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Production Improvement of Epothilone B by *Sorangium cellulosum* by Mutation of Strain and Optimization of the Fermentation Process

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With the aim of obtaining the high producing strain of epothilones B, we mutated *Sorangium cellulosum* SoF5-76 with the combination treatment of UV rays and N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG) to improve the yields. Epothilone B yield was improved from 35.24mg/L to 79.83 mg/L of the broth resulting in a high yielding mutant. Then, Plackett-Burman design and the response surface methodology (RSM) were applied to optimize fermentation process of the high producing mutant. The obtained optimal fermentation conditions were potato starch 4.8 g / L, skim milk powder 2.3g / L, glucose 0.5g / L, soybean powder 2g / L, magnesium sulfate 2g / L, chlorine calcium 2g / L, EDTA-Fe^{3+} 2mL / L, trace elements 0.5mL / L, absorbent resin 2%, pH7.4, liquid volume 50mL/ 250mL, inoculation amount 8%, temperature 30°C. Under this optimal condition, epothilone B production reached up to 108.67mg / L that was 36.13% higher than preliminary conditions. It is the higher yield of epothilone B in the epothilone producing strains reported in literatures.

Key words: *Sorangium cellulosum*, Epothilone B, Mutation, Optimization, Response surface methodology.

Epothilones which are secondary metabolites naturally produced by the myxobacterium *Sorangium cellulosum* have attracted considerable attention in recent years as a novel anticancer agents[1-2]. They share a mechanism of action similar to that of antitumor drug paclitaxel (Taxol) in their ability to stabilize microtubule and lead to cell death[3-4]. And epothilones have superior features relative to Taxol. They are more water soluble, less toxic and more effective against tumors resistant to Taxol[5]. Therefore, epothilones are known as one of the most potential anti-tumor agent.

However, the low levels of epothilones produced by Sorangium cellulosum is a major obstacle to the development of epothilones as marketable drugs. Generally, yield improvement has been achieved in myxobacteria by coordinated strain improvement and process optimization[6]. Classical strain improvement has relied on mutation breeding of high-producing strains of epothilone B, heterologous expression of epothilone and recombinant DNA technologies have already improved the process of fermentation optimization. Although heterologous expression of epothilone have been successful[7-8], the yield of epothilone have not increased but reduced because of its toxic effects on the host cell. Hence, mutation breeding of high-producing strains is still the most effective method.

Process optimization is also important for yield improvement of epothilones. Different strategies can be used for the fermentation process optimization. Conventional "one -variable-at-a-time" approach was simple and used more often

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than other methods, but it is time-consuming and incapable of detecting the true optimum, especially to the interactions among the factors[9]. The statistical approach such as Plackett-Burman design and Box-Behnken design were proved to be useful and had been widely used to optimize the fermentation process of microorganisms[10-12].

In this paper, *Sorangium cellulosum* SoF5-76 obtained by genome shuffling improvement[13] was treated by combination mutagenesis of UV and NTG to capable of producing higher yields of epothilone B. Then fermentation process of the mutant was optimized by the statistical methods for the higher production.

MATERIALS AND METHODS

Chemicals

Most of the reagents for culture media were from recognized commercial suppliers, XAD-16 resin and the standard product of epothilone B was from Sigma (St. Louis, MO, USA). These were of either reagent grade (for culture media) or analytical grade (for analysis).

Strains, media and culture conditions

Sorangium cellulosum SoF5-76 is an epothilone B-producing strain obtained through genome shuffling improvement after screening from the soil and epothilone B production was 35.24mg/L[14]. The strain was routinely inoculated on M26 agar[15] at 30°C for 5 days and then cells of this strain were cultured in liquid M26 medium for 60 hours at 30°C and shaken at 170rpm in 250ml Erlenmeyer flask. This culture was used as the seed for fermentation. Then 10mL of seed culture was transferred into a 250 mLshake-flask containing 50ml fermentation medium(potato starch 3.9g / L, skim milk powder 2.2g / L, glucose 1g / L, soybean powder 1.5g / L, magnesium sulfate 2.5g / L, chlorine calcium 1.3g / L, EDTA-Fe^{3+} 3mL / L, trace elements 0.5mL / L, absorbent resin 2%, pH7.4). This culture was incubated for another 6d at 30° by shaking at 200 rpm.

