Biochemical Engineering Journal 178 (2022) 108274

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Short communication

Improved ethanol fermentation by promoter replacement of zinc responsive genes *IPL1*, *PRP6* and *RTC1* in *Saccharomyces cerevisiae*

Hong-Qi Chen [a], Ming-Ming Zhang [a], Qi Xing [a], Pei-Liang Ye [a], Tomohisa Hasunuma [b,c], Akihiko Kondo [b,c], Xin-Qing Zhao [a,*]

[a] State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[c] Engineering Biology Research Center, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

ARTICLE INFO

Keywords:
Saccharomyces cerevisiae
zinc responsive gene, promoter replacement
acetic acid stress tolerance, inhibitors in lignocellulosic hydrolysate

ABSTRACT

Zinc sulfate is an important mineral nutrient for yeast stress tolerance. In this study, *IPL1, PRP6*, and *RTC1* whose expression are responsive to zinc sulfate, were investigated on their effects on inhibitor tolerance and ethanol fermentation by *Saccharomyces cerevisiae*. Yeast strains were developed through replacing native promoters by the constitutive *PGK1* promoter for these genes. The engineered yeast strains showed improved ethanol fermentation in the presence of acetic acid and mixed inhibitors. Meanwhile, enhanced ethanol titer from corncob hydrolysate was achieved, and up to 19.5% more ethanol was produced using the engineered yeast strain carrying the *RTC1* promoter replacement. The results in this study provides basis for further engineering yeast strains to improve efficiency of lignocellulosic biorefinery.

1. Introduction

Production of fuel ethanol using lignocellulosic biomass benefits sustainable development [1]. However, inhibitors, such as acetic acid, formic acid, 5-Hydroxymethylfurfural (5-HMF), and furfural, present in cellulosic hydrolysate are a major challenge for economic production [2]. Among the inhibitors, acetic acid is present at relatively high concentrations, and is known to exert toxicity to growth and metabolism [3]. Therefore, it is of great importance to improve inhibitor tolerance of microbial strains for efficient lignocellulosic biorefinery.

Budding yeast *Saccharomyces cerevisiae* is widely studied for cellulosic ethanol fermentation, and improvement of its tolerance to inhibitors is highly desirable [4]. Zinc ion is important for cell growth and metabolism. Previous studies also revealed that zinc sulfate exerts positive effect on yeast stress tolerance [5,6]. Further transcriptomic analyses identified zinc responsive genes related to yeast stress tolerance, such as *SET5*, *ADY2* and *ADE17*, and improved ethanol production was achieved [7–9].

In our previous study, different promoter activities of various genes were revealed under stress conditions [10]. It was demonstrated in another study that stress-driven promoters can be employed to enhance robustness and production performance of *S. cerevisiae* [11]. Therefore, it is of interest to investigate and engineer promoters of yeast zinc responsive genes under stress conditions and improve bioproduction.

In this work, we focused on *IPL1*, *PRP6* and *RTC1*, whose protein expression was considerably elevated by zinc sulfate supplementation when yeast was grown in the presence of acetic acid, which was revealed by our comparative proteomic analysis. The functions of these three genes are related to spindle checkpoint regulation, RNA splicing, and TOR signaling, respectively [12–14]. We employed promoter replacement strategy, changing the native promoters by the constitutive *PGK1* promoter (*PGK1p*) to obtain engineered strains. Our results provide basis for further engineering yeast robustness for cellulosic biorefinery.

2. Materials and methods

2.1. Strains and culture medium

The model yeast *S. cerevisiae* S288c was used as the parent strain. All strains used in this study were listed in Table S1. Yeast strains were propagated in YPD medium, and ethanol fermentation was performed using the fermentation medium described previously [7]. The inhibitors

* Corresponding author.
E-mail address: xqzhao@sjtu.edu.cn (X.-Q. Zhao).

https://doi.org/10.1016/j.bej.2021.108274
Received 14 September 2021; Received in revised form 12 November 2021; Accepted 14 November 2021
Available online 21 November 2021
1369-703X/

