

Improvement of robustness and ethanol production of ethanologenic *Saccharomyces cerevisiae* under co-stress of heat and inhibitors

Ying Lu · Yan-Fei Cheng · Xiu-Ping He ·
Xue-Na Guo · Bo-Run Zhang

Received: 6 April 2011 / Accepted: 7 June 2011
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Abstract Bioethanol is an attractive alternative to fossil fuels. *Saccharomyces cerevisiae* is the most important ethanol producer. However, yeast cells are challenged by various environmental stresses during the industrial process of ethanol production. The robustness under heat, acetic acid, and furfural stresses was improved for ethanologenic *S. cerevisiae* in this work using genome shuffling. Recombinant yeast strain R32 could grow at 45°C, and resist 0.55% (v/v) acetic acid and 0.3% (v/v) furfural at 40°C. When ethanol fermentation was conducted at temperatures ranging from 30 to 42°C, recombinant strain R32 always gave high ethanol production. After 42 h of fermentation at 42°C, 187.6 ± 1.4 g/l glucose was utilized by recombinant strain R32 to produce 81.4 ± 2.7 g/l ethanol, which were respectively 3.4 and 4.1 times those of CE25. After 36 h of fermentation at 40°C with 0.5% (v/v) acetic acid, 194.4 ± 1.2 g/l glucose in the medium was utilized by recombinant strain R32 to produce 84.2 ± 4.6 g/l of ethanol. The extent of glucose utilization and ethanol concentration of recombinant strain R32 were 6.3 and 7.9 times those of strain CE25. The ethanol concentration produced by recombinant strain R32 was 8.9 times that of strain CE25 after fermentation for 48 h under 0.2% (v/v) furfural stress at 40°C. The strong physiological robustness and

fitness of yeast strain R32 support its potential application for industrial production of bioethanol from renewable resources such as lignocelluloses.

Keywords *Saccharomyces cerevisiae* · Ethanol production · Thermotolerance · Acetic acid tolerance · Genome shuffling

Introduction

Ethanol is well known as a novel and attractive alternative to fossil fuels. Bioethanol production from renewable resources such as lignocelluloses is thought to be cost-effective, reproducible, and leads to lower emissions [9, 19]. *Saccharomyces cerevisiae* is the most important ethanol producer. However, there are some barriers to using this yeast in the industrial production of ethanol from lignocellulosic biomass. Inhibitors formed in the hydrolysis process of lignocellulose, including furan derivatives, weak acids, and phenolic compounds, have deleterious effects on both the cell growth and ethanol synthesis of *S. cerevisiae* [1, 13]. On the other hand, the optimal temperature for most of the hydrolytic enzymes of lignocellulose is approximately 45–50°C, whereas the optimal temperature for cell growth and fermentation of *S. cerevisiae* is about 30°C. Therefore there is a need to develop thermotolerant microorganisms capable of growth and fermentation at elevated temperatures compatible with optimal cellulase and hemicellulase activities to reduce the cost of cooling during fermentation [6]. Thus, improving the robustness of *S. cerevisiae* under the stress of heat and inhibitors is very important for the effective conversion of lignocellulose to ethanol.

The thermotolerance of ethanologenic yeast has been improved by using strategies of adaptation [2], mutagenization

Y. Lu · Y.-F. Cheng · X.-P. He (✉) · X.-N. Guo · B.-R. Zhang
The Laboratory of Molecular Genetics and Breeding of Yeast,
Institute of Microbiology, Chinese Academy of Sciences, Beijing
100101, People's Republic of China
e-mail: hexp@im.ac.cn

Y. Lu
Department of Applied Chemistry, School of Natural Sciences,
Anhui Agricultural University, No. 130 West Changjiang Road,
Hefei, Anhui 230036, People's Republic of China

[23, 25], protoplast fusion [5, 7], and genome shuffling [24]. For improving the acetic acid tolerance of yeast, strategies of adaptation [17], mutagenization [20], freeze-thaw [27], and genome shuffling [28] were also used. In these studies, the highest concentration of acetic acid that yeast strain could tolerate was reported to be 0.8% (v/v) at 30°C [28], and the highest temperature for yeast growth was 55°C [24]. However, few reports have so far described *S. cerevisiae* strains which could produce high concentrations of ethanol under co-stress of heat and acetic acid.

