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# Mutant Selection of *Hahella chejuensis* KCTC 2396 and Statistical Optimization of Medium Components for Prodigiosin Yield-Up

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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, a mutant of *Hahella chejuenesis* KCTC 2396, assigned M3349, was developed by an antibiotic mutagenesis using chloramphenicol. When cultured in Sucrose-based Marine Broth medium (SMB), M3349 could produce prodigiosin at 1.628±0.06 g/L, while wild type producing at 0.658±0.12 g/L under the same conditions. To increase the yield of prodigiosin production by M3349, significant medium components were determined using a two-level Plackett-Burman statistical design technique. Among fourteen components included in SMB medium, NaCl, $Na_2SiO_3$, $MgCl_2$, $H_3BO_3$, $Na_2HPO_4$, $Na_2SO_4$, and $CaCl_2$ were determined to be important for prodigiosin production. The medium formulation was finally optimized using a Box-Behnken design as follows: sucrose 10.0, peptone 8.0, yeast extract 2.0, NaCl 10.0, $Na_2SO_4$ 12.0, $CaCl_2$ 1.8, $MgCl_2$ 0.7 g/L; and $H_3BO_3$ 22.0, $Na_2HPO_4$ 20.0, $Na_2SiO_3$ 8.0 mg/L. The predicted maximum yield of prodigiosin in the optimized medium was 2.43 g/L by the Box-Behnken design, while the practical production was 2.60±0.176 g/L, which was 3.9 times higher than wild type with SMB Medium (0.658 g/L).**

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

Natural red-pigment prodigiosin, produced by some eubacterial strains including *Serratia marcescens* (Lewis and Corpe, 1964), *Pseudomonas magnesiorubra* and *Vibrio psychroerythrus* (D'Aoust and Gerber, 1974) has been known as a compound showing a broad range of cytotoxic activity to human cells (Fürstner, 2003). *Hahella chejuensis* KCTC 2396 isolated from marine sediment in Cheju island, Korea (Lee *et al.,* 2001), was characterized to produce prodigiosin, later which was demonstrated to have a high algicidal activity against *Cochlodinium polykrikoides*, a major red-tide microalga (Jeong *et al*., 2005).

Some mutagenic strategies were applied to many microbial strains to produce their mutants capable of producing higher levels of secondary metabolites (Anwar *et al*., 1996; Szengyel *et al*., 2000; Liming and Xueliang, 2004; Chand *et al*., 2005; Skomarovsky *et al*., 2005). For example, fungal strain *Aspergillus niger* UMIP 2564, producing citric acid, was randomly mutated by different doses of UV-irradiation and chemical mutagens (Walid *et al.,* 2007). Another fungus *Penicillium janthinellum* NCIM 1171 was subjected to a treatment of ethyl methane sulfonate (EMS) and UV-irradiation, leading to several mutants producing a higher amount of cellulase (Adsul *et al*., 2007).

To improve the prodigiosin production, *Serratia marcescens* was investigated under different culture conditions such as temperature, pH (Sole *et al*., 1994), carbon and nitrogen source (Alonzo *et al*., 1979), and NaCl concentration (Rjazantseva *et al*., 1994). Practical experiments for optimizing the prodigiosin production were carried out changing each single factor, while maintaining the other factors at a constant level. It was shown clearly that they did not represent a combined effect of all the factors mentioned above and required additionally a large number of experiments to determine an optimized culture condition. However, by using a statistical experimental design such as Plackett-Burman (Plackett and Burman, 1946) and Box-Behnken methodology, all the parameters could be optimized by eliminating those limitations in a single factor optimization process (Stanbury *et al*., 1997).

To increase the production of prodigiosin, a mutant strain M3349 of *Hahella chejuenesis* KCTC 2396 was developed, the medium components for prodigiosin production were optimized for M3349 using statistical designed experiment.

