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Statistical Approach to Development of Culture Medium for Ansamitocin P-3 Production with *Actinosynnema pretiosum* ATCC 31565

BANDI, SRINIVASULU, YOON JUNG KIM, SOON OK SA, AND YONG-KEUN CHANG*

Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

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Abstract The Plackett-Burman design and the response surface method (RSM) with a central composite design (CCD) were employed to develop a culture medium for ansamitocin P-3 production using *Actinosynnema pretiosum* ATCC 31565. Among the 11 nutrients tested using the Plackett-Burman design, two carbon sources, sucrose and dextrin, and two nitrogen sources, polypeptone and yeast extract, were selected. Optimization of the concentrations of the selected nutrients was then performed using RSM with CCD. After two rounds of RSM, the optimum concentrations (%w/v) of sucrose, dextrin, polypeptone, and yeast extract were identified as 4.5, 4.5, 0.16, and 0.89, respectively. The maximum ansamitocin P-3 titer was 45.2 mg/l with the optimized medium, which was about 6 times higher than that (7.315 mg/l) obtained with an R_2YE medium before optimization.

Key words: Ansamitocin P-3, *Actinosynnema pretiosum*, medium development, Plackett-Burman design, central composite design, response surface method

Ansamitocin P-3 is a potent antitumor compound, belonging to the maytansinoid group, originally isolated from African plants [23, 27]. Maytansinoid-producing microorganisms have also been isolated [3, 14, 16, 19]. Maytansinoids exhibit a potent cytotoxic activity and have demonstrated a strong antitumor activity when delivered in a conjugate form with a cell-binding agent [24, 25]. Therefore, such conjugates have pharmaceutical potential for the treatment of various cancers. A few reports have already appeared on the metabolic and physiological properties, and purification of maytansinoids from fermentation broth of *A. pretiosum* ATCC 31565 [13, 15, 34]. In addition, the Floss group [36] recently revealed a maytansinoids biosynthetic gene cluster from *A. pretiosum* ATCC 31565. However, no reports have yet appeared on systematic medium development for ansamitocin production.

Medium development for effective metabolite production plays a key role in fermentation processes, and high product yields can only be achieved based on the design of a proper production medium. Balanced concentrations of nutrients are required for proper growth and a high level of product formation. However, in conventional optimization, only one factor is changed at a time, while keeping all the other factors at a fixed level, and this is repeated for different levels of the chosen factor and the other factors, which is both time consuming and laborious, plus the optimum levels identified are often not reliable [2, 6, 10]. Applying statistical experimental design approaches to optimizing nutritional factors and culture conditions has been recently introduced and successfully employed for the production of primary metabolites [1, 9, 31, 35] and secondary metabolites [5, 8, 23, 28]. Such statistical techniques have also been employed for the optimization of enzymatic reactions [18, 26], parameters for effective food preservation [21], the fermentation process for wine production [32], and removal of sodium in fermented food composts [4].

Accordingly, in this study we attempted to select prominent nutrients influencing ansamitocin production using the Plackett-Burman design [30], then further optimize their concentrations based on a central composite design (CCD) and the response surface methodology (RSM) [2, 6, 7, 10, 20] for ansamitocin P-3 production using *A. pretiosum* ATCC 31565.

Materials and Methods

Chemicals

The chemicals used for cultivation, such as dextrin, maltose, cotton-seed flour, and malt extract, were all purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Yeast extract,

*Corresponding author
Phone: 82-42-869-3927; Fax: 82-42-869-3910;
E-mail: ychang@kaist.ac.kr

glucose, and agar were from DIFCO Laboratories (Detroit, MI, U.S.A.), and methanol and ethyl acetate were of HPLC purity (Merck, Germany).

