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Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance

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ABSTRACT

In this study, a systemic analysis was initially performed to investigate the relationship between fermentation-related stress tolerances and ethanol yield. Based on the results obtained, two elite *Saccharomyces cerevisiae* strains, Z8 and Z15, with variant phenotypes were chosen to construct strains with improved multi-stress tolerance by genome shuffling in combination with optimized initial selection. After three rounds of genome shuffling, a shuffled strain, YZ1, which surpasses its parent strains in osmotic, heat, and acid tolerances, was obtained. Ethanol yields of YZ1 were 3.11%, 10.31%, and 10.55% higher than those of its parent strains under regular, increased heat, and high gravity fermentation conditions, respectively. YZ1 was applied to bioethanol production at an industrial scale. Results demonstrated that the variant phenotypes from available yeast strains could be used as parent stock for yeast breeding and that the genome shuffling approach is sufficiently powerful in combining suitable phenotypes in a single strain.

1. Introduction

Fuel ethanol, one of the most common renewable energy sources, is primarily produced from sugars and starches by industrial *Saccharomyces cerevisiae* yeast strains (Sanchez and Cardona, 2008). In industrial alcoholic fermentation, yeast cells are exposed to numerous environmental stresses, including osmotic stress, oxidation, ethanol, and low pH, all of which impose potential constraints on yeast growth and viability (Blieck et al., 2007; Laopaiboon et al., 2009; Zhao and Bai, 2009). For ethanol production to take place, yeast strains must be able to withstand these stresses and still function at economically and technically acceptable standards (Marullo et al., 2009; Zhao and Bai, 2009). Therefore, improving the multi-stress tolerance of yeast strains is crucial to their usefulness in industrial production.

In *S. cerevisiae*, stress tolerance is generally controlled by multiple loci widely distributed throughout the cellular genome. Many of these genes are not characterized well enough to determine cellular phenotypes (Attfield, 1997; Causton et al., 2001; Zhao and Bai, 2009). Thus, it is impossible to efficiently improve the multi-stress tolerance of yeast strains using rational strategies, such as gene manipulation. To overcome this obstacle, genome shuffling procedures may be used (Zhang et al., 2002). These procedures offer the advantage of simultaneous genetic changes at different positions throughout the entire genome without the need for gene regulatory network information. This novel approach has been proven effective for complex trait improvements in some microorganisms (Otte et al., 2009; Shi et al., 2009; Zheng et al., 2010).

In heredity and breeding of yeast strains (including genome shuffling), high throughput and effective screening methods may yield mutant strains with the desired traits (Zuzuarregui and del Olmo, 2004). To this end, culture media (i.e., agar plates) containing trait-relevant inhibitors were broadly applied in the initial selection of desired tolerance phenotypes (Limtong et al., 2007; Shi et al., 2009; Sridhar et al., 2002). Unfortunately, there is neither a systemic analysis of the relationship between fermentative performance and stress resistance nor standard procedures in yeast strain screening.

The first aim of this study is to investigate the relationship between stress resistance and ethanol production in yeast strains. The second aim is to construct industrial yeast strains with improved multi-tolerance and fermentation performance. As a prerequisite step, tolerance to multiple stresses and fermentation of 15 *S. cerevisiae* strains were assayed. Based on the results obtained from the assay, the elite strains Z8 and Z15 were chosen to undergo genome shuffling in combination with optimized initial selection. A shuffled strain YZ1, which had significantly improved multiple stress tolerance and fermentation performance, was obtained after

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three rounds of genome shuffling. Finally, the mechanism for multiple stress tolerance improvement was investigated.

2. Methods

2.1. Strains and media

The *S. cerevisiae* Z1–15 are all diploid strains. Z1–3 were isolated from a soil sample, Z4–9 were received from Henan Tianguan Fuel Ethanol Co. Ltd., and Z10–12 were isolated from Angel active dry yeasts. The genetic background of Z13–15 (stored by our laboratory) was *S. cerevisiae* var. ellipsoideus. The growth medium (YPD) contained: 1% yeast extract, 2% peptone, 2% glucose (w/v), and had a pH of 5.5. The fermentation medium was prepared from corn meal via the double enzyme hydrolyzed method (Graves et al., 2007).

2.2. Stress tolerance test

For the tolerance tests, yeast cells were pre-cultured in YPD and then inoculated in 25 mL YPD with specified stress factors to an initial OD_{600} of 0.05. Stress conditions were as follows: (A) high sugar content stress, 30% glucose (w/v); (B) heat stress, incubation at 42 °C; (C) oxidative stress, 4 mM H_2O_2; (D) ethanol stress, 7% ethanol (v/v); (E) low pH stress, culture pH was adjusted to 2.5 using HCl; (F) salt stress, 0.8 M NaCl; (G) acetic acid stress, 4 g/L acetic acid; and (H) complex stress conditions (CSC), 20% glucose (w/v), 5% ethanol (v/v), and incubated at 38 °C. Growth was followed for 12 h, and cultures for each condition were performed in triplicates. Two measurements, biomass formation (measured by OD_{600}) and viable counts (cells were plated onto YPD agar plates and counts were recorded after 2–3 days), were used to assess the tolerance of the yeast strains. Stress tolerance was calculated as the percentage of biomass formation or viable counts compared to that of the unstressed control (YPD at 30 °C) for each stressor of each strain. Although some differences in the exact figures were observed between these two measurements, the results of the stress tolerance comparison and statistical analysis, obtained using the two methods, agreed substantially. Considering the smaller standard deviation of the OD_{600} method and limited space available, the results of the tolerance test and statistical analysis based on the viable counts method are supplied as Supplementary material (Supplementary data 1).

