

Developing New Sacchariferous Starters for Liquor Production Based on Functional Strains Isolated from the Pits of Several Famous *Luzhou-flavor* Liquor Brewers

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ABSTRACT

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Two new sacchariferous starters were developed based on functional strains isolated from the pits of several famous Chinese *Luzhou-flavor* liquor brewers and a genetically engineered mold strain previously constructed in this laboratory. Compared with traditional *Daqu*, the new starters, especially with the genetically engineered strain, possessed higher abilities of saccharification and fermentation as well as a higher alcohol yield ratio and percent conversion. Applying these new starters in liquor production has advantages that include easy preparation, convenient operation and time-savings and thus can greatly elevate the efficiency of liquor production. The taste and flavor of the liquors obtained with the new starters were comparable to those produced with traditional *Daqu*. This study further contributes to the investigation of the mechanisms of traditional liquor production and the improvement of process control. It also demonstrates the possibility of producing high quality traditional or new-style liquors in different locations.

Key words: brewing, Chinese liquor, *Daqu*, *Luzhou-flavor* liquor, new sacchariferous starters

INTRODUCTION

Chinese liquor is not only popular in China but is also well known worldwide. Among the main types of Chinese distilled liquors, *Luzhou-flavor* liquor is the most accepted one, of which many famous brands, such as *Wuliangye*, *Luzhou-laojiao* and *Quanxing*, are manufactured in Sichuan province. Produced from the distillation of *Zaopei* (a mixture of fermented grains such as sorghum, corn, wheat and rice), *Luzhou-flavor* liquor is generally de-

scribed as highly flavored, sweet and refreshing³. The microbes growing in *Zaopei*, which come from Chinese *Daqu*, pit mud and the fermentation environment, are considered to play key roles during the fermentation and contribute highly to the flavor and taste of the final product.

Daqu, a special starter culture for liquor brewing in China, is roughly equivalent to malt and yeast for beer fermentation¹⁶. Traditional *Daqu* is prepared by a natural inoculation of molds, yeasts and bacteria as well as their growth on the grains. The preparation of *Daqu* includes complicated procedures including material mixing, shaping, ripening and drying. The whole process is rather time-consuming, and 3–4 months is usually required. On the other hand, as microbes in *Daqu* come from the natural environment, their composition is complicated and depends heavily on experience, weather and a number of geographical factors. The traditional preparation method of *Daqu* not only makes it impossible to produce high quality traditional liquor in different locations, but also results in unstable qualities of the sacchariferous starter and the liquor.

Developing a starter based on a pure microbe culture, named *Fuqu* in China, is a promising way to increase liquor productivity. However, liquors obtained with *Fuqu* often suffer from a low quality flavor and taste, which may be attributed to the lack of various functional microbes in the starters. In recent years, isolation and analysis of microorganisms in *Daqu* and *Zaopei* of famous distilleries has received considerable attention and an increasing body of knowledge in this aspect has been accumulated^{8,10,16,17}. This sheds some light on elucidating the mechanisms of Chinese liquor production, and also shows the possibilities of developing new starters with functional microbes. With these new starters and appropriate environment controls, production of high quality traditional liquors in the future may be not confined only to their places of origin.

The aim of this research was to develop a new sacchariferous starter for *Luzhou-flavor* liquor production based on functional strains isolated from the pits of famous Chinese distillers elevating liquor productivity while maintaining flavor and taste.

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MATERIALS AND METHODS

Strains

The strains of bacteria, mold and yeast were isolated from the *Zaopei* in the pits of several famous Chinese distilleries including Sichuan *Quanxing* distillery Co., Ltd. and *Luzhoulaojiao* Co., Ltd. In total, more than 1000 strains were obtained, among which 6 bacteria, 7 yeasts and 3 molds were selected according their characteristics and abilities for liquor production. These strains, together with a genetically engineered mold strain previously constructed in this laboratory (ZZH) with high starch utility under acidic condition, were used to develop the new starters. Detailed information for the construction of strain ZZH can be found in the literature¹⁵. Briefly, two target genes, *asaA* (coding for an acid stable α -amylase) and *glaA* (coding for a glucoamylase) obtained from a mold strain, together with a regulation gene were transferred into a plasmid. The plasmid was then transferred into the same mold strain to obtain a recombinant with multi-copies of the target genes.