Strain and Culture Conditions

A. pretiosum ATCC 31565 was grown on YMG agar plates at 28°C for 7 days. The composition of YMG agar (% w/v) was yeast extract 0.4, malt extract 1.0, glucose 0.4, and agar 2.0, with a pH of 7.4. The cultures were preserved at 4°C and subcultured every 4 weeks. For inoculum preparation, colonies of spores scrapped from the plates into sterile water were transferred into 50 ml of YMG broth and incubated at 28°C for 48 h while being shaken at 220 rpm. The composition of YMG broth used was the same as that for YMG agar, except for agar. For the flask cultures, 2 ml of the cell broth was inoculated into 25 ml of R_2YE medium [17], as a control medium, in a 250-ml Erlenmeyer flask. A sterile solution of L-valine as a precursor was added to the culture medium to give a 0.3% (w/v) concentration. The total culture volume was adjusted to 30 ml with water. The basal composition of the medium to be developed (% w/v) was K_2HPO_4 0.05, $Fe_2SO_4 \cdot 7H_2O$ 0.0002, and $CaCO_3$ 0.5, except for carbon and nitrogen sources. Carbon and nitrogen sources were to be identified by the Plackett-Burman method and their concentrations were to be optimized by the response surface method. The initial pH of the medium was 7.2. The culture flasks were incubated at 28°C while being shaken at 220 rpm for 9 days. At the end of the cultivation, 5 ml of whole broth was extracted with 5 ml of the ethyl acetate for 1 h. The extract was then filtered through a 0.22 μm membrane filter and analyzed for ansamitocin P-3 by HPLC. All the experiments were performed in triplicate and the average values were reported.

Analysis of Ansamitocin P-3

A Hitachi HPLC system with a C_{18} Reverse Phase column (Kento make, Japan) was used for ansamitocin P-3 analysis. The injection volume was 20 μl. The sample was eluted with a mobile phase comprising 65% methanol and 35% water at a flow rate of 1.0 ml/min. The chromatogram was monitored at 254 nm. Ansamitocin P-3 (a gift of Prof. Heinz Floss, University of Washington, U.S.A.) was used to construct the calibration curve.

STATISTICAL-DESIGN

Plackett-Burman Design for Nutrient Factors Screening

The total number of experiments carried out according to the Plackett-Burman method is k+1, where k is the number of factors (medium components) to be evaluated. Each factor is represented at two levels, the high level designated by (+), and the low level designated by (−). The number of positive signs in a row is equal to (k+1)/2, whereas the number of negative signs is equal to (k−1)/2, and a column should contain equal numbers of positive and negative signs. In the present work, the effects of eleven factors (k=11) were evaluated in twelve experiments. Among these 11 factors, only carbon and nitrogen sources in this study were selected based on existing literature on ansamitocin biosynthesis [15, 34]. The choice of signs for the first row was arbitrary and contained 6 positive signs and 5 negative signs (Table 1). In the second row, the level of the first factor (F_1) had the same sign as that of the last factor (F_{11}) of the first (or previous) row, F_2 had the same sign as F_1 in the first row, F_3 had the same sign as F_2 in the first row, and so on. The same procedure was repeated for the subsequent rows. The last row contained all negative signs.

Table 1. Plackett-Burman experimental design to evaluate factors affecting ansamitocin P-3 production.

