Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(1):283-288

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and improvement of *Saccharomyces cerevisiae* for producing the distilled liquor

Guo-li Gong*, Li-yun Ma, Xue-feng Chen

College of Life Science and Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi, China

---

ABSTRACT

Various strains of Saccharomyces cerevisiae were isolated and characterized in an effort to screen for higher ethanol production strains from Daqu that is the starter culture of the Chinese liquor fermentation process, contains numerous enzymes and microbes. More than 200 colonies were isolated and characterized. A total of 67 yeast isolates were characterized as higher ethanol producers. The higher ethanol producers, 13-3, 13-10, 13-13 and 13-21, were selected out and prepared to protoplasts which were then mutagenized using UV-irradiation. The mutagenized protoplasts were further subjected to recursive protoplast fusions and the fusants were screened on selective medium containing ethanol of various concentrations. Furthermore, the production of ethanol was greatly increased by the genome shuffling. Many fusants showed higher ethanol yields (from 10.1% to 13.9%, v/v) than the original strain (9.5%, v/v). For two selected fusants, G13105 and G13110, showed the best fermentation characteristic. G13105 produced the highest level of ethanol, 13.9% (v/v) within 60 h fermentation, while G13110, 13.2% (v/v).

Keywords: *Saccharomyces cerevisiae,* Strain improvement, Genome shuffling, Distilled liquor

---

INTRODUCTION

Chinese Spirit production is a complex process in which three important process were took place: the production of Daqu as a starter culture, inoculating the sorghum must with the starter and the fermentation process of the spirit.

Daqu is the starter culture of the famous traditional Chinese liquor, prepared by a natural inoculation of molds, bacteria and yeasts on the grains and contains numerous enzymes and microbes.

The most important volatile compounds formed during fermentation that affect the organoleptic characteristics of Chinese Spirit are higher alcohols, esters and carbonyl compounds. Most of which are mainly produced by yeast metabolites. The yeast responsible for alcoholic fermentation in Spirit making is usually introduced into the must from Daqu and the fermentation environment and considered to play key roles during fermentation [1].

In traditional and industrial Spirit making, no external sources of *Saccharomyces cerevisiae* are added to the fermented sorghum, so the fermentation process generally occurs slowly and is difficult to control, and result in the low level alcohol production. To avoid these problems, the use of selected *Saccharomyces cerevisiae* strains is of interest to provide uniformity in the final product for industrial Spirit production. In addition, selected indigenous local *Saccharomyces cerevisiae* strains, can be used to obtain better quality wine than with only natural fermentation [2,3]. Use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermented process would reduce distillation costs and hence the profitability of the overall process. The disadvantages of brewing at lower ethanol production level could be overcome by using more robust yeast strains with higher ethanol capacity and tolerance to the associated stress conditions.

In alcoholic fermentations, many attempts have been made to enhance alcoholic fermentation using *Saccharomyces cerevisiae*, such as induction of alcohol-tolerant cells, screening of alcohol-tolerant mutants and alteration of nutritional conditions [4,5]. A number of compounds, including unsaturated fatty acids and sterols, proteins, amino acids, vitamins and metal ions, have improved alcoholic fermentations [6]. Complex medium, such as Jerusalem artichoke juice, also enhanced alcoholic fermentation by increasing yeast ethanol tolerance [7,8] .

Genome shuffling by recursive protoplast fusion of the mutants with different phenotypes is an efficient method for improving the production of metabolities by microbes [1,9]. The technique was demonstrated to be successful in increasing the production of tylosin in *Streptomyce fradiae* [1]acid tolerance in *Lactobacillus* [10]and even improved the degradation of pentachlorophenol in *Sphingobium chlorophenolicum* [11] .There have been no reports of genome shuffling or protoplast fusion in yeast.

In this paper, yeast strains from the post-spaceflight Daqu were isolated and characterized. In addition, we developed a protoplast fusion protocol and used the genome shuffling technique to improve the production of ethanol by *Saccharomyces cerevisiae*.

EXPERIMENTAL SECTION

Media and culture conditions
Growth medium (YPD) contained: 1% yeast extract, 2% peptone, 2% glucose, pH 6.5.

YPD Agar (0.4% yeast extract, 0.5% peptone, 2% glucose, 2% agar; pH 5.5).

Regeneration medium: YPD plus KCI (0.6 M) and 2% agar.

Fermentation medium (YPD-1): for ethanol production consisted of: 1% yeast extract, 2% peptone, 25% glucose, 0.6% $(NH_4)_2 SO_4$, 0.15% KH_2PO_4, pH 5.5.

