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Optimization of fermentation conditions for pristinamycin production by immobilized *Streptomyces pristinaespiralis* using response surface methodology

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Abstract Response surface methodology was used to optimize the fermentation conditions for the production of pristinamycin by immobilization of *Streptomyces pristinaespiralis* F213 in shaking flask cultivation. Seed medium volume, fermentation medium volume and shaking speed of seed culture were found to have significant effects on pristinamycin production by the Plackett-Burman design. The steepest ascent method was adopted to approach the vicinity of optimum space, followed by central composite design for further optimization. A quadratic model was built to fit the pristinamycin production. The optimum conditions were found to be seed medium volume of 29.5 ml, fermentation medium volume of 28.8 ml, and shaking speed of seed culture at 204 rpm. At the optimum conditions, a production of 213 mg/l was obtained, which was in agreement with the maximum predicted pristinamycin yield of 209 mg/l. This is the first report on pristinamycins production by immobilized *S. pristinaespiralis* using response surface methodology.

Keywords: fermentation conditions, immobilization, pristinamycin, response surface methodology, *Streptomyces pristinaespiralis*

INTRODUCTION

Pristinamycin, which was first discovered in France in 1962, is produced by *Streptomyces pristinaespiralis* (Corvini et al. 2000; Voelker and Altaba, 2001; Corvini et al. 2004). Pristinamycin consists of approximately 30% pristinamycin I (PI_A, PI_B, PI_C) and 70% pristinamycin II (PII_A, PII_B). Because these compounds bind to 23S rRNA of the 50S ribosomal subunit of bacteria and thereby inhibit protein synthesis (Ng and Gosbell, 2005), pristinamycin exhibits a prolonged post-antibiotic effect, and has been considered as an alternative for infections due to penicillin- and macrolide-resistant *S. pneumonia* (Qadri et al. 1997). So there is a growing interest of pristinamycin production in terms of both the strain improvement and the fermentation process optimization. In the last decades, attention has been paid mainly to cloning and analysis of genes involved in the pristinamycin synthesis (Sezonov et al. 1997; Bamas-Jacques et al. 1999; Jin et al. 2010). Whereas so far, few reports have been found on conditions optimization of pristinamycin fermentation. Under conventional batch conditions, the pristinamycins yield with the buffered synthetic medium was about 100 mg/l by a spontaneous mutant (Corvini et al. 2004).

Fermentation of immobilized microbial cells has recently gained much attention among many biotechnological approaches, because of its advantage over conventional free cell systems in respect to retention of high cell density, operational stability, higher efficiency of catalysis, higher volumetric productivity and lower shear stress (Adinarayana et al. 2005; Givry et al. 2008). The adsorption in porous material such as polyurethane foam (PUF) is a very simple immobilization used in a liquid

fermentation (de Ory et al. 2004; Quezada et al. 2009). Many examples of microbial cells immobilization on PUFs have been reported in literatures, such as bacteria (de Ory et al. 2004), microalgae (Yamaguchi et al. 1999), basidiomycetes (Guimaraẽs et al. 2005), ascomycetes (Hama et al. 2006) and Cyanobacteria (Chetsumon et al. 1993). It is well known that designing proper culture conditions is a prerequisite in the production of metabolites (Maia et al. 2001). Nevertheless, the optimization of fermentation conditions for pristinamycin production by immobilized cell has not been made so far.

The conventional optimization method, one-factor-at-a-time approach, is time consuming and incapable of detecting the true optimum, especially due to the complex interactions among various physicochemical parameters (Wang et al. 2008). Response surface methodology (RSM) has overcome these drawbacks, therefore it can evaluate the relative significance of several variables simultaneously, especially in the presence of complex interactions. As a result, RSM is used popularly to solve multivariate problems and has proved to be powerful and useful for the optimization of the target metabolites production (Rahman et al. 2004; Katapodis et al. 2006; Li et al. 2006; Sayyad et al. 2007).

In the present study, a surface response methodology was applied to optimize fermentation conditions for pristinamycins production using *S. pristinaespiralis* immobilized on PUFs. The Plackett-Burman (PB) design was used at first to screen critical factors from a number of process variables, then the steepest ascent method to approach the experimental design space, and finally central composite design (CCD) was applied to further estimate the relationship between the variables and response as well as optimize the levels. The optimal conditions of seed medium volume, fermentation medium volume and shaking speed of seed culture for maximum pristinamycin yield have been quantified with response surface methodology.

