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ORIGINAL PAPER

Optimization of medium composition for actinomycin X2 production by *Streptomyces spp* JAU4234 using response surface methodology

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Abstract The effects of cultivation medium compositions including soybean meal, peptone, soybean oil and cornstarch for actinomycin X2 production by *Streptomyces spp* JAU4234 were accessed by using response surface methodology. The 2^4 full factorial designs and the paths of steepest ascent were effective in searching for the major factors of actinomycin X2 production. In this study, cornstarch and soybean oil showed negative effect on actinomycin X2 production based on the first-order regression coefficients derived from MINITAB software. Subsequently, a central composite design for optimization was further investigated. Preliminary studies showed that soybean meal and peptone were believed to be the major factors for actinomycin X2 production. Estimated optimum compositions for the production of actionmycin X2 were as follows (g/l): soybean meal 21.65 and peptone 9.41, and result in a maximum actionmycin X2 production of 617.4 mg/l. This value was closed to the 612 mg/l actionmycin X2 production from actual experimental observations. The yield of actionmycin X2 was increased by 36.9% by culturing the strain *Streptomyces spp* JAU4234 in the nutritionally optimized fermentation medium.

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Introduction

Actinomycins are chromopeptide lactone antibiotics produced by a number of *Streptomyces* strains and by some strains of *Micromonospora* [1–4]. Among the actinomycins, actinomycin D has been studied most extensively, and is widely used as an anti-tumor drug [5–8]. Actinomycin X2 is structurally related to actinomycin D and differs only in the amino acid content of the peptide side chains, but its medicinal properties have not been well investigated. Whereas actinomycin D has been shown to have higher activity toward human leukemia cell lines such as HL-60 cells, actinomycin X2 shows higher cytotoxicity toward HL-60 cells than even actinomycin D [9]. This result encourages further research on actinomycin X2 that would be valuable.

The conventional research technique of media optimization manipulated a single parameter per trial, which was not only time-consuming and expensive, but also failed to consider the interactions between different factors, and hence, the one-factor approach frequently failed to identify the optimal conditions for the bioprocess [10]. Response surface methodology has overcome the limitation of classical methods and has proved to be powerful and useful for the optimization of the wanted metabolites production [11–13]. Response surface methodology can be used to evaluate the relative significance of several factors. This method can especially be used in the presence of complex interactions [14].

For widespread field use, large quantities of actinomycin X2 preparation of a high potency are required. Apart from

the development of better strains by the genetic approach, e.g., repeated rounds of mutation and selection [15, 16], DNA shuffling [17] and genome shuffling [18], another way to successful commercialization of metabolite is the development of an optimal fermentation condition. Williams and Katz [19] developed a chemically defined medium with a view to reaching high concentration of actinomycin D (500–600 mg/l) with *Streptomyces parvulus* ATCC 12434. Using the same medium and strain, Dalili and Chau [20] achieved maximum actinomycin D concentration of around 50, 70 and 80 mg/l by employing batch, fed batch and continuous processes, respectively, in an airlift bioreactor. However, few studies have been carried out and have improved the culture conditions of actinomycin X2 fermentation, in particular the carbon and nitrogen sources for enhancing actinomycin X2 production. The aim of this work was to apply the full factorial design, followed by the response surface methodology to optimize the culture medium for actinomycin X2 production by *Streptomyces spp* JAU4234. The major variables affecting the performance of the culture in terms of actinomycin X2 production as a function of the levels of carbon (cornstarch, soybean oil) and nitrogen (soybean meal, peptone) sources were investigated.

Materials and methods

Microorganism

A culture of *Streptomyces spp* JAU4234 was obtained from the College of Life Sciences and Technology, Jiangxi Agricultural University, China, maintained on slants of potato-dextrose agar medium at 37 °C and subcultured every 30 days.

