IMPROVED XYLOSE FERMENTATION BY EXPRESSION OF A PUTATIVE XYLOSE TRANSPORTER ENCODING GENE *HXT2.4* IN *SACCHAROMYCES CEREVISIAE*

BY

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THESIS

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Abstract

Saccharomyces cerevisiae is considered one of the promising microorganisms in lignocellulosic bioethanol production. Unfortunately *S. cerevisiae* cannot consume xylose, a pentose sugar which comprises almost 30% of lignocellulosic biomass. Metabolic and genetic engineering methods were used to develop *S. cerevisiae* that could consume xylose. However in *S. cerevisiae*, pentose sugars can only enter the cell through native hexose transporters which have two orders of magnitude lower affinities toward pentose sugar than hexose sugar. Thus pentose uptake is a limiting step in xylose fermentation using *S. cerevisiae*. In order to solve this problem, we introduced putative xylose transporter gene *HXT2.4* from natural xylose consuming *Scheffersomyces stipitis* into engineered xylose consuming *S. cerevisiae*. Xylose consumption by the *HXT2.4* expressing *S. cerevisiae* was tested through fermentation. To prove that *HXT2.4* indeed enhanced the flux of xylose into the yeast cell, intracellular xylose and xylitol concentrations were measured using 100% methanol quenching and extraction. The results showed that the *HXT2.4* expressing *S. cerevisiae* could accumulate 10% more xylose and 40% more xylitol than the control strain. Sugar uptake kinetic parameters were determined using ^{14}C-labeled xylose. The results showed higher V_{max} of *HXT2.4* expressing *S. cerevisiae* than control strain. Introduction of *HXT2.4* may improve xylose fermentation by engineered *S. cerevisiae* depending on strain background. More efficient transport of pentose sugar can improve the utilization of xylose, which will allow the development of an efficient xylose fermenting *S. cerevisiae* for the production of bioethanol from lignocellulose.

To my family and friends

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Chapter 1. Overview of xylose fermentation and pentose transport in *S. cerevisiae*

1.1 Biofuels and Lignocellulosic Biomass

1.1.1 Biofuels

Biofuels have been around us since ancient times. Wood and olive oil are the early known types of biofuels used by mankind for centuries. At the beginning of the automobile era in the early 1920's, Henry Ford planned to fuel his Model T's with ethanol. However, discovery of large deposits of petroleum kept gasoline and diesel prices low for decades, and thus biofuels were largely forgotten. The rising heavy industry and increased usage of fossil fuels augmented global warming and greenhouse gas emission. Lashof et al. analyzed the contribution of greenhouse gases in global warming, concluding that CO_2 emission accounts for 80% of the contribution to global warming (1990). A report published in 1990 thoroughly studied emission of CO_2 from transportation fuel, where it showed that 30% of CO_2 is coming from transportation emissions (De Luchi). Along with environmental problems, the unstable price and high dependence of the US on oil suppliers created an urgent need for alternatives to fossil fuels. Plant biomass offers an attractive alternative to produce cost efficient and sustainable transportation fuel through fermentation. One of the developed industrial processes in this respect is the production of fuel ethanol. Ethanol can be easily blended with gasoline up to 10% and used in cars without the need to modify engines. Furthermore blended gasoline produces less CO_2. Although CO_2 is released during the fermentation, the net effect is offset by the uptake of carbon gases by the plants which produce biomass for ethanol fermentation (Wang et al., 1980).

1.1.2 Lignocellulosic Biomass

Ethanol is the main biofuel produced in large amounts by *Saccharomyces cerevisiae* fermentation of starch and sugars from corn and sugar cane, common biomass feedstocks. Wild type *S. cerevisiae* strains readily ferment glucose, mannose and fructose via the Embden Meyerhof pathway of glycolysis while galactose is fermented via the Leloir pathway (van Maris et al., 2006). Since corn and sugar cane are used as food, it's vital to produce ethanol from non-food feedstocks such as lignocellulosic biomass. When the US enacted the Energy Independence and Security Act of 2007, the US government set a goal to produce 36 billion gallons of renewable fuel by 2022, with 16 billion to be obtained from cellulosic ethanol to address energy security and climate change concerns (Energy Independence and Security Act of 2007). Every year, the world agricultural industry accumulates vast amounts of lignocellulosic crop residues which comprise more than half of the world agricultural phytomass. Corn stover, sugarcane bagasse, rice and wheat straws are the most promising and abundant cellulosic feedstock derived from agricultural plant residues. Around 10-30% of the total dry weight in common woody agricultural residue consists of xylose (Galbe et al., 2007).

Sulfite pumping of hardwood is the main process by which xylose from lignocellulosic biomass are obtained. Depending on the process condition, sulfite pretreatment can recover up to 95 % of xylose from lignocellulose (Galbe et al., 2007). However, the main host microorganism used for bioethanol production, *S. cerevisiae*, cannot effectively utilize xylose, thus metabolic engineering of the host microorganism is needed.

Despite the fact that lignocellulosic biomass is an attractive substrate for bioethanol production, its rigid and complex structure remains a major economic and technical problem to lignocellulosic based biofuel production.

1.2 Lignocellulosic Biofuel Production Using *Saccharomyces cerevisiae*

1.2.1 *S. cerevisiae* is a Preferred Microorganism For Biofuel Production

Due to high yields of ethanol, high ethanol and inhibitor tolerance and ease of genetic manipulations, *S. cerevisiae* is the most attractive candidate for lignocellulosic bioethanol production (van Maris et al., 2006). In addition, *S. cerevisiae* is already used in industrial scale production of ethanol from glucose. Moreover high resistance of *S. cerevisiae* to low fermentation pH is useful to prevent bacterial contamination during fermentation. Unfortunately *S. cerevisiae* is not able to utilize pentose sugars extracted from lignocellulosic biomass (Jeffries 2006).

Yeast species like *Candida utilis*, *Pichia stipitis* (Wang et al., 1980) and *Pachysolen tannophilus* (Du Preez et al., 1984) are well known for their ability to efficiently consume xylose, however low productivity of ethanol, low tolerance to inhibitors and lack of genetic tools for these microorganisms make them incompatible for industrial lignocellulosic ethanol production. Therefore, *S. cerevisiae* is the best strain to engineer for efficient pentose utilization (Jeffries et al., 2004).

1.2.2 Xylose Utilization by *S. cerevisiae*

The main strategy for engineering xylose consuming *S. cerevisiae* is the introduction of heterologous genes responsible for pentose utilization from natural xylose consuming yeast (Jeffries et al., 2004; Kim et al., 2012; Yu et al., 1995). Xylose utilization requires initial reduction and oxidation steps which are not available in *S. cerevisiae*. The best natural xylose utilizing yeast *S. stipitis* has served as a good source of genes for engineering *S. cerevisiae* to consume xylose. The *XYL1*, *XYL2* and *XYL3* coding for xylose reductase (XR), xylose dehydrogenase (XDH) and xylulose kinase (XK) were expressed in *S. cerevisiae* (Fig 2.1). The

resulting transformants were able to utilize xylose by converting it to xylitol and then to xylulose and xylulose 5-phosphate which enters the pentose phosphate pathway and glycolysis (Kim et al., 2012; Yu et al., 1995; Hector et al., 2008). Expression of fungal (*Piromycies*) xylose isomerase (XI) gene is another approach to engineer xylose utilization in *S. cerevisiae* which converts xylose directly to xylulose bypassing the oxidoreductase step (Fig 2.1). A lot of effort has been focused on improving the xylose utilization pathway in *S. cerevisiae* over the past few decades; however efficient fermentation of xylose with engineered *S. cerevisiae* is limited by several issues including redox imbalance, low influx to pentose phosphate pathway and lack of xylose specific transporters in *S. cerevisiae* (Hector et al., 2008).

Recently Kim et al. developed a very efficient xylose consuming strain of *S. cerevisiae* using *XYL1*, *XYL2* and *XYL3* coding for XR, XDH and XK from *S. stipitis* which allows oxidative use of xylose with production of ethanol and xylitol. The strain was able to convert xylose to xylitol using *XYL1*, *XYL2* and NAD^+ as a cofactor. The produced xylitol was converted to xylulose through the *XYL3* gene. The engineered strain was able to consume xylose with a specific xylose consumption rate of 0.83 g xylose/g cell/h and an ethanol yield 0.37g/g (2013). Kuyper et al. developed a strain (RWB 202 AFX) that could consume xylose under aerobic conditions with a yield of 0.42 g/g (2004) by expressing evolved XI which converts xylose directly to xylulose.

1.2.3. Xylose Transportation by *S. cerevisiae*

S. cerevisiae transports xylose using hexose transporters (hxt1p-hxt7p, Gal2p, Snf3p) through facilitated transportation (Maier et al., 2002; Sedlak et al., 2004). Many of the transporters contain 12 putative transmembrane domains, which is similar to most transporters (Leandro et al., 2009). Engineered *S. cerevisiae* mainly ferment xylose through high affinity

hexose transporters Hxt 4, Hxt 5, Hxt 7 and Gal2 (Sedlak et al., 2004). However, the affinity of those transporters to glucose (K_m=1-28mM) is much higher than to xylose (K_m=49-300 mM), thus making xylose assimilation strongly inhibited by the presence of glucose (Kotter et al., 1993).

Xylose uptake could be a limiting step in engineered xylose consuming *S. cerevisiae*. Overexpression of native transporters in xylose consuming *S. cerevisiae* did not show any significant improvement in xylose fermentation (Sedlak et al., 2004). However, laboratory evolutionary engineering of *S. cerevisiae* aimed to improve its native transporters resulted in significant xylose uptake by engineered *S. cerevisiae* (Kuyper et al., 2005). This result suggests that improvement of xylose uptake through overexpression of pentose transporters could improve xylose fermentation in engineered *S. cerevisia*e (Gardonyui et al., 2003).

