Response Surface Methodology for Optimization of the Erythromycin Production by Fed-Batch Fermentation Using an Inexpensive Biological Nitrogen Source

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A novel source of biological nitrogen produced by multi-strain fermentation was developed for erythromycin production. The nitrogen composition of corn steep liquor, biological nitrogen and soybean flour was optimized by using Response surface methodology (RSM) firstly. A Box-Behnken design was used to estimate the optimum nitrogen composition for the production of erythromycin as follows: corn steep liquor: $\gamma = 5.1$ g L^{-1}, biological nitrogen $\gamma = 5.96$ g L^{-1} and soybean flour $\gamma = 24.17$ g L^{-1}. Based on the optimum nitrogen source, the constant glucose fed-batch and pH control fed-batch strategies were used to further optimize the erythromycin production in 50 L stirred bioreactor, respectively. It was found that pH control could serve as an effective control strategy for erythromycin production, and the maximum erythromycin production was 8528 U mL^{-1} at 190 h. This work demonstrates that the new medium formulation based on a cheap nitrogen source, biological nitrogen, is a potential alternative for economic erythromycin production on a large scale.

Key words:

Biological nitrogen, erythromycin production, fed-batch strategy, medium optimization, response surface methodology, *Saccharopolyspora erythraea*

Introduction

Erythromycin is a macrolide antibiotic produced by the fermentation of *Saccharopolyspora erythraea,* which was used for treatment of many infectious diseases caused by some bacteria.[1–3] Erythromycin has also been recommended as an alternative in patients who are allergic to penicillin or in cases of penicillin failure. Recently, erythromycin and its semi-synthetic derivatives are widely used in medicine. There is a great need to supply the market with a large amount of high-quality and low-cost erythromycin products.

Erythromycin fermentation is a classic antibiotic fermentation process. Despite some efforts in using classical mutagenesis techniques and metabolic engineering strategies for erythromycin strain improvement,[4,5] the optimization of fermentation medium still plays an important role in the production and productivity of erythromycin and the cost of raw materials/products. In our previous study, soybean flour was used as the main nitrogen source for erythromycin production,[6] however the price of the soybean flour has risen 30 ~ 40 % in China presently. The erythromycin production needs an economic nitrogen source to reduce the raw material costs. Biological nitrogen (trade name: Xindansu, produced by Beijing Jinruikang Biotech Co. Ltd), as a novel nitrogen source, is produced by multi-strain fermentation using agricultural byproducts (soybean meal, cottonseed meal, and rapeseed meal) as the main raw material. Compared with conventional nitrogen products such as soybean cake flour, cottonseed cake flour, and fish meal, biological nitrogen possesses more stable quality, cheap and high nitrogen content, and the price of biological nitrogen is 50 % cheaper than soybean flour in China.

Statistical experimental designs have been used for several decades and it is an efficient approach to find out the optimal conditions for targeted response. Response surface methodology (RSM) is a useful statistical technique for investigating and optimizing a medium and process, and has been used successfully to optimize some antibiotic fermentation processes.[7–9] In this work, biological nitrogen was used as a novel nitrogen source for erythromycin production. Response surface methodology (RSM) was applied to optimize the nitrogen source composition using the Box-Behnken design. Based on the optimal nitrogen source composition, a different fed-batch strategy including constant glucose fed-batch and pH control fed-batch was further used to optimize the erythromycin production in a

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50 L stirred bioreactor. The aim of this work was to study erythromycin fermentation under the influence of a cheap nitrogen source, while the information obtained is useful to large-scale fermentation of *Saccharopolyspora erythraea* for efficient production of erythromycin at low cost.

