Research Paper 研究报告

Medium optimization for nitrogen fixer *Paenibacillus* sp. 1–49

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Abstract: [**Objective**] *Paenibacillus* sp. 1–49 is a nitrogen-fixing bacterium that has the potential as a fertilizer. However, it grows poorly ($OD_{600} \leqslant 1$) in both mineral medium and rich medium. To achieve high-yield biomass, we optimized fermentation medium for *Paenibacillus* sp. 1–49. [**Methods**] Plackett-Burman Design and Central Composite Design in response surface methodology were used to optimize medium composition. [**Results**] The optimal fermentation medium contained: (per liter) 36.22 g Sucrose, 5.31 g Tryptone, 10.92 g Yeast Extract, 0.51 g $MgSO_4$, 3.5 g NaCl, 0.02 g Na_2MoO_4 and 0.02 g $FeSO_4$. The maximum OD_{600} of 10.280±0.009 was obtained in shake flask fermentation, reached 94.6% of the predicted value. [**Conclusion**] We succeeded in using a response surface methodology to optimize the fermentation medium for *Paenibacillus* sp. 1–49. Our results will be useful for large-scale fermentation of this strain and other *Paenibacillus* spp..

Keywords: *Paenibacillus* sp. 1–49, central composite design, response surface methodology

Paenibacillus is a genus of facultative anaerobic, endospore-forming bacteria within the Firmicute division. The genus *Paenibacillus* was originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 [1]. At that time, it encompassed 11 species, including the 3 N_2-fixing species *P. polymyxa*, *P. macerans* and *P. azotofixan*. *Paenibacillus* currently comprises more than 120 named species, more than 20 of which have nitrogen fixation ability, including the following 8 novel species described by our laboratory: *P. sabinae* [2],

P. zanthoxyli [3], *P. forsythia* [4], *P. sonchi* [5], *P. sophorae* [6], *P. jilunlii* [7], *P. taohuashanense* [8] and *P. beijingensis* [9]. Some nitrogen-fixing *Paenibacillus*, such as *P. polymyxa*, are used in agriculture and horticulture [10]. However, many members of these *Paenibacillus* strains grow poorly in both mineral medium and in rich medium such as LB medium. It is generally accepted that bacterial growth is greatly affected by culture medium components, such as carbon, nitrogen and salt sources, and the fermentation conditions [11]. In conventional method, the medium is

optimized by changing one factor at a time while keeping others at a constant level, which is laborious and often leads to wrong conclusions. Multivariate experiments are designed not only to reduce the number of necessary experiments in the optimization process, but also to produce more defined results than those available by univariate strategies. Plackett-Burman (P-B) and response-surface methodology (RSM) are multivariate analyzing tools comprising of mathematical and statistical techniques for generating empirical models that obtained importance for optimizing production conditions of industrially high value products such as chemicals, enzymes, and for studying enzyme kinetics. The statistical tool has been used in many biotechnological processes namely optimization of culture conditions, enzyme production, ethanol production, and biomass production [12–14]. In this study, RSM was used to optimize the culture medium for obtaining the greatest bacterial growth rate during flask fermentation process of *Paenibacillus* sp. 1–49.

1 Materials and Methods

1.1 Organism and culture conditions

The nitrogen-fixing bacterium *Paenibacillus* sp. 1–49 was isolated by our laboratory from the soil samples collected from Shaanxi Province, China. And this strain was maintained on LD medium (per liter) containing 10 g Tryptone, 5 g Yeast Extract and 2.5 g NaCl with agar at pH 7.0 at 4 °C and –70 °C for short and long term storage, respectively. The seed culture was prepared by inoculating single colony from the LD medium plate. In order to obtain fresh and stable strains, the clones were transferred into a new plate by streak culture for several times. After

incubation for 2 or 3 days at 30 °C, the single colony from the flat plate was transferred to 50 mL LD seed medium in a 250 mL shake flask and incubated at 30 °C for 18 hours on a rotary shaker (200 r/min). The cells growth was monitored by absorbance measurement at 600 nm with a UV-visible spectrophotometer. For shake flask culture, the liquid medium was sterilized in the autoclave at 121 °C for 20 min, and pH was adjusted to 7.0 before autoclaving.

