DISCOVERY AND CHARACTERIZATION OF PENTOSE-SPECIFIC TRANSPORTERS IN *SACCHAROMYCES CEREVISIAE*

BY

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THESIS

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Abstract

Saccharomyces cerevisiae is considered one of the most promising organisms for ethanol production from lignocellulosic feedstock. Unfortunately, pentose sugars, which make up to 30% of lignocellulose, cannot be utilized by *S. cerevisiae*. Pentoses can only enter yeast cells through hexose transporters, which have two orders of magnitude lower affinities for pentose sugars. Additionally, inefficient pentose uptake has been shown to be the limiting step for some D-xylose metabolizing yeast strains.

In this thesis, we report the discovery of seven active pentose transporters from pentose assimilating fungal species *Pichia stipitis* and *Neurospora crassa* based on sequence homology with the glucose/xylose/H^+ symporter (*GXS1*) in *Candida intermedia*. These transporters were cloned and heterologously expressed in *S. cerevisiae* and their sugar uptake activities were studied by analysis of intracellular sugar accumulation using HPLC.

Among the seven active transporters, one L-arabinose-specific and two D-xylose-specific transporters were identified. These transporters were functionally expressed and properly localized in *S. cerevisiae* as indicated by HPLC analysis and immunofluorescence microscopy, respectively. Kinetic parameters of the transporters were determined using a ^{14}C-labeled sugar uptake assay. Sugar uptake assay in un-buffered cell suspension indicated the sugar uptake through these three transporters was not coupled with proton uptake, revealing that these three sugar transporters are facilitators.

Introduction of these pentose-specific transporters may facilitate pentose sugar utilization in *S. cerevisiae* by improving pentose uptake. More efficient utilization of pentose sugars will lower the cost for lignocellulosic ethanol production.

To my family and friends

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Table of Contents

Chapter 1. Overview of Pentose Transport in *Saccharomyces cerevisiae*

1.1 Biofuels and Pentose Sugar Utilization

1.1.1 Biofuels

In the past few decades, rapid economic growth and development of modern technologies has drastically altered the way many people live. Large quantities of non-renewable natural resources, most notably fossil fuels, are consumed everyday in order to keep up with an ever-growing demand for energy. As a consequence, large amounts of green house gases are released into the atmosphere every day. The increasing price and limited supply of crude oil has created an energy supply crisis [1]. To meet the increasing demand for energy and raw materials for sustainable economic growth, an alternative, sustainable energy source needs to be developed. Biomass-derived energy sources, namely biofuels, are an attractive candidate as alternative energy sources due to their inherent advantages in economic, environmental, and energy-security considerations. In the 2007 State of Union Address, President Bush set an ambitious goal to reduce gasoline usage in the United States by 20 percent over the next 10 years through the use of biofuels [2].

Biofuels, which include bioethanol, biodiesel, and biogas, are a class of energy sources derived from plant materials, or 'biomass'. Bioethanol is currently one of the most widely used biofuels, as it can be blended with regular gasoline or, in certain dedicated engines, be burned without any additional fuel-source additives [1].

1.1.2 Preferred Feedstock for Biofuels Production

Traditionally, bioethanol is produced by fermentation of sugar or starch containing food crops. To farther reduce the product cost of bioethanol, researchers are working to use non-food lignocellulosic biomass as the feedstock for ethanol production, as the cultivation of lignocellulosic biomass typically requires lower input of energy, fertilizer, pesticide and herbicide [3].

For production of cellulosic ethanol, biomass feedstocks are first hydrolyzed to release individual sugars, and then microorganisms are added to ferment these sugars into ethanol. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. The hemicellulose components makes up to 20~30% of lignocellulosic biomass, and is primarily composed of five-carbon sugars (pentoses) such as D-xylose and L-arabinose [4]. Unfortunately, the industrial microorganism currently used for large scale production of ethanol, *Saccharomyces cerevisiae,* cannot utilize pentoses contained in the hydrolysates of the hemicellulose component of biomass feedstocks [5]. The incomplete utilization of sugar substrates present in hydrolysates of lignocellulosic biomass leads to an elevated bioethanol production cost, making this environmental-friendly energy alternative less economically competitive when compared to fossil fuels [6, 7].

1.1.3 Preferred Microorganism for Biofuels Production

Saccharomyces cerevisiae, also known as baker's yeast, has been used for the bioconversion of hexose sugars into ethanol for thousands of years. Additionally, it is the most widely used microorganism for large scale industrial fermentation of D-glucose into ethanol. *S. cerevisiae* is a very suitable candidate for bioconversion of lignocellulosic biomass into biofuel [8]. It has a well studied genetic and physiological background, high productivity of ethanol, and

high tolerance to ethanol and inhibitors present in biomass hydrolysates [9]. The low fermentation pH of *S. cerevisiae* can also help prevent bacterial contamination during the fermentation. Unfortunately, *S. cerevisiae* cannot utilize pentose sugars contained in the hydrolysates of the biomass feedstock's hemicellulose component [5].

1.1.4 Pentose Utilization by *Saccharomyces cerevisiae*

Complete substrate utilization is one of the prerequisites to render cellulosic ethanol processes economically competitive [6, 7]. Unfortunately, the most widely used ethanol producing microorganism, baker's yeast (*Saccharomyces cerevisiae*), cannot ferment pentose sugars into ethanol. On the other hand, there are many naturally existing fungal and bacterial species which can utilize pentose sugars efficiently. However, these microorganisms are not compatible with industrial ethanol fermentation due to their complicated physiology and low ethanol productivity [10].

One of the most practical solutions to this problem is to introduce a pathway from a pentose assimilating organism into baker's yeast. [11-13] Over the past few decades, pentose utilization pathways from various species have been transferred into *S. cerevisiae* allowing fermentation of D-xylose and L-arabinose [14-18]. However, efficient conversion of pentose sugars into biofuels is limited by multiple issues including cellular redox imbalance, low influx of the pentose phosphate pathway, and lack of efficient pentose transport into the cell [5].

1.2 Rationale of Pentose Transporter Study in *S. cerevisiae*

1.2.1 *S. cerevisiae* Lacks an Efficient Xylose Transport System

Sugar uptake through corresponding sugar transporters is the first step of sugar utilization. *S. cerevisiae* has a complicated glucose uptake system composed of 17 hexose transporters and several sensors and regulators. The hexose transporters family consists of both high-affinity and low-affinity transporters. Expression of different kind of transporters are regulated by sensors and regulators according to various sugar concentrations in the environment [19]. In contrast, *S. cerevisiae* lacks an efficient transport system for the uptake of pentose sugars. Pentose sugars can only enter *S. cerevisiae* cells through the hexose uptake system but with two orders of magnitude lower affinity [13]. As a result, D-xylose uptake in D-xylose-assimilating yeast strains is very slow and inhibited by D-glucose present in the environment.

D-Xylose uptake has been found to be the limiting step in some recombinant D-xylose assimilating *S. cerevisiae* strains [10]. Significant improvement of D-xylose uptake activity has also been found in an engineered *S. cerevisiae* strain obtained through evolutionary engineering aimed for efficient D-xylose fermentation [20]. Therefore, improving D-xylose uptake activity by introducing more efficient D-xylose transporters can be a viable strategy to facilitate D-xylose utilization in recombinant *S. cerevisiae* strains [21, 22].

1.2.2 Overexpression of Xylose Transporters Has Been Shown to Improve Xylose Utilization in *S. cerevisiae*

Nature has evolved many pentose assimilating fungal strains in which both low-affinity and high-affinity sugar transport systems are present for pentose uptake [14, 23, 24]. 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 [23]. It has also been observed that overexpression of the Gxf1 transporter can improve fermentation performance in recombinant D-xylose-utilizing *S. cerevisiae* [25]. Strains expressing heterologous D-xylose transporters from *Arabidopsis thaliana* showed up to a 2.5-fold increase in D-xylose consumption rate and a 70% increase in ethanol production rate [5]. Overexpression of a 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 [26].

1.2.3 Xylose Assimilating Fungi *Neurospora crassa* and *Pichia stipitis* are Good Candidate Source for Novel Pentose Specific Transporters

Neurospora crassa and *Pichia stipitis*, two organisms which have complete genomic sequence data available, can utilize pentose sugar very efficiently [27, 28]. In *Pichia stipitis*, three D-glucose/D-xylose transporters (SUT1-3) were cloned and characterized at the molecular level [29]. Overexpression of SUT1 has been shown to facilitate D-xylose consumption and ethanol production in recombinant D-xylose assimilating *S. cerevisiae* [26]. However, as a D-xylose assimilating filamentous fungus species, no pentose transporter has been identified from *Neurospora crassa* so far.

Molecular characterization of the D-glucose/D-xylose-H^+ symporter from *Candida intermedia* unveiled a group of putative, previously uncharacterized, fungal sugar transporters [26]. With the sequence of the characterized D-xylose symporter, it is also possible to discover new sugar transporters based on sequence homology from genome-sequenced fungal species such as *Neurospora crassa* and *Pichia stipitis*.

1.3 Project Overview

The project aimed to discover, characterize and engineer novel pentose-specific transporters in *S. cerevisiae*, and improve the pentose utilization in recombinant *S. cerevisiae* strains by improving pentose sugar uptake.

First, we wish to discover pentose-specific transporters in pentose-assimilating fungal species *N. crassa* and *P. stipitis* using the newly characterized D-xylose-H^+ symporter from *C. intermedia* as a probe.

Second, we would like to characterize the newly discovered pentose-specific transporters. We wish to study the heterologous expression and the functionality of the pentose-specific transporter by investigating their cellular localization, proton coupling type, and kinetic parameters.

Third, we wish to introduce the pentose-specific transporters into recombinant *S. cerevisiae* strains and test if the introduction of the transporters will help the pentose sugar utilization. Considering uptake inhibition is partially responsible for the D-glucose repression during mixed sugar fermentation, we are also interested in knowing to what extent the introduction of pentose-specific transporters will help alleviate the D-glucose repression.

Finally, if the transport efficiency of the heterologous transports were not satisfactory, we would like to engineer the newly discovered transporters using directed evolution to improve their specificity, affinity and capacity.

