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Genome Shuffling of *Saccharomyces cerevisiae* for Improving Thermotolerance and Glycerol Production

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In this study, the thermotolerant and high glycerol-producing *Saccharomyces cerevisiae* strain F3/24 was obtained after three rounds of genome shuffling of UV and NTG mutants derived from the original strain *Saccharomyces cerevisiae* NAU-ZH-GY1. The recombinant *S. cerevisiae* strain F3/24 could grow well at 45 °C. At temperature higher than 39°C, recombinant strain F3/24 always gave higher cell growth and glycerol production. In fermentation medium containing 200g/l glucose and 5g/l corn steep liguor powder, glycerol yield of the shuffled strain F3/24 was 75.3, 72.4, 70.5 and 68.7g/l at 32 ºC, 39 ºC, 42 ºC and 45 ºC for 64h, respectively.

Key words: Genome shuffling, *Saccharomyces cerevisiae*, Glycerol, Thermotolerance.

Glycerol (glycerin) is a colorless, odorless, hygroscopic, sweet tasting, viscous liquid that is a by product of biodiesel production [1].. It is a simple alcohol with many uses in the cosmetic, food, tobacco, pharmaceutical, pulp and leather industries or as a feedstock for the production of various chemicals [2]. It is also a potential application as a gluconeogenic substrate for ruminants [3]. Moreover, it can be converted to glucose in the liver of cattle and can provide energy for cellular metabolism [4].

Although most commercial glycerol is produced by chemical synthesis, there are instances when biological synthesis by yeast is significant. The well-known yeast *Saccharomyces cerevisiae* is the most important glycerol-producing yeast[5]. Moreover, in North America and China, *Saccharomyces cerevisiae* species are also registered in the Generally Recognised As Safe (GRAS) list and are used as probiotics. They are increasingly used in ruminant nutrition as feed additives to improve feed efficiency and performance and, at the same time, to prevent health disorders [6,7]. However, the optimum temperature for maximum glycerol production by commercial yeast strains of *Saccharomyces cerevisiae* varies between 22-32ºC and *Saccharomyces cerevisiae* strains do not normally grow at temperatures >40°C nor do they produce glycerol at such temperatures[5, 8,9]. In the rumen condition, the rumen temperature is usually between 39 and 40.5 ºC [10], *S. cerevisiae* can not play its important role for the reason of the rumen temperature. Therefore, If the thermotolerance and high-yield glycerol *Saccharomyces cerevisiae* strains are successfully constructed, it can be efficiently used to ferment the starch, sugar, farm and sideline products as the raw materials into glycerol in vitro and can continue to play its

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biological role in vivo. These feed additives with the dual role of bio-glycerol and probiotics for ruminants will have a wide development prospect.

Genome shuffling serves as a genome-wide approach for improving desirable phenotypes without the knowledge of the genetic determinants or for network information about those phenotypes[11]. This method has been demonstrated as an effective method to increase enthanol production and multiple tolerance of *Saccharomyces cerevisiae*[12-15]. However, there has not been report that improving thermotolerance and glycerol production of *Saccharomyces cerevisiae* by genome shuffling.

In this study, firstly, the mutant strains of *Saccharomyces cerevisiae* with the property of thermotolerance and slightly improving glycerol production were obtained by using the traditional mutagenesis method, and then we demonstrated the application of genome shuffling to these mutants for further constructing high temperature resistance and high glycerol productivity excellent *Saccharomyces cerevisiae*. These study can lay the foundation for the future development of novel probiotics to prevent and treat the energy negative balanced diseases of the transition dairy cows.

MATERIALS AND METHODS

Bacterial strains and media

Strain

Saccharomyces cerevisiae NAU-ZH-GY1 was isolated from the unprocessed honey of a bee-keeping factory and identified by our lab and preserved in China General Microbiological Culture Collection Center, Spawn preservation number CGMCC No.4551. It can produced more glycerol (about 22.4 g l^{-1}) at its the most suitable fermentation temperature (32°C-33°C), but cannot ferment well at high temperature (higher than 37°C).

