OPTIMIZATION OF LIPOPEPTIDE PRODUCTION BY *BACILLUS AMYLOLIQUEFACIENS* B15 USING RESPONSE SURFACE METHODOLOGY

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In this study, we increased concentration of lipopeptides by improving culture medium components and fermentation conditions, thereby laying the foundation for the industrial production of antibacterial lipopeptides. By studying optimized carbon and nitrogen sources, Plackett-Burman design, steepest ascent experiment and response surface methodology, for its medium and fermentation conditions were optimized. After the optimization, the optimized culture medium components and fermentation conditions were as follows: glucose 36.28g/L, yeast extract powder 12.77g/L,$MgSO_4$ 0.5 g/L,KCl 0.5 g/L,KH_2PO_4 1.0 g/L,$FeSO_4$ 0.15mg/L,$MnSO_4$ 5.0 mg/L,$CuSO_4$ 0.16 mg/L,inoculation quantity 4%,initial pH 6.82, temperature 37°C,time 51.69 h,rotation speed 225 r/min and loaded liquid 100 ml. Under these conditions, the yield of lipopeptides increased substantially.

Introduction

Since Arima *et al*[1] first discovered that *Bacillus subtilis* could produce surfactin, researchers have paid attention on selecting lipopetides producing strains, especially among *Bacillus*. Lipopeptide is a kind of biosurfactants which is composed of hydrophilic peptide chain and lipophilic aliphatic hydrocarbon chain. Except for surface activity, some lipopeptides also have anti-fungal, anti-bacterial, anti-viral, anti-tumor and other biological activities [2]. In recent years, there were a large number of studies which indicated that besides *B*. subtilis, *B. amyloliquefaciens* [3][4], *B. lichemformis* [5], *B. circulan* [6], *B. cereus* [7] and *B. pumilus* [8] and etc. could also produce antimicrobial lipopeptides.

Lipopeptides are cyclic compounds which has a β- amino group or β- hydroxy fatty acid in the peptide part. Based on different amino acid sequence and branches of fatty acids, lipopeptide are divided into three categories: (1) iturin, including iturin A, mycosubtilin and bacillomycin; (2) Fengycin, including plipastatin; (3) surfactin, the most researched in the lipopetides family, including many compounds with biological activity[9][10].

We obtained a bacterium B15 from the skin of grape, which was identified as *B. amyloliquefaciens*,could produce lipopeptides. In this study, we optimized lipopeptides production by *B. amyloliquefaciens* B15 using carbon and nitrogen sources optimization, Plackett-Burman design, steepest ascent design, and Box-Behnken design.

Materials and methods

Microorganism

B. amyloliquefaciens B15 isolated from the grape skin was stored at China National Research Institute of Food & Fermentation Industries. *Growth medium and culture conditions*

Potato Dextrose Agar (PDA);

Nutrient Broth (NB);

Landy medium: Glucose 30.0 g,L-Sodium Glutamate5.0 g,$MgSO_4$ 0.5 g,KCl 0.5 g,KH_2PO_4 1.0 g,$FeSO_4$ 0.15mg,$MnSO_4$ 5.0mg,$CuSO_4$ 0.16 mg,H_2O1000 mL,pH 7.0

The inoculated flask with NB medium (a 250 mL Erlenmeyer flask, liquid volume 100 mL) was incubated on an orbital shaker at 120 rpm and 37^{O}C for 24 h.

The seeding culture was inoculated by 4% in Landy medium (a 250 mL Erlenmeyer flask, liquid volume 100 mL) at 180 rpm and 30 ^{O}C for 48 h.

Crude extraction of lipopeptides

In this study, lipopeptides from *B. amyloliquefaciens* B15 were extracted using a developed method (Lee SC et al., 2007) [11]. Briefly, the suspension was centrifuged at 4^{O}C and 8, 000 rpm for 20 min and bacterial cells were discarded. The cell-free supernatant was adjusted to pH 2 with 6 M HCl, and incubated overnight at 4^{O}C. The pellet was recovered by centrifugation at 8, 000 rpm for 20 min, extracted with methanol, shaked in the shaker at 25^{O}C and 120 rpm for 30 min, and filtered by polytetrafluoroethylene membrane.

