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Research Article

Expression of *Candida intermedia* GXF1 Improves Xylose Transport in *Saccharomyces cerevisiae*

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ABSTRACT

The heterologous expression of the GXF1 xylose transporter from *Candida intermedia* was investigated in *Saccharomyces cerevisiae* DTY165 to evaluate the role of xylose transport in a strain lacking engineered xylose-metabolizing pathways. Introduction of *GXF1* led to increased xylose uptake and improved growth when xylose was used as the sole carbon source, as evidenced by a higher specific growth rate and faster depletion of extracellular xylose compared to the empty vector control. Enhanced intracellular accumulation of xylose further supported the contribution of *GXF1* to transport efficiency. Despite improved xylose uptake, ethanol production from xylose remained low, and only small increases in ethanol levels were observed in both glucose- and xylose-containing media. These findings indicate that improving xylose transport alone is not sufficient to achieve substantial ethanol production in *S. cerevisiae*. Instead, effective conversion of xylose to ethanol will likely require additional engineering of downstream xylose-utilization pathways. Overall, this study highlights the importance of coupling enhanced xylose transport with metabolic pathway optimization for meaningful ethanol production.

Keywords: Bioethanol; *Candida intermedia*; GXF1; *Saccharomyces cerevisiae*; Xylose transport

Introduction

Increased global demand for renewable energy has grown interest in bioethanol as a fuel alternative due to being sustainable and as derived from green masses. While first-generation ethanol derived from corn and sugarcane raises concerns over food security, second-generation bioethanol produced from lignocellulosic biomass offers a non-food-based environmentally friendly option (Karp & Richter, 2011). Nepal, known as an agriculturally resourceful country, produces high quantities of lignocellulosic residues including rice husks, wheat straw and sugarcane bagasse (NV et al., 2006). Such by-products are largely underutilized, and this gives a valuable opportunity as feedstocks for bioethanol production. Effective conversion of plant biomass requires the extraction and hydrolysis of its main biomass which are embedded in the highly recalcitrant structure of the plant cell wall. This complex matrix is predominantly composed of cellulose (40–

50%), hemicellulose (25–35%) and lignin (15–20%) (Jordan et al., 2012; dos Santos et al., 2016). Cellulose is made up of glucose chains, the most abundant sugar in biomass, whereas hemicellulose contains xylose, a five-carbon sugar and the second most prevalent monomer.

Saccharomyces cerevisiae is the primary yeast employed for industrial ethanol production, utilizing glycolysis to convert sugars into pyruvate under anaerobic conditions (Figure 1). This pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase, followed by reduction to ethanol via alcohol dehydrogenase, regenerating NAD^+ in the process—steps that form the core of ethanol fermentation industries (Walker, 1998). *S. cerevisiae* application in industrial sectors is favoured by its several advantageous traits including the Generally Recognized as Safe (GRAS) status, food-grade production, ethanol tolerance, stress resilience, ease of genetic modification and adaptability to large-scale fermentation (Steensels et al., 2014; Walker & Walker, 2018; Parapouli et al., 2020). These kinds of features collectively make *S. cerevisiae* an ideal host for ethanol production and industrial fermentation applications, with strain selection often focusing on ethanol productivity and robustness under stress conditions. Despite being one of the preferred industrial ethanol producers, *S. cerevisiae* lacks a dedicated xylose transport system.

As a result, during lignocellulosic biomass utilization, the pentose fraction of lignocellulosic hydrolysates remains largely underutilized, leading to reduced ethanol yields. Engineering yeast with heterologous xylose transporters from other organisms, therefore, represents a promising strategy to enhance xylose uptake. Beyond ethanol production, improved xylose transport capacity also has broader implications for the biosynthesis of value-added products such as xylitol, xylonate and biopolymers (Qiao et al., 2021; van Arsdale et al., 2024; Regmi & Bhattarai, 2025; Queiroz et al., 2025). Sugar uptake in cells is facilitated by transmembrane transporter proteins, which enable the movement of substrates between the extracellular and intracellular spaces. Among these, the major facilitator superfamily (MFS) is widely distributed across all domains of life and includes a variety of transporters with different mechanisms: uniporters transport a single substrate, symporters couple substrate movement to ions, and antiporters exchange two distinct substrates in opposite directions (Marger & Saier, 1993; Forrest et al., 2011; Reddy et al., 2012; Quistgaard et al., 2016). Within the MFS, several subfamilies contain well-characterized xylose transporters including GAL2, HXT, GXF1 and GXS1 (Hamacher et al., 2002; Maier et al., 2002; Sedlak & Ho, 2004; Leandro et al., 2008; Runquist et al., 2009; Fonseca et al., 2011; Young et al., 2011; Aeling et al., 2012; Young et al., 2012; Young et al., 2014).