MATERIALS AND METHODS

Microorganism and medium

The *Streptomyces pristinaespiralis* strain F213 derived from the chemical mutagenesis of wild-type *S. pristinaespiralis* ATCC25486 was used in the study and the spore solution of the strain was cryopreserved in 20% (v/v) glycerol at -80ºC, which was 5 x 10^6 spores/ml. The seed medium (pH 7.0) was composed of (g/l): soluble starch 15, glucose 10, soybean flour 15, peptone 5, yeast extract 5, KNO_3 2.5, NaCl 2, and $CaCO_3$ 4. For fermentation of immobilized mycelia, the production medium (pH 6.5) was composed of (g/l): soluble starch 40, glucose 10, soybean flour 25, peptone 5, yeast extract 3, fish extract 10, $(NH_4)_2SO_4$ 1.5, $MgSO_4 \cdot 7H_2O$ 3.5, KH_2PO_4 0.2, $CaCO_3$ 4. Both media were sterilized for 30 min at 121ºC.

Microorganism culture and immobilization

Commercial polyurethane foam (PUF, Qitai Foam Co. Ltd, Shanghai, P.R. China) was used as the carrier and cut in the same length (1 cm), thickness (0.5 cm) and different width, the size of the PUFs were determinated by volume. The PUFs were soaked and swollen in 95% alcohol for 24 hrs to remove impurities and washed several times with sufficient distilled water to remove the alcohol. The PUFs were dried in vacuum oven at 80ºC for 8-10 hrs and added to the seed medium before sterilization.

The fermentation condition before optimization was as follows: for seed culture, 1 ml of spore solution was inoculated into a 250 ml shaking flask containing 30 ml seed medium, in which the size and amount of PUFs was 0.35 cm^3 and 17.5 cm^3/100 ml. After incubation for 42 hrs with shaking at 240 rpm at 28ºC, then 1.5 cm^3 PUFs on which mycelia were immobilized were transferred with sterilized tweezers to 30 ml fermentation medium in 250 ml flasks. The fermentation was carried out at 25ºC with shaking at 210 rpm for 72 hrs.

Analytical procedures

To determinate yield of pristinamycin, one volume of the whole fermentation broth containing mycelia and PUFs was directly mixed with two volumes of methanol for 1 hr. After centrifugation (4000 x *g*, 10

min), the supernates were analyzed by high-performance liquid chromatography using a Hypersil C18 column (4.6 by 250 mm), 0.1 M potassium phosphate buffer (pH 2.9) and acetonitrile (55:45, v/v) as the mobile phase, 1 ml/min flow rate with detection at 206 nm. Commercial pristinamycin from Rhone-Poulenc Rorer Co. (Montrouge, France) was used as a reference standard.

Experimental design and data analysis

The preliminary single-factor experiments revealed that the major variables affecting the pristinamycin production were seed medium volume, carrier amount, seed age, carrier size, inoculum volume of fermentation, fermentation medium volume, shaking speed of both seed culture and fermentation culture. These variables were chosen for further optimization.

Plackett-Burman design (PBD)

PBD was used to screen the most important factors influencing pristinamycin production. The experimental design with the name, symbol code, and level of the variables is shown in Table 1. Each independent variable is represented in two levels, high and low, which are denoted by (+) and (-), respectively. Three dummy variables were studied in 12 experiments to calculate the standard error. Pristinamycin fermentation was carried out in duplication and the average value was taken as the response. Usually, the variable with *P*-value of < 0.05 was considered to have a significant effect on the response and was selected for further optimization.

Path of the steepest ascent experiment

To find the neighbourhood of the optimum condition quickly, we used the method of the steepest ascent. The experiments were applied to determine a suitable direction by increasing or decreasing the variables according to the results obtained from the Plackett-Burman design (Gheshlaghi et al. 2005).

Central composite design (CCD)

To describe the optimum culture conditions to enhance the pristinamycin production, the response surface methodology was performed with central-composite design. The levels of each variable and the design matrix are given in Table 2. The low, middle, and high levels of each variable were designated as -1.682, -1, 0, and 1, 1.682, respectively.

Statistical analysis

The Design Expert software (Version 7.0.0, Stat-Ease, Minneapolis, USA) was used for the experimental design and the analysis of variance (ANOVA) for the data. The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was tested by an F-test. The significance of the regression coefficients was determined by a t-test.

