JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 2595-2604

Enhancing Pyocyanin Production by *Pseudomonas aeruginosa* RS11 using Response Surface Methodology

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(Received: 03 May 2014; accepted: 25 June 2014)

The objective of this study was to maximize production of pyocyanin by *Pseudomonas aeruginosa* RS11using response surface methodology. Plackett-Burman design (PBD) was used to select the operational parameters influencing the optimization process. The most effective variables (pH, yeast extract, and K_2SO_4) were chosen for further optimization using Box-Behnken design. The optimal fermentation parameters for enhancing PYO production were found to be pH 5.8, K_2SO_4 20 g/l and yeast extract 1.5 g/l. The model predicted a PYO yield of 15.55 μg/ml. Verification of the optimization showed that the maximum PYO yield reached 17.7 μg/ml. Up to our knowledge, this is the first report on PYO optimization by *Pseudomonas aeruginosa* RS11 using response surface methodology. The yield after optimization was 2.1-fold higher than the unoptimized medium. The response surface methodology (RSM) with the Box–Behnken design was a useful tool for achieving the high yield of pyocyanin by *Pseudomonas aeruginosa* RS11.

Key words: Pyocyanin, *Pseudomonas aeruginosa*, Optimization, Plackett-Burman, Response surface methodology.

Phenazines comprise a large group of nitrogen-containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of functional groups present [1] . More than 100 different phenazine structural derivatives have been identified in nature[2], which exhibit broad-spectrum activity against numerous bacteria and fungi [3]. These include pyocyanin (5-N-methyl-1-hydroxy-phenazine)(PYO), phenazine-1-carboxylic acid (PCA),1-hydroxyphenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN)[1,4]. Phenazines continue to be used for many diverse applications; as electron acceptors and donors, as components of fuel cells, as environmental sensors and biosensors, and as central components of antitumor compounds [1,5] .

Pseudomonas aeruginosa found in all sources of environments, produces a variety of phenazines. It is the only organism of the pseudomonads and other non-fermenting Gram-negative bacilli known to be capable of producing pyocyanin [6,7], making its presence helpful in identifying the organism. Pyocyanin showed antagonistic activity against various pathogens, food spoilage bacteria and suppressed the growth of fungi like *Aspergillus fumigatus* and *Candida albicans* [7,8.] Several studies reported that *P. aeruginosa* not only showed bio-control activity against a wide range of phytopathogens but also increased plant growth rate [7,9]. *P. aeruginosa* formulations were not only effective in plant

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growth, but also increased resistance against soyabean stunt virus (SSV)[10].

In the development of any microbial fermentation process medium optimization is an early and essential step, and plays an important role in enhancement of metabolite production [11]. The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechology, due to its propensity and relevance [12]. Response surface methodology (RSM) is a useful statistical technique for investigation and optimization of complex processes. It is a collection of mathematical and statistical techniques, and is widely used in different biotechnological processes to study the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments[11]. Indeed, RSM enables the optimum values of the factors to be determined and also predicts the response of the process under optimized conditions[13]. To the best of our knowledge, there are scant in the literature regarding the use of statistical methods for optimization of pyocyanin production from *P. aeruginosa*.

The objective of the present study was to optimize PYO production by a local *Pseudomonas aeruginosa* RS11. Firstly, Plackett–Burman experimental design was used to evaluate the likely effects of the medium. Subsequently, the factors that had significant effects were optimized using response surface methodology.

MATERIALS AND METHODS

Microorganism

Pseudomonas aeruginosa RS11 was isolated from rhizosphere soil collected from Siwa Oasis, Egypt.

Chemicals

Ingredients of media specific for cultivation were all of analytical grade obtained from recognized chemical suppliers. Fine chemicals were obtained from Sigma Chemical Company, St Louis, USA. Solvents used throughout this study were all of analytical grade chemicals. Bacterial filters used were 0.22µm pore- size cellulose acetate membrane (Corning, USA).

Samples collection and preparation

Garden, rocky and rhizosphere soils were collected from different places in sterile plastic bags and quickly transferred to the laboratory. One gram of each was suspended in 10 ml sterile distilled water and shaken at 120 rpm for 30 min [14] .

