African Journal of Biotechnology Vol. 10(53), pp. 11058-11070, 14 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1352
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Optimization of epothilone B production by *Sorangium cellulosum* using multiple steps of the response surface methodology

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Accepted 5 August, 2011

The anticancer compound epothilone B is biosynthesized by the myxobacterium *Sorangium cellulosum*; however, the fermentation characteristics for epothilone production in *Sorangium cellulosum* have not yet been reported. In this study, medium components for the production of epothilone B in the So0157-2 strain of *S. cellulosum* were statistically screened and optimized. First, the nutrients in the fermentation medium were optimized in one-factor-at-a-time, Plackett–Burman design, and Box–Behnken design experiments. Afterwards, three nutritional parameters were selected for the optimization of epothilone B production in shaking flask cultures using a central composite response surface methodology design; a polynomial equation model that related the medium components and epothilone B yield was established. The data were further analyzed using response surface plots and canonical mathematical model analyses with the SAS 8.0 software. After optimized, the yield of epothilone B increased to 82.0 ± 3 mg/l, 7.2-fold higher than the initial yield (11.3 ± 0.4 mg/l).

Key words: Myxobacterium, *Sorangium cellulosum,* epothilone B, statistical optimization, response surface methodology.

INTRODUCTION

Epothilones, which are naturally produced by the myxobacterium *Sorangium cellulosum* (Gerth et al., 1996; Höfle et al., 1996), are anticancer agents that mimic the anticancer mechanisms of paclitaxel (that is, microtubule stabilization) (Bollag et al., 1995; Kowalski et al., 1997). Up to now, there are at least five epothilones or chemically modified derivatives of epothilone undergoing evaluation in clinical trials (Figure 1) (Altmann, 2005; Larkin and Kaye, 2006), and Ixabepilone (BMS-247550) has already been authorized for clinical use by the U.S. Food and Drug Administration (Fornier, 2007).

However, the production efficiency of epothilones in *Sorangium* is poor. Several reports have attempted to improve the production of epothilones. For example, the gene cluster that encodes the epothilone production pathway has been genetically engineered into a *Myxococcus xanthus* strain (Julien and Shah, 2002); the production medium for epothilone D was thereafter optimized for the transgenic strain, and 85 mg/l in a 22-day semi-continuous fermentation was obtained (Frykman et al., 2005). Previously, we also made mutations in epothilone-producing *Sorangium* strains to improve production (Gong et al., 2007). However, the effects of culture medium components on the production of epothilones in natural *Sorangium* producers have not been reported, most likely due to the difficulties in manipulating *S. cellulosum* strains (Reichenbach and Dworkin, 1992). Myxobacteria are unique among bacteria because they have complex social living patterns; they feed in groups, move in swarms, germinate myxospores in a cell density-dependent manner, and develop multi-cellular fruiting bodies (Shimkets, 1990). In this study, we investigated the relationships between the production of epothilone B in *S. cellulosum* strain So0157-2 and the composition of the culture medium and further optimized the concentration for this compound.

In a conventional process optimization, factors that may be involved in production are initially screened and are then optimized using different techniques (Plackett and

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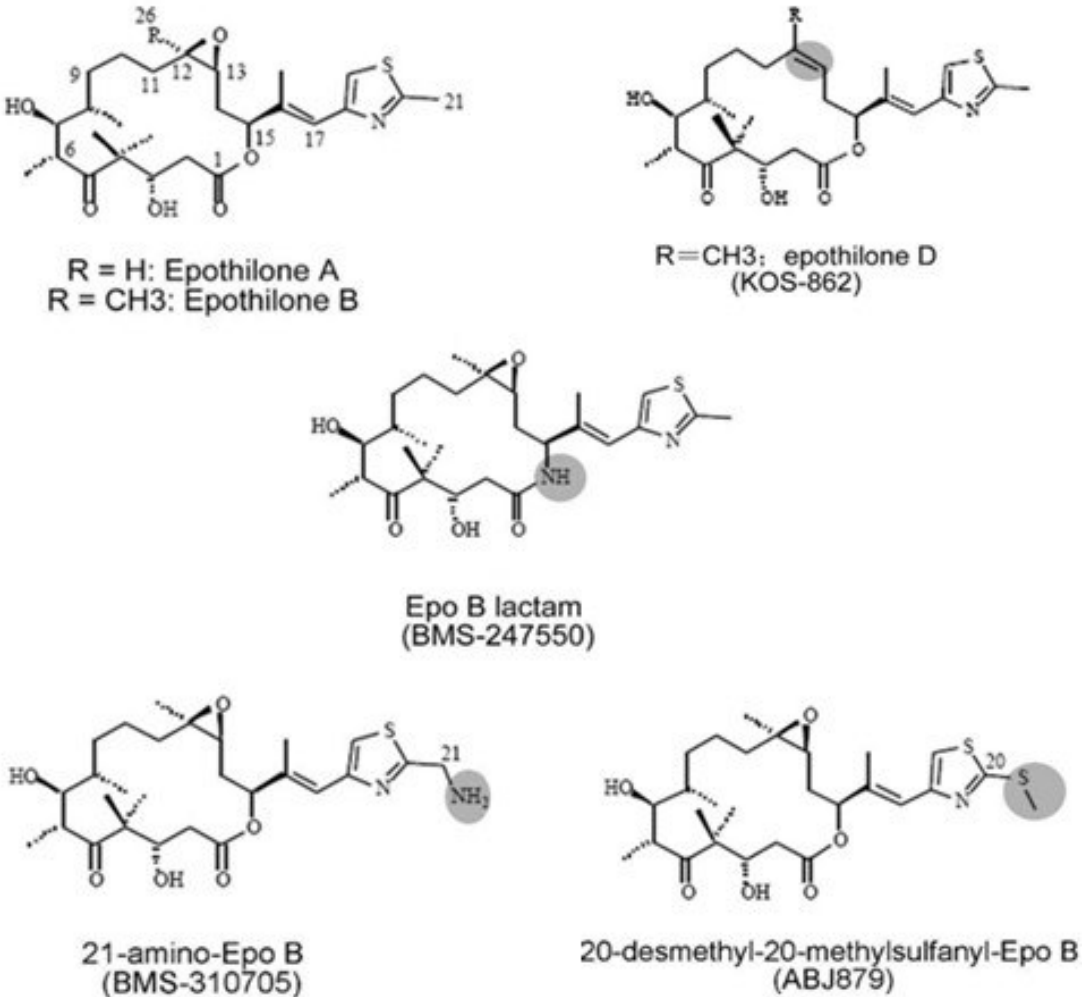