The existence of natural xylose consuming yeast, which has both low affinity and high affinity pentose specific transporters, is well known. The species *S. stipitis* and *C. intermedia* are considered the best xylose fermenting yeast (Jeffries et al., 2006; Leandro et al., 2006). Studies have been conducted to express heterologous transporters from natural xylose consuming yeast in engineered *S. cerevisiae* with the aim to improve xylose fermentation. Leandro et al. (2006) isolated *GXS1* glucose/xylose symporter and *GXF* facilitator from *C. intermedia* which showed xylose uptake capability, yet exhibiting low affinity to xylose. The studies by Katahira et al. (2008), Tanino et al. (2012), Saloheimo et al. (2007), Runquist et al. (2010) and Du et al. (2010) focused on heterologous expression of transporters in recombinant *S. cerevisiae* with an aim to improve xylose uptake rate. *GXF1* and *GXS1* gene overexpression from *C. intermedia* improved xylose consumption rate with high ethanol yield in XI harboring strains (Tanino et al., 2012). The kinetic characterization of transporters from *S. stipitis* and *C. intermedia* in recombinant *S.*

cerevisiae showed increased xylose transport performance compared with the control strain harboring native transporters (Runquist et al., 2010). Du et al. identified and characterized two xylose specific transporters Xyp29 and An25 from *S. stipitis* and *N. crassa* respectively; however overexpression of those transporters in recombinant *S. cerevisiae* harboring a xylose utilizing pathway did not improve xylose uptake rate (2010).

1.3 Rationale of Putative Xylose Transporter Study and Thesis Overview

1.3.1 *S. stipitis* is a Good Candidate Source for Putative Pentose Transporter Study

S. stipitis is a haploid, homothallic yeast which is closely related to endosimbiotic yeast found in the gut of beetles and has the highest native capacity for xylose fermentation of any known microbe (van Dijken et al., 1986). Genome sequencing of *S. stipitis* revealed that it possesses a considerable amount of genes responsible for lignocellulose assimilation and fermentation. *S. stipitis* contains a specifically arranged combination of β-glucosidases and β-endoglucosidases in close proximity to sugar transporters (Jeffries et al., 2007). Slow cellobiose fermentation by an engineered *S. cerevisiae* strain expressing *HXT2.4* transporter and *BGL5* β-glucosidases from *S. stipitis* has been reported (Nelson et al., 2010). This suggests that each cluster could have a putative sugar transporter. In addition, Ha et al., (2013) reported that putative transporter Hxt2.4 from *S. stipitis* is able to efficiently transport cellobiose into engineered *S. cerevisiae* which originally could not transport the disaccharide. We hypothesized that if Hxt2.4 is able to transport cellobiose, it's possible that it can also transport xylose. According to this hypothesis, we can isolate a multisugar transporter with capability to transport both xylose and cellobiose, which can allow efficient cofermentation of those sugars in engineered *S. cerevisiae.* Moreover, improving xylose specificity could alleviate glucose repression, making glucose and xylose cofermentation more efficient. According to this

hypothesis, a putative xylose transporter encoded by *HXT2.4* from chromosome 1 of *S. stipitis* was chosen for evaluation and characterization in engineered *S. cerevisiae*.

1.3.2 Thesis Overview

The aim of this thesis is to characterize a putative pentose transporter Hxt2.4 from *S. stipitis* by expressing it in engineered xylose fermenting *S. cerevisiae* strains. In order to characterize Hxt 2.4 transporter, we would like to express *HXT2.4* in two engineered *S. cerevisiae* strains with different xylose fermentation capabilities. The first strain, DX123, is not optimized to use xylose and has a slow fermentation rate as compared to the second strain, SR8, which has optimized xylose utilization with a high rate of fermentation. Moreover, in order to prove that expression of *HXT2.4* indeed improves xylose uptake rate, intracellular xylose and xylitol concentrations were measured. Finally, we investigated cellular localization and kinetic parameters of *HXT2.4* expressing *S. cerevisiae*.

Chapter 2. Improvement of xylose fermentation by overexpression of Hxt2.4 transporter in *S. cerevisiae*

2.1 Introduction

The fermentation of pentose sugar like xylose by *S. cerevisiae* can be performed by introducing the xylose pathway from native xylose consuming yeast *Scheffersomyces stipitis*. However *S. cerevisiae* does not possess sugar transporters which are specific to xylose. Hence, xylose enters the yeast cell through nonspecific hexose transporters which have low affinities to xylose. Thus identification of xylose specific transporters is important to improve xylose fermentation and cofermentation ability for pentose and hexose sugars. In order to solve these problems, several research groups attempted to overexpress putative transporters from natural xylose consuming fungi like *N. crassa*, *S. stipitis* and *C. intermedia* in *S. cerevisiae* (Du et al., 2010; Runquist et al., 2010; Leandro et al., 2006). Transporter kinetics of the putative transporters Gxf1, Sut1 and At5g59250 have been investigated using ^{14}C- labeled xylose to evaluate the effect of the transporter in engineered *S. cerevisiae*. The expression of transporters in engineered *S. cerevisiae* improved xylose uptake kinetics (Runquist et al., 2010). These results showed that putative xylose transporters can improve xylose uptake kinetics in recombinant *S. cerevisiae*. In addition the genome sequence of *S. stipitis* revealed that it possesses several different transporters which can transport cellobiose or xylose located near the enzymes that can degrade lignocellulose (Jeffries et al., 2007). Based on that knowledge, Ha et al. attempted to overexpress the *HXT2.4* gene from *S. stipitis* in *S. cerevisiae* harboring a cellobiose utilizing pathway (Ha et al., 2013). The study showed that Hxt2.4 transporter is able to transport cellobiose (Ha et al., 2013). According to evidence that Hxt2.4 has cellobiose transport capability, we hypothesized that it can transport xylose and improve xylose fermentation in engineered *S. cerevisiae*. To investigate our hypothesis, we overexpressed

HXT2.4 in two engineered xylose fermenting *S. cerevisiae* strains and performed fermentation experiments using xylose. The experimental results showed improvement in xylose fermentation. To further confirm our hypothesis, we measured intracellular xylose and xylitol concentrations using HPLC and checked accurate localization of the transporter in the membrane. The results showed that Hxt2.4 can correctly localize in the cell membrane and *HXT2.4* overexpression can improve xylose uptake depending on strain background. Xylose uptake kinetics of *HXT2.4* expressing *S. cerevisiae* were also studied. Sugar uptake rates follow saturation kinetics, and the Michaelis-Menten model is used to describe sugar uptake kinetics by yeast cells expressing *HXT2.4* and a control strain with V_{max} as the maximum sugar uptake rate and a half of the V_{max} as the sugar affinity or K_m (Lee et al., 2002; Coons et al., 1995). *HXT2.4* expressing *S. cerevisiae* showed improved V_{max} with high K_m.

2.2 Results

2.2.1 Construction of an Integration Plasmid and Fermentation Experiments

We used two different strains of xylose fermenting *S. cerevisiae*, DX123 and SR8 exhibiting different xylose fermentation capabilities. DX123 was constructed by integration of *XYL1*, *XYL2* and *XYL3* in to the *S. cerevisiae* D452-2 strain (Sugiyama and Nikawa, 2001). DX123 could consume xylose slowly and accumulate large amounts of xylitol (Kim et al., 2012). The SR8 strain is engineered for efficient xylose fermentation through rational and combinatorial approaches. In addition to optimized integration of *XYL1*, *XYL2* and *XYL3* genes into the genome, laboratory evolutionary engineering was performed on SR8, which allowed it to consume xylose efficiently with low accumulation of xylitol (Kim et al., 2013).

In order to check the effect of *HXT2.4* overexpression on xylose fermentation, an integration plasmid with *HXT2.4* under the control of a strong and constitutive promoter was

constructed. The pRS426 plasmid harboring *HXT2.4* with PGK promoter and CYC1 terminator was used as a template to PCR amplify the PGK-*HXT2.4*-CYC1 cassette. The amplified fragment was then cloned into the pITY3 integration vector which integrates the target gene randomly into Ty sites of *S. cerevisiae* via the G418 resistance marker (*KanMX*) (Parekh et al., 1996).

The integration vector containing *HXT2.4* expression cassette and empty plasmid as a control were linearized with XhoI restriction enzyme and transformed to both DX123 and SR8 strains using high efficiency transformation (Gietz et al., 2007). The transformants were plated on YPD with G418 antibiotic at a concentration of 200µl/mL. The growing transformants resulted in DX-H and SR8-HXT2.4 strain, respectively (Fig 2.2) (Gietz et al., 2007).

To examine the effect of *HXT2.4* transporter on xylose fermentation by DX-H and SR8-HXT2.4, fermentation experiments were performed using YP media with either 20g/L or 40g/L of xylose. Both DX-H and SR8-HXT2.4 were grown on YPD media for 12 hrs and inoculated into 25mL YPX 20g/L or YPX 40g/L media at OD_{600}~1. The fermentation profile of SR8-HXT2.4 did not show improvement in both xylose consumption and final ethanol concentration in YPX 40g/L (Fig 2.3). The DX-H strain could not consume all the xylose in the same conditions due to high accumulation of acetate which is toxic in concentration more than 1.5 g/L (data is not shown). Under fermentation conditions with YPX 20g/L, DX-H showed better xylose consumption (improved by 20%), faster ethanol production (improved by 7%) and growth rate (improved by 50%) than the control strains (Fig 2.4). Interestingly DX-H started to produce ethanol from the beginning of fermentation whereas the control strains had a 13 hrs lag period.

