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ORIGINAL PAPER

Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*

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Abstract Genome shuffling is a powerful strategy for rapid engineering of microbial strains for desirable industrial phenotypes. Here we improved the thermotolerance and ethanol tolerance of an industrial yeast strain SM-3 by genome shuffling while simultaneously enhancing the ethanol productivity. The starting population was generated by protoplast ultraviolet irradiation and then subjected for the recursive protoplast fusion. The positive colonies from the library, created by fusing the inactivated protoplasts were screened for growth at 35, 40, 45, 50 and 55°C on YPD-agar plates containing different concentrations of ethanol. Characterization of all mutants and wild-type strain in the shake-flask indicated the compatibility of three phenotypes of thermotolerance, ethanol tolerance and ethanol yields enhancement. After three rounds of genome shuffling, the best performing strain, F34, which could grow on plate cultures up to 55°C, was obtained. It was found capable of completely utilizing 20% (w/v) glucose at 45–48°C, producing 9.95% (w/v) ethanol, and tolerating 25% (v/v) ethanol stress.

Keywords Ethanol production · Genome shuffling · *Saccharomyces cerevisiae* · Thermotolerance

Introduction

The use of ethanol as a liquid fuel has been increasing in many parts of the world [2, 14, 35, 37]. Although many microorganisms have been exploited for ethanol production, *Saccharomyces cerevisiae* still remains as the prime species [3]. Typical yeast fermentations require that the temperature be maintained between 30 and 35°C to maximize ethanol production [8]. Maintaining the optimum fermentation temperature requires expensive cooling systems in tropical countries where average temperatures are usually high throughout the year. Therefore, it would be economically and technically advantageous to ferment at higher temperatures. This, however, would require a yeast strain that could produce maximum ethanol levels at these higher temperatures.

There are a limited number of reports on the selection of yeasts that are able to grow and ferment at or above 40°C. Researchers have reported to improve the yeast fermentation using UV [27, 28], chemical mutagenesis [31], adaptations [4, 22], and protoplast fusion [10, 12, 16]. In these studies, the maximum growth temperature was 45°C.

Classical strain improvement methods have succeeded in obtaining many industrial strains, but it is time-consuming and laborious for many repeated rounds of random mutation and selection methods. Recently, an efficient technology named genome shuffling has made a major advance in the construction of mutants with distinctly significant improved phenotype [29]. Genome shuffling allows many parental strains with certain phenotypic improvements recombined through recursive protoplast fusion, and a library of shuffled strains with genetic exchange is achieved by the repetition of this process. This offers the advantage of simultaneous genetic changes at different positions throughout the entire genome without the necessity for genome sequence information [26]. This approach has also been successfully used to improve the acid tolerance in *Lactobacillus* [21, 25, 34], degradation of pentachlorophenol in *Sphingobium chlorophenolicum* [7] and production of hydroxycitric acid in *Streptomyces* [13].

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In this study, genome shuffling was used to rapidly improve the thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae* SM-3, whose protoplasts were mutagenized with ultraviolet (UV) irradiation and then used for recursive protoplast fusion. A novel mutant strain F34 with improved thermotolerance and ethanol tolerance was isolated after three rounds of genome shuffling. Its ethanol production was also increased. Furthermore, the behavior of this stain was investigated in shake-flasks.

Materials and methods

Microbial strains and media

Saccharomyces cerevisiae SM-3, a haploid strain (*MAT*α), was isolated from *Maotai-flavor* liquor starter culture [33] and stored in our laboratory. It exhibited good growth ability at 35°C, and produced more ethanol than the other isolates, so it was selected as the starting strain.

SM-3 was maintained on YPD solid medium at −4°C. At least two generations of precultures were required just before the experiments.

Growth medium (YPD) contained (w/v): 1% yeast extract, 2% peptone, 2% glucose (pH 5.5).

YPD solid medium contained (w/v): 1% yeast extract, 2% peptone, 2% glucose, 2% agar (pH 5.5) [19].

Regeneration medium (RM) was YPD supplemented with KCl (0.6 M), $CaC1_2$ (25 mM) and agar (2%, w/v).

