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Response surface methods for optimizing *Saccharopolyspora spinosa*, a novel macrolide producer

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SUMMARY

Strain A83543, recently identified as *Saccharopolyspora spinosa*, was cultured in a variety of media to optimize macrolide titer. Response surface methodology (RSM) was used to improve the fermentation medium and to characterize the microorganism's response to systematic variations in medium composition. Three sequential RSM studies on wild-type A83543 and two high macrolide-producing mutants showed that each strain produced maximum titers in nearly identical fermentation media. No obvious differences in nutrient requirements were evident in the three strains indicating little interaction between mutational change and medium composition through at least two cycles of mutagenesis. The overall increase in macrolide titer starting from the wild-type organism in the original fermentation medium to the second-generation mutant in the optimized medium was over 25-fold.

INTRODUCTION

Strain A83543, identified as *Saccharopolyspora spinosa*, is a new species of actinomycete which produces a large family of macrolide compounds [1,2]. Two major components, A83543A and A83543D, are of primary interest due to their mosquito larvicidal activity. The tetracyclic ring system of A83543 (Fig. 1) was shown to be derived from acetate and propionate units, suggesting biosynthesis through a polyketide pathway [3].

Fermentation yield improvement for the combined titer of A83543A and A83543D was studied empirically using response surface methodology (RSM). Reports on the use of RSM to optimize biochemical reactions and microbial processes remain relatively scarce. This situation is changing, however, with the advent of personal computers and statistical programs for the facile processing of experimental data [4]. Swanson et al. [5] reported on the use of RSM to formulate a mathematical model for industrial-scale saccharification of corn starch hydrolysate using commercial enzymes. RSM has also been applied to the optimization of primary and secondary metabolites in fermentation processes. Optimization of fumaric acid production [6] and the production of gibberellic acid from whey permeate by *Gibberella fujikuroi* [7] are two respective examples. Recently published reports on the use of mathematical models for the improvement of metabolite titers by fermentation include asperlicin production in *Aspergillus alliaceus* [8] and the production of a novel polyketide, herboxidiene, by *Streptomyces chromofuscus* [9]. RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantitated using RSM.

For A83543 macrolide production, five levels (concentrations) of four fermentation medium components (glucose, cottonseed flour, peptonized milk and corn steep

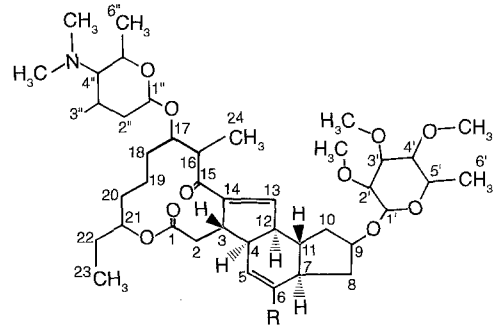

Fig. 1. Structure of A83543. For A83543A, R = H; for A83543D, R = CH_3.

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liquor) were studied using RSM. Three strains of *Sa. spinosa*, the wild-type and two successive high-titer mutants, were tested for combined A83543A and A83543D production in response to systematic variations in medium composition imposed by the response surface design. This paper reports that the optimal formulation for the highest titer mutant was also optimal for lower titer strains, showing that mutagenesis contributed more to the increase in macrolide production than did medium re-formulation. Therefore, it is likely that continued evaluation of new high-titer mutants can be done adequately in the present fermentation medium.

MATERIALS AND METHODS

Microorganisms

Saccharopolyspora spinosa A83543.0 (original soil isolate), A83543.3 (NRRL 18537) and A83543.5 (NRRL 18539) were used throughout this study. The latter two strains are *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced isolates originating from the wild-type A83543.0. Culture stocks were maintained under liquid nitrogen; 1 ml of the thawed stock was used to inoculate 50 ml of vegetative medium in a 250-ml shake-flask.

