Optimization of *Actinomycetes* Changbai No.2 Liquid Fermentation by Response Surface Method

Ning YANG[1], Yong WANG[1,a*],Yu-bo WANG[2], Li ZHANG[1], Yong LU[1], Quan-jie CHEN[1], Wen-ge ZHANG[3], Dan-hua SU[4] and Ruo-peng LU[4]

[1] University of Science and Technology Liaoning, Anshan 114051 P. R.China

[2] Shenyang Agricultural University, Shenyang, 110161 P. R. China

[3] Anshan Normal University Affiliated Medical School, Anshan, 114001 P. R. China

[4] Qianshan Datun Town Agricultural Technology Extension Station, Anshan 114000 P. R.China

[a]wy2002866@126.com

*Corresponding author

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Abstract. Actinomycetes are affected by external conditions during fermentation, especially the secondary metabolites, the influence of the external conditions is very large. In order to produce the most abundant pigment, the method of response surface was used to optimize the liquid fermentation condition of the actinomycetes Changbai No.2. A single factor was used to determine the best single factor, and the Plackett-Burman experiment was used, and statistical software SPSS 17.0 for statistical processing, there are three main influencing factors: the concentration of starch, fermentation time and temperature. Finally, using Box - Behnken design to get the best value of these three factors is: the concentration of starch is 18.42g/L, fermentation time is 6.46 days, the temperature is 26.19℃. Postoptimality, the absorbance of the Changbai No.2 fermentation liquid was 2.098.

Introduction

Microbial pigment is a natural pigment, it's extracted from microorganisms, not limited by resources, time, and space, unparalleled advantages of plant pigments and animal pigments.[1] Microbial fermentation is used to produce a variety of pigments. [2] The pigment is the secondary metabolite of actinomycetes, and the production of secondary metabolites will be affected by different cultivation conditions. Therefore, the optimization of culture medium and fermentation conditions of actinomycetes is a very important part of industrial production, which is an indispensable link from laboratory to industrial production. [3]

Response surface methodogy(RSM)has been widely used in the optimization of fermentation culture medium, optimization of culture conditions and enzymatic hydrolysis and other biochemical reaction optimization and model establishment. [4,5] In this experiment, Plackett-Burman experiment and Box-Behnken experiment were used to optimize the composition of fermentation medium to improve the yield of secondary metabolites pigment of actinomyces. [6,7]

Materials and methods

Materials

Bacterial strain

Tested strain: Actinomycetes strain Changbai No.2 (separation, screening and preservation from the soil of Changbai mountain).

Culture medium

Liquid fermentation medium: Gause's No.1 liquid medium.

methods

Single-factor experiment

Single factor test was used to optimize the fermentation process parameters such as culture medium, fermentation temperature, fermentation time, liquid loading quantity, inoculation quantity and pH value. Each time three parallel experiments were conducted, the average was averaged. The fermentation liquid was centrifuged for 30min under the condition of 4000r/min. The lower cell body was then placed in the mortar, and the quartz sand and a small amount of fermentation liquid were added to grind, and then the centrifuge was used after 20min. The absorbance at 527nm was determined by mixing the supernatant liquid with the upper cleaning liquid after the first centrifuge. Then determine the best condition for optimal single factor.

Design experiment of response Surface Placket-Burman (P-B). [8]

Selected on the basis of single factor experiment, effect on fermentation of larger factors to optimize experiment, these factors are: starch, potassium nitrate, culture temperature, pH value, fermentation time and fluid volume. Using P-B program test, the absorbance of pigment in the fermentation liquid was used as the evaluation index, and three parallel experiments were performed in each group, and the average value was taken at the time of measurement. It is concluded that the above factors have the most influence on the production of the *actinomycetes* Changbai No.2. The six main factors that will affect the fermentation of *actinomycetes* Changbai No.2 are the main components of P-B design. First of all, the high level of (1) and the low level (-1) are set, in which the maximum data is 1.25 times the minimum data, with absorbance as the response value Y. P-B design experiment was shown in Table 1.1. The data were statistically processed using SPSS 17.0 software, and the obtained data was analyzed with a single factor variance, and the difference was significant with $P < 0.05$. Select 3 significant factors that affect the absorbency.

Tab.1.1 Fermentation conditions for screening Plackett-Burman experimental design factors and levels

Experimental factor	Unit	code	(-) low level	(+)high level
starch	g/L	X1	18	22.5
nitrate of potash	g/L	X2	0.9	1.1
the volume of liquid	mL	X7	80	100
fermentation time	d	X8	5.5	7
temperature	℃	X9	24	30
pH	—	X11	6	7.5

Box-Behnken (B-B) experimental design

According to the conclusion of P-B, the optimum value of selected factors was determined by software B-B experiment design.