1.2 Screening of critical media components using a Plackett-Burman design

The Plackett-Burman factorial design was used to select significant medium components affecting the growth of *Paenibacillus* sp. 1–49. Flask-shaking fermentation condition was designed by Minitab16 statistical software (State College, PA, USA). Previous studies showed that Sucrose, Tryptone, Yeast Extract, Magnesium Sulfate, Ferric Sulfate, Potassium Nitrate, Sodium Molybdate were effective medial components for process modeling. A total of fifteen process variables comprising of seven medial components (Sucrose, Tryptone, Yeast Extract, Magnesium Sulfate, Ferric Sulfate, Potassium Nitrate, Sodium Molybdate) and inoculum size were studied in Plackett-Burman screening experiments.

Each variable was represented at 2 levels, upper (high, +), and lower (low, −) levels of the range covered by each variable and the response (Table 1). Experimental responses were analyzed by first order model by the following equation $Y = \beta_0 + \Sigma\beta_i x_i$ ($i=1......k$) where y is the response for OD_{600}, β_0 is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. A first-order model could be obtained from the regression results of fractional factorial experiment. This model

describes the interaction among factors and it is used to screen and evaluate important factors that influence the response. From the regression analysis of the variables, the factors having significant effect on the biomass were further optimized by RSM.

1.3 Method of steepest ascent

Table 1. Range of different factors investigated with Plackett-Burman

Variables	Fermentation factors	Low level (−)	High level (+)
X_1	Sucrose /(g/L)	15.00	30.00
X_2	Na$_2$MoO$_4$ /(g/L)	0.02	0.06
X_3	KNO$_3$ /(g/L)	1.00	3.00
X_4	MgSO$_4$ /(g/L)	0.20	0.60
X_5	Inoculum size (%)	4.00	5.00
X_6	FeSO$_4$ /(g/L)	0.02	0.06
X_7	Tryptone /(g/L)	5.00	10.00
X_8	Yeast Extract /(g/L)	0	5.00

Because the operating conditions are normally far from the optimum response settings, the experiment needs to move from the current operating conditions to the optimum region in the most efficient way. In order to establish an effective fitting equation, steepest ascent design was conducted to obtain the optimum region of the maximum OD_{600}. Step size was designed separately according to the effect of significant factors which were screened by the Plackett - Burman method while the other factors were maintained at the low (−) level.

1.4 Optimization of medium with the response surface method

Central composite design (CCD) and response methodology (RSM) were employed for random experimental design. The second-order model used to fit the response to the independent variables is shown in Equation (1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{j-1} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2$$

Equation (1)

Where Y is the predicted response, x_i and x_j are the coded independent factors that influence the response variable Y, β_0 is the intercept, β_i represents the linear effect of x_i, β_{ij} represents the interaction between x_i and x_j, and β_{ii} represents the quadratic effect of x_i. Minitab16 Software (State College, PA, USA) was used for the experimental design and regression analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by an F test. The significance of the regression coefficients was tested by t test.

2 Results and Discussion

2.1 Screening of significant factors by Plackett-Burman design

Eight factors, including seven medial components (Sucrose, Tryptone, Yeast Extract, Magnesium Sulfate, Ferric Sulfate, Potassium Nitrate and Sodium Molybdate) and inoculum size, as listed in Table 1 in the Plackett-Burman model, were used to test on the influence of bacterial growth rate during shaking flask fermentation process. The results from fifteen experiments revealed that the combination of Sucrose, Tryptone, Yeast Extract and inoculum size produced the highest bacterial growth rate. Furthermore, regression coefficients and standard error coefficient were determined by using the software of Minitab (Table 2). The P value was less

than 0.1 implied that model terms were significant. From the experimental data, five of these variables could distinctly affect the OD_{600} of the culture and these were taken as the main factor affecting the density of bacterial strains by significant analysis. Among them, Sucrose (P=0.014), Magnesium Sulfate (P=0.026), Yeast Extract (P=0.003), and inoculum size (P=0.020) exerted positive effect, whereas the amount of Tryptone (P=0.084) exerted negative effects on the OD_{600}. After screening of the influence factors, the linear regression equation which took the OD_{600} as the response values was obtained and the model was given in Equation (2).

