

Optimized xylose isomerase uptake and expression level in *Saccharomyces cerevisiae* for improving ethanol production

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Abstract: The ability to engineer the yeast *Saccharomyces cerevisiae* to efficiently convert lignocellulosic biomass to ethanol remains a considerable challenge. Here, we propose a new reprogrammable strategy to optimize the expression level of the xylose isomerase (*XI*) gene with the induction of mutations in *S. cerevisiae* to improve efficient ethanol production and productivity. We sought to fine-tune the xylose uptake and catabolism abilities of *S. cerevisiae* during fermentation by improving efficiency of the xylose transporter, which was fused with four copies of the *XI* gene under the control of different promoters to obtain recombinant yeast strains. In fermentation experiments, the optimized strain CW9 cultured in yeast extract-peptone (YP) medium containing approximately 65 g/L glucose and 55 g/L xylose produced consistent ethanol yields of 0.45 g/g total sugar in about 72 h, which was close to 90% of the theoretical yield. These promising results indicate that strain CW9 is the best producer of ethanol from mixed sugar when synthetically regulating the xylose assimilation pathway. Overall, this study provides an optimal method to control *XI* expression levels to find better conditions for enhancing biofuel production.

Keywords: xylose; promoter elements; transporter; ethanol; *Saccharomyces cerevisiae*

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1 Introduction

The cost-effective production of second-generation bioethanol requires efficient utilization of the most abundant glucose source to yield a significant fraction of the xylose obtained after hydrolysis of certain types of lignocellulosic biomass. The yeast *Saccharomyces cerevisiae*, which has been traditionally used for industrial ethanol production owing to its high rate of glucose fermentation, shows high tolerance to ethanol, inhibitors, and other industrial process conditions, and does not efficiently metabolize pentose sugars, particularly xylose. Therefore, numerous xylose-utilizing *S. cerevisiae* strains have been established using intensive research and genetic engineering approaches to efficiently utilize xylose, resulting in higher overall ethanol yield and productivity (Bengtsson et al., 2009; Olofsson et al., 2011).

Xylose assimilation by different *S. cerevisiae* strains differs only with respect to the pathways used for xylose: the oxidoreductive pathway with the NAD(P)H-dependent xylose reductase (XR) and the NAD⁺-dependent xylitol dehydrogenase (XDH) from *Scheffersomyces stipitis* (Johansson et al., 2001; Hou et al., 2007), or the isomerization pathway with xylose isomerase (*XI*) from fungal and bacterial genes (Brat et al., 2009; Madhavan et al., 2009). However,

an imbalance in the NADH/NADPH cofactor ratio in the XR and XDH pathways can result in variable production levels of xylitol, and therefore reduce ethanol yields. Some recombinant strains were constructed by chromosomal integration of codon-optimized prokaryotic *XI* from *Clostridium phytofermentans* in the xylose utilization pathway (Brat et al., 2009; Demeke et al., 2013). Furthermore, the *XI* pathway in the fungus *Piromyces* sp., which shows higher activity, could functionally enable yeast cells to ferment xylose efficiently (Kuyper et al., 2015). Nevertheless, this *XI* activity requires further evolution or adaptation in the recombinant strain to allow for anaerobic growth on xylose (Lee et al., 2012; Zhou et al., 2012). Furthermore, overexpression of the rate-controlling xylulokinase (*XK*) gene (Eliasson et al., 2000) and the genes *TAL1*, *TKL1*, *RPE1*, and *RKII* involved in the non-oxidative part of the pentose phosphate pathway (PPP) have been reported to be beneficial for improving xylose uptake, in addition to the overexpression of uptake systems for transporting xylose (Young et al., 2012; Kuyper et al., 2015). Thus, xylose utilization in an *S. cerevisiae* strain expressing *Piromyces XI* has been successfully improved with the use of combined metabolic and evolutionary engineering strategies.

However, the co-fermentation of xylose and glucose remains a challenge, and yeast can generally only utilize xylose

after depletion of glucose owing to glucose repression during the uptake of xylose into the cells. This is because xylose is taken up by *S. cerevisiae* cells via the glucose uptake systems (Saloheimo et al., 2007), and xylose uptake is competitively inhibited by glucose because of preference of the transporters for glucose. To date, some specific xylose transporters have been functionally expressed in yeast, which could specifically mediate the uptake of xylose into *S. cerevisiae* cells to some degree but were not able to effectively transport hexoses or were still somewhat inhibited by glucose (Subtil et al., 2012). Recently, the mutant hexose transporter *HXT7* (F79S), which was established through a laboratory evolution regime, was reported to show increased xylose uptake rates that allowed the *S. cerevisiae* strain to grow rapidly with xylose, and even under mixed glucose and xylose conditions (Apel et al., 2016). In addition, substitution of the N-terminal lysine residues in the endogenous hexose transporters *HXT1* and *HXT36*, which function as specific xylose transporters that are subjected to catabolite degradation, resulted in their improved retention at the cytoplasmic membrane in the absence of glucose, leading to improved xylose fermentation (Nijland et al., 2016). An artificial complex between an endogenous sugar transporter and a heterologous xylose isomerase in *S. cerevisiae* resulted in accelerated xylose consumption and substantially diminished xylitol production, with a concomitant increase in the production of ethanol (Thomik et al., 2017). Therefore, increasing bioethanol production during mixed glucose and xylose fermentation through efficient modification of the xylose utilization pathway in *S. cerevisiae* strains after consuming sufficient amounts of glucose to support yeast cell growth may require the following conditions: increasing the expression levels and activities of heterologous proteins, regulating the redox balance, realizing a proper metabolic burden, and accumulation of less toxic intermediates (Salis, et al., 2009; Bond-Watts et al., 2011).