2.2.2 Cellular Localization of Hxt2.4 Transporter

The correct localization and folding inside of the cell membrane is highly important for proper function of a heterologous transporter. As Hxt2.4 transporter originated from *S.stipitis*, it can be localized differently in *S. cerevisiae.* In order to ensure correct localization of the transporter in the cell membrane of *S. cerevisiae*, a plasmid containing *HXT2.4* with a fluorescent protein (GFP) fusion at the C-terminus was used (Ha et al.,, 2013). The plasmid was transformed to the D452-2 strain. The experiment confirmed that HXT2.4 was correctly folded in the cell membrane. The results and methods of this experiment are described in Ha et al., 2013.

2.2.3 Analysis of Intracellular Xylose and Xylitol Accumulation

We hypothesized that if DX-H has increased xylose uptake, it should accumulate higher amounts of intracellular xylose and xylitol than the control strain. To verify this hypothesis we measured intracellular xylose and xylitol concentrations of DX-H and DX123 strains using methanol extraction and high performance liquid chromatography (HPLC). Because the DX-H strain is already harboring a xylose utilizing pathway, we decided to use 100% methanol quenching to stop cell metabolism prior to extracting xylose and xylitol from the yeast cells.

In this study we used DX123 and DX-H strains and a slightly modified metabolite extraction method described in Sellick et al. (2011). The cells were grown in YPD media and inoculated into YPX 20g/L media at OD_{600}~10, then incubated for 9 hrs at 30^{o}C and 80rpm. 1mL of OD_{600}~10 cell culture samples were collected after incubation and added directly to 49mL of 100% methanol at -40 ^{o}C for quenching. The cell suspension was immediately mixed and incubated for 1min at room temperature followed by centrifugation at 5000g for 10min at -9^{o}C (Fig 2.5). The cell pellet was then used to extract xylose and xylitol using two 100%

methanol extractions followed by a single water extraction (Fig 2.6) (Sellick et al., 2011). Extracted xylose and xylitol were dried and resuspended in 100μL water. Suspensions were analyzed using high performance liquid chromatography (1200 series; Agilent Technologies) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA).

The results showed that DX-H accumulated approximately 10% higher xylose and 50% higher xylitol than the control strain DX123 (Fig. 2.7) which indicates increased xylose uptake by DX-H. However intracellular xylose and xylitol concentrations in SR8-HXT2.4 did not show any difference, which can be explained by the fact that the xylose pathway in SR8-HXT2.4 is optimized to efficiently convert xylose into ethanol. Therefore xylose and xylitol did not accumulated in high amounts in both SR8-HXT2.4 and SR8 (data is not shown).

2.2.4 ^{14}C- Labeled Xylose Experiment to Determine V_{max} and K_m

Xylose uptake kinetics of DX123 and DX-H strains were measured. ^{14}C- labeled xylose at different concentrations was used as a substrate. In order to perform the assay, cells were grown on YPD media and washed twice in potassium phosphate (pH 6.8) buffer. The cells then were resuspended in the same buffer and ^{14}C- labeled xylose at different concentrations was added. The reaction started by mixing the cell suspension and ^{14}C- labeled xylose in a 50mL tube followed by incubation for 10 sec (accurately timed) and quenching by ice cold water. The reaction mixture was washed twice and filtered prior to counting in a liquid scintillation counter. The measured xylose uptake rate was plotted against substrate concentration and fitted to the Michaelis-Menten model (Fig 2.8). V_{max} and K_m were determined using Lineweaver-Burk plots (Fig 2.9) (Lee et al., 2002; Coons et al., 1995). Because both DX123 and DX-H also have native hexose transporters, which can transport xylose, the determined kinetic parameters refers to the

overall xylose uptake by all transporters. Xylose uptake kinetic parameters of DX123 showed a higher affinity to the substrate with K_m=281.85 mM than DX-H (K_m=1474mM), however V_{max} of the DX123 strain was lower (V_{max}=0.014mmole/h/g DCW) than the DX-H strain (V_{max}=0.07 mmole/h/g DCW) (Table 2.1)

2.3 Discussion

We overexpressed *HXT2.4* in two xylose fermenting strains DX123 and SR8. The pITY3 integration plasmid with *HXT2.4* under control of the PGK promoter and CYC1 terminator was used. The fermentation profile of *HXT2.4* expressing SR8 strain did not show any improvement in xylose fermentation; however overexpression of *HXT2.4* in the DX123 strain showed improvement both in xylose uptake rate and ethanol production rate in YPX 20g/L (Fig 2.4). Hxt2.4 transports xylose into the cell through facilitated diffusion, thus transport is dependent on intracellular and extracellular sugar concentration. The greater the difference in concentration gradient between extracellular and intracellular compartments, the better the xylose diffusion occurs. Because SR8-HXT2.4 has an optimized internal xylose utilizing pathway, intracellular xylose concentration remains very low. In another words, xylose is immediately converted to xylitol and channeled to the pentose phosphate pathway and glycolysis. Therefore low internal xylose concentration increased the flux of xylose in the SR8-HXT2.4 strain through its native transporters, thus the effectiveness of Hxt2.4 transporter was not observed (Fig 2.10). From this observation we can conclude that xylose transportation in SR8 is not the limiting step. DX-H has a xylose fermenting pathway with only a single copy of *XYL1, XYL2* and *XYL3* integrated in the genome, thus xylose fermentation is slow. However overexpression of *HXT2.4* in DX-H showed better performance of xylose fermentation in comparison with control DX123 and DX123

expressing empty pITY3 plasmid. It's worthy to mention that pITY3 plasmids integrate randomly in the genome; having DX123 with an empty pITY3 plasmid as a control eliminated the possibility that integration of the plasmid itself could improve xylose fermentation due to random changes in the genome structure (Parekh et al., 1996). Because internal assimilation of xylose in DX-H is slow, the concentration of intracellular xylose remained high which makes native hexose transporters less efficient in transporting xylose, therefore expression of *HXT2.4*, which has better V_{max}, could improve fermentation in the DX-H strain (Fig 2.10).

Expression of GFP tagged *HXT2.4* revealed that the transporter correctly localizes in the cell membrane, which suggests that some inherent sequence exists in *HXT2.4* ORF that direct the protein to localize correctly in the cell membrane. It was well known that transporters from other filamentous fungi like *N. crassa* or *C. intermedia* can be correctly expressed and localized in the membrane of *S. cerevisiae* (Du et al., 2010; Katahira et al., 2008; Leandro et al., 2006). Furthermore, sugar transporters from species like *Arabidopsis thaliana*, which is a plant genus, can also be expressed and localized correctly in the *S. cerevisiae.* These facts indicate that transporter localization mechanism is preserved in different species (Hector et al., 2008).

To further characterize the Hxt2.4 transporter, the DX-H strain was used for intracellular xylitol and xylose concentration measurements. Because putative xylose transporters should increase xylose uptake rate, we hypothesized that the strain with Hxt2.4 transporter should accumulate a higher amount of intracellular xylose and xylitol in comparison to the control strain. Other studies also used a similar approach to verify xylose transport through putative pentose transporters with some difference in methodology (Du et al., 2010). The results showed that the DX-H strain had 10% more xylose and 50% more xylitol accumulation (Fig 2.7) than the

control DX123 strain. This result further proves that *HXT2.4* gene expression is able to increase xylose flux into the cell.

The intracellular measurement of xylose and xylitol concentration in engineered *S. cerevisiae* expressing *HXT2.4* resulted in higher xylitol and xylose accumulation. This observation suggests that Hxt2.4 increases the flux of xylose in to the cell. For further confirmation, we performed a ^{14}C -labeled sugar uptake assay. The experiment demonstrated that DX-H had a 5 fold lower affinity (K_m) to xylose than DX123, however the maximum rate (V_{max}) was 5 fold higher in DX-H. Because DX123 native transporters have a low maximum rate and not optimized internal xylose utilizing pathway, high xylose concentrations will saturate the transporters, reducing the transport of xylose. The high maximum rate of Hxt2.4 transporter in DX-H will compensate xylose saturation of native transporters thus facilitating xylose uptake. Even though the overall xylose affinity of DX-H is lower than the control strain, higher maximum rate of DX-H than the control strain increased xylose uptake rate which showed high intracellular xylose and xylitol accumulation (Fig. 2.7). The experiment suggests that Hxt2.4 is a non-specific xylose transporter due to high K_m. Further studies are needed to measure the sole Hxt2.4 kinetic characteristics and substrate preference.

S. cerevisiae evolved to efficiently consume glucose, thus the whole internal metabolism of *S. cerevisiae* is controlled by the presence of glucose, also known as glucose repression (Salusjarvi et al., 2008). The glucose repression phenomenon down regulates the utilization of other sugars including xylose. Therefore *S. cerevisiae* will consume glucose first and after that will consume any other sugar present in the medium. Because sugar extraction from lignocellulose releases a considerable amount of glucose, it's highly important to engineer *S. cerevisiae* with xylose specific transporters which can improve cofermentation of glucose and

xylose, thus alleviating glucose repression in engineered *S. cerevisiae*. Also, according to this study and the previous study (Ha et al., 2013), Hxt2.4 can transport multiple sugars with different properties. For example Ha et al., 2013 confirmed that Hxt2.4 can transport cellobiose (disaccharide), and this study confirmed that Hxt2.4 can improve xylose (monosaccharide) transport. The multisugar transport properties of Hxt2.4 could give advantages in fermenting lignocellulosic hydrolyzates because it has different types of sugars, allowing cofermentation and higher productivity of ethanol. This makes Hxt2.4 an interesting target for future studies with a goal to isolate multisugar transporters.

