Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*

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Abstract
The heterologous expression of a highly functional xylose isomerase pathway in *Saccharomyces cerevisiae* would have significant advantages for ethanol yield since this pathway bypasses cofactor requirements found in the traditionally-used oxidoreductase pathways. However, nearly all reported xylose isomerase-based pathways in *S. cerevisiae* suffer from poor ethanol productivities, low xylose consumption rates, and poor cell growth when compared with an oxidoreductase pathway and additionally often require adaptive strain evolution. Here, we report on the directed evolution of the *Piromyces sp.* xylose isomerase (*xylA*) for use in yeast. After three rounds of mutagenesis and growth-based screening, we isolated a variant containing six mutations (E15D, E114G, E129D, T142S, A177T, and V433I) that exhibited a 77% increase in enzymatic activity. When expressed in a minimally engineered yeast host containing a *gre3* knockout, and *tal1* and *XKS1* overexpression, the strain expressing this mutant enzyme improved aerobic growth rate by 61 fold and both ethanol production and xylose consumption rates by nearly 8 folds. Moreover, this mutant enzyme enabled ethanol production by these yeasts in an oxygen-limited fermentation condition, unlike the wild-type enzyme. Under micro-aerobic conditions, ethanol production rates of the strain expressing the mutant xylose isomerase were considerably higher than previously reported values for yeast harboring a xylose isomerase pathway, and also comparable to the strains harboring an oxidoreductase pathway. Consequently, this study shows the potential to evolve a xylose isomerase pathway for more efficient xylose utilization.
Introduction

Efficient utilization of all available carbon in lignocellulosic biomass is one of the major challenges preventing economically viable biofuels production (1, 45). Commonly used organisms for biofuel production such as the yeast *Saccharomyces cerevisiae* are unable to natively utilize the pentose sugars, which comprises a substantial portion of lignocellulosic biomass (29, 49). Among these pentose sugars, xylose is the most abundant in commonly studied biomass sources. Thus, the ability to improve xylose catabolism and conversion in a recombinant host such as *S. cerevisiae* would substantially improve the prospect of biofuels and biochemicals production.

Recently, our group has improved xylose (D-xylose) catabolism through the introduction of heterologous molecular transporters, a key rate determining step in xylose catabolism, especially at low concentrations (50). Once this xylose is transported into the cell, one of two main heterologous pathways can be utilized (49). The first pathway, with oxidoreductase-based chemistry, has been well-established in yeast through the heterologous expression of (at a minimum) a xylose reductase (XR) and xylitol dehydrogenase (XDH) leading to the ability of *S. cerevisiae* to utilize xylose (3, 11, 13, 14, 26, 33, 36). However, this pathway is inherently limited by a cofactor imbalance with the xylose reductase utilizing NADPH and the xylitol dehydrogenase utilizing NAD+ which leads a diversion of metabolic flux toward undesired products as a compensation reaction and decreases ethanol yield (49). Recent work has focused on modifying the cofactor preference of these enzymes to make them more compatible and establish an oxidation-reduction cycle (37, 47). However, even with matching cofactor specificities, the oxidoreductase pathway requires cofactors which may limit overall pathway
throughput. In all of these cases, the yield of ethanol from xylose still remains suboptimal when compared with native xylose utilizers.

A second, alternative pathway for xylose catabolism mainly exists in bacteria and rare yeasts. This isomerase-based pathway has no cofactor requirements and thus could lead to higher theoretical yields (0.51 g ethanol / g xylose) since no by-product is necessarily produced to compensate co-factor imbalance. By comparison, experimental ethanol yields using the oxidoreductase and xylose isomerase pathways in anaerobic conditions have been shown to be between 0.09 - 0.23 (6, 29) and near 0.43 g ethanol/g xylose, respectively (16). For this reason, there is considerable interest in improving a xylose isomerase-based pathway in *S. cerevisiae* with a particular focus on improving both cell growth rate and xylose consumption rate. Recent reports of successful expression of xylose isomerase genes from *Piromyces sp.* (19), *Orpinomyces sp.* (28), and *Clostridium phytofermentans* (8) in *S. cerevisiae* raise the prospect of efficient xylose fermentation. Furthermore, researchers have applied adaptive evolutionary engineering (21), optimized metabolic flux by introducing/overexpressing xylose transporter and/or overexpressing downstream pathway (20, 27), and employed bioprospecting to identify other putative xylose isomerase enzymes (8, 34). In all of these cases, extensive downstream overexpression and/or evolutionary engineering is required to improve cell growth and xylose consumption. Even still, these levels are not yet comparable with strains expressing an oxidoreductase pathway (4, 16, 45).

Beyond the assembly of xylose catabolic pathways, xylose isomerase is an important enzyme for the food industry, especially in the production of high-fructose corn syrup. For these applications, xylose isomerase has been extensively studied (5) to improve thermal stability (30, 42), pH optimum (23), and substrate preference (31). However, these studies were mainly
focused on obtaining a xylose isomerase that (i.) has different optimum temperature and pH range, 60 – 80 °C and pH 7.0 - 9.0, respectively (44), than those for conventional ethanol fermentation, (ii.) are expressed in *E. coli* rather than *S. cerevisiae* (2), and (iii.) are found to be unsuccessfully expressed (40) or to be inactive at mesophilic temperature (46) in *S. cerevisiae* mainly due to protein misfolding (12). Moreover, later attempts to improve the xylose isomerases for ethanol fermentation, such as cold-adaptation (25) and optimizing expression level (35), were unsatisfactory for use in constructing a functional xylose catabolic pathway in yeast.

