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# Study on Primary Influencing Factors of Flavor Components Produced by *Bacillus* sp. Isolated from Black Part of Maotai Daqu

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**Abstract:** Microbes are major contributors to the unique fragrance of Maotai liquor, in which *Bacillus* sp. is recognized as the main flavor-producing strain. However, studies with respect to the importance, sensitivity and optimal ranges of the factors influencing the flavor components secreted by *Bacillus* sp. have not been reported. In this paper, a thermophilic flavor-producing strain *Bacillus* sp., isolated from the black part of Maotai Daqu, was chosen as the representative to study how the factors (inoculum size, temperature and incubation time) influenced the production of flavor components (acetic acid, 2, 3-butanediol and ethyl oleate). The optimal combination of factors as well as the quadratic mathematical model of flavor-producing process were obtained through central composite design analysis by batch tests. Results showed that the simulation accuracy of nonlinear equations for the production of three different types of flavor components was 96.00, 87.76, and 90.54%, respectively. Temperature was the most significant factor influencing the production of flavor components, followed by incubation time, and inoculum size showed the smallest impact. In conclusion, the optimized zones of the three factors were as follows: temperature of 38°C~45°C, incubation time of 13~15 d, inoculum size of 14~17%.

## 1. Introduction

Maotai liquor is a kind of fermentation product of microorganism under certain environment and nutrient condition. The change of environmental factors leads to the change of the quantity and population structure of microorganism, and subsequently leads to the change of the composition and flavor of the fermented products [1]. Therefore, it is meaningful to research how the factors influence the relationship between microorganisms and the flavor components production in Maotai liquor. However, the underlying mechanism about this relationship is difficult to understand due to the complicated microflora and high biodiversity in fermentation of Maotai liquor. In order to explore this mechanism in an easier way, we have to choose a single representative strain which has a profound impact on the fermentation process as the research subject.

Among these complex bacterial groups, Bacillus sp. is the dominant group as well as the main functional group of Maotai flavor. Wang et al. and Yao et al [2-3]. studied the metabolites of the representative bacteria of Bacillus sp., and the relationship between these bacteria (such as Bacillus subtilis and Bacillus licheniformis) and flavor substances in Maotai liquor, and believed Bacillus played an important role in fermentation process. Thus Bacillus was chosen as the strain in this research.

It is commonly believed that the flavor components in Maotai liquor are derived from Daqu, which is the raw material of Maotai liquor and the starter of fermentation containing abundant microorganisms. Furthermore, our preliminary study showed that the central black component was the most important part of Maotai Daqu, since it usually possesses a bacterial community and better sauce flavor due to the high temperature and low air permeability [4]. Thus the central black component was chosen as the material to isolate Bacillus in this study.

Characteristics of liquor style are closely related to its chemical constituents. The main components of liquor are ethanol, water, and other trace components (e.g., alcohols, acids, esters, aldehydes, phenols) which determine the flavor and taste of liquor. Therefore, accurate measure of these trace components is the key to the quality control of liquor. Gas chromatography-mass spectrometry (GC-MS) is an advanced new separation technology with high selectivity, high separation efficiency, fast analytical speed, high sensitivity and low sample dosage. It has been widely used in the analysis of trace components of wine [5-6]. In this paper, GC-MS was used to determine the fermentation extracts of flavor-producing strain.

The traditional optimization method of influencing factors is to change one variable and fix other variables to carry out experiments. However, this method is time-consuming and laborious. Compared to traditional method, response surface method (RSM) can accurately describe the relationship between the factors and the response value, determine the interaction between the factors by using the reasonable experiment design, polynomial fit the correlations between the factors and levels in the multifactor experiment, and find the optimal process parameters through the analysis of regression equation[7-8].

The bacterial metabolism and flavor-producing characteristics are obviously affected by the environmental factors. Among all the factors, inoculation size, culture temperature and incubation time are the key environmental factors. The change of these key environmental factors will drastically affect the metabolism of microorganisms and the species and yield of metabolites. The study of the factors which influence the relationship between microbes and soy flavor substances is not only beneficial to improve the production process of Mao-flavor liquor and the quality of liquor body, but also to increase the product types of Mao-flavor liquor. Meanwhile, some unknown synergistic effect and interactive metabolic patterns could be found. However, up to now, relevant studies are few.

