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Research article

Adaptive evolution and selection of stress-resistant *Saccharomyces cerevisiae* for very high-gravity bioethanol fermentation

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ABSTRACT

Background: In industrial yeasts, selection and breeding for resistance to multiple stresses is a focus of current research. The objective of this study was to investigate the tolerance to multiple stresses of *Saccharomyces cerevisiae* obtained through an adaptive laboratory evolution strategy involving a repeated liquid nitrogen freeze–thaw process coupled with multi-stress shock selection. We also assessed the related resistance mechanisms and very high-gravity (VHG) bioethanol production of this strain.

Results: Elite *S. cerevisiae* strain YF10-5, exhibiting improved VHG fermentation capacity and stress resistance to osmotic pressure and ethanol, was isolated following ten consecutive rounds of liquid nitrogen freeze–thaw treatment followed by plate screening under osmotic and ethanol stress. The ethanol yield of YF10-5 was 16% higher than that of the parent strain during 35% (w/v) glucose fermentation. Furthermore, there was upregulation of three genes (*HSP26*, *HSP30*, and *HSP104*) encoding heat-shock proteins involved in the stress response, one gene (*TPS1*) involved in the synthesis of trehalose, and three genes (*ADH1*, *HXK1*, and *PFK1*) involved in ethanol metabolism and intracellular trehalose accumulation in YF10-5 yeast cells, indicating increased stress tolerance and fermentative capacity. YF10-5 also showed excellent fermentation performance during the simultaneous saccharification and fermentation of VHG sweet potato mash, producing 13.40% (w/v) ethanol, which corresponded to 93.95% of the theoretical ethanol yield.

Conclusions: A multiple-stress-tolerant yeast clone was obtained using adaptive evolution by a freeze–thaw method coupled with stress shock selection. The selected robust yeast strain exhibits potential for bioethanol production through VHG fermentation.

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1. Introduction

Bioethanol, as a clean and renewable energy source, is considered a good alternative to fossil fuels. There is considerable research interest in the production of ethanol through microbial fermentation. Among many fermentative microorganisms that have been used for ethanol production, the yeast *Saccharomyces cerevisiae* is the best ethanol producer. However, during ethanol fermentation, *S. cerevisiae* cells are subject to various stresses, especially during very high-gravity (VHG) fermentation. In VHG fermentation, when high sugar concentrations (in excess of 250 g glucose l^{-1}) are used, high ethanol titers can be obtained (generally above 15% v/v) [1,2]. However, the hyperosmotic stress that results from high sugar and ethanol concentrations leads to a decrease in yeast cell proliferation and viability, ultimately resulting in a decrease in the efficiency of ethanol production [3]. Therefore, the selection and engineering of more robust yeast strains with properties tailored are critical for the efficient completion of VHG fermentation.

In *S. cerevisiae*, tolerance to environmental stresses is known to be a complex phenotype influenced by multiple genes, many of which are not well characterized [4]. Thus, the simultaneous improvement of yeast tolerance to multiple stresses by rational design with genetic and metabolic engineering techniques is challenging. To overcome this obstacle, several random mutational approaches using evolutionary engineering/adaptive evolution have been used to select *S. cerevisiae* mutants with improved performance in terms of multiple stress tolerance and fermentation [5,6,7,8,9]. Such methods can be used to obtain microorganisms with desired phenotypes not present in their genetic background or with a complex, multi-gene basis [10].

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Sweet potato (*Ipomoea batatas* Lam.) is a relatively ideal feedstock for bioethanol production, as it generally contains 20–30% starch, and it is produced at a rate of approximately 130 million tons/year in China [11]. Although VHG fermentation technology has been applied to ethanol production from cereal grains, sweet sorghum, sugar beet syrup, and potato [1,12,13,14,15,16], its application to sweet potato has rarely been reported [17]. Therefore, to compete with conventional grain-based ethanol production, there is a need for yeast strains that produce ethanol effectively under VHG sweet potato mash conditions.

In this study, an adaptive evolution strategy involving a repeated liquid nitrogen freeze–thaw process coupled with multi-stress shock selection was used to select *S. cerevisiae* clones with stress resistance and improved fermentation capacity under VHG conditions. Quantitative real-time PCR (qPCR) was used to analyze the transcriptional responses of the resulting strain, *S. cerevisiae* YF10-5, under VHG fermentation conditions. Functional analysis of the selected genes may provide important information regarding the mechanisms involved in stress tolerance and fermentation capacity of *S. cerevisiae*. The stability and application of YF10-5 for bioethanol production by simultaneous saccharification and fermentation of VHG sweet potato mash were also evaluated.

2. Materials and methods

2.1. Yeast strains, media, and growth conditions

S. cerevisiae Y-1 (CCTCC M206111) was isolated from wine lees and used for this study. The strain was maintained on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 4°C. YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) was used for precultivation at 30°C with aeration and agitation (200 rpm). Fermentation medium was prepared as follows (all are expressed in g compound l^{-1}): yeast extract, 5.0; peptone, 5.0; $(NH_4)_2SO_4$, 1.5; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.65; $CaCl_2$, 2.8; inositol, 0.85; thiamin, 0.35; pyridoxine, 0.004; nicotinic acid, 0.004; para-aminobenzoic acid, 0.007; biotin, 0.000024, and pantothenate, 0.005. Glucose was added at various concentrations. Fermentation medium containing 35% (w/v) glucose was used for the screening of stress-tolerant strains. All other cultivation procedures were performed in yeast minimal medium (YMM) containing 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose in Erlenmeyer flasks under aerobic conditions at 30°C and 150 rpm. Raw fresh sweet potato (Shangshu19) was provided by the Sichuan Academy of Agricultural Sciences of China.

