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Statistical Optimization of Medium Components for the Production of Prodigiosin by *Hahella chejuensis* KCTC 2396

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Prodigiosin is a natural red pigment with algicidal activity against *Cochlodinium polykrikoides*, a major harmful red-tide microalga. To increase the yield of prodigiosin production by *Hahella chejuensis* KCTC 2396, significant medium components were determined using a two-level Plackett-Burman statistical design technique. Among 12 components included in basal medium, $NaHCO_3$, Na_2SiO_3, NH_4NO_3, Na_2SO_4, and $CaCl_2$ were determined to be important for prodigiosin production. The medium formulation was finally optimized using a Box-Behnken design as follows: 1% sucrose; 0.4% peptone; 0.1% yeast extract; and (g/l): NaCl, 20.0; Na_2SO_4, 9.0; $CaCl_2$, 1.71; KCl, 0.4; and (mg/l): H_3BO_3, 10.0; KBr, 50.0; NaF, 2.0; $NaHCO_3$, 45.0; Na_2SiO_3, 4.5; NH_4NO_3, 4.5. The predicted maximum yield of prodigiosin in the optimized medium was 1.198 g/l by the Box-Behnken design, whereas the practical production was 1.495 g/l, which was three times higher than the basal medium (0.492 g/l).

Keywords: *Hahella chejuensis*, prodigiosin, statistical experimental design

Hahella chejuensis KCTC 2396 was originally isolated from marine sediment collected in Cheju Island, Korea [6]. It is able to concomitantly produce a red pigment, prodigiosin, which has been shown to be highly algicidal against *Cochlodinium polykrikoides*, a major red-tide microalga in the Korea coastal region [5]. For several decades, prodigiosin has been known to be a natural compound showing a broad range of cytotoxic activity [4], and is also produced by *Vibrio psychroerythrus* [3], *Seratia marcescen*, *Pseudomonas magnesiorubra*, and other eubacteria [7]. Recently, prodigiosin has been considered effective as a biological control agent against harmful algae in natural marine environments; therefore, prodigiosin should be produced in large quantities to be able to meet future needs.

To increase the production yield of prodigiosin, *Serratia marcescens* was investigated while varying culture conditions including temperature, pH [11], carbon and nitrogen sources [1], and NaCl concentration [10]. A practical experiment for optimization of a single factor, while maintaining the other factors at constant levels, does not represent the combined effects of all the factors involved. In addition to the large number of experiments required, the optimal values obtained from such experiments are unreliable. Plackett-Burman design is a well-established and widely used statistical design technique for the screening of medium components [9]. Using a statistical experimental design such as Plackett-Burman and Box-Behnken methodologies, all the parameters can be optimized, eliminating the limitations of a single factor optimization process [12].

There have been few reports on statistical optimization for the production yield-up of natural pigment. *Serratia marcescens* SMΔR was investigated under modified LB medium to improve the prodigiosin production. However, the prodigiosin production was 0.79 g/l [13]. We optimized for the first time the medium components for higher prodigiosin production using statistical designed experiments. In this study, using a statistical optimization method, we aimed to determine the optimal medium composition to substantially increase the production yield of prodigiosin by strain KCTC 2396.

Materials and Methods

Organisms and Basal Culture Conditions

Hahella chejuensis KCTC 2396 was precultured in Zobell medium [14] at 25°C for 24 h. The seed culture (2%) was used as an inoculums into 20 ml of basal medium (1% sucrose; 0.4% peptone; 0.1% yeast extract; and (g/l): NaCl, 20.0; Na_2SO_4, 4.06; $CaCl_2$, 1.14; KCl, 0.69; KBr, 0.1; NaF, 0.1; $NaHCO_3$, 0.2; and (mg/l): KH_2PO_4,

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50; H_3BO_3, 27.5; SrCl, 26.0; Na_2SiO_3, 2.0; NH_4NO_3, 2.0) in a 100-ml culture flask, and cultured at 25°C for 72 h.

Optimization Procedure

The optimization of medium components for prodigiosin production by *H. chejuensis* KCTC 2396 was accomplished in two stages.