By the time this manuscript was prepared, we have successfully cloned and screened 18 putative pentose transporters and discovered two D-xylose-specific transporters and one L-arabinose-specific transporter. All the three pentose-specific transporters were correctly

localized in the *S. cerevisiae* cell membrane, and they were determined to be low affinity pentose-facilitators.

However, we have not observed the benefit of overexpressing of D-xylose-specific transporters, probably due to the low transport efficiency or the slow pentose utilization rate in the recombinant *S. cerevisiae* strains. To solve this problem, we are working on the engineering of the pentose-specific transporters and improvement of the pentose utilization pathway at the same time.

1.4 Conclusions and Outlook

Biofuels, especially lignocellulose derived alcohols, are under extensive investigation due to the increasing concerns about energy security, sustainability and global climate change [30]. Consequently, bioconversion of plant derived lignocellulosic material into ethanol has drawn more and more attention for being an attractive candidate for its potential to eventually replace fossil fuels. *Saccharomyces cerevisiae*, also known as baker's yeast, has been used for bioconversion of hexose sugars into ethanol for thousands of years, and is the most widely used microorganism for large scale industrial fermentation of D-glucose into ethanol. *S. cerevisiae* is a very suitable candidate for bioconversion of lignocellulosic biomass into biofuels. Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. Hemicellulose makes up to 20~30% of the lignocellulosic biomass and is primarily composed of the five-carbon sugars D-xylose and L-arabinose [4]. Unfortunately, *S. cerevisiae* cannot utilize pentose sugars from the hemicellulose hydrolysates [5].

Pentose utilization pathways from D-xylose-assimilating organisms have been transferred into *S. cerevisiae*, allowing fermentation of D-xylose and L-arabinose [14-18]. However, efficient conversion of pentose sugars into biofuels is limited by multiple issues including cellular redox

imbalance, low influx of the pentose phosphate pathway, and a lack of efficient pentose uptake into the cell [5].

S. cerevisiae lacks an efficient transport system for pentose uptake. Pentose sugars can only enter *S. cerevisiae* cells through the hexose uptake system but with two-orders of magnitude lower affinity [13]. D-xylose uptake has been found to be the limiting step in 'fast' D-xylose utilizing *S. cerevisiae* strains [10]. In contrast, native D-xylose utilizing yeasts have been shown to use both low-affinity and high-affinity D-xylose transport systems for D-xylose and L-arabinose uptake [14, 23]. It was shown that introducing D-xylose transporters can improve D-xylose utilization in *S. cerevisiae* [5, 25, 26]. *Neurospora crassa* and *Pichia stipitis* can utilize pentose sugars very efficiently and the complete genome sequences of these organisms are available [27, 28]. As the D-glucose/D-xylose symporter from *Candida intermedia* has been characterized at the molecular level [23], it is possible to discover and characterize more pentose transporters in pentose assimilating fungal species such as *Neurospora crassa* and *Pichia stipitis*. Introducing heterologous transporters with high-affinity and high-capacity for pentose sugars into *S. cerevisiae* together with the development of a more efficient pentose utilization pathway will make the creation of cellulosic ethanol a more economically competitive process.

1. 5 References

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Chapter 2. Discovery of Pentose-Specific Transporters in *S. cerevisiae*

2.1 Introduction

Pentose uptake is the first step of pentose utilization in *Saccharomyces cerevisiae*. Currently, pentose sugars can only enter yeast cells through hexose transporters which have two orders of magnitude lower affinity and capacity, suggesting that pentose uptake can be a limiting step for pentose utilization. To solve this problem, researchers have tried to overexpress different sugar transporters in *S. cerevisiae* to facilitate pentose uptake. Leandro *et al.* discovered two D-glucose/D-xylose transporters from *Candida intermedia* and characterized them at the molecular level [1]. Among these two transporters, Gxs1 was identified as a D-glucose/D-xylose-H^+ symporter, which became the first active D-xylose-H^+ symporter characterized at the molecular level in *S. cerevisiae*. Based on the gene sequence of *GXS1* and the recently available genome sequence of pentose-assimilating fungal species *Neurospora crassa* and *Pichia stipitis*, more possible putative pentose transporters have been identified using BLAST search basing on sequence homology. These putative transporters were cloned and introduced into a sugar transporter knockout strain and tested for their pentose uptake activity. Seven active transporters were identified through a HPLC-mediated sugar uptake assay. Among the active transporters, two D-xylose-specific and one L-arabinose-specific transporters have been discovered.

2.2 Results

2.2.1 Genome Mining for Putative Pentose Transporters

Characterization of D-glucose/D-xylose symporter *GXS1* revealed a very important piece of information regarding the sequence/function relationship of D-xylose transporters in yeast. Comparison of the amino acid sequence of *GXS1* with recently available genome sequence information of *N. crassa* and *P. stipitis* may lead to discovery of additional D-xylose transporters active in *S. cerevisiae* [1]. To search for candidates for pentose transporter research, proteins sharing high sequence identity with Gsx1 were investigated using BLAST (http://www.ncbi.nlm.nih.gov/). In order to identify more candidates, uncharacterized putative L-arabinose-proton symporter from *Pichiaa stipitis* (*AUT1*, locus tag PICST_87108) was also used as a template for the BLAST search. The BLAST searches for putative transporters in *N. crassa* and *P. stipitis* were performed separately.

The results of the BLAST searches were screened to eliminate any protein with known activity of D-glucose uptake or activity other than sugar uptake. Using a cut-off of 25% minimum identity, 17 putative transporters were identified from the BLAST searches (Table 2.1). Together with *AUT1* from *Pichia stipitis*, 18 putative transporters were cloned. These putative transporters shared 25~50% identity with either *GSX1* from *Candida intermedia* or *AUT1* from *Pichia stipitis*. All 17 putative transporters were annotated to be either sugar uptake proteins or hypothetical proteins with unknown activity. D-glucose transporter *SUT1* and *SUT2* from *P. stiptis* were also cloned for comparison.

2.2.2 Cloning of Putative Pentose-Specific Transporters

N. crassa and *P. stipitis* were cultivated in rich media supplemented with D-xylose or L-arabinose as the sole carbon source. Next, total RNA was isolated from the fungal strains and reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Putative transporters were amplified directly from cDNA through polymerase chain reaction (PCR), if possible. Because the regulation mechanism and expression pattern were not clear for pentose transporters in fungal species, cDNAs encoding for putative transporters were not always obtainable despite alteration of cultivation conditions. In this case, primers were designed according to the corresponding cDNA sequence from GenBank and were used to amplify gene sequences using genomic DNA as a template. Overlap-extension PCR was then used to get the corresponding full-length gene without introns.

The above-mentioned PCR products were then cloned into a pRS424 shuttle vector (New England Biolabs, Beverly, MA) with a HXT7 promoter and a HXT7 terminator using the DNA assembler method [2]. Yeast plasmids isolated from transformants were retransferred into *E. coli* DH5α. Isolated *E. coli* plasmids were first checked by diagnostic PCR using the primers originally used to amplify the transporter genes. The entire open reading frames were then submitted for sequencing to confirm the correct construction of plasmids.

2.2.3 Transporter Activity Assay for Putative Pentose Transporters

To determine the pentose uptake ability of yeast strains expressing putative pentose transporters, intracellular accumulation of D-xylose and L-arabinose was measured using high performance liquid chromatography (HPLC) [3]. It was reported that a *S. cerevisiae* strain without the D-xylose assimilating pathway is still able to uptake and accumulate D-xylose within the cell, though it cannot metabolize it further for growth or fermentation [4]. D-xylose

accumulated within yeast cells will be partially converted to xylitol due to the presence of endogenous aldose reductase [5]. This work also revealed that L-arabinose can be accumulated in *S. cerevisiae* strain. Similar to D-xylose, a portion of the L-arabinose accumulated will also be converted into L-arabitol by an endogenous aldose reductase. D-xylose, xylitol, L-arabinose, and L-arabitol can all be extracted using osmosis followed by analysis using HPLC.[3]

In this study, the sugar transporter deletion strain EBY.VW4000 was used for the pentose sugar uptake assay. The EBY.VW4000 strain has concurrent knockout of more than 20 sugar transporters and sensors including *HXT1-17* and *GAL2* [6]. Growth on D-glucose as the sole carbon source was completely abolished in this strain, while uptake of maltose through a different sugar transport system is retained. EBY.VW4000 also exhibits minimal D-xylose and L-arabinose uptake under current assay conditions, which makes it a suitable strain for pentose uptake assay. Plasmids expressing putative transporters were transferred into the EBY.VW4000 strain using a standard lithium acetate method [7] and single colonies were used for measurement of sugar uptake activity. EBY.VW4000 strains harbouring putative pentose transporters were first grown up in SC-Trp media supplemented with 2% maltose. Cells were then harvested and washed before transferring into fresh YPA media supplemented with 2% D-xylose or L-arabinose. Samples of cell culture were collected after incubation with pentose sugars for 24 hours. Cell pellets were washed to remove extracellular sugars and resuspended in water and then shook at 37 °C for two days in order to extract intracellular sugars via osmosis. Supernatants of cell suspension were filtered and analyzed using a HPLC (Shimadzu, Kyoto, Japan) equipped with an HPX-87C column (Bio-Rad Laboratories, Hercules, CA) and an ELSD-LTII detector (Shimadzu). The pentose uptake ability of putative pentose transporters was determined by summation of the concentration of intracellular pentose sugars and corresponding sugar alcohols.

Since D-glucose will be metabolized once it enters a yeast cell, D-glucose transport activity cannot be determined by measuring the intracellular D-glucose concentration. However, the fact that the EBY.VW4000 strain cannot grow on media with D-glucose as the sole carbon source linked the D-glucose uptake ability with cell survival. If the putative pentose transporter has D-glucose uptake activity, it should complement the transporter knockout in the EBY.VW4000 strain, resulting in cell survival on media with D-glucose as the sole carbon source.

Using HPLC in combination with the functional assay, several putative pentose transporters were identified to have uptake activity towards D-glucose, D-xylose, and L-arabinose. Introduction of Xyp37, Xyp33, An29-2, and Xy31 restored cell growth of the EBY.VW4000 strain on D-glucose. At the same time, Xyp37, Xyp33, and An29-2 also exhibited a D-xylose uptake activity, while AN29-1 and Xy31 showed L-arabinose uptake activity at the same time (Figure 2.1).