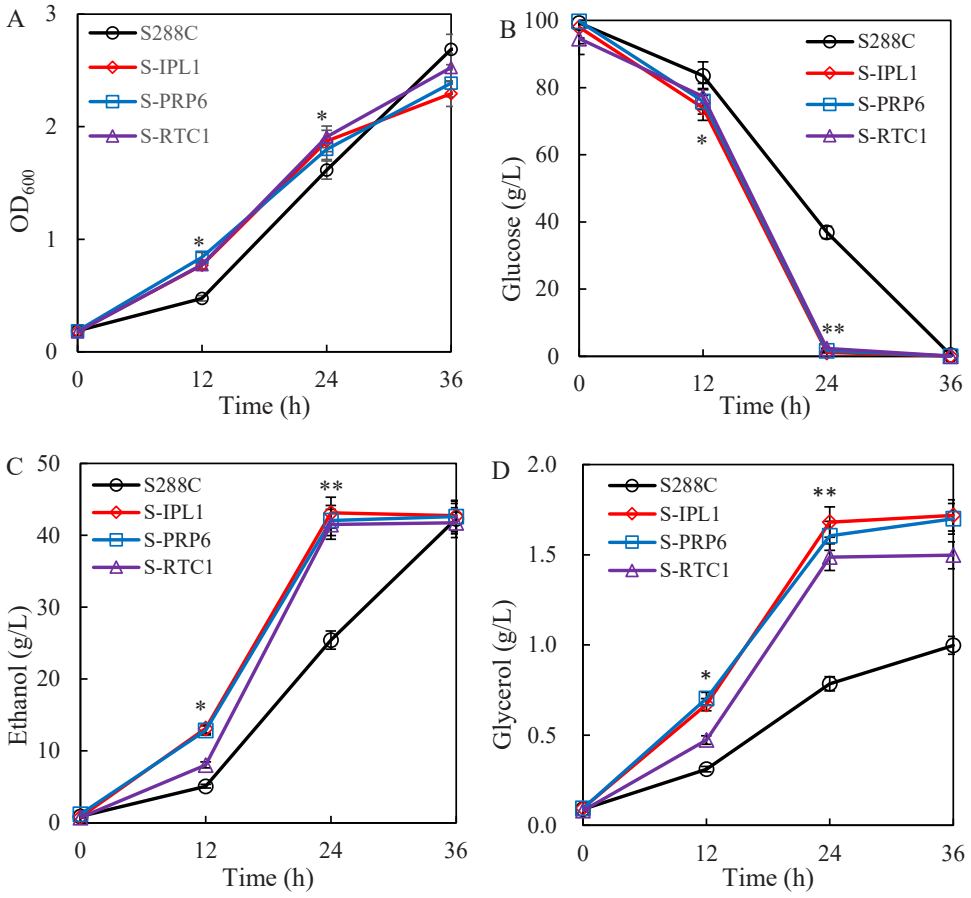

Fig. 1. Increased ethanol fermentation of yeast strains under 5 g/L acetic acid stress.
Yeast strains were cultured in fermentation medium containing 5 g/L acetic acid. Samples were taken at 12 h interval, and cell growth (A), glucose consumption (B), ethanol production (C) as well as glycerol (D) were monitored. Statistical significance was evaluated using Student's-*t*-test, *** $p < 0.001$, ** $p < 0.01$; * $p < 0.05$.

used were 5 g/L acetic acid, and simulated mixed inhibitors (4.33 g/L acetic acid, 0.34 g/L formic acid, 0.36 g/L 5-HMF, and 0.53 g/L furfural) [7]. The corncob hydrolysate contained 100 g/L glucose, 10.21 g/L xylose, 1.45 g/L acetic acid, 0.22 g/L formic acid, and 4 g/L peptone was added to support adequate yeast growth.

2.2. Construction of recombinant yeast strains

All primers for PCR used in this study were listed in Table S2. The scheme of the strain development was shown in Fig. S1. The plasmid DNA pHO [15] was used as the template. *PGK1p* was amplified by polymerase chain reaction with the primer P_{PGK1}-F as forward primer, and primers P_{PGK1}-IPL1-R, P_{PGK1}-PRP6-R and P_{PGK1}-RTC1-R were used as reverse primers, respectively. Correspondingly, the *KanMX4* cassette was amplified with the forward primers K+IPL1-F and K+P_{PGK1}-R, and reverse primer K+P_{PGK1}-R, respectively. Next, *PGK1p* and the *KanMX4* cassette were used as templates to amplify homologous recombination fragment by overlap extension PCR [16] for replacing the native promoter region of *PRP6*, *IPL1,* and *RTC1* with a pair primers K+PRP6-F and P_{PGK1}-IPL1-R, K+IPL1-F and P_{PGK1}-PRP6-R, K+RTC1-F and P_{PGK1}-RTC1-R, respectively. Subsequently, the successfully constructed cassettes were transformed into the *S. cerevisiae* S288c by the chemical method and transformants confirmed by diagnostic PCR (primers of K-F and K-R) as described previously (Fig. S2) [17]. All primers were listed in Table S2. The confirmed recombinant yeast strains were named as S-PRP6, S-IPL1 and S-RTC1, respectively.