$$Y = 4.72 + 0.676X_1 - 0.146X_2 - 0.211X_3 + 0.582X_4 + 0.622X_5 - 0.106X_6 - 0.411X_7 + 1.20X_8$$

<div align="right">Equation (2)</div>

As shown in the analysis of variance, the values of Prob (P)>F was 0.003 in variance analysis model. This implied that the regression equation reached a very significant level. The R^2 coefficient obtained, of 94.3%, suggests that it is a reliable model and is attributed to a high correlation. The relatively high-adjusted determination coefficient (R^2_{Adj}=0.8680) indicates that 86.8% of the variability from experimental data can be explained by this regression model. Considering the inoculation amount of bacteria in a single system could not affect the

Table 2. Partial regression coefficients and analysis of their significance

Factor	Regression coefficient	Standard error coefficient	t-value	P-value
Constant	4.7198	0.1813	26.04	0.000
X_1	0.6761	0.1981	3.41	0.014
X_2	−0.1465	0.1981	−0.74	0.488
X_3	−0.2113	0.1981	−1.07	0.327
X_4	0.5821	0.1981	2.94	0.026
X_5	0.6224	0.1981	3.14	0.020
X_6	−0.1056	0.1981	−0.53	0.610
X_7	−0.4105	0.1981	−2.07	0.084
X_8	1.1968	0.2417	4.95	0.003

highest density, the four remaining optimum variables (Sucrose, Magnesium Sulfate, Yeast Extract and Tryptone) were further evaluated by a method of steepest ascent design.

2.2 Method of steepest ascent

Based on the model equation obtained, the path of steepest ascent experiment was conducted to find the more accuracy domain towards the optimum region. According to the effect of the significant factors screened from the Plackett-Burman experiment, compensation was designed for steepest ascent to search for the maximum value of OD_{600}, while the rest was maintained at "−" level. Results are shown in Table 3.

Table 3. Experimental results of steepest ascent method

No.	c(sucrose)/(g/L)	c(yeast eXtract)/(g/L)	c(tryptone)/(g/L)	c(MgSO$_4$·7H$_2$O)/(g/L)	OD_{600}
1	18	1	7.2	0.2	4.684±0.012
2	22	3	6.4	0.4	6.040±0.009
3	26	5	5.8	0.6	8.045±0.010
4	30	7	5	0.8	10.060±0.006
5	34	9	4.2	1.0	9.540±0.003

The central point for this Central Composite Design (CCD) experiment was reallocated as follow: Sucrose 30 g/L, Tryptone 5 g/L, Yeast Extract 7 g/L, MgSO$_4$ 0.8 g/L.

2.3 Optimization of fermentation medium for

Paenibacillus sp. 1–49 using RSM

To search for the optimum combination of these components in the culture medium, a Central Composite Design (CCD) experimental plan was carried out. Their suitable concentration ranges were preliminarily determined. Table 4 shows various combinations used and the corresponding OD_{600}. The

Table 4. CCD design and response values

Run	Variables				OD_{600}
	X_1 (Sucrose)	X_7 (Tryptone)	X_8 (Yeast Extract)	X_4 (MgSO$_4$)	
1	–2	0	0	0	9.066
2	0	0	–2	0	6.987
3	0	2	0	0	7.910
4	2	0	0	0	8.645
5	0	–2	0	0	6.487
6	0	0	0	2	7.531
7	0	0	0	0	9.685
8	0	0	0	–2	9.868
9	0	0	2	0	10.397
10	0	0	0	0	10.000
11	0	0	0	0	9.988
12	–1	–1	1	1	7.454
13	1	–1	–1	–1	7.130
14	–1	–1	–1	–1	6.424
15	1	1	–1	–1	7.790
16	–1	1	1	–1	9.177
17	1	–1	1	–1	10.210
18	1	1	1	1	9.470
19	0	0	0	0	9.871
20	1	–1	1	1	9.970
21	1	1	1	–1	8.990
22	0	0	0	0	9.821
23	–1	–1	1	–1	7.583
24	–1	1	1	1	9.620
25	1	–1	–1	1	7.180
26	1	1	–1	1	8.100
27	0	0	0	0	10.124
28	–1	1	–1	–1	7.965
29	–1	–1	–1	1	7.273
30	–1	1	–1	1	8.129

