Appl Biochem Biotechnol (2010) 160:1084–1093
DOI 10.1007/s12010-009-8552-9

Improved Production of Ethanol by Novel Genome Shuffling in *Saccharomyces cerevisiae*

Lihua Hou

Received: 8 December 2008 / Accepted: 29 January 2009 /
Published online: 13 February 2009

Abstract Fermentation properties under the control of multiple genes of industrial *Saccharomyces cerevisiae* strain are difficult to alter with traditional methods. Here, we describe efficient and reliable genome shuffling to increase ethanol production through the rapid improvement of stress resistance. The strategy is carried out using yeast sexual and asexual reproduction by itself instead of polyethylene glycol-mediated protoplast fusion. After three rounds of genome shuffling, the best performing strain S3-10 was obtained on the special plate containing a high ethanol concentration. It exhibits substantial improvement in multiple stress tolerance to ethanol, glucose, and heat. The cycle of fermentation of S3-10 was not only shortened, but also, ethanol yield was increased by up to 10.96% compared with the control in very-high-gravity (VHG) fermentations. In total, S3-10 possesses optimized fermentation characteristics, which will be propitious to the development of bioethanol fermentation industry.

Keywords Ethanol · Genome shuffling · Industrial strain · *Saccharomyces cerevisiae*

Abbreviations

PEG	polyethylene glycol
EMS	ethyl methane sulfonate
CFU	colony-forming units
HPLC	high-performance liquid chromatography
FCAS	flow-cytometry analysis
VHG	very high gravity

Introduction

Due to the imminent decline in the availability of global fossil energy and increasing concern over environment, partial substitution of fossil fuel with bioethanol has become an

L. Hou (✉)
Department of Biochemical Engineering, School of Chemical Engineering and Technology,
Tianjin University, Tianjin 300072, People's Republic of China
e-mail: lhhou@tju.edu.cn

important renewable energy strategy. The demand of ethanol requires the engineering of new strains that can produce ethanol more efficiently. However, the ethanol and osmotic stress tolerance, the sugar-to-ethanol conversion rate, and the speed of fermentation are complicate phenotypes controlled by multiple genes [1]. For example, it is conjectured that more than 250 genes are involved in the ethanol tolerance of yeast [2]. These traits are difficult to manipulate directly by classical breeding, metabolic engineering, or other genetic methods with specific genes or pathways as target [3]. Hence, employing the whole genome engineering strategy will be a productive way to manipulate yeast strains, especially for industrial yeast strains with higher stress tolerances [4].

Genome shuffling, using the recursive multiparental protoplast fusion [5], is an accelerated evolutionary approach by which the preferred multiple traits are achieved. The strategy has been successfully applied in prokaryotic and eukaryotic cells [6–9]. However, genome shuffling in previous studies was carried out on the base of protoplast fusion. The hybrids would be unstable due to the genetic background of the parents that diverges in eukaryotes [10]. Therefore, protoplast formation and fusion induced by polyethylene glycol (PEG) cannot be implemented with high efficiency. Furthermore, the process of regeneration is laborious and time-consuming as it requires selection via many generations under nonselective conditions. Besides, some useful mutations were not obtained in the initial selection of variants. Genome shuffling cannot greatly accelerate the improvement of more phenotypes of microorganisms [11].

In this study, novel genome shuffling was used to increase ethanol production of industrial *Saccharomyces cerevisiae* strains by improving stress tolerance. Unlike most other microorganisms, *S. cerevisiae* strains have both stable haploidy and diploidy state. Thus, the genome can be shuffled using yeast meiosis and conjugation. The starting population was generated by ethyl methane sulfonate (EMS) mutagenesis and then subjected for the recursive sporulation of diploid cells [12] and cross of haploid cells. After three rounds of genome shuffling, the best performing strain was isolated on the plates with a high ethanol concentration.

Materials and Methods

Yeast Strains and Growth Conditions

The strain WT used in this study was the diploid industrial strains TH-AADY of *S. cerevisiae* (Angel Yeast, China).

Serial dilution assay was carried out to evaluate the stress tolerance. The log-phase cells were added to 1 ml fresh yeast peptone dextrose (YPD) medium (2% peptone, 1% yeast extract, and 2% glucose). After cultivation at 30 °C for 2 h, the cells were serially diluted ($10^{-1} \times 10^7$ cells/ml). Three microliters of the indicated dilutions were then spotted on YPD plates containing 15% (*v*/*v*) ethanol, 30% (*w*/*v*) glucose, and nonselective (YPD) plates, respectively. The plates were incubated at 30 °C or 40 °C for 3 days.