2.3. Flask fermentation

Strains culture and fermentation as reference [8]. The fermentation was performed at 30 °C with shaking at 150 rpm. Yeast cell growth, residual sugar, ethanol and glycerol were recorded.

Table 1
Ethanol production of the engineered yeast strains under various stress conditions comparing with that of the control strain[a].

Parameter	5 g/L Acetic acid				Mixed inhibitors				Corncob hydrolysate			
	CK	R	I	P	CK	R	I	P	CK	R	I	P
T (h)	36	24	24	24	48	36	36	36	36	24	24	24
S_R (g/L)	0.4	1.51	1.11	2.1	0.19	0.3	0.13	0.14	9.7	1.3	1.3	1.2
Ep (g/L)	42.3	41.5	43.1	42.6	42.4	44.1	42.4	44.2	33.9	40.5	39.3	38.8
Q (g/L/h)	1.17	1.73	1.79	1.77	0.88	1.22	1.18	1.23	0.94	1.68	1.63	1.61
$Y_{E/S}$ (g/g)	0.42	0.42	0.43	0.43	0.42	0.44	0.42	0.44	0.36	0.40	0.39	0.39

[a] *T*: fermentation time; S_R: residual glucose; E_P: ethanol produced; *Q*: ethanol productivity; $Y_{E/S}$: ethanol yield, g(ethanol)/g(glucose/sugars); CK: *S. cerevisiae* S288C; R: *S. cerevisiae* S-RTC1; I: *S. cerevisiae* S-IPL1; P: *S. cerevisiae* S-PRP6.

