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Optimization of pyocyanin production from *Pseudomonas aeruginosa* JY21 using statistical experimental designs

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Abstract: *Pseudomonas aeruginosa* isolate JY21 (GenBank accession number, KF922508) which produces pyocyanin was tested as biocontrol agent for its antagonistic effect on the *in vitro* growth of plant pathogens namely, *Alternaria* sp, *Pythium* sp, *Phytophthora infestans*, *Rhizoctonia solani* and *Sclerotium* sp. Sequential optimization strategy, based on statistical experimental designs, was implemented for enhancing production of pyocyanin as biocontrol agent. For screening of various fermentation factors significantly influencing pyocyanin production, Plackett–Burman design was used. Fourteen different factors (variables) with two-level including fermentation conditions and medium constitution were chosen to perform this optimization process. pH, KCl and $MgCl_2.6H_2O$ were found to be the most significant variables affecting pyocyanin concentration. A near optimum medium formulation was obtained using this method, pyocyanin concentration achieved on this medium was 217.5 µg/ml. Response surface methodology (RSM) was adopted to acquire the best process conditions. In this respect, Box–Behnken design with three-level was employed. The optimal levels of the three variables as obtained from the maximum point of the polynomial model were estimated using the Solver function of Microsoft Excel tools and JMP program, and found to be: pH 8.2, KCl, 1.25 g/l and $MgCl_2.6H_2O$, 4.2 g/l with a predicted pyocyanin concentration of 245.39 µg/ml.
Keyword: *Pseudomonas aeruginosa*, pyocyanin, optimization, experimental designs.

Introduction

Phenazines compounds produced by fluorescent pseudomonas are low-molecular-weight ("secondary") metabolites including nitrogen-containing heterocyclic pigments. The most widely studied phenazine-producing fluorescent pseudomonad is *P. aeruginosa* which produces a variety of redox-active phenazine compounds, including pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) [**1**]. Almost all phenazines exhibit broad spectrum activity against plant pathogenic bacteria and fungi, from 90 to 95% of *P. aeruginosa* produce pyocyanin [**2**], this compound produced in the rhizosphere of plants play an important role in the biological control activity of *P. aeruginosa* against *Fusarium* wilt of chickpea and *Pythium* dampingoff of bean [**3**].

Statistical experimental designs such as Plackett–Burman (PB) [**4**] can collectively optimize all the affecting parameters to eliminate the limitations of a single-factor optimization process. PB design provides a fast and effective way of screening a large number of factors and identifying the significant ones, thereby, saving time and maintaining convincing information on each component [**5**].

Plackett–Burman comprises one type of two-level screening design. This design allows for the study of up to n – 1 factors where n is the number of trials. Typical Plackett–Burman design consists of 8, 12, 16, 20 and 24 trials [**6**]. To choose factor settings for any two-level screening design, one should consider the following criteria: (i) the factor range ideally should contain the optimum response for that factor; (ii) the range should be wide enough for any effect or trend to be exposed; and (iii) the range should avoid combinations of low and high factor settings which are likely to produce an outright process failure. Factors level selection can be a difficult part of the experimental process. Experience, prior experimentation, and the literature can be valuable resources for choosing factor settings [**7**].

In Plackett–Burman, low and high factor settings are coded as -1 and +1, respectively, so the factors are computationally equivalent. The coded arrangement for Plackett–Burman design of any size may be found in statistical software packages or in the experimental design literature [**6, 8, 9**].

Statistical software, such as Excel and JMP can be used to quickly calculate parameter estimates and perform a statistical analysis of the data. Like the interpretation of any slope, the estimate is the expected change I product titer for every unit increase in the coded factor level. Therefore, two times the factor estimate represents the change in titer over the entire factor range (-1 to +1). The Change in response over the entire range is called the main effect of a given factor. The sign of a factor estimate indicates, on average, which factor setting results in higher titers. A large estimate, either positive or negative, indicates that a factor has a large impact on titer, while an estimate close to zero means that a factor has little or no effect [**7**].