Mutation method

UV mutagenesis: cells of the *Sorangium cellulosum* SoF5-76 train were cultured in liquid M26 medium for 36~48h, and then centrifuged, collected the cells and washed 2~3 times with physiological saline. After stirred in Erlenmeyer flask with glass beads, got cell suspension, the concentration of which was about 10^6 cells in a volume of 1.0 ml. The Petri dish with 5ml cell suspension was placed on magnetic stirrer and irradiated by UV light of 15 W placed at 30cm from it. After irradiation certain time (30, 60, 90, 120, 150s), inoculated in M26 medium and cultured for 2~4h without light. Then culture was diluted, patched onto VY/2 plates and incubated for 5 days at 30°C. Non-irradiated cell suspension was as a control, observed the growth and plotted mortality curves.

NTG mutagenesis, cells of the *Sorangium cellulosum* SoF5-76 train were cultured in liquid M26 medium for 36~48h, and then centrifuged, collected the cells and washed 2 times with phosphate buffer of 0.1mol/L and pH6.0. After stirred in Erlenmeyer flask with glass beads, got cell suspension, the concentration of which was about 10^6 cells in a volume of 1.0 ml. 5ml cell suspension was transferred into 10ml centrifuge tube, adding NTG solution, and the final concentration of NTG was 200, 400, 600, 800, 1000 ug/mL. After shaking for 30 min at 30°C, mutagenesis was immediately terminated by centrifugation and washing with cold physiological saline. Inoculated in M26 medium and cultured for 2~4h without light. Then culture was diluted, patched onto VY/2 plates and incubated for 5 days at 30. Non-irradiated cell suspension was as a control, observed the growth and plotted mortality curves.

Determination of epothilone B content

After incubation for 6 d at at 30°C by shaking at 200rpm, the Amberlite XAD-16 resin was collected from the fermentation culture, washed with water, air-dried, and extracted by 50 ml methanol with shaking for 24 hours. The extract was concentrated under vacuum at 40°C, and then redissolved in 500 ul methanol for HPLC analysis[16]. A waters-2487 HPLC System equipped with a YWG C_{18} column(250×4.6mm) was employed to analyze the epothilone B concentration. The mobile phase consisted of methanol/H_2O (65/35,v/v), and the flow-rate was 1.0ml/min. The wavelength of UV detector was set at 249nm and the volume of each injection was 20 ul.

Experiment design and data analysis

Plackett-Burman design

A Plackett-Burman design was used to determine the most important factors influencing

epothilone B production and remove the dispensable anes to conclude a smaller and more manageable set of factors[9]. The Plackett-Burman method allows evaluation of N variables in N+1 experiments and each factor was examined at two levels:(-1)for a low level and(+1) for a high level. In this study, 13 factors were tested, The experimental programme was designed using Statistical Analysis System, version 9.2(SAS 9.2). The yield of epothilone B was listed as the response variable.

Path of steepest ascent

In order to approach the optimal values region of the three most significant factors screened by Plackett-Burman design, path of steepest ascent can achieve. According the results of Plackett-Burman design, the steps size was designed, and the experimental design used for study is shown in Table 4.

Box-Behnken design

A Box-Behnken design was employed to optimize the three most significant factors screened by Plackett-Burman design for enhancing epothilone B production. The three factors were investigated at three diffirent levels (-1,0,+1) on the basis of the Steepest ascent experiment, and the experimental design used for study is shown in Table 6.

RESULTS AND DISCUSSION

Mutation breeding of high-producing strains of epothilone B

UV mutagenesis

To select the appropriate time of UV treatment, lethal effect of UV for different irradiation time on the strain was investigated. The results were shown in Fig. 1. Fatality rate of the strain showed an increasing tendency with the increasing of the irradiation time of UV and the value was closed to 100% when the irradiation time of UV was 80 s. Because the best mortality for screening mutant strain should be controlled within the range of 70% to 85%, so the optimal irradiation time of UV was determined as 50 s.