2.3. Genome shuffling

Genome shuffling was implemented by three rounds of sporulation and hybridization. In the first round of genome shuffling, the original strains Z8 and Z15 were grown in YPD to an OD_{600} of 1 and harvested by centrifugation. The resulting cells were washed twice with sterilized water and sporulated in 200 mL flasks containing 50 mL of the sporulation medium (1% NaAc, 0.2% yeast extract, 0.1% glucose, and 0.2% KCl, at pH 6.0) oscillating at 200 rpm, at 28 °C. The spores were purified and crossed using a method described previously (Hou, 2009). About 600 hybrids from Z8 to Z15 were mixed, diluted, and then spread onto selection plates (YPD with 20% glucose and 5% ethanol, cultured at 38 °C) for primary screening of tolerance hybrids. One hundred of the fast-growing colonies on the selection plates were chosen for shake-flask fermentation analysis under heat (42 °C) and high gravity (300 g/L glucose). Four hybrids with the best fermentation performances, i.e., produced more ethanol than did all parent strains at the end of fermentation and had glucose/ethanol conversion rates higher than those of all parents strains, advanced to the next round of genome shuffling. Sporulation, crossing, and selection of hybrids in the next two rounds of genome shuffling were similar to those of the first round of genome shuffling, but the cultured temperatures for initial selection was adjusted to 39 and 40 °C, respectively.

2.4. Ergosterol and trehalose content

Yeast cells were cultivated in YPD medium at 30 °C with shaking at 150 rpm. They were harvested at the log phase to extract total sterol. Ergosterol concentrations were measured using an HPLC system equipped with a reverse-phase column (Endo et al., 2009). Trehalose was extracted from chilled and washed cells with cold 0.5 M trichloroacetic acid and estimated using the anthrone method (Blieck et al., 2007). Samples for dry weight analysis were washed with distilled water and dried at 80 °C for 12 h.

2.5. Reactive oxygen species accumulation, catalase, and superoxide dismutase activity

Stationary phase cells were collected by centrifugation, washed three times with YPD, and then transferred to YPD with 15 mM of H_2O_2 at 30 °C for 1 h. Cells were collected by centrifugation and washed twice with 0.1 M PBS (pH 7.4) to remove residual H_2O_2, before staining with 10 μg/mL of dichlorodihydrofluorescein diacetate (DCFH-DA) at 30 °C for 1.5 h. The cell viability and reactive oxygen species (ROS) accumulation labeled by DCFH-DA were observed and imaged using a confocal laser scanning microscope (CLSM).

The catalase (CTA) activity of the cell extract was assayed by measuring the decrease in A240 using H_2O_2 as a substrate in 0.05 M PBS (pH 7.0) at 25 °C. One unit of CTA activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of H_2O_2 at 25 °C in 1 min. The activity of superoxide dismutase (SOD) was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system using a commercial assay kit (Nanjing Jiancheng Biological Products, Nanjing, China). One SOD activity unit is defined as the amount of enzyme that causes 50% inhibition in 1 mL reaction solution per mg protein (Peng et al., 2009). The Yeast Buster™ Protein Extraction Reagent was used to release proteins from yeast cells according to the manufacturer's instructions (Novagen). The protein concentration of the crude cell extract was measured by BCA protein assay reagent (Pierce).

2.6. Fermentation and metabolites

Anaerobic fermentation was performed in 500 mL Erlenmeyer flasks containing 200 g of converted mash (pH 5). Yeast cells were aerobically pre-cultured in YPD for 20 h at 30 °C, and 0.05 g of cells (dry weight) were transferred to the fermentation media. Three fermentation conditions, (Fr) with 224.5 g/L glucose at 34 °C, (Fh) with 224.5 g/L glucose at 42 °C, and (Fo) with 299.5 g/L glucose at 34 °C, were used in this study.

Samples for metabolite measurement were immediately centrifuged, filtered through 0.45 mm filters, and stored at −20 °C until analysis. Glucose and ethanol concentrations were measured on an Aminex HPX-87H column (Bio-Rad) at 60 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The compounds were detected using a refractive index detector.

2.7. Statistical analysis

All stress tolerances data were standardized by expressing the biomass formation and viable counts for the stress cultures as percentages of the biomass and viable counts relative to the unstressed control. Ethanol yield data were expressed as a volume

ratio. Means and standard errors were calculated from three replicates experiments of each strain. Hierarchical cluster analysis and bivariate correlation analyses were performed using the software package SPSS 15.0 (Palazzuoli et al., 2010; Sun et al., 2006). The correlation coefficient was calculated for pairs of stress and between ethanol yields and each stress. Significance levels for each correlation were assigned based on ANOVA.

3. Results and discussion

3.1. Stress tolerance and ethanol yield of 15 S. cerevisiae strains

Stress tolerance of the 15 strains varied widely, especially for heat, H_2O_2, and acetic acid stress, where up to 7.5-fold differences existed between the least and most tolerant strains (Table 1 and Table S1). Nevertheless, no single strain was tolerant to all stressors. Z15 is the most generally stress-tolerant strain, demonstrating the highest recorded tolerance in 30% glucose, 0.8 M NaCl, heat, and low pH stress treatments. Strain Z11 showed the highest tolerance to ethanol and acetic acid, whereas strain Z1 had the highest tolerance to H_2O_2. Low tolerances to heat and H_2O_2 were found for strain Z6. Strain Z1 was the most sensitive to salt and acetic acid. The dendrogram (Fig. 1 and Fig. S1) based on stress tolerances in the hierarchical cluster analysis distinctly discriminates differences among the yeast strains. The 15 strains were divided into four groups using a standard measure value of 10.

