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# In-Depth Two-Stage Transcriptional Reprogramming and Evolutionary Engineering of *Saccharomyces cerevisiae* for Efficient Bioethanol Production from Xylose with Acetate

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*Supporting Information*

**ABSTRACT:** In order to achieve rapid xylose utilization in the presence of acetate, improved yeast strains were engineered for higher bioethanol production. First, a six-gene cluster, including *XYL1*/*XYL2*/*XKS1*/*TAL1*/*PYK1*/*MGT05196*, was generated by using an in-depth two-stage (glucose and xylose) transcription reprogramming strategy in an evolutionary adapted strain of CE7, resulting in two improved engineered strains WXY46 and WXY53. Through a combined screening of xylose and glucose stage-specific promoters between tricarboxylic acid (TCA)/HSP and constitutive types, respectively, WXY46 with the constitutive promoters showed a much higher ethanol yield than that of WXY53 with the TCA/HSP promoters. Second, an optimized strain WXY74 was obtained by using more copies of a six-gene cluster, which resulted in a higher ethanol yield of 0.500 g/g total sugars with acetate conditions. At last, simultaneous saccharification and co-fermentation were performed by using the evolved WXY74 strain, which produced 58.4 g/L of ethanol from wheat straw waste and outperformed previous haploid XR-XDH strains.

**KEYWORDS:** *Saccharomyces cerevisiae, two stage transcription reprogramming, xylose, copy number variation, simultaneous saccharification and co-fermentation*

## 1. INTRODUCTION

Escalation in energy consumption, depletion of fossil fuel reserves, and emerging environmental impacts have motivated many researchers to seek sustainable alternative energy resources. One such archetype, bioethanol, is considered the most viable option owing to the abundant availability of the raw material and to its environmentally benign effects.[1] Moreover, compared to gasoline, bioethanol has also some unique properties such as higher octane number, higher heats of vaporization, and higher flame speed.[2] In terms of current production, the United States, Brazil, and China produces about 15 000, 7000, and 875 million gallons/year, respectively.[3] In fact, gasoline mixed with 10% bioethanol will be utilized throughout the country in China by the year 2020. Certainly, there is an urgent need to improve ethanol yield for a green environment and high economic interests.

The process of producing bioethanol from various renewable resources such as corn stover, rice straw, and orange waste[4−6] is considered to have a notably low environmental impact, thereby contributing to a clean environment.[7] *Saccharomyces cerevisiae* is a well-characterized eukaryotic model microorganism, a yeast widely used in the production of second-generation biofuels.[8−10] Native *S. cerevisiae* efficiently uses glucose to produce ethanol; however, it cannot naturally utilize xylose, which is a major component in lignocellulosic hydrolysates and potentially important for ethanol production.[4,11,12] Therefore, manipulation of the xylose utilization pathway in *S. cerevisiae* via metabolic engineering should contribute to increased ethanol yields.[13] Generally, a major limiting factor that impedes *S. cerevisiae* growth and metabolism, as well as its production of ethanol, is acetate. Lignocellulosic hydrolysate contains various inhibitors like acetate which greatly affect the sugar utilization and cell growth.[10,14,15] Therefore, a metabolic engineering strategy is necessary to construct a *S. cerevisiae* strain that is acclimatized to an acetate environment in order to achieve efficient xylose utilization and thus higher ethanol production.

*S. cerevisiae* has two metabolic pathways that utilize xylose: the XR-XDH-XK pathway, which produces xylitol as a byproduct and the XI-XK pathway.[13,16] As a consequence, the resulting xylulose is converted into xylulose 5-phosphate by native yeast *XKS1*. Moreover, *TAL1* is a key gene of nonoxidative pentose phosphate pathway, markedly affecting ethanol metabolism.[17,18] The metabolism of both glucose and xylose produces pyruvate, a substrate of Pyk1. Pyk1 displays a significant level of regulation over both the growth rate and direction of the carbon flux toward ethanol formation in

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Table 1. All Engineered Yeast Strains Used in This Work

| strains | alias/its construct number | genotype descriptions | resource |
|---|---|---|---|
| WXY18 | | MATαura3::PGK1p-XR4m-PGK1t/PGK1p-XDH-PGK1t/ADH1p-XK-XKS1t/ADH1p-RPE1-RPE1t/PGK1p-RKI1-CYC1t/ADH1p-TAL1-ADH1t/KGD1p-TAL1-TAL1t/PGK1p-XYL1-ADH1t/PGK1p-XYL2-PGK1t/PGK1p-XKS1-PGK1t | [10] |
| WXY39 | 1 | WXY18, nat1::tTDH3p-AckA-TPI1t/ADH1p-PTA-PGIt/PGK1p-adhE-PGK1t | this work |
| WXY40 | 2 | WXY18, hygB::PGK1p-ACS2-PGK1t | this work |
| WXY41 | 3 | WXY18, hygB::PGK1p-PMA1-RPS2t/TEF2p-HAA1-tTDH3t | this work |
| WXY42 | 4 | WXY18, nat1::tTDH3p-CimA-TPI1t/ADH1p-LeuCD-PGIt/PGK1p-LeuB-PGK1t | this work |
| CE1 | | evolutionary engineering for WXY18 | this work |
| CE2 | | evolutionary engineering for CE1 | this work |
| CE3-7 | | continuous evolutionary engineering for evolved strain CE (2–6) | this work |
| WXY43-45 | | CE7, his3::PGK1 (tTDH3, DDI2)p-PYK1 | this work |
| WXY46-47 | 1-2Z (5–6) | CE7, hygB::T1-Z1, T2-Z2, T3-Z3, T4-Z4 (KL) | this work |
| WXY48-49 | 1-2YH (7–8) | CE7, hygB::T11, T21, T31, T41 (T42) | this work |
| WXY50-51 | 3-4YH (9–10) | CE7, hygB::T11, T21, T32, T41 (T42) | this work |
| WXY52-53 | 5-6YH (11–12) | CE7, hygB::T11, T22, T31, T41 (T42) | this work |
| WXY54-55 | 7-8YH (13–14) | CE7, hygB::T11, T22, T32, T41 (T42) | this work |
| WXY56-57 | 9-10YH (15–16) | CE7, hygB::T12, T21, T31, T41 (T42) | this work |
| WXY58-59 | 11-12YH (17–18) | CE7, hygB::T12, T21, T32, T41 (T42) | this work |
| WXY60-61 | 13-14YH (19–20) | CE7, hygB::T12, T22, T31, T41 (T42) | this work |
| WXY62-63 | 15-16YH (21–22) | CE7, hygB::T12, T22, T32, T41 (T42) | this work |
| WXY64 | 23 | CE7, hygB::T1-Z1, T2-Z2, T3-Z3, T4-Z4/G418::T11, T22, T31, T42 | this work |
| WXY65 | 24 | CE7, hygB::T1-Z1, T2-Z2, T3-Z3, T4-Z4/G418::T12, T21, T32, T42 | this work |
| WXY66 | 25 | CE7, hygB::T1-Z1, T2-Z2, T3-Z3, T4-Z4/G418::T12, T22, T32, T41 | this work |
| WXY67 | 26 | CE7, hygB::T11, T22, T31, T42/G418::T12, T21, T32, T42 | this work |
| WXY68 | 27 | CE7, hygB::T11, T22, T31, T42/G418::T11, T22, T31, T42 | this work |
| WXY69 | 28 | CE7, hygB::T12, T21, T32, T42/G418::T12, T21, T32, T42 | this work |
| WXY70 | 1Z1Z/29 | CE7, hygB::T1-Z1, T2-Z2, T3-Z3, T4-Z4/G418::T1-Z1, T2-Z2, T3-Z3, T4-Z4 | this work |
| WXY71 | 1Z1Z1Z/30 | WXY70, AbA::T1-Z1, T2-Z2, T3-Z3, T4-Z4 | this work |
| WXY72-73 | delta7, 9/31 | WXY70, ABA::T1-Z1, T2-Z2, T3-Z3, T4-Z4 | this work |
| WXY74-75 | rdna8, 12/31 | WXY70, ABA::T1-Z1, T2-Z2, T3-Z3, T4-Z4 | this work |
| WXY74E | *rDNA-evo* | evolutionary engineering for better WXY74 | this work |

