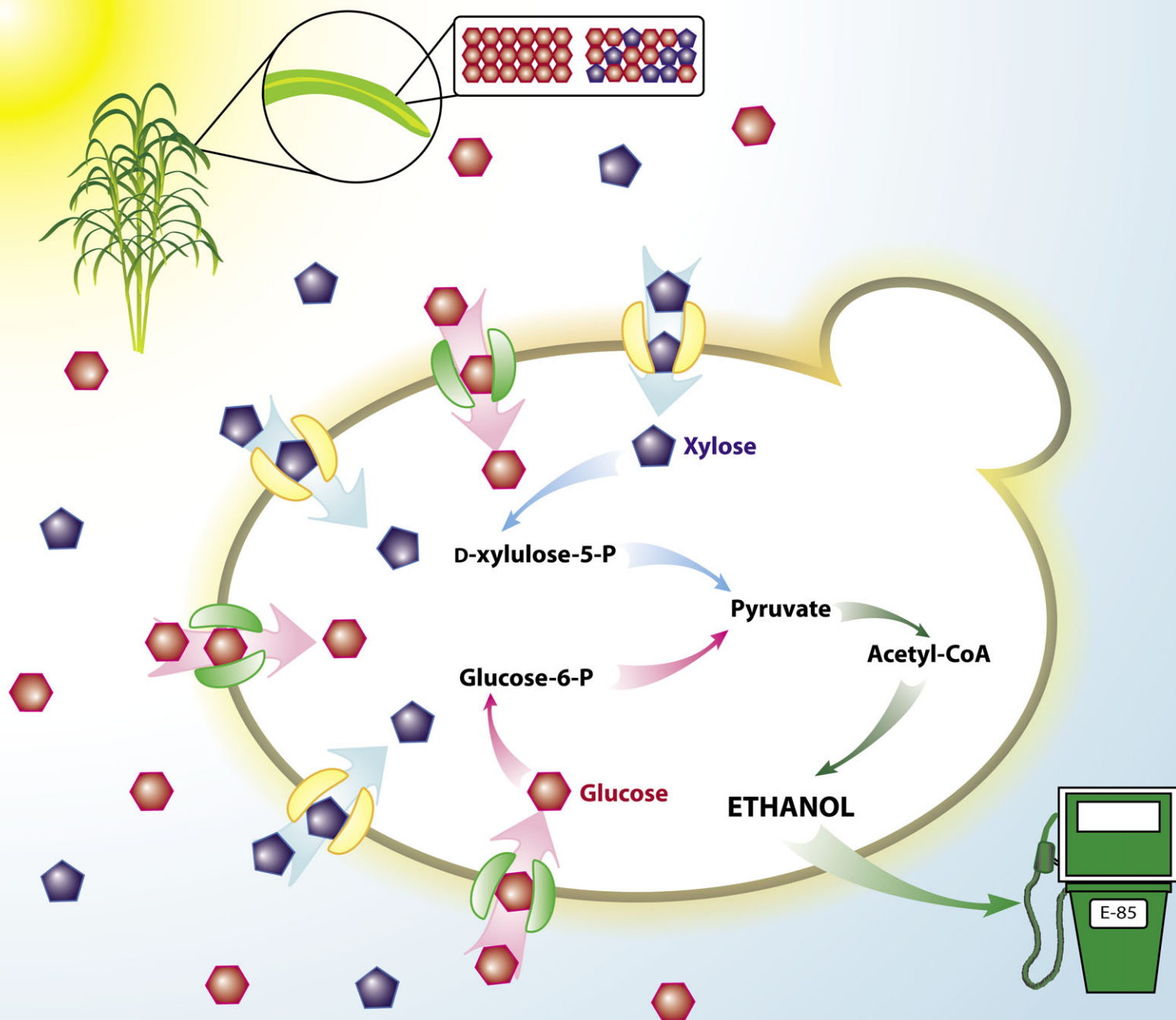


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Discovery and characterization of novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*

Discovery and characterization of novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*†

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Saccharomyces cerevisiae is considered one of the most promising organisms for ethanol production from lignocellulosic feedstock. Unfortunately, pentose sugars, which comprise up to 30% of lignocellulose, cannot be utilized by wild type *S. cerevisiae*. Heterologous pathways were introduced into *S. cerevisiae* to enable utilization of D-xylose, the most abundant pentose sugar. However, the resulting recombinant *S. cerevisiae* strains exhibited a slow growth rate and poor sugar utilization efficiency when grown on D-xylose as the sole carbon source. D-xylose uptake is the first step of D-xylose utilization. D-xylose can only enter yeast cells through hexose transporters, which have two orders of magnitude lower affinity towards D-xylose compared to hexoses. It was also shown that inefficient pentose uptake is the limiting step in some D-xylose metabolizing yeast strains. Here we report the cloning and characterization of two novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*. These two transporters were identified from a total of 18 putative pentose transporters. They were functionally expressed and properly localized in *S. cerevisiae* as indicated by HPLC analysis and fluorescence confocal microscopy, respectively. Kinetic parameters of the D-xylose-specific transporters were determined using a ¹⁴C-labeled sugar uptake assay. Use of pentose-specific transporters should improve D-xylose consumption and ethanol production in fast D-xylose assimilating strains, thereby lowering the cost of lignocellulosic ethanol production.