Genome shuffling was believed to be an effective approach to produce genetic diversity of the population by facilitating recombination among members of a diverse selected population [10, 21]. This technique has been successfully used to improve the tylosin production of *Streptomyces fradiae* [31], acid tolerance of *Lactobacillus* [21], L-lactic acid production of the fusant of *Lactobacillus delbrueckii* and *Bacillus amyloliquefaciens* [12], and ethanol production of *S. cerevisiae* [11]. In the present study, genome shuffling was applied to improve the acid tolerance and thermotolerance of *S. cerevisiae* simultaneously. After three rounds of shuffling, a recombinant strain with improved multiple-stress tolerance and enhanced ethanol production was isolated. The fermentation performance of the recombinant strain under different stress was analyzed and reported.

Materials and methods

Microorganism and cultivation media

Saccharomyces cerevisiae CE25 (CGMCC 2.1418), an industrial ethanologenic yeast strain, was purchased from China General Microbiological Culture Collection Center (CGMCC). Yeast cells were usually grown in yeast extract/peptone/dextrose (YPD) medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) at 30°C or other temperatures for different aims. Fermentation experiments were carried out in ethanol fermentation medium (EFM) composed of 6 g/l yeast extract, 10 g/l peptone, 5 g/l carbamide, 1 g/l potassium dihydrogen phosphate, 1.5 g/l magnesium sulfate, 0.55 g/l calcium chloride, and 200 g/l glucose. For protoplast preparation and fusion, YPDS medium (YPD containing 1 M sorbitol) was also used.

Mutagenesis and isolation of mutants

A loopful of cells taken from a slant was inoculated into 30 ml of YPD medium in a 250-ml Erlenmeyer flask and grown at 30°C and 180 rpm for 16 h. Ten milliliters of cells suspension was centrifugated at 3,000×g for 5 min, and washed twice with distilled water. Then the cells were

suspended in 1 ml of sterile 0.05 M phosphate buffer (pH 7.0), to which 30 µl of diethyl sulfate (DES) was added. This culture was incubated for 1 h at 30°C with shaking at 180 rpm. The mutagenesis was stopped by transferring 0.1 ml of the treated cells to 5 ml of sterile sodium thiosulfate (50 g/l). The percentage of cells that survived after the DES treatment was determined to be about 10%. The cells that were not treated with DES were used as control.

For selection of thermotolerant mutants, the serial dilutions of DES-treated cells and original cells were spread on YPD agar plates and incubated at various temperature. The single colonies that showed better thermotolerance than the original strain were selected, diluted, and then spotted onto YPD plates and incubated at different temperature (41, 42, or 43°C) for 48 h. The mutants which expressed higher thermotolerance than the original strain were picked out for fermentation at different temperature to determine ethanol production. The mutants with higher thermotolerance and ethanol production were selected for genome shuffling.

For isolation of mutants with improved acetic acid tolerance, the DES-treated cells and untreated cells were spotted onto YPD plates containing various concentrations of acetic acid and incubated at 37°C for 48 h. The colonies with higher acetic acid tolerance than the original cells were picked out to verify their acetic acid tolerance and determine ethanol production in the EFM with various concentrations of acetic acid at 37°C. The mutants with improved acetic acid tolerance and ethanol production were selected as the parental strains for the genome shuffling.

Genome shuffling

For protoplast preparation and fusion, yeast cells were cultured in 30 ml of YPD medium at 30°C for 18 h with shaking at 180 rpm. Yeast cells were harvested by centrifugation at 3,000×g for 5 min, washed twice with distilled water, and incubated in 0.2 M phosphate buffer (pH 7.0) containing 0.01 M β-mercaptoethanol for 10 min at 30°C. Cells were harvested and then resuspended in 0.2 M phosphate buffer (pH 7.0) containing 1 M sorbitol and 1% (w/v) lyophilized snail enzyme. After 1 h of incubation at 30°C with shaking at 180 rpm, fresh protoplasts were harvested and washed with 0.2 M phosphate buffer (pH 7.0) containing 1 M sorbitol.