yeast.[52] When *PYK1* is up-regulated, the pyruvate metabolic stream produces ethanol; in the case of downregulation, the pyruvate mostly enters the tricarboxylic acid (TCA) cycle, which favors the production of citric acid.[19−21] A recent study found that citrate inhibits pyruvate kinase; hence, citrate accumulation limits nitrogen-limited glycolytic efflux and ethanol production in yeast.[22] A *MGT05196* transporter is responsible for the transport of xylose without glucose inhibition and hence is a key prerequisite for efficient mixed glucose/xylose metabolism.[23] In addition, some promising transporters may have better xylose transport capacity.[24−26] In fact, our transcriptome analysis showed that the xylose transporter *MGT05196* has improved fermentation efficiency due to its increased expression levels. Therefore, we chose

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these six genes as a whole cluster including basic xylose utilization pathway XR-XDH-XK, two key rate limiting genes *TAL1* and *PYK1*, an efficient transporter *MGT05196* to further xylose metabolism. The number of copies of one or more genes can affect its expression. Gene copy number can differ among individuals because of genetic duplications, giving rise to copy number variations (CNVs), which natural selection can act upon.[27] Copy number is generally positively correlated with expression levels, often producing a beneficial gene dosage effect[28] to yeast fitness although they can disrupt the stoichiometric balance in molecular networks.[29] Therefore, a six gene cluster of CNVs was tested to control gene expression in xylose metabolism.

Furthermore, some yeast metabolic process inhibitors from cellulosic hydrolysate, such as acetate, furaldehydes, and phenolic derivatives were previously studied by transcriptomics analysis.[30−32] For example, deletion of *CAT8* was found to be comparable with the inactivation of 26 TCA cycle genes that direct the metabolic flux to the fermentation state.[33] Comparative transcriptome analysis revealed that three genes encoding plasma membrane carboxylic acid transporters (*ADY2*, *ATO1*, and *YCR010*) improved growth of *S. cerevisiae* with acetate, which ultimately led to increased ethanol production.[34]

Previously, we developed a two-stage transcriptional reprogramming (TSTR) strategy to optimize the expression of key genes at both glucose fermentation stage and xylose fermentation stage. Our experimental results demonstrate the proposed TSTR strategy in yeast by synthetically regulating the xylose assimilation pathway toward efficient xylose fermentation.[13] Here, we apply a deep TSTR strategy to reprogram the expression of six-gene cluster[13] at an optimal level for efficient utilization of both glucose and xylose. In addition, we further adopted four representative promoters including *CIT1*, *ACO1*, *MDH1*, and *PDA1* from a total of 26 TCA promoters compared to that of previous promoter *KGD1* to drive xylose metabolism.

Strains showed increasing bioethanol conversion from glucose and xylose after the in-depth TSTR modification of the six-gene cluster. Furthermore, an evolutionary engineering approach was utilized to improve the acetate resistance of strains in an industrial purpose. The transcriptome analysis of engineered strains was then conducted, which revealed the expression pattern of key genes and the six xylose metabolism genes. This information helps to understand the mechanism of efficient glucose and xylose metabolism by yeast. Finally, some superior industrial strains were obtained after validation by domestication of industrial hydrolysate and simultaneous saccharification and co-fermentation (SSCF) processes in fermenters.[35]

## 2. MATERIALS AND METHODS

**2.1. Strains, Plasmids, Media, and Growth Conditions.** The recombinant haploid yeast *S. cerevisiae* strains and plasmids generated for this study, as well as primer information, are summarized in Tables S1−S3. Plasmid construction is described in the Supporting Information. The strain construction process is also given in the Supporting Information. Yeast cells were routinely cultivated at 30 °C in yeast peptone[36] medium (10 g/L yeast extract and 20 g/L peptone) with 20 g/L of glucose. The yeast nitrogen base (YNB) without amino acids (Difco, Franklin Lakes, NJ, USA) medium and the antibiotic *natMX* or *hphNT1* marker genes were prepared as previously described.[13] The evolved and engineered yeast strains CE7 and WXY46 were deposited in the China General Microbiological Culture Collection Center (CGMCC) (nos. 15567 and 15568, respectively). In addition, we have constructed some engineered yeast strains (Table 1 and Figure S1).