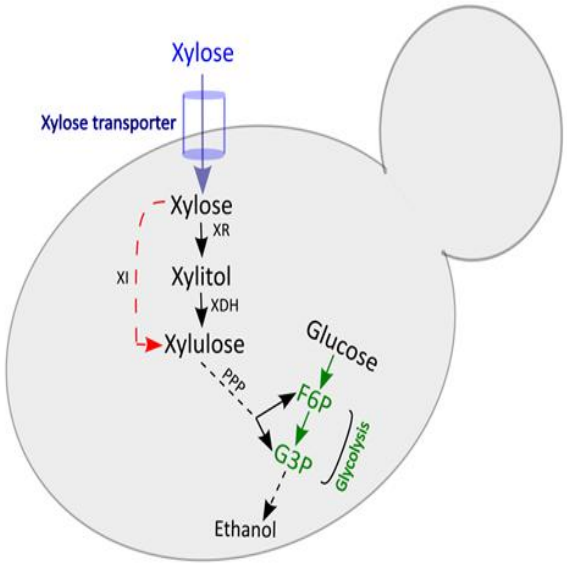

Figure 1: Schematic diagram of xylose metabolism in *S. cerevisiae*. XR: xylose reductase, XDH: xylitol dehydrogenase, XI: xylose isomerase, PPP: Pentose phosphate pathway, F6P: Fructose 6-phosphate, G3P: Glyceraldehyde 3-Phosphate. The red line indicates the heterologous pathway.

In *S. cerevisiae*, native hexose transporters such as Hxt1p, Hxt2p, Hxt4p, Hxt5p, Hxt7p and Gal2p can import xylose, but their low affinity for xylose and strong preference for glucose restrict efficient fermentation of mixed sugars (Farwick et al., 2014; Bracher et al., 2018). To overcome these limitations, heterologous xylose transporters have been expressed in engineered strains, often in combination with downstream metabolic pathway modifications, leading to significant improvements in xylose uptake and utilization. The fungus *Candida intermedia* possesses two different glucose/xylose transporters. GXF1 encodes a glucose/xylose facilitator, whereas GXS1 encodes a glucose/xylose proton symporter, with K_m values of 50 mM and ~0.4 mM respectively (Leandro et al., 2006). Expression of GXF1 could enhance xylose transport kinetics thereby boosting recombinant *S. cerevisiae* growth (Leandro et al., 2006; Runquist et al., 2009; Runquist et al., 2010; Fonseca et al., 2011; Parachin et al., 2011; Young et al., 2011). In *S. cerevisiae*, xylose transport across the plasma membrane is less efficient, posing a major obstacle for biomass conversion (Reznicek et al., 2015). This study specifically evaluates the contribution of the GXF1 membrane transporter to xylose uptake and growth in a non-xylose engineered *S. cerevisiae*, without modification of downstream xylose metabolizing pathways. By isolating the role of transport capacity, the

work aims to clarify how enhanced xylose uptake alone influences cellular growth and ethanol formation.

Materials and Methods

Strains, plasmid and culture conditions

Escherichia coli DH10B was used for plasmid propagation cultured in the Luria-Bertani (LB) medium at 37 °C. *Saccharomyces cerevisiae* DTY165 (leucine auxotroph) was used as host for functional expression studies cultured in YPD or synthetic complete (SC) medium at 30 °C. *E. coli* was gifted by Boles Lab, University of Frankfurt, Germany. And, *S. cerevisiae* strain was gifted by Clemens Lab, University of Bayreuth, Germany. The expression plasmid pGXF1 containing *Candida intermedia* GXF1 gene (1664 bp) and LEU2 selectable marker was gifted by Prof. Dr. Paula Gonçalves (University of Lisbon, Portugal).

