Improving Acetic Acid and Furfural Resistance of Xylose-Fermenting *Saccharomyces cerevisiae* Strains by Regulating Novel Transcription Factors Revealed via Comparative Transcriptomic Analysis

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ABSTRACT Acetic acid and furfural are the two prevalent inhibitors coexisting with glucose and xylose in lignocellulosic hydrolysate. The transcriptional regulations of *Saccharomyces cerevisiae* in response to acetic acid (Aa), furfural (Fur), and the mixture of acetic acid and furfural (Aa_Fur) were revealed during mixed glucose and xylose fermentation. Carbohydrate metabolism pathways were significantly enriched in response to Aa, while pathways of xenobiotic biodegradation and metabolism were significantly enriched in response to Fur. In addition to these pathways, other pathways were activated in response to Aa_Fur, i.e., cofactor and vitamin metabolism and lipid metabolism. Overexpression of Haa1p or Tye7p improved xylose consumption rates by nearly 50%, while the ethanol yield was enhanced by nearly 8% under acetic acid and furfural stress conditions. Co-overexpression of Haa1p and Tye7p resulted in a 59% increase in xylose consumption rate and a 12% increase in ethanol yield, revealing the beneficial effects of Haa1p and Tye7p on improving the tolerance of yeast to mixed acetic acid and furfural.

IMPORTANCE Inhibitor tolerance is essential for *S. cerevisiae* when fermenting lignocellulosic hydrolysate with various inhibitors, including weak acids, furans, and phenols. The details regarding how xylose-fermenting *S. cerevisiae* strains respond to multiple inhibitors during fermenting mixed glucose and xylose are still unknown. This study revealed the transcriptional regulation mechanism of an industrial xylose-fermenting *S. cerevisiae* strain in response to acetic acid and furfural. The transcription factor Haa1p was found to be involved in both acetic acid and furfural tolerance. In addition to Haa1p, four other transcription factors, Hap4p, Yox1p, Tye7p, and Mga1p, were identified as able to improve the resistance of yeast to these two inhibitors. This study underscores the feasibility of uncovering effective transcription factors for constructing robust strains for lignocellulosic bioethanol production.

KEYWORDS transcriptome, *Saccharomyces cerevisiae*, mixed sugars fermentation, multiple inhibitors, transcription factors

Lignocellulose biomass has high cellulose and hemicellulose content. Fermenting it into bioethanol is an effective way to cope with resource and energy crises (1). Pretreatment and hydrolysis are needed to break down the structure of lignocellulosic biomass and release the fermentable sugars (mainly glucose and xylose) before it can be fermented by *Saccharomyces cerevisiae* (2, 3). However, various inhibitors (weak acids, furans, and phenols) are generated during pretreatment and hydrolysis (4–6). By integrating the xylose metabolic pathway, *S. cerevisiae* could be endowed with xylose fermentation capacity (7, 8). These xylose-fermenting strains should have strong tolerance to inhibitors; otherwise, they cannot be applied to industrial lignocellulose

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bioethanol production. Manipulating inhibitor tolerance-related genes or metabolic pathways that could be mined via investigating the molecular response to inhibitors is an effective strategy to improve the inhibitor tolerance of those strains.

To date, studies on the response of xylose-fermenting strains to lignocellulosic hydrolytic inhibitors are limited compared with those of traditional *S. cerevisiae* strains that can ferment only glucose. Several researchers have reported that the transcriptional profiles of *S. cerevisiae* had larger differences when fermenting different sugars (glucose, xylose, or mixed glucose and xylose) in the presence or absence of specific inhibitors (9–13). Therefore, it is of great significance to reveal the response mechanisms and further to improve the tolerance of xylose-fermenting *S. cerevisiae* strains to multiple inhibitors when mixed sugars are fermented, as glucose, xylose, and different inhibitors coexist in lignocellulose hydrolysates.

Acetic acid and furfural are two typical inhibitors in lignocellulosic hydrolysates (14, 15), and they suppress the cell growth rate, sugar consumption rate, ethanol yield, and ATP production of *S. cerevisiae* when fermenting glucose (16, 17). The pathways involved in carbohydrate metabolism, amino acid metabolism, signal transduction, and material transport were affected by acetic acid (11, 18, 19), while those involved in aldehyde reduction, pentose phosphate pathway, transcriptional and translational control, or stress responses were affected by furfural (19–22). There were reports on the response of *S. cerevisiae* to mixed acetic acid and furfural; however, these studies used glucose but not mixed glucose and xylose as a fermenting sugar (16).

Since global gene expression of *S. cerevisiae* would be reprogrammed under inhibitor stress, it is difficult to improve the inhibitor tolerance by regulating only one or limited numbers of genes. Transcription factors (TFs), which regulate the expression of a series of genes, are considered to be more effective for inhibitor stress improvement. To date, the inhibitor tolerance of some *S. cerevisiae* strains when fermenting glucose was improved by overexpression of some TFs, such as Haa1p (23), Sfp1p, and Ace2p (16) for acetic acid tolerance, and Sfp1p, Ace2p (16), Msn2p (24), and Yap1p (25) for furfural tolerance. It is necessary to mine effective TFs for constructing inhibitor-tolerant strains for mixed glucose and xylose fermentation.