**2.2. Strain Co-Fermentation Using Experiments with Simulated Industrial Mixed-Sugar.** Single colonies from a plate were first cultured at 30 °C and 200 rpm shaking overnight in 100 mL flasks containing 20 mL of YNB medium with an appropriate carbon source and nutritional components as previously described.[13] Next, yeast cells were pelleted by centrifugation at 845 RCF for 5 min at 4 °C, followed by two washes with sterile water, and were then transferred into 250 mL flasks containing 100 mL of synthetic drop-out medium supplemented with simulated corn stover hydrolysate containing 40 g/L xylose, 80 g/L glucose, and 3 g/L acetate with an initial culture $OD_{600}$ of 1.0. Fermentation maintained at pH 5.0 with 1 M NaOH was performed at 30 °C with 150 rpm shaking. All flasks were covered (depending on the growth conditions), and fermentation samples were collected from the cultures at specific time intervals as previously described.[13] All fermentations were carried out in triplicate.

**2.3. Metabolite Analysis and Quantification.** Cell growth was monitored by measuring the $OD_{600}$, and cell dry weight values were determined as previously described.[37] The consumed glucose, xylose, and acetate, as well as xylitol and ethanol production were analyzed by high-performance liquid chromatography (HPLC) with a refractive index detector and an Aminex HPX-87H column.[13] The column and detector temperatures were set at 30 and 50 °C, respectively, and 5 mM $H_2SO_4$ served as the mobile phase at a flow rate of 0.4 mL/min.

**2.4. Transcriptome and Quantitative Reverse Transcription Polymerase Chain Reaction Analyses.** RNA-seq was carried out for transcriptome analysis using strain s288c as a reference. Samples were taken from the batch fermentations, and pellets were formed from them using the procedure previously described.[13] Extracted total RNA samples, enriched mRNA fragments, and the cDNA library were provided to BGI Tech (Shenzhen, China) for sequence analysis using the Illumina HiSeq 2000 platform. The sample library was prepared using QC steps, as previously described.[13] Expression levels were normalized as reads per kilobase of exon region per million mapped read values. The raw data from the transcriptional analysis and the processed gene data are available from the NCBI Gene Expression Omnibus[38] database (accession number PRJNA484057). The expression levels of target genes were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using *ACT1* (Actin1) as a reference gene, as previously described.[11]

**2.5. Evolutionarily Engineered Yeast Strains.** Repeated batch cultivation of yeast strain WXY18 was performed in a simple microaerobic cultivation system; 5 mL 2% YPX cultures were maintained at 30 °C and with shaking at 200 rpm. Serial transfer was performed by alternating cultivation in YPX with acetate; cultures with an initial $OD_{600}$ of 0.2 were incubated with increasing concentrations of acetate, ranging from 3 to 12 g/L. For five days, cultures were inoculated by spinning down 5 mL of the prior culture with acetate, washing the pellet twice with water, and resuspending it in YPX prior to inoculation. At periodic intervals, the repeated cultures were selected for independent evolution in liquid culture to propagate the several fastest-growing colonies. In total, the evolution process lasted approximately four years until satisfactory growth was achieved. At the end of the process, potential clones were re-streaked and tested individually for growth in a xylose medium.

*S. cerevisiae* strain WXY74 was used as the initial haploid strains for adaptive acclimation and fermentation. Wheat straw was pretreated using the dry acid pretreatment method,[39,40] and the pretreated solids were aerobically biodetoxified in a 15 L bioreactor to remove the inhibitors generated during the dry acid pretreatment.[41] The pretreated and biodetoxified lignocellulose feedstock solid wheat straw was saccharified into a liquid hydrolysate slurry over a period of 48 h at 50 °C, pH 4.8, with 15 FPU/g DM of cellulose in specially designed 5 L bioreactors equipped with a helical ribbon impeller, as previously described.[42] Following centrifugation at 9391 RCF for 10 min, the wheat straw hydrolysate was obtained by removing the insoluble solids. Next, the hydrolysate was sterilized at 115 °C for 20 min, filtered with filter paper, and supplemented with 2 g/L $KH_2PO_4$, 2 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4$, and 10 g/L yeast extract. Briefly, the yeast seed and treated wheat straw hydrolysate at 10.0% (v/v) (20 mL) were concurrently fed into the 100 mL shake flask and cultured at 30 °C and 200 rpm for 24 h in a shaker for daily transfer. Samples were taken every 24 h and centrifuged at 15 871 RCF for 5 min, and the supernatant containing glucose, xylose, ethanol, and glycerol was analyzed as previously described.[42]

**2.6. Simultaneous Saccharification and Co-Fermentation.** The seed broth of target *S. cerevisiae* strain WXY74 was prepared using the treated wheat straw hydrolysate. Briefly, the yeast seed and treated wheat straw hydrolysate at 10.0% (v/v) (20 mL) for 24 h transfer were added to a 1 L shake flask containing 200 mL treated wheat straw hydrolysate and then cultured in a shaker at 30 °C and 200 rpm for 24 h. The yeast seed broth was then used for the SSCF step. The pretreated and biodetoxified wheat straw stover material was loaded (30% (w/w) solid loading) into the 5 L bioreactors equipped with a helical ribbon impeller, followed by pre-hydrolysis for 12 h at 50 °C, pH 4.8, once the cellulase was added. The temperature was then reduced to 30 °C, and the *S. cerevisiae* seed broth was inoculated into the 5 L bioreactor at a 10% (v/v) inoculum concentration at pH 5.5 to start the SSCF, which continued for 72 h. The pH was increased to 5.5 by addition of a 5 M NaOH solution. Samples were taken periodically for analysis of glucose, xylose, ethanol, and glycerol by HPLC as described in the previous section.