Ethanol yields also varied among the strains. Z6 (Group D) was clearly the strain with the lowest ethanol yield under regular and high-gravity conditions. The strain that performed worst under high temperature was Z7. Strain Z13 had the maximum ethanol production under regular conditions. The highest ethanol yield under high heat and high-gravity conditions were generated by strains Z8 and Z15, respectively. Overall, strains Z8 and Z9 produced more ethanol under high temperature conditions, whereas the strains in Group C exhibited better performance under regular and highly osmotic conditions.

3.2. Correlation between stress tolerance and ethanol yield

During breeding of yeast strains, fermentation-related stress conditions are usually applied for mutant strain selection because it is almost impossible to test the fermentative behavior of every strain from the mutant library. To develop a simple test for the initial selection of desired traits, a correlation analysis was performed on the stress-tolerance data to investigate whether or not tolerance to one type of stress was related to tolerance to another and to ethanol yield. Although evidence accumulated in recent years suggests the existence of an integrating mechanism (i.e., heat shock proteins, trehalose, and catalase) that senses and responds to different forms of stress (Estruch, 2000), the correlations among the stress tolerances were not as obvious as expected. Of the 21 possible correlations between each of the stressors, only three common positive correlations were found with 1% or 5% level of significance (Table 2 and Table S2). The correlations among pH 2.5, 30% glucose, and 0.8 M NaCl treatments implied that the injury or repair mechanism associated with tolerance to low pH stress is also associated with tolerance to osmotic stress. The loss of H^+-ATPase activity, which confers sensitivity to weak acids and low extracellular pH, also results in a decreased tolerance to high ionic and osmotic conditions in yeast strains (Mccusker et al., 1987).

Ethanol is one of the most predominant inhibitors of yeast cells in the latter part of the fermentation process (Dinh et al., 2009; Salgueiro et al., 1988). The ability to tolerate this type of stress is

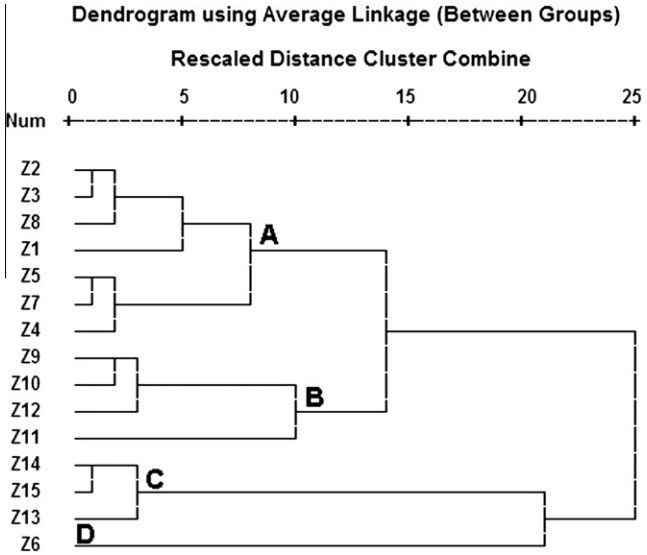

Fig. 1. Relationship among 15 yeast strains based on stress tolerance is depicted as a dendrogram obtained on the between-group linkage method of hierarchical cluster analysis. Fifteen yeast strains were divided into four clusters: A, B, C, and D by the standard measure value 10. Values were standardized by *Z* scores.

Table 1
Multiple stress tolerances and ethanol yields of the 15 *S. cerevisiae* strains.

