

Uniform experimental design optimization of liquid fermentation Baijiu mixed fermentation

Wei Peng¹, Jinmei Peng¹, Luling Lan¹, Huazhi Li², Xinqiang Ning^{1,*}

¹ College of Bioengineering, Sichuan University of Science & Engineering, Yibin 644000, China

² Zizhong Yinshan Hongzhan Industry Co. Ltd., Neijiang 641000, China

*Corresponding Author: 1257201571@qq.com

ABSTRACT

In this study, corn alcohol fermentation mash was used as raw material, and different microorganisms were added to improve the flavor of liquid fermentation Baijiu. The optimum fermentation conditions of liquid fermentation Baijiu were optimized by uniform experimental design. The optimum fermentation conditions were as follows: *Saccharomyces cerevisiae*: non-*Saccharomyces cerevisiae*=3.8:1, yeast inoculation amount was 0.65 g/g, *Lactobacillus* inoculation amount was 1%, yeast inoculation time was 26.5 h, *Lactobacillus* inoculation time was 0.5 h, the total acid content was 1.943 g/L, the estimated total acid yield was 2.036 g/L, and the relative deviation was 0.077%. Compared with the three control groups, the total acid yield increased by 27.39%, 12.36% and 19.05% respectively.

KEYWORDS

Liquid fermentation Baijiu; Uniform experimental design; *Pichia pastoris*; *Lactobacillus casei*

1. INTRODUCTION

Liquid fermentation method can also be called "one-step method", which is a liquor brewing method that keeps raw materials in liquid state from cooking, saccharification, fermentation to distillation [1]. Compared with solid-state liquor, liquid fermentation has the advantages of saving food [2], high liquor yield, high utilization rate of raw materials [3], high degree of mechanization, simple process equipment [4], etc., but at the same time, liquid-state liquor also has problems such as uneven quality and lax quality [5], and because of the single microorganism in the fermentation process, enzymes and metabolites are different compared with solid-state fermentation, liquid-state fermented liquor lacks Error: reference source not found, which makes its flavor not full enough, and its taste is weak and spicy. At present, the measures to improve the quality of liquid fermentation Baijiu mainly include: solid-liquid combination method [6], solid-liquid blending method [7], flavoring method [8] [9], mixed fermentation of various bacteria [10] and so on. Multi-strain mixed fermentation is a mixed fermentation of various microorganisms. By using its synergistic effect, it can not only improve the flavor of wine, but also improve the wine yield.

The method of "Uniform Design" was put forward by Academician Fang Kaitai and Academician Wang Yuan in 1970s by using pseudo-Monte Carlo method. When selecting experimental points, it only considered that the experimental points were fully "evenly distributed" within the experimental range, and did not consider "neat comparability" [11]. Because it did not consider "neat comparability", it greatly reduced the number of experiments and achieved a better effect of finding the optimal experimental conditions. Because uniform design is based on a specific robust regression

model, it can not only analyze the main effects among experimental factors in the regression model, but also analyze the interactive effects among factors [12] [13]. Uniform experimental design method is widely used in optimization experiments of complex factors, and has been highly recognized at home and abroad.

Because there are many factors affecting the fermentation effect in the process of multi-strain mixed fermentation, the uniform experimental design method is a better exploration tool for how to obtain the optimal fermentation conditions efficiently. In this study, *Saccharomyces cerevisiae*, *Pichia pastoris* and *Lactobacillus casei* were mixed to ferment liquid fermentation Baijiu, and the fermentation conditions were optimized by uniform experimental design principle, in order to obtain a better fermentation combination, which provided theoretical basis for improving the quality of liquid fermentation Baijiu by multi-strain mixed fermentation.

2. MATERIALS AND METHODS

2.1. Material

(1) Mature mash: an alcohol factory in Neijiang.

(2) Strains: Anqi's yeast and Farkey yeast powder: an alcohol factory in Neijiang; *Pichia pastoris*: A strain collected in a laboratory of Sichuan University of Light Chemical Technology; *Lactobacillus casei*: Sichuan Microbial Resources Platform Strain Preservation Association Center (SICC 1.1343).

2.2. Medium

(1) Yeast medium (YPD medium)[14]: Yeast extract 10.0 g, glucose 20.0 g, peptone 20.0 g, agar 20.0 g, distilled water 1000 ml, sterilized for 20 min under 0.1 MPa.

(2) *Lactobacillus* medium (MRS medium) [14]: Peptone 10.0 g, sodium acetate 5.0 g, beef extract 10.0 g, diamine citrate 2.0 g, yeast extract 5.0 g, dipotassium hydrogen phosphate 2.0 g, manganese sulfate 0.25 g, magnesium sulfate 0.58 g, glucose 20.0 g, Tween 1.0 ml, agar 20.0 g and distilled water 1000 ml.