The rest of putative pentose transporters failed to enable D-glucose uptake in the EBY.VW4000 strain. However, the EBY.VW4000 strains harbouring Xyp29 and An25 were able to uptake and accumulate D-xylose within the cells, indicating that they are D-xylose-specific transporters. Similarly, the EBY.VW4000 strain harbouring Xyp32 was able to accumulate L-arabinose, indicating that it may be an L-arabinose-specific transporter (Figure 2.3).

2.3 Discussion

Using the Gxs1 from *Candida intermedia* and Aut1 from *Pichia stipitis* as probes, 17 putative transporters were identified using BLAST searches based on protein sequence identity. These putative pentose transporters shared 50-25% sequence identity with either Gxs1 or Aut1. Interestingly, Aut1, which was annotated as an L-arabinose transporter, failed to show any sugar

uptake activity in the sugar uptake assay. However, among putative pentose transporters found using Aut1 as a probe, both An29-2 and An25 exhibited a D-xylose uptake activity. Similarly, using the D-xylose transporter Gxs1 as a probe, both D-xylose transporter and L-arabinose transporters can be identified. All the active transporters shared less than 40% identity with the probes, indicating the pentose transporter family is not highly conserved.

Sugar transport activity exhibited by the cloned putative pentose transporters indicated that the constructs of sugar transporters under the HXT7 promoter on the multicopy pRS424 shuttle plasmid are functional. The fact that sugar transporters were active without adding any additional signal peptide suggested some inherent sequence may exist within the encoding region of sugar transporters to direct expressed sugar transporters to localize in the cell membrane and fold in a functional manner. Interestingly, several putative transporters from filamentous fungus *N. crassa* also showed sugar uptake activity in *S. cerevisiae*. Note that it is not surprising that D-xylose 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* [1, 8]. In fact, sugar transporters from more distinct species like *A. thaliana* can also be expressed and correctly localized in the *S. cerevisiae* cell membrane [9]. This fact may indicate that different species may use similar mechanism for directing transporter proteins into the cell membrane.

After the first round of the sugar uptake activity assay using HPLC, seven putative transporters were found to be functionally expressed in *S. cerevisiae*. Among these seven transporters, two appear to be potentially D-xylose-specific while the other could be L-arabinose-specific. If the specificities of these three transporters are further confirmed, they will be the first group of heterologous pentose-specific transporters in *S. cerevisiae*.

Pentose-specific transporters are very useful for creation of recombinant *S. cerevisiae* capable of simultaneously utilizing pentose sugars with high efficiency. Many researchers have been working very hard to increase the efficiency of pentose utilization in *S. cerevisiae*. Matsushika *et al.* reported the construction of a recombinant industrial yeast strain which can completely ferment a mixture of 45 g/L D-glucose and 45 g/L D-xylose within 24 hours [10]. As the pentose utilization pathway becomes more and more efficient in *S. cerevisiae*, the insufficient uptake of pentose sugars will be the limiting step in pentose utilization. Since the pentose-specific sugar transporters are highly specific towards pentose sugars, it would be less likely for them to be inhibited by D-glucose in the cell media. The introduction of pentose-specific transporters may provide a pentose transport system that is independent of D-glucose concentration.

Furthermore, it was shown that D-glucose serves as a very important signaling compound in the regulation of metabolic fluxes in *S. cerevisiae* in addition to being a preferred carbon source. In the presence of D-glucose, pathways involved in utilization of pentose sugars will be down regulated. This regulation event made *S. cerevisiae* to utilize D-glucose prior to other carbon sources, such as pentose sugars. As part of the regulation process, the presence of D-glucose represses the uptake of D-xylose [11]. By introducing an independent pentose-specific sugar uptake system, the down regulation of pentose uptake in the presence of D-glucose may be eliminated, and glucose repression on pentose sugar utilization may be alleviated.

The putative pentose transporters with both glucose and pentose transport activities can be considered candidates for engineering increased activity towards pentose sugar uptake via directed evolution. Mutations can be introduced into these transporters through error-prone PCR, resulting in the creation of a library that can be transferred to transporter knockout strains

capable of pentose utilization. The library can be enriched in pentose media, after which fast growth mutants can be isolated and analyzed (Figure 2.3).

The overexpression of active heterologous xylose-specific transporters in *S. cerevisiae* strains with a D-xylose utilization pathway was also investigated. Unfortunately, the advantage of a pentose-specific transporter overexpression cannot be observed despite alteration of expression strategies, cultivation conditions, and choice of the pentose 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 [12]. It has been observed that transporter overexpression strains display a slower growth rate even when D-glucose is used as a carbon source (data not shown). Second, the pentose uptake activity of wild-type *S. cerevisiae* through hexose transporters is much higher than pentose uptake activity of a certain pentose transporter overexpressed in the hexose transporter knockout strain. The low sugar transport activity of newly discovered pentose transporters may make it hard to observe the improvement of sugar uptake ability. Third, even if the introduction of new pentose transporters could improve the uptake of pentose sugars into *S. cerevisiae* cells, the benefit of pentose sugar utilization can only be observed when the pentose utilization pathway is efficient enough to make the 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 [13].

2.4 Conclusions and Outlook

Pentose uptake is the first step of pentose utilization by *S. cerevisiae*. Pentose sugars enter *S. cerevisiae* cells through hexose transporters at an affinity two orders of magnitude lower

than hexose sugars. In order to facilitate pentose utilization in *S. cerevisiae* by the introduction of pentose transporters, eighteen putative transporters from *N. crassa* and *P. stipitis* were identified based on sequence homology with *GXS1* and *AUT1*.

Open reading frames of putative transporters were amplified and cloned into the pRS424 multicopy shuttle plasmid under the HXT7 promoter and terminator using the DNA assembler method. Resulted constructs were first confirmed using diagnostic PCR and then the whole open reading frames were sequenced.

Correct constructs of plasmid-encoding putative pentose transporters were then transferred into the transporter knockout strain EBY.VW4000 for the assessment of sugar uptake activities. These assays checked intracellular sugar accumulation using HPLC. Among 18 cloned putative transporters, Xyp37, Xyp33, An29-2, Xy31 were identified to have glucose transport activity. Xyp37, Xyp33 and An29-2 also had D-xylose transport activity, while An29-1 and Xy31 displayed L-arabinose uptake activity at the same time. In additional, two D-xylose-specific transporters, An25 and Xyp29, were identified together with one L-arabinose transporter Xyp32.

When this research project was initiated, only limited information was available on the pentose transporters. *GXS1* was the only D-xylose-H^+ symporter characterized at the molecular level in *S. cerevisiae*. Through sequence homology with the newly available genomic sequence of *N. crassa* and *P. stipitis*, seven novel pentose transporters were identified. Using the identified transporters as probes, more pentose-specific transporters could be discovered from various species. These pentose-specific transporters could aid in the systematic analysis of this important family in sugar transporters in *S. cerevisiae*.

2.5 Materials and Methods

2.5.1 Strains, Media and Cultivation Conditions

S. cerevisiae CEN.PK2-1C (MATα *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8*^{c}) was purchased from Euroscarf (Frankfurt, Germany), and was used for manipulation of recombinant DNA in yeast. Sugar transporter knockout strain EBY.VW4000 (CEN.PK2-1c *Δhxt1-17 Δstl1 Δagt1 Δydl247w Δyjr160c Δgal2*) was a gift from Eckhard Boles [6]. *Escherichia coli* DH5α (cell media facility, University of Illinois at Urbana-Champaign, Urbana, IL) was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% of Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% of ammonium sulfate, 0.05% of amino acid drop out mix). YPA media supplemented with 2% of sugar was used to grow yeast strain harboring no plasmids (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate). *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were cultured at 30 °C and 250 rpm for aerobic growth. Yeast strains were grown under aerobic condition for cell multiplation if not specified. *E. coli* strains were cultured at 37 °C and 250 rpm if not specified. All restriction enzymes were purchased from New England Biolabs (Ipwich, MA). All chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.5.2 Cloning of Putative Pentose-specific Transporters Using DNA Assembler Method

Most of the cloning work was done using the DNA assembler method via the yeast homologous recombination mechanism [2]. pRS424-HXT7-GFP plasmids were used for cloning of putative pentose-specific transporters. In this plasmid, a HXT7 promoter, a GFP

gene flanked with *Eco*RI sites at both ends, and a HXT7 terminator were assembled into the pRS424 shuttle vector (New England Biolabs) linearized by *Cla*I and *Bam*HI. PCR products of the putative pentose-specific transporters flanked with DNA fragments sharing sequence identity to the HXT7 promoter and terminator were co-transferred into CEN.PK2-1C with *Eco*RI digested pRS424-HXT7-GFP using the standard lithium acetate method [7]. (See Table 2.3 for list of primers used for the cloning of putative pentose-specific transporters. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose (Figure 2.4).

To confirm the proper construction of plasmids using the DNA assembler method, yeast plasmids were isolated using Zymoprep Yeast Plasmid Miniprep II (Zymo Research, Orange, CA). Yeast plasmids were then transferred into *E. coli* DH5α, which were plated on LB plates containing 100 mg/L ampicillin. Single colonies of *E. coli* transformants were then inoculated in LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR. All constructs of heterologous pentose transporters were submitted for DNA sequencing to confirm the correct sequence (UIUC Core Sequencing Facility, Urbana, IL). The DNA sequencing results were compared with gene sequences from NCBI using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI).

2.5.3 Analysis of Intracellular Accumulation of Pentose Sugars

Single colonies of the EBY.VW4000 strain expressing a target transporter were inoculated in culture tubes containing 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 hours of growth and resuspended in YPA medium

supplemented with 2% D-xylose or L-arabionse to a final OD_{600} of 10. At 30 min, 60 min, 120 min, and 24 hours, 5 mL of each 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 then incubated at 37 °C with 250 rpm agitation for 2 days to extract intracellular sugars. Resulted 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 (Shimadzu) following the manufacturer's protocol. The sugar uptake activity was calculated to be mg sugar extracted through osmosis per mL of cell culture at OD~10.