Materials

Grains (mainly sorghum), auxiliary raw materials (mainly chaff), tap water, the new starters developed in this study and traditional *Daqu* were used for the brewing process. *Daqu* and the raw liquor sample for comparison were kindly provided by *Quanxing* distillery Co., Ltd.

Brewing and distillation

Starter development was carried out in 250 mL flasks, the scale-up and preparation of starters for liquor brewing in pit were conducted in 3000 mL flasks, and liquor brewing was performed in an artificial pit developed previously in this laboratory¹³. All experiments were carried out in triplicate.

In liquor brewing, the prepared raw materials were fermented for 20 days (when using new starters) or for about one month in the pit (when using *Daqu*) after several procedures including material mixing and grain stewing, details of which have been described in a previous paper¹³. In all fermentations, 100 kg of material, with a 16% (w/w) starch content, was used. After brewing, the fermented grains were removed and a distillation performed to obtain the distilled liquor.

Analytical methods

Phenotypic characterizations of the isolated strains were carried out according to the literature^{1,2,7}. Alcohol content in *Zaopei* was determined at 600 nm by potassium dichromate-spectrophotometry¹². The alcohol in the distilled liquor was determined directly using an alcoholometer (Liminju Glass Instrument Company, Hejian City, Hebei Province) and a conversion table of temperature and concentration. After the assessment of alcohol, the percent conversion of starch and alcohol yield ratios for starters was calculated as follows:

$$\text{Percent conversion of starch (\%)} = \frac{\text{actual liquor yield}}{\text{theoretical liquor yield}} \times 100$$

$$\text{Alcohol yield ratio (\%)} = \frac{\text{actual liquor yield}}{\text{total amount of starch}} \times 100$$

Saccharification ability for *Daqu* or starter was assessed as follows:

The enzyme extracted from *Daqu* or starter with a buffer solution (pH 4.6) consisting of HAc and NaAc at 35°C for 1 h was added to a soluble starch solution. After incubation at 35°C for 15 min, the glucose in the saccharified solution was analyzed using the dinitrosalicylic acid (DNS) method. Saccharification ability was expressed by the production of glucose (mg) by 1g *Daqu* or starter per hour.

Liquefaction ability for *Daqu* or starter was measured as follows:

The enzyme extracted from *Daqu* or starter with a buffer solution (pH 6.0) consisting of Na₂HPO₄ and citric acid at 40°C for 1 h was added to an active starch solution. The solution was then incubated at 60°C until the starch was entirely liquefied, which was indicated by the change in color from blue to red-brown when sample from the liquefied starch solution was added to an iodine solution. Liquefaction ability was calculated by the liquefied starch (g) by 1 g *Daqu* or starter per hour.

The aromatic compounds were determined using a GC-960 Gas Chromatograph (Haixin Chromatographic Instrument Ltd., Shanghai) equipped with a FID detector and DNP filled column. The column was a stainless helix column (2m × Φ3mm, filled with 6201 red supporter with 80~100 holes and a mixed stationary liquid of 15% dinonyl phthalate and 6% Tween-80). The injector, detector and column temperatures were set at 125°C, 120°C and 90°C, respectively. The carrier gas was N₂, and N₂, H₂ and air flow-rates were set at 20 mL/min, 20 mL/min and 230 mL/min, respectively^{4,11,14}. The chromatogram was analyzed using a N2010 workstation (Zhida Information Engineering Ltd., Zhejiang University).

Sensory appraisals for liquors

Sensory appraisals for liquors were performed according to a reference⁶. Briefly, liquor samples were evaluated by ten tasters and the average scores of color, flavor, taste and style of the samples, as well as their summations were given.