Equal numbers of protoplasts obtained from different mutants were mixed, centrifuged, and resuspended in 0.2 M phosphate buffer (pH 7.0) with 30% (w/v) polyethylene glycol (PEG; mol. wt 4,000) and 0.01 M CaCl₂. After incubation for 40 min at 30°C, the fused protoplasts were harvested and resuspended in phosphate buffer containing 1 M sorbitol. The serial dilutions were spread on YPDS plates with 0.45% (v/v) acetic acid, and incubated at 38°C for 48 h. The colonies appearing under these conditions were

selected to analyze their tolerance to acetic acid and ethanol production at different temperature. The strains with higher ethanol productivity were selected for the next round of genome shuffling.

After each round of shuffling, the acetic acid concentration in YPDS solid medium or the culture temperature used for selection was increased. The strains with improved stress tolerance and ethanol productivity were employed for the subsequent rounds of genome shuffling with the methods described above. The colonies from each round of genome shuffling were saved for further analysis.

Ethanol fermentation experiments

Yeast cells were pre-cultured in 250-ml Erlenmeyer flasks containing 50 ml of YPD medium at 37°C and 180 rpm for 18 h. Ten milliliters of this culture was inoculated into 100 ml of EFM containing different concentrations of acetic acid or furfural in 250-ml Erlenmeyer flasks. Fermentation was firstly conducted at 150 rpm for 6 h and then at 80 rpm under micro-aerobic conditions (the Erlenmeyer flask was covered with a silicon rubber plug) at the required temperature. Fermentation experiments were performed in triplicate. The cell growth, ethanol production, and glucose consumption were monitored periodically.

Analytical methods

Determination of dry cell weight (DCW) was performed as described previously [8]. The biomass was determined as the gram of DCW per liter of culture. Concentrations of glucose and ethanol were analyzed by an SBA-40C biosensor (Institute of Biology, Shandong Academy of Sciences, China). Fermentation broth was centrifuged for 5 min at 3,000×g. Supernatant was diluted 500-fold with water and analyzed using the biosensor. The 1.0 g/l glucose or 0.5 g/l ethanol solution was used as standard. For determination of intracellular trehalose, yeast cells were collected by centrifugation and washed twice with ice-cold distilled water, and subsequently the trehalose was extracted with cold 0.5 M trichloroacetic acid. Trehalose content was determined using the anthrone method [15].

Genetic stability analysis

Yeast cells were firstly transferred onto YPD slants for 10 generations. Each generation of the strain was cultivated at 30°C for 48 h. Yeast cells from the tenth generation were cultivated in 5 ml of YPD medium for 18 h at 30°C on a rotary shaker. Cultures were diluted and spread on YPD plates. After cultivation for 48 h at 30°C, 100 single colonies were chosen to analyze their thermotolerance, acetic acid tolerance, and fermentation characteristics.

Statistical analysis

All fermentation cultures were run in triplicate in 250-ml Erlenmeyer flasks and all determinations were conducted in triplicate with mean values given. All data were analyzed statistically using Data Analysis and Technical Graphics, origin 6.0 (Microcal Software Inc.).

Results

Improvement of robustness of *S. cerevisiae* by genome shuffling

After comparison of the thermotolerance, acetic acid tolerance, and ethanol production of 44 ethanologenic yeast strains, an industrial ethanologenic *S. cerevisiae* CE25 which could tolerate 0.45% acetic acid at 30°C, had a maximum temperature for growth of 41°C, and had high ethanol productivity at 30°C was chosen as the initial strain. Yeast cells of CE25 were treated with chemical mutagen (DES). The DES-treated cells were screened for their thermotolerance, acid tolerance, and ethanol production. As shown in Table 1, two mutants marked as MT1 and MT2, which could grow at 42°C and tolerate 0.5% acetic acid at 30°C, were obtained that exhibited similar ethanol production as strain CE25 at 37°C. Meanwhile, some acetic acid-tolerant mutants which could grow on YPD plate (pH 4.5) containing 0.6% acetic acid at 30°C were obtained and analyzed further for tolerance to acetic acid at 37°C. The highest acetic acid concentration that these mutants could tolerate at 37°C was 0.5% (v/v). After comparison of ethanol production of the acetic acid-tolerant mutants in EFM with 0.4% (v/v) acetic acid at 37°C, two mutants marked as MA3 and MA4 that showed improvement of ethanol production were obtained.