Isolation of pyocyanin producing *Pseudomonas aeruginosa*

For isolation of *Pseudomonas* strains, diluted extracts were plated on casamino acid (CAA) [15] King's media A (KMA) or King's media B (KMB) [16] of pH 7. The plates were incubated at 37° C for 48 h and a number of morphologically different fluorescent colonies developed were selected using a UV lamp (366 nm) [17] . Selected colonies were picked using sterile tooth picks, purified by successive streaking using the standard spatial streaking method and incubated for 24 h at 42 ºC. Pyocyanin producers were recognized by the formation of deep blue coloration of the medium around the bacterial colonies[18].

Screening and selection of optimal pyocyanin production medium

P. aeruginosa PYO producing strains were grown on KMA agar plates and a single colony was used to inoculate a 250-ml Erlenmeyer flask containing 50 ml broth of KMA. The flasks were kept on a rotary shaker at 150 rpm for 24h at 37°C, functioning as the inoculum (OD_{600} =0.45). Subsequently, 1 ml was inoculated in 100-ml Erlenmeyer flasks containing 25 ml of three different production medium (CAA, KMB and KMA). The cultures were incubated at 37°C for 2 days. Samples were collected from each fiask and assayed for pyocyanin concentration.

Optimization by sequential strategy of experimental design

Optimization was performed by a two-phase experimental design. The first phase was to adjust the levels or select the signal variables, using Plackett–Burman design. In the second phase, levels of the variables, which had significant influences on the production process, were further studied using response surface methodology.

Plackett-Burman design

Plackett–Bruman design [19]is a very useful technique for picking the most important factors from a list of candidate factors and to verify if the investigated levels were in adequate range. Seven independent variables were screened in eight combinations organized according to the design matrix. The high (+1) and low (-1) ranges selected

for tested variables are given in Table 1. Statistica software version 6.0 (Statsoft, USA) was used to generate a set of 8 experimental designs. All trials were performed in duplicates and the averages of pyocyanin observation results were treated as the responses. The effect of each variable on the response, E, was determined according to the main effect equation:

$$E = \frac{\text{Total response at high level} - \text{total response at low level}}{\text{Number of trials}}$$

A main effect figure with a positive sign indicates the influence of the variable upon PYO production is greater at a high concentration, and when negative, the influence of the variable is greater at a low concentration.

Response surface methodology

In order to determine the optimum levels of key variables for PYO production, the response surface methodology (RSM) was applied, using Box- Behnken design [20]. This process involves three important steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. The optimum concentration and interaction of the three signal parameters selected by the above PB design, namely yeast extract, K_2SO_4 and pH were studied. Each factor was studied at three different levels, low (-1), medium (0) and high (+1). A set of 16 experiments with three factors and four replicates at the centre was generated. The full experimental plan with respect to their values in actual and coded forms is listed in **Table 3**. All the experiments were carried out in duplicate and the average of PYO production obtained was taken as the response (Y). The relationship between dependent and independent variables is explained by the following second-order polynomial equation:

$$Y = \beta_0 + \Sigma \beta_1 X_1 + \Sigma \beta_2 X_2 + \Sigma \beta_3 X_3 + \Sigma \beta_{12} X_1 X_2 + \Sigma \beta_{13} X_1 X_3 + \Sigma \beta_{23} X_2 X_3 + \Sigma \beta_{11} X^2_1 + \Sigma \beta_{22} X^2_2 + + \Sigma \beta_{33} X^2_3$$

Where, Y is the predicted pyocyanin concentration; X_1 ,X_2, X_3 are independent variables (pH ,yeast extract and K_2SO_4); α_0 is the intercept term, β_i , β_2 and β_3 are the linear coefficients, β_{12} , β_{13} and β_{23} are second order interaction coefficients; β_{11}, β_{22} and β_{33} are quadratic coefficients, and X_1, X_2 and X_3 were coded independent variables.