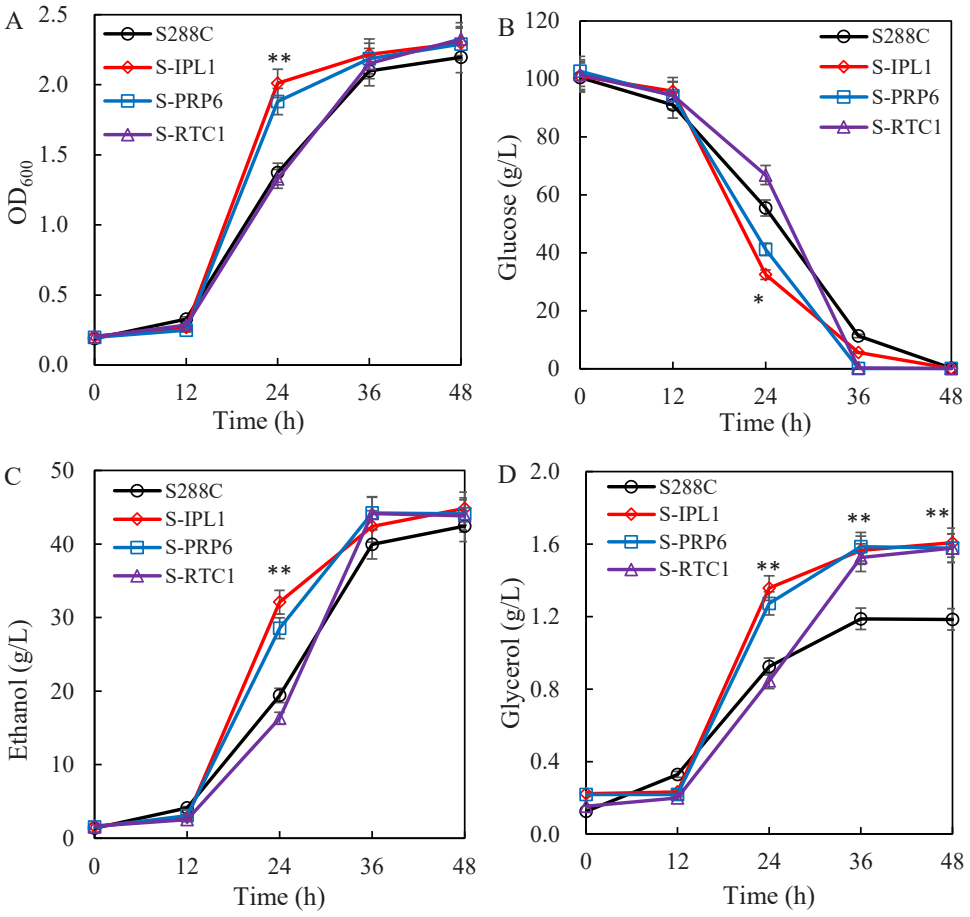

Fig. 2. Comparison of fermentation performance of the strains in mixed inhibitors.
The mixed inhibitors include 4.33 g/L acetic acid, 0.34 g/L formic acid, 0.53 g/L furfural and 0.36 g/L HMF. Samples were taken every 12 h, and the corresponding cell growth (A), residual sugar (B), ethanol production (C) and glycerol (D) were recorded. Statistical significance was determined using Student's-*t*-test, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

2.4. Analysis of fermentation performance

The concentrations of glucose, ethanol, acetic acid and glycerol in the samples were determined by high performance liquid chromatography system according to the previously described method [7].

2.5. Real-time quantitative PCR (RT-qPCR) assays

S. cerevisiae cells were harvested at log phase (OD_{600} is about 1.5). Total RNA extracted and RT-qPCR were carried out as showed in the reference [18]. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [19]. All primers used were listed in Table S3.

3. Results and discussion

3.1. Effects of the promoter replacement on ethanol fermentation

The engineered yeast strains showed better ethanol fermentation under 5 g/L acetic acid stress (Fig. 1). Specifically, the fermentation time was shortened by 12 h in the recombinant strains. Among the strains, the best fermentation performance was obtained in S-IPL1, followed by S-PRP6 and S-RTC1. The highest ethanol productivity in S-IPL1 is 1.75 g/L/h, 53.0% higher than that of the wild-type strain (Table 1).

Improved ethanol production performance of the engineered strains was also observed in the presence of mixed inhibitors (Fig. 2). Lag phase of all the strains were 12 h due to the strong inhibition of mixed inhibitors, and promoter replacement did not shorten the lag phase. Among the recombinant strains, S-PRP6 and S-IPL1 showed better growth than S288c, and reached nearly the maximum biomass after 24 h, whereas S-RTC1 grew similarly to the control strain (Fig. 2A). Interestingly, S-RTC1 showed faster glucose consumption and ethanol production at 36 h than that of the wild-type strain, although it showed the same growth profile (Fig. 2A-C), and 19.5% more ethanol was produced. All the engineered strains produced more glycerol than the control strain (Fig. 2D).

We further detected transcription of the three genes in the recombinant yeast strains under different conditions. Under stress free condition, promoter replacement did not increase transcription of *RTC1* and *PRP6*, in contrast, under stress condition, transcription levels of all the three genes were up-regulated by the promoter replacement (Fig. 3A, Fig. S3). We found increased transcription of the three genes in the presence of mixed-inhibitor when compared to that under the control condition without stress (Fig. S4), suggesting the regulation of the native promoters of the three genes under stresses. We also examined transcription of the common stress responsive genes [3] in the recombinant yeast strains relative to that of the control strain in the mixed inhibitors. The results revealed that promoter replacement to engineer the transcription of *PRP6*, *IPL1* or *RTC1* led to up-regulated transcription of the stress responsive genes to various extents under stress, including *SET5* and *ADE17* (Fig. 3B).