## 3. RESULTS AND DISCUSSION

**3.1. Creation of Acetate-Tolerant Yeast Strains Based on Evolutionary and Metabolic Engineering.** We previously constructed WXY18, a yeast strain with an increased rate of xylose consumption, to solve the problem of cellulosic hydrolysate's severe hindrance of yeast growth and intracellular mixed sugar fermentation. However, WXY18 is susceptible to inhibition by acetate and produces less ethanol from a mixture of 50 g/L glucose, 50 g/L xylose, and 3 g/L acetate than under the same conditions without acetate (Figure S2), a finding consistent with a previous report.[10] To improve acetate consumption during mixed sugar fermentation, we followed a reported modification[43] to WXY18 and observed no improvement in ethanol production (data not shown). Then, we constructed four yeast strains, WXY39−WXY42, expressing various acetate utilization pathway genes in WXY18 (Figure S3) and displaying either low or rapid xylose consumption (Figure S4A−E). Strain WXY39 and WXY40 had lower/higher efficiency for co-consumption of xylose and acetate than strain WXY18[43] (Figure S4A−C). WXY41 and WXY42 consumed more xylose and acetate, respectively and had 21% higher ethanol yield than WXY18 (Figure S4A,D,E).[44]

Several reports have described increased tolerance of *S. cerevisiae* strains to fermentation inhibitors acquired through long-term adaptation and directed evolution.[14,45] This ability to create synergy between rational metabolic and laboratory evolutionary engineering by appropriate tailoring of the experimental setup is essential for harnessing natural selection to fine-tune yeast metabolism. Our evolutionary engineering approach took over four years and resulted in the isolation of acetate-adapted strains CE1−CE7 derived from WXY18 with gradual improvement in comparative fermentations. Figure 1A shows fermentation profiles of strains CE1−CE5 under a mixture of 50 g/L glucose, 50 g/L xylose, and 3 g/L acetate expressed as the residual xylose and acetate and the produced ethanol, among which CE5 produced the highest ethanol yield. Figure 1B shows fermentation profiles of strains CE5−CE7 in simulated industrial mixed-sugar conditions containing 80 g/L glucose, 40 g/L xylose, and 3 g/L acetate. CE7 cells consumed more xylose and produced more ethanol than CE5 in the presence of 3 g/L acetate under mixed sugar conditions and were able to produce 51.6 g/L ethanol, reaching an ethanol yield of 0.434 g/g total sugar within 48 h.

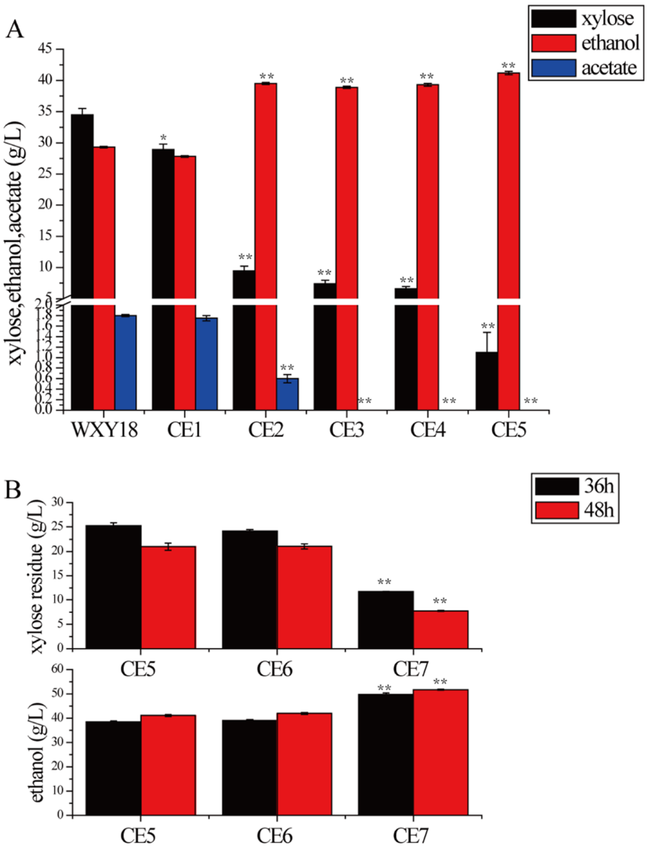

**Figure 1.** (A) Fermentation profiles (ethanol production yields and residual xylose and acetate) of control strain WXY18 and evolved strains CE1−CE5 at 48 h. Fermentation was performed in YNB medium with 50 g/L glucose, 50 g/L xylose, and 3 g/L acetate. (B) Fermentation profiles (ethanol production yields and residual xylose) of control strain CE5 and evolved strains CE6 and CE7 in YNB medium with 80 g/L glucose, 40 g/L xylose, and 3 g/L acetate. The error bars in all figures represent the standard deviations of the biological triplicates. (**$P < 0.01$; *$P < 0.05$.)

**3.2. Optimizing Expression of Six Xylose Metabolism Genes To Achieve Efficient Xylose Consumption.** In order to promote metabolic efficiency in mixed sugars with acetate, three engineered strains WXY43−WXY45 were derived from CE7, applying TSTR and CNV strategies. These strains showed improved xylose consumption, probably owing to the appropriate transcription level of *PYK1* driven by the constitutive promoters *PGK1* and *TDH3* or a new inducible promoter, *DDI2*, using 10 mmol/L cyanamide.[46] In addition, the *DDI2* promoter was preferred over the *PGK1* and *TDH3* promoters. The best of the three strains, WXY45, reached approximately a productivity of 0.45 g ethanol/g total sugar (Figure S5).

Next, we investigated combined genetic strategies to improve xylose utilization. We constructed 12 expression vectors using the Golden Gate Assembly to optimize xylose metabolism including a balanced expression of *XYL1/XYL2/XKS1/TAL1* for the xylose utilization pathway, overexpression of newly introduced sugar transporter *MGT05196*,[23] and the key gene *PYK1* as an output in glycolysis driven by candidate