Media and growth conditions

The vegetative medium was composed of the following ingredients (g/l): glucose (10.0), N-Z-Amine A (Sheffield) (30.0), yeast extract (3.0) and $MgSO_4 \cdot 7H_2O$ (2.0) in deionized water with no pH adjustment. Strains were cultured for 48 h on a 250 rpm rotary shaker at 30 °C. One ml of vegetative culture (3.3% v/v) was used to inoculate all subsequent fermentation media. The initial fermentation medium was composed of the following ingredients (g/l): glucose (40.0), Proflo cottonseed flour (Traders Protein) (40.0), peptonized milk nutrient or PMN (Sheffield) (15.0), corn steep liquor or CSL (5.0), methyl oleate or MEO (40.0) and $CaCO_3$ (5.0) in tap water with pH adjustment to 7.0. The medium was dispensed into 250-ml Erlenmeyer flasks (30 ml per flask) and capped with foam closures (Bellco Glass Co., Vineland, NJ). All media were sterilized by autoclaving at 121 °C for 20 min. Strains were cultured for up to 7 days at 30 °C on a 250 rpm rotary shaker.

Plackett-Burman screening design

A Plackett-Burman screening design was initially used to detect improvements in A83543 titers [10,11]. Two levels (concentrations) of each of the following variables were studied for their effects on macrolide production: glucose (10 and 40 g/l), soluble starch (10 and 30 g/l), Proflo (10 and 30 g/l), PMN (0 and 20 g/l), CSL (0 and 10 g/l), MEO (10 and 40 g/l) and $CaCO_3$ (0 and 5 g/l).

Response surface optimization design

Based on the results obtained from the Plackett-Burman screen, four medium components were studied further using a Box-Wilson Central Composite Design [12]. In this design, all four variables (medium components) could be tested simultaneously using five different levels of each component (Table 1).

If all combinations of medium components (each at their five respective levels) were tested, the total number of shake-flask cultures required would be 625. Because Box-Wilson is a fractional design, the total number of runs required was 31, including seven replicates of the center point.

Analytical methods

The primary response measured was 'total titer', the combined quantities of A83543A and A83543D. Samples were prepared by diluting whole broth (1:4) with acetonitrile, vortexing and allowing to stand at room temperature for 15 min followed by filtration through a 0.45-micron membrane. Quantitative HPLC was accomplished with a C-18 reversed-phase column (Waters Nova-Pak RCM 8 x 100 mm, 4 micron). The flow rate was 4.0 ml/min. Samples were eluted with a mobile phase consisting of acetonitrile/methanol/1.0% (w/v) aqueous ammonium acetate (45:45:10). The eluent was monitored at 250 nm with a detector setting of 0.1 AUF. Other responses measured, but not modeled, included final culture pH, residual glucose and packed cell volume (PCV). Residual glucose was determined by reaction with hexokinase (Sigma Chemical Co., St. Louis, MO) and PCV (% v/v) was determined by centrifugation of 10 ml of whole broth at 2200 rpm for 15 min. Multiple regression analysis of total titer was done with the aid of two computer programs, XSTAT (Wiley Interscience, New York, NY) and RS Discover (BBN, Cambridge, MA).

TABLE 1

Combinations of medium components

Medium component (variable)	Concentrations used (g/l)				
Glucose	20	40	60	80	100
Proflo	10	20	30	40	50
PMN	10	15	20	25	30
CSL	1	5.5	10	14.5	19

Factors held constant: methyl oleate, 40 g/l; $CaCO_3$, 5 g/l; tap H_2O; pH 7.0; 30 °C, 250 rpm, 7 days.

RESULTS

Initial fermentation medium

Mean total titer (A83543A and A83543D), harvest pH, PCV and residual glucose were determined from six replicate shake-flask cultures of each strain after 7 days growth (Table 2).

Plackett-Burman screening (A83543.0)

Sixteen shake-flask runs were used to estimate the main effects of seven fermentation medium components (Table 3). The last column in the table shows the total macrolide titer obtained after growth for 7 days. The largest effects on total titer (based on the magnitude of the regression coefficients) were seen by changing the concentrations of glucose, Proflo, PMN, methyl oleate and $CaCO_3$ (Table 4). Only the glucose effect was significant at the 95% confidence limit. Strains A83543.3 and A83543.5 responded similarly to A83543.0 in Plackett-Burman studies except that total titers were higher (data not shown). It was decided to proceed with a Box-Wilson optimization design, varying glucose, Proflo, PMN and CSL, while holding methyl oleate constant at 40 g/l and $CaCO_3$ constant at 5 g/l. Methyl oleate and $CaCO_3$ were not tested further because their effects on total titer were previously shown to be insignificant over a broad range of concentrations (data not shown).

