Directed Evolution of Xylose Specific Transporters to Facilitate Glucose-Xylose Co-Utilization

Meng Wang,1 Chenzhao Yu,2 Huimin Zhao1,3,4
1Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
2School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
3Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
4Departments of Chemistry, Bioengineering, and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

ABSTRACT: A highly active xylose specific transporter without glucose inhibition is highly desirable in cost-effective production of biofuels from lignocellulosic biomass. However, currently available xylose specific transporters suffer from low overall activity and most are inhibited by glucose. In this study, we applied a directed evolution strategy to engineer the xylose specific transporter AN25 from Neurospora crassa with improved xylose transportation capacity. After four rounds of directed evolution using two different strategies, we obtained an AN25 mutant AN25-R4.18 with 43-fold improvement in terms of xylose transportation capacity while maintaining its high xylose specificity. In addition, glucose inhibition was almost completely eliminated in the final evolved mutant. We demonstrated that improved xylose transportation of AN25 mutants in the exponential growth phase led to significant improvement of xylose consumption in high cell-density fermentation. Finally, we showed that AN25 mutant AN25-R4.18 can enable relatively efficient glucose-xylose co-utilization in high concentrations of mixed sugars.

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KEYWORDS: directed evolution; xylose specific transporter; glucose-xylose co-utilization

Introduction

The hope of replacing fossil fuels with biofuels has provided a strong motivation for both academic and industrial researches (Buijs et al., 2013; Kallio et al., 2014; Peralta-Yahya et al., 2012; Rabinovitch-Deere et al., 2013; Stephanopoulos, 2007). Currently, cost-effective lignocellulosic biofuel production is a popular target because lignocellulosic biomass is abundant, cheap, and not a food source (Kim and Dale, 2004; Limayem and Ricke, 2012). As one of the primary microbial cell factories in industrial fermentation, Saccharomyces cerevisiae (Weber et al., 2010) can efficiently convert glucose from lignocellulosic biomass to bioethanol. However, native S. cerevisiae does not readily consume D-xylose, which is the second most abundant sugar.

After decades of researches, efficient xylose fermentation has been partially achieved by introducing a heterologous xylose utilization pathway (Kim et al., 2013), balancing cofactors (Watanabe et al., 2007), and optimizing the flux of the xylose utilization pathway (Du et al., 2012; Latimer et al., 2014) in S. cerevisiae. However, efficient xylose utilization was achieved only when xylose was used as the sole carbon source. In a more industrial relevant setting, in which both glucose and xylose are present, xylose utilization initiates only after the depletion of glucose (Carlson, 1999; Trumbly, 1992). The diauxic shift is mostly due to the biased sugar uptake system in the native S. cerevisiae. All native sugar transporters in S. cerevisiae have significant higher preference to glucose than xylose (Young et al., 2011). The diauxic shift is very common among xylose-consuming species ranging from bacteria, such as Escherichia coli (Deutscher, 2008), to yeast, such as Pichia stipites (Delgenes et al., 1989). However, it is not desirable in glucose-xylose cofermentation since it will reduce the productivity of biofuels production.

To address such limitation, a xylose specific transporter that can efficiently transport xylose in the presence of high glucose concentration is highly desirable. Nonetheless, previous efforts in discovering native xylose specific transporter turned out to be less than fruitful (Leandro et al., 2009; Young et al., 2010). Most native sugar transporters have high preference towards glucose and high
glucose inhibition on xylose transportation (Leandro et al., 2009; Young et al., 2011). In addition, only a handful of xylose specific transporters have been discovered and they suffer from very low overall activities (Du et al., 2010). While bioprospecting failed, directed evolution has been used to engineer xylose specific transporters (Farwick et al., 2014; Nijland et al., 2014; Wang et al., 2015; Young et al., 2012). These directed evolution efforts have been focused on engineering highly active glucose transporters with xylose transportation capacity towards xylose specific transporters by reducing their glucose transportation capacities (Young et al., 2014) as well as glucose inhibition (Farwick et al., 2014; Nijland et al., 2014). Although several xylose specific transporters have been obtained, their common drawback is dramatically decreased xylose transportation capacities (Farwick et al., 2014; Nijland et al., 2014; Young et al., 2014) and most of them have significant glucose inhibition (Young et al., 2014).