Primer design and PCR amplification

Oligonucleotide primers was needed for the amplification and verification of the GXF1 gene. The primer sets (YTUF1F and YTUF1R) and (CiGXFL1 and CiGXFR1) were designed using NCBI primer designing tool. All primers were synthesized by Macrogen (South Korea). Primer sequences and their specific applications are listed in Table 1.

Transformation and verification of transformant

The pGXF1 plasmid was first transformed into *E. coli* competent cells using the heat-shock method (Sambrook & Russell, 2001). Transformants were selected on LB agar supplemented with 100 µg/ml ampicillin. Plasmid DNA was isolated from *E. coli* using an alkaline lysis method (Sambrook & Russell, 2001) for subsequent analysis and yeast transformation. For expression in yeast, isolated pGXF1 plasmid was transformed into *S. cerevisiae* via electroporation (Becker & Guarente, 1991). Transformants were selected by plating on synthetic complete (SC) medium lacking leucine.

Verification of transformation was performed through a multi-step process. Putative *E. coli* transformants were initially confirmed by the presence of plasmid DNA, visualized via agarose gel electrophoresis. This plasmid DNA was used to transform yeast, with successful observations of colonies in SC agar plate without leucine supplement indicates successful transformation. Yeast transformants were further verified by PCR amplification of yeast DNA using the internal GXF1-specific primers (CiGXFL1 and CiGXFR1), with successful transformation confirmed by the appearance of the expected 163 bp amplicon. The presence of GXF1 insert was further confirmed by Southern blot analysis (Southern, 1975) of yeast DNA using a DIG-labeled probe generated with the CiGXFL1 and CiGXFR1 primers set. Briefly, the PCR product was separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a biotin-labeled GXF1-specific probe. Hybridization was detected using a streptavidin-alkaline phosphatase conjugate and a chromogenic substrate. After confirmation, yeast transformants were subsequently used for functional expression analysis, including growth kinetics on various sugar substrates.

Growth kinetics assay

For growth assay, colonies of pGXF1 transformed (hereafter referred as GXF1 transformed) and empty vector transformed (hereafter referred as control) into *S. cerevisiae* DTY165 were inoculated into YEP medium containing 20 g/l of either glucose or xylose as the sole carbon source. Cultures were incubated at 30°C with shaking (180 rpm). Growth was monitored by measuring the optical density at 600 nm at 3-hour intervals for 36 hours. The specific growth rate (µ/h) was calculated from the exponential phase of growth. For the intracellular xylose accumulation assay, yeast grown in liquid culture was pelleted, and the cells were broken by freezing them, and finally, glass beads were used to vortex to break the cells (Stowers & Boczko, 2007). The concentrations of substrates and products (glucose, xylose and ethanol) were determined using colorimetric assays. For each assay, a standard curve was prepared using known concentrations of the pure compound, and sample concentrations were calculated based on the standard curve. Glucose concentration in the culture medium was quantified using the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar (Miller, 1959). Xylose concentration was specifically quantified using the phloroglucinol assay (Eberts et al., 1979). Ethanol concentration was determined using a potassium dichromate-based method (Seo et al., 2009). All the tests were done in duplicate.

Table 1: Oligonucleotide primers used in the study.

Primer	5'-3' sequence	Restriction sites	Expected size
CiGXFL1	CTTTGCTTCCACCTTCGTTG	-	163 bp
CiGXFR1	AGTGTTGGAGGTCTCGTTGG	-	

YTUF1F	GACTA ***GAGCTCCATATG*** TCACAAGATTCGCATTC	NdeI/SacI	1600 bp
YTUF1R	TACTGT ***GGATCC*** CAT***TCTAGA*** TTAAACCTGTTCGTCGGTG	XbaI/BamHI	

Data analysis

Data are presented as the mean of biological duplicates. Statistical comparisons between the growth rate of control and GXF1 transformed strains were performed using an unpaired two-tailed Student's t-test. In the present study, given the limited number of biological replicates, statistical analysis was used to support observed trends rather than to draw definitive quantitative conclusion. A p-value of ≤0.05 was considered statistically significant.

Results and Discussion

Confirmation of pGXF1 transformation in *E. coli* and *S. cerevisiae*

The successful construction of recombinant *S. cerevisiae* strains expressing the GXF1 transporter was systematically confirmed through a multi-tiered molecular analysis, confirming the presence and maintenance of the expression plasmid.