Several promoters showing strong activity based on our test libraries were previously used to express the key enzymes *XI* and *XK* to optimize xylose metabolic pathways, resulting in improved xylose utilization (Yuan et al., 2017). Based on this background, we used these same promoters in the present study to evaluate the effect of genetic modifications on improving xylose utilization in *S. cerevisiae* strains expressing the *XI* pathway. First, we constructed two yeast multi-copy expression vectors harboring four non-oxidative PPP genes, which were transformed into the xylose assimilation pathway. Second, we introduced two promoters that show strong activity, *pPGK1* and *pHXT7*, capable of optimizing high expression levels of mutated *XI* and *XK* in the cytoplasm of the yeast four-gene strain so as to tailor the *XI* pathway toward obtaining a higher ethanol yield. Third, we designed novel fusion proteins using xylose-transporting hexose transporters, including hexogenous *HXT7* and heterogeneous *GXS1*, with a mutated *XI* gene to improve the xylose uptake rate in the cell membrane in combination with the cytoplasmic *XI* strains mentioned above. As a supplementary molecule, the addition of the endogenous transporter *HXT7* may ensure the proper uptake of xylose and glucose. The characteristics of the resulting recombinant *S. cerevisiae* strains with higher ethanol production were studied to systematically determine the fac-

tors affecting the metabolic pathway flux of *XI*, such as copy number variations and different cell locations. The optimized yeast *XI* strain (designated as strain CW9) showed an ethanol yield that was nearly 90% of the theoretical maximum within about 72 h.

2 Materials and Methods

2.1 Yeast strains, Plasmids, and Media

In this study, the *S. cerevisiae* haploid strains used were derived from the diploid industrial strain YC-DM (Angel Yeast, China). The *Scheffersomyces stipitis* genomic DNA was extracted from Sch. stipitis CBS6054 (Jeffries et al., 2007). Yeast cells were propagated in 2% YPD medium (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L). YNB medium without amino acids (Difco) supplemented with 2% (w/v) glucose and certain amino acids (according to the requirement of each strain), and 5-fluoroorotic acid medium (5-FOA) were used for yeast growth and the screening of correct transformants. The yeast strains, plasmids, and primers used in this study are listed in Tables 1, 2, and 3, respectively. *S. cerevisiae* strains were maintained at 80°C in a stock medium composed of YP and 20% glycerol. The methods and physical map for the construction of the four plasmids pCYTO1-XI, pCYTO2-XI, pPM-HXT7-XI, and pPM-GXS1-XI are provided in the Supplementary Materials and in Supplementary Figures S1-S4. We adopted the yeast codon-optimized version of the mutated *XI* gene (see the Supplementary Materials).

Table 1. Plasmids used in this study.

Plasmids	Marker and description	Reference
pUC18-RKUR	<i>AMP</i> ,	Kong et al., 2017
pBluescript SKP	<i>AMP</i>	Stratagene, La Jolla, CA
5-Final	<i>AMP</i> , PGK1p-RK11-CYC1t/ADH1p-TAL1-ADH1t/ PGK1p-RPE1-CYC1t/ADH1p-TKL1-ADH1t	Xiong et al., 2011
pGU-3X	<i>AMP</i> , ADH1p-XR-ADH1t/PGK1p-XDH-PGK1t/ PGK1p-XK-PGK1t	Xiong et al., 2011
pCYTO1-XI	<i>AMP</i> , PGK1p-XI ^{6m} -PGK1t/PGK1p-XK-XKt	This work
pCYTO2-XI	<i>AMP</i> , HXT7p-XI ^{6m} -PGK1t/PGK1p-XK-XKt	This work
pPM-HXT7-XI	<i>AMP</i> , PGK1p-HXT7(ORF)-XI ^{6m} -PGK1t/PGK1p-XK-XKt	This work
pPM-GXS1-XI	<i>AMP</i> , PGK1p-GXS1 ^{1m} -XI ^{6m} -PGK1t/PGK1p-XK-XKt	This work

2.2 Construction of Yeast *XI* Strains

To overexpress the four genes *RPE1*, *RK11*, *TAL1*, and *TKL1*, the plasmid "5-final", carrying a selective marker of the G418 kit, was digested with *HpaI*. The digested fragment was used to transform the yeast *S. cerevisiae* haploid strain to obtain strain CW1 with all four genes integrated (Xiong et al., 2011).