Preparation of seed culture

Spore suspension of *Streptomyces spp* JAU4234 was prepared from actively growing slants in sterile water and diluted to a concentration of 1×10^8 CFU/ml. A total of 1 ml of the spore preparation was inoculated to conical flasks containing 30 ml seed medium (10 g cornstarch, 30 g maize meal, 10 g soybean meal, 0.5 g $(NH_4)_2SO_4$, 6 g $CaCO_3$ and 2 g bean oil in 1,000 ml distilled water, adjusted to pH 8.0). These cultures were incubated at 30 °C for 48 h in a shaker incubator at 200 rpm.

Flask fermentation

All experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml media. The basic medium for fermentations consisted of 20 g maize meal, 20 g glucose, 2.5 g KNO_3, 2.5 g $(NH_4)_2SO_4$, 0.3 g KH_2PO_4, 3 g NaCl, 6 g $CaCO_3$ and varying amounts of cornstarch, bean oil, soybean meal and peptone in 1,000 ml distilled water, adjusted to pH 6.5. These flasks were inoculated with 5% seed culture and incubated at 30 °C for 96 h on a rotary shaker at 200 rpm.

Experimental design

Four cultivation medium compositions were studied for actinomycin X2 production including cornstarch, bean oil, soybean meal and peptone concentrations. This was studied at the center of the design to find the accuracy of results of statistical experimentation. At the beginning of the studies, a 2^4 full factorial design was effective in searching for the direction of the optimum domain (Table 1). Actinomycin X2 production for each shake-flask run was determined after a fermentation period of 96 h, and the experimental data was subjected to the segregate of each factor and their interactions. The actual concentration of each medium component was coded to facilitate multiple regression analysis. From the experimental results, an approximate polynomial's relationship for each dependent variable was obtained. If the fitted first-order model was adequate, a series of single experiments had to be performed along the path of steepest ascent toward the optimum region. From here, the new region of the central point can be detected in which the desirable values of the response were suspected to be within the boundaries of the operability region.

Finally, to describe the nature of the response surface in the optimum region, a central-composite design was performed. The levels of each factor are given in Table 2. The model fitted to the centred data on the response of actinomycin X2 production were of second-order polynomial function. MINITAB for windows, Release 14 software (Minitab Inc., Pennsylvania, USA) was used for statistical experimental design, for the analysis of the results and for drawing the response surface. All experiments were repeated at least three times. Standard deviations did not exceed 5% of average values.

Table 1 Assigned concentration of variables of different levels of the 2^4 full factorial design

Symbol	Factor	Coded level		
		−1	0	+1
x_1	Soybean meal (g/l)	7.5	10	12.5
x_2	Cornstrach (g/l)	10	20	30
x_3	Peptone (g/l)	4	5	6
x_4	Soybean oil (g/l)	0	5	10

Table 2 Coding and assigned concentration of variables of different levels of the central-composite design ($\alpha = 1.414$)

Symbol	Factor	Coded level				
		$-\alpha$	-1	0	$+1$	$+\alpha$
x_1	Soybean meal (g/l)	16.465	17.5	20	22.5	23.535
x_3	Peptone (g/l)	7.586	8	9	10	10.414

Actinomycin X2 estimation

Actinomycin X2 was determined by reverse phase HPLC (Hewlett Packard series 1050, Agilent, USA), using a Zorbax RX-C8 (4.6 mm × 25 cm, Agilent) column, 45% acetonitrile in MilliQ water as the mobile phase, 1.0 ml/min flow rate with detection at 254 nm [9]. All the actinomycin X2 estimations were performed repeatedly.

Results

Carbon (cornstarch, soybean oil) and nitrogen (soybean meal, peptone) sources were studied to evaluate the approximate polynomial (first-order model) for all dependent variables, explaining their effects on the actinomycin X2 production by *Streptomyces spp* JAU4234. Table 3 presents the experimental design and results of 2^4 full factorial design experiments. The actinomycin X2 yield varied markedly with the condition tested, in the range of 349–577 mg/l. The lower values of actinomycin X2 production were obtained when minimal levels of peptone and soybean meal were used (assays 1, 3, 9 and 11). Actinomycin X2 yields higher than 500 mg/l were obtained when peptone and soybean meal were adjusted to the highest levels (assays 6, 8, 14 and 16). These results suggested that these variables strongly affect the actinomycin X2 production.