NTG mutagenesis

During NTG mutagenesis, firstly lethal effect of NTG concentration on the strain was examined when mutagenesis time was fixed at 30 min. Usually the concentration of NTG treatment with bacteria was within the range of 100~1000ug/mL[12]. Different concentration (200,400,600,800,1000 ug/ mL) of NTG was investigated and the results was shown in Fig. 2. Then lethal effect of mutagenesis time (10,30,50,70min) of NTG on the strain was examined in the preferred final concentration of NTG. The experimental results were shown in Fig 3.

Fig. 2 and 3 showed that fatality rate of the strain presented an increasing tendency with the increasing of the final concentration and treatment time of NTG. When the concentration of NTG was 800ug/mL, the fatality rate was closed to 100% and the value was 85% when the concentration of NTG was 400ug/mL. As to lethal effect of mutagenesis time of NTG on the strain, the fatality rate was 80% when treatment time of NTG was 30min. To obtain satisfactory results of mutagenesis, the final concentration and treatment time of NTG corresponding the fatality rate within the range of 80% to 85% was preferred. Hence, the optimal conditions of NTG mutagenesis were determined as final concentration of 400ug/mLand treatment time of 30min.

Combination mutagenesis with UV and NTG and genetic stability test

After combination mutagenesis with UV and NTG, the strain was inoculated into fermentation medium and carried out in the shake-flask cultures. Epothilone B production was determined by HPLC. Ultimately, a high epothilone B producing mutant SoF5-H23 was obtained and production was 79.83mg/L, which was 1.27 times higher than that of original strain SoF5-76(35.24 mg/L).

After 5 times continuous passage culture of the high epothilone B producing mutant SoF5-H23, fermentation experiment was carried out in the shake-flask cultures. Epothilone B production was determined by HPLC and the results were shown in Table 1. The results indicated that this mutant SoF5-H23 has good stability of producing epothilone B.

Optimization of fermentation conditions of epothilone B by mutant strain

Selection of significant variables by Plackett-Burman Design(PBD)

The importance of the thirteen factors, potato starch, skim milk powder, glucose, soybean powder, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, EDTA-Fe^{3+}, trace elements , XAD-16 resin, pH 7.4, liquid volume,

inoculum size, temperature for epothilone B production was investigated by PBD. The results showed the effects of these factors on the response and significant levels in Tables 2 and 3.

According to statistical analysis of the data by Statistical Analysis System (SAS) software, the results showed that potato starch (X_1), skim milk powder (X_4), pH (X_{12}) and temperature (X_{15}) had confidence levels above 95% (p<0.05) and were considered to influence epothilone B production significantly. Due to four factors would significantly increase the number of trials in response surface experiments, temperature (X_{15}) was less significant than other three significant factors, the three variables potato starch (X_1), skim milk powder (X_4), pH (X_{12}) were selected and their optimal levels were identified further using response suface methodology.

The path of steepest ascent

PBD results showed that the effect of potato starch and skim milk powder were positive (t>0), pH (X_{12}) was negative (t<0). Increasing the concentration of potato starch and skim milk powder and decreasing pH might result in higher production of epothilone B. Experimental design of the steepest ascent and corresponding results were showed in Table 4. The results showed that optimal levels of significant factors was between 0+2Δ and 0+3Δ, so level of 0+3Δ was selected as center level of Box-Behnken design.

