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Optimization of the Fermentation Medium of *Streptomyces ahygroscopicus* Δ*nysB*, a Tetramycin Producing Strain, by the Response Surface Method

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Abstract: *Streptomyces ahygroscopicus* Δ*nysB* is a genetically engineered strain that produces tetramycin by blocking the competition nystatin pathway. Response Surface Methodology (RSM) was used to optimize its fermentation medium composition. Taking the yield of tetramycin as the response value, the effects of corn starch, glucose, fish peptone, $(NH_4)_2SO_4$, KH_2PO_4, and CH_3COONa on the yield of tetramycin were investigated by the Plackett-Burman method and three main factors of corn starch, glucose and $(NH_4)_2SO_4$, were determined. The path of the steepest ascent test was used to approach the maximum response region and the center point was determined. Based on this, the three-factor three-level test was designed by the Box-Behnken center combination. Quadratic regression analysis was performed to calculate the medium with the highest yield of tetramycin and the optimal fermentation medium for tetramycin was formulated as corn starch 25.08 g/L, soybean cake meal 30 g/L, glucose 37.70 g/L, NaCl 0.2 g/L, MgSO4 0.2 g/L, K_2HPO_4 0.1 g/L, $FeSO_4$ 0.2 g/L, $CaCO_3$ 5 g/L, $(NH_4)_2SO_4$ 0.24 g/L. The yield of tetramycin in the optimized medium reached 167.19 mg/L, which was close to the predicted value of the model. The yield of tetramycin was significantly increased, which was 5.5 times of that before optimization. The results provide a basis for improving the production level of tetramycin.

Keywords: Tetramycin, Medium Optimization, Plackett-Burman Method, Box-Behnken Design, Response Surface Methodology

Introduction

Tetramycin is a twenty-six-member polyene macrolide with a conjugated tetraene structure in the molecule, consisting of A and B components (Chen *et al*., 2021). These antibiotics interact with ergosterol on the surface of fungal cells through conjugation and form ion channels on the plasma membrane, which causes the loss of cell contents and eventually leads to fungal cell death. The main antifungal components in the secondary metabolite of Streptomyces *ahygroscopicus* are the polyene macrolide antibiotics tetramycin and nystatin. It has been found that the same precursor is used in the synthesis of tetramycin and nystatin and the production of nystatin can be improved by blocking the tetramycin pathway in *S. ahygroscopicus* and changing the precursor flow to the nystatin pathway (Ren *et al*., 2014). *S. ahygroscopicus* Δ*nysB* (Δ*nysB*), a high tetramycin-producing strain, was obtained by blocking a key gene *nysB* in nystatin synthesis, with the production improvement of tetramycin to 1.27 times (Chen *et al*., 2021).

For the new engineered strain, optimizing the amount of gradually metabolized carbon source (Aharonowitz and Demain, 1978), readily metabolized carbon source (Medeot *et al*., 2017), organic nitrogen source (Papizadeh *et al*., 2020; Venkateswarulu *et al*., 2017), phosphorus source, inorganic nitrogen source (Kominek, 1972) and the precursor (Byun *et al*., 1973) in the fermentation medium can achieve its best performance. Plackett-Burman (PB) design is an effective method to evaluate the relative importance of medium components to fermentation products, which can quickly and efficiently screen out the most important influencing factors. Box-Behnken Design (BBD) for Response Surface Analysis (RSM) is a robust and efficient statistical method for the optimization of multiple factors that act together to produce a certain effect value.

Science Publications

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Studies on blocking the nystatin pathway in *S. ahygroscopicus*, changing the precursor flow to the tetramycin pathway and further optimizing the medium composition to make tetramycin highly productive have not been reported. The PB design, path of steepest ascent test, and BBD response surface analysis were used to optimize the feasible fermentation medium of high-producing strain Δ*nysB* (Al-Madboly and Khedr, 2017; Sorour *et al*., 2023), which could pave the foundation for improving the industrial production of tetramycin.

Materials and Methods

Strain and Cultural Conditions

S. ahygroscopicus Δ*nysB*, a tetramycin producer, was generated from the wild-type *S. ahygroscopicus* by disrupting the key gene *nysB* in the nystatin pathway. *Saccharomyces cerevisiae* ATCC 9763 strains, used for antifungal activity test, were purchased from http://www.bzwzw.com /index.php. The culture medium and culture conditions of *Streptomyces* strains were followed as described by Cui *et al*. (2015). Spore culture with Gause's synthetic agar medium (20.0 g/L soluble starch, 0.5 g/L $K_2HPO_4·3H_2O$, 0.5 g/L $MgSO_4·7H_2O$, 0.5 g/L NaCl, 1.0 g/L KNO_3, 0.01 g/L $FeSO_4$, 1.0 g/L beef-extract, 1.5 g/L Agar, pH 7.0-7.2), at 28°C cultivation for 6-7 d. After the spores matured, the spore mass was added into 30 mL of seed medium (6.0 g/L peptone, 6.0 g/L yeast extract, 20.0 g/L $C_6H_{12}O_6$, and 10.0 g/L NaCl, pH 7.0-7.2) and incubated at 28°C, shaking at 220 rpm for 24-28h. Seed solution was used to inoculate the fermentation medium to 10% (v/v) and incubated for 96 h. *Saccharomyces* cerevisiae was incubated in MD medium (2.0 g/L peptone, 1.0 g/L yeast extract, 2.0 g/L $C_6H_{12}O_6$ and 2.15 g/L Agar, pH 7.0-7.2) at 28°C for 24 h.

