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Optimization of Cultural Conditions for Vinegar of Litchi (*Litchi chinensis* Sonn.) in Liquid State Fermentation

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Abstract In order to enhance the value of industry application of litchi, fermentation experiment of litchi vinegar was conducted to optimize process parameters. The technology of alcohol fermentation was carried out to investigate the effects of parameters such as original sugar content, yeast inoculation amount, pH value and fermentation temperature. A Box-Behnken experimental design of 3-factor and 3-level was used to build the secondary multivariate regression model related to the yield of acid including inoculation amount, original alcohol content and fermentation temperature, and explore the effect of process parameters of acetic acid fermentation above on the yield of acid in the light of response surface plots. Finally the fermentation process of its acetic acid fermentation was optimized using Box-Behnken methodology. Results were as follows. The optimal process of its alcohol fermentation was as follows: original sugar content, 16%; yeast inoculation amount, 5%; pH value, 3.5 and fermentation temperature, 30~32 °C. The optimal process of its acetic acid fermentation optimized by response surface methodology was as follows: inoculation amount, 10%; original alcohol content, 7.0% and fermentation temperature, 30 °C, in the optimal condition the yield of acetic acid was 52.45 g/L. The secondary multivariate regression model showed as follows. The effects of inoculation amount and original alcohol content were non-significant, and that of fermentation temperature extremely significant. The interaction of inoculation amount and original alcohol content was extremely significant. In conclusion, the regression model was accurate and reliable; the optimal fermentation technology of litchi vinegar was advisable.

Keywords: *litchi, vinegar, fermentation, process optimization*

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1. Introduction

Litchi (*Litchi chinensis* Sonn.) is an evergreen sub-tropical fruit crop belonging to the family *Sapindaceae*, has been widely planted in South-East Asia, especially in China for many years.). Owing to its bright colour and delicious taste, litchi has been gradually accepted by consumers and has established great popularity in the international market. It is rich in nutrition and it has very high value on food and medical care [1,2,3]. The nutritive value and pleasant rose flavour of litchi fruits make its juice a delicacy. Litchi fruits provide carbohydrates, organic acids (i.e. lactic, acetic, succinic, citric and phosphoric), vitamin C, aroma compounds (β-damascenone, linalool, furaneol, ethyle hexanoate, geraniol, etc.) [4] and minerals like calcium, magnesium, potassium, phosphorus and iron (Mahajan and Goswami [5] and the consumption of litchi fruit would meet 2-4% of the dietary reference intakes [6]. Fruit vinegar is the kind of fruit beverage, fermentation by acetic bacteria, and retains abundant nutrient component such as vitamin, mineral substance and amino acid. It is universally acknowledged in academic circle that fruit vinegar is not only superior to conventional grain vinegar, but also carries many physiological and health care functions. Using litchi as raw material to develop litchi vinegar, not only is an important way to improve the efficiency of litchi industry, but also can increase the types of vinegar. Box-Behnken method has the capacity to obtain high accuracy in short time [7,8], and the response surface method is often used to optimize process and predict response value by multiple quadratic regression equation [9]. Currently the yielding acid rate and volatile aroma are still the main direction of further study, and technology progress is nearly still for better quality of volatile aroma. Therefore, the process optimization of acetic acid fermentation of vinegar was carried out using response surface methodology to build the secondary multivariate regression model related to the yield of acid and explore the effect of process parameters on the yield of acid in the light of response surface plots in order to provide reference for further development and utilization of litchi.

2. Materials and Methods

2.1. Materials and Regents

Litchi was purchased from Gaozhou Genzi litchi garden in Maoming city, Guangdong, after transported into the laboratory, selected and washed for use in the picking day. Highly active dry yeast of *Saccharomyces cerevisiae* was purchased from Angel Yeast Co., China. *Acetobacter pasteuriem* (AS.1.41) was the strain preserved by the Microbial Culture Collection Center of Guangdong Institute of Microbiology (GIMCC). Glucose (Wujiang Jinfeng Fine Chemicals Co., LTD, China) and yeast extract (Guangdong Huankai Microbial Sci. &Tech, Co. LTD, China) were biochemical reagent, others were domestic analytic reagents as follows: calcium carbonate (Tianjin Baishi Chemical Industry Co., China), 95% alcohol (Tianjin Baishi Chemical Industry Co., China), citric acid (Tianjin Baishi Chemical Industry Co., China), sodium erythorbate of 0.075% (Tianjin Guangfu Technology Development Co,. LTD, China) and sodium metabisulfite (Tianjin Damao Chemical Reagent Factory, China).

