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ORIGINAL PAPER

Application of oxygen uptake rate and response surface methodology for erythromycin production by *Saccharopolyspora erythraea*

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Abstract A process for efficient production of erythromycin by *Saccharopolyspora erythraea* using statistical designs and feeding strategy was developed. The critical nutrient components were selected in accordance with fractional factorial design and were further optimized via response surface methodology. Three significant components ($ZnSO_4$, citric acid threonine) were identified for the optimization study. The optimum levels of these significant variables were determined with Box–Behnken design, which were $ZnSO_4$ 0.039 g/l, citric acid 0.24 g/l and threonine 0.42 g/l, respectively. A novel feeding strategy based on oxygen uptake rate (OUR) measurement was developed successfully to increase the flux of erythromycin biosynthesis, in which the optimized nutrient components was fed in the 50 l stirred bioreactor when OUR began to decline at 46 h. The maximum erythromycin production reached 10,622 U/ml, which was 11.7% higher than the control in the same cultivation conditions. It was the first report to integrate physiological parameter OUR and statistical methods to optimize erythromycin production.

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Introduction

Erythromycin, a prominent member of the macrolide antibiotics, is produced by the fermentation of *Saccharopolyspora erythraea*. It's semi-synthetically modified derivatives, such as Azithromycin, Roxithromycin and Clarithromycin, are widely used in the treatment of many infectious diseases [1–3]. The global demand for erythromycin is increasing tremendously and the cost-effective production of erythromycin is greatly needed. One of the strategies to achieve this goal is to carry out optimization of medium recipe and its process, and further improve the erythromycin production and productivity.

In our previous work, a novel bioreactor system was designed to monitor mass flux based on metabolic flux analysis in a fermentation process, and successfully applied to the optimization and scale-up of some industrial fermentation process for penicillin, chlortetracycline, inosine and guanosine, etc. [4]. Some physiological parameters, for example, oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), and respiratory quotient (RQ), were used for assessing metabolic fluxes, and employed as an on-line metabolic indicator of the physiological state of the cells in the bioreactor, and designed some operation strategies for optimizing fermentation process [5, 6].

Statistic experimental designs have been used for several decades, which can be adopted at different phases of an optimization strategy, such as, for screening critical medium components or for looking for the optimal process conditions. The use of experimental factorial design and

response surface methodology (RSM) has been successfully applied to optimize media and cultural conditions in some fermentation processes for the production of primary and secondary metabolites [7–9]. In this work, we report for the first time a sequential optimization strategy of critical components for erythromycin production. First, fractional factorial design was employed to screen the most significant medium components affecting erythromycin production. Second, Box–Behnken design was applied to determine the optimum level of each of the significant medium components. According the profiles of on-line physiological parameter oxygen uptake rate (OUR), a novel feeding strategy was developed successfully to feed the optimum nutrient components for improvement of erythromycin production in a bioreactor with multi-parameter monitoring system. The information obtained in this work should be useful for the efficient production of erythromycin by the submerged fermentation of *S. erythraea* on a large scale.

Materials and methods

Microorganism and culture conditions

S. erythraea No.8 from HEC Biochem. Co. Ltd. producing erythromycin in submerged culture was used. Agar slants were inoculated with spores and incubated at 32 °C for 7 days, and then used as inoculum for seed culture. The seed culture was grown in a 500-ml shake flask containing 50 ml of liquid medium and incubated at 32 °C on a rotary shaker (220 rpm) for 7 days. The fermentation cultivation was inoculated at 10%(v/v) of the above seed culture medium and kept at 32 °C and 220 rpm.

Experimental design

Fractional factorial design

For screening purpose, five independent variables (i.e., $ZnSO_4$, Na_2MoO_4, citric acid, $CoCl_2$ and threonine) were screened in eight combinations organized according to the fractional factorial design in shake flasks. Table 1 displayed the five variables and their levels selected and the result of erythromycin production was shown in the Table 2. All trials were performed in triplicate and the average of erythromycin production observations were treated as responses.

Box–Behnken design

For elucidating the optimal concentrations of the most significant independent variables screened by fractional factorial design, a Box–Behnken design was applied which was a RSM. As presented in Table 3, $ZnSO_4$ (X_1), Citric acid (X_2), and threonine (X_3) were prescribed into three levels coded (−1, 0, +1). According to the applied design, fifteen combinations were executed and their experimental results were fitted with a second-order polynomial equation of Eq. (1) by a multiple regression technique.