2.2. Freeze–thaw treatment and screening of stress-resistant yeasts

Cultures of *S. cerevisiae* at the late exponential phase (approximately 1×10^8 cells per ml) were collected in 2-ml micro-vial tubes and directly immersed in liquid nitrogen for 30 min before thawing in a 30°C water bath for 10 min. Afterwards, yeast cells were inoculated into YMM and incubated at 30°C until the late exponential phase for subsequent successive freeze–thaw treatments.

After repeated freeze–thaw cycles (up to 10) were performed, cells were diluted and plated on YPD agar plates containing 12% (v/v) ethanol and 30% (w/v) glucose and incubated at 30°C until the appearance of colonies. Ethanol was added to cooled YPD agar at the time of pouring, and plates were sealed with a parafilm to prevent ethanol volatilization. All plates were allowed to solidify at room temperature and used immediately for screening the stress-resistant strains. Well-grown colonies were selected and pre-incubated with the YPD liquid medium until reaching the exponential growth phase. Then, the cells were incubated in the fermentation medium containing 35% (w/v) glucose. The residual glucose and produced ethanol concentrations were analyzed as described below.

2.3. Analytical methods

The optical density at 620 nm (OD_{620}) was measured to monitor cell growth. The glucose concentration was determined by the 3,5-dinitrosalicylic acid (DNS) method [18]. The ethanol concentration was analyzed with a gas chromatograph (FULI 9770, China) equipped with a GDX103 packed column (FULI), a flame ionization detector, and a computing integrator system at a column temperature of 95°C, injection temperature of 150°C, and detector temperature of 150°C, respectively. Before analysis, all samples were centrifuged at 4000 × *g* for 5 min to remove solid particles, and *n*-propanol (final concentration, 2% v/v) was used as an internal standard [19]. Ethanol productivity (g l^{-1} h^{-1}) was calculated as the ratio between the final ethanol concentration and total fermentation time. Ethanol yield (%) was calculated as the percentage of produced ethanol out of the maximal theoretical yield (0.511 g g^{-1}) based on the amount of glucose present in the fermentation medium.

2.4. Analysis of yeast stress tolerance

To assess tolerance to particular stresses, yeast cells were preincubated in YPD liquid medium to the exponential growth phase, and the OD_{620} value was adjusted to 1.0 for all cultures. Then, serial dilution of the cultures was carried out. To determine yeast tolerance to osmotic stress, 5 μl of each serial dilution was spotted onto YPD plates containing glucose concentrations of 30%, 40%, or 50% (w/v). For ethanol stress analysis, serial dilutions of exponential cultures were spotted onto YPD agar plates containing ethanol concentrations of 12%, 16%, or 20% (v/v); each plate was sealed with a parafilm and put in a plastic bag to prevent ethanol volatilization. All plates were incubated at 30°C until colonies appeared (4 d). For heat stress analysis, serial dilutions of cells were spotted onto YPD agar plates, and the resulting plates were incubated at 37°C or 40°C. The parent strain Y-1 was used as a control.

The cell growth of YF10-5 and Y-1 in YPD liquid medium in shake flasks (100 ml) under stress shock was determined and monitored for various time points (12, 24, 36, and 48 h). The flasks were sealed with rubber stoppers and a plastic film. Stress shock protocols for heat, ethanol, and osmotic stresses involved the following: incubation at 40°C, growth at 20% (v/v) ethanol, and growth at 50% (w/v) glucose in medium, respectively.

2.5. q-PCR

Cells were collected in the logarithmic phase (24 h) and stationary phase (48 h) following VHG fermentation with 35% glucose and washed with RNase-free water. Total RNA was extracted and purified using the RNAprep pure Cell/Bacteria Kit (Tiangen, Beijing, China) with DNase I treatment, according to the instructions of the manufacturer. The concentration and purity of RNA were determined by spectrophotometric analysis based on the absorbance ratio at 260/280 nm. RNA obtained from the parent strain Y-1 following VHG fermentation with 35% glucose was used as a control sample. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

qPCR was performed using SsoFast Evagreen Supermix (Bio-Rad) on a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). The cycle conditions were as follows: initial denaturation at 98°C for 2 min, followed by 40 cycles of 98°C for 5 s, 54°C for 15 s, and an increase in temperature from 65 to 95°C in intervals of 5 s to generate a melting curve. Relative gene expression levels were analyzed by comparison to the internal reference gene *ACT1*. Primer sequences used for qPCR analysis were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are given in Table 1. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method [20]. All experiments were performed in triplicate.

Table 1
Primers used for quantitative real-time PCR.

Primer	Sequence 5′ → 3′	Amplicon (bp)	Gene/ORF	Reference/Source
ACT-F	TGTTACTCACGTCGTTCCAAT	103	*ACT1*	[21]
ACT-R	GATCTTCATCAAGTAGTCAGTCAA			
HSP12-F	CGCAGGTAGAAAAGGATTCG	105	*HSP12*	[22]
HSP12-R	TCGGCCTTGTCAGTGATGTA			
HSP26-F	CTTGTCCCTGTTCCCATCTG	109	*HSP26*	[22]
HSP26-R	GACACCAGGAACCACGACTT			
HSP30-F	TTGGACTGGTGTTCAAGCTG	117	*HSP30*	[22]
HSP30-R	CAGGACAAGAACCAGGCAAT			
HSP104-F	TGAAGTCGCTGAACCAAGTG	125	*HSP104*	[22]
HSP104-R	TGGCTAATTGAGCAGCAGTG			
TPS1-F	AACTTTCAAGGGCTGCAAGA	114	*TPS1*	[22]
TPS1-R	CTCCATTCTGGATGCTCGTT			
ADH1-F	GTCGGTGCTGTTCTAAAGGC	114	*ADH1*	This study
ADH1-R	GCTTCAATAGCGGCTTCGG			
HXK1-F	CCCAGCTTCCCAAAACAAGA	116	*HXK1*	This study
HXK1-R	TGGCCTTCGACATTTGGAATA			
PFK1-F	TCGCAAGGTATTGACGCTTTG	109	*PFK1*	This study
PFK1-R	TGGCCATTCGTGTCTGAAAA			
PYK1-F	GATGTCGATTTGCCAGCTTTG	115	*PYK1*	This study
PYK1-R	GAAGACCATGTGGACACCGTT			

