Improving acetic acid and furfural resistance of Saccharomyces cerevisiae by regulating novel transcriptional factors revealed via comparative transcriptome

Bo Li[1], Li Wang[1], Ya-Jing Wu[1], Zi-Yuan Xia[1], Bai-Xue Yang[1], and yueqin tang[1]

[1]Sichuan University

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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 S. cerevisiae in response to acetic acid (Aa), furfural (Fur), and the mixture of acetic acid and furfural (Aa_Fur) while fermenting with glucose and xylose were revealed. The pathways classified as carbohydrate metabolism were significantly enriched in response to Aa, while the pathways belonged to xenobiotics biodegradation and metabolism were significantly enriched in response to Fur. In addition to these pathways, some new pathways were activated in response to Aa_Fur, i.e., cofactors and vitamins metabolism, and lipid metabolism. Overexpression of Haa1p or Tye7p improved xylose consumption rate by nearly 50%, while the ethanol yield enhanced by nearly 8%. Further co-overexpression of Haa1p and Tye7 resulted in 59% increase in xylose consumption rate and 12% increase in ethanol yield, revealing the beneficial effects of Haa1p and Tye7p on improving the tolerance of yeast to mixed fermentation inhibitors.

Keywords

Transcriptome, *Saccharomyces cerevisiae* , Mixed sugars fermentation, Multiple inhibitors, Transcription factors

1 Introduction

Lignocellulose biomass has high cellulose and hemicellulose content. Fermenting it into bioethanol is an effective way to cope with resource and energy crisis (Shi et al. 2020; Cheah et al. 2020). Pretreatment and hydrolysis are needed to break down the structure of lignocellulosic biomass and release the fermentable sugars (mainly glucose and xylose) before it could be fermented by *Saccharomyces cerevisiae* (Du et al. 2020; Zhou et al. 2018). However, various inhibitors (weak acids, furans, and phenols) are generated during pretreatment and hydrolysis (Nandal et al. 2020; Zhang et al. 2019). By integrating the xylose metabolic pathway, *S. cerevisiae* could be endowed with xylose fermentation capacity (Wu et al. 2020; Zhang et al. 2018). These xylose-fermenting strains are supposed to have strong tolerance to inhibitors, otherwise, they cannot be applied to industrial lignocellulose bioethanol production.

Compared with the researches on xylose fermentation capacity of *S. cerevisiae* , the researches on response and tolerance of xylose-fermenting *S. cerevisiae* strains to lignocellulosic hydrolytic inhibitors are limited. Most researches on inhibitor response and tolerance of *S. cerevisiae* were conducted under the condition using glucose as carbon source. Several studies have reported that the transcriptional profiles of *S. cerevisiae* had larger differences when fermenting with different sugars (glucose, xylose, or mixed glucose and xylose) in the presence or absence of specific inhibitors (Henriques et al. 2017; Li et al. 2020; Mira et al. 2010; Matsushika et al. 2014; Zeng et al. 2016). Therefore, it is of great significance to reveal the response mechanisms

and further improving the tolerance of xylose-fermentable *S. cerevisiae* strains to multiple inhibitors when fermenting with mixed sugars.

Acetic acid and furfural are two typical inhibitors in hydrolysates (Jönsson and Martín 2016). It is reported that the specific growth rate, ethanol yield, and ATP production of *S. cerevisiae* were significantly suppressed by acetic acid or furfural stress when fermenting glucose (Chen et al. 2016; Luo et al. 2018). The pathways involved in carbohydrate metabolism, amino acid metabolism, signal transduction, and material transport were significantly enriched under acetic acid stress (Mollapour and Piper 2007; Li and Yuan 2010; Mira et al. 2010); while genes involved in aldehyde reduction, pentose phosphate pathway, transcriptional and translational control or stress responses were differentially expressed under furfural stress (Gorsich et al. 2006; Liu et al. 2008; Liu and Moon 2009; Li and Yuan 2010). As *S. cerevisiae* responses differently to acetic acid and furfural, it is particularly significant to reveal the response and improve the tolerance of *S. cerevisiae* to mixed acetic acid and furfural that coexist in hydrolysate. There are some reports on mixed acetic acid and furfural to date, however, these studies used glucose instead of mixed glucose and xylose as fermenting sugar (Chen et al. 2016).