Fermentation medium (FM) contained (w/v): 1% yeast extract, 2% peptone, 20% glucose, 0.6% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, pH 5.5.

Protoplast formation buffer (PB) consisted of 0.01 M Tris–HCl, pH 6.8, 20 mM $MgCl_2$, and 0.5 M sucrose as a stabilizer.

Preparation of protoplast

Strains were cultured at 30°C for 24 h in 10 ml YPD. Cells were harvested by centrifugation, washed twice with distilled water and incubated in PB buffer containing 0.01 M *β*-mercaptoethanol for 30 min at 30°C. Cells were collected and then resuspended in PB buffer containing 2% (w/v) snail enzyme (purchased from Lianxing Biochemical Reagent Company, Tianjin, China) for enzymatic digestion of the cell wall. The cell suspension was shaken at 100 rpm for 60 min at 30°C. The efficiency of protoplast formation was determined by microscopy.

Protoplast mutagenesis and mutant screening

After digestion for 1 h at 30°C, the fresh protoplasts were washed twice with PB buffer. About 5 ml buffer of the yeast protoplasts was irradiated with a Phillips TUV-30-W-254 nm Lamp (Phillips, The Netherlands) for 30 s at a distance of 20 cm (cell density was adjusted to 10^8 cells/ml). The treated protoplasts were kept in the dark for 2 h to avoid photo-reactivation repair, and then spread on RM agar plates. The plates were incubated at 30°C for 3 days. The colonies were selected, diluted and then spread on YPD plates containing 15% (v/v) ethanol (each plate was sealed with plastic film to prevent ethanol volatilization) and incubated at different high temperatures (38–45°C) for 36 h, the fast grown colonies were picked off for shake-flask analysis to determine their ethanol production individually. The mutants with higher ethanol productivities were selected as the starter for genome shuffling. Viability was expressed as a percentage of colony-forming units of the heat and ethanol treatment compared with an untreated control for each culture of the strains.

Genome shuffling

The protoplasts of the UV mutants were prepared as previously described. Equal number of protoplasts from different populations of these UV mutants were mixed and divided equally into two parts. One part was inactivated with UV for 10 min, and the other was heat treated at 60°C for 30 min. Both inactivated protoplasts were mixed in a cell ratio of 1:1, centrifuged, and resuspended in PB contained 40% (v/v) polyethylene glycol (PEG6000) and 0.01 M $CaCl_2$. After gently shaking for 15 min at 30°C to allow the protoplast fusion, the fused protoplasts were centrifuged, washed and resuspended in PB, and serial dilutions were regenerated on RM plates containing 20% (v/v) ethanol at required high temperatures (40–55°C) for 2–4 days. The colonies appearing under these conditions were selected to carry out shake-flask analysis and the strains with higher ethanol productivity were selected and named F1. Three successive rounds of protoplast fusion were carried out, and after each round, the ethanol concentration of the plates and the incubated temperature used for selection were increased. Samples from each round of the fusion strains (F1, F2 and F3) were saved for further analysis and used as sources of protoplasts for the subsequent rounds of genome shuffling, which were carried out using the same methods.

Ethanol tolerance test

To ensure the ethanol tolerance of the selected strains, these selected isolates were cultured in YPD liquid medium supplemented with various concentrations of ethanol (0% control, 10, 15, 20 and 25%, v/v). Yeast inocula were prepared as described previously and inoculated at an initial cell density of 2×10^6 cells/ml, and then incubated at 40°C for 48 h. Samples were removed, diluted, and plated on YPD

agar. After incubation at 40°C for 3 days, the colonies appearing on the plates were counted. Viability was expressed as a percentage of colony-forming units of the ethanol treatment compared with an untreated control for each culture of the strains.

Shake-flask analysis

Colonies from YPD plates were used to inoculate YPD medium and incubated overnight with shaking at 150 rpm at 30°C. The 200 ml FM medium in shake-flasks (500 ml) was inoculated with 10 ml of the overnight cultures and shaken at 100 rpm at required temperatures. The fermentation flasks were fitted with stoppers to vent CO_2 through a water trap and allowed to ferment for up to 72 h. Each strain was cultured in three shake-flasks. Fermentations were monitored by determining the glucose and ethanol concentrations in the culture.

