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Effects of Sucrose, Phosphate, and Calcium Carbonate on the Production of Pikromycin from *Streptomyces venezuelae* S

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Polyketide secondary metabolites share common precursor pools, acyl-CoA. Thus, the effects of engineering strategies for heterologous and native secondary metabolite production are often determined by the measurement of pikromycin in *Streptomyces venezuelae*. It is hard to compare the effectiveness of engineering targets among published data owing to the different pikromycin production media used from one study to the other. To determine the most important nutritional factor and establish optimal culture conditions, medium optimization of pikromycin from *Streptomyces venezuelae* ATCC 15439 was studied with a statistical method, Plackett-Burman design. Nine variables (glucose, sucrose, peptone, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $CaCO_3$) were analyzed for their effects on a response, pikromycin. Glucose, K_2HPO_4, and $CaCO_3$ were determined to be the most significant factors. The path of the steepest ascent and response surface methodology about the three selected components were performed to study interactions among the three factors, and the fine-tune concentrations for maximized product yields. The significant variables and optimal concentrations were 139 g/l sucrose, 5.29 g/l K_2HPO_4, and 0.081 g/l $CaCO_3$, with the maximal pikromycin yield of 35.5 mg/l. Increases of the antibiotics production by 1.45-fold, 1.3-fold, and 1.98-fold, compared with unoptimized medium and two other pikromycin production media SCM and SGGP, respectively, were achieved.

Keywords: *Streptomyces venezuelae*, pikromycin, media optimization, response surface methodology

Introduction

Large numbers of secondary metabolites such as antibiotics and antitumor drugs are produced by microorganisms, and about 7600 are known to be produced by *Streptomyces* species [3]. Fast and non-aggregating growth characteristics that allow a large cell mass, and efficient profiling of primary and secondary metabolites have given attention to *Streptomyces venezuelae*, producing mainly pikromycin and methymycin, as being a good heterologous expression host [30]. Pikromycin is a polyketide secondary metabolite, a 14-membered cyclized macrolactone narbonolide with a desosamine that has hydroxylation at a specific carbon [21]. Owing to the nature of polyketide sharing common acyl-CoA precursors, results of engineering strategies for enhancing the heterologous or native secondary metabolite productions are often screened by measurement of the antibiotic pikromycin [22, 32].

The production of secondary metabolites with *Streptomyces venezuelae* is often conducted in many different media. The strain is inoculated from the fermentation medium to three different production media for pikromycin alone [8, 9, 20], which results in altered metabolic behaviors leading to changes in titers of product yields. Since the titers and

growth conditions are different from one another, it complicates research conduction and data comparisons, such as microarray and ChIP data, as a result. Because gene manipulations of *Streptomyces* species are difficult and limited compared with that of other microorganisms like *Escherichia coli* [5], resolving this complication is crucial for selecting a right engineering target for host generation.

The aim of this research was to determine the most significant factors and create an optimized medium for the production of pikromycin in *Streptomyces venezuelae*, and increase the yield at the same time. In order to do so, Plackett-Burman design and a statistical method, response surface methodology, were applied to identify and fine-tune the concentrations of significant medium components.

Materials and Methods

Bacterial Strain and Culture Conditions

Streptomyces venezuelae ATCC 15439, a pikromycin- and methymycin-producing wild type, was used for the medium optimization study. Chemicals were purchased from BD (San Jose, CA, USA) and Junsei (Tokyo, Japan). Liquid R2YE complex medium (10 g glucose, 103 g sucrose, 5 g yeast extract, 0.1 g Difco casamino acids, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 2 ml of a trace element solution (composed of 200 mg $FeCl_3 \cdot 6H_2O$, 40 mg $ZnCl_2$, 10 mg $MnCl_2 \cdot 4H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 ml of 0.5% KH_2PO_4, 4 ml of 5 M $CaCl_2 \cdot 2H_2O$, and 15 ml of 20% L-proline in 1 L of distilled water), 5.73 g *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, and 7 ml of 1 N NaOH in 1 L of distilled water) was used for propagation of the strain. Productions of pikromycin was performed in non-optimized medium (20 g glucose, 103 g sucrose, 10 g peptone, 2.5 g $(NH_4)_2SO_4$, 2.5 g K_2HPO_4, 2.5 g KH_2PO_4, 1 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $CaCO_3$ in 1 L of distilled water).