2.6. Analysis of trehalose

Trehalose was extracted from washed cells with cold 0.5 M trichloroacetic acid, and levels were estimated by the anthrone method as previously described [23]. Cell dry weight determination was performed by filtration of a known volume of sample through a 0.45-μm pore size polyamide membrane, followed by drying at 105°C until constant weight.

2.7. Simultaneous saccharification and fermentation of sweet potato mash

Fresh sweet potato (Shangshu19) tubers (unpeeled) were washed and mashed using a Philips Juicer HR2826 (Royal Philips Electronics Co., Ltd., The Netherlands). Sweet potato mash was then liquefied with thermostable α-amylase (Liquozyme Supra, 90 KNU g^{-1}) at an optical concentration of 0.12 KNU per g sweet potato mash and incubated at 85°C for 20 min in a water bath. After liquefaction, the mash was cooled to 30°C, and glucoamylase (Suhong GA II 500 AGU g^{-1}, 1.60 AGU per g sweet potato mash), ammonium sulfate (0.15% w/w), and *S. cerevisiae* inoculum (10% v/w) were simultaneously added. Simultaneous saccharification and fermentation was performed in a 250-ml Erlenmeyer flask containing 100 g of sweet potato mash at 30°C with shaking at 150 rpm.

3. Results and discussion

3.1. Screening of stress-resistant yeasts

Generally, multiple cycles of random genetic perturbation and selection are performed sequentially, resulting in various genetic alterations, including those that are phenotypically advantageous [24]. Tolerance to high osmotic pressure and to high ethanol concentrations is an important characteristic for yeasts in VHG fermentation. To obtain such yeasts, ten rounds of liquid nitrogen freeze–thaw treatments were performed, resulting in 36 colonies derived from YPD agar plates containing 12% (v/v) ethanol and 30% (w/v) glucose after 4 d of cultivation. Among these colonies, ethanol fermentation tests were performed in a medium containing 35% (w/v) glucose. The ethanol concentrations, residual glucose, ethanol yield, and ethanol productivity were monitored, and five colonies with higher fermentation capacities than the parent strain *S. cerevisiae* Y-1 (Table 2) were selected and numbered YF10-1, YF10-2, YF10-3, YF10-4, and YF10-5. Among these strains, YF10-5 exhibited the highest fermentation capacity and produced the highest ethanol concentration at 14.58% (w/v), which was 16% higher than that of the parent strain. Based on the results of the screening tests and VHG ethanol fermentation assays, the robust variant strain YF10-5 was selected for further study. Moreover, good stability of the higher ethanol productivity of YF10-5 under VHG conditions was observed after eight cycles of repeated batch fermentation experiments (data not shown).

3.2. Multi-stress tolerance of yeast strains to osmotic, ethanol, and heat stress

Osmotic stress is the first challenge for yeast cells that must be overcome at the beginning of VHG fermentation. Analysis of yeast cell survival under osmotic stress could provide useful information regarding their ability to initiate growth and carry out VHG fermentation rapidly. In the stress tolerance analysis, non-stressed Y-1 cells were grown on YPD agar plates as a control to understand the effect of each stress condition on cell viability. As shown in Fig. 1, the growth of YF10-5 on 50% (w/v) glucose YPD agar plates was less affected than that of the control, indicating that the selected strain exhibited improved osmotic stress resistance following the repeated freeze–thaw treatments. By contrast, the parent strain Y-1 grew poorly owing to its high sensitivity to high osmotic pressure. Similarly, during growth in liquid medium, YF10-5 cells grew more rapidly than the parental Y-1 cells (Fig. 2).

Ethanol is one of the predominant inhibitors of yeast cells in the latter phase of the fermentation process [25]. Resistance to this type of stress is considered a key factor in achieving high ethanol concentrations. Fig. 1 shows the results of experiments carried out on YPD agar plates containing 12%, 16%, and 20% (v/v) exogenous

Table 2
Residual glucose, ethanol concentration, ethanol productivity, and ethanol yield after 60 h of fermentation in medium containing 35% glucose at 30°C.

Strain	Glucose (% w/v)	Ethanol (% w/v)	Productivity (g l^{-1} h^{-1})	Ethanol yield (%)
Y-1	7.57 ± 0.38	12.50 ± 0.02	2.08 ± 0.00	71.53 ± 2.16
YF10-1	6.00 ± 1.24	13.25 ± 0.13	2.21 ± 0.02	74.65 ± 0.01
YF10-2	6.07 ± 0.95	13.20 ± 0.59	2.20 ± 0.01	74.53 ± 0.03
YF10-3	5.78 ± 0.21	13.76 ± 0.41	2.29 ± 0.01	82.06 ± 0.04
YF10-4	5.91 ± 1.10	13.58 ± 0.54	2.26 ± 0.00	77.67 ± 0.02
YF10-5	5.50 ± 0.29	14.58 ± 0.43	2.43 ± 0.10	83.00 ± 0.19

Results are the mean ± standard deviation of three independent experiments.