## 2. Material and methods

*2.1 Sampling*

Samples of central black component of Maotai Daqu were collected from KWEI CHOW MOUTAI CO. LTD. located in Zunyi City, Guizhou Province [4]. PDA medium was used as the isolation medium, that is, take 200 g potato, chopped peeled wash, add 1000 ml ddH2O to boil for 30 minutes. Four layers of gauze filtration, 20 g glucose and 15-20 g Agar were added, fully dissolved and sterilized at 121℃ for 30 min.

*2.2. Screening, identification and cultivation method of the cultivable flavor-producing bacteria*

The formula of wheat culture medium was as follows: the wheat was crushed into wheat grain and wheat flour for 100 g each, add 50% ddH2O, then sterilized at 121℃ for 30 min, then cooled for later use. The high temperature Daqu was inoculated in PDA plate medium and cultured at 55℃ for 24 hours. The single colony was selected for purification, and the thermophilic bacteria solution was added to wheat culture medium to cultivate in the temperature gradient of 37℃-46℃-55℃ for 6 days, the bacteria which could produce good flavor of sauce in this medium were selected as the high-temperature flavor-producing strain. PCR amplification and sequence analysis of 16S rDNA was performed in the method described by our previous study [4].

*2.3. Detection of flavor substances in different fermentation products by GC-MS*
Fermentation treatment: the screening bacteria strain was inoculated in wheat medium by 10%, and cultivated at 37℃-46℃-55℃ gradient, 2 days at each gradient. After 2 cycles the flavor substances were extracted.Extraction and treatment of flavor substances: The flavoring substances were extracted using anhydrous ethanol. Anhydrous ethanol was added to submerge the fermentation medium and shocked 3 h, this operation was repeated 3 times and then all the extracts were combined together. Finally, the extracts were concentrated using the rotary evaporators and dried with anhydrous sodium sulfate.GC-MS analysis was performed in a DM-FFAP GC system coupled with a HP MSD5973 N quadruple mass spectrometer. The experimental details were described in our previous study [4].

*2.4. Central composite design*
Three factors (inoculation size, culture temperature and incubation time) closely related to the growth and metabolism of the bacterial strain were selected as the influence factors. The specific values were given to these 3 factors at different levels which were shown in Table 1.

Table 1. Quantitative value of the coded parameter levels.

| Factors | Factor level | | | | |
|---|---|---|---|---|---|
| | -2 | -1 | 0 | 1 | 2 |
| A: inoculum size (%) | 5 | 10 | 15 | 20 | 25 |
| B: temperature (℃) | 20 | 30 | 40 | 50 | 60 |
| C: incubation time (d) | 5 | 10 | 15 | 20 | 25 |

Using the central composite option of the Design Expert 8.0.6 software, the center compound design matrix of 19 experiments consisting of 3 factors, 6 axial points and 5 central points is designed, and the distance α of each axis point from the center is the software system default value of 2.0. Data analysis is performed using Minitab 16.1.1 and Design Expert 8.0.6.

## 3. Results and discussion

*3.1 The molecular identification of the cultivable flavor-producing strain*
Figure 1 shows the N-J phylogenetic tree of isolated strain (MT04) and some referenced bacterial 16S rRNA sequences retrieved from GeneBank. It shows that the bacterium MT04 (serial number: KF798318) selected from the black part of the Maotai Daqu belongs to *Bacillus* sp.

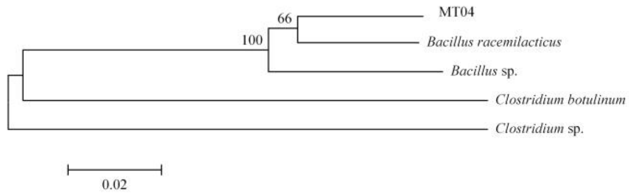


Figure 1. A neighbor joining phylogenetic tree showing the phylogenetic relationships of the bacterial 16S rRNA gene sequences of MT04 to those related sequences from the GenBank database. Scale 0.02 represents the distance of evolution. Bootstrap replications of 1,000 were used.

*3.2 Analysis of the GC-MS results*
Based on the analysis of 19 different treatments fermentation experiments of Bacillus sp. MT04 and the flavor substances of Maotai liquor, it was found that, acid, alcohols, esters and furfural substances

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appeared in the fermented products, e.g., acetic acid, propionic acid, isoamyl acid, palmitic acid, linoleic acid, 3-furfural, 2, 3-butanediol, ethyl palmitate, ethyl oleate and ethyl linoleate.

Acetic acid, as a kind of organic acid flavor substances, can make wine with acetic acid odor, refreshing with swee t[2]. Therefore, the acetic acid was chosen as the representative of organic acid flavor substances, and the influence of each factor on its yield change was studied, in order to determine the different effects of each factor, and provide reference data for the subsequent blending regulation of the flavor substances of organic acids.