In our previous study, a flocculating industrial *S. cerevisiae* strain s6 with efficient glucose and xylose fermentation capacity was constructed (26). In the present study, as shown in Fig. S1 in the supplemental material, the response mechanisms of strain s6 to acetic acid, furfural, and their mixture when fermenting mixed glucose and xylose were studied by comparative transcriptome analysis. Potential TFs, which may be related to the tolerance of acetic acid and furfural, were screened out. Five of them were overexpressed using the CRISPR/Cas9 gene engineering method (27–30), and their contribution to the tolerance of acetic acid and furfural were evaluated. The results of the present study contributed to the understanding of the response mechanisms of *S. cerevisiae* to hydrolysate inhibitors, and tolerance-related TFs could be applied to the construction of robust strains for lignocellulosic bioethanol production.

RESULTS

Batch fermentation results of strain s6 under different stress conditions. The effects of different concentrations of acetic acid (Aa; 2.4, 3.6, 4.8, and 6.0 g/liter) and furfural (Fur; 1.0, 1.9, 3.9, and 5.8 g/liter) on the fermentation of *S. cerevisiae* s6 were studied using 10% YPDX medium (10 g/liter yeast extract, 20 g/liter peptone, 60 g/liter glucose, and 40 g/liter xylose [pH 5]). The range of concentration of acetic acid and furfural was based on reported data and the robustness of strain s6. As shown in Fig. S2 in the supplemental material, under Aa stress, the glucose consumption was not inhibited, while the xylose consumption was significantly inhibited. The residual xylose concentration was approximately 14.94 g/liter after 24 h of fermentation under the condition with 2.4 g/liter Aa. Under Fur stress, both glucose consumption and xylose consumption were inhibited. The residual xylose concentration was approximately 8.65 g/liter after 24 h of fermentation under the condition with 1.9 g/liter Fur.

FIG 1 Cell growth (A) and glucose, xylose, and ethanol concentrations (B) of *S. cerevisiae* strain s6 during batch fermentation with mixed glucose and xylose under acetic acid (Aa; 2.4 g/liter), furfural (Fur; 1.9 g/liter), and mixed acetic acid and furfural (Aa_Fur; 2.4/1.9 g/liter) stress conditions. Black squares, control without inhibitor; red circles, Aa; blue upwards triangles, Fur; olive green downwards triangles, Aa_Fur.

Based on these results, the effect of mixed acetic acid and furfural (Aa_Fur; 2.4/1.9 g/liter) on the fermentation of strain s6 was further studied (Fig. 1). The groups with only Aa (2.4 g/liter) and only Fur (1.9 g/liter) were considered the comparisons; the group without inhibitor was considered the control (C). The glucose was depleted in the first 4 h of fermentation under all conditions. The cell growth rate and xylose consumption rate decreased under Aa_Fur stress compared with those in the comparison and control groups. The residual xylose concentration was 19.35 g/liter after 24 h fermentation in the Aa_Fur group, which was 29.52% higher than that under Aa stress and 123.70% higher than that under Fur stress (t test; $P < 0.001$). In all inhibition groups, the ethanol concentrations at 24 h were lower than that of the control, while the ethanol yields based on consumed sugar were higher than that of the control group (t test; $P < 0.05$) (see Table S1 in the supplemental material).

Transcriptome profile of *S. cerevisiae* s6 under different stress conditions.

Considering the carbon catabolite repression effect (31) and xylose consumption rate, cell samples collected at 6 h (xylose fermentation phase) were subjected to RNA extraction and transcriptome sequencing. In total, 6,440 gene transcripts were identified from alignment with *S. cerevisiae* S288C (see Fig. S3 and Table S2 in the supplemental material). The transcription levels of *ADY2*, *ATO2*, *BTN2*, *ENO1*, *ENO2*, and *HSP30* in all 12 samples were analyzed by reverse transcription and quantitative real-time PCR (RT-qPCR), and the results were consistent with the results of transcriptome analysis, suggesting that the transcriptomic results were reliable (see Fig. S4 in the supplemental material).

FIG 2 Radar map of the significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in Aa versus C, Fur versus C, and Aa_Fur versus C groups. The radar map (A) was based on the *P* value of each KEGG pathway (statistical significance at a *P* value of <0.05), and the radar map (B) was based on the enrichment ratio of each KEGG pathway. The values in the middle of radar maps A and B represent the *P* value (decreased by 0.1 from the center [0.7] to the outside [−0.1]) and the enrichment ratio (increased by 0.1 from the center [−0.1] to the outside [0.7]) of each KEGG pathway, respectively.

The gene expression profiles of the three inhibition groups (Aa, Fur, and Aa_Fur) were respectively compared with that of the control group (C). The numbers of differentially expressed genes (DEGs) were 308, 249, and 521 under Aa, Fur, and Aa_Fur stress, respectively. The upregulated DEGs were 177, 218, and 314, and the downregulated DEGs were 131, 31, and 207 under Aa, Fur, and Aa_Fur stress, respectively. The number of DEGs in the Aa_Fur group was 69.16% more than that in the Aa group, and 109.24% more than that in the Fur group, indicating that the dual inhibitors had more pleiotropic effects at the transcriptional level.

