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Construction of *asm2* Deletion Mutant of *Actinosynnema pretiosum* and Medium Optimization for Ansamitocin P-3 Production Using Statistical Approach

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Abstract Ansamitocin P-3 is a potent antitumor agent produced by *A. pretiosum*. A deletion mutant of *A. pretiosum* was constructed by deleting the *asm2* gene, a putative transcriptional repressor. The deletion mutant showed a 9-fold enhanced ansamitocin P-3 productivity. The response surface method with central composite design was employed to further optimize the culture medium composition for ansamitocin P-3 production by the deletion mutant. The concentrations of four medium ingredients, dextrin, maltose, cotton seed flour, and yeast extract, which have been reported as major components for ansamitocin production, were optimized through a series of flask culture experiments. The optimum concentrations of the selected factors were found to be dextrin 6.0%; maltose 3.0%; cotton seed flour 0.53%; and yeast extract 0.45%. The maximum titer of ansamitocin P-3 was 78.3 mg/l with the optimized composition, about 15-folds higher than the unoptimized titer of 5.0 mg/l obtained with YMG medium.

Key words: *asm2* deletion mutant of *Actinosynnema pretiosum*, ansamitocin P-3 production, medium optimization, central composite design, response surface method

To date, improvement of *Actinomycetes* strains to obtain high-titer cultures is usually achieved by random mutagenesis and selection techniques. However, recent advances in *Actinomycete* molecular biology, and knowledge of antibiotic biosynthetic pathways and gene clusters, offer genetic engineering as an alternative approach in a targeted manner. Engineered *Streptomyces* strains have been constructed, in which gene dosage has been increased by introducing extra copies of genes encoding pathway enzymes [41], gene expression has been enhanced by introducing positive-acting, pathway-specific regulatory genes [50, 57], or cell growth and fermentation properties have been improved by adding foreign protein genes such as a bacterial hemoglobin gene, *vhb* [4, 40]. In each of these cases, genetic engineering has resulted in a significant overproduction of the desired metabolite, demonstrating the utility of this recombinant DNA approach for strain improvement. Following the genetic engineering of a superior production strain, the fermentation conditions including the medium composition need to be reoptimized to fully exploit the increased potential of the strain developed. The conventional method of media optimization involves changing one factor while keeping all other factors at a fixed level, which is both laborious and time consuming, and the optimum levels are not always reliable [2, 9, 14]. The application of a statistical experimental design approach for the optimization of nutritional factors is one of the recent techniques that have been successfully applied to the production of various bioproducts [1, 6, 12, 13, 17, 24, 27, 30–32, 34, 37, 38, 42, 43, 45, 47, 48, 51, 52].

Ansamitocin P-3, a potent antitumor compound, belongs to the family of maytansinoids [11], which were originally isolated from African plants [33]. A microorganism producing maytansinoids was subsequently isolated and originally classified as a *Nocardia* species, but later redesignated as *Actinosynnema pretiosum* ssp. *pretiosum* ATCC 31309. Furthermore, a mutant strain, *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565, was derived from ATCC 31309 by mutagenesis [3, 19, 22]. Maytansinoids have a potent antitumor activity when delivered in conjugate forms with tumor-cell binding agents [35, 36]. These conjugates have pharmaceutical potential for the treatment

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of various cancers. A few reports have appeared on the metabolic and physiological properties and the purification of maytansinoids from the fermentation broth of mutant *Actinosynnema pretisoum* ATCC 31565 [18, 22, 58]. Recently, the maytansinoids biosynthetic gene cluster from *Actinosynnema pretiosum* ATCC 31565 has been cloned, sequenced, and analyzed [59]. In addition, several potential regulatory and transport genes have been identified. The functions of many of these genes in ansamitocin biosynthesis have been probed by gene inaction or heterologous expression experiments [10, 26, 44, 56, 59].

In this study, a mutant of *Actinosynnema pretiosum* was constructed by deleting the *asm2* gene, one of the putative regulatory genes. Then, medium composition was optimized for ansamitocin P-3 production by this mutant, by employing a statistical method, the response surface methodology (RSM) with central composite design (CCD) [8, 28].

MATERIALS AND METHODS

Strains

A. pretiosum ssp. *auranticum* ATCC 31565 was obtained from the American Type Culture Collection. *E. coli* strains of DH10B (Stratagene, CA, U.S.A.) and ET12567/pUZ8002 [39] were used throughout the study as a routine cloning host and a transient host for *E. coli–A. pretiosum* intergeneric conjugation, respectively.

Construction of the *asm2* Deletion Mutant

Isolation of total genomic DNA of *A. pretiosum* and Southern blotting analysis were carried out as described elsewhere [59]. General cloning procedures and DNA manipulation were performed according to Sambrook and Russel [53]. A 2.34-kb PstI-EcoRI DNA fragment carrying the *aac(3)IV* gene for apramycin resistance and the RK2 replication oriT origin was extracted from pOJ446 [5], blunt-ended by treatment with Klenow enzyme, and cloned into pBluescript KS-/(ScaI, SspI) to create pHGF9050 as a gene-replacement vector. To truncate *asm2*, the 0.69-kb KpnI-XhoI and 0.87-kb EcoRI-XhoI DNA fragments that carry the N-terminus of *asm2* (64 aa) and the C-terminal part of *asm2* (7 aa), respectively, were ligated into a 1.56-kb KpnI-EcoRI fragment (Fig. 1). The assembled fragment was then cloned onto pHGF9050 to generate pHGF9422, in which the 195–620th nucleotides of *asm2* were replaced by 28 nucleotides of 5'-ATACCGTCGACCTCGAGGTC-GACGGTAT-3', which were introduced with the cloning linker (Fig. 1). pHGF9422 was transformed into *E. coli* ET12567/pUZ8002 through electroporation and selected with apramycin on LB agar plates. A transformant colony was picked and grown in LB medium containing apramycin (50 mg/l), chloramphenicol (25 mg/l), and kanamycin (50 mg/l) at 37°C for 12 h. Cells were then collected, washed with