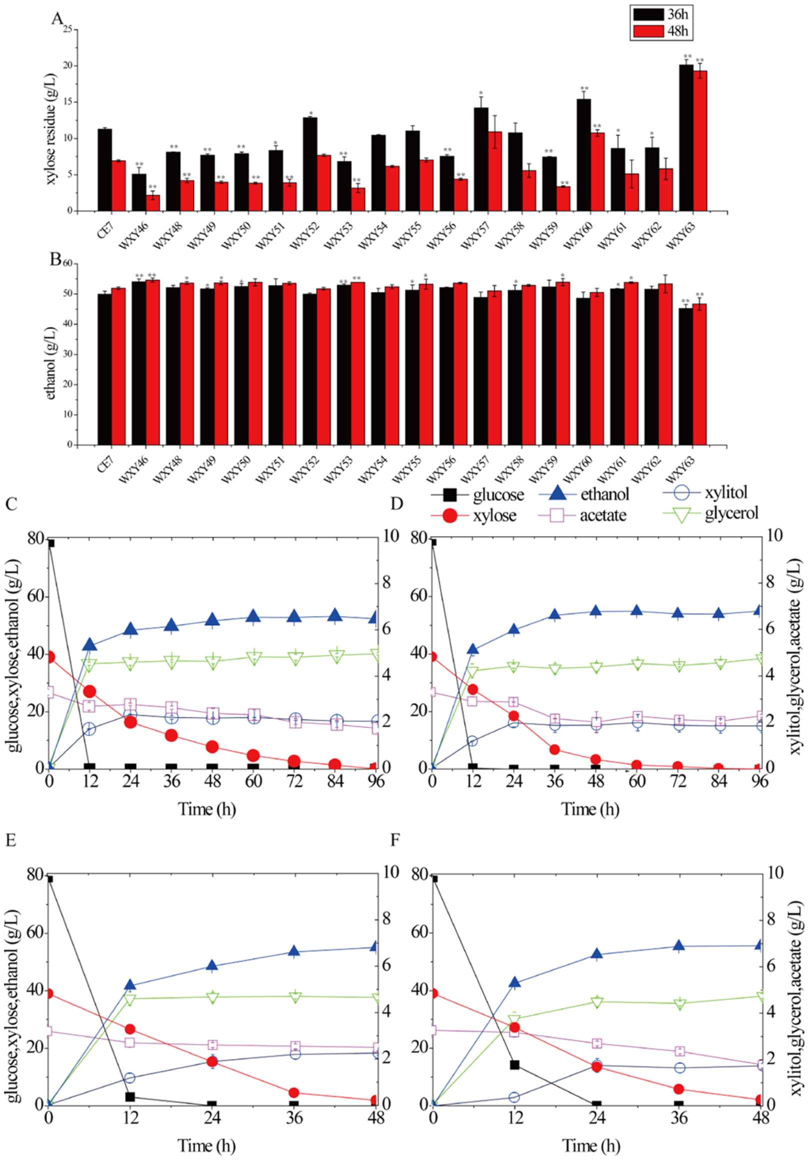

**Figure 2.** Comparison of fermentation profiles for 18 strains, including control strain CE7, WXY46, and WXY48−WXY63, for (A) residual xylose and (B) ethanol production, at 36 and 48 h in YNB medium with 79.3 g/L glucose, 38.9 g/L xylose, and 3.2 g/L acetate. Comparison of fermentation profiles for (C) CE7 and (D) WXY53 at 96 h, and (E) WXY46 and (F) WXY70 at 48 h, in YNB medium with 79.3 g/L glucose, 39.0 g/L xylose, and 3.2 g/L acetate. (**$P < 0.01$; *$P < 0.05$.)

constitutive promoters (glucose stage) and inducible promoters (xylose stage), such as TCA-type (environmental $O_2$ response) and HSP-type (environmental temperature response) promoters (Figure S6). From the different promoter−gene combinations designed, we obtained 17 strains: WXY46, containing six genes driven by constitutive promoters, and WXY48−WXY63, in which six genes were driven by inducible promoters from the control strain CE7 (Tables 1 and S2).

These 18 differentially engineered strains along with CE7 were used for fermentation for 48 h under industrial fermentation conditions simulating those for hydrolyzing corn stalk (Figure 2A−D). The fermentation results showed that the candidate strains WXY46, WXY53, WXY59, WXY61, and WXY62 produced higher ethanol yields than CE7 and the other 12 engineered strains. WXY46 produced the highest ethanol yield, 55.1 g/L, at 48 h, indicating that under simulated industrial fermentation conditions, the six xylose metabolism genes *XYL1*, *XYL2*, *XKS1*, *TAL1*, double *MGT05196*, and *PYK1* driven by constitutive promoters such as *PGK1* and *ADH1* are more efficient than genes driven by inducible promoters from *HSP12*, *HSP26*, *ACO1*, and *PDA1*. Higher expression levels for the xylose metabolism driven by constitutive strong promoters may be very necessary during the inhibitor effects from acetate stress. In contrast, inducible promoters may contribute a more balanced xylose metabolism under same conditions. This is not consistent with our previous report under only a mixed sugar metabolism, suggesting that the TSTR strategy is variously balanced with different fermentation conditions. Four strains—CE7, WXY46, WXY53, and WXY59—were subjected to the same fermentation conditions except without acetate, over 96 h (Figure S7). They consumed glucose completely within 12 h and most of the xylose within 24 h, and produced up to 51.6 g/L ethanol; this indicates that the acetate response affects key players during xylose fermentation. We found that the transcription

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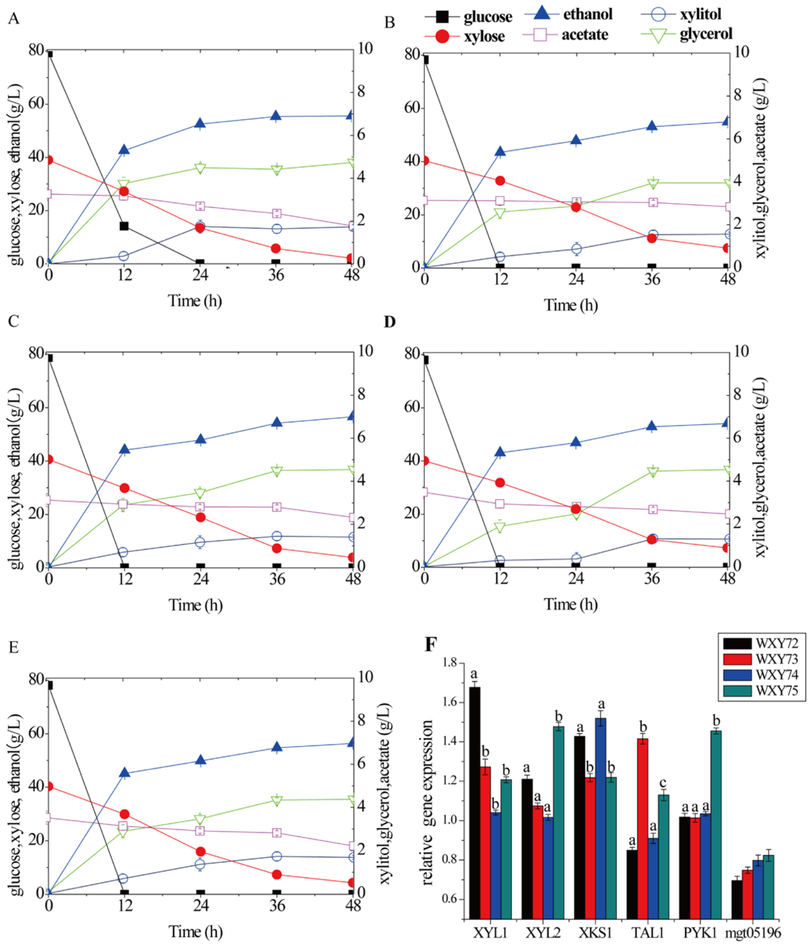