Litchi fruit had abundant nutrient constituent, containing varieties of vitamins, organic acids and quantities of free arginine and serine [4,5]. In the study, litchi was famous species in Maoming, Guangdong which was tested in the Laboratory. Its edible part was tested in 78.26-80.23% of the whole fruit, content of soluble solid in 18.23-20.21% and content of titratable acid in 2.4-2.6 mg/g.

2.2. Activating and Enlarging of Dry Yeast

Dry yeast was taken 3 g into litchi juice adjusted to the sugar content of 6% at 35-38 °C to hydrate and activate for 30 min, then the activate liquid was taken into the electro-heating constant temperature cultivator (DHP-9162, Shenzhen Sanli Instrument Co., China) at 32°C to activate for 2 h until quantities of bubble produced [10].

2.3. Enlarging Cultivation of *Acetobacter pasteuriem* AS.1.41

Active culture medium of AS.1.41 was firstly made up as follows: yeast extraction of 1%, glucose of 1% and calcium carbonate of 1%. Secondly active culture medium was heated for absolutely dissolution and sterilization, and lastly 95% alcohol of 2% was added into the medium. In bacterial-free environment AS.1.41 was inoculated in active culture medium, and cultivated in constant temperature cultivator for 24 h at 32 °C for use [11].

2.4. Material Treatment and Enzymolysis

Ripe and sound litchi was selected and scalded for 2 min after removing peel and seed. Then litchi was stayed in color fixative fluid for 3 min and then squeezed using a family juicer. The color fixative fluid was the following ratio: citric acid of 0.1% (additive amount), sodium erythorbate of 0.075% (additive amount) and sodium metabisulfite of 0.25% (additive amount). Litchi juice was kept at 45 °C for 3 h in water bath with 1% pectase and 1% cellulase added in, and filtered into triangular flasks of 250 mL.

2.5. Alcohol Fermentation of Vinegar

Litchi juice was adjusted to sugar content of 12-18% measured by Saccharimeter (RX-700α, ATAGO Scientific Instrument Co., Japan) and pH value of 3.0-4.5 measured by pH meter (PHS-3D type, Hangzhou Hua Chuang Scientific Instrument Co., China) and then sterilized for 30 min at 65 °C using pasteurization. Bacterial-free juice was added yeast active liquor of 3-6% into and kept at 28-34 °C for alcohol fermentation. Finally the change of alcohol content was monitored by desktop electronic alcohol meter (DA-650 type, Kyoto Electronics Manufacturing Co., Ltd, Japan) every day until alcohol content kept constant.

2.6. Acetic Acid Fermentation of Vinegar

Acetic acid fermentation of litchi vinegar was carried out by Box-Behnken method as the following steps. Enlarging culture fluid of *Acetobacter pasteuriem* AS.1.41 was added into alcohol fermentation fluid for acetic acid fermentation according to the adding rate of 9-11%. The alcohol fermentation fluid was adjusted to make original alcohol content 5-9% before and the temperature was controlled ranging from 28-32 °C. The change of acetic acid content was monitored by NaOH titration of related reference [12] every day until acetic acid content kept changeless.

2.7. Filter and Sterilize of Vinegar

Diatomite was added into litchi vinegar. Litchi vinegar was stirred and placed still for 1-2 h. At the arrival of filter layer of 0.5-1.0 cm and no distinct turbidity, the litchi vinegar was filtered in vacuum condition and filter liquor was collected and sterilized using pasteurization method for 30 min at 65 °C [13].

2.8. Single Factor Experiment of Alcohol Fermentation

Alcohol fermentation of litchi vinegar was studied using single factor experiment as the following steps. In single factor experiment the sugar content was set 12%, 14%, 16% and 18%; pH was controlled in 3.0, 3.5, 4.0 and 4.5; then additive volume was limited in 3%, 4%, 5% and 6%; at last the temperature was in 4 levels namely 28 °C, 30 °C, 32 °C and 34 °C.