The global gene expression of *S. cerevisiae* would be reprogrammed under hydrolysate inhibitor stress. It is difficult to improve the inhibitor tolerance by regulating only one or limited numbers of genes. Transcriptional factors (TFs), which can regulate the expression of a series of genes, are considered to be key regulators of hydrolysate inhibitor stress. Overexpression of TFs Haa1p (Cunha et al. 2018), Sfp1p, and Ace2p (Chen et al. 2016) was found effective for improving the resistance of *S. cerevisiae* to acetic acid, while overexpression of Sfp1p, Ace2p (Chen et al. 2016), Msn2p (Sasano et al. 2012), and Yap1p (Kim et al. 2013) could improve furfural resistance when fermenting with glucose. Thus, revealing potential TFs which contribute to inhibitor tolerance is necessary when fermenting with mixed sugar to construct robust strains for industrial bioethanol production.

In our previous study, a flocculating industrial *S. cerevisiae*strain s6 with efficient glucose and xylose fermentation capacity was constructed (Zeng et al. 2017). In the present study, by comparative transcriptome analysis, the response mechanisms of s6 to acetic acid, furfural, and their mixture were studied through the fermentation of mixed glucose and xylose. Potential TFs that may be related to inhibitor tolerance of acetic acid and furfural were screened out. Five of them were overexpressed using CRISPR/Cas9 gene engineering method (Lander 2016; Stovicek et al. 2017; Zhang et al. 2014), and their contribution to the tolerance of acetic acid and furfural were studied. The results of this study contributed to the understanding of the response mechanisms of *S. cerevisiae* to hydrolysates inhibitors, and the TFs verified its effectiveness of inhibitor tolerance could be applied to the construction of strains for lignocellulosic bioethanol production.

2 Materials and methods

2.1 Strains, primers, and plasmids

All the plasmids and strains used and constructed in this study were listed in Table 1. Target sequences and primers were shown in Table 2, Table S1, and Table S2. *Escherichia coli* DH5α was used for gene cloning and manipulation.

2.2 Media

2% YPD-agar plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 15 g/L agar) was used for strain activation. 5% YPD medium (10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose) was used for strain pre-cultivation. 10% YPDX medium (10 g/L yeast extract, 20 g/L peptone, 60 g/L glucose, and 40 g/L xylose, pH 5) was used for batch fermentation. 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/L yeast extract, 10 g/L peptone, and 10 g/L NaCl) supplemented with ampicillin (100 ng/mL) or kanamycin (100 ng/mL) was used for *E. coli* DH5α transformation.

2.3 Batch fermentation and RNA extraction and sequencing

The batch fermentation was conducted in biological triplicates under the condition with and without inhibitors as previously described (Li et al. 2020). 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), acetic acid (40 mM), furfural (20 mM), and mixed acetic acid and furfural (40+20 mM) groups. Total RNA was extracted from biological triplicates using the Takara 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. RNA-seq was conducted on HiSeq platform, and the method was previously described (Li et al. 2020). Three biological duplicates were sequenced for each fermentation condition.

2.4 Reverse transcription and real-time quantitative PCR (RT-qPCR)

To verify the accuracy of RNA-seq data, RNA samples used for transcriptome sequencing were also used for quantification of mRNA copies of six genes, *ADY2* , *ATO2* , *BTN2* , *ENO1* ,*ENO2,* and *HSP30,* with varying transcript abundance (Table S2). The cDNA was obtained by reverse transcription using total RNA as template via the Takara PrimeScriptTM RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara Biomedical Technology, Beijing). The qPCR was performed according to the manufacture's manual of Takara SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Takara Biomedical Technology, Beijing), and the copy number of each gene was normalized using *ACT1* expression level as a reference. The fold change was determined by the $2^{-\Delta\Delta^{\tilde{}}T}$ method (Livak and Schmittgen 2001). 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.