## Materials and Methods

### Bacterial strain and cultural condition

*Hahella chejuensis* KCTC 2396 strain was grown on Marine agar (Difco, USA) at 25°C for 72 h. To prepare a seed culture, a single colony was transferred to 20 ml of SMB medium (sucrose 10.0, peptone 8.0, yeast extract 2.0, NaCl 19.45, $MgCl_2$ 5.9, $Na_2SO_4$ 3.24, $CaCl_2$ 1.8, KCl 0.55, $Na_2CO_3$ 0.16, $FeCl_3{\cdot}6H_2O$ 0.1 g/L; and KBr 80.0, $SrCl_2$ 34.0, $H_3BO_3$ 22.0, $Na_2HPO_4$ 8.0, $Na_2SiO_3$ 4.0, NaF 2.4, $NH_4NO_3$ 1.6 mg/L), and cultured at 25°C for 24 h. Prodigiosin production were carried out in 2 liter baffles flask, each medium containing 500 ml medium on a shaker of 120 rpm at 25°C

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for 96 h. Inoculum size of the seed culture was 2% (v/v).

### Mutagenesis

Pre-cultured wild type cells were adjusted to an $OD_{600}$ value of 0.3. One hundred microliters of the cell suspension were inoculated onto Marine agar containing various kinds of antibiotics (ampicillin, carbenicillin, chloramphenicol, erythromycin, kanamycin, neomycin, penicillin, streptomycin, and tetracyclin) at different concentrations (0.65~100 μg/ml). After incubation at 25°C for 72 h, grown cells on the corresponding antibiotic plates were selected as spontaneous antibiotic-resistant mutants. For further experiments, the grown mutants were isolated as a single colony, and stored in 20% glycerol at -80°C.

Chemical mutagenesis was carried out with EMS (Sigma, USA). In detail, 100 μl of the KCTC 2396 suspension ($OD_{600}$=0.3) was inoculated in 1 ml SMB medium supplemented with EMS at a final concentration of 0, 0.1, 0.2, or 0.3 M, and further incubated at 25°C for 2 h. After incubation, $Na_2S_2O_3$ (6%, v/v) was added to the culture as a stop solution, and centrifuged at 10,000×g for 10 min at 4°C. The supernatant was removed, and the cell pellet was dissolved in saline solution (0.85% NaCl). The EMS-treated wild type cells were diluted in saline solution at serial dilution rates ($10^{-1}$, $10^{-2}$, and $10^{-3}$), which were plated on Marine agar and incubated. After incubation at 25°C for 72 h, the grown cells on the plates were selected as EMS-survived mutants. For further experiments, the mutants were isolated as a single colony, and stored under the same conditions.

### Identification of significant nutrient components

Plackett-Burman design (Plackett and Burman, 1946), an efficient tool for the screening of nutrient component was used to found factors that significantly influenced production (Naveena *et al*., 2005). Based on the design, fourteen nutrient components of SMB medium were examined at two levels, low level (-) and high level (+), as shown in Table 1, Resulting in a first order model, $Y=\beta_0+\sum\beta_i X_i$, where $Y$ is the predicted response (prodigiosin production), $\beta_0$ is the model intercept and $\beta_i$ is the linear coefficient, and $x_i$ 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.

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

| Variables | Medium components | + Values (g/L) | - Values (g/L) |
|---|---|---|---|
| $X_1$ | $FeCl_3$ | 0.1 | 0.01 |
| $X_2$ | NaCl | 19.45 | 1.945 |
| $X_3$ | $MgCl_2$ | 5.90 | 0.59 |
| $X_4$ | $Na_2SO_4$ | 3.240 | 0.324 |
| $X_5$ | $CaCl_2$ | 1.80 | 0.18 |
| $X_6$ | KCl | 0.55 | 0.055 |
| $X_7$ | $Na_2CO_3$ | 0.16 | 0.016 |
| $X_8$ | KBr | 0.08 | 0.008 |
| $X_9$ | $SrCl_2$ | 0.034 | 0.0034 |
| $X_{10}$ | $H_3BO_3$ | 0.022 | 0.0022 |
| $X_{11}$ | $Na_2SiO_3$ | 0.004 | 0.0004 |
| $X_{12}$ | NaF | 0.0024 | 0.00024 |
| $X_{13}$ | $NH_4NO_3$ | 0.0016 | 0.00016 |
| $X_{14}$ | $Na_2HPO_4$ | 0.008 | 0.0008 |