2.9. Process Optimization of Acetic Acid Fermentation

It was the first to determine preliminary range of acetic acid fermentation variables through single-factor experiment and related references [14,15], then a Box-Behnken design with three independent variables (X_1, inoculation amount; X_2, original alcohol concentration; X_3, fermentation temperature) at three levels was performed. The independent variables and their levels were listed out (Table 1).

Table 1. Analysis factors and levels of Box-Behnken design

Factor	Level		
	-1	0	1
X_1 (inoculation amount,%)	9	10	11
X_2 (original alcohol content,%)	5	7	9
X_3(fermentation temperature, °C)	28	30	32

2.10. Statistical Analysis

All the measurements were in triplicate. Computer graphics and response surface analysis were achieved by Excel 2003 and Design-Expert software.

3. Result and Analysis

3.1. Effect of Litchi Sugar Content

Sugar content presents a very strong impact in alcohol fermentation, which has much to do with the growth and metabolism of yeast [16]. Original sugar contents ranging from 12% to 18% were shown (Figure 1) to impact the alcohol yield in following given conditions: inoculation amount of 5%, temperature of 30 °C and pH value of 3.5. Alcohol content was constantly increasing in former period before the 6th day. In 6th day the alcohol contents started to stay still and it was right now to measure the alcohol content of different fermentation conditions. The alcohol content reached the peak of 7.4%Vol in the condition of original sugar content of 16%, alcohol contents of other fermentation fluids were 5.2%Vol, 6.8%Vol, 7.0%Vol in order. In summary, increasing sugar content was originally helpful to enhance alcohol content noticeably, while excessive sugar had negative effect on the accumulation of alcohol. Given above, the sugar content of 16% was the most suitable condition of alcohol fermentation, the product was abundant in wine flavor with the highest alcohol content. Previous fruit wine fermentation was inclined to control original sugar content in 18-24% [9,11] and original sugar content above 20% was mainly used in litchi wine fermentation [17]. However, high original sugar content often led to excessive accumulation of alcohol which was disadvantageous for following acetic acid fermentation in addition to dropping cost up [11]. Besides, low original sugar content was less used in fruit wine fermentation whose influence was still unclear. Hence, original sugar contents of 12-18% were explored in the study to make the influence of original sugar content clear. Eventually, it was 16% that was determined to be the optimal original sugar content in alcohol fermentation phase of litchi vinegar, in which alcohol content was higher than others and corresponding to the demand of acetic acid fermentation of litchi vinegar without exceptional dilution procedure. Additionally, original sugar contents of 14-18% with higher alcohol content were considered to be used for further optimization, and 12% was out of consideration for its excessively low alcohol content below 5.5%.

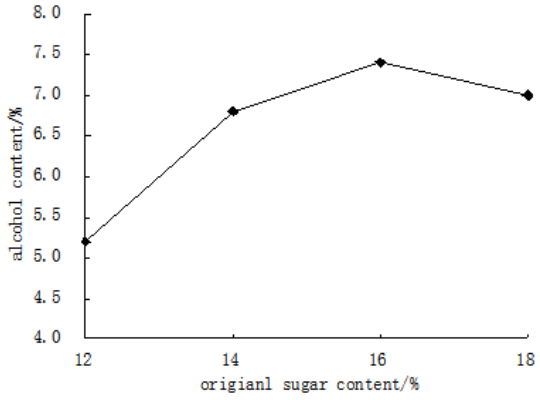

Figure 1. Effect of original sugar content on the changes of alcohol during fermentation period