The quality of the fit of the polynomial model equation was expressed by the coefficient of determination, R^2 which measures the goodness fit of regression model. The fitted polynomial equation was then expressed in the form of contour and surface plots in order to illustrate the relationship between the response and the experimental levels of each of the variables tested in this study. Experimental design, analysis of results and graphs were conducted using Statistica software version 6.0 (Statsoft. Inc, USA).

Analytical procedures

Extraction, quantification and purification of pyocyanin

Following growth in KMA, 5ml culture sample were centrifuged, and supernatant was extracted subsequently with 3ml of chloroform. The mixed sample was agitated vigorously and centrifuged at 12,000 rpm for 5 min. The blue chloroform solution was transferred into a new tube containing 1 ml of 0.2 N HCl to extract pyocyanin into the acidic solution. The mixture was agitated vigorously and centrifuged at 12,000 rpm for 5 min. The pyocyanin was partitioned to the HCl aqueous phase. The upper layer was collected and the absorbance of deep red acid solution of 1- hydroxy phenazine was measured at 520 nm and the pyocyanin concentration was calculated according to the following equation [21].

Pyocyanin concentration (µg/ml) = OD_{520} x 17.07

To the deep red acid solution, 0.4M borate-NaOH buffer, pH 10 was added until the color changed to blue and the pyocyanin again extracted in 5 ml chloroform. Pyocyanin was purified by repeated cycles of chloroform extraction of the basic (blue) form followed by aqueous extraction of the acid (red) form of the compound as previously described [22]. The latter was evaporated to a stream of cold air and the product was crystallized. The pyocyanin was solubilized in H_2O and sterilizied by ultra-filtration using 0.22 µm membrane filters before use.

RESULTS AND DISCUSSION

Isolation of PYO-producing pseudomonads

Fluorescent pseudomonads are prominent in the aerobic microflora of the rhizosphere of many plants [23]. Specific iron-deficient medium (King's medium A) was

Table 1. Minimum and maximum ranges of the seven parameters used in the PB run

Factors	Level	
	-1	+1
Peptone(g/l)	2	30
$MgCl_2$(g/l)	0.14	2.4
K_2SO_4(g/l)	1	20
Glycerol(g/l)	1	20
Yeast extract(g/l)	1	5
pH	6.5	7.5
Medium volume (ml /flask)	10	75

recommended for the isolation of the fluorescent pseudomonads [24]. Out of nineteen bacterial colonies showing characteristic yellow fluorescent pigment and different morphotypes, only thirteen were able to grow and produce pyocyanin around the colonies on KMA which confirmed their identity as *Pseudomonas aeruginosa*. The most widely used criteria for distinguishing *P. aeruginosa* from closely related organisms is by the production of pyocyanin pigment [5]. Moreover, growth at elevated temperature 42 °C was used by many workers for the selective isolation of *Pseudomonas aeruginosa* [25]. Based on these two criteria, five isolates were selected for pyocyanine production in liquid media.

Table 2. Screening of variables using PB design and their ranking affecting pyocyanin production

Factors	PYO production	
	E value	Rank
Peptone (g/l)	-1.54	4
$MgCl_2$(g/l)	-1.48	5
K_2SO_4(g/l)	+4.38	2
Glycerol (g/l)	+1.41	6
Yeast extract (g/l)	-2.94	3
pH	-5.17	1
Medium volume (ml/flask)	+0.085	7

Screening and selection of optimal production medium

Many types of media are used for selective isolation, maintenance and enhancement of pyocyanin (PYO) production by *P. aeruginosa* [26]. Liquid culture screening is an attractive approach because it is more adaptable to simultaneous detection of multiple metabolites [27]. Therefore, a preliminary experiment was conducted to select the production medium. Selected strains (GS 4, RS 9, RS 11, RS 12, and RS 13) were allowed to grow in liquid portions of either King's medium A, King's medium B or casamino acid medium. Data illustrated in **Fig. 1** indicate that in general, King's Medium A enhanced pyocyanin production. These results are

Table 3. Experimental design using Box-Behnken of three independent variables with their actual and coded values and four center points showing the experimental response