In this study, in order to obtain an efficient xylose specific transporter without glucose inhibition, we sought to improve the xylose transportation capacity of a native xylose specific transporter using directed evolution. We chose our previously discovered xylose specific transporter AN25 from Neurospora crassa as a target (Du et al., 2010). We applied two different directed evolution strategies in our first three rounds of directed evolution and improved the xylose transportation capacity of AN25 by 45-fold. In the last round of directed evolution, we obtained mutant AN25-R4.18 with significantly reduced glucose inhibition. The $K_m$ value of AN25-R4.18 towards D-xylose was 5.4-fold lower than that of wild type AN25, while the $V_{max}$ value of AN25-R4.18 was twofold higher. We demonstrated that the improvement of xylose transportation capacities in each round of directed evolution led to increased xylose utilization rates in high cell-density xylose fermentation. In addition, high xylose specificity of AN25 was preserved during four rounds of directed evolution. Finally, to illustrate that AN25 mutant can enable glucose-xylose co-utilization, we co-expressed glucose transporter HXT14 with mutant AN25-R4.18 and achieved simultaneous and relatively efficient glucose-xylose co-fermentation in S. cerevisiae.

### Materials and Methods

#### Strains, Plasmids, Media, and Cultivation Conditions

*S. cerevisiae* HZ848 (MATα, ade2-1, Δura3, 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 *S. cerevisiae* MW01 (CEN.PK2-1C Δhxt1-17 Δstl1 Δagt1 Δydl247w Δyjr160c ∆gal2, aka VW4000, a gift from Dr. Eckhard Boles in Goethe University Frankfurt) was used as the host for sugar transporter characterization, directed evolution and high cell-density fermentation. See supplementary material for more details on media and cultivation conditions.

#### Plasmid Construction

To construct helper plasmid pRS416-X-helper for transporter cloning and mutants library creation, single copy plasmid harboring the xylose utilization pathway, pRS416-X (Du et al., 2012), was linearized by SacII. Helper plasmid pRS416-X-helper was first constructed by inserting HXT7p promoter and HXT7t terminator into linearized pRS416-X by the DNA assembler method (Shao et al., 2009). The cloning of the wild-type AN25 and its mutants were performed by inserting PCR amplified AN25 gene fragments into SacII linearized pRS416-X using DNA assembler method. See supplementary material for detailed plasmids construction. All plasmids were summarized in Table I.

#### Directed Evolution

Four rounds of directed evolution were carried out using a screening method similar to what was described elsewhere (Young et al., 2012), except in the fourth round, both glucose and xylose were used as carbon sources. Cell growth assay was performed to validate selected positive mutants and the high cell-density fermentation assay was used to characterize best mutants in each round of directed evolution. See supplementary material for detailed description of our directed evolution method, cell growth assay, and high cell-density fermentation assay.

### Table I. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pRS416-X</td>
<td>pRS416-xylose pathway</td>
<td>Du et al. (2012)</td>
</tr>
<tr>
<td>pRS416-X-helper</td>
<td>pRS416-X-HXT7p-SacII-HXT7t</td>
<td>This study</td>
</tr>
<tr>
<td>pRS416-X-helper-AN25WT</td>
<td>wild type AN25</td>
<td>This study</td>
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<tr>
<td>pRS416-X-helper-AN25-R1.1</td>
<td>First round mutant #1 of AN25</td>
<td>This study</td>
</tr>
<tr>
<td>pRS416-X-helper-AN25-R1.4</td>
<td>First round mutant #4 of AN25</td>
<td>This study</td>
</tr>
<tr>
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<td>Combined all amino acid mutations from R1.1 and R1.4</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
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<tr>
<td>pRS416-X-helper-AN25-E141D</td>
<td>Single mutation E141D of AN25</td>
<td>This study</td>
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<td>Single mutation S257I of AN25</td>
<td>This study</td>
</tr>
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<td>Single mutation S280G of AN25</td>
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<td>pRS416-X-helper-AN25-R4.18</td>
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<td>Cloned glucose transporter HXT14</td>
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<tr>
<td>pRS416-X-helper-HXT7</td>
<td>Cloned glucose transporter HXT7 as a positive control</td>
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[14C]-Xylose Uptake Assay