### 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 (Box and Behnken, 1960). 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: $Y=\beta_0+\sum\beta_i X_i+\sum\beta_{ij}X_iX_j+\sum\beta_{ii}X_j^2$, where $Y$ is the predicted response (prodigiosin production), and $\beta_0$, $\beta_i$, $\beta_{ij}$, and $\beta_{ii}$ are the constant and regression coefficients of the model, with $X_i$ and $X_j$ representing the independent nutrient components. The statistical software Minitab (v. 13.1, Minitab Inc., USA) 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 wild type culture broth was added to 1 ml 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.

## Results and Discussion

### Mutant selection for prodigiosin yield-up

Through the antibiotic mutagenesis, a sum of 12 strains was randomly selected from wild type cells grown on SMB plate containing each antibiotic: 9 mutants (M1-M5 and M9-M12) at 50 μg/ml of chloramphenicol and 3 mutants (M6, M7, and M8) at 6.25 μg/ml of kanamysin. Also, 3 mutants (M13, M14, and M15) were randomly selected from wild type cells induced by EMS treatment (0.2 M). By measuring the prodigiosin yield using the spectrometric assay described in 'Materials and Methods', M3 could produce the highest amount of prodigiosin among these 15 mutants, and was finally selected for further study to increase the production yield. Subsequently, a stabilized mutant, assigned M3349, was obtained after three generations on SMB plate supplemented with chloramphenicol (50 μg/ml). When cultured in SMB, M3349 was able to produce prodigiosin at a concentration of 1.628±0.06 g/L, while wild type produced at 0.658±0.12 g/L under the same conditions (Fig. 1).

### Identification of significant nutrient components on prodigiosin production

To eliminate some nutrient components having no significant effects on the prodigiosin production, each of the fourteen

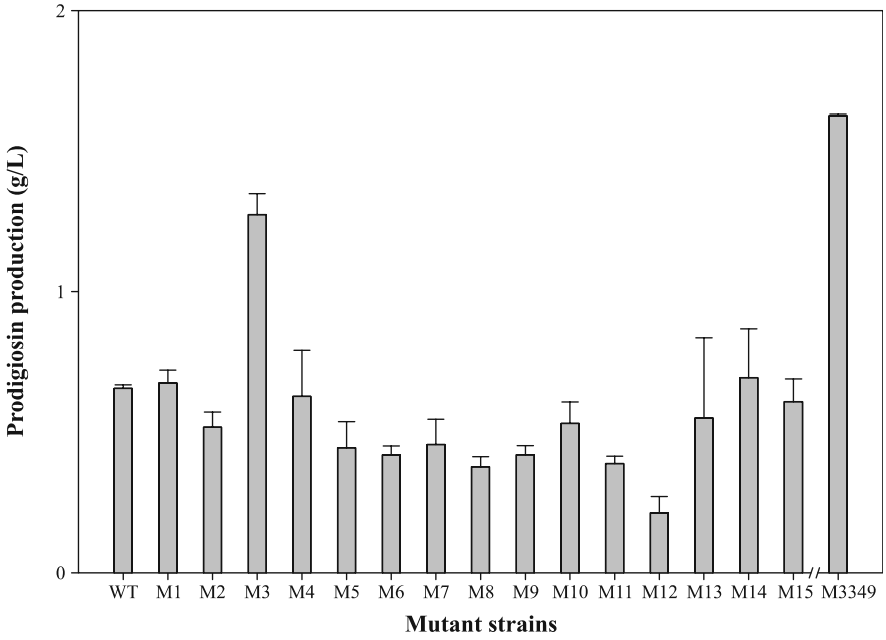


**Fig. 1.** Prodigiosin production of selected mutant strains. All strains were incubated on 20 ml of SMB medium in culture flask at 25°C for 72 h.