The strain was seed cultured in 100 ml of R2YE, 30°C with shaking at 200 rpm for 18 h. Then 200 mg of wet weighted cells was inoculated in 50 ml of non-optimized medium and grown at 30°C and 200 rpm shaking for 60 h. The maximum production time point was different from one medium to another, due to different nutritional sources and concentrations. The main culture period was determined from preliminary experiments with a common pikromycin production medium, SCM. No significant increase in pikromycin production was observed after 60 h of the culture, and thus that time point was used throughout the experiments for comparison of pikromycin production from different media.

Quantification of Pikromycin Production

A 15 ml aliquot of the cultures was harvested by centrifugation at 2,840 ×*g* for 10 min. Pikromycin was extracted from supernatants with 2 volumes of ethyl acetate. The extract was dried using a rotary vacuum evaporator and reconstituted with 0.68 ml of methanol. The 20 µl of the samples was analyzed by high-performance liquid chromatography (HPLC; YL-9100; Younglin, Korea). A reverse phase C_{18} column from Waters (Milford, MA, USA) was used with a linear gradient from solvent A (80:20 water / 80% acetonitrile in 5 mM ammonium acetate and 0.05% acetic acid) to solvent B (20:80 water / 80% acetonitrile in 5 mM ammonium acetate and 0.05% acetic acid) over 25 min at a flow rate of 1 ml/min. Detection was made at 220 nm, and the pikromycin corresponding peak was confirmed by LCQ-LC/MS.

Experimental Design and Data Analysis

From preliminary studies testing various C and N sources, osmotic stress, buffers, and fatty acids, one medium component from each source that resulted in the most pikromycin production was selected. Glucose, sucrose, peptone, ammonium sulfate, monobasic and dibasic phosphate, sodium chloride, magnesium sulfate, and calcium carbonate were selected for further optimization.

Minitab 14.12 (Minitab Inc., Pensylvania, USA) was used to design and analyze the data throughout the experiments. To identify the most significant variables in pikromycin production, the Plackett-Burman Design (PBD) was employed. Eleven variables, nine components, and two dummies, were included in the design of a total of 12 experimental sets. The detailed design of the PBD matrix is listed in Table 1. Two dummy variables, which had no chemical relevance to the production of pikromycin, were included to calculate random measurement errors. The random measurement errors were used to determine the significance of real values by calculating *F* values of the *F*-test from the Minitab software.

The PBD results were reinterpreted to study correlations between pikromycin production and cell mass. With the same PBD matrix design (Table S1), the significance of the factors was observe with cell mass as response.

The path of the steepest ascent was performed to set up basal concentrations of media components selected from the PBD to be used in a central composite design (CCD). The path of the steepest ascent allowed rapid movement towards the optimum of variable concentrations. The amounts were altered by increasing or decreasing according to the result of the PBD. The combination of variable concentrations that resulted in maximum production indicated that it was at the point near optimum [14].

Response surface methodology (RSM), one of the CCD methods, was performed to find the optimal concentrations of variables determined to be important from the PBD to maximize the production of pikromycin. The matrix design and levels of components are listed in Table 4.

Results

Identification of the Most Significant Variables with PBD

PBD experiments tested the importance of the nine factors listed in Table 2. The result showed effects of variables to the response, pikromycin. Effects of sucrose, K_2HPO_4, and $CaCO_3$ were (+) 6.346, (+) 4.686, and (-) 3.578, respectively,

Table 1. PBD matrix design, real values of each coded variable, and responses.