Quantization of lipopeptides by HPLC

The quantization of lipopeptides by high-performance liquid chromatography (HPLC) was conducted according to previous reports with some modifications [12, 13].

The method of quantization of lipopeptides by high-performance liquid chromatography (HPLC) was used in this experiment, and this method was first introduced by Zhu zhen[12] and D. Vitullo[13], et al. And we made some changes: lipopeptides Lipopeptides was detected by HPLC (C18 reversed-phase column 4.6 mm×250 mm×0.5 μm, 110A). And the column temperature was 30 OC, the UV monitor wavelength was 210 nm, the flow rate was 1.0 ml / min, and the injection volume was 20 μl. When detecting iturin A was detected, using 0.1% of trifluoroacetyl acid (TFA) was added in acetonitrile-H2O (2:3, v/v) as eluent for 20 min. The peak area (x) and iturin A (y) for the standard curve was: y = 7E-05x + 11.88 (R^2 = 0.9913). When detecting surfactin, using 0.1% of trifluoroacetyl acid (TFA) was added in acetonitrile-H2O (4:1, v/v) as eluent for 30 min. The peak area (x) and surfactin (y) for the standard curve was: y = 0.0001x + 21.945 (R^2 = 0.9964). According to the above two equationsthe standard curve based on the peak area, the content of iturin A and surfactin could be calculatedobtained.

Antifungal activity

The antimicrobial activity was detected according to a previous report with some modifications [14]. Botrytis cinerea (stored in 4°C) was inoculated on PDA slope. After incubated for 7 days at 25°C, spore suspension was prepared with a sterile buffer containing 0.5% NaCl, 15% glycerin, and 1% Tween-20. 200 μL spore suspension was spread on PDA plates and set aside the plates for 3 h for punching. 80 μL lipopeptides crude extract was injected in each hole and methanol was injected as a solvent control. The plates were incubated for 5 days at 25°C and then the diameters of antimicrobial circle were measured.

Effects on lipopeptides production and antifungal activity by carbon and nitrogen sources

Glucose and L-glutamate in the basic fermentation medium were replaced by carbon sources (maltose, fructose, sucrose, xylose, soybean flour, millet flour and corn flour) and nitrogen sources (L- glutamate, peptone, yeast extract, beef extract powder, soybean flour, urea, ammonium sulfate and ammonium nitrate), respectively. The content and antimicrobial activity of surfactin were measured by previous methods.

Medium optimization design

Plackett-Burman Design

In the present study, the medium components and fermentation conditions were screened by Plackett-burman design for fourteen variables at two levels. The fourteen factors were as follows: glucose (X1), yeast extract powder (X2), $MgSO_4$ (X3), KCl (X4), KH_2PO_4 (X5), $FeSO_4$ (X6), $MnSO_4$ (X7), $CuSO_4$ (X8), inoculation quantity (X9), initial pH (X10), temperature (X11), fermentation time (X12), rotation speed (X13), and loaded liquid (X14). All experiments were designed by Design-Expert V8.0, and carried out in triplicate and the averages of lipopeptides production were taken as response (table 1).

Steepest ascent experiment

According to the result of Plackett-Burman design, the most significant factors which affected the lipopeptides production were selected. The path of steepest ascent experiment (including changes in direction and the step size) was determined by identifying the effect of every significant factor, and the

conditions which could produce the maximum lipopeptides were obtained.

Box-behnken Design

In this study, four key variables with three concentration levels were adopted. Design-Expert V8.0.6.1 was used for the experimental design and statistical analysis.