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2.6 References

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2.7 Tables

Blast search result using *AUT1* (Locus tag PICST_87108) from *Pichia stipitis* as a probe

Name:	Origin:	% identity with AUT1	Annotation:	Length (cDNA)	Locus Tag
Ap31 (SUT2)	*P. stipitis*	31	Sugar UpTake (tentative)	1653	ABN66266
Ap26	*P. stipitis*	26	Sugar transporter	1404	XP_001387242
AN49	*N. crassa*	49	hypothetical protein NCU01494 similar to MFS sugar transporter	2025	EAA26691
AN41	*N. crassa*	41	hypothetical protein NCU09287 similar to galactose-proton symport	1968	EAA28903
AN29-2	*N. crassa*	29	hypothetical protein NCU04963 similar to MFS monosaccharide transporter	1584	EAA30175
AN28-3	*N. crassa*	28	hypothetical protein NCU02188 conserved hypothetical protein	1458	EAA30346
AN25	*N. crassa*	25	sugar transporter	1653	EAA35128

Blast search result using *GSX1* from *Candida intermedia* as a probe

Name:	Origin:	% identity with GSX1	Annotation:	Length (cDNA)	Locus Tag
Xy50	N. crassa	50	hypothetical protein NCU04537	1620	EAA26741
Xy31	N. crassa	31	hypothetical protein NCU06138 similar to MFS monosaccharide transporter"	1752	EAA30764
Xy33	N. crassa	33	hypothetical protein NCU00988 similar to MFS quinate transporter" D-xylose transporter; PRK10077 Sugar (and other) transporter; pfam00083	1614	EAA34662
Xyp37 (SUT3)	P. stipitis	37	Sugar UpTake (tentative)	1653	ABN67990
Xyp33 (XUT3)	P. stipitis	33	sugar transporter, putative Xylose UpTake (tentative) Predicted transporter (major facilitator superfamily) with sequence similarity to similar;	1656	EAZ63115
Xyp32 (XUT1)	P. stipitis	32	sugar transporter, high affinity, putative "Xylose UpTake (tentative); D-xylose transporter; PRK10077	1701	ABN67554
Xyp30 (STL1)	P. stipitis	30	sugar transporter, strongly conserved; no transcript evident	1587	ABN65745
Xyp31 (XUT2)	P. stipitis	31	Sugar transporter, xylose transporter (tentative) similarity to GXS1 (STL1);	1404	AAVQ01000002
Xyp29 (STL12) (XUT6)	P. stipitis	29	Sugar transporter, putative (STL12) xylose uptake (tentative)	1635	ABN68560
Xyp30-1 (HGT3)	P. stipitis	30	high affinity xylose transporter (putative)	1587	ABN68686
Xyp28 (XUT7)	P. stipitis	28	xylose transporter, high affinity, putative	1251	EAZ63044

Table 2.1 Putative pentose transporters from BLAST search

Name:	Forward Primer	Reverse Primer
SUT2	caaaaagttttttaattttaatcaaaaaatgtcctcacaagatttacc	gatcatgaattaataaaagtgttcgcaaactaaacttgctcttgctcttttgc
Ap26	caaaaagttttttaattttaatcaaaaaatgaagtattttcaaatctgg	gatcatgaattaataaaagtgttcgcaaatgagaacaaatcgtcaatggc
AN49	caaaaagttttttaattttaatcaaaaaatggcgtcgaacccaacgaac	gatcatgaattaataaaagtgttcgcaaactacaccttcccagcagcatcc
AN41	caaaaagttttttaattttaatcaaaaaatgggccacaatccagacctg	gatcatgaattaataaaagtgttcgcaaactaagaattaggagcattaac
AN29-2	caaaaagttttttaattttaatcaaaaaatgaagccatttctggggctc	gatcatgaattaataaaagtgttcgcaaactacgactcccgattacctcc
AN28-3	caaaaagttttttaattttaatcaaaaaatgtccgccatcgtcgtgaccg	gatcatgaattaataaaagtgttcgcaaactaaaccttctcatgctcatgc
AN25	caaaaagttttttaattttaatcaaaaaatggcgcctccaaagttcctgg	gatcatgaattaataaaagtgttcgcaaattaacgtgtttccttggtg
Xy50	caaaaagttttttaattttaatcaaaaaatggagcacgatcactccgcctcc	gatcatgaattaataaaagtgttcgcaaagaattcttactcgctatcgctgcggttc
Xy31	caaaaagttttttaattttaatcaaaaaatggaattcggtggcggaggcggctccggc	cgcccatgtgtttctgaaaggctggcatggc
	cagcctttcagaaacacatgggcgaatacgatccg	cacccatacccgtgataaaccgtccggcaag
	cggtttatcacgggtatgggtgtcggatcc	ggtcagcttctggattgactacggaaccaac
	cttggaagagatggacatgatcttcggatccg	gatcatgaattaataaaagtgttcgcaaagaattcttagacatgctgagaatagtg
Xy33	caaaaagttttttaattttaatcaaaaaatgggtctttcgataggaaatagg	gatcatgaattaataaaagtgttcgcaaagaattcttagacatgctgagaatagtg
Xyp37	caaaaagttttttaattttaatcaaaaaatgtcctcacaagatttaccctcg	gatcatgaattaataaaagtgttcgcaaagaattcctaaacttgctcttgctc
Xyp33	caaaaagttttttaattttaatcaaaaaatgagagaagttggtattcttg	gatcatgaattaataaaagtgttcgcaaagaattcttattctgacatttcaatc
Xyp32	caaaaagttttttaattttaatcaaaaaatgcacggtggtggtgacggtaac	gatcatgaattaataaaagtgttcgcaaagaattcttatttttcaacgtggtagac
Xyp30	caaaaagttttttaattttaatcaaaaaatggcatatcttgattggttaac	gatcatgaattaataaaagtgttcgcaaagaattcttaagagctgctggatgcgttttc

Table 2.2 (continued on next page)

Xyp31	caaaaagttttttaattttaatcaaaaaatgaagtattttcaaatctgg	gatcatgaattaataaaagtgttcgcaaagaattcttatgagaacaaatcgtcaatgg
Xyp29	caaaaagttttttaattttaatcaaaaaatgtccagtgttgaaaaaagtgctc	gatcatgaattaataaaagtgttcgcaaagaattcttagctgatgttttcgacatg
Xyp30-1	caaaaagttttttaattttaatcaaaaaatgtcttcgttattgactaacg	gatcatgaattaataaaagtgttcgcaaagaattctcattccatctcattcaacttg
Xyp28	caaaaagttttttaattttaatcaaaaaatgacttttgcagttaacttgtat	gatcatgaattaataaaagtgttcgcaaagaattcttagtccaaatcgtccaaatcg
AUT1	caaaaagttttttaattttaatcaaaaaatgagtgctgacgaaaaagtcgctg	gatcatgaattaataaaagtgttcgcaaagaattcctactcgacataagagacttc

Table 2.2 List of primers for putative pentose transporters cloning

2.8 Figures

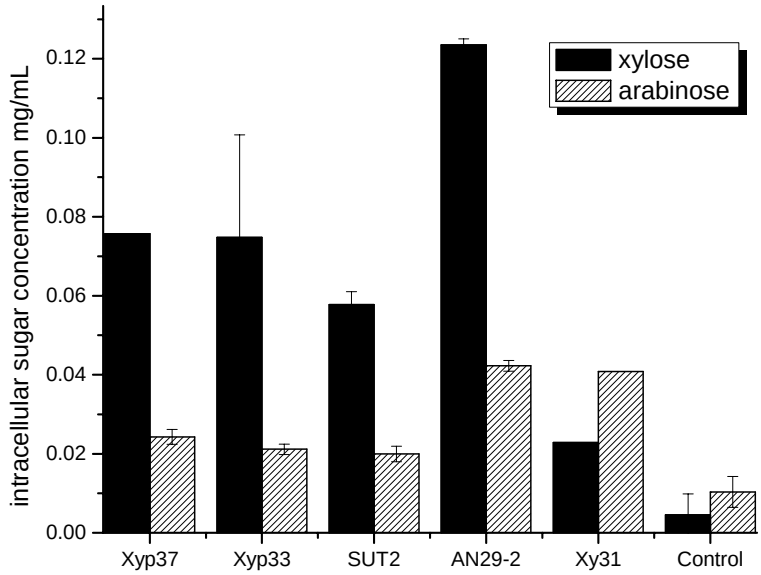

Figure 2.1 Putative pentose transporters with glucose uptake activity. Transporter-expressing plasmids were introduced into transporter knockout strains to analyze the intracellular accumulation of D-xylose and L-arabinose. After one day incubation of D-xylose or L-arabinose, sugar accumulated in the cells were extracted via osmosis. The sugar uptake activity of sugar transporters were determined as mg/mL sugar extracted from 1 mL of cell suspension of OD~10. D-Glucose uptake activity of transporters is determined by the ability of survival on media containing D-glucose as the sole carbon source. This figure showed the pentose sugar uptake activity of cloned transporters with confirmed D-glucose uptake activity.