2.5 Transcriptome data analysis

The transcriptome data can be accessed through the SRA accession number PRJNA553647 and PRJNA640954. The analysis was conducted as previously described (Li et al. 2020). Quantified gene expression results used FPKM (fragments per kilobase of exon per million reads mapped) as unit. The gene filtered with a threshold of false discovery rate (FDR) < 0.05 and an absolute $\log_2$ fold change (Sample B/Sample A) of [?] 1 was considered as 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$P < 0.05$ and enrichment ratio > 0.1 were considered to be significantly enriched. 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 number of DEGs regulated by the TF to the number of total DEGs.

2.6 gRNA plasmid construction

Linearized plasmid backbone and gRNA insert were assembled together to form the guideRNA (gRNA) plasmid. The linearized plasmid backbone was amplified by PCR with primer 6005/6006 using pMEL13 as the template (Mans et al. 2015). The gRNA insert (120 bp) was made up of upstream homologous arm (tgR-F, 50 bp), downstream homologous arm (tgR-R, 50 bp), and complementary sequence (20 bp), which was synthesized in GENEWIZ (Suzhou, China) (Fig. S1). The complementary sequence was located in the upstream of the PAM (NGG) site in the promoter regions of genes of *HAA1* , *HAP4* ,*YOX1* , *TYE7* , and *MGA1* , respectively, which was designed using the yeastriction 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 manufacture's manual of Gibson Assembly® Master Mix (New England Biolabs, Beverly, MA, USA). Each plasmid was transformed into *E. coli* DH5α. After sequencing, the plasmids with correct sequence

insertion were used for the subsequent yeast transformation.

2.7 Repair fragment amplification

The strength of the promoter of *UBI4*(P_{UBI4}, 590 bp) was moderated and kept stable under all studied fermentation conditions (Table S1 and S3). P_{UBI4} was used as repair fragment to replace the promoters of *HAA1* ,*HAP4* , *YOX1* , *TYE7* , and *MGA1* , respectively. The repair fragment was amplified using s6 genome as template, and it contained upstream homologous arm, downstream homologous arm, and P_{UBI4} sequence (Fig. S1).

2.8 Yeast transformation

The LiAc method was used for yeast transformation (Gietz and Woods et al. 2002). Cas9 plasmid was first transformed into *S. cerevisiae*s6. The gRNA plasmid and repair fragment (P_{UBI4}) were then transformed into yeast with 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 the reported method (Mans et al. 2015). The resulted transformants were used for the followed fermentation evaluation.

2.9 Evaluation of inhibitor tolerance of transformants

Fermentation using synthetic medium and pretreated straw slurry were conducted to evaluate the performance of the transformants. Batch fermentation using the synthetic medium was conducted as reported (Li et al, 2020). Straw slurry was pretreated using a two-step steam explosion method. Each kilogram of pretreated straw 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. Pre-saccharification and simultaneous saccharification fermentation were performed. 120 g-pretreated slurry (pH 5.0) with 20% solid content was added to a 500-mL flask together with cellulase CTec3 (30 FPU/g-cellulose) and penicillin (100 mg/kg-pretreated slurry) and presaccharified for 11 h in a shaker (50 °C, 200 rpm). 100 g-presaccharified slurry (pH 5.0) was transferred into a 300-mL flask, and fresh cells (0.05 g dry cell weight (DCW)) pre-cultivated using 5% YPD medium were inoculated. Flasks were incubated in a thermostat water bath (35 °C, 200 rpm). All fermentation experiments were conducted in biological duplicate. The differences between the groups of discrete variables were evaluated by *t*test, while a value of $P < 0.05$ was considered statistically significant.

2.10 Analytical methods

The concentrations of residual glucose, xylose and ethanol were determined as previously described (Tang et al. 2006). Glucose and xylose were determined by HPLC equipped with a fluorescence detector (RF-10AXL). Ethanol was measured by GC with an FID detector and 2-propanol was used as the internal standard.

3 Results

3.1 Batch fermentation results of strain s6 under different stress conditions

The effects of different concentrations of acetic acid (Aa, 40, 60, 80 and 100 mM) and furfural (Fur, 10, 20, 40 and 60 mM) on *S. cerevisiae* s6 were studied using 10% YPDX medium. As shown in Fig. S2, 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/L after 24 h fermentation under the condition with 40 mM Aa. Under Fur stress, both the glucose consumption and the xylose consumption were inhibited. The residual xylose concentration was approximately 8.65 g/L after 24 h fermentation under the condition with 20 mM Fur.