3.2. Effect of Inoculation Amount

Inoculation amount is also a very important factor during alcohol fermentation, where there is a subtle function relationship between product and strain, for the reason it is excessively vital to analysis the relationship [18]. It was showed the effect of different inoculation amounts on the alcohol yield in the following conditions: sugar content of 16%, temperature of 30 °C and pH value of 3.5 (Figure 2). Alcohol content was steadily increasing in early period and came to the steady level gradually in 6th day, so the 6th day was treated as the terminal point of alcohol fermentation and in 6th day the alcohol contents of different fermentation conditions were measured. The condition which inoculation amount was 16% pulled alcohol content up to the highest level of 7.3%Vol, alcohol contents of fermentation fluid were all lower from 5.6%Vol to 6.9%Vol in inoculation amount conditions of 3%, 4%, 6%. The reason may lay in that low inoculation amount will limit the growth of fermenting yeast, otherwise high inoculation amount will promote the overgrowth of fermenting yeast and suppress alcohol fermentation. In a word, inoculation amount of 3-5% had upgrading effect on the alcohol, more inoculation amount on contrast decreased alcohol content. Therefore, the best condition of alcohol fermentation was to select inoculation amount of 5%, the product was superior in wine flavor with the most accumulation of alcohol. Inoculation amount is very important factor, so many researches [19,20] would take it into consideration unexceptionally. However, inoculation amount was just the factor that ranged fiercely without a certain range. The reason may lay in species difference and method difference of activation and cultivation of *Saccharomyces cerevisiae*. According to the activation method above, inoculation amounts of 3-6% were studied in the study to explore their influence, and inoculation amount of 5% was selected as the optimal one with the highest alcohol level of 7.3%, proving that experiments of inoculation amount were advisable and fitted well with the demand of following acetic acid fermentation.

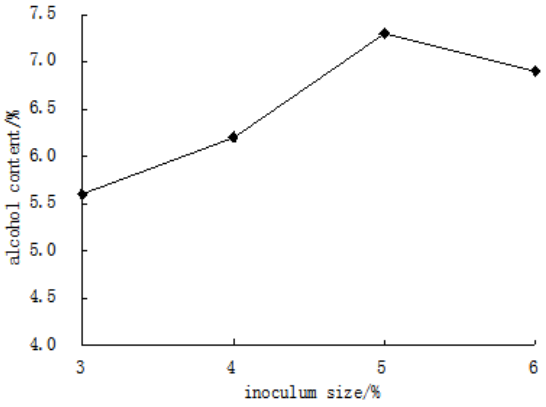

Figure 2. Effect of inoculation amount on the changes of alcohol during fermentation period

3.3. Effect of initial pH Value

pH works in many respects such as affecting enzymatic activity and the process of metabolism reaction, strain will also be faced with a series of limitations to suppress its reproduction and growth in unfavorable pH condition,

reasonably suitable pH is dispensable in fermentation process [21]. The effect tendency chart of different pH values of 3.0, 3.5, 4.0, 4.5 was drawn (Fig. 3) on the alcohol yield in the following conditions: sugar content of 16%, temperature of 30 °C and inoculation amount of 5%. The change of alcohol content was firstly steadily increasing in early period, secondly gradually increasing slowly and finally steady in the late period, in 6th day the alcohol contents of different fermentation conditions were measured. The alcohol content reached 7.3%Vol when pH value was 3.5, other alcohol contents were in the lower level of 7.0%Vol and the lowest of 6.2%Vol. The growth and fermentation of fermenting yeast has different suitable pH, so the selection of pH has the consideration to balance growth and fermentation of fermenting yeast. Therefore, pH value of 3.5 was selected to be the most suitable for alcohol fermentation. Just as was shown in Fig. 3, pH limited in 3.0-4.0 was the suitable pH condition with the alcohol content of 7.0-7.3%, and pH of 3.5 was the most suitable pH condition. Compared with others researches [22], same pH range was obtained. Generally speaking, fruit wine was in want of suitable pH condition, and pH range of 3.0-4.0 was universally used in many studies of fruit wine. In addition, when pH was 4.5, alcohol content was suffering from sharp drop, turning out that unsuitable pH condition had strong negative effect on the alcohol content.