Malt extract agar(MEA)((g l^{-1})

Malt extract 15g, yeast extract 3g, glucose 10g, agar 20g. pH5.4. Yeast Extract Peptone Dextrose Medium (YPD) (g l^{-1}): Yeast Extract 5g, Peptone 10g, glucose 20g, agar 20g. Seed culture medium(g l^{-1}): glucose 100g, urea 3g, yeast extract 4g. Fermentation medium(FM)(g l^{-1}): glucose 140g, urea 2.14g, KH_2PO_4 0.4g, $MgSO_4$.7 H_2O 0.25g, NaCl 40g, the pH was not adjusted. Regeneration medium (RM) was YPD supplemented with KCl (0.6 mol l^{-1}), $CaC1_2$ (25 m mol l^{-1}) and agar (2%, w/v). Protoplast formation buffer (PB) consisted of 0.01 mol l^{-1} Tris-HCl, pH 6.8, 20 m mol l^{-1} $MgCl_2$, and 0.5 mol l^{-1} sucrose as a stabilizer.

Mutagenesis and mutant screening

The cells of *Saccharomyces cerevisiae* NAU-ZH-GY1 were mutagenized with nitrosoguanidine (NTG) and ultraviolet (UV) irradiation respectively. The growing NAU-ZH-GY1 cultures(16h) were washed after centrifugation, re-suspended in 0.1 mol l^{-1} phosphate buffer of pH7.0 and adjusted to 10^8 cells per ml by a turbidometric method. About 10 ml of NAU-ZH-GY1 cultures were transferred to sterile petriplates and irradiated for 6 min with a UV lamp of 30W at a distance of 30 cm, the percentage of cells that survived after the UV treatment was determined to be about 5%. For NTG mutagenesis, 5 ml of NAU-ZH-GY1 cultures were added with 5 ml of NTG(2.5 mg ml^{-1}), this culture was incubated for 1 h at 30°C with shaking at 180 rpm, the percentage of cells that survived was determined to be about 1%. Both of the cells after treated by UV and NTG were serially diluted in buffer and plated onto YPD plates and incubated at different high temperatures (38-45°C) for 48 h, the fast grown colonies that showed better thermotolerance than the original strain were picked off for shake-flask analysis to determine their glycerol production individually. The mutants with higher productivities were obtained and taken as the starter for genome shuffling.

Genome shuffling

Genome shuffling was carried out using modified described methods.The protoplasts of the UV and NTG mutants were prepared as previously described. 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 4,000×g for 5 min, washed twice with distilled water, and incubated in 0.2 M phosphate buffer (pH 7.0) containing 0.01 mol l^{-1} β-mercaptoethanol for 10 min at 30°C. Cells were harvested and then resuspended in protoplast formation buffer and 1% (w/v) lyophilized snail enzyme. After 40 min of incubation at 30°C with shaking at 180 rpm, fresh protoplasts were harvested and washed with protoplast formation buffer.

Equal number of protoplasts from different populations of the UV and NTG mutants

were mixed and divided equally into two parts. One part was inactivated with UV irradiation for 40 min, and the other was heat treated at 60°C for 40min, Both inactivated protoplasts were mixed in a cell ratio of 1:1 centrifuged, and resuspended in 50 µl, 0.2 mol l^{-1} phosphate buffer(pH 7.0) with 30%(w/v) polyethylene glycol(PEG; mol. wt 6000) and 0.01 mol l^{-1} $CaCl_2$. After incubation for 10min at 34°C, the fused protoplasts were harvested and resuspended in protoplast formation buffer. The serial dilutions were spread on RM plates and incubated at required high temperatures (40–45°C) for 4-7d. The colonies appearing under the conditions were selected to analyze their glycerol production. The strains with higher glycerol productivity were selected for the next round of genome shuffling. After each round of shuffling, the culture temperature used for selection was increased. The strains with improved thermotolerance and glycerol 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.

Shake-flask analysis

For shake-flask experiments, all fermentations were carried out as described by Zhuge (Zhuge *et al*., 2001). The 200 FM medium in 500 ml gauze-covered flasks was inoculated with 10 ml of the 16h cultures. Fermentations were carried out over a period of 64h at required temperatures on a reciprocating shaker at 180 rpm. Each strain was cultured in three shake-flasks.

Analytical methods

Glycerol concentration was confirmed with HPLC(Shimadzu Liquid Chromatograph, model RID-10A refractive index detector) using an Aminex HPL-87H(Bio-Rad, Hercules, CA) with a differential refractive index detector, operated at 55ºC, with 5mM H_2SO_4 as the mobile phase at 0.6ml min^{-1} flow rate.