The fermentation medium was prepared from corn powder by the double enzyme hydrolyzed method [13]. The medium contained different concentrations of glucose, 0.5 g/l $(NH_4)_2HPO_4$, and 0.5 g/l K_2HPO_4. Anaerobic batch cultivations were performed in the cap-covered flasks with a working volume of 150 ml at 200 rpm and 30 °C. Inoculum was cultured in YPD media at 30 °C until OD_{600} reached 1.0. Then, preculture with the certain number of cells was transferred to the above flasks. During fermentations, pH 5 was maintained by regulation with 2 mol/l NaOH.

Sampling and Analysis

Samples were taken to determine cell number and colony-forming units (CFU). CFU per milliliter was determined by counting the number of colonies and multiplying the average by the dilution factor. In the meanwhile, supernatant analysis was conducted to measure concentrations of glucose, glycerol, and ethanol by high-performance liquid chromatography (HPLC; HP1100, Japan). XDB-C8 column (Agilent, America) for determination of ethanol was eluted with 0.25 mmol/l H_2SO_4 (pH 3.1) at 30 °C. Glycerol and glucose were analyzed using ZORBAX carbohydrate column (Agilent) and eluted with 75% acetonitrile at 30 °C. The flow rate was all 1 ml/min. Detection was performed via differential refractive index detector.

EMS Treatment

EMS treatment was carried out as described by Lawrence [14] with some modifications. The log-phase cells were recovered and washed twice with 0.1 mol/l potassium phosphate buffer, pH 7.0. Subsequently, above 10 ml buffer containing 5×10^8 cells in a hood was treated with EMS and incubated at 200 rpm and 30 °C for 30 min. An equal volume of a freshly made 5% solution of sodium thiosulfate was then added to stop EMS mutagenesis. The mutant cells were collected and washed twice with sterile water. The resulting cells were appropriately diluted and spread onto YPD plates, culturing at 30 °C for 2 days to allow for colony formation.

Efficient Sporulation

Yeast efficient sporulation was performed by means of a modified method [14]. The mutant cells of the initial population or each round of shuffling were grown to OD_{600}=1.0 in YPD medium and harvested by centrifugation (3,000 rpm, 5 min). Afterwards, the cells were washed three times with sterilized water and incubated in the 200-ml shake flasks with 60 ml of YPK medium (20 g/l peptone, 10 g/l yeast extract, 10 g/l KAc) at 200 rpm and 28 °C for 1 day. The resulting cells were washed three times with sterile water, followed by sporulation in the 200-ml flasks containing 60 ml of the medium (1% KAc, 0.1% yeast extract, 0.05% glucose, 0.005% adenosine, 0.005% uridine, 0.01% tryptophan, 0.01% leucine, and 0.01% histidine) at 200 rpm and 28 °C.

Spore Purification

Yeast spore purification was carried out using modified described methods [12]. Sporulated cultures of the appropriate diploid cells were harvested by centrifugation (3,000 rpm, 10 min) and resuspended in softening buffer (10 mmol/l dithiothreitol, 100 mmol/l Tris–SO_4, pH 9.4) at 5 OD_{600} per milliliter and incubated at 30 °C for 10 min. Subsequently, the cells and spores were collected, as above, and resuspended in spheroplasting buffer (1 mol/l sorbitol, 10 mmol/l potassium phosphate, pH 7.2) at 25 OD_{600} per milliliter. Zymolyase-20T (Seikagaku America) was then added to a concentration of 0.5 mg per OD_{600}, and the spheroplasting reaction was carried out at 30 °C for 30 min. The above suspension was spun at 3,000 rpm for 10 min and washed once with 0.5% (*v*/*v*) Triton X-100. The resulting spore pellets were resuspended in the same solution whose amount was one fourth the volume of spheroplasting buffer used in the previous step, followed by brief sonication to disperse the spores.

Random and Adequate Cross

The spores were cultivated in the 200-ml flasks containing 40 ml liquid YPD at 100 rpm and 30 °C. The culture of 100 μl was removed every 4 h and diluted with sterile water. The appropriately diluted cells were plated on YPD plates, and then the mating type of these colonies were tested by switching assays [15].