KEGG enrichment analysis for total DEGs in each group. As shown in Fig. 2, 11, 16, and 16 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were significantly enriched in Aa versus C (308 DEGs), Fur versus C (249), and Aa_Fur versus C (521) groups, respectively ($P < 0.05$). There were 25 specific pathways when pathways from these three groups were combined into a whole (Fig. 2A). These specific pathways could be classified as carbohydrate metabolism, energy metabolism, amino acid metabolism, xenobiotic biodegradation and metabolism, cofactor and vitamin metabolism, signal transduction, biosynthesis of other secondary metabolites, and lipid metabolism. The common pathways significantly enriched in three inhibition groups indicated that carbohydrate metabolism (see Fig. S5 in the supplemental material), amino acid

metabolism (see Fig. S6 in the supplemental material), and xenobiotic biodegradation and metabolism played important roles in response to acetic acid and/or furfural. Aside from these common pathways, amino sugar and nucleotide sugar metabolism, inositol phosphate metabolism, and pentose and glucuronate interconversions were significantly enriched in response to Aa. Tyrosine metabolism, degradation of aromatic compounds, naphthalene degradation, metabolism of xenobiotics by cytochrome P450, drug metabolism—cytochrome P450, and retinol metabolism were significantly enriched in response to Fur. Butanoate metabolism, bisphenol degradation, thiamine metabolism, and linoleic acid metabolism were significantly enriched in response to Aa_Fur.

Under Aa_Fur stress, the enrichment ratios of these 25 specific pathways were higher than those under Aa or Fur stress (Fig. 2B; see also Table S3 in the supplemental material), except tyrosine metabolism, degradation of aromatic compounds, naphthalene degradation, metabolism of xenobiotics by cytochrome P450, and drug metabolism—cytochrome P450. These exceptional pathways were classified as xenobiotic biodegradation and metabolism and were significantly enriched under Fur stress. Combined with the *P* value of each pathway, it could be concluded that more DEGs were involved in carbohydrate metabolism in response to Aa, whereas more DEGs were involved in xenobiotic biodegradation and metabolism in response to Fur. Besides those DEGs involved in carbohydrate metabolism and xenobiotic biodegradation and metabolism, more DEGs participating in cofactor and vitamin metabolism and lipid metabolism were revealed in response to Aa_Fur. These results indicated that strain s6 adopted distinct regulatory mechanisms to reprogram the cell metabolism in response to Aa, Fur, and Aa_Fur. Moreover, under Aa_Fur stress, the response of s6 was more complicated than that under Aa or Fur stress.

KEGG enrichment analysis for the DEGs shared with inhibitors and exclusive in Aa_Fur versus C groups. The distribution of the DEGs in each group was revealed by Venn diagram (Fig. 3A and C). For the DEGs that were shared under two and three stress conditions, the 215 (63, 104, and 48) co-upregulated DEGs were mainly involved in carbohydrate metabolism and in cofactor and vitamin metabolism (Fig. 3B). The 101 (88, 6, and 7) co-downregulated DEGs were mainly involved in energy metabolism, xenobiotic biodegradation and metabolism, and amino acid metabolism (Fig. 3D). For the exclusive DEGs in response to Aa_Fur (99 upregulated and 106 downregulated; 39.35% of total 521 DEGs) (Fig. 3), pathways such as thiamine metabolism, terpenoid backbone biosynthesis, glyoxylate and dicarboxylate metabolism, methane metabolism, and 2-oxocarboxylic acid metabolism were significantly enriched. They are classified as cofactor and vitamin metabolism and terpenoid and polyketide metabolism.

Transcription factors in response to different stress conditions. The transcription factors (TFs) that have been experimentally shown to regulate the expression of target genes were selected to do statistical analysis. DEGs under Aa (308), Fur (249), and Aa_Fur (521) stress were regulated by 195, 189, and 202 TFs, respectively. Among these TFs, 10, 4, and 18 TFs were differently expressed, and 7, 4, and 15 of them were upregulated, respectively (see Fig. S7 and Table S4 in the supplemental material).

A schematic diagram of regulatory network was constructed based on the differently expressed TFs, DEGs, and enriched metabolism pathways (Fig. 4). The DEGs regulated by TF of Mga1p (red) were involved in all of the critical pathways. The DEGs regulated by Hap4p and Tye7p (green) were involved in seven critical pathways, while those regulated by Haa1 and Yox1p (blue) were involved in six critical pathways. These top-ranked and differently expressed TFs may be potential targets for constructing inhibitor tolerant strains with the consideration of their enrichment ratio and log₂ fold change (log₂FC) value.

Overexpression of target TFs and evaluation of stress tolerance. Five TFs, namely Haa1p, Hap4p, Yox1p, Tye7p, and Mga1p, were overexpressed to investigate their relationship with acetic acid and furfural tolerance. Given the fact that the expression quantity (fragments per kilobase of exon per million reads mapped [FPKM]) of *UBI4* was much higher than that of each target TF's genes and was kept stable under different stress conditions (see Table S5 in the supplemental material), the P_{HAA1} , P_{HAP4} , P_{YOX1} ,

FIG 3 Venn diagrams (A and C) and KEGG enrichment histograms (B and D) of differentially expressed genes (DEGs) in Aa versus C, Fur versus C, and Aa_Fur versus C groups. (A and B) Upregulation DEGs; (C and D) downregulation DEGs. The sum of 63, 104, and 48 (215) represents upregulated DEGs, and the sum of 88, 6, and 7 (101) represents downregulated DEGs shared by the two and three compared groups. The numbers 99 and 106 represent the upregulated and downregulated DEGs, respectively, that were exclusively revealed in response to Aa_Fur.

P_{TYE7} and P_{MGA1} in strain s6 were replaced by P_{UBI4} respectively (see Fig. S8 in the supplemental material). Five engineered strains were constructed and successively named s6H3, s6P5, s6Y1, s6T3, and s6M1 (Table 1).