Confirmation of transformants in *E. coli*

The *E. coli* transformation was observed by the appearance of approximately 60 ampicillin-resistant colonies in LB agar plate containing ampicillin, contrasted with the complete absence of growth in the control plate, and this demonstrated successful selection. Alkaline lysis of plasmid DNA isolation and its gel electrophoresis further confirmed the presence of plasmid DNA. This plasmid DNA was then used to transform *S. cerevisiae* to make transgenic yeast expressing GXF1.

Confirmation of *S. cerevisiae* transformants

The successful transformation of pGXF1 plasmid into leucine auxotroph *S. cerevisiae* DTY165 was first indicated by functional complementation with transformants growing on synthetic medium lacking leucine. This phenotype is contingent upon the presence of the episomal plasmid containing LEU2 selectable marker. Crucially, the control failed to grow under the same selective conditions, confirming that growth was plasmid-dependent. Further molecular analyses confirmed the presence of the plasmid within the yeast cells. Isolation of plasmid DNA was done from the GXF1 transformed yeast cells. The DNA was then used as a template to perform the PCR using two different primer sets as listed in Table 1. The Figure 2 clearly shows the observation of expected size bands using two different set of primers.

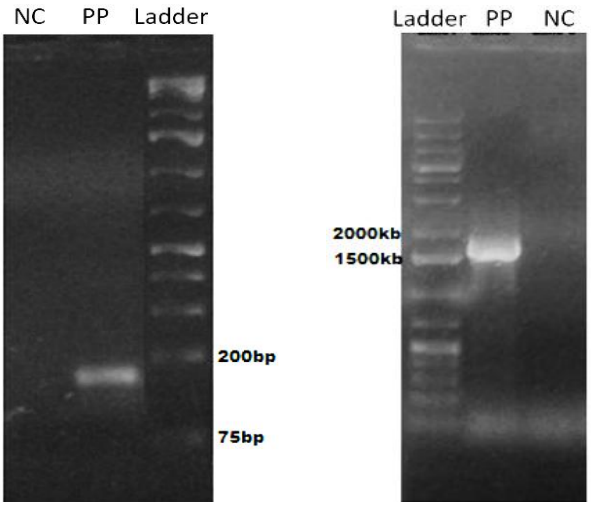

Figure 2: PCR of GXF1 transformed yeast DNA using different primers CiGXFL1 and CiGXFR1, expected size 163 bp (left), and YTUF1F and YTUF1R, expected size ~1600 bp (right). NC, PP, Ladder refers to negative control, PCR product and 1 Kb plus ladder (Thermoscientific) respectively. Lane 2 is the PCR product of GXF1 transformed yeast cells.

Finally, Southern blot analysis using a biotin-labeled GXF1 probe detected a clear hybridization signal in DNA isolated from the GXF1 transformed *S. cerevisiae* strain, while no signal was observed in the control strain (Figure 3).

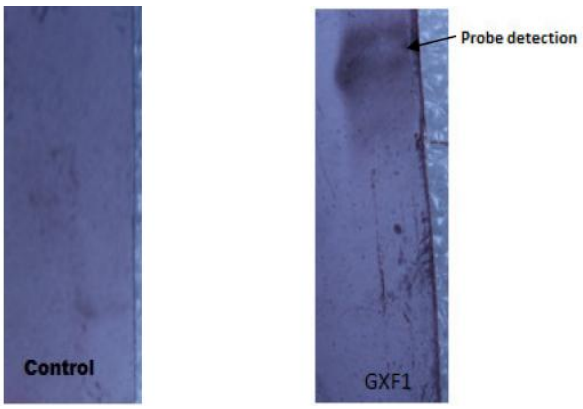

Figure 3: Confirmation of yeast transformation by Southern blotting.

This result confirms the successful introduction of the GXF1 gene into the yeast host. The molecular evidence obtained from Southern blotting complements the

phenotypic selection and supports the identification of the recombinant strain carrying the GXF1 expression plasmid. The episomal plasmid system enabled consistent maintenance of the GXF1 construct during strain validation, providing a stable genetic background for subsequent functional analyses of xylose uptake.