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

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 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

Table 1 Process variables and levels in the fractional factorial designs

Factors (g/l)	Symbol	Coded variable level	
		−1	1
$ZnSO_4$	X_1	0.02	0.03
Na_2MoO_4	X_2	0.2	0.3
Citric acid	X_3	0.1	0.2
$CoCl_2$	X_4	0.01	0.02
Threonine	X_5	0.2	0.3

Table 2 Fractional factorial designs for 5 variables and 8 trials

Trial	X_1	X_2	X_3	X_4	X_5	Erythromycin (U/ml)
1	−1	−1	−1	1	1	3,276[a]
2	1	−1	−1	−1	−1	3,014
3	−1	1	−1	−1	1	3,140
4	1	1	−1	1	−1	3,220
5	−1	−1	1	1	−1	2,741
6	1	−1	1	−1	1	3,037
7	−1	1	1	−1	−1	2,437
8	1	1	1	1	1	3,032

[a] The data were calculated from three independent samples

Table 3 Process variables and levels in the Box–Behnken designs

Factors (g/l)	Symbol	Coded variable level		
		−1	0	1
$ZnSO_4$	X_1	0.02	0.03	0.04
Citric acid	X_2	0.1	0.3	0.5
Threonine	X_3	0.2	0.4	0.6

regression significance was tested by a t test. The SAS software (version 9.0 by SAS Institute Inc., NC, USA) was used for regression and graphical analyses of the data obtained.

Fed-batch fermentation in 50-l stirred bioreactors

The bioreactor used was a 30 l (working volume) agitated bioreactor with 3 six-bladed turbine impellers and equipped with devices to monitor and control more than 14 online measurable parameters, which was designed by Shanghai Guoqiang Bioengineering Equipment Co. Ltd, China [4]. The stirred reactor was aerated through a ring sparger. Dissolved oxygen (DO) level was set above 30% of air saturation and controlled by adjusting agitation speed and aeration rate during fermentation. The culture temperature and the inoculum size were the same as in shake flasks. Samples were taken every 8 h for the analyses of PMV, erythromycin production, NH_2–N, and total sugar concentration. A fed-batch process was applied to increase the flux of erythromycin biosynthesis, in which the above optimal nutrient components was fed in the bioreactor one trial and reached the predicted concentrations when the value of OUR began to fall down at 46 h. The reactor fermentation under each condition was repeated twice, and the data shown represent the mean values with the standard deviations.

Determination of cell biomass (PMV) and total sugar

For the determination of cell biomass (PMV), 10 ml ferment broth was taken as sample each time, after removal of supernatant by centrifugation (4,000g, 10 min), PMV was calculated as: the volume of precipitate/10-ml ferment broth. The total sugar level was assayed by the Fehling method.

Assay of erythromycin production

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 0.1 mol/l hydrochloric acid. The aqueous phase fraction was separated with great care, and further mixed with sulphate acid for 3 min. Its absorbance was measured at 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 cylinder plate assay method (China Pharmacopoeia 2005).

Results and discussion

Evaluation of the nutrient components affecting erythromycin production

The biosynthesis of erythromycin was involved in a universal central pathway of glucose catabolism [10]. Some divalent ions (Zn^{2+}, Mg^{2+}, Mn^{2+}, etc.) as a cofactor can regulate the enzyme activity of metabolic pathway [11, 12]. Many secondary metabolites were derived from amino acids which were usually used as precursor for enhancing production of antibiotics [13]. A total of five variables were analyzed with regard to their effects on erythromycin production using a fractional factorial design (Table 1). The design matrix selected for the screening of significant variables for erythromycin production and the corresponding results were shown in Table 2. The variables evidencing statistically significant effects were screened with ANOVA analysis (Table 4). The results of the P values test revealed that $ZnSO_4$, citric acid, and threonine were the most significant factors (Table 4). The optimum levels of the three variables were further determined by an RSM design.

Optimization of the critical nutrient components by Box–Behnken design

The design matrix and the corresponding results of RSM experiments to determine the effects of the three independent variables ($ZnSO_4$, citric acid, threonine) were shown in Table 5. Regression analysis was performed to fit the response function (erythromycin production) with the experimental data. From the variables obtained (Table 5), the model was expressed by Eq. (2), which represented erythromycin production (y) as a function of $ZnSO_4$ (X_1), citric acid (X_2), and threonine (X_3) concentrations.

$$\begin{aligned} y = {} & 2958.333 + 64.125X_1 - 7.125X_2 - 22.5X_3 \\ & + 78.20833X_1^2 - 165.25X_1X_2 + 157X_1X_3 \\ & - 106.2917X_2^2 + 119.5X_2X_3 - 337.0417X_3^2 \end{aligned} \quad (2)$$