Tetramycin Analytical Methods

Fermentation Liquid Treatment

Culture broths were centrifuged at 3000 rpm for 10 min to obtain the mycelia and then washed once with distilled water. Similar volumes of methanol were added to the water-washed mycelia. Samples were shaken for 15 min at 37°C to extract the products. After centrifuging at 3000 rpm for 10 min, supernatants containing the products were used for evaluating the antifungal activity.

Examination of Antifungal Activity

Since there is no tetramycin standard in the market, the nystatin standard (Sangon Biotech, Shanghai, Code: A600390-0005) was used as a comparison. The nystatin standard was dissolved with methanol and prepared into standard solutions with concentrations of 1, 0.6, 0.36, 0.216, and 0.1296 g/L, respectively, which were used to draw the standard curve (concentration to inhibition zone) and calculated the titer of tetramycin by a bioactivity assay. The antifungal activity of the fermentation broth was determined by the K-B method using *Saccharomyces cerevisiae* as the test organism. MD medium (100 mL) was heated and melted, cooled to 48-50°C and the test organisms were added. After the medium was solidified, the paper (φ = 6 mm) with 10 μL samples was attached to the MD medium and cultured at 28°C for 24 h. The inhibition zone was measured and the titer was calculated by biology software.

Medium Optimization

Single-Factor Experimental Design

The fermentation medium of wild strain of *S. ahygroscopicus* was used as the initial fermentation medium, with the components of corn starch 8 g/L, corn power 20 g/L, soybean cake meal 30 g/L, glucose 20 g/L, NaCl 0.2 g/L, $MgSO_4$ 0.2 g/L, K_2HPO_4 0.1 g/L, $FeSO_4$ 0.2 g/L, $CaCO_3$ 5 g/L, $(NH_4)_2SO_4$ 0.42 g/L, pH 7.0-7.2. Gradually metabolized carbon source, readily metabolized carbon source, organic nitrogen source, phosphorus source, inorganic nitrogen source, and the precursor were selected as single factors for investigation, respectively. Corn flour, lactose, soluble starch, and corn starch, at a concentration of 32.5 g/L, were chosen as the only gradually metabolized carbon sources, respectively. The maltose, sucrose, and glycerol, at a concentration of 20 g/L, were used as the only readily metabolized carbon source, respectively. The soybean cake meal, peptone, yeast extract powder, cooked soybean meal, raw soybean meal, fish peptone, beef extract paste, and beef extract powder, with a concentration of 30 g/L, were selected as the only organic nitrogen source, respectively. $(NH_4)_2SO_4$, CH_3COONH_4, and NH_4Cl, with a concentration of 0.42 g/L, were selected as the only inorganic nitrogen sources, respectively. K_2HPO_4 and KH_2PO_4 were used as the only phosphorus sources at a concentration of 0.1 g/L. CHOONa, CH_3COONa, and mannitol, were used as the only precursors at a concentration of 0.15 g/L, respectively.

Plackett-Burman Design

Plackett-Burman design was used to assess the significant effect of each factor on the efficacy value with a minimum number of experiments. Based on the results of the single-factor experiment, six factors (X_1-corn starch, X_2-glucose, X_3-fish peptone, X_4-$(NH_4)_2SO_4$, X_5-KH_2PO_4 and X_6-CH_3COONa) were selected as a variable in the design. Two levels were set for each variable, the initial concentration was taken as the low level (-1) and a high level (+1) was defined as 1.5 times the initial concentration (Table 1). The fermentation titer of tetramycin was taken as the response value (mg/L) and the results were analyzed by Minitab18 software. Table 2, a PB design, with N = 12, was selected for the experiment and the average was taken from three replicates.