Figure 1. Epothilone and its structural analogues. Shadows show the modified groups based on the epothilone structure.

Burman, 1946; Box and Hunter, 1957; Lewis et al., 1999; Wang and Wan, 2009). The optimization techniques include non-statistical techniques, such as one-factor-at-a-time, and statistical techniques, such as the Plackett-Burman (PB) design, Box-Behnken (BB) design, fractional factorial design (FFD), and central composite design (CCD). The response surface methodology (RSM) is a statistical method that determines the relationships between at least one measured dependent response and a number of input factors. Compared to other optimization techniques, RSM has several advantages because it requires fewer experimental trials, is suitable for multiple factor experiments, searches for relationships between factors, will identify the most suitable conditions, and is able to predict responses; thus, RSM has been employed widely for the optimization of medium components (Kipan et al., 1999; Adinarayana and Ellaiah, 2002; Wang and Lu, 2004; Liu et al., 2005; Claucia et al., 2006; Sharma et al., 2007; Bajaj et al., 2009; Ghanem et al., 2009; Palaniyappan et al., 2009).

As the impact of nutrients for epothilone synthesis had not been investigated exhaustively, we assumed that the biosynthesis of epothilone by *S. cellulosum* So0157-2 would highly improve when we determine the best nutritional conditions. Furthermore, significant differences of antitumor activities between epothilone A and B, and their corresponding derivatives drive multidisciplinary studies to increase B ratio from the mixture (Rowinsky and Calvo, 2006), therefore, our objective was to optimize medium components for increasing the production of epothilone B in *S. cellulosum* So0157-2 in this study. The optimization steps were based on statistical design methods and included the following stages: (a) screening of multiple nutrient components in one-factor-at-a-time experiments; (b) elucidating the relationships between medium components and epothilone B production using a PB design; (c) optimizing the significant components using a BB design to produce a new medium; (d) elucidating the effects of the new medium on epothilone B production using a FFD; and (e)

Table 1. Designs and results of one-factor-at-one-time.

Carbon source	Epothilone B titer (mg/l)	Nitrogen source	Epothilone B titer (mg/l)	Other	Epothilone B titer (mg/l)
Dextrin	32.0±1.2	Fish flour peptone	7.9±0.3	Acetate	16.5±0.3
Soluble starch	24.4±0.5	Soy peptone	21.0±0.3	Propionate	17.9±0.5
Cottonseed powder	21.5±0.8	Casitone	11.0±0.5	Cysteine	20.5±0.7
Maltose	25.2±0.4	Tryptone	9.2±0.2	Serine	27.8±0.7
Lactose	28.9±1.2	Peptone	27.3±1.0	Threonine	27.6±1.2
Sucrose	33.1±1.2	Yeast powder	3.2±0.1		
Cellubiose	15.8±0.4	Instant dry yeast	25.2±1.0		
Glycerol	5.3±0.2	Cotton seed powder	28.9±1.0		

(Control) potato starch; glucose; soy powder; V_{B12} 11.3±0.4mg/l.

optimizing three significant nutritional parameters for the production of epothilone B using a CCD.

MATERIALS AND METHODS

Strain and growth conditions

Sorangium cellulosum So0157-2 is an epothilone-producing strain and is routinely cultured on M26 agar at 30°C (Nguimbi et al., 2003). For convenience, cells of this strain were cultured in liquid M26 medium for 3 days and then frozen in aliquots (Gong et al., 2007). Each cryo-vial contained 1×10^9 cells in a volume of 1.0 ml. The fermentation medium (EPM) (Gong et al., 2007) was used for the production of epothilone B. The EPM medium contained 2.0 g of potato starch, 2.0 g of glucose, 2.0 g of soy powder, 1.0 g of slim milk powder, 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of $CaCl_2$, 1.0 ml of EDTA-Fe^{3+} solution (0.01 g/l), 1.0 ml of trace element solution (Reichenbach and Dworkin, 1992), 0.5 mg of V_{B12}, 1000 ml of distilled water, and 2% (v/v) Amberlite XAD-16 resin at pH 7.2. One cryo-vial was used to inoculate 50 ml of M26 medium in a 250-ml Erlenmeyer flask, which was then agitated at 30°C for 3 days. This culture was used as the seed for fermentation. The composition of the production medium varied according to the experimental design. The pH of the medium was adjusted to 7.2 using a 20% KOH solution before autoclaving at 121°C for 20 min. A one-ml aliquot of the inoculum was transferred into each 250-ml flask containing 50 ml of production medium.

Determination of epothilone B production capacity

After shaking at 200 rpm at 30°C for 10 days, the Amberlite XAD-16 resin was harvested from the culture, washed with water, air-dried, and extracted with 50 ml of methanol. Next, the extract was concentrated under a vacuum at 40°C and then re-dissolved in 10 ml of methanol for HPLC analysis (Gong et al., 2007). Each experiment was performed three times.