Strains	Mean relative biomass formation in stress ± s.e.m.								Ethanol yield[a](v/v%)		
	30% Glu	Heat	H_2O_2	Ethanol	pH 2.5	NaCl	HAc	CSC	Yr	Yh	Yo
Z1	0.191 ± 0.001	0.653 ± 0.006	0.737 ± 0.011	0.210 ± 0.003	0.164 ± 0.001	0.206 ± 0.002	0.080 ± 0.001	0.439 ± 0.004	12.21 ± 0.054	9.45 ± 0.054	13.55 ± 0.051
Z2	0.176 ± 0.002	0.703 ± 0.005	0.555 ± 0.007	0.259 ± 0.003	0.134 ± 0.001	0.238 ± 0.002	0.217 ± 0.003	0.441 ± 0.003	12.18 ± 0.057	9.37 ± 0.047	13.77 ± 0.049
Z3	0.183 ± 0.001	0.592 ± 0.006	0.668 ± 0.009	0.277 ± 0.003	0.197 ± 0.002	0.275 ± 0.002	0.115 ± 0.002	0.400 ± 0.007	12.43 ± 0.061	9.30 ± 0.042	14.07 ± 0.075
Z4	0.204 ± 0.004	0.401 ± 0.005	0.502 ± 0.003	0.190 ± 0.001	0.253 ± 0.001	0.244 ± 0.005	0.244 ± 0.003	0.333 ± 0.007	11.80 ± 0.041	8.50 ± 0.001	13.41 ± 0.043
Z5	0.164 ± 0.003	0.436 ± 0.005	0.521 ± 0.006	0.236 ± 0.003	0.246 ± 0.001	0.250 ± 0.002	0.214 ± 0.002	0.346 ± 0.003	11.81 ± 0.043	9.01 ± 0.041	13.54 ± 0.047
Z6	0.190 ± 0.002	0.230 ± 0.002	0.227 ± 0.001	0.168 ± 0.001	0.324 ± 0.002	0.301 ± 0.002	0.216 ± 0.002	0.257 ± 0.003	11.40 ± 0.046	8.99 ± 0.037	12.98 ± 0.039
Z7	0.189 ± 0.001	0.245 ± 0.003	0.552 ± 0.007	0.235 ± 0.003	0.184 ± 0.001	0.210 ± 0.002	0.170 ± 0.001	0.204 ± 0.003	12.40 ± 0.051	8.88 ± 0.037	13.53 ± 0.047
Z8	0.185 ± 0.001	0.658 ± 0.007	0.626 ± 0.009	0.308 ± 0.004	0.250 ± 0.001	0.241 ± 0.003	0.267 ± 0.002	0.553 ± 0.005	12.53 ± 0.043	9.86 ± 0.071	13.96 ± 0.046
Z9	0.210 ± 0.002	0.660 ± 0.008	0.550 ± 0.008	0.255 ± 0.002	0.176 ± 0.001	0.289 ± 0.002	0.323 ± 0.004	0.558 ± 0.006	12.51 ± 0.043	9.77 ± 0.071	14.09 ± 0.046
Z10	0.182 ± 0.001	0.542 ± 0.001	0.571 ± 0.009	0.225 ± 0.001	0.179 ± 0.001	0.350 ± 0.003	0.454 ± 0.006	0.357 ± 0.004	11.91 ± 0.042	9.21 ± 0.052	13.22 ± 0.045
Z11	0.219 ± 0.005	0.634 ± 0.007	0.362 ± 0.003	0.317 ± 0.004	0.226 ± 0.003	0.310 ± 0.003	0.603 ± 0.007	0.599 ± 0.007	12.20 ± 0.049	9.75 ± 0.065	14.17 ± 0.069
Z12	0.200 ± 0.001	0.723 ± 0.007	0.336 ± 0.005	0.240 ± 0.001	0.155 ± 0.001	0.404 ± 0.004	0.424 ± 0.004	0.503 ± 0.007	11.95 ± 0.061	9.23 ± 0.062	13.50 ± 0.076
Z13	0.250 ± 0.003	0.660 ± 0.008	0.233 ± 0.005	0.239 ± 0.003	0.282 ± 0.001	0.374 ± 0.002	0.328 ± 0.002	0.590 ± 0.008	12.65 ± 0.065	9.47 ± 0.049	14.38 ± 0.076
Z14	0.223 ± 0.002	0.680 ± 0.008	0.293 ± 0.006	0.200 ± 0.001	0.358 ± 0.004	0.445 ± 0.006	0.319 ± 0.002	0.570 ± 0.005	12.61 ± 0.064	9.28 ± 0.053	13.87 ± 0.051
Z15	0.270 ± 0.002	0.740 ± 0.009	0.294 ± 0.006	0.201 ± 0.001	0.398 ± 0.005	0.448 ± 0.007	0.306 ± 0.002	0.641 ± 0.006	12.64 ± 0.065	9.60 ± 0.071	14.63 ± 0.082

[a] Yr, Yh, and Yo represent the ethanol yields under regular, increased heat, and high-gravity conditions, respectively, as mentioned in the Section 2. The fermentations were terminated when any one of the 15 strains exhausted the sugar (residual glucose <2.5 g/L) under regular conditions or when ethanol could no longer be produced under increased heat and high-gravity conditions. Means and standard errors were calculated from three replicates of fermentation experiments for each strain.

Table 2
Correlation analysis of stress tolerance and ethanol yield ($n = 15$). Data represent the correlation coefficient (r). Probability (P) was assigned based on ANOVA.

	Heat	Ethanol	H_2O_2	30% Glucose	pH 2.5	0.8 M NaCl	HAc	CSC
Ethanol	0.387 ($P = 0.154$)							
H_2O_2	0.004 ($P = 0.989$)	0.337 ($P = 0.219$)						
30% Glucose	0.409 ($P = 0.131$)	−0.171 ($P = 0.541$)	−0.639 ($P = 0.010$)					
pH 2.5	−0.045 ($P = 0.874$)	−0.425 ($P = 0.114$)	−0.659 ($P = 0.008$)	0.634* ($P = 0.011$)				
0.8 M NaCl	0.452 ($P = 0.091$)	−0.242 ($P = 0.384$)	−0.729 ($P = 0.001$)	0.699** ($P = 0.004$)	0.577* ($P = 0.024$)			
HAc	0.311 ($P = 0.260$)	0.324 ($P = 0.238$)	−0.476 ($P = 0.073$)	0.349 ($P = 0.202$)	0.066 ($P = 0.814$)	0.535* ($P = 0.040$)		
Yr	0.594* ($P = 0.020$)	0.383 ($P = 0.159$)	0.057 ($P = 0.841$)	0.539* ($P = 0.038$)	0.162 ($P = 0.564$)	0.260 ($P = 0.349$)	0.000 ($P = 0.999$)	0.652** ($P = 0.008$)
Yh	0.718** ($P = 0.003$)	0.619* ($P = 0.014$)	0.036 ($P = 0.857$)	0.323 ($P = 0.240$)	0.003 ($P = 0.992$)	0.208 ($P = 0.456$)	0.329 ($P = 0.232$)	0.788** ($P = 0.000$)
Yo	0.659** ($P = 0.008$)	0.433 ($P = 0.107$)	−0.167 ($P = 0.551$)	0.703** ($P = 0.003$)	0.312 ($P = 0.257$)	0.367 ($P = 0.179$)	0.189 ($P = 0.499$)	0.809** ($P = 0.000$)

* Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

considered a key factor in achieving high ethanol concentrations, which result in considerable energy savings during the distillation of the fermentation broth. Although some general anti-stress proteins, such as Ctt1 and Sod2, can be induced to relieve toxicity from ethanol, heat, osmotic pressure and H_2O_2 (Mollapour et al., 2008; Pampulha and Loureirodias, 1989), ethanol tolerance failed to significantly correlate with the tolerances to these stressors (Table 2 and Table S2). These weak correlations suggest that improving the cross-tolerance to ethanol and other stressors through specific gene manipulation (even general anti-stress factors) is difficult. In addition, no strong correlation was found between ethanol tolerance and ethanol yield, except when fermentation was performed under increased heat ($P < 0.01$) (Table 2 and Table S2).This may result from the synergistic action of heat stress on the adverse effects of ethanol.

In the results, tolerance to heat was the only characteristic that correlated well with ethanol yield under all conditions (Table 2 and Table S2), which indicates that heat-related tolerance factors, including trehalose and heat shock proteins, play a role in providing cross-protection against multiple stressors during ethanol fermentation (Attfield, 1997; Estruch, 2000; Zhao and Bai, 2009). Conversely, to simulate industrial conditions, the fermentation temperature in this study was set to 34 or 42 °C, which is higher than the optimal growth temperature and may strengthen the relationship between heat tolerance and ethanol yield to a certain extent. Considering this statistical result, heat tolerance became the most crucial criterion for the primary selection of elite strains in this study.

The other stressor that significantly correlated with ethanol yield was 30% glucose, especially under high gravity (Table 2 and Table S2). Nevertheless, tolerance to 0.8 M NaCl failed to significantly correlate with Yo, which may partly be due to the different response mechanisms of yeast to ionic (NaCl) and non-ionic (glucose) osmotic stress. Remarkably, tolerance to the complex stress condition (CSC) showed much stronger correlations with ethanol yield (Table 2 and Table S2) compared to any single stress tolerance. This result suggests that ethanol fermentation process is synergistically controlled by multiple stressors and that optimized complex stress is more suitable for the initial selection of yeast strains with better fermentation performance than single stresses.

3.3. Improved multi-tolerance and fermentation performance after genome shuffling

In the current bioethanol industry, fermentation is usually conducted from an economic standpoint under severe conditions (with 22–28 Brix sugar at 33–38 °C), which impose simultaneous and sequential environmental stresses on yeast cells. Consequently, only strains with the capacity to respond and adapt to various stressors are able to complete the fermentation task efficiently (Cakar et al., 2005; Sanchez and Cardona, 2008). However, during the long period of natural evolution and artificial selection of industrial *S. cerevisiae* strains, a huge genetic variability among individuals was formed, resulting in stress tolerance diversity. As shown in Table 1 and Table S1, no single strain had the highest resistance to all stressors. In this study, the genome shuffling approach, which can efficiently eliminate deleterious mutations and combine desired traits from parent strains (Gong et al., 2009), was used to improve multiple stress tolerance and fermentation performance in *S. cerevisiae* strains. Considering the complementarity of tolerance and fermentation characteristics, strains Z8 and Z15 were selected as original parent strains. After three rounds of shuffling, a mutant strain designated as YZ1, which grew faster on the screening plate and had the best fermentation performance, was chosen for further study.

Fig. 2a and Fig. S1 show that YZ1 exceeded both parent strains in 30% glucose, heat, low pH, and 0.8 M NaCl stress tolerance ($P < 0.05$). In contrast, the capacity of YZ1 to tolerate H_2O_2, ethanol, and acetic acid was close to that of Z8 but significantly higher than that of Z15 ($P < 0.05$). These results indicate the multi-stress tolerance of YZ1.

Strains Z8, Z15, and YZ1 were also evaluated in an industrial fermentation medium under regular, heat, and high-gravity conditions. Compared to the two parent strains, YZ1 had a slightly faster fermentation rate during the middle and late periods and produced more ethanol (3.11%, $P < 0.05$) within 59 h under regular conditions (Fig. 3a, b). A decrease in performance was observed under high temperature (Fig. 3c, d) and high-gravity conditions (Fig. 3e, f), but the shuffled strain YZ1 was noticeably more tolerant to environmental stresses. The ethanol yield of YZ1 (11%) was 10.31% higher than that of Z8 under increased heat ($P < 0.01$). To the best of our knowledge, few reports have described *S. cereviseae* strains producing more than 10.5% (v/v) ethanol at 42 °C with industrial feedstock. Furthermore, compared to Z15, YZ1 improved ethanol yields by 10.55% under high sugar condition within 90 h ($P < 0.01$). To date, the shuffled strain YZ1, which showed significant superiority (2–5% improved glucose/ethanol conversion rate) over some traditional industrial yeast strains (i.e., Z8 and Z15), has produced an excess of 1 million tons of ethanol for Henan Tianguan Fuel Ethanol Co. Ltd., China.