The four mutants MT1, MT2, MA3, and MA4 were used as the starting population for genome shuffling. After each round of genome shuffling, the wild-type strain CE25, mutants, and recombinant strains were tested for their ethanol production under different conditions (Table 2). After the first round of shuffling, about 60 colonies were selected from the YPD plate containing 0.45% (v/v) acetic acid at 38°C. The fermentation characteristics of different yeast strains in the EFM containing 0.4% (v/v) acetic acid at 38°C were compared, and three colonies marked as R11, R12, and R13 that exhibited improved ethanol productivity were chosen as the population for the second round of genome shuffling (Table 2).

After the second round of shuffling, about 32 colonies were selected from the YPD plate containing 0.5% (v/v) acetic acid at 39°C. Two colonies marked as R21 and R22 were isolated from the second shuffled library owing to

Table 1 Fermentation of 200 g/l glucose by the wild-type strain and mutants for 48 h under different fermentation conditions

Strain	37°C			37°C with 0.4% (v/v) acetic acid		
	Biomass (g/l)	RS (g/l)	EC (g/l)	Biomass (g/l)	RS (g/l)	EC (g/l)
CE25	11.2 ± 0.7	1.8 ± 0.2	84.2 ± 1.2	6.2 ± 0.3	65.7 ± 0.5	56.4 ± 0.5
MT1	11.5 ± 0.2	1.2 ± 0.5	85.4 ± 0.7	7.9 ± 0.4	53.7 ± 0.6	65.2 ± 0.2
MT2	12.3 ± 0.4	1.3 ± 0.3	84.9 ± 0.7	7.7 ± 0.4	55.7 ± 0.5	63.8 ± 0.4
MA3	11.4 ± 0.3	1.3 ± 0.2	84.7 ± 1.2	8.4 ± 0.4	42.7 ± 0.3	70.5 ± 0.4
MA4	11.7 ± 0.6	1.3 ± 0.2	85.2 ± 0.2	8.5 ± 0.3	43.2 ± 0.5	70.2 ± 0.4

Results are represented as average and standard deviation of data from three independent batch cultures

RS residual sugar, EC ethanol concentration

Table 2 Comparison of growth and ethanol production of the wild-type strain, mutants, and recombinant strains under different fermentation conditions

Strain	38°C with 0.4% acetic acid		39°C with 0.45% acetic acid		40°C with 0.5% acetic acid	
	μ_{max} (h ⁻¹)	EC (g/l)	μ_{max} (h ⁻¹)	EC (g/l)	μ_{max} (h ⁻¹)	EC (g/l)
CE25	0.15 ± 0.05	50.2 ± 0.7	0.14 ± 0.02	36.7 ± 0.6	0.13 ± 0.02	10.8 ± 1.6
MT1	0.18 ± 0.03	60.2 ± 0.6	0.15 ± 0.08	51.3 ± 1.2	0.14 ± 0.06	41.8 ± 0.5
MT2	0.18 ± 0.08	60.5 ± 0.8	0.14 ± 0.02	50.2 ± 0.6	0.14 ± 0.02	37.9 ± 0.6
MA3	0.19 ± 0.03	67.2 ± 0.3	0.17 ± 0.04	57.2 ± 0.7	0.16 ± 0.05	47.9 ± 0.2
MA4	0.19 ± 0.05	67.9 ± 0.5	0.17 ± 0.02	58.2 ± 0.3	0.16 ± 0.03	46.7 ± 0.2
R11	0.22 ± 0.05	83.1 ± 0.5	0.19 ± 0.04	65.8 ± 0.7	0.17 ± 0.02	56.2 ± 1.1
R12	0.22 ± 0.02	82.8 ± 0.4	0.19 ± 0.04	66.7 ± 0.3	0.17 ± 0.04	56.3 ± 0.4
R13	0.22 ± 0.05	81.9 ± 1.2	0.19 ± 0.01	66.3 ± 0.2	0.17 ± 0.03	55.7 ± 0.7
R21	–	–	0.21 ± 0.01	81.2 ± 0.7	0.19 ± 0.03	70.8 ± 0.3
R22	–	–	0.21 ± 0.03	80.6 ± 0.4	0.19 ± 0.01	69.2 ± 0.5
R31	–	–	–	–	0.21 ± 0.01	81.3 ± 0.3
R32	–	–	–	–	0.22 ± 0.05	83.5 ± 0.5
R33	–	–	–	–	0.22 ± 0.01	82.3 ± 1.5