Analytical method

Ethanol concentration was analyzed by GLC [Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol (PEG-20 M), carrier gas: nitrogen, 90°C isothermal packed column, injection temperature 160°C, flame ionization detector temperature 230°C; C-R7 Ae plus Chromatopac Data Processor] and isopropanol was used as an internal standard [20].

Glucose was determined using a Spectra-Physics SP8100 high-performance liquid chromatograph (HPLC) incorporating a Bio-Rad oligosaccharide column (Aminex HPX-42A), measuring 300 by 7.8 mm, a Micromeritics model 771 refractive index detector, and a Spectra-Physics SP4270 computing integrator[8].

DNA extraction and estimation

The nucleic acid extraction and estimation were performed as described [11]. Calf thymus DNA (Sigma, USA) was used to prepare standard curves.

Reproducibility of the results

In this work, all experiments were performed in triplicate and all determinations were done in duplicate with mean values given.

Results

Formation of protoplasts

After cell wall hydrolysis, yeast cells were stained with methylene blue and checked for formation of protoplasts. As cell wall was lost, membrane bound oxidases were also lost and oxidation of methylene blue dye to white color did not take place. Protoplasts were also observed for their spherical shape and hypotonic rupture with the help of microscope. Using the protocol described in the "Materials and methods", the efficiency for preparation of protoplasts reached nearly 100%, and the regeneration rate was more than 75%.

Protoplast mutagenesis and mutant screening

Genome shuffling practically mimics the features of natural evolution through the recursive genetic recombination. Thus, an improved starting point for breeding was required [25]. UV irradiation was used to develop the first population of thermotolerant mutants of wild strain SM-3. The protoplast cells of the wild strain SM-3 were exposed to UV irradiation at 254 nm for 30 s when an approximate 90% killing was obtained. After UV radiation, they were incubated at 38–45°C on RM agar plates containing 15% (v/v) ethanol on which the wild-type SM-3 could not exist.

The irradiated cells being both thermotolerant and ethanol tolerant were selected. There were 21 UV mutant strains selected from the protoplast mutant library that were capable of growing above 40°C on 15% ethanol (v/v) RM plates.

During the subsequent screening in shake-flask evaluations, two UV mutants (UV1 and UV2) were selected from these 21 UV mutants. They could grow up to 43°C (Fig. 1a) and exhibited further improved ethanol production than SM-3 after 48 h of fermentation (Table 1).

Genome shuffling

Genome shuffling is dependent upon the recursive fusion of protoplasts to allow recombination. This recursive strategy permits obtaining the phenotype of interest quickly. The high frequency of protoplast formation and regeneration is the basis of the efficiency of genome shuffling. Using the protocol described in the "Materials and methods", the efficiency for preparation of protoplasts reached nearly 100%, and the regeneration ratio was more than 75%.

Recombination within a selected population amplifies the genetic diversity of the population by creating new mutant combinations, and thereby improves the performance of individuals within the population. Genome shuffling accelerates directed evolution by facilitating recombination among members of a diverse selected population [25]. Strains UV1 and UV2 were used as the starting population for genome shuffling. After the first fusion, 200 colonies were selected according to their thermotolerance and ethanol tolerance, and were further assayed for ethanol production in FM-liquid culture above 45°C. We found