Selection of Mutant Cells

After concentrations of ethanol were determined at the end of fermentation with 30% (*w*/*v*) glucose as carbon source, the spent media were collected by spinning down cells and debris. Ethanol was removed from the media, recovered via distilling apparatus, and analyzed by HPLC. Although ethanol cannot be distilled completely from an aqueous solution, the remaining ethanol in the media had no effect on the selection of the desired mutant strains. The resulting media were adjusted to pH=4.2 with 2 mol/l NaOH and added with 1.5% agar, followed by sterilizing at 115 °C for 20 min. The special plates, made from the spent media and the different concentrations of ethanol by volume, were used to screen the mutant strains with high stress resistance. The concentration of ethanol in the plates was the summation of the concentration of ethanol left after distilling and the concentration of ethanol added after autoclaving.

Results

Novel Genome Shuffling

To determine the optimal mutagen doses of diploid industrial strain, 2.5%, 3.0%, 3.5%, and 4.0% (*v*/*v*) of EMS were tested. It is found that the lethal rate of four different doses was 75%, 88%, 94%, and 99%, respectively. Most efficient mutagenesis in haploid cells occurs when a mutagen confers a high frequency of mutations in the range of 50% to 90% lethality. Consequently, 3.5% and 4.0% (*v*/*v*) of EMS was excluded according to the lethal rate of four different doses. Here, diploid strains were induced to obtain dominant mutations, including many of the mutations to stress resistance [16]. Besides, diploid cells have a high survival rate than haploid cells when the cells are treated with the mutagen. To gain the frequency of mutations in diploid cells, 3% (*v*/*v*) EMS was thus used as the optimal mutagen doses. After that, all resulting mutant diploid cells were plated onto the special plates containing 8% (*v*/*v*) ethanol at 30 °C for 3 days. The 183 colonies were obtained from the plates and used as the starting population. When wild-type diploid cells were spread onto these special plates, as control, no colonies were observed. The plates were incubated at 30 °C; therefore, chances were very little for ethanol evaporation to occur. Further, the plates were airproofed to preclude the possibility of selection for mutants better able to metabolize ethanol.

Novel shuffling was carried out within the initial population using yeast sexual and asexual reproduction by itself. The process of sporulation continued until the efficiency of sporulation was about 60%. Random and adequate cross of 24 h ensured that the rate of cross was about 80%. All new zygotes were then cultivated in 50-ml, cap-covered flasks with a working volume of 10 ml fermentation medium at 200 rpm and 30 °C for about 8 days until a few living cells remained in the culture medium. When CFU per milliliter was about 100, the cells were harvested and subjected to next round of shuffling.

Fermentation media containing 20%, 25%, and 30% (*w*/*v*) glucose was used for enrichment of the mutant cells of the first, second, and third rounds of shuffling, respectively. After enrichment in fermentation media of the third round of shuffling, the resulting cells were harvested and spread onto the special plates containing 15% ethanol by volume. The plates were then cultivated for 4 days, and 18 colonies were obtained. As a control, none of the colony was observed when the initial mutagenic strains along with the first- and second-round shuffled strains were also spread onto the special plates containing 15% (*v*/*v*) ethanol, respectively. After that, the 18 mutant strains were evaluated in fermentation media at initial glucose of 30% (*w*/*v*). It was found that the stress tolerance and ethanol production of the 18 mutant strains were better than those of the control strain WT and the starting population (data not shown). The mutant strain S3-10 with the strongest stress resistance and the highest ethanol production was selected. The remainder of this study focuses on the selected strain S3-10 because it provides the most desirable phenotype with respect to elevated alcohol tolerance and ethanol production.

Morphology and Ploidy of the Selected Strain

Cell morphology such as size and shape was observed using phase-contrast microscope. The microscopic observation indicated that it was not a contaminant. No significant variation in cell shape was found. However, the cell size of S3-10 (about 4–7 μm diameter) is slightly larger than that of WT (about 4–6 μm diameter). Regarding whether S3-10 had a different ploidy than the parent strains, analysis of switching assays suggested that it was a diploid yeast strain. To further examine the ploidy of S3-10, flow-cytometry analysis (FCAS) was performed [17]. The log-phase cells were fixed with 70% EtOH and stained with propidium iodide, which bound DNA quantitatively and emitted red fluorescence with an intensity corresponding to DNA content [18]. Figure 1 displays ploidy state of S3-10 and WT as determined by FCAS. The data further demonstrated that S3-10 was a diploid yeast strain.