Materials and methods

Microorganism and culture conditions

Saccharopolyspora erythraea No. 8 from Yidu HEC Biochem. Co. Ltd. (Hubei province, China) was employed to produce erythromycin in submerged culture. Agar slants were inoculated with spores and incubated at $\vartheta = 32$ °C for 7 days, and then used for seed culture inoculation. For seed cultures, the medium composition was (γ/g L^{-1}): starch 30, soybean flour 15, NaCl 5, $(NH_4)_2SO_4$ 2, pH 7.0. The seed culture was grown in a 500 mL shake flask containing 50 mL of liquid medium and incubated at $\vartheta = 32$ °C on a rotary shaker (220 rpm) for 2 days. For fermentation, the medium as the control, had the following concentration of each nutrient (γ/g L^{-1}): soluble starch 40, dextrin 30, soybean meal 32, corn steep liquor 15, $CaCO_3$ 5, NaCl 2, pH 7.0. The fermentation cultivation was inoculated at $\varphi = 10$ % ($1 \cdot 10^8$ individual mL^{-1}) of the above seed culture medium and kept at $\vartheta = 32$ °C and 220 rpm for 7 days.

Materials

Corn steep liquor was obtained from Yichang Huacheng Fermentation Co., while soybean flour was obtained from Yichang Mingyuan Technology Co., Hubei province, China. Biological nitrogen was purchased from Beijing Jinruikang Biotech Co. Ltd China. The mass fraction of protein in corn steep liquor, soybean flour and biological nitrogen reached $w = 22.5$ %, 42 % and 53.7 % by Kjeldahl nitrogen method,[10] respectively.

Box-Behnken designs

The RSM used in the present study is a Box-Behnken design involving three different nitrogen factors (corn steep liquor, biological nitrogen, and soybean flour), the other culture medium components were same as the control media. As presented in Table 1, corn steep liquor (X_1), biological nitrogen (X_2), and soybean flour (X_3) were prescribed in three levels coded (−1, 0, + 1). According to the applied design, fifteen combinations were implemented and their experimental results were fitted with a second-order polynomial equation of eq. (1) by a multiple regression technique.

Table 1 – *Process variables and level in Box-Behnken designs*

Variable	Symbol	Coded variable level		
		−1	0	1
corn steep liquor (γ/g L^{-1})	X_1	5	10	15
biological nitrogen (γ/g L^{-1})	X_2	4	6	8
soybean flour (γ/g L^{-1})	X_3	20	25	30

$$y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

Where y is the dependent variable (erythromycin production), b_0 is the regression coefficient at center point, and b_1, b_2, and b_3 are linear coefficients, and b_{11}, b_{22}, and b_{33} are quadratic coefficients. The coefficients and the optimum concentration were calculated using the SAS software (version 9.0 by SAS Institute Inc., NC, USA). The fitness of the second-order model was expressed by the regression coefficient r^2 and its statistical significance was determined by an F-test. The significance of each coefficient was determined using T-test.

Fed-batch fermentation in a 50 L fermenter

The fermenter used was a $V = 50$ L stirred tank bioreactor with a working volume of $V = 30$ L, having three six-bladed turbine impellers and equipped with different sensors and devices to monitor and control more than 14 on-line measurable parameters, which was designed by Shanghai Guoqiang Bioengineering Equipment Co., Ltd China.[11] The culture temperature and the inoculum size were the same as in shake flasks. Samples were taken every 8 hours for the analyses of cell growth (PMV), erythromycin production, residual sugar and total sugar concentration.

Constant glucose fed-batch fermentation

For constant glucose feeding experiments, a constant glucose fed-batch process with a pulse feeding of $\gamma = 300$ g L^{-1} of highly concentrated glucose solution into the stirred bioreactor was conducted to increase the glucose concentration to $\gamma = 0.1$ g L^{-1}, when the glucose concentration fell down to $\gamma = 0.1$ g L^{-1} in the broth. The addition rate can be adjusted by interval glucose off-line assay.

pH control fed-batch fermentation

For feeding experiments with pH control, a pH control fed-batch process with a pulse feeding of $\gamma = 300$ g L^{-1} of highly concentrated glucose solu-

tion into the stirred bioreactor was conducted to control the pH between 6.9 and 7.0, when the pH was above 6.95 in the fermentation broth.