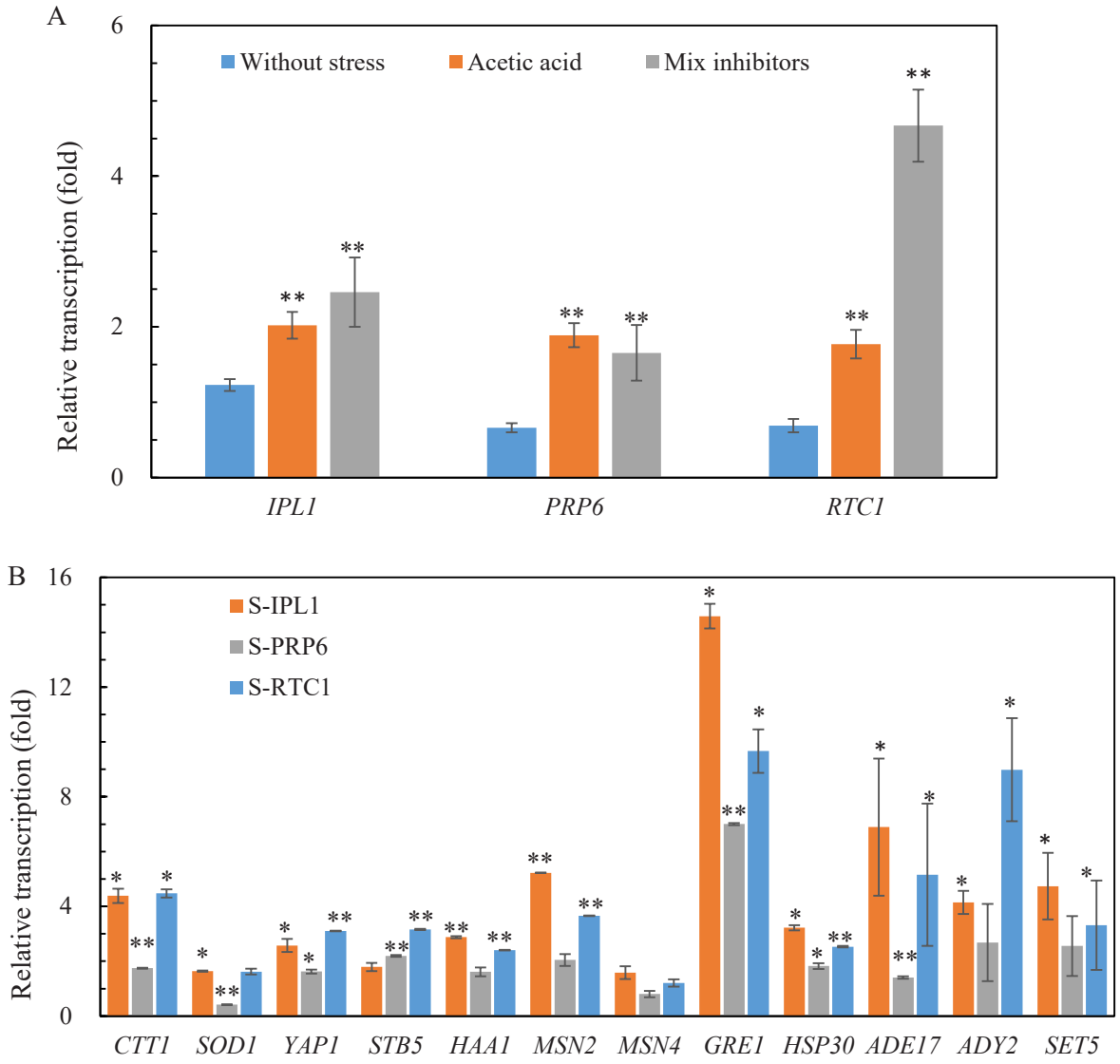

Fig. 3. Transcription of *IPL1*, *PRP6*, and *RTC1* as well as the selected stress-related genes in yeast strains under control condition and stress conditions. A, Transcription of *RTC1*, *PRP6* and *IPL1* in the engineering strains with or without stress compared to the parent strain *S. cerevisiae* S288c; B, Expression of stress-related genes in the yeast strains grown in the mixed inhibitors. Statistical significance was calculated using Student's-*t*-test, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

3.2. Effects of the promoter replacement on ethanol fermentation from corncob hydrolysate

There is no difference in growth of engineering strains compared with S288c (Fig. 4A) in the corncob hydrolysate. However, the recombinant strains consumed glucose within 24 h, faster than the wild-type strain S288c (Fig. 4B). Improved ethanol production was achieved in the recombinant strains (Fig. 4C). Interestingly, all the engineered strains consumed acetic acid, but the wild-type strain accumulated acetic acid instead (Fig. 4D). In addition, increased glycerol production was found in the recombinant strains, but no difference was found for formic acid (Fig. 4E, F). All the fermentation results of engineered strains are summarized in Table 1. The highest ethanol production titer from corncob hydrolysate was achieved using the engineered yeast strain carrying the *RTC1* promoter replacement, and 19.5% higher ethanol was produced. The corresponding productivity is 78.7% higher than that of the control strain. This is the first report on enhancement of inhibitor tolerance using these three genes. We found that promoter activities of these three genes were considerably elevated when the strains were grown in the mixed inhibitors, suggesting that these genes are stress responsive genes, and that the promoters of these genes can be used for dynamic control of gene expression as previously reported [11].