Isolation of yeasts
The Daqu sample was carried out from liquor- production factory in Guizhou, China. Samples (10 g) were mixed with 90 ml sterile saline solution, soaked at 4°C for 30 min and homogenized with a Stomacher Lab Blender 400 (Seward Medical, London, UK). Serial dilutions of homogenate were surface plated on yeast-malt extract agar, YM-agar, which was supplemented with 0.01% chlortetracycline (Sigma, St. Louis, MO, USA) and 0.01% chloramphenicol (Sigma) to prevent bacterial growth. In addition, 0.02% Triton-X 100 (BDH Laboratory Supplies, Poole, England) was used to prevent the spreading of fungal colonies. All isolates were named as S-M series and stored for further evaluation.

Isolation and streaking were repeated on malt extract and Rose Bengal streptomycin agar at pH 4.5 until pure cultures were obtained. Colony characters of the pure yeast isolates were examined and cultures were stored in YPD agar medium under sterile mineral oil at 4°C.

Characteristics of yeast isolated
In the present study, the simplified identification methods [12,13] were used and these were based mainly on the morphological and physiological characteristics of the isolates.

Fermentation experiments by yeast isolates under laboratory conditions.

Yeast isolates subcultured on YPD agar plates were used as seed cultures for fermentations. Cultures were incubated at 30°C in YPD Growth medium in a rotary shaker.

Fermentation medium: YPD, 1% yeast extract, 2% peptone, 20% glucose, pH 5.5.

The seed cultures were inoculated into fermentation medium YPD with a rate of 2×10^6 cells/ml, cells were counted with a haemocytometer. Fermentations were carried out in 500 ml flasks, incubated in a rotary water bath at 30°C with mild shaking (100 rpm) sampled periodically.

Yeast inoculum: 2×10^6 cells/ml was added in 500 ml shake-flasks containing airlocks. The flasks were incubated in a rotary shaker at 30°C with mild shaking (100 rpm) and sampled periodically.

Genome shuffling
According to the ethanol production capacity, yeasts with the higher ethanol yields were selected out as the starting strain for the subsequently protoplast fusion protocol and genome shuffling process.

Cells were harvested in the mid-log phase of growth, washed twice with distilled water and incubated in 0.15 M phosphate buffer (pH 5.4) containing 0.01M

β-mercaptoethanol for 30 min at 28°C. Cells were collected and then resuspended in 0.15 M phosphate buffer (pH 5.4) containing 0.7 M mannitol and 2% (w/v) snail enzyme for enzymatic digestion of the cell wall. The cells were shaken at 100 rpm for 60 min at 30°C. The efficiency of protoplast formation was determined by microscopy.

After digestion for 1 h at 30°C, the fresh protoplasts were washed twice with 0.15 M phosphate buffer (pH 5.4) containing 0.7 M mannitol. About 5 ml buffer of the yeast protoplast was irradiated with a Phillips TUV-30-W-254 nm Lamp (Phillips, The Netherlands) for 0.5, 1 and 1.5 min at a distance of 20 cm. The treated protoplasts were kept in the dark for 2 h to avoid photoreactivation repair. (To calculate the viability, 0.1ml of the protoplast dilution was spread on the surface of a regeneration YPD agar plate at the same time and incubating at 30°C).

The mutagenized fresh protoplasts were mixed and resuspended in 0.15 M phosphate buffer (pH 5.4) containing 40% (v/v) polyethylene glycol (PEG6000) and 0.01 M $CaCl_2$. After gentle shaking for 15 min at 30°C to allow the protoplast fusion, 15 ml fresh buffer was added and the cells were centrifuged (2000rpm, 20 min) at 4°C. The fusants re-collected and re-suspended in the same buffer, containing 0.7 M mannitol, and immediately spread on regeneration plates (YPD plus KCI 0.6 M and 2% agar). The regeneration plates were incubated at 30°C for 4–7 days.

In fermentation, the YPD medium was inoculated with the selected fusants to give final cell concentrations of approximately 2×10^6 cells/ml. The fermentation was operated at 30 °C under static condition. Seed cultures were processed as described above.

Samples were taken aseptically at 12 h intervals to determine the alcohol concentration, residual glucose. The samples Ethanol was determined by densitometry at 20°C after distillation. The fermentation broth was centrifuged at 7000 rpm for 10 min. The supernatant was then determined for total residual sugar by a phenol-sulfuric acid method. In addition, yeast growth was determined by measuring cell numbers with a haemocytometer after 0, 12, 24, 48, 60 and 72 h incubation.