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

| Variables | Medium components | Effect | Standard error | *t*-Statistics | *P*-value |
|---|---|---|---|---|---|
| $X_1$ | $FeCl_3$ | -0.1546 | 0.0217 | -3.56 | 0.021 |
| $X_2$ | NaCl | 0.8439 | 0.0217 | 19.45 | 0.002 |
| $X_3$ | $MgCl_2$ | 0.6273 | 0.0217 | 14.45 | 0.008 |
| $X_4$ | $Na_2SO_4$ | 0.5384 | 0.0217 | 12.41 | 0.007 |
| $X_5$ | $CaCl_2$ | 0.5709 | 0.0217 | 13.16 | 0.001 |
| $X_6$ | KCl | -0.1976 | 0.0217 | -4.55 | 0.018 |
| $X_7$ | $Na_2CO_3$ | -0.1570 | 0.0217 | -3.62 | 0.022 |
| $X_8$ | KBr | -0.1151 | 0.0217 | -2.65 | 0.016 |
| $X_9$ | $SrCl_2$ | -0.3097 | 0.0217 | -7.14 | 0.013 |
| $X_{10}$ | $H_3BO_3$ | 0.5622 | 0.0217 | 12.96 | 0.003 |
| $X_{11}$ | $Na_2SiO_3$ | 0.4846 | 0.0217 | 11.17 | 0.004 |
| $X_{12}$ | NaF | -0.2384 | 0.0217 | -5.49 | 0.033 |
| $X_{13}$ | $NH_4NO_3$ | -0.1326 | 0.0217 | -3.06 | 0.017 |
| $X_{14}$ | $Na_2HPO_4$ | 0.0706 | 0.0217 | 1.63 | 0.002 |

different nutrient components included in SMB medium preparation were 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 < 0.01 were accepted as significant factors affecting the production of prodigiosin. It was found that *P*-value of $FeCl_3 \cdot 6H_2O$, KCl, $Na_2CO_3$, KBr, $SrCl_2$, NaF, and $NH_4NO_3$ were > 0.01, indicating that these seven elements are not significant factors on prodigiosin production compared with other factors. Therefore, these elements were considered to have no effects on prodigiosin production, and eliminated from further study.

To select nutrient components having more effects on prodigiosin yield, the seven nutrient components, selected through a preliminary Plackett-Burman experiment, were re-tested using a secondary Plackett-Burman design (Table 3). As a result of the secondary experiments, a Plackett-Burman experimental design matrix for the seven components was obtained, which showed the variable (nutrient components) with two levels of concentrations for each variable and the corresponding predicted prodigiosin production (Table 4). Consequently, the Effect values, standard errors, *t*-values and

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

| Variables | Medium components | + Values (g/L) | - Values (g/L) |
|---|---|---|---|
| $X_1$ | NaCl | 25 | 2.5 |
| $X_2$ | $MgCl_2$ | 12 | 1.2 |
| $X_3$ | $CaCl_2$ | 5 | 0.5 |
| $X_4$ | $H_3BO_3$ | 0.1 | 0.01 |
| $X_5$ | $Na_2SO_4$ | 8 | 0.8 |
| $X_6$ | $Na_2SiO_3$ | 0.02 | 0.002 |
| $X_7$ | $Na_2HPO_4$ | 0.04 | 0.004 |

**Table 4.** Plackett-Burman design matrix of selected nutrient components and observed prodigiosin production

| Trials No. | Variables[a], Levels[b] | | | | | | | Prodigiosin production (g/L) |
|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | |
| 1 | + | − | − | + | − | + | + | 1.817 |
| 2 | + | + | − | − | + | − | + | 1.298 |
| 3 | + | + | + | − | − | + | − | 1.552 |
| 4 | − | + | + | + | − | − | + | 0.326 |
| 5 | + | − | + | + | + | − | − | 1.362 |
| 6 | − | + | − | + | + | + | − | 1.665 |
| 7 | − | − | + | − | + | + | + | 1.800 |
| 8 | − | − | − | − | − | − | − | 0.657 |
| 9 | + | − | − | + | − | + | + | 1.155 |
| 10 | + | + | − | − | + | − | + | 1.062 |
| 11 | + | + | + | − | − | + | − | 1.545 |
| 12 | − | + | + | + | − | − | + | 0.284 |
| 13 | + | − | + | + | + | − | − | 1.279 |
| 14 | − | + | − | + | + | + | − | 1.572 |
| 15 | − | − | + | − | + | + | + | 1.581 |
| 16 | − | − | − | − | − | − | − | 0.699 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.060 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.138 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.334 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.214 |

[a] $X_1$, NaCl; $X_2$, $MgCl_2$; $X_3$, $CaCl_2$; $X_4$, $H_3BO_3$; $X_5$, $Na_2SO_4$; $X_6$, $Na_2SiO_3$; $X_7$, $Na_2HPO_3$; 0 was center point

[b] +, high concentration of variable; −, low concentration of variable; 0, intermediate concentration of variable.