Run No	Factors tested											Response
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	P-3 (mg/l)
1	+1 (2.0)	−1 (1)	+1 (5)	−1 (1)	−1 (0)	−1 (0.01)	+1 (0.1)	+1 (0.1)	+1 (1.0)	−1 (0.01)	+1 (0.5)	22.907
2	+1 (2.0)	+1 (3)	−1 (1)	+1 (5)	−1 (0)	−1 (0.01)	−1 (0.01)	+1 (0.1)	+1 (1.0)	+1 (1.0)	−1 (0.05)	32.976
3	−1 (0.5)	+1 (3)	+1 (5)	−1 (1)	+1 (0.5)	−1 (0.01)	−1 (0.01)	−1 (0)	+1 (1.0)	+1 (1.0)	+1 (0.5)	13.466
4	+1 (2.0)	−1 (1)	+1 (5)	+1 (5)	−1 (0)	+1 (1.0)	−1 (0.01)	−1 (0)	−1 (0.01)	+1 (1.0)	+1 (0.5)	12.506
5	+1 (2.0)	+1 (3)	−1 (1)	+1 (5)	+1 (0.5)	−1 (0.01)	+1 (0.1)	−1 (0)	−1 (0.01)	−1 (0.01)	+1 (0.5)	15.084
6	+1 (2.0)	+1 (3)	+1 (5)	−1 (1)	+1 (0.5)	+1 (1.0)	−1 (0.01)	+1 (0.1)	−1 (0.01)	−1 (0.01)	−1 (0.05)	49.716
7	−1 (0.5)	+1 (3)	+1 (5)	+1 (5)	−1 (0)	+1 (1.0)	+1 (0.1)	−1 (0)	+1 (1.0)	−1 (0.01)	−1 (0.05)	19.408
8	−1 (0.5)	−1 (1)	+1 (5)	+1 (5)	+1 (0.5)	−1 (0.01)	+1 (0.1)	+1 (0.1)	−1 (0.01)	+1 (1.0)	−1 (0.05)	45.756
9	−1 (0.5)	−1 (1)	−1 (1)	+1 (5)	+1 (0.5)	+1 (1.0)	−1 (0.01)	+1 (0.1)	+1 (1.0)	−1 (0.01)	+1 (0.5)	42.199
10	+1 (2.0)	−1 (1)	−1 (1)	−1 (1)	+1 (0.5)	+1 (1.0)	+1 (0.1)	−1 (0)	+1 (1.0)	+1 (1.0)	−1 (0.05)	31.845
11	−1 (0.5)	+1 (3)	−1 (1)	−1 (1)	−1 (0)	+1 (1.0)	+1 (0.1)	+1 (0.1)	−1 (0.01)	+1 (1.0)	+1 (0.5)	12.344
12	−1 (0.5)	−1 (1)	−1 (1)	−1 (1)	−1 (0)	−1 (0.01)	−1 (0.01)	−1 (0)	−1 (0.01)	−1 (0.01)	−1 (0.05)	0

F_1, glucose; F_2, maltose; F_3, dextrin; F_4, sucrose; F_5, yeast extract; F_6, soybean flour; F_7, casamino acids; F_8, polypeptone; F_9, corn steep liquor; F_{10}, cotton seed flour; F_{11}, soytone.

( ): concentration, % w/v.

All the experiments were carried out in triplicate and the averages of ansamitocin P-3 titers were taken as the responses. Table 1 illustrates the factors under investigation as well as the level of each factor used in the experimental design. The Plackett-Burman design is based on a first-order model,

$$y=\beta_0+\sum_i \beta_i f_i \quad (1)$$

where y is the response (product titer), f_i is the level of the i^{th} factor, β_0 is the intercept, and β_i is the coefficient of f_i. The model does not describe the interaction among the factors. It was used to evaluate and screen the factors that influence the ansamitocin production. The software package named "Design-Expert" (Stat-Ease, Inc., Minneapolis, MN, U.S.A.) was used to estimate the significance levels of the selected factors.

Experimental Design and Optimization by RSM

RSM consists of a group of empirical techniques that can evaluate the relationships existing between a cluster of experimental variables (or independent variables) and the measured response (or dependent variable), according to one or more selected criteria. In this study, the independent variables were the nutritional factors screened earlier by the Plackett-Burman method. *A priori* knowledge and an understanding of the process and process variables under investigation were also necessary for achieving realistic results.

CCD was applied to select the 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 the CCD design, the total number of experimental runs is 2^k+2k+n_0, where k is the number of independent variables and n_0 is the number of experiments repeated at the center point [6]. In CCD, the natural value of the i^{th} variable, X_i, is normalized for coding, as in

$$x_i=(X_i-X_i^x)/\Delta X_i \quad (2)$$

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

The RSM model used in this study was

$$y=b_0+\sum_i b_i x_i+\sum_i\sum_j b_{ij}x_i x_j+\sum b_{ii}x_i^2+e \quad (3)$$

where y is the measured response, b_0 is the intercept, and b_i, b_{ij}, and b_{ii} are the coefficients or 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 is the modeling error.

A software package called 'STATISTICA' (StatSoft Inc., Tulsa, OK, U.S.A.) was used for the regression and graphical analysis of the experimental data obtained. The optimum values of the variables were obtained by solving the regression equation and analyzing the response surface contour plots [20].