One-factor-at-a-time experiment

The production of epothilone B by *Sorangium cellulosum* So0157-2 in EPM medium was 11.3 mg/l. To increase the yield of epothilone B, some nutrient sources (Table 1) were evaluated using the one-factor-at-a-time design based on EPM medium. The substitutes for the complex carbon source potato starch were dextrin, soluble starch and cottonseed powder. Sucrose, maltose, lactose, glycerol and cellubiose were selected as potential simple carbon substitutes for glucose. Complex nitrogen substitutes for soy powder included fish flour peptone, peptone, yeast powder, instant dry yeast, cottonseed powder, soy peptone, casitone and tryptone. In addition, acetate, propionate, cysteine, serine and threonine were used as the substitutes for V_{B12} at concentrations of 40 μ mol/l each. All results were obtained from triplicate experiments.

Plackett-Burman design

The PB design, a two-level experimental design method, is a popular method for process improvement in which the relevant factors are selected from a long list of multitudinous factors (Kennedy and Krouse, 1999). In this study, 17 factors (Table 2) were tested on the basis of single factor experiment results and the components of EPM medium for further culture medium optimization. Each factor was tested at high (+1) and low (–1) concentrations (Table 2). The experimental protocol was designed using Statistical Analysis System, version 8.0 (SAS 8.0). The yield of epothilone B was listed as the response variable. All experiments were performed in triplicate.

Box-Behnken design

From the aforementioned PB design experiments, three components—soy powder (X3), glucose (X4) and sucrose (X5) (for each, $p < 0.05$)—were selected for further optimization by BB design. The other factors were adjusted according to the *t*-test and *p*-values of the PB experiment, that is, 3.0 g of dextrin, 1.0 g of slim milk powder, 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of $CaCl_2$, 2 ml of EDTA-Fe^{3+} solution, 0.5 ml of trace element solution, 1000 ml of distilled water, and 2% (v/v) Amberlite XAD-16 resin at pH 7.2. The BB design in SAS 8.0 was used to optimize the concentration of the three factors selected from the PB experiment. Each factor was tested at three levels: sucrose, 0.04, 0.06, and 0.08% (ΔX_5 = 0.02%); glucose, 0.04, 0.06, and 0.08% (ΔX_4 = 0.02%); and soy powder, 0.15, 0.2, and 0.25% (ΔX_3 = 0.05%). All experiments were performed in triplicate. This part of the study included 15 experiments (Table 3). The response variable, epothilone B yield, was analyzed using SAS 8.0.

Fractional factorial design

Based on the aforementioned experiments, we obtained a preliminarily optimized medium (GSM): 3.0 g of dextrin, 0.5 g of sucrose, 0.8 g of glucose, 1.7 g of soy powder, 1.0 g of slim milk

Table 2. Code value of factors; estimate; standard error; *t*-value and *p*-value for the Plackett-Burman design.

Factor	Code value		Estimate	Standard error	*t*-value	*p*-value
	-1	1				
X1: (blank)			-1.0833	0.97587	-1.1101	0.3479
X2: Potato starch (%)	0.05	0.10	2.2333	0.97587	2.2886	0.1061
X3: Soy powder (%)	0.10	0.20	3.2167	0.97587	3.2962	0.0459
X4: Glucose (%)	0.05	0.06	4.3167	0.97587	4.4234	0.0215
X5: Sucrose (%)	0.05	0.06	3.4333	0.97587	3.5182	0.0390
X6: Dextrin (%)	0.10	0.30	2.7000	0.97587	2.7668	0.0698
X7: (blank)			2.3333	0.97587	2.391	0.0967
X8: Peptone (%)	0.05	0.10	-0.0667	0.97587	-0.068315	0.9498
X9: Cotton seed powder (%)	0.05	0.10	-1.8167	0.97587	-1.8616	0.1596
X10: Instant dry yeast (%)	0.05	0.10	-5.1667	0.97587	-5.2944	0.0131
X11: Slim milk powder (%)	0.10	0.20	-0.2000	0.97587	-0.20495	0.8507
X12: $MgSO_4 \cdot 7H_2O$ (%)	0.38	0.75	-1.0167	0.97587	-1.0418	0.3741
X13: $CaCl_2$ (%)	0.15	0.30	-1.5000	0.97587	-1.5371	0.2219
X14: EDTA-Fe^{3+} (mg/l)	1.0	2.0	2.4667	0.97587	2.5277	0.0856
X15: (blank)			-1.6000	0.97587	-1.6396	0.1996
X16: Trace element solution (mg/l)	1.0	2.0	-2.6500	0.97587	-2.7155	0.0728
X17: pH	7.0	9.0	-1.9500	0.97587	-1.9982	0.1396
X18: Serine (μ mg/l)	40	80	1.2667	0.97587	1.298	0.2851
X19: Threonine (μ mg/l)	40	80	-4.0667	0.97587	-4.1672	0.0251
X20: XAD-16 resin (%)	2	4	-2.1667	0.97587	-2.2202	0.1130

powder, 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of $CaCl_2$, 2 ml of EDTA-Fe^{3+} solution, 0.5 ml of trace element solution, 1000 ml of distilled water, and 2% (v/v) Amberlite XAD-16 resin at pH 7.2. Furthermore, the concentrations of 10 factors (Table 4) in this medium were optimized using the FFD in SAS 8.0. Each factor was tested at high (+1) and low (–1) concentrations, which were a 1.25-fold increase or decrease, respectively (Table 4). All experiments were performed in triplicate. The epothilone B yield was used as the response variable. Three components, dextrin (X2), slim milk powder (X6) and $MgSO_4 \cdot 7H_2O$ (X8), each with *p*-values < 0.05, were selected for further optimization using the CCD.
8.5