Xylose uptake assay was performed using the oil-stop method. The sugar uptake rate was calculated to be mmol sugar transported per hour per gram dry cell weight (DCW). The Michaelis–Menten equation was fitted to the sugar uptake rate and substrate concentration data by non-linear regression. See supplementary material for detailed description of the xylose uptake assay.

Results

First Round of Directed Evolution to Improve Xylose Transportation of AN25

Previously, we identified and characterized a xylose specific transporter AN25 from N. crassa (Du et al., 2010). Unlike most other native sugar transporters, AN25 is highly xylose specific and does not transport glucose at all. However, AN25 suffers from very low overall activity, which barely supports cell growth of sugar transporters knockout strain. Nonetheless, high xylose specificity is a rare and advantageous property among sugar transporters. We sought to improve xylose transportation capacity of AN25 while maintaining its xylose specificity using directed evolution. Similar to a previous report (Young et al., 2012), we coupled the xylose transportation capacity of a sugar transporter with cell growth when xylose was used as the sole carbon source. We screened a library of 10^4 AN25 mutants based on colony size and cell growth activity. From the first round of directed evolution, we obtained two AN25 mutants (AN25-R1.1 and AN25-R1.4) that had the highest exponential growth rates (the specific growth rate measured in the exponential phase of cell growth) in xylose compared to wild type AN25 (AN25WT) (Fig. 1). AN25-R1.1 was a quadruple mutant (S257I, S280G, Y453C, and E494V) (Supplementary Table S2) with 12.3-fold improvement in terms of exponential growth rate in xylose. AN25-R1.4 was a double mutant (T63A and E141D) (Supplementary Table S2), which conferred 14.6-fold higher xylose transportation capacity. While the xylose transportation capacities were significantly improved, mutants AN25-R1.1 and AN25-R1.4 still did not support cell growth in glucose (data not shown), which indicated that high xylose specificity was not compromised in those two mutants.

Mutational Analysis and Combinatorial Engineering of First Round Mutants

In directed evolution, the next round of evolution usually uses the best mutant from the previous round as the template. However, a high throughput screening similar to the first round of directed evolution had similar activities, we decided to combine the beneficial mutations from those two mutants and create a better mutant before the next round of random mutagenesis. To assess which mutation might be beneficial, we first generated all six single mutations appeared in AN25-R1.1 and AN25-R1.4 (AN25-T63A, AN25-E141D, AN25-S257I, AN25-S280G, AN25-Y453C, and AN25-E494V). One possibility is that other mutations were either neutral or synergistically contributed to the improved activity of AN25-R1.1 and AN25-R1.4.

To combine beneficial mutations, we constructed a combinatorial library of the mutations from AN25-R1.1 and AN25-R1.4 using the staggered extension process (StEP) method (Zhao et al., 1998). We performed a high throughput screening similar to the first round of directed evolution and denoted as the second round of evolution. Compared to the parent strains, we identified thirteen faster growing mutant strains (Supplementary Table S3) and mutant AN25-R2.StEp29 conferred the highest exponential growth rate in xylose. AN25-R2.StEp29 was also better than AN25-R1.(1 + 4), which contained all six amino acids mutations from the two parents. AN25-R2.StEp29 is a triple mutant (T63A, S280G, and Y453C) (Supplementary Table S2), in which T63A came from AN25-R1.4 while S280G and Y453C were inherited from AN25-R1.1. Both parent mutants contributed to AN25-R2.StEp29 and provided a 41-fold improvement compared to AN25WT (Fig. 1). See Supplementary Figure S1 and supplementary discussion on frequencies of single mutations in all thirteen faster growing mutants.