2.3. Instruments and Equipment

DGX-9073B-2 constant temperature blast drying oven, Shanghai Fuma Experimental Equipment Co., Ltd.; LRH-250 Biochemical Incubator, Shanghai Yiheng Scientific Instrument Co., Ltd.; SW-CJ-2FD ultra-clean workbench, Suzhou Antai Air Technology Co., Ltd.; ZWY-1102 Constant Temperature Cultivation Oscillator, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.; LDZF-30L vertical high pressure steam sterilization pot, Shanghai Shen'an Medical Equipment Factory.

2.4. Experimental Methods

2.4.1. Strain activation

YPD culture medium and MRS culture medium about 1/3 of the volume of the test tube were added into the test tube respectively. After cooling on the inclined plane, *Pichia pastoris* and *Lactobacillus casei* were inoculated on the ultra-clean worktable (sterilized by ultraviolet light for half an hour in advance), and then put into a constant temperature incubator at 30°C for 2 days, and then expanded culture was carried out after repeated three times

2.4.2. Determination of total acid content in fermented samples

Refer to GB/T 10345-2007, the total acid content in fermentation samples was detected.

The calculation formula is shown in (2-1):

$$X = \frac{c \times m \times 60}{50.0} \quad (2-1)$$

Table 1. Meaning of each letter in formula

Alphabet	Representative meaning	Unit
X	Mass concentration of total acid (calculated by acetic acid	Gram per liter (g/L)
c	Standard concentration of sodium hydroxide titration	Moles per liter
V	Volume of sodium hydroxide standard titration solution	milliliters (mL)
60	Molar mass of acetic acid	Gram per mol (g/mol)
50.0	The volume of wine sample was selected for determination	milliliters (mL)

2.4.3. Uniform Experimental Design of liquid fermentation Baijiu Fermentation by Mixed Bacteria

According to the principle of uniform experimental design, the correlation between the total acid content of raw materials after mixed fermentation and five experimental factors, namely, *Saccharomyces cerevisiae*: non-*Saccharomyces cerevisiae*, yeast inoculation amount, *Lactobacillus* inoculation amount, yeast inoculation time and *Lactobacillus* inoculation time, was investigated. The five factors were arranged at 10, 10, 10, 5 and 5 levels respectively. The investigation level table of each factor is shown in Table 2. U10 (103×52) mixed level uniform design table was selected in the experiment. Using DPS data processing system, the uniform experimental design of each factor in mixed bacteria fermentation in Table 1 was carried out, and the uniform experimental design table was obtained as shown in Table 3.

150 mL mash was placed in a 250 mL conical flask, and the ratio of Angel yeast to Falquay yeast was 7:3. The other fermentation conditions were designed according to the uniform experimental design table. All the experimental samples were placed in a 150 r/min, 30°C shaking table. After fermentation for 3 days, the total acid and total acid content were determined.

Table 2. Uniform experienced factor level table

factor	<i>Saccharomyces cerevisiae</i> : Non- <i>Saccharomyces cerevisiae</i>	Yeast inoculation amount g/g	Inoculation amount of lactic acid bacteria/%	Yeast inoculation time/h	Inoculation time of lactic acid bacteria/h
1	1: 0	0.002	0	0	0
2	1: 1	0.0025	0.5	12	12
3	1: 2	0.003	1	18	18
4	2: 1	0.0035	1.5	24	24
5	3: 1	0.004	2	36	36
6	4: 1	0.0045	2.5		
7	5: 1	0.005	3		
8	6: 1	0.0055	3.5		
9	8: 1	0.006	4		
10	10: 1	0.0065	4.5		

Table 3. Uniform design scheme table

factor	Saccharomyces cerevisiae (X ₁)	Yeast inoculation amount g/g (X ₂)	Lactobacillus inoculation amount/% (X ₃)	Yeast inoculation time/h (X ₄)	Lactobacillus inoculation time/h (X ₅)
1	10: 1	0.0065	2	18	24
2	5: 1	0.003	1	0	36
3	6: 1	0.005	0.5	36	12
4	1: 2	0.006	3	0	12
5	3: 1	0.0055	4	24	36
6	2: 1	0.0025	1.5	24	0
7	1: 0	0.0045	0	12	18
8	8: 1	0.004	3.5	12	0
9	1: 1	0.0035	2.5	36	24
10	4: 1	0.002	4.5	18	18

2.5. Data Analysis

In uniform experimental design, DPS 7.05 is used for data processing, SPSS 26 and Excel 2016 are used for mathematical processing and analysis, and Origin 2018 is used for graphic drawing.