Identification of Significant Nutrient Components

The Plackett-Burman design, an efficient tool for the screening of medium components [8, 9], was used to find the nutrient components significantly influencing prodigiosin production from KCTC 2396. Based on the design, 12 nutrient components of basal medium were examined at two levels, low level (-) and high level (+), as shown in Table 1, resulting in a first-order model, $\mathbf{Y}=\beta_0+\Sigma\beta_i\mathbf{X}_i$, where $\mathbf{Y}$ is the predicted response (prodigiosin production), β_0 is the model intercept, β_i is the linear coefficient, and $\mathbf{X}_i$ is the level of the independent variable. This model does not describe interaction among factors (nutrient components), and is used only to screen and evaluate important factors influencing the response.

Optimization of Selected Nutrient Components

In order to optimize the concentrations of the nutrient components previously selected through the experiment using the Plackett-Burman design, a Box-Behnken design was applied [2]. The quantities of the nutrient components were coded into three levels: (-), (0), and (+) for low, intermediate, and high concentrations, respectively. For prediction of the optimal concentrations, a second-order polynomial model was designed to describe the relationship between the independent variables (nutrient components) and the response: $\mathbf{Y}=\beta_0+\Sigma\beta_i\mathbf{X}_i+\Sigma\beta_{ij}\mathbf{X}_i\mathbf{X}_j+\Sigma\beta_{ii}\mathbf{X}_j^2$, where $\mathbf{Y}$ is the predicted response (prodigiosin production), and β_0, β_i, β_{ij}, and β_{ii} are the constant and regression coefficients of the model, with $\mathbf{X}_i$ and $\mathbf{X}_j$ representing the independent nutrient components. The statistical software Minitab (v. 13.1; Minitab Inc., U.S.A.) was used for the experimental design and for regression analysis of the data obtained.

Prodigiosin Assay

For determination of the concentration of prodigiosin, 500 µl of KCTC 2396 culture broth was added to 1 ml of acidic ethanol (pH 3.0 with HCl), and thoroughly mixed by continuous shaking for 20 min. A red-colored supernatant was obtained by centrifugation at 10,000 ×*g* for 5 min. The supernatant was diluted with acidic ethanol, and the absorbance value at 535 nm was determined. The concentration of prodigiosin was determined with a standard curve of the purified prodigiosin.

Table 1. The nutrient components and test levels for the Plackett-Burman experiment.

Variables	Medium components	+ value[a] (g/l)	- value (g/l)
X_1	NH_4NO_3	0.002	0.0002
X_2	H_3BO_3	0.0275	0.00275
X_3	$CaCl_2$	1.14	0.114
X_4	KH_2PO_4	0.05	0.005
X_5	KBr	0.1	0.01
X_6	KCl	0.69	0.069
X_7	$NaHCO_3$	0.2	0.02
X_8	NaF	0.003	0.0003
X_9	Na_2SiO_3	0.002	0.0002
X_{10}	Na_2SO_4	4.06	0.406
X_{11}	SrCl	0.026	0.0026
X_{12}	NaCl	20	2.0

[a]+ value indicates the concentration of trace elements in the basal medium.

Results and Discussion

Selection of Significant Nutrient Components

To eliminate nutrient components having no significant effects on the prodigiosin production, each of the 12 different elements included in the basal medium preparation was tested. Table 2 represents the effect, standard error, *t*-statistics, and *P*-value for each nutrient component. The nutrient components were screened and those with a *P*-value of <0.1 were accepted as significant factors affecting the production of prodigiosin. It was found that the *P*-value of SrCl and KH_2PO_4 were >0.1, indicating that these two elements are not significant factors on prodigiosin production compared with the other factors. In addition, since SrCl and KH_2PO_4 showed a low effect value, both of these elements were considered to have no effects on prodigiosin production and were eliminated from further study. In contrast, the effect value of NaCl was higher (265.0) than other nutrient components on prodigiosin production. Physiological data previously obtained showed that the optimal concentration of NaCl for KCTC 2396 is 2.0%, and this strain was unable to grow in the absence of NaCl [7]. Therefore, NaCl was considered to be a factor required for growth and prodigiosin production of KCTC 2396, and the concentration of NaCl was fixed at 2.0% for further study.