*P*-values for the seven components were calculated as shown in Table 5. Finally the polynomial model describing the correlation between the seven components and the yield of prodigiosin was presented as follows: $Y_{(production)}=1.22838-0.1555X_1-0.16523X_2-0.01213X_3-0.04597X_4+0.22398X_5+0.35742X_6-0.06296X_7$; where $Y$ is predicted production (prodigiosin production), and $X_1$–$X_7$ are the coded values of NaCl, $MgCl_2$, $CaCl_2$, $H_3BO_3$, $Na_2SO_4$, $Na_2SiO_3$, and $Na_2HPO_4$. Analysis of the regression coefficients of the seven nutrient components, showed *P*-values for $CaCl_2$ and $H_3BO_3$ that were above 0.1, indicating that these components were insignificant for prodigiosin production compared with others (Table 5).

**Table 5.** Statistical analysis of selected nutrient components using Plackett-Burman design

| Variables | Medium components | Effect | Standard error | *t*-Statistics | *P*-value |
|---|---|---|---|---|---|
| $X_1$ | NaCl | 0.311 | 0.042793 | 3.64 | 0.004 |
| $X_2$ | $MgCl_2$ | -0.13047 | 0.042793 | -1.53 | 0.055 |
| $X_3$ | $CaCl_2$ | -0.02426 | 0.042793 | -0.28 | 0.782 |
| $X_4$ | $H_3BO_3$ | -0.09194 | 0.042793 | -1.08 | 0.35 |
| $X_5$ | $Na_2SO_4$ | 0.44797 | 0.042793 | 5.24 | 0.000 |
| $X_6$ | $Na_2SiO_3$ | 0.71484 | 0.042793 | 8.36 | 0.000 |
| $X_7$ | $Na_2HPO_4$ | -0.12592 | 0.042793 | -1.47 | 0.069 |

**Table 6.** Test levels of selected significant nutrient components for Box-Behnken optimization

| Variables | Variables code | + (g/L) | 0 (g/L) | − (g/L) |
|---|---|---|---|---|
| $Na_2SiO_3$ | $X_{1(+)}$ | 0.08 | 0.044 | 0.008 |
| $Na_2SO_4$ | $X_{2(+)}$ | 12 | 6.6 | 1.2 |
| NaCl | $X_{3(+)}$ | 35 | 19.25 | 3.25 |
| $MgCl_2$ | $X_{4(+)}$ | 7 | 3.85 | 0.7 |
| $Na_2HPO_4$ | $X_{5(+)}$ | 0.02 | 0.011 | 0.002 |

In summary, the five nutrient components (NaCl, $MgCl_2$, $Na_2SO_4$, $Na_2SiO_3$, and $Na_2HPO_4$) were finally selected as having a positive effect on prodigiosin yield based on their *P*-values (<0.1) and Effect values (+ or –). These results indicate that the Plackett-Burman design was a powerful tool for identification of the nutrient components significantly affecting the prodigiosin production.