Xylose uptake profile

The growth profile of GXF1-transformed and control *S. cerevisiae* strains on glucose was comparable throughout the cultivation period (Result not shown). A slight advantage was observed in GXF1-transformed cells (μ = 0.019/h) compared to the control (μ = 0.018/h). This minor difference may be attributed to the dual substrate specificity of GXF1, which functions as both a xylose and hexose transporter (Leandro et al., 2006), thereby facilitating glucose uptake in addition to xylose.

Growth in xylose as a carbon source

In contrast, the difference between the strains (GXF1-transformed and control) was more pronounced when xylose was provided as the sole carbon source (20 g/l). Both strains exhibited a brief lag phase of approximately 9 hours, but their growth trajectories diverged significantly thereafter (Figure 4). Control cells displayed negligible further growth, reflecting the inherent inability of wild-type *S. cerevisiae* to efficiently metabolize xylose (Barnett et al., 1983). By comparison, the GXF1-transformed strain showed sustained exponential growth between 9 and 30 hours and reached a higher final biomass yield than the control strain (Figure 4).

The quantitative growth analysis supported this phenotypic difference. The specific growth rate of the GXF1-transformed (μ = 0.022/h) was nearly fourfold higher than that of the control (μ = 0.00572/h), representing a 249.65% increase. An independent t-test confirmed the statistical significance of this difference ($p = 0.0015$, $\alpha = 0.05$), supporting that the observed growth enhancement is associated with GXF1 expression. Thus, the introduction of the GXF1 transporter appears to enhance xylose uptake and utilization, which is associated with improved growth under the conditions tested. These findings suggest that sugar transport represents an important factor influencing xylose utilization and indicate that GXF1 expression can contribute to improved xylose uptake in *S. cerevisiae*. The slight increase in xylose-based growth observed in the control strain may be explained by the promiscuous activity of native hexose and galactose transporters, which can exhibit weak xylose transport activity (Hamacher et al., 2002; Sedlak & Ho, 2004; Young et al., 2011).

Previous studies have reported even higher growth rates following GXF1 expression—for example, an increase from μ = 0.070 to 0.081/h (Parachin et al., 2011). These enhanced performances likely result from additional genetic modifications, including overexpression of non-oxidative pentose phosphate pathway (PPP) genes, co-expression of XR, XDH, and XK, and deletion of GRE3. In contrast, the present study employed a simple haploid laboratory strain without further pathway engineering, which explains the lower, yet still significant, improvement in growth rate (μ = 0.022/h).

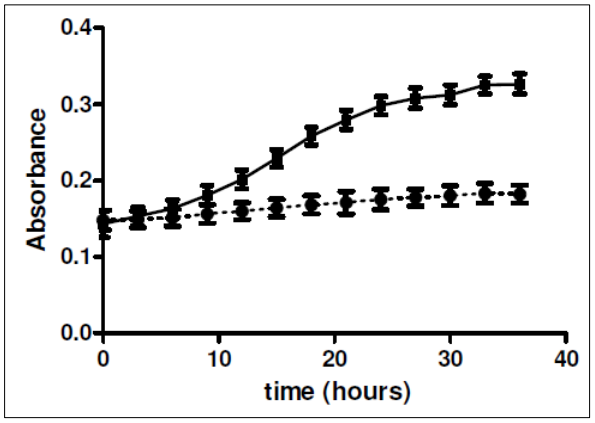

Figure 4: Growth kinetics of GXF1-transformed (solid line) and control (dotted line) *S. cerevisiae* in xylose as a carbon source.

Extracellular and intracellular xylose content

Xylose transport efficiency was evaluated by monitoring both extracellular depletion (Figure 5A) and intracellular accumulation (Figure 5B) over 30 hours using phloroglucinol assay. In both cases, GXF1-transformed *S. cerevisiae* showed markedly improved uptake compared to control cells. Extracellular xylose concentration decreased more rapidly in GXF1 transformants with a 28.3% reduction (19.50 to 13.97 mg/ml) compared to only 12.5% in control cells (19.73 to 18.26 mg/ml), a difference that was statistically significant ($p = 0.015$) (Figure 5A).