Results and Discussion

Screening of Carbon and Nitrogen Sources by the Plackett-Burman Method

The evaluation of eleven nutrients for ansamitocin P-3 production was carried out based on 12 experimental runs, and the results are shown in Table 1. The ansamitocin P-3 titer exhibited a variation from 0 to 49 mg/l, reflecting the importance of the medium components and their optimization for attaining a higher production. The statistical analysis is given in Table 2 in terms of the coefficient values, and their t- and p-values, where a positive t-value means a positive influence. The decreasing order of the positive effectiveness of the nutritional factors was polypeptone, yeast extract, soybean flour, sucrose, glucose, dextrin, and corn steep liquor. All these factors were significant at a 99% level ($p<0.01$). Meanwhile, soytone also had a significant effect, but negatively. Thus, among the significant factors, two nitrogen sources, yeast extract and polypeptone, and two carbon sources, sucrose and dextrin, were selected for P-3 production. Although glucose did exhibit a slightly higher significance level over dextrin, dextrin was selected because of its slower uptake, which is generally better for secondary metabolites production.

Medium Composition Optimization by CCD and RSM

The concentrations of sucrose, dextrin, polypeptone, and yeast extract were optimized by employing CCD and RSM. For the CCD, a 2^4 factorial design with eight star points and six replicates at the central points were used to fit a second-order polynomial RSM model (Eq. 3),

Table 2. Statistical analysis of Plackett-Burman design experiment.

Factors	Coefficients	t-values	p
Intercept	24.850		
F_1, glucose	**2.655**	**8.991**	<0.01*
F_2, maltose	−1.018	−3.448	
F_3, dextrin	**2.442**	**8.2715**	<0.01
F_4, sucrose	**3.137**	**10.625**	<0.01
F_5, yeast extract	**8.160**	**27.634**	<0.01
F_6, soybean flour	**3.152**	**10.675**	<0.01
F_7, casamino acids	−0.293	−0.992	
F_8, polypeptone	**9.465**	**32.054**	<0.01
F_9, corn steep liquor	**2.282**	**7.730**	<0.01
F_{10}, cotton seed flour	−0.035	−0.118	
F_{11}, soytone	−5.099	−17.269	<0.01

*$p<0.01$ means a 99% significance level.

Table 3. Natural values of independent variables according to coded values in first-round CCD.

Variables	Coded values (S, D, P, Y)				
	-2	-1	0	+1	+2
Sucrose (%w/v), S	0.5	2.5	4.5	6.5	8.5
Dextrin (%w/v), D	0.5	2.5	4.5	6.5	8.5
Polypeptone (w/v), P	0.01	0.11	0.21	0.31	0.41
Yeast Extract (%w/v), Y	0.05	0.25	0.45	0.65	0.85

meaning that 30 experiments were required. The coded and natural values of the variables at various levels are given in Table 3.

The experimental results based on the first CCD with 30 experiments are given in Table 4. The P-3 titers at the central points were very low compared with those at the other conditions.

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

Run no.	S	D	P	Y	Coefficients assessed by	Ansamitocin P-3 (mg/l)	
						Observed responses	Predicted responses
1	-1	-1	-1	-1	Fractional 2^4 factorial design	3.657	6.615
2	1	-1	-1	-1		1.585	3.413
3	-1	1	-1	-1		6.64	6.857
4	1	1	-1	-1		1.311	-1.079
5	-1	-1	1	-1		6.614	7.979
6	1	-1	1	-1		3.351	2.186
7	-1	1	1	-1		19.728	17.355
8	1	1	1	-1		0	6.826
9	-1	-1	-1	1		41.507	36.354
10	1	-1	-1	1		33.003	35.256
11	-1	1	-1	1		31.529	32.574
12	1	1	-1	1		26.434	26.742
13	-1	-1	1	1		30.529	32.800
14	1	-1	1	1		27.655	29.111
15	-1	1	1	1		38.308	38.154
16	1	1	1	1		32.807	29.729
17	-2	0	0	0	Star points (8 points)	23.387	24.075
18	2	0	0	0		14.692	12.449
19	0	-2	0	0		20.07	17.939
20	0	2	0	0		18.224	18.799
21	0	0	-2	0		15.205	15.448
22	0	0	2	0		21.599	19.801
23	0	0	0	-2		0	-2.856
24	0	0	0	2		48.484	49.785
25	0	0	0	0	Central points	19.785	24.119
26	0	0	0	0		20.294	24.119
27	0	0	0	0		27.177	24.119
28	0	0	0	0		25.872	24.119
29	0	0	0	0		27.703	24.119
30	0	0	0	0		23.885	24.119