RESULTS AND DISCUSSION

Optimization by PBD

Based on our previous single-factor experiments, the importance of the eight culture conditions, namely, seed medium volume, carrier amount, inoculum amount, seed age, fermentation medium volume, shaking speed of both seed culture and fermentation culture for the pristinamycin production was analyzed by PBD. The experimental design and corresponding pristinamycin yields were shown in Table 1, whereas Table 3 shows the effects of these factors on the response and significant levels.

Based on the statistical analysis, fermentation medium volume, with a probability value of 0.022, was determined to be the most significant factor, followed by shaking speed of seed culture (0.047), and seed medium volume (0.053), so these three factors were considered in the further optimization. In the

results, R^2 was found to be 0.9602, which means the model could explain 96.02% of the total variations in the system.

Optimization by the path of the steepest ascent experiment

PBD results indicated that the effect of seed medium volume was positive, whereas that of fermentation medium volume and shaking speed of seed culture was negative. Thus it is predicted that increasing seed medium volume (X_1), while decreasing fermentation medium volume (X_2) and shaking speed of seed culture (X_3) should result in a higher production of pristinamycin. The centre point of the PBD has been considered as the origin of the path. The response for this point was determined as the average of responses for all the runs. From the results of the path of the steepest ascent, it is clearly seen that the yield profile shows a maximum 202 mg/l at run 2 (Table 4). It suggested that this point might be near the region of the maximum pristinamycin yield response. Consequently, this point was chosen as the central point of CCD.

Optimization by response surface methodology

Based on the identification of variables by the PBD and the steepest ascent method, the experiments were performed according to a CCD experimental plan together with experimental results (Table 5). In order to predict the maximum pristinamycin production corresponding to the optimum levels of the three variables, a second-order polynomial model was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second-order polynomial model in coded unit explains the role of each variable and their second-order interactions in producing pristinamycin. All terms, regardless of their significance, were included in the following second-order polynomial equation:

$$y = 209.07 - 12.59\,x_1 - 25.05\,x_2 - 5.8\,x_3 - 4.57\,x_1x_2 - 11.17\,x_1x_3 + 9.08\,x_2x_3 - 42.44\,x_1^2 - 54.46\,x_2^2 - 17.33\,x_3^2$$

[Equation 1]

Where Y is the predicted pristinamycin production, X_1 is the seed medium volume, X_2 is the fermentation medium volume and X_3 is the shaking speed of seed culture. The predicted level of pristinamycin production at each experimental point calculated by the regression equation was shown in Table 5 along with the observed data.

Furthermore, the results of the second-order response surface model in the form of analysis of variance (ANOVA) were shown in Table 6. The P-value was used as a tool for checking the significance of each coefficient. The smaller the P-value, the more significant is the corresponding coefficient (Rahman et al. 2004). The Fisher's F-test with a very low probability value [(P_{model} > F) = 0.0002] demonstrated that the model was highly significant. Among the model terms, X_2, x_1^2, x_2^2 and x_3^2 had significant effects on pristinamycin production with a probability of not less than 95%, however, X_1, X_3 and the interaction terms of X_1, X_2, and X_3 seemed to be insignificant.

The fitness of the model can be checked by the determination coefficient (R^2) and the adjusted determination coefficient (Adj R^2). Here the value of R^2 for (Equation 1) was 0.9232, implying that 92% of the variability in the response could be explained by the model. The value of Adj R^2 was 0.8542 and it was also high enough to advocate for the significance of the model. The model also indicated statistically insignificant lack of fit [(P_{model} > F) = 0.9403], so the model was supposed to be adequate for prediction within the range of variables employed. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared (Tanyildizi et al. 2006; Zhu et al. 2007) and the lower reliability of the experiment is usually indicated by high value of CV (Rahulan et al. 2009). In the present case, the lower value of CV (19.28%) indicates a better precision and reliability of the experiments performed. The normal plot of residuals was shown in Figure 1, as the most important diagnostic for the model, the normal probability plot of the residuals, came up by default. A linear pattern verified normality in the error term, *i.e.*, there were no signs of any problems in the data (Wang and Liu, 2008).