Based on these results, the effect of mixed acetic acid and furfural (Aa_Fur, 40+20 mM) on the fermentation of strain s6 was further studied (Fig. 1). The groups with only acetic acid (Aa, 40 mM) and only furfural (Fur, 20 mM) were considered as the comparisons; the group without inhibitor was considered as the control (C). As shown in Fig. 1, compared with the comparison and control groups, the cell growth rate and xylose

consumption rate were lower under Aa_Fur stress. The residual xylose concentration was 19.35 g/L after 24 h fermentation in 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, $P < 0.05$) (Table S4).

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

Considering the carbon catabolite repression effect (Gancedo et al. 1998) and xylose consumption rate, cell samples collected at 6 h (xylose fermentation phase) were subjected to RNA extraction and transcriptome sequencing. Totally 6440 genes analyzed after the transcriptome data were aligned with *S. cerevisiae* S288C (Fig. S3 and Table S5). The transcription levels of *ADY2* , *ATO2* , *BTN2* ,*ENO1* , *ENO2* , and *HSP30* in all 12 samples were analyzed by RT-qPCR, and the results were consistent with the results of transcriptome analysis, suggesting that the transcriptomic results were reliable (Fig. S4).

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 Aa_Fur group was 69.16 % more than that in Aa group, and 109.24 % more than that in Fur group, indicating that the toxic effect was much serious under Aa_Fur stress.

3.3 KEGG enrichment analysis for total DEGs in each group

As shown in Fig. 2, 11, 16 and 16 KEGG pathways were significantly enriched in Aa *vs.* C (308 DEGs), Fur *vs.* C (249) and Aa_Fur *vs.* C (521) groups, respectively ($P < 0.05$). There were 25 specific pathways when combined these pathways into a whole (Fig. 2A). They belonged to carbohydrate metabolism, energy metabolism, amino acid metabolism, xenobiotics biodegradation and metabolism, cofactors and vitamins metabolism, signal transduction, biosynthesis of other secondary metabolites, and lipid metabolism. Among these 25 specific pathways, six pathways were significantly regulated in all three inhibition groups, which were carbon metabolism, glycolysis/gluconeogenesis, starch and sucrose metabolism, pentose phosphate pathway, biosynthesis of amino acids, and chloroalkane and chloroalkene degradation. These results indicated that carbohydrate metabolism, amino acids metabolism, and xenobiotics biodegradation and metabolism played important roles in response to acetic acid and/or furfural. Aside from these common metabolic pathways, amino sugar and nucleotide sugar metabolism, inositol phosphate metabolism, and pentose and glucuronate interconversions were only 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 only enriched in response to Fur. Butanoate metabolism, bisphenol degradation, thiamine metabolism, and linoleic acid metabolism were only 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 and Table S6), 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 xenobiotics biodegradation and metabolism, which were significantly enriched under Fur stress. Combined w ith the$P$ value of each pathway, it could be concluded that more DEGs participating in carbohydrate metabolism were revealed under Aa stress, and more DEGs participating in xenobiotics biodegradation and metabolism were revealed under Fur stress. Besides of those DEGs involved in carbohydrate metabolism, and xenobiotics biodegradation and metabolism, more DEGs participating in cofactors and vitamins 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.

3.4 KEGG enrichment analysis for the DGEs shared with inhibitors and exclusive in Aa_Fur

***vs.* C group**

The distribution of the DEGs in each group was revealed by venn diagram (Fig. 3A and 3C). For the DEGs that shared under Aa, Fur, and Aa_Fur stress conditions, the 215 co-upregulated DEGs were mainly involved in carbohydrate metabolism (starch and sucrose metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway), and cofactors and vitamins metabolism (retinol metabolism) (Fig. 3B). The 101 co-downregulated DEGs were mainly involved in energy metabolism (sulfur metabolism), xenobiotics biodegradation and metabolism (bisphenol degradation, chloroalkane and chloroalkene degradation), and amino acid metabolism (lysine biosynthesis, cysteine and methionine metabolism) (Fig. 3D). These results were consistent with the results of KEGG enrichment analysis for the total DEGs (Fig. 2), suggesting that regulating the expression of these pathways might contribute to the inhibitory resistance of yeast.