The fermentation performances of these engineered strains were evaluated by batch fermentation using 10% YPDx (see Fig. S9 in the supplemental material). Glucose was depleted by all of the strains in the first 4 h. The fermentation performance of these strains was compared after 18 h of fermentation for the group without inhibitors and after 24 h for the groups with inhibitors (Fig. 5). The overexpression of the five TFs induced a slight effect on cell growth under conditions with a single inhibitor. However, under Aa_Fur stress, the cell growth rates were significantly decreased after the overexpression of *HAA1*, *HAP4*, and *YOX1*, and improved after the overexpression of *TYE7* and *MGA1*. The xylose consumption rates and ethanol yields of s6H3, s6T3, s6P5, and s6Y1 were enhanced to different degrees under all conditions (with/without inhibitors), while that of s6M1 was improved under only three inhibition conditions. Among them, s6H3 had the best fermentation ability; its xylose consumption rate and ethanol yield were improved by 39.81% and 10.40%, respectively, under Aa stress and by 17.45% and 6.43% under Fur stress. Under Aa_Fur stress, the strains ranked in order of xylose consumption rate and ethanol yield

FIG 4 Schematic diagram of the regulatory network involving transcription factors (TFs), core genes, and regulatory elements in response to acetic acid and furfural of *S. cerevisiae* s6. The numbers listed under the secondary classification of KEGG pathway represent the number of subpaths contained in it. These pathways are consistent with the pathways listed in the radar map (Fig. 2). TFs involved in eight, seven, and six KEGG pathways were successively highlighted in red, green, and blue.

from high to low were s6H3, s6T3, s6P5, s6M1, s6Y1, and s6. Compared with s6, the xylose consumption rates of s6H3 and s6T3 were improved by 51.88% and 47.09%, and the ethanol yields were enhanced by 8.78% and 8.56%, respectively. The fermentation performance of s6H3 suggested that Haa1p was not only involved in acetic acid tolerance (23), but also contributed to furfural resistance. The fermentation results of the other four engineered strains indicated that Hap4p, Yox1p, Tye7p, and Mga1p were related to the resistance of *S. cerevisiae* to acetic acid and furfural.

Since overexpressing *HAA1* or *TYE7* significantly enhanced fermentation performance, strain s6H3T10, overexpressing both *HAA1* and *TYE7*, was constructed (Table 1). When 10% YPDx with mixed acetic acid and furfural (2.4/1.9 g/liter) was used for the fermentation, the xylose consumption rate and ethanol yield of s6H3T10 were enhanced by 58.54% and 11.64%, 4.39% and 2.63%, and 7.78% and 2.84% compared with s6, s6H3, and s6T3, respectively, after 24 h-fermentation (Fig. 6A). The fermentation capacity of s6H3, s6T3, and s6H3T10 were evaluated using pretreated corn stover slurry (20% solid content, 2.82 g/kg of acetic acid, 1.53 g/kg of formic acid, 0.21 g/kg of furfural, 0.37 g/kg of 5-hydroxymethylfurfural [5-HMF], and 2.33 g/kg of total phenols). By presaccharification and

TABLE 1 Plasmids and strains

Plasmid or strain	Description	Reference or source
Plasmids		
pMD19-T	Amp ^r	TaKaRa
pMD19-T- <i>ACT1</i>	<i>ACT1</i> inserted pMD19-T	This study
pMD19-T- <i>ADY2</i>	<i>ADY2</i> inserted pMD19-T	This study
pMD19-T- <i>ATO2</i>	<i>ATO2</i> inserted pMD19-T	This study
pMD19-T- <i>BTN2</i>	<i>BTN2</i> inserted pMD19-T	This study
pMD19-T- <i>ENO1</i>	<i>ENO1</i> inserted pMD19-T	This study
pMD19-T- <i>ENO2</i>	<i>ENO2</i> inserted pMD19-T	This study
pMD19-T- <i>HSP30</i>	<i>HSP30</i> inserted pMD19-T	This study
Cas9-NAT	Amp ^r ; <i>Cas9</i> ; <i>NAT1</i>	29
pMEL13	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-CAN1.Y</i>	30
pMEL13- <i>HAA1</i>	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-HAA1</i>	This study
pMEL13- <i>HAP4</i>	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-HAP4</i>	This study
pMEL13- <i>YOX1</i>	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-YOX1</i>	This study
pMEL13- <i>TYE7</i>	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-TYE7</i>	This study
pMEL13- <i>MGA1</i>	Amp ^r ; 2 μ m origin, <i>KanMX</i> , <i>gRNA-MGA1</i>	This study
Strains		
s6	Gene-engineered xylose-fermenting diploid <i>Saccharomyces cerevisiae</i> strain	26
s6H3	s6; Replacement of promoter P_{HAA1} to P_{UBI4}	This study
s6P5	s6; Replacement of promoter P_{HAP4} to P_{UBI4}	This study
s6Y1	s6; Replacement of promoter P_{YOX1} to P_{UBI4}	This study
s6T3	s6; Replacement of promoter P_{TYE7} to P_{UBI4}	This study
s6M1	s6; Replacement of promoter P_{MGA1} to P_{UBI4}	This study
s6H3T10	s6H3; Replacement of promoter P_{TYE7} to P_{UBI4}	This study

simultaneous saccharification and fermentation, the ethanol concentrations of s6, s6H3, s6T3, and s6H3T10 were approximately 44.50, 46.30, 46.73, and 47.50 g/liter, respectively, after 120 h of fermentation (Fig. 6B). Compared with that of s6, the ethanol yields (based on total sugar in pretreated slurry) of s6H3, s6T3, and s6H3T10 were increased by 4.06%, 5.03%, and 6.76%, respectively. These results indicated that the simultaneous overexpression of *HAA1* and *TYE7* further improved fermentation performance under inhibitor stresses, though the degree of improvement was limited.