Here, we report the first directed evolution study of a xylose isomerase gene (*xylA* from *Piromyces sp.*) for improved specific enzyme activity at the conditions tested, cell growth, xylose consumption rate, and ethanol production in the yeast *S. cerevisiae*. Directed evolution is an efficient approach for tailoring proteins that require refined functions such as higher stability, tolerance, substrate specificity, and product selectivity of protein. The iterative application of this method allows for proteins with significantly improved function to be easily obtained in a short period of time (7). To this end, we subjected the *xylA* gene to iterative rounds of random mutagenesis (aided by error-prone PCR) followed by selection for increased cell growth on xylose as a sole carbon source. After three rounds of mutagenesis and selection, we obtained an improved mutant of xylose isomerase that can offer a promising starting point for further strain engineering to improve xylose catabolism.
Materials and methods

Strains and culture conditions

*S. cerevisiae* strain BY4741-S1 deleted in *gre3* (Mat *a*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YHR104w::kanMX4, p415TEF-tal1) was used as a host strain in this study. This strain was obtained by transforming *S. cerevisiae* BY4741 *gre3* knockout strain (supplied by Zhihua Li, The University of Texas at Austin) with *tal1* from *S. spititis* cloned into a p415 vector under the control of the TEF promoter (32). Yeast strains were routinely propagated at 30 °C in yeast synthetic complete (YSC) medium composed of 6.7 g/L yeast nitrogen base, 20g/L glucose, and CSM-Leu-Trp-Ura, CSM-Leu-His-Ura or CSM-His-Leu-Trp-Ura (MP Biomedicals, Solon, OH).

Mutant selection, growth characterizations, and ethanol fermentation were conducted in the identical medium except that 20 g/L or 40 g/L xylose was added as a carbon source. *Escherichia coli* strain DH10β was used for all cloning and plasmid propagation. DH10β was grown at 37 °C in Luria-Bertani (LB) Broth supplemented with 50 μg/mL of ampicillin. All strains were cultivated with 225 RPM orbital shaking. Yeast and bacterial strains were stored at -80°C in 15% glycerol.

Construction of *xylA* mutant library

A library of randomly mutated xylose isomerase sequences was generated by error-prone PCR. A yeast codon-optimized version of xylose isomerase gene (*xylA*) from *Piromyces sp.* was synthesized by Blue Heron (Bothell, WA) and cloned into the Mumberg plasmid p416-GPD (32) to form p416-GPD-XI. Error-prone PCR of xylose isomerase gene was conducted with the primer set S004 xylA-f (5'-CCGGGCTGCAGGAATTCATG-3') and S004 xylA-r (5'-
GCGTGACATAACTAATTACATGACTCGAGTTA-3') using GeneMorph II Random Mutagenesis Kit (Stratagene, La Jolla, CA). According to the directions, libraries using low (0-4.5 mutations/kb), medium (4.5-9 mutations/kb), and high (9-16 mutations/kb) mutagenesis rates were cloned to achieve a library size of 1 x 10^5. The randomly amplified xylose isomerase genes were digested with EcoRI and XhoI (New England Biolabs, Ipswich, MA) and ligated into EcoRI and XhoI site of p416-GPD. The mutant plasmid library was transformed into E. Coli DH10β using standard electroporation protocols (38). The resulting E. coli library was harvested from the petri-dishes, isolated using the Zippy Plasmid Miniprep Kit (Zymo Research, Irvine, CA) and retransformed into S. cerevisiae BY4741-S1. Yeast transformation was conducted using Frozen EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA) according to the manufacturer’s instructions to achieve a one to three times coverage of the original mutagenesis library. This process and procedure for mutagenesis was repeated for each of the three rounds of directed evolution. These yeast libraries were allowed to grow for three days on plates before being scraped off and harvested for selection. These cells were then pooled and subjected to growth-based enrichment.

Mutant selection

To enrich fast-growing transformants, cells from the yeast library were cultured and serial-transferred into 20 ml of fresh YSC medium with xylose as a sole carbon source in a loosely closed 50 ml falcon tube. Serial transfer was repeated every 4 to 5 days using 10% inoculums. After seven rounds of serial transfers, cells from the final culture were plated onto YSC medium with xylose and the largest colonies were isolated. In total, 40 cells were selected from these plates for further characterization. Cell growth of isolated variants was compared in 5
ml of YSC medium with xylose in a 14 ml culture tube and the fastest growing variants were initially selected. The vectors from these promising mutants were isolated, sequenced, and retransformed. A second growth rate measurement against control confirmed that the growth rate increase was due to the mutant xylose isomerase and not background adaptation of the host strain. The mutant xylose isomerase conferring the highest growth rate on xylose was used as the template for the next round of mutagenesis and selection. In total, two additional rounds of iterative random mutagenesis and the selection were conducted. The best performing mutant was termed \textit{xylA}^*1 (1\textsuperscript{st} round mutant), \textit{xylA}^*2 (2\textsuperscript{nd} round mutant), and \textit{xylA}^*3 (3\textsuperscript{rd} round mutant), respectively, and the re-transformed strains expressing these mutant xylose isomerase are termed S1A1, S1A2, and S1A3, respectively. The number of serial transfers was reduced from seven to five in the second and the third round of the selection processes.

\textbf{Site-directed mutagenesis of identified mutations}

Site-directed mutations were conducted to confirm the beneficial mutations in the xylose isomerase mutant. A series of six individual back mutations were made using the third round mutant xylose isomerase (\textit{xylA}^*3) by using Quikchange II kit (Stratagene, La Jolla, CA) and transformed into \textit{S. cerevisiae} to identify beneficial mutations on the basis of cell growth. Once identified, the beneficial mutations were introduced to wild type xylose isomerase to confirm the improved cell growth phenotype on xylose.