2.4 Materials and Methods

2.4.1 Strain and Plasmid Construction

S cerevisiae strains DX123 (D452-2 *ura3*::*URA3* pSR6-X123), SR8 (*ald6*::*AUR1-C* pAUR_d_ALD6*his1*::*HIS1* pSR3-X23, *ura3*::*URA3* pSR6-X123, *leu2*::*LEU2* pYS10) (Kim et al., 2013) and D452-2 (*MATα leu2 ura3 his3*) (Hosaka et al., 1992) were used for putative transporter overexpression. *Escherichia coli DH5* (*F_ recA1 endA1 hsdR17 [rK _mK_] supE44thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation. In order to construct the integration vector, the *HXT2.4* gene was amplified by PCR from the pRS426 PGK – *HXT2.4* – CYC1(Ha et al., 2012) plasmid using forward primer HXT-F (`5 – GGCGGATCCA AAAATGTCTGACAAACTTCACAACATCAAG–3`) with SacI enzyme site and the reverse primer HXT-R (`5-GGCGTCGACATAAT CAGGTATAATTTATTGACTAAAGCT TAG-3`) with SalII enzyme site. The PCR product was digested with SacI and SalII (New England Biolabs) and ligated to pITY3 integration plasmid linearized with the same restriction enzymes (Parekh et al., 1996). The plasmid was linearized using XhoI restriction enzyme (New England Biolabs) and transformed to *S. cerevisae*

strains DX123 and SR8 using standard Lithium-Acetate transformation (Gietz et al., 2007). The resulting transformation mixtures were plated to YPD plates containing G418 antibiotic at a concentration 200μg/mL (Invitrogen).

2.4.2 Media and Cell Cultures

E. coli was grown in Luria- Bertani medium with addition of either ampicillin (50μg/mL) or kanamycin (50μg/mL). The cells were cultivated at 37 ^{o}C degrees and 250 rpm. *S. cerevisiae* cells were cultivated in in YP medium (10g/l yeast extract and 20g/l peptone) containing glucose (YPD) or xylose (YPX). The concentrations of the sugars were displayed as numbers following their initials (e.g., YPX 40g/L, YP medium containing 40g/l xylose), except YPD, which contained 20g/l glucose.

2.4.3 Fermentation Experiments

The yeast strains were grown on YPD medium at 30 ^{o}C and 250 rpm to prepare inoculum for YPX 20g/L or YPX 40g/L fermentation experiments. Cells were harvested at mid-exponential phase and washed with deionized water prior to inoculation. All flask fermentations were conducted using 25 mL YPX 20g/L or YPX 40g/L in a 125 mL flask at 30 ^{o}C and 80rpm (microaerobic) with an initial optical density at 600nm ~ 1.0. All fermentations were done in duplicates.

2.4.4 Intracellular Xylose and Xylitol Measurement

Single colonies of DX123 expressing *HXT2.4* gene and DX123 (control) were inoculated in 5mL YPD medium. The cultures were harvested at mid exponential phase and reinnoculated to 10mL YPX 20g/L in 50 mL flask with initial optical density at 600nm (OD_{600}) ~10. The cells were incubated at 30^{o}C and 80 rpm for 10 hrs. 1mL cell cultures normalized to OD_{600} ~10 were used. The cell cultures were quenched by direct addition of 49mL of 100% methanol precooled

to -40 ^{o}C in a 50mL conical tube and incubated for 10 min at room temperature. The quenching mixture was centrifuged at -9 ^{o}C for 10 min. The supernatant was removed completely by vacuum aspiration. Cell pellets were resuspended in 500µl of 100 % methanol, transferred to micro centrifuge tubes and immediately snap frozen in liquid nitrogen. The mixture was thawed, vortexed and centrifuged for 1 min at 800g. The supernatants were transferred to new microcentrifuge tubes. The cell pellets were used for a second round of extraction by performing the same steps mentioned prior. The supernatants were removed and pooled with the previous methanol extracts and the cell pellets were resuspended with sterile deionized water. The cell mixtures were snap frozen in liquid nitrogen and thawed at room temperature and centrifuged for 1min at 800g. The supernatants were added to the previous pooled methanol extracts. The extracts were dried in a vacuum centrifuge (Sellick et al., 2011). The dry metabolite pellet was resuspended with 100µl of deionized water and analyzed by high performance liquid chromatography (HPLC) (1200 series, Agilent technologies) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomex Inc., Torrance, CA). The column was eluted with 0.005N H_2SO_4 at flow rate of 0.6mL/min at 50^{o}C.

2.4.5 Analytical Methods

Cell growth was monitored at OD_{600} using a UV-visible spectrophotometer (BioMate 5; Thermo Spectronic, NY). Xylose, xylitol, glucose, glycerol, acetate, and ethanol concentrations were determined using high performance liquid chromatography (HPLC) (1200 series, Agilent Technologies) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6mL/min at 50°C.

2.4.6 ^{14}C- Labeled Xylose Uptake Assay

^{14}C- Labeled xylose was purchased from American Radiolabeled Chemicals (St. Louis, MO) as a solution in 90% ethanol. The radiolabeled sugar was dried in a chemical hood and resuspended in water prior to use. The radiolabeled sugar concentrations 350mM, 250mM, 100mM, 50mM and 25mM were prepared using methods described by Coons et al., (1995). Cell cultures in exponential phase were harvested and washed three times with potassium phosphate buffer (pH=6.7). The washed cells were resuspended in 5mL potassium phosphate buffer (pH=6.7) to final OD_{600}~23 and divided into two aliquots, one of which was placed on ice and the other equilibrated in a 30 ^{o}C water bath for 5min. Four aliquots of 200µl cell suspension were dried at 60 ^{o}C for 24hrs to determine the cell dry weight. The sugar uptake was initiated by addition of 160µl of cell suspension into 40µl radiolabeled xylose in 50mL conical tubes for 10 seconds. The xylose uptake was stopped by vigorous addition of 10mL ice cold water. The zero time point sample was obtained by adding ice-cold water and cell suspension simultaneously into the radiolabeled xylose containing conical tube. The mixtures were immediately filtered through a Whatman GF/C filter (Whatman, Florham Park, NJ) presoaked in 40% xylose solution and washed twice with 10mL ice cold water. The filter was then placed into 3mL of a scintillation cocktail (ThermoFisher Scientific) and counted using the Beckman LS6500 scintillation counter (Beckman Coulter, Brea, CA) for 1min. All data points were measured in duplicates. Sugar uptake rates were calculated as mmol sugar transported per hour per gram of cell dry weight (Du et al., 2010; Lee et al., 2002; Coons et al., 1995). The measured xylose uptake rates were plotted against substrate concentration and fitted to the Michaelis-Menten model, V_{max} and K_m were measured using Lineweaver-Burk plots.

2.5 Figures

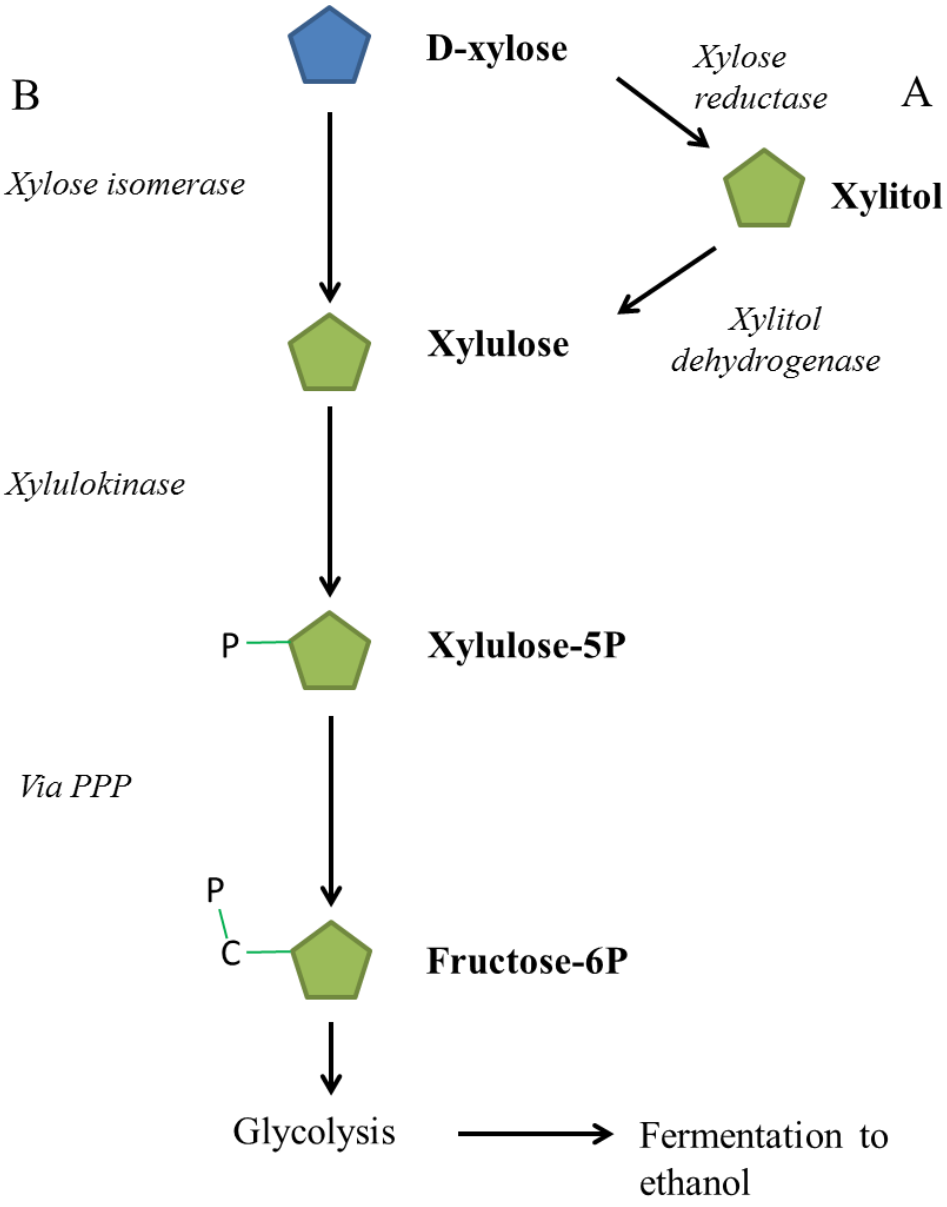


Fig 2.1 General overview of the xylose utilizing pathway in recombinant *S. cerevisiae.* (A) Oxidoreductase pathway through *XYL1* (XR) and *XYL2* (XDH). (B) Isomerization pathway through XI.