Further Engineering of AN25 to Reduce Its Glucose Inhibition

After the second round of directed evolution using combinatorial engineering, we performed another round of directed evolution to further improve the xylose transportation capacity of AN25. Using AN25-R2.StEp29 as the template, we performed error-prone PCR and created a random mutagenesis library with more than 1 × 10^4 mutants. The best mutant AN25-R3.65 identified in this round of directed evolution only provided a marginal improvement (12% compared to AN25-R2.StEp29). So far, our engineered AN25 mutants maintained high xylose specificity and cannot support cell growth in glucose (data not shown). However, glucose inhibition is a common trait in native and engineered highly active xylose transporters (Young et al., 2012, 2014), which hampers their application in glucose-xylose co-fermentation. Therefore, we tested whether AN25-R3.65 also had glucose...
inhibition. By comparing the exponential cell growth rate in xylose containing medium with or without the presence of glucose, we found that AN25-R3.65 indeed suffered from marginal glucose inhibition (30% reduced growth rate) (Fig. 2). Glucose inhibition is a significant drawback, because it decreases or completely abolishes the xylose transportation in the presence of glucose in the co-fermentation condition. Therefore, we decided to perform another round of directed evolution to reduce the glucose inhibition of AN25-R3.65. Directed evolution has been used to reduce glucose transportation and inhibition of hexose transporters (Farwick et al., 2014; Nijland et al., 2014). In these studies, in order to develop a high throughput screening method, the glycolysis pathway must be disrupted so that glucose only acts as an inhibitor of xylose transportation instead of a carbon source for cell growth. However, in our case, the disruption of glycolysis pathway is not necessary, because AN25 and its variants do not transport glucose. We generated a random mutagenesis library of AN25-R3.65 using error-prone PCR. After screening more than $1 \times 10^4$ mutants, we obtained mutant AN25-R4.18. We determined the exponential growth rate of AN25-R4.18 in both xylose only medium and the mixed xylose-glucose medium. As a result, AN25-R4.18 maintained 96% xylose transportation capacity in xylose only medium compared to AN25-R3.65 (Fig. 1). On the other hand, in the presence of 20 g/L glucose, the xylose transportation of AN25-R4.18 is 30% higher than that of AN25-R3.65 and almost completely eliminated glucose inhibition (2.3% glucose inhibition). Such property may significantly improve the performance of AN25-R4.18 in the xylose-glucose co-fermentation. After the fourth round of directed evolution, we attempted another round of random mutagenesis and screening to further improve xylose transportation capacity. Unfortunately, no better mutant can be obtained. Then we determined the kinetic parameters of AN25WT, AN25-R3.65, and AN25-R4.18 via the $^{14}$C-labeled D-xylose uptake assay (Table II). Compared to wild-type AN25, our best mutant AN25-R4.18 has 5.4-fold higher affinity towards D-xylose and twofold higher $V_{max}$ which is likely the reason for the improved growth rate of AN25-R4.18 in xylose containing medium. In addition, we found that the $K_i$ value of glucose inhibition for AN25-R3.65 was 53.9 ± 6.4 mM while no glucose inhibition was observed for AN25-R4.18. These results are consistent with our previous conclusions based on cell growth rates.

### AN25 Mutants Improved Xylose Consumption in High Cell-Density Fermentation

Since the sugar uptake kinetics in the exponential phase might be different from high cell-density fermentation that is more relevant to industrial applications (Young et al., 2012, 2014), we sought to evaluate the performance of AN25 mutants under high cell-density fermentation in rich YPA medium containing 10 g/L xylose with an initial OD$_{600}$ of 10. As illustrated in Figure 3A and Supplementary Figure S2, AN25WT barely consumed any xylose during the tested period. It was likely that the slow growth of AN25WT only required a very small amount of xylose consumption, which cannot be detected compared to high xylose concentration background. On the other hand, every tested AN25 mutants showed clear xylose consumption phenotype. Unlike the reported engineering effort on C. intermedia GXS1 (Young et al., 2012), the exponential growth rate data was consistent with high cell-density fermentation in our study. Note that in our fourth round of directed evolution, we screened for mutants with reduced glucose inhibition, so it is not surprising that AN25-R4.18 and AN25-R3.65 had very similar xylose consumption rate.