\textbf{In vitro xylose isomerase activity measurements}

Xylose isomerase activities from cell extracts were assayed by measuring the decrease of NADH in a 1 ml of reaction mixture at 340 nm using a spectrophotometer (18).
mixture contained 100 mM Tris-HCl buffer (pH 7.5), 0.15 mM NADH, 10 mM MgCl₂, and 2 U sorbitol dehydrogenase (Roche, Mannheim, Germany). The cell extracts were prepared from the yeast transformants cultivated until early exponential growth phase in selective medium by using YPER Plus Dialyzable Yeast Protein Extraction Reagent (Thermo Scientific, Rockford, IL). The protein contents of cell extracts were determined by the method of Bradford assay using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Enzyme assays were performed in biological triplicate and 25 to 500 mM xylose was used to determine kinetic parameters.

**Fermentation assays and Growth Analysis**

Ethanol fermentations were performed at high yeast optical density in oxygen-limited conditions and micro-aerobic conditions. For oxygen-limited conditions, cells were grown at 30°C in 50 ml of YSC medium containing 40g/L of xylose as a sole carbon source in a 125 ml flask. For micro-aerobic conditions, cells were grown at 30°C in 40 ml of YSC medium with 20g/L of xylose in a sealed 50 ml falcon tube. For both of these fermentations, a one day old pre-culture (grown on glucose) was pelleted and re-suspended in xylose medium and inoculated at an initial OD of 20. Aerobic growth rate analysis was performed at low yeast optical density while culturing cells at 30°C in 5ml of YSC medium with 20g/L of xylose in a 14 ml culture tubes. Xylose concentrations were measured using an YSI 7100 Multiparameter Bioanalytical System (YSI Life Sciences, Yellow Springs, OH) and ethanol concentrations were measured using Ethanol Assay, UV-method Kit (R-Biopharm, Darmstadt, Germany). Cell density was measured spectrophotometrically at 600 nm absorbance, from which cell dry weight was calculated for the ethanol production and xylose consumption rates. One OD₆₀₀ unit was
considered as 0.17 g cells/L (14). Fermentation and growth assays were performed in biological triplicate.

**Protein structure prediction for xylose isomerase**

The three-dimensional structure of the xylose isomerase protein was predicted by Swiss-Model (http://swissmodel.expasy.org), a web-based server for automated comparative modeling of three dimensional protein structures (41). The crystal structure of *Thermotoga neapolitana* [PDB code 1A0E (9)] was used as a template. The active site of xylose isomerase was predicted using the computer program PHYRE (a protein fold recognition server; http://www.sbg.bio.ic.ac.uk/phyre/ (17)). The predicted protein model and mutations were visualized using Pymol version 1.3.

**Results**

**Identification of a Xylose Isomerase Mutant Conferring Increased Cell Growth on Xylose**

In this study, we sought to utilize directed evolution to improve the poor growth and xylose consumption rates usually associated with xylose isomerase-based pathways in heterologous *S. cerevisiae*. First, the xylose isomerase gene (*xylA*) from *Piromyces sp.* was synthesized as a codon-optimized version for *S. cerevisiae* to maximize translational efficiency. This optimal version was then used as a template for random mutagenesis afforded by error-prone PCR using the GeneMorph II Random Mutagenesis kit. Specifically, a library size of $1 \times 10^5$ members (as measured by independent *E. coli* colonies post-transformation) was created
using a range of mutation rates. This library was cloned into a yeast expression vector behind the strong GPD promoter for high level expression.

To create a suitable host strain for selection and testing of this xylose isomerase library, the standard haploid yeast BY4741 was chosen as a background. Two additional genetics changes were selected for this host strain. First, a gre3 knockout strain of BY4741 was chosen as Gre3p is an aldose reductase that has been previously shown to inhibit xylose isomerase by nonspecifically producing xylitol from xylose (43). Second, the overexpression of a heterologous transaldolase, tall from S. stipitis was chosen since this protein has been previously been reported to be beneficial for xylose utilization in S. cerevisiae (14). The overexpression of downstream pathway enzymes to insure proper flux is advantageous for selection phenotypes (37). To achieve overexpression of tall, this gene was cloned behind a TEF promoter in the p415 plasmid. The resulting strain containing both the gre3 knockout and the heterologous tall expression was named BY4741-S1. Finally, the native chromosomal copy of S. cerevisiae xylulokinase (XKS1) was maintained as opposed to overexpressing a heterologous copy from S. stipitis. Previous work has shown that strong overexpression of heterologous xylulokinase can be toxic to cells, especially with non-optimal xylose catabolic pathways, thus we sought to initially avoid this improper balance of enzyme levels in this strain (15).

The mutant xylose isomerase library was transformed into S. cerevisiae BY4741-S1 and screening/selection was conducted on the basis of growth rate advantage. To this end, pools of cells were serially subcultured in xylose media for between 5 and 7 serial transfers. Following this selection, many mutant strains were isolated and tested for growth (a sampling of 20 from each round provided in Fig. 1). The mutant conferring the most improved growth phenotype was subjected subsequent rounds of mutation and selections. This process was repeated for a
total of three rounds of mutagenesis and selection. At each step, improved cell growth
phenotypes of selected mutants were confirmed through retransformation of the xylose
isomerase mutant gene into a fresh host strain to exclude any adaptive changes in the genomic
DNA of isolated strains. It should be noted that cell adaptation can provide an additional means
of improving the xylose utilizing cells created here. For example, the isolated strain after third
round of mutation showed almost double the growth rate compared to the retransformed strain.
Thus, the ultimate production strain will utilize the synergy between pathway engineering and
adaptive laboratory evolution.