A number of tools may be used to help assess the significance of each process factor. These include *P* values, normal plots and Pareto charts. The most common means of assessing significance is the *P* value. The *P* value is the probability that the magnitude of a parameter estimate is due to random process variability [**8**]. A low *P* value indicates a real or significant effect and provides a baseline for deeming some factors critical and others less important. A second method for assessing significance is the normal probability plot. Parameter estimates not significantly different from zero will form a straight line on the normal probability plot. Estimates which deviate from the line are deemed significant. JMP software and other statistical packages will construct normal plots and identify critical factors. Finally, a Pareto chart of estimates can be constructed to judge the relative importance of factors simply by the magnitude of the parameter estimates [**9**].

Response surface methodology, which includes factorial design and regression analysis, helps in evaluating the effective factors, building models to study the interaction between the variables, and selecting the optimum conditions of variables or desirable responses [**10**]. The most popular response surface methodologies are central composite and Box-Behnken designs.

Box-Behnken design is an efficient and creative three-level composite design for fitting second-order response surfaces. It is an independent quadratic design. The methodology is based on the construction of balance designs which are rotatable and enable each factor level to be tested several times. Each factor or independent variable can be placed at one of three equally spaced values (coded as –1, 0, and +1). In this design the treatment combinations are at the midpoints of edges of the cubical design region and at the center. Box-Behnken designs provide excellent predictability within the spherical design space and require fewer experiments [**11**].

Production of L-asparaginase was enhanced by *P. aeruginosa* in solid-state culture using Box-Behnken design [**12**]. Thermostable lipase production by *Geobacillus thermoleovorans* was optimized in shake-flask cultures using Box-Behnken experimental design [**13**]. Production of uricase was improved from *P. aeruginosa* by optimization of process parameters through statistical experimental designs (Plackett–Burman and Box-Behnken designs) [**5**].

The objective of this study was for optimization culture conditions for pyocyanin (1-hydroxy-5-methyl phenazine) production from *P. aeruginosa* through statistical experimental designs including Plackett–Burman design and Box-Behnken design.

Materials and methods

Microorganism

The bacterial isolate used in this study is *P. aeruginosa* isolate JY21 which was isolated from rhizosphere of potato, and was obtained from El-Minoufiya. *P. aeruginosa* isolate JY21 was identified according to 16S rRNA gene sequencing with GenBank accession number KF922508.

Pyocyanin assay

The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution [**14**]. A 5ml sample of culture broth was extracted with 3 ml of chloroform and then reextracted into 1 ml of 0.2 N HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm using a spectrophotometer (Pharmacia Biotech, England). Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant were determined by multiplying the extinction at 520 nm (E_{520}) by 17.072 [**15**].

Statistical designs

Plackett–Burman design

For screening purpose, various medium components and culture parameters have been evaluated. Based on the Plackett–Burman factorial design, each factor was examined at two levels: -1 for a low level and +1 for a high level [**6**]. This design is practical specially when the investigator is faced with a large number of factors and is unsure which settings are likely to be nearer to optimum responses [**7**]. Table 1 illustrates the factors under investigation as well as levels of each factor used in the experimental design, whereas Table 2 represents the design matrix.

Table 1. Media components and test levels for Plackett–Burman experiment.

Variable	Variable code	Low level (-1)	High level (+1)
pH	X1	7	8
Incubation time (h)	X2	24	72
Temperature (°C)	X3	30	37
Inoculum size (%)	X4	2	5
Peptone (g/l)	X5	2	20
$MgCl_2.6H_2O$ (g/l)	X6	0.4	4
D-L.Alanin (g/l)	X7	0.1	1
Glycerol (ml)	X8	2	20
Glucose (g/l)	X9	2	20
K_2SO_4 (g/l)	X10	1	10
K_2HPO_4(g/l)	X11	0.01	0.1
$FeSO_4.7H_2O$ (mM)	X12	0.01	0.1
$MgSO4.7H_2O$ (g/l)	X13	0.1	1
KCl (g/l)	X14	0.1	1

Table 2. Randomized Plackett-Burman experimental design for evaluating factors influencing pyocyanin production by *Pseudomonas aeruginosa* JY21.