Improved Tolerance of the Selected Strain to Several Stress

To examine the effect of several adverse conditions on yeast strains, serial dilution assay was carried out under several stress conditions (heat, ethanol, and osmotic stress). Figure 2 shows the results of the selected strain S3-10 and the control strain WT under different stress conditions. Significant differences between S3-10 and WT can be observed in ethanol and osmotic stress resistance. Although thermotolerance of S3-10 was slightly stronger,

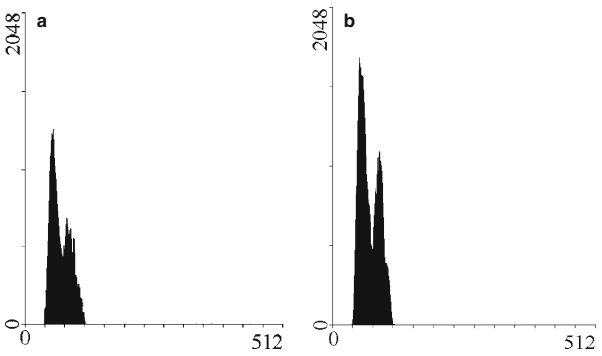

Fig. 1 FCAS of the selected strain S3-10 (**a**) and the control strain WT (**b**). The *horizontal axis* indicates DNA content and the *vertical axis* indicates number of cells. The *first and second peaks* in the histogram represents prereplication and postreplication cells, respectively

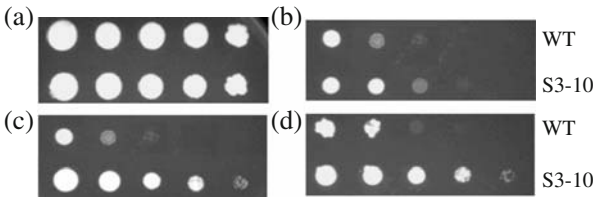

Fig. 2 Serial dilution assay of the selected strain S3-10 and the control strain WT on different plates. **a** Nonselective (YPD) plates and incubation at 30 °C; **b** nonselective (YPD) plates and incubation at 42 °C; **c** YPD plates containing 15% (*v*/*v*) ethanol and incubation at 30 °C; **d** YPD plates containing 30% (*w*/*v*) glucose and incubation at 30 °C. Experiments were carried out at least in triplicate. One representative experiment is shown

ethanol and osmotic stress tolerance of S3-10 was greater than the control. The result demonstrated that genome shuffling modified in this study could greatly accelerate the improvement of more phenotypes of yeast. Thermotolerant breeding of yeast strain would be focused on in our future work since thermotolerance is another key trait in ethanol fermentation.

Fermentations of the Selected Strain

The selected strain S3-10 and the control strain WT was evaluated using fermentation media with an initial concentration of 30% glucose. Figure 3 shows fermentation details including cell count, CFU, consumption of glucose, formation of glycerol, and production of ethanol. As can be seen from Fig. 3a, b, important differences were present between the strains S3-10 and WT. The growth characteristics of S3-10 with a prolonged exponential growth phase were superior to those of WT. It was interesting that cell number of S3-10 was inferior (Fig. 3a), but CFU was superior (Fig. 3b) compared with the control.

Table 1 indicates dry weight, residual sugar, and ethanol production at the end point of fermentation. Biomass of S3-10 was higher than that of WT, suggesting the metabolic activity of S3-10 was stronger than the control. In the case of glucose consumption, improvement in glucose utilization of S3-10 was achieved (Fig. 3c), resulting in the faster overall consumption of substrate and the faster overall ethanol production. The cycle of fermentation shortened from 49 to 42 h, thus reducing fermentation cost relative to the control. At the end of fermentation, remaining sugar of S3-10 decreased 51.32% compared with the control. Moreover, obvious difference in glycerol formation of the two strains was observed (Fig. 3d). It was noted that S3-10 provided up to 10.96% improvement in ethanol yield compared with the control.