### AN25 Mutants Enabled Glucose-Xylose Co-fermentation

The biggest advantage of using AN25 as a template for directed evolution is that AN25 is a xylose specific transporter that does not transport glucose. However, after three rounds of directed evolution, we found that AN25-R3.65 suffered from significant glucose inhibition (Fig. 2). It was likely that glucose merely acted as an inhibitor, which competitively bound to the sugar binding site but could not be transported (Sun et al., 2012). Such glucose inhibition phenomenon was reported in the native xylose specific transporter XyE (Henderson, 1990; Henderson and Maiden, 1990; Lam et al., 1980) as well as several engineered sugar transporters (Young et al., 2014). Our fourth round of directed evolution successfully eliminated glucose inhibition. To evaluate whether glucose inhibition was also reduced under the high cell-density fermentation condition, we performed glucose-xylose co-fermentation with AN25-R3.65 and AN25-R4.18 (Fig. 3B and Supplementary Fig. S3). Clearly, no glucose was consumed by either mutant, which indicated that our evolution effort did not compromise the high xylose specificity of AN25. In addition, AN25-R4.18 had a higher xylose consumption.
rate than AN25-R3.65 in the presence of glucose. It seems that AN25-R3.65 suffered from a prolonged lag phase (~24 h) compared to AN25-R4.18, which was likely due to the high glucose inhibition of AN25-R3.65. To the best of our knowledge, it is the first time that a *S. cerevisiae* strain was demonstrated to only consume xylose while no glucose was utilized under high cell-density glucose-xylose co-fermentation.

Because wild type AN25 and its mutants did not transport glucose, we could not demonstrate glucose-xylose co-utilization using *S. cerevisiae* MW01 strain carrying only the AN25 mutant. To mitigate this problem, we introduced a plasmid containing a weak glucose transporter HXT14 to support glucose transportation in the sugar transporter knockout strain. Plasmid pRS424-HXT7p-HXT14-HXT7t was co-transformed with pRS416-X-helper-AN25-R4.18 to *S. cerevisiae* MW01 strain (named AN25-R4.18+HXT14 in Fig. 3C), while pRS414-HXT7p-HXT14-HXT7t and pRS416-X-helper were also co-transformed as the control strain (named control (HXT14 only) in Fig. 3C). As shown in Figure 3C and Supplementary Figure S4, the control strain consumed glucose relatively rapidly while only a limited amount of xylose was used after glucose was significantly depleted. It was consistent with literature report that HXT14 was a glucose transporter with very low xylose transportation capacity (Young et al., 2011). On the contrary, when AN25-R4.18 was introduced in addition to HXT14, the resulted strain consumed glucose and xylose simultaneously. Similarly, glucose-xylose co-utilization was observed when sugar concentrations were much higher (70 g/L of glucose and 40 g/L of xylose, which are the typical concentrations in cellulosic hydrolysates) (Supplementary Fig. S5). Our results clearly demonstrated that the xylose transportation capacity of a xylose specific transporter can be significantly improved while maintaining its high xylose specificity. It is a significant step towards achieving efficient and simultaneous glucose-xylose co-fermentation in a *S. cerevisiae* stain with a full set of sugar transporters.