The inhibitor resistance of s6 and engineered strains was assayed by their growth on agar plates with acetic acid and/or furfural (Fig. 6C). The engineered strains had better survival than s6 under all stress conditions. Strain s6H3T10 showed significantly better growth among these engineered strains, indicating that overexpressing both *HAA1* and *TYE7* was helpful for cell growth under inhibitor stresses.

DISCUSSION

Considering that acetic acid and furfural are typical inhibitors that generally coexist in lignocellulosic hydrolysates, the response mechanism to these two inhibitors of *S. cerevisiae* s6 when fermenting mixed glucose and xylose was investigated. The fermentation ability of s6 was severely inhibited under Aa_Fur stress compared to that under Aa or Fur stress (Fig. 1). More DEGs were involved in response to Aa_Fur, which not only increased the diversity of the enriched metabolic pathways but also increased the enrichment ratio of each enriched pathway (Fig. 2 and 3). The response of s6 was more complicated under Aa_Fur stress, possibly due to both the dual inhibitors and the higher inhibitory effect.

By the reports to date, the transcription response of *S. cerevisiae* has been found to be different in the presence of different inhibitors. However, this research has mainly focused on revealing the inhibitor response mechanism when glucose was fermented. Mira et al. (11) found that pathways, including mitochondrial ribosomal protein,

FIG 5 Fermentation performances of strains s6, s6H3, s6P5, s6Y1, s6T3, and s6M1. The fermentation was performed using 10% YPD medium without inhibitor (A), with acetic acid (2.4 g/liter) (B), with furfural (1.9 g/liter) (C), and with mixed acetic acid and furfural (2.4/1.9 g/liter) (D), respectively. The growth rate, xylose consumption rate, and ethanol yield of each engineered strain were compared with those of strain s6. Values and standard deviations were calculated from three repeated samples. ★★, $P < 0.01$; ★, $P < 0.05$.

transmembrane transport process, and sensing-, signaling-, and uptake-related pathways, play pivotal roles in response to Aa. The pathways related to carbohydrate metabolic process and to transcriptional and translational control were reported crucial in response to Fur (16, 19, 20). Chen et al. (16) reported that the pathways of transmembrane transport and cellular amino acid metabolic process were significantly enriched in response to Aa_Fur. Compared with these enriched pathways when glucose was fermented, in the present study, when mixed glucose and xylose were fermented, more pathways related to carbohydrate metabolism were enriched under Aa stress, and more pathways related to xenobiotic biodegradation and metabolism were significantly enriched under Fur stress. Besides these pathways, those related to cofactor and vitamin metabolism and to lipid metabolism were further significantly enriched in response to Aa_Fur. These results indicated that the stress response of *S. cerevisiae* was impacted by the type of sugars fermented.

Transcriptional profiling revealed that strain s6 adopted distinct endogenous genetic regulatory mechanisms to reprogram cell metabolism under different stress conditions.

FIG 6 Evaluation of inhibitor tolerance of strains by batch fermentation using 10% YPDX medium containing mixed acetic acid and furfural (2.4/1.9 g/liter) (A) and pretreated corn stover slurry (B), and by spot assay (C). Cells (initial optical density at 600 nm [OD₆₀₀] of 1, 10-fold serial dilutions from left to right) were spotted onto 2% YPD-agar plates with and without acetic acid, furfural, or mixed acetic acid and furfural. The spotted agar plates were incubated at 30°C for 48 h or 96 h.

However, from the key enriched pathways (Fig. 2), it could be concluded that the strain tends to upregulate the pathways related to energy production to alleviate stress damage, such as central carbon metabolism (see Fig. S5 in the supplemental material). The pathways related to amino acid metabolism were downregulated, suggesting that reduced amino acid metabolism might be one of the reasons for the decreased fermentation capacity of strain under inhibitor stress conditions (see Fig. S6 and Table S3 in the supplemental material).

Manipulating target TFs is an effective approach to improve the tolerance of *S. cerevisiae* to inhibitors in hydrolysates (32). In the present study, the engineered strains overexpressing TFs of Haa1p, Hap4p, Yox1p, Tye7p, and Mga1p, respectively, showed improved resistance to Aa, Fur, and Aa_Fur (Fig. 5 and 6). Haa1p is a well-known

transcription factor involved in tolerance of weak acids (9, 23). In the present study, Haa1p also stands out under Fur stress; that there were many commonalities in core DEGs, key pathways, and significantly differentially expressed TFs in response to acetic acid and furfural might be one of the reasons (Fig. 2 to 4). In addition, both acetic acid and furfural have oxidative properties, which may lead to a similar response in *S. cerevisiae* (16, 33, 34).

The relationship of the other four TFs, namely Hap4p, Yox1p, Tye7p, and Mga1p, with inhibitor resistance was revealed in the present study. The genes and pathways regulated by these four TFs were located in critical positions under all of the stress conditions (Fig. 4). Hap4p is a transcriptional activator and global regulator of respiratory gene expression (35). Yox1p is a homeobox transcriptional repressor that participates in cell cycle regulation (36). Tye7p can activate glycolytic genes and may function as a transcriptional activator in Ty1-mediated gene expression (37). The protein encoded by *MGA1* is similar to heat shock transcription factor and is related to pseudohyphal growth (38). The function of these four TFs is directly or indirectly related to the stress response, which may be one of the reasons why the overexpression of these TFs enhanced the inhibitor resistance of *S. cerevisiae* s6. Comparing the present study (fermentation of mixed glucose and xylose) with the previous studies (fermentation of glucose only), the types of the revealed TFs were different, while some of the previously reported TFs, such as Sfp1p, Ace2p, Msn2p, and Yap1p, also had higher enrichment ratios in the present study (see Fig. S10 in the supplemental material) (16, 24, 25). These results indicate that comparative transcriptomic analysis is a powerful tool to discover TFs as potential targets for constructing robust strains.