Optimization of the factors with Box-Behnken experiments and Statistical analysis

Box-Behnken design (BBD) was employed to determine the optimal levels of the three selected

Table 1. Genetic stability of the strain SoF5-H23

Subculture times	1	2	3	4	5
Epothilone B yield (mg/L)	75.23	78.12	79.13	76.87	79.35

Table 2. The Plackett-Burman design variables (in clded levels) with Epothilone B as response

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	Epothilone B yield (mg/L)
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	93.71
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	86.29
3	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	45.74
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	52.85
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	96.36
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	72.26
7	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	75.13
8	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	89.47
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	51.73
10	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	80.15
11	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	57.13
12	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	68.18
13	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	54.02
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	46.58
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	58.29
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	43.19
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	66.52
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	65.37
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	42.37
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	38.56

factors that affected the production of epothilone B. The respective low and high levels with the coded levels for the variables are defined in Table 5, experimental design and results are shown in Table 6. The epothilone B yield displayed a considerable variation from 70.32 to 108.36 mg/L depending on the changes of variables. Based on the results of BBD experiments, a second-order polynomial regression model between epothilone B yield and the tested independent variables was derived by software of Statistical Analysis System (SAS) as follows(Equation 1):

$$Y=107.55+8.94X_1-6.33X_2-1.24X_3-12.59X_1^2-7.94X_1X_2-4.25X_1X_3-12.61X_2^2+3.3X_2X_3-11.83X_3^2 \quad ...(1)$$

Where Y represents the yield of epothilone B, and X_1, X_2 and X_3 are the coded values of the test variables potato starch, skim milk powder and pH.

The statistical significance of Equation (1) was evaluated by F-test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 7. The ANOVA of the quadratic regression model demonstrated that the model is statistically valid, evident from the high model *F*-value (32.49) and the very low *p*-value (0.0007) indicating the model was significant. The success of the model could be checked by the determination coefficient R^2 of 98.32% which was consistence with the adjusted R^2_{adj} of 95.29%. The R^2 value indicated that the total variation of 98.32% for epothilone B yield was attributed to the tested independent variables and could be explained by the model. Normally, the closer R^2-value is to 1, the better it predicts the response. The present R^2-value reflected a very good fitness between the observed and predicted responses. The coefficient

Table 3. Levels of variables and analysis of the main effect for Plackett-Burman.
** Statistically significant at 95% of confidence level*

No.	Variables	Level		*t*-value	*p*-value	Order of importance
		-1	1			
X_1	Potato starch (g/L)	3	4	24.82	0.0001	1*
X_2	Glucose (g/L)	0.5	1	-0.40	0.7161	11
X_3	Soybean powder (g/L)	1	2	0.65	0.5627	8
X_4	Skim milk powder (g/L)	1	2	12.79	0.0010	2*
X_5	Blank	-1	1	0.93	0.4222	
X_6	$MgSO_4 \cdot 7H_2O$ (g/L)	2	4	-1.91	0.1516	5
X_7	$CaCl_2$ (g/L)	1	2	1.04	0.3760	6
X_8	EDTA-Fe^{3+}(mL/L)	1.5	3	0.63	0.5749	9
X_9	Trace elements (mL/L)	0.5	1	-0.25	0.8194	12
X_{10}	XAD-16 resin (v/v)	2%	4%	0.52	0.6394	10
X_{11}	Blank	-1	1	-0.43	0.6967	
X_{12}	pH	7	9	-5.12	0.0144	3*
X_{13}	Liquid volume (mL)	40	50	0.98	0.3981	7
X_{14}	Inoculum size (v/v)	5%	10%	0.18	0.8708	13
X_{15}	Temperature (°C)	24	30	3.49	0.0396	4*
X_{16}	Blank	-1	1	1.42	0.2501	

Table 4. Experimental design and results of steepest ascent

Steplength	Potato starch (g/L)	Skim milk powder (g/L)	pH	Epothilone B yield (mg/L)
0	3	1	9	75.21
0+1Δ	3.5	1.5	8.5	84.50
0+2Δ	4	2	8	98.13
0+3Δ	4.5	2.5	7.5	103.27
0+4Δ	5	3	7	94.53
0+5Δ	5.5	3.5	6.5	79.76

Table 5. Experimental variables and levels for Box-Behnken design

	Factor	Level		
		-1	0	1
X_1	Potato starch /(g/L)	4	4.5	5
X_2	Skim milk powder /(g/L)	2	2.5	3
X_3	pH	7	7.5	8

Table 6. Design and results of Box-Behnken. *X_1=(x_1-4.5)/0.5 X_2=x_2-2.5/0.5 X_3=(x_3-7.5)/0.5

