OPTIMIZATION OF ETHANOL FERMENTATION WITH REDUCING SUGARS FROM CAMELLIA (*CAMELLIA OLEIFERA*) SEED MEAL USING RESPONSE SURFACE METHODOLOGY

Ling PENG [a,b*]

[a] Department of Food Science, College of Chemistry and Bio-Engineering, Yichun University, Yichun, China
[b] CAS Key Laboratory of Bio-Based Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, China

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Camellia seed meal is studied as raw material for fermentation of ethanol. In the experiments, the effect of different variables, such as the ratio of calcium to magnesium ion, ammonium chloride addition ratio, and yeast addition ratio on ethanol yield of the reducing sugar fermentation from Camellia seed meal were investigated by a single-factor test and a response surface methodology, respectively. The results indicated that the optimal fermentation conditions of the reducing sugar from Camellia seed meal were the ratio of calcium and magnesium ion 1:1, the ammonium chloride addition ratio 0.70%, and the yeast addition ratio 0.4%. The yield of ethanol was 95.4% under the optimum fermentation conditions (1:1, 0.70% ammonium chloride, 0.4% yeast) given by using response surface methodology. Moreover, variation coefficient was 0.59%, which showed the results were significantly consistent with the predicted value of response surface methodology. It indicated that response surface methodology was reliable to optimize the fermentation conditions of ethanol yield.

Key words: *camellia seed meal, reducing sugar, fermentation, ethanol, optimization, response surface methodology*

Introduction

The investigation on biomass materials has been a fascinating topic of research mainly because it has great potential to produce biofuels. Today, the fossil fuels provide about 80% of global energy requirement which is estimated to grow by 37% by 2024 according to International Energy Report 2014 [1]. Constantly over-exploitation of existing petroleum fuel resources to match current energy demand has resulted that these energy sources exhausted rapidly [2, 3]. Meanwhile, the prices of crude oil are continuing to rise along with continuous emissions of GHG by their burning up, which caused various adverse influences on human health as well as Earth's ecology [4, 5]. Consequently, it is crucial to expose new alternative energy sources which should be sustainable, inexhaustible, efficient, environmental friendly and economically applicable [6, 7]. Among lots of substitute energy sources, biofuel specially bioethanol have obtained greater consideration all over the world because the biofuel are acknowledged the most renewable and environment-friendly energy source [8].

*Author's, e-mail: pling1981@126.com

At the present, one of the most important biofuels is bioethanol, a sustainable product obtained from energy crops and lignocellulosic material. In this sense the seed meal of *Camellia Oleifera* has been researched as an useful biomass to produce biofuels [1]. There are about 5 million hectares of *Camellia Oleifera* forests in China. About 202 million tons of mature Camellia seed, 40 million tons of Camellia oil, and 162 million tons of Camellia seed meal can be produced every year [9]. Up to now, China has become the largest country producing Camellia seed in the world [10]. Camellia seed meal is a by-product of Camellia oil industry, which contains polymersaccharide and lignocellulosic material that are suitable source for the obtention of sugars. The polymersaccharide of Camellia seed meal is mainly made up of various monosaccharide, such as xylose, glucose, mannan, galactose and so on. After the physical, chemical, or biological pre-treatments for Camellia seed meal to release fermentable sugars, this material can be fermented to produce ethanol [1].

The fermentation of biomass is a key step in the yield of ethanol. In this study, Camellia seed meal was take as raw material, and after serious pre-treatment, such as acid pre-treatment, bacterial pre-treatment, and enzyme pre-treatment, followed by removing inhibitor, Camellia seed meal could be degraded into reducing sugar. The present manuscript focused on the optimization of fermentation conditions to improve the yield of ethanol [1]. Therefore, the effect of the extraction factors were examined by a single-factor test and a response surface methodology, in order to achieve optimal fermentation conditions of reducing sugars from the seed meal of *Camellia Oleifera* [10].