RESULTS AND DISCUSSION

Selecting functional microorganisms for starter development

The main function of bacteria in liquor brewing is to produce acids, which can react with the alcohols and form esters. These acids and esters are important to form the flavor and taste of the liquor. Additionally, some bacteria can produce enzymes including cellulase, gelatin liquefaction enzymes and pectinase, which are important for material degradation and also contribute to the flavor of the final product⁹. Based mainly on abilities of starch degradation and acid production, six strains of bacteria were selected (Table I). All these strains of *Lactobacillus* sp. possessed a high ability to degrade starch. Strains LII-2-H-11, LII-2-H-14, LII-4-H-1, LII-6-H-26 had high acid productivity, promoting the formation of esters and thus increasing the liquor flavor. Among these strains, LII-2-H-11, LII-2-H-14, LII-4-H-1 and LII-6-H-26 were aerobes;

Table I. Phenotypic characteristics of the selected bacteria^a.

Strain	Starch degradation	Gelatin liquefaction	Pectinase production	Cellulase production	Acid production	Genus
LII-2-H-11	+++++	+	+	+	+++++	<i>Lactobacillus</i> sp.
LII-2-H-14	+++	+	-	+	+++	<i>Lactobacillus</i> sp.
LII-4-H-1	+++	+	-	-	+++	<i>Lactobacillus</i> sp.
LII-6-H-26	+++++	+++	-	-	+++	<i>Lactobacillus</i> sp.
LII-6-JX-13	+++++	-	-	-	++	<i>Lactobacillus</i> sp.
LII-7-JX-13	+++++	-	-	-	++	<i>Lactobacillus</i> sp.

^a+ represents the size of transparent circle on the plate; -, not detected

Table II. Phenotypic characteristics of the selected yeast^a.

Strain	Acid production	Ester production	Alcohol production	Genus
LI-8	+++	+++++	+++	<i>Citeromyces</i> sp.
LI-30	+++++	++	++++	<i>Candida</i> sp.
L2-5	+++	++	+	<i>Debaryomyces</i> sp.
Q-18	+++	+	++	<i>Citeromyces</i> sp.
LII-2-2	-	+++++	++	<i>Pichia</i> sp.
J-E8	++++	+++	+++++	<i>Saccharomyces</i> sp.
Q-2	++++	++++	+++	<i>Oosporidium</i> sp.

^a+ represents the size of transparent circle on the plate; -, not detected

Table III. Phenotypic characteristics of the selected mold^a.

Strain	Starch degradation	Gelatin liquefaction	Cellulase production	Genus
LI-3-6	++++	++	-	<i>Aspergillus</i> sp.
LI-1-2	+++++	+	+	<i>Aspergillus</i> sp.
LII-4-6	++	++++	-	<i>Penicillium</i> sp.
ZZH	+++++	-	-	<i>Aspergillus</i> sp.

^a+ represents the size of transparent circle on the plate; -, not detected

while LII-6-JX-13 and LII-7-JX-13 were facultative anaerobes. Therefore, adding these strains to the starter helps meet the requirements of liquor production, which has both aerobic and anaerobic stages.

The yeast contributed mainly to the formation of ethanol during the fermentation but yeasts also have the ability to produce acids and esters and thus are important for liquor flavor formation¹⁷. With the consideration of their productivities of alcohol, acids and esters, seven yeast strains were selected (Table II). These strains showed high productivities for ester, acid or alcohol. Addition of these strains to a compound starter should therefore increase both the alcohol productivity and liquor flavor.

Mold, which exists abundantly in naturally prepared *Daqu*, plays a key role in starch degradation by supplying simple sugars for the fermentation by the yeast. In brewing with starchy material, the ability of starch degradation by mold determines the utility of the material. Taking into account their abilities of starch degradation, three mold strains were selected (Table III). Most of these strains possessed high abilities for starch degradation. Additionally, strain LII-4-6 had a high ability for gelatin liquefaction and strain LI-1-2 had the ability to produce cellulase. These abilities are helpful for the degradation of material such as wheat and sorghum.

Strain ZZH is a genetically engineered mold constructed previously in this laboratory, details of which can be found in the literature¹⁵. The main characteristic of this strain is its high ability for starch utilization under acidic condition, which is important for liquor production, as brewing is normally performed largely with a pH value lower than 4.5¹³.

Table IV. The abilities of saccharification and liquefaction of different sacchariferous starters.