experimental results revealed that this mathematical tool could satisfactorily explain the effects of the variables concentration (Sucrose, Yeast Extract, Tryptone and Magnesium Sulfate) in biomass of *Paenibacillus* sp. 1–49 in shake-flask cultures. The *P*-value blew 0.05 indicated that test parameter was significant at 5% level of significance. The regression coefficients, *t* and *P* values for all linear, interaction and quadratic effects of the variables were shown in Table 5. It was shown that Tryptone (X_7) and Yeast Extract (X_8) were significant in linear effect of bacterial density, Sucrose (X_1) and $MgSO_4$ (X_4) were not significant. All of the four factors were significant in surface effect of bacterial density. As for in interaction effect, both Sucrose (X_1) Tryptone (X_7) and Sucrose (X_1) Yeast Extract (X_8) were significant but not the rest. Analysis of variance (ANOVA) for the Quadratic model was shown in Table 6. Value of "Model Prob (*P*)>F" was 0.001 implied that the model was high significant. In this case X_1, X_7, X_8 and X_4 are significant model terms. The "Lack of Fit Prob>*F* value" of 0.002 showed that the lack of fit is insignificant. The R^2 coefficient obtained, of 0.8791, suggests that it is a reliable

Table 5. Coefficient estimates by the regression quadratic model

Source of variation	Regression coefficient	Standard error coefficient	*t*-value	*P*-value
Constant	9.9148	0.2473	40.090	0.000
X_1	0.3644	0.2473	1.474	0.161
X_7	0.7386	0.2473	2.986	0.009
X_8	1.6086	0.2473	6.504	0.000
X_4	–0.2289	0.2473	–0.926	0.369
X_1X_1	–1.1142	0.4627	–2.408	0.029
X_7X_7	–2.7712	0.4627	–5.989	0.000
X_8X_8	–1.2777	0.4627	–2.762	0.015
X_4X_4	–1.2702	0.4627	–2.745	0.015
X_1X_7	–1.5742	0.6058	–2.599	0.020
X_1X_8	1.0993	0.6058	1.815	0.009
X_1X_4	–0.1818	0.6058	–0.300	0.768
X_7X_8	–0.4843	0.6058	–0.799	0.437
X_7X_4	0.2168	0.6058	0.358	0.725
X_8X_4	–0.2047	0.6058	–0.338	0.740

S=0.605790, PRESS = 31.1892, R^2=0. 8791, R^2_{Adj}=76.62%

model and that 87.91% of the sample variation of biomass is attributed to a high correlation between the independent variables. Only 12.09% of the total variation of biomass was not explained by the model. The relatively high-adjusted determination coefficient

Table 6. Analysis of variance for the quadratic model of CCD design

Source	DF	Seq SS	Adj SS	Adj MS	*F*-value	*P*-value
Model	14	40.0090	40.0090	2.8578	7.79	0.001
Linear	4	19.9095	19.9095	4.9774	13.56	0.001
Square	4	16.0565	16.0565	4.0141	10.94	0.000
Interaction	6	4.0430	4.0430	0.6738	1.84	0.159
Residual error	15	5.5047	5.5047	0.3670	–	–
Lack of fit	10	5.3848	5.3848	5.385	22.45	0.002
Pure error	5	0.1199	0.1199	0.0240	–	–
Total	29	45.5137	–	–	–	–

S=0.605790, PRESS=31.1892, R^2=0. 8791, R^2_{Adj}=76.62%.