**Figure 3.** Comparison of fermentation profiles for (A) WXY70, (B) WXY72, (C) WXY73, (D) WXY74, and (E) WXY75 at 48 h in YNB medium with 80 g/L glucose, 40 g/L xylose, and 3 g/L acetate. (F) Comparison of transcription levels of six key genes, *XYL1*, *XYL2*, *XKS1*, *TAL1*, *PYK1*, and *MGT05196*, in control strain WXY70 and strains WXY72–WXY75 as measured by qRT-PCR. (Markers a, b, and c show different groups with statistical significance; $P < 0.05$ between groups.)

level of *Kluyveromyces lactis PYK1* is higher than that of *S. cerevisiae PYK1* in vitro (data not shown). However, strain WXY47, expressing heterologous *K. lactis PYK1* (Table 1), displayed fermentation capabilities only comparable to those of WXY46 with *S. cerevisiae PYK1* (Figure S8).

To further improve fermentation efficiency, we transformed additional expression clusters using antibiotic G418 for selection into the above-mentioned improved strains, and thereby obtained new engineered strains WXY64–WXY70 (Table 1). Under simulated industrial fermentation conditions, strains WXY46 and WXY70 achieved ethanol yields of 0.467 and 0.470 g/g total sugar, respectively (Figure 2E,F). These fermentation results showed that in WXY70, an additional copy of the six-gene cluster markedly inhibited glucose metabolism at 12 h and subsequently improved xylose consumption and slightly increased the ethanol yield over that of WXY46. Similarly, we transformed a second additional six-gene cluster using the antibiotic ABA into WXY70 to yield new engineered strain WXY71 containing three six-gene clusters with the aim of obtaining superior capability. Under simulated industrial fermentation conditions, strains WXY70 and WXY71 consistently achieved ethanol yields of 0.469 and 0.468 g/g total sugar, respectively.

### 3.3. Increasing CNVs of Expression Clusters To Maximize Xylose Utilization, and Analyzing Relative Transcriptome Profiles.

To investigate whether an optimized number of six-gene clusters can improve xylose metabolism, we attempted to increase six-gene CNVs in WXY70 by random integration into multiple copies of rDNA sites or Delta DNA.[47] Eight positive clones were obtained: Delta6, Delta7 (WXY72), Delta8, Delta9 (WXY73), rDNA8 (WXY74), rDNA9, rDNA10, and rDNA12 (WXY75). As shown in Figures 3A–E and S9, among these eight strains, WXY74 and WXY73 showed the highest and lowest ethanol yield, 0.500 and 0.461 g/g total sugars, respectively, and WXY73 showed the strongest xylose utilization capability. We analyzed the transcription levels of six key genes in four strains (with WXY70 as the control strain) by qRT-PCR to explore the underlying mechanism(s) of the increased fermentation performance and gene expression cluster CNVs.

As shown in Figure 3F, the strain with the highest ethanol yield, WXY74, had high expression levels of *XYL1* and *XKS1*, which could explain its high ethanol yield of 0.500 g/g total sugars. We predicted that unbalanced expression of *XYL1*, *XYL2*, and *XKS1* would cause unbalanced xylose metabolism and further accumulation of byproducts. Additionally, high expression levels of *TAL1* or *PYK1* could promote more xylose or mixed sugars toward the ethanol metabolic pathway, respectively.