Experimental

Materials and reagents

Camellia seed meals were collected from Qinglong technology company (Yichun, China) and after oven-drying at 40 °C for 24 hours, the dry meals were milled into powder by a pulveriser (XB-02, Guohua Machinery Co., Shanghai China) and passed through a 20-mesh sieve. The meal powder was exacted to remove the oil by petroleum ether and the resulting deoiled meal powder was oven-dried again for 24 hours at 40 °C, then stored in the refrigerator at –20 °C until required.

Calcium chloride, magnesium chloride, ammonium chloride, ethanol standard, glucose standard, cellulose having multiple enzyme activities were purchased from Sigma Chemical Co., (Sent Louis, Mo., USA). Concentrated sulfuric acid, analytical grade phenol were purchased from Tianjin Sunshine Co., (Tianjin, China). Yeast (*Saccharomyces cerevisiae*) was purchased form Anqi Yeast Limited Co., (Hubei, China). *Phanerochaete chrysosporium* was purchased from Guangzhou Culture Collect Center (Guangzhou, China). All other chemicals were analytical grade and purchased from Nanchang Xinyuan Chemical Reagent Co., (Nanchang, China). Distilled water was used to prepare the various solutions.

Experiment process

As shown in fig. 1, the powder of deoiled Camellia seed meal was firstly pre-treated with the sulfuric acid solution, followed by the bacterial pre-treatment using *Phanerochaete chrysosporium*, and then were sterilized and added with cellulose to make an enzyme pre-treatment. After those three pre-treatments, the inhibitor for the following fermentation was removed and then all the filtrate and residue were collected for the final ethanol fermentation with yeast (*Saccharomyces cerevisiae*).

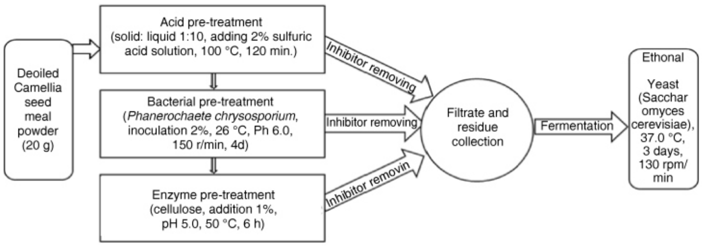

Figure 1. The process of ethanol fermentation with reducing sugar from Camellia seed meal

Analysis of reducing sugars and ethanol

The amount of the reducing sugars was determined using 3.5-dinitrosalicylic acid (DNS) reagent by the Ghose method (1987). The amount of ethanol was quantified with GC-MS (GCMS-QP2010 of Shimadzu) using a DB-5 capillary column on previously described method [11].

Establish of standard curve

Different volumes (0, 0.25, 0.5, 1.0, 1.25, 1.5, 2.0, 2.25, 2.5 mL) of the stock standard solutions of glucose were transferred into a test tube with a stopper and diluted with water to a metered volume (15.0 mL), respectively. After colorimetric reaction of glucose absorbance of prepared solution at the maximum absorption wavelength was measured by UV spectrophotometer. The linear relationship of absorbance and glucose content could be then obtained by regression analysis:

$$Y = 0.8858\,X + 0.0360$$

where Y is the absorbance of prepared solution at 540 nm and X [%] – the mass concentration of glucose. The correlation coefficient, R2, is 0.9977.

Experimental design

At first, a serious of single-factor experiments, such as ratio of calcium to magnesium ion(calcium chloride/magnesium chloride, w/w), ammonium chloride addition ratio, and yeast addition ratio were carried out to evaluate the effects of these factors on the yield of ethanol and all the ratios previously mentioned were based on the dry weight of camellia seed meal. Then, a three-level, three-factor Box-Behnken design (response surface methodology) was employed for optimizing the process. The yield of ethanol was used as the indices in evaluating the results of fermentation. The independent factors and levels of experimental design with observed values for the response are given in tabs. 1 and 2. The factors and levels of ethanol fermentation conditions were determined on the basis of single-factor experiments, such as ratio of calcium to magnesium ion, ammonium chloride addition ratio, and yeast addition ratio [12]. Calculation formula of ethanol yield was:

$$\text{Ethanol yield} \quad \frac{\text{Actual content of ethanol}}{(\text{Total content of reducing sugar} \quad \text{residue content of reducing sugar}) \quad 0.511}$$