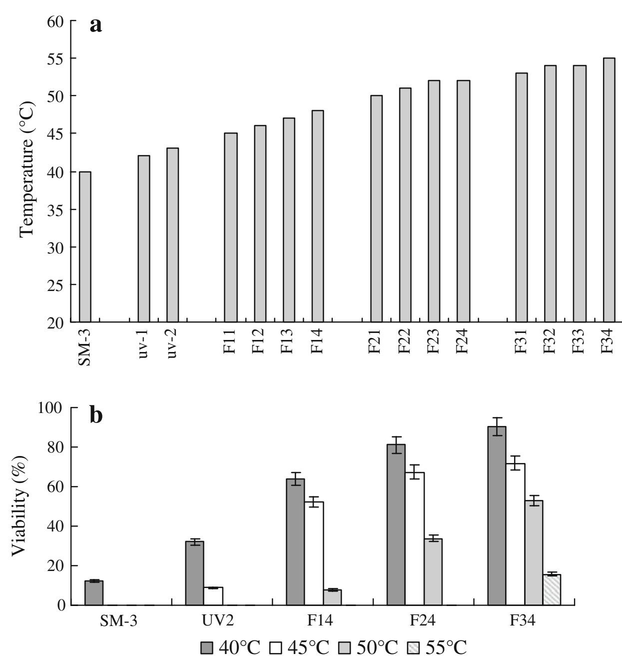

Fig. 1 **a** Comparison of SM-3, UV mutants and shuffled strains for their maximum growth temperatures. **b** Effects of different high temperatures on cell viability of SM-3, UV mutants and shuffled strains. The strains were cultured on YPD plates containing 15% ethanol

four isolates (F11, F12, F13 and F14) that exhibited further improved thermotolerance (Fig. 1a; ethanol productivity Table 1). These four isolates (F11, F12, F13 and F14) were pooled and used as the population for the second round of genome shuffling.

After the second protoplast fusion of the selected isolates (F11, F12, F13 and F14), 200 colonies were screened for their thermotolerance and ethanol productivity, another four colonies (F2) were obtained and used for the next round of genome shuffling. These four isolates (F21, F22, F23, and F24) were found to be more tolerant to high temperatures, and they could grow at temperatures above 50°C (Fig. 1a). They produced more ethanol than did the F1 strains in shake flasks (Table 1).

After the third fusion, four colonies (F3) were obtained from the third shuffled library. These isolates (F31, F32, F33, and F34) could tolerate temperatures above 53°C (Fig. 1a), but the ethanol productivities began to decrease at 50°C in shake-flasks (Table 1).The best performing shuffled strain from F3, F34, which could grow up to 55°C on YPD plate, was selected for the subsequent fermentation.

A control experiment was carried out by plating the selected populations of UV mutants and F1 without exposure to PEG on the YPD plates containing ethanol and incubating at different high temperatures (above 40°C). This was to determine whether acclimatization effect could lead to adaptive growth at high temperatures. In contrast to the shuffled strains, no colonies were found on the control plates during the same cultivation period.

The ethanol tolerance of the F2 and F3 strains was also significantly improved (Fig. 2). Therefore, the differences of thermotolerance, ethanol tolerance and ethanol production between the shuffled strains and mutated strains are obvious.

Table 1 Fermentation of 200 g/l glucose in fermentation medium (FM) after 48 h by shuffled strains, UV mutants and SM-3 at different temperatures

Strain	Temperature (°C)							
	35		40		45		50	
	RS (g/l)	EY(g/l)	RS (g/l)	EY (g/l)	RS (g/l)	EY (g/l)	RS (g/l)	EY (g/l)
SM-3	68.75 ± 0.05	60.53 ± 0.06	110.73 ± 0.05	33.27 ± 0.05	–	–	–	–
UV1	61.42 ± 0.06	65.44 ± 0.05	92.9 ± 0.05	43.4 ± 0.07	–	–	–	–
UV2	52.31 ± 0.05	70.21 ± 0.05	94.8 ± 0.08	44.9 ± 0.06	–	–	–	–
F11	18.3 ± 0.05	84.25 ± 0.06	85.47 ± 0.04	55.04 ± 0.08	94.61 ± 0.05	40.76 ± 0.04	–	–
F12	27.47 ± 0.07	81.43 ± 0.03	83.38 ± 0.04	53.0 ± 0.06	93.1 ± 0.07	43.2 ± 0.33	–	–
F13	21.43 ± 0.06	86.72 ± 0.05	47.11 ± 0.02	67.61 ± 0.10	73.24 ± 0.05	47.97 ± 0.05	–	–
F14	18.27 ± 0.08	88.98 ± 0.06	42.19 ± 0.05	71.23 ± 0.09	60.12 ± 0.11	59.34 ± 0.08	–	–
F21	9.82 ± 0.07	91.54 ± 0.05	35.43 ± 0.08	78.73 ± 0.08	59.64 ± 0.03	63.24 ± 0.04	85.66 ± 0.05	50.51 ± 0.05
F22	9.29 ± 0.06	93.65 ± 0.07	32.32 ± 0.06	80.15 ± 0.05	40.62 ± 0.05	69.53 ± 0.05	80.21 ± 0.07	52.21 ± 0.03
F23	10.30 ± 0.05	92.14 ± 0.05	25.48 ± 0.05	82.72 ± 0.05	37.41 ± 0.06	75.91 ± 0.05	73.51 ± 0.04	60.71 ± 0.05
F24	8.95 ± 0.05	93.15 ± 0.06	23.37 ± 0.08	89.91 ± 0.03	33.37 ± 0.07	79.64 ± 0.04	72.09 ± 0.10	63.35 ± 0.08
F32	4.14 ± 0.05	95.03 ± 0.05	12.65 ± 0.05	91.33 ± 0.05	12.12 ± 0.05	90.35 ± 0.05	64.52 ± 0.08	68.24 ± 0.05
F34	3.46 ± 0.02	98.46 ± 0.05	8.36 ± 0.05	95.54 ± 0.05	4.92 ± 0.05	95.05 ± 0.05	61.34 ± 0.05	73.53 ± 0.05