Statistical analysis

All data were analyzed statistically using Data Analysis and Technical Graphics, origin 6.0 (Microcal Software Inc.).

RESULTS

Strain Mutagenesis and mutant screening

For thermotolerance of wild-type NAU-ZH-GY1, the temperature of 39 ºC was its threshold and strain could hardly grow on the PYD. Firstly, an initial library of *S. cerevisiae* with improving thermotolerance and glycerol characters were got

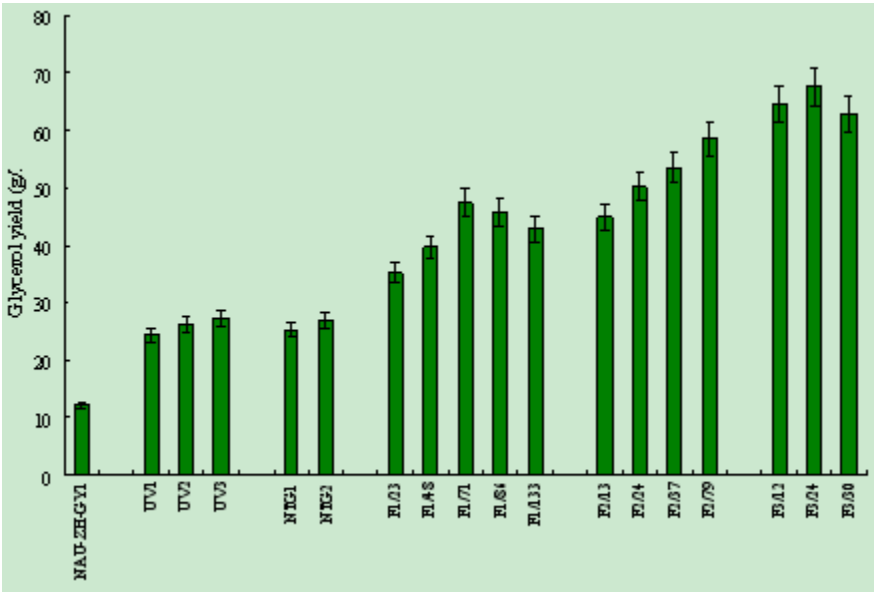

Fig. 1. Improvement of glycerol yield by mutation and genome shuffling. One to three rounds of genome shuffling were used to improve glycerol yield of S. cerevisiae NAU-ZH-GY1. The selected population after three rounds of genome shuffling exhibited the improved glycerol yield of more than 67.6 g/l. The Bar represents with standard deviation of less than 10%

by UV and NTG mutagenesis methods for three times. There were 143 UV mutants and 132 NTG mutants could grow on the YPG plates at the temperature 40 ºC. But there were only 7 UV mutants and 6 NTG mutants with the characteristic of improving glycerol productivity, selected five mutants with higher glycerol production among these mutants and named UV-1, UV-2, UV-3, NTG-1 and NTG-2. There five selected mutants showed glycerol production of 24.3, 26.2, 27.3, 25.2, 26.9 g/l respectively at 40ºC (Fig 1), which increased by 8.48%, 16.96%, 21.88%, 12.50% and 20.09% compared with that of the parental strain NAU-ZH-GY1 (22.4 g/l) at 32 ºC. The characterization of high glycerol productivity was maintained after at least 10 transfers in FM liquid medium in shake-flask. Consequently, these five mutants were used as the starting population for genome shuffling.

Improvement of thermotolerance and glycerol yield by Genome shuffling

Five mutants(UV-1, UV-2, UV-3, NTG-1 and NTG-2), which with high glycerol productivity and thermotolerance property were subjected to three rounds of protoplast fusion. After each round, 3-6 individual colonies with thermotolerance were selected according to the impoved one from the comparison with the best UV and NTG mutants on PYG plates. These mutants were individually tested