Determination of cell biomass (Packed mycelium volume, PMV)

For the determination of cell biomass (PMV), 10 mL fermentation broth was taken as sample each time, after removal of supernatant by centrifugation ($a_c = 4\ 000 \cdot g$, 10 min), PMV was calculated as: the volume of precipitate/10 mL fermentation broth.

Determination of the glucose, total sugar and residual sugar concentration

The glucose concentration was measured by the glucose oxidase method.[12] The standard reagents were purchased form Shanghai Feiheng Medical Technology Co., Ltd China. The total sugar and residual sugar were assayed by Fehling method.[13]

Assay of erythromycin production and components

The concentration of total erythromycin production was measured by the modified colorimetric method. After removing the biomass and insoluble ingredients, the fermentation broth was extracted with butyl acetate. Extracted erythromycin was mixed with the $c = 0.1$ mol L^{-1} hydrochloric acid. The aqueous phase fraction was separated with great care, and further mixed with anhydrous sulfuric acid for 3 min. Its absorbance was measured at $\lambda = 498$ nm with a spectrophotometer. To confirm the production of erythromycin, the fermentation broth samples at the end of fermentation were further bioassayed against *Bacillus pumilus* CMCC(B) 63202 using the cylinder plate assay method (China Pharmacopoeia 2005).

The components of erythromycin were determined by the HPLC method (JASCO PU2080, Japan), Hypersil BDS-C18 column (4 mm × 250 mm, 5 μm, Elite, China), mobile phase: mixture of acetonitrile and $c = 0.025$ mol L^{-1} potassium hydrogen phosphate ($\Psi = 60 : 40$, and flow-rate $Q = 0.9$ mL min^{-1} using UV detector at $\lambda = 215$ nm.[14]

Results and discussion

Box-Behnken design

The design matrix and actual production of erythromycin obtained in the experiments were shown in Table 2. The regression coefficients and significance levels of the model representing erythromycin production were given in Table 3. It was evident from Table 3 that the model terms, X_1, X_2, X_3, X_1^2, X_2X_3 were significant ($P < 0.05$). The corn steep liquor (X_1) was more significant than other variables of biological nitrogen (X_2) and soybean flour (X_3). The regression equation coefficients were calculated and data fitted to a second-order polynomial equation. The response of erythromycin production (y) can be expressed in terms of the following regression equation:

Table 2 – *Box-Behnken design for three independent variables with results*

Trial	Variable			Erythromycin/U mL^{-1}
	X_1	X_2	X_3	
1	−1	−1	0	2862[a]
2	−1	1	0	2848
3	1	−1	0	2372
4	1	1	0	1789
5	0	−1	−1	2276
6	0	−1	1	2481
7	0	1	−1	2403
8	0	1	1	1272
9	−1	0	−1	2793
10	1	0	−1	2484
11	−1	0	1	2477
12	1	0	1	1974
13	0	0	0	2213
14	0	0	0	2115
15	0	0	0	2227

[a] The data were calculated from three independent samples.

Table 3 – *Significance of regression results from the data of response surface design (RSM) experiments*

Parameters	Parameter estimate	*T*-value	Pr > |*t*|
X_1	−295.125	−5.733	0.002
X_2	−209.875	−4.077	0.009
X_3	−219	−4.254	0.008
$X_1 \cdot X_1$	303.375	4.004	0.010
$X_1 \cdot X_2$	−142.25	−1.954	0.108
$X_1 \cdot X_3$	−48.5	−0.666	0.535
$X_2 \cdot X_2$	−20.625	−0.272	0.796
$X_2 \cdot X_3$	−334	−4.588	0.006
$X_3 \cdot X_3$	−56.375	−0.744	0.490

$$y = 2185 - 295.125X_1 - 209.875X_2 - 219X_3 + \\ + 303.375X_1^2 - 142.25X_1X_2 - 48.5X_1X_3 - \\ - 20.625X_2^2 - 334X_2X_3 - 56.375X_3^2 \quad (2)$$

in which X_1 was corn steep liquor, X_2 was biological nitrogen, and X_3 was soybean flour.