Runs	Variables											Pikromycin (mg/l)
	A	B	C	D	E	F	G	H	I	J[a]	K[a]	
1	1	1	-1	1	-1	-1	-1	1	1	1	-1	16.3
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	30.6
3	-1	1	-1	-1	-1	1	1	1	-1	1	1	15.7
4	1	1	-1	1	1	-1	1	-1	-1	-1	1	30.3
5	1	-1	-1	-1	1	1	1	-1	1	1	-1	5.6
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	17.3
7	-1	-1	1	1	1	-1	1	1	-1	1	-1	28.5
8	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.5
9	1	-1	1	-1	-1	-1	1	1	1	-1	1	9.8
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.0
11	1	-1	1	1	-1	1	-1	-1	-1	1	1	1.2
12	-1	1	1	1	-1	1	1	-1	1	-1	-1	13.7

[a]Dummy factors. Nothing was added for the two to study errors.

Codes	Glucose	Sucrose	Peptone	$(NH_4)_2SO_4$	K_2HPO_4	KH_2PO_4	NaCl	$MgSO_4 \cdot 7H_2O$	$CaCO_3$
(g/l)	A	B	C	D	E	F	G	H	I
-1	14	72.1	7	2	1.8	1.8	0.7	0.7	0.14
1	26	134	13	3	3.3	3.3	1.3	1.3	0.26

which meant that sucrose and K_2HPO_4 had positive, and $CaCO_3$ had negative, effects to the response. The three components were also determined to be the most significant in pikromycin production, based on the low *P*-values (<0.1). R^2 was found to be 0.9754, meaning that 97.54% of the total variations could be explained by the model. On the Pareto chart of the standardized effects (Fig. 1), the minimal effects were presented towards lower fields, near 0, and the maximal effects towards upper fields.

The importance of the nine factors (Table S2), when the response was cell mass, indicated that $CaCO_3$ was positively significant, but sucrose was negatively significant towards cell mass (Fig. S1), which was totally opposite from that of when pikromycin was the response.

Table 2. Effects of variables to the response, pikromycin.

Factors	Effect	Coefficient	*T*	*P*
Glucose	2.678	1.339	1.14	0.372
Sucrose	12.692	6.346	5.41	0.033
Peptone	5.122	2.561	2.18	0.161
$(NH_4)_2SO_4$	1.905	0.953	0.81	0.502
K_2HPO_4	9.372	4.686	3.99	0.057
KH_2PO_4	-5.825	-2.912	-2.48	0.131
NaCl	5.942	2.971	2.53	0.127
$MgSO_4 \cdot 7H_2O$	5.562	2.781	2.37	0.141
$CaCO_3$	-7.155	-3.578	-3.05	0.093

R^2 = 97.54%; R^2 (adj) = 86.48%.

Path of the Steepest Ascent

Concentrations of sucrose and K_2HPO_4 were increased, but that of $CaCO_3$ was decreased because $CaCO_3$ was

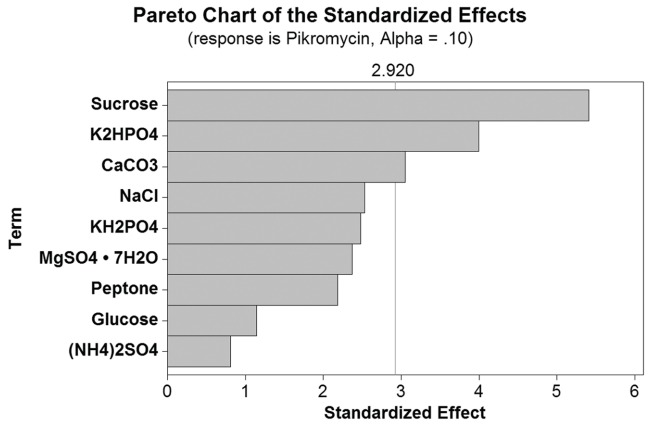

Fig. 1. Pareto chart of the standard effects of the tested nine factors to pikromycin production.

