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OPTIMIZATION OF CULTURE VARIABLES FOR THE PRODUCTION OF CYTOTOXIC YELLOW PIGMENT USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In our study a Central Composite Rotatable Design (CCRD) combined with Response Surface Methodology (RSM) was employed for determining the optimum conditions for the production of a cytotoxic yellow pigment from *Streptomyces griseoaurantiacus* JUACT 01**.** *S. griseoaurantiacus* JUACT 01was cultured using solid state fermentation and pigment was extracted with ethyl acetate. A total of 20 sets of experiments were developed to obtain the second order polynomial equations which was used to predict the yellow pigment yield in terms of independent variables namely pH, temperature and incubation time. The optimal conditions for pigment production were found to be with a pH of 4.0, temperature of 15˚C and time duration of 7 days. The amount of pigment produced was found to be 1.3 fold greater than pigment produced by non optimized media**.**

Keywords: Anticancer, CCRD, RSM, *Streptomyces griseoaurantiacus* JUACT 01, Yellow Pigment

INTRODUCTION

A number of bacterial species produce vast variety of pigments that are important for cellular physiology and survival. Many of these pigments were found to be having bioactive potentials **[1]**. In particular, Actinomycetes are the producers of a large number of natural products with antibiotic, anticancer and immunosuppressive

properties. Due to such diverse and promising activities, the pigments can play a vital role in pharmaceutical research. Pigment production in microorganisms is greatly affected by several factors like pH, temperature, incubation time and other media components such as carbon source, nitrogen source etc. **[2]**. Every pigment producing organism or strain has its own unique requirement for these factors for maximum pigment production.

Optimization of physicochemical parameters and the effect of their interaction can be effectively performed with the help of statistical tools like Response Surface Methodology and Central Composite Rotatable Design in various biotechnological and industrial processes **[3]**. A well designed study is often more important than the actual analysis. Because no statistical analysis can use a poorly designed experiment. RSM has enjoyed immense development and wide-ranging applications since the seminal papers of BOX and Wilson **[4]** and Box and Draper **[5]**. A vital component of RSM is the fitting of an appropriate empirical model, usually a first/second order polynomial model to observed responses which depend on several explanatory variables. As the pigment isolated from *Streptomyces griseoaurantiacus* JUACT 01was found to possess promising anti cancer activities (unpublished results. Data is not shown), we have made an attempt to statistically optimize the media conditions in order to obtain maximum pigment yield. Hence, to examine the nature of the Response Surface in the vicinity of the optimum operating conditions, in our research we used second order polynomial to observe the pigment yield with explanatory variables pH, temperature and incubation time at three levels. There is an enormous array of flexible and robust designs for such response surface modeling and which are immediately available for the practitioners like orthogonal designs for the first order polynomial models, Box-Behbken, Small Composite and Central Composite Rotatable Design for the second order polynomials **[6]**.

MATERIALS AND METHODS

Isolation and identification of the pigment producing organism

During the systematic screening for bioactive Actinomycetets a pale yellow pigmented strain, namely *Streptomyces griseoaurantiacus* JUACT 01 was isolated from Lal bagh (12.95˚N, 77.59˚ E) Bangalore, India. The isolation was carried out by serial dilution and spread plate techniques. Serial dilution was performed with sterile distilled water and 10^{-1} to 10^{-5} dilutions were plated on Starch Casein Nitrate Agar (SCNA) plates containing

20mg/L of tetracycline and 50mg/L of fluconezole to inhibit the growth of unwanted bacteria and fungi respectively. The plates were then incubated at 28°C for 15 days to allow sporulation and pigmentation, and were subsequently sub cultured for future use. Morphological characterization was performed based on the specifications given by International Streptomyces project (ISP) **[7]**. The microscopic examination was performed by Gram staining method **[8]**. The observed structures were compared with Bergey's Manual of determinative Bacteriology, 9th edition. For molecular characterization, the 16S r DNA alalysis was performed **[9]** and the data was deposited in GenBank with accession number **KJ774106**.