3.4. Mechanisms for multi-tolerance improvement in the shuffled strain YZ1

Over the past few years, numerous studies have been performed to determine stress injuries and responses in *S. cerevisiae* strains, both from the perspective of its prominence in ethanol production and its role as a model eukaryote (Aguilera et al., 2006; Causton et al., 2001; Dinh et al., 2009; Hirasawa et al., 2007). The physiological consequences of the stresses encountered by yeast during

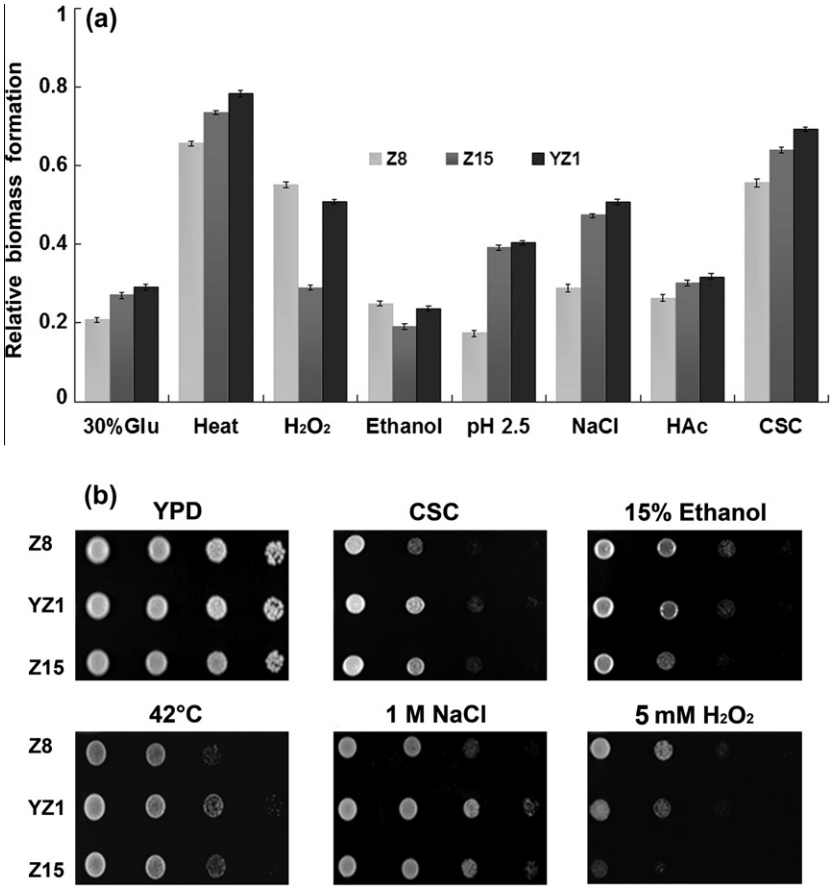

Fig. 2. (a) Comparison of the stress tolerances of strains Z8, Z15, and YZ1 in liquid medium. Stress tolerance was taken as the percentage of biomass formation (measured by OD_{600}) after the stress treatment compared with that of unstressed control. The values represent the mean of three independent experiments and bars indicate SD. (b) A serial dilution assay was performed to evaluate the stress tolerances of Z8, Z15, and YZ1. Yeast cells were grown to the mid-exponential phase and collected by centrifugation. Cells (3×10^8 cells/mL) were serially diluted 10-fold, and 3 μL of the dilutions were then spotted onto YPD plates under the indicated stress condition. Growth was monitored over 2–4 days at 30 °C or at a given temperature. Experiments were performed in triplicates. One representative experiment is shown.

ethanol fermentation include the disruption of membrane integrity and processes, loss of internal homeostasis, injury to macromolecules, and oxidative damage, among others. In this study, the ergosterol content, trehalose accumulation, and antioxidation factors of Z8, Z15, and YZ1 were compared to investigate the mechanisms for multi-tolerance improvement in YZ1.

3.4.1. Ergosterol content

Inhibition of growth and viability by some stresses, such as ethanol and acetic acid, was expected partly because they affected various transport systems and modified the fluidity of the plasma membrane (Aguilera et al., 2006; Hirasawa et al., 2007; Lloyd et al., 1993). Ergosterol, a ubiquitous component of cellular membranes required to maintain the fluidity and functioning of the cellular membrane in yeast, has also been reported to be directly correlated with the tolerance of yeast *S. cerevisiae* yeast to ethanol and other stressors (Aguilera et al., 2006; Endo et al., 2009). Compared to the two parent strains, the ergosterol content of YZ1 (6.15 mg/g dry weight) was slightly less than that of Z8, but 32.9% higher than that of Z15 ($P < 0.01$) (Fig. 4). These results correlated well with the tolerance of the strains Z8, Z15, and YZ1 to ethanol, which implies that ergosterol may act as a determinant of ethanol tolerance in the three strains.

3.4.2. Trehalose accumulation

Previous studies have revealed that trehalose is an important compound involved in the survival of *S. cerevisiae* when exposed to various severely stressful environments, such as heat and hyperosmotic pressure. The protective role of this disaccharide lies in maintaining membrane stability and preventing protein denaturation under stress (Francois and Parrou, 2001; Samokhvalov et al., 2004). However, the trehalose threshold required for stress tolerance is around 5% (w/w), and, above this level, yeast strains experience no direct benefits by accumulating more trehalose (Lewis et al., 1997). The tolerance test showed that strains YZ1 and Z15 had higher tolerance to osmotic pressure and heat than Z8 (Fig. 2 and Fig. S2). Fig. 5a shows that the amount of intracellular trehalose of YZ1 was similar to that of Z15, but higher than that of Z8 with and without stresses. Specifically, trehalose accumulated in YZ1 was 20.2% and 33.9% higher than those of Z8 in the pro-stationary phase (20 h) and after heat treatment (Fig. 5b), respectively ($P < 0.05$). Nevertheless, this gap became surprisingly wider (73.5%, $P < 0.01$) after treatment with 1 M NaCl for 1 h (Fig. 5b). The different levels of stress response reflected by trehalose accumulation may partly explain why yeast strains YZ1 and Z15 are more tolerant to heat and osmotic stress.