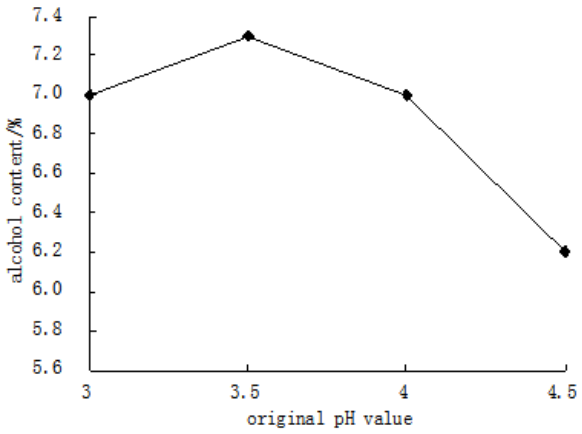

Figure 3. Effect of different pH value on the changes of alcohol during fermentation period

3.4. Effect of Temperature

Temperature is of great importance to determine fermentation quality with other process parameters. Suitable temperature is necessary to maintain normal reproduction, growth and metabolism of strain [23]. The change of alcohol content was shown (Figure 4) with fermentation temperature in the following conditions: sugar content of 16%, inoculation amount of 5% and pH value of 3.5. The change of alcohol content was firstly increasing in early period and secondly steady in the late period, the 6th day was the end to ferment and the alcohol contents of different fermentation conditions were measured. The alcohol content also reached 7.2-7.3%Vol with fermentation temperature of 30-32 °C, other alcohol contents were both far below 7.0%Vol. Thus, alcohol fermentation was suitable to ferment in the condition of 30-32 °C. Generally speaking, alcohol fermentation was always carried out in broad temperature range. Different optimal temperatures were determined in many studies which were mainly around 30°C [24]. However, there were also studies pointing out the lower temperature, such as 20-25°C, also could be applied to produce alcohol [25]. If comparing their difference, the reason may result from different strain of *Saccharomyces cerevisiae* and fermentation condition. As for the used strain of *Saccharomyces cerevisiae* in the study, previous studies have presented the same temperature condition [26], comparable with the obtained one in the study. In addition to those above, there were conclusions to be summarized that unsuitable temperature had strong negative effect on the alcohol content and higher temperature of 34 °C decreased alcohol content more remarkably than lower temperature of 28 °C.

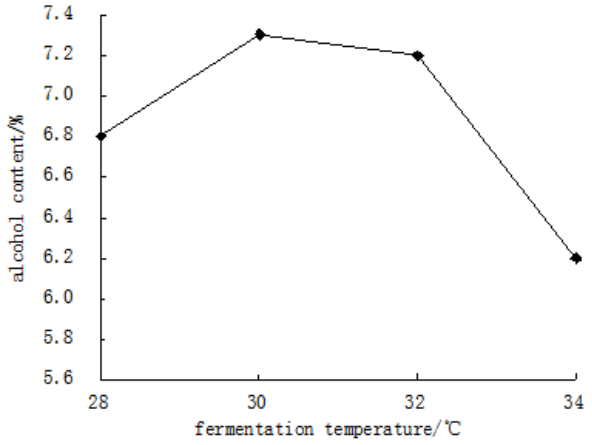

Figure 4. Effect of different temperature on the changes of alcohol during fermentation period

In summary, the technology study of alcohol fermentation of litchi vinegar was carried out and the purpose was to determine the optimal technology condition for better fermentation performance in the light of the results of the single factor test. The technology condition was as follows: original sugar content of 16%, inoculation amount of 5%, and original pH value of 3.5 and fermentation temperature of 30-32 °C. Accordingly, the alcohol content was of great capacity to reach 7.3-7.4%.

3.5. Response Surface Experiment of Acetic Acid Fermentation

The response surface method (RSM) consists of a group of mathematical and statistical procedures that can be used to study the relationship between one or more responses and a number of independent variables. In addition to analyzing the effect of independent variables, the experimental methodology generates a mathematical model that accurately describes the overall process [27,28]. Statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components. RSM has already been successfully applied for the optimization of media and culture conditions in many cultivation processes for the production of primary and secondary metabolites [29,30] i.e., ethanol [31].

The response surface experiment (RSE) was used for the process optimization of acetic acid fermentation and the results were shown (Table 2).