Pigment production by solid state fermentation

Solid state fermentation was adopted for pigment production as described previously **[10]** with minor variations. *Streptomyces griseoaurantiacus* JUACT 01 strain was allowed to grow and sporulate on SCNA plate for 10 days at 28˚C. The spores were scraped from the plate and inoculated into 25ml of seed medium (ISP2) and incubated at 28°C for 2 days on a shaker incubator at 200rpm. For solid state fermentation 10 g of oat flakes were used as substrate with 5% (v/v) seed. The substrate with 10ml of water was taken in conical flask and sterilized. The seed (5mL) was inoculated and incubated at 28°C for 10 days under static conditions.

Extraction of pigment

The fermented biomass was homogenised using mortar and pestle with 50ml of 70% methanol and centrifuged at 10000 rpm for 20 minutes. The supernatant was filtered and pigment was extracted with equal volumes of ethyl acetate. The mixture was shaken in a rotary shaker overnight and allowed to stand for an hour. The organic phase was carefully separated and dried to obtain the pigment extract. To further, purify the pigment Thin Layer Chromatography (TLC) was performed with methanol: hexane (7:3) as the solvent system **[11]**. Pigment quantity was determined by measuring the optical density of the sample at 410 nm using UV spectrosphotometer as the pigment was found to have maximum absorbance at 410nm.

Experimental design and optimization by response surface methodology

A vital component of Response Surface Methodology (RSM) is fitting of an appropriate empirical model, usually first/second order polynomial model, to observe responses which depend on several explanatory variables in order to examine the nature of the response surface in the vicinity of the optimum operating

conditions. RSM comprises a body of methods for exploring the optimum conditions through experimental methods. In our study, a CCRD was employed for determining the optimum conditions for maximum pigment yield. It is an effective alternative to the factorial design. The CCRD gives almost as much information as a three level factorial, and requires much fewer tests than the full factorial and has been shown sufficient to describe the majority of steady-state process responses. The number of tests required for the CCRD includes 2^k factors with its origin at the center, 2k points fixed axially at a distance, say alpha, from the centre to generate the quadratic terms, and replicate tests at the centre where k is the number of variables. The axial points are chosen such that they allow rotatability which ensures that the variance of the model precision is constant at all points equidistant from the design centre. Replicates of the test at the centre are very important as they provide an independent estimate of the experimental error. For three variables, the recommended number of tests at the center is six. Hence the total number of tests required for 3 variables is 2^3+2x3+6 = 20

The CCRD is an effective design that is ideal for sequential experimentation and allows a reasonable amount of information for testing the lack of fit while not involving a usually large number of design points. It was first announced by Box and Wilson in 1951, and is well suited for fitting a quadratic surface, which usually works well for the process optimization **[12-15]**. The experimental results were analyzed using Design Expert 8.1, and the regression model was proposed. pH, temperature, incubation time were chosen as three independent variables in the process. Accordingly, the CCRD matrix of 20 experiments covering the full design of five factors were for building quadratic models as shown in **Table 1, [16]**. The experimental data obtained from the CCRD model experiments can be represented in the form of the following equation.

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j + e_i \quad \rightarrow \text{Equation (1)}$$

Where Y is the predicted response, n is the number of factors, $x_i$and x_j are the coded variables, b_0 is the off-set term; $b_i, b_{ii} and, b_{ij}$ are the first order, quadratic ,and interaction effects, respectively; i and j are the index numbers for factors; e_i is the residual error **[17 and 18]**.

The quality of the polynomial model was expressed by the coefficient of determination, name,R^2 and Adj R^2 .The statistical significance was verified with dequate precision ration by F test **[18]** (Rauf *et al*., 2008). According to the obtained experimental data, the levels of the three

main parameters investigated in this study are presented in **Table 1**. For statistical calculations, the variables Xi (the real value of an independent variable) according to the following equation:

$X_i = \frac{(X_a - X_o)}{\partial X}$ → Equation (2)

Where X_0 is the value of X_a at the center point, and ∂X represents the step size **[19 and 20]**.

Central composite rotatable design for optimization of culture variables for pigment production

A central composite design was set up to determine the optimum level of three physiochemical parameters. The effect of pH, temperature, incubation time on the pigment production was analyzed at five experimental levels : -alpha,-1,0,1, alpha, where alpha= $2^{(n/4)}$ where n = no of parameters (here it is 3) and '0' corresponds to the central level. Therefore in our problem we took alpha = 1.682. In order to describe the response surfaces, a five level, three variable central composite design was adopted in this study. The three independent variables and their levels for 20 experiments in the CCRD are shown in **Table 1**.