RS Residual sugar, *EY* ethanol yield, – no or too weak fermentation

Data represent the mean ± standard deviations of three independent fermentations

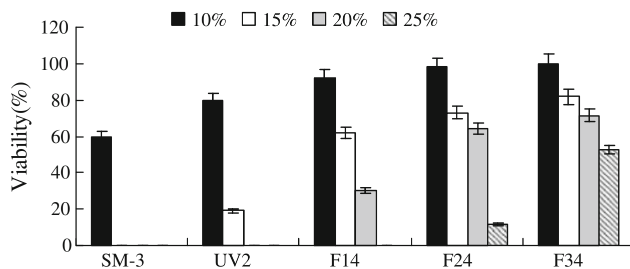

Fig. 2 Effects of different ethanol concentrations on cell viability of SM-3, UV mutants and shuffled strains

We also compared the genome shuffling method with traditional repetitive UV mutagenesis. To estimate the contribution of traditional repetitive UV mutagenesis, protoplasts were prepared, mutagenized and regenerated. Mutants with improved tolerance were selected. Then the protoplasts of these selected cells were mutagenized, regenerated and selected again. This procedure was repeated ten times. Only six strains with slight improvement in thermotolerance were selected, and their maximum growth temperature was 44°C, showing approximately the same ethanol production as UV1 and UV2. The results indicate that genome shuffling method is much more effective than the traditional repetitive UV mutagenesis to improve the complex phenotypes of microorganisms.

Improvement of thermotolerance during three rounds of genome shuffling

The UV mutants and shuffled strains were screened for individuals with improved thermotolerance. Mutant UV2 showed the highest viability in mutants obtained after UV irradiation, while strain F14, F24 and F34 showed the highest viability at high temperatures in all shuffled strains after the first, second and third round of genome shuffling, respectively. These strains were selected and their maximum growth temperatures were assessed (Fig. 1a).

A decrease in viability of these strains was observed when the temperature was increased from 40 to 55°C, but the shuffled strains were noticeably more resistant to high temperatures than SM-3 and the UV mutants (Fig. 1b). The selected shuffled strains obtained from each round of shuffling showed higher viability than their parent strains from the former rounds of shuffling at various high temperatures. All these selected shuffled strains had the ability to grow above 45°C on plates. When the temperature was increased to 45°C, the viability of shuffled strains remained high, whereas that of SM-3 decreased to zero. After three rounds of shuffling, when the temperature was increased to 55°C, the shuffled strain F34 showed a viability of 15.7%, whereas all the other strains lost their viability (Fig. 1b).