Discussion

During very-high-gravity fermentations (VHG), yeast cells are exposed to greater glucose stress in the first stage and greater ethanol stress at the last stage compared with conventional fermentations [19]. Yeast cells also encounter other environmental stress, such as a decrease in the amount of nutrients and the accumulation of by-products produced by its normal fermentation [20]. The stress leads to a decrease in cell growth and the production of ethanol [21]. Therefore, enhancing the stress resistance which was closely related to ethanol productivity was a useful approach to improve fermentation performance

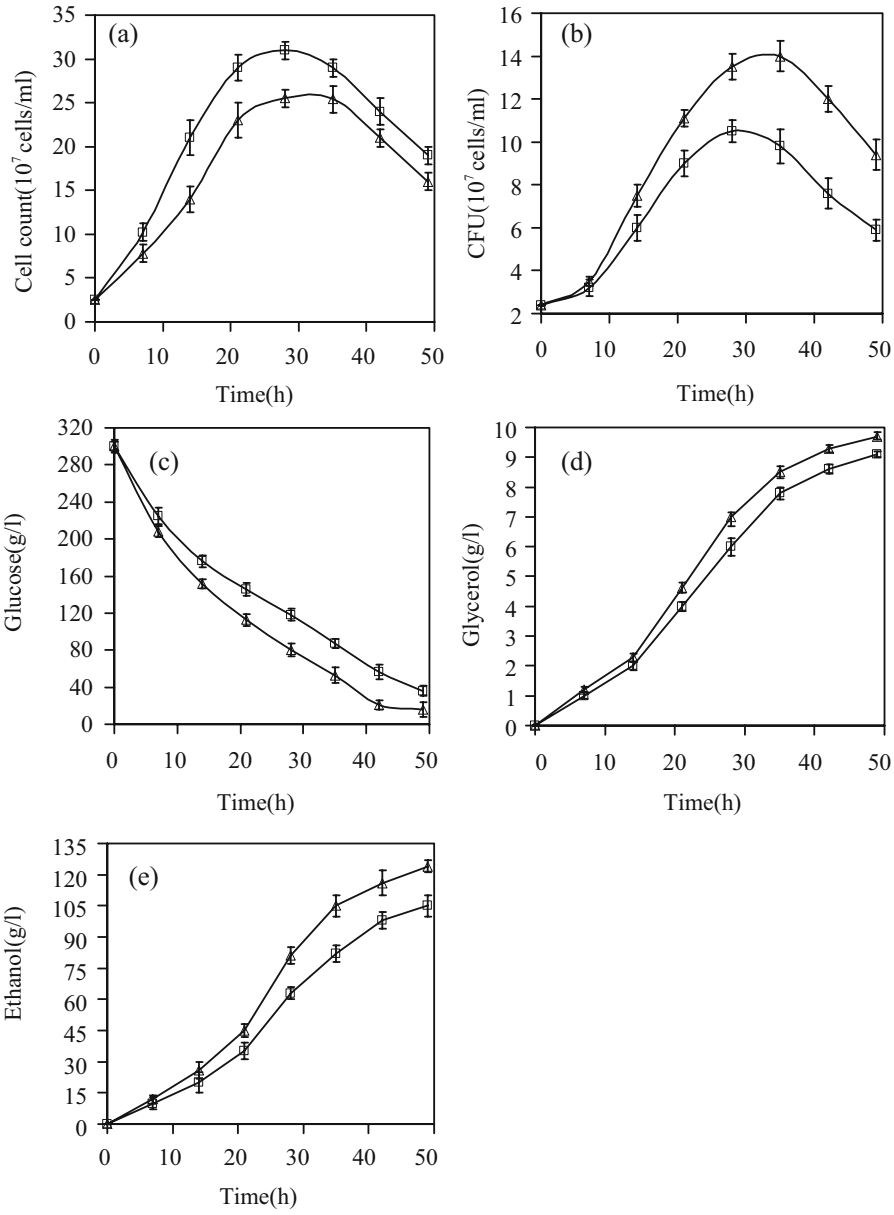

Fig. 3 Changes in measured parameters of OD_{600} (**a**), colony-forming units (**b**), consumption of glucose (**c**), formation of glycerol (**d**), and ethanol production (**e**) of S3-10 (*white triangle*) and WT (*white square*) during fermentations with 30% (*w*/*v*) glucose as carbon source. Data are averages from three independent experiments. *Error bars* are the maximum and minimum of duplicate experiments

[1]. Here, the novel genome shuffling was applied to enhance ethanol production of the industrial strain by improving the stress tolerance.

Lethal mutation in diploid yeast cells can be compensated by other mutations, whereas in haploid cells, it probably causes cell death. Thus, the initial population from

Table 1 Fermentation parameters of the selected strain S3-10 and the control strain WT at the end of fermentations.