The growth rates of the retransformed strains (S1A1, S1A2, and S1A3) expressing the
best mutant xylose isomerase isolated from each round (xylA*1, xylA*2, and xylA*3 from the
first, second and third round, respectively) are shown in Table I compared to strains (S1A)
harboring the wild type xylose isomerase gene (xylA). Growth on xylose is not observed in a
control strain (S1C) harboring a blank plasmid as wild-type yeast cells lack a pathway to
catabolize xylose. The growth rates on xylose progressively increased through the successive
rounds of directed evolution (Table I). The S1A3 imparted a 9 fold increase in growth rate
compared to S1A.

Enzymatic Assay of Xylose Isomerase Mutants

As demonstrated above, these mutant xylA genes were able to increase the growth rate of
yeast cells on xylose, thus we sought to understand the underlying mechanism of this
improvement using in vitro kinetics assays. To do so, total protein was extracted from these
strains and enzyme assays were conducted using a spectrophotometric-based coupled enzyme
system (18). Similar to the growth rates, the enzyme activities of xylose isomerase mutants
increased progressively with the rounds of mutagenesis and selection (Table II). The \textit{xylA}*3 had a 77% increase in $V_{\text{max}}$ compared to \textit{xylA} (0.094 \text{ \(\mu\)mol min\(^{-1}\) mg protein\(^{-1}\) compared to 0.053 \text{ \(\mu\)mol min\(^{-1}\) mg protein\(^{-1}\)} for \textit{xylA}*3 and \textit{xylA}, respectively). It should be noted that the $V_{\text{max}}$ value measured here for \textit{xylA} is consistent with previously reported values (8). In contrast, the $K_{m}$ values did not show the expected trend of decreasing $K_{m}$ values, which refers to high substrate affinity leading high enzyme activity, with increased cell growth (the $K_{m}$ for \textit{xylA}*3 is significantly higher than the wild-type value). Nevertheless, these $K_{m}$ values (averaging around 100 mM) are quite high, highlighting the root cause of difficulties with the isomerase system. Therefore, the improved cell growth seems to be derived mainly by increased $V_{\text{max}}$ rather than decreased $K_{m}$ of mutant xylose isomerase.

**Identification of Critical Mutations in Isolated Xylose Isomerase Mutants**

In addition to enzyme analysis, these improved xylose isomerase genes were sequenced to identify the mutant residues responsible for improved activity. In each round of mutagenesis, two amino acid substitutions occurred which resulted in six mutations in the \textit{xylA}*3 third round mutant: E15D, E114G, T142S, E129D, A177T, and V433I (Table II).

The cumulative amino acid substitutions obtained in this third round mutant were next investigated to identify which were necessary and sufficient for the improved performance of \textit{xylA}*3. To accomplish this, single back-mutations of each amino acid substitution in the \textit{xylA}*3 were conducted to identify the essential nature of each of these six mutated sites. The strains expressing \textit{xylA}*3 with single back mutations of amino acid at the position 15 and 142, which were obtained during the 2\textsuperscript{nd} and 3\textsuperscript{rd} round respectively, showed 46% and 32% decrease in aerobic growth rates on xylose compared to the strain expressing \textit{xylA}*3. This suggests that both
E15D and T142S are necessary mutations that strongly contribute to the improved performance of mutant xylose isomerase (Fig. 2). The remaining four mutations appeared to be neutral mutations as their back substitution did not have an impact on enzyme performance as measured by resulting growth rate. The beneficial effect the E15D and T142S mutations were confirmed by single and double point mutations of using xylA as a template. This test was conducted to determine whether these mutations either alone or in conjunction were sufficient to obtain the same phenotype as the isolated mutant. As shown in Fig. 2, single substitution of either amino acid at the position 15 or 142 was not enough to confer improved activity, but substitutions at both position resulted in nearly the same performance as xylA3* in aerobic growth rates. As a result, the improved performance of xylA3* seems to be the result of synergistic mutations at position 15 and 142.

**Xylose Isomerase Mutants Improve Xylose Consumption and Ethanol Production**

We next evaluated the xylose consumption and ethanol production rates enabled by these xylose isomerase mutants using a series of high-cell density (OD = 20) batch fermentations in both oxygen-limited and micro-aerobic conditions. For the oxygen-limited conditions, 50 ml was cultured in a covered 125 ml flask. In these conditions, xylose consumption rates increased progressively through the rounds of directed evolution with S1A3 increasing xylose consumption rates by nearly 90% over the S1A (Fig. 3B). As described above, the control strain (S1C) harboring a blank plasmid does not grow and thus consumed no xylose. Interestingly, only S1A2 and S1A3 produced measurable ethanol levels in this condition (Fig. 3A). The S1C, S1A, and S1A1 did not produce any ethanol (Fig. 3A). Furthermore, S1A3 had a relatively short lag time for ethanol production whereas S1A2 started producing only after 95 hours of fermentation
Thus, the identified mutant xylose isomerase (especially xylA*3) enables ethanol production capacity in these oxygen-limited conditions. As a second test for ethanol production, we utilized less aerobic conditions (specifically, a micro-aerobic condition) afforded by culturing 40 ml in a 50 ml sealed vial. These conditions were expected to be more favorable for ethanol fermentations and likewise resulted in all strains producing ethanol at significantly increased rates (Table I). Ethanol production capacity was greatly increased in strains harboring these mutant versions of xylose isomerase (Fig. 4A) as was xylose utilization rates (Fig. 4B). The S1A1 produced ethanol at the rate of 0.0041 g ethanol g cell\(^{-1}\) h\(^{-1}\) in this condition which represents an increase of 28% relative to that of S1A (0.0032 g ethanol g cell\(^{-1}\) h\(^{-1}\)). In addition, the ethanol yield in S1A1 (0.50 g ethanol / g xylose) was close to the theoretical value of 0.51 g/g. Subsequent mutants of xylose isomerase resulted in higher rates of ethanol production with an 88% and 75% increase over S1A for S1A2 and S1A3, respectively. It is interesting that in this more micro-aerobic condition, the performance of S1A2 and S1A3 were quite similar. Although S1A3 produced ethanol at a slightly lower rate (0.0056 g ethanol g cell\(^{-1}\) h\(^{-1}\)) than S1A2 (0.0060 g ethanol g cell\(^{-1}\) h\(^{-1}\)), the xylose consumption rate was slightly higher in S1A3 (0.0126 versus 0.0123 g xylose g cell\(^{-1}\) h\(^{-1}\)) (Fig. 4A and B). Despite the increased rate, ethanol yields in these strains were slightly lower than S1A1 (0.49 and 0.45 g ethanol / g xylose for S1A2 and S1A3 respectively). Collectively, these results along with the production profiles (Fig. 4) suggest that downstream metabolic flux is limited in the cells harboring these improved xylose isomerase genes in these micro-aerobic conditions.
Improved performance of mutant xylose isomerase by additional engineering