Results

Effects on lipopeptides production and antifungal activity by carbon and nitrogen sources

As Fig. 1 and Fig. 2 showed, different carbon and nitrogen sources had significant

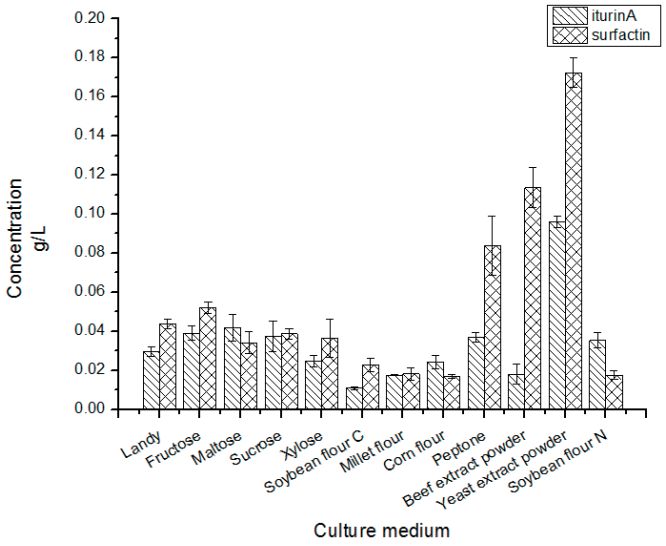

Fig. 1 - Effects of carbon sources and nitrogen sources on the production of lipopeptides of B15

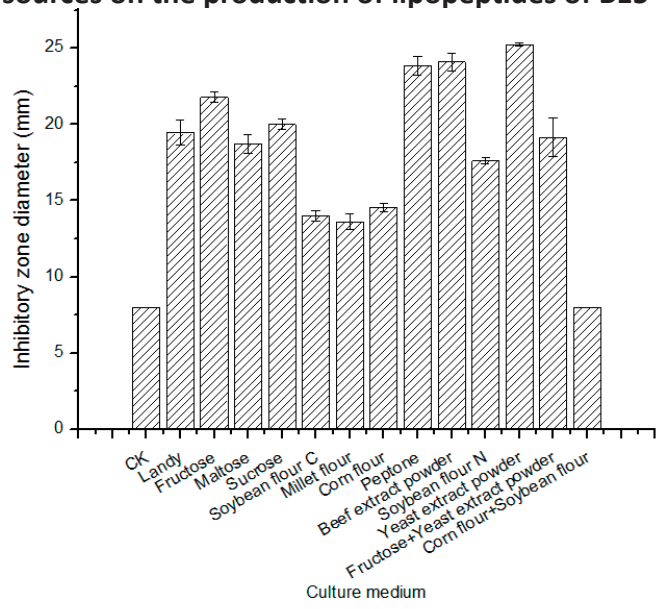

Fig. 2 - Effects of carbon sources and nitrogen sources on the inhibitory zone diameter

effects on the lipopeptides production. When glucose and yeast extract powder were used as carbon and nitrogen sources respectively, lipopeptides production was significantly higher than other sources. The production of iturin A and surfactin was 0.09283 g/L and 0.18583 g/L, respectively, and the inhibition zone diameter was 25.22 mm. In order to reduce cost, fructose and yeast extract powder, corn flour and soybean flour were also used as carbon and nitrogen sources, but the effects of the both groups were not good.

Plackett-Burman Design

According to the Plackett-Burman Design, the lipopeptides production was detected. The design and result were shown in table 1.

From the regression analysis results in table 2, X12 (Time) > X2 (Yeast extract powder) > X1 (Glucose) > X10 (Initial pH) were the most significant four factors. Among them, the glucose, yeast extract powder and initial pH showed positive effects, whereas the fermentation time showed the opposite. Therefore, the factors of glucose, yeast extract powder, and initial pH were selected as significant factors in the next experiment.