Table 1: PB experimental factor level coding

Sequence number	Factor	Standard -1 (g/L)	+1 (g/L)
X_1	Corn Starch	32.500	48.750
X_2	Glucose	20.000	35.000
X_3	Fish peptone	30.000	45.000
X_4	$(NH_4)_2SO_4$	0.420	0.630
X_5	KH_2PO_4	0.100	0.150
X_6	CH_3COONa	0.150	0.225

Table 2: Levels of factors and responses to PB design

Sequence number	X_1	X_2	X_3	X_4	X_5	X_6	Titer (mg/L)
1	1	1	1	-1	1	-1	88.725
2	-1	-1	-1	-1	-1	-1	78.390
3	-1	1	1	-1	-1	1	89.505
4	1	-1	1	1	-1	-1	60.840
5	-1	1	-1	1	1	1	91.065
6	-1	-1	1	1	1	-1	73.710
7	-1	-1	1	-1	1	1	75.465
8	1	-1	-1	-1	-1	1	66.885
9	1	-1	-1	1	1	1	66.105
10	1	1	1	1	-1	1	85.215
11	1	1	-1	-1	1	-1	88.725
12	-1	1	-1	1	-1	-1	89.115

Path of Steepest Ascent Method

The response surface fitting equation can only be established when the factors are close to the optimal level, so the path of the steepest ascent method was used to test the optimal level range of the key factors (according to the positive and negative effects of significant factors in the PB test results and the base value was taken). The factors with positive effects were set at higher values and the factors with negative effects were set at lower values.

Box-Behnken Design

Based on the PB design and path of the steepest ascent method, a response surface test with three factors and three levels, a total of 17 sets of experiments containing five replications of the central point design, was designed using Box-Behnken's central test principle. The tetramycin titer in fermentation was selected as the response value. Design expert 8.0.6 software was used for quadratic regression fitting of the experimental data, model establishment, and variance analysis to obtain the optimal fermentation medium formula.

To predict the medium factor combination with the highest tetramycin titer, a multivariate quadratic equation model with tetramycin titer as the response value was obtained based on the equation:

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

where, Y is the predicted tetramycin titer, β_0 is a constant coefficient, β_i, β_{ii}, and β_{ij} are the first-order, quadratic coefficients of X_i and effect of interaction respectively and ε is the experimental error (random).

Statistical Analysis

All the assays were performed in triplicate. The experimental data were statistically tested by Analysis of Variance (ANOVA) by Duncan's multiple range tests by SPSS statistical software version 19.0 (SPSS Inc, Chicago, IL, USA) and results were expressed as mean ± standard error. $p<0.05$ indicated significant effects.

Results and Discussion

Single-Factor Experimental Design

Figures 1-6, the fermentation titer was obtained by software from antifugal data in the single factor test. Among the gradually metabolized carbon source group, the highest tetramycin titer was 69.266 g/L of corn starch and thus corn starch was selected as the best gradually metabolized carbon source. An excessive concentration of corn starch leads to an increase in the viscosity of the medium and reduces dissolved oxygen, thus inhibiting the growth of the microorganisms. In the readily metabolized carbon source group, the highest tetramycin titer was 68.636 g/L of maltose, maltose can be hydrolyzed into two glucose molecules by maltase, which can effectively provide energy for the growth and metabolism of microorganisms. But glucose, with a similar titer, was instead selected

due to the high cost of maltose. The high concentration of glucose not only causes the imbalance of the carbon and nitrogen ratio of the medium but also makes the pH of the medium too low in the process of fermentation, which affects the activity of the microorganisms and in turn affects the synthesis of the secondary metabolites (Li *et al*., 2018). In the organic nitrogen source group, the highest tetramycin titer was 75.313 g/L of fish peptone. Fish peptone is rich in small molecule peptides, amino nitrogen, vitamins, amino acids, and other nutrients that provide essential nitrogen and nutrients for the microorganisms. Therefore, fish peptone was selected as the best organic nitrogen source. In the inorganic nitrogen source and phosphorus source groups, the highest tetramycin titer was 79.690 g/L of $(NH_4)_2SO_4$ and 78.087 g/L of K_2HPO_4. In addition to providing nitrogen and carbon sources for microbial growth, $(NH_4)_2SO_4$ and K_2HPO_4 also regulate the pH of the fermentation medium. In the precursor group, the highest tetramycin titer was 86.417 g/L of CH_3COONa. CH_3COONa provided a sufficient precursor for the synthesis of tetramycin.