Ninety-nine upregulated DEGs (31.53% of total) and 106 downregulated DEGs (51.21%) were exclusively revealed in response to Aa_Fur (Fig. 3A and 3C).The KEGG enrichment analysis was performed for these exclusive DEGs (Fig. 3B and 3D). Thiamine metabolism, terpenoid backbone biosynthesis, glyoxylate and dicarboxylate metabolism, methane metabolism, 2-oxocarboxylic acid metabolism *etc.* , which are mainly classified as cofactors and vitamins metabolism, and terpenoids and polyketides metabolism, were significantly enriched.

3.5 Transcription factors in response to different stress conditions

The transcription factors (TFs) analysis was performed on the total DEGs under Aa, Fur, and Aa_Fur stress, respectively, using YEASTRACT database. Under Aa (308 DEGs), Fur (249 DEGs), and Aa_Fur (521 DEGs) stress, the DEGs were regulated by 195, 189, and 202 TFs, respectively. Among these TFs, 10, 4, and 18 TFs were differently expressed in response to Aa, Fur, and Aa_Fur, respectively. The number of upregulated TFs were 7, 4, and 15, and the downregulated TFs were 3, 0, and 3 successively (Fig. S5 and Table S7).

Based on the differently expressed TFs, DEGs, and enriched metabolism pathways, a schematic diagram of regulatory network was constructed in response to Aa, Fur, and Aa_Fur (Fig. 4). The DEGs regulated by TF of Mga1p (red), similar to heat shock transcription factor (Lorenz and Heitman 1998), were involved in all the critical pathways. The DEGs regulated by Hap4p and Tye7p (green), acting as transcriptional activators (Löhning and Ciriacy 1994; Zampar et al. 2013), were involved in seven critical pathways except lipid metabolism. The DEGs regulated by Haa1 and Yox1p (blue) were involved in six critical pathways. Haa1p, acting as a transcriptional activator (Cunha et al. 2018), was not involved in the regulation of DEGs in energy metabolism and signal transduction. Yox1p, acting as a homeobox transcriptional repressor (Pramila et al. 2002), was not involved in the regulation of DEGs in cofactors and vitamins metabolism and lipid metabolism. These top ranked and differently expressed TFs may be potential targets for constructing inhibitor tolerant robust strain with consideration of their enrichment ratio and $\log_2$FC value.

3.6 Overexpression of target TFs and the evaluation of stress tolerance

TFs, Haa1p, Hap4p, Yox1p, Tye7p, and Mga1p were overexpressed to verify their relationship with acetic acid and furfural tolerance. Given the fact that the expression quantity (FPKM) of *UBI4* was much higher than those of each target TFs genes and was kept stable under different stress conditions (Table S3), the P_{HAA1} , P_{HAP4} , P_{YOX1} , P_{TYE7} , and P_{MGA1} in strain s6 were replaced by P_{UBI4} , respectively. Five engineered strains were constructed and successively named as s6H3, s6P5, s6Y1, s6T3, and s6M1 (Table 1).

The performances of these engineered strains were evaluated by batch fermentation using 10% YPDX under four conditions: without inhibitor (0 mM), acetic acid (40 mM), furfural (20 mM), and mixed acetic acid and furfural (40+20 mM), respectively (Fig. 5 and S6). Glucose was depleted in the first 4 h for all strains in all experimental groups (Fig. S6). The fermentation performance of the strains was compared via the growth rate, xylose consumption rate, and ethanol yield (Fig. 5). These values were calculated after 18 h fermentation of the group without inhibitors, and 24 h fermentation of three groups with inhibitors.