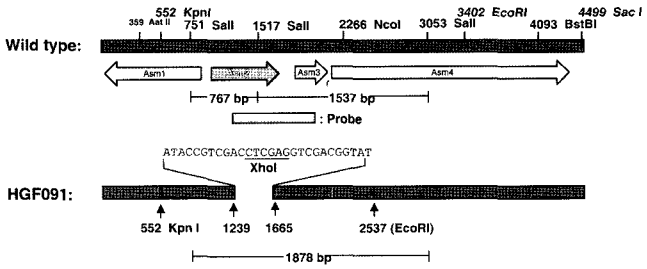

Fig. 1. Organization of *asm2* and part of the ansamitocin gene cluster.

The locations of ORFs identified in the region are shown as arrows. The internally deleted region of *asm2*, which was replaced with 28 oligonucleotides, is shown below (connoted with HGF091) in comparison with the wild-type strain. The green bar gives the region labeled with ^{32}P-dCTP to probe the extracted genomic DNA and validate the expected SalI fragments from the wild-type (0.77 and 1.54 kb) and the HGF091 (1.88 kb) strains, respectively.

3×5 ml 2×YT, mixed with 5 ml of log-phase fresh culture of *A. pretiosum*, and plated onto YMG plates supplemented with 10 mM $MgCl_2$. The YMG medium contained 0.4% yeast extract, 1% malt extract, and 0.4% glucose at pH 7.3. After incubation at 37°C for 16 h, the plates were overlaid with 1 ml of distilled water containing 1 mg of apramycin and 1 mg of nalidixic acid, and cultivated at 30°C. Exconjugants attributed to the homologous recombination

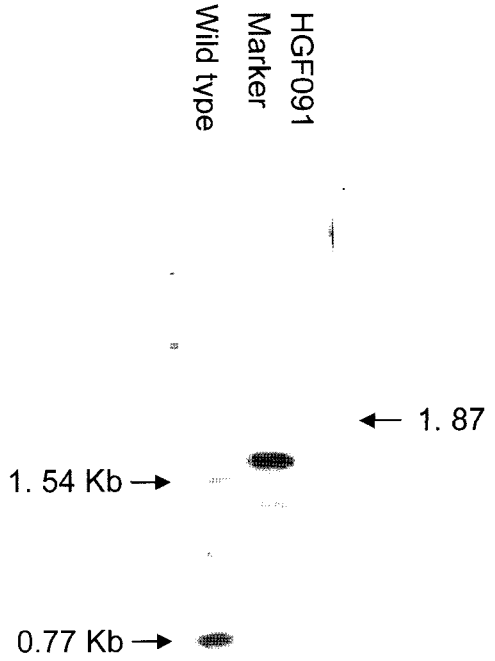

Fig. 2. Southern-blotting analysis.

The total genomic DNA prepared from the *A. pretiosum* wild-type and the *asm2* mutant HGF091 was treated with the SalI restriction enzyme, separated by electrophoresis on a 0.8% agarose gel, and blotted onto a nylon membrane. The signals revealed by the ^{32}P-labeled probe are indicated.

between the delivered pHGF9422 and *A. pretiosum* were detectable in 2–3 days. The individual conjugants were selected and transferred onto YMG broth for three more rounds of relaxed cultivation. A dilution series of the harvested mycelia was prepared and plated again on nonselective YMG agar plates. After sufficient growth, these colonies were replicated on YMG agar plates containing apramycin. Colonies growing on the nonselective, but not on the selective agar plates were isolated, and the total genomic DNA was prepared and analyzed by Southern blotting (Fig. 2) to determine whether the *asm2* gene had been replaced by its corresponding inactivated version or the colony was a revertant to the wild-type. The mutant was designated as HGFG091.

Biological Assay of Ansamitocin P-3

Filobasidium uniguttulatum IFO 0699 was used as the test organism for the bioassay of ansamitocin P-3 [20]. A single colony of the strain was transferred into 2 ml of Trypticase Soy Broth (TSB), incubated at 28°C for 60 h, seeded into 100 ml of TSB agar at 42°C, and then poured onto Petri dishes. The crude culture broth of *A. pretiosum* was extracted twice with the same volume of ethyl acetate, the extract evaporated *in vacuo*, and the residue dissolved in methanol. The assay was carried out by the filter paper disk method. The crude extracts of the *A. pretiosum* wild-type and HGF091 strains, and standard ansamitocin P-3 were diluted, spotted on 8-mm diameter filter paper disks, air dried, and placed on the assay agar plates, which were incubated at 28°C for 8 h. The dose response of ansamitocin was determined by plotting the average diameter of the clear-edged inhibition zone against the antibiotic concentration.

Flask Cultures of the Wild-Type Strain and the Deletion Mutant

In seed cultures for inoculum preparation, colonies of spores scraped from plates into sterile water were transferred into 50 ml of YMG broth (the composition of YMG broth was the same as YMG agar except for agar) and incubated at 28°C for 48 h in a shaking incubator. As control experiments, flask cultures for ansamitocin P-3 production by the *asm2* deletion mutant and the wild-type strain were performed in 500-ml EM flasks containing 100 ml of YMG medium (the control medium) for 74 h.