Steepest ascent experiment

According to the results of the Plackett-Burman design, the path of steepest ascent experiment was as follows: glucose, yeast extract and initial pH showed positive effects, the additive content should be increased in accordance with a certain gradient; the fermentation time showed a negative effect, and itwhich should be reduced in accordance with a certain gradient. The remaining 10 factors were added as initial additive content. In order to determine optimal ranges of four significant factors, antibacterial antimicobial lipopeptides production was detected. The results were shown in table 3.

The situation of lipopeptide production changes with each factor varies was shown in Table 3. When the glucose, yeast extract powder, initial pH, fermentation time were 36 g/L, 12 g/L, 7.0 and 48 h, respectively, the response value is the maximum. Therefore, these conditions were selected as the central point.

Box-Behnken Design

Glucose, yeast extract powder, initial

Table 1

Design and result of Plackett-Burman design

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	Lipopeptides Y $g \cdot L^{-1}$
1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	0.2311
2	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	0.1714
3	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	0.1655
4	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	0.1156
5	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	0.2871
6	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	0.2054
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.1100
8	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	0.2063
9	-1	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	0.1877
10	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	0.2794
11	1	1	-1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	0.1490
12	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	0.1351
13	1	-1	1	1	1	1	1	-1	1	-1	1	-1	-1	-1	0.1711
14	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	0.1544
15	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	0.2144
16	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	0.2255
17	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	0.1142
18	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	0.1722
19	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	0.1235
20	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	0.1030

Table 2

Regression analysis results of Plackett-Burman experiment

Source	Levels		Effect	F value	***P* value Prob>F**	Sequence
	Low(-1)	High(+1)				
X_1	30	40	+0.021	17.8547	0.0083**	3
X_2	10	14	+0.023	18.0229	0.0081**	2
X_3	0.4	0.6	+3.716E-003	0.3384	0.5860	12
X_4	0.4	0.6	+3.590E-003	0.4449	0.5343	10
X_5	0.8	1.2	+3.277E-003	0.4177	0.5466	11
X_6	0.12	0.18	+0.012	5.8888	0.0596*	6
X_7	4	6	-9.987E-003	3.8798	0.1060	7
X_8	0.12	0.18	+9.843E-003	3.7693	0.1099	8
X_9	2	3	-5.266E-004	0.0108	0.9213	14
X_{10}	6.5	7.5	+0.019	14.0979	0.0132**	4
X_{11}	25	35	+3.473E-003	0.4693	0.5238	9
X_{12}	48	72	-0.026	23.3531	0.0047**	1
X_{13}	150	225	+0.014	7.1390	0.0442**	5
X_{14}	70	100	+5.666E-004	0.0125	0.9154	13

Table 3

Design and results of the steepest ascent experiment

Run	Glucose, g/L	Yeast extract power, g/L	Initial pH	Time, h	Lipopeptides production, g/L
1	30	8	6.0	72	0.0702
2	33	10	6.5	60	0.1425
3	36	12	7.0	48	0.2319
4	39	14	7.5	36	0.1729
5	42	16	8.0	24	0.0881

Table 4

Levels and coding of Box-Behnken design

Factor	Source	Levels		
		-1	0	+1
X1	Glucose g/L	33	36	39
X2	Yeast extract powder g/L	10	12	14
X3	Initial pH	6.5	7.0	7.5
X4	Time (h)	36	48	60

Table 5

Design and results of Box-Behnken experiment

Run	X1	X2	X3	X4	Lipopeptides production g/L
1	0	0	0	+1	0.3312
2	0	+1	0	0	0.3087
3	-1	+1	0	0	0.3171
4	0	+1	0	-1	0.2662
5	+1	+1	0	0	0.3206
6	0	0	1	+1	0.2010
7	0	0	0	0	0.3087
8	0	-1	0	-1	0.2469
9	0	-1	+1	0	0.2285
10	0	+1	-1	0	0.3188
11	0	-1	0	+1	0.2802
12	0	0	-1	+1	0.2802
13	0	0	+1	-1	0.2586
14	-1	-1	0	0	0.3009
15	0	-1	-1	0	0.2892
16	-1	0	-1	0	0.2742
17	0	0	0	0	0.3387
18	-1	0	0	+1	0.3018
19	0	0	-1	-1	0.2087
20	-1	0	+1	0	0.2371
21	+1	0	-1	0	0.3107
22	+1	0	0	+1	0.2721
23	+1	-1	0	0	0.3082
24	0	+1	+1	0	0.2329
25	+1	0	0	-1	0.2447
26	-1	0	0.000	-1	0.2578
27	+1	0	+1	0	0.2485