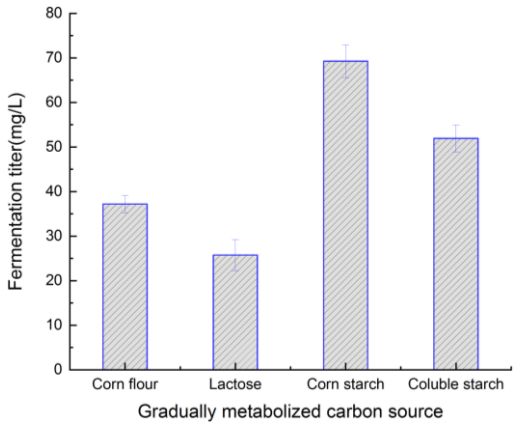


Fig. 1: Effects of gradually metabolized carbon source on fermentation titer

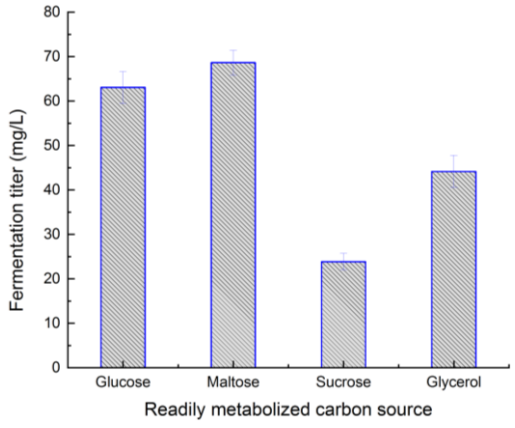


Fig. 2: Effects of readily metabolized carbon source on fermentation titer

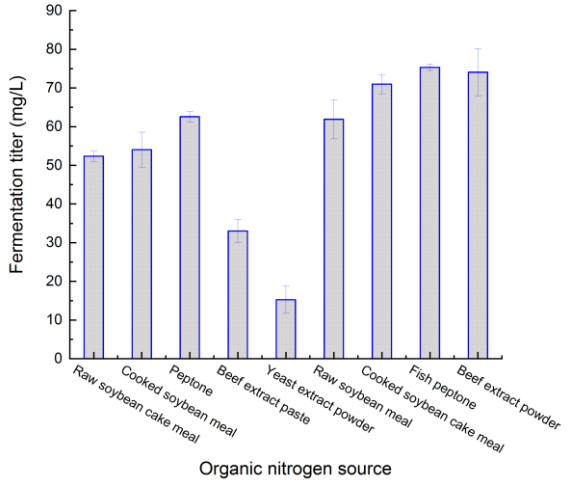


Fig. 3: Effects of organic nitrogen source on fermentation titer

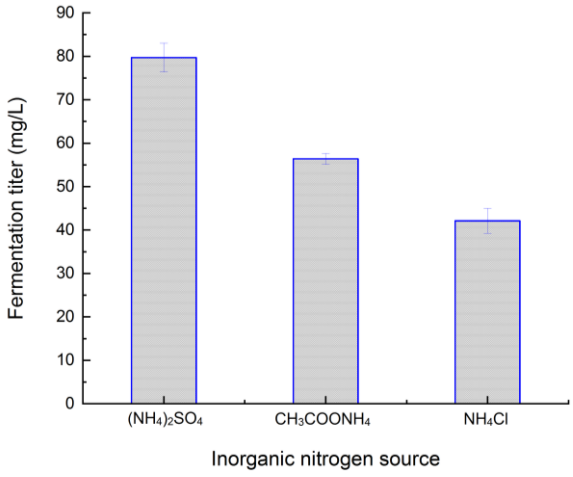


Fig. 4: Effects of inorganic nitrogen source on fermentation titer

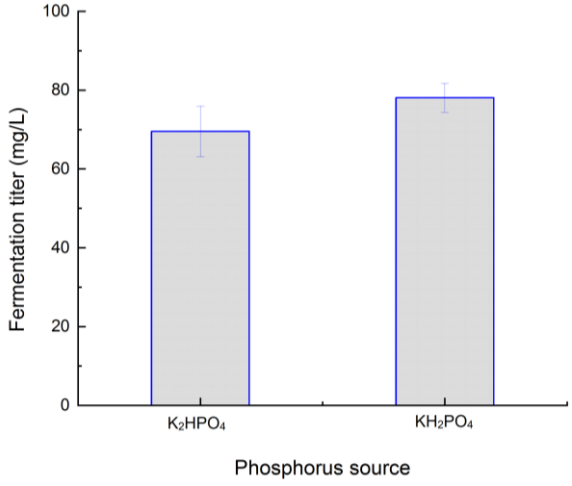


Fig. 5: Effects of phosphorus source on fermentation titer

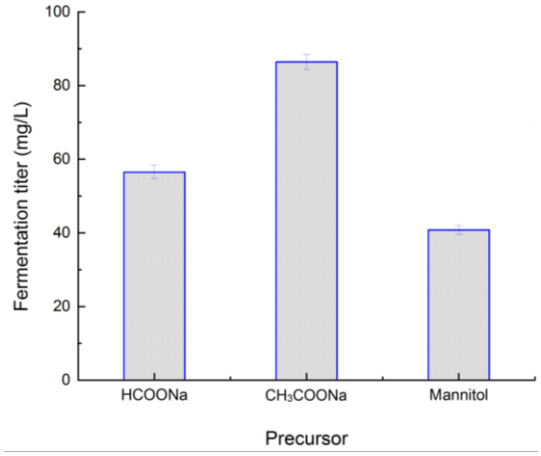


Fig. 6: Effects of precursor on fermentation titer

Plackett-Burman Experimental Design and Results

According to the results of the single-factor experiment, the significant influences of six factors in the medium were screened by PB experimental design. The low level of each factor was taken as the concentration of each component in the initial fermentation medium and the high level was 1.5 times of the low level. The results are shown in Table 2.