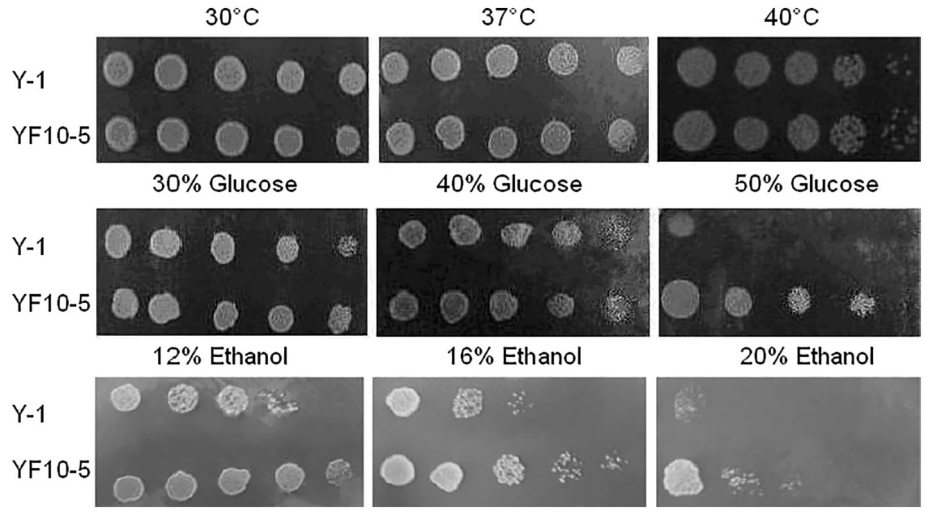

Fig. 1. Osmotic, ethanol, and heat stress tolerance of strains Y-1 and YF10-5. Yeast cells were grown to the exponential phase and collected by centrifugation. Tenfold serial dilutions of each culture ($OD_{620} = 1.0$) were spotted onto YPD plates under the indicated stress conditions. Plates were incubated at 30°C until colonies appeared (4 d). Experiments were performed in triplicate. One representative experiment is shown.

ethanol. All yeast strains showed high tolerance to 12% ethanol. However, the parental Y-1 strain was unable to grow on ethanol plates containing 20% ethanol, while YF10-5 was able to grow on these plates, indicating that YF10-5 had acquired improved ethanol stress resistance. Higher cell growth rates of YF10-5 were also observed in liquid medium (Fig. 2). Sharma [26] has reported an overlap between osmotolerance and ethanol tolerance in *S. cerevisiae*, suggesting that improved osmotolerance may be one of the factors contributing to the improved ethanol tolerance of YF10-5.

Moreover, when yeast cells respond to one particular form of stress, they often acquire cross-resistance to other types of stress [27]. We also tested whether YF10-5 exhibited improved tolerance to heat stress. However, as shown in Fig. 1 and Fig. 2, both YF10-5 and the parental Y-1 strain grew similarly at 40°C, respectively. This was consistent with cell growth analysis under 50°C heat shock for an hour in which the YF10-5 strain showed a cell viability of 40.0% and Y-1 exhibited 40.6% (data not shown). These results suggest that the multi-stress tolerance of YF10-5 to high osmotic pressure and ethanol did not confer improved thermal tolerance but that YF10-5 did maintain the original high temperature tolerance of the Y-1 strain.

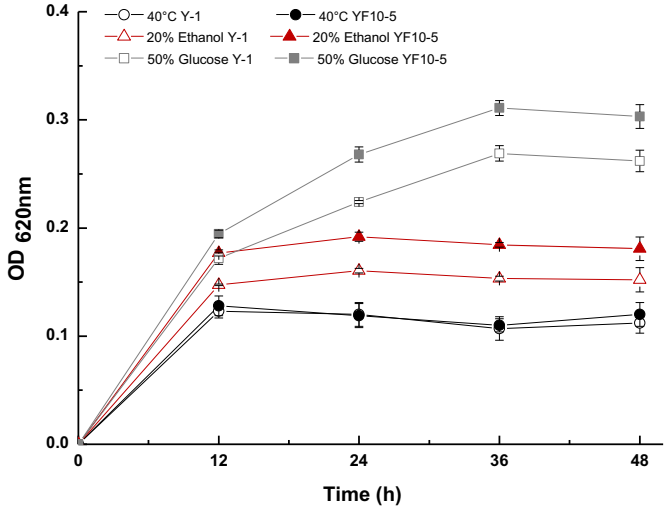

Fig. 2. Cell growth of YF10-5 and Y-1 in YPD medium after stress treatment (40°C, 20% ethanol, and 50% glucose). The values shown represent the mean values of three replicate experiments. 40°C.

3.3. Analysis of relative transcription levels by qPCR

The induced expression of trehalose and heat-shock proteins (HSPs) in yeast cells has been shown to correlate with stress tolerance [28]. To better understand the transcriptional response of *S. cerevisiae* YF10-5 to multiple stresses and ethanol fermentation under VHG conditions (35% glucose), samples of YF10-5 and Y-1 were collected during fermentation at the logarithmic phase (24 h) and stationary phase (48 h), and the mRNA levels of *TPS1*, *HSP12*, *HSP26*, *HSP30*, *HSP104*, *ADH1*, *HXK1*, *PFK1*, and *PYK1* were evaluated by qPCR (Fig. 3).