The design matrix of the variables in both coded and actual units along with the response values are presented in **Table 2** , an empirical second order polynomial model (*) was used to fit the data with three factors.

RESULT AND DISCUSSION

The Actinomycete producing the cytotoxic yellow pigment was identified as *Streptomyces griseoaurantiacus* JUACT 01. The non optimized method of solid state fermentation resulted in 0.9 g/L of the yellow pigment.

RSM was used for obtaining a relationship between factors and the response for optimizing the response. **Table 2** depicts a complete 2^3 factorial design with four center points in cube, and six axial points and two center points in axial. According to the RSM results, polynomial regression modeling was performed on the responses of the corresponding coded values of the three different process variables, and the results were evaluated. The predicted response(Y) for the yellow pigment yield of samples were obtained using Equation (3)

$$\mathbf{Y = 0.6582 - 0.2117\ X_1 - 0.1977\ X_2 - 0.0021\ X_3 - 0.0950\ X_1 \times X_1 - 0.1454\ X_2 \times X_2 - 0.1489\ X_3 \times X_3 + 0.1736\ X_1 \times X_2 - 0.0299\ X_1 \times X_3 + 0.0059\ X_2 \times X_3}$$ → Equation (3)

A statistical approach using a CCRD was used for optimum yield for yellow pigment and for determining the interaction between three independent factors. For response surface methodology involving CCRD, a total of 20 experiments were conducted for

three factors at five levels with three replicates at center point. **Table 1** provides a list of independent variables and coded factor levels. The number of experiments required (N) is given by the expression:

$2^k(2^3 = 8; star\ points) + 2k\ (2x3 = 6\ ;axial\ points{+}6\ center\ pints\ ;6\ replications = 20)$

RSM is appropriate to identify the optimal yield of the yellow pigment. The design used for optimization and observed responses for 20 experiments are given in **Table 2**. In Eq (3), Y is the yellow pigment yield; and X_1, X_2 and X_3 are the corresponding coded variables of pH, temperature and incubation time respectively.

Analysis of variance (ANOVA) values for the quadratic regression model obtained from CCRD employed in the optimization of yellow pigment yield are listed in Table 3. On the basis of the experimental values, statistical testing was carried out using Fisher's test for ANOVA. The statistical significance of the second-order equation revealed that the regression is statistically significant ($P < 0.0001$). **Table 3** depicts the significance of the regression coefficients and ANOVA for the regression model, respectively. The fit of the models were controlled by the coefficient of determination R^2. Based on the ANOVA results, these models report high R^2 value of 95.66%. Also an acceptable agreement with the adjusted determination coefficient is necessary. In this study, the Adj-R^2 value 91.75% was found. The values of R^2 and Adj-R^2 advocate a high correlation between the observed values and the predicted values. This indicates that the regression model provides an excellent explanation of the relationship between the independent variables and the response.

From the Eq(3) , the optimal coded values of pH, temperature and time were estimated - 3.536 ,-2.665 and 0.294 respectively and the corresponding maximum yield 1.297g/L with actual values 4.0 pH, 15˚C at about 7 days, respectively. The three dimensional RSM and contour plots were obtained and the interaction among the three factors was studied and optimum value for each factor which gives maximum pigment production was determined. **Figures 1-3** show the relative effect of two factors when the third factor is kept at the central level. **Figure 1** shows the relative effects of two factors namely, temperature and incubation time in combinations when the pH is kept at central level. It clearly shows a strong degree of 3D surface curvature where the optimum can be easily determined. In **Figure 2**, the 3D surface plot shows the interaction between pH and incubation time when the temperature is kept at the central level. Similarly, in **Figure 3** the interaction

between the temperature and time is plotted with time kept at the central level. All the three plots show higher degree of curvature indicating that the optimum value can easily be determined. **Tanyildizi *et al*., (2005) [21]** showed that the maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. There were reports on elliptical contours being obtained when there is a perfect interaction between the independent variables **[22]**. The statistical design approach using the response surface methodology was used to study the interactive effects of pH, temperature and incubation time on the pigment production by *Streptomyces griseoaurantiacus* JUACT 01.