Run	X_1	X_2	X_3	Epothilone B yield (mg/L)
1	-1	-1	0	70.32
2	-1	1	0	73.06
3	1	-1	0	107.52
4	1	1	0	78.49
5	0	-1	-1	95.35
6	0	-1	1	83.05
7	0	1	-1	76.56
8	0	1	1	77.46
9	-1	0	-1	71.28
10	1	0	-1	94.23
11	-1	0	1	80.51
12	1	0	1	86.47
13	0	0	0	108.36
14	0	0	0	106.07
15	0	0	0	108.21

Table 7. ANOVA of regression model. *SS, sum of squares; DF, degree of freedom; MS, mean square

Source	DF	SS	MS	*F*	*Pr*>*F*
Model	9	2805.46	311.72	32.49	0.0007
Error	5	47.97	9.59		
Lack of fit	3	44.69	14.90	9.08	0.1008
Pure Error	2	3.28	1.64		
Total	14	2853.43			

R^2 =98.32%R^2_{adj}=95.29% *CV*=3.53

Table 8. Test of significance for regression coefficient

Term	Estimate	Std Err	*t*	*Pr* > | *t* |
X_1	8.94	1.10	8.17	0.0004
X_2	-6.33	1.10	-5.78	0.0022
X_3	-1.24	1.10	-1.13	0.3084
X_1^2	-12.59	1.61	-7.81	0.0006
X_1X_2	-7.94	1.55	-5.13	0.0037
X_1X_3	-4.25	1.55	-2.74	0.0407
X_2^2	-12.61	1.61	-7.82	0.0005
X_2X_3	3.3	1.55	2.13	0.0863
X_3^2	-11.83	1.61	-7.34	0.0007

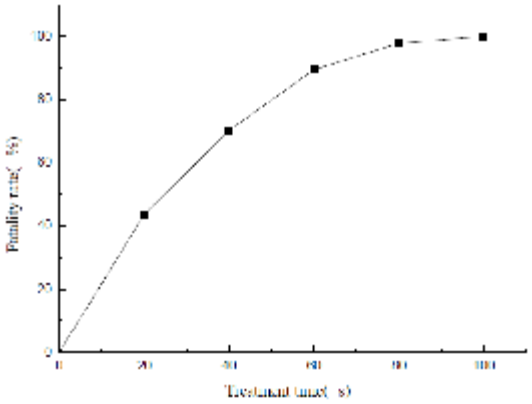

Fig. 1. Effect of UV on strain fatality rate

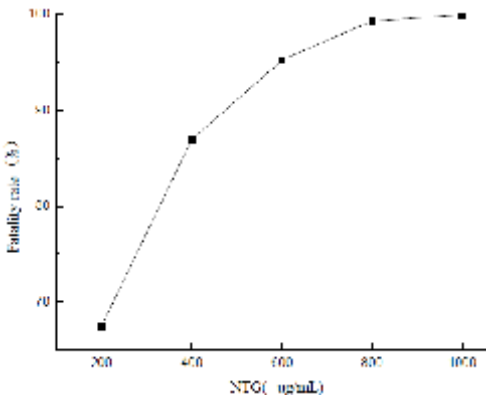

Fig. 2. Effect of concentration of NTG on strain fatality rate

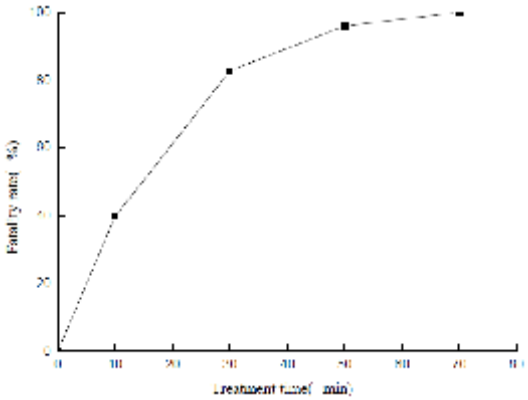

Fig. 3. Effect of mutagenesis time of NTG on strain fatality rate

of variation (*CV*) indicates the degree of precision with which the treatments were compared[17]. A low value of *CV* (3.53) showed the experiments conducted were precise and reliable[18] .The lack-of- fit measured the failure of the model to represent the data in the experiment domain at points which were not include in the regression[19]. The *p*-value for lack-of- fit (0.1008) indicated it is not significant relative to the pure error, which confirmed the validity of the model.