In the medium optimization experiments for the *asm2* deletion mutant, 2 ml of seed culture was inoculated into 25 ml of the basal medium in a 250-ml Erlenmeyer flask. The composition of the basal culture medium used in this study was K_2HPO_4 0.05%, $Fe_2SO_4 \cdot 7H_2O$ 0.0002%, and $CaCO_3$ 0.5%. Its pH was 7.2. Three ml of filter-sterilized solution of L-valine as a side chain precursor was added to the culture medium to 0.3% w/v of final concentration. The total culture volume was adjusted to 30 ml. The concentrations of dextrin, maltose, cotton seed flour, and yeast extract in the culture medium were varied according to the design specified in Tables 2 and 4. The culture flasks were incubated for 9 days at 28°C in a shaking incubator.

All the experiments were carried out in triplicate.

Analysis of Ansamitocin P-3

At the end of the culture, 5 ml of whole broth was extracted with 5 ml of ethyl acetate for 1 h. The extract was filtered through a 0.22-μm membrane filter and then analyzed for ansamitocin P-3 titer by using a high-performance liquid chromatograph (HPLC, Hitachi, Japan) with a C_{18} reverse-phase column (Kento, Japan). Detection was done at 254 nm. The injection volume was 20 μl. The eluant contained 65% methanol and 35% water, and its flow rate was 1.0 ml/min. The authentic ansamitocin P-3 sample used to construct a calibration curve was a gift from Takeda Chemical Co., Osaka, Japan.

Experimental Design and Optimization by RSM

A priori knowledge and understanding of the process and the process variables under investigation are necessary for achieving realistic results. In this study, the independent variables were the concentrations of the carbon and nitrogen sources: dextrin, maltose, cotton seed flour, and yeast extract, selected from the literature [21, 58].

CCD was applied for selecting levels of the independent variables in carrying out experimental runs. CCD maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments. According to CCD design, the total number of experimental runs was 2^k+2k+n_0, where k is the number of independent variables and n_0 is the number of repetitions of experiments at the center point [9]. In CCD, the natural value of the i^{th} variable X_i was normalized for coding, as in

$$x_i=(X_i-X_i^X)/\Delta X_i \quad (1)$$

where x_i is the coded value, X_i^X is the natural value of the i^{th} independent variable at the center point, and ΔX_i is the scaling factor.

The RSM model used in this study was

$$y=b_0+\sum_i b_i x_i+\sum_i\sum_j b_{ij}x_i x_j+\sum b_{ii}x_i^2+e \quad (2)$$

where y is the measured response (ansamitocin titer in this study), b_0 the intercept, and b_i, b_{ij}, and b_{ii} are coefficients or the measures of the effects of x_i, x_ix_j, and x_i^2, respectively. The term x_ix_j represents the first-order interactions between x_i and x_j ($i<j$), and e denotes the modeling error. In this study, $x_1 \equiv \underline{D}$ (coded value of dextrose concentration), $x_2 \equiv \underline{M}$ (maltose), $x_3 \equiv \underline{C}$ (cotton seed flour), and $x_4 \equiv \underline{Y}$ (yeast extract).

A software package called STATISTICA (StatSoft Inc., US) was used for the regression and graphical analysis of the experimental data. The optimum values of the process variables were obtained by solving the regression equation

and also by analyzing the response surface contour plots [28].

RESULTS AND DISCUSSION

Construction of *asm2* Deletion Mutant

Based on sequence similarity, the predicted Asm2 protein belongs to a group of bacterial transcription regulatory proteins that bind DNA via a helix-turn-helix (HTH) motif. These proteins, including AcrR, BetI, Bm3R1, EnvR, QacR, MtrR, TcmR, TetC, TetR, Ttk, YbiH, and YhgD, have similar molecular weights, ranging from 21 to 25 kDa [23, 49, 55]. Many of them function as repressors that control the level of susceptibility to hydrophobic antibiotics and detergents. The intensive studies of TetR, a tetracycline inducible repressor, have offered a model of the effect-inducible systems for the transcription regulation known to date. With the rationale that *asm2* could play an important role in the regulation of ansamitocin production, we carried out an experiment to truncate the functional Asm2 protein. An inactivated version of the *asm2* gene was generated by replacing the internal 195–620th nucleotides of the coding sequence with 28 nucleotides, which were introduced with the pHGF9050 gene-replacement vector. To reduce the restriction limitation and increase the recombination efficiency, the replacing vector pHGF9050 was passed through *E. coli* ET12567/pUZ8002, a DNA-methylation-deficient strain, and then introduced into wild-type *A. pretiosum* by intergeneric conjugation. Successive selection first for single-crossover integrants (resistance to apramycin) and then for a second-step recombination gave a double-crossover mutant (sensitive to apramycin) in which the functional *asm2* gene had been replaced with the inactivated version. This mutant grew normally compared with the wild-type strain, but produced elevated levels of ansamitocin up to about 4–5-fold, depending on conditions, as demonstrated by bioassay with *Filobasidium uniguttulatum* IFO 0699. Through HPLC analysis, about 9-fold increase in ansamitocin titer was observed with the *asm2* deletion mutant strain (5.0 mg/l) over the wild strain (0.53 mg/l). The mutant strain was used in the subsequent work on the optimization of the medium for ansamitocin production.

Table 1. Natural values of the independent variables according to coded values in the first round of CCD.

Variables	Coded values (D, M, C, Y)				
	−2	−1	0	+1	+2
Dextrin (%w/v), D	1.0	3.0	5.0	7.0	9.0
Maltose (%w/v), M	1.0	2.0	3.0	4.0	5.0
Cotton seed flour (%w/v), C	0.1	1.3	2.5	3.7	4.9
Yeast extract (%w/v), Y	0.1	0.3	0.6	0.8	1.0

Production Medium Optimization by Statistical Design

The concentrations of dextrin, maltose, cotton seed flour, and yeast extract were optimized by employing CCD and RSM methods. In the CCD, a 2^4 factorial design with eight star points and six replicates at the central points were employed to fit the second-order polynomial RSM model (Eq. 2), which indicated that 30 experiments were required. The coded and natural values of the variables are given in Table 1.