The data were analyzed by Minitab18 software to obtain partial regression coefficients and significance tests. A six-variable regression equation was obtained as follows:

$$Y = 79.479 - 3.396\,X_1 + 9.246\,X_2 - 0.569\,X_3 - 1.804\,X_4 + 1.154\,X_5 - 0.439\,X_6$$

where, Y, X_1, X_2, X_3, X_4, X_5, and X_6 are tetramycin titer, corn starch, glucose, fish peptone, $(NH_4)_2SO_4$, KH_2PO_4, and CH_3COONa, respectively. According to the results of ANOVA in Table 3, the p-values of corn starch and glucose are less than or equal to 0.05, so these two factors have a significant effect on the tetramycin titer. As an important nutrient in the growth process of microorganisms, carbon sources can provide energy for the growth of strains, accelerate the transmission of substances to the cells and the rate of cell metabolism, and promote the proliferation of strains. Because $(NH_4)_2SO_4$ is closer to these two components than the p-value of the other factors, it thus will also be considered as the third influencing factor on the response value of tetramycin titer. In the metabolic process of microorganisms, nitrogen is mainly converted into nucleic acids, amino acids, and components of the cell wall, which are the main nutrients for the growth of microorganisms. Taking into account that the titer of gradually metabolized carbon source, readily metabolized carbon source and inorganic nitrogen source have been determined and the other single factors affecting tetramycin fermentation efficacy are not significant and thus the three factors identified from the path of steepest ascent method were used in order to save experimental resources. Table 3, the effect values were negative for corn starch and $(NH_4)_2SO_4$ and positive for glucose.

Path of Steepest Ascent Method Design and Results

In order to obtain better response values, the path of the steepest ascent was designed in combination with the PB test results to approximate the maximum response region. Based on the group with the best PB test results as the center, the concentrations of corn starch and $(NH_4)_2SO_4$ were successively reduced with 2.5 g/L and 0.125 g/L as the step rate, respectively and the concentration of glucose was increased with 2.5 g/L as the step rate. Table 4, the fermentation titer of tetramycin reached the maximum of 96.945 mg/L when the concentrations of corn starch, glucose, and $(NH_4)_2SO_4$ reached 25, 37.5, and 0.25 g/L, respectively, indicating that the optimal formula for tetramycin production was near the third group of experiments.

Table 3: Partial regression coefficients and significance tests for the Plackett-Burman design

Project	Effect	Coefficient	Std Error	T-value	p-value	Variance Inflation factor
		79.479	0.831	95.67	0.000	
Corn Starch	-6.792	-3.396	0.831	-4.09	0.009	1.00
Glucose	18.492	9.246	0.831	11.13	0.000	1.00
Fish Peptone	-1.137	-0.569	0.831	-0.68	0.524	1.00
$(NH_4)_2SO_4$	-3.607	-1.804	0.831	-2.17	0.082	1.00
KH_2PO_4	2.307	1.154	0.831	1.39	0.224	1.00
CH_3COONa	-0.877	-0.439	0.831	-0.53	0.620	1.00

Table 4: Path of steepest ascent method experimental design and results

	Mass concentration of impact factor (g/L)			
Number	Corn starch	Glucose	$(NH_4)_2SO_4$	Titer (mg/L)
1	35.0	27.5	0.750	60.026
2	32.5	30.0	0.625	70.907
3	30.0	32.5	0.500	75.543
4	27.5	35.0	0.375	78.228
5	25.0	37.5	0.250	96.945
6	22.5	40.0	0.125	71.750

Table 5: Box-Behnken experimental design and results

	Mass concentration of impact factor (mg/mL)			
Number	Corn Starch	Glucose	$(NH_4)_2SO_4$	Titer (mg/L)
1	20	35.0	0.25	96.7
2	30	37.5	0.30	96.8
3	25	37.5	0.25	165.9
4	25	37.5	0.25	179.0
5	20	37.5	0.30	107.8
6	30	35.0	0.25	90.1
7	25	37.5	0.25	167.0
8	25	35.0	0.30	108.8
9	20	37.5	0.20	84.9
10	25	37.5	0.25	171.7
11	20	40.0	0.25	130.9
12	25	37.5	0.25	156.4
13	25	40.0	0.20	120.9
14	30	37.5	0.20	125.7
15	25	40.0	0.30	115.6
16	25	35.0	0.20	144.8
17	30	40.0	0.25	108.3

Table 6: Response surface quadratic term model ANOVA

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F	
Model	13716.060000	9	1524.0070	7.260498	0.0080	significant
A-Corn Starch	0.045000	1	0.0450	0.000214	0.9887	
B-Glucose	155.761300	1	155.7613	0.742060	0.4175	
C-$(NH_4)_2SO_4$	279.661300	1	279.6613	1.332330	0.2863	
AB	64.000000	1	64.0000	0.304901	0.5980	
AC	670.810000	1	670.810	3.195795	0.1170	
BC	235.622500	1	235.6225	1.122525	0.3246	
A^2	6774.790000	1	6774.7900	32.275670	0.0007	
B^2	1926.001000	1	1926.0010	9.175629	0.0191	
C^2	2442.980000	1	2442.9800	11.638560	0.0113	
Residual	1469.328000	7	209.9039			
Lack of Fit	1194.668000	3	398.2225	5.799498	0.0613	not significant
Pure Error	274.660000	4	68.6650			
Cor Total	15185.390000	16				
R-Squared	0.903241					
Adj R-Squared	0.778836					
Adeq Precision	7.468388					