Response surface optimization

Thirty-one shake-flask runs were required to estimate the effects of the 5^4 factor matrix (five concentrations of four medium components or 625 combinations). The total titer response for each of the A83543 strains is shown in Table 5. The actual concentration of each medium component was coded to facilitate multiple regression analysis (Table 6).

The total titer for each shake-flask run was determined after a growth period of 7 days. Runs 25 through 31 are replicates of the center point of the model and these replicates were used to estimate the amount of pure error in the model. The center point of the model is coded as 0,0,0,0 and, for these studies, represents fermentations containing glucose at 60 g/l, Proflo at 30 g/l, PMN at 20 g/l and CSL at 10 g/l. The settings selected for the center point represent the 'best guess' as to where the optimum for each medium component lies.

Fermentations representing the center point in the model were monitored daily over the entire 7-day growth period (Fig. 2). Results showed that A83543.5 produced about 4-fold more macrolide than the parent strain, A83543.0, and that macrolide production began to level off in all three strains after 7 days.

Multiple regression analysis for total titer in A83543.5 resulted in the prediction equation: $y = 813.6 + 90.5X_1 - 150.1X_2 - 21.9X_3 + 60.3X_4 - 41.1X_1X_2 - 9.8X_1X_3 + 26.7X_1X_4 + 21.7X_2X_3 - 79.1X_2X_4 + 28.2X_3X_4 - 119.2X_1^2 - 112.8X_2^2 - 36.5X_3^2 - 47.7X_4^2$, where y = predicted total titer and X_1, X_2, X_3 and X_4 = coded levels for glucose, Proflo, PMN and CSL, respectively. The regression coefficients for glucose and CSL were large and significant (Table 7). Therefore, higher titers should result from increases in glucose and CSL over the experimental range. The large negative coefficient for Proflo indicates that total titer decreases with increasing levels of Proflo.

The coefficients also indicate the existence of a negative interaction (antagonism) between Proflo and CSL. This suggests that the use of high concentrations of both components in a medium formulation should be avoided to obtain optimal macrolide titers. A negative interaction also exists between glucose and Proflo.

The last four coefficients in the multiple regression

TABLE 2

Mean total titer, harvest pH, PCV and residual glucose in the initial fermentation medium

Strain	Titer (μg/ml)	pH	PCV (% v/v)	Glucose (% v/v)
A83543.0	35 ± 14	7.5 ± 0.3	41 ± 2.9	0.11 ± 0.08
A83543.3	77 ± 23	7.6 ± 0.2	39 ± 2.9	0.00 ± 0.00
A83543.5	268 ± 55	7.9 ± 0.2	38 ± 3.8	0.00 ± 0.00

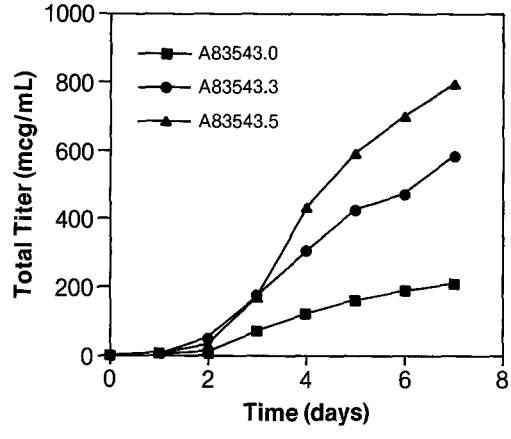

Fig. 2. Comparison of total titer over a 7-day period for A83543.0, A83543.3, and A83543.5. The medium used represents the geometrical center point in the Box-Wilson Central Composite Design. The ingredients were (g/l): glucose (60), Proflo (30), PMN (20), CSL (10), MeO (40), and $CaCO_3$ (5). The fermentations were run in triplicate; standard deviations for all strains were less than 10%. See text for abbreviations.