Table 4 Analysis of variance (ANOVA) for fractional factorial designs

Parameters	DF	MS	F	Pr > F
X_1	1	62,658.00	6.88	0.119
X_2	1	7,140.13	0.78	0.469
X_3	1	246,051.10	27.04	0.035
X_4	1	51,520.50	5.66	0.140
X_5	1	143,648.00	15.79	0.058
Model	5	102,203.60	11.23	0.084
Error	2	9,098.56		

Table 5 Box–Behnken design for three independent variables with results

Trial	Variable			Erythromycin (U/ml)
	X_1	X_2	X_3	
1	−1	−1	0	2,734[a]
2	−1	1	0	2,972
3	1	−1	0	3,219
4	1	1	0	2,796
5	0	−1	−1	2,664
6	0	−1	1	2,302
7	0	1	−1	2,489
8	0	1	1	2,605
9	−1	0	−1	2,789
10	1	0	−1	2,577
11	−1	0	1	2,508
12	1	0	1	2,924
13	0	0	0	2,971
14	0	0	0	2,924
15	0	0	0	2,980

[a] The data were calculated from three independent samples

Table 6 Regression results from the data of response surface design (RSM) experiments

Parameters	Parameter estimate	*T* value	Pr > |t|
X_1	64.125	2.441	0.058
X_2	−7.125	−0.271	0.797
X_3	−22.500	−0.856	0.431
$X_1 \times X_1$	78.208	2.023	0.099
$X_1 \times X_2$	−165.250	−4.448	0.007
$X_1 \times X_3$	157.000	4.226	0.008
$X_2 \times X_2$	−106.292	−2.749	0.040
$X_2 \times X_3$	119.500	3.217	0.023
$X_3 \times X_3$	−337.042	−8.717	0.000

Table 7 Analysis of variance (ANOVA) of the model representing the erythromycin production according to the experimental design

Source	DF	SS	MS	*F*	Pr > *F*
Model	9	790,794.70	87,866.08	15.92	0.003
Linear	3	37,352.25	12,450.75	2.26	0.199
Quadratic	3	488,495.20	162,831.70	29.50	0.001
Cross product	3	264,947.30	88,315.75	16.00	0.005
Error	5	27,600.92	5,520.18		
Lack of fit	3	25,792.25	8,597.42	9.51	0.097
Pure error	2	1,808.67	904.33		
Total	14	818,395.60			

$R^2 = 96.63\%$, Adjusted $R^2 = 90.56\%$, *SS* sum of squares, *DF* degree of freedom, *MS* mean square