Model verification test

In order to further verify the predictive value of response surface method, the best predicted value after optimization was fermented, and each experiment was set up with 3 repetitions, and the average value was obtained. By comparing the experimental values with the predicted values, we can determine whether the optimal fermentation conditions obtained by the response surface method are accurate and reliable, and whether they are of practical value.

Results and analysis

Results and analysis of Placket-Burman test

In the 12 experiments designed by Design-Expert.V.8.0.6 software, the experiment was carried out according to the combination of experimental components and experimental conditions designed in table 2.1, and three parallel tests were designed for each group. Then according to the experiments, the experimental conditions for training, training is completed, according to the treatment method of fermented liquid fermented liquid before, and then determine the absorbance of fermented liquid, after processing will be parallel to the three groups of experiments take its average.

Tab.2.1 Experimental design and result of Plackett-Burman

component	Starch g/L	Potassium nitrate g/L	Fermentation time d	Culture temperature ˚C	pH	With fluid volume mL	Absorbance
1	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.933
2	1.00	1.00	-1.00	1.00	1.00	1.00	2.400
3	1.00	-1.00	1.00	1.00	1.00	-1.00	1.933
4	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	1.633
5	-1.00	1.00	-1.00	1.00	1.00	-1.00	2.00
6	-1.00	-1.00	1.00	-1.00	1.00	1.00	1.533
7	-1.00	1.00	1.00	1.00	-1.00	-1.00	1.667
8	-1.00	1.00	1.00	-1.00	1.00	1.00	1.633
9	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.933
10	1.00	-1.00	1.00	1.00	-1.00	1.00	1.967
11	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.800
12	1.00	1.00	-1.00	-1.00	-1.00	1.00	1.967

According to the data obtained in the software Placket-Burman table, the data is processed by the statistical software SPSS 17.0, which can be used to establish a model. The specific data is shown in table 2.2 below, three distinct items can be drawn from the chart, because the effect of $Pr<0.05$ is significant, the impact of $Pr<0.01$ is significant. And because P(X1)=0.005, P(X3)=0.013, P(X4)=0.011，therefore X1、X3、X4 that is the significant term in this model. Starch, fermentation time and culture temperature were used as three significant factors influencing fermentation culture. The three significant items were further optimized by the experimental design of the response surface.

Tab.2.2 The significant of factors

model	Non-standardized coefficient		The standard coefficient	t	Pr＞| t|
	B	Standard error	A trial version		
Starch	0.125	0.27	0.584	4.679	0.005
Potassium nitrate	0.058	0.27	0.273	2.185	0.081
Fermentation time	-0.100	0.27	-0.468	-3.744	0.013
Culture temperature	0.106	0.27	0.496	3.968	0.011
pH	0.37	0.27	0.173	1.387	0.224
With fluid volume	0.23	0.27	0.109	0.876	0.421

Tab.2.3 Regression analysis of variance table

model	Sum of squares	df	The mean square	Value of F	significant
Regression	0.507	6	0.085	9.854	0.12[a]
Residual error	0.043	5	0.009		
Total	0.550	11			

a. prediction variables: (constants), With fluid volume,pH,Culture temperature,Fermentation time,Potassium nitrate,starch .

Tab.2.4 Model summary

model	R	R^2	Adjust R^2	Standard estimation error
1	0.960[a]	0.922	0.828	0.9260

a. prediction variables: (constants), With fluid volume,pH,Culture temperature,Fermentation time,Potassium nitrate,starch .

Analysis of the results of response surface Box-Behnken experiment

The 3 significant items obtained by the Placket-Burman test were the influencing factors of this experiment, other culture medium formulations and culture conditions remain unchanged, according to the response surface software Box-Behnken designed the experimental scheme, then a series of experiments were carried out using the experiment, the fermentation fluid was fermentated according to the experimental scheme, and the results of the experiment were averaged by three parallel experiments. After culture, the fermentation liquid was treated accordingly, and the absorbance was determined at the wavelength of 527nm. Finally, the results of the experiment are obtained, as shown in table 2.5 and 2.6:

Tab.2.5 Box-Behnken experimental results analysis

Run	A starch（g/L）	B Fermentation time（d）	C Culture temperature（˚C）	Y absorbance
1	20.00	8.00	20.85	2.003
2	20.00	6.40	27.80	2.23
3	15.00	6.40	20.85	2.067
4	15.00	4.80	27.80	2.02
5	20.00	6.40	27.80	2.197
6	20.00	4.80	20.85	2.25
7	20.00	6.40	27.80	2.23
8	25.00	8.00	27.80	1.7
9	20.00	6.40	27.80	2.25
10	20.00	6.40	27.80	2.167
11	15.00	6.40	34.75	2.033
12	25.00	4.80	27.80	1.8
13	20.00	8.00	34.75	1.933
14	25.00	6.40	34.75	1.833
15	25.00	6.40	20.85	1.867
16	20.00	4.80	34.75	2.1
17	15.00	8.00	27.80	1.967

Tab.2.6 Analysis of experimental results of Box-Behnken

Sources of variance	Sum of squares	Degree of freedom	The mean square	Value of F	Pr>F
model	0.45	9	0.050	21.18	0.0003 significant
A-starch	0.098	1	0.098	41.34	0.0004
B-Fermentation time	0.04	1	0.040	16.89	0.0045
C-Incubation temperature	0.01	1	0.010	4.36	0.0752
AB	0.000	1	0.000	0.23	0.6446
AC	0.000	1	0.000	0.000	1.0000
BC	0.002	1	0.002	0.67	0.4392
A^2	0.23	1	0.23	95.49	<0.0001
B^2	0.052	1	0.052	21.72	0.0023
C^2	0.004	1	0.004	1.87	0.2135
Residual item	0.017	7	0.002	—	—
Loss of quasi item	0.012	3	0.004	3.83	0.1138 not significant
error	0.004	4	0.001	—	—
Sum	0.47	16	—	—	—

Verification test of the model

It can be seen from the above table that the Pr of the model is 0.003 and Pr<0.01, so it's remarkable, its misfit is 0.1138, misfit>0.05, it is not obvious that the model is missing. The R^2 of the complex correlation coefficients of the model is 0.9646, it is good to show the fitting of the data between the experiment and the actual measurement; The adjusted R2 is 0.190, its numerical change is related to starch concentration, fermentation time and culture temperature; The variation coefficient C.V can represent the accuracy of the model, the smaller the C.V, the more accurate it is, in this experiment, C.V=2.39%, indicates that the operation accuracy of the experiment is relatively high.

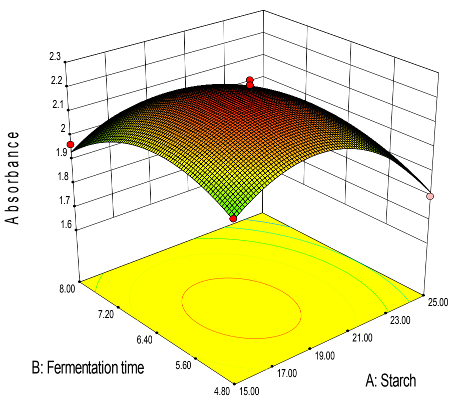
The regression equation was obtained by using the experimental data of the software design-expert. V.8.0.6: Absorbance=2.12-0.11A-0.071B-0.036C-0.012AB+0.000AC+0.020BC-0.23A^2 -0.11B^2 -0.033C^2.

A partial derivative of this equation for different independent variables, the following equations can be obtained

$$0.11+0.012B+0.46A=0$$
$$0.071+0.012A-0.02C+0.22B=0$$
$$0.036-0.02B+0.066C=0$$

Through this system, A=-0.25, B=0.38, C=-0.43, the actual value of each factor can be obtained by conversion: the concentration of starch is 18.42g/L, the fermentation time was 6.46 days, temperature is 26.19˚C.

Response surface analysis diagram by software Design-Expert.V. 8.0.6:



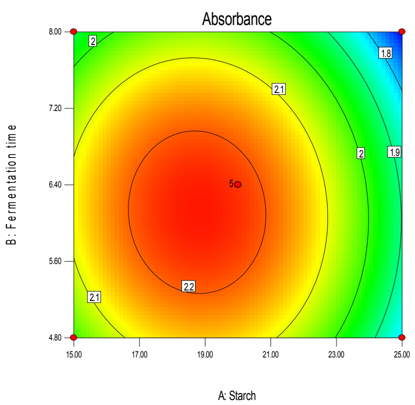


Fig.2.1 Starch and fermentation time effect on the pigmentof response surface and contour plots