Trials	Variables/ levels						
	pH		Yeast extract		K_2SO_4		PYO
	Coded value	Actual value	Coded value	Actual value (g/l)	Coded value	Actual value(g/l)	concentration (µg/ml)
1	0	6	-1	0	-1	10	8.84
2	0	6	-1	0	1	30	10.12
3	0	6	1	2	-1	10	10.54
4	0	6	1	2	1	30	12.42
5	-1	5.5	0	1	-1	10	11.02
6	-1	5.5	0	1	1	30	13.28
7	1	6.5	0	1	-1	10	11.5
8	1	6.5	0	1	1	30	13.23
9	-1	5.5	-1	0	0	20	11.6
10	-1	5.5	1	2	0	20	14.2
11	1	6.5	1	0	0	20	10.26
12	1	6.5	1	2	0	20	12.3
13	0	6	0	1	0	20	15.44
14	0	6	0	1	0	20	15.55
15	0	6	0	1	0	20	15.47
16	0	6	0	1	0	20	15.55

similar to that obtained by Hernandez et al.[3] and this compound is known to be regulated by iron which is limited in King's Medium A. Strain RS11 gave maximum PYO concentration and was then selected for optimization studies.

Time –course of pyocyanin production

Data in **Fig.2** reveal that strain RS11 grown on KMA, produced pyocyanin in early exponential phase of growth (24 h) and continued to increase until the cells reached stationary phase. Maximum production (8.48µg/ml) was achieved after 4 days. Shaking did neither show a pronounced effect on cell growth nor pyocyanin production, since almost the same result was recorded (data not shown). Pyocyanin production was indicated by change in color to bluish green. The change in color of the pigment to deep pink was observed upon addition of chloroform and 0.2 N HCl (**Fig.3**), confirming the identity of the pigment as pyocyanin [28]. In addition, UV -spectrophotometric analysis revealed a maximum absorbance at 278 nm (data no shown), which was in accordance with results reported by Sudhakar et al.[5] .

Optimization by Plackett-Burman design

For improvement of PYO production by *P. aeruginosa* RS11, seven factors, namely glycerol, K_2SO_4, peptone, yeast extract, $MgCl_2$, pH, and volume of the culture medium were screened using the Plackett–Burman design. These factors were ranked for their effect on the response based on their E values (Table 2). Yeast extract, pH, and K_2SO_4 were found to be signal factors. Yeast extract and pH affected the response at a negative level, while K_2SO_4 affected the response at a positive level.

A large contrast mean, either positive or negative, indicates that a factor has a large impact on titre; while a mean close to zero means that a factor has little or no effect. A positive value of a main effect indicates that the higher concentrations of that variable are ideal for enhancing PYO production, whereas negative values indicate the opposite. On the basis of the analysis of the regression coefficients of the 7 variables, K_2SO_4 and glycerol positively affected PYO production. Data depict that a higher glycerol concentration resulted in increasing of PYO production. In *P. aeruginosa*, the production of pyocyanin and other phenazines is stimulated during growth on glycerol as carbon source[29,30]. Yeast extract, pH, and K_2SO_4 were found to be signal factors. Peptone, $MgCl_2$, yeast extract, and pH showed a negative effect. However, the volume of the culture medium had no obvious effect on PYO production.

Response surface methodology (RSM)

The main objective of response surface analysis is to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum production[13]. On the basis of the Plackett–Burman results, the three variables yeast extract, K_2SO_4 and pH were selected for optimization studies using RSM. The three key factors were examined at three

Table 4. Analysis of variance (ANOVA) for the experimental results of the Box-Behnken design (Quadratic Model)

Factor	Sum squares	Degree of freedom	Mean square	*F*-value	*p*-value
X_1	0.28322	1	0.28322	89.674	.002496
X_2	7.98848	1	7.98848	2529.334	.000017
X_3	5.31480	1	5.31480	1682.788	0.000032
X^2_1	2.67323	1	2.67323	846.404	0.000089
X^2_2	26.93610	1	26.93610	8528.580	0.000003
X^2_3	23.57103	1	23.57103	7463.121	0.000003
X_1X_2	0.07840	1	0.07840	24.823	0.015543
X_1X_3	0.07023	1	0.07023	22.235	0.018060
X_2X_3	0.09000	1	0.09000	28.496	0.012852
$X_2X^2_3$	0.05120	1	0.05120	16.211	0.027532
$X_3X^2_2$	0.08611	1	0.08611	27.265	0.013661
$X_1X^2_3$	1.68361	1	1.68361	533.070	0.000178
Error	0.00984	3	0.00316		
Total	71.9579	15			