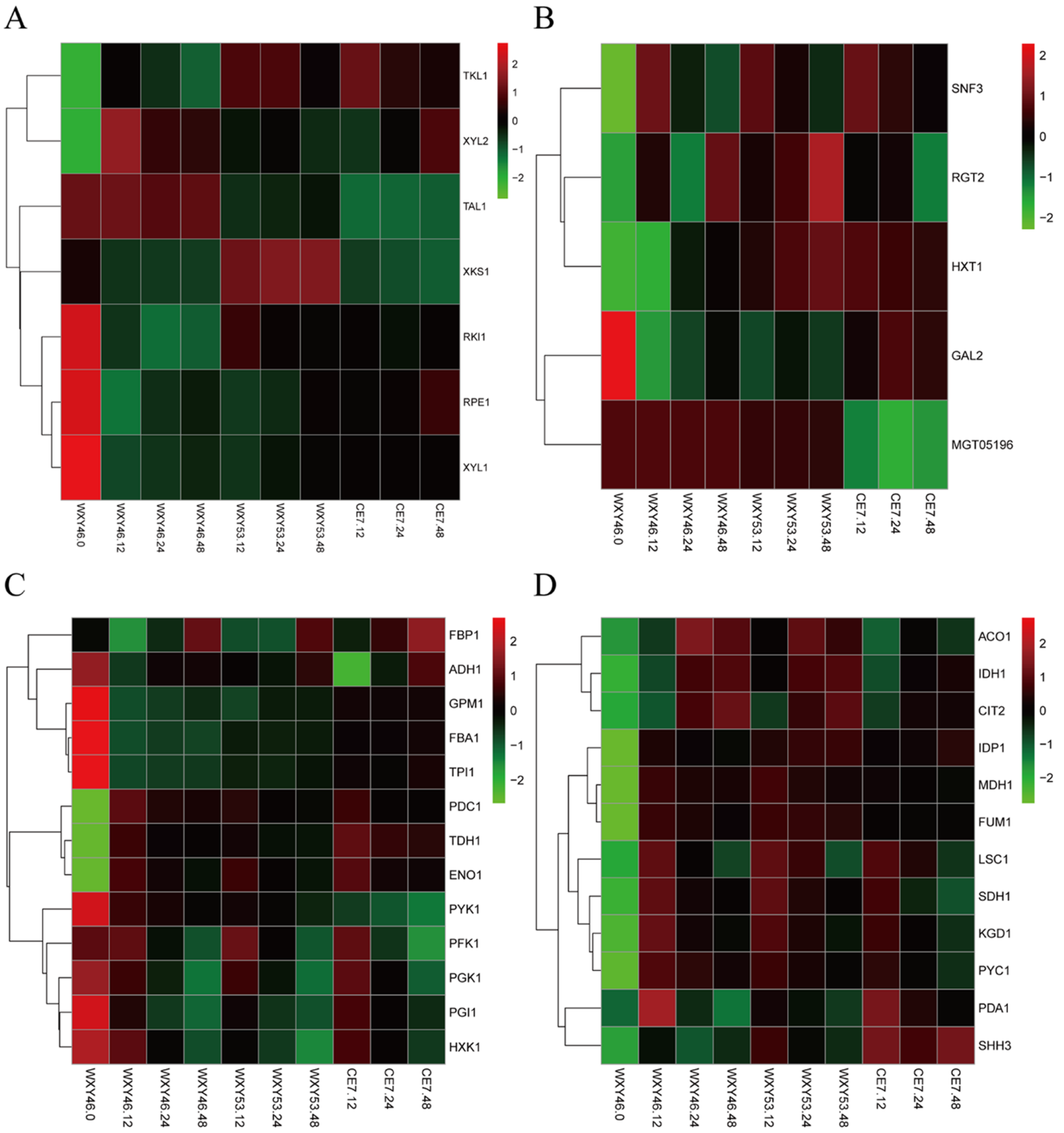

**Figure 4.** (A,B) Hierarchical clustering of the xylose utilization pathway and transporter time course expression profiles at 0 h (WXY46 only), 12, 24, and 48 h for strains CE7, WXY46, and WXY53. (C,D) Hierarchical clustering of the main glycolysis and TCA cycle time course expression profiles at 0 h (WXY46 only), 12, 24, and 48 h for strains CE7, WXY46, and WXY53.

To provide insight into the genes differentially expressed in strains WXY46 and WXY53, which result in fermentation capabilities vastly different from those of CE7, we profiled their transcriptome by RNA-seq. The expression levels of the main known genes, *TAL1*, *PYK1*, and *MGT05196*, increased when the strains exhibited a gradual increase in xylose fermentability (WXY53 is better than CE7, and WXY46 is the best of the three) (Figure 4A−C), suggesting that they play an important role in the regulation of xylose metabolism. WXY53 produced more ethanol than CE7, suggesting that our proposed combined TSTR promoters such as TCA promoters like *ACO1* and *PDA1* and HSP promoters like *HSP12* and *HSP26*, including high and low expression levels, could effectively drive xylose pathway genes. In all strains, the expression levels of *CIT2* were higher at 36 and 48 h than at 12 h (Figure 4D). These results are consistent with our previous TSTR results and other reports,[13,48] which indicate that an efficient xylose phase, via optimization of *CIT2* expression levels, requires the energy provided by the respiration pathway and a balanced metabolic flux toward the increased ethanol-fermentation pathway. Figure S10 shows a circle diagram of the RNA-seq results for the whole-genome sequencing of WXY46 and the comparative transcriptome analysis of strains CE7, WXY46, and WXY53.

The transcription levels of six genes of *PYK1*, *TAL1*, *XYL1*, *XYL2*, *XKS1*, and *MGT05196* of WXY70 showed a decreasing trend from 12, 24 to 48 h. Compared to WXY70, WXY74 has higher transcription levels of *TAL1*, *XYL1*, *XYL2*, and *MGT05196* at 12 h. The transcription levels of the six genes of WXY74 were slightly higher at 48 h than at 24 h, and such fluctuations were relatively stable; the transcription level of the six genes of WXY72 remained stable within 24−48 h, rather than the continuous decrease of WXY70 (Figure 5). This indicates that strains with a higher and stable expression level of multicopies of six-gene clusters have more efficient xylose utilization and ethanol production.

### 3.4. SSCF of Wheat Straw Hydrolysate for Co-Utilization of Xylose and Glucose Using Evolutionary

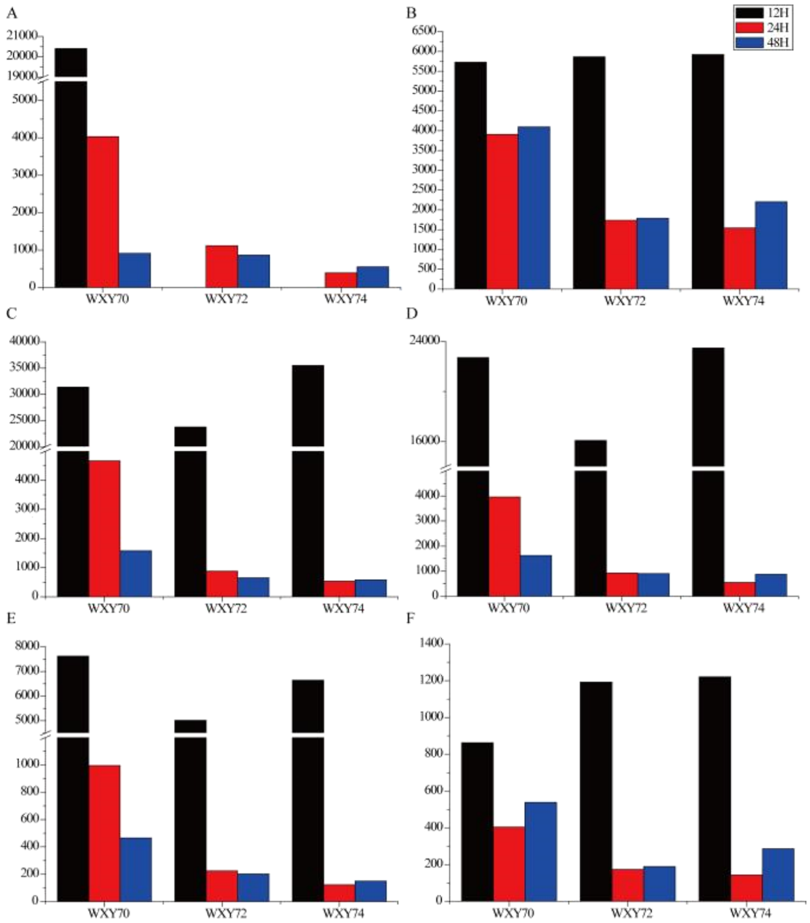

**Figure 5.** Transcriptome data for six metabolic genes. (A) *PYK1*, (B) *TAL1*, (C) *XYL1*, (D) *XYL2*, (E) *XKS1*, and (F) *MGT05196*. The ethanol yield of WXY72 and WXY74 was 0.499 and 0.500 g/g total sugar, respectively. WXY74E produced 58.4 g/L of ethanol by SSCF.