Introduction

Biofuels are under intensive investigation due to increasing concerns about energy security, sustainability, and global climate change.¹ Biological conversion of plant-derived lignocellulosic materials into biofuels has been regarded as an attractive alternative to chemical production of fossil fuels.^{2,3} *Saccharomyces cerevisiae*, also known as baker's yeast, has been used for bioconversion of hexose sugars into ethanol for thousands of years. It is also the most widely used microorganism for large scale industrial fermentation of D-glucose into ethanol. *S. cerevisiae* is an excellent organism for bioconversion of lignocellulosic biomass into biofuels.⁴ It has a well-studied genetic and physiological background, ample genetic tools, and high tolerance to ethanol and inhibitors present in lignocellulosic hydrolysates.⁵ Moreover, the low fermentation pH of *S. cerevisiae* can also prevent bacterial contamination. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. The hemicellulose component comprises 20–30% of lignocellulosic biomass, and it is primarily composed of five-carbon sugars (pentoses) such as D-xylose and L-arabinose.⁶ Unfortunately, wild type *S. cerevisiae* can not utilize pentose sugars.⁷ To overcome this limitation, pentose utilization pathways from pentose-assimilating organisms

have been introduced into *S. cerevisiae*, allowing fermentation of D-xylose and L-arabinose.^{8–13} However, efficient conversion of pentose sugars into biofuels is limited by multiple issues, including the cellular redox imbalance, the low influx to the pentose phosphate pathway, and the lack of efficient pentose transport into the cell.⁷

D-Xylose uptake through sugar transporters is the first step of D-xylose utilization in *S. cerevisiae*. However, D-xylose can only enter *S. cerevisiae* cells through the hexose uptake system, and with two orders of magnitude lower affinity than hexoses.¹⁴ As a result, D-xylose uptake in D-xylose-assimilating yeast strains is very slow and inhibited by D-glucose present in the growth media. D-Xylose uptake has been found to be the limiting step in some recombinant D-xylose-assimilating *S. cerevisiae* strains.¹⁵ Significant improvement of D-xylose uptake activity has also been found in an engineered *S. cerevisiae* strain for efficient D-xylose fermentation that was obtained through evolutionary engineering.¹⁶ Therefore, it is hypothesized that improving D-xylose uptake activity by introducing more efficient D-xylose transporters should enhance D-xylose utilization efficiency in recombinant *S. cerevisiae* strains.^{13,17}

Nature has evolved many pentose-assimilating fungal species in which both low affinity and high affinity sugar transport systems are present for pentose uptake.^{18–20} To improve D-xylose uptake, heterologous D-xylose transporters were introduced into recombinant *S. cerevisiae* strains. Leandro and coworkers discovered one high affinity D-xylose/D-glucose symporter (*GXS1*) and one low affinity D-xylose/D-glucose facilitator (*GXF1*) from *Candida intermedia* and characterized them in *S. cerevisiae* at the molecular level.¹⁸ It was observed that overexpression of the *Gxf1* transporter

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improved fermentation performance in a recombinant D-xylose-utilizing *S. cerevisiae* strain.²¹ Similarly, *S. cerevisiae* strains overexpressing heterologous D-xylose transporters from *Arabidopsis thaliana* showed up to 2.5-fold increased D-xylose consumption and 70% increased ethanol production.⁷ In addition, overexpression of the D-glucose transporter SUT1 from *Pichia stipitis* was also shown to improve ethanol productivity during D-xylose and D-glucose co-fermentation by a D-xylose-assimilating *S. cerevisiae* strain.²² However, none of these D-xylose transporters were D-xylose specific.

Motivated by the above studies, we sought to discover D-xylose specific transporters from pentose-assimilating fungal species. Using the DNA sequence of the newly characterized D-glucose/D-xylose symporter from *Candida intermedia* as a probe, we identified 18 putative pentose transporters from *Neurospora crassa* and *Pichia stipitis* and through a series of molecular biology and biochemical analysis experiments, we discovered two novel D-xylose-specific transporters.