$(R_{\text{Adj}}^2=0.7662)$ accounts for significance of the model. This revealed that the equation is a suitable model to describe the response of experiment pertaining to cell density (OD_{600}). Data were analyzed by linear multiple regression using Minnitab 16 software (State College, PA, USA) and the Equation (3) was obtained.

$$Y = 9.9148 + 0.3644X_1 + 0.7386X_7 + 1.6086X_8 \\ - 0.2289X_4 - 1.1142X_1X_1 - 2.7712X_7X_7 \\ - 1.2777X_8X_8 - 1.5742X_1X_7 + 1.0993X_1X_8 \\ - 0.1818X_1X_4 - 0.4843X_7X_8 + 0.2168X_7X_4$$

Equation (3)

Where Y is the predicted response (OD_{600}), X_1, X_7, X_8 and X_4 are the symbols of concentration of Sucrose, Yeast Extract, Tryptone and Magnesium Sulfate respectively.

The response surfaces of fermentation medium for maximum OD_{600} of *Paenibacillus* sp. 1–49 have been established according to the model.

Figure 1 shows the mutual influences of Sucrose and Tryptone on OD_{600} of *Paenibacillus* sp. 1–49 when the concentration of Yeast Extract and MgSO$_4$ were under optimal condition.

Figure 2 shows the mutual influences of Sucrose and Yeast Extract on OD_{600} when the concentration of Tryptone and MgSO$_4$ were under optimal condition.
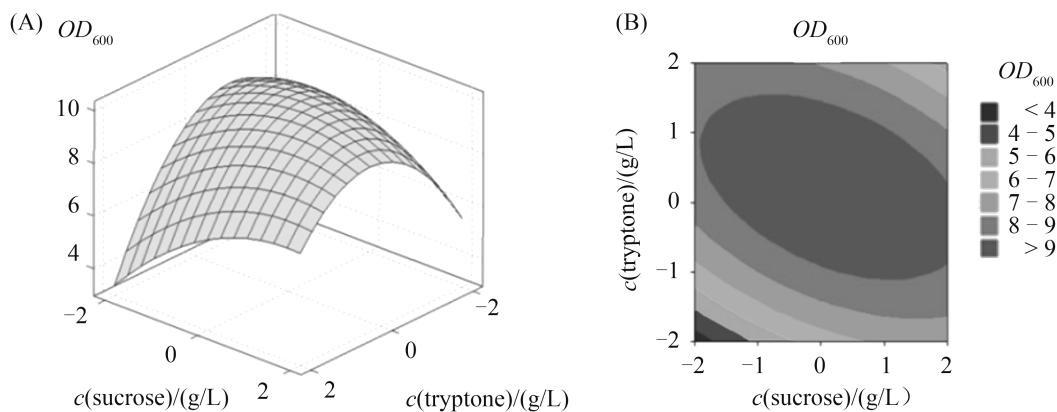
Figure 1. Response surface and contour line of the influence of sucrose and tryptone on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: Response surface graph of mutual-influence for sucrose and tryptone on OD_{600}; B: The contour plot between sucrose and tryptone on OD_{600}.

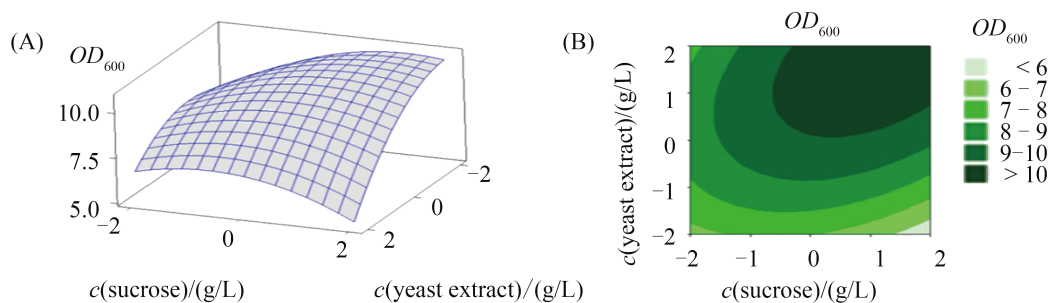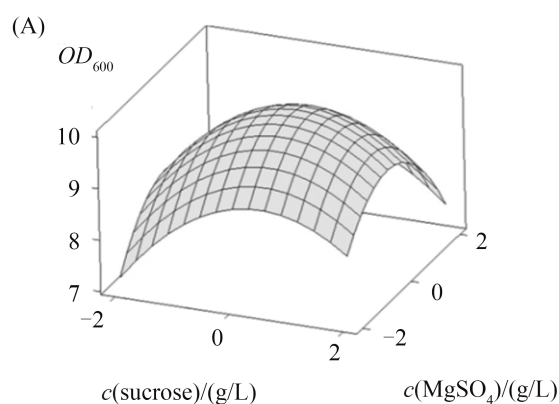Figure 2. Response surface and contour line of the influence of sucrose and yeast extract on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: Response surface graph of mutual-influence for sucrose and yeast extract on OD_{600}; B: The contour plot between sucrose and yeast extract on OD_{600}.