To select nutrient components having more effects on prodigiosin yield, the nine nutrient components, selected through the preliminary Plackett-Burman experiment, were

Table 2. Statistical analysis of nutrient components using the initial Plackett-Burman experiment.

Variables	Medium components	Effect	Standard error	*t*-statistic	*P*-value
X_1	NH_4NO_3	-90.5	24.08	-1.88	0.075
X_2	H_3BO_3	-95.8	24.08	-1.99	0.060
X_3	$CaCl_2$	736.2	24.08	15.29	0.000
X_4	KH_2PO_4	29.2	24.08	0.61	0.551
X_5	KBr	130.0	24.08	2.70	0.014
X_6	KCl	189.6	24.08	3.94	0.001
X_7	$NaHCO_3$	101.1	24.08	2.10	0.049
X_8	NaF	-191.8	24.08	-3.98	0.001
X_9	Na_2SiO_3	116.4	24.08	2.42	0.025
X_{10}	Na_2SO_4	-123.4	24.08	-2.56	0.019
X_{11}	SrCl	0.7	24.08	0.02	0.988
X_{12}	NaCl	265.0	24.08	5.50	0.000

Table 3. Selected nutrient components and test levels for the secondary Plackett-Burman experiment.

Variables	Medium components	+ value (g/l)	- value (g/l)
X_1	$CaCl_2$	5	0.5
X_2	NaF	0.02	0.002
X_3	KCl	4	0.4
X_4	KBr	0.5	0. 05
X_5	Na_2SO_4	20	2
X_6	Na_2SiO_3	0.01	0.001
X_7	$NaHCO_3$	1	0.1
X_8	H_3BO_3	0.1	0.01
X_9	NH_4NO_3	0.01	0.001

retested using a secondary Plackett-Burman design. Selected nutrient components and test levels were obtained, which showed the variables (nutrient components) with two levels of concentrations for each variable (Table 3). Consequently, the effect values, standard errors, *t*-values, and *P*-values for the nine components were calculated as shown in Table 4. Finally, the polynomial model describing the correlation between the nine components and the yield of prodigiosin was presented as follows: $Y_{(production)}=1{,}025.3-123.3X_1+10.3X_2+6.6X_3+15.2X_4-143.1X_5-187.3X_6+358.7X_7-34.9X_8-170.3X_9$; where Y is the predicted production and X_1–X_9 are the coded values of $CaCl_2$, NaF, KCl, KBr, Na_2SO_4, Na_2SiO_3, $NaHCO_3$, H_3BO_3, and NH_4NO_3. Analysis of the regression coefficients of the nine nutrient components showed *P*-values for NaF, KCl, KBr, and H_3BO_3 that were above 0.05, indicating that these components were insignificant for prodigiosin production compared with others (Table 4).

In summary, five nutrient components ($CaCl_2$, Na_2SO_4, Na_2SiO_3, $NaHCO_3$, and NH_4NO_3) were finally selected as having a positive effect on prodigiosin yield based on their *P*-values (<0.05) and effect values (+ or –). These results indicate that the Plackett-Burman design is a powerful tool for identification of the nutrient components significantly affecting the prodigiosin production.

Table 4. Statistical analysis of selected nutrient components using Plackett-Burman design.

Variables	Medium components	Effect	Standard error	*t*-statistics	*P*-value
X_1	$CaCl_2$	-246.7	45.00	-2.74	0.015
X_2	NaF	20.5	45.00	0.23	0.823
X_3	KCl	13.2	45.00	0.15	0.885
X_4	KBr	30.4	45.00	0.34	0.740
X_5	Na_2SO_4	-286.2	45.00	-3.18	0.006
X_6	Na_2SiO_3	-374.6	45.00	-4.16	0.001
X_7	$NaHCO_3$	717.3	45.00	7.97	0.000
X_8	H_3BO_3	-69.3	45.00	-0.78	0.450
X_9	NH_4NO_3	-340.6	45.00	-3.78	0.002

Table 5. Box-Behnken optimization of selected significant nutrient components.