The data was analyzed using MINITAB software, and the model equation of the first order was given as follows:

$$\text{Actinomycin X2 (mg/l)} = 446.813 + 50.062x_1 - 3.812x_2 + 39.688x_3 - 1.313x_4. \quad (1)$$

This equation showed that soybean meal (x_1) was the most significant factor, with its coefficient effect being most pronounced. The second most conspicuous change was the response of peptone (x_3). Cornstarch (x_2) and soybean oil (x_4), however, at the experimental level gave opposite effect on the actinomycin X2 production. Interestingly, this equation indicated that nitrogen sources strongly affected actinomycin X2 production, whilst carbon sources were considered to be insignificant under this experimental condition. As can be seen from Table 4, the factors

Table 3 Experimental design and results of full factorial design

Run	x_1	x_2	x_3	x_4	Actinomycin X2 (mg/l)
1	−1	−1	−1	−1	381
2	1	−1	−1	−1	450
3	−1	1	−1	−1	349
4	1	1	−1	−1	468
5	−1	−1	1	−1	410
6	1	−1	1	−1	577
7	−1	1	1	−1	420
8	1	1	1	−1	550
9	−1	−1	−1	1	350
10	1	−1	−1	1	445
11	−1	1	−1	1	390
12	1	1	−1	1	427
13	−1	−1	1	1	430
14	1	−1	1	1	547
15	−1	1	1	1	435
16	1	1	1	1	523
17	0	0	0	0	456
18	0	0	0	0	445
19	0	0	0	0	439
20	0	0	0	0	448

Table 4 Analysis of variance and model-fitting results for the full factorial design

Term	Effect	Coef	SE Coef	*T* ratio	*P* value
Constant		446.813	2.776	160.93	0.000*
x_1	100.125	50.062	2.776	18.03	0.000*
x_2	−7.625	−3.812	2.776	−1.37	0.263
x_3	79.375	39.688	2.776	14.29	0.001*
x_4	−2.625	−1.313	2.776	−0.47	0.669
$x_1 \times x_2$	−6.125	−3.062	2.776	−1.10	0.351
$x_1 \times x_3$	23.875	11.938	2.776	4.30	0.023
$x_1 \times x_4$	−16.125	−8.063	2.776	−2.90	0.062
$x_2 \times x_3$	−3.875	−1.937	2.776	−0.70	0.535
$x_2 \times x_4$	4.625	2.313	2.776	0.83	0.466
$x_3 \times x_4$	−0.875	−0.437	2.776	−0.16	0.885
$x_1 \times x_2 \times x_3$	−3.375	−1.687	2.776	−0.61	0.586
$x_1 \times x_2 \times x_4$	−12.875	−6.438	2.776	−2.32	0.103
$x_1 \times x_3 \times x_4$	−5.375	−2.688	2.776	−0.97	0.404
$x_2 \times x_3 \times x_4$	−4.625	−2.313	2.776	−0.83	0.466
$x_1 \times x_2 \times x_3 \times x_4$	9.875	4.937	2.776	1.78	0.173
Ct Pt		−4.812	6.208	−0.78	0.495

$R^2 = 0.994$, R^2 (adj) $= 0.967$

* Significant at the 99% level

Coef coefficient, *SE Coef* standard error of coefficient, *Ct Pt* central point

soybean meal (x_1) and peptone (x_3) were found to be significant at the probability level of 99% for actinomycin X2 production. It was found that the coefficient of determination R^2 of the model was calculated to be 0.994 (Table 4). This indicated that the model explained 99.4% of the variability in the data. This statistical significance of the equation model was also confirmed by F-test, which was 160.93.