Studies were conducted to examine the effect of added ethanol on cell growth by the superior fusants isolates compared with their parental yeast under laboratory conditions. The yeast isolates were grown in media with 1.25% (w/v) glucose and varying quantities of ethanol added aseptically at concentrations of 0% (control), 4%, 6%, 8%, 10% and 12% (v/v). Yeast inocula were prepared as described previously and inoculated at an initial cell density of 2×10^6 cells/ml prior to incubation at 30°C for 48 h in a shaking water bath at 50 rpm. In addition, yeast growth was determined by measuring cell numbers with a haemocytometer after 0, 4, 8, 16, 24, 32, 40 h incubation.

RESULTS AND DISCUSSION

Selection and characterisation of indigenous yeast strains from Daqu starter
In an attempt to isolate naturally-occurring yeasts with better performance in fermentation, over 1000 isolates were collected from the Daqu.

A total of 67 yeast strains with alcoholic fermentation capabilities at least over 2% (v/v) ethanol were selected for subsequently study.

Yeasts which performed well in the fermentation of sugars were further evaluated to test their alcohol producing capabilities in YPD medium under laboratory conditions. The highest alcohol yield of over 9% (v/v) was produced by the yeast isolates 13-3, 13-10, 13-13 and 13-21. However, only the strains 13-3 and 13-10 showed thermotolerant fermentation characteristics at 43°C. Indigenous *Saccharomyces* yeasts with very high ethanol producing capabilities in the natural environment are thought to be very rare. It is possible that the *S. cerevisiae* yeasts isolated from Daqu with very high ethanol producing capabilities may be mutagenized during the aerospace flight process. For another possible reason, the yeasts were all isolated from the industrial Daqu starter culture, whose fermentation temperature was above 45°C, the high temperature environment is expected to exert a natural selection pressure on organi13s to evolve as thermotolerant strains. Yeasts in these areas undergo an irregular pattern of thermal adaptation cycling which may lead to mutation producing thermotolerant ones.

Genome shuffling

Since the aim of this study is to find a fusant that has high ethanol productivity and was thermotolerant, we screen those fusants based on following criteria: first, it must grow very well in ethanol selecting medium; second, it must grow well at 43°C. Based on those criteria, we decided to keep fusants 13-3, 13-10, 13-13 and 13-21 for further analysis.

After exposure to UV treatments, the viability of yeast cells declined with the time exposure to UV increased. When the exposure time increased to 12min, the viability dropped to 0. So we choose 8 min as the UV-mutation time.

Using the protocol provided in the Materials and Methods, the efficiency for preparation of protoplasts reached nearly 100%, and the regeneration ratio was more than 60%.

The conditions for the yeast fusion were also optimized, including degrees of polymerization and concentrations of PEG (polyethylene glycol), fusion time and temperature. Using the optimized fusion conditions provided in Materials and Methods, the fusion ratio, measured with a microscope, was about 10% at 5 min, and then increased to 50% at 10 min, 80% at 15 min, and after that time increased more slowly. During the fusion process, the protoplasts stuck together, allowing the pla13a membranes to dissolve at the points of contact and fusion of the protopla13ic contents took place. Finally, the fused protoplasts became single, large and round or oval shaped structures. On the selective regeneration plate, colonies were observed after 7 days.

We evaluated the production of ethanol by more than 1,000 regenerated fusants, and obtained many fusants with high yields of ethanol. After being subcultured for more than 100 generations, 21 fusants, produced much higher ethanol than the initial strain and were shown to be stable. The ethanol yields and the thermotolerant phenotypes of these fusants are shown in Table 1.

Tab. 1 Ethanol production of mutants of genome shuffling

Strains	Parent strains	Alcohol (% v/v)
G13-105	13-3 13 -10 13-13 13-21	13.9
G13 -110	13-3 13 -10 13-13 13-21	13.2
G13-10	13-3 13 -10 13-13 13-21	12.3
G13-20	13-3 13 -10 13-13 13-21	12.7
G13-4	13-3 13 -10 13-13 13-21	10.7
G13-5	13-3 13 -10 13-13 13-21	10.1
G13-46	13-3 13 -10 13-13 13-21	10.9
G13-67	13-3 13 -10 13-13 13-21	12.0
G13-22	13-3 13 -10 13-13 13-21	12.6
G13-17	13-3 13-13 13-21	12.9
G13-19	13-3 13-13 13-21	11.2
G13 1-5	13-3 13-13 13-21	11.4
G13 1-45	13-3 13-13 13-21	11.7
G13 1-1	13-3 13-13 13-21	10.9
G13 1-10	13-3 13-13 13-21	11.8
G13 1-48	13-3 13-13 13-21	12.7
G13 1-28	13-13 13-21	10.5
G13 1-32	13-13 13-21	12.3
G13 1-52	13-13 13-21	12.5
G13 1-21	13-13 13-21	11.1
G13 1-24	13-13 13-21	10.7

All of the fusants had high ethanol productivity than their parent, and could grow well at 43°C, suggesting that they had inherited those superior properties from their parental strains (Figure 1).