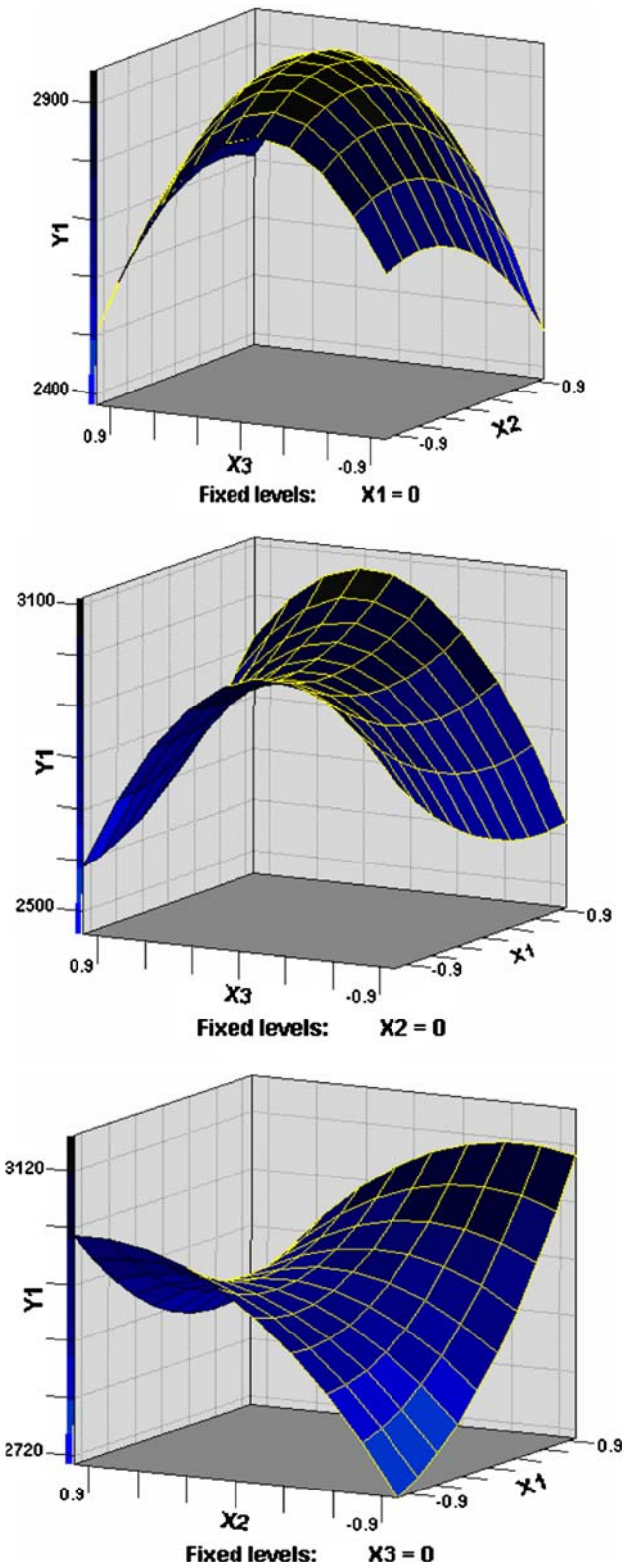

Fig. 1 The response of erythromycin production as a function of $ZnSO_4$ (X_1), citric acid (X_2) and threonine (X_3) based on the Box–Behnken experimental results

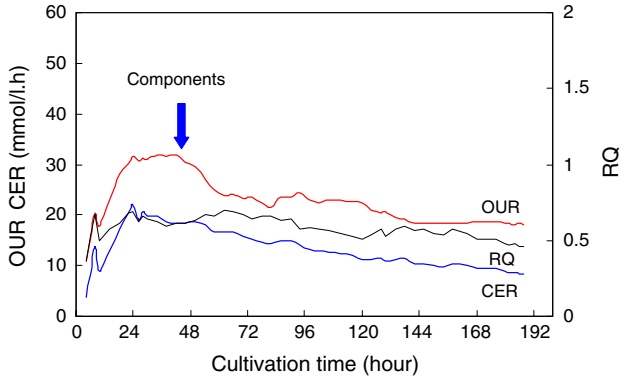

Fig. 2 Time course of physiological parameters OUR, CER, and RQ during submerged cultivation of S. *erythraea* in 50-l stirred bioreactor

The quadratic model in Eq. (2) with 9 terms contained 3 linear terms, 3 quadratic terms and 3 two-factorial interactions. The ANOVA analysis was evident from Table 6 that the model terms, X_1X_2, X_1X_3, *and* X_2X_3 were significant ($P < 0.05$). These results indicated that the interaction between variables were remarkable. The model presented a high regression coefficient ($R^2 = 0.9663$) from Table 7. This value indicated a high degree of correlation between the experimental and the predicted values. The experimental data were fitted into the Eq. (2), and the response surface plot was shown in Fig. 1 that the erythromycin production reached its maximum at a combination of coded level 0.948 (X_1, $ZnSO_4$), −0.302 (X_2, citric acid) and 0.093 (X_3, threonine) by ridge analysis of SAS software. The model predicted a maximum response of erythromycin 3,134 U/ml at levels of ZnSO4 0.04 g/l and citric acid 0.24 g/l and threonine 0.42 g/l as optimized nutrient components.

Feeding strategy in stirred bioreactor

Under these optimized conditions, an experiment was performed under the predicted conditions in 50-l stirred bioreactor. Time course of physiological parameters OUR, CER and RQ was shown in Fig. 2. After inoculation, OUR and CER increased quickly to the maximum value at 46 h, then decreased gradually until the end of fermentation. It was indicated that the cells grew quickly before 46 h, after that, the cells started to enter the stationary phase, and turned to secondary metabolite biosynthesis [14]. A novel feeding strategy based on OUR measurement, was developed successfully to increase erythromycin production, in which the optimized nutrient components was fed to the broth at 46 h when OUR began to decline on-line. Kinetics profiles of erythromycin biosynthesis under feeding the critical nutrient components are shown in Fig. 3. The maximum cell growth of feeding the critical components