Thus, based on the model equation obtained, Eq. (1), the path of steepest ascent was to increase peptone concentration and soybean meal concentration in order to improve the actinomycin X2 production. The cornstrarch and soy oil concentrations were fixed at 10 and 0 g/l, respectively. The values of actinomycin X2 production obtained in these experiments (Table 5) were very similar to those achieved in assays 6, 8, 14 and 16 (Table 3). These results indicated that we were working in the neighborhood of optimum actinomycin X2 production.

New levels of nitrogen sources were chosen as factors (Table 2) after the path of steepest ascent experiment. Soybean meal (x_1) and peptone (x_3) were the most effective variables for promoting the production of actinomycin X2 and their concentrations were further optimized using the central composite design. Experiments 1−8 were performed at different combinations, and those from 9 to 13 were under the same conditions (Table 6). The actual yield obtained in the experiments is also given in Table 6. By analysis for the regression on the experimental data, the quadratic equation model is presented below:

$$\text{Actinomycin X2(mg/l)} = 605 + 25.82x_1 + 18.64x_3 - 21x_1^2 - 26x_3^2 + 4.5x_1x_3. \quad (2)$$

Statistical significance of the second-order model equation was checked by an F-test (Table 7). The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.986, indicating that 98.6% of the variability in the response can be explained by the model. This revealed that Eq. (2) was a suitable model to describe the response of the experiment pertaining to actinomycin X2 production. The response taken from Table 6 reveals that the linear term of soybean meal (x_1), peptone (x_3) and quadratic coefficients of x_2^2 and x_3^2 have remarkable effects on the actinomycin X2 yield with low P-values of less than 0.01. This also can be seen from the surface plot and the contour plot for these components as shown in Figs. 1 and 2 (see below). By moving along the two axes (Fig. 1), for example, it can be demonstrated that increasing the levels of x_1 and x_3 from the percentages has a conspicuous effect on overall linkage. As a result, a stationary ridge shape was observed in the surface plot of these two components. According to Eq. (2), it was confirmed again that peptone and soybean meal were believed to be the major factors for actinomycin X2 production by *Streptomyces spp* JAU4234 among the variables studied.

The above observations indicated that the maximum production of actinomycin X2 was obtained when the concentration of soymeal powder and peptone were at about 20 and 9 g/l, respectively. According to the optimized mathematical model, the optimal levels of the two nutrients were: soymeal powder 21.65 g/l and peptone 9.41 g/l, and the corresponding maximum yield of actinomycin X2 were 617.4 mg/l. It was clear that the optimal values obtained from response surface graph and contour

Table 5 Design of experiments to obtain the steepest ascent path and corresponding actinomycin X2 yields

Assay number	Soybean meal (g/l)	Peptone (g/l)	Actinomycin X2 (mg/l)
1	10	5	450
2	12.5	6	482
3	15	7	533
4	17.5	8	585
5	20	9	606
6	22.5	10	601
7	25	11	583

Table 6 The design and results of the central-composite experiments

Run	x_1	x_3	Actinomycin X2 (mg/l)
1	−1	−1	524
2	−1	1	545
3	1	−1	561
4	1	1	600
5	−1.414	0	523
6	1.414	0	604
7	0	−1.414	522
8	0	1.414	585
9	0	0	605
10	0	0	603
11	0	0	601
12	0	0	610
13	0	0	606

Table 7 Analysis of variance and regression for the results of the central-composite design

Source	DF	Seq SS	Adj SS	Adj MS	F ratio	P value
Regression	5	15089.5	15089.53	3017.91	97.18	0.000*
Residual error	7	217.4	217.39	31.06		
Total	12	15306.9				

R^2 = 0.986, R^2 (adj) = 0.986

* Significant at the 99% level

DF degrees of freedom, *Seq SS* sequential sum of squares, *Adj SS* adjusted sum of squares, *Adj MS* adjusted mean squares