Box-Behnken Experimental Design and Results

According to the results of the PB experiment and the path of the steepest ascent experiment, the significant influencing factors and central points were determined. The three significant factors were corn starch, glucose, and $(NH_4)_2SO_4$ and the central points were 25 g/L, 37.5 g/L, and 0.25 g/L, respectively. The BBD plan consisting of 17 experiments with 3 factors and in triplicate at the center was performed to fit a quadratic model. The step size was 5 g/L for corn starch, 2.5 g/L for glucose, and 0.05 g/L for $(NH_4)_2SO_4$. The experimental design and results are shown in Table 5. The quadratic polynomial regression model of corn starch (A), glucose (B), and $(NH_4)_2SO_4$ (C) was generated as shown in the equation:

$$Y = 16+0.075\times A+4.4125\times B-5.9125\times C-4\times A\times B -12.95\times A\times C+7.675\times B\times C-40.1125\times A^2-21.387\times B^2-24.0875\times C^2$$

The analysis of variance is shown in Table 6. The f-value of the model is 7.26 and values of "Prob > *F*" were 0.008 and less than 0.0500 indicating model terms are significant. This relatively low probability (<10%) is troubling. The *F*-value of 0.0613 suggests that the lack of fit is

not significant relative to the pure error, demonstrating a good-fitted model (De Freitas *et al*., 2019). Within the selected range of independent variables, the model fits the experimental data well and can be used for the analysis of the changes between the response values as well as the interrelationships. A larger model correlation coefficient R^2 indicates a better fit (Mohanty and Jena, 2018), as can be seen in Table 6, the model correlation coefficient $R^2 = 0.9032$ indicating that the model fits well. Adeq Precision is the ratio of signal to noise ratio, a ratio greater than 4 is desirable and a precision value of 7.468 indicates that the signal is adequate. The above data indicate that the fitted regression equation is well adapted and the selected model can analyze and predict the changes in the titer of tetramycin. As shown in Table 6, with $p<0.05$, the quadratic coefficients (A^2, B^2, and C^2), had significant effects. The others, the linear coefficient (A, B, and C) and interaction coefficients (AB, AC, and BC) with $p>0.05$, had insignificant effects. Furthermore, the influence of the component on the tetramycin titer follows the sequence: C-$(NH_4)_2SO_4$, B-glucose and A-corn starch.

The normal probability of the residuals of the model was also analyzed, when the probability distribution of the residuals is on a straight line and the residuals are not regular with the distribution of the predicted values, it proves that the model performance is good. Figure 7, it can be seen that the probability distribution of the residuals is almost on a straight line and there is no pattern in the distribution of the residuals and the predicted values, which indicates that the model performance is good.

The type of contour can be observed that the interaction between the two single factors is first insignificant and when the shape is oval means significant, while circular means insignificant (Coello *et al*., 2002; Tang *et al*., 2022; Falah *et al*., 2021; Tang *et al*., 2019; Rodrigues *et al*., 2019; Kahani *et al*., 2020). Clearly, an increase in corn starch or glucose led to an elevation of the titer of tetramycin at the initial stage of the reaction (Fig. 8). The contour plots and 3D response surface plots in Fig. 9 indicated the effects of corn starch and $(NH_4)_2SO_4$. Observably, the titer of tetramycin improved with the increase of corn starch or $(NH_4)_2SO_4$ at first but did not rise significantly with further increase of either parameter. Figure 10 shows the titer of tetramycin as a function of glucose and $(NH_4)_2SO_4$. The titer of tetramycin increased with the increasing glucose and $(NH_4)_2SO_4$ at the beginning but decreased afterward.

The optimized concentrations of the above three major factors obtained by design expert 8.0.6.1 were: Corn starch at 25.08 mg/mL, glucose at 37.70 mg/mL, and $(NH_4)_2SO_4$ at 0.24 mg/mL with the predicted maximum tetramycin titer of 168.517 mg/L. An additional set of experiments was conducted to evaluate the model's accuracy.