**Homology Modeling Based Mutation Analysis**

After four rounds of directed evolution, seven beneficial mutations have been identified, which led to an AN25 mutant with 43-fold improvement of xylose transportation and significantly reduced efficiency.
glucose inhibition. To better understand the molecular basis of those seven mutations, we built a homology model based on the crystal structure of XylE (Protein Data Bank Access Number: 4GBZ) from E. coli using Molecular Operating Environment (Chemical Computing Group, Montreal, Canada). XylE belongs to the major facilitator superfamily and acts as a xylose/H⁺ symporter (Henderson, 1990; Henderson and Maiden, 1990; Lam et al., 1980). The co-crystal structures of XylE with xylose or glucose were obtained (Sun et al., 2012), which may provide valuable insights on the activity and specificity of wild type AN25 and its mutants. In addition, XylE is a xylose specific transporter and glucose only acts as an inhibitor of XylE. Such property of XylE is strikingly similar to the mutant AN25-R3.65. Despite the relatively low sequence identity (27%) between AN25 and XylE, superimposition of the homology model of AN25 and the crystal structure of XylE indicated the high structural resemblance except for some shortened loop regions (Supplementary Fig. S6). As shown in Figure 4, mutations S280G, Y453C, A94T, E235G, and F332S were far away from the solvent-accessible channel, which suggested that they were not directly involved in sugar recognition and transportation. These five mutations may be involved in protein stability or dynamics. On the other hand, mutations T63A and I299T were part of the solvent-accessible channel that may have direct contact with the sugar molecule during the sugar transportation process. Residue T63 of AN25 belongs to the conserved transmembrane segment 2 (TM2). It is intriguing that multiple sequence alignment of sugar transporters (Supplementary Fig. S7) indicated that threonine residue is an exception rather than a common residue at this position (residue 63 according to AN25). Most sugar transporters have a valine or an isoleucine at this position. Mutation T63A changed a hydrophilic side chain to a hydrophobic side chain, which is similar to other sugar transporters. The change of polarity at position 63 of AN25 may explain the significantly improved xylose transportation capacity. However, it is not clear whether the threonine at residue 63 was responsible for the low activity of AN25.

Residue I299 is part of the conserved transmembrane segment 7 (TM7), which plays a critical role in controlling activity and specificity of sugar transporters. In the case of XylE, it was proposed that, three polar residues (Q288, Q289, and N294) and an aromatic residue Y298 of TM7 contributed to the recognition of xylose (Sun et al., 2012). More importantly, we recently discovered a YYX(T/P) motif (Supplementary Fig. S8) that controls activity and specificity of sugar transporters (unpublished data). The highly conserved YYX(T/P) motif belongs to TM7 and forms part of the solvent-accessible channel. We have demonstrated that mutating two tyrosine residues of the motif to phenylalanine respectively improved glucose transportation capacity across several different sugar transporters. We also illustrated that by engineering the fourth position in the YYX(T/P) motif, the sugar specificity of transporter was significantly altered or even reversed towards xylose. According to the multiple sequence alignment (Supplementary Fig. S8), the YYX(T/P) motif is YYFP in AN25, and it is right before residue I299. Our discovery of the important YYX(T/P) motif and the fact that the next residue I299 of AN25 contributed to glucose inhibition supported our conclusion that this part of TM7 had significant interactions with the sugar molecule during transportation and can be targeted as “hot spot” for future sugar transporter engineering effort.

**Discussion**

In the presence of glucose, xylose uptake is the rate-limiting step in xylose utilization by S. cerevisiae. Native sugar transporters of S. cerevisiae have much higher specificity towards glucose than xylose (Leandro et al., 2009; Young et al., 2011). In order to achieve
simultaneous glucose-xylose co-utilization, an alternative xylose transportation system that is highly active, xylose specific and not inhibited by glucose is highly desirable. Previous studies have been devoted to eliminating glucose transportation and glucose inhibition in hexose transporters while maintaining their xylose transportation capacity (Farwick et al., 2014; Nijland et al., 2014; Young et al., 2012, 2014). Young et al. (2014) have demonstrated that glucose transportation of hexose transporters such as GXS1 can be eliminated by rewiring the G-G/F-XXX-G motif. However, the best mutant, albeit highly xylose specific, failed to consume any xylose under a glucose-xylose co-fermentation condition. It is likely due to the high glucose inhibition and the low overall activity of the mutant transporter. Farwick et al. (2014) discovered that single mutation N376F can eliminate both glucose transportation and glucose inhibition in GAL2 and resulted in a xylose specific transporter. Nonetheless, the xylose transportation capacity of mutant GAL2-N376F was significantly reduced, which was likely the reason that no xylose fermentation or glucose-xylose co-fermentation was demonstrated. Interestingly, adaptation was used to engineer a quadruple hexokinase deletion S. cerevisiae strain to grow on xylose in the presence of glucose (Nijland et al., 2014). As a result, mutation N367A (same position as N376 of GAL2) of Hxt36 transporter was identified to be responsible for xylose uptake in the presence of glucose. Although the \( V_{\text{max}} \) of Hxt36 was reduced by \( \sim 50\% \) (similar to that of GAL2-N376F), marked glucose-xylose co-fermentation was illustrated in a low sugar concentration setting (5 g/L glucose and 5 g/L xylose).