Results and discussion

Single-factor experiments

Effect of the ratio of calcium to magnesium ion on ethanol fermentation. The influence of the ratio of calcium to magnesium ion on ethanol fermentation was carried out at ammonium

Table 1. Levels and factors of response surface analysis for ethanol fermentation from reducing sugar of Camellia seed meal

Level	A	B	C
	Ratio of calcium to magnesium ion	Ammonium chloride addition ratio [%]	Yeast addition ratio [%]
–1	1:2	0.50	0.30
0	1:1	0.75	0.40
1	3:2	1.00	0.50

Table 2. The design and experimental results of response surface analysis for ethanol fermentation from reducing sugar of Camellia seed meal

Number	A	B	C	Y
	Ratio of calcium to magnesium ion	Ammonium chloride addition ratio [%]	Yeast addition ratio [%]	Ethanol yield [%]
1	1:1	0.50	0.30	86.8
2	3:2	0.75	0.50	84.7
3	1:1	0.75	0.40	95.9
4	1:1	1.00	0.30	87.0
5	1:1	0.75	0.40	95.3
6	1:1	1.00	0.50	81.8
7	1:2	1.00	0.40	79.5
8	1:2	0.75	0.50	79.4
9	1:2	0.50	0.40	81.7
10	3:2	1.00	0.40	83.1
11	1:1	0.75	0.40	94.1
12	1:1	0.75	0.40	95.1
13	3:2	0.50	0.40	92.3
14	1:2	0.75	0.30	81.2
15	1:1	0.75	0.40	94.2
16	1:1	0.50	0.50	87.9
17	3:2	0.75	0.30	90.9

chloride addition ratio 0.75%, yeast addition ratio 0.40%, fermentation pH 5.0. The results were shown in fig. 2(a). The ethanol yield went up from 76.12 % to 92.87% with the ratio of calcium to magnesium ion increasing from 1:10 to 1:1, respectively. However, when liquid-solid ratio was more than 1:1, the ethanol yield decreased gradually. The ratio of calcium to magnesium ion played an important role in the metabolism of living cells. Therefore, the appropriate ratio of calcium to magnesium ion was around 1:1.

Effect of ammonium chloride addition ratio on ethanol fermentation. Figure 2(b) showed the influence of ammonium chloride addition ratio on ethanol fermentation, carried out

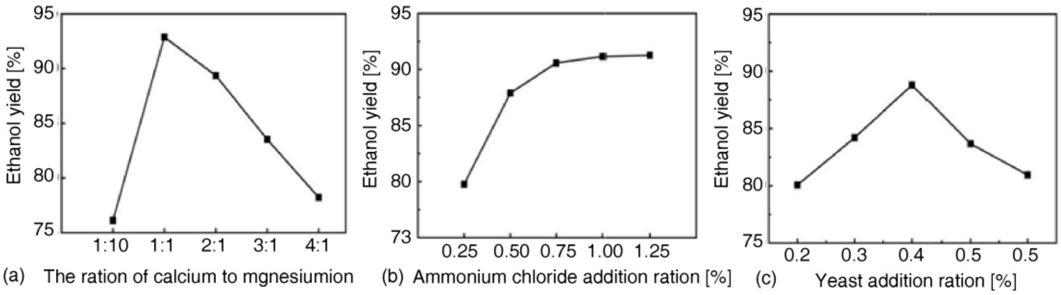

Figure 2. Effect of various factors on ethanol yield; (a) effect of the ratio of calcium to magnesium ion, (b) effect of ammonium chloride addition ratio, and (c) effect of yeast addition ratio

under similar conditions with the ratio of calcium to magnesium ion 2:1, yeast addition ratio 0.40%, fermentation pH 5.0. The source of nitrogen element played an important role in yeast fermentation, which could improve the fermentation process. From fig. 2, it would been seen that the yield of ethanol kept increasing sharply with the ammonium chloride addition ratio increasing from 0.25% to 0.75%. But when the range of ammonium chloride addition ratio was from 0.75% to 1.25%, the increasing of ethanol became very slow. Therefore, considering the producing cost and ethanol yield, the appropriate ratio of ammonium chloride should be around 0.75%.