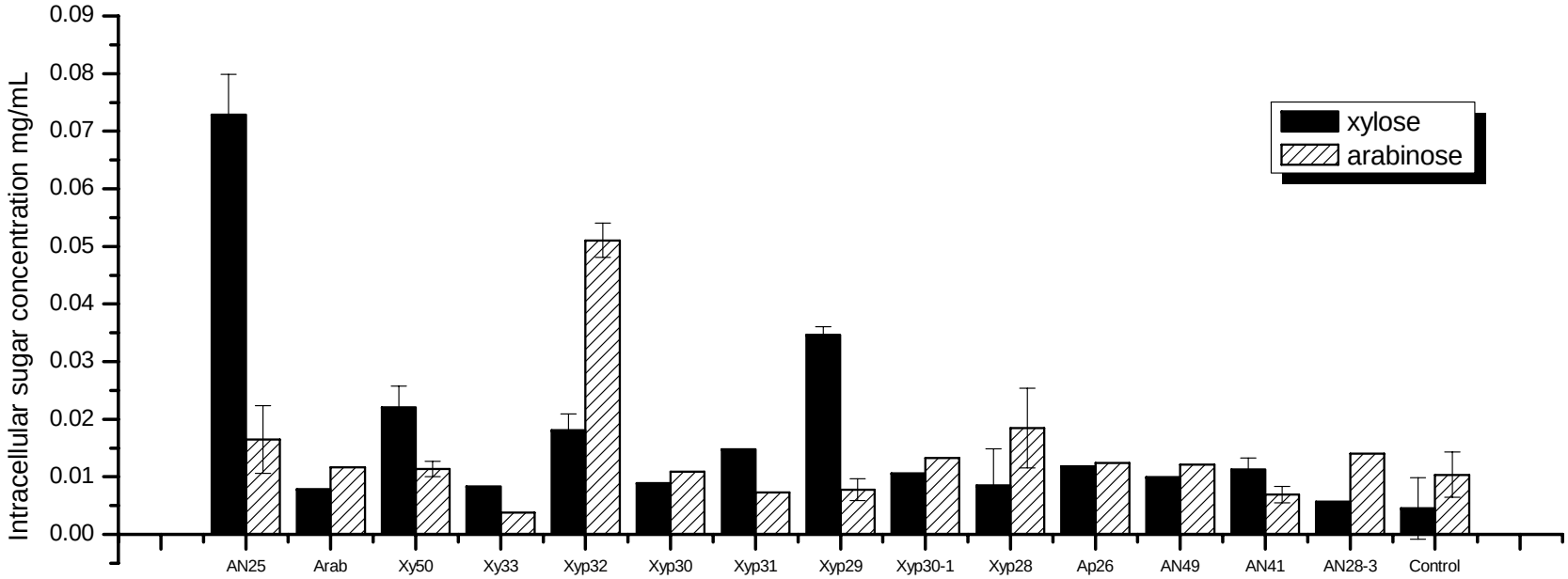

Figure 2.2 putative pentose transporters without glucose uptake activity. Transporter-expressing plasmids were introduced into transporter knockout strains to analyze the intracellular accumulation of D-xylose and L-arabinose. After one day incubation of D-xylose or L-arabinose, sugar accumulated in the cells were extracted via osmosis. The sugar uptake activity of sugar transporters were determined as mg/mL sugar extracted from 1ml of cell suspension of OD~10. D-Glucose uptake activity of transporters is determined by the ability of survival on media containing D-glucose as the sole carbon source. This figure showed the pentose sugar uptake activity of cloned transporters without D-glucose uptake activity.

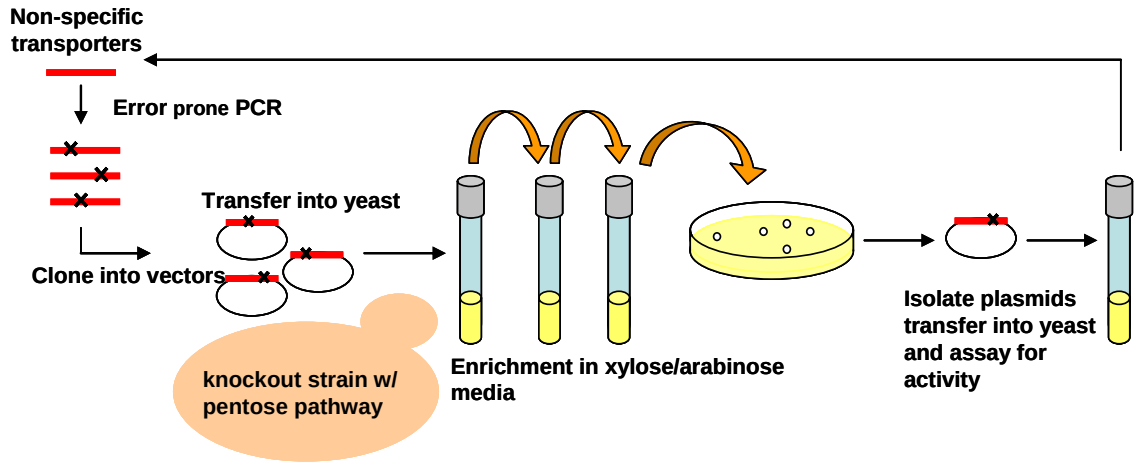

Figure 2.3 Strategy for transporter engineering using directed evolution. Using a transporter with pentose uptake activity as a template, mutations were introduced via error-prone PCR. The resulting PCR products were cloned into plasmid and transferred into a yeast transporter knockout strain with pentose utilization pathway. The population with improved transporter efficiency can be enriched in media with pentose sugar as sole carbon source. After several rounds of enrichment, the culture can be plated and the plasmids can be isolated from single colonies and transferred into transporter knockout strain to be assayed for sugar uptake activity. The resulted mutants with improved sugar uptake efficiency can be used as templates for the next round of library creation and directed evolution. The process can be repeated till the efficiency of the mutant is satisfactory or no more improvement can be observed.

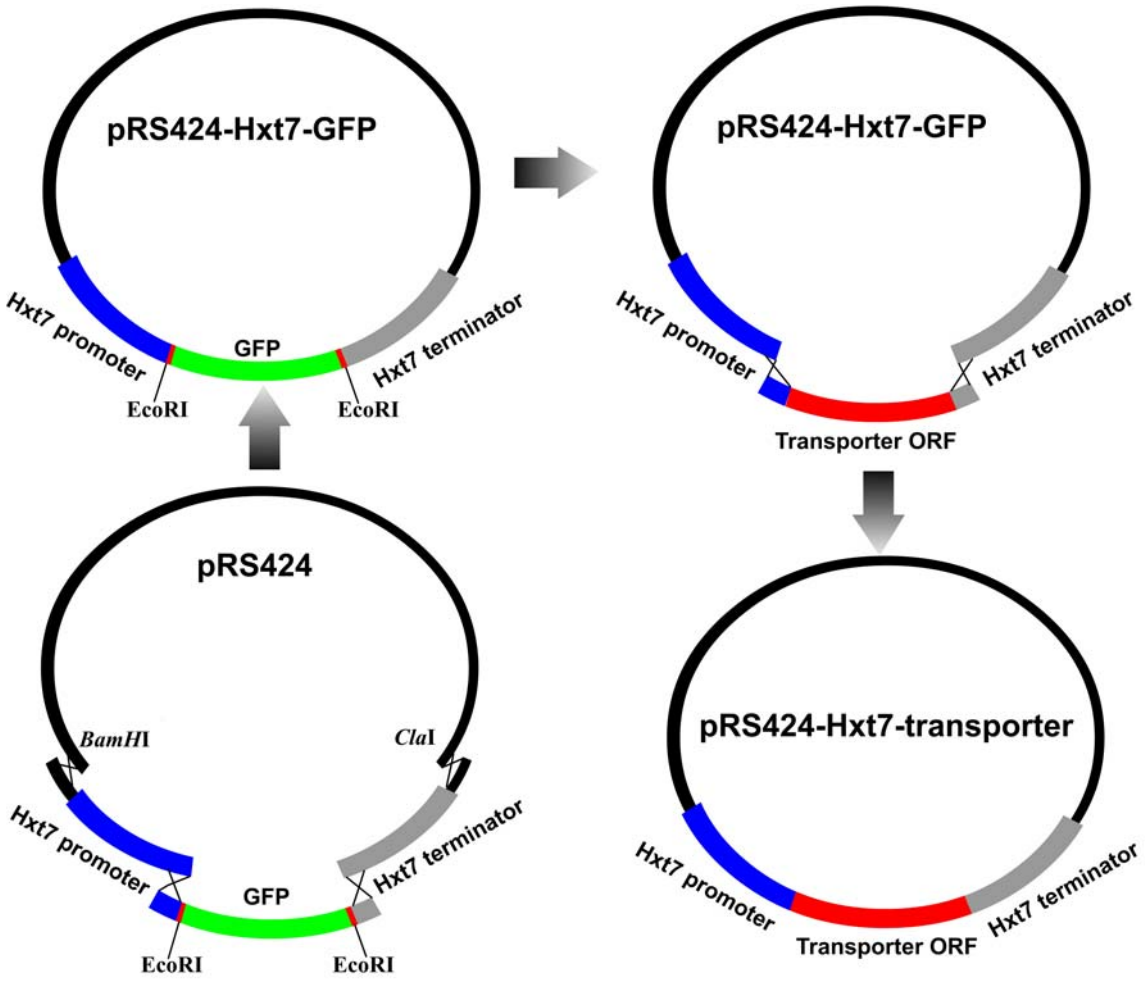

Figure 2.4 Cloning of putative pentose transporters using DNA assembler method. pRS424-HXT7-GFP plasmids were used for cloning of putative pentose-specific transporters. In this plasmid, a HXT7 promoter, a GFP gene flanked with *Eco*RI sites at both ends, and a HXT7 terminator were assembled into the pRS424 shuttle vector linearized by *Cla*I and *Bam*HI. PCR products of the putative pentose-specific transporters flanked with DNA fragments sharing sequence identity to the HXT7 promoter and terminator were co-transferred into CEN.PK2-1C with *Eco*RI digested pRS424-HXT7-GFP using the standard lithium acetate method.

Chapter 3. Biochemical Characterization of Pentose-Specific Transporters

3.1 Introduction

S. cerevisiae lacks an efficient transport system for pentose sugar uptake. Pentose sugars can only enter *S. cerevisiae* cells through the hexose uptake system, which occurs at a affinity two-orders of magnitude lower than normal hexose uptake [1]. In contrast, native D-xylose utilizing yeasts have been shown to use both low-affinity and high-affinity pentose sugar transport systems for D-xylose and L-arabinose uptake [2, 3].

Recent research has shown that introducing D-xylose transporters can improve D-xylose utilization by *S. cerevisiae* [4-6]. Despite the significance of pentose transporters for pentose utilization by *S. cerevisiae*, very limited research has been done so far to characterize novel pentose transporters in *S. cerevisiae*. Leandro *et al.* discovered two new D-glucose/D-xylose transporters from *Candida intermedi*a and characterized them at the molecular level. Among the two D-glucose/D-xylose transporters, one is a D-xylose-H^+ symporter while the other is D-xylose facilitator [2].

There are a number of properties that contribute the different performance of sugar transporters. First, since sugar transporter has to be correctly folded and localized into the cell membrane to be active, transporter localization is very important when introducing a sugar transporter into another species. Second, there are two types of transporters in *S. cerevisiae*, symporter and facilitator. For symporters, sugar uptake is coupled with proton uptake and ATP is needed in order to generate the proton gradient for sugar uptake. In contrast, sugar uptake is not coupled with proton uptake in facilitators; therefore, no ATP is required to be consumed for

sugar uptake with facilitator transporters. Usually, symporters exhibit higher sugar uptake affinity when compared to facilitators [2]. Third, similar to enzymatic reaction kinetics, the kinetics of sugar uptake can be described using a non-linear model; when sugar transport rate is plotted against extracellular sugar concentrations, the maximum sugar uptake rate is defined as V_{max} for a certain sugar transporter and the sugar concentration at which the uptake rate is half of the maximum sugar uptake rate is defined as K_m [7].