The significance of each coefficient was determined by a student t-test and the p-values, as listed in Table 5, where the larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient [2]. The results show that the first-order effects of sucrose and yeast extract were more significant than those of dextrin and polypeptone (the p-value for S, $p_S < 0.001$; $p_Y < 2\times10^{-11}$; $p_D < 0.77$; and $p_P < 0.17$). However, the second-order effects of sucrose, dextrin, and polypeptone were negatively significant, as evident from their respective p-values ($p_S^2 < 0.055$; $p_D^2 < 0.059$; and $p_P^2 < 0.036$), indicating that at higher concentrations all the factors, except for yeast extract, repressed the antibiotic synthesis. Furthermore, a significant interaction was observed between dextrin and polypeptone ($p_{DP} < 0.02$).

Seven terms were selected, the intercept and six terms with a p-value lower than 0.06, and Eq. (4) represents the relationship between the P-3 titer and the selected terms:

$$y = 24.12 - 2.9S + 13.16Y + 2.28DP - 1.46S^2 - 1.44D^2 - 1.6P^2 \quad (4)$$

where y is the response, that is, the antibiotic concentrations, and S, D, P, and Y are coded levels of the variables sucrose (S), dextrin (D), polypeptone (P), and yeast extract (Y), respectively.

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

$$y = -9.88 + 1.83S + 0.82D + 15.9P + 65.8Y + 11.4DP - 0.36S2 - 0.36D2 - 160P^2 \quad (5)$$

To check for the presence of optimum values of the variables within the ranges tested, contour graphs were plotted (Fig. 1). Two-dimensional contour plots of the response surface as a

Table 5. Model coefficients values and their significances (first-round CCD and RSM).

Terms	Coefficients	t-values	p
Intercept	24.1193	15.9746	7.96×10^{-11}
S	-2.9065	-3.8500	0.0015
D	0.2152	0.2850	0.7795
P	1.0881	1.4413	0.1700
Y	13.1605	17.4329	$\mathbf{2.2\times10^{-11}}$
SD	-1.1837	-1.2803	0.2199
SP	-0.6478	-0.7007	0.4942
SY	0.5261	0.5690	0.5777
DP	2.2832	2.4694	0.0260
DY	-1.0055	-1.0875	0.2939
PY	-1.2296	-1.3299	0.2034
$\mathbf{S^2}$	-1.4644	-2.0737	0.0557
$\mathbf{D^2}$	-1.4375	-2.0356	0.0598
$\mathbf{P^2}$	-1.6237	-2.2994	0.0363
Y^2	-0.1637	-0.2319	0.8197