Central composite design

A five-variable CCD was used to optimize the important variables selected by the FFD. Table 5 lists the design matrix of the experiment according to the 2^3 full factorial design. The central points of these three components were 0.24% dextrin (X2), 0.12% slim milk powder (X6) and 0.08% $MgSO_4 \cdot 7H_2O$ (X8), and the rest of the factors were as follows: 0.5 g of sucrose, 0.8 g of glucose, 1.7 g of soy powder, 1.0 g of $CaCl_2$, 2 ml of EDTA-Fe^{3+} solution, 0.5 ml of trace element solution, 1000 ml of distilled water, and 2% (v/v) Amberlite XAD-16 resin at pH 7.2. The epothilone B yield was used as the response variable in the different cycles of runs and was analyzed using SAS 8.0. Twenty experiments were performed in triplicate, and each of the central points was repeated six times.

RESULTS

Modifying nutrient components based on EPM medium

Table 1 lists the effects of different nutrient components on epothilone B production in the one-factor-at-a-time experiments. Dextrin and sucrose were the best carbon sources for epothilone B production. These two carbon sources, as well as potato powder and glucose, were used in further PB design optimization experiments. Suitable nitrogen sources for the production of epothilone B included soy peptone, soy powder, instant dry yeast and cottonseed powder; these were used as nitrogen sources in further optimization experiments. Accordingly, 17 factors (Table 2), seven from the above one-factor-at-a-time experiments (dextrin, sucrose, peptone, instant dry yeast, cottonseed powder, serine, and threonine) and 10 from the components of the EPM culture medium, were used in further PB design optimizations.

Table 3. Box–Behnken design and results.

Run	X5: Sucrose (%)	X4: Glucose (%)	X3: Soy powder (%)	Epothilone B titer(mg/l)
1	0.04	0.04	0.20	34.1
2	0.04	0.06	0.15	47.5
3	0.04	0.06	0.25	26.5
4	0.04	0.08	0.20	44.9
5	0.06	0.04	0.15	36.8
6	0.06	0.04	0.25	27.3
7	0.06	0.08	0.15	42.8
8	0.06	0.08	0.25	32.6
9	0.08	0.04	0.20	36.5
10	0.08	0.06	0.15	42.3
11	0.08	0.06	0.25	24.7
12	0.08	0.08	0.20	44.4
13	0.06	0.06	0.20	43.8
14	0.06	0.06	0.20	43.8
15	0.06	0.06	0.20	43.3

Table 4. Code value of factors; estimate; standard error; *t*-value and *p*-value for fractional factorial experiment.

Factor	Code value		Estimate	Standard error	*t*-value	*p*-value
	-1	1				
X1: (blank)			0.775	2.81297	0.2755	0.8088
X2: Dextrin (%)	0.24	0.375	26.525	2.81297	9.4295	0.0111
X3: Sucrose (%)	0.04	0.063	11.35	2.81297	4.0349	0.0563
X4: Glucose (%)	0.064	0.1	5.775	2.81297	2.0530	0.1765
X5: Soy powder (%)	0.136	0.213	-3.875	2.81297	-1.3776	0.3022
X6: Slim milk powder (%)	0.08	0.125	-12.2	2.81297	-4.3371	0.0493
X7: (blank)			-3.3	2.81297	-1.1731	0.3615
X8: $MgSO_4 \cdot 7H_2O$ (%)	0.08	0.125	13.375	2.81297	4.7548	0.0415
X9: $CaCl_2$ (%)	0.08	0.125	-1.275	2.81297	-0.4533	0.6948
X10: EDTA-Fe^{3+} (ml/l)	1.6	2.5	-7.6	2.81297	-2.7018	0.1140
X11: Trace element solution (ml/l)	0.4	0.625	0.1	2.81297	0.0356	0.9749
X12: pH	7	9	-3.25	2.81297	-1.1554	0.3673
X13: (blank)			-6.025	2.81297	-2.1419	0.1655

Screening important factors for epothilone B production by the Plackett–Burman design

In the PB design, epothilone B production was used as the response variable (Table 6). According to the probabilities (Table 2) ($p < 0.05$), two factors (instant dry yeast and threonine) significantly negatively influenced production ($t < 0$). The epothilone B yield was reduced when these two factors were included in the culture medium. Factors that positively influenced the production of epothilone B with a high probability ($p < 0.05$) included soy powder, glucose and sucrose; these were selected for further optimization by RSM. According to the *t*-tests and *p*-values, the other medium components, including potato powder, dextrin, peptone, cottonseed powder, slim milk powder, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, EDTA-Fe^{3+}, trace element solution, pH, serine and XAD-16 resin, did not significantly influence epothilone B production ($p > 0.05$).

Determining elements of the GSM medium by the Box–Behnken(BB) design

To detect combination effects, BB experiments were performed (Table 3) according to the above PB design. The production of epothilone B (the response variable)

Table 5. Central composite design and results.