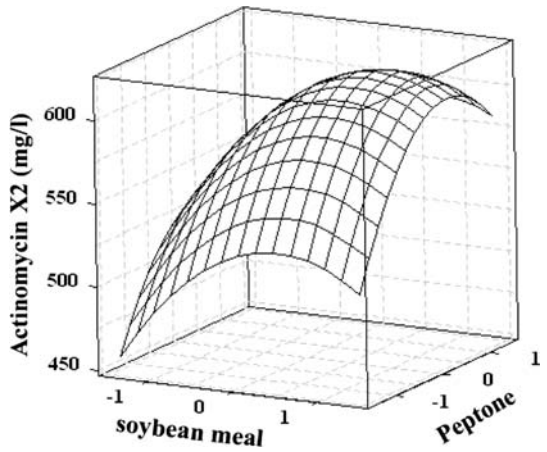

Fig. 1 Response surface graph of actinomycin X2 affected by soybean meal and peptone concentrations. The plot was obtained with the central-composite design

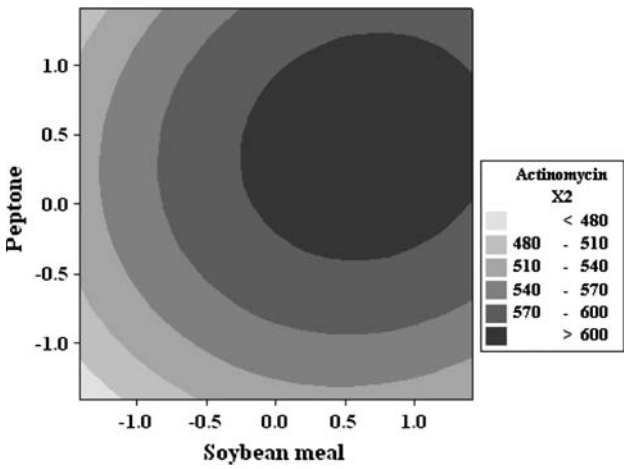

Fig. 2 Contour plot showing actinomycin X2 in response to varying concentrations of soybean meal and peptone

plot were almost consistent with those obtained from optimized mathematical equation. In order to verify the predicted results, experimental rechecking was performed using a medium under the optimized nutrients levels, and the experimental value was 612 mg/l, suggesting that experimental and predicted values of actinomycin X2 yield were in good agreement.

Discussion

As we all know, optimization of culture condition is a critical process to exploit the full capacity of better strains. Souza et al. [21] optimized the complex medium for the production of actinomycin D by *Streptomyces parvulus* and obtained high levels of product (approximately 530 mg/l) in shake-flask fermentation. Using the same strain and optimization medium, the yield of actinomycin D achieved was 1.53 g/l in a 14-l stirred tank under suitable aeration (1.5 vvm) and agitation (500 rpm) conditions [22]. Liu et al. [13] reported their results on optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide (EPS) production by *Agaricus blazei*. Under their optimized medium composition in shake flasks, the experimental biomass and EPS yields were 10.78 and 354.2 mg/l, respectively, which were 141 and 152% of those obtained from nonoptimal medium. The results in a 30 l stirred tank bioreactor also showed that the optimized culture medium could enhance both biomass (13.91 g/l) and EPS (363 mg/l) production. In the present study, two nutrients were identified to be the most influencing components for enhancing actinomycin X2 production by using full factorial design, and then their optimal concentrations were obtained by using response surface methodology. The production of actinomycin X2 increased to 612 mg/l under the optimum conditions, with 36.9% increase compared to the nonoptimized medium. On comparison with relevant references [9, 23], a significant higher yield of actinomycin X2 was obtained in our studies. It was evident that the response surface methodology had the advantage of identifying the most significant medium composites and their optimal levels, and thus was useful for operating the fermentation towards the accumulation of wanted metabolites. To our knowledge, this is the first report of enhancing actionmycin X2 production using response surface methodology. However, this paper is only an attempt to demonstrate the applicability of statistical theories to the study of actinomycin X2 fermentation processes, and the next step for enhancing actinomycin X2 yield should be the production in a bioreactor under optimized conditions.

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