Effect of yeast addition ratio on ethanol fermentation. The effect of yeast addition ratio on ethanol fermentation was shown in fig. 2(c). The conditions, under which the experiments were carried out, were: the ratio of calcium to magnesium ion 2:1, ammonium chloride addition ratio 0.75%, fermentation pH 5.0. The addition ratio of yeast was a key step in the process of ethanol fermentation. When the addition ratio of yeast was too small, efficiency of ethanol fermentation would not be enough. But if the addition was too large, the nutrients would become lack to prevent fermentation. It was obvious that the most optimal yeast addition ratio was about 0.4%.

Analysis of response surface

Response surface methodology used in this study is an efficient statistical technique for modelling and optimization of multiple variables to predict the best conditions with a minimum number of experiments [10].

The levels of ethanol yield with fermentation of reducing sugar in Camellia (*Camellia Oleifera*) seed meal from the seventeen sets of variable combinations showed in tab. 2 were fit into a quadratic regression equation by using the software Design-Expert 8.0. The estimated values of constant coefficients and analysis of variance were shown in tab. 3, and the regression model for ethanol yield, *Y*, was predicted as follows [13]. In the regression equation, the negative quadratic coefficient indicated that the parabolic opening of the equation was downward, had a maximum point and could be optimally analysed.

$$\text{R1} \quad 94.92 \quad 3.65\ A \quad 2.16\ B \quad 1.51\ C \quad 1.75\ A\ B$$
$$1.10\ A\ C \quad 1.58\ B\ C \quad 6.30\ A^2 \quad 4.47\ B^2 \quad 4.57\ C^2$$

where A is the ratio of calcium to magnesium ion, B – the ammonium chloride addition ratio, and C – the yeast addition ratio.

Table 3. The ANOVA of ethanol yield with fermentation of reducing sugar in Camellia seed meal

Source of variation	Sum of squares	Degree of freedom	Mean aquare	F-value	P-value	Significant
Model	566.97	9.00	63.00	54.51	<0.0001	***
A	106.58	1.00	106.58	92.21	<0.0001	***
B	37.41	1.00	37.41	32.37	0.0007	***
C	18.30	1.00	18.30	15.83	0.0053	**
AB	12.25	1.00	12.25	10.60	0.0139	*
AC	4.84	1.00	4.84	4.19	0.0800	
BC	9.92	1.00	9.92	8.59	0.0220	*
A2	166.98	1.00	166.98	144.48	<0.0001	***
B2	84.22	1.00	84.22	72.87	<0.0001	***
C2	88.03	1.00	88.03	76.17	<0.0001	***
Residual error	8.09	7	1.16			
Lack of it	5.76	3	1.92	3.30	0.1394	Not significant
Pure error	2.33	4	0.58			
Total	575.06	16				

*** – extremely significant ($P < 0.001$), ** – very significant ($P < 0.01$), * – significant ($P < 0.05$)

Table 4. Analysis of model reliability

Item	Value
Standard deviation	1.08
Mean	87.70
Coefficient of variation, [%]	1.23
PRESS	95.84
R-squared	0.9859
Adjusted R-squared	0.9678
Predicted R-squared	0.8333
Adeq. precision	18.110