The experimental results based on the first set of CCD design are given in Table 2. The P-3 titers at the central points appeared to be very low compared with those at other levels of variables.

To check for the presence of optimum values of the variables within the ranges tested, contour graphs were plotted (Fig. 3). Two-dimensional contour plots of the response surface as a function of two factors at a time, holding all others at fixed levels (zero, for instance), were

Table 2. First-round CCD consisting of 30 planned experiments along with observed experimental data.

Run no.	D	M	C	Y	Coefficients assessed by	Ansamitocin P-3 (mg/l) Observed responses
1	−1	−1	−1	−1	Fractional 2^4 factorial design	43.3
2	1	−1	−1	−1		61.3
3	−1	1	−1	−1		41.7
4	1	1	−1	−1		65.6
5	−1	−1	1	−1		1.02
6	1	−1	1	−1		8.97
7	−1	1	1	-1		1.10
8	1	1	1	-1		10.3
9	−1	−1	−1	1		15.5
10	1	−1	−1	1		31.2
11	−1	1	−1	1		1.94
12	1	1	−1	1		22.4
13	−1	−1	1	1		1.04
14	1	−1	1	1		1.01
15	−1	1	1	1		2.24
16	1	1	1	1		1.03
17	−2	0	0	0	Star points (8 points)	2.95
18	2	0	0	0		5.87
19	0	−2	0	0		1.34
20	0	2	0	0		4.45
21	0	0	−2	0		36.0
22	0	0	2	0		1.97
23	0	0	0	−2		1.50
24	0	0	0	2		7.60
25	0	0	0	0	Central points	6.66
26	0	0	0	0		5.77
27	0	0	0	0		5.78
28	0	0	0	0		6.49
29	0	0	0	0		4.72
30	0	0	0	0		4.96

useful in understanding the interaction between the two factors. The optimum point is confined in the smallest ellipse in the contour diagrams. Figure 3 indicates that the optimum values of maltose and yeast extract were within the tested ranges, whereas the optimum value of dextrin and cotton seed flour were out of the tested ranges.

Based on these results, another set of experimental runs was carried out with the central points for dextrin increased from 5.0% to 6.0%, for cotton seed flour decreased from 2.5% to 0.6%, and for yeast extract decreased from 0.55% to 0.45%. The coded and natural values of the variables at various levels are given in Table 3. The design of experiments and the resulting P-3 titers with this set of experiments are presented in Table 4.

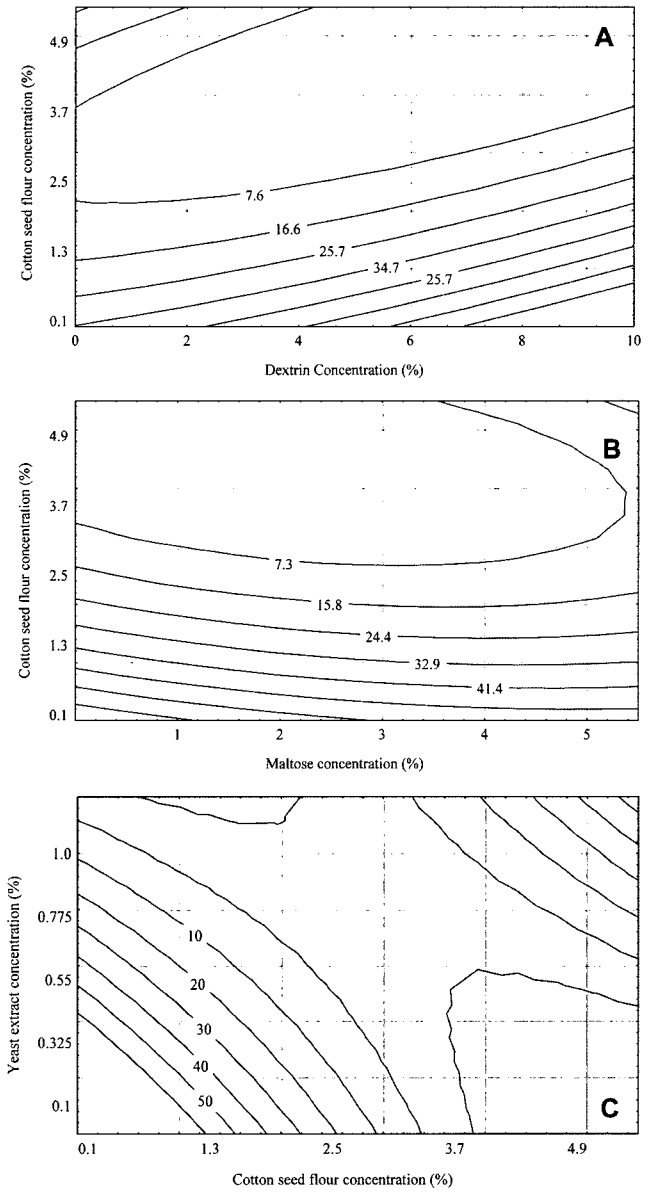

Fig. 3. Contour plots of ansamitocin P-3 concentration (mg/l), showing the effects of dextrin and cotton seed flour (**A**), maltose and cotton seed flour (**B**), and cotton seed flour and yeast extract (**C**).

Table 3. Natural values of the independent variables according to coded values in the second round of CCD.