In this chapter, the D-xylose-specific transporters, An25 and Xyp29, together with the L-arabinose-specific transporter Xyp32, will be characterized for their cellular localization, type, and kinetic parameters.

3.2 Results

3.2.1 Cellular Localization of Pentose-Specific Transporters

Sugar transporters are trans-membrane proteins, and correct folding and localization in the cell membrane is required for functionality. Since no signal peptide was specifically added when the putative pentose transporters were cloned, it is important to ensure that the pentose-specific transporters are correctly localized in the cell membrane. This is particularly true for putative transporters like An25 cloned from filamentous fungi *Neurospora crassa*, which exhibits a very different physiology when compared to *S. cerevisiae*.

To study the cellular localization of pentose-specific transporters in *S. cerevisiae*, An25, Xyp29, and Xyp32, were fused with a green fluorescent protein (GFP) at their C-terminus via a GS-linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser). The resulting plasmids were transferred into wild-type *S. cerevisiae* strain CEN.PK2-1c and fluorescent images were taken using a

confocal microscope (Core Facilities, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL).

As shown in Figure 3.1, Yeast strains expressing D-xylose-specific transporters showed a distinctive fluorescent halo at the cell periphery. For An25 and Xyp32, almost all the GFP fluorescence appeared in the cell membrane, while a big portion of fluorescence of Xyp29 expressing cells remained in the cytoplasma. 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 expression of the transporter is not optimized or stable.

3.2.2 Type of Pentose-specific Transporters

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 affinities for sugars. Alternatively, sugar uptake through facilitators is not coupled with proton transport, and facilitators usually exhibit low sugar uptake affinities [2]. Symporter assays were performed for An25, Xyp29, and Xyp32 in the EBY.VW4000 strain. No elevation of pH in un-buffered cell suspensions was observed for any of the pentose transporters, indicating that pentose sugar uptake through these transporters was not coupled with proton transport (Figure 3.2). In other words, An25 and Xyp29 are D-xylose facilitators while Xyp32 is an L-arabinose facilitator.

3.2.3 Intracellular Accumulation of Pentose Sugar

Intracellular accumulation of both D-xylose and L-arabinose in the EBY.VW4000 strain expressing Xyp29, An25, and Xyp32 was also measured using HPLC. Cell cultures with 30 min,

60 min, 120 min, and 24 hour incubation with pentose sugars were sampled and analyzed using HPLC. As shown in Figure 3.3, EBY.VW4000 strains expressing Xyp29 and An25 exhibited D-xylose uptake activity during 24 hour incubation, while EBY.VW4000 strains expressing Xyp32 only exhibited L-arabinose uptake activity.

3.2.4 Kinetic Parameters of Pentose-specific Transporters

To further confirm that Xyp29, An25, and Xyp32 are actually pentose-specific transporters with no D-glucose uptake activity, ^{14}C-labeled sugar uptake assays were performed using ^{14}C-labeled D-glucose, D-xylose, and L-arabinose as substrates. As a result, D-glucose and L-arabinose uptake activity in the EBY.VW4000 strain expressing only Xyp29, An25, and Xyp32 were too low to measure under assay conditions used to determine pentose sugar uptake kinetics of both transporters (data not shown).

Using ^{14}C-labeled sugar uptake assay, kinetic parameters of pentose transport through An25, Xyp29, and Xyp32 were determined. It has been tested that under the assay condition, sugar uptake is within a linear range for the first 60 seconds (data not shown). The EBY.VW4000 strains expressing An25, Xyp29, or Xyp32 were incubated with D-xylose or L-arabinose for 40 or 60 seconds (exactly timed), and sugar uptake was stopped by the addition of ice-cold water. The reaction mixture was then filtered and washed before counting with a liquid scintillation counter. Sugar uptake rate and substrate concentration were fitted into a Michaelis–Menten equation by non-linear regression using OriginPro 8.1 (OriginLab Corporation, Northampton, MA). The K_m towards D-xylose for the transporter knockout strain expressing only An25 or Xyp29 were 175.74±21.36 mM and 55.96±9.37 mM, respectively. The V_{max} of EBY.VW4000 strains harboring An25 and Xyp29 were 0.03666±0.00287 mmol/h/gram dry cell

weight and 0.04147±0.00232 mmol/h/gram dry cell weight, respectively. For the EBY.VW4000 strain harboring Xyp32, the K_m and V_{max} towards L-arabinose were determined to be 48.00± 13.18 mM and 0.005577±0.001632 mmol/h/gram dry cell weight.

3.3 Discussion

The correct localization of pentose-specific transporters was confirmed using fluorescence imaging of the transporter-GFP fusion proteins. The correct localization of pentose-specific transporters from *N. crassa* and *P. stipitis* further confirmed the addition of an extra signal peptide is not required for heterologous expression of sugar transporters. As it was shown in Figure 2.1, most of An25 and Xyp32 fusion proteins are localized in the cell membrane of *S. cerevisiae* while a large portion of Xyp29-GFP fusion proteins are retained in the cytoplasm. This result indicated further improvement of expression and localization of Xyp29 may be necessary for it to function efficiently. The fact that not all the cells with transporter-GFP fusion proteins were visible during the fluorescence imaging suggested the expression level of transporters were not stable and optimized. This problem may be solved by altering the expression level and/or integrating the transporter genes into the genome of recombinant *S. cerevisiae*.

^{14}C-labeled sugar uptake assays, 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, while Xyp32 is only responsible for L-arabinose uptake. Of note, most sugar transporters studied in yeast for D-xylose uptake still have higher uptake activity towards D-glucose than D-xylose [2, 4]. Only *Trxlt1* from *Trichoderma reesei* exhibited

D-xylose-specific uptake activity after its adaptive evolution [8]. Our data indicates that Xyp29 and Xyp32, from *P. stipitis*, together with An25, from *N. crassa*, are the first three experimentally confirmed naturally occurring pentose-specific transporters introduced to *S. cerevisiae*.

In naturally occurring D-xylose-assimilating fungal species, both high-affinity D-xylose-proton symport systems and low-affinity D-xylose facilitated diffusion systems were present. The K_m value of these two systems were determined to be 0.4~4 mM for the symport system and around 140 mM for the facilitate diffusion system [2, 9]. These values are close to the affinity of the D-glucose uptake system in *S. cerevisiae*, with a 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 of D-xylose uptake in *S. cerevisiae* are only 190 mM for the high-affinity system and 1.5 M for the low-affinity system [1]. The affinities of our newly discovered pentose-specific transporters are lower when compared to the high-affinity D-xylose uptake system in naturally occurring D-xylose-assimilating yeast. However, when compared with the D-xylose uptake system in wild-type *S. cerevisiae*, the newly discovered pentose-specific transporters still showed higher affinities towards D-xylose. Especially for Xyp29 and Xyp32, the K_m towards pentose sugars were only one-fourth of the value for wild-type *S. cerevisiae*.

All three pentose-specific transporters were determined to be sugar facilitators. This result is consistent with the fact that kinetic parameters of these three pentose-specific transporters is similar to those of the low-affinity D-xylose facilitated diffusion system in naturally occurring pentose-assimilating yeasts [2, 3]. Despite the fact symporters have higher affinities towards D-xylose, overexpression of symporters may not always facilitate the 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 [4, 6]. Furthermore, The K_m values of 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* [4, 6]. It is very possible that D-xylose fermentation can be improved by introducing these newly characterized D-xylose-specific transporters.

3.4 Conclusions and Outlook

Two D-xylose-specific transporters and one L-arabinose-specific transporter were discovered from 18 putative pentose transporters. Identified pentose-specific transporters were cloned downstream a HXT7 promoter on the pRS424 multicopy plasmids, and their pentose uptake activities were confirmed by checking intracellular sugar accumulation using HPLC.

The cellular localization of pentose-specific transporters was studied by using transporter-GFP fusion proteins and confocal microscopy. All three pentose-specific transporters were expressed, correctly folded, and localized in the cell membrane.

To determine the type of pentose specific transporters, transporters were expressed in the EBY.VW4000 strains. Any changes in pH of the un-buffered solution during sugar uptake were recorded using a pH meter equipped with data collection software. None of the three pentose-specific transporters showed elevated pH together with sugar uptake, indicating that they are all pentose facilitators.

The specificities of the three newly cloned sugar transporters were further confirmed using a ^{14}C-labeled sugar uptake assay. The K_m and V_{max} values for all three pentose specific

transporters were determined using a ^{14}C-labeled sugar uptake assay. All three transporters are low-affinity transporters, but their affinity towards pentose sugars are still higher compared to endogenous hexose transporters in *S. cerevisiae*.

The overexpression of pentose-specific transporters may help pentose utilization in recombinant *S. cerevisiae* strains when the pentose utilization pathway is fast enough. Pentose-specific transporters may be more beneficial for mixed sugar fermentation by pentose-assimilating recombinant *S. cerevisiae*, as the strict substrate specificity may eliminate the inhibition of D-xylose uptake when D-glucose is present and thus alleviate the D-glucose repression.

3.5 Materials and Methods

3.5.1 Strains, Media, and Cultivation Conditions

S. cerevisiae CEN.PK2-1C (MATα *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^{c}*) was purchased from Euroscarf (Frankfurt, Germany), and was used for manipulation of recombinant DNA in yeast. Sugar transporter knockout strain EBY.VW4000 (CEN.PK2-1c *Δhxt1-17 Δstl1 Δagt1 Δydl247w Δyjr160c Δgal2*) was a gift from Eckhard Boles [10]. *Escherichia coli* DH5α (cell media facility, University of Illinois at Urbana-Champaign, Urbana, IL) was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% of Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% of ammonium sulfate, 0.05% of amino acid drop out mix). YPA media supplemented with 2% of sugar was used to grow yeast strains harboring no plasmids (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate). *E. coli* strains were cultured in Luria broth (ThermoFisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were cultured at 30 °C and 250

rpm for aerobic growth. Yeast strains were grown under aerobic condition for cell multiplification if not specified. *E. coli* strains were cultured at 37 °C and 250 rpm if not specified. All restriction enzymes were purchased from New England Biolabs (Ipwich, MA). All chemicals were purchased from Sigma Aldrich (St. Louis, MO) or ThermoFisher Scientific (Pittsburgh, PA).