Parameter	WT	S3-10
Dry weight (g/l)	19.12±0.23	21.87±0.28
Residual sugar (g/l)	31.07±0.45	15.12±0.86
Ethanol production (g/l)	105.80±1.02	118.83±1.16

The table shows the mean values and standard deviation of at least three independent experiments (variance analysis is ≤0.01)

mutated diploid cells contains more beneficial mutations, which are probably not maintained in the case of mutated haploid cells used as the starting population. In the present work, the rate of mating is high since meiosis and conjugation is a natural way for the sexual reproduction of yeast. On the contrary, the maximal fusion rate of protoplasts is only 10.5% when the PEG concentration is 35% [22]. Furthermore, the regeneration process increases the formation probability of polyploid yeast strains which appear to enhance chromosome loss level [23]. The regeneration process needs at least five batch growth cycles (about 20 generations). Besides, the selection approaches in this study provided better conditions to screen the cells with higher stress tolerance. Through the selection of enrichment, only the cells with higher stress resistance associated with desirable phenotypes can survive in the environment of decreasing of carbon source and increasing of ethanol. Moreover, the special plates made from the spent media and supplemented with ethanol are used to isolate the desired progeny. The methods of selection presented here enabled genome shuffling to greatly accelerate the improvement of more phenotypes involved in ethanol production and ethanol and osmotic stress tolerance. As such, the novel genome shuffling is more efficient than that based on PEG-mediated protoplast fusion.

The fermentation results (Fig. 3a, b) suggested high cell number of yeast strains was not essential to high viable cell count. Low number of cells resulted in less glucose consumed for cell growth and more substrates converted towards ethanol. On the other hand, high viable cell count indicated high resistance to greater glucose and ethanol stress, which was the most important reason of elevated ethanol production (Fig. 3e) during VHG fermentations [21]. The longer exponential growth phase and stronger metabolic activity of S3-10 are also key reasons of the increase in ethanol production. Besides, little residual sugar (Fig. 3c and Table 1) was not only a saving of substrate, but it was also advantageous in the downstream processing [24]. Additionally, higher production of glycerol (Fig. 3d) resulted in stronger stress tolerance, which is another important reason of high-level production of ethanol.

In conclusion, the success of novel genome shuffling depends on the selection of initial variants, the efficiency of the genetic shuffling during meiosis and conjugation, and the power of the selection approaches. The selected strain S3-10 possesses optimized fermentation characteristics under VHG conditions which will be propitious to the development of the starch- and sugar-based ethanol industry. In addition, the strategy can be explored to improve other desired traits in yeast strains which can also undergo efficient mating and sporulation.

Acknowledgments The author particularly thanks Prof. P. Ma for constructive advice on this work. The research was supported by the National Natural Science Foundation of China (no. 30470849).