Trial	pH	Incubation time	Temperature	Inoculum size	Peptone	$MgCl_2.6H_2O$	Alanine	Glycerol	Glucose	K_2SO_4	K_2HPO_4	$FeSO_4.7H_2O$	$MgSO_4.7H_2O$	KCl	Pyocyanin concentration (µg/ml)
1	+1	+1	+1	+1	+1	+1	+1	+1	-1	-1	-1	-1	-1	1-	19.29
2	+1	+1	+1	+1	+1	+1	+1	-1	-1	-1	-1	-1	-1	1-	12.55
3	+1	+1	+1	+1	+1	+1	-1	-1	-1	-1	-1	-1	-1	1+	26.97
4	+1	+1	+1	+1	+1	-1	-1	-1	-1	-1	-1	-1	+1	1+	19.18
5	+1	+1	+1	+1	-1	-1	-1	-1	-1	-1	-1	+1	+1	1 +	9.05
6	+1	+1	+1	-1	-1	-1	-1	-1	-1	-1	+1	+1	+1	1 +	4.95
7	+1	+1	-1	-1	-1	-1	-1	-1	-1	+1	+1	+1	+1	1+	20.03
8	+1	-1	-1	-1	-1	-1	-1	-1	+1	+1	+1	+1	+1	1+	9.5
9	-1	-1	-1	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	1 +	6.2
10	-1	-1	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	1+	1.37
11	-1	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	1-	0.85
12	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	1-	2.39
13	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	-1	1-	5.12
14	-1	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	-1	-1	1-	2.48
15	-1	+1	+1	+1	+1	+1	+1	+1	+1	-1	-1	-1	-1	1-	1.93
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1-	0

Plackett–Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta i x i \quad (1)$$

where Y is the response (pyocyanin concentration), β_0 is the model intercept and βi is the linear coefficient, and xi is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. The maximum number of variables that can be evaluated in one design is equal to the number of individual experiments minus one. In the present work, fourteen variables were screened in sixteen experiments. All experiments were carried out in triplicate and the mean of pyocyanin concentration was taken as a response (Table 2). The variables whose confidence levels were higher than 90% were considered to significantly influence pyocyanin concentration.

Box–Behnken design

In order to describe the nature of the response surface in the experimental region, a Box–Behnken design [**16**] was applied. As presented in Table 3, factors of highest confidence levels were prescribed into three levels, coded -1, 0, and +1 for low, middle and high concentrations (or values), respectively. Table 4 represents the design matrix of a 14 trials experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response (pyocyanin concentration). For the three factors this equation is:

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

where Y is the predicted response, β_0 model constant; X_1, X_2 and X_3 independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross product coefficients and β_{11}, β_{22} and β_{33} are the quadratic coefficients. Microsoft Excel 2007 was used for the regression analysis of the experimental data obtained.

Table 3. The levels of variables chosen for the Box–Behnken optimization experiment.

Variable	Variable code	-1	0	+1
pH	X1	8	8.5	9
KCl (g/l)	X2	0.5	1	1.5
$MgCl_2.6H_2O$ (g/l)	X3	3.5	4	4.5

Table 4. Box–Behnken factorial experimental design, representing the response of pyocyanin concentration as influenced by pH, KCl and $MgCl_2.6H_2O$.

Trial	pH	KCl	$MgCl_2.6H_2O$	Pyocyanin concentration (µg/ml)	
				Measured	Predicted
1	-1	-1	0	195.4	197.9
2	+1	-1	0	181.3	184.95
3	-1	+1	0	240.25	236.6
4	+1	+1	0	189	186.5
5	-1	0	-1	210.8	205.4
6	+1	0	-1	193.1	186.5
7	-1	0	+1	224.6	231.2
8	+1	0	+1	181.6	187
9	0	-1	-1	189.5	192.4
10	0	+1	-1	194.9	204
11	0	-1	+1	206.1	197
12	0	+1	+1	228.7	225.8
13	0	0	0	236.4	236.2
14	0	0	0	236	236.2

The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Experiments were performed in triplicate and mean values are given.