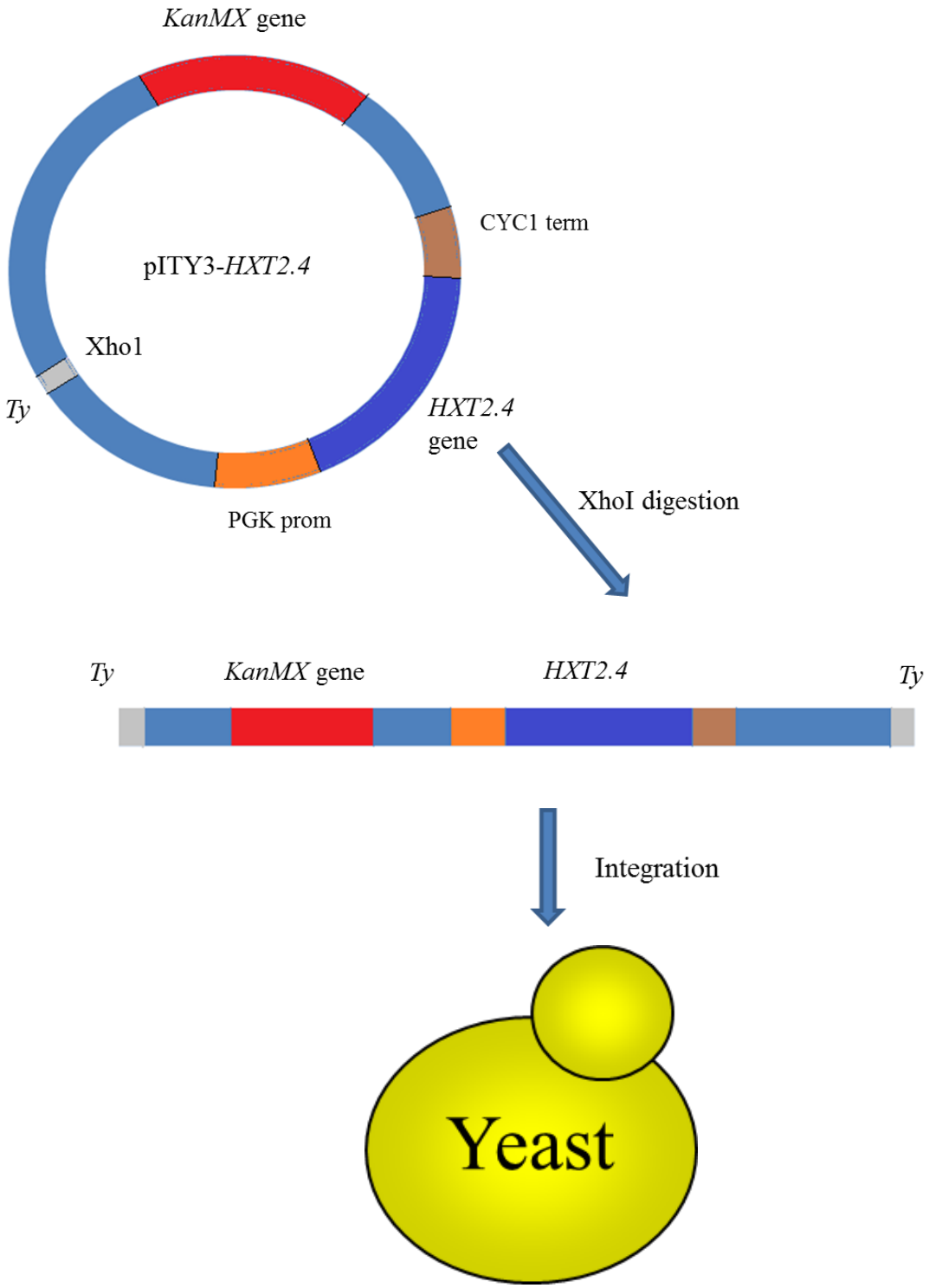


Fig 2.2 Integration of pITY3-*HXT2.4* into DX123 strain using standard lithium-acetate method. The pITY3-*HXT2.4* plasmid was linearized using XhoI prior transformation to the yeast.

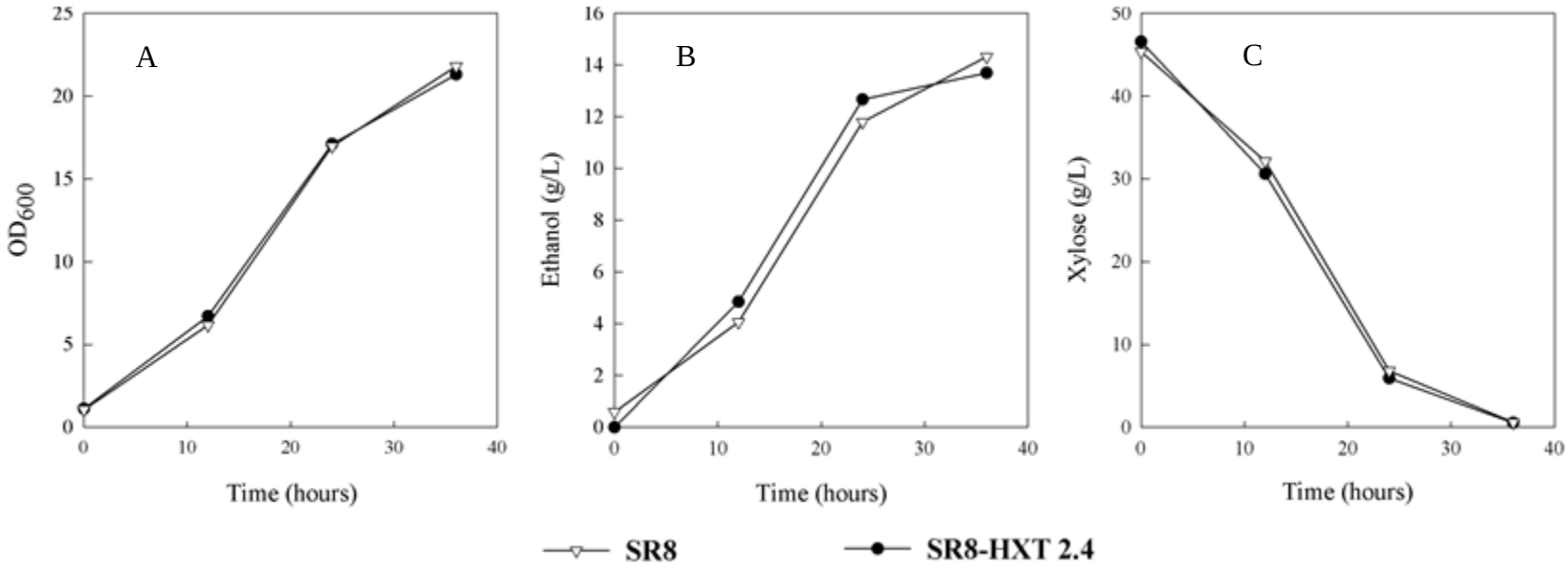


Fig 2.3 Fermentation profiles of *S. cerevisiae* harboring *HXT2.4* putative xylose transporter in YP media containing 20g/L xylose. (A) Optical density at 600nm. (B) Ethanol production rate. (C) Xylose uptake rate. All fermentations were performed under an oxygen-limited condition (100rpm). Initial cell density was adjusted to OD_{600}~1.