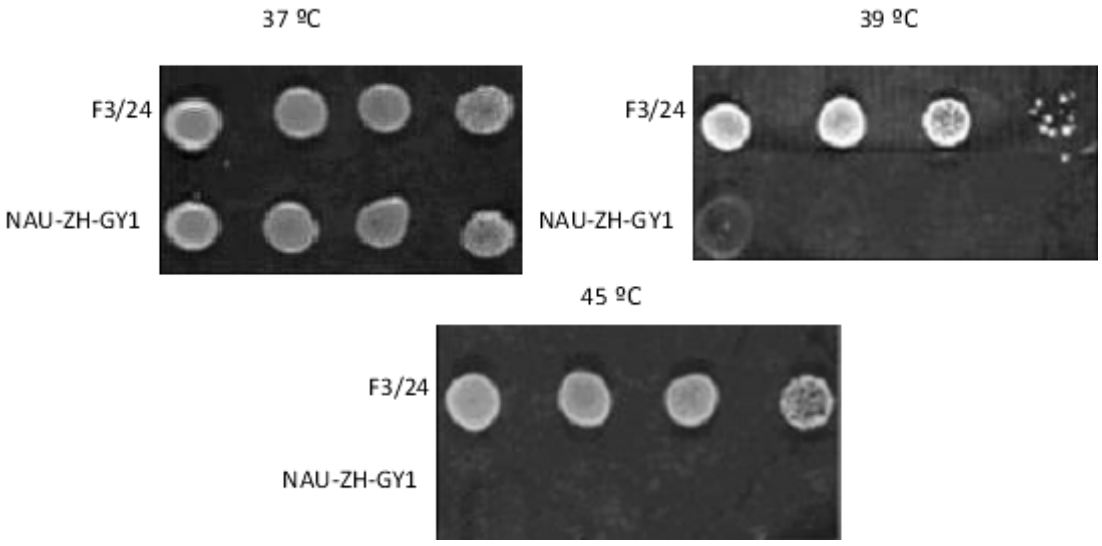

Fig. 2. Comparison of yeast cells under different temperature. Mid-exponential cultures(OD_{600} of 1.0) of recombinant strain F3/24 and original strain NAU-ZH-GY1 were serially diluted, and 5 µl of each dilution 10^{-1}- 10^{-4}(from left to right) was spotted onto YPD plates and incubated at indicated temperatures

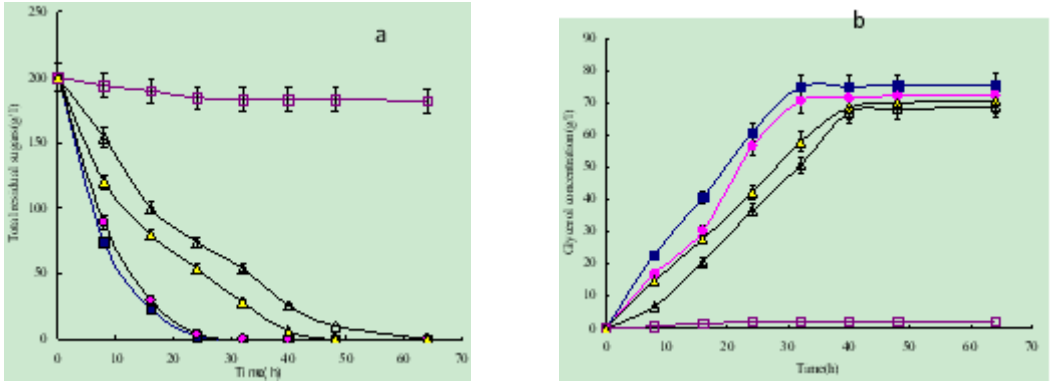

Fig. 3. Fermentation kinetics of F3/24 at different temperatures during fermentation: 50°C (open square), 45°C (open triangle), 42°C(closed triangle), 39°C (closed circle), 32°C(closed square). The error bars represent the standard deviations

for improved glycerol productivity in comparison with UV and NTG mutants and NAU-ZH-GY1 on FM liquid medium. Again, 3-6 colonies from each isolate showing improved thermotolerance and glycerol productivity were selected. These colonies were then pooled and subjected to the next round of genome shuffling. With each round of genome shuffling up to 3 rounds, apparent thermotolerance and glycerol productivity increased as shown in Fig. 1. In the first round of genome shuffling, 157 colonies which can grow at 42 ºC were selected and tested for glycerol production. The glycerol production were ranged from 34 to 46 g l^{-1} at 42 ºC in the FM liquid medium. Six recombinants(F1/23, F1/48, F1/71, F1/86 and F1/133) that exhibited a further improvement in the yield of glycerol (35.2, 39.6, 47.5, 45.7 and 42.8 g l^{-1}, respectively, Fig. 1) were used as the starter strains for the second round of genome shuffling.