Results and discussion

Genome mining and cloning of putative D-xylose-specific transporters from *P. stipitis* and *N. crassa*

To discover novel D-xylose-specific transporters, the gene encoding the D-glucose/D-xylose symporter GXS1¹⁸ was used as a probe in a BLAST search (<http://www.ncbi.nlm.nih.gov/>) against the sequenced genomes of *N. crassa* and *P. stipitis*. In order to identify more candidates, an uncharacterized putative L-arabinose-proton symporter from *Pichia stipitis* (*AUT1*, locus tag PICST_87108) was also used as a query for the BLAST search. The results were examined to eliminate any protein with known D-glucose uptake activity or activity other than sugar uptake. Using a cutoff of 25% sequence identity, 17 putative pentose transporter genes were identified (ESI, Table S1†), in addition to *AUT1* from *Pichia stipitis*. These putative pentose transporter genes shared 25–50% identity with either *GXS1* from *Candida intermedia* or *AUT1* from *Pichia stipitis*. All 18 putative pentose transporters were annotated as either sugar uptake proteins or hypothetical proteins with unknown activity. The D-glucose transporter genes *SUT1* and *SUT2* from *P. stipitis* were also cloned for comparison.

N. crassa and *P. stipitis* were cultivated in rich media supplemented with either D-xylose or L-arabinose. Total RNA was isolated and reverse transcribed into cDNA. Polymerase chain reaction (PCR) was used to amplify the putative transporter genes directly from cDNA. However, because the regulation mechanism and expression pattern were unknown for pentose transporters in fungal species, cDNAs encoding the putative pentose transporters were not always obtainable despite alteration of cultivation condition. In this case, primers were designed according to the corresponding cDNA sequences from GenBank and used to amplify the exons using genomic DNA as a template. Overlap-extension PCR was then used to assemble the exons into the full-length genes. The resulting PCR products were cloned into the pRS424 shuttle vector containing a *HXT7* promoter and a *HXT7* terminator using the DNA assembler method.²³ Yeast plasmids isolated from

transformants were retransformed into *E. coli* DH5 α , and isolated *E. coli* plasmids were first checked by diagnostic PCR using the primers used to amplify the original transporter genes. The entire open reading frames were submitted for sequencing to confirm the correct construction of the plasmids.

Identification of D-xylose-specific transporters

To determine the D-xylose uptake ability of yeast strains overexpressing putative pentose transporters, intracellular accumulation of D-xylose was measured using high performance liquid chromatography (HPLC).²⁴ It was reported that a *S. cerevisiae* strain without a D-xylose assimilating pathway is still able to uptake and accumulate D-xylose within the cell, though it cannot further metabolize it for growth or fermentation.²⁵ D-Xylose accumulated within yeast cells will be partially converted to xylitol due to the presence of an endogenous aldose reductase.²⁶ Both D-xylose and xylitol can be extracted using osmosis and analyzed using HPLC.²⁴

The sugar transporter deletion strain EB.Y.VW4000 was used for the D-xylose uptake assay. The EB.Y.VW4000 strain has a concurrent knockout of more than 20 sugar transporters and sensors including *HXT1-17* and *GAL2*.²⁷ Growth on D-glucose as the sole carbon source is completely abolished in this strain, while uptake of maltose through a different sugar transport system is retained. The EB.Y.VW4000 strain also exhibits minimal D-xylose uptake under HPLC assay conditions, which makes it a suitable host for the D-xylose uptake assay. Plasmids overexpressing the cloned putative pentose transporter genes were transferred into the EB.Y.VW4000 strain using the standard lithium acetate method²⁸ and single colonies were used for measurement of the sugar uptake activity. Cells were first grown up in SC-Trp media supplemented with 2% maltose and then harvested and washed before being transferred into fresh YPA media supplemented with 2% D-xylose. Samples of cell culture were collected after 24 h incubation with D-xylose. Cell pellets were washed to remove extracellular sugar, resuspended in water, and shaken at 37 °C for two days to extract intracellular sugar by osmosis. Supernatants of cell suspensions were filtered and analyzed by HPLC. The D-xylose uptake ability of putative pentose transporters was determined by summation of intracellular D-xylose and xylitol concentrations.

Since D-glucose will be metabolized once it enters a yeast cell, the D-glucose transport activity cannot be determined by measuring intracellular D-glucose concentration. However, the fact that the EB.Y.VW4000 strain cannot grow on media with D-glucose as the sole carbon source links the D-glucose uptake ability with cell survival. If the putative pentose transporter has D-glucose uptake activity, it should lead to cell growth of the EB.Y.VW4000 strain on media with D-glucose as the sole carbon source.