The expression of the improved xylose isomerase in the minimally engineered strain of \textit{S. cerevisiae} BY4741 still showed limited cell growth, xylose consumption, and ethanol production presumably due to insufficient downstream metabolic flux. To further improve the xylose fermentation performance, the xylose isomerase enzymes (mutant and wild-type) were cloned into a high copy plasmid p426-GPD (32) and co-transformed into \textit{S. cerevisiae} BY4741-S1 along with an additional plasmid expressing the xylulokinase \textit{XKS1} driven by the TEF promoter (32). The resulting strains were then named BY4741-S2AK, S2A2K, and S2A3K expressing \textit{xylA}, \textit{xylA*2}, and \textit{xylA*3}, respectively. It should be noted that although these strains will have an expected higher downstream flux when compared to \textit{S. cerevisiae} BY4741-S1, they are still relatively minimally engineered when compared with other strains commonly described in literature that contain many pentose phosphate pathway enzyme overexpressions (Table I). Therefore, this experiment was intended to demonstrate the potential of the improved xylose isomerase enzyme as a promising starting point for further strain engineering.

As shown in Fig. 5, the overexpression of xylulokinase significantly increased the aerobic growth rates in the strains expressing the various xylose isomerase enzymes (S2AK, S2A2K, and S2A3K expressing \textit{xylA}, \textit{xylA*2}, and \textit{xylA*3}, respectively) compared to the strains without xylulokinase overexpression (S2A, S2A2, and S2A3 expressing \textit{xylA}, \textit{xylA*2}, and \textit{xylA*3}, respectively). The improvement imparted by xylulokinase overexpression was most profound in strains harboring the mutant xylose isomerase variants. The aerobic growth rate of S2A3K (0.061 ± 0.00) was 20 fold higher than the strain without the xylulokinase overexpression and nearly 61 fold higher than a strain simply overexpressing wild-type xylose isomerase.
Finally, we tested the performance of the strain expressing the 3rd round mutant xylose isomerase enzyme with xylulokinase overexpression (S2A3K) in a micro-aerobic xylose fermentation as described above. In these high-cell density batch fermentations, S2A3K produced ethanol at the rate of 0.024 g ethanol g cell\(^{-1}\) h\(^{-1}\) and consumed xylose at the rate of 0.057 g xylose g cell\(^{-1}\) h\(^{-1}\), which represents an increase of nearly 8 folds relative to those of S1A (0.0032 g ethanol g cell\(^{-1}\) h\(^{-1}\) and 0.007 g xylose g cell\(^{-1}\) h\(^{-1}\) S1A) (Fig. 6 and Table I). These production rates make this strain comparable to the rates obtained in strains harboring the traditional oxidoreductase pathway (Table I). The ethanol yield in these fermentations was 0.42 g ethanol / g xylose which is significantly higher than the oxidoreductase pathway.

**Discussion**

This study demonstrates that a xylose isomerase pathway in *S. cerevisiae* can be improved by directed evolution. In doing so, the minimally engineered strain expressing the improved xylose isomerase outperformed the strain expressing wild type xylose isomerase in terms of ethanol production by nearly 90%, xylose consumption by 80%, and aerobic growth rate by 9 fold. These improvements were achieved by a 77% increase in *in vitro* catalytic activity for this enzyme. Combined with one additional downstream enzyme overexpression (xylulokinase), the strain expressing the mutant xylose isomerase exhibited a 61 fold increase in aerobic growth and an 8 fold increase in both ethanol production and xylose consumption rates. These improvements clearly show the potential of this mutant as a promising starting point for further strain engineering for efficient xylose fermentation.

The increased enzyme activity seems to be resulted from a series of identified mutations in both the active site and monomer-binding contacts. Fig. 7 shows three-dimensional protein
structure modeling predicted based on the *Themotoga neapolitana* xylose isomerase [PDB code 1A0E (9)], which was available and shows high sequence similarity (65%) and identity (51%) to xylA, suggested that improved enzyme activity is likely due to two main factors: (i.) increased substrate-enzyme interaction by mutations (Thr142 and Ala177) near the active site (His 102) and (ii.) increased enzyme stability caused by mutations near the monomer-binding contacts (Glu15 and Val433). This theory is supported by the fact that T142S and E15D were both necessary for the improved xylose isomerase performance of the xylA*3 (Fig. 2). It is of interest that mutations near the monomer-binding contacts occurred in the early rounds of mutagenesis and selection, prior to active site mutations. Previous reports suggest that xylose isomerase is only active when in the form of a dimer and tetramer (24). Based on the evolutionary trajectory identified in this work, it seems that increased stability of xylose isomerase dimer/tetramer through mutations near the monomer-binding sites was a prerequisite for increased enzyme activity and active site mutations. The instability of dimerized xylose isomerase is one of the possible explanations for previously poor expression and activity of this enzyme in *S. cerevisiae* (44).