Variables	Medium components	+ (g/l)	0 (g/l)	- (g/l)
X_1	$NaHCO_3$	2	1.1	0.2
X_2	Na_2SiO_3	0.005	0.00275	0.0005
X_3	NH_4NO_3	0.005	0.00275	0.0005
X_4	Na_2SO_4	10	5.5	1
X_5	$CaCl_2$	2.5	1.375	0.25

Optimization of Screened Medium Components for Prodigiosin Production

Based on the results obtained by Plackett-Burman experimental design, $NaHCO_3$, $CaCl_2$, Na_2SO_4, Na_2SiO_3, and NH_4NO_3 were selected as significant nutrient components for prodigiosin production and were subsequently subjected to further study using a Box-Behnken design. Table 5 shows the selected nutrient components tested for Box-Behnken optimization, the values of which were calculated by linear multiple regression using Minitab software. The following equation was obtained: $Y_{(production)}=369-33.06X_1-3.35X_2-8.53X_3+75.65X_4+24.66X_5+11.19X_1^2-28.72X_2^2-28.44X_3^2-15.30X_4^2-50.35X_5^2-52.86X_1, X_2-10.19X_1, X_3+18.42X_1, X_4+28.33X_1, X_5-13.15X_2, X_3+37.33X_2, X_4+25.52X_2, X_5+27.79X_3, X_4-32.31X_3, X_5-14.56X_4, X_5$; where Y is the predicted response (prodigiosin production), and X_1–X_5 are the values of $NaHCO_3$, Na_2SiO_3, NH_4NO_3, Na_2SO_4, and $CaCl_2$, respectively. At the model level, the correlation measurement for the estimation of the regression equation is the coefficient R^2. The value of R^2, being a measure of the fit of the model, is 0.977 for prodigiosin production, which indicates that about 2.3% of the total variation is not explained by prodigiosin production.

Presenting experimental results in the form of response surface plots showing the effects of NH_4NO_3, Na_2SO; Na_2SiO_3, Na_2SO_4; $NaHCO_3$, Na_2SO_4; $NaHCO_3$, $CaCl_2$; and Na_2SiO_3, $CaCl_2$ at different concentrations of the other two variables are shown in Fig. 1. The statistical optimal values of variables are obtained when moving along the major and minor axies of the contour, and the response at the center point yields maximum prodigiosin production. Through the study response of surface plots and Box-Behnken experimental design, the optimal concentrations of X_1–X_5 ($NaHCO_3$, Na_2SiO_3, NH_4NO_3, Na_2SO_4, and $CaCl_2$) were determined to be 0.45, 0.0045, 0.0045, 9.0, and 1.7115 g/l, respectively.

When using the optimized culture medium (1% sucrose; 0.4% peptone; 0.1% yeast extract; and (g/l): NaCl, 20.0; Na_2SO_4, 9.0; $CaCl_2$, 1.71; KCl, 0.4; and (mg/l): H_3BO_3, 10.0; KBr, 50.0; NaF, 2.0; $NaHCO_3$, 45.0; Na_2SiO_3, 4.5; NH_4NO_3, 4.5) for a higher production yield of prodigiosin by KCTC 2396, the maximum yield of prodigiosin was predicted to be 1.198 g/l, and the yield obtained from the