The regression equation obtained from the ANOVA showed that the regression was statistically significant ($P < 0.05$) at 95 % of confidence level (Table 4). The model presented a high regression coefficient ($r^2 = 0.9566$), which indicated that 95.66 % of the variability in the response could be explained by the model. The experimental data were fitted into eq. (2), and Fig. 1 illustrated the response surface plot that the erythromycin production reached its maximum at a combination of coded level −0.986 (X_1, corn steep liquor), −0.015 (X_2, biological nitrogen) and −0.164 (X_3, soybean flour) by canonical analysis of SAS software. The model predicted a maximum response of erythromycin 2798 U mL^{-1} at levels of corn steep liquor $\gamma = 5.1$ g L^{-1}, biological nitrogen $\gamma = 5.96$ g L^{-1} and soybean flour $\gamma = 24.17$ g L^{-1} as optimized nitrogen components. To confirm the above prediction, further experiments using optimized nitrogen components media (as predicted) and previous media (as control) were performed under the same cultivation conditions. Table 5 shows actual maximum

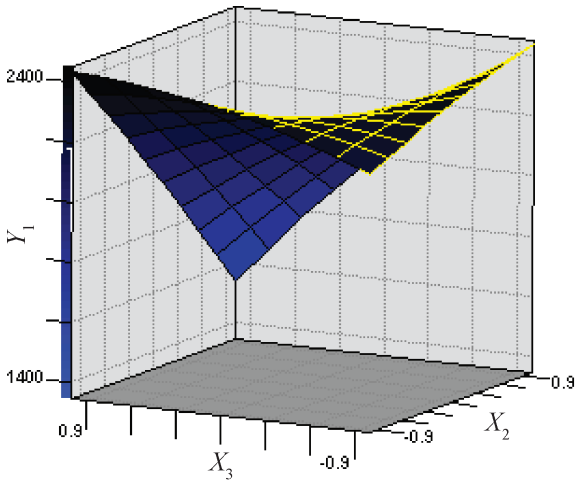
Table 4 – *Analysis of variance (ANOVA) for the parameters of response surface methodology fitted to second-order polynomial equation*

Source	DF	SS	MS	*F*	Pr > *F*
model	9	2 338 326.00	259 814.00	12.26	0.006
linear	3	1 432 858.00	477 619.40	22.53	0.002
quadratic	3	368 894.20	122 964.70	5.80	0.044
cross Product	3	536 573.30	178 857.80	8.44	0.021
error	5	105 997.30	21 199.45		
lack of fit	3	98 549.25	32 849.75	8.82	0.103
pure error	2	7 448.00	3 724.00		
total	14	2 444 323.00			

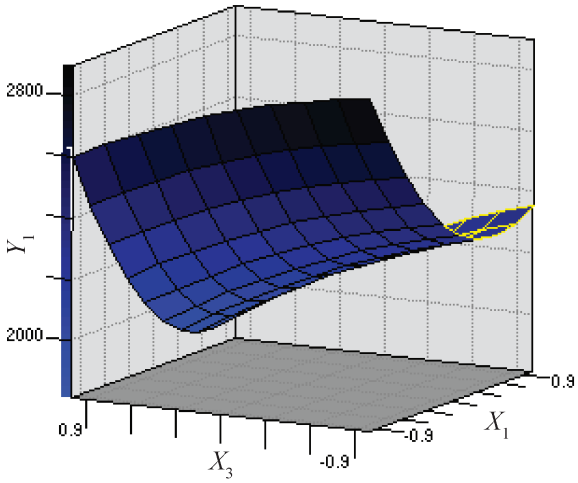
$r^2 = 95.66$ %; adjusted $r^2 = 87.86$ %; SS, sum of squares; DF, degree of freedom; MS, mean square