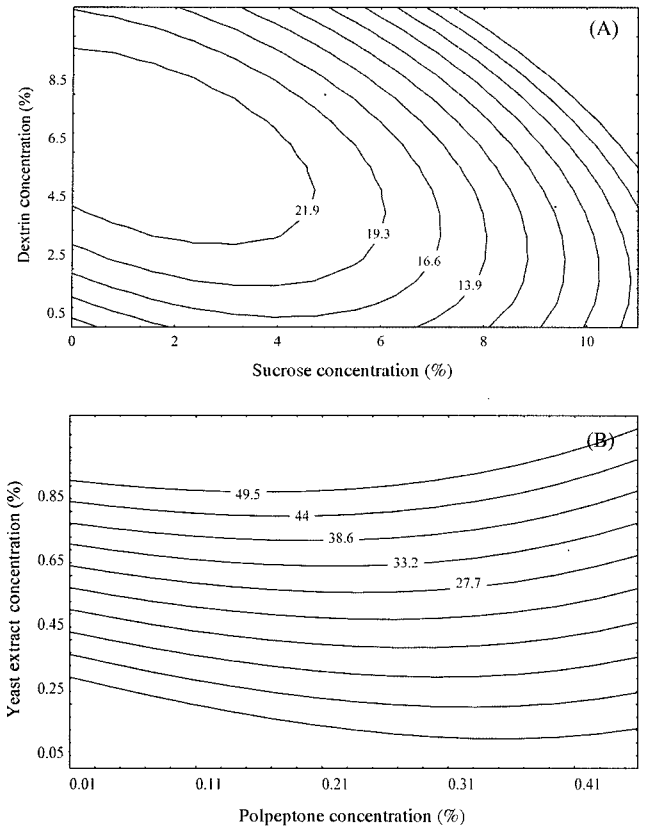

Fig. 1. Contour plots of ansamitocin P-3 concentration (mg/l): Effects of sucrose and dextrin (A), and polypeptone and yeast extract (B).

function of two factors at a time, while holding all others at fixed levels (zero, for instance), were found to be useful in understanding the interaction between the two factors. Furthermore, the optimum point was confined within the smallest ellipse in the contour diagrams. The plots (Fig. 1) indicated that the optimum values for sucrose, dextrin, and polypeptone were within the tested ranges, whereas the optimum value for yeast extract was outside the tested range.

Therefore, based on these results, another set of experiments was carried out where the center point for yeast extract was increased from 0.45% to 0.85%. The coded and natural values of the variables at various levels are given in Table 6,

Table 6. Natural values for independent variables according to coded values in second-round CCD.

Variables	Coded levels				
	−2	−1	0	+1	+2
Sucrose (S, %w/v)	0.5	2.5	4.5	6.5	8.5
Dextrin (D, %w/v)	0.5	2.5	4.5	6.5	8.5
Polypeptone (P, %w/v)	0.01	0.11	0.21	0.31	0.41
Yeast Extract (Y, %w/v)	0.05	0.45	0.85	1.25	1.65

Table 7. Second-round CCD consisting of 30 planned experiments, along with observed experimental data and data predicted by the RSM model.

Run no.	S	D	P	Y	Coefficients assessed by	Ansamitocin P-3 (mg/l) Observed response	Predicted responses
1	−1	−1	−1	−1	Fractional 2^4 factorial design	20.543	14.056
2	1	−1	−1	−1		16.042	15.220
3	−1	1	−1	−1		19.787	19.122
4	1	1	−1	−1		3.125	6.696
5	−1	−1	1	−1		19.551	19.802
6	1	−1	1	−1		16.435	20.484
7	−1	1	1	−1		22.532	26.006
8	1	1	1	−1		18.578	13.098
9	−1	−1	−1	1		21.794	21.305
10	1	−1	−1	1		34.749	31.588
11	−1	1	−1	1		36.141	32.406
12	1	1	−1	1		35.319	29.099
13	−1	−1	1	1		4.609	1.352
14	1	−1	1	1		16.456	11.152
15	−1	1	1	1		18.737	13.590
16	1	1	1	1		3	9.801
17	−2	0	0	0	Star points (8 points)	23.401	28.600
18	2	0	0	0		25.521	25.975
19	0	−2	0	0		4.703	9.484
20	0	2	0	0		12.326	13.198
21	0	0	−2	0		27.714	33.889
22	0	0	2	0		20.86	20.338
23	0	0	0	−2		5.249	3.475
24	0	0	0	2		0	7.427
25	0	0	0	0	Central points	46.239	43.550
26	0	0	0	0		44.132	43.550
27	0	0	0	0		42.352	43.550
28	0	0	0	0		40.673	43.550
29	0	0	0	0		42.742	43.550
30	0	0	0	0		45.167	43.550

and the experimental design and resulting P-3 titers with this set of experiments are presented in Table 7.