Variables	Coded levels				
	-2	-1	0	+1	+2
Dextrin (D, %w/v)	1.0	3.5	6.0	8.5	11.0
Maltose (M, %w/v)	1.0	2.0	3.0	4.0	5.0
Cotton seed flour (C, %w/v)	0.0	0.30	0.6	0.9	1.2
Yeast extract (Y, %w/v)	0.05	0.25	0.45	0.65	0.85

The significance of each coefficient was determined by Student *t*-test and *p*-values, which are listed in Table 5. The larger the magnitude of the *t*-value and the smaller the *p*-value, the more significant is the corresponding coefficient [2]. The results showed that the first-order effect of cotton seed flour was more significant than those of dextrin, maltose, and yeast extract (the *p*-value for $\underline{C}$, $p_{\underline{C}}<0.024$; $p_{\underline{D}}<0.98$; $p_{\underline{M}}<0.49$; and $p_{\underline{Y}}<0.31$). The second-order effects of dextrin, maltose, cotton seed flour, and yeast extract were negatively significant as was evident from their respective *p*-values ($p_{\underline{D}}^2<0.0008$; $p_{\underline{M}}^2<0.0003$; $p_{\underline{C}}^2<0.00008$, and $p_{\underline{Y}}^2<0.00007$). This shows that all factors at higher concentrations repress the antibiotic synthesis. Other investigators have also reported such effects of carbon and nitrogen repression on various antibiotic productions [54, 16]. No significant interaction was observed between the factors.

Only 6 terms in Eq. (2) were selected; the intercept and five terms with a *p*-value lower than 0.05. Equation (3) represents the relationship between the P-3 titer and the selected terms.

$$y=78.2-6.5\underline{C}-10.1\underline{D}^2-11\underline{M}^2-13\underline{C}^2-13.2\underline{Y}^2 \quad (3)$$

The above equation was recast in terms of the natural values (% w/v) of the independent variables and represented as follows:

$$y=-210.8+19.39D+66M+151.62C+297Y -1.616D^2-11M^2-144.4C^2-330Y^2 \quad (4)$$

The contour plots (Fig. 4) clearly show no interaction between the factors.

Verification of Model

The optimum values of the independent variables were obtained by solving the regression equation (Eq. 4): dextrin 6.0%; maltose 3.0%; cotton seed flour 0.53%; and yeast extract 0.45%, with the maximum predicted P-3 titer of 79.0 mg/l. For verification, shake flask experiments under the optimum conditions were carried out, and 78.3 mg/l of P-3 titer was obtained. This experimental finding agrees closely to the model prediction. The ansamitocin titer

Table 4. Second-round CCD consisting of 30 planned experiments along with observed experimental data and the data predicted by the RSM model.

Run no.	D	M	C	Y	Coefficients assessed by	Ansamitocin P-3 (mg/l)	
						Observed response	Predicted responses
1	-1	-1	-1	-1	Fractional 2^4 factorial design	52.7	45.2
2	1	-1	-1	-1		47.4	37.5
3	-1	1	-1	-1		41.4	33.4
4	1	1	-1	-1		40.8	31.0
5	-1	-1	1	-1		37.6	34.9
6	1	-1	1	-1		24.4	27.1
7	-1	1	1	-1		34.5	31.1
8	1	1	1	-1		31.0	28.6
9	-1	-1	-1	1		51.7	39.9
10	1	-1	-1	1		48.0	42.2
11	-1	1	-1	1		43.0	31.2
12	1	1	-1	1		50.3	38.7
13	-1	-1	1	1		15.7	16.3
14	1	-1	1	1		24.8	18.5
15	-1	1	1	1		20.0	15.6
16	1	1	1	1		24.8	23.1
17	-2	0	0	0	Star points (8 points)	25.0	37.7
18	2	0	0	0		26.8	37.5
19	0	-2	0	0		29.2	37.8
20	0	2	0	0		15.7	30.6
21	0	0	-2	0		13.0	39.3
22	0	0	2	0		16.2	13.3
23	0	0	0	-2		22.1	30.9
24	0	0	0	2		5.47	20.1
25	0	0	0	0	Central points	76.6	78.2
26	0	0	0	0		77.9	78.2
27	0	0	0	0		79.4	78.2
28	0	0	0	0		79.3	78.2
29	0	0	0	0		78.4	78.2
30	0	0	0	0		77.3	78.2

Table 5. Model coefficient values and their significances (second round of CCD and RSM).

Terms	Coefficients	*t*-values	*p*
Intercept	78.15	15.06	0.00000
D	-0.06	-0.02	0.9806
M	-1.81	-0.69	0.4958
C	**-6.50**	**-2.51**	**0.0242**
Y	-2.69	-1.04	0.3149
DM	1.31	0.41	0.6861
DC	-0.03	-0.01	0.9938
DY	2.50	0.78	0.4429
MC	2.01	0.63	0.5355
MY	0.77	0.24	0.8123
CY	-3.32	-1.05	0.3124
D^2	**-10.14**	**-4.17**	**0.0008**
M^2	**-10.99**	**-4.53**	**0.0003**
C^2	**-12.95**	**-5.34**	**0.00008**
Y^2	**-13.17**	**-5.43**	**0.00007**