3. RESULTS AND ANALYSIS

3.1. Experimental Design Results Of Uniform Fermentation Of Liquid Fermentation Baijiu

With total acid as the analysis index, DPS software was used to analyze the results of uniform fermentation experiment of liquid fermentation Baijiu with mixed bacteria. The results are shown in Table 4.

Table 4. Uniform experienced results table

factor	Saccharomyces cerevisiae (X ₁)	Yeast inoculation amount g/g (X ₂)	Lactobacillus inoculation amount/% (X ₃)	Yeast inoculation time/h (X ₄)	Lactobacillus inoculation time/h (X ₅)	Total acid content (g/L) (Y)
1	10: 1	0.0065	2	18	24	1.751
2	5: 1	0.003	1	0	36	1.588
3	6: 1	0.005	0.5	36	12	1.914
4	1: 2	0.006	3	0	12	1.533
5	3: 1	0.0055	4	24	36	1.588
6	2: 1	0.0025	1.5	24	0	1.914
7	1: 0	0.0045	0	12	18	1.697
8	8: 1	0.004	3.5	12	0	1.912
9	1: 1	0.0035	2.5	36	24	1.698
10	4: 1	0.002	4.5	18	18	1.806

The data in Table 2-4 were analyzed by quadratic polynomial stepwise regression analysis with uniform design experimental processing software DPS 8.0, and the regression equation between total acid content and various fermentation conditions was obtained as follows:

$$Y=0.5561+3.9251X_1-0.0032X_4+0.0008X_5-2.6145X_1^2+0.0001X_4^2-0.0004X_5^2-0.0102X_1X_4+1.3506X_2X_4$$

The fitting degree test of the equation is shown in Table 5, the correlation coefficient $R = 1$, the determination coefficient $R^2 = 0.9999$, the residual standard deviation $S = 0.0023$, the adjusted correlation coefficient $R_a = 0.9999$, p value = 0.0118 < 0.05, F value = 4317.1599. For F test, $F > F_{0.05(5,1)}$. Durbin-Watson statistic $d = 1.7097$, Durbin-Watson statistic equals 2 for normal distribution. The closer its value is to 2, the more normal the residual is, and the model is appropriate, otherwise the model is inappropriate [15][16]. It can be seen from these statistical parameters that this regression equation can well fit the relationship between total acid yield and the addition ratio of *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae*, yeast inoculation amount, Lactobacillus inoculation amount, yeast addition time and Lactobacillus addition time, and this regression equation is remarkable and accurate.

Table 5. Test of fitting degree of equation

R	R _a	F value	P value	S	d	R ²
1	0.9999	4317.1599	0.01	0.0023	1.7097	0.9999

The relevant statistical results of regression analysis of this equation are shown in Tables 2-6 and 2-7. It can be seen from Table 6 that the total acid yield is directly proportional to the addition ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* and the addition time of Lactobacillus, and inversely proportional to the addition time of yeast, which can appropriately reduce the addition time of yeast and increase the addition ratio of *Saccharomyces cerevisiae* to non-*Saccharomyces cerevisiae* and the addition time of Lactobacillus to maximize the total acid yield. As can be seen from Table 7, the fitting error value of the equation is very small, which shows that the accuracy of the fitting model is high [17].

Table 6. Regression analysis results and statistical test of parameters

	Partial correlation	T-test value	P value
$R(Y, X_1) =$	0.9998	50.72	0.0004
$R(Y, X_4) =$	-0.9942	9.21	0.0116
$R(Y, X_5) =$	0.9312	2.55	0.1251
$R(Y, X_1^2) =$	-0.9998	46.66	0.0005
$R(Y, X_4^2) =$	0.9983	17.09	0.0034
$R(Y, X_5^2) =$	-0.9995	30.72	0.0011
$R(Y, X_1X_4) =$	-0.9983	17.30	0.0033
$R(Y, X_2X_4) =$	0.9987	19.62	0.0026

Table 7. Regression Equation Fitting Value and Fitting Error

Sample	Observation value	Fitted value	Fitting error
1	1.751	1.7518	-0.0008
2	1.588	1.5879	0.0001
3	1.914	1.9145	-0.0005
4	1.533	1.5329	0.0001
5	1.588	1.5870	0.0010
6	1.914	1.9154	-0.0014
7	1.697	1.6969	0.0001
8	1.912	1.9132	-0.0012
9	1.698	1.6962	0.0018
10	1.806	1.8048	0.0012

According to the path coefficients of the regression equation (Table 8), the effects of various fermentation conditions on total acid content can be obtained: X_1 (5.5037) > X_1^2 (4.8997) > X_5^2 (1.1639) > X_1X_4 (0.6325) > X_2X_4 (0.5631) > X_4^2 (0.3574) > X_4 (0.2765) > X_5 (0.0734), that is, among the mixed fermentation conditions of liquid fermentation Baijiu, yeast addition time has the greatest influence on total acid yield, followed by Lactobacillus addition time, and *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* addition ratio has the smallest influence [18].