## Optimization of selected medium components for prodigiosin production

Based on the results obtained by Plackett-Burman experimental design, $Na_2SiO_3$, $Na_2SO_4$, NaCl, $MgCl_2$, and $Na_2HPO_4$ were selected as significant nutrient components for prodigiosin production and were subsequently subjected to a further study using a Box-Behnken design. Table 6 shows the selected nutrient components tested for Box-Behnken optimization, the values of which were calculated by linear multiple regression using a Minitab software. The following equation was obtained: $Y_{(production)}=2.2772-0.0310X_1-0.1320X_2-0.2997X_3+0.0116X_4+0.0735X_5-0.0098X_1^2+0.0273X_2^2-0.6117X_3^2+0.0253X_4^2-0.0517X_5^2-0.1546X_1X_2+0.3141X_1X_3+0.0482X_1X_4+0.0993X_1X_5+0.0186X_2X_3-0.0959X_2X_4+0.1902X_2X_5+0.0858X_3X_4-0.3242X_3X_5-0.0636X_4X_5$; where $Y$ is the predicted response (prodigiosin production), and $X_1$-$X_5$ are the values of $Na_2SiO_3$, $Na_2SO_4$, NaCl, $MgCl_2$, and $Na_2HPO_4$, 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 0.927 for prodigiosin production, which indicates that about 7.3% of the total variations are not explained by the