Table 2. The results of Box-Behnken experiments

No.	Code level			Yield of acid (g/L)
	X_1	X_2	X_3	
1	-1	-1	0	44.75
2	1	-1	0	47.68
3	-1	1	0	46.83
4	1	1	0	44.13
5	-1	0	-1	47.17
6	1	0	-1	47.01
7	-1	0	1	45.16
8	1	0	1	44.33
9	0	-1	-1	47.41
10	0	1	-1	45.88
11	0	-1	1	46.12
12	0	1	1	45.58
13	0	0	0	52.25
14	0	0	0	52.56
15	0	0	0	52.62

Design-Expert software was used for the regression analysis of results and presented the secondary multivariate regression equation: $Y=-1074.09031+76.93542\times X_1+15.98354\times X_2+46.20562\times X_3-0.70375\times X_1\times X_2-0.083750\times X_1\times X_3+0.061875\times X_2\times X_3-3.47958\times X_1^2-0.78740\times X_2^2-0.76990\times X_3^2$, in which response value was set as Y value(yield of acid, g/L) and variables were X_1 (inoculation amount,%), X_2 (original sugar content, %) and X_3 (temperature, °C).

$P_{model}<0.01$ claimed the model was extremely significant and $P_{lack\ of\ fit}=0.0773>0.05$ (Table 3) turned out lack of fit was non-significant, in which lack of fit was the probability of non-fit between actual value and theoretical value of the model. R^2_{adj} was 0.9639, declaring 96.39% of response value changes could be explained; R^2 was 0.9871 and near to R^2adj, declaring the degree of fit fine and low error. Summarily speaking, the whole model can be used for the optimization of fermentation process.

Table 3. Variance analysis for regression model

Source	Sum of squares	Free degree	Mean square	F	*p*-value	Significant
Model	115.76	9	12.86	42.56	0.0003	**
X_1	0.072	1	0.072	0.24	0.6457	
X_2	1.57	1	1.57	5.18	0.0718	
X_3	4.93	1	4.93	16.31	0.0099	**
X_1X_2	7.92	1	7.92	26.22	0.0037	**
X_1X_3	0.11	1	0.11	0.37	0.5689	
X_2X_3	0.25	1	0.25	0.81	0.4092	
X_1^2	44.70	1	44.70	147.91	<0.0001	**
X_2^2	36.03	1	36.03	121.19	<0.0001	**
X_3^2	35.02	1	35.02	115.86	<0.0001	**
Residual error	1.51	5	0.30			
Lack of fit	1.43	3	0.48	12.11	0.0773	
Pure error	0.079	2	0.039*			
Sum	117.27	14				
			R^2=0.9871	R^2adj=0.9639		

*Significant difference (P<0.05), **Extremely significant difference (P<0.01).

3.6. Analysis of Interaction of Response Surface

It was shown (Figure 5a) that the increasing of original alcohol content made the yield of acid rise firstly and then decrease, and the change of inoculation amount strongly affected the effect tendency of original alcohol content on the yield of acetic acid; the increasing of inoculation amount made the yield of acetic acid rise firstly and then decrease, and the effect of inoculation amount was strongly affected with original alcohol content changing. Thus, the interaction of inoculation amount and original alcohol content was extremely significant, which was corresponding to the extremely significant interaction of X_1X_2 (Table 3). It was shown (Figure 5b) that the yield of acid rose firstly and then decreased when increasing inoculation amount, and the change kept the same when changing temperature; the improvement of temperature made the yield of acetic acid rise firstly and then decrease, and the current kept the same with the change of inoculation amount. Therefore, the interaction of inoculation amount and temperature was non-significant, which was corresponding to the non-significant interaction of X_1X_3 (Table 3). It was shown (Figure 5c) that the enhancement of original alcohol content made the yield of acetic acid rise firstly and then decrease, and the change of temperature didn't change the current of the yield; the improvement of temperature made the yield of acetic acid rise firstly and then decrease, and the current kept the same with the change of original alcohol content. Thus the interaction of temperature and original alcohol content was non-significant, which was corresponding to the non-significant interaction of X_2X_3 (Table 3).

There were many studies using response surface methodology (RSM) to carry out statistical optimization of fruit vinegar process and explore the interaction of process parameters each other [32,33]. In many studies of fruit vinegar optimization by response surface methodology (RSM), there was optimization study of persimmon vinegar revealed significant interaction of fermentation temperature and inoculation amount, which was different [34]. Another study introduced the effect of process parameters each other on suisho pear vinegar by response surface methodology (RSM) [35]. The study showed that fermentation temperature and inoculation amount had extremely significant effect each other. Meanwhile, it was different that fermentation temperature and alcohol content also had extremely significant interaction. Eventually, alcohol content and inoculation amount were determined in the study to be non-significant. Given above, different optimization studies using response surface methodology were inclined to present different conclusion. The reason may lay in level difference when setting levels of process parameter using response surface methodology.