The plasmids pCYTO1-XI and pCYTO2-XI, containing two genes for *XI* and *XK*, were digested with *AscI* and *XmaI* and then transformed into strain CW1, creating the resulting yeast strains CW2 and CW3, respectively. Moreover, the plasmids pPM-GXS1-XI with a fusion protein of *GXS1* and *XI*, and pPM-HXT7-XI containing a fusion protein of *HXT7* and *XI* were digested by *AscI*-*SacI* and then transformed into the strain CW1, creating the resulting engineered strains CW4 and CW5, respectively. Moreover, the engineered strain CW6 was obtained by introducing pCYTO2-XI

Table 2. Strains used in this study.

Strains	Genotypes	References
YC-DM	MAT a/a	Angel Yeast, China
CBS6054	<i>Sch. stipitidis</i> CBS 6054	Jeffries et al., 2007
WT	<i>MA Ta ura3</i>	This work
CW1	WT, 5-Final	This work
CW2	CW1, <i>ura3</i> ::pCYTO1-XI	This work
CW3	CW1, <i>ura3</i> ::pCYTO2-XI	This work
CW4	CW1, <i>ura3</i> ::pPM-HXT7-XI	This work
CW5	CW1, <i>ura3</i> ::pPM-GXS1-XI	This work
CW6	CW2, <i>ura3</i> ::pCYTO1-XI::pCYTO2-XI	This work
CW7	CW4, <i>ura3</i> ::pPM-GXS1-XI	This work
CW8	CW4, <i>ura3</i> ::pPM-HXT7-XI	This work
CW9	CW4, <i>ura3</i> ::pPM-HXT7-XI::pPM-GXS1-XI	This work

into strain CW2 (Table 2). The yeast transformants were isolated on minimal medium lacking uracil and were verified for the correct integration of the RKUR cassette with diagnostic polymerase chain reaction. The isolates showing overexpression of the targeted gene were incubated on FOA plates to select a loop-out of the *URA3* gene through homologous recombination between the repeated sequences flanking the *URA3* gene in the integration cassette (Kong et al., 2007; Xiong et al., 2011).

Moreover, the plasmid pPM-GXS1-XI or pPM-HXT7-XI previously digested by *AscI-SacI* was used for transformation of the target strain CW6, creating the resulting engineered strain CW7 or CW8, respectively. At the same time, the engineered strain CW9 was obtained by introducing pPM-HXT7-XI into strain CW7 (Table 2). Isolation and verification of the transformants and subsequent loop-out of the vector sequence containing the *URA3* gene were performed essentially as described above.

2.3 Fermentation Experiments

The recombinant yeast strains were first cultivated aerobically in 2% YPD medium overnight at 30°C. The overnight-grown cells were centrifuged at 3000 rpm and 4°C and then washed twice with sterile water. Yeast cells were transferred to 250-mL shake flasks with 100 mL of YP medium containing 55 g/L xylose and 65 g/L glucose to ferment at 30°C with continuous shaking at 200 rpm. Initial cell densities were adjusted to an optical density at 600 nm of 0.2. All flasks were sealed with sealing film (Parafilm M, USA) after the yeast cells were harvested. The cultures were initially aerobic and then gradually became oxygen-limited as cultivation proceeded. All of the flask fermentation experiments were performed in triplicate, and less than 5% variation was observed between independent fermentations.

2.4 Analysis of Substrates and Metabolites

Concentrations of substrates and metabolites in the culture broth, including glucose, xylose, xylitol, and ethanol, were analyzed using high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Series USA), containing an Aminex HPX-87 H ion exchange column (BioRad, USA) and a refractive index detector (Waters 2414 RI detector). Samples were centrifuged in 2-mL microcentrifuge tubes at 14,000 rpm for 5 min. The supernatant was filtered using 0.2- μ m filters, and the filtered samples were stored at -20°C. The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6

mL/min. The column temperature and detection temperature were maintained at 30°C and 50°C, respectively.

2.5 Determination of Specific XI Activity

The specific XI activity from cell extracts was assayed using a modified method (Zhou et al., 2012). The reaction mixture (1 mL) contained 100 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH, 10 mM MgCl₂, and 2 U sorbitol dehydrogenase (Roche, Mannheim, Germany), as well as 20 mL of equilibrated fresh cell extract at 30°C for 5 min. The protein contents of the cell extracts were determined using the Pierce 660 nm Protein Assay kit (Thermo Scientific). The reaction was started by the addition of xylose to a final concentration of 500 mM. A molar extinction coefficient of 6.25 mM⁻¹ cm⁻¹ at 340 nm for NADH was used to calculate the specific XI activity, expressed as units per milligram of protein. One unit corresponds to the conversion of 1 μ M of xylose into xylulose per minute under the specified assay conditions.