The significance of the regression coefficients was tested by a t-test and the results was shown in Table 8. The p values less than 0.05 indicate that model terms are significant while values greater than 0.1 shows that the model terms are not significant. Among the independent factors, linear term of potato starch X_1 and skim milk powder (X_2) were very significant. The positive coefficient X_1 showed a linear effect to increase epothilone B production while the negative coefficient X_2 had an inverse relation. The quadratic term and the interaction of the three variables also had a significant effect on epothilone B yield.

Response surface and contour plots analyses

The three-dimensional(3D) response surface and two-dimensional(2D) contour plots were plotted to explain the interactions of independent variables and the optimum values of tested variables required for epothilone B production(Figures 4-6). Each figure shows the effect of two independent variables while the third factor was fixed at zero level. The 2D contour plots provided a visual interpretation of the interaction between two variables. A circular contour plot indicates that the interactions between the corresponding variables are negligible. An elliptical nature of the contour plots shows that the interactions between the corresponding variables are significant[19].

By analyzing the 3D response surface and 2D contour plots, the corresponding point to the maximum of epothilone B production should locate on the peak of the response surface, which projected in the smallest ellipse in the contour diagram. According to ridge analysis by Statistical Analysis System (SAS) software, the optimal

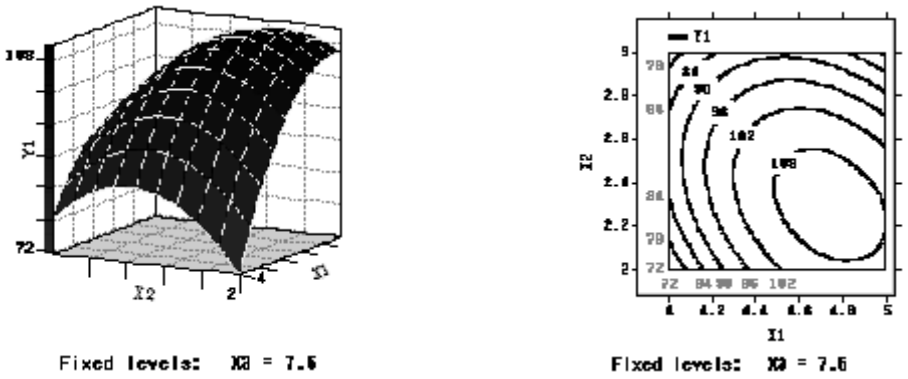

Fig. 4. Response surface plot and contour plot of the function Y= f(X_1, X_2)

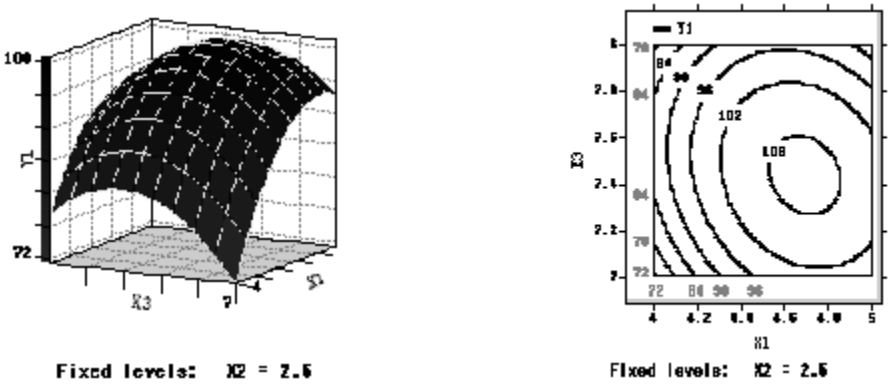

Fig. 5. Response surface plot and contour plot of the function Y= f(X_1, X_3)