pH and fermentation time were considered as independent variables in the Box-Behnken design. The levels of factors, coded values, experimental design and results were shown in Table4 and Table5.

Lipopeptides production was considered as the response value, according to the results of Box-Behnken experiment (table 5), the regression analysis was analyzed by Design-Expert8.0 (table 6). The quadric multiple regression equation is:

$Y=0.33+0.001325A+0.006836B-0.023C+0.015D-0.0095AB-0.006275AC-0.00415AD-0.0063BC-0.00115BD-0.032CD-0.011A2-0.011B2-0.049C2-0.042D2$, $R^2=0.8584$

As table 6 showed, C, CD, C^2, D^2 were highly significant, D was significant, and the model was highly significant, whereas the lack of fit was not significant. It indicated that the model was suitable for the analysis of this experiment.

Based on the above analysis of experimental data, lipopeptides production was forecasted. When glucose, yeast extract powder, initial pH and fermentation time were 36.28 g/L, 12.77 g/L, 6.82, 51.69 h, respectively, the predicted value of the lipopeptides yield was 0.3356 g/L. Verification test was conducted under these optimal conditions. Lipopeptides production was 0.3309 g/L, which was very close to the predicted value.

Conclusions

Medium carbon and nitrogen sources and fermentation conditions were optimized by means of Plackett-Burman design, Steepest ascent test and Box-Behnken design. The best solution of glucose, yeast extract powder,

Table 6

The regression analysis of Box-Behnken experiment

Source	SS	df	mf	F value	P value prob>F
A-Glucose	2.107E-005	1	2.107E-005	0.048	0.8308
B-Yeast extract powder	5.200E-004	1	5.200E-004	1.18	0.2991
C-initial pH	6.311E-003	1	6.311E-003	14.29	0.0026**
D-Time	2.448E-003	1	2.448E-003	5.54	0.0364*
AB	3.610E-006	1	3.610E-006	8.177E-003	0.9294
AC	1.575E-004	1	1.575E-004	0.36	0.5614
AD	6.889E-005	1	6.889E-005	0.16	0.6998
BC	1.588E-004	1	1.588E-004	0.36	0.5599
BD	3.419E-006	1	3.419E-006	7.744E-003	0.9313
CD	4.167E-003	1	4.167E-003	9.44	0.0097**
A^2	5.868E-004	1	5.868E-004	1.33	0.2714
B^2	5.405E-004	1	5.405E-004	1.22	0.2902
C^2	0.012	1	0.012	27.27	0.0002**
D^2	7.489E-003	1	7.489E-003	16.96	0.0014**
Model	0.032	14	2.294E-003	5.20	0.034**
Residual	5.298E-003	12	4.415E-004		
Lack of Fit	4.848E-003	11	4.407E-004	0.98	0.6660
Pure Error	4.500E-004	1	4.500E-004		
Cor Total	0.037	26			

*Note: *and **indicated significance at 0.05 and 0.01 respectively.*

initial pH and fermentation time was 36.28 g/L, extract 12.77 g/L, 6.82 and 69 h, respectively. The yield of lipopeptides increased from 0.2686 g/L to 0.3309 g/L through the optimized solution, whose efficiency increased 23.19%. And it had a significant increase compared with the Landy medium with L- Sodium Glutamate as nitrogen source.