Statistical analysis of data

Data of pyocyanin concentration were subjected to multiple linear regressions using Microsoft Excel 2007 to estimate *t* values, *P*-values and confidence levels which is an expression of the *P*-value in percent. The optimal value of pyocyanin concentration was estimated using the solver function of Microsoft Excel tools and JMP program.

Results

Evaluation of factors affecting pyocyanin production

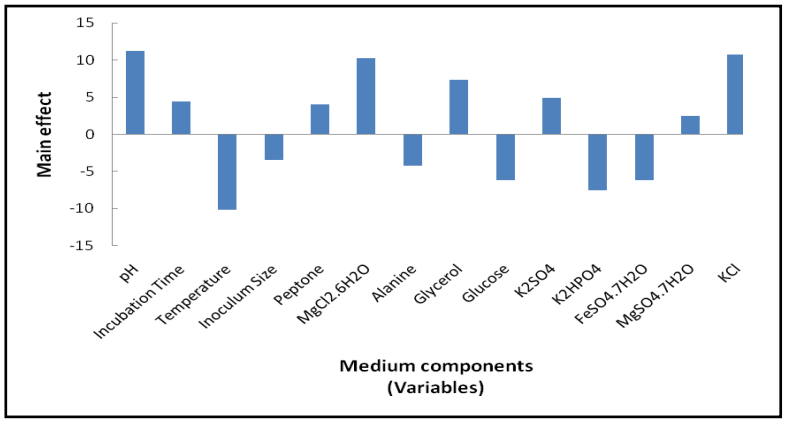

Fig. 1. Effect of environmental factors on pyocyanin production by *Pseudomonas aeruginosa* isolate JY21 in terms of concentration (µg/ml).

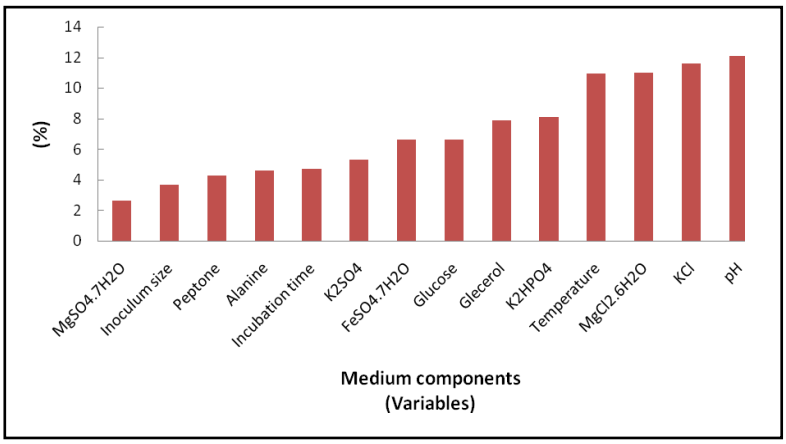

Fig. 2. Pareto chart rationalising the effect of each variable on pyocyanin production by *Pseudomonas aeruginosa* isolate JY21 in terms of concentration (µg/ml).

In the first approach, the Plackett–Burman design was applied to reflect the relative importance of various fermentation factors as described in Table 1. Fourteen different factors (variables) including fermentation conditions and medium constitution were chosen to perform this optimization process. The means of pyocyanin concentration for the different trials are given in µg/ml and shown in Table 2. The main effect of each variable upon pyocyanin concentration was estimated as the difference between both the means of measurements made at the high level (+1) and that of those taken at the low level (-1) of that factor. Data in Table 2 show a wide variation from 0 to 26.97 µg/ml of pyocyanin concentration. The main effects of the examined factors on the pyocyanin concentration were calculated and presented graphically in Fig. 1. Analysis of the regression coefficients of the 14 variables, namely, pH, incubation time, peptone, $MgCl_2.6H_2O$, glycerol, K_2SO_4, $MgSO4.7H_2O$ and KCl had shown positive effect on pyocyanin production. However temperature, inoculum size, alanine, glucose, K_2HPO_4 and $FeSO_4.7H_2O$ were found to contribute negatively. Fig. 2 shows the ranking of factor estimates in a Pareto chart.