The performance of the identified xylose isomerase mutant was tested in two high-cell density fermentation assays—oxygen-limited and micro-aerobic conditions. In micro-aerobic conditions, the xylose consumption and ethanol production rates of S1A3 (0.0126 g xylose g cell\(^{-1}\) h\(^{-1}\) and 0.0056 g ethanol g cell\(^{-1}\) h\(^{-1}\), respectively) were considerably higher than previously reported values for yeast cells harboring a xylose isomerase pathway (Table 1). As an example, when wild-type *Piromyces sp. xylA* was expressed in *S. cerevisiae* CEN.PK (TMB3066) background with AXSI, TAL1, TKLI, RPE1, RKII, HXT7 overexpression and gre3 deletion, xylose consumption rate and ethanol production rates were reported as 0.005 g xylose g cell\(^{-1}\) h\(^{-1}\).
and 0.002 g ethanol g cell\(^{-1}\) h\(^{-1}\), respectively (16). Thus, the results presented here—obtained strictly by overexpression of a mutant xylose isomerase along with \textit{tal1} from \textit{S. stipitis} and \textit{gre3} deletion—are over 2 to 3 fold higher in these metrics. Moreover, ethanol production rate of S1A3 are also comparable to strains expressing an oxidoreductase pathway (Table I). Most studies on ethanol fermentation with \textit{S. cerevisiae} expressing oxidoreductase pathways have reported ethanol production rates in the range of 0.007 – 0.043 g ethanol g cell\(^{-1}\) h\(^{-1}\) in anaerobic condition, which depend on host strains, downstream gene overexpression, and reactor type (37, 45), though the highest reported ethanol production rate of 0.23 g ethanol g cell\(^{-1}\) h\(^{-1}\) was achieved by random mutagenesis of xylose reductase (37). Based on the efforts reported here, the ethanol production rates of a xylose isomerase-based pathway are now comparable to early reports on the oxidoreductase pathway.

The improvements of xylose consumption and ethanol production of strain expressing mutant xylose isomerase are substantial especially since they were achieved without the need for as extensive pathway and metabolic engineering in the host strain. This implies that xylose consumption rate and ethanol production rate are expected to be further increased by additional metabolic/evolutionary engineering. In previous research as shown in Table I, xylose consumption and ethanol production rates of strains expressing wild type xylose isomerase from \textit{Piromyces sp.} were improved by overexpressing downstream genes (3 fold increase in ethanol production rate) or evolutionary engineering (36 fold increased in aerobic growth rate) in a bioreactor. By overexpressing one additional enzyme, xylulokinase, we successfully demonstrated that additional metabolic engineering would significantly improve xylose fermentation performances (Table I and Fig. 5 and 6). The S2A3K showed higher aerobic growth rate than the evolved-industrial strain expressing xylose isomerase from \textit{Clostridium sp.} (7)
and the strain expressing wild-type xylose isomerase from *Piromyces sp.* with extensive engineering of downstream genes and xylose transporter (15). Moreover, the ethanol production rate of S2A3K (0.024 g ethanol g cell^{-1} h^{-1}) was comparable to strains with an oxidoreductase pathway, which are usually in the range of 0.007 – 0.043 g ethanol g cell^{-1} h^{-1} in anaerobic conditions (35, 44). Given that the better performance was obtained without extensive strain engineering, it is expected that rates and yields could be further improved by the overexpression of other enzymes involved in pentose phosphate pathway or xylose transporters. Thus, the improved xylose isomerase reported here could serve as a critical starting point for further strain engineering to boost ethanol yields and productivity.

In oxygen limited conditions, the improvement of ethanol production is also important as this mutant pathway enabled ethanol production. *S. cerevisiae* strains S1A2 and S1A3 produced ethanol whereas S1A did not produce any ethanol. Moreover, xylose consumption rates in these conditions were increased by nearly 90% with S1A3. Ethanol fermentation is typically an anaerobic process that converts sugar to ethanol. Although *S. cerevisiae* can produce ethanol in aerobic condition by respiro-fermentation, ethanol is usually not the by-product in aerobic condition (48). As a result, efficient industrial ethanol fermentors are mainly operated under anaerobic condition (39); however, intermittent aeration is required to maintain *S. cerevisiae* viability and ethanol productivity during long period of fermentation. In the aerobic or oxygen-limited condition created by aeration, ethanol productivity decreases (39). Hence, an idealized strain for ethanol fermentation will produce high concentrations of ethanol in anaerobic condition and is less vulnerable to lower yields under aerobic or oxygen-limited condition. To this end, the *S. cerevisiae* with improved xylose isomerase pathway reported here exhibited both of these properties with a rapid initiation of ethanol production under oxygen-limited condition.
and a strong ethanol production under more anaerobic conditions. Thus, this pathway could form
the foundation of a promising host for industrial ethanol fermentation.