Table 8. Path coefficient of regression equalization

factor	Path coefficient
X_1	5.5037
X_4	-0.2765
X_5	0.0734
X_1^2	-4.8997
X_4^2	0.3574
X_5^2	-1.1639
X_1X_4	-0.6325
X_2X_4	0.5631

3.2. Validation Experiment Of Optimal Fermentation Conditions

By using DPS software, the optimal fermentation conditions were obtained as follows: *Saccharomyces cerevisiae*: non-*Saccharomyces cerevisiae* = 3.8:1, the inoculation amount of yeast was 0.65g/g, the inoculation amount of Lactobacillus was 1.0213%, the inoculation time of yeast was 26.3835 h, the inoculation time of Lactobacillus was 0.554 h, and the estimated total acid yield was 2.036 g/L, which was better than the total acid yield of each group in uniform experiment. In order to facilitate the actual experiment, taking *Saccharomyces cerevisiae*: non-*Saccharomyces cerevisiae* = 3.8:1, yeast inoculation amount 0.65 g/g, Lactobacillus inoculation amount 1%, yeast inoculation time 26.5 h, Lactobacillus inoculation time 0.5 h as the conditions for verification experiment, the verification results are shown in Table 9. The total acid content was 1.943 g/L, the predicted total acid yield was 2.036 g/L, and the relative deviation was 0.077%. The accuracy of this experiment is high.

Table 9. verifications the experiential results

	X_1	X_2	X_3	X_4	X_5	Measured value (Y_1)	Predicted value (Y_2)	Relative deviation/%
1	3.8:	0.0065	1	26.5	0.5	1.732	2.036	0.215
2	3.8:	0.0065	1	26.5	0.5	2.048	2.036	0.008
3	3.8:	0.0065	1	26.5	0.5	2.048	2.036	0.008

Saccharomyces cerevisiae, *Saccharomyces cerevisiae* and non-*Saccharomyces cerevisiae* (*Pichia pastoris*), *Saccharomyces cerevisiae* and *Lactobacillus casei* were added in Group A, *Saccharomyces cerevisiae* and *Pichia pastoris* were added in Group B, *Saccharomyces cerevisiae* and *Lactobacillus casei* were added in Group C, and three groups were set in parallel in each group. The experimental results are shown in Table 10. The total acid yield of *Saccharomyces cerevisiae* group was the lowest, and the total acid yield of *Saccharomyces cerevisiae* and *Pichia pastoris* group was the highest.

Table 10. Control group experiential results

	Saccharomyces cerevisiae: Non-Saccharomyces cerevisiae	Yeast inoculation amount g/g	Inoculation amount of lactic acid bacteria/%	Yeast inoculation time/h	Inoculation time of lactic acid bacteria/h	Total acid content (g/L)	Comparison of optimal conditions/%
A	1: 0	0.05	0	0	0	1.373	27.39
B	5: 1	0.05	0	0	0	1.530	12.36
C	1: 0	0.05	2	24	0	1.491	19.05

Note: Group A: *Saccharomyces cerevisiae*, Group B: *Saccharomyces cerevisiae*+*Pichia pastoris*, Group C: *Saccharomyces cerevisiae*+*Lactobacillus casei*

4. CONCLUSION

In this experiment, the mash of corn alcohol fermentation was used as raw material, and different microorganisms were added to improve the flavor of liquid fermentation Baijiu. In this study, DPS software was used for uniform experimental design to obtain different experimental conditions. The total acid content was analyzed as an index to explore the optimal technological conditions of multi-bacterial fermentation of liquid fermentation Baijiu. The optimal process conditions for mixed fermentation of liquid fermentation Baijiu were obtained by uniform design experiment: *Saccharomyces cerevisiae*: non-*Saccharomyces cerevisiae* = 3.8:1, yeast inoculation amount 0.65 g/g, *Lactobacillus* inoculation amount 1%, yeast inoculation time 26.5 h, *Lactobacillus* inoculation time 0.5 h. The total acid content was 1.943 g/L, the predicted total acid yield was 2.036 g/L, and the relative deviation was 0.077%. Compared with the three control groups, the total acid yield increased by 27.39%, 12.36%, and 19.05%, respectively. It shows that mixed fermentation of liquid fermentation Baijiu can greatly increase the content of acids in liquor body compared with single strain fermentation, which provides a theoretical basis for improving the quality of liquid fermentation Baijiu by mixed fermentation of multiple strains.

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