R^2 = 0.9998; Adj-R^2 = 0.99

different levels (-, 0 and +), with 16 independent trials (Table 3). The other components of the medium were added in their optimum concentration obtained from Plackett-Burman and treated as constant factors. Data revealed a considerable variation in PYO production, depending on the levels of the three independent variables in the medium (Table 3). The highest amount of PYO was observed in the runs representing the center points (trials 13-16) and the lowest amount was observed in trial 1 (8.84 µg/ml).

A second order regression equation showed the dependence of PYO production on the medium constituents. The parameters of the equation were obtained by multiple regression analysis of the experimental data. An empirical relationship between the response and the screened variables was expressed in terms of second-order polynomial equation:

$$Y = 82.65 + 27.8X_1 + 4.2X_2 + 2.2X_3 - 2.18X^2_1 - 1.59X^2_2 - 0.052X^2_3 - 0.09X_1X_2 - 0.25X_1X_3 + 0.04X_2X_3 - 0.0006X_2X^2_3 - 0.006X_3X^2_2 + 0.006X_1X^2_3$$

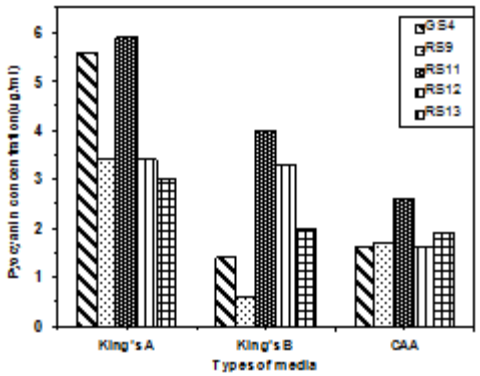

Fig. 1. Effect of different culture media on pyocyanin production by the most potent *Pseudomonas aeruginosa* strains after incubation at 37 °C for 2 days

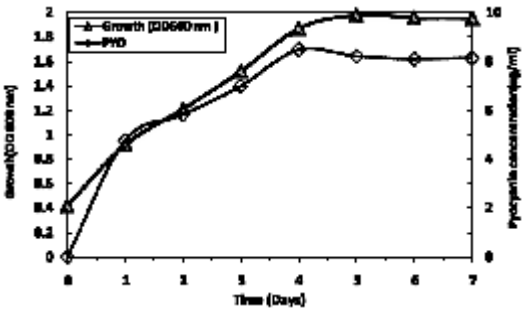

Fig. 2. Time–course of growth and pyocyanin production by *Pseudomonas aeruginosa* RS11 grown in KMA under static conditions at 37 °C

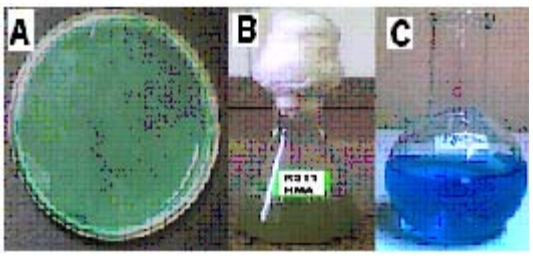

Fig. 3. Growth of *P. aeruginosa* RS11 on (A) KMA plate incubated for 48 h at 37°C and (B) KMA after 4 days.(C) purified pyocyanin obtained from the same bacterium