Sucrose, K_2HPO_4, and $CaCO_3$ were determined to be significant, while C and N sources were not.

determined to have negative effects on pikromycin production (Table 3). All the other components were fixed at the concentrations of the non-optimized medium. When the concentrations of sucrose, K_2HPO_4, and $CaCO_3$ were 154.5, 3.75, and 0.1 g/l respectively, the production of pikromycin was maximal, 28.5 mg/l. This point was chosen as a clue to set up basal concentrations for further optimization.

Table 3. Design of path of the steepest ascent, and the results.

Runs	Variables			Pikromycin
	Sucrose (g/l)	K_2HPO_4 (g/l)	$CaCO_3$ (g/l)	(mg/l)
1	103	2.5	0.2	19.3
2	133.9	3.25	0.14	23.8
3	154.5	3.75	0.1	28.5
4	175.1	4.25	0.06	26.3
5	195.7	4.75	0.02	25.1
6	206	5	0	19.0

Table 4. RSM matrix design, real values of variables, and the results.

Run	Variables			Pikromycin
	Sucrose	K_2HPO_4	$CaCO_3$	(mg/l)
1	1.633	0	0	24.4
2	0	0	-1.633	28.4
3	0	-1.633	0	37.0
4	0	1.633	0	26.1
5	0	0	1.633	30.4
6	0	0	0	30.4
7	0	0	0	25.8
8	-1.633	0	0	25.1
9	0	0	0	25.4
10	-1	1	-1	25.0
11	-1	-1	1	32.5
12	1	1	1	22.9
13	0	0	0	25.7
14	1	-1	-1	26.8
15	0	0	0	24.0
16	0	0	0	26.4
17	-1	-1	-1	25.6
18	-1	1	1	21.9
19	1	-1	1	26.0
20	1	1	-1	20.5

Factors (g/l)	Codes	-1.633	-1	0	1	1.633
Sucrose	X1	104.0	123.6	154.5	185.4	205.0
K_2HPO_4	X2	5.3	6.0	7.0	8.1	8.7
$CaCO_3$	X3	0.049	0.055	0.065	0.075	0.081

Response Surface Methodology

Specific interactions between the three variables with response to pikromycin production were studied with RSM (Fig. 2). Effects of the three components, sucrose, K_2HPO_4, and $CaCO_3$, were analyzed by *t*-test and *P*-values as shown in Table 5. A regression model with R^2 higher than 0.9 meant that the result had high correlation between predicted and experimental values of the response [10]. It also meant that the model could explain 91.2% of the total variations. This model can be expressed as Eq. (1).