Improved ethanol tolerance of the selected strain

The improvement of ethanol tolerance of *Saccharomyces cerevisiae* is important for industrial ethanol fermentations. We measured the viability of the parent strain SM-3 and its derived mutants (UV and shuffled strains) to different concentrations of ethanol in the culture media and found that all shuffled strains had considerably improved their tolerance to ethanol stresses (Fig. 2). Increasing the ethanol concentration to 25% (v/v) resulted in a decrease in cell viability. Shuffled strains were noticeably more resistant to ethanol stress than SM-3 and UV mutant. The selected strains obtained from each round of shuffling showed higher viability than those from the former rounds of shuffling. When the concentration of ethanol was increased to 25% (v/v), the F34 strain showed a cell viability of 52.62% and F24 exhibited 11.47%, while the others lost their viability.

When the temperature was increased to 55°C and the concentration of ethanol was increased to 25% (v/v), the F34 showed a viability of 9.4%, whereas all the other strains lost their viability.

Glucose fermentation of the selected strains

In our present study, considerable improvement in the ethanol production of the selected shuffled strains was achieved after genome shuffling. Typical industrial ethanol fermentations are performed using up to 20% (w/v) fermentable sugar. Therefore, all the selected strains were tested for their ability to ferment 20% (w/v) glucose at 35–50°C. The results of the shake-flask fermentation experiment of SM-3, UV mutant and shuffled strains carried out in FM at different high temperatures are shown in Table 1. The shuffled strains produced markedly more ethanol compared to the UV mutants or wild-type SM-3, and the ethanol production of selected strains obtained from each round of shuffling was higher than those of their parent strains from the former rounds of shuffling at different high temperatures. Ethanol fermentations of both the wild-type strain SM-3 and UV mutants were inhibited by temperatures above 45°C; however, the shuffled strains (F1, F2, and F3) could ferment glucose and produce ethanol at temperatures above 45°C (Table 1). Ethanol concentrations produced by these strains were highest at 35–40°C, and decreased at temperatures above 40°C. However, lower but appreciable ethanol of 5.05–7.35% (w/v) was produced by shuffled strains F2 and F3 at 50°C. It was also noticed that large amounts of glucose, 6.13–8.56% (w/v), remained after 48 h fermentation at 50°C in the medium of the thermotolerant strains. Though growth and ethanol production were decreasing above 45°C, the F2 and F3 cells remained viable at temperatures above 50°C (Fig. 1a).

These results clearly show that *Saccharomyces cerevisiae* strain F34 is the most promising strain for high-temperature fermentation under these conditions, producing the maximum amount of ethanol, utilizing all of the available glucose up to 45°C and having good cell viability up to 55°C. Thus, this strain was used for subsequent studies.

Glucose fermentation of F34

The effect of increasing the temperature from 45 to 50°C on 20% (w/v) glucose fermentation by strain F34 is shown in Fig. 3. It can be seen that increasing the temperature resulted in a decrease in the rate and extent of glucose utilization and ethanol production. In fact, at 45°C, complete utilization of the 20% (w/v) glucose is observed, producing approximately 9.95% (w/v) ethanol and resembling the fermentation profile at 48°C with 9.33% ethanol produced. At 50°C, however, this strain did not completely ferment the glucose, producing about 8.2% (w/v) ethanol and leaving 4% glucose in the medium after 72 h of fermentation. Fermentations conducted for longer periods of time at these higher temperatures did not result in further increase in ethanol production.