Starters	Saccharification ability (mg glucose/g starter h)	Liquefaction ability (g starch/g starter h)
Starter A	5208	1.3
Starter B	9042	4.3
<i>Daqu</i>	2600	Not detected

Developing compound starters for liquor production and the scale-up of starter preparations

Referring to the compositions of microbes in traditional *Daqu*, two new starters (starter A and starter B) were developed with selected strains (Table I-III).

In starter A, 4% of the culture was selected molds (the amounts of all strains were equal, the same below) except for strain ZZH, 2.4% was the bacterial culture and 5% was the yeast culture. After the cultivation at 37°C for 4 days, starter A was obtained.

In starter B, 4% mold culture (strain ZZH was involved) was cultivated at 28°C for 2 days, then 2.4% bacterial culture and 5% yeast culture were added. After cultivation at 28°C for another 2 days, starter B was obtained.

Comparison among different starters showed that the new starters had much higher abilities of saccharification and liquefaction than traditional *daqu*, and the abilities of starter B were the highest (Table IV). This can be attributed to the concentration of functional strains in the new starters, and the supplementation with strain ZZH which appeared to increase the abilities of saccharification and liquefaction. As these abilities determine the efficiency of liquor production, applying these new starters could significantly shorten the period of liquor brewing.

Scale-up of the starter preparation was a necessity for commercial liquor production. To optimize operation parameters for starter preparation, effects on the abilities of saccharification and liquefaction of new starters by temperature, cultivation time and inocula were assessed using an orthogonal test (Tables V and VI). The optimal parameters for the preparation of starter A were as follows: temperature 37°C, cultivation time 4 days and 3% inocula, with cultivation time as the predominant factor. The optimal parameters for the preparation of starter B were temperature 28°C, cultivation time 4 days and 1% inocula, and temperature was the predominant factor. Though the saccharification ability of starter B decreased slightly, the enzyme abilities of new starters were maintained satisfactorily after scale-up.

Table V. Orthogonal test for the scale-up of starter A.

No.	A Temperature (°C)	B Time (d)	C Inocula (%)	Saccharification ability (mg glucose/g starter h)	Fermentation ability (g CO ₂ /100g grain 72h)
1	28	2	0.5	1759.59	3.42
2	28	4	1	2911.05	4.72
3	28	6	3	2018.33	2.87
4	37	2	1	2076.55	3.21
5	37	4	3	5045.80	6.72
6	37	6	0.5	2264.15	2.95
7	45	2	3	2173.58	2.54
8	45	4	0.5	3803.77	3.02
9	45	6	1	575.74	1.72
I	58.7	47.7	54.3		
II	59	73.7	50		
III	44.7	41	58		
R	14.3	32.7	8		

Table VI. Orthogonal test for the scale-up of starter B.

No.	A Temperature (°C)	B Time (d)	C Inocula (%)	Saccharification ability (mg glucose/g starter h)	Fermentation ability (g CO ₂ /100g grain 72h)
1	28	2	0.5	2205.59	3.25
2	28	4	1	7921.04	5.22
3	28	6	3	7027.98	4.87
4	37	2	1	4076.55	4.21
5	37	4	3	4045.17	3.72
6	37	6	0.5	2264.15	1.95
7	45	2	3	2146.58	2.44
8	45	4	0.5	2803.77	1.02
9	45	6	1	1575.74	0.72
I	67.7	52.3	36.7		
II	54	53.3	53.3		
III	24.7	40.7	56.3		
R	43	12.6	19.6		

Applying new starters for liquor production in an artificial pit

With new starters and traditional *Daqu*, liquor brewing was performed in an artificial pit¹³ using grains as the substrate. The alcohol yield ratios and percent conversions obtained with different starters were calculated after measuring the alcohol produced (Table VII). Starter B had the highest alcohol yield ratio and percent conversion among the three starters, and both starter A and B had a higher alcohol yield ratio and percent conversion than traditional *Daqu*. This showed that starters composed with selected functional strains, especially with the addition of the engineered mold strain ZZH, could significantly increase material utilization.

Flavor, taste, color and style are important indices in evaluating the quality of Chinese liquor, for which sensory appraisal is a widely accepted method⁶. Table VIII demonstrates that the scores of the liquors obtained with the new starters were close to the scores of the liquor produced with traditional *Daqu*. This showed that applying the new starters, especially starter B, in brewing could maintain liquor quality.