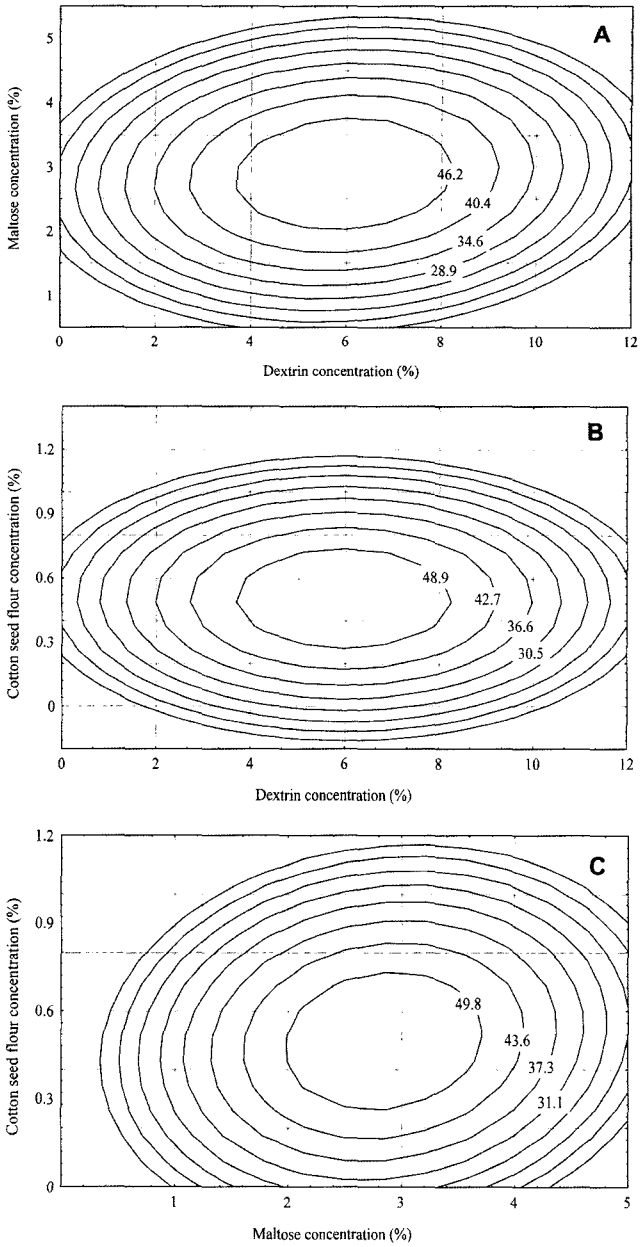

Fig. 4. Contour plots of ansamitocin P-3 concentration (mg/l), showing the effects of dextrin and maltose (**A**), dextrin and cotton seed flour (**B**), maltose and cotton seed flour (**C**), and maltose and yeast extract (**D**).

by flask cultures with the optimized medium in this study was comparable to the maximum titer reported recently as 86.3 mg/l in a bioreactor culture of a mutant of *Actinosynnema pretiosum* ATCC 31565, although the strain and culture environment were quite different [18].

The accuracy of the RSM model was analyzed by analysis of variance (ANOVA), and the results are summarized in Table 6. The F-value is the ratio of the mean square due to regression (MSR) to the mean square due to error (MSE).

Table 6. Analysis of variance (ANOVA) for the final RSM model.

Source of variations	Sum of squares	Degrees of freedom (d.f)	Mean square	$F_{statistic}$ (MSR/MSE)	Prob. (*P*)
Regressions	12,572.3 (SSR)	14	898.0 (MSR=SSR/d.f)	5.56	0.001
Error	2,422.5 (SSE)	15	161.5 (MSE=SSE/d.f)		
Total	14,994.9 (SST)				

Determination coefficient R^2=0.8384; Correlation coefficient R=0.9156.

The F-value in this study ($F_{statistic\ (14,\ 15)}$=5.56) was greater than the tabulated $F_{(14,15)}$ (=3.57) [2, 8, 15]. The low value of probability (P<0.001) demonstrates high significance of the regression model [2, 28]. The goodness of fit of the model was checked by the determination coefficient (R^2), the value of which was 0.8385 in this study, and t indicates that only 16.16% of the total variations is not explainable by the regression model, justifying a good correlation [9].

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References

1. Abdel-Fattah, Y. R. and Z. A. Olama. 2002. L-Asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: Evaluation and optimization of culture conditions using factorial designs. *Process. Biochem.* **38:** 115–122.
2. Aknazarov, S. 1982. *In* Kafarov, V. (ed.). *Experiment Optimization in Chemistry and Chemical Engineering.* Mir Publications, Moscow.
3. Asai, M., E. Mizuta, M. Izawa, K. Haibara, and T. Kishi. 1979. Isolation, chemical characterization and structure of ansamitocin, a new antitumor ansamycin antibiotic. *Tetrahedron* **35:** 1079–1085.
4. Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai. 1996. Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes. *Biotechnol. Bioeng.* **52:** 109–121.
5. Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116:** 43–49.
6. Bloor, S. and R. R. England. 1981. Elucidation and optimization of the medium constituents controlling antibiotic production by the cyanobacterium *Nostoc muscorum*. *Enz. Microb. Technol.* **13:** 76–81.
7. Bandi, S., Y. J. Kim, S. O. Sa, and Y.-K. Chang. 2005. Statistical approach to development of culture medium for ansamitocin P-3 production with *Actinosynnema pretiosum* ATCC 31565. *J. Microbiol. Biotechnol.* **15:** 930–937.
8. Box, G. E. P. and K. B. Wilson. 1951. On the experimental attainment of optimum conditions. *J. Roy. Stat. Soc.* **B13:** 1–45.
9. Box, G. E. P. 1978. *In* Hunter, W. G. and Hunter, J. S. (eds.). *Statistics for Experiments*, pp. 291–334. John Wiley and Sons, New York.
10. Carroll, B. J., S. J. Moss, L. Bai, Y. Kato, S. Toelzer, T. W. Yu, and H. G. Floss. 2002. Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual "glycolate" chain extension unit in ansamitocin biosynthesis. *J. Am. Chem. Soc.* **124:** 4176–4177.
11. Cassady, J. M., K. K. Chan, H. G. Floss, and E. Leistner. 2004. Recent developments in the maytansinoid antitumor agents. *Chem. Pharm. Bull.* **52:** 1–26.
12. Chang, Y. N., J. C. Huang, C.-C. Lee, I. L. Shih, and Y. M. Tzeng. 2002. Use of response surface methodology to optimize culture medium for production of lovastatin by *Monascus ruber*. *Enz. Microb. Technol.* **30:** 889–894.
13. Chen, Q. H., G. Q. He, and M. A. M. Ali. 2002. Optimization of medium composition for the production of elastase by *Bacillus* sp. EL 31410 with response surface methodology. *Enz. Microb. Technol.* **30:** 667–672.
14. Cochran, W. G. 1957. *In* Cox, G. M. (ed.). *Experimental Designs* 2nd Ed., pp. 346–354. John Wiley and Sons. New York.
15. Devore, J. L. 1995. *Probability and Statistics for Engineering and the Sciences*, 4th Ed. p. 710. Duxbury Press; An International Thomson Publishing Company, US.
16. Dulaney, E. 1948. Observation on *Streptomyces griseous* II. Nitrogen sources for growth and streptomycin production. *J. Biotechnol.* **56:** 305–313.
17. Elibol, M. 2004. Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3(2) with response surface methodology. *Process. Biochem.* **39:** 1057–1062.
18. Fulston, M., A. L. Stefanska, and J. E. Thirkettle. 2003. Methods for ansamitocin production. US Patent 6, 573, 074.
19. Hasegawa, T., S. Tanida, K. Hatano, E. Higashide, and M. Yoneda. 1983. Motile actinomycetes: *Actinosynnema pretiosum* subsp. *pretiosum* sp. *nov.*, subsp. *nov.*, and *Actinosynnema pretiosum* subsp. *auranticum* subsp. *nov. Int. J. Syst. Bacteriol.* **33:** 314–320.
20. Hatano, K., E. Higashide, and M. Yoneda. 1984. Bioassay of ansamitocin P-3, an antitumor antibiotic. *Agric. Biol. Chem.* **48:** 1899–1890.