Using both HPLC and the functional assay, several putative pentose transporters were identified to have uptake activity towards D-glucose, D-xylose, or both. Introduction of Xyp37, Xyp33, An29-2 and Xy31 restored cell growth of the EB.Y.VW4000 strain on D-glucose. At the same time, Xyp37, Xyp33, An29-2 also exhibited D-xylose uptake activity.

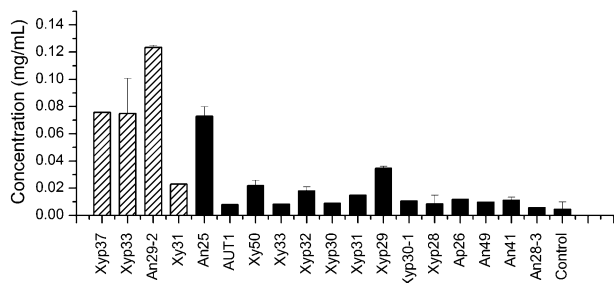


Fig. 1 Intracellular D-xylose accumulation of EBY.VW4000 strains harboring various putative pentose transporters. Shaded columns: putative transporter with D-glucose uptake activity. Solid columns: putative transporters without D-glucose uptake activity. The intracellular D-xylose accumulation was determined as mg sugar extracted through osmosis per mL of cell culture at $OD_{600} \sim 10$. Assay was performed in triplicates for different constructs. Error bar indicates the standard deviation of the triplicates.

The rest of the putative transporters failed to enable D-glucose uptake in the EBY.VW4000 strain. However, the EBY.VW4000 strains harboring Xyp29 and An25 were able to uptake and accumulate D-xylose within the cells, indicating that they are D-xylose-specific transporters (Fig. 1).

Characterization of D-xylose-specific transporters in *S. cerevisiae*

To further confirm that Xyp29 from *P. stipitis* and An25 from *N. crassa* are actually D-xylose-specific transporters with no D-glucose uptake activity, the ^{14}C -labeled sugar uptake assay was performed using ^{14}C -labeled D-glucose, D-xylose and L-arabinose as substrates. It was found that the D-glucose and L-arabinose uptake activities of the EBY.VW4000 strain over-expressing only Xyp29 and An25 are too low to be measured under assay conditions used to determine D-xylose uptake kinetics of both transporters (data not shown). Intracellular accumulation of both D-xylose and L-arabinose in the EBY.VW4000 strains expressing Xyp29 or An25 was also measured using HPLC. Cell cultures with 30 min, 60 min, 120 min, and 24 h incubation with pentose sugars were sampled and analyzed. As shown in Fig. 2, the EBY.VW4000 strains expressing Xyp29 or An25 only exhibited D-xylose uptake activity during 24 h incubation. The ^{14}C -labeled sugar uptake assay together with HPLC analysis of intracellular sugar accumulations confirmed that among the three most abundant monosaccharides in lignocellulosic hydrolysates, D-glucose, D-xylose, and L-arabinose, Xyp29 and An25 are responsible only for D-xylose uptake. Of note, most sugar transporters studied in yeast for D-xylose uptake have higher uptake activity towards D-glucose than D-xylose.^{18,22} Only the sugar transporter homologue *Trx1* isolated from *Trichoderma reesei* after adaptive evolution exhibited D-xylose-specific uptake activity.²⁹ Our data indicates that Xyp29 from *P. stipitis* and An25 from *N. crassa* are the first two experimentally confirmed naturally occurring D-xylose-specific transporters introduced to *S. cerevisiae*.

Kinetic parameters of D-xylose-specific transporters

Using the ^{14}C -labeled sugar uptake assay, kinetic parameters of D-xylose transport through An25 and Xyp29 were

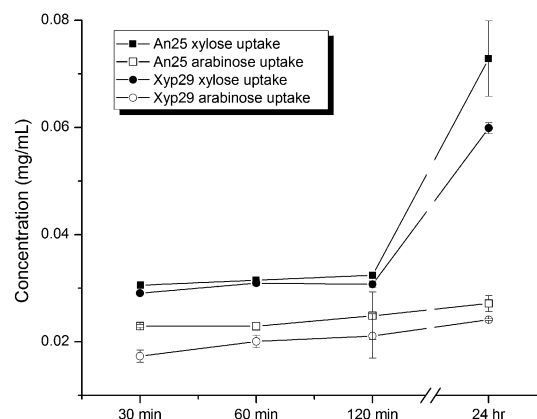


Fig. 2 Intracellular sugar accumulation of EBY.VW4000 strains harboring D-xylose-specific transporters. (■) D-xylose accumulation by An25, (□) L-arabinose accumulation by An25, (●) D-xylose accumulation by Xyp29, (○) L-arabinose accumulation by Xyp29. Assay was performed in triplicates for different constructs. Error bar indicates the standard deviation of the triplicates.