The P-values determine the significant of each coefficient. A higher significant of the corresponding coefficient is indicated by a lower P-value, a lower significant of the corresponding coefficient is indicated by a higher P-value [14]. It could be noticed from tab. 3 that the P-values of model, A and B items were less than 0.001, which meant that the predicted model and the items of A and B were extremely significant. Meanwhile, the P-value of C item was less than 0.01 indicating this item was very significant. Furthermore, the P-value of lack of it was 0.1394 (>0.05, not significant), which meant this model had a good fit with the experiments and the experiment errors were little. From tab. 4, analysis of model reliability showed that correlation coefficient, R2, was 0.9859; adjustment coefficient, R2Adj, was 0.9678, which meant only less than 4% of ethanol yield results did not match the model, and coefficient of variation was only 1.23%, indicating good reproducibility. Overall, the model could be used to optimize the ethanol fermentation processing of reducing sugar from Camellia seed meal [15].

The response surface is formed by the two-factor interaction among the variables. When the slope of the response surface is steeper, the greater the effect of the processing condition on the response value. In contrast, when the slope of the response surface is smoother, it indicates that the effect of the processing condition on the response value is smaller [16]. Meanwhile, the contour line is elliptical, indicating that the interaction of the variables is significant.

In contrast, the contour line is round, which means that the interaction of the variables is not significant.

The interaction effect of each variable was analysed by quadratic model, and the response surface and contour line were obtained. As shown in fig. 3, the influence of each factor and its interaction value on the response value could be visually seen. Figures 3(a) and 3(b) described the interaction effect between the ratio of calcium to magnesium ion and ammonium chloride addition ratio on ethanol yield, with another one variable unchanged (0.4% yeast addition ratio). As the ratio of calcium to magnesium ion (holding ammonium chloride addition ratio constant) or ammonium chloride addition ratio (holding the ratio of calcium to magnesium ion constant) increased, the trend of ethanol yield firstly went up then decreased. Similarly, figs. 3(c) and 3(d) and figs. 3(e) and 3(f) showed the interaction effect between the ratio of calcium to magnesium ion and yeast addition ratio, and between ammonium chloride addition ratio and