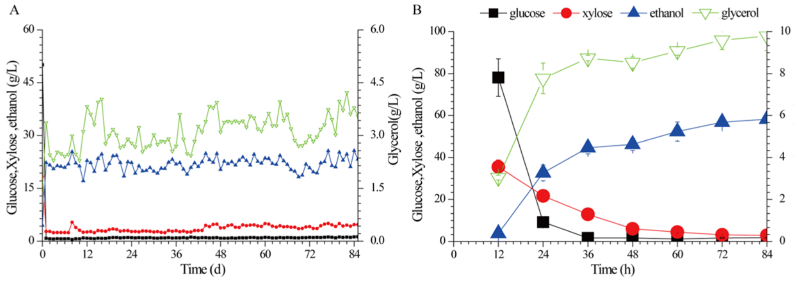

**Figure 6.** Glucose concentrations of the yeast seed mixtures WXY74E and treated wheat straw hydrolysate at 10.0% (v/v) was 50.50 g/L. Evolutionary engineering in hydrolysate at a solid loading of a 15% wheat straw stover for target improved strain (A) WXY74E (*rDNA-evo*). Time-dependent ethanol profiles of SSCF for strain (B) WXY74E.

**and Metabolic Engineered Strains of *S. cerevisiae*.** Currently, lignocellulose is used to produce bio-based chemicals, such as ethanol, by anaerobic fermentation. Because of the complexity of the components of the wheat straw hydrolysate, inhibitor accumulation occurs during the pretreatment step. Although some inhibitors can be removed after detoxification, the remaining inhibitors still negatively affect cell growth during fermentation. An adaptive domestication strategy is commonly used to allow target strains to adapt to the industrial hydrolysate fermentation environment. As shown in Figure 6A,B, glucose concentrations of the yeast seed mixtures WXY74E and treated wheat straw hydrolysate at 10.0% (v/v) was 50.50 g/L; the initial xylose concentration was 19.73 g/L; the initial ethanol concentration was 4.93 g/L; and the initial glycerol concentration was 1.22 g/L. Commonly used measures of the target strain using 15% solid-loading wheat straw hydrolysate reflect the utilization of glucose and xylose as well as ethanol and glycerol production. The strain could utilize 15% wheat straw stover hydrolysate to produce ethanol directly (Figure 6A). During the initial transfer process, the three strains could not stably utilize 15% wheat straw stover hydrolysate to enrich ethanol; however, with adaptive evolution, glucose and xylose release, and the increase in ethanol and glycerol, production remained within a stable range. This indicates that the strain had adapted to the 15% solid wheat straw detoxified hydrolysate environment and that SSCF could be carried out to further evaluate this strain.

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Following evolutionary engineering, the evolved new strain WXY74E (*rDNA-evo*) was evaluated by SSCF. In the prehydrolysis stage, glucose increased with the saccharification time, using the dose of cellulose with 9.71% (W/W) in fermentation medium, whereas xylose was constant with saccharification time because most of the xylan was already converted to xylose and oligoxylan in the pretreatment step. Glucose and xylose levels were approximately 80 and 35 g/L, respectively, with a saccharification time of 12 h and an optimum saccharification temperature of cellulose at 50 °C. During the subsequent SSCF stage with the three strains, the initial glucose was rapidly converted into ethanol within 24 h, and then glucose from cellulose hydrolysis began to be utilized. Xylose conversion gradually decreased with fermentation time. Finally, at a cellulose dose of 15 mg total protein/g cellulose, the strain produced 58.4 g/L of ethanol with an ethanol yield of 65.1% and achieved a xylose conversion rate of 91.4%, respectively (Figure 6). These results demonstrate that improved WXY74E produced 58.4 g/L of ethanol with 65.1% of the theoretical ethanol yield, which is higher than that previously reported for haploid XR-XDH strains by SSCF (Table S1). Approximately half of the ethanol was produced from the initial glucose released during prehydrolysis, and the other half was produced from xylose and glucose released during SSCF.[49] This adapted *S. cerevisiae* strain, WXY74E, was evaluated by SSCF and exhibited satisfactory fermentation capacity.

In summary, metabolic and evolutionary engineering was performed in acetate-tolerant *S. cerevisiae* for improving ethanol yield via rapid xylose utilization from wheat straw waste. A six-gene expression cluster was generated using our developed TSTR strategy and optimized acetate metabolic pathway via the expression of key genes. Compared with other improved engineered yeast strains with higher xylose utilization efficiencies, our resulting optimized engineered strains have the advantage of four years of long-term domestication with good acetate tolerance and higher ethanol yields. The pretreatment of lignocellulosic materials like corn waste often releases main compounds such as acetic acid; good fermentation productivity using lignocellulosic hydrolysate can also be achieved through evolutionary engineering. Long-term adaptation of the yeast to stimulated lignocellulosic hydrolysates has been shown to improve its acetate tolerance, and thus its performance in (stimulated) lignocellulose fermentation. The best reported industrial yeast strains in other labs containing the xylose utilization pathway can consume both glucose and xylose with an ethanol yield of 0.46 or 0.47 g/g.[50,51] After employing transcriptome analysis, the six-gene expression cluster was optimized for obtaining improved ethanol yields from mixed sugars consisting of 80 g/L glucose and 40 g/L xylose with the presence of 3 g/L acetate in the medium, which was close to 99% of the theoretical yield. Additionally, the genetically engineered industrial strain demonstrated efficacious xylose utilization and achieved an ethanol titer of 58.4 g/L by SSCF. In short, our proposed target strain WXY74 has a higher sugar to ethanol conversion rate as compared to other reports, indicating that it would be a top candidate for industrial applications, and it opens a new gateway for efficient bioethanol production.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b05095.

> Construction process of the relevant plasmids and the sources of the different numbered engineered strains, fermentation data for some engineered strains and an outline map of the relationship between the constructed strains, and name of the plasmid and the primer used (PDF)

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### Notes

The authors declare no competing financial interest.

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