This study presented a significantly improved xylose isomerase pathway in *S. cerevisiae*
through the use of directed evolution without the need for extensive pathway engineering. The
xylose isomerase-based pathway is an advantage for yeast fermentation as it bypasses the
imbalanced cofactor issues that plague the oxidoreductase pathway. The third round mutant
created here has now alleviated xylose isomerase as the rate limiting step in this xylose catabolic
pathway. Beyond this enzyme, it will be necessary to optimize the downstream genes involved
in xylose metabolic pathway (downstream approach) and introduce efficient xylose transporter
(upstream approach) to further improve xylose fermentation efficiency of engineered *S.
cerevisiae* (29, 49). In further strain engineering, it will be important to balance the upstream
and downstream enzymatic activity since isomerization of xylose to xylulose is reversible and
xylose formation is more favorable than xylulose formation at equilibrium by 80:20 (10).

Regardless, the xylose isomerase pathway has long-been considered an attractive alternative to
the oxidoreductase pathway for expression in yeast. The results presented here demonstrate that
this enzyme can be evolved for improved function in yeast leading to more efficient cell growth,
xylose uptake rates, and ethanol fermentation rates—three phenotypes that were limited with the
wild-type enzyme.

Acknowledgments

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47. Watanabe, S., A. Abu Saleh, S. P. Pack, N. Annaluru, T. Kodaki, and K. Makino. 2007. Ethanol production from xylose by recombinant Saccharomyces cerevisiae expressing protein-
engineered NADH-preferring xylose reductase from *Pichia stipitis*. Microbiology **153**:3044-3054.


Table and Figure Legends

Table I: Comparison of literature based growth and fermentation rates with xylose as a sole carbon source. Fermentation performance of S. cerevisiae BY4741-S1A, S1A1, S1A2, S1A3 and S2A3 were compared with previously reported results. The strains developed here have 10 to 30 fold and 3 to 10 fold higher rates than previously reported engineered strains for xylose isomerase pathways and oxidoreductase pathways, respectively. Legend: n.a.: not available.

Table II: Enzyme Kinetics and sequence analysis of xylA mutants. The wild type (xylA) and mutant xylose isomerase: first (xylA*1), second (xylA*2), and third (xylA*3) round mutant were expressed in S. cerevisiae and in vitro xylose isomerase activity was measured and reported. Sequence analysis reveals the mutations identified in each round. Error bars represent the standard deviation of biological triplicates.

Figure 1: Comparison of relative growth rate of isolated mutants (selected). The growth rate of strains isolated during the first (white), second (grey), and third (black) rounds of mutagenesis (µ*) were compared to those of the wild type (µ°). Twenty representative strains are shown out of the 40 tested for each round. The average growth rate of these isolated mutants increased progressively with the round of mutagenesis. The isolated strain with the highest growth rate in each round was selected.
Figure 2: Identification of critical mutations by site-directed mutation. Aerobic cell growth rate (µ) of strains expressing the third round mutant with single back mutation (grey) and wild type with point mutation (black) were compared to that of the strain expressing the third round mutant xylose isomerase (µₒ). The back mutations at the amino acid position 15 and 142 resulted in 46% and 32% decrease in growth rates. Combining these two positions improved the performance of wild type xylose isomerase in aerobic growth on xylose. Error bars represent the standard deviation of biological triplicates.

Figure 3: Oxygen-limited fermentation tests with xylA mutants. Ethanol production (A) and xylose consumption (B) profiles were measured in oxygen-limited conditions for S. cerevisiae BY4741-S1C (○), S1A (●), S1A1 (▲), S1A2 (◆), and S1A3 (■). Ethanol production was only present in the second and third round mutant. Xylose consumption was likewise increased in these strains. Error bars represent the standard deviation of biological triplicates.

Figure 4: Micro-aerobic fermentation tests with xylA mutants. Ethanol production (A) and xylose consumption (B) profiles were measured in micro-aerobic conditions for S. cerevisiae BY4741-S1C (○), S1A (●), S1A1 (▲), S1A2 (◆), and S1A3 (■). Ethanol production was increased over oxygen-limited conditions with both the second and third round having similar productivities. Total improvement in ethanol production was nearly 90% using these mutants. Xylose consumption was increased progressively with the round of directed evolution in these strains. Error bars represent the standard deviation of biological triplicates.
Figure 5: Aerobic growth rates of the strains expressing xylose isomerase with xylulokinase overexpression. Growth rates were measured for the strain expressing wild type (xylA), 2nd (xylA*2) and 3rd round (xylA*3) mutant xylose isomerase with xylulokinase overexpression (black) and compared to those without xylulokinase overexpression (grey). The improved growth rate of the strain expressing xylA*3 (0.061 ± 0.001) was 5 fold higher than that of the strain expressing xylA (0.013 ± 0.001). Total improvement of the best strain compared with the strain simply expressing wild-type xylA is 61 fold. Error bars represent the standard deviation of biological triplicates.

Figure 6: Micro-aerobic fermentation tests with xylulokinase overexpression. Ethanol production (closed square) and xylose consumption (open square) profiles were measured in micro-aerobic conditions for S. cerevisiae BY4741-S2A3 (solid line) and S2A3K (dotted line). Ethanol production was increased by xylulokinase overexpression. Total improvement in both ethanol production and xylose consumption rates were nearly 8 folds when the mutant xylose isomerase mutant was co-expressed with heterologous xylulokinase. Error bars represent the standard deviation of biological triplicates.