21. Hatano, K., M. Nakamishi, and S. I. Akiyama. 1982. Method for the production of antibiotic C-15003 P-3. US Patent 4, 356, 265.
22. Higashide, E., M. Asai, K. Ootsu, S. Tanida, Y. Kozai, T. Hasegawa, T. Kishi, Y. Sugino, and M. Yoneda. 1977. Ansamitocin, a group of novel maytansinoid antibiotics with antitumour properties from *Nocardia*. *Nature (London)* **270:** 721–722.
23. Hillen, W. and C. Berens. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu. Rev. Microbiol.* **48:** 345–369.
24. Houng, J. Y., H. F. Hsu, Y. H. Liu, and J. Y. Wu. 2003. Applying the Taguchi robust design to the optimization of the asymmetric reduction of ethyl 4-chloro acetoacetate by Bakers' yeast. *J. Biotechnol.* **100:** 239–250.
25. Jeong, D. H., K. D. Park, S. H. Kim, K. R. Kim, S. W. Choi, J. T. Kim, K. H. Choi, and J. H. Kim. 2004. Identification of *Streptomyces* sp. producing antibiotics against phytopathogenic fungi, and its structure. *J. Microbiol. Biotechnol.* **14:** 212–215.
26. Kato, Y., L. Bai, Q. Xue, P. Revill, T.-W. Yu, and H. G. Floss. 2002. Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. *J. Am. Chem. Soc.* **124:** 5268–5269.
27. Kenneth, W. Y. M., G. S. Y. Miranda, and K. T. Wah. 1995. Formulation and optimization of two culture media for the production of tumor necrosis factor-b in *Escherichia coli*. *J. Chem. Tech. Biotechnol.* **62:** 289–294.
28. Khuri, A. I. and J. A. Cornell. 1987. Response Surface Designs. Marcel Dekker Inc., New York.
29. Kim, H., H. J. Eom, J. S. Lee, J. S. Han, and N. S. Han. 2004. Statistical optimization of medium composition for growth of *Leuconostoc citreum*. *Biotechnol. Bioprocess. Eng.* **9:** 278–284.
30. Kim, M. W., C. S. Shin, H. J. Yang, S. H. Kim, H. Y. Lim, C. H. Lee, M. K. Kim, and Y. H. Lim. 2004. Naltriben analogues as peptide anticancer drugs. *J. Microbiol. Biotechnol.* **14:** 881–884.
31. King, V. A. 1993. Studies on the control of the growth of *Saccharomyces cerevisiae* by using response surface methodology to achieve effective preservation at high water activities. *Int. J. Food Sci. Technol.* **28:** 519–529.
32. Krishna, S. H., A. P. Sattur, and N. G. Karanth. 2001. Lipase-catalyzed synthesis of isoamyl isobutyrate - optimization using a central composite rotatable design. *Process Biochem.* **37:** 9–16.
33. Kupchan, S. M., Y. Komada, W. A. Court, G. J. Thomas, R. M. Smith, A. Karim, C. J. Gilmore, R. C. Haltiwanger, and R. F. Bryan. 1972. Maytansine, a novel antileukemic ansa macrolide from *Maytenus ovatus*. *J. Am. Chem. Soc.* **94:** 1354–1356.
34. Lai, L. S. T., C. C. Pan, and Y. M. Tzeng. 2003. The influence of medium design on lovastatin production and pellet formation with a high-producing mutant of *Aspergillus terreus* in submerged cultures. *Process Biochem.* **38:** 1317–1326.
35. Lidino, C. A., R. V. J. Chari, L. A. Bourret, N. L. Kedersha, and V. S. Goldmacher. 1997. Folate-maytansinoids: Target-selective drugs of low molecular weight. *Int. J. Cancer* **73:** 859–864.
36. Liu, C., B. M. Tadayoni, L. A. Bourret, K. M. Mattocks, S. M. Derr, W. C. Widdison, N. L. Kedersha, P. D. Ariniello, V. S. Goldmacher, and J. M. Lambert. 1996. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. *Proc. Nat. Acad. Sci. USA* **93:** 8618–8623.
37. Liu, J. Z., L. P. Weng, Q. L. Zhang, H. Xu, and L. N. Ji. 2003. Optimization of glucose oxidase production by *Aspergillus niger* in a bench top bioreactor using response surface methodology. *World J. Microbiol. Biotechnol.* **19:** 317–323.
38. Ma, A. Y. M. and B. Ooraikul. 1986. Optimization of enzymatic hydrolysis of canola meal with response surface methodology. *J. Food Proc. Preserv.* **10:** 99–113.
39. MacNeil, D. J., K. M. Gewain, C. L. Ruby, G. Dezeny, H. Gibbons, and T. MacNeil. 1992. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111:** 61–68.
40. Magnolo, S. K., D. L. Leenutaphong, J. A. DeModena, J. E. Curtis, J. E. Bailey, J. L. Galazzo, and D. E. Hughes. 1991. Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of bacterial hemoglobin. *Bio-Technology* **9:** 473–476.
41. Malmberg, L.-H., W.-W. Hu, and D. H. Sherman. 1993. Precursor flux control through targted chromosomal insertion of the lysine-aminotranferase (*lat*) gene in cephamycin C biosynthesis. *J. Bacteriol.* **175:** 6916–6924.
42. Manohar, B. and S. Diwakar. 2006. Applications of surface plots and statistical designs to selected lipase catalysed esterification reactions. *Process Biochem.* (in press).
43. Momen, B., P. D. Anderson, and J. A. Helms. 2001. Application of response surface methodology and ANOVA to detect pollution effects on photosynthetic response under varying temperature and light regimes. *Forest Ecol. Manage.* **152:** 331–337.
44. Moss, S. J., L. Bai, S. Toelze, B. J. Carroll, T. Mahmud, T.-W. Yu, and H. G. Floss. 2002. Identification of Asm19 as an acyltransferase attaching the biologically essential ester side chain of ansamitocins using *N*-desmethyl-4,5-desepoxymaytansinol, not maytansinol, as its substrate. *J. Am. Chem. Soc.* **124:** 6544–6545.
45. Murthy, M. V. R., E. V. S. Mohan, and A. K. Sadhukhan. 1999. Cyclosporin-A production by *Tolypocladium inflatum* using solid state fermentation. *Process Biochem.* **34:** 269–280.
46. Park, H. S., S. H. Kang, H. J. Park, and E. S. Kim. 2005. Doxorubicin productivity improvement by the recombinant *Streptomyces peucetius* with high-copy regulatory genes cultured in the optimized media composition. *J. Microbiol. Biotechnol.* **15:** 66–71.
47. Park, J. C., D. M. Ha, and M. G. Kim. 1996. Modified response surface methodology (MRSM) for phase equilibrium-theoretical background. *Korean J. Chem. Eng.* **13:** 115–122.
48. Park, J. S., B. H. Kim, J. H. Lee, E. S. Seo, K. S. Cho, H. J. Park, H. K. Kang, S. K. Yoo, M. S. Ha, H. J. Chung,