TPS1 encodes a subunit of the trehalose synthase complex that affects trehalose synthesis [29]. The Tps1 protein has been identified as a key player in yeast survival in response to temperature, oxidative, and desiccation stress [30]. As shown in Fig. 3, transcription of the *TPS1* gene in YF10-5 was enhanced under VHG fermentation challenge compared with that in the parental strain, with expression levels almost 4-fold and 7-fold higher at 24 and 48 h, respectively. Similar upregulation of *TPS1* was previously observed in *S. cerevisiae* under ethanol, heat, and osmotic stress conditions [22,31–33]. Pereira et al. [34] also reported that *TPS1*-deficient *S. cerevisiae* showed poor tolerance to heat and alcoholic stresses. Moreover, we observed an accumulation of trehalose in YF10-5 yeast cells during VHG fermentation (Fig. 4). These results suggest that the accumulation of trehalose is an adaptive response of yeast cells under VHG fermentation stress.

HSPs act primarily as molecular chaperones, preventing protein aggregation and playing important roles in yeast resistance to adverse environmental conditions [35]. As shown in Fig. 3, expression of three of the four examined HSP genes, namely, *HSP26*, *HSP30*, and *HSP104*, was upregulated at 24 h and 48 h in YF10-5 cells compared with levels in the parental strain Y-1. These three genes showed similar expression profiles, and their expression in cells at the stationary phase was significantly higher than that at the logarithmic phase. Among these genes, *HSP26* exhibited the greatest increase in expression (2.79- and 3.82-fold at 24 and 48 h, respectively). In contrast, for the fourth HSP gene, *HSP12*, expression first increased by 2.70-fold at 24 h and then decreased by 0.91-fold at 48 h compared to control levels. It has been shown that mutants lacking *HSP12*, *HSP26*, *HSP30*, or *HSP104* are hypersensitive to ethanol stress, suggesting the important role of these genes in conferring enhanced ethanol tolerance [22,36,37]. Moreover, Cashikar et al. [38] found that an *HSP26*/*HSP104* double-mutant was hypersensitive to heat shock, suggesting their cooperative role in adaptation to heat stress.

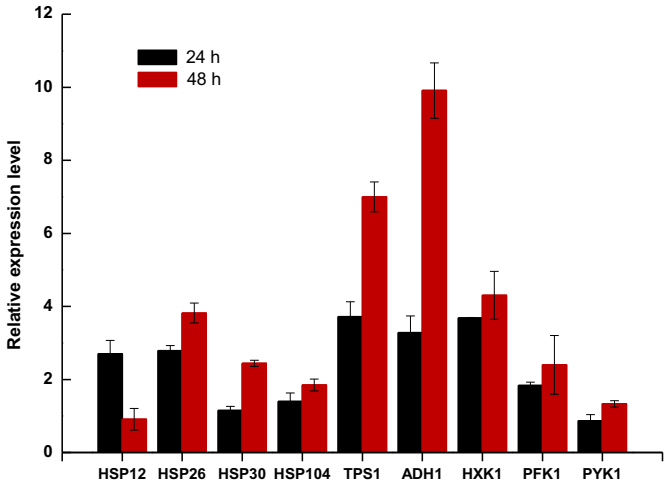

Fig. 3. Relative transcript levels of genes involved in stress tolerance and ethanol fermentation in YF10-5 and Y-1 under VHG fermentation (35% glucose). The results are shown as the mean values of three independent experiments.

Consistent with these findings, the enhanced transcription of HSP genes in the selected YF10-5 strain under VHG fermentation with a high glucose concentration (35%) suggests the important role of HSPs in tolerance to multiple stresses.

The transcriptional dynamics of genes encoding key enzymes in the glycolysis pathway, such as *HXK1*, *PFK1*, *PYK1*, and *ADH1*, were also assessed to investigate their role in the regulation of the fermentative capacity of YF10-5. Three genes, namely, *HXK1*, *PFK1*, and *ADH1*, were upregulated at 24 h and 48 h after exposure to the harsh VHG fermentation environment (Fig. 3). In particular, *ADH1* was highly upregulated by 9.91-fold at 48 h. The enhanced transcription of *PFK1* has also been observed in *S. cerevisiae* during must fermentation in the presence of sulfite stress [39]. In contrast, *PYK1*, encoding pyruvate kinase, was downregulated at 24 h and then upregulated at 48 h compared with expression in the parental Y-1 strain. The results suggested that the enhanced gene expression of *HXK1*, *PFK1*, *ADH1*, and *PYK1* in YF10-5 is critical to better complete the VHG ethanol fermentation. Therefore, the enhanced expression of genes encoding trehalose biosynthesis enzymes, HSPs, and key glycolysis enzymes contributes to the multi-stress tolerance of YF10-5 and indirectly to improved cell growth and synergistic efficiency during VHG ethanol fermentation.

3.4. Intracellular trehalose accumulation

It is well known that yeast cells accumulate trehalose when exposed to various severe environmental stresses such as high osmotic pressure, high ethanol concentration, and high temperature [40–42]. Trehalose is important for maintaining cell longevity, avoiding mitochondrial mutation, and improving ethanol production [43]. As shown in Fig. 4, during 35% (w/v) glucose VHG fermentation,