Run	X2: dextrin (%)	X6: slim milk powder (%)	X8: $MgSO_4 \cdot 7H_2O$ (%)	Epothilone B titer (mg/l)
1	0.12	0.06	0.04	27.4
2	0.12	0.06	0.12	31.5
3	0.12	0.18	0.04	22.1
4	0.12	0.18	0.12	21.8
5	0.36	0.06	0.04	45.9
6	0.36	0.06	0.12	50.9
7	0.36	0.18	0.04	72.3
8	0.36	0.18	0.12	65.6
9	0.038	0.12	0.08	13.1
10	0.44	0.12	0.08	73.0
11	0.24	0.019	0.08	51.9
12	0.24	0.22	0.08	45.9
13	0.24	0.12	0.013	55.2
14	0.24	0.12	0.15	59.6
15	0.24	0.12	0.08	41.5
16	0.24	0.12	0.08	48.9
17	0.24	0.12	0.08	55.6
18	0.24	0.12	0.08	53.2
19	0.24	0.12	0.08	54.9
20	0.24	0.12	0.08	54.6

from the BB experiments was used to establish the following polynomial model through a multiple regression analysis, and the statistical significance of the model was verified with a variance analysis using SAS 8.0.

$$Y=43.63–0.64\times X_5+3.75\times X_4–7.28\times X_3–1.64\times X_5\times X_5–0.73\times X_4\times X_5–2.01\times X_4X_4+0.85\times X_3\times X_5–0.18\times X_3\times X_4–6.74\times X_3\times X_3$$

Where, Y is the epothilone B production; X_3 is the soy power; X_4 is the glucose and X_5 is the sucrose.

The high *F*-value and the very low probability (Table S1 (Appendix)) ($p < 0.05$) indicates that the experimental model agrees well with the experimental results. Furthermore, the response surface regression showed that the linear coefficient of the polynomial model is highly significant ($p < 0.05$), but the quadratic coefficient has little significance ($p > 0.05$); the cross coefficient of the medium components was not significantly associated with production ($p >> 0.05$). The determination coefficient ($R^2 = 0.92$) in the experimental model suggested that experimental predictions agreed well with results. The precision and reliability of the experiments were confirmed by the relatively low coefficient of variation (CV = 9.21%).

The significance of each coefficient in the experimental model was determined by the *t*-value and the *p*-value using SAS 8.0 (Table S2 (Appendix)). High *t*-values and low *p*-values indicate that glucose and soy powder had highly significant effects on epothilone B production; however, the effect of sucrose was not significant. Student's *t*-test of each coefficient of the model showed that two linear and one quadratic coefficients had significant effects ($p < 0.05$), but the interactive effect of the three factors was not significant. Comparison of the coefficients in the experimental model also revealed descending levels of significance for the three factors: soy powder > glucose > sucrose.

The effect of these three medium components on epothilone B production was further analyzed using 3D response surface plots, which are graphical representations of the regression model. By simulating the experimental results using the empirical model, these plots efficiently identified the optimal values for the variables. From these plots, it is straightforward to determine the interactions between any two factors and to locate their optimum levels (Figure 2). When epothilone B production was observed as a response variable for the interaction of glucose and sucrose as variables and soy powder as the central point, there is an enhancement in epothilone B production at glucose and sucrose concentrations between the central and maximal levels (Figure 2a). Because epothilone B production decreased beyond this range, the maximal epothilone B production could be obtained at the optimal values of glucose and sucrose. The same procedure was followed for other culture medium components to determine the optimal values of each component (Figure 2b and 2c). Therefore, the experimental model has a stationary point, and the predictive yield of epothilone B is the maximal

Table 6. Plackett-Burman design and results.

[a]Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17	X18	X19	X20	Epothilone B titer (mg/l)
1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	16.5
2	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	11.8
3	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	19.7
4	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	24.9
5	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	18.4
6	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	27.6
7	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	37.5
8	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	31.5
9	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	23.6
10	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	1	18.4
11	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	-1	24.4
12	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	18.4
13	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	19.7
14	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	18.4
15	-1	1	-1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	27.6
16	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	20.5
17	1	-1	1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	-1	22.3
18	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	-1	19.2
19	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	-1	13.1
20	-1	1	1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1	22.3
21	-1	-1	-1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	1	14.4
22	-1	-1	1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	1	15.2
23	-1	-1	-1	-1	1	-1	1	-1	-1	1	1	-1	-1	1	1	-1	1	-1	1	1	14.4
24	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	21.8

a: X1: (blank); X2: Potato starch; X3: Soy powder; X4: Glucose; X5: Sucrose; X6: Dextrin; X7: (blank); X8: Peptone;X9: Cotton seed powder; X10: Instant dry yeast; X11: Slim milk powder; X12: $MgSO_4 \cdot 7H_2O$; X13: $CaCl_2$; X14: EDTA-Fe^{3+};X15: (blank); X16: Trace element solution; X17:pH; X18: Serine; X19: Threonine; X20: XAD-16 resin.

Table S1. The results of the regression analysis of Box–Behnken design.

Source	DF	Sum of square	*F*-value	*p*-value
Model	9	725.7332	6.55	0.0261
Linear	3	540.6125	14.64	0.0066
Quadratic	3	180.0057	4.87	0.0604
Cross product	3	5.1150	0.14	0.9328
Error	5	61.5642		
Lack of fit	3	61.3975	245.59	0.0041
Pure error	2	0.16667		
Total	14	787.2973		

R^2 =0.9219, Coefficient of variation (CV) =9.2133.

Table S2. Student's *t*-test of Box–Behnken design.

Parameter	Estimate	Standard error	*t*-value	*p*-value
INTERCEPT	43.63	2.03	21.54	<0.0001
X5	-0.64	1.24	-0.51	0.6292
X4	3.75	1.24	3.02	0.0293
X3	-7.29	1.24	-5.87	0.0020
X5*X5	-1.64	1.83	-0.90	0.4099
X4*X5	-0.73	1.75	-0.41	0.6966
X4*X4	-2.02	1.83	-1.10	0.3197
X3*X5	0.85	1.75	0.48	0.6485
X3*X4	-0.18	1.75	-0.10	0.9244
X3*X3	-6.74	1.83	-3.69	0.0141

value at the stationary point.