As shown in Fig. 5, compared with s6, the cell growth rates of the five engineered strains were not significantly improved under all four conditions studied. Under the condition without inhibitor, the fermentation

performance of the engineered strains was enhanced except that of the strain s6M1. Under three inhibitor stress conditions, the fermentation performance of all the engineered strains was enhanced, while the improvement level was different. Among five engineered strains, s6H3 had the best fermentation ability under three inhibitor stress conditions. Compared to s6, the xylose consumption rate and ethanol yield were improved by 39.81 % and 10.40 % under acetic acid stress, and 17.45% and 6.43% under furfural stress, respectively. Under mixed acetic acid and furfural stress, as regards the xylose consumption rate and ethanol yield, strains ranked from high to low were s6H3, s6T3, s6P5, s6M1, s6Y1, and s6. Compared with strain s6, the xylose consumption rates of the top two strains s6H3 and s6T3 were improved by 51.88 % and 47.09 %, and the ethanol yield were enhanced by 8.78 % and 8.56 %, respectively. Under all the three inhibition conditions, strain s6H3 effectively fermented mixed sugars, suggesting that Haa1p was not only involved in tolerance to acetic acid (Cunha et al. 2018; Henriques et al. 2017), but also contributed to furfural resistance. Compared with s6, the fermentation performance of s6P5, s6Y1, s6T3, and s6M1 were all improved, suggesting that these TFs were related to the resistance of *S. cerevisiae* to acetic acid and furfural.

Since overexpressing *HAA1* or *TYE7* significantly enhanced the inhibitor tolerance to mixed acetic acid and furfural, the strain s6H3T10 overexpressing both *HAA1* and *TYE7* was further constructed (Table 1). The xylose consumption rate and ethanol yield of s6H3T10 were enhanced by 4.39 % and 2.63 %, 7.78 % and 2.84 %, and 58.54 % and 11.64 %, compared with s6H3, s6T3, and s6, respectively, after fermenting 24 h under the condition with mixed acetic acid and furfural (Fig. 6A). This result indicated that the simultaneous overexpression of *HAA1* and *TYE7* further improved the inhibitor tolerance, but the improvement degree was to some extent limited. Strains s6, s6H3, s6T3, and s6H3T10 were also evaluated by fermenting pretreated straw slurry (Fig. 6B). Compared with s6, the ethanol yield (based on total sugar in pretreated straw slurry) of s6H3, s6T3 and s6H3T10 was increased by 4.06%, 5.03%, and 6.76%, respectively, after fermenting 120 h.

4 Discussion

The fermentation performance of s6 was severely inhibited under Aa, Fur, and Aa_Fur stress conditions. The inhibition of Aa_Fur was more serious than that of Aa or Fur (Fig. 1). The comparative transcriptional analysis revealed that global genes and pathways were involved in response to these stress conditions, and the response was more complicated under Aa_Fur stress compared with Aa or Fur stress (Fig. 2 and 3). Compared with DEGs under Aa or Fur stress, more genes were noted as DEGs under Aa_Fur stress. These DEGs not only increased the diversity of the enriched metabolic pathways, but also increased the enrichment ratio of each pathway.

By the reported to date, the transcriptional response of *S. cerevisiae* is found to be different in response to different inhibitors. However, these researches mainly concentrated on revealing the inhibitor response mechanism when glucose was fermented. Mira et al. (2010) found that the pathways, such as mitochondrial ribosomal proteins, transmembrane transport processes, sensing, signaling, and uptake-related pathways, played pivotal roles in response to Aa. The pathways related to carbohydrate metabolic process, transcriptional and translational control were reported crucial in response to Fur (Chen et al. 2016; Gorsich et al. 2006; Li and Yuan 2010). Chen et al. (2016) reported that the pathways, such as transmembrane transport, cellular amino acid metabolic process were significantly enriched in response to Aa_Fur. However, compared with these reported enriched pathways when glucose was fermented, in the present study with the fermentation of mixed glucose and xylose, more pathways related to carbohydrate metabolism were enriched under Aa stress, and more pathways related to xenobiotics biodegradation and metabolism were significantly enriched under Fur stress. Under Aa_Fur stress condition, besides carbohydrate metabolism, and xenobiotics biodegradation and metabolism, the pathways related to cofactors and vitamins metabolism, and lipid metabolism were also significantly enriched. These results indicated that the inhibitor stress response of *S. cerevisiae* was impacted by the type of fermented sugar.