2.6 Directed Evolution

All of the engineered XI strains obtained were used to initiate a directed evolution procedure to improve the xylose utilization rate and ethanol production, which consisted of the same three stages according to a recent evolution method (Zhou et al., 2012). In brief, the cells were first grown aerobically in YPX medium through sequential batch cultivation. After about 70 generations of evolution with the same dry cell weight, the isolated single strain displayed improved aerobic growth. Further evolution of the cell was carried out using the same sequential batch cultivation setup in YNBX medium from a micro-aerobic condition to anaerobic condition. The resulting evolved strain should show improved anaerobic growth. At the third stage, evolution of the whole strain was performed under continuous anaerobic cultivation using a xylose gradient as the limiting substrate.

3 Results and Discussion

3.1 Enhanced XI Activities for Key XI Strains

A previous study showed that metabolic/evolutionary engineering for improved growth on xylose in a yeast strain led to increased activity of the heterologous *Piromyces XI* gene expressed from a plasmid (Brat et al., 2009). To test whether a similar change might have occurred in our strains, we measured XI activity in cell extracts of the key XI strains constructed: CW2, CW6, and CW9. The specific XI activity in CW9 and CW6 was 3- and 2-fold higher than that of the precursor strain CW2, respectively (Figure 1). This marked improvement in XI activity, up to 0.55 U/mg protein, could be a crucial reason for the improved xylose fermentation rate observed in the CW6 or CW9 strain, which is consistent with the results of previous similar reports for evolutionarily engineered strains (Shen et al., 2012; Zhou et al., 2012;

Table 3. Primers used in this study.

Primer name	Sequence
ADH2p-F	5'-AAACCCGGTACCGGCGCGCCAGGGATTTGTCTTCATTAA
ADH2p-R	5'-GGGCCC <u>CTCGAGGTTTAAAC</u> TGTGTATTACGATATAGTTA
PGK1p-F1	5'-AAACCC <u>GTTTAAAC</u> AGGCATTTGCAAGAATTACT
PGK1p-R1	5'-GGGCCC <u>GTCGACCATAIG</u> TGTTTTATATTTGTTGTA AAAAG
XI-F	5'-AAATTT <u>GTCGAC</u> ATGGCTAAGGAATACTTCCC
XI-R	5'-GGGCCC <u>ATCGATTTAATTAA</u> TTATTGGTACATAGCGATGA
PGK1t-F	5'-GGGCCC <u>TAAATTAA</u> ATTGAATTGAATTGAAATCG
PGK1t-R	5' -AAACCC <u>GCTAGC</u> GTTGCAAGTGGGATGAGCTT
RUR-F	5'- GGGCCC <u>GCTAGC</u> GAGCAGCATAAACGACTGCT
RUR-R	5'- GGGCCC <u>CTGCAG</u> ACGCTCAATGTTGTTTCATGA
PGK1p-F2	5'- ATATAT <u>CTGCAG</u> AGGCATTTGCAAGAATTACT
PGK1p-R2	5'- GGGCCC <u>GGATCC</u> TTTGAATATGTATTACTTGG
ADH2t-F	5'- ATGCAT <u>GCGGCCGC</u> TGTTTTATATTTGTTGTA AAAAG
ADH2t-R	5'- GGATCC <u>GAGCTCCCCGGG</u> GTATAGAATTATATAACTTG
XK-F	5'- CCCTTT <u>GGATCC</u> ATGTTGTGTTTCAGTAATTCA
XK-R	5'- CCCGGG <u>GCGGCCGC</u> ACTCTTCATTTATTGAATTTT
CRE1 p-F	5' -AAATTT <u>GCGCGGCC</u> TTGACTGTAATTGCCGAATA
CRE1 p-R	5' - GGGCCC <u>GTTTAAAC</u> GCCAATGAATGAATTTTGC
Cre1t-F	5' -ATATAT <u>GCGGCCGC</u> TGTGCTGCAAAATTTCCAG
Cre1t-R	5' -CCCAAA <u>CCCGGG</u> CTGTTGAGTTGGCGTATGCA
HXT7 p-F	5' -GGGCCC <u>GTTTAAAC</u> TCTAGTTTCTGCCTTAAACA
HXT7 p-R	5' -GGGCCC <u>CATATG</u> TTTTGGATTA AAAATTA AAAA
FPS1p-F	5'- AAACCC <u>GCGCGGCC</u> TCCATATATGTTAAAATGCT
FPS1p-R	5'- CCCGGG <u>GTTTAAAC</u> GTCTTTTAATGCATTAGAAATGT
FPS1t-F	5'- CCCTTT <u>GCGGCCGC</u> GAAAACAGACAAGAAAAAGA
FPS1t-R	5'- GCGCGC <u>CCCGGG</u> AGAAAAGCATAGATGGTATT
HXT70-F	5'- GGGCCC <u>CATATGATGTCACAAGACGCTGCTAT</u>
HXT7-XI-R	5'- GAAGTATTCCTTAGCCATTTGGTGCTGAACATTCTCT
XI-HXT7-F	5'- AGAGAATGTTTCAGCACCAAAAATGGCTAAGGAATACTTC
XIO-R	5'- GGGCCC <u>TAAATTAA</u> TTATTGGTACATAGCGATGA
PGK1p-F3	5'- GGGCCC <u>CTGCAG</u> AGGCATTTGCAAGAATTACT
PGK1p-R3	5'- GGGCCC <u>GGATCC</u> TGTTTTATATTTGTTGTA AAAAG
GXS10-F	5'- GGGCCC <u>GGATCC</u> ATGGGTTTGGAGGACAATAGA
GXS1-XI-R	5'- GGGAAGTATTCCTTAGCCATAACAGAAGCTTCTTCAGACA
XI-GXF1-F	5'- TGTCTGAAGAAGCTTCTGTATGGCTAAGGAATACTTCCC
XIO-R2	5'- AAATTT <u>CCCGGG</u> TTATTGGTACATAGCGATGA
AU-F	5'- GCGCGC <u>CCCGGG</u> GCGAATTTCTTATGATTTAT
AU-R	5'- AAATTT <u>GATATC</u> CACATGTGTTTTAGTAAAC