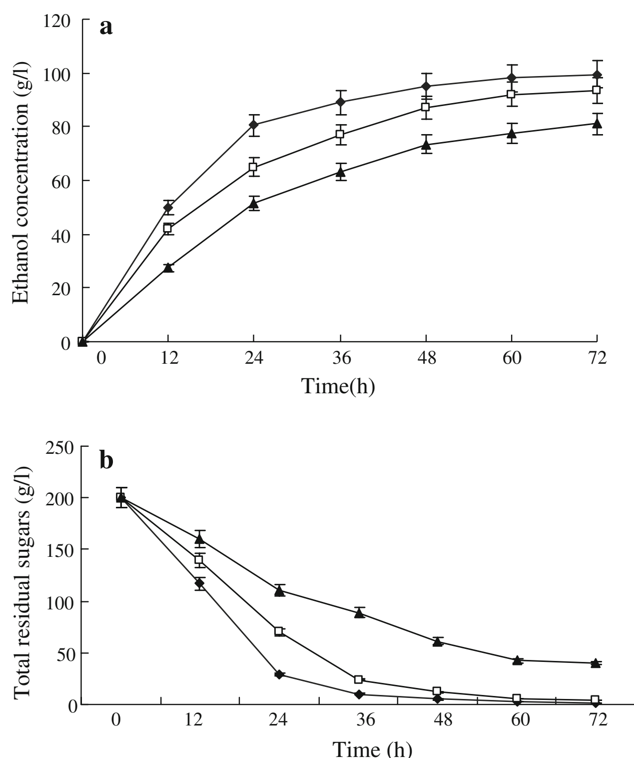

Fig. 3 Fermentation kinetics of F34 at different temperatures during fermentation: 50°C (*closed triangle*), 48°C (*open square*), 45°C (*closed diamond*). The *error bars* represent the standard deviations. **a** Ethanol production. **b** Total residual sugars

DNA contents

The DNA contents of some mutant strains were measured, and they were 5.089, 5.144, 6.289 and 7.447 mg/g cells for UV1, F14, F24 and F34, respectively; however, there were no significant differences between DNA contents of these shuffled strains from the different rounds of genome shuffling. The results indicated that complete addition of the chromosomes of parent strains did not occur during the genome shuffling process.

The genetic stability of the F24 and F34

To check the genetic stability of F24 and F34, we cultured them for 50 generations and measured the thermotolerance, ethanol tolerance and ethanol production of every other generation. All the generations showed similar tolerance and production as the initial strain, suggesting that F24 and F34 are genetically stable and suitable for industrial production.

Discussion

As is already known, the high temperatures inhibit both the growth and fermentation of *Saccharomyces cerevisiae*. In the present study, we successfully improved the thermotolerance, ethanol tolerance and ethanol production of strain SM-3 by genome shuffling technique combined with protoplast mutagenesis. Although protoplast fusion is broadly applicable, since the early 1970s, to our knowledge, this is the first report using genome shuffling to construct thermotolerant yeast with good fermentation characteristics successfully. The results of the present study indicate that genome shuffling is a powerful means to rapidly improve the complex phenotypes of microorganisms, whether haploid or polyploid, while still maintaining their robust growth.

Our results also suggest that it is possible that the optimal genomic profiles of three phenotypes become compatible, and it is easy to operate by protoplast mutagenesis and inactivated parental protoplasts fusion. This improved genome shuffling could reduce time for screening fusants and improve work efficiency.

As a single *Saccharomyces cerevisiae* strain, SM-3 was the starting point of the evolution program, an improved population was required for genome shuffling. Classical method such as UV mutagenesis was sufficient to generate improved populations of genetically diverse strains, with slight improvements in thermotolerance (Fig. 1a), and ethanol production (Table 1). Genome shuffling of these populations by three rounds of recursive pooled protoplast fusion generated a new population of strains with further

improvements in thermotolerance (Fig. 1), ethanol tolerance (Fig. 2) and ethanol production (Table 1); the third shuffled population (F3) contained members that could grow on YPD plates at 53–55°C (Fig. 1). The successive improvement of populations that had undergone successively more recombination (F3 > F2 > F1; Fig. 1a) illuminates the importance of recombination in the improvement process.

The high-ethanol concentrations produced by F34 at 45–48°C (Fig. 3) varied between 95 and 100% of the maximum theoretical amount of ethanol obtainable from 20% (w/v) glucose. These results indicate the potential advantages this culture can offer for use in industrial ethanol production in hot regions, where temperatures frequently reach above 40°C. It may also be of great importance in the simultaneous saccharification and fermentation of polymeric carbohydrates such as cellulose and sorghum, whose saccharification has an optimum temperature in the range of 45–50°C. When coupled with fermentation using non-thermotolerant yeast, lower temperatures have to be used to prevent yeast inhibition or inactivation. Such temperature limitation results in a consequent decrease in ethanol production. Thus, using this F34 strain can avoid this limitation.