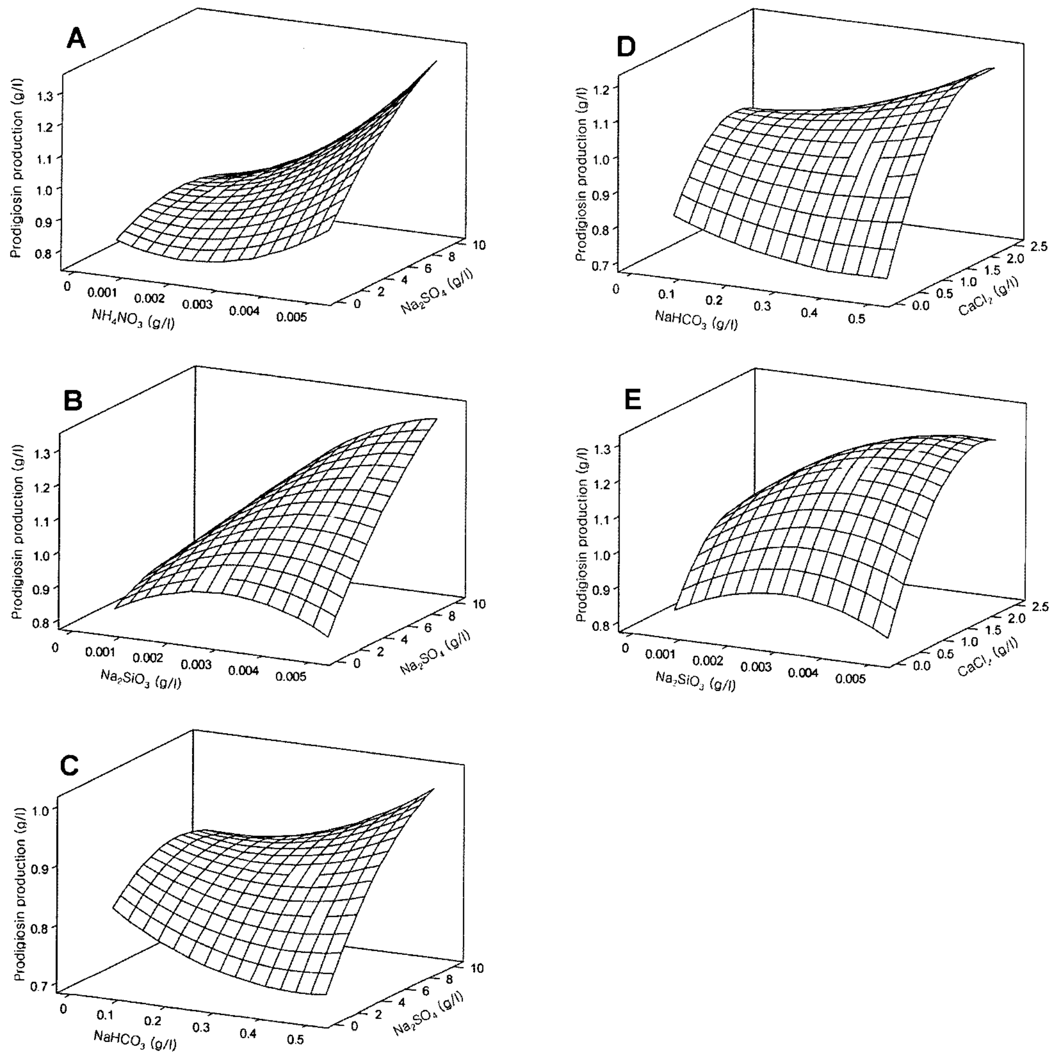

Fig. 1. Three-dimensional response surface plot for the effect of (**A**) NH_4NO_3, Na_2SO_4; (**B**) Na_2SiO_3, Na_2SO_4; (**C**) $NaHCO_3$, Na_2SO_4; (**D**) $NaHCO_3$, $CaCl_2$; and (**E**) Na_2SiO_3, $CaCl_2$ on prodigiosin production (g/l).