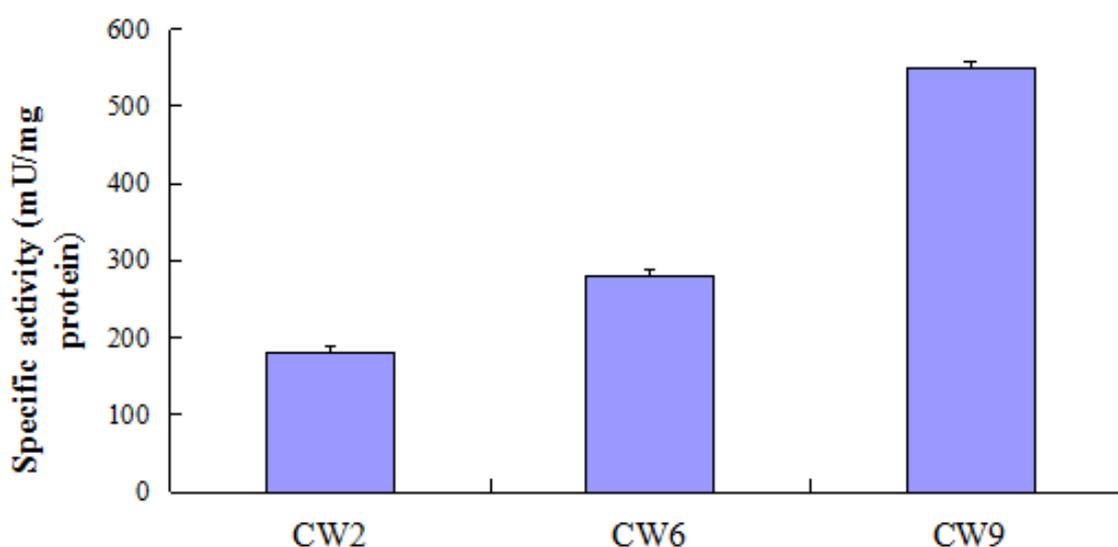


Figure 1. XI activities of three XI strains. The specific XI activities were measured in the cell extracts of strains CW2, CW6, and CW9. Error bars represent the standard deviations from triplicate experiments.

Demeke et al., 2013). These results confirmed that increased copy numbers of XI genes contribute to improved xylose utilization in the evolved strains by integration into the yeast genome. This further confirms that high XI activity is indeed beneficial for improving the xylose fermentation rate, but that achieving this advantage requires the introduction of six mutations in the genome of a given strain.

3.2 Fermentation Profile from Mixed Sugars for Engineered Strains with XI Located in the Cell Cytoplasm

We combined different promoters based on the expression of the yeast codon-optimized version of mutated XI (see [Supplementary Materials](#)) with an evolutionary engineering approach, and developed five industrial *S. cerevisiae* strains that could efficiently convert xylose to ethanol with a rational yield and productivity level. The fermentation profiles of the five strains (CW2-6) obtained through sequential metabolic/evolutionary engineering were evaluated in YPX medium containing 65 g/L glucose and 55 g/L xylose (Figure 2). Strains CW2, CW3, and CW6 consumed 100% glucose and 39.5 g/L (72.5%), 42.2 g/L (74.4%), and 44.9 g/L (82.4%) xylose after 48 h, and produced 36.7 g/L, 41.0 g/L, and 44.2 g/L ethanol with 7 g/L, 5.1 g/L, and 3.8 g/L of xylitol formation, respectively. After 84 h, CW2 consumed 97.6% xylose and produced a maximum ethanol concentration of 42.2 g/L with 7.8 g/L xylitol (Figure 2A). However, CW3 and CW6 consumed 96.1% and 97.6% xylose and produced a maximum ethanol concentration of 45.5 g/L and 47.9 g/L with 6.1 g/L and 4.7 g/L of xylitol, respectively, after 72 h (Figure 2B and 2E). The yields of ethanol and xylitol from total sugar for the three strains (CW2, 3, 6) were 0.35 g/g, 0.38 g/g, and 0.40 g/g, and 0.07 g/g, 0.05 g/g, and 0.04 g/g,