The co-overexpression of *HAA1* and *TYE7* (strain s6H3T10) was more favorable for improving inhibitor resistance than only overexpressing *HAA1* (strain s6H3) or *TYE7* (strain s6T3) (Fig. 6). However, the enhancement was not as significant as expected for the complementary effect of Haa1p and Tye7p, even though only 31 of 254 total DEGs and 178 of 1,632 total genes were coregulated by these two TFs (see Fig. S11 in the supplemental material). This may relate to the regulation model of these two TFs, which should be deeply studied by comparative transcriptome analysis for strains s6H3, s6T3, and s6H3T10. The ethanol yield of strain s6H3T10 was about 0.44 g/g, which was increased by 6.76% compared to the starting strain s6 when the pretreated corn stover slurry containing various inhibitors (total concentration, >7.26 g/kg slurry) was fermented. Although strains reported to date have different ethanol yields, in the range of ~0.30 to 0.48 g/g, due to the diversity and heterogeneity of the lignocellulosic hydrolysates (39), strain s6H3T10 showed a strong inhibitor resistance compared to those reported for *S. cerevisiae* strains (39, 40), suggesting its industrial application potential in lignocellulosic ethanol production.

The starting strain used in the present study is an industrial *S. cerevisiae* strain engineered for xylose metabolism, which originally has better tolerance to different inhibitors (41, 42). It is a big challenge to further improve its inhibitor tolerance, which was not significantly improved though approaches such as physical mutagenesis, acclimation under inhibitor stresses, or the combination of them (data not shown). However, in the present study, its inhibitor tolerance was significantly improved by overexpressing target TFs mined via comparative transcriptome analysis, suggesting the effectiveness of TF manipulation and the strategy used on inhibitor improvement of industrial *S. cerevisiae*. Among the differentially expressed TFs (Fig. 4; see also Fig. S7 in the supplemental material), only five of them were evaluated by overexpression. The others should be systemically investigated in future studies to reveal their relationship with inhibitor tolerance. In addition, the function of these target TFs should be evaluated through studies of both overexpression and knockout of the genes of these target TFs.

High production cost is still the core problem of industrial production of lignocellulosic bioethanol. Constructing robust xylose-fermenting strains able to resist various inhibitors is critical for achieving high ethanol productivity and yield, which would markedly reduce the production cost. The present study provided an effective strategy

TABLE 2 Target sequences used in yeast transformation

Target ^a	Target type ^b	Sequence ^c
gRNA insert		
tgR-F		TGCGCATGTTTCGGCGTTCGAACTTCTCCGAGTGAAAGATAAATGATC
tgR-R		GTTGATAACGGACTAGCCTTATTTAACTTGCTATTCTAGCTCTAAAAC
HAA1	CS	AAGCTTTTCCAAGGAAAAG AGGG
HAP4	CS	GATGGACCTAAACAACCAC ACGG
YOX1	CS	GTGGTGACCACAGAACAAC AGG
TYE7	CS	GGCGAACTGTGAACGTGCGC AGG
MGA1	CS	GCAGTAGATATCCATATCGA AGG
Repair fragment		
UBI4	F	CTTCAAATAACCCGTTCA
	R	AATCTATTAGTTAAAGTA
HAA1	RF F	<u>AGTTCACGTTTTATAAGTCTTCCGTTCTCTTTATCAGTCTTCAAATAACCCGTTCA</u>
	RF R	<u>ACCTCTCACAGGCATACCTTTATGCCATTTATCAAGACCATAATCTATTAGTTAAAGTA</u>
	V _p F	ACAGCACCAGCACTTGATTG
	V _p R	AAGCGTTGATCTGTATGAT
HAP4	RF F	<u>AAATTATATATATTGTAAGCTATTAAGTAATCTCCTATATCAATTTCTCTTGCTTAACCTCAAATAACCCGTTCA</u>
	RF R	<u>AAAAATGGTTACTACGAGGGCGACTAGCGGAGGCCTGTAGTAGAAAAAGTCTTTGCGGTCATAATCTATTAGTTAAAGTA</u>
	V _p F	CGGATCTTGCACGCGGTAAG
	V _p R	TCCTCTACCAGGTCTTGGTC
YOX1	RF F	<u>ATATGCCTTGGCATTTTAACAAGACTCTATGTTGTGATTTAGTATGATTGGGTGAACTTCAAATAACCCGTTCA</u>
	RF R	<u>ATTTCCGTTCCCGATAGAAGACTAGATAGAGAAGGTAGCATTTTGTCTTGTGACATAATCTATTAGTTAAAGTA</u>
	V _p F	GCTTCTGCAACTGTATGTCCAACC
	V _p R	CAAGGCGCTTCCACAGATC
TYE7	RF F	<u>CTTACCCTAGTAGTAATTCATTGTGCCACGTACGAACCGTACAATGGGATTCTGCCCTTCAAATAACCCGTTCA</u>
	RF R	<u>TTCAGGTTTAATTAAGTAGTTTCGCTAGATCTAACATTTCTGTCTAAAATAGAGTTTCAATCTATTAGTTAAAGTA</u>
	V _p F	CCTTCTGGAGTGGGATTGAG
	V _p R	GATGCAGAAAGAACTGACGA
MGA1	RF F	<u>GAAAAAGGAGGAACCGAAAAAATAATCTTTCAGTTGTTGACCCGTAGAGCAGAAAAATGGACTTCAAATAACCCGTTCA</u>
	RF R	<u>ATTTACTTCCGGTTCAGAGAAGATTGCATGAAGTTGGTGACGAAAGTTTTAGGTTGCATAATCTATTAGTTAAAGTA</u>
	V _p F	CGCTTTGGCTTGGGCTTACG
	V _p R	CCATTGTTGGCAGAAGATTG