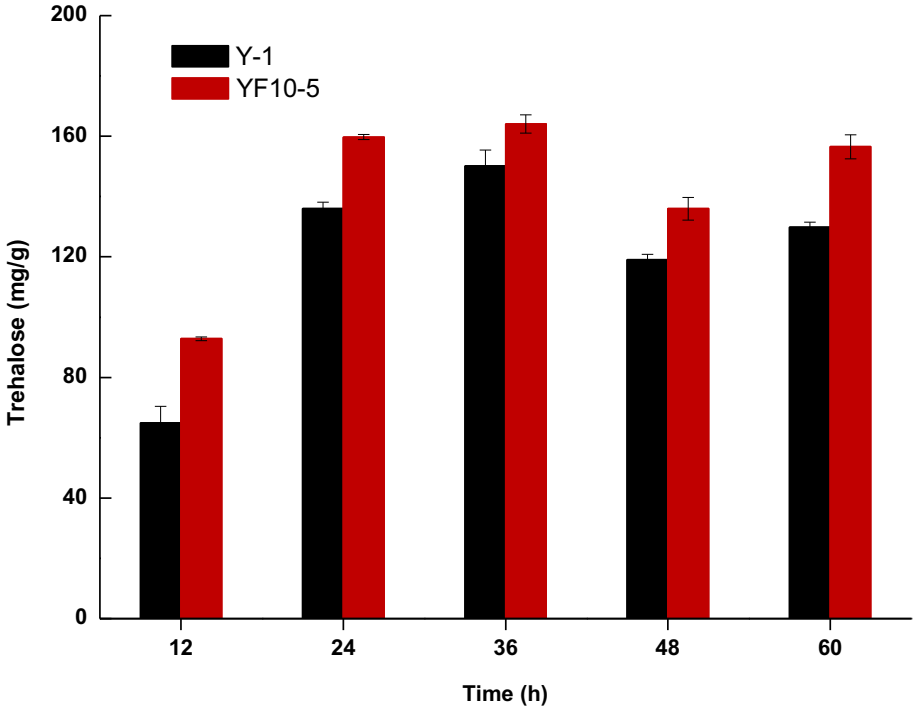

Fig. 4. Trehalose accumulation in strains Y-1 and YF10-5 during VHG fermentation (35% glucose). The results are shown as the mean values of three independent experiments.

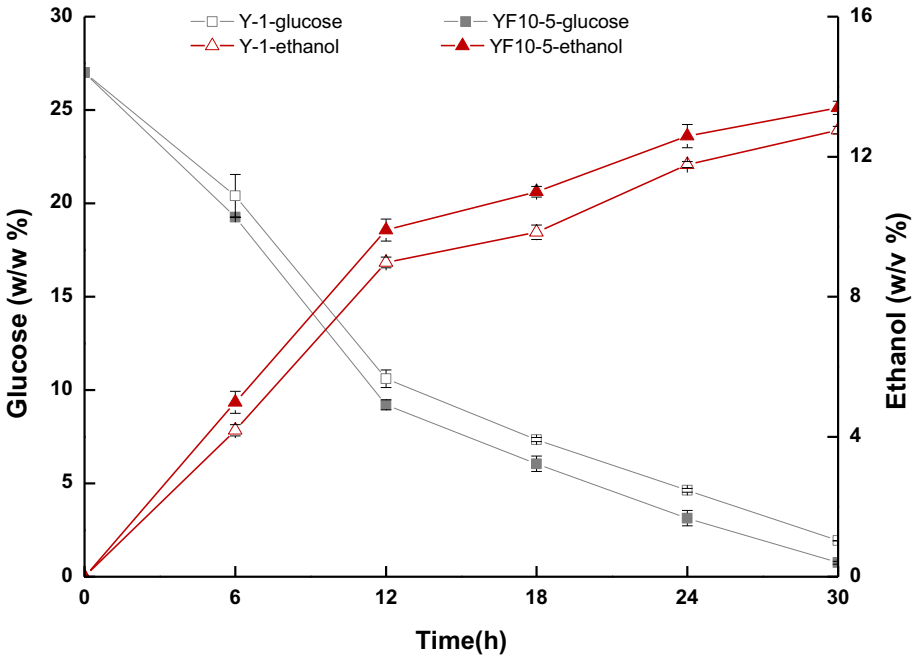

Fig. 5. Time courses of ethanol and glucose concentrations during VHG-simultaneous saccharification and fermentation of sweet potato mash. The results are shown as the mean values of three independent experiments.

the amount of intracellular trehalose that accumulated in the YF10-5 strain was higher than that in the parent strain. Specifically, trehalose accumulation in YF10-5 was 42.9% and 20.5% higher than that in Y-1 in the early (12 h) and late (60 h) stages of VHG fermentation, respectively. This increase in intracellular trehalose accumulation in response to stress may partly explain why yeast strain YF10-5 is more tolerant to osmotic and ethanol stress and has a higher VHG fermentative capacity. Furthermore, stress-resistant isolates such as YF10-5 may be good candidates for further metabolic engineering efforts.

3.5. Simultaneous saccharification and fermentation of sweet potato mash

To further evaluate the fermentation efficiency of mutant YF10-5, simultaneous saccharification with glucoamylase and fermentation was performed using VHG sweet potato mash. The initial total reducing sugar of the sweet potato mash was 27.2% (w/w). As shown in Fig. 5, among the two strains tested, YF10-5 showed better fermentation performance and produced a greater amount of ethanol (13.40% w/v) from sweet potato mash within 30 h, with a residual reducing sugar content of 0.74% (w/v). In contrast, the parent strain produced 12.75% (w/v) ethanol, with a residual reducing sugar content of 1.73% (w/v) due to its poor stress resistance and fermentation capacity. The ethanol productivity and ethanol yield of YF10-5 were 4.47 g l^{-1} h^{-1} and 93.95%, respectively (Table 3). These results indicate that the selected robust yeast strain YF10-5 may be useful in VHG-simultaneous saccharification and fermentation processes.

Table 3
The fermentation performance of Y-1 and YF10-5 during VHG-simultaneous saccharification and fermentation of sweet potato mash at 30°C.