The results of the statistical analysis are given in Table 8, where the first-order effect of polypeptone was the most significant. The second-order effects of sucrose, dextrin, polypeptone, and yeast extract were also highly significant, as evident from their respective p-values, indicating that all the nutritional factors at higher concentrations repressed the antibiotic synthesis. Other reports on various antibiotic productions have also noted such carbon and nitrogen repression effects [12, 33]. A significant interaction was observed between sucrose and dextrin, and between polypeptone and yeast extract as well.

Eight terms were selected, the intercept and seven terms with a p-value less than 0.05. As a result, the following RSM equation was obtained.

Table 8. Model coefficients values and their significances (second-round CCD and RSM).

Terms	Coefficients	t-values	p
Intercept	43.550	19.327	5.1×10^{-12}
S	-0.656	-0.582	0.56891
D	0.928	0.824	0.42275
P	**-3.388**	**-3.007**	**0.00884**
Y	0.988	0.877	0.39432
SD	**-3.397**	**-2.462**	**0.02640**
SP	-0.121	-0.087	0.93149
SY	2.279	1.652	0.11928
DP	0.284	0.206	0.83942
DY	1.508	1.093	0.29152
PY	**-6.425**	**-4.656**	**0.00031**
$\mathbf{S^2}$	**-4.066**	**-3.857**	**0.00154**
$\mathbf{D^2}$	**-8.052**	**-7.640**	$\mathbf{1.5\times10^{-6}}$
$\mathbf{P^2}$	**-4.109**	**-3.899**	**0.00142**
$\mathbf{Y^2}$	**-9.525**	**-9.038**	$\mathbf{1.8\times10^{-7}}$

$$y=43.55-3.88P-3.39SD-6.42PY-4.06S^2-8.05D^2-4.11P^2-9.52Y^2 \quad (6)$$

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

$$y=-246.59+2.96S+21.93D+275.27P+438.33Y-0.85SD-160.62PY-1.01S2-2.01D2-411P2-238Y^2 \quad (7)$$

The contour plots (Fig. 2) clearly revealed an interaction between sucrose and dextrin, and between polypeptone and yeast extract. They also showed the optimum values for sucrose at around 4.5%, dextrin at 4.5%, polypeptone at 0.15–0.18%, and yeast extract at 0.85%–0.9%.

Verification of Model

The optimum values for the independent variables were obtained by solving the regression equation (Eq. 7), and the optimal concentrations were as follows: 4.5% of sucrose, 4.5% of dextrin, 0.16% of polypeptone, and 0.89% of yeast extract for the maximum predicted P-3 titer of 44.3 mg/l. For verification, shake-flask experiments were carried out under the optimum conditions, and a P-3 titer of about 45.2 mg/l was obtained, which was close to the model prediction of 44.3 mg/l.

The accuracy of the RSM model (Eq. 6) was analyzed by an analysis of variance (ANOVA), and the results are summarized in Table 9, where the F-value is the ratio of the mean square due to regression (MSR) to the mean square due to error (MSE). Generally, if the model is a good predictor of experimental results, the calculated