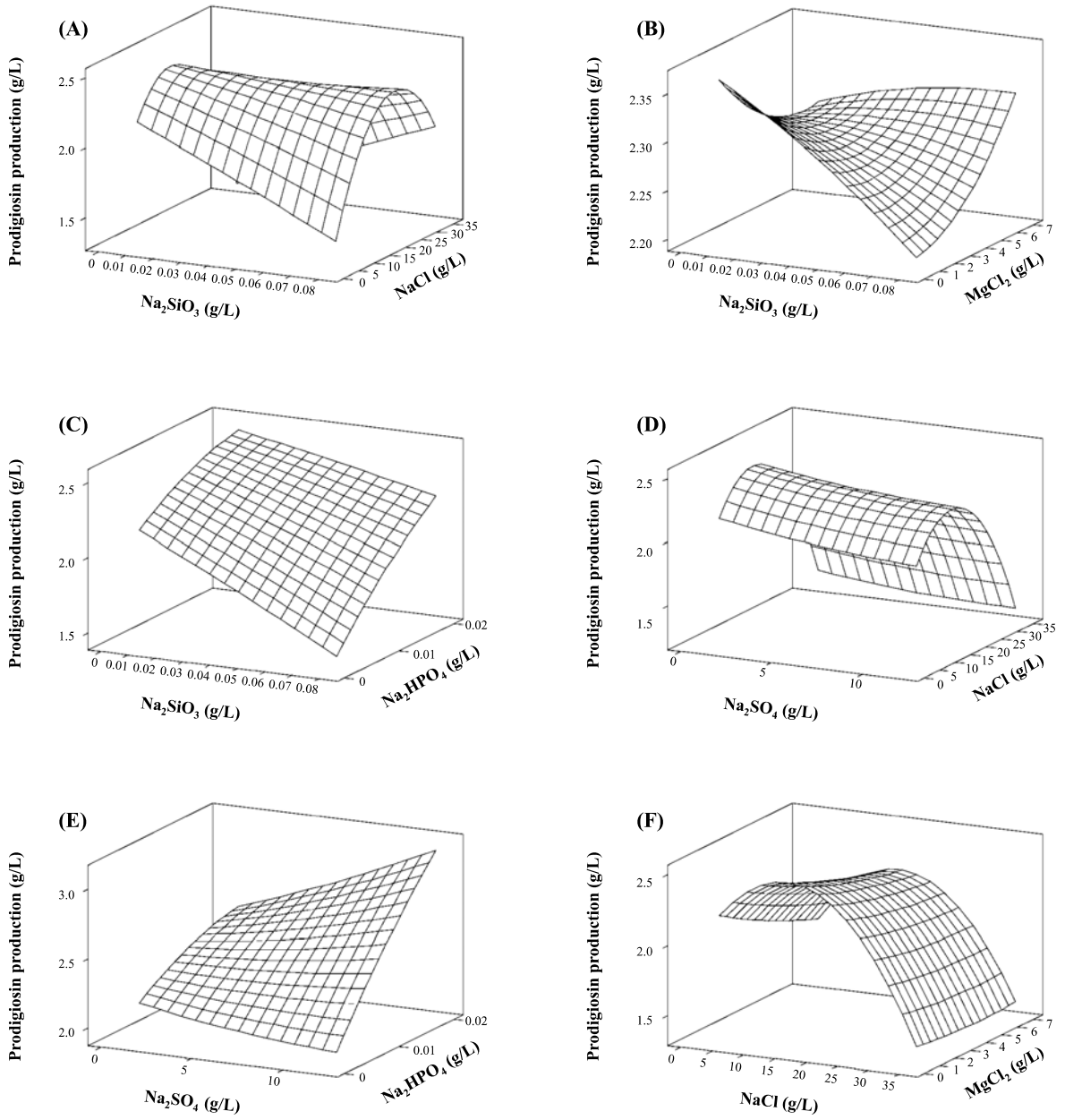


**Fig. 2.** Three-dimensional response surface plot for the effect of (A) $Na_2SiO_3$, NaCl (B) $Na_2SiO_3$, $MgCl_2$ (C) $Na_2SiO_3$, $Na_2HPO_3$ (D) $Na_2SO_4$, NaCl (E) $Na_2SO_4$, $Na_2HPO_3$ (F) NaCl, $MgCl_2$ on prodigiosin production (g/L).

prodigiosin production.

Presenting experimental results in the form of response surface plots showing the effect of $Na_2SiO_3$, NaCl; $Na_2SiO_3$, $MgCl_2$; $Na_2SiO_3$, $Na_2HPO_4$; $Na_2SO_4$, NaCl; $Na_2SO_4$, $Na_2HPO_4$; NaCl, $MgCl_2$ at different concentrations of the other two variables are shown in Fig. 2. The statistical optimal values of variables are obtained when moving along the major and minor axis of the contour and the response at the center point yields maximum prodigiosin production. Though the study response of surface plots and Box-Behnken experimental design, the optimal concentration of $X_1$-$X_5$ ($Na_2SiO_3$, $Na_2SO_4$, NaCl, $MgCl_2$, and $Na_2HPO_4$) were determined to be 0.008, 12, 10, 0.7, and 0.02 g/L, respectively.

When using the optimized culture medium (sucrose 10.0, peptone 8.0, yeast extract 2.0, NaCl 10.0, $Na_2SO_4$ 12, $CaCl_2$ 1.8, $MgCl_2$ 0.7 g/L; and $H_3BO_3$ 22.0, $Na_2HPO_4$ 20.0, $Na_2SiO_3$ 8.0 mg/L) for a higher production yield of prodigiosin by M3349, the maximum yield of prodigiosin was predicted to be 2.43 g/L, while the yield obtained from the practical experiment was 2.60±0.176 g/L, 3.9 times higher than wild type with SMB medium (0.658 g/L) and the yield of prodigiosin by M3349 with SMB medium was 1.628 g/L (Fig. 3).

There have been few reports on statistical optimization for the production yield-up of natural pigment. *Serratia marces-*

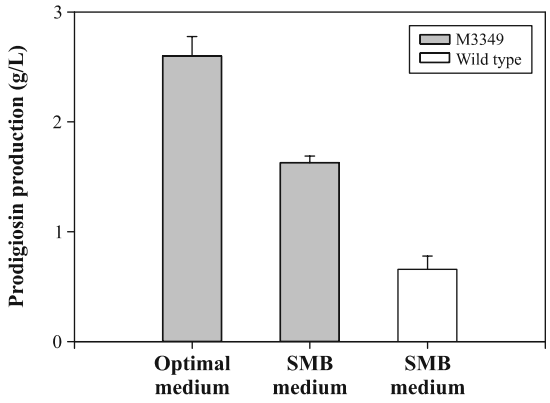


**Fig. 3.** Prodigiosin production by M3349 and wild type in each medium. Prodigiosin production were carried out in 2 L baffles flask, each medium containing 500 ml medium on a shaker of 120 rpm at 25°C for 96 h. Three experiments were carried out and the error bar indicates standard deviations from the means.

*cens* SMΔR was investigated under modified LB medium to improve the prodigiosin production. However, the prodigiosin production was 0.79 g/L (Wei and Chen, 2005). We optimized for the first time the medium components for higher prodigiosin production using statistical designed experiments. Plackett-Burman and Box-Behnken 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 type and relative amounts of main medium components.

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