Variable	Y-1	YF10-5
Total reducing sugar (% w/w)	27.2 ± 0.18	27.2 ± 0.18
Ethanol (% w/v)	12.75 ± 0.11	13.40 ± 0.18
Residual reducing sugar (% w/w)	1.73 ± 0.01	0.74 ± 0.08
Fermentation time (h)	30	30
Productivity (g l^{-1} h^{-1})	4.21 ± 0.04	4.47 ± 0.06
Ethanol yield (%)	90.03 ± 0.47	93.95 ± 0.21

Results are the mean ± standard deviation of three independent experiments.

3.6. Conclusions

Multiple cycles of freeze–thaw treatment followed by osmotic pressure and ethanol stress shock selection produced the robust yeast strain YF10-5, which exhibits improved osmotolerance, ethanol tolerance, and fermentation capacity during VHG fermentation compared to those of the parent strain. Furthermore, YF10-5 exhibited upregulation of three genes encoding HSPs (*HSP26*, *HSP30*, and *HSP104*) involved in the stress response, one gene (*TPS1*) involved in the synthesis of trehalose, and three genes (*ADH1*, *HXK1*, and *PFK1*) involved in ethanol metabolism, as well as increased intracellular trehalose accumulation. YF10-5 showed significantly improved fermentation performance during VHG-simultaneous saccharification and fermentation with sweet potato mash, producing 13.40% (w/v) ethanol. Thus, this strain has potential for VHG bioethanol production.

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Conflict of interest

The authors declare no conflict of interest.

References

[1] Bai FW, Anderson WA, Moo-Young M. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 2008;26(1):89–105. https://doi.org/10.1016/j.biotechadv.2007.09.002 PMid: 17964107.