The predicted maximal epothilone B yield and the coded value for each factor were obtained by a canonical analysis of the response surface using SAS. The coded values for the three factors sucrose, glucose and soy powder were -0.581, 1.060 and -0.591, respectively and the predicted epothilone B production was 48.0 mg/l. After translating these coded values, the concentrations of sucrose, glucose and soy powder were calculated as 0.5, 0.8 and 1.7 g/l, respectively; validation experiments were performed in triplicate in shaking flasks, and the resulting yield, 46.5 ± 1.2 mg/l epothilone B, indicated that the experimental model could be employed to predict epothilone B production.

Screening for key factors of GSM medium by fractional factorial design

To search for the optimal GSM medium components for epothilone B production, experiments were designed using the FFD, in which epothilone B yield was used as the response variable (Table 7). The positive influence factors on epothilone B production ($p < 0.05$) included dextrin (X2), slim milk powder (X6) and $MgSO_4 \cdot 7H_2O$ (X8), which were selected for further optimization in CCD (Table 4). In addition, the results of the response surface curvature analysis showed that all variables had significant effects on the yield of epothilone B and that the value of each variable was in the maximum response region. Therefore, we determined that the central point of the CCD was 0.24% dextrin (X2), 0.12% slim milk powder (X6) and 0.08% $MgSO_4 \cdot 7H_2O$ (X8) , instead of the path with the steepest ascent. Because the remaining factors (sucrose, glucose, soy powder, $CaCl_2$, EDTA-Fe^{3+}, trace element solution and pH) did not significantly influence epothilone B production ($p > 0.05$), the concentrations of these factors were not changed in the next experiments.

Determining the optimal medium by central composite design

To observe the effects of combinations of components, experiments were designed using a CCD (Table 5), according to the results obtained via FFD. The results from the variance analysis were obtained (Table S3 (Appendix)). The regression equation showed that the epothilone B yield is an empirical function of the test variables in coded units:

$$Y=51.69+17.03\times X_2+1.17\times X_6+0.70\times X_8-4.55\times X_2\times X_2+7.01\times X_2\times X_6-2.48\times X_6\times X_6-0.69\times X_2\times X_8-2.01\times X_6\times X_8+0.52\times X_8\times X_8$$

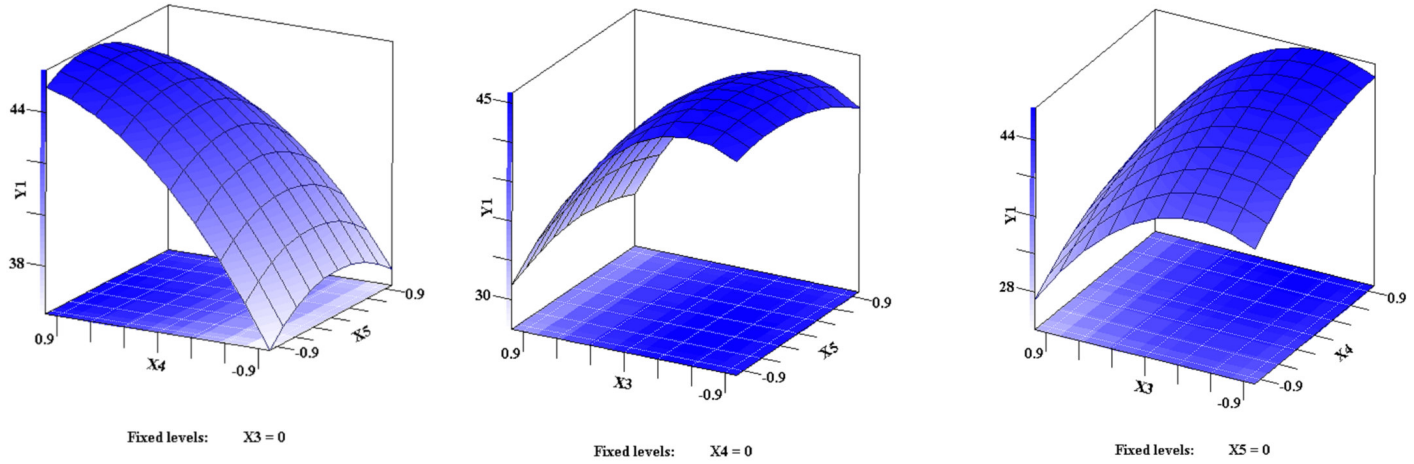


Figure 2. Response surface plots of glucose (X4) and sucrose (X5) with soy powder (X3) as the central point (A), soy powder (X3) and sucrose (X5) with glucose (X4) as the central point (B), and soy powder (X3) and glucose (X4) with sucrose (X5) as the central point (C). Y1: epothilone B production (mg/l).

Where; Y is the epothilone B production; X_2 is the dextrin; X_6 is the slim milk powder and X_8 is the $MgSO_4 \cdot 7H_2O$.

The high *F*-value and the very low probability ($p < 0.05$) indicated that the experimental model agrees well with the experimental results. The response surface regression showed that the linear coefficient of the polynomial model is highly significant ($p < 0.05$), while the cross product and quadratic coefficient are less significant ($p > 0.05$). This suggests that there are some subtle interactions among the three factors. The coefficient of determination ($R^2 = 0.92$) in the experimental model also suggests that experimental predictions agreed well with results. The precision and reliability of the experiments were confirmed by the relatively low coefficient of variation (CV = 13.84%).