determined. It was observed that under the assay conditions, sugar uptake is linear with respect to time for the first 60 s (data not shown). The EBY.VW4000 strains expressing An25 or Xyp29 were incubated with D-xylose for 40 or 60 s, and sugar uptake was stopped by addition of ice-cold water. The reaction mixture was then filtered and washed before measurement using a liquid scintillation counter. The Michaelis–Menten equation was fitted to the sugar uptake rate and substrate concentration data by non-linear regression. The K_m values towards D-xylose for the EBY.VW4000 strains harboring only An25 or Xyp29 were 175.7 ± 21.4 mM and 56.0 ± 9.4 mM, respectively. The corresponding V_{max} values were 36.7 ± 2.9 $\mu\text{mol/h/gram}$ dry cell weight (DCW) and 41.5 ± 2.3 $\mu\text{mol/h/gram}$ DCW, respectively.

In naturally occurring D-xylose-assimilating fungal species, both the high affinity D-xylose-proton symport system and the low affinity D-xylose facilitated diffusion system are present. The K_m values of these two systems were determined to be 0.4–4 mM for the symport system and around 140 mM for the facilitated diffusion system.^{18,20} In comparison, the D-glucose uptake system in *S. cerevisiae* has K_m of 1.5 mM for the high affinity system and 20 mM for the low affinity system. Unfortunately, the D-xylose uptake affinity of wild-type *S. cerevisiae* is two orders of magnitude lower compared to D-glucose. The K_m values for D-xylose uptake in *S. cerevisiae* are only 190 mM for the high affinity system and 1.5 M for the low affinity system.¹⁴ The affinities of our newly discovered D-xylose-specific transporters are lower when compared to the high affinity D-xylose uptake system in naturally occurring D-xylose-assimilating fungal species. However, when compared with the D-xylose uptake system in the wild-type *S. cerevisiae*, An25 and Xyp29 showed higher affinity towards D-xylose. This is especially true for Xyp29, where the K_m towards D-xylose is only about one fourth of the value of the wild-type *S. cerevisiae*.

The K_m values of the D-xylose-specific transporters are also close to those of Gxf1 ($K_m = 88$ mM) and Sut1 ($K_m = 145$ mM), which have been shown to improve D-xylose fermentation in

recombinant *S. cerevisiae*.^{21,22} Thus, D-xylose fermentation may be improved by introducing these newly characterized D-xylose-specific transporters into *S. cerevisiae*.

Cellular localization of D-xylose-specific transporters

Sugar transporters are transmembrane proteins; correct folding and localization in the cell membrane is required for them to be functional. Since no signal peptide was specifically added when the putative pentose transporters were cloned, it was important to ensure that the D-xylose-specific transporters were correctly localized in the cell membrane. This is particularly true for putative pentose transporters like An25 that are cloned from the filamentous fungi *N. crassa*, which exhibits a very different physiology compared to *S. cerevisiae*. To study the cellular localization of D-xylose-specific transporters in *S. cerevisiae*, An25 and Xyp29 were fused with a green fluorescent protein (GFP) at their C-termini via a GS-linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Ser). The resulting plasmids were transferred into the wild-type *S. cerevisiae* strain CEN.PK2-1C, and fluorescent images were taken using a confocal microscope (Andor Technology, Belfast, Northern Ireland).

Yeast strains overexpressing D-xylose-specific transporters showed a distinctive fluorescent halo at the cell periphery (Fig. 3). For An25, almost all the GFP fluorescence appeared in the cell membrane, while a large portion of fluorescence of Xyp29 overexpressing cells remained in the cytoplasm. This may indicate inefficient export of the transporter due to an elevated expression of the membrane protein. It has also been noticed that not all the cells showed fluorescence (data not shown), indicating that the expression of the transporter was not optimized or stable. Further improvement of transporter expression can be achieved through altering the expression level and/or integrating the transporter genes into the genome of recombinant *S. cerevisiae*.