Figure 7: Structure predictions for xylose isomerase active site in wild-type and mutant (xylA*3). The smaller size of S142 in the xylose isomerase mutant could loosen structural inhibition. In addition, T177 in the xylose isomerase mutant seems to open the active site, thereby allowing xylose to more easily interact with this binding pocket. The mutations near active site of xylose isomerase in predicted three-dimensional structures of mutant (A) and wild type xylose isomerase (B). The mutation sites (S142 and T177) are shown in red and their wild
type counterpart residues are shown in yellow. The F101, H102, and D103 residues, active site residues are shown in blue and sky-blue.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Strain description</th>
<th>Aerobic growth rate (h⁻¹)</th>
<th>Xylose consumption rate (g/g⁻¹ h⁻¹)</th>
<th>Ethanol production rate (g/g⁻¹ h⁻¹)</th>
<th>Ethanol yield (g/g⁻¹)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>BY4741-S1A</td>
<td><em>Piromyces</em> XI expressing strain overexpressing <em>tal1</em> and <em>Agre3</em></td>
<td>0.001</td>
<td>0.007</td>
<td>0.0032</td>
<td>0.46</td>
<td>This study</td>
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<td>BY4741-S1A1</td>
<td><em>Piromyces</em> XI mutant (<em>xylA</em>¹) strain overexpressing <em>tal1</em> and <em>Agre3</em></td>
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<td>0.0082</td>
<td>0.0041</td>
<td>0.50</td>
<td>This study</td>
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<td>BY4741-S1A2</td>
<td><em>Piromyces</em> XI mutant (<em>xylA</em>²) strain overexpressing <em>tal1</em> and <em>Agre3</em></td>
<td>0.003</td>
<td>0.0123</td>
<td>0.0060</td>
<td>0.49</td>
<td>This study</td>
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<tr>
<td>BY4741-S1A3</td>
<td><em>Piromyces</em> XI mutant (<em>xylA</em>³) strain overexpressing <em>tal1</em> and <em>Agre3</em></td>
<td>0.009</td>
<td>0.0126</td>
<td>0.0056</td>
<td>0.45</td>
<td>This study</td>
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<tr>
<td>BY4741-S2A3K</td>
<td><em>Piromyces</em> XI mutant (<em>xylA</em>³) strain overexpressing <em>tal1</em>, <em>XKS1</em> and <em>Agre3</em></td>
<td>0.061</td>
<td>0.057</td>
<td>0.024</td>
<td>0.42</td>
<td>This study</td>
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<td>CEN.PK-RWB 202</td>
<td><em>Piromyces</em> XI expressing strain</td>
<td>0.005</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(19)</td>
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<tr>
<td>CEN.PK-RWB202-AFX</td>
<td>Evolved strain from CEN.PK- RWB 202 for 160 days in xylose medium</td>
<td>0.18</td>
<td>0.340</td>
<td>0.140</td>
<td>0.42</td>
<td>(22)</td>
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<td>CEN.PK-RWB217</td>
<td><em>Piromyces</em> XI expressing strain overexpressing <em>XKS1</em>, <em>TKL1</em>, <em>RPE1</em>, <em>RKI1</em> and <em>Agre3</em></td>
<td>0.22</td>
<td>1.060</td>
<td>0.456</td>
<td>0.43</td>
<td>(20)</td>
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<tr>
<td>CEN.PK-TMB3066</td>
<td><em>Piromyces</em> XI expressing strain overexpressing <em>XKS1</em>, <em>TKL1</em>, <em>RPE1</em>, <em>RKI1</em>, <em>HXT7</em> and <em>Agre3</em></td>
<td>0.02</td>
<td>0.005</td>
<td>0.002</td>
<td>0.43</td>
<td>(16)</td>
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<tr>
<td>Strain</td>
<td>Description</td>
<td>n.a.</td>
<td>0.06</td>
<td>0.007</td>
<td>0.12</td>
<td>(37)</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
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<tr>
<td>BWY10XyI</td>
<td><em>Clostridium</em> XI expressing strain (evolved industrial strain in xylose medium)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.03</td>
<td>0.43</td>
<td>(8)</td>
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<td>CEN.PK2-1C -TMB 3424</td>
<td>XR/XDH expressing strain overexpressing <em>XKS1</em>, <em>GAL2</em>, <em>TKL1</em>, <em>RPE1</em>, <em>RKII</em> and Agre3</td>
<td>n.a.</td>
<td>0.06</td>
<td>0.007</td>
<td>0.12</td>
<td>(37)</td>
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### Table II.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>$V_{\text{max}}$ (µM mg protein$^{-1}$ min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th></th>
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<tbody>
<tr>
<td>xylA</td>
<td>0.053 ± 0.007</td>
<td>86.97 ± 6.56</td>
<td>-</td>
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<tr>
<td>xylA*1</td>
<td>0.064 ± 0.001</td>
<td>89.98 ± 3.93</td>
<td>E129D, V433I</td>
</tr>
<tr>
<td>xylA*2</td>
<td>0.083 ± 0.002</td>
<td>50.18 ± 7.08</td>
<td>E129D, V433I, E15D, E114G</td>
</tr>
<tr>
<td>xylA*3</td>
<td>0.094 ± 0.006</td>
<td>168.39 ± 11.98</td>
<td>E129D, V433I, E15D, E114G, T142S, A177T</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing relative growth rate (µ* / µ₀) for three rounds.]

- 1st round
- 2nd round
- 3rd round

Relative growth rate (µ* / µ₀)

0
5
10
15
20
25
30
35
40

1st round  2nd round  3rd round
Figure 2

![Graph showing relative growth rate (µ/µo)]

- xylA3* with single back mutation
- xylA with site mutation

- xylA3*
- D129E
- I433V
- D15E
- G114E
- S142T
- T177A
- xylA
- E15D
- T142S
- E15D/T142S

Relative growth rate (µ/µo)
Figure 3

A

Ethanol concentration (g/L)

Time (h)

B

Xylose concentration (g/L)

Time (h)
Figure 4

A

B
Figure 6

![Graph showing changes in Ethanol and Xylose concentrations over time.](http://aem.asm.org/)
Figure 7

A              B