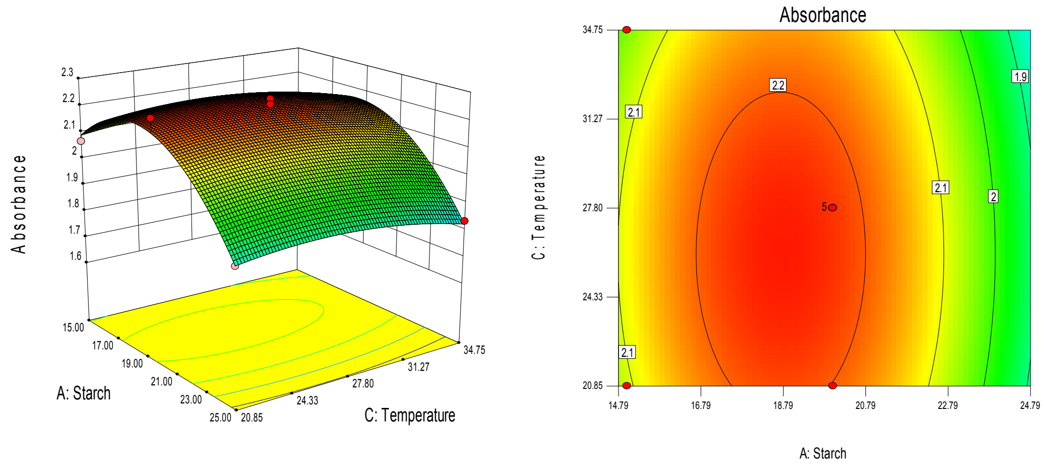


Fig.2.2 Starch and culture temperature effects on the pigment response surface and contour plots

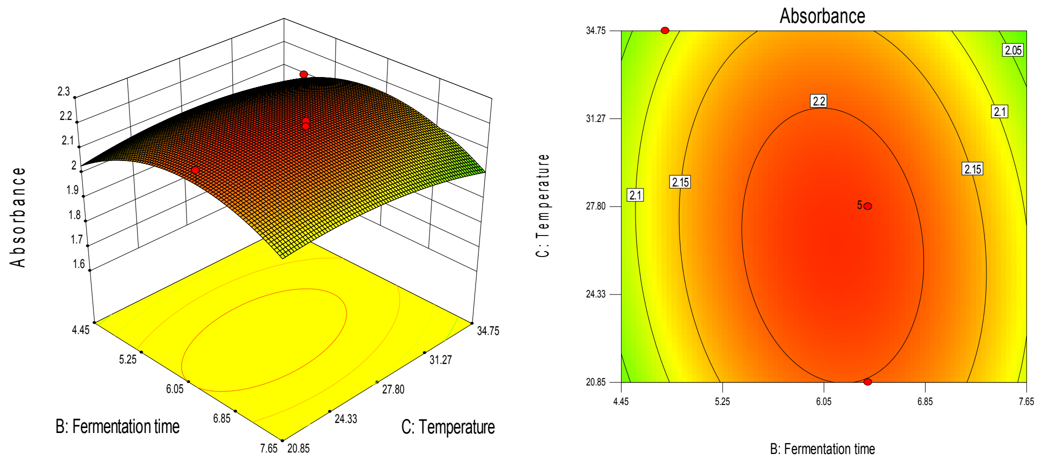


Fig.2.3 Culture temperature and fermentation time effects on the pigment diameter response surface and contour plots

Response surface figure is the graphical expression of the regression equation, as can be seen from the response surface analysis, the size of the absorbance value Y is increased with the increase of the values of two factors, first reduce the trend, after various interaction factor has a best combination to maximize the Y value. According to the regression equation of Design-Expert.V.8.0.6 software, the best predictive value is: The concentration of starch is 18.42g/L，the fermentation time was 6.46 days, Temperature is 26.19℃, the absorbance is calculated at 2.098， then the actual absorbance is 2.1, which can be obtained by repeated experiments. The difference between the two values is small, and the error is small.

Conclusion

Single factor optimization experiment results show: The factors that influence the fermentation liquid are starch, potassium nitrate, culture temperature, acid alkalinity, fermentation time and liquid loading.

On the basis of single factor experiment, using the response surface method on the metabolism of actinomycetes long white 2 pigment, the optimization of fermentation conditions, established the fermented liquid of pigment content and starch concentration in the culture medium, fermentation time and temperature of three factors quadratic polynomial regression model, and verified experiment proves that the model is reasonable and reliable.

The optimal culture conditions for the determination of *actinomycetes* Changbai No.2 metabolism were: The concentration of starch is 18.42 g/L, The fermentation time was 6.46 days, temperature is 26.19 ˚C. In this condition, the absorbance of pigment in the actinomycete Changbai No.2

fermentation liquid was calculated as 2.098, and the actual absorbance obtained by repeated experiments was 2.1. The experimental results show that the model is basically consistent with the test results, and the model can predict Y values reasonably and effectively, which lays the foundation for industrial production.

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