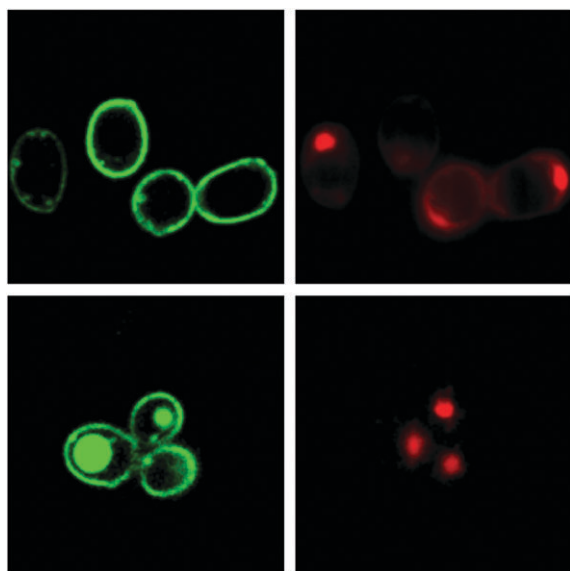


Fig. 3 GFP fluorescence images of transporter localization. First row from left to right: An25-GFP fluorescence, An25 nuclei; second row from left to right: Xyp29-GFP fluorescence, Xyp29 nuclei.

It is not surprising that D-xylose-specific transporters from other fungal species can be correctly expressed and localized in *S. cerevisiae*, given the fact that several transporters from various fungal species have already been introduced into *S. cerevisiae*.^{18,22} In fact, sugar transporters from more distinct species like *A. thaliana* can also be expressed and correctly localized in the *S. cerevisiae* cell membrane.⁷ This fact may indicate that different species use similar signal peptides for directing transporter proteins into the cell membrane.

An25 and Xyp29 are D-xylose facilitators

There are two types of sugar transporters in *S. cerevisiae*, symporters and facilitators. For symporters, sugar uptake is coupled with proton uptake. Sugar symporters usually exhibit high affinity toward sugars. On the other hand, sugar uptake through facilitators is not coupled with proton transport, and facilitators usually exhibit low sugar uptake affinities.¹⁸

Symporter assays were performed for An25 and Xyp29 in the EBY.VW4000 strain. No elevation of pH in un-buffered cell suspensions was observed for any of the D-xylose-specific transporters, indicating that D-xylose uptake through these transporters was not coupled with proton transport (ESI, Fig. S2†). In other words, An25 and Xyp29 are D-xylose facilitators.

This result is consistent with the fact that the kinetic parameters of An25 and Xyp29 are similar to those of the low affinity D-xylose facilitated diffusion system in naturally occurring D-xylose-assimilating yeasts. Despite the fact that symporters have higher affinities towards D-xylose, overexpression of symporters may not always facilitate sugar utilization by D-xylose-assimilating strains due to the ATP requirement to create the proton gradient. In fact, most of the transporters shown to be beneficial for D-xylose fermentation are facilitators.^{21,22}

Overexpression of D-xylose-specific transporters

The overexpression of active heterologous D-xylose-specific transporters in *S. cerevisiae* strains containing the D-xylose utilization pathway was also investigated. Unfortunately, the advantage of D-xylose-specific transporter overexpression could not be observed despite alteration of expression strategies, cultivation conditions, and choice of the D-xylose utilization pathway (data not shown). There are several possible reasons. First, the overexpression of membrane proteins, such as sugar transporters, could affect the integrity of the cell membrane and consequently hamper cell growth.³⁰ It was observed that transporter overexpression strains displayed a slower growth rate, even when D-glucose was used as a carbon source (data not shown). Second, the D-xylose uptake activity of wild-type *S. cerevisiae* through hexose transporters is much higher than that of the hexose transporter knockout strain expressing a particular D-xylose transporter. The low sugar transport activity of newly discovered D-xylose-specific transporters may make it hard to observe the improvement of sugar uptake ability. Third, even if the introduction of new D-xylose-specific transporters could improve the uptake of D-xylose into *S. cerevisiae* cells, the benefit of D-xylose utilization can only be observed when the D-xylose utilization pathway is efficient enough to make sugar uptake the limiting step. It has been shown that the effect of overexpression of sugar

transporters depends on the strain background and cultivation conditions.³¹

Conclusions

A total of 18 putative D-xylose transporters were identified through bioinformatic analysis using the newly characterized D-glucose/D-xylose symporter GXS1 from *C. intermedia* as a probe sequence. They were cloned and overexpressed individually in a hexose transporter knockout strain for sugar uptake assays. Together with a functional assay for D-glucose uptake, two D-xylose-specific transporters named An25 and Xyp29 were identified from *N. crassa* and *P. stipitis*, respectively.

An25 and Xyp29 are D-xylose-specific transporters with K_m towards D-xylose of 176 mM and 56 mM, respectively. Fluorescence imaging of C-terminal GFP-fused transporters showed that both transporters can be expressed and correctly localized in *S. cerevisiae*. D-Xylose uptake through An25 and Xyp29 in un-buffered solution did not result in an elevation of pH, indicating that these two transporters are facilitators.