The second round of genome shuffling produced 94 colonies that could grow at 44 ºC were assayed for glycerol production. After glycerol production tested at 44 ºC, five of these recombinants (F2/13, F2/24, F2/37 and F2/79) showed a further improvement in the yield of glycerol (44.9, 50.2, 53.4 and 58.6 g ml^{-1}, respectively, Fig. 1). These were subjected to a third round of genome shuffling producing 34 colonies that could grow at 45 ºC were assayed for glycerol production. Three recombinants (F3/12, F3/24 and F3/30) had a further improvement in yield of glycerol (64.5, 67.6 and 62.8 g l^{-1}, respectively, Fig. 1) over the second round. Strain F3/24 yielded 67.6 g $l^{-1}$of glycerol, which is about 2-fold higher than that of the initial strain NAU-ZH-GY1.

As indicated in Fig. 2, thermotolerance of *S. cerevisiae* was improved by using genome shuffling. The recombinant strain F3/24 had the same viability as the original strain NAU-ZH-GY1 at 37°C. However, when the temperature was increased to 45°C, the recombinant strain F3/24 still retained high viability whereas strain NAU-ZH-GY1 could not grow any more.

Characterization of glucose consumption and glycerol production of F3/24

The glycerol production and glucose consumption by strain F3/24 and NAU-ZH-GY1 under the anaerobic conditions at the temperature 32 ºC, 39 ºC, 42 ºC, 45 ºC and 50 ºC on contained 200 g l^{-1} glucose and 5 g l^{-1} corn steep liguor powder fermentation liquid was shown in Fig. 3. It can be seen that increasing the temperature resulted in a decreasing in the rate and extent of glucose utilization and glycerol production. At 32 ºC and 39 ºC fermented for 32h by strain F3/24, the 200 g l^{-1} sugars was all consumed and the glycerol production was 75.3 g $l^{-1}$and 72.4 g l^{-1}, respectively. At 42 ºC and 45 ºC, the fermentation time were extended by 16h and 24h than that at 32 ºC respectively when the 200 g l^{-1} sugar was completely consumed and the glycerol production were 70.5 g $l^{-1}$and 68.7g/l at the end of fermentation. At 50 ºC, there was almost no glycerol production.

The genetic stability of the F3/24

To check the genetic stability of F3/24, it was cultured for 30 generations and was measured its thermotolerance and glycerol production of every other generation. All the generations showed similar tolerance and production as the initial strain, suggesting that F3/24 are genetically stable and suitable for industrial production.

DISCUSSION

As is alrealy known, the high temperatures inhibit both the growth and fermentation of yeast, improving thermotolerance of the *Saccharomyces cerevisiae* strains can help in reducing cooling costs, distillation costs and have faster fermentation rates and also help in decreasing contamination chances during fermentation[16,17] . In this study, we successfully improved the thermotolerance and glycerol production of strain NAU-ZH-GY1 by genome shuffling technique combined with conventional mutagenesis methods.

Although UV and NTG is broadly applicable to improve the phenotype, including improving the thermotolerance[17], to our knowledge, this is the first report that using genome shuffing to construct thermotolerant yeast with good glycerol yield successfully.

Recent studies have shown that the biochemical mechanism of the tolerance to the adverse environmental conditions outside world is very complex, the multiple resistance phenotypes are not controlled by one or a few genes, they involves a large number of gene products and their associated metabolic pathways[18-20]. Before the mechanisms of external

stress on yeast cells were clarified, it is very difficult to use genetic engineer to improve the tolerance performance of the yeast[21]. Morever, The mechanisms of the metabolic pathway to produce glycerol of *Saccharomyces cerevisiae* also involved multiple enzymes and genes[22,23]. As a result, it is difficult to improve thermotolerance and productivity of the cell with direct genetic manipulation through the introduction and control of specific genes.

The results of the present study indicate that genome shuffing is a powerful means to rapidly improve the complex phenotypes of microorganisms, whether haploid or polyploid, while still maintaining their robust growth.

CONCLUSION

In this study, the application of genome shuffling combined with UV and NTG traditional mutagenesis method was successful used to breed of the strain with thermotolerance character and high glycerol production. This thermotolerant glycerol-producing *Saccharomyces cerevisiae* strain would lay a foundation for further studying its thermotolerance mechanism and its application in the production practice.

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