^atgR-F, upstream homologous arm of guide RNA (gRNA); tgR-R, downstream homologous arm of gRNA.

^bCS, complementary sequence; RF, repair fragment; V_p, verification primer; F, forward primer; R, reverse primer.

^cBoldface indicates the PAM (NGG) site, and single underlines indicate the homologous arm.

for robust strain construction. The novel TFs verified as effective in the present study can be applied to the construction of other xylose-fermenting industrial *S. cerevisiae* strains for lignocellulosic bioethanol production.

MATERIALS AND METHODS

Strains, primers, and plasmids. All of the plasmids and strains used and constructed in this study were listed in Table 1. Target sequences and primers were shown in Table 2 and in Tables S6 and S7 in the supplemental material. *Escherichia coli* DH5 α was used for gene cloning and manipulation.

Media. A 2% YPD-agar plate (10 g/liter yeast extract, 20 g/liter peptone, 20 g/liter glucose, and 15 g/liter agar) was used for strain activation. Five percent YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 50 g/liter glucose) was used for strain precultivation. Ten percent YPD medium (10 g/liter yeast extract, 20 g/liter peptone, 60 g/liter glucose, and 40 g/liter xylose [pH 5]) with and without acetic acid (Aa), furfural (Fur), or mixed acetic acid and furfural (Aa_Fur) was used for batch fermentation. A 2% YPD-agar plate with and without Aa, Fur, or Aa_Fur were used for plate spot assay. A 2% YPD-agar plate supplemented with nourseothricin (NAT; 50 ng/ml) and Geneticin (G418; 100 ng/ml) was used for yeast transformation. LB medium (5 g/liter yeast extract, 10 g/liter peptone, and 10 g/liter NaCl) supplemented with ampicillin (100 ng/ml) or kanamycin (100 ng/ml) was used for *E. coli* DH5 α transformation.

Batch fermentation and RNA extraction and sequencing. The fermentation performance of *S. cerevisiae* s6 were evaluated using 10% YPD medium containing 2.4, 3.6, 4.8, and 6.0 g/liter Aa, and 1.0, 1.9, 3.9, and 5.8 g/liter Fur, respectively. Then, batch fermentation was conducted in three independent biological replicates using 10% YPD medium with and without Aa (2.4 g/liter), Fur (1.9 g/liter), or Aa_Fur (2.4/1.9 g/liter). The batch fermentation method was described previously (13). The initial cell inoculum was 0.5 g dry cell weight (DCW) per 100 ml. If necessary, acetic acid and furfural were added into sterilized medium. Flasks were incubated in a thermostat water bath (35°C). Broth in flasks was stirred (200 rpm) using a magnetic stirring system.

Cells used for RNA extraction were collected at 6 h from the control (without inhibitor, C), Aa (2.4 g/liter), Fur (1.9 g/liter), and Aa_Fur (2.4/1.9 g/liter) groups. Total RNA was extracted from biological

triplicates using the yeast RNAiso kit (TaKaRa Biomedical Technology, Beijing) according to the manufacturer's protocol. The quality and concentration of total RNA were measured by NanoDrop 2000/2000C (Thermo Scientific, USA) and agarose gel electrophoresis. Transcriptome sequencing (RNA-seq) was conducted on HiSeq platform, and the method was previously described (13). Three independent biological replicates were sequenced for each fermentation condition.

Reverse transcription and quantitative real-time PCR. To verify the accuracy of RNA-seq data, RNA samples used for transcriptome sequencing were also used for quantification of mRNA copies by reverse transcription and quantitative real-time PCR (RT-qPCR). Six genes, *ADY2*, *ATO2*, *BTN2*, *ENO1*, *ENO2*, and *HSP30*, with varied transcript abundance, were chosen to quantify the relative expression levels (see Table S7 in the supplemental material). The cDNA was obtained by reverse transcription using total RNA as a template via the TaKaRa PrimeScript reverse transcription reagent kit with Genomic DNA (gDNA) Eraser (Perfect Real Time) (TaKaRa Biomedical Technology). The qPCR was performed according to the manufacturer's manual for SYBR *Premix Ex Taq II* (Tli RNaseH Plus; TaKaRa Biomedical Technology), and the copy number of each gene was normalized using *ACT1* expression level as a reference. The fold change was determined by the $2^{-\Delta\Delta CT}$ method (43). Each sample was run in triplicate, and each group was repeated three times. The value of RT-qPCR presented is the mean of the triplicate results.