Correspondingly, intracellular accumulation was significantly higher in GXF1- transformed strains ($p = 0.035$), increasing by 15.8% (0.152 to 0.176 mg/ml) versus only 5.9% (0.151 to 0.158 mg/ml) in control cells (Figure 5B). A strong negative correlation ($r = –0.99$) between the extracellular depletion and the intracellular accumulation confirmed that xylose transport kinetics were tightly coupled. The plasmid-based expression of a xylose transporter from *Arabidopsis thaliana* increased intracellular xylose accumulation in a strain

not engineered for xylose utilization (Hector et al., 2008).

However, it should be noted that in this study, the decrease in xylose content is not same as the increase in xylose content (Figure 5). Approximately 5.53 mg/ml of xylose was consumed from the medium, whereas only 1.47 mg/ml accumulated intracellularly due to the presence of pGXF1. The portion of xylose not accounted for intracellularly may have contributed, at least in part, to ethanol production or could have been diverted toward other metabolites, such as xylitol (Zhu et al., 2021).

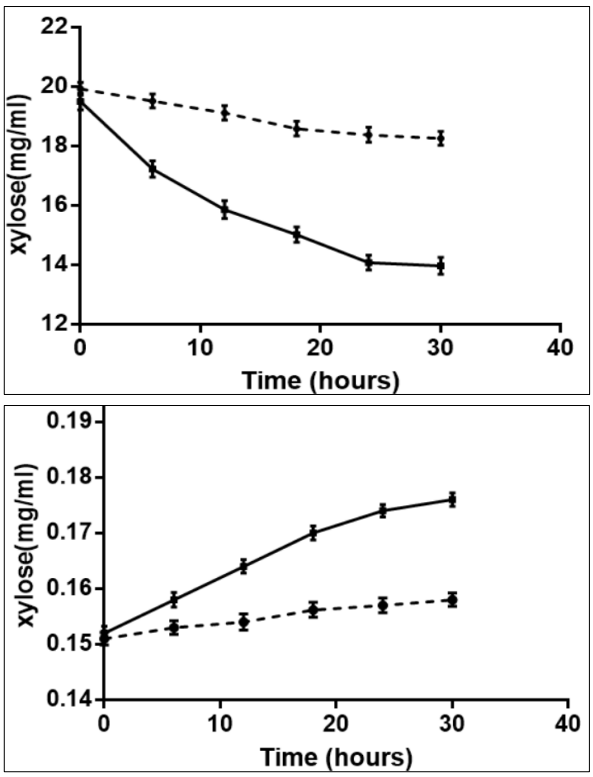

Figure 5: The extracellular **(**upper**)** and intracellular **(**lower**)** xylose concentrations of GXF1-transformed *S. cerevisiae* (solid line) and control (dotted line), measured using the phloroglucinol assay.

Ethanol production

Ethanol production, estimated by the dichromate method, measured at 30 hours, was marginally higher in GXF1-transformed strains compared to controls on both glucose and xylose substrates (Figure 6). On glucose, ethanol titers reached 0.396 mg/ml in the GXF1-transformed versus 0.368 mg/ml in the control cells (7.6% increase). On xylose, ethanol levels were 0.0902 mg/ml and 0.0863 mg/ml respectively representing only a 4.5% improvement. Although GXF1 expression enhanced sugar utilization, the ethanol gains remained modest, particularly under xylose conditions.

Ethanol production was only modestly increased in GXF1-transformed strains, with yields on glucose showing a slight improvement and xylose-derived ethanol remaining nearly fourfold lower (Figure 6). These results suggest that sugar transport is not the only limiting step for ethanol biosynthesis in *S. cerevisiae*. For glucose, this is expected since the yeast already harbors a broad repertoire of efficient hexose transporters (Boles & Hollenberg, 1997; Wieczorke et al., 1999; Diderich et al., 2001). Furthermore, glucose serves as the preferred carbon source and exerts strong regulatory control through carbon catabolite repression, which ensures its prioritized consumption while delaying utilization of other alternative sugars (Gancedo, 1998; Santangelo, 2006; Belinchón & Gancedo, 2007a, 2007b; Conrad et al., 2014).