2, 3-butanediol is a common flavor additive, which added in wine can make the body become woolly alcohol, and bring the bouquet sweet. Its catalytic dehydrogenation of two acetyl form of 2, 3-octanol is a high value of spices, it can bring a unique flavor of the sauce when added to the white liquor [5]. Therefore, it was studied as a representative of the lower alcohols in this paper.

Ethyl oleate, which is one of the three kinds of advanced fatty acid ethyl ester (ethyl oleate, linoleic acid ethyl ester and palmitic acid ethyl ester), is one of the important flavor of liquor. Regardless of low or high alcohol, both are required to maximize the preservation of high fatty acid ethyl ester content. Ethyl oleate can endow liquor with floral and fruity aromas, fatty acids and vegetable oil odor. Therefore, the paper chose ethyl oleate as the representative of fatty acid ethyl ester.

### *3.3 Regression equation models of the three representative flavoring substances*

By using Design Expert, 19 groups of experiments were deployed, and the nonlinear regression equation models which simulate Bacillus sp. fermentation of acetic acid, 2, 3-butanediol and ethyl oleate respectively were obtained as follows (independent variable A, B and C are inoculation size, temperature and incubation time respectively, dependent variable Y is the product yield).

$$Y(\text{acetic acid}) = 19672.76+190.37A+1110.37B-926.25C-285.00AB+338.75AC+663.50BC-86.97A2-3509.84B2-3524.59C2\ (R2=96\ \%) \quad (1)$$

$$Y(\text{2, 3-butanediol}) = 979.09-11.06A-91.31B-30.81C-4.62AB-15.12AC+54.63BC-25.58A2-255.95B2-78.70C2\ (R2=87.76\ \%) \quad (2)$$

$$Y(\text{ethyl oleate}) = 740.91+40.69A+63.31B-43.19C+0.88AB-0.87AC-6.62BC-82.55A2-93.80B2-61.67C2\ (R2=90.54\ \%) \quad (3)$$

The regression coefficients of the three response surface models were 96%, 87.76% and 90.54% respectively, indicating that the changes of model response values (the yield of the acetic acid, 2, 3-butanediol and ethyl oleate) are 96%, 87.76% and 90.54% respectively from the selected independent variables (inoculation size, temperature, and incubation time). It also shows that these three models can explain 96%, 87.76% and 90.54% of the experimental data. Due to the high regression coefficients, the regression equation can accurately describe the real relationship between the independent variable and the response value.

The factor of temperature shows the most significant impact on all three flavor substances. High temperature has a positive effect on the production of acetic acid and ethyl oleate, but a negative effect on the production of 2, 3-butanediol. High inoculum size has a same effect as temperature does on these three flavor components, but the significance is the lowest. High incubation time has a negative effect on all the flavor components, which indicates that short cultivation time is conducive to the increase of flavor production. The significance of incubation time is a little higher than that of inoculum size.

### *3.4 Optimized conditions for three flavor components*

According to the CCD analysis, the maximum value of acetic acid, 2, 3-Butanediol and ethyl oleate, and their corresponding conditions are listed in Table 2.

Table 2. Maximum value and optimized condition for flavor components.

| Response value | Inoculum size (%) | Temperature (℃) | Inoculation time (days) | Yields (peak area) |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Acetic acid | 14.75 | 40.80 | 14.80 | 19740.00 |
| 2, 3-butanediol | 14.40 | 38.00 | 13.70 | 993.03 |
| Ethyl oleate | 16.25 | 43.50 | 13.15 | 765.14 |

It shows that the three influencing factors for the maximum yield of acetic acid, 2, 3-butanediol and ethyl oleate are not the higher the better or the lower the better. Based on the analysis of typical metabolic pathway of acetic acid, 2, 3-butanediol and ethyl oleate of *Bacillus* sp., it may be that the *Bacillus* sp. grows well under this condition, and the secretion of the amylase and protease is stronger, which is more beneficial to decompose and use the macromolecule polysaccharide such as starch in wheat medium to produce acetic acid, 2, 3-butanediol and ethyl oleate to provide the prerequisite substances. However, the temperature value of the maximum yield of ethyl oleate is higher than the first two substances. Ethyl oleate is a macromolecule ester containing high carbon atoms, and is generally synthesized by the interaction of oleic acid and ethanol. The difference of its maximum yield value is probably because that the *Bacillus* sp. can produce oleic acid as the raw material of ethyl oleate in this temperature, and it is beneficial to the synthesis reaction of ethyl oleate.