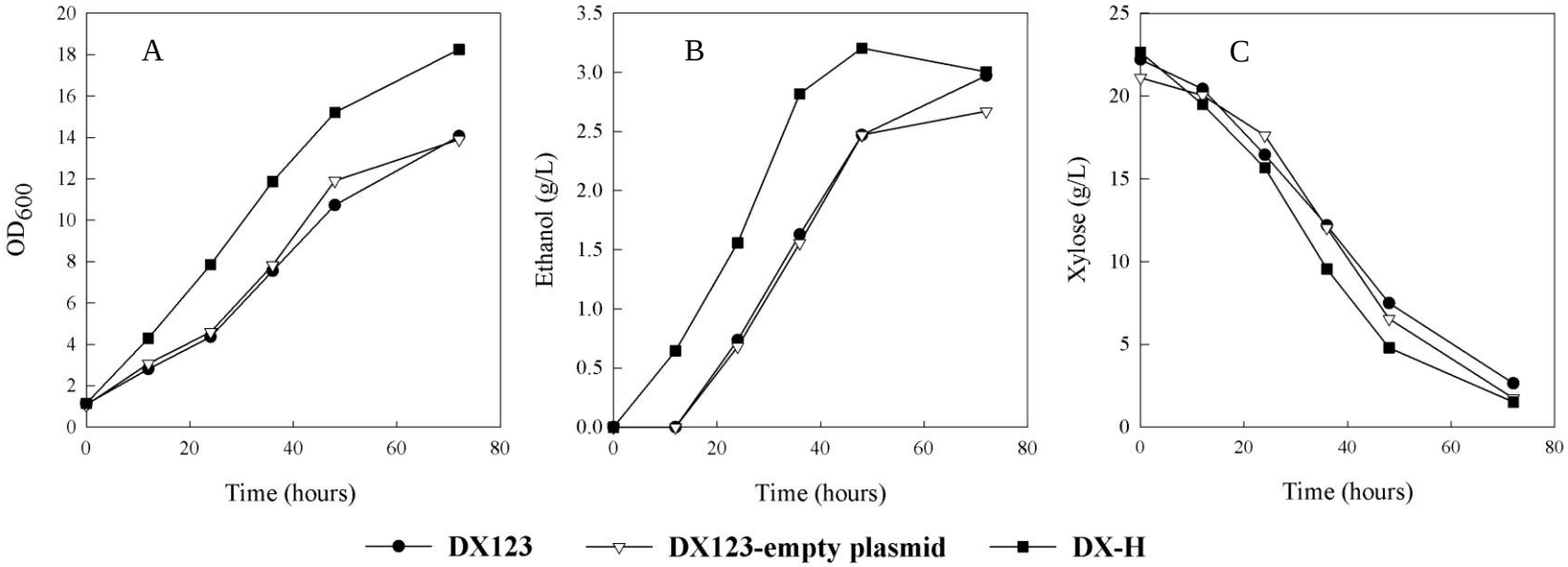


Fig 2.4 Fermentation profiles of *S. cerevisiae* harboring *HXT2.4* putative xylose transporter in YP media containing 20g/L xylose. (A) Optical density at 600nm. (B) Ethanol production rate. (C) Xylose uptake rate. All fermentations were performed under an oxygen-limited condition (100rpm). Initial cell density was adjusted to OD_{600}~1.

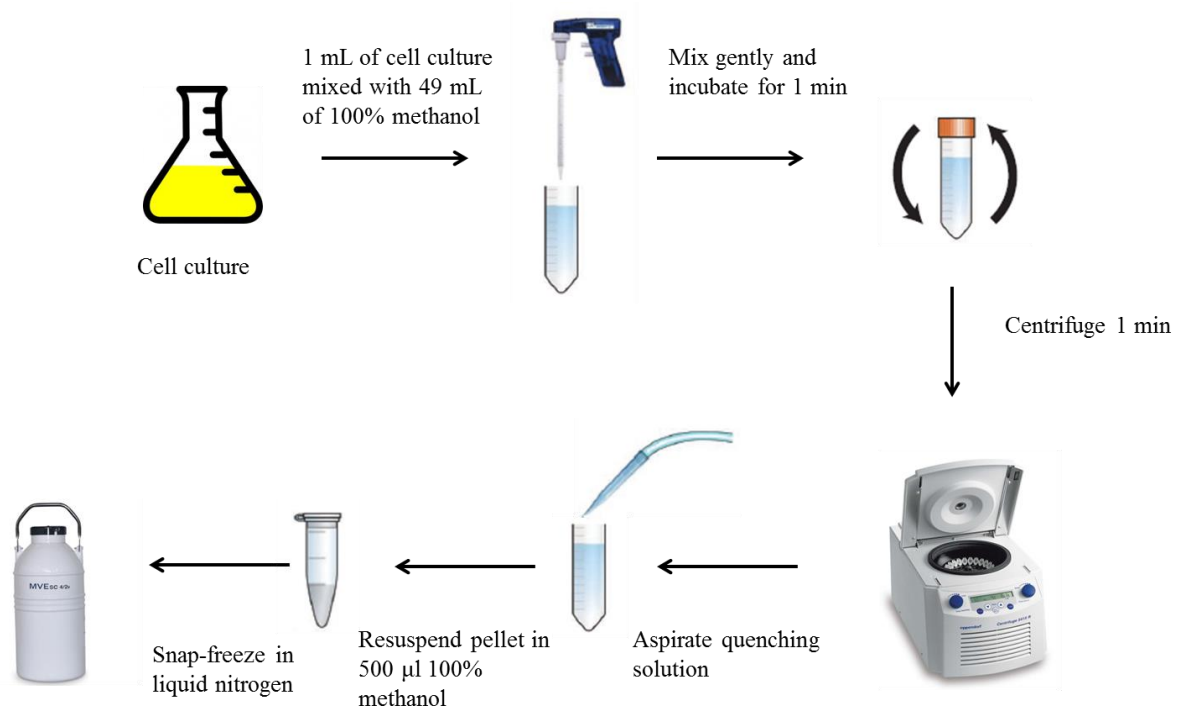


Fig 2.5 Overview of cell quenching protocol using 100% methanol.1mL cell was mixed with 49mL of -40^{o}C 100% methanol and then centrifuged. The quenching solution was removed and 100% methanol was added for extraction.

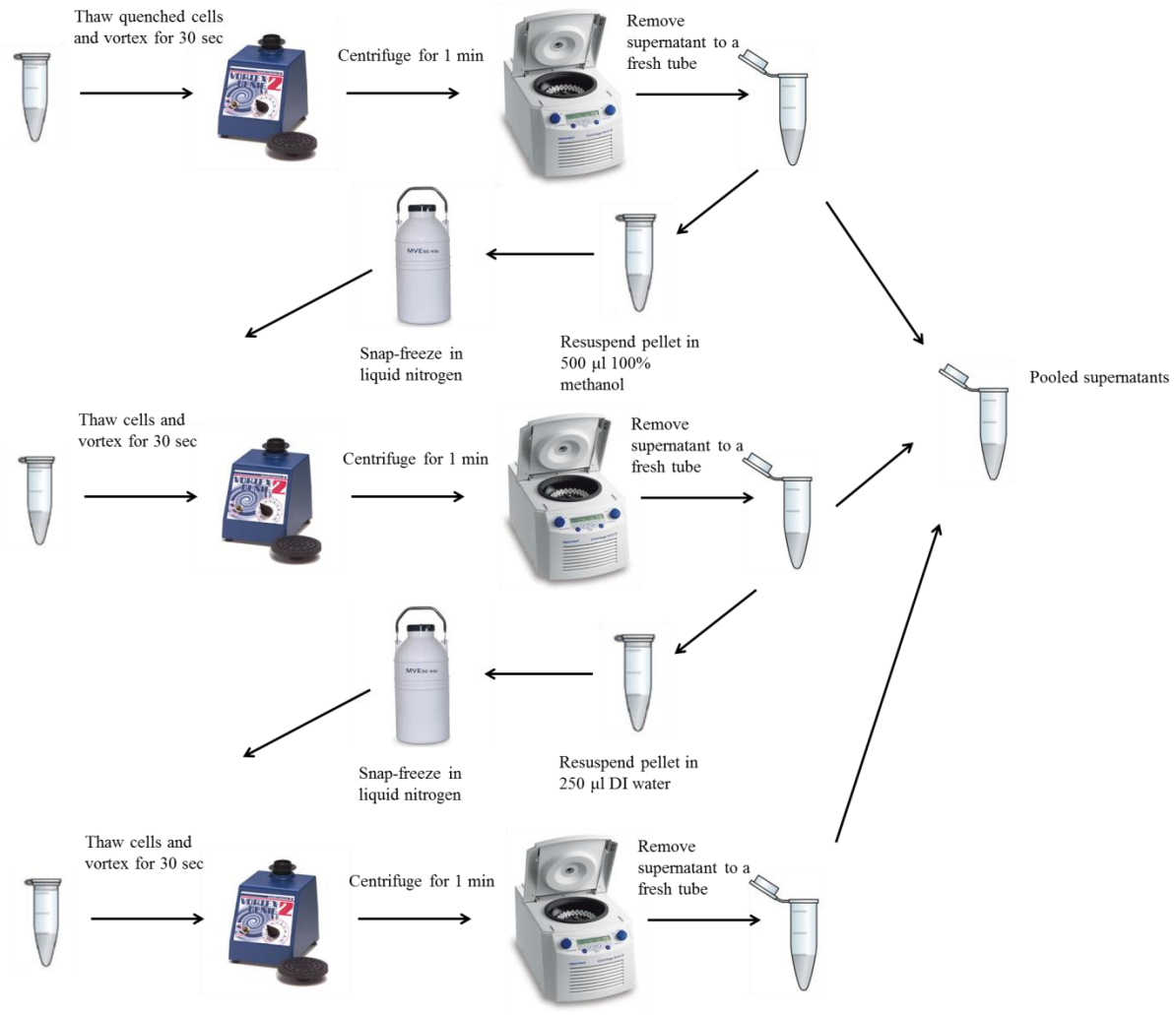


Fig 2.6 Xylose and xylitol extraction protocol using 100% methanol and snap-freezing in liquid nitrogen.