The significance of each coefficient in the experimental model was determined by the *t*-values and the probabilities (*p*-value) using SAS 8.0 (Table S4 (Appendix)). Student's *t*-test of each coefficient of the model showed that one linear, one quadratic and one cross product coefficient had significant effects on epothilone B production ($p < 0.05$). Dextrin had a significant effect on the production of epothilone B. There is some interaction between dextrin and slim milk powder for epothilone B production. Comparing the value of each coefficient in the experimental model also revealed descending significances of the three factors, from dextrin to slim milk powder to $MgSO_4 \cdot 7H_2O$. Accordingly, 3D graphs were generated for the pairwise combination of the three factors by keeping the third factor as the central point (Figure 3). By simulating the experimental results using the empirical model, these plots efficiently identified the optimal values for the variables. When epothilone B production was observed as a response variable with the interaction between dextrin and slim milk powder as variables with $MgSO_4 \cdot 7H_2O$ at the central point, there was an enhancement in epothilone B production at dextrin and slim milk powder concentrations between the central and the maximal levels (Figure 3a). The maximal epothilone B production could be obtained at the optimal values of dextrin and slim milk powder. The same procedure (Figure 3b and 3c) was used to determine the optimal values for each medium component. Therefore, the experimental model had a stationary point, and the predicted yield of epothilone B was maximal at the stationary point.

The predicted maximal epothilone B production

Table 7. Fractional factorial design and results.

[b]Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	Epothilone B titer(mg/l)
1	-1	-1	-1	-1	1	-1	-1	1	-1	1	1	-1	1	33.1
2	1	-1	-1	-1	-1	-1	1	1	1	1	-1	1	1	32.8
3	-1	1	-1	-1	-1	1	-1	1	1	-1	1	1	-1	59.5
4	1	1	-1	-1	1	1	1	1	-1	-1	-1	-1	-1	62.3
5	-1	-1	1	-1	-1	1	1	-1	-1	1	1	1	-1	26.1
6	1	-1	1	-1	1	1	-1	-1	1	1	-1	-1	-1	23.4
7	-1	1	1	-1	1	-1	1	-1	1	-1	1	-1	1	63.6
8	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	66.5
9	-1	-1	-1	1	-1	1	1	-1	1	-1	-1	-1	1	20.1
10	1	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	21.4
11	-1	1	-1	1	1	-1	1	-1	-1	1	-1	1	-1	50.5
12	1	1	-1	1	-1	-1	-1	-1	1	1	1	-1	-1	65.3
13	-1	-1	1	1	1	-1	-1	1	1	-1	-1	1	-1	63.1
14	1	-1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	64.3
15	-1	1	1	1	-1	1	-1	1	-1	1	-1	-1	1	71.3
16	1	1	1	1	1	1	1	1	1	1	1	1	1	57.5

b: X1: (blank); X2: Dextrin; X3: Sucrose; X4: Glucose; X5: Soy powder; X6: Slim milk powder; X7: (blank); X8: $MgSO_4 \cdot 7H_2O$; X9: $CaCl_2$; X10: EDTA-Fe^{3+} X11: Trace element solution; X12: pH; X13: (blank).

Table S3. The results of the regression analysis of central composite design.

Source	DF	Sum of square	*F*-value	*p*-value
Model	9	4796.13	12.47	0.0002
Linear	3	3988.30	31.10	<0.0001
Quadratic	3	378.25	2.95	0.0848
Cross product	3	429.58	3.35	0.0638
Error	10	427.46		
Lack of fit	5	279.84	1.90	0.2498
Pure Error	5	147.6150		
Total	19	5223.59		

R^2=0.9182; Coefficient of variation (CV) =13.8386.

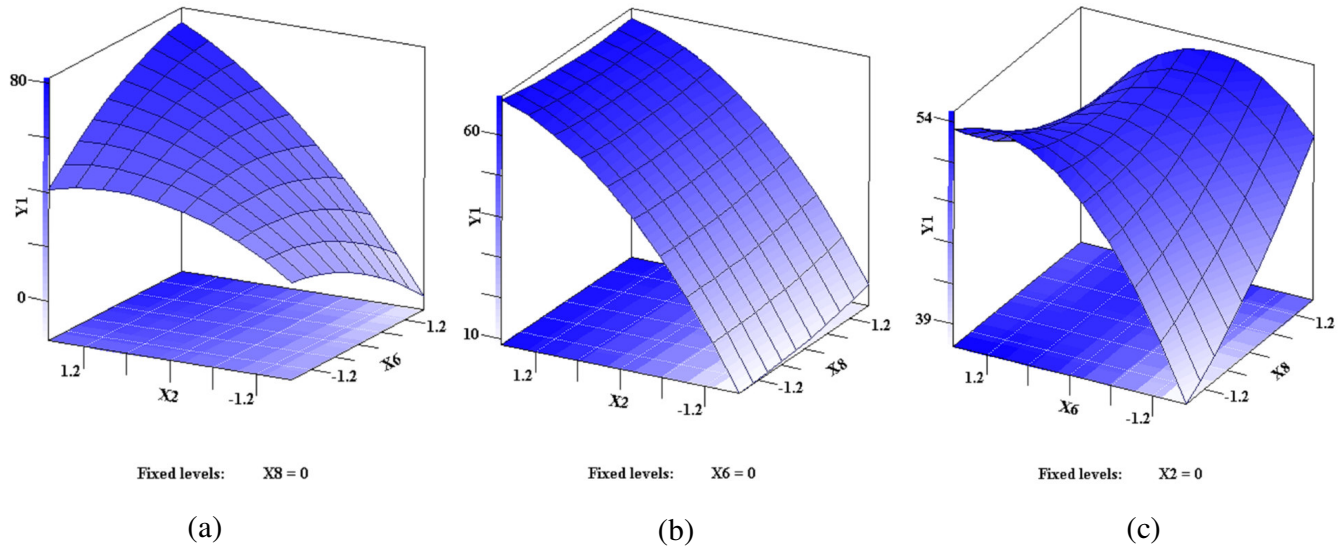
and the coded value for each factor were obtained by a canonical analysis of the response surface using SAS. The coded values of the three factors, dextrin, slim milk powder and $MgSO_4 \cdot 7H_2O$, were 3.240, 2.367 and 6.029, respectively, and the predicted epothilone B production was 82.8 mg/l. After translating these coded values, the concentrations of dextrin, slim milk powder and $MgSO_4 \cdot 7H_2O$ were calculated as 6.3, 2.6 and 3.2 g/l, respectively. The calculated optimum conditions for epothilone B yield were verified by culturing So0157-2 in the optimized conditions in triplicate shaking flasks. Under these conditions, So0157-2 produced an epothilone B yield of 82.0 ± 3 mg/l, which agreed with the predicted value of 82.8 mg/l. These data suggest that the model is valid for optimizing epothilone B yield.