Fermentation experiments were conducted in EFM containing 200 g/l of glucose under different conditions for 48 h. Results are represented as average and standard deviation of data from three independent batch cultures

EC ethanol concentration, μ_{max} maximum specific growth rate, – not detected

their fermentation characteristics in the EFM containing 0.45% (v/v) acetic acid at 39°C (Table 2).

After the third shuffling, the resulting populations were screened on YPD plate containing 0.5% (v/v) acetic acid at 40°C. The fermentation test was conducted in EFM containing 0.5% (v/v) acetic acid at 40°C. As shown in Table 2, three colonies marked as R31, R32, and R33 showed significant improvement in ethanol production; of these recombinant strain R32 gave the highest ethanol production and genetic stability. As a control, in the whole process of genome shuffling, parent strain was also cultivated under the same selection conditions, but no colony was observed for adaptation and/or evolution.

As indicated in Fig. 1, multiple-stress tolerance of ethanologenic *S. cerevisiae* was improved by using genome shuffling. The recombinant strain R32 had the same viability as the original strain CE25 at 37°C. However, when the

temperature was increased to 45°C, the recombinant strain R32 still retained high viability whereas strain CE25 could not grow any more. Meanwhile, recombinant strain R32 showed much higher tolerance to acetic acid and furfural than strain CE25.

Ethanol fermentation at different temperature

Ethanol fermentations of original strain CE25 and recombinant strain R32 were conducted in EFM containing 200 g/l glucose at 30, 37, 40, and 42°C. When fermentation was carried out at 30 and 37°C, recombinant strain R32 and original strain CE25 gave similar ethanol production and glucose consumption profiles (Fig. 2a). When fermentation temperature was increased to 40°C, recombinant strain R32 showed both higher cell growth and ethanol production than the original strain CE25. The specific cell growth rate

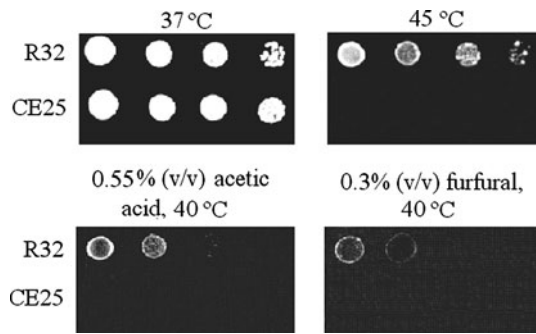


Fig. 1 Growth of yeast cells under different stresses. Mid-exponential cultures (OD_{600} of 0.8) of recombinant strain R32 and original strain CE25 were serially diluted, and 4 μ l of each dilution 10^{-1} – 10^{-4} (from left to right) was spotted onto YPD plates, or YPD plates containing 0.55% (v/v) acetic acid or 0.3% (v/v) furfural and incubated at indicated temperatures

of R32 was 1.44-fold that of CE25. After 30-h fermentation, the extent of glucose utilization and ethanol production of recombinant R32 were increased by 35.1 ± 0.3 and $35.7 \pm 0.6\%$, respectively, as compared with those of CE25. The average ethanol production rate of recombinant R32 reached $2.83 \pm 0.04 \text{ g l}^{-1} \text{ h}^{-1}$, higher than that of original strain CE25 ($1.52 \pm 0.06 \text{ g l}^{-1} \text{ h}^{-1}$) (Fig. 2b). More distinct differences between recombinant strain R32 and original strain CE25 were observed at 42°C. The specific growth rate of R32 was 1.53-fold that of CE25. After 42 h of fermentation at 42°C, the extent of glucose utilization and ethanol concentration of R32 reached $93.8 \pm 0.7\%$ and $81.4 \pm 2.7 \text{ g/l}$, which were respectively 3.4 and 4.1 times those of CE25 (Fig. 2c). The recombinant strain R32 synthesized more trehalose than the original strain CE25 under heat stress, and the degradation rate of trehalose in R32 cells was lower than that of CE25 cells (Fig. 3), which maybe contribute to the thermotolerance of recombinant strain R32.