However, we have not reproducibly observed the benefit of overexpressing the D-xylose-specific transporters in *S. cerevisiae*, probably due to the low transport efficiency or the slow D-xylose utilization rate in recombinant *S. cerevisiae* strains. To solve this problem, we are attempting to increase the efficiency of D-xylose-specific transporters and optimize the D-xylose utilization pathway in parallel.

An25 and Xyp29 are the first two experimentally confirmed naturally occurring D-xylose-specific transporters. By comparing the amino acid sequence of characterized D-xylose-specific transporters with other putative sugar transporters available in the public databases, more D-xylose-specific transporters may be discovered. This new group of sugar transporters will facilitate D-xylose utilization by recombinant *S. cerevisiae* strains. More efficient D-xylose utilization will significantly reduce the production cost of chemicals and fuels from lignocelluloses.

Experimental

Strains, media, and cultivation conditions

S. cerevisiae CEN.PK2-1C (MAT α *leu2-3,112 ura3-52 trp1-289 his3- Δ 1 MAL2-8^c*) was purchased from Euroscarf (Frankfurt, Germany) and used for manipulation of recombinant DNA in yeast. The sugar transporter knockout strain EBY.VW4000 (CEN.PK2-1C Δ *hxt1-17* Δ *stl1* Δ *agt1* Δ *yd1247w* Δ *yjr160c* Δ *gal2*) was a gift from Eckhard Boles.²⁷ *Escherichia coli* DH5 α (Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL) was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic drop-out media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.083% amino acid drop out mix). YPA media supplemented with 2% sugar was used to grow yeast strain harboring no plasmids (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate). *E. coli* strains were cultured in Luria broth (LB) (Fisher Scientific, Pittsburgh, PA).

S. cerevisiae strains were cultured at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition. Yeast strains were grown under aerobic condition for cell multiplication and assays. *E. coli* strains were cultured at 37 °C and 250 rpm unless specified otherwise. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

Plasmid and strain construction

Most of the cloning work was carried out using the yeast homologous recombination mediated DNA assembler method.²³ pRS424-HXT7-GFP plasmid was used for cloning of putative pentose transporters. In this plasmid, the HXT7 promoter, the GFP gene flanked with the *EcoRI* sites at both ends, and the HXT7 terminator were assembled into the pRS424 shuttle vector (New England Biolabs) linearized by *ClaI* and *BamHI*. PCR products of the putative pentose transporters flanked with DNA fragments sharing sequence identity to the HXT7 promoter and terminator were co-transferred into CEN.PK2-1C with *EcoRI* digested pRS424-HXT7-GFP using the standard lithium acetate method. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose (ESI, Fig. S3a†).

The fusion proteins of the D-xylose-specific transporters with GFP at the C-terminus were constructed for the transporter localization study. A GS-linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser) was introduced between the transporter and the GFP. The GS-linker was added to the N-terminus of the GFP open reading frame by a PCR primer, resulting in a PCR product of GS-linker-GFP flanked with nucleotide sequence homologous to the transporters at the 5'-end and the HXT7 terminator at the 3'-end. Transporter genes were amplified from the original pRS424-HXT7-transporter constructs to generate DNA fragments of the transporters flanked with nucleotide sequences identical to the HXT7 promoter at the 5'-end and GS-linker-GFP at the 3'-end. These two fragments were then co-transferred into CEN.PK2-1C with pRS424-HXT7-GFP digested with *EcoRI*. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose (ESI, Fig. S3b†).

To confirm the proper construction of plasmids using the DNA assembler method, yeast plasmids were isolated using the Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Orange, CA). Yeast plasmids were then transferred into *E. coli* DH5 α , which were plated on LB containing 100 mg/L ampicillin. Single colonies of *E. coli* transformants were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion. All constructs of heterologous transporters were submitted for DNA sequencing to confirm the correct construction (Core Sequencing Facility, University of Illinois at Urbana-Champaign, Urbana, IL). The DNA sequencing results were compared with gene sequences in databases using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI). Sequences of cloned An25 and Xyp29 are slightly different from the entries from NCBI. Amino acid sequence alignment

of cloned An25 and Xyp29 with corresponding entries at NCBI are shown in ESI, Fig. S4.†

Symporter assays

To determine the transporter type, the pH change of the EBY.VW4000 strain overexpressing D-xylose-specific transporters was measured in un-buffered cell suspension after the addition of D-xylose using a Seven Multi pH meter equipped with an USB communication module and Direct pH software (Mettler Toledo, Columbus, OH). Plasmids encoding D-xylose-specific transporters were transferred into the EBY.VW4000 strain followed by plating on SC-Trp plates supplemented with 2% maltose. Single colonies were inoculated into 2 mL SC-Trp medium supplemented with 2% maltose. Seed culture was then used to inoculate a 400 mL culture in 2 L flask. The culture was harvested at OD ~ 1 and washed twice with ice-cold water. Cell pellet was resuspended in 4 mL of water and kept on ice before use. For the symporter assay, the pH electrode was immersed in a water-jacketed, 50 mL beaker that was kept at 25 °C and with magnetic stirring. To the beaker, 23 mL of deionized water and 1 mL of yeast cell suspension equilibrated at 25 °C was added. The pH was adjusted to 5 and a baseline was obtained. The pH change was recorded with addition of 1 mL 50% sugar solution at pH 5. An elevation of pH following the addition of the sugar solution suggests a sugar-proton symport behavior of the target sugar transporter.