3.4.3. Antioxidation capacity

Reactive oxygen species, which may be derived from exposure to H_2O_2, heat, ethanol or organic acids, appear to play a somewhat common role in stress-induced damage (Lee et al., 1999; Piper, 1995).Yeast cells certainly generate antioxidant defenses (i.e., CTA and SOD) under different types of stresses (Lee et al., 1999). In accordance with their tolerance to H_2O_2 (Fig. 2b), Z15 accumu-

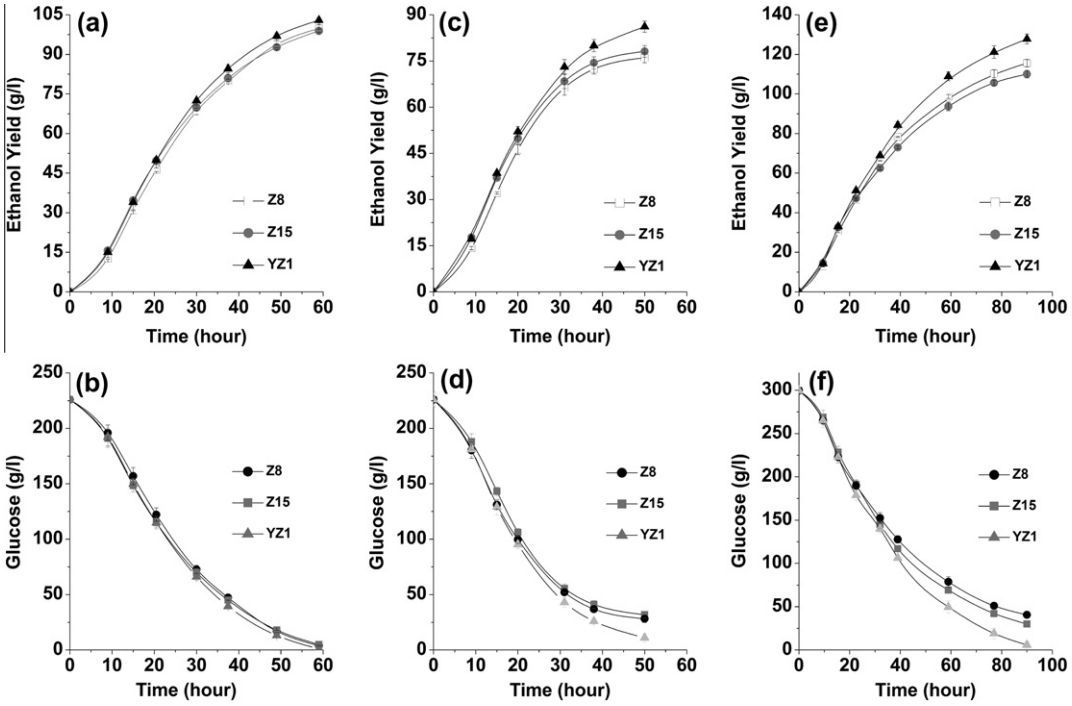

Fig. 3. Ethanol yield and glucose consumption curve of Z8, Z15, and YZ1 under (a, b) regular, (c, d) heat, and (e, f) high gravity fermentation conditions.

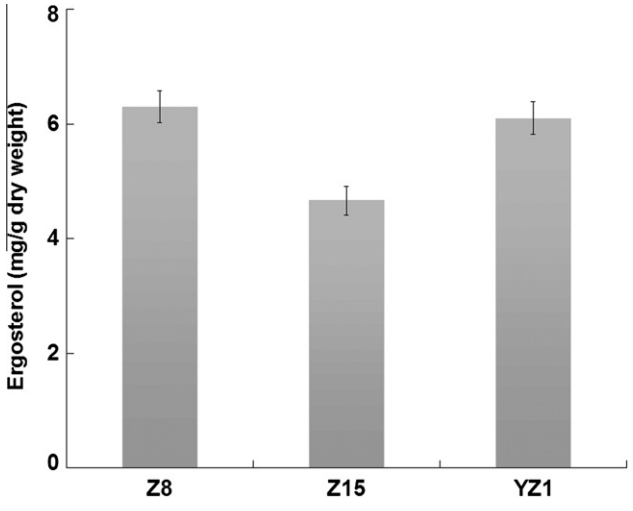

Fig. 4. Ergosterol content of parent strains Z8 and Z15 and the shuffled strain YZ1.