respectively. Strains CW2 and CW3 had very similar maximum sugar consumption rates and ethanol production rates under anaerobic conditions. During this fermentation process, the strains CW4 and CW5 showed maximum ethanol production of 45.8 g/L and 46.5 g/L with 4.3 g/L and 2.2 g/L of xylitol within 72 h, respectively (Figure 2C and 2D). Their xylose utilization capabilities displayed a slightly improved fermentation profile compared to those of CW2 or CW3 (Figure 2). Therefore, improving the flux of xylose into the cell via fusing the expressed transporter with the XI gene could help to achieve more efficient uptake for metabolism. These results indicate that our experimental design resulted in an improved impact of the fusion protein for the relative activity of strains CW4 and CW5. In brief, strain CW6 with two copies of XI showed slightly improved fermentation performance compared to that of the other four strains. The co-fermentation comparisons from a sugar mixture for the five XI strains are summarized in Table 4.

CW3 showed slightly higher ethanol production and reduced xylitol formation compared to CW2, which was due to the moderate XI expression level from the promoter of the xylose-transporting hexose transporter *HXT7* for CW3 grown in mixed sugar compared with the promoter of *PGK1*. Fusing the expressed transporter with the XI gene for CW4 and CW5 resulted in more efficient xylose utilization, suggesting a potential advantage of expressing a fusion protein compared to expressing the same copy number of the XI and transporter genes. The higher ethanol-producing capability in CW6 could be explained by the fact that two copies of the plasmid carrying the XI gene were integrated into the genome, leading to higher XI expression levels in the yeast cell cytoplasm. This also indicates that rapid xylose consumption requires a synergistic interaction resulting from different promoters, driving high XI activity in the genome,

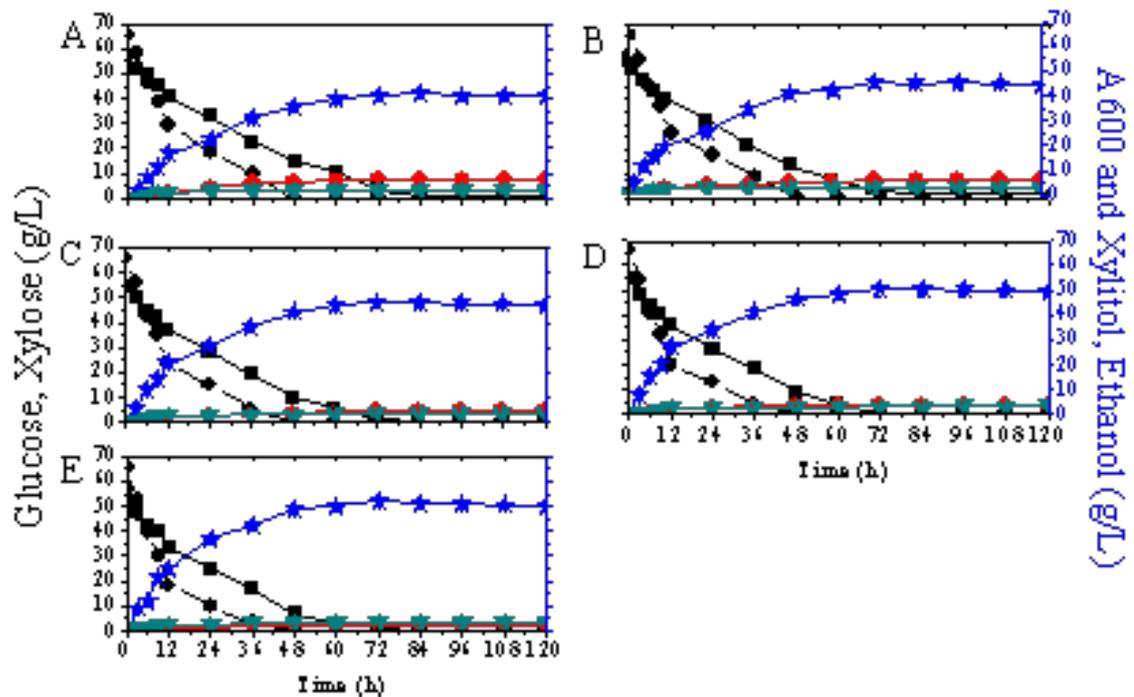


Figure 2. Time-dependent ethanol fermentation profiles of the engineered strains CW2 (A), CW3 (B), CW4 (C), CW5 (D), and CW6 (E) in YPDX medium with 55 g/L xylose and 65 g/L glucose. Symbols: black squares, xylose; black circles, glucose; red circles, xylitol; blue stars, ethanol; cyan triangles, biomass. Error bars represent the standard deviations from triplicate experiments.

Table 4. Fermentation profile of five *XI* strains, CW2-6.