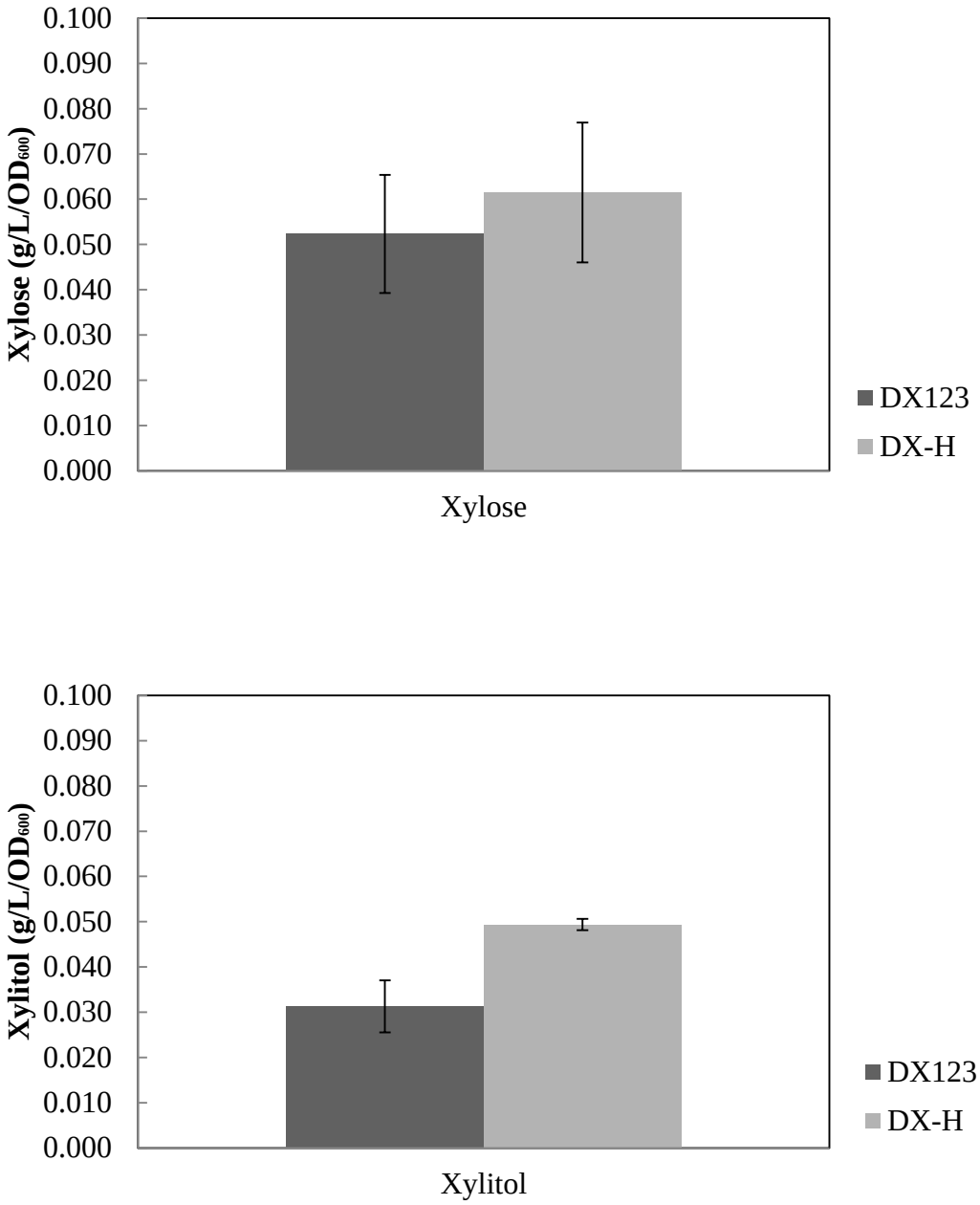


Fig 2.7 Intracellular measurement of xylose and xylitol in DX123 (control) and DX-H (overexpressing *HXT2.4* putative xylose transporter) strains. The cells were incubated for 10hrs at OD_{600} ~10 in YP medium with 20g/L xylose. The intracellular xylose and xylitol were extracted by 100% cold methanol quenching and extraction. Xylose and xylitol were measured using HPLC. The experiment was done in triplicates.

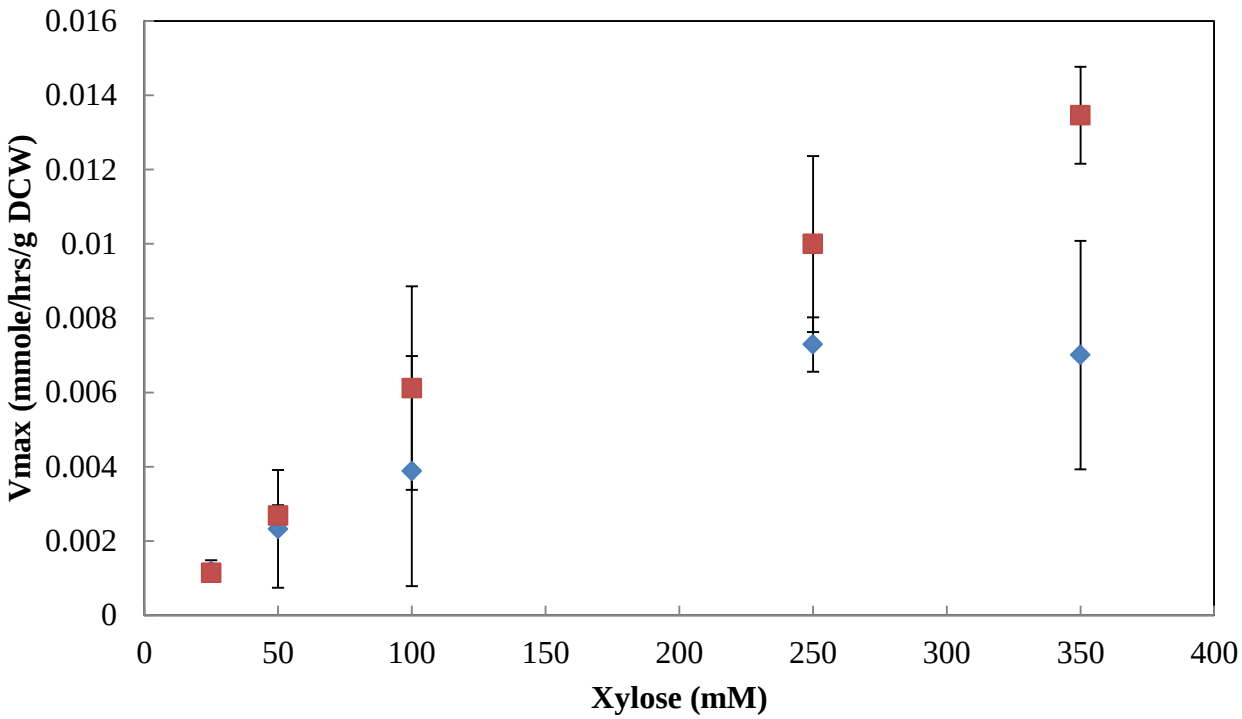
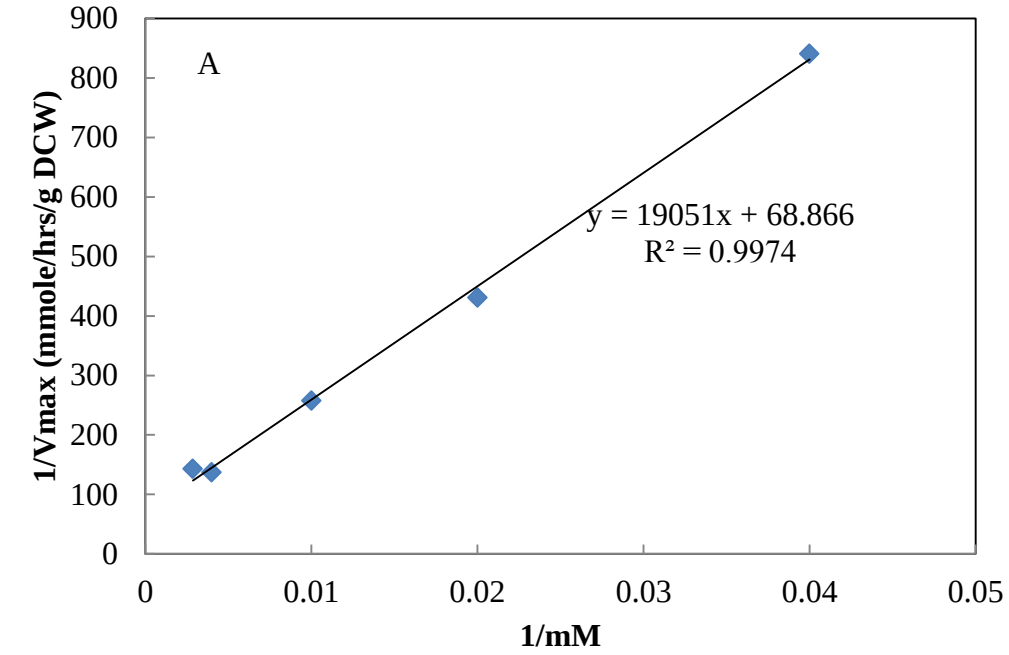


Fig 2.8 ^{14}C-Labeled xylose uptake rates of DX123 (diamond) and DX-H (square) strains. The measured xylose uptake rates were plotted against substrate concentration and fitted to the Michaelis-Menten equation.