### *3.5 Analysis of CCD model*

Figure 2 shows the normal probability plot and residual plot of the fermentation product yield of acetic acid. The data points are distributed in a straight line in the plot of residuals in figure 2a, which supports the adequacy of the least-squares fit. The data points are evenly distributed on the upper and lower sides of the x axis in figure 2b, which indicates that the variance is independent, and do not depend on the change of the yield. This supports the fitting of least squares once more. The characteristics of normal probability plot and residual plot for 2, 3-butanediol and ethyl oleate are as same as that of acetic acid, and thus are not shown in this paper.

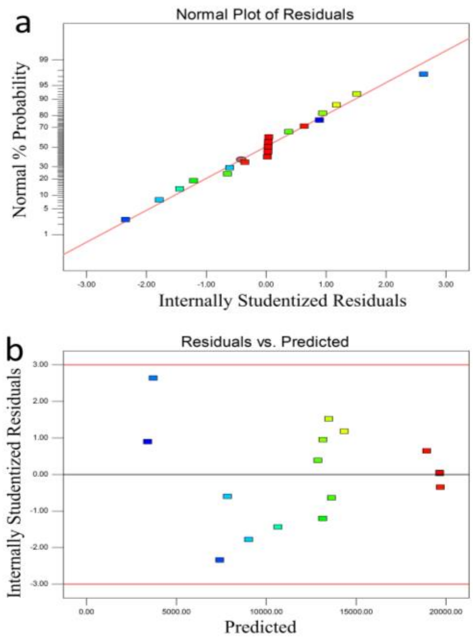


Figure 2. Normal probability plot (a) and residual plot (b) for production model of acetic acid.

The response surface equation about the yield of fermentation products of *Bacillus* sp. contains three independent variables and their squares and interactions. It is impossible to visualize all the interaction and complexity of these factors. But the three-dimensional response surface can reflect the interaction of response surface model and its three factors in a certain extent [7, 8]. Figure 3 shows the three-dimensional response surface plot of three kinds of substances content changes.

It can be seen from the graph, the change extent of acetic acid production is very large when changing the temperature and incubation time as well as fixing the inoculation size (figure 3a). However, there is little change of acetic acid production when changing the inoculation size. The subpoint of the maximum value of acetic acid is located in the central zone of axis of temperature and incubation time (third graph in figure 3a), which indicates that the range of independent variable chosen in our test is suitable. As for the production of 2, 3-butanedio, only the independent variable of temperature shows obvious effect on dependent variable. However, as for production of ethyl oleate, all three factors of inoculation size, temperature and incubation time greatly impact the change of independent variable. According to the CCD analysis above, the optimum condition of three factors achieving the maximum value for three kinds of flavoring substance were synthesized as follows: temperature of 38~45℃, time of 13~15 d, inoculation size of 14~17%.

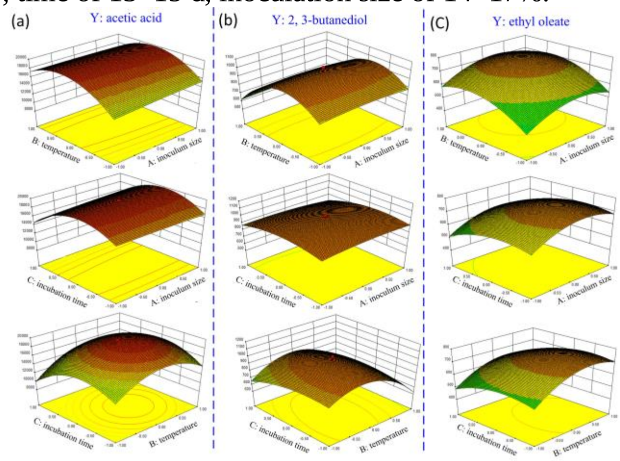


Figure 3. Three-dimensional surface plot of the empirical model for flavor components of *Bacillus* sp. MT04 (a: acetic acid; b: 2, 3-butanediol; c: ethyl oleate).

## 4. Conclusion

The simulation degree of the nonlinear regression equation models for the three flavor components were as high as 96%, 87.76%, 90.54% respectively, which indicated that the response surface analysis was highly reliable. The effects of temperature on the production of three flavor components were the most significant, the incubation time was the second significant, and the inoculation size was the least. In conclusion, the optimized zones of the three factors were as follows: temperature was 38℃~45℃, incubation time was 13~15 d, inoculum size was 14~17%.

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