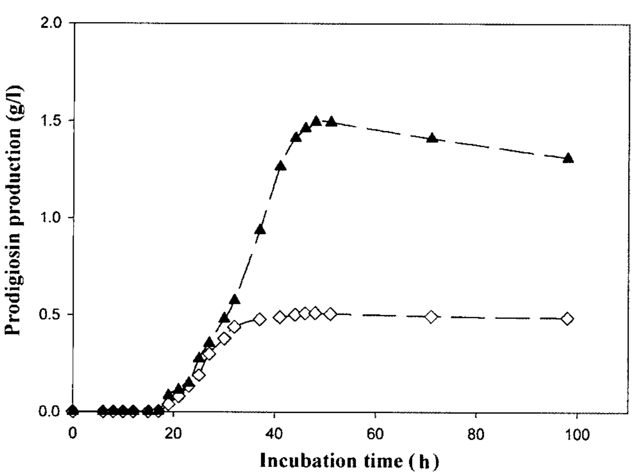

Fig. 2. Time course for prodigiosin production using the optimal designed medium. ▲, optimal medium; ◇, basal medium.

practical experiment was 1.495 g/l, three times higher than with basal medium (0.492 g/l) (Fig. 2).

The Plackett-Burman and Box-Behnken statistical methods were found to be very useful for the determination of relevant variables, such as medium components, for further optimization. These methods made it possible to consider a large number of variables and avoid laborious and time-consuming, repeated experiments. The use of these techniques has been proven helpful to optimize the types and relative amounts of main medium components.

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REFERENCES

1. Alonzo, V., M. E. Scoglio, and L. Mangione. 1979. Effect of the carbon/nitrogen ratio on the antibiotic activity of prodigiosin. *Bacteriol. Virol. Immunol.* **71:** 3–15.
2. Box, G. E. P. and D. W. Behnken. 1960. Some new three level designs for the study of quantitative variables. *Technometrics* **2:** 455–475.
3. D'Aoust, J. Y. and N. N. Gerber. 1974. Isolation and purification of prodigiosin from *Vibrio psychroerythrus*. *J. Bacteriol.* **118:** 756–757.
4. Fürstner, A. 2003. Chemistry and biology of roseophilin and the prodigiosin alkaloids: A survey of the last 2500 years. *Chem. Int. Ed. Engl.* **42:** 3582–3603.
5. Jeong, H., J. H. Yim, C. Lee, S. H. Choi, Y. K. Park, S. H. Yoon, *et al.* 2005. Genomic blueprint of *Hahella chejuensis*, a marine microbe producing an algicidal agent. *Nucleic Acids Res.* **33:** 7066–7073.
6. Lee, H. K., J. Chun, E. Y. Moon, S. H. Ko, D. S. Lee, H. S. Lee, and K. S. Bae. 2001. *Hahella chejuensis* gen. nov., sp. nov., an extracellular-polysaccharide-producing marine bacterium. *Int. J. Syst. Evol. Microbiol.* **51:** 661–666.
7. Lewis, S. M. and W. A. Corpe. 1964. Prodigiosin-producing bacteria from marine sources. *Appl. Microbiol.* **12:** 13–17.
8. Naveena, B. J., M. Altaf, K. Bhadriah, and G. Reddy. 2005. Selection of medium components by Plackett-Burman design for production of L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresour. Technol.* **96:** 485–490.
9. Plackett, R. L. and J. P. Burman. 1946. The design of optimum multifactorial experiments. *Biometrika* **37:** 305–325.
10. Rjazantseva, I. N., I. N. Andreeva, and T. I. Ogorodnikova. 1994. Effect of various growth conditions on pigmentation of *Serratia marcescens*. *Microbes* **79:** 155–161.
11. Sole, M., N. Rius, A. Francia, and J. G. Loren. 1994. The effect of pH on prodigiosin production by nonproliferating cells of *Serratia marcescens*. *Lett. Appl. Microbiol.* **19:** 341–344.
12. Stanbury, P. F., A. Whitaker, and S. J. Hall. 1997. *Principles of Fermentation Technology*, pp. 93–122. 2th Ed. Aditya Books, New Delhi.
13. Wei, Y. H. and W. C. Chen. 2005. Enhanced production of prodigiosin-like pigment from *Serratia macescens* SMΔR by medium improvement and oil-supplementation strategies. *J. Biosci. Bioeng.* **99:** 616–622.
14. ZoBell, C. E. 1946. *Marine Microbiology*, XV, pp. 240. Chronica Botanica Co., Waltham, Massachusetts, U.S.A.