The ethanol yields (13.9%, v/v) of the highest-producing fusant was G13-105 (a fusant from two yeasts with higher ethanol producing capability and two yeasts could grow well at 43°C) was about 4.3% higher than the parent strain 13-3; another was G13-110, about 3.6% higher than 13-3. It is important to note that, while the absolute differences between the fusants and the staring strain may seem 13all to non-specialists, they are in fact quite important for the brewer. Due to the large scale of today's fermentation industry, even a 1% improvement in production efficiency represents a highly significant advantage.

Besides the yield, the fusant G13-105 also grew faster in liquid fermentation medium than all the parent strains. In addition, the fusant also produced ethanol earlier than the initial highest ethanol producer 13-3, and the peak yield of ethanol occurred as early as 60h of the cultivation, whereas the initial strain 13-3 peaked after 72 h.

G13-105 showed the best performance in terms of maximum ethanol production within shortest fermentation time, producing nearly 13.2% (v/v) ethanol at 43°C in 48 h and 13.9% (v/v) in 72 h, and G13-110 producing nearly 13.2% (v/v) ethanol at the same fermentation conditions in 65 h.

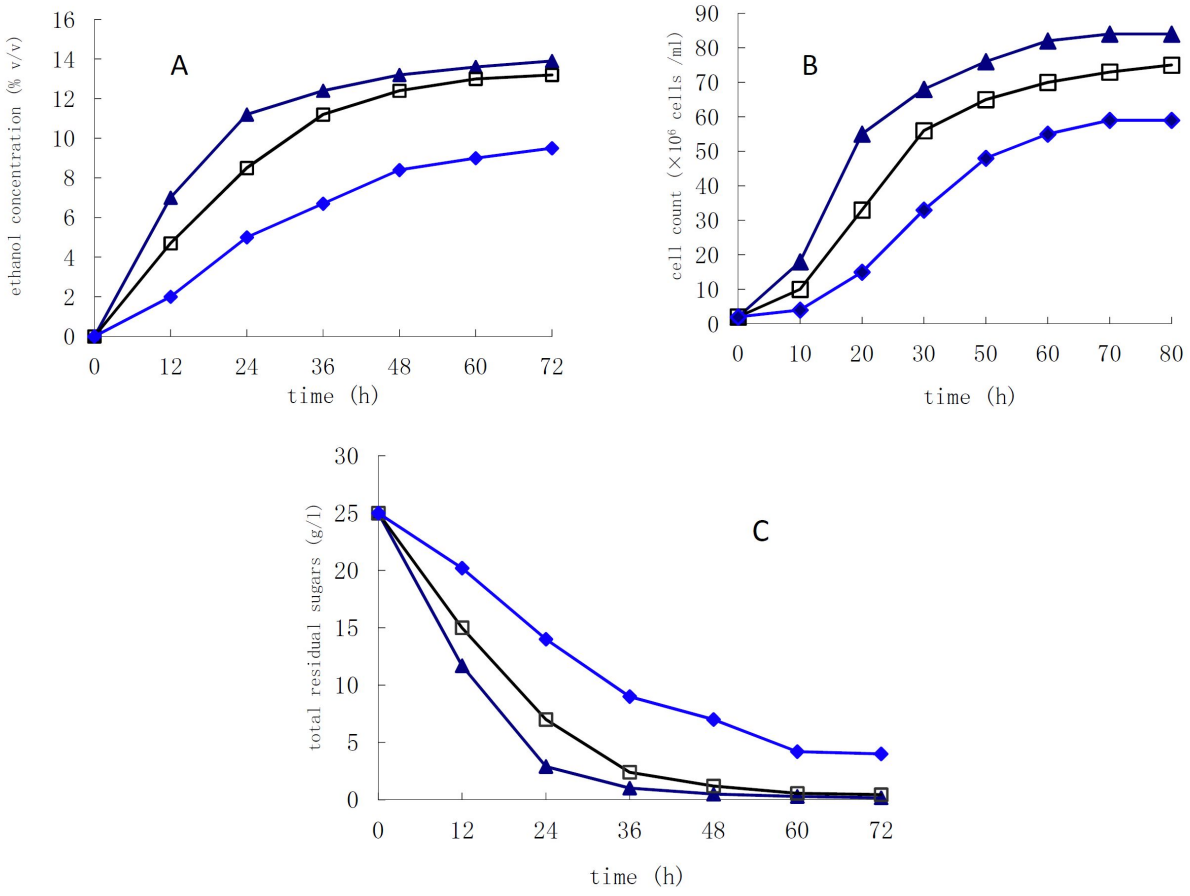

Fig.1 Fermentation kinetics by different yeasts at 43°C during fermentation (G13-105(▲), G13-110 (□), 13-3 (◆), a. Kinetics of alcohol production, b. growth curve, c. total residual sugars)

Tab. 2 Effect of ethanol on the growth of strain

Yeast isolate	Ethanol (% v/v)	Cell no. (10^6/mL) Hours after inoculation			
		16	24	32	40
G13-105	0	36	47	59	62
	4	35	42	52	62
	6	21	38	43	54
	8	19	35	40	52
	10	16	26	39	45
	12	11	11	19	20
G13-110	0	32	44	50	49
	4	28	40	47	45
	6	14	30	35	42
	8	11	21	24	36
	10	10	22	24	24
	12	7	14	16	16
13-3	0	15	34	42	50
	4	14	32	39	43
	6	11	25	29	33
	8	10	14	17	17
	10	3	4	8	8
	12	2	6	6	6

The possibility that the fusants G13-105 and G13-110 performed much better in test fermentations compared with their parent strain due to its greater alcohol tolerance was evaluated. For both yeasts, glucose consumption decreased with increasing exogenous ethanol concentrations. However, fusants G13-105 and G13-110 utilized higher levels of

glucose than their parent strain 13-3 at the same levels of ethanol, indicating that the fusants exhibits higher ethanol tolerance than 13-3.