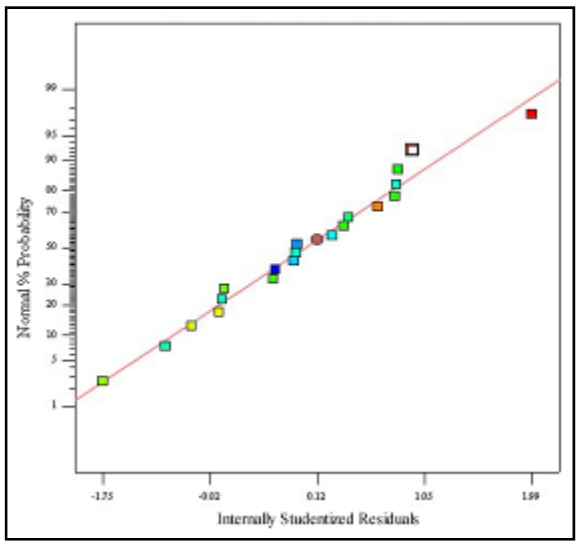

Fig.1 Normal plot of residuals.

The 3D response surface curve and 2D contour plot are generally the graphical representation of the regression equation. The three dimensional response surface and their corresponding 2D contour plots for the pristinamycin production against any two independent variables while the other independent variable maintained at zero levels were presented in Figure 2 and Figure 3. The graphical representation provides a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between test variables (Rahulan et al. 2009). The optimum value of each variable was located based on the hump in the three-dimensional plot, or from the central point of the corresponding contour plot.

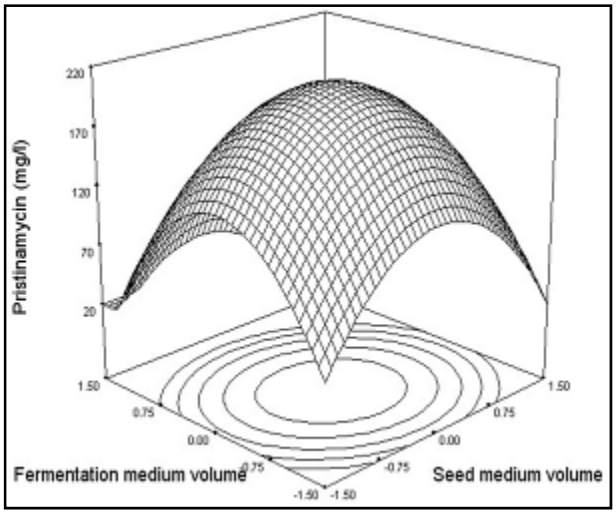

Fig. 2 Response surface plot of pristinamycin production expressed as a function of the seed medium volume and fermentation medium volume.

Figure 2 depicted the three dimensional plot and its respective contour plot showing the response surface from the interaction between seed medium volume (X_1) and fermentation medium volume (X_2) while keeping other variable at its zero level. It can be seen from Figure 2, when fermentation medium volume was at a fixed level, the pristinamycin production increased with the increasing volume of seed

medium but decreased beyond the range. On the contrary, when seed medium volume was at a fixed level, the effect of fermentation medium volume on the production response was similar to that of seed medium volume. These indicated that maximum pristinamycin could be obtained in the middle volume of both seed medium and fermentation medium. The elliptical nature of the contour plots stated clearly that the interaction between seed medium volume and fermentation medium volume were significant.

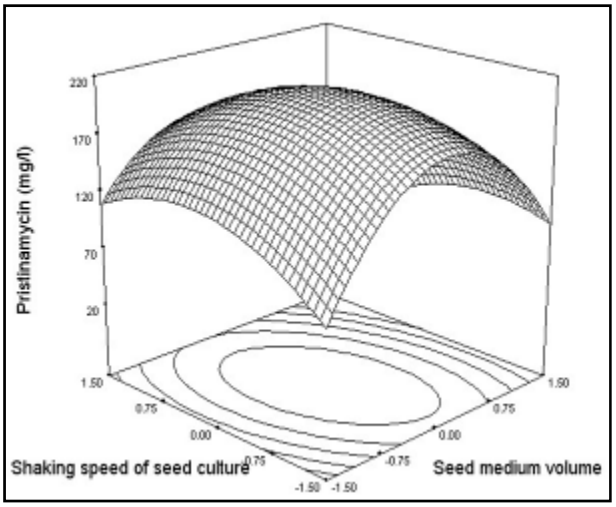

Fig. 3 Response surface plot of pristinamycin production expressed as a function of seed medium volume and shaking speed of seed culture.