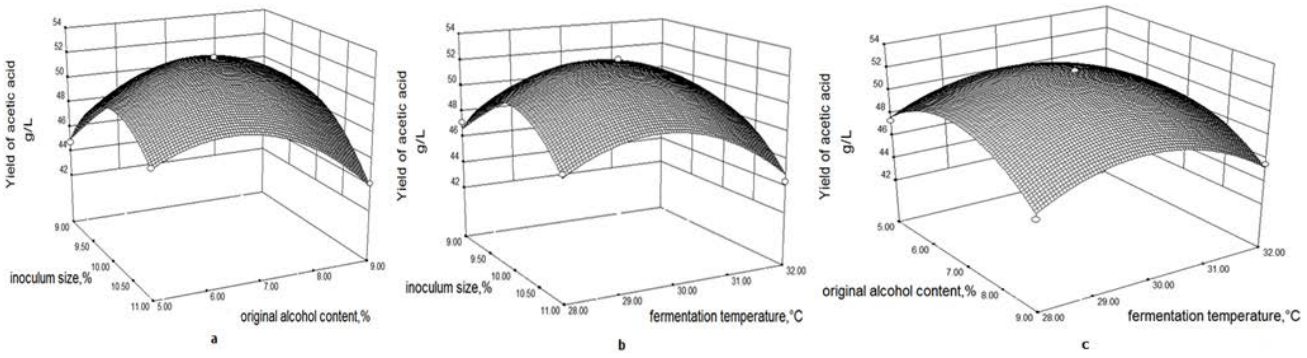

Figure 5. The response surface of technology parameters of acetic acid fermentation of litchi

3.7. Identification of Response Surface Model

Design-Expert soft was used to analyze response surface experiment results, formulate a regression equation fitting the variables and response surface value and obtain the optimal process parameters of acetic acid fermentation. The optimal process parameters were as follows: inoculation amount of 10.01%, original alcohol content of 6.85% and temperature of 29.74°C, and predicted yield was 52.54 g/L in the optimal fermentation condition. Now the fermentation process parameters were simplified into the following process: inoculation amount, 10%, original alcohol content of 7% and temperature of 30 °C, accordingly the yield was 52.47 g/L. 3 test experiments were also carried out according to the best fermentation process parameters. The result showed the mean acetic acid yield was 52.45 g/L and relative error was low, declaring response surface method was advantageous to optimize process parameters of acetic acid fermentation.

4. Conclusion

The purpose of the research was to study the fermentation of litchi vinegar including alcohol fermentation and acetic acid fermentation. Firstly the optimal parameters of alcohol fermentation were determined as follows: original sugar content of 16%, inoculation amount of 5% and pH value of 3.5, temperature of 30-32 °C; and the optimization of acetic acid fermentation was carried out using Box-Behnken methodology and then the optimal process was determined in following optimal conditions: inoculation amount of 10%, original alcohol content of 7% and temperature of 30 °C, at last the yield was 52.45 g/L. Secondly the research built the regression equation which was as follows: $Y=-1074.09031+76.93542\times X_1+15.98354\times X_2+46.20562\times X_3-0.70375\times X_1\times X_2-0.083750\times X_1\times X_3+0.061875\times X_2\times X_3-3.47958\times X_1^2-0.78740\times X_2^2-0.76990\times X_3^2$. The action of single factor and interaction of double factors on the yield of vinegar were eventually shown as follows: the effects of inoculation amount and original alcohol content were non-significant (both $P>0.05$), that of fermentation temperature was extremely significant ($P<0.01$); the interaction of inoculation amount and original alcohol content was extremely significant ($P<0.01$), on contrast, the interaction of others were non-significant. ($P>0.05$). Given above the regression model is accurate and reliable, the optimal fermentation technology of litchi vinegar is steady and advisable and the study is greatly favorable to upgrade the value for the industry application of litchi vinegar.

Acknowledgment

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