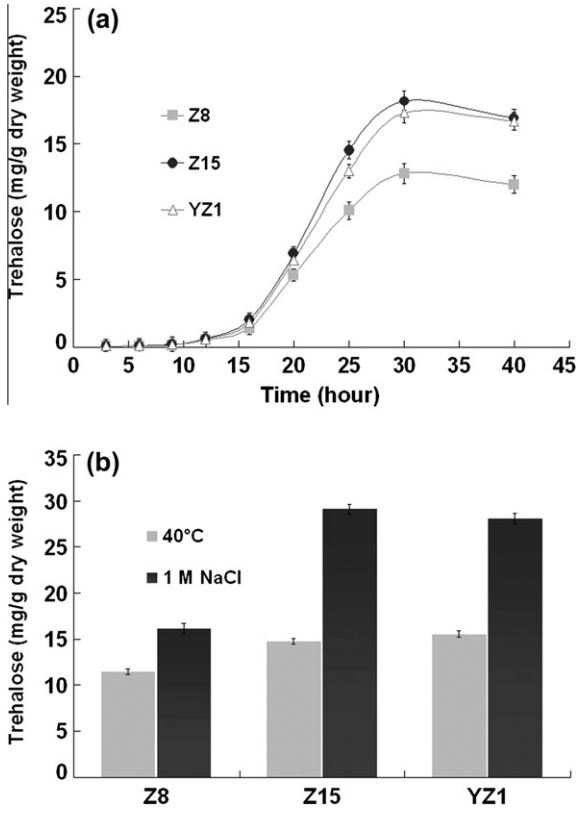

Fig. 5. Trehalose accumulation in strains Z8, Z15, and YZ1 (a) during growth and (b) after treatment with heat and osmotic pressure. Yeast cells were cultured in 25 mL YPD at an initial OD_{600} of 0.05 in a 30 °C atmosphere at 200 rpm. At the pre-stationary phase (20 h), 2 mL of the sample was extracted and cultured at 42 °C or treated with 1 M NaCl for 1 h before the measurement of trehalose content.

lated more cellular ROS than either Z8 or YZ1 after H_2O_2 treatment (Fig. 6a), Strain Z15 showed a similar total SOD activity but much less CTA activity during growth compared to Z8 and YZ1 (Fig. 6b). The CTA activity in Z15 was only 39.9% of that in Z8 at 16 h. In addition, unlike Z8 and YZ1, CTA and SOD activities of Z15 were not induced by H_2O_2 but were instead slightly decreased (Fig. 6c). Failure of Z15 to respond to ROS may offset its superiority in stress tolerance (i.e., more trehalose content), because induced antioxidant activities have been proven to be crucial in relieving ROS damage caused by several kinds of stress (Causton et al., 2001; Hirasawa et al., 2007).

From the above results, the shuffled strain YZ1 seemed to be closer to the parent strain Z15 in tolerance traits. However, after three rounds of genome shuffling, the tolerance of YZ1 to ethanol and oxidative damage increased significantly compared to Z15. Enhancement of ergosterol content and antioxidation capacity may partly account for these improvements. As a result, YZ1

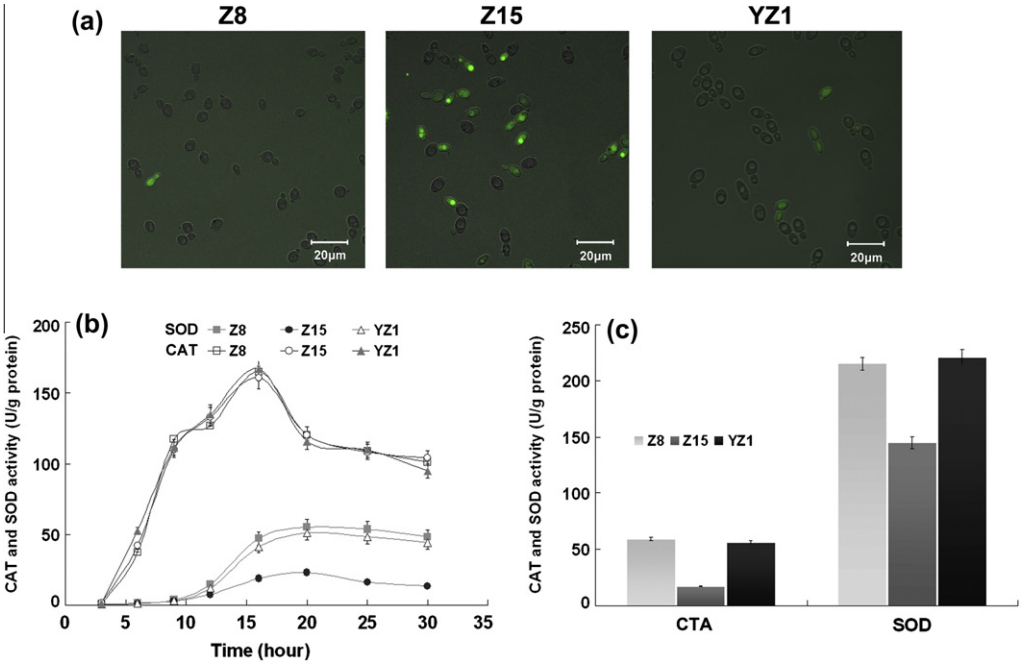

Fig. 6. (a) Qualitative analysis of ROS accumulation in Z8, Z15, and YZ1, which were observed by staining with DCFH-DA after treatment with 15 mM H_2O_2 for 1 h. Three fields of view from each coverslip were randomly chosen, and one representative is shown. The total CTA and SOD activities of Z8, Z15, and YZ1 (b) during growth and (c) after treatment with H_2O_2. Cells were cultured in 25 mL YPD with an initial OD_{600} of 0.05 in a 30 °C atmosphere at 200 rpm. To a 2 mL sample, 15 mM H_2O_2 was added at 16 h, after which sample was cultured for 1 h before determination of enzymatic activity.

surpassed its parent strains in multiple stress tolerance and fermentation performance, although levels of some main stress-resistant factors did not exceed those of either Z8 or Z15.

4. Conclusion

In this report, the correlation between stress tolerance and ethanol yield were systematically analyzed to facilitate parent strain selection and development of an optimized initial screening condition for the construction of *S. cerevisiae* strains with improved complex traits. Through three rounds of genome shuffling of the original strains Z8 and Z15, we obtained a shuffled strain YZ1, which combines the improved stress tolerance from the parent strains and significantly improved fermentation performances. This study demonstrated that variant phenotypes from available industrial yeast strains could be used as parent stock for yeast breeding through genome shuffling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.09.122.

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