Research article

Adaptive evolution and selection of stress-resistant *Saccharomyces cerevisiae* for very high-gravity bioethanol 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 \( \text{L}^{-1} \)) 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].
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, and 2% glucose) and used for precultivation at 30°C with aeration and agitation (200 rpm). Fermentation medium was prepared as follows (all are expressed in g per 1 L medium): yeast extract, 5.0; peptone, 5.0; (NH₄)₂SO₄, 1.5; KH₂PO₄, 1.5; MgSO₄·7H₂O, 0.65; CaCl₂, 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/w) glucose was used for the screening of stress-tolerant strains. All other culture 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 × 10⁸ 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 (OD₆₂₀) 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 (FULL), 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 OD₆₂₀ 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 SoFast 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⁻ΔΔC T method [20]. All experiments were performed in triplicate.
alterations, including those that are phenotypically advantageous [24].

Selection are performed sequentially, resulting in various genetic

3. Results and discussion

Higher fermentation capacities than the parent strain

(125 g/L) were selected and numbered YF10-1, YF10-2, YF10-3, YF10-

500 AGU g^-1 mash was cooled to 30°C, and glucoamylase (Liquozyme Supra, 90 KNU g^-1) was added. Simultaneous saccharification and fermentation of sweet potato mash

2.7. Simultaneous saccharification and fermentation of sweet potato mash

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 concentration is considered a key factor in achieving high ethanol productivity [25]. Resistance to this type of stress is important for 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 productivity. As shown in Fig. 1, the growth of YF10-5 on 50% (w/v) glucose 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

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' → 3'</th>
<th>Amplicon (bp)</th>
<th>Gene/ORF</th>
<th>Reference/Source</th>
</tr>
</thead>
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<tr>
<td>ACT-F</td>
<td>TGTTACAGCTGCTTTCAAT</td>
<td>103</td>
<td>ACT1</td>
<td>[21]</td>
</tr>
<tr>
<td>ACT-R</td>
<td>GATCTTCATCAGTAGCTGAC</td>
<td>105</td>
<td>HSP12</td>
<td>[22]</td>
</tr>
<tr>
<td>HSP12-F</td>
<td>CCAGGCTGAAAAGGATTG</td>
<td>109</td>
<td>HSP26</td>
<td>[22]</td>
</tr>
<tr>
<td>HSP12-R</td>
<td>TGGCAAGCCGAGGGTGA</td>
<td>117</td>
<td>HSP30</td>
<td>[22]</td>
</tr>
<tr>
<td>HSP30-F</td>
<td>TGGGAAGTGTGGTCAGTCTG</td>
<td>114</td>
<td>HPS1</td>
<td>[22]</td>
</tr>
<tr>
<td>HPS1-R</td>
<td>CAGGACAGAAGACCGCAAAT</td>
<td>116</td>
<td>HXX1</td>
<td>[22]</td>
</tr>
<tr>
<td>HXX1-F</td>
<td>CGCGTCTGGCTCTTTAGCCG</td>
<td>116</td>
<td>HXX1</td>
<td>This study</td>
</tr>
<tr>
<td>HXX1-R</td>
<td>CACGTCGTGCTCAGGAAAACAAGA</td>
<td>114</td>
<td>HYY1</td>
<td>This study</td>
</tr>
<tr>
<td>HYY1-F</td>
<td>TCACAGTGGTTGCTCAGG</td>
<td>117</td>
<td>HYY1</td>
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<td>HYY1-R</td>
<td>TACGTCGTGCTCAGGAAAACAAGA</td>
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<tr>
<td>HYY1-F</td>
<td>TACGTCGTGCTCAGGAAAACAAGA</td>
<td>115</td>
<td>HYY1</td>
<td>This study</td>
</tr>
</tbody>
</table>

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, 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

The ethanol concentration, residual glucose, ethanol yield, and ethanol productivity were monitored, and viability of the yeast cells was analyzed by enumerating the yeast cell numbers.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose (% w/v)</th>
<th>Ethanol (% w/v)</th>
<th>Productivity (g L^-1 h^-1)</th>
<th>Ethanol yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td>12.50 ± 0.02</td>
<td>2.08 ± 0.00</td>
<td>71.53 ± 2.16</td>
<td></td>
</tr>
<tr>
<td>V-10</td>
<td>12.00 ± 0.13</td>
<td>2.00 ± 0.02</td>
<td>74.65 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V-10-1</td>
<td>12.00 ± 0.13</td>
<td>2.00 ± 0.01</td>
<td>74.65 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>V-10-2</td>
<td>12.00 ± 0.13</td>
<td>2.00 ± 0.01</td>
<td>82.06 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>V-10-3</td>
<td>12.50 ± 0.02</td>
<td>2.00 ± 0.01</td>
<td>77.67 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>V-10-4</td>
<td>12.50 ± 0.02</td>
<td>2.00 ± 0.01</td>
<td>83.00 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

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

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. 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_{600}=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.](image1)

![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.](image2)
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,
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.

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, HKX1, 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