Figure 3 shows the mutual influences of Sucrose and MgSO$_4$ on OD_{600} when the concentration of

Tryptone and Yeast Extract were under optimal condition.

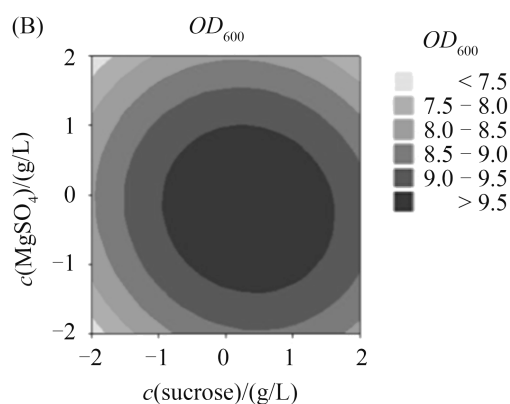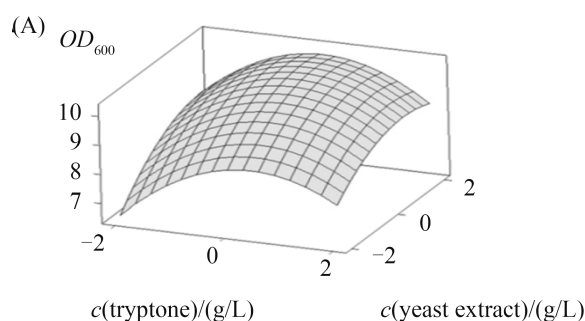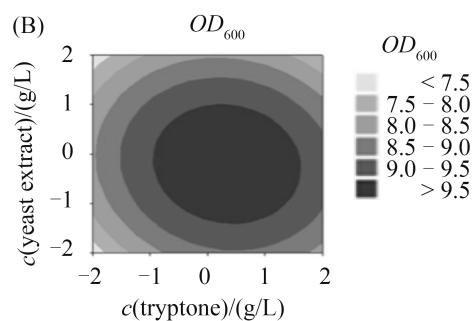Figure 3. Response surface and contour line of the influence of sucrose and MgSO$_4$ on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: response surface graph of mutual-influence for sucrose and MgSO$_4$ on OD_{600}; B: The contour plot between sucrose and MgSO$_4$ on OD_{600}.

Figure 4 shows the mutual influences of Tryptone and Yeast Extract on OD_{600} when the

concentration of Sucrose and MgSO$_4$ were under optimal condition.

Figure 4. Response surface and contour line of the influence of tryptone and yeast extract on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: response surface graph of mutual-influence for tryptone and yeast extract on OD_{600}; B: the contour plot between tryptone and yeast extract on OD_{600}.

Figure 5 shows the mutual influences of Tryptone and MgSO$_4$ on OD_{600} when the concentration of Sucrose and Yeast Extract were under optimal condition.

Figure 6 shows the mutual influences of MgSO$_4$ and Yeast Extract on OD_{600} when the concentration of Sucrose and Tryptone were under optimal condition.

On the basis of these response surfaces, the optimal fermentation medium for the maximal maximum bacterial density is composed of 33.62 g/L Sucrose, 5.31 g/L Tryptone, 10.92 g/L Yeast Extract and 0.51 g/L MgSO$_4$. The maximal OD_{600} of 10.867 is predicted.