Ethanol fermentation under different acetic acid stress

The fermentation profiles of recombinant strain R32 and original strain CE25 were compared under different acetic acid stress at 37 or 40°C. When fermentation was conducted in EFM containing 0.1, 0.2, or 0.3% (v/v) acetic acid at 37°C, no obvious difference in fermentation kinetics was observed between R32 and CE25 (data not shown). When acetic acid concentration was increased to more than 0.4%, strain R32 displayed much higher viability and ethanol production than strain CE25 both at 37 and 40°C. At 37°C, strain R32 consumed 200 g/l of glucose completely after 36 h under 0.5% acetic acid stress to produce 86.8 g/l ethanol (Fig. 4a). The extent of glucose utilization and ethanol production of R32 were 3.0 and 3.1 times those of CE25. When fermentation was conducted in EFM containing

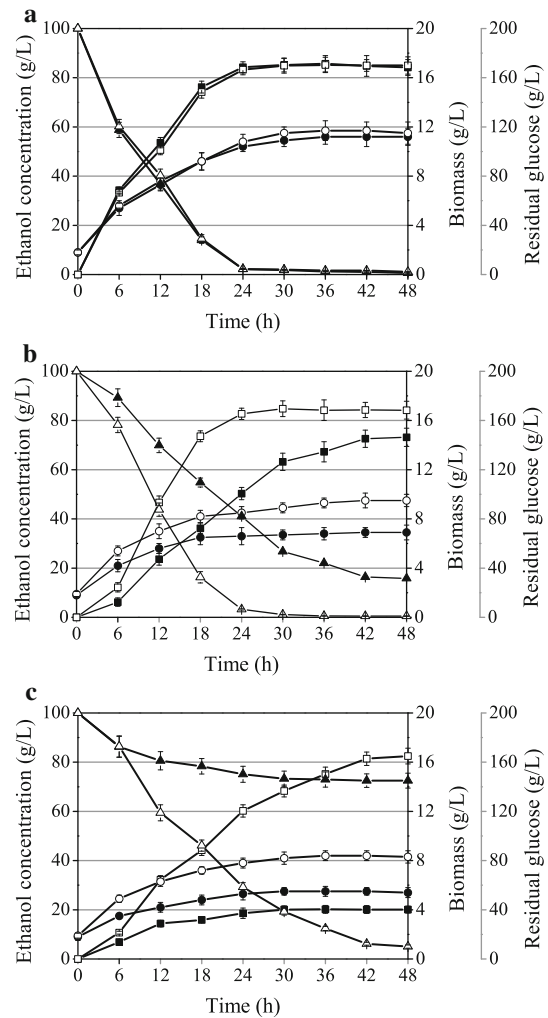


Fig. 2 Comparison of fermentation kinetics of original strain CE25 and recombinant strain R32 at different temperatures: **a** 37°C; **b** 40°C; **c** 42°C. Original strain CE25 (filled squares ethanol, filled triangles residual glucose, filled circles biomass), recombinant strain R32 (open squares ethanol, open triangles residual glucose, open circles biomass). Values are means of three replications \pm standard deviation

0.5% acetic acid at 40°C, the original strain CE25 exhibited very poor growth ability which resulted in much lower glucose utilization and ethanol production. However, for recombinant strain R32, $97.2 \pm 0.6\%$ of glucose in the medium was utilized after 36 h to produce $84.2 \pm 4.6 \text{ g/l}$ of ethanol (Fig. 4b). The extent of glucose utilization and ethanol production of recombinant strain R32 were 6.3 and 7.9 times those of strain CE25.