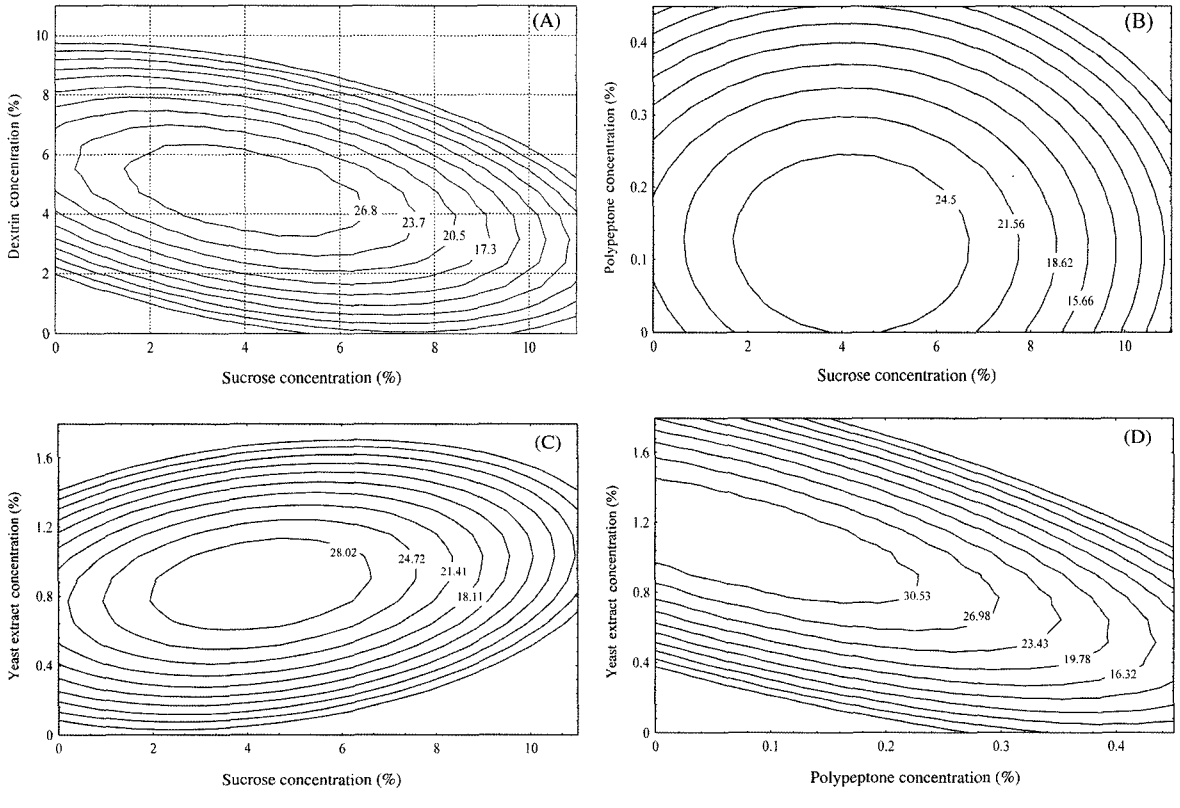

Fig. 2. Contour plots of ansamitocin P-3 concentration (mg/l): Effects of sucrose and dextrin (A), sucrose and polypeptone (B), sucrose and yeast extract (C), and polypeptone and yeast extract (D).

Table 9. Analysis of variance (ANOVA) for the final RSM model.

Source of variations	Sum of squares	Degrees of freedom	Mean square	$F_{statistic}$ (MSR/MSE)	Prob. (P)
Regressions	5221.346 (SSR)	14	372.953 (MSR=SSR/d.f)	12.24	9.1×10^{-6}
Error	456.997 (SSE)	15	30.466 (MSE=SSE/d.f)		
Total	5678.343 (SST)				

Determination coefficient R^2=0.9195; Correlation coefficient R=0.9589.

F-value, $F_{statistic}$, should be several times the tabulated F-value. In this study, the F-value ($F_{statistic\ (14,\ 15)}$=12.24) was much greater than the tabulated $F_{(14,\ 15)}$ (=3.57) [12, 31, 36]. Furthermore, the low probability value (P<0.00009) demonstrated the high significance of the regression model [2, 20]. The fitness of the model was also checked by the determination coefficient (R^2), where the value of the determination coefficient (R^2=0.9195) indicated that only 8% of the total variations was not explained by the regression model, thereby confirming a good correlation between the independent variables [6].

The present work clearly demonstrated the effectiveness of using the Plackett-Burman design to identify critical nutritional factors and RSM with CCD to experimentally determine the optimum medium composition leading to a high production of the antibiotic ansamitocin P-3. After two rounds of optimization using CCD and RSM, the optimal concentrations of carbon and nitrogen sources were identified as 4.5% sucrose, 4.5% dextrin, 0.16% polypeptone, and 0.86% yeast extract. Based on the optimized medium composition, an ansamitocin concentration of 45.2 mg/l was obtained, which was about 6 times higher than that (7.315 mg/l) obtained with an R_2YE medium before optimization.

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