TABLE 3

Plackett-Burman screening results for strain A83543.0

Run[a] No.	Media components (g/l)							Total titer (μg/ml)
	glucose	starch	Proflo	PMN	CSL	MEO	$CaCO_3$	
1	40.0	30.0	30.0	20.0	0.0	40.0	0.0	63.0
2	40.0	30.0	30.0	0.0	10.0	10.0	5.0	54.0
3	40.0	30.0	10.0	20.0	0.0	40.0	5.0	117.0
4	40.0	10.0	30.0	0.0	10.0	40.0	0.0	85.0
5	10.0	30.0	10.0	20.0	10.0	10.0	0.0	34.0
6	40.0	10.0	30.0	20.0	0.0	10.0	5.0	87.0
7	10.0	30.0	30.0	0.0	0.0	40.0	0.0	23.0
8	40.0	30.0	10.0	0.0	10.0	10.0	0.0	49.0
9	40.0	10.0	10.0	20.0	0.0	10.0	0.0	63.0
10	10.0	10.0	30.0	0.0	0.0	10.0	5.0	15.0
11	10.0	30.0	10.0	0.0	0.0	40.0	5.0	45.0
12	40.0	10.0	10.0	0.0	10.0	40.0	5.0	70.0
13	10.0	10.0	10.0	20.0	10.0	40.0	5.0	100.0
14	10.0	10.0	30.0	20.0	10.0	40.0	0.0	17.0
15	10.0	30.0	30.0	20.0	10.0	10.0	5.0	21.0
16	10.0	10.0	10.0	0.0	0.0	10.0	0.0	30.0

[a] Each run represents a single shake-flask fermentation.

analysis provide an estimate of the 'curvature' in the response to each medium component over the experimental range. The negative sign for the coefficients in Table 7 indicates that the total titer is predicted to decline in media formulations where the concentrations of glucose, Proflo, PMN and CSL deviate away from the center point of the model. Multiple regression analysis for strains A83543.0 and A83543.3 showed the same general trends as those described for the high-titer mutant, A83543.5 (data not shown).

Multiple regression coefficients can be used to predict optimum titers within the experimental range (see equation above). The maximum total titer predicted by the model for A83543.0, A83543.3 and A83543.5 was 223, 625 and 999 μg/ml, respectively (Table 8). The medium composition needed to obtain the maximum titer for each strain was nearly identical.

Verification of the empirical model (A83543.5)

The optimized medium formulation for A83543.5 as determined by the empirical model was (g/l): glucose (68), Proflo (22), PMN (25) and CSL (14.5), with methyl oleate held at 40 g/l and $CaCO_3$ held at 5 g/l. The total titer

TABLE 4

Regression coefficients for total titer

Coefficient	Term	Standard error	*t*-Value	Confidence coef. < > 0[a] (%)
54.56	1 (constant)	4.985	10.95	99.9
18.94	glucose	4.985	3.799	99.4
−3.813	starch	4.985	0.7648	51.8
−8.937	Proflo	4.985	1.793	89.0
8.187	PMN	4.985	1.642	86.0
−0.8125	CSL	4.985	0.1630	21.9
10.44	MEO	4.985	2.094	93.3
9.063	$CaCO_3$	4.985	1.818	89.5

[a] Confidence figures are based on 8 degrees of freedom.

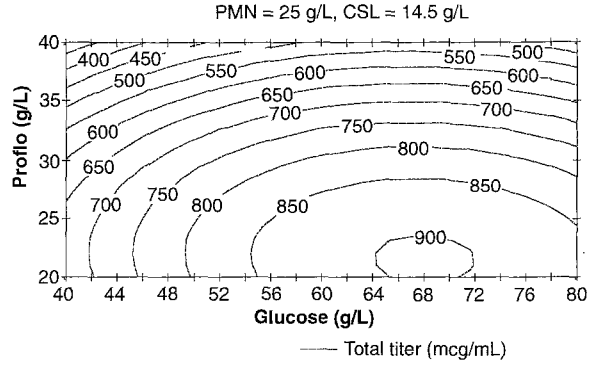

Fig. 3. Contour plot showing total titer in response to varying concentrations of Proflo and glucose. Factors held constant were PMN (25 g/l) and CSL (14.5 g/l). The strain depicted is high-titer mutant A83543.5.