The polynomial model describing the correlation between the 14 factors and the pyocyanin concentration could be presented as follows:

$$Y_{activity} = 8.86625 + 5.601X_1 + 2.186X_2 - 5.078X_3 - 1.708X_4 + 1.991X_5 + 5.116X_6 - 2.124X_7 + 3.660X_8 - 3.078X_9 + 2.461X_{10} - 3.758X_{11} - 3.073X_{12} + 1.221X_{13} + 5.376X_{14}$$

on the basis of the calculated *t*-values and confidence level (%) (Table 5), pH, KCl, $MgCl_2.6H_2O$ and glycerol which have confidence level > 90% were found to be the most significant variables affecting pyocyanin concentration. The analysis of variance using ANOVA test was generated and summarized in Table 6 which gives $P = 0.069$. This indicates that there is a statistically significant relationship between the variables at 95% confidence level. The *R*-squared statistic indicates that the model as fitted explains 0.999 of the variability in pyocyanin concentration. The adjusted *R*-squared statistic is 0.991.

Other variables with less significant effect were not included in the next optimization experiment, but instead were used in all trials at their (-1) level and (+1) level, for the negatively contributing variables and the positively contributing variables, respectively.

According to these results, a medium of the following composition is expected to be near optimum: Peptone, 20 g/l; $MgCl_2.6H_2O$, 4 g/l ; glycerol, 20 ml; K_2SO_4, 10 g/l; $MgSO4.7H_2O$, 1 g/l; KCl, 1 g/l; pH, 8; incubation time, 72h; temperature, 30°C and inoculum size 2%. The pyocyanin concentration achieved on this medium was 217.5 µg/ml.

Optimization of the culture conditions by Box–Behnken design

In order to approach the optimum response region of the pyocyanin production in terms of concentration (µg/ml), significant independent variables (pH, X1; KCl, X2; and $MgCl_2.6H_2O$, X3) were further explored, each at three levels. Table 4 depicts the design matrix of the coded variables together with the experimental results of pyocyanin concentration. All cultures were performed in triplicate and the mean of observations was used.

Table 5. Statistical analysis of Plackett–Burman design showing coefficient values, *t*- and *P*-values for each variable on pyocyanin production.

Variable	Coefficient	*t*-statistic	*P*-value	Confidence level (%)
Intercept	8.86625			
PH	5.601429	11.1242772	0.057074561	94.29254394
Incubation time (h)	2.186429	4.3421847	0.144100269	85.5899731
Temperature (°C)	-5.07857	-10.085898	0.062914173	93.70858274
Inoculum size (%)	-1.70857	-3.3931741	0.182452626	81.75473745
Peptone (g/l)	1.991429	3.95492027	0.157664511	84.23354894
$MgCl_2.6H_2O$ (g/l)	5.116429	10.161081	0.062451654	93.7548346
D-L.Alanin (g/l)	-2.12429	-4.6214318	0.13566229	86.43377096
Glycerol (ml)	3.660714	7.96396708	0.079521332	92.04786678
Glucose (g/l)	-3.07857	-6.1139549	0.103211792	89.67882078
K_2SO_4(g/l)	2.461429	4.88832685	0.128460268	87.15397317
K_2HPO_4(g/l)	-3.75857	-7.4644155	0.084782479	91.52175212
$FeSO_4.7H_2O$ (mM)	-3.07357	-6.1040251	0.103376761	89.66232394
$MgSO4.7H_2O$ (g/l)	1.221429	2.42572226	0.248931384	75.10686161
KCl (g/l)	5.376429	10.6774336	0.059449508	94.05504924

Table 6. ANOVA test of Plackett-Burman experiments.