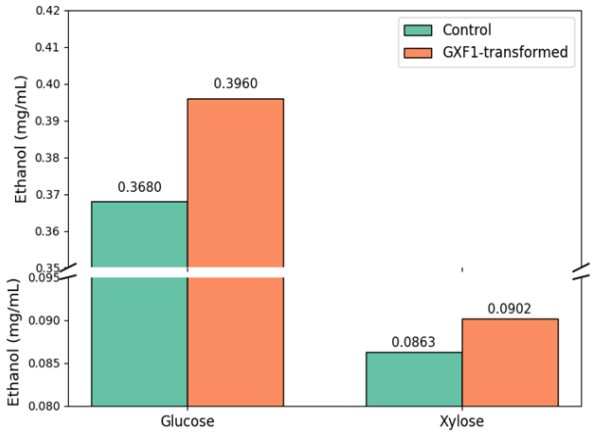

Figure 6: Ethanol production by GXF1-transformed and control yeast using glucose or xylose. The ethanol production was measured by dichromate test.

In the present study, the ethanol yield from xylose was likely constrained by downstream metabolic inefficiencies rather than transport capacity. Previous studies have shown that overexpression of GXF1 accelerates xylose uptake but often diverts carbon into xylitol due to the redox imbalance inherent in the XR/XDH pathway (Runquist et al., 2009; Fonseca et al., 2011). Even after the deletion of the major xylose reductase gene (GRE3) in yeast, residual xylose reductase activity can still sustain xylitol formation, leaving ethanol production largely unaffected. Higher ethanol titers have only been achieved when transport engineering was combined with rational or non-rational metabolic engineering. For example, co-expression of XYL1, XYL2, and XKS1 with a plant xylose transporter enhanced ethanol production to 1.5 g/l (Hector et al., 2008), while introduction of xylose isomerase, xylulokinase, and the *Pichia stipitis* SUT1 transporter, followed by adaptive evolution, yielded up to 0.43 g ethanol per gram xylose (Madhavan et al., 2009). Adaptive laboratory evolution has also proven effective in improving xylose consumption rates in GXF1-expressing strains (Diao et al., 2013). Beyond transporter integration, optimization of the xylose reductase (XR) node, particularly introducing cofactor preference for NADH instead of NADPH, has been

shown to reduce xylitol accumulation and increase ethanol yield (Olofsson et al., 2011). Cofactor balancing is thus a critical strategy, as highlighted in recent studies on tailoring redox metabolism for improved microbial performance (Regmi et al., 2024).

In our study, the absence of a dedicated xylose-metabolizing background strain may have likely further constrained flux through the pentose phosphate pathway, limiting ethanol conversion. Although xylitol accumulation was not quantified, it remains a plausible factor contributing to the modest increase in ethanol observed in GXF1-transformed yeast. Overall, these findings indicate that while GXF1 enhances substrate uptake, ethanol production remains restricted by downstream metabolic bottlenecks. The ethanol concentrations obtained (~0.09 mg/ml on xylose vs. ~0.40 mg/ml on glucose) reflect the long-standing challenge of efficient pentose fermentation in *S. cerevisiae*. Transporter engineering alone, therefore, seems insufficient; and to achieve industrially relevant yields will require integrated strategies that couple transporter enhancement with redox balancing, pathway optimization, and process-level improvements. Such a systems-level approach is essential for unlocking the full potential of xylose fermentation and advancing lignocellulosic bioethanol production using *S. cerevisiae*. Interpretation of these findings should take into account the limited number of biological replicates and the use of colorimetric assays for metabolite estimation. Accordingly, the observed differences are discussed as indicative trends rather than definitive quantitative effects.

Conclusion

Expression of the *Candida intermedia* xylose transporter GXF1 in *Saccharomyces cerevisiae* DTY165 enhanced xylose uptake, intracellular accumulation, and growth on xylose as the sole carbon source. The GXF1-transformed strain exhibited a higher specific growth rate and faster extracellular xylose depletion than the control. However, ethanol production from xylose remained low, indicating that improvement in xylose transport alone is not sufficient to support substantial ethanol formation. The study was conducted in a non-xylose-engineered *S. cerevisiae* background and relied on colorimetric assays for metabolite quantification, which limits quantitative resolution. Nevertheless, these findings demonstrate that enhanced xylose transport can contribute to improved xylose utilization, while highlighting the need for additional engineering of downstream xylose-metabolizing pathways to enable more effective conversion of xylose to ethanol.

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