Figure 4 showed the effect of fermentation medium volume (X_2) and shaking speed of seed culture (X_3) on pristinamycin production while the third variable was fixed at its middle level. It was obvious that higher level of fermentation medium volume and lower shaking speed of seed culture would not increase the yield of pristinamycin. This may be that low dissolved oxygen results in the inhibitory effects to pristinamycin synthesis. When fermentation medium volume was at higher level (exceed 33 ml), pristinamycin production varied little with the change of shaking speed levels in seed culture. It was also noticed that pristinamycin production increased rapidly first and then decreased with gradually increasing value in fermentation medium volume, while shaking speed of seed culture was fixed at a certain level. This indicated that the requirements of dissolved oxygen and shearing strength to mycelia were quite high in pristinamycin fermentation by immobilized *Streptomyces pristinaespiralis* F213.

Figure 3 depicted the effect of seed medium volume (X_1) and shaking speed of seed culture (X_3) on pristinamycin production while fermentation medium volume was fixed at its zero level. As shown in Figure 3, when seed medium volume maintained at moderate level (27.5 ~ 32.5 ml), pristinamycin production varied little with the change of shaking speed of seed culture. However, pristinamycin production was sensitive to the change of shaking speed of seed culture, it tended to decrease rapidly with the increasing or decreasing shaking speed of seed culture, when the volume of seed medium was oversize or undersize.

Validation of the optimized condition

Based on the quadratic model, the optimal values of each test variables in coded levels were as follows: X_1 = - 0.11, X_2 = - 0.24 , and X_3= - 0.19, whose actual values were seed medium volume 29.5 ml, fermentation medium 28.8 ml, and shaking speed of seed culture 204 rpm. The model predicted that the production of pristinamycin could reach 209 mg/l under the optimal condition. To verify the predicted result, validation experiment was carried out in triplicate test and the observed experimental production of pristinamycin was 213 ± 9 mg/l, which was closer to the predicted response. However the pristinamycin yield was 91 mg/l before optimization, a 1.34-fold increase had been obtained.

The RSM design applied in the present investigation have been successfully used in many metabolite production for optimization of immobilization conditions (Sankpal and Kulkarni, 2002; Aybastier and Demir, 2010; Liu et al. 2010). However, to the best our knowledge, there are no reports of optimization of pristinamycin production by immobilized *S. pristinaespiralis* using statistical experimental design.

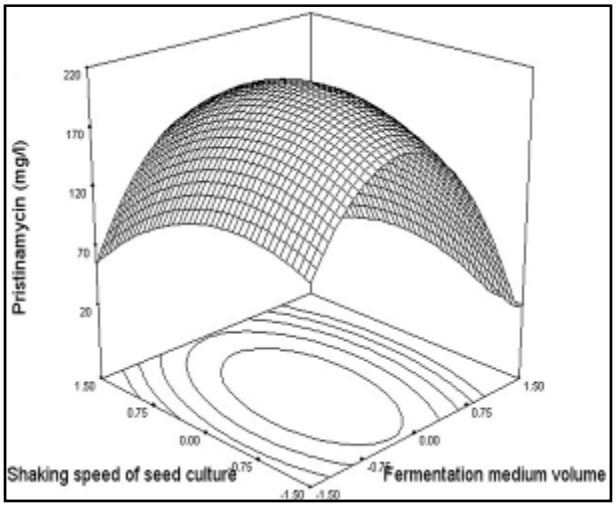

Fig. 4 Response surface plot of pristinamycin production expressed as a function of fermentation medium volume and shaking speed of seed culture.

CONCLUDING REMARKS

Response surface methodology used in this investigation suggested the importance of dissolved oxygen supply for pristinamycin production in immobilization fermentation. A highly significant quadratic polynomial obtained by the CCD was very useful for determining the optimal conditions with significant effects on pristinamycin production. Validation experiments were also performed to verify the accuracy of the model, and the results indicated that the predicted value agreed with the experimental values well. A maximum pristinamycin yield of 213 mg/l was achieved, which was 2.34-fold higher than that before optimization, but it was lower than the reported yield of 412 mg/l (Xu et al. 2009). The less yield may be due to the difference of the strain used: the strain used in this study is a mutant strain, while that in the literature is a recombinant created from genome shuffling. Thus, for the fermentation of immobilized *S. pristinaespiralis* on PUFs, the response surface methodology was found to be a favourable strategy to optimize fermentation conditions for pristinamycin production. Being convenient and effective, this method might be useful in optimization of the immobilized fermentation for the overproduction of other metabolites.

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Tables

Table 1. The Placker-Burman design variables (in coded levels) with yield of pristinamycin as response.