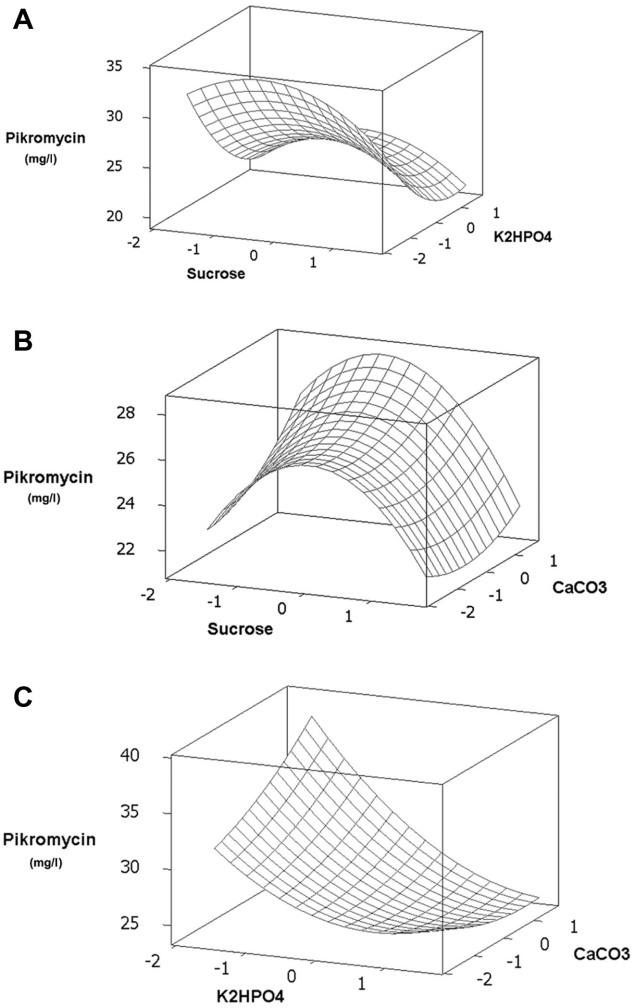

Fig. 2. 3D surface graph of sucrose *vs.* K_2HPO_4 (**A**), sucrose *vs.* $CaCO_3$ (**B**), and K_2HPO_4 *vs.* $CaCO_3$ (**C**) for the response, pikromycin.

Table 5. Significance and regression coefficients for RSM.

Term	Coef.[a]	SE Coef.[a]	*T*	*P*
Constant	26.2912	0.7033	37.384	0.000
X1	-0.7551	0.4723	-1.599	0.149
X2	-2.9028	0.4723	-6.146	0.000
X3	0.6478	0.4723	1.372	0.207
X1*X1	-1.4306	0.4745	-3.015	0.017
X2*X2	1.1231	0.4745	2.367	0.045
X3*X3	0.3112	0.4745	0.656	0.530
X1*X2	0.2138	0.6097	0.351	0.735
X1*X3	-0.2737	0.6097	-0.449	0.665
X2*X3	-0.8513	0.6097	-1.396	0.200

R^2 = 91.2%; R^2 (adj) = 79.1%.

[a]Coef. = Coefficient.

$$Y = 26.2912 - 0.7551\ X_1 - 2.9028\ X_2 + 0.6478\ X_3 - 1.4306\ X_1{*}X_1 + 1.1231\ X_2{*}X_2 + 0.3112\ X_3{*}X_3 + 0.2138\ X_1{*}X_2 - 0.2737\ X_1{*}X_3 - 0.8513\ X_2{*}X_3 \quad (1)$$

Analysis of variance (Table 6) also provided the reliability of the model from the statistically significant regression ($P < 0.01$), and the statistically insignificant lack of fit ($P = 0.778$). As a result, the model was determined to be reliable and adequate to optimize the production of pikromycin.

Validation of the Optimized Medium

The full quadratic model from RSM predicted that the maximum production of pikromycin was 38.6 mg/l with 139 g/l sucrose, 5.29 g/l K_2HPO_4, and 0.081 g/l $CaCO_3$. Validation experiments with the optimized condition resulted in 35.5 ± 0.866 mg/l of pikromycin, which was in near agreement. The yield from unoptimized medium was 24.4 mg/l, meaning a 1.45-fold increase was achieved. Moreover, 1.98-fold and 1.29-fold increases in pikromycin production also had been obtained, compared with SGGP (17.9 ± 2.43 mg/l) and SCM (27.4 ± 1.05 mg/l), respectively. Production of pikromycin from the optimized medium was 34.1 ± 0.852 mg/l per gram cell, which was 4.19-fold and 3.2-fold higher than that of SGGP and SCM, respectively.

Discussion

Various stress conditions applied during growth of *Streptomyces* species are known to alter secondary metabolite production. It has been reported that secondary metabolism in *Streptomyces coelicolor* begins during the pre-sporulating stage of the cell cycle [33], which means that the time point of the secondary metabolite production can be actively altered by the availability of nutrients. Nutrient sources may also change the metabolism, in that different carbon and nitrogen sources result in various titers [35].