3.5.2 Plasmid Construction of Transporter-GFP Fusion Using DNA Assembler Method

C-terminal fusion of D-xylose-specific transporters with the GFP fusion were constructed for the transporter localization study. A GS-linker consisting of amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser was introduce 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 with the transporters at the 5'-end and the HXT7 terminator at the 3'-end (See Table 3.1 for primer sequences for construction of transporter-GFP fusion). Transporter genes were amplified from the original pRS424-HXT7-transporter constructs to generate DNA fragments of the transporters flanked with nucleotide sequence identical to the HXT7 promoter at the 5'-end and the GS-linker-GFP at the 3'-end. These two fragments were then co-transferred into CEN.PK2-1C with pRS424-HXT7-GFP digested with *Eco*RI. The resulting transformation mixture was plated on SC-Trp supplemented with 2% D-glucose (Figure 3.4).

To confirm the proper construction of plasmids using the DNA assembler method, yeast plasmids were isolated using Zymoprep Yeast Plasmid Miniprep II (Zymo Research, Orange, CA). Yeast plasmids were then transferred into *E. coli* DH5α, which were plated on LB plates containing 100 mg/L ampicillin. Single colonies of *E. coli* transformant were then

inoculated into the LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR. All constructs containing heterologous transporters were submitted for DNA sequencing to confirm the correct construction (UIUC Core Sequencing Facility, Urbana, IL). The DNA sequencing results were compared with gene sequences in the NCBI database using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI).

3.5.3 Determination of Type of Transporters

To determine the transporter type, pH change of the EBY.VW4000 strain expressing D-xylose-specific transporters was measured in un-buffered cell suspension containing D-xylose, L-arabinose, or maltose using a Seven Multi pH meter equipped with an USB communication module and Direct pH software (Mettler Toledo, Columbus, OH). Plasmids encoding pentose-specific transporters were transferred into the EBY.VW4000 strain followed by plating on the SC-Trp plates supplemented with 2% maltose. Single colonies were inoculated with 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. The 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 50 mL water-jacketed beaker kept at 25 °C and provided 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 base line 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. As it was reported that pH in un-buffered *S. cerevisiae*

cell suspension should go up with the addition of maltose, 1 mL of 50% maltose solution was added to the un-buffered cell suspension to make sure the pH recording system is functional [3]. pH elevations of all the constructs were observed, indicating that the pH recording system can monitor the transient pH change in the experimental setting (Figure 3.5).

3.5.4 GFP Fluorescence Imaging of Transporter Expressing Live Cells

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 the SC-Trp plates with 2% maltose. Single colonies were inoculated in 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 florescence images were taken using an Andor Technology Revolution System Spinning Disk Confocal Microscope (Core Facilities, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL). Images were processed using Imaris image analysis and visualization software (Bitplane, Saint Paul, MN).

3.5.5 Analysis of Intracellular Accumulation of Pentose Sugars

Single colonies of the EBY.VW4000 strain expressing a target transporter was inoculated in 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 2 days of growth and resuspended in YPA medium supplemented with 2% D-xylose or L-arabinose to a final OD_{600} of 10. At 30 min, 60 min, 120 min, and 24 hours, 5 mL of culture samples were 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 a 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 (Shimadzu) following the manufacturer's protocol. The sugar uptake activity was calculated to be mg sugar extracted through osmosis per mL of cell culture at OD~10.

3.5.6 ^{14}C-labeled Sugar Uptake Assay

^{14}C-labeled D-glucose, L-arabinose, and D-xylose were purchased from American Radiolabeled Chemicals (St. Louis, MO) as solutions in 90% ethanol. Radio-labelled sugars were first dried in a chemical hood and then resuspended in water. Sugar solutions at a concentration of 1.33 M, 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 cultures at the exponential phase were harvested and washed twice with ice-cold water and resuspended to about 60 mg dry cell weight 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 hours to determine the cell dry weight. The rest of cell suspension was kept on ice before use. For sugar uptake assay, 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 seconds (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 (ThermoFisher 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. Sugar uptake rate was calculated to be mmol sugar transported per hour per gram of dry cell weight.

3.6 References

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3.7 Tables

Name		Forward primer	Reverse primer
AN25	AN25	caaaaagttttttaattttaatcaaaaaatggcgcctccaaagttcctgg	ctcatagaacctccacctccagaacctccacctccacgtgtttccttggtgttaatac
	GFP	cacgtggaggtggaggttctggaggtggaggttctatgagtaaaggagaagaacttttc	gaattaataaaagtgttcgcaaagaattcctatttgtatagttcatccatg
Xyp29	AN29	caaaaagttttttaattttaatcaaaaaatgtccagtgttgaaaaaagtgctc	ctcatagaacctccacctccagaacctccacctccgctgatgttttcgacatgctc
	GFP	cagcggaggtggaggttctggaggtggaggttctatgagtaaaggagaagaacttttc	gaattaataaaagtgttcgcaaagaattcctatttgtatagttcatccatg
Xyp32	Xyp32	caaaaagttttttaattttaatcaaaaaatgcacggtggtggtgacggtaac	ctgatgtctaccacgttgaaaaaggaggtggaggttctggaggtggaggttctatgag
	GFP	gaaaaaggaggtggaggttctggaggtggaggttctatgagtaaaggagaagaacttttc	gaattaataaaagtgttcgcaaagaattcctatttgtatagttcatccatg

Table 3.1 List of Primers for Construction of Transporter-GFP Fusion

3.8 Figures

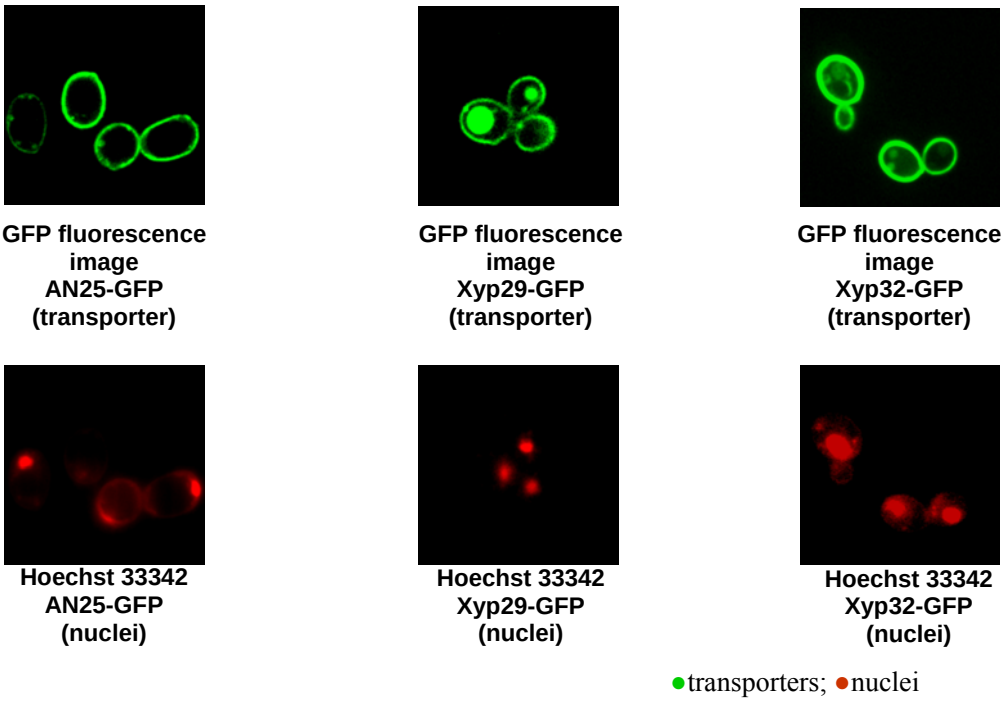

Figure 3.1 GFP fluorescence images of transporter localization. Fluorescence image of transporter-GFP fusion protein (upper row) and cell nuclei dyed with Hoechst 33342 (lower row) are shown of *S. cerevisiae* strain CEN.PK2-1C harboring transporter-GFP fusion protein expressing plasmids.

