose specificity.

Our discovery of mutant HXT2-T339P-M420I with high xylose specificity was an accident. The high glucose specificity of both single mutations (HXT2-T339P and HXT2-M420I) illustrated the synergistic effect of both mutations in reducing glucose transport and inhibition. During the preparation of this manuscript, several studies also demonstrated glucose-xylose co-utilization using engineered sugar transporter HXT36-N367A (Nijland et al. 2014), Mgt05196p-N360F (Wang et al. 2015), and a highly mutated Gal2 (Reznicek et al. 2015). Here we demonstrated that our engineered transporter HXT2-T339P-M420I, which contained mutations at different positions compared to these previously reported engineered transporters, enabled genuine glucose-xylose co-fermentation with similar glucose and xylose consumption rates. It indicated that there is more than one way to alter the specificity of sugar transporters to facilitate glucose-xylose co-utilization. In our co-fermentation experiment, xylose consumption occurred when glucose concentration was still relatively high (at least >2 g/L). Therefore,
the co-utilization phenotype is not due to the rapid depletion of glucose which resulted in unbalanced glucose/xylose ratio leading to favored xylose transport. Instead, the simultaneous utilization of glucose and xylose suggested HXT2-T339P-M420I can transport glucose and xylose with similar rate under the co-fermentation condition. Such property of the mutant transporter is highly desired considering glucose concentration is always high in continuous fermentation. Indeed, sugar consumption rate is very low in the co-fermentation, which is due to the low activity of the engineered transporter. However, directed evolution can be applied to improve the activity of mutant transporters to achieve a more efficient glucose-xylose co-fermentation.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

All the experiments were performed by MW and CY. All authors (MW, CY and HZ) contributed to designing the experiments, writing the manuscript and have approved the final manuscript.

**Acknowledgements**

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References


Table 1. Kinetic parameters of selected sugar transporters

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N.D.: Not determined.
Figure legends

Figure 1. Multiple sequence alignment of sugar transporters. Amino acid number is assigned according to Ec.XylE.

Figure 2. Characterization of sugar transporters and their mutants. (A) Exponential growth rates and sugar specificity of HXT14WT and its mutants. (B) Characterization of the tyrosine to phenylalanine mutation in HXT10, GAL2 and HXT2. (C) Engineering the fourth position in the YYX(T/P) motif to alter substrate specificity of sugar transporters. (D) Glucose-xylose co-fermentation using S. cerevisiae MW01 strain carrying an engineered sugar transporter. Asterisks indicate no measurable cell growth. Specificity is defined by exponential growth rate in glucose divided by that of xylose.

Figure 3. Homology modeling of HXT14. (A) Side view of HXT14; Y362, Y363 and T365 are located at the same helix as part of a solvent-accessible channel. N387 is in close contact with glucose. (B) Top view of the molecular surface of HXT14. Red (solvent-accessible channel surface formed by YYGT motif of HXT14). Green (Glucose).
Figure 1

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Figure 2
Figure 3