We found changes of genes related to stress tolerance, including *CTT1*, *YAP1*, *STB5*, *MSN2* [20–22], in the engineered strains in the mixed inhibitor condition. Although the phenotype of *PRP6* engineered strain is similar to that of the other two strains (Figs. 1, 2, 4), we found that changes of the stress tolerance related genes in this strain is different. For example, no changes in *MSN2*, *HAA1* and *SOD1* were

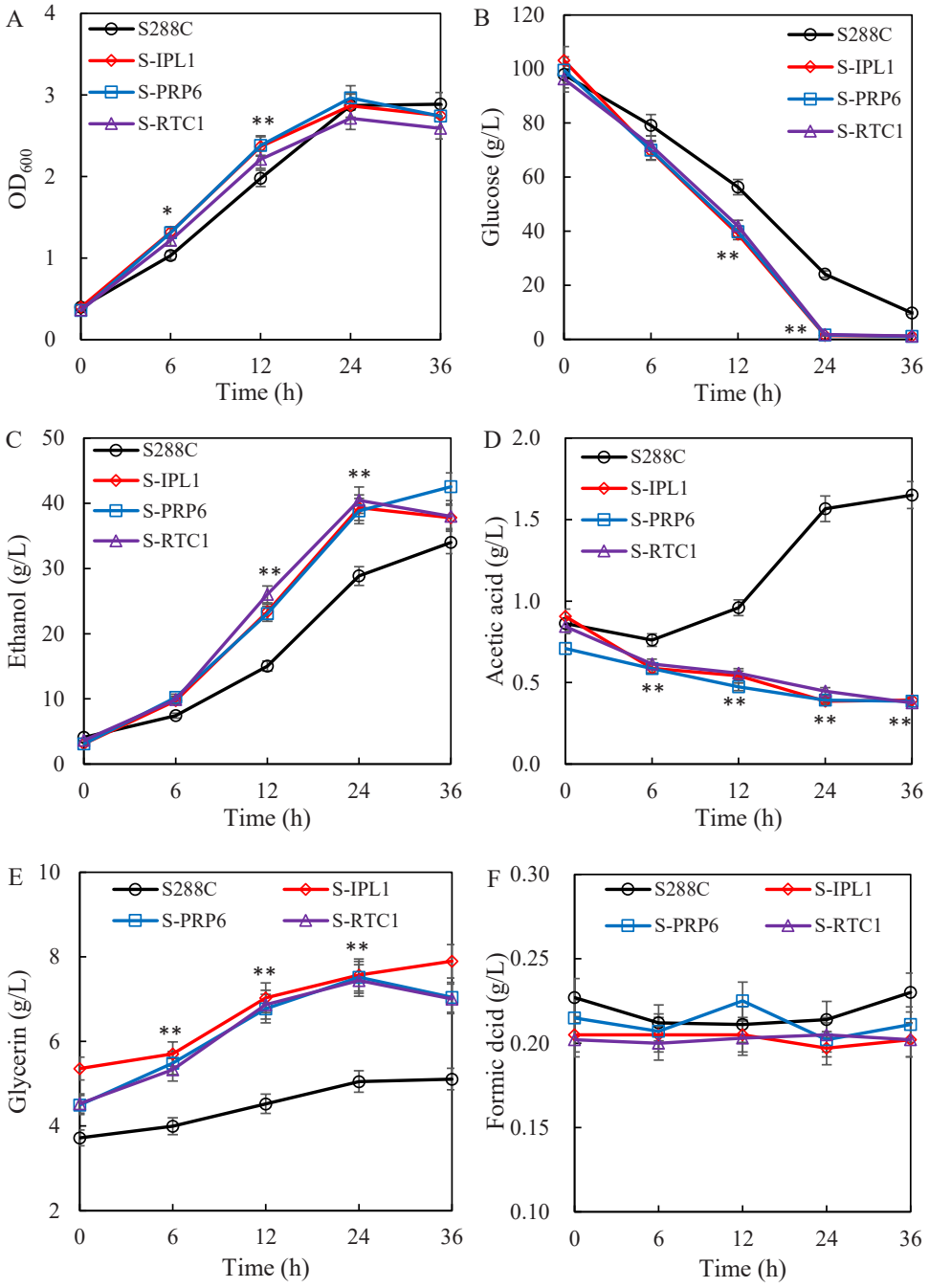

Fig. 4. Ethanol fermentation by the yeast strains using corncob hydrolysate.
Cell growth (A), residual sugar (B), ethanol (C), acetic acid (D), glycerol (E) and formic acid (F) were recorded in the corncob hydrolysate. Statistical significance was examined using Student's-*t*-test, *** $p < 0.001$, ** $p < 0.01$ and *$p < 0.05$.

found (Fig. 3B). It will be interesting to further study the regulation of *PRP6* for its unique functional mechanism.