Analysis of variance					
Source	**Degree of freedom**	**Sum of squares**	**Mean square**	**F-ratio**	***P*-value**
Regression	14	1047.199171	74.7999408	126.4359183	0.069605
Residual	1	0.591603571	0.59160357		
Total	15	1047.790775			

R-squared = 0.999435, Adjusted *R*-squared = 0.991531

Presenting experimental results in the form of surface plots, Fig. 3a show that lower levels of pH support high pyocyanin concentration levels. On the other hand, higher levels of pyocyanin concentration were attained with increasing the concentration of KCl and $MgCl_2.6H_2O$ in the medium (Fig. 3b and Fig. 3c). For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the experimental results (linear optimization algorithm) of pyocyanin concentration (Table 7):

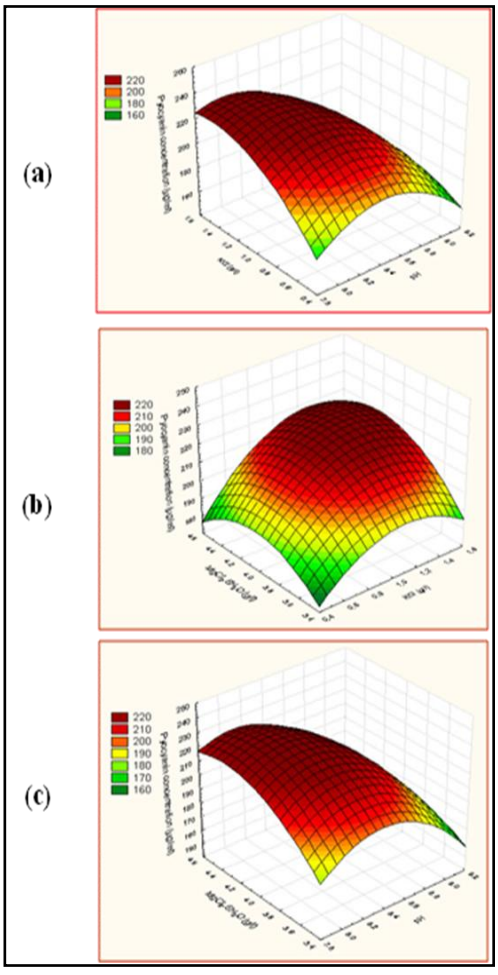

Fig. 3. Three dimensional response surface representing pyocyanin production by *Pseudomonas aeruginosa* isolate JY21 in terms of concentration (µg/ml) as affected by culture conditions.

$$Y_{activity} = 236.2 - 15.756X_1 + 10.068X_2 + 6.587X_3 - 9.28X_1X_2 - 6.325X_1X_3 + 4.3X_2X_3 - 18.493X_1^2 - 16.218X_2^2 - 15.181X_3^3$$

where, X_1, X_2, and X_3 are culture pH, KCl, and $MgCl_2.6H_2O$, respectively. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficient *R* and the

determination coefficient R^2. The closer the value of R is to 1, the better is the correlation between the measured and the predicted values. In this experiment, the value of R was 0.968 for pyocyanin concentration. This value indicates a high degree of correlation between the experimental and the predicted values. The value of determination coefficient R^2 = 0.938 for pyocyanin concentration, being a measure of fit of the model, indicates that about 6.2% of the total variations are not explained by pyocyanin concentration.

Analysis of variance using ANOVA test in Box-Behnken experiment was generated and summarized in Table 8 at P = 0.039. Since the P-value indicated in the ANOVA table is less than 0.05, it is concluded that there is a statistically significant relationship among the studied variables at the 95% confidence level (P = 0.05).

Table 7. Statistical analysis of Box-Behnken design showing coefficient value, *t*-statistic and *P*-value for each variable on pyocyanin production in terms of concentration (µg/ml) concentration.

Variable	Coefficient	*t*-statistic	*P*-value	Confidence level (%)
Intercept	236.2			
x1	-15.7563	-4.65055	0.009657474	99.03425265
x2	10.06875	2.971852	0.041068863	95.89311368
x3	6.5875	1.94434	0.123758817	87.62411832
x1x2	-9.2875	-1.93836	0.124611341	87.53886594
x1x3	-6.325	-1.32007	0.257285514	74.27144857
x2x3	4.3	0.897439	0.420218179	57.97818212
x1x1	-18.4938	-3.45228	0.026000166	97.39998341
x2x2	-16.2188	-3.0276	0.038871869	96.1128131
x3x3	-15.1813	-2.83393	0.047156754	95.28432458

Table 8. ANOVA test of Box-Behnken experiments.