To further understand the fragrance composition of the liquors produced with the new starters, aromatic compounds in the liquors obtained in the artificial pit were analyzed (Table IX). In samples produced with the new starters, especially in those produced with starter A, abundant aromatic compounds were detected with relatively high contents of ethyl acetate and ethyl butyrate and a low content of ethyl lactate. Liquors obtained with new start-

Table VII. The alcohol yield ratios and percent conversions for liquor productions with different starters in an artificial pit.

Liquor production with different starters	Alcohol yield ratio (%)	Percent conversion of starch (%)
Starter A	35.9	63.2
Starter B	44.7	78.6
<i>Daqu</i>	31.3	55.6

ers had a higher ester content and a lower acetaldehyde content than those produced with traditional *Daqu*. All these characteristics are required for *Luzhou-flavor* liquors. Therefore, the new starters have the potential to produce a high quality liquor.

Hexanoic acid and ethyl hexanoate, which are abundant in *Luzhou-flavor* liquor products, were not detected either in the liquors obtained with the new starters or in those produced with traditional *Daqu*. This may be attributed to the lack of mud in the artificial pit, as the pit mud is known to be closely related to the growth of hexanoate bacteria⁵. In addition, some necessary steps for liquor production used in distilleries, including aging and blending, were not adopted in our experiments due to experimental limitations. This might have influenced the flavor and taste of liquors obtained with new starters, thus the qualities of liquor obtained in our study cannot be compared directly to those of high quality products. This supposition can be supported by the results of the sensory appraisals and flavor ingredient analyses in Tables VIII and IX, where no significant differences were found between the liquor produced with new starter and that with

Table VIII. Sensory appraisals for liquors obtained with different starters.

Distilled liquor obtained with different starters	Color (out of 10)	Flavor (out of 25)	Taste (out of 50)	Style (out of 15)	Total score (out of 100)
Starter A	8.25	16.31	30.23	11.56	66.35
Starter B	8.17	16.67	31.67	12.00	70.51
Daqu	8.93	16.46	33.89	13.51	72.79

Table IX. Comparison of flavor ingredients in different liquors (mg/100 mL).

Components	Liquors obtain with different starters in an artificial pit			Highly flavored liquor products	
	Starter A	Starter B	Daqu	LLT liquor ^a	QX raw liquor ^b
Formic acid	16.4	n.d. ^c	13.5	n.d.	9.6
Acetic acid	39.5	n.d.	7.1	64.3	38.9
Propionic acid	1.6	n.d.	1.9	n.d.	2.1
Butyric acid	2.1	n.d.	6.1	1.2	19.1
Isovaleric acid	0.3	n.d.	n.d.	n.d.	1.2
Hexanoic acid	n.d.	n.d.	n.d.	82.8	57.9
Lactic acid	36.1	n.d.	4.6	37.8	21.0
Ethyl hexanoate	n.d.	n.d.	n.d.	189.0	186.0
Ethyl acetate	123.0	142.6	37.2	124.0	135.4
Ethyl butyrate	10.2	27.9	31.4	24.0	20.7
Ethyl lactate	17.5	25.6	15.0	141.0	98.4
Isobutyl alcohol	25.5	15.4	23.1	12.0	50.4
Normal butanol	3.0	1.2	14.2	8.6	7.8
Isoamyl alcohol	92.1	54.7	79.0	34.6	55.8
Propyl alcohol	32.5	8.9	34.7	15.5	25.1
Sec-butyl alcohol	2.0	n.d.	1.6	2.8	11.4
Acetaldehyde	16.0	18.3	33.0	18.6	37.1
Acetal	21.8	0.9	42.1	122.1	84.7

^a Published data for high quality liquor produced by *Luzhou Laojiao* Co., Ltd.

^b Raw liquor sample provided by *Quanxing* distillery Co., Ltd.

^c Not detected.

traditional *Daqu*. Therefore, we have reason to believe that applying new starters for industry liquor brewing with pit mud and full steps, should result in liquor of a quality comparable to, or even higher, than that obtained with traditional *Daqu*.

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