GFP fluorescence imaging

Plasmids encoding D-xylose-specific transporters with C-terminal GFP fusion were transferred into the CEN.PK2-1C strain and the cells were plated on SC-Trp plates with 2% maltose. Single colonies were inoculated into 2 mL of SC-Trp liquid medium supplemented with 2% maltose. Cell culture was harvested at the exponential phase. In a centrifuge tube, 250 µL of cell culture was stained with 10 µL Hoechst 33342 nuclei dye (Invitrogen, Carlsbad, CA) for 10 min at room temperature. A small droplet of cell culture was then transferred onto a piece of cover glass and fluorescence images were taken using an Andor Technology Revolution System Spinning Disk Confocal Microscope (Andor technology, Belfast, Northern Ireland). Images were processed using Imaris image analysis and visualization software (Bitplane, Saint Paul, MN).

Intracellular accumulation of D-xylose

A single colony of the EBY.VW4000 strain overexpressing a target transporter was inoculated into a culture tube with 2 mL SC-Trp medium supplemented with 2% maltose. Seed culture was then used to inoculate a 50 mL culture in a 250 mL flask. Cell culture was harvested by centrifugation after 24 h of growth and resuspended in YPA medium supplemented with 2% D-xylose or L-arabinose to a final OD₆₀₀ of 10. At 30 min, 60 min, 120 min, and 24 h, 5 mL of culture sample was taken for measuring intracellular sugar concentrations. Culture samples were washed twice with ice-cold water and resuspended in 3 mL of deionized water. Cell suspensions were incubated at 37 °C with 250 rpm agitation for 2 days to extract intracellular sugars. The resulting cell suspension was filtered through a 0.22 µm PES filter (Corning, Lowell, MA) before HPLC analysis. Sugar and corresponding sugar alcohol

concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87C column (Bio-Rad Laboratories, Hercules, CA) and Shimadzu ELSD-LTII low temperature-evaporative light scattering detector following the manufacturer's protocol. The sugar uptake activity was calculated as mg sugar extracted through osmosis per mL of cell culture at OD ~ 10 (ESI, Fig. S5†).

¹⁴C-labeled sugar uptake assay

¹⁴C-labeled D-glucose, L-arabinose, and D-xylose were purchased from American Radiolabeled Chemicals (St. Louis, MO) as solutions in 90% ethanol. Radiolabeled sugars were first dried in a chemical hood and then resuspended in water. Sugar solutions at concentrations of 1.33 M and 1 M with specific radioactivity of approximately 40 000 dpm/µL, and at concentrations of 500 mM, 350 mM, 250 mM, 100 mM, and 50 mM with specific radioactivity of about 20 000 dpm/µL were used for the sugar uptake assay. Cell culture at the exponential phase was harvested and washed twice with ice-cold water and resuspended to about 60 mg DCW per mL in 100 mM Tris-Citrate buffer at pH 5. Three aliquots of 160 µL cell suspension were dried at 65 °C for 24 h to determine the cell dry weight. The rest of the cell suspension was kept on ice before use. For the sugar uptake assay, the cell suspension was equilibrated at 30 °C for 5 min before the assay. In a 50 mL conical tube, 160 µL of cell suspension was mixed with 40 µL of radio-labeled sugar solution for 40 or 60 s (accurately timed). Reaction was stopped by addition of 10 mL ice-cold water delivered by a syringe. The zero time point sample was obtained by adding ice-cold water and cell suspension simultaneously into a culture tube containing the radio-labeled solution. The mixture was filtered immediately through a Whatman GF/C filter (Whatman, Florham Park, NJ) pre-soaked in 40% sugar solution, and washed with 15 mL of ice-cold water. The filter was then placed in 3 mL of Econo I scintillation cocktail (Fisher scientific) and counted using a Beckman LS6500 scintillation counter (Beckman Coulter, Brea, CA) for 1 min. All data points were measured by three independent experiments. The sugar uptake rate was calculated to be mmol sugar transported per hour per gram DCW.

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