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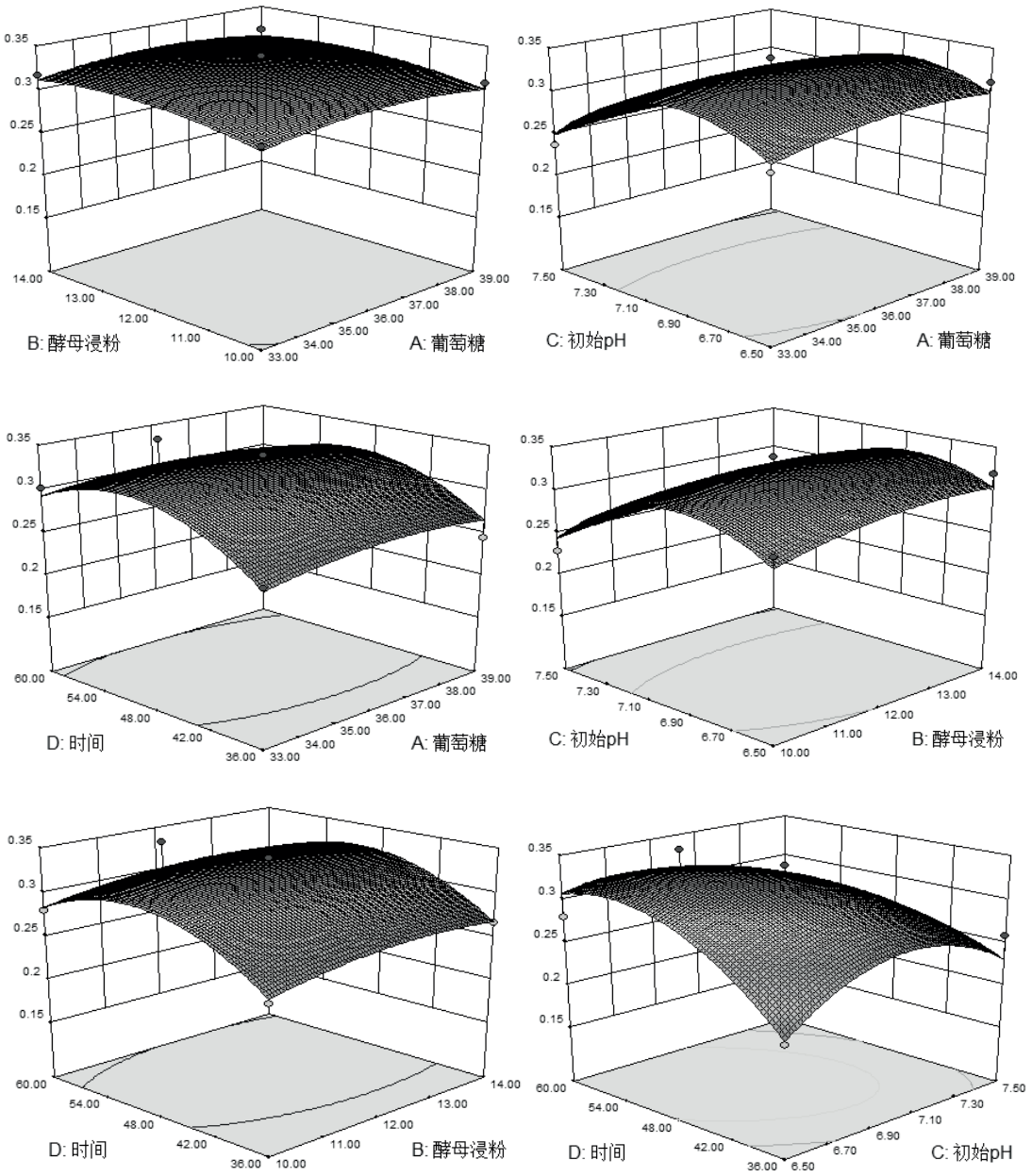

Fig. 3 - The response surface graph between the various factors

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