An essential element for microorganisms, which applies to all living organisms, is phosphorus. Formation of a fuel compound ATP [27], DNA and RNA synthesis [25], and many other biochemical processes occurring in living creatures require phosphorus [16]. A primary source of phosphorus in bacterial cultures is K_2HPO_4 and KH_2PO_4. K_2HPO_4 was determined to be a more suitable source of phosphorus to produce pikromycin from the results of PBD. It is expected that a precursor of desosamine from the glycone portion of pikromycin, glucose-1-phosphate [31], can better be synthesized from K_2HPO_4. Alternatively, optimization of pH by altering the ratio of monobasic and dibasic phosphate [15] may have dedication to the increase of the pikromycin production as reported in the pH-sensitive production of actinorhodin with *Streptomyces coelicolor* [6, 18].

Like phosphorus, sucrose is an important carbon source

Table 6. Analysis of variance test of the regression model.

Source	DF[a]	Seq SS[b]	Adj SS[b]	Adj MS[c]	*F*	*P*
Regression	9	180.736	180.736	20.082	6.75	0.007
Linear	3	125.547	125.547	41.849	14.07	0.001
Square	3	48.426	48.426	16.142	5.43	0.025
Interaction	3	6.762	6.762	2.254	0.76	0.548
Residual Error	8	23.792	23.792	2.974		
Lack-of-Fit	5	10.591	10.591	2.118	0.48	0.778
Pure Error	3	13.201	13.201	4.4		
Total	19	270.181				

[a]DF = Degrees of Freedom.

[b]SS = Sum of Squares.

[c]MS = Mean Square.

for plants and some phytopathogenic bacteria [4]. However, it is used somewhat differently in cultures of *Streptomyces* species. Sucrose cannot be utilized with the species [26] as it is often used as one of the compounds reducing osmotic stress, one of the key components in secondary metabolism [17, 24, 29]. A previous report indicated an absence of sucrose banished production of the antibiotic actinorhodin in *Streptomyces coelicolor*, and an optimization of sucrose for the production medium has been further studied [13]. In the case of this study, effective amounts of sucrose is given, for 20 g of glucose is 139 g of sucrose. Less or over that sucrose concentration would decrease the production of pikromycin as it did with that of actinorhodin in *Streptomyces coelicolor* [12].

Balancing primary versus secondary metabolism is inevitable for maximizing yields of target polyketide metabolite production, and one way to do so is optimizing the availability of calcium ions. Calcium ions are often used for increases of enzymatic stability [7, 28]. However, calcium is known to decrease secondary metabolite production. From past studies, increases in calcium ion supplement resulted in a larger total mass of *Streptomyces coelicolor*, but the opposite was observed with the production of actinorhodin [1]. Secondary metabolism shares common precursor and cofactor pools like acetyl-CoA and NADPH [11, 19], where calcium may have contributions to balancing fluxes of the compounds toward growth and secondary metabolism, as previous reports and the result of PBD in this study indicate. $CaCO_3$ is determined to have negative effects on pikromycin production but positive effects on cell mass. In other words, some loss in cell mass should be endured for gain in pikromycin production. Thus, the concentrations of calcium carbonate was minimized to a satisfactory level with RSM for the optimum balance between growth and pikromycin production. There was some loss in the total cell mass, but productivity increased by 3~4 folds.

As the RSM method was applied in many recent optimization research [2, 23, 34], it was successfully used to investigate relationships between sucrose, K_2HPO_4, and $CaCO_3$. It also resulted in the fine-tuning of the three component concentrations for maximum yield of pikromycin antibiotic.

In conclusion, medium optimization of pikromycin production was achieved by the statistical designs of experiments in this study. First, PBD was applied to select the most significant factors. In the second step, CCD was used to fine-tune the optimal concentrations of the selected variables. With the optimal concentrations at 139 g/l sucrose, 5.29 g/l K_2HPO_4, and 0.081 g/l $CaCO_3$, a maximum pikromycin yield of 35.5 mg/l was achieved. This was a 1.45-fold increase compared with the non-optimized medium, and 1.98-fold and 1.29-fold increases were achieved compared with SGGP and SCM, respectively. Thus, our medium may be employed to other studies related to pikromycin production.

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