TABLE 5

Total titer response for strains A83543.0, A83543.3, and A83543.5 after 7 days growth in 25 media defined by the Box-Wilson Central Composite Design

Run No.	Controlled factors (coded)				Total titer (μg/ml)		
	glucose	Proflo	PMN	CSL	A83543.0	A83543.3	A83543.5
1	−1.000	−1.000	−1.000	−1.000	183.0	504.0	526.0
2	1.000	−1.000	−1.000	−1.000	145.0	448.0	644.0
3	−1.000	1.000	−1.000	−1.000	65.0	119.0	272.0
4	1.000	1.000	−1.000	−1.000	100.0	361.0	427.0
5	−1.000	−1.000	1.000	−1.000	158.0	388.0	332.0
6	1.000	−1.000	1.000	−1.000	112.0	463.0	418.0
7	−1.000	1.000	1.000	−1.000	57.0	107.0	284.0
8	1.000	1.000	1.000	−1.000	93.0	322.0	318.0
9	−1.000	−1.000	−1.000	1.000	166.0	465.0	614.0
10	1.000	−1.000	−1.000	1.000	117.0	462.0	501.0
11	−1.000	1.000	−1.000	1.000	96.0	213.0	287.0
12	1.000	1.000	−1.000	1.000	141.0	385.0	343.0
13	−1.000	−1.000	1.000	1.000	83.0	451.0	582.0
14	1.000	−1.000	1.000	1.000	199.0	544.0	957.0
15	−1.000	1.000	1.000	1.000	67.0	147.0	288.0
16	1.000	1.000	1.000	1.000	101.0	352.0	321.0
17	−2.000	0.000	0.000	0.000	61.0	116.0	144.0
18	2.000	0.000	0.000	0.000	159.0	518.0	623.0
19	0.000	−2.000	0.000	0.000	158.0	434.0	684.0
20	0.000	2.000	0.000	0.000	42.0	124.0	134.0
21	0.000	0.000	−2.000	0.000	136.0	497.0	700.0
22	0.000	0.000	2.000	0.000	117.0	610.0	729.0
23	0.000	0.000	0.000	−2.000	120.0	493.0	593.0
24	0.000	0.000	0.000	2.000	178.0	585.0	746.0
25	0.000	0.000	0.000	0.000	201.0	574.0	781.0
26	0.000	0.000	0.000	0.000	202.0	630.0	561.0
27	0.000	0.000	0.000	0.000	226.0	502.0	948.0
28	0.000	0.000	0.000	0.000	217.0	593.0	875.0
29	0.000	0.000	0.000	0.000	109.0	548.0	756.0
30	0.000	0.000	0.000	0.000	186.0	655.0	914.0
31	0.000	0.000	0.000	0.000	197.0	755.0	742.0

TABLE 6

Code for concentration of each medium component

Actual concentration (g/l)				Coded levels
glucose	Proflo	PMN	CSL	
20	0	10	1.0	−2
40	20	15	5.5	−1
60	30	20	10.0	0
80	40	25	14.5	+1
100	50	30	19.0	+2

for ten replicate shake-flask cultures grown in the optimized formulation was 1037 μg/ml with a standard deviation of 93.9 μg/ml (Table 9). These results confirm the predictions made by the empirical model. No verification trials were done on A83543.0 or A83543.3 because these strains are less interesting from the point of view of macrolide productivity.

Response surface plots (A83543.5)

The relationship between any two factors and total titer can be quantitated and displayed in the form of a contour plot or map (Fig. 3). For example, the interaction between

TABLE 7

Regression coefficients for total titer in strain A83543.5; $R^2 = 92.7\%$

Coefficient	Term	Standard error	t-Value	Confidence coef. $< > 0$[a] (%)
813.6	1 (constant)	41.25	19.72	99.9
90.48	glucose	19.75	4.582	99.9
−150.1	Proflo	19.75	7.603	99.9
−21.90	PMN	19.75	1.109	69.7
60.31	CSL	19.75	3.054	99.3
−41.09	glucose + Proflo	24.73	1.662	88.2
−9.844	glucose + PMN	24.73	0.3981	32.8
26.72	glucose + CSL	24.73	1.080	68.4
21.72	Proflo + PMN	24.73	0.8783	58.4
−79.09	Proflo + CSL	24.73	3.198	99.5
28.16	PMN + CSL	24.73	1.139	71.0
−119.2	glucose^2	18.17	6.561	99.9
−112.8	Proflo^2	18.17	6.211	99.9
−36.47	PMN^2	18.17	2.007	94.1
−47.72	CSL^2	18.17	2.626	98.4

[a] Confidence figures are based on 13 degrees of freedom; highest and lowest values for the center point replicates were omitted for regression analysis.