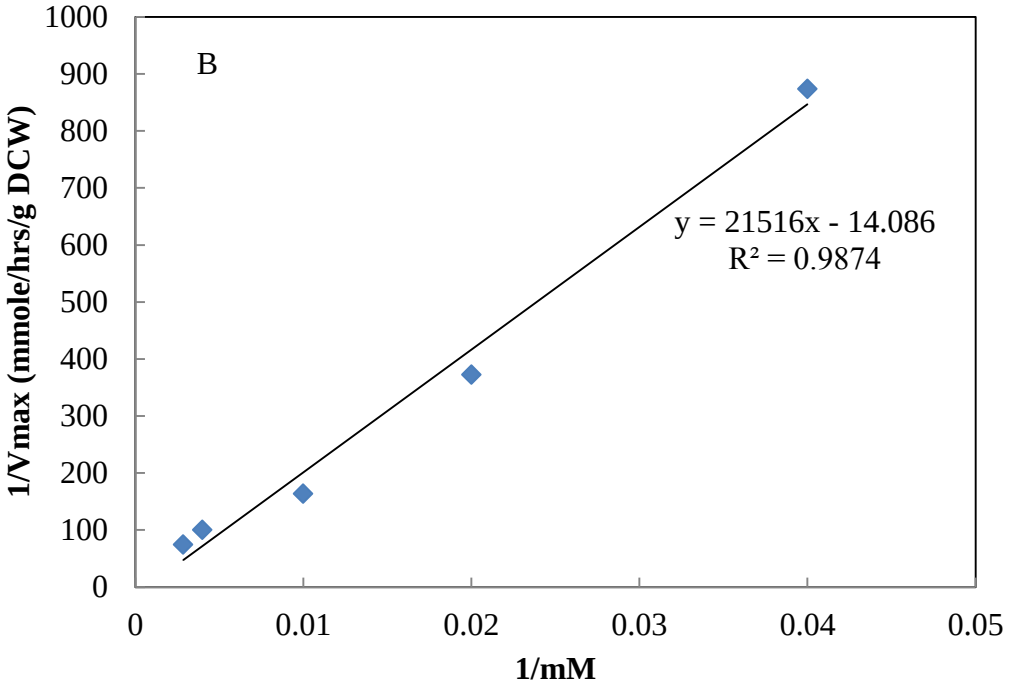


Fig 2.9 ^{14}C-Labeled xylose uptake rates of DX123 (A) and DX-H (B) strain plotted according to Lineweaver–Burk plot to measure K_m and V_{max}.

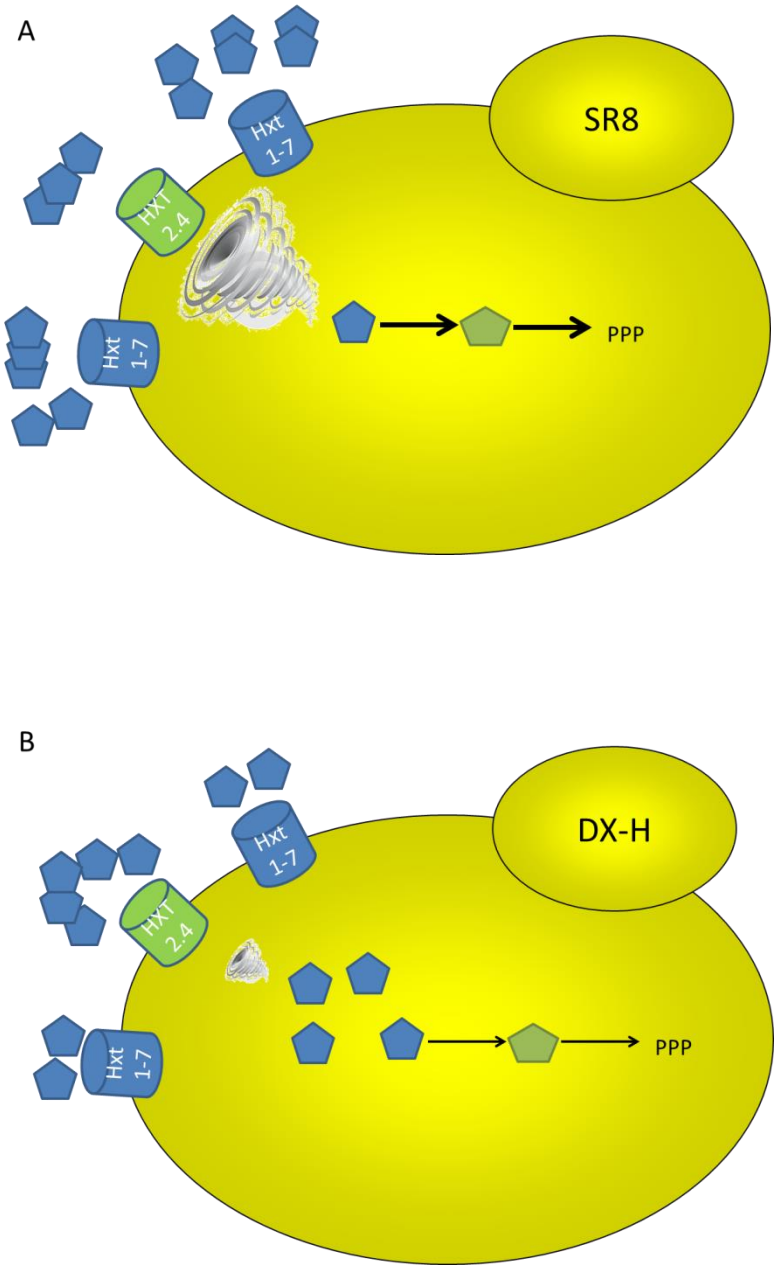


Fig 2.10 Illustration of increased xylose (blue pentagon) uptake by SR8-HXT2.4 (A). Presence of low intracellular xylose due to optimized xylose utilizing pathway in SR8-HXT2.4 strain facilitates extracellular xylose diffusion into the cell via native sugar transporters. In other words, xylose uptake in SR8 resembles the vacuum, which lead to less efficient xylose transport by Hxt2.4. However, the not optimized DX-H strain has high intracellular xylose which results in less efficient xylose uptake by native sugar transporters, so the Hxt2.4 transporter performs better (B).

Table 2.1 Calculated xylose uptake kinetics of DX123 and DX-H strains using **Lineweaver–Burk plot**

Strain	**V_{max}**	**K_m**
DX123	0.014mmole/hrs/g DCW	281.85 mM
DX-H	0.07 mM/hrs/g DCW	1474 mM

Chapter 3. Conclusions

Increased concerns about greenhouse gas emission and energy security augmented research in the field of biofuels in the last decades. Lignocellulosic biomass offers an attractive alternative to produce cost effective and environmentally friendly biofuel. Currently ethanol is the most widely produced biofuel. *S. cerevisiae* is a suitable host for production lignocellulosic ethanol because it has been used for thousands of years to convert hexose sugars into ethanol. Ethanol production industries use *S. cerevisiae* for large scale industrial fermentations. Unfortunately, *S. cerevisiae* cannot consume pentose sugars like xylose which comprise approximately 30% of hemicellulose, one of the main components of lignocellulose (Hector et al., 2008).

The main strategy to allow *S. cerevisiae* fermentation of xylose is the introduction of a xylose utilizing pathway from natural xylose fermenting microorganisms (Jeffries et al., 2004; Kim et al., 2012; Yu et al., 1995). However, redox imbalance and lack of xylose specific transporters are limiting xylose fermentation (Hector et al., 2008).

It's well-known that xylose enters the cell through native hexose transporters which have lower affinity toward xylose (Kotter et al., 1993) than hexose sugars. In order to solve this problem, many studies have been conducted to introduce heterologous transporters from different microorganism like *S. stipitis* and *N. crassa* (Katahira et al., 2008; Tanino et al., 2012; Saloheimo et al., 2007; Runquist et al., 2010; Du et al., 2010). In this study we investigated the overexpression of putative xylose transporter gene *HXT2.4* from *S. stipitis* in engineered xylose fermenting *S. cerevisiae* strains DX123 and SR8. We hypothesized that expression of *HXT2.4* can improve xylose fermentation in recombinant *S. cerevisiae* strains. The overexpression of *HXT2.4* in optimized xylose consuming *S. cerevisiae* SR8 showed no difference in comparison

to the control strain; however when we overexpressed *HXT2.4* in not optimized DX123 strain (DX-H), the fermentation phenotype showed some improvement in both xylose uptake rate and ethanol production rate. Further analysis of intracellular xylose and xylitol concentrations in DX-H and DX123 strains revealed that DX-H has more intracellular xylose and xylitol than the control strain. Xylose uptake kinetics were measured using ^{14}C- labeled xylose to characterize and confirm our hypothesis. Because DX and DX-H strains expressing Hxt2.4 transporter also have their own native hexose transporters, the determined kinetic parameters refer to the overall xylose uptake by all transporters. The results demonstrated that the DX-H strain has higher V_{max} than the control strain. This result explains the high flux of xylose into the cell. However, the low affinity (K_m) of DX-H strain suggests that Hxt2.4 is not a xylose specific transporter. Hxt2.4 is a very interesting transporter for future studies due to its multisugar transport capability. Several recommendations for the future studies can be suggested: 1) Further kinetic studies are needed to determine xylose uptake kinetics in solely Hxt2.4 transporter expressing *S. cerevisiae* lacking all other hexose transporters (*hxt* null strain). 2) Mutagenesis of Hxt2.4 transporter to improve xylose affinity could be a good strategy to develop xylose specific transporter. 3) Transport capability of other sugars like mannose, maltose, galactose, fructose, sucrose and arabinose should be investigated in an *HXT2.4* expressing *S. cerevisiae.*

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