[2] Thomas KC, Hynes SH, Ingledew WM. Practical and theoretical considerations in the production of high concentration of alcohol by fermentation. Process Biochem 1996; 31(4):321–31. https://doi.org/10.1016/0032-9592(95)00073-9.
[3] Stanley D, Bandara A, Fraser S, et al. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol 2010;109(1):13–24. https://doi.org/10.1111/j.1365-2672.2009.04657.x PMid: 20070446.
[4] Zhao XQ, Bai FW. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J Biotechnol 2009;144(1):23–30. https://doi.org/10.1016/j.jbiotec.2009.05.001 PMid: 19446584.
[5] Çakar ZP, Seker UO, Tamerler C, et al. Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res 2005;5(6–7):569–78. https://doi.org/10.1016/j.femsyr.2004.10.010 PMid: 15780656.
[6] Wei P, Li Z, Lin Y, et al. Improvement of the multiple-stress tolerance of an ethanologenic *Saccharomyces cerevisiae* strain by freeze–thaw treatment. Biotechnol Lett 2007;29(10):1501–8. https://doi.org/10.1007/s10529-007-9419-1 PMid: 17541503.
[7] Mans R, Daran JG, Pronk JT. Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. Curr Opin Biotechnol 2018;50:47–56. https://doi.org/10.1016/j.copbio.2017.10.011 PMid: 29156423.
[8] Turanlı-Yıldız B, Benbadis L, Alkım C, et al. *In vivo* evolutionary engineering for ethanol-tolerance of *Saccharomyces cerevisiae* haploid cells triggers diploidization. J Biosci Bioeng 2017;124(3):309–18. https://doi.org/10.1016/j.jbiosc.2017.04.012 PMid: 28552194.
[9] Gurdo N, Novelli Poisson GF, Juárez AB, et al. Improved robustness of an ethanologenic yeast strain through adaptive evolution in acetic acid is associated with its enzymatic antioxidant ability. J Appl Microbiol 2018;125(3):766–76. https://doi.org/10.1111/jam.13917 PMid: 29770550.
[10] Perrone G, Tan S, Dawes I. Reactive oxygen species and yeast apoptosis. BBA-Mol Cell Res 2008;1783(7):1354–68. https://doi.org/10.1016/j.bbamcr.2008.01.023 PMid: 18298957.
[11] Li SZ, Chan-Halbrendt C. Ethanol production in (the) People's Republic of China: potential and technologies. Appl Energy 2009;86(1):S162–9. https://doi.org/10.1016/j.apenergy.2009.04.047.
[12] Watanabe T, Srichuwong S, Arakane M, et al. Selection of stress-tolerant yeasts for simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash to ethanol. Bioresour Technol 2010;101(24):9710–4. https://doi.org/10.1016/j.biortech.2010.07.079 PMid: 20705456.
[13] Larnaudie V, Rochón E, Ferrari MD, et al. Energy evaluation of fuel bioethanol production from sweet sorghum using very high gravity (VHG) conditions. Renew Energy 2016;88:280–7. https://doi.org/10.1016/j.renene.2015.11.041.
[14] Joannis-Cassan C, Riess J, Jolibert F, et al. Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup. Biomass Bioenergy 2014;70:165–73. https://doi.org/10.1016/j.biombioe.2014.07.027.
[15] Kawa-Rygielska J, Pietrzak W. Ethanol fermentation of very high gravity (VHG) maize mashes by *Saccharomyces cerevisiae* with spent brewer's yeast supplementation. Biomass Bioenergy 2014;60:50–7. https://doi.org/10.1016/j.biombioe.2013.10.028.
[16] Appiah-Nkansah NB, Zhang K, Rooney W, et al. Ethanol production from mixtures of sweet sorghum juice and sorghum starch using very high gravity fermentation with urea supplementation. Ind Crop Prod 2018;111:247–53. https://doi.org/10.1016/j.indcrop.2017.10.028.
[17] Zhang L, Chen Q, Jin YL, et al. Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). Fuel Process Technol 2010;91(12):1845–50. https://doi.org/10.1016/j.fuproc.2010.08.009.
[18] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31(3):426–8. https://doi.org/10.1021/ac60147a030.
[19] Antony JC. Malt beverages and malt brewing materials: gas chromatographic determination of ethanol in beer. J Assoc Off Anal Chem 1984;67:192–3.
[20] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2_T^{-\Delta\Delta C}$ method. Methods 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMid: 11846609.
[21] Chen LY, Widom J. Mechanism of transcriptional silencing in yeast. Cell 2005;120(1):37–48. https://doi.org/10.1016/j.cell.2004.11.030 PMid: 15652480.
[22] Ma MG, Liu ZL. Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol 2010;10:169–88. https://doi.org/10.1186/1471-2180-10-169 PMid: 20537179.
[23] Lillie SH, Pringle JR. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J Bacteriol 1980;143(3):1384–94. [PMid: 6997270].
[24] Nevoigt E. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 2008;72(3):379–412. https://doi.org/10.1128/MMBR.00025-07 PMid: 18772282.
[25] Dinh TN, Nagahisa K, Yoshikawa K, et al. Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioprocess Biosyst Eng 2009;32:681–8. https://doi.org/10.1007/s00449-008-0292-7 PMid: 19125301.
[26] Sharma SC. A possible role of trehalose in osmotolerance and ethanol tolerance in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 1997;152(1):11–5. https://doi.org/10.1111/j.1574-6968.1997.tb10402.x PMid: 9228764.
[27] Park JI, Grant CM, Attfield PV, et al. The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS–cyclic AMP signal transduction pathway. Appl Environ Microbiol 1997; 63(10):3818–24. [PMid: 9327544].
[28] Estruch F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 2000;24(4):469–86. https://doi.org/10.1111/j.1574-6976.2000.tb00551.x PMid: 10978547.
[29] Hazell BW, Kletsas S, Nevalainen H, et al. Involvement of *CIF1* (*GGS1*/*TPS1*) in osmotic stress response in *Saccharomyces cerevisiae*. FEBS Lett 1997;414(2):353–8. https://doi.org/10.1016/S0014-5793(97)01033-8.
[30] Petitjean M, Teste MA, François JM, et al. Yeast tolerance to various stresses relies on the trehalose-6P synthase (Tps1) protein, not on trehalose. J Biol Chem 2015;290: 16177–90. https://doi.org/10.1074/jbc.M115.653899 PMid: 25934390.
[31] Alexandre H, Ansanay-Galeote V, Dequin S, et al. Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Lett 2001;498(1): 98–103. https://doi.org/10.1016/S0014-5793(01)02503-0.
[32] Mensonides FIC, Hellingwerf KJ, Mattos MJTD, et al. Multiphasic adaptation of the transcriptome of *Saccharomyces cerevisiae* to heat stress. Food Res Int 2013;54(1): 1103–12. https://doi.org/10.1016/j.foodres.2012.12.042.
[33] Hirasawa T, Ashitani K, Yoshikawa K, et al. Comparison of transcriptional responses to osmotic stresses induced by NaCl and sorbitol additions in *Saccharomyces cerevisiae* using DNA microarray. J Biosci Bioeng 2006;102(6):568–71. https://doi.org/10.1263/jbb.102.568 PMid: 17270724.
[34] Pereira MD, Eleutherio ECA, Panek AD. Acquisition of tolerance against oxidative damage in *Saccharomyces cerevisiae*. BMC Microbiol 2001;1(11). https://doi.org/10.1186/1471-2180-1-11 PMid: 11483159.
[35] Piper P. The yeast heat shock response. In: Hohmann S, Mager WH, editors. Yeast stress responses. New York: Chapman & Hall; 1997. p. 75–99.
[36] Sanchez Y, Parsell DA, Taulien J, et al. Genetic evidence for a functional relationship between Hsp104 and Hsp70. J Bacteriol 1993;175:6484–9. https://doi.org/10.1128/jb.175.20.6484-6491.1993 PMid: 8407824.
[37] Sales K, Brandt W, Rumbak E, et al. The LEA-like protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. Biochim Biophys Acta 2000;1463(2):267–78. https://doi.org/10.1016/S0005-2736(99)00215-1.
[38] Cashikar AG, Duennwald M, Lindquist SL. A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. J Biol Chem 2005;280(25):23869–75. https://doi.org/10.1074/jbc.M502854200 PMid: 15845535.
[39] Nadai C, Campanaro S, Giacomini A, et al. Selection and validation of reference genes for quantitative real-time PCR studies during *Saccharomyces cerevisiae* alcoholic fermentation in the presence of sulfite. Int J Food Microbiol 2015;215:49–56. https://doi.org/10.1016/j.ijfoodmicro.2015.08.012 PMid: 26325600.
[40] Yoshiyama Y, Tanaka K, Yoshiyama K, et al. Trehalose accumulation enhances tolerance of *Saccharomyces cerevisiae* to acetic acid. J Biosci Bioeng 2015;119(2):172–5. https://doi.org/10.1016/j.jbiosc.2014.06.021 PMid: 25060731.
[41] Ding J, Huang X, Zhang L, et al. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2009;85:253–63. https://doi.org/10.1007/s00253-009-2223-1 PMid: 19756577.
[42] Zhang M, Shi J, Jiang L. Modulation of mitochondrial membrane integrity and ROS formation by high temperature in *Saccharomyces cerevisiae*. Electron J Biotechnol 2015;18(3):202–9. https://doi.org/10.1016/j.ejbt.2015.03.008.
[43] Trevisol ET, Panek AD, Mannarino SC, et al. The effect of trehalose on the fermentation performance of aged cells of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2011;90:697–704. https://doi.org/10.1007/s00253-010-3053-x PMid: 21243352.