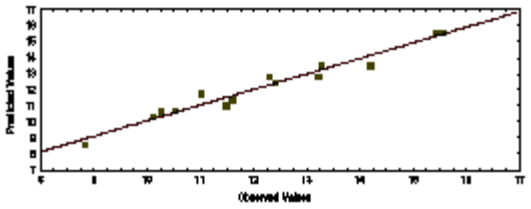

Fig. 4. Plot of predicted vs. actual PYO production by *P. aeruginosa* RS11

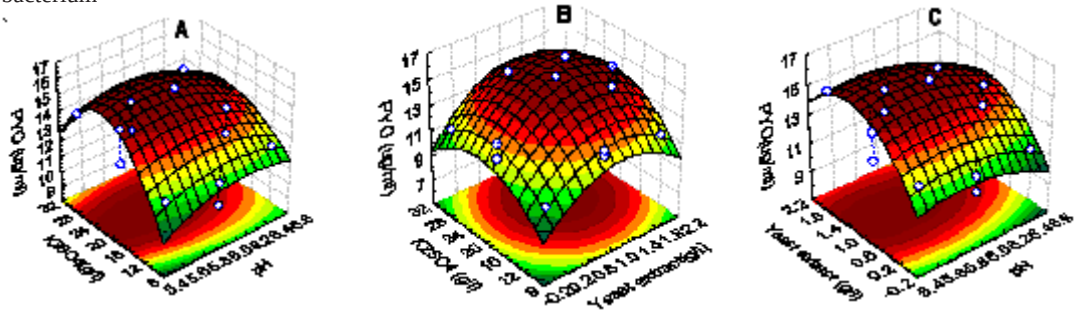

Fig. 5. Response surface and contour plots of the interaction of (A) K_2SO_4 and pH,(B) K_2SO_4 and yeast extract and (C) pH and yeast extract on pyocyanin production by *P. aeruginosa* RS11

where Y is the predicted PYO yield, X_1, X_2 , X_3 are the coded values of pH, yeast extract and K_2SO_4 respectively.

The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the R^2 value was calculated to be 0.9998, indicating that 99.98% of the total variability in the response could be explained by the model. A regression model with R^2 close to 1.0 is considered of a very high correlation [31]. Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for predicting PYO production. The value of the adjusted determination coefficient (Adj R^2 = 0.99) confirmed the significance of the model as well. This result confirms the validity of the statistical method [32]. Figure 4 represents the predicted vs. actual values of PYO concentration. The clustering of the points around the diagonal line indicates a satisfactory correlation between the experimental data and the predicted values, confirming the robustness of the model. Furthermore, an analysis of variance (ANOVA) for the response surface quadratic model is presented in Table 4. The *p*-values are used as a tool for checking the significance of each coefficient, which also indicate the interaction strength between each independent variable; the smaller the *p*-values, the more significance of the corresponding coefficient. The responses revealed that all the three linear coefficients of tested variables, their interaction terms, and

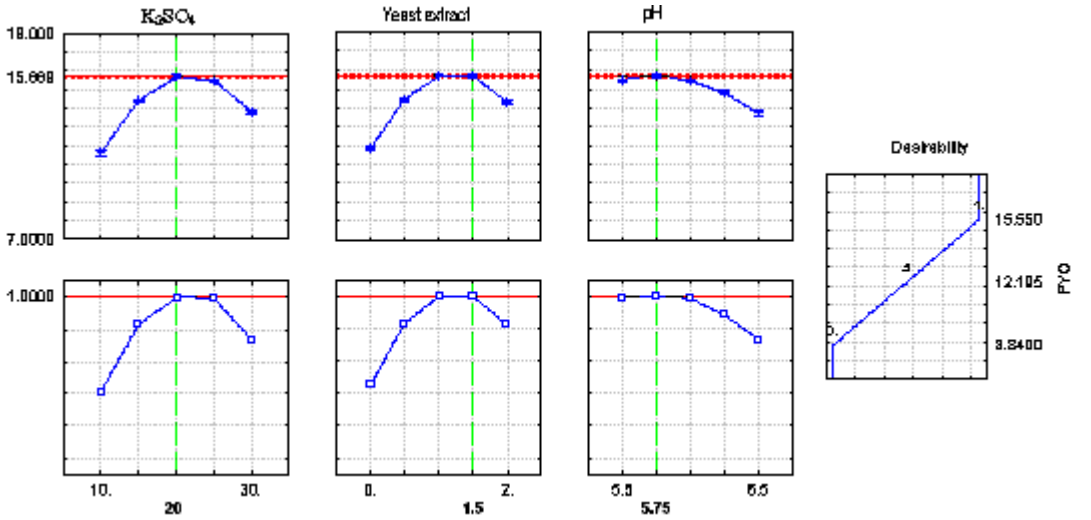

Fig. 6. Profiles for predicted values and desirability for different variables based on Box- Behnken experiment

quadratic coefficients were significant model terms ($p < 0.01$) and had remarkable effects on the overall production.