Parameters	CW2	CW3	CW4	CW5	CW6
Initial glucose concentration (g/L)	65.5±0.01	65.5±0.02	65.2±0.00	65.4±0.01	65.5±0.01
Initial xylose concentration (g/L)	54.5 ± 0.01	54.5 ± 0.00	54.6± 0.01	54.7 ± 0.01	54.5 ± 0.02
Maximum ethanol concentration (g/L)	42.2 ± 0.02	45.5 ± 0.01	45.8 ± 0.01	46.5 ± 0.00	47.9 ± 0.01
Specific glucose consumption rate (g/g DCW/h)	1.86 ± 0.01	1.98 ± 0.02	2.43 ± 0.01	2.61 ± 0.00	3.25 ± 0.01
Specific xylose consumption rate (g/g DCW/h)	0.71 ± 0.00	0.74 ± 0.01	0.90 ± 0.02	0.95 ± 0.01	1.10 ± 0.00
Specific ethanol production rate (g/g DCW/h)	1.08 ± 0.00	1.12 ± 0.01	1.35 ± 0.00	1.36 ± 0.01	1.42 ± 0.02
Ethanol yield (g/g total sugar)	0.35 ± 0.00	0.38 ± 0.01	0.38 ± 0.00	0.39 ± 0.02	0.4 ± 0.01
Xylitol yield (g/g total sugar)	0.07 ± 0.00	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00

Table 5. Fermentation profile of XI strains CW7, CW8, and CW9.

Parameters	CW7	CW8	CW9
Initial glucose concentration (g/L)	65.5 ± 0.02	65.5 ± 0.01	65.5 ± 0.01
Initial xylose concentration (g/L)	54.5 ± 0.00	54.4 ± 0.02	54.5 ± 0.01
Maximum ethanol concentration (g/L)	50.2 ± 0.02	51.8 ± 0.01	54 ± 0.01
Specific glucose consumption rate (g/g DCW/h)	3.31 ± 0.02	3.43 ± 0.00	3.58 ± 0.01
Specific xylose consumption rate (g/g DCW/h)	1.26 ± 0.00	1.29 ± 0.01	1.34 ± 0.01
Specific ethanol production rate (g/g DCW/h)	1.43 ± 0.00	1.55 ± 0.02	1.76 ± 0.01
Ethanol yield (g/g total sugar)	0.42 ± 0.01	0.43 ± 0.00	0.45 ± 0.01
Xylitol yield (g/g total sugar)	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00

which is consistent with a previous report showing that high xylose utilization could only be partially attributed to the high activity of *XI* (Zhou et al., 2012).

3.3 Anaerobic Fermentation of the Improved Strains Expressing *XI* in the Cell Plasma Membrane

Next, we evaluated the fermentation profiles of three continuous *XI* strains (CW7-9) located in the cell plasma membrane (PM), in comparison with the parent strain CW6, located in the cell cytoplasm, based on the transporter locations and copy number variations of *XI* expression. Co-fermentations in rich YP medium containing 65 g/L glucose and 55 g/L xylose were used to compare the performance of strains CW7-9 established through sequential metabolic/evolutionary engineering steps (Figure 3). The three engineered PM *XI* strains CW7-9 partially consumed 65 g/L glucose after 36 h, and completely consumed 55 g/L xylose after 72 h, producing high ethanol yields (0.42 g/g, 0.43 g/g, and 0.45 g/g total sugar, respectively) and low xylitol yields (0.04 g/g, 0.02 g/g, and 0.02 g/g total sugar, respectively) (Figure 3A-C and Table 5). The PM strain CW8 showed higher ethanol production compared with CW7, suggesting that CW8, harboring the endogenous fusion protein *XI&XI*^{6m}, had slightly faster glucose and xylose consumption rates compared to CW7, harboring the exogenous fusion protein *GXS1&XI*^{6m} (Figure 3A, 3B and Table 5). Strains CW8 and CW9 showed an improved maximum sugar consumption rate and ethanol production rate compared to CW7, respectively (Table 5). The strain showing the best performance in this study (CW9) consumed all of the glucose and more than 99.7% of the xylose in approximately 72 h, with an ethanol yield of 0.45 g/g total sugar, equivalent to 90% of the maximum theoretical ethanol yield (Figure 3C and Table 5).

Although CW6 showed improved xylose consumption and

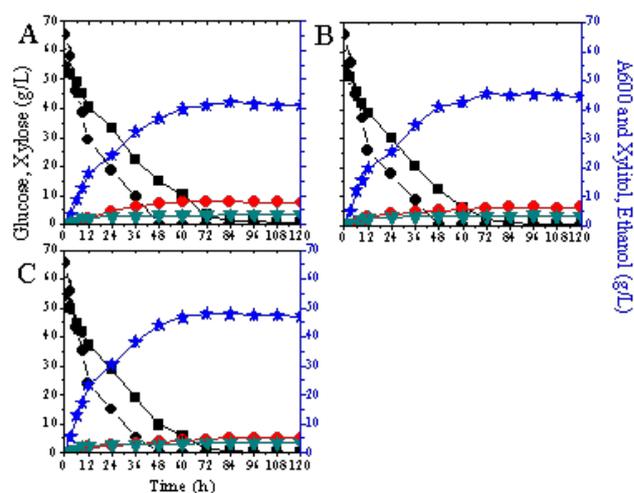


Figure 3. Time-dependent ethanol fermentation profiles of engineered strains CW7 (A), CW8 (B), and CW9 (C) in YPDX medium with 55 g/L xylose and 65 g/L glucose. Symbols: black squares, xylose; black circles, glucose; red circles, xylitol; blue stars, ethanol; cyan triangles, biomass. Error bars represent the standard deviations from triplicate experiments.