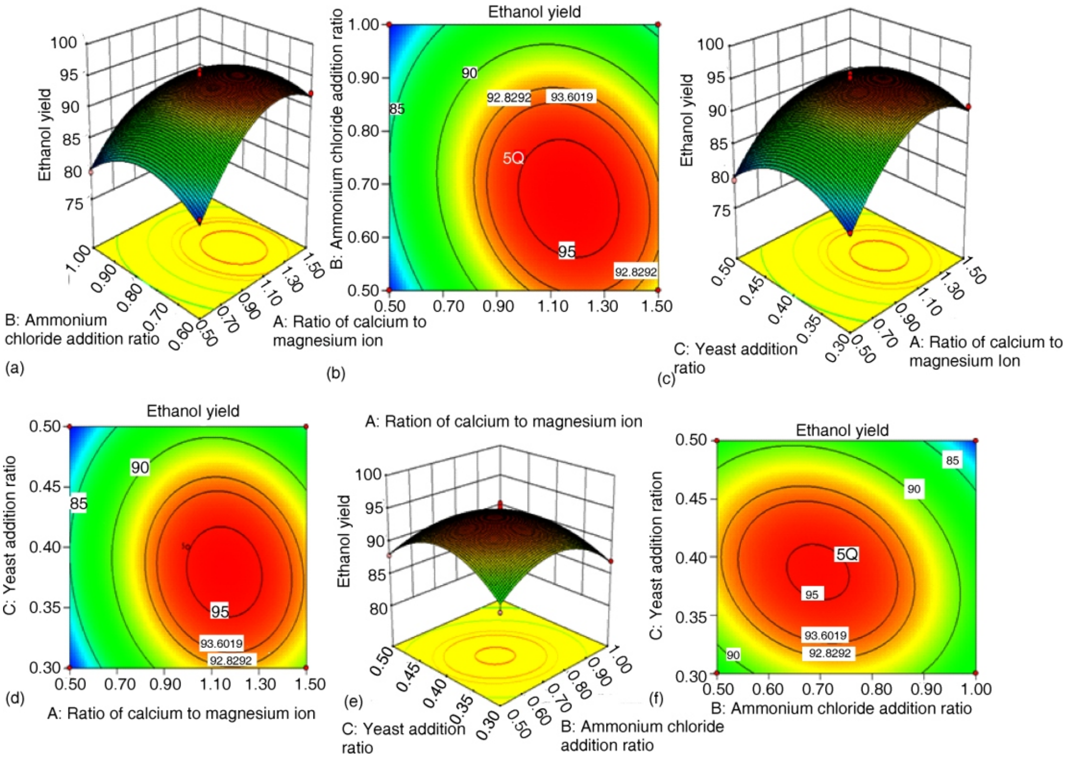

Figure 3. Corresponding response surfaces and contour lines for ethanol yield from the reducing sugar in Camellia seed meal; (a) response surface of interaction effect between the ratio of calcium to magnesium ion and ammonium chloride addition ratio on ethanol yield, (b) contour line of interaction effect between the ratio of calcium to magnesium ion and ammonium chloride addition ratio on ethanol yield, (c) response surface of interaction effect between the ratio of calcium to magnesium ion and yeast addition ratio on ethanol yield, (d) contour line of interaction effect between the ratio of calcium to magnesium ion and yeast addition ratio on ethanol yield, (e) response surface of interaction effect between ammonium chloride addition ratio and yeast addition ratio on ethanol yield, and (f) contour line of interaction effect between ammonium chloride addition ratio and yeast addition ratio on ethanol yield

yeast addition ratio on ethanol yield, respectively. According to the significant differences in the variables, the sharpness of the response surface and the shape of the contour line, it can be found that the order of impacts of different variables on ethanol yield was A (the ratio of calcium to magnesium ion) >B (ammonium chloride addition ratio) C (yeast addition ratio), the effect of linear and quadratic term of A, B, and C on ethanol yield were extremely significant (***) and the influence of the interaction effects between A and B, and between B and C were significant (*) which illustrated the relationship between variables and the result of ethanol yield was not simply the linear relationship, but presented interactive function, and suggested that they were significant for the optimization of ethanol fermentation from the reducing sugar in the Camellia seed meal [17].

Based on the model, the optimum fermentation conditions for reducing sugar to obtain ethanol were determined by regression analysis. The maximum predicted yield of ethanol of 95.97% was obtained under the optimum fermentation conditions of 1.17:1, 0.68%, and 0.38% for the ratio of calcium to magnesium ion, ammonium chloride addition ratio and yeast addition ratio, respectively. In order to verify the reliability of the predicted quadratic model, three parallel replication experiments were carried out under the similar fermentation conditions aforementioned of the ratio of calcium to magnesium ion 1:1, ammonium chloride addition ratio 0.70% and yeast addition ratio 0.40%. The average of the results of ethanol yield was 95.4%, and coefficient of variation was 0.59%, which was not significantly different from the predicted value. These results indicated that the quadratic model was reliable and response surface methodology could be used to optimize the fermentation conditions of ethanol yield.

Conclusion

The conditions of fermentation with reducing sugar from Camellia seed meal were optimized using response surface methodology. This method could effectively predict the optimum process conditions and the final yield of ethanol. At first, fermentation conditions for ethanol with reducing sugar from Camellia seed meal were analysed and successfully optimized by single-factor experiments and response surface methodology, respectively. The effect of the ratio of calcium to magnesium ion, ammonium chloride addition ratio, and yeast addition ratio on ethanol yield were significant. The order of impacts of different variables was the ratio of calcium to magnesium ion > ammonium chloride addition ratio > yeast addition ratio. Optimized fermentation conditions were:

calcium and magnesium ion 1:1,
ammonium chloride addition ratio 0.70%, and
yeast addition ratio 0.40%.

Under the optimum conditions, the optimal ethanol yield was 95.4%.

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