Transcriptome data analysis. Transcriptome analysis was conducted as previously described (13). Quantified gene expression results used fragments per kilobase of exon per million reads mapped (FPKM) as the unit. Genes filtered with a threshold of false discovery rate (FDR) of <0.05 and an absolute $\log_2$ fold change (sample B/sample A) of ≥ 1 were considered differentially expressed genes (DEGs). The gene function was annotated by searching in *Saccharomyces* Genome Database (SGD). The DEGs were shown on the KEGG pathway map according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The KEGG pathway terms were further enriched using KOBAS, and those with a *P* value of <0.05 and an enrichment ratio of >0.1 were considered to be significantly enriched. The *P* value was calculated based on the hypergeometric distribution. The enrichment ratio of each KEGG pathway was the number of DEGs involved in each KEGG pathway to the number of total genes involved in each KEGG pathway. The DEGs were used to search for TFs that have been experimentally shown to regulate the expression of the genes from documented associations in the YEASTRACT database. The enrichment ratio of each TF was the ratio of the number of DEGs regulated by the TF to the number of total DEGs.

gRNA plasmid construction. Linearized plasmid backbone and guide RNA (gRNA) insert were assembled together to form the gRNA plasmid. The linearized plasmid backbone was amplified by PCR with primer 6005/6006 using pMEL13 as the template (30). The gRNA insert (120 bp) was made up of the upstream homologous arm (tgR-F, 50 bp), the downstream homologous arm (tgR-R, 50 bp), and complementary sequence (20 bp), which was synthesized by Genewiz (Suzhou, China) (see Fig. S8 in the supplemental material). The complementary sequence was located in the upstream of the PAM (NGG) site in the promoter region of *HAA1*, *HAP4*, *YOX1*, *TYE7*, or *MGA1*, respectively, which was designed using the Yeastricion tool at <http://www.e-crisp.org> (Table 2).

The integration of the gRNA into the linearized backbone was conducted using Gibson assembly according to the manufacturer's manual for Gibson assembly mastermix (New England Biolabs, Beverly, MA). Each plasmid was transformed into *E. coli* DH5 α . After sequencing, the plasmids with correct sequence insertion were used for the subsequent yeast transformation.

Repair fragment amplification. The strength of the promoter of *UBI4* (P_{UBI4} , 590 bp) was moderated and kept stable under all studied fermentation conditions (see Tables S5 and S6 in the supplemental material). P_{UBI4} was used as a repair fragment to replace the promoter of *HAA1*, *HAP4*, *YOX1*, *TYE7*, or *MGA1*. The repair fragment was amplified using the s6 genome as the template, and it contained the upstream homologous arm, the downstream homologous arm, and the P_{UBI4} sequence (see Fig. S8 in the supplemental material).

Yeast transformation. The LiAc method was used for yeast transformation (44). Cas9 plasmid was first transformed into *S. cerevisiae* s6. The gRNA plasmid and repair fragment (P_{UBI4}) were then transformed into yeast harboring Cas9 plasmid. Transformants grown on 2% YPD plates containing appropriate NAT and G418 were subjected to the confirmation of correct promoter replacing by PCR and sequencing. Cas9 and gRNA plasmids were removed from the transformants according to a previously reported method (30). The resulted transformants were used for the followed fermentation evaluation.

Evaluation of fermentation performance and inhibitor tolerance of transformants. The fermentation performance of s6 and transformants were evaluated using 10%YPDX medium with and without Aa (2.4 g/liter), Fur (1.9 g/liter), or Aa_Fur (2.4/1.9 g/liter) and pretreated corn stover slurry. The evaluation using pretreated corn stover slurry were performed by presaccharification and simultaneous saccharification and fermentation. Corn stover was pretreated using a two-step steam explosion method. Each kilogram of pretreated slurry (20% solid content) had 93.88 g of glucose, 14.81 g of xylose, 2.82 g of acetic acid, 1.53 g of formic acid, 0.21 g of furfural, 0.37 g of 5-hydroxymethylfurfural (5-HMF), and 2.33 g of total phenols. Pretreated slurry (120 g, pH 5.0) was added to a 500-ml flask together with cellulase CTec3 (30 filter paper units [FPU]/g cellulose) and penicillin (100 mg/kg-pretreated slurry) and presaccharified for 11 h in a shaker (50°C, 200 rpm). Presaccharified slurry (100 g, pH 5.0) was transferred into a 300-ml flask, and fresh cells (0.05 g dry cell weight [DCW]) precultivated using 5% YPD medium were inoculated. Flasks were incubated in a thermostat water bath (35°C, 200 rpm). All fermentation experiments were conducted in three independent biological replicates.

The effect of acetic acid and furfural on the cell growth of s6 and transformants were conducted using plate spot assay. Cells were grown on 2% YPD liquid medium at 30°C overnight, the 10-fold serial dilutions of each sample (initial optical density at 600 nm [OD_{600}] of 1) were spotted onto the 2% YPD-agar plate with and without Aa (2.4 g/liter), Fur (1.0 g/liter), or Aa_Fur (2.4/1.0 g/liter).

Analytical methods. The concentrations of glucose, xylose, and ethanol were determined as previously described (45). Glucose and xylose were determined by high-performance liquid chromatography (HPLC) equipped with a fluorescence detector (RF-10AXL). Ethanol was measured by gas chromatography (GC) with a flame ionization detector (FID), and 2-propanol was used as the internal standard. The differences in discrete variables between the groups were evaluated by Student's *t* test using IBM SPSS Statistics, while a *P* value of <0.05 was considered statistically significant.

Data availability. Transcriptome data can be accessed through SRA accession numbers PRJNA553647 and PRJNA640954.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2 MB.

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We declare no conflicts of interest.

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