DISCUSSION

Generally, each organism has its own nutritional requirement for compound production (Elibol, 2004). The yield of epothilones in *Sorangium* strains is low (Gerth et al., 1996). Probably because of manipulation limitations (Frykman et al., 2002; Julien and Shah, 2002; Park et al., 2008), there is no report regarding to the optimization of media to improve the epothilone production in the natural *Sorangium* producers. In the past decade, researchers attempted to bypass the limitation by heterologously expressing the epothilone biosynthetic genes in other fermentation-friendly hosts, such as *Streptomyces coelicolor* (Tang et al., 2000), *Escherichia coli*. (Mutka et al., 2006) or *Myxococcus xanthus* (Julien and Shah,

Table S4. Student's *t*-test of central composite design.

Parameter	Estimate	Standard error	*t*-value	*p*-value
INTERCEPT	51.69	2.6665	19.46	<0.0001
X2	17.03	1.7692	9.63	<0.0001
X6	1.17	1.7692	0.66	0.5226
X8	0.70	1.7692	0.39	0.7024
X2*X2	-4.55	1.7223	-2.64	0.0246
X6*X2	7.01	2.3115	3.03	0.0126
X6*X6	-2.48	1.7223	-1.44	0.1799
X8*X2	-0.69	2.3115	-0.30	0.7722
X8*X6	-2.01	2.3115	-0.87	0.4044
X8*X8	0.52	1.7223	0.30	0.7680



(a) (b) (c)

Figure 3. Response surface plots and contour plots of dextrin (X2) and slim milk powder (X6) with $MgSO_4 \cdot 7H_2O$ (X8) as the central point (A), dextrin (X2) and $MgSO_4 \cdot 7H_2O$ (X8) with slim milk powder (X6) as the central point (B), and slim milk powder (X6) and $MgSO_4 \cdot 7H_2O$ (X8) with dextrin (X2) as the central point (C). Y1: epothilone B production (mg/l).

2002; Frykman et al., 2005). However, compared to that in natural *Sorangium* producers, the yield is in trace amount in *S. coelicolor* (Tang et al., 2000), or did not significantly improved in *M. xanthus* after optimization or in long-time continuous fermentation process (Lau et al., 2002; Frykman et al., 2005). Although, the nutrient regulation of epothilone production in heterologous and native strains had been compared of the tolerance of ammonium, phosphate and iron ions (Regentin et al., 2003), the effects of nutritional factors on the epothilone production, to our knowledge, have not yet been considered in details. This study focused on the nutrient regulation in native epothilone production strains So0157-2 and methodically investigated the nutritional factors that affected the production of epothilone B. Although, compositions of complex carbon and nitrogen sources were not definite, we paid close attention to them owing to their excellent enhancing capacity for epothilone production.

Variables and interactions between variables were tested, identified and quantified in terms of epothilone B yield responses using the RSM multiple processes (Liu and Wang, 2007). Response surface plots of different models (Wang and Lu, 2004) provided a method to visualize the interactions of nutrients and the optimum concentration of each nutrient required for maximum epothilone production. Because Box–Behnken design and central composite design is useful only for a small number of variables (up to five), and is impractical for a majority of variables (Mohana et al., 2008), reduction of the initial variable number was performed using Plackett–Burman design and fractional factorial design. Sucrose, glucose and soy powder were firstly selected from seventeen tested variables for BB design, then dextrin,

slim milk powder and $MgSO_4 \cdot 7H_2O$ were taken for CCD of RSM to assess their effects on epothilone B production in *S. cellulosum* 0157-2.

In this study, the GSM medium was selected on the base of BB design, in which two components dextrin and sucrose that had not appeared in previously epothilone synthetic medium. The estimate of dextrin in PB design was higher than that of potato starch which was the only complex carbon source in EPM medium. Accordingly, we have taken dextrin instead of potato starch and increased the concentration to 3.0% in GSM medium. From the model of FFD and CCD, dextrin was found to be significant at both linear and quadratic levels. This implies that the substrate acted as a limiting factor for the production of epothilone B. Although, slim milk powder failed to show direct effects on the compound production, its interaction effect with dextrin was significant. The results suggest that sufficient dextrin produced rich acetyl-CoA, providing precursors and ATPs for epothilone biosynthesis.

According to the analysis of the model, the optimal concentrations of the medium components for epothilone B production were determined. Epothilone B production in the optimal medium was 82.0 ± 3 mg/l, which is 7.2-fold higher than that observed in the initial EPM medium by *S. cellulosum* So0157-2.

ACKNOWLEDGEMENTS

This work was financially supported by National Science Foundation for Distinguished Young Scholars (No. 30825001), National Natural Science Foundation (No. 30671192), National High Technology Research and Development program (No. 2007AA021501 and 2006AA09Z415), and Shandong Science foundation (No. 2008BS02010) of China.

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