References

1. Steinmeta, L. M., Sinha, H., Richaeds, D. R., Spiegelman, J. I., Oefner, P. J., McCusker, J. H., et al. (2002). Dissecting the architecture of a quantitative trait locus in yeast. *Nature*, *416*, 326–330. doi:10.1038/416326a.
2. Boulton, B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (1996). Yeast and biochemistry of ethanol fermentation. In B. Boulton, V. L. Singleton, L. F. Bisson, & R. E. Kunkee (Eds.), *Principles and practices of winemaking* (pp. 139–172). New York: Chapman and Hall.
3. Stephanopoulos, G. (2002). Metabolic engineering by genome shuffling. *Nature Biotechnology*, *20*, 666–668. doi:10.1038/nbt0702-666.
4. Alper, H., Moxley, J., Nevoigt, E., Fink, G. R., & Stephanopoulos, G. (2006). Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science*, *314*, 1565–1568. doi:10.1126/science.1131969.
5. Zhang, Y. X., Perry, K., Vinci, V. A., Powell, K., Stemmer, W. P. C., & Cardayre, S. B. (2002). Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature*, *415*, 644–646. doi:10.1038/415644a.
6. Patnaik, R., Louie, S., Gavrilovic, V., Stemmer, W. P. C., Ryan, C. M., & Cardayre, S. (2002). Genome shuffling of lactobacillus for improved acid tolerance. *Nature Biotechnology*, *20*, 707–712. doi:10.1038/nbt0702-707.
7. Dai, M. H., & Copley, S. D. (2004). Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum ATCC 39723*. *Applied and Environmental Microbiology*, *70*, 2391–2397. doi:10.1128/AEM.70.4.2391-2397.2004.
8. Hida, H., Yamada, T., & Yamada, Y. (2007). Genome shuffling of *Streptomyces sp. U121* for improved production of hydroxycitric acid. *Applied Microbiology and Biotechnology*, *73*, 1387–1393. doi:10.1007/s00253-006-0613-1.
9. Wei, P., Li, Z., He, P., Lin, Y., & Jiang, N. (2008). Genome shuffling of ethanologenic yeast *Candida krusei* for improved acetic acid tolerance. *Biotechnology and Applied Biochemistry*, *49*, 113–128. doi:10.1042/BA20070072.
10. Giudici, P., Solieri, L., Pulvirenti, A. M., & Cassanelli, S. (2005). Strategies and perspectives for genetic improvement of wine yeasts. *Applied Microbiology and Biotechnology*, *66*, 622–628. doi:10.1007/s00253-004-1784-2.
11. Yu, L., Pei, X., Lei, T., Wang, Y., & Feng, Y. (2008). Genome shuffling enhanced l-lactic acid production by improving glucose tolerance of lactobacillus rhamnosus. *Journal of Biotechnology*, *134*, 154–159. doi:10.1016/j.jbiotec.2008.01.008.
12. Herman, P. K., & Rine, J. (1997). Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle. *The EMBO Journal*, *16*, 6171–6181. doi:10.1093/emboj/16.20.6171.
13. Kong, Q. X., Cao, L. M., Zhang, A. L., & Chen, X. (2007). Overexpressing *GlT1* in *gpd1Δ* mutant to improve the production of ethanol of *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *73*, 1382–1386. doi:10.1007/s00253-006-0610-4.
14. Lawrence, C. W. (2004). Guide to yeast genetics and molecular and cell biology. *Methods in enzymology* Part A. Elesevier academic press NK, 194.
15. Houston, P., Simon, P. J., & Broach, J. R. (2004). The *saccharomyces cerevisiae* recombination enhancer biases recombination during interchromosomal mating-type switching but not in interchromosomal homologous recombination. *Genetics*, *166*, 1187–1197. doi:10.1534/genetics.166.3.1187.
16. Spencer, J. F. T., & Spencer, D. M. (1996). Yeast Protocols: Methods in Cell and Molecular Biology, Mutagenesis in yeast. 17–18.
17. Carlson, C. R., Grallert, B., Bernander, R., Stokke, T., & Boye, E. (1997). Measurement of nuclear DNA content in fission yeast by flow cytometry. *Yeast (Chichester, England)*, *13*, 1329–1335. doi:10.1002/(SICI)1097-0061(199711)13:14<1329::AID-YEA185>3.0.CO;2-M.
18. Ormerod, M. G., & Kubbies, M. (1992). Cell cycle analysis of asynchronous cells by flow cytometry using bromodeoxyuridine label and Hoechst-propidium iodide stain. *Cytometry*, *13*, 678–685. doi:10.1002/cyto.990130703.
19. Rautio, J. J., Huuskonen, A., Vuokko, H., Vidgren, V., & Londesborough, J. (2007). Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression. *Yeast (Chichester, England)*, *24*, 741–760. doi:10.1002/yea.1510.
20. Cardona, F., Carrasco, P., Perez-Ortin, J. E., Olmo, M., & Aranda, A. (2007). A novel approach for the improvement of stress resistance in wine yeasts. *International Journal of Food Microbiology*, *28*, 83–91. doi:10.1016/j.ijfoodmicro.2006.10.043.
21. Jones, R. P. (1989). Biological principles for the effects of ethanol. *Enzyme and Microbial Technology*, *11*, 130–153. doi:10.1016/0141-0229(89)90073-2.

22. Zhao, K., Ping, W., Zhang, L., Liu, J., Lin, Y., Jin, T., et al. (2008). Screening and breeding of high taxol producing fungi by genome shuffling. *Science in China Series C-Life Sciences*, *51*, 222–231. doi:10.1007/s11427-008-0037-5.
23. Mayer, W. V., & Aguilera, A. (1990). High levels of chromosome instability in polyploids of *Saccharomyces cerevisiae*. *Mutation Research*, *231*, 177–186. doi:10.1016/0027-5107(90)90024-X.
24. Devantier, R., Pedersen, S., & Olsson, L. (2005). Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. *Applied Microbiology and Biotechnology*, *68*, 622–629. doi:10.1007/s00253-005-1902-9.