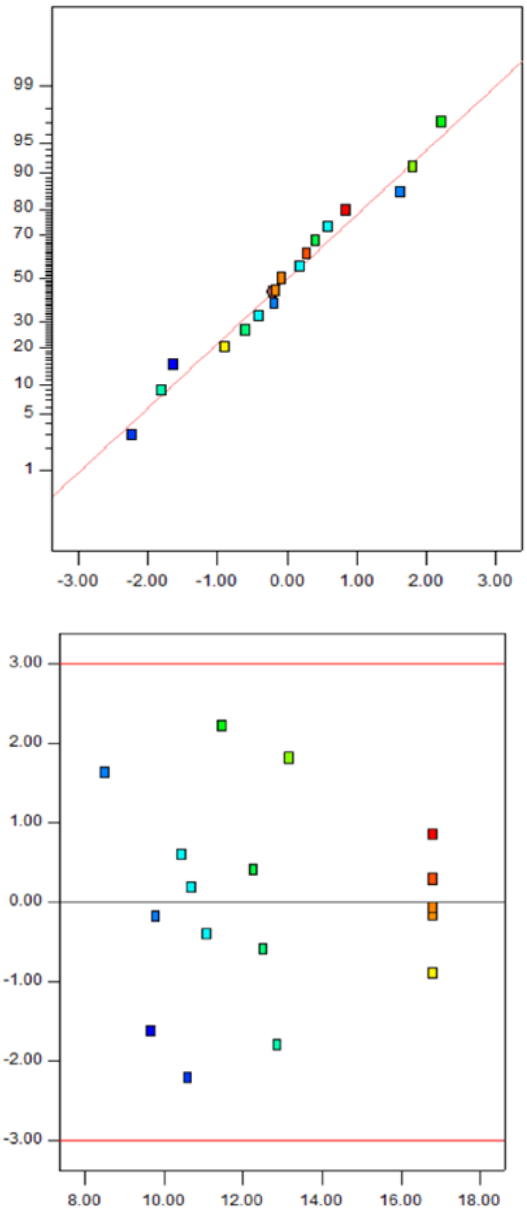
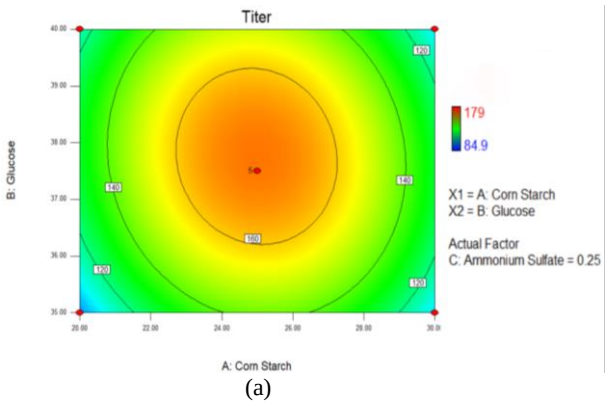
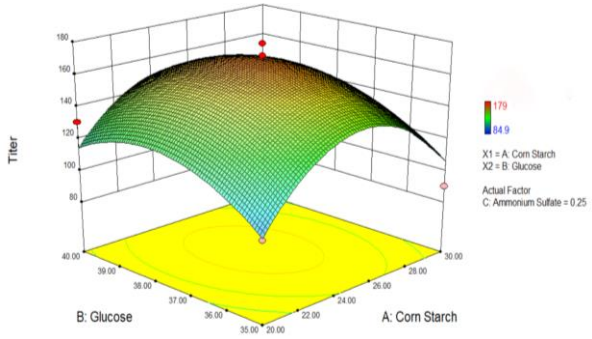


Fig. 7: Residuals and predicted values

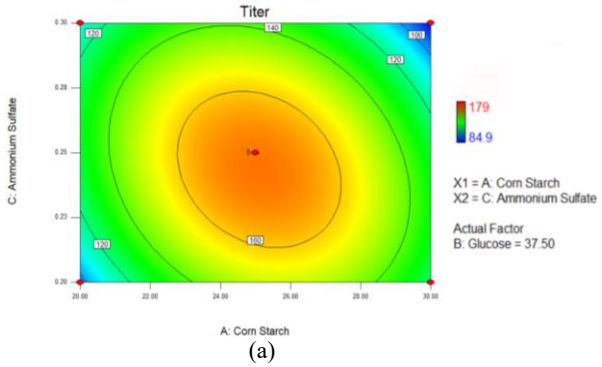


(a)

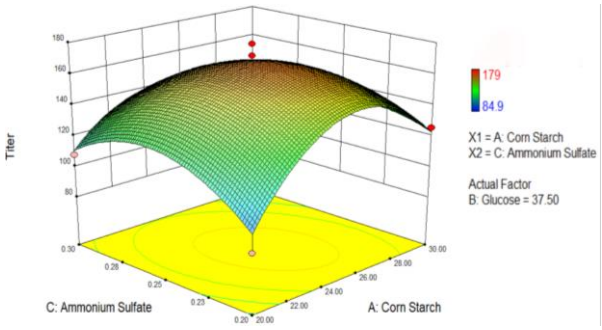


(b)

Fig. 8: The effect of corn starch and glucose on the tetramycin titer; A. Contour plots; B. Response surfaces