The modification of target TFs is a desirable approach to improve the tolerance of *S. cerevisiae* to hydrolysate inhibitors (Alriksson et al. 2010). In the present study, the engineered strains overexpressing TFs of Haa1p, Hap4p, Yox1p, Tye7p, and Mga1p, respectively, showed improved tolerance to Aa, Fur, and Aa_Fur (Fig. 5). Haa1p is a well-known transcription factor involved in tolerance of weak acid (Cunha et al. 2018; Henriques

et al. 2017). In the present study, Haa1p also stands out under furfural stress. The reasons why Haa1p contributes to the tolerance of furfural could be explained as follows. First, there were many commonalities in core DEGs, key pathways, and significantly differentially expressed TFs in response to acetic acid and furfural (Fig. 2, 3 and 4). Second, both acetic acid and furfural have the oxidative properties, which may lead to a similar response in *S. cerevisiae* (Allen et al. 2010; Chen et al. 2016; Zhang et al. 2017).

The relationship of the other four TFs, Hap4p, Yox1p, Tye7p, and Mga1p, with the inhibitor tolerance, was first revealed in the present study. The genes and pathways regulated by these four TFs are in critical positions under all the stress conditions (Fig. 4). Hap4p is a transcriptional activator and global regulator of respiratory gene expression (Zampar et al. 2013). Yox1p is a homeobox transcriptional repressor, which participates in cell cycle regulation (Pramila et al. 2002). Tye7p can activate glycolytic genes and may function as a transcriptional activator in Ty1-mediated gene expression (Löhning and Ciriacy 1994). The protein encoded by *MGA1* is similar to heat shock transcription factor and related to pseudohyphal growth (Lorenz and Heitman 1998). The function of these four TFs is directly or indirectly related to the stress response, which may be at least one of the reasons why the regulation of these TFs enhanced the inhibitor resistance of *S. cerevisiae* s6. Comparing the present study (fermentation of mixed glucose & xylose) with the previous studies (fermentation of glucose only), the TFs related to inhibitor tolerance were different, while some of the previous reported TFs also had higher enrichment ratios in the present study, such as Sfp1p, Ace2p, Msn2p, and Yap1p (Fig. S7) (Chen et al. 2016; Sasano et al. 2012; Kim et al. 2013). These results indicated the comparative transcriptomic analysis is a powerful tool to discover TFs as potential targets for constructing robust strains.

The inhibitor tolerance of the strain s6H3T10, overexpressing both*HAA1* and *TYE7* , was further enhanced, compared with that of strain s6H3 or s6T3 (Fig. 6). This result suggested that the co-overexpression of these two TFs was more favorable for improving the inhibitor resistance. However, compared with the strain s6H3 or s6T3, the enhancement of the inhibitor tolerance of strain s6H3T10 was limited. Even though only 31 DEGs among total DEGs and 178 genes among total genes were co-regulated by these two TFs (Fig. S8), the complementary effect of these two TFs was not as significant as expected.

In the present study, by comparative transcriptome analysis, many TFs were revealed having potential relationship with inhibitor tolerance, but only five of them were evaluated by overexpression. The relationship with inhibitor tolerance of other differentially expressed TFs (Fig. S5) and those TFs regulating DEGs (Fig. 4) under different inhibitor conditions should be systemically investigated in the future study. In addition, the molecular mechanism of inhibitor tolerance regulation of these TFs should be deeply studied using engineered *S. cerevisiae*strains overexpressing these TFs or knocking out these TFs.

5 Conclusion

The response mechanism of *S. cerevisiae* s6 to acetic acid and furfural were revealed by comparative transcriptome analysis in mixed glucose and xylose fermentation. The carbohydrate metabolism, amino acid metabolism, xenobiotics biodegradation and metabolism played key roles in response to acetic acid and furfural. Haa1p was identified as a pivotal regulator to improve the resistance to furfural. Four TFs, Hap4p, Yox1p, Tye7p, and Mga1p were first time identified as regulators in response to acetic acid and furfural. The genetic engineering method in this study could be used as an effective strategy for constructing robust strains for industrial bioethanol production.

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Compliance with ethical standards

Conflict of interest

The authors have declared no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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