Recently, we also demonstrated that xylose specific transporters can be obtained by engineering a YYX(T/P) motif of sugar transporters (unpublished data). Similarly, glucose-xylose co-utilization was enabled by mutant HXT2-T339P-M420I in 10 g/L glucose and 10 g/L xylose mixture. All published studies demonstrated that engineering highly active glucose transporters is a successful strategy, however, all engineered transporters have lower xylose transportation capacity compared to their wild types and most still suffer from significant glucose inhibition. In this study, we took a different approach by using xylose specific transporter AN25 as the starting point and improved its xylose transportation capacity by 43-fold through four rounds of directed evolution. The major advantage of using a xylose specific transporter was that high xylose specificity was no longer the screened phenotype, which seems to be hard to achieve without compromising the xylose transportation capacity of sugar transporters. Our study suggested that the high xylose specificity can be maintained during multiple rounds of directed evolution while the xylose transportation capacity can be significantly improved. In addition, S. cerevisiae strain with a disrupted glycolysis pathway was not necessary for our engineering strategy.

Glucose inhibition on xylose uptake is a very common property in sugar transporters. In native glucose transporters, glucose is a preferred substrate so that xylose can only be transported after the depletion of glucose. On the other hand, in the case of xylose specific transporters, such as XyIE from E. coli (Lam et al., 1980), engineered GXS1 (Young et al., 2014) and our AN25-R3.65, glucose is not a substrate and acts only as an inhibitor. Interestingly, the glucose inhibition of XyIE and GXS1 mutant is so severe that no xylose consumption can be observed in the presence of glucose. However, AN25-R3.65 only suffered moderate inhibition, which only delayed instead of completely shutting down xylose consumption. In addition, glucose inhibition of AN25-R3.65 can be almost completely eliminated by mutation I299T. Mutating arginine in the “hot spot” (N376 of GAL2) seems to be an effective means to reduce glucose transportation and glucose inhibition in S. cerevisiae hexose transporters. However, at least 50–70% xylose transportation capacity was lost (Farwick et al., 2014; Nijland et al., 2014), which significantly reduced the effectiveness of resulted transporters in glucose-xylose co-fermentation. However, mutation I299T in AN25-R4.18 did not decrease the xylose transportation capacity at all, which enabled relatively efficient glucose-xylose co-fermentation in our study. Therefore, it is possible that the same position at I299 of AN25 can serve as an alternative “hot spot” for eliminating glucose inhibition in the engineering of other sugar transporters. Noted, unlike N376 of GAL2, I299 of AN25 is not a highly conserved residue among all sugar transporters (Supplementary Fig. S8). Therefore, saturation mutagenesis on this position couple with screening might be required in order to obtain sugar transporter mutants with reduced glucose inhibition. It was surprising that I299 of AN25 resided next to our previously discovered YYX(T/P) motif, which controls the activity and specificity of sugar transporters. But the fact that our studies of different transporters converged towards the same region, which is clearly part of the solvent-accessible channel of sugar transporters, strongly suggested that this region plays an important role in sugar transportation and merits significant further investigations.

In previous sugar transporter engineering studies, the improved exponential growth rates and the xylose consumption rates under high cell-density fermentation were not always consistent (Young et al., 2012). It is likely because the metabolic states are different between the exponential growth phase and the stationary phase. It could be a potential problem, because all current directed evolution methods relied on screening cells in the exponential growth phase (Farwick et al., 2014; Nijland et al., 2014; Wang et al., 2015; Young et al., 2012), while the most desired phenotype is xylose consumption in the stationary phase. However, in our study, the exponential growth rate data and xylose fermentation data were highly consistent. Each round of directed evolution improved both the growth rate in the exponential phase and the xylose consumption rate in high cell-density fermentation.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.