Analysis of variance					
Source	**Degree of freedom**	**Sum of squares**	**Mean square**	**F-ratio**	***P*-value**
Regression	9	5642.929	626.9921	6.827713303	0.039999506
Residual	4	367.3219	91.83047		
Total	13	6010.251			

R-squared = 0.938884
Adjusted *R*-squared = 0.80137

The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the Solver function of Microsoft Excel tools and JMP program, and found to be: pH 8.2, KCl, 1.25 g/l and $MgCl_2.6H_2O$, 4.2 g/l with a predicted pyocyanin concentration of 245.39 µg/ml (Fig. 4).

Based on the results obtained from the Plackett–Burman and Box-Behnken designs the following medium composition is expected to be near the optimum: Peptone, 20 g/l; $MgCl_2.6H_2O$, 4.2 g/l ; glycerol, 20 ml; K_2SO_4, 10 g/l; $MgSO_4.7H_2O$, 1 g/l; KCl, 1.25 g/l; pH, 8.2; incubation time, 72h; temperature, 30°C and inoculums size 2%. The pyocyanin concentration achieved on this medium was 311.1µg/ml.

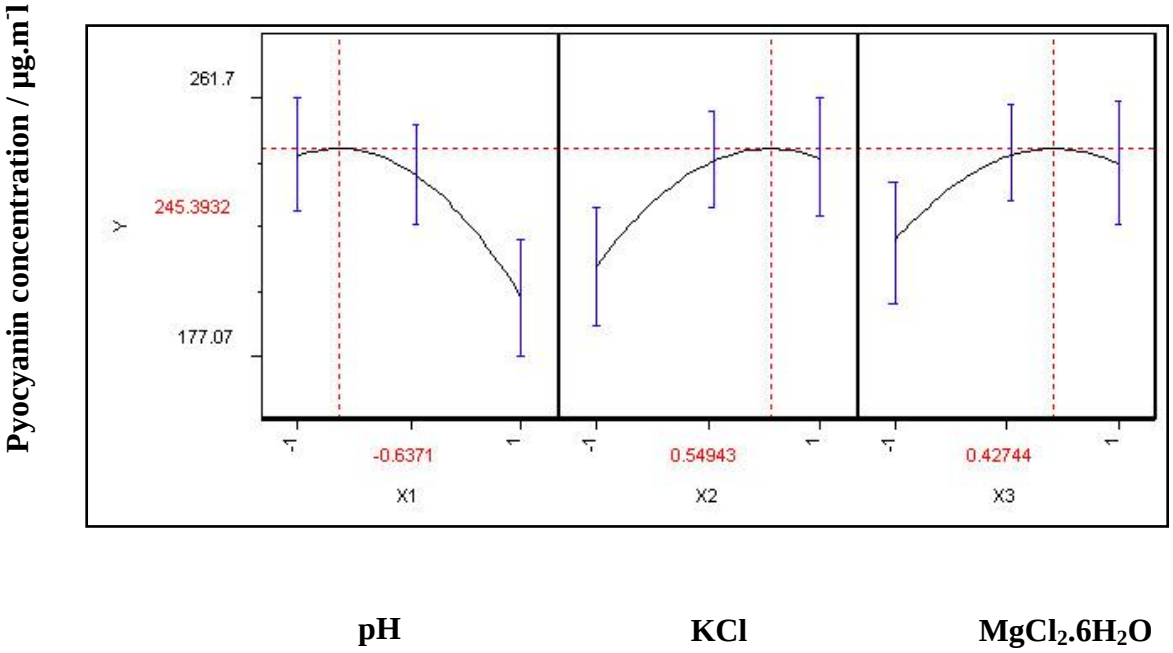

Fig. 4. JMP profile showing the predicted optimal levels of three components, X1, pH; X2, KCl and X3, $MgCl_2.6H_2O$ along with the predicted pyocyanin concentration.