Table 5 – *Changes of erythromycin and component A production with optimized nitrogen components media and control*

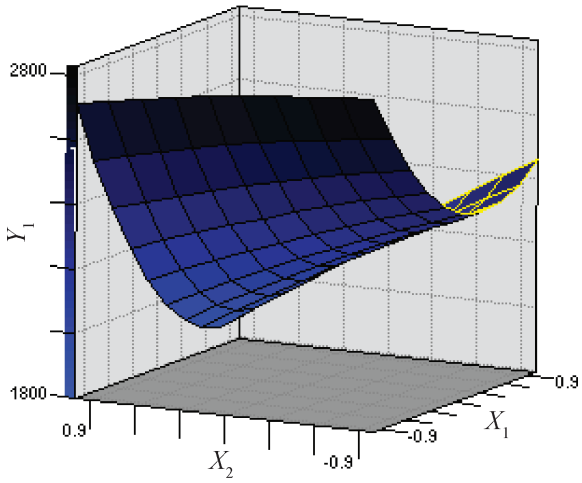
Media	Erythromycin/ U mL^{-1}	Erythromycin A/ U mL^{-1}
optimized media	2757	2164
control	2917	2304



Fixed levels: $X_1 = 0$



Fixed levels: $X_2 = 0$



Fixed levels: $X_3 = 0$

Fig. 1 – *Response of erythromycin production as a function of corn steep liquor (X_1), biological nitrogen (X_2) and soybean flour (X_3) based on the Box-Behnken experimental results*

erythromycin production of 2757 U mL^{-1} according to the results of the Box-Behnken designs, and similar to the results of the control. The results indicated that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. Compared with the control media, the optimized nitrogen components media can be selected to reduce the raw material costs.

Fed-batch fermentation in stirred bioreactor

Based on the optimal nitrogen media, two different fed-batch strategies were further developed to optimize the erythromycin production in $V = 50$ L stirred bioreactor. Time course of constant glucose fed-batch and pH control fed-batch is shown in Fig. 2, and Fig. 3, respectively. The culture pH changed constantly when glucose began to feed from 72 hour, and decreased from 6.89 at 100 hour to 6.47 at the end of fermentation with constant glucose fed-batch strategy. Fig. 4 clearly shows that the glucose feeding rate with constant glucose fed-batch was higher than that using pH control

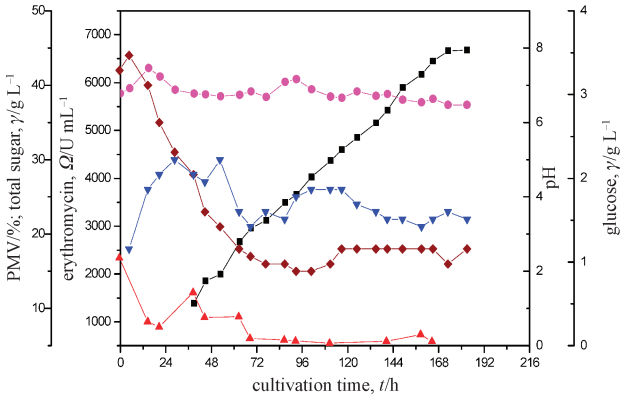


Fig. 2 – *Time course of cell growth by PMV(▼), total sugar (◆), glucose (▲), pH (●) and erythromycin production (■) during constant glucose fed-batch fermentation in stirred bioreactor*