Run	Variable levels											Pristinamycin (mg/l)
	A	B	D_1[a]	D	E	D_2	G	H	D_3	J	K	
1	1	-1	1	1	-1	1	1	1	-1	-1	-1	161
2	1	1	1	-1	-1	-1	1	-1	1	1	-1	173
3	-1	-1	-1	1	-1	1	1	-1	1	1	1	151
4	-1	1	1	-1	1	1	1	-1	-1	-1	1	186
5	-1	-1	1	-1	1	1	-1	1	1	1	-1	108
6	1	-1	-1	-1	1	-1	1	1	-1	1	1	105
7	1	1	-1	1	1	1	-1	-1	-1	1	-1	170
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	105
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	196
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	128
11	1	1	-1	-1	-1	1	-1	1	1	-1	1	172
12	1	-1	1	1	1	-1	-1	-1	1	-1	1	235

[a]D_1-D_3 were dummy variables; other symbols were the same as those in Table 3.

Table 2. Coded and real values of variables in central composition design (α = 1.682).

variables	Level of variables				
	−α	−1	0	+1	+ α
X_1: seed medium volume (ml)	21.59	25	30	35	38.41
X_2: fermentation medium volume (ml)	21.59	25	30	35	38.41
X_3: shaking speed of seed culture (rpm)	159.54	180	210	240	260.46

Table 3. The Placker-Burman design for screening variables in pristinamycin production.

Factors	Code	Low level (−1)	High level (+1)	Effect	t-Test	*P*-value Prob>F	Confidence Level (%)
Seed medium volume (ml)	A	25	35	28.933	4.184	0.053	94.74
Carrier amount (cm^3/100ml)	B	15	22	-9.63	-1.392	0.298	70.17
Seed age	D	36	48	-3.87	-0.559	0.632	36.78
Carrier size (cm^3)	E	0.25	0.5	-2.53	-0.365	0.750	25.02
Inoculum volume of fermentation (cm^3)	G	1	2	-12.04	-1.741	0.224	77.63
Fermentation medium volume (ml)	H	25	35	-46.09	-6.666	0.022	97.82
Shaking speed of seed culture (rpm)	J	190	230	-30.73	-4.444	0.047	95.29
Shaking speed of fermentation culture (rpm)	K	200	240	16.263	2.352	0.143	85.70

R^2 = 96.02%, R^2 (adj) = 85.40%.

Table 4. Experimental design and response of the steepest ascent path experiments.

Run	Factors								Pristinamycin (mg/l)
	X_1 (ml)	X_2 (ml)	X_3 (rpm)	B[a]	D	E	G	K	
1	25	35	240	15	36	0.25	1	240	121
2	30	30	210						202
3	35	25	180						144
4	40	20	150						133

[a]The symbols B, D,E,G,K were the same as those in Table 3.

Table 5. Experimental result of central composition design showing observed and predicted pristinamycin production.

Run	X_1	X_2	X_3	Pristinamycin (mg/l)	
				Observed	Predicted
1	-1	-1	-1	143	132
2	-1	1	1	107	101
3	-1	-1	1	136	124
4	-1	1	-1	84	72
5	1	-1	-1	143	138
6	1	-1	1	85	86
7	1	1	-1	59	61
8	1	1	1	44	45
9	-1.682	0	0	91	110
10	1.682	0	0	72	680
11	0	-1.682	0	86	97
12	0	1.682	0	9	13
13	0	0	-1.682	159	170
14	0	0	1.682	146	150
15	0	0	0	231	209
16	0	0	0	169	209
17	0	0	0	186	209
18	0	0	0	224	209
19	0	0	0	255	209
20	0	0	0	192	209

Table 6. ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F Value	*P*-value Prob > F
Model	76899.76	9	8544.418	13.187	0.0002
X_1	2116.934	1	2116.934	3.267	0.1008
X_2	8550.057	1	8550.057	13.196	0.0046
X_3	446.5	1	446.500	0.689	0.4258
$X_1 X_2$	157.975	1	157.975	0.244	0.6321
$X_1 X_3$	962.508	1	962.508	1.485	0.2509
X_2X_3	645.303	1	645.303	0.996	0.3418
X_1^2	25989.01	1	25989.014	40.110	< 0.0001
X_2^2	42979.31	1	42979.305	66.331	< 0.0001
X_3^2	4314.174	1	4314.174	6.658	0.0274
Residual	6479.475	10	647.947		
Lack of Fit	1156.868	5	231.374	0.217	0.9403
Pure Error	5322.607	5	1064.521		
Total	83379.24	19			

*R - Sq = 0.9232; CV = 19.28%; R- Sq (adjust) = 0.8542.