ethanol production by expressing two copies of the mutant *XI* gene in the cell cytoplasm compared to strains CW2-5, a certain amount of xylose was still left unfermented, and the rate of xylose utilization by strain CW6 was too low to achieve viable ethanol production. Therefore, obtaining a faster xylose consumption rate and ethanol production rate could be expected through additional metabolic/evolutionary engineering. The yield of ethanol obtained with PM strain CW8 derived from strain CW6 was slightly higher than that obtained with the similar PM strain CW7. Therefore, the sustained improvement in the rate of xylose fermentation and ethanol production for CW8 and CW7 might be explained by the presence of a suitable combination of an important fusion protein with *XI* introduced by a xylose transporter gene such as endogenous *HXT7* or exogenous *GXS1*.

By overexpressing two additional copies of the mutated *XI* gene in the PM for CW9, we demonstrated that the gradual engineering of mutated *XI* through exploitation of the two different fusion protein strategies described above could lead to the highest xylose-to-ethanol conversion yield. The improved enzyme activity was observed from the six mutations of the *XI* gene (E15D, E114G, E129D, T142S, A177T, and V433I). It seems plausible that the molecular transport of xylose into the cell, upon fast uptake depending on the expressed transporters *HXT7* or *GXS1*, first aggregates into the dimer, which is partially active. This dimer can then associate into the tetramer, resulting in the fusion of *XI*s forming the ordered dimer/tetramer structure. This observation is also likely to be due to the effects of some mutations near the monomer-binding sites. Basically, fusing the xylose uptake by the expressed transporters and xylose utilization by subsequent *XI* appears to be a rational spatial arrangement that can be used to quickly and effectively rewire xylose metabolism. The improved *XI* reported herein could serve as a critical starting point for further strain engineering. It

is likely that a combination of the strong promoter of *PGKI* and the glucose/xylose transporter promoter of *HXT7* in the cell cytoplasm work together to regulate mutated *XI* to boost ethanol yields and productivity. Moreover, other exogenous, endogenous, or intrinsic xylose transporters in the cell PM could jointly improve xylose uptake and utilization efficiency. Thus, the rapid fermentation and complete sugar attenuation observed was likely due to the high level of xylose uptake. Xylose transport was also possibly improved because of the combined effect of the xylose transporter with mutated *XI*, which is consistent with the results of a recent study (Thomik et al., 2017). Therefore, the improvement of xylose transport is likely attributed to changes in the transporter expression level.

Zhou et al. (2012) confirmed that a high expression level of *XI* could significantly promote a rapid xylose assimilation rate. Their engineered strains exhibiting high xylose assimilation rates harbored a large number of *XI* gene copies, which is consistent with the high transcription level and enzymatic activity of *XI*. In the present study, the increase in the ethanol yield for CW6 or CW9 was in fact slightly higher than that expected from xylose conversion alone for CW2/CW3/CW4/CW5 or CW7/CW8, respectively. However, Demeke et al. (2007) inferred that high *XI* activity was likely the main, but not the only, reason for fast xylose assimilation capacity. The xylose consumption rate and ethanol production rate of our optimized strain CW9 were improved by moderately overexpressing four copies of a mutated *XI* gene, without imposing a greater metabolic burden on the xylose assimilation pathway. This indicates that achieving both rapid xylose consumption and high ethanol production requires a synergistic interaction between high *XI* expression levels and appropriate *XI* mutation in the genome. In summary, we successfully optimized the PPP genes involved in the xylose utilization pathway (downstream approach), and introduced an efficient xylose transporter to fuse with a mutated *XI* gene (upstream approach) to further improve the xylose fermentation efficiency of engineered *S. cerevisiae*.

4 Conclusion

We describe the establishment of optimized strains of the yeast *S. cerevisiae* to improve *XI* uptake using an evolutionary engineering approach. Specifically, this strategy involves the introduction of specific mutations in the *XI* gene with fusion of exogenous promoters during fermentation. The results showed that the best-performing strain CW9 improved *XI* uptake to increase ethanol production at 90% of the theoretical yield. The strategy proposed and results obtained demonstrate the value of targeting the xylose assimilation pathway for enhancing biofuel production in engineered yeast strains. The improved *XI* reported herein could serve as a critical starting point for further strain engineering and help increase bioethanol production in a cost-effective manner.

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Author contributions

Mei Zhang and Wen-Jing Fan performed this study. Jing-Yu Wang and Li-Min Cao designed this experiment. Li-min Cao wrote this manuscript.

Conflict of Interest

No conflict of interest was reported by all authors.

Electronic Supplementary Materials

This article contains supplementary materials in the following website:

<http://ojs.udspub.com/index.php/aeb/rt/suppFiles/462/0>

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