Discussion

Based on this fact, a sequential optimization strategy was implemented in order to improve pyocyanin production by *P. aeruginosa* isolate JY21 through three steps. The first step involves screening experiment by applying Plackett-Burman fractional factorial design to address the most significant factors affecting production among the studied variables. In screening the factors affecting production of certain secondary metabolite, it is very important to test as mush factors as possible and to identify the significance of each of them. Plackett-Burman design offers a good, fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component. Although, otherwise, interaction is not included in this design, it is not of first priority in the screening program to examine the interaction between these large numbers of variables. Out of these, only the most effective factors with positive significance were selected for further optimization and those which showed high negative effect on the bioprocess were dropped in all subsequent experiments. This indicates the effectiveness of the Plackett-Burman design as a tool for elucidating the most important variables affecting the response when more than five factors are under investigation [**12**].

The wide variation in pyocyanin concentration reported here as from 0 to 26.97 µg/ml using Plackett-Burman design reflects the importance of medium optimization to attain higher productivity. According to analysis of the regression coefficient, t-test and *P*-value, pH, KCl, $MgCl_2.6H_2O$ and glycerol, out of fourteen variables tested, were found to be the most significant variables affecting pyocyanin concentration in the medium with a *P*-value of 0.057, 0.059, 0.062 and 0.079. Showing that pH had a high significant positive effect on pyocyanin production is consistent with the results of [**14**] who reported that pyocyanin production was observable at a pH higher than 7 and when final pH value of the medium became higher than about 9, the cultural solution became viscous and pyocyanin production was halted. Also, KCl, $MgCl_2.6H_2O$ and glycerol showed high significant positive effect on pyocyanin production. Among the tested carbon sources, glycerol was considered to be most favorable for pyocyanin production. On the other hand, the inhibitory effect of glucose as an alternative carbon source on pyocyanin production was previously reported by [**14**] who reported that when glucose (higher than 2%) was substituted for glycerol pyocyanin was not produced at all. However, when it was supplemented with CaCO3, glucose was found to be as good as glycerol as a carbon source for pyocyanin production.

The medium of the following composition Peptone, 20 g/l; $MgCl_2.6H_2O$, 4 g/l ; glycerol, 20 ml; K_2SO_4, 10 g/l; $MgSO4.7H_2O$, 1 g/l; KCl, 1 g/l was found to be the most potent medium for pyocyanin production giving 217.5 µg/ml, 72 h post-inoculation, 30 ^{0}C, pH 8 and inoculum size of 2%.

The second step in sequential optimization strategy aimed at finding the optimum levels of the most significant variables identified in the first step through non-linear optimization algorithm and the application of response surface methodology. Response surface methodology is one of the most widely applied statistical methods for optimization of enzyme production and reported in many studies [**17, 5**]. Applying Box–Behnken design to optimize the selected factors for maximal production is an efficient method that tests the effect of factors interaction. Besides, it converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. Thses experimental designs are recommended to the microbial industry sponsors for maintaining high efficiency and profitable bioprocesses [**5**]. In order to approach the optimum response region of pyocyanin production, significant independent variables (pH, KCl and $MgCl_2.6H_2O$) were further studied, each at three levels: -1, 0 and +1. Three dimensional response surfaces plots of these variables showed that lower levels of pH support high pyocyanin concentration levels. Attaining higher levels of pyocyanin concentration with increasing the concentration of KCl and $MgCl_2.6H_2O$ in the medium indicates that these two factors are limiting. The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the Solver function of Microsoft Excel tools and JMP program, and found to be: pH 8.2; KCl, 1.25 g/l and $MgCl_2.6H_2O$, 4.2 g/l with a predicted pyocyanin concentration of 245.39 µg/ml.

When optimal conditions realized from the optimization experiment were experimentally verified and compared to the predicted optimum of model, the experimental pyocyanin concentration was 311.1 µg/ml while the predicted value from the polynomial model was 245.39 µg/ml.

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