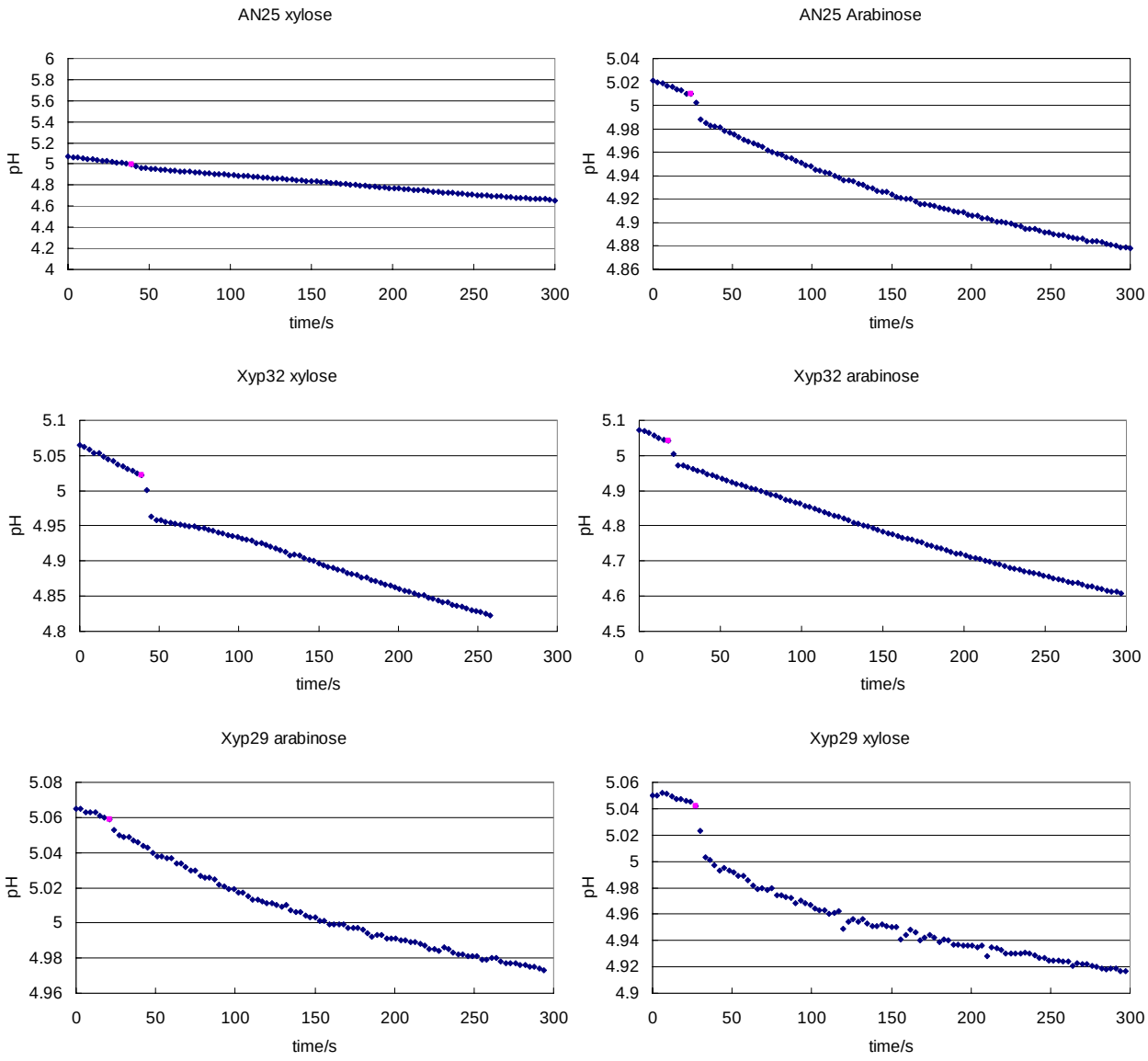

Figure 3.2 Symporter assay of AN25, Xyp29 and Xyp32. pH change in un-buffered cell suspension of *S. cerevisiae* EBY.VW4000 strains harboring pentose-specific transporter expressing plasmids. The pink point in figures indicated when the sugar solution is added into the system. As it shown the figures above, none of the transporter expressing strains showed elevated pH with the sugar uptake.

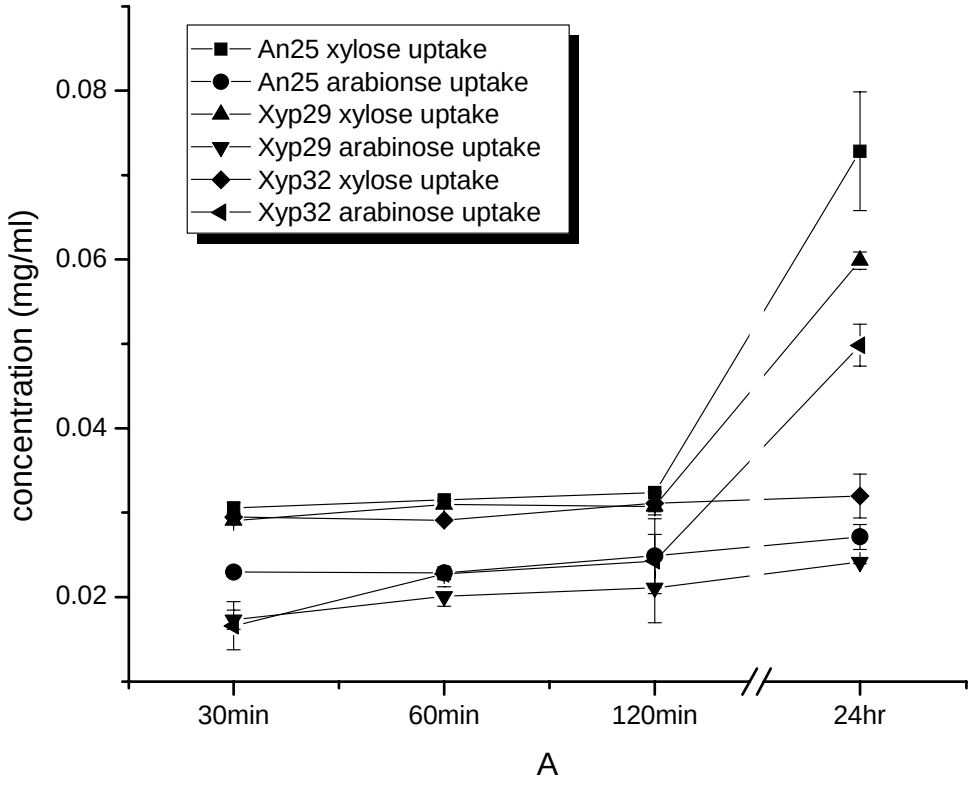

Figure 3.3 Pentose uptake of AN25, Xyp29 and Xyp32. *S. cerevisiae* stains EBY.VW4000 harboring pentose-specific transporter expressing plasmids are used to test the pentose uptake activity of transporters. After various length of incubation with D-xylose or L-arabinose, sugar accumulated in the cells was extracted via osmosis. The sugar uptake activity of sugar transporters were determined as mg/mL sugar extracted from 1ml of cell suspension of OD~10.

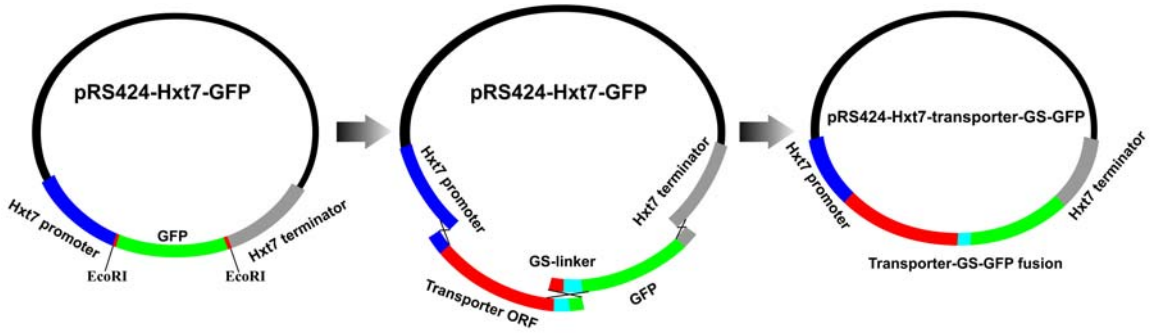

Figure 3.4 Construction of plasmids containing transporter-GFP fusion proteins using the DNA assembler method. The GS-linker was added to the N-terminus of the GFP open reading frame by a PCR primer, resulting in a PCR product of GS-linker-GFP flanked with nucleotide sequence homologous with the transporters 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 sequence identical to the HXT7 promoter at the 5'-end and the GS-linker-GFP at the 3'-end. These two fragments were then co-transferred into CEN.PK2-1C with pRS424-HXT7-GFP digested with *Eco*RI. The resulting transformation mixture was plated on SC-Trp supplemented with 2% D-glucose.

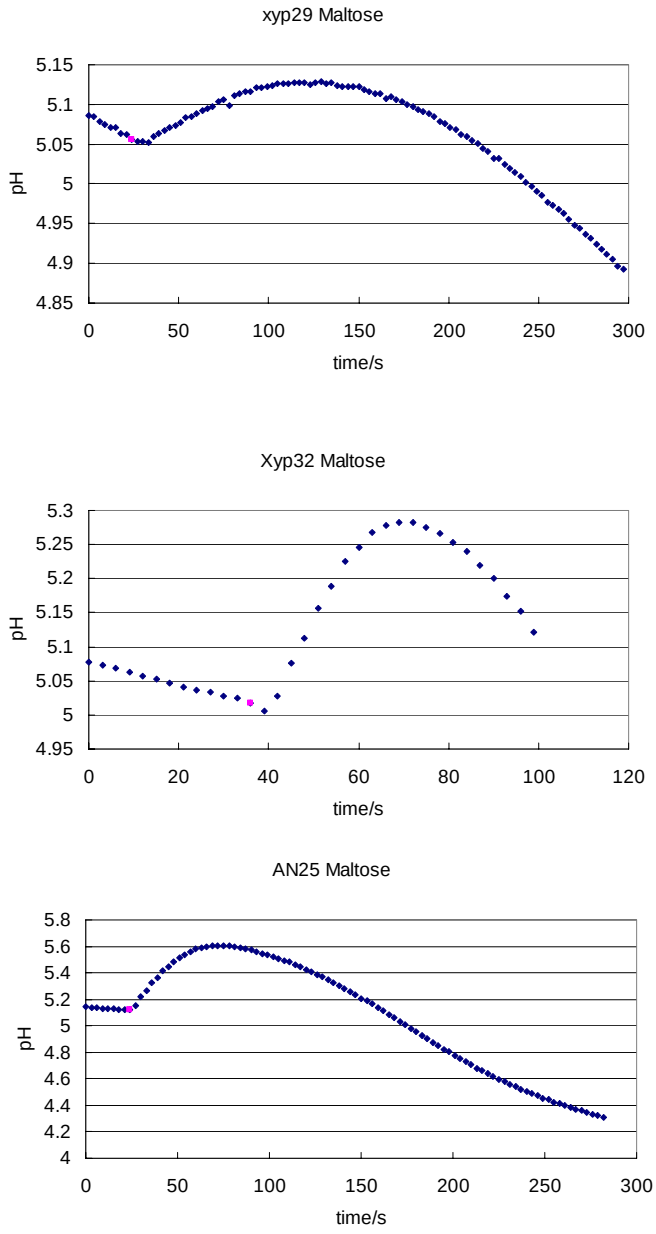

Figure 3.5 pH changes in the un-buffered cell suspension after the addition of maltose. As it was reported that pH in un-buffered *S. cerevisiae* cell suspension should go up with the addition of maltose, 1 mL of 50% maltose solution was added to the un-buffered cell suspension to make sure the pH recording system is functional. pH elevations of all the constructs were observed, indicating that the pH recording system can monitor the transient pH change in the experimental setting.