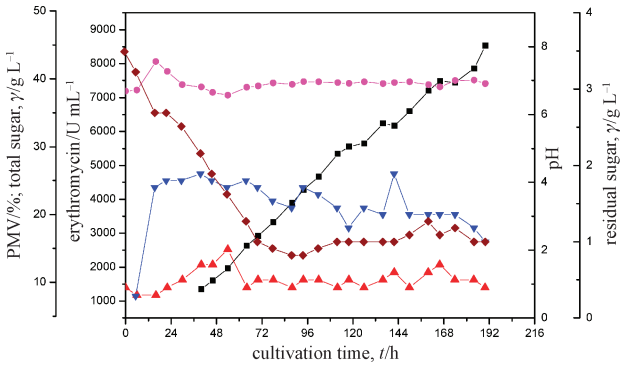


Fig. 3 – *Time course of cell growth by PMV(▼), total sugar (◆), glucose (▲), pH (●) and erythromycin production (■) during pH control fed-batch fermentation in stirred bioreactor*

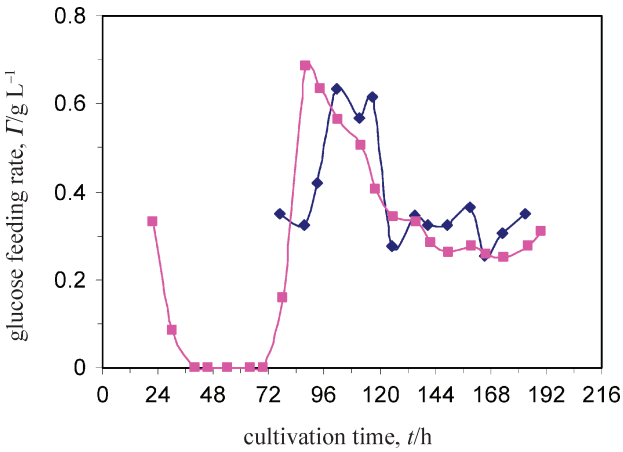


Fig. 4 – *Changes of feeding glucose rate under different fed-batch strategies. Symbols for pH control fed-batch (■) and constant glucose fed-batch (◆).*

fed-batch from 100 hour to the end of fermentation. The maximum erythromycin production was 6677 U mL^{-1}, and 8528 U mL^{-1} when these two strategies were used, respectively. The results indicated that whereas residual glucose concentration with constant glucose fed-batch was below $\gamma = 0.1$ g L^{-1} at the period of erythromycin biosynthesis, the metabolism of glucose still produced some acidic substance and induced pH value decline, and the biosynthesis of erythromycin was further inhibited. It was obvious that pH control was favorable for the erythromycin production.

Table 6 displays the changes of erythromycin components with constant glucose fed-batch and pH control fed-batch strategy. The main component of erythromycin A with pH control fed-batch was higher than constant glucose fed-batch, and the results were consistent with above chemical assay results. The impurity components of erythromycin B and C have not obviously increased with two different fed-batch strategies.

Table 6 – *Changes of erythromycin components by HPLC under different fed-batch strategies*

Strategy	Cultivation time *t*/h	Erythromycin/U mL^{-1}		
		A	B	C
constant glucose fed-batch	183	4205	167	226
pH control fed-batch	190	6145	145	473

Conclusion

An economically feasible raw material was beneficial to improve the production and productivity of industrial fermentation products. In this work, a novel renewable product of biological nitrogen

was used as cheap nitrogen source for economical production of erythromycin. Box-Behnken design was used to optimize the nitrogen source in shake flask in order to obtain the optimum nitrogen components. The different fed-batch strategy was further investigated based on the optimum nitrogen source medium on a bioreactor scale. The pH control fed-batch strategy was beneficial for erythromycin production. The fundamental information obtained in this work should benefit further development of economic process for erythromycin production on plant scale. Such work may also be helpful to other antibiotic fermentation for secondary metabolites production.

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List of symbols

b – coefficients in eq. (1)
c – molar concentration, mol L^{-1}
Q – volume flow rate, mL min^{-1}
r^2 – regression coefficient
V – volume, mL, L
t – cultivation time, h
w – mass fraction, %
γ – mass concentration, g L^{-1}
Γ – feeding rate, g L^{-1} h^{-1}
φ – volume fraction, %
Ψ – volume ratio
λ – wavelength, nm
ϑ – temperature, °C

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