D. L. Cho, D. F. Day, and D. Kim. 2003. Optimization of novel glucanhydrolase production of *Lipomyces starkeyi* KSM 22 by statistical design. *J. Microbiol. Biotechnol.* **13:** 993–997.

49. Pan, W. and B. G. Spratt. 1994. Regulation of the permeability of the gonococcal cell envelope by the mtr system. *Mol. Microbiol.* **11:** 769–775.
50. Perez-Llarenak, F. J., P. Liras, A. Rodriguez-Garcia, and J. F. Martin. 1997. A regulatory gene (ccaR) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: Amplification results in overproduction of both β-lactam compounds. *J. Bacteriol.* **179:** 2053–2059.
51. Rao, K. J., C. H. Kim, and S. K. Rhee. 2000. Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. *Process Biochem.* **35:** 639–647.
52. Rosi, I. 1987. Wine fermentation by immobilized yeast: an optimization study. *In* Costamagna, L. and Bertuccioli, M. (eds.). Flavor Science and Technology; John Wiley and Sons, New York.
53. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Lab. Press, New York.
54. Sathosh, A., H. Ogawa, and Y. Satomura. 1976. Regulation of *N*-acetyl kanamycin amidohydrolase in the iodiophase in kanamycin fermentation. *Agric. Biol. Chem.* **40:** 191–196.
55. Shaw, G. C. and A. J. Fulco. 1993. Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site on the barbiturate-inducible cytochrome P450BM-3 gene of *Bacillus megaterium*. *J. Biol. Chem.* **268:** 2997–3004.
56. Spiteller, P., L. Bai, G. Shang, B. J. Carroll, T.-W. Yu, and H. G. Floss. 2003. The post-polyketide synthase modification steps in the biosynthesis of the antitumor agent ansamitocin by *Actinosynnema pretiosum*. *J. Am. Chem. Soc.* **125:** 14236–14237.
57. Stutzman-Engwall, K. J., S. L. Otten, and C. R. Hutchinson. 1992. Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. *J Bacteriol.* **174:** 144–154.
58. Tanida, S., T. Hasegawa, K. Hatano, E. Highashide, and M. Yoneda. 1980. Ansamitocins, maytansinoids, antitumor antibiotics producing organism, fermentation and antimicrobial activities. *J. Antibiot. (Tokyo)* **32:** 192–198.
59. Yu, T.-W., L. Bai, D. Clade, D. Hoffmann, S. Toelzer, K. Q. Trinh, J. Xu, S. J. Moss, E. Leistner, and H. G. Floss. 2002. The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. *Proc. Nat. Acad. Sci. USA* **99:** 7968–7973.