2.4 Optimization and model verification
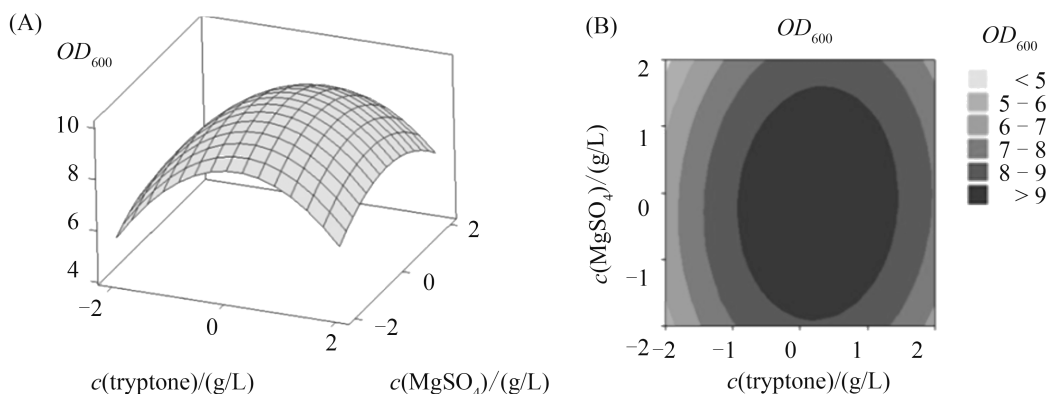
From the study of plots, the maximum OD_{600} of

Figure 5.   Response surface and contour line of the influence of tryptone and MgSO$_4$ on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: response surface graph of mutual-influence for tryptone and MgSO$_4$ on OD_{600}; B: the contour plot between tryptone and MgSO$_4$ on OD_{600}.
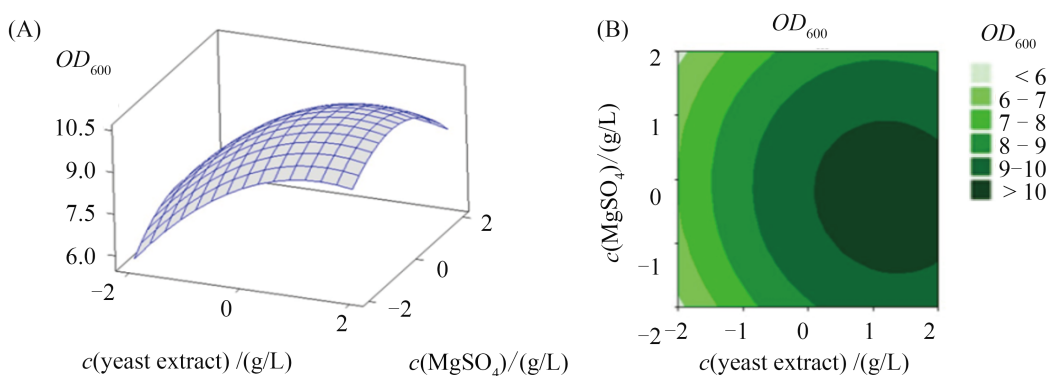

Figure 6.   Response surface and contour line of the influence of yeast extract and MgSO$_4$ on cell growth rate (OD_{600}) of *Paenibacillus* sp. 1–49. A: response surface graph of mutual-influence for yeast extract and MgSO$_4$ on OD_{600}; B: the contour plot between yeast extract and MgSO$_4$ on OD_{600}.