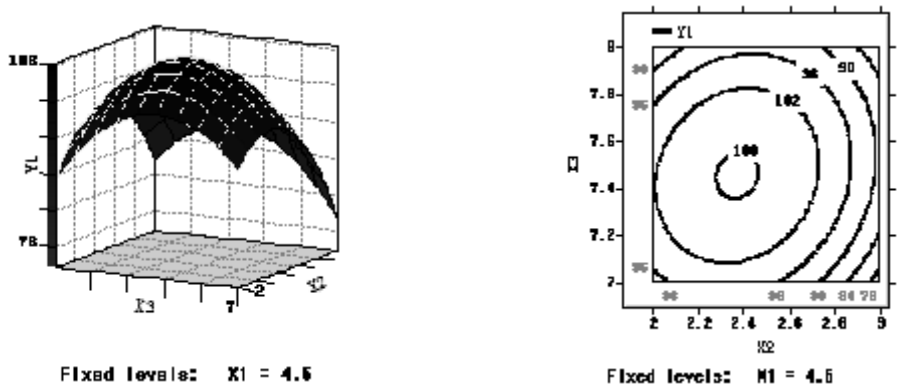

Fig. 6. Response surface plot and contour plot of the function Y= f(X_2, X_3)

values of the tested factors in their coded level were X_1=0.514228, X_2=-0.43058 and X_3=-0.20042. The actual corresponding value of potato starch, skim milk powder and pH were 4.7571g/L, 2.2847g/L and 7.3998. The maximum predicted value of epothilone B yield was 111.42mg/L.

Validation experiments

To verify the fitness of the model equation for predicting the optimum response value, validation experiments in shake flasks were carried out in triplicate tests under the determined optimum fermentation condition. The mean experimental value of epothilone B yield was 108.67mg/L which had a good agreement with the predicted value (111.42mg/L). This result shows a good predicting pattern of the model to the experimental data, confirming the model was validity and adequate for reflecting the expected optimization.

CONCLUSION

Strain breeding of industrial microbiology plays an important role in the fermentation industry, which is the key to determine the industrial productive value and the success of fermentation process. In this paper, to obtain the high epothilone B-producing strains, the starting strain were treated with combined mutation of UV and NTG.

A high epothilone B producing mutant SoF5-H23 was screened from *Sorangium cellulosum* SoF5-76 by combination treatment with UV and NTG. This mutant has good stability of producing epothilone B and production was 79.83mg/L, which was 1.27 times higher than that of original strain. Plackett-Burman design and the response surface methodology (RSM) were applied to optimize medium for fermentation and conditions. The obtained optimal fermentation conditions were potato starch 4.8 g / L, skim milk powder 2.3g / L, glucose 0.5g / L, soybean powder 2g / L, magnesium sulfate 2g / L, chlorine calcium 2g / L, EDTA-Fe^{3+} 2mL / L, trace elements 0.5mL / L, absorbent resin 2%, pH7.4, liquid volume 50mL/ 250mL, inoculum size 8%, temperature 30°C. Under this optimal conditions, epothilone B production reached up to 108.67mg / L which is the highest yield of epothilone B on home and overseas and was 36.13% higher than preliminary conditions.

In order to improve the yield of epothilone B, breeding of high epothilone B-producing strains made production epothilone B increased from 35.24mg/L to 79.83mg/L (increased 1.27-fold) and then the fermentation process was optimized. Under this optimal conditions, epothilone B production reached up to 108.67mg / L, improved 36.13%. The strain mutagenesis is a key to increase the production of epothilone B in whole process. Analysis of the reasons for the increase of epothilone B yield may be as follows: the starting strain of mutation improvement in this study was obtained by genome shuffling with four wild strains as the original strains [14] which contain more diverse genetic information. Positive mutation effect was cumulated by genome shuffling and may stand out by combination treatment with UV and NTG and fermentation optimization in this paper. Hence, we got high epothilone B yield. This paper shows that the traditional biological engineering technology still has a significant value in the field of industrial microorganisms.

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