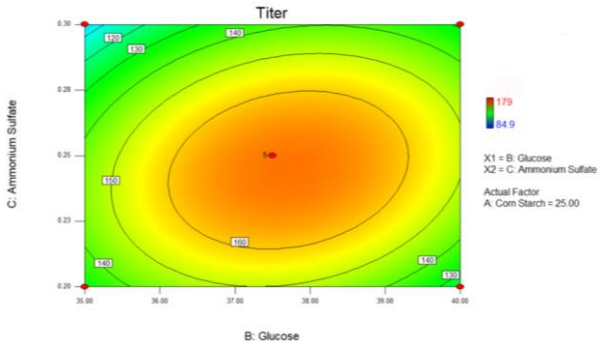


(a)

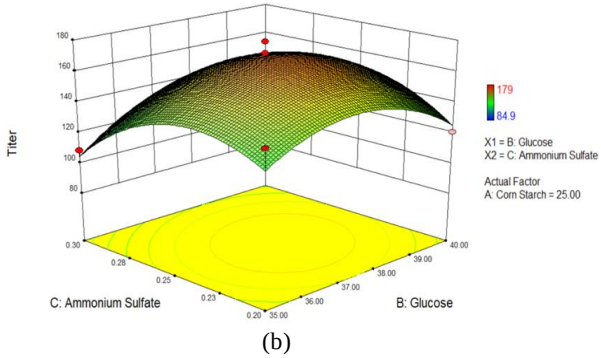


(b)

Fig. 9: The effect of corn starch and $(NH_4)_2SO_4$ on the tetramycin titer; A. Contour plots; B. Response surfaces



(a)



(b)

Fig. 10: The effect of glucose and $(NH_4)_2SO_4$ on the tetramycin titer; A. Contour plots; B. Response surfaces

The validation test was repeated three times under the optimized fermentation conditions and the results of the original fermentation medium were used as the control. The results showed that the tetramycin titer under the optimized conditions was 167.91±21.75 mg/L which was basically the same as that of the predicted value of 168.517 mg/L. The titer of tetramycin in the basic fermentation medium was 30.42±5.03 mg/L. The titer of tetramycin in the optimized tetramycin fermentation medium increased 5.5-fold compared with the original fermentation medium ($p<0.05$) and the experimental data fitted well with the predicted values, which indicated that the established model was effective. The optimized medium was corn starch 25.08 g/L, soybean cake meal 30 g/L, glucose 37.70, NaCl 0.2, $MgSO_4$ 0.2, K_2HPO_4 0.1, $FeSO_4$ 0.2, $CaCO_3$ 5 and $(NH_4)_2SO_4$ 0.24 g/L. Compared with the original medium, the corn flour component is removed. The corn flour usually exists as a large particle in the fermentation medium, which will cut the mycelium during rotation. At the same time, the product extraction is unfavorable after the fermentation and the new formula is more conducive to industrial production.

There are various means of improving antibiotic yield, including mutation of strains by physical or chemical means, optimization of medium composition, or modification of strains by means of genetic engineering. Cui *et al*. (2015) provided additional copies of ttmRIV, a pathway-specific positive regulator of tetramycin genomic transcription, which increased tetramycin production by 3.3 times. Chen *et al*. (2021) constructed tetramycin A high-yield strains by blocking the precursor competitive biosynthetic gene cluster, disrupting tetramycin B biosynthesis and overexpressing the tetramycin pathway regulator, increasing tetramycin A production by 2.36 times. As a result, the yield of tetramycin was increased by 5.5 times, indicating that for tetramycin fermentation, the optimization of the medium to increase the yield is an effective method. Although this experiment optimized the medium formulation with higher tetramycin yield, some shortcomings still deserve further exploration at industrial production levels.

Conclusion

Medium optimization was carried out for the tetramycin-producing strain *Streptomyces ahygroscopicus* Δ*nysB* by single factor experiment, PB experiment, the path of steepest ascent experiment, and response surface method were used to optimize the fermentation medium. It was determined that corn starch, glucose, and $(NH_4)_2SO_4$ had the most significant effect on the yield of tetramycin, so the above components were selected for optimization. Finally, the optimal fermentation medium was determined as corn starch 25.08 g/L, soybean cake powder 30 g/L, glucose 37.70 g/L, NaCl 0.2, $MgSO_4$ 0.2 g/L, K_2HPO_4 0.1 g/L, $FeSO_4$ 0.2 g/L, $CaCO_3$ 5 and $(NH_4)_2SO_4$ 0.24 g/L. The yield of tetramycin in the optimized medium reached 167.19 mg/L, which was very close to the predicted value of the model. The yield of tetramycin was significantly increased to 5.5 times that before optimization, which indicates medium optimization was an effective method to enhance the production of tetramycin. The research on the optimization of Δ*nysB* fermentation medium lays the foundation for the industrial production of tetramycin with better economic benefits and development prospects.

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Author's Contributions

Xiao Wang: Designed, performed the experiments, wrote drafted, and revised the manuscript. Engaged in the process of gathering experiment-related supplies and did some experiments.

Yao Dong, Hao Cui and Yawen Gu: Analyzed the experimental data and revised the manuscript.

Xin Chen: Engaged in the process of gathering experiment-related supplies and did some experiments.

Ethics

This article is an original manuscript, all authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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