In the previous studies, we reported that transcription of *SET5*, *ADY2* [7,8] and *ADE17* was affected by zinc sulfate addition, and we also proved that overexpression of *ADE17* and *SET5*, as well as disruption of *ADY2* led to better fermentation performance under stress [7,9]. We found increased transcription of *ADE17* and *SET5* in the engineered strains, as well as *ADY2*, indicating possible different regulatory network in the engineered strains. The strains developed in this study showed comparable fermentation performance with the above-mentioned strains. We developed yeast strains by replacing the endogenous promoters of *PRP6*, *IPL1* and *RTC1* with *PGK1p*, and the engineered yeast strains showed improved growth and ethanol fermentation in the presence of acetic acid as well as mixed inhibitors under the conditions employed in this study. Meanwhile, higher ethanol production from corncob hydrolysate was achieved using the engineered yeast strains. Our study provides a novel strategy for improving yeast robustness and cellulosic biorefinery.

CRediT authorship contribution statement

Hong-Qi Chen performed the experiments, wrote the draft of the manuscript and revised the manuscript; Ming-Ming Zhang, Qi Xing and Pei-Liang Ye helped strain construction and revision of the manuscript; Tomohisa Hasunuma and Akihiko Kondo participated in discussion of the results; Xin-Qing Zhao conceived the project, designed and

supervised the experiments and contributed to manuscript preparation and revision.

Declaration of Competing Interest

These authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by financial support from the State Key Research and Development Program, China (No. 2021YFC2101300), National Science Foundation of China (No. 21978168), and Natural Science Foundation of Shanghai, China (19ZR1427500). The authors thank Professor Yu Deng in Jiang Nan University for helpful discussion.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2021.108274.

References

[1] C.G. Liu, Y. Xiao, X.X. Xia, X.Q. Zhao, L. Peng, P. Srinophakun, F.W. Bai, Cellulosic ethanol production: progress, challenges and strategies for solutions, Biotechnol. Adv. 37 (2019) 491–504.

[2] R.R. Kumar, K. Arora, S. Goswami, A. Sakhare, B. Singh, V. Chinnusamy, S. Praveen, MAPK enzymes: a ROS activated signaling sensors involved in modulating heat stress response, tolerance and grain stability of wheat under heat stress, 3 Biotech 10 (2020) 380.

[3] J.T. Cunha, A. Romaní, C.E. Costa, I. Sá-Correia, L. Domingues, Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions, Appl. Microbiol. Biotechnol. 103 (2019) 159–175.

[4] M.M. Zhang, H.Q. Chen, P.L. Ye, S. Wattanachaisaereekul, F.W. Bai, X.Q. Zhao, Development of robust yeast strains for lignocellulosic biorefineries based on genome-wide studies, in: I. Sá-Correia (Ed.), Yeasts in Biotechnology and Human Health: Physiological Genomic Approaches, Springer International Publishing, Cham, 2019, pp. 61–83.

[5] K.S. Ismail, T. Sakamoto, T. Hasunuma, X.Q. Zhao, A. Kondo, Zinc, magnesium, and calcium ion supplementation confers tolerance to acetic acid stress in industrial *Saccharomyces cerevisiae* utilizing xylose, Biotechnol. J. 9 (2014) 1519–1525.

[6] C. Wan, M. Zhang, Q. Fang, L. Xiong, X. Zhao, T. Hasunuma, F. Bai, A. Kondo, The impact of zinc sulfate addition on the dynamic metabolic profiling of *Saccharomyces cerevisiae* subjected to long term acetic acid stress treatment and identification of key metabolites involved in the antioxidant effect of zinc, Met.: Integr. Biometal Sci. 7 (2015) 322–332.

[7] M.M. Zhang, X.Q. Zhao, C. Cheng, F.W. Bai, Improved growth and ethanol fermentation of *Saccharomyces cerevisiae* in the presence of acetic acid by overexpression of *SET5* and *PPR1*, Biotechnol. J. 10 (2015) 1903–1911.