Ethanol fermentation under furfural stress

The fermentation profiles of recombinant strain R32 and original strain CE25 under furfural stress at 40°C were compared (Fig. 4c). When fermentation experiments were conducted in EFM containing 0.2% (v/v) furfural at 40°C,

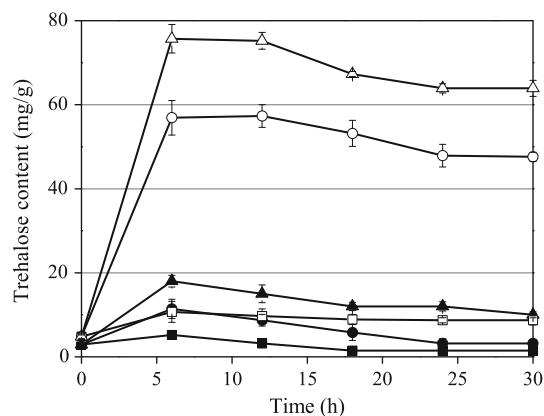


Fig. 3 Intracellular trehalose contents of original strain CE25 and recombinant strain R32 at different temperatures. Original strain CE25 (filled squares 37°C, filled circles 40°C, filled triangles 42°C), recombinant strain R32 (open squares 37°C, open circles 40°C, open triangles 42°C). Values are means of three replications \pm standard deviation

very weak cell growth and ethanol production were observed for original strain CE25 during the initial 18 h, and no more cell growth, glucose consumption, and ethanol synthesis occurred in the following time. Under the same fermentation conditions, recombinant strain R32 exhibited much higher cell viability than strain CE25. After 48 h of fermentation under furfural stress at 40°C, strain R32 produced 78.2 ± 2.6 g/l of ethanol by consumption 172.2 ± 1.6 g/l of glucose. The ethanol production of recombinant strain R32 was 8.9 times that of strain CE25.

Discussion

During ethanol fermentation, yeast cells are challenged by temperature fluctuation. In the tropics or in summer, large cooling costs are required to maintain the optimal temperature for fermentation. Also, simultaneous saccharification and fermentation (SSF) technology is economically advantageous in fuel ethanol production from lignocellulose, which requires yeast strains with higher thermotolerance. In this work, thermotolerance of an industrial *S. cerevisiae* was improved by using genome shuffling. Recombinant strain R32 could give high ethanol production at temperatures ranging from 30 to 42°C. The superior cell growth of recombinant strain R32 over the original strain CE25 under heat stress resulted in much higher glucose utilization and ethanol production. Thermotolerance in *S. cerevisiae* is mediated by the induction of a set of stress response genes such as heat shock protein (Hsp) genes. Heat induces the production and activation of heat shock proteins by heat shock transcription factors and other cofactors in *S. cerevisiae* [4, 29], which endows yeast cells with thermotolerance. In addition, if trehalose accumulates in the cell,