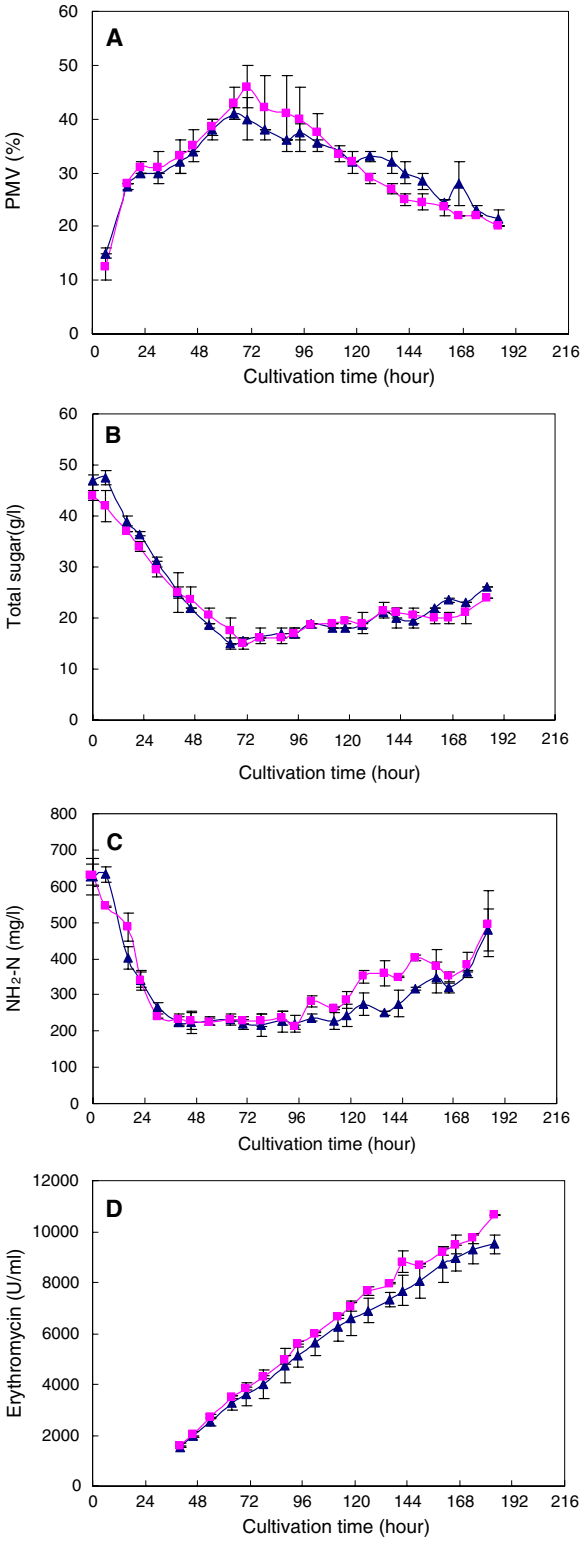

Fig. 3 Time course of cell growth by PMV (**a**), total sugar (**b**), NH_2–N (**c**) and erythromycin production (**d**) during submerged cultivation of S. *erythraea* with feeding critical nutrient components and control in 50-l stirred bioreactor. Symbols for feeding components (*filled square*) and control (*filled triangle*). The *error bars* in the figure indicate the standard derivations from two independent samples

was 46% at 70 h, which was higher than that (41% at 64 h) of the control. Compared with the control, the consumption of total sugar and NH_2–N were quite similar in the whole process of fermentation, whereas the rate of erythromycin biosynthesis was obviously higher than the control. The maximum erythromycin production reached 10,622 U/ml, which was 11.7% higher than the control under the same cultivation conditions. This indicated that the flux of secondary metabolites biosynthesis was enhanced by feeding the critical nutrient components.

Conclusions

Important parameter monitored on-line is a valuable method for optimizing the fermentation process. The oxygen uptake rate (OUR) is one of the pivotal physiological parameter, and characterizes the activity of microbial metabolism, which can be correlated with other parameters CER, RQ, and DO, etc., and was used for optimizing the fermentation course [4, 15]. During the guanosine fermentation course, the shifts of metabolic flux was observed by the correlation of OUR with other parameters [4]. Palomares [6] also reported that OUR can be utilized to access the role of glucose and glutamine in the metabolism of infected insect cell cultures. In order to increase the flux of erythromycin biosynthesis, a variety of nutrient components which were usually used as cofactors or precursors were selected to optimize in this work. The five variables were tested using fractional factorial design, and three variables (i.e., $ZnSO_4$, citric acid, threonine) exerted significant effects on erythromycin biosynthesis. Box–Behnken design was further employed to optimize critical nutrient components, and the optimum levels of each variable were as follows: $ZnSO_4$ 0.039 g/l, citric acid 0.24 g/l and threonine 0.42 g/l. A novel feeding strategy based on on-line parameter OUR was successfully developed to perform dynamic optimization in stirred bioreactor. The maximum erythromycin production was 10,622 U/ml, which was 11.7% higher than the control under the same cultivation conditions. The fundamental information obtained in this work should be favorable for further development of efficient process for erythromycin production on a large scale.

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