Increasing the fermentation temperature to 50°C resulted in a decrease in the rate and extent of ethanol production of F34 (Fig. 3). Although the fermentations did not reach completion under these conditions, the ethanol values obtained at such high temperatures by a *Saccharomyces* strain have not been previously reported and represent a significant development in the area of high-temperature fermentations with this yeast genus.

Yeasts respond to the physical effects of high temperatures (increased membrane fluidity) by changing their fatty acid composition [5, 30]. With increasing temperature, the proportion of saturated fatty acids that esterified into membrane lipids increases at the expense of unsaturated acyl chains [6]. This decrease in fatty acid unsaturation with increasing growth temperature serves to maintain optimal membrane fluidity for cellular activities. Yeast ethanol tolerance is also strictly related with lipid composition of cell membranes [36]. In addition, increasing the growth temperature results in induced transient synthesis of heat-shock proteins [17, 32]. The induction of heat-shock proteins has been shown to play an important role in conferring increased thermal and ethanol cross tolerance in various microorganisms [23]. This laboratory is currently investigating the role of membrane lipids and heat-shock proteins during ethanol production at higher temperatures of these thermotolerant strains.

Successful works on selection of *Saccharomyces cerevisiae* for their ability to produce ethanol at high temperatures have been reported by several investigators. For example, D'Amore et al. [8] selected a strain of *Saccharomyces* capable of completely utilizing 15% glucose at 40°C and producing 6.4% (w/v) ethanol. However, when the glucose concentration was increased to 20%, this strain could not completely utilize the sugar, producing only 7.0% (w/v) ethanol. Some mutants of *Saccharomyces cerevisiae* capable of growing at 35°C have also been reported as thermotolerant yeasts [16, 24]. Abdel-Fattah et al. [1] isolated a *Saccharomyces cerevisiae* strain that could grow up to 43°C and produce ethanol concentrations of 6.8–8.0% (w/v). Kiransree et al. [19] reported the isolation of four thermotolerant strains of the *Saccharomyces cerevisiae*, the maximum ethanol yields obtained from 150 g/l glucose were 75, 60 and 58 g/l using the best strain VS_3 at 30, 40 and 44°C, respectively. The four isolates could tolerate temperatures above 44°C though growth and ethanol production was decreasing at this temperature. Efforts were made to further improve their thermotolerance and ethanol tolerance by treating them with UV radiation [28]. The maximum ethanol yields produced by the mutant of VS_3 strain from 250 g/l glucose were 98 and 62 g/l at 30 and 40°C, respectively. In another research, VS_3 strain was reported to show 12% (w/v) ethanol tolerance [18]. Balakumar et al. [4] reported the isolation and improvement of a thermotolerant *Saccharomyces cerevisiae* strain. The ethanol produced from 100 g/l glucose by this strain was 46 g/l (36 h), 38 g/l (48 h) and 26 g/l (48 h) at 40, 43 and 45°C, respectively, in rich nutrient medium, and with increased temperature, the fermentative ability quickly decreased. Jin et al. [15] obtained a thermotolerant *Saccharomyces cerevisiae* strain that could grow and ferment at 40°C, and maintain viable at 50°C for 10 min. Edgardo et al. [9] reported the selection of a *Saccharomyces cerevisiae* strain that could grow and ferment glucose at 42°C. Glucose-to-ethanol conversion yield by this strain was 75% of the theoretical value at 40°C. However, few reports have described *Saccharomyces cerevisiae* strain of being both themotolerant and ethanol tolerant and having high-ethanol production at the same time at temperatures up to 55°C.

In conclusion, we, for the first time, explored the genome shuffling technique to improve the thermotolerance, ethanol tolerance and ethanol productivity of yeast, and combined this technique with protoplast mutagenesis. With the technique we developed, we obtained *Saccharomyces* yeast strain F34 which can effectively ferment 20% (w/v) glucose up to 45–48°C within 48 h, while maintaining high-cell viability up to 55°C. This strain can also tolerate 25% (v/v) ethanol stress. Further evaluations of the strain under scaled-up conditions are planned to evaluate the suitability for practical use in alcohol distilleries.

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