The three-dimensional response surface plots and their corresponding contour plots are the graphical representations of the regression equation, from which the PYO production is generated for the pair-wise combination of the three factors, with one variable kept constant at its optimum level and with variation of the other two variables within the experimental range. 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.5A. represents the interaction between K_2SO_4 and pH. Phenazine synthesis by *P. aeruginosa* RS11 was strongly influenced by K_2SO_4. The concentration of PYO was positively correlated with the K_2SO_4 in the medium. Similar results were also observed in another report, where PYO production was enhanced with the addition of K_2SO_4 [33], this is due to the higher level of sulfate required for optimal pigment synthesis. On the contrary, increasing the K_2SO_4 concentration 10-fold (0.8 to 8 mM) had little effect on phenazine-1-carboxamide production by *P. chlororaphis* PCL1391 [29]. Fig. 5B represents the interaction between K_2SO_4 and yeast extract. Increasing concentrations of K_2SO_4 and yeast extract had positive influence on PYO production. Yeast extract

is a highly source of protein with an excellent amino acids profile, essential vitamins and minerals, some of which were the precursor of PYO synthesis. The fact that PYO contains nitrogen is a logical explanation for the stimulatory effect of N-containing compounds on PYO production. Higher concentrations of peptone and yeast extract decreased PYO level indicating that the ratio of carbon source to nitrogen concentration plays a key role in metabolic flow. Similar to *P. aeuroginosa* RS11, the production of phenazine-1-carboxylic acid (PCA) by *P. fluorescens* 2-79 [34], *P. chlororaphis* SPB1217[35], *P.*sp. M18G [30], and PCN production by *P. chlororaphis* PCL1391 [29] were found to be stimulated by amino acid. Fig. 5C depicts the three dimensional plot and its respective contour plot showing the response surface from the interaction between yeast extract and pH. As shown, when yeast extract was maintained at moderate level, PYO production varied a little with the change of pH value. However, PYO production was sensitive to the change of pH, it tended to decrease rapidly with the increasing or decreasing pH, when the yeast level was oversize or undersize. It was obvious that higher pH resulted in a drastic decrease in PYO yield; moderate initial pH (approximately 5.8) appeared to be the most favorable for production.

The maximal predicted PYO production was determined by the fitted model as 15.55 µg/ml when the yeast extract, initial pH, and K_2SO_4 concentration were 1.5g/l, 5.8, and 20g/l, respectively (Fig. 6). The desirability factor of closer to 1 indicates that the process had maximum efficiency under those operating conditions. The average of experimental validation of the model produced 17.7 µg/ml PYO. The excellent correlation between predicted and experimental values supported the validity and accuracy of the mathematical model. Up to our knowledge, this is the first report for PYO production using sequential optimization strategy through statistically experimental design.

CONCLUSION

In this study, the statistically based experimental designs proved to be an effective tool in optimizing the medium for PYO production by *Pseudomonas aeruginosa* RS11 and indicated that nutritional status of the growth medium plays an important role in the biosynthesis of PYO. A statistical screening procedure via a Plackett-Burman design was adopted to select the main factors affecting PYO production. Analyses of Plackett Burman design results demonstrate that, yeast extract, K_2SO_4, and pH were the more important independent variables. Optimization of these three selected variables through Box-Behnken design showed a maximum PYO concentration of 17.7 µg/ml. The production yield is approximately 2.1 much higher than the original production. This suggests the effectiveness of the sequential strategy of the experimental design in bioprocess optimization with a large gain of cost and time. In fact, response surface methodology was demonstrated in many literature studies as an efficient tool to optimize metabolites production by several strains.

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