Proflo and glucose can be studied and the optimal region(s) can be identified while holding the other two factors, PMN and CSL, constant. Fig. 3 shows that a single optimal region exists within the experimental range of Proflo and glucose. A titer of greater than 900 μg/ml is predicted when Proflo is held between 20 and 23 g/l and glucose is held between 66 and 70 g/l. The prediction applies when PMN is held at 25 g/l and CSL is held at 14.5 g/l.

The same relationship between Proflo and glucose can be depicted three-dimensionally (Fig. 4). The three-dimensional plot emphasizes the necessity for Proflo to remain at low levels in the fermentations (20–23 g/l) in order for titer to increase significantly with increasing glucose.

TABLE 8

Maximum total titers predicted by the response surface model

Strain	Titer predicted (μg/ml)	Optimal medium predicted			
		glucose (g/l)	Proflo (g/l)	PMN (g/l)	CSL (g/l)
A83543.0	223	77	28	20	14.5
A83543.3	625	67	25	22	12.0
A83543.5	999	68	22	25	14.5

TABLE 9

Total titer for ten replicate shake-flask cultures of A83543.5 in the predicted optimal fermentation medium

Trial replicate	Final pH	A83543A[a]	A83543D[a]	Total titer[a]
1	7.5	804	162	966
2	7.5	860	123	983
3	7.5	838	119	957
4	7.5	884	156	1040
5	7.5	1046	177	1223
6	7.5	811	141	932
7	7.5	835	151	986
8	7.5	934	152	1086
9	7.5	998	169	1167
10	7.5	861	148	1009

[a] Concentration in μg/ml.

DISCUSSION

Optimization of media for A83543 wild-type and two successive high-titer mutants using response surface methods resulted in almost identical medium formulations for the three strains. Under optimal fermentation conditions, there was a 5-fold increase in total macrolide titer when comparing the wild-type strain (A83543.0) to the second-generation mutant (A83543.5). The overall increase in macrolide production, comparing A83543.0 in the initial fermentation medium (before optimization) to the A83543.5 mutant after response surface optimization was 25-fold. A83543.5 produced approx. 1 mg of macrolide/ml in the optimized medium.

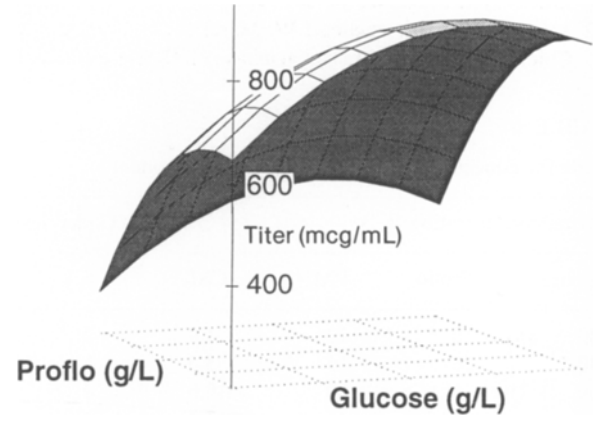

Fig. 4. Three-dimensional response surface of contour map in Fig. 3. Total titer appears on the vertical axis.

The RSM model was confirmed for A83543.5 by growing ten replicate shake-flask cultures under the predicted optimal settings for glucose, Proflo, PMN and CSL. The observed mean titer was 1037 ± 94 μg/ml compared to a predicted mean titer of 999 μg/ml.

The practice of fermentation yield improvement through cycles of mutation and medium optimization has not been well documented. In the case of A83543 wild-type and the mutants presented here, there is little interaction between mutation and the medium re-formulation process. Interaction would be expected if high-titer mutants possessed changes in their basic physiology; e.g., rate of glucose utilization or level of proteolytic activity. The fact that the optimum medium was the same for all three strains strongly suggests that the mutations involved here are rather specific for the increased biosynthesis of A83543A and A83543D.

This report shows how RSM can be used to formulate an empirical model for the production of A83543 macrolides. The validity of the model was checked with one of three strains which produces high macrolide titers. The optimized, RSM-derived, medium represents the best medium for the continued evaluation of high-titer strains.

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