Paenibacillus sp. 1–49 was obtained when the optimal concentrations of 33.62 g/L Sucrose, 5.31 g/L Tryptone, 10.92 g/L Yeast Extract and 0.51 g/L MgSO$_4$ were used for flask fermentation at 30 °C, 200 r/min for 18 hours. To verify the predicted results, the triplicate verification experiments were performed under the optimized nutrients levels. The maximum OD_{600} was obtained to 10.280±0.009, which reached 94.6% of the predicted value. As a result, the models developed were considered to be accurate and reliable for predicting the biomass of OD_{600} for *Paenibacillus* sp. 1–49. Figure 7 described the bacterial growth curve.
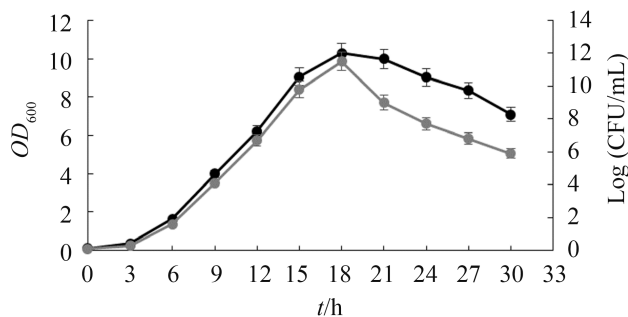

Figure 7.   Growth of *Paenibacillus* sp. 1–49 in optimized medium by flask fermentation at 30 °C, 200 r/min, for 30 hours. Each point represents the mean of three independent experiments ± 1 SEM. The black dot: values of OD_{600}; the gray dot: log colony-forming units (CFU)/mL.

As well known, many strains of bacilli, mainly species of the genera *Bacillus* and *Paenibacillus*, displaying important plant growth promoting (PGP) characteristics were isolated and identified in the past decades [15]. At the same time, numerous *Bacillus* and *Paenibacillus* strains express plant growth promoting activities and a number of these strains have already been commercially developed as biological fungicides, insecticides, and nematicides or generic plant growth promoters [16]. Nitrogen-fixing *Paenibacillus* species have the potential use as biofertilizer in agriculture. It is a genus of gram-positive, facultative anaerobic, endospore-forming bacterium. They can survive in strict environmental conditions and are easy to be stored. So, the use of these strains in agriculture has been viewed as promising prospects in the future. A nitrogen-fixing bacterium *Paenibacillus* sp. 1–49 was newly isolated from soil samples had relatively high nitrogenase activity. It can be used as a kind of potential microbial fertilizer.

In order to obtain large biomass production as bacterial agents, fermentation is a general method for industrial biological processes. Many medium experiments include group factors such as culture media components and the fermentation conditions [17]. The present study shows that the RSM based on CCD established an efficient model to describe the shake flask fermentation process of *Paenibacillus* sp. 1–49. The high similarity between the observed value and the predicted ones implied that the RSM was an accurate tool to optimize the culture medium for biomass production of *Paenibacillus* sp. 1–49 in fermentation industry. By utilizing the statistical methodology, the optimum OD_{600} was obtained to 10.280±0.009. Under optimized culture conditions, the OD_{600} was dramatically increased, almost 10-fold

than in both mineral medium and rich medium. In particularly, the culture temperature and time was both decreased too. This process will show the direction to find the optimum condition in further experimentation.

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利用响应面法优化固氮类芽孢杆菌*Paenibacillus* sp. 1–49的发酵培养基

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摘要：【目的】固氮类芽孢杆菌*Paenibacillus* sp. 1–49是本实验室分离的具有潜在生物肥料价值的微生物菌剂。该菌在常见的培养基中不能高效生长($OD_{600}\leqslant 1$)。本文拟优化发酵培养基成分以获得最大菌体生物量。【方法】运用响应面分析中的因素筛选实验设计和最陡爬坡实验以及中心复合设计法，对菌株的发酵培养基进行了响应面优化。【结果】利用响应面优化法最终确定了菌株最佳培养基：蔗糖36.22 g/L，Tryptone 5.31 g/L，Yeast Extract 10.92 g/L，$MgSO_4$ 0.51 g/L，NaCl 3.5 g/L，Na_2MoO_4 0.02 g/L，$FeSO_4$ 0.02 g/L。通过摇瓶发酵后菌体OD_{600}=10.280，达到预测值的94.6%。【结论】利用响应面法成功地对*Paenibacillus* sp. 1-49的发酵培养基进行了优化，为该菌株和其他类芽孢杆菌的大规模发酵提供了参考。

关键词：*Paenibacillus* sp.1-49，中心复合设计，响应面优化

(本文责编：张晓丽)