[8] M.M. Zhang, K.Y. Zhang, M.A. Mehmood, Z.K. Zhao, F.W. Bai, X.Q. Zhao, Deletion of acetate transporter gene *ADY2* improved tolerance of *Saccharomyces cerevisiae* against multiple stresses and enhanced ethanol production in the presence of acetic acid, Bioresour. Technol. 245 (2017) 1461–1468.

[9] M.M. Zhang, L. Xiong, Y.J. Tang, M.A. Mehmood, Z.K. Zhao, F.W. Bai, X.Q. Zhao, Enhanced acetic acid stress tolerance and ethanol production in *Saccharomyces cerevisiae* by modulating expression of the de novo purine biosynthesis genes, Biotechnol. Biofuels. 12 (2019) 116.

[10] L. Xiong, Y. Zeng, R.Q. Tang, H.S. Alper, F.W. Bai, X.Q. Zhao, Condition-specific promoter activities in *Saccharomyces cerevisiae*, Microb. Cell Fact. 17 (2018) 58.

[11] L. Qin, S. Dong, J. Yu, X. Ning, K. Xu, S.-J. Zhang, L. Xu, B.-Z. Li, J. Li, Y.-J. Yuan, C. Li, Stress-driven dynamic regulation of multiple tolerance genes improves robustness and productive capacity of *Saccharomyces cerevisiae* in industrial lignocellulose fermentation, Metab. Eng. 61 (2020) 160–170.

[12] K. Shirk, H. Jin, T.H. Giddings Jr., M. Winey, H.G. Yu, The Aurora kinase Ipl1 is necessary for spindle pole body cohesion during budding yeast meiosis, J. Cell Sci. 124 (2011) 2891–2896.

[13] S.W. Stevens, J. Abelson, Purification of the yeast U4/U6.U5 small nuclear ribonucleoprotein particle and identification of its proteins, Proc. Natl. Acad. Sci. USA 96 (1999) 7226–7231.

[14] N. Panchaud, M.P. Péli-Gulli, C. De Virgilio, Amino acid deprivation inhibits TORC1 through a GTPase-activating protein complex for the Rag family GTPase Gtr1, Sci. Signal. 6 (2013) ra42.

[15] L.Y. He, X.Q. Zhao, X.M. Ge, F.W. Bai, Identification and functional study of a new FLO10-derivative gene from the industrial flocculating yeast SPSC01, J. Ind. Microbiol. Biotechnol. 39 (2012) 1135–1140.

[16] Y. Lu, S. Xiao, M. Yuan, Y. Gao, J. Sun, C. Xue, Using overlap-extension PCR technique to fusing genes for constructing recombinant plasmids, J. Basic Microbiol. 58 (2018) 273–276.

[17] M. Bergkessel, C. Guthrie, Chemical transformation of yeast, Methods Enzym. 529 (2013) 311–320.

[18] C. Ma, X. Wei, C. Sun, F. Zhang, J. Xu, X. Zhao, F. Bai, Improvement of acetic acid tolerance of *Saccharomyces cerevisiae* using a zinc-finger-based artificial transcription factor and identification of novel genes involved in acetic acid tolerance, Appl. Microbiol. Biotechnol. 99 (2015) 2441–2449.

[19] M.-A. Teste, M. Duquenne, J.M. François, J.-L. Parrou, Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*, BMC Mol. Biol. 10 (2009) 99.

[20] B. Li, L. Wang, Y.J. Wu, Z.Y. Xia, B.X. Yang, Y.Q. Tang, Improving acetic acid and furfural resistance of xylose-fermenting *Saccharomyces cerevisiae* strains by regulating novel transcription factors revealed via comparative transcriptomic analysis, Appl. Environ. Microbiol. 87 (2021).

[21] N.S.B. Mat Nanyan, H. Takagi, Proline homeostasis in *Saccharomyces cerevisiae*: how does the stress-responsive transcription factor Msn2 play a role? Front. Genet. 11 (2020) 438.

[22] N.P. Mira, S.F. Henriques, G. Keller, M.C. Teixeira, R.G. Matos, C.M. Arraiano, D. R. Winge, I. Sá-Correia, Identification of a DNA-binding site for the transcription factor Haa1, required for *Saccharomyces cerevisiae* response to acetic acid stress, Nucleic Acids Res. 39 (2011) 6896–6907.