Alcohol tolerance test (Table 2) shows the results of the alcohol tolerance tests of the two fusants and their parents. Similarly, the fusants G13-105 and G13-110 had a higher rate of multiplication in the presence of ethanol than their parent strain 13-3 (Table 3).The viability of each of two fusants indicated that they all had a higher alcohol tolerance than their parents. The alcohol tolerance test might lead to the same result that the higher ethanol productivity of the yeast depended on higher ethanol tolerance.

CONCLUSION

In this paper, yeasts with better fermentation features were isolated from Daqu. Then, we improved the ethanol producing capability of yeast by Genome shuffling combined with protoplast mutagenesis. With the strain improving technique we developed, we obtained two fusants (G13-105 and G13-110) with enhanced production of ethanol, which was 13.9% and 13.2% (v/v) respectively. With the fusants we obtained, we can improve ethanol yields in the fermented process and reduce distillation costs and hence improve the quality of liquor.

REFERENCES

[1] Zhang Y. X., Perry K., Vinci V. A., et al. *Nature*. **2002**, 415: 644–646.
[2] Vieira E. D., Andrietta M., Andrietta S. R. *Braz J Microbiol*. **2013**, 44(2):551-8.
[3] Enquist-Newman M., Faust A. M., Bravo D. D., et al. *Nature*. **2013**, [Epub ahead of print].
[4] Shiroma S., Jayakody L. N., Horie K., et al. *Metab Eng*. **2013**, 21C:26-33.
[5] Thomas D. S., Hossack J. A., Rose A. H. *Arch. Microbiol*. **1978**, 17: 239–245.
[6] Casey G. P. and Ingledew M. *Crit Rev Microbiol*. **1986**,13: 219–290.
[7] Rosa M. F., Sá-Correia I., Novais J. M. *Biotechno. Lett.* **1987**, 9: 441–444
[8] Rosa M. F., Sá-Correia I., Novais J. M. *Biotechnol Bioeng*. **1988**, 31: 705–710.
[9] Stephanopoulos G. *Nat Biotechnol*, **2002**, 20: 660–668.
[10] Patnaik R., Louie S., Gavrilovic V. et al. *Nat Biotechnol.* **2002,** 20: 707–712.
[11] Dai M. H., and Copley S. D. *Appl Environ Microbiol.* **2004,** 70: 2391–2397.
[12] Kurtzman C. P. and Fell J. W. *The Yeasts. A Taxonomic Study, 3rd edn*. Elsevier Publishers, Amsterdam, Netherlands. **1998.**
[13] Barnett C. D., Payne R. W., Yarrow D. *Yeasts: Characteristics and Identification,* CUP, Cambridge. **1983**.