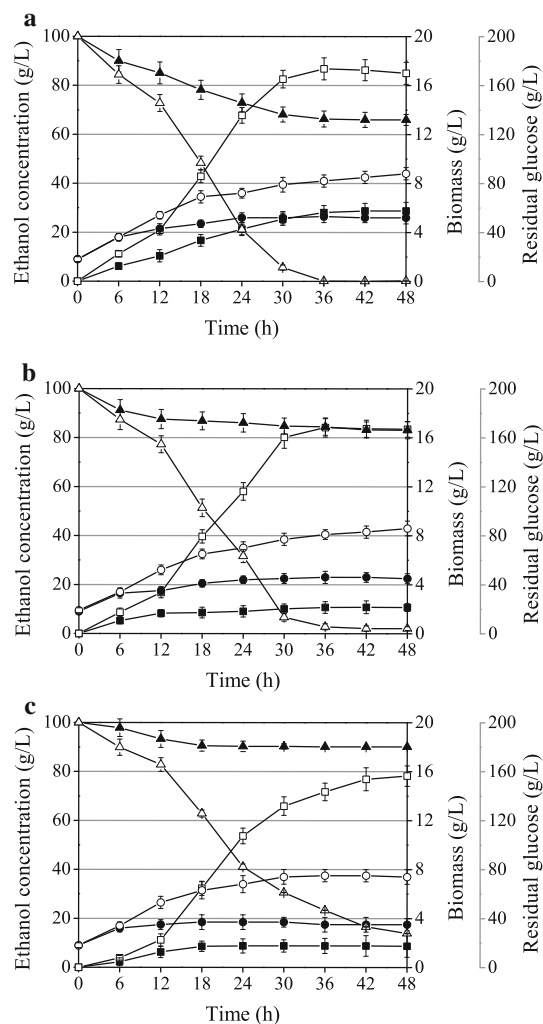


Fig. 4 Comparison of fermentation kinetics of original strain CE25 and recombinant strain R32 under co-stress of heat and acetic acid stress or furfural stress: **a** with 0.5% (v/v) acetic acid at 37°C; **b** with 0.5% (v/v) acetic acid at 40°C; **c** with 0.2% (v/v) furfural at 40°C. Original strain CE25 (filled squares ethanol, filled triangles residual glucose, filled circles biomass), recombinant strain R32 (open squares ethanol, open triangles residual glucose, open circles biomass). Values are means of three replications \pm standard deviation

thermotolerance in yeast can also be induced in an Hsp-independent process [3]. The accumulation of trehalose in recombinant strain R32 seems to contribute to the thermotolerance or tolerance to other stresses.

Acetic acid is not only a by-product in many fermentation processes, but also the main inhibitor formed in the hydrolysis process of lignocellulose. The intake of acetic acid produces cell acidification and intracellular acetate anion (CH_3COO^-) accumulation, which is involved in inhibition of the glycolytic enzymes in yeast cells [14]. At the general culture temperature of yeast (30°C), the inhibitory effect of acetic acid on cell growth is more effective than on fermentation performance [22, 30]. In this work, both cell

growth and ethanol production of original strain CE25 at 37 and 40°C were hampered by acetic acid. The specific cell growth rate ($\text{g l}^{-1} \text{h}^{-1}$) and the specific ethanol productivity ($\text{g g DCW}^{-1} \text{h}^{-1}$) of CE25 at 37°C were decreased by 51.6 and 30.7% respectively by acetic acid, whereas the specific cell growth rate and the specific ethanol productivity of CE25 at 40°C were decreased by 42.1 and 80.5% respectively by acetic acid. Such a disadvantage may result from the co-stress of heat and acetic acid. For recombinant strain R32, only the biomass synthesis was inhibited by acetic acid, and the specific cell growth rate at 37 and 40°C was decreased by 30.3 and 15.4% respectively by acetic acid. Interestingly, the specific ethanol productivity of strain R32 was increased by 46.5 and 14.9% at 37 and 40°C respectively by acetic acid. These results indicate that 37 or 40°C is not heat stress for recombinant strain R32. In *S. cerevisiae*, except the specific stress response, there are common general stress responses to different environmental stress, which are dynamic and complex processes under the control of multiple loci broadly distributed throughout the genome of yeast strains [18]. The damage induced by heat, acetic acid, or furfural on yeast cells shares certain common characteristics, such as the accumulation of reactive oxygen species, membrane damage, chromatin and actin damage, and accumulation of unfolded proteins, which subsequently results in triggering of common stress responses to protect yeast cells from serious damage. Yeast strains with high tolerance to heat and acetic acid may also hold resistance to furfural or other environmental stress [16, 26]. In this research, in addition to high tolerance to heat and acetic acid, recombinant strain R32 also exhibits much higher cell growth and ethanol production than original strain CE25 under 0.2% furfural stress at 40°C. The strong physiological robustness and fitness of yeast strain R32 support its potential application for industrial production of bioethanol from renewable resources such as lignocelluloses.

For *S. cerevisiae*, there are some challenges in the lignocellulose-to-ethanol conversion process, including tolerance to environmental stresses and utilization of xylose. The results in this study indicate that ethanol production of *S. cerevisiae* under multiple stress can be increased largely by improvement of stress tolerance. The detailed mechanisms underlying the multiple-stress tolerance of the recombinant strain R32 need to be further investigated. Xylose is the second most abundant sugar in lignocellulose hydrolysates, so we suggest that metabolic engineering approaches should be used to modify the yeast strain obtained in the current work to develop robust yeast strains for both glucose and xylose alcoholic fermentation.

Acknowledgments The authors would like to acknowledge the financial support of Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX1-YW-11-C4).

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