CONCLUSION

Many physical, environmental and chemical factors have profound effect on pigment production. The conventional approach of optimization of physicochemical parameters by one-at-a time-approach not only is time consuming but also has the limitation of ignoring the effects caused due to the interactions by various factors affecting pigment yield. In the current study we could successfully utilize the statistical tools to optimize the parameters pH, temperature and incubation time for the maximum production of the cytotoxic yellow pigment from *Streptomyces griseoaurantiacus* JUACT 01.

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Table 1: Variables and their levels for CCRD

Independent variable	-α	-1	0	1	α
pH (X_1)	5.85	6.4	7.2	8	8.55
Temperature (X_2)	21.59	25	30	35	38.41
Time(X_3)	6.59	10	15	20	23.41

Table 2: Experimental design predicted and observed pigment yield in CCRD experiments

Trial Number	CODED VALUES			ACTUAL VALUES			Quantity of pigment g/L	
	pH (X_1)	Temperature °C(X_2)	Incubation Time Days(X_3)	pH (X_1)	Temperure° C(X_2)	Incubation Time in Days(X_3)	Observed	Predicted
1	-1	-1	-1	6.4	25	10	0.723	0.822975
2	1	-1	-1	8	25	10	0.143	0.122975
3	-1	1	-1	6.4	35	10	0.002	0.071225
4	1	1	-1	8	35	10	0.012	0.051225
5	-1	-1	1	6.4	25	20	0.816	0.867025
6	1	-1	1	8	25	20	0.012	0.047025
7	-1	1	1	6.4	35	20	0.014	0.138775
8	1	1	1	8	35	20	0.009	-0.001225
9	-1.682	0	0	5.85	30	15	0.913	0.74445322
10	1.682	0	0	8.55	30	15	0.014	0.03801322
11	0	-1.682	0	7.2	21.59	15	0.631	0.5720314
12	0	1.682	0	7.2	38.41	15	0.011	-0.1007686
13	0	0	-1.682	7.2	30	6.59	0.311	0.2391636
14	0	0	1.682	7.2	30	23.41	0.311	0.2320992
15	0	0	0	7.2	30	15	0.654	0.66
16	0	0	0	7.2	30	15	0.654	0.66
17	0	0	0	7.2	30	15	0.654	0.66
18	0	0	0	7.2	30	15	0.654	0.66
19	0	0	0	7.2	30	15	0.654	0.66
20	0	0	0	7.2	30	15	0.654	0.66

Table 3: Analysis of Variance (ANVOA) regression model for yellow pigment yield Response Surface Regression: y – Pigment yield- y versus pH (X_1), Temperature (X_2) and Incubation time (X_3)

Source	Degree of freedom	Sum of squares	Mean Squares	F-Value	P-Value
Model	**9**	**2.03123**	**0.225692**	**24.49**	**0**
Linear	**3**	**1.14572**	**0.381907**	**41.43**	**0**
X_1	**1**	**0.61198**	**0.611979**	**66.4**	**0**
X_2	**1**	**0.53368**	**0.53368**	**57.9**	**0**
X_3	**1**	**0.00006**	**0.000062**	**0.01**	**0.936**
Square	**3**	**0.63693**	**0.21231**	**23.03**	**0**
$X_1 \times X_1$	**1**	**0.13022**	**0.130223**	**14.13**	**0.004**
$X_2 \times X_2$	**1**	**0.30483**	**0.304827**	**33.07**	**0**
$X_3 \times X_3$	**1**	**0.31983**	**0.319827**	**34.7**	**0**
2-Way Interaction	**3**	**0.24858**	**0.08286**	**8.99**	**0.003**
$X_1 \times X_2$	**1**	**0.24117**	**0.241165**	**26.16**	**0**
$X_1 \times X_3$	**1**	**0.00714**	**0.00714**	**0.77**	**0.399**
$X_2 \times X_3$	**1**	**0.00028**	**0.000276**	**0.03**	**0.866**
Error	**10**	**0.09217**	**0.009217**		
Lack-of-Fit	**5**	**0.09217**	**0.018434**	*	*
Pure Error	**5**	**0**	**0**		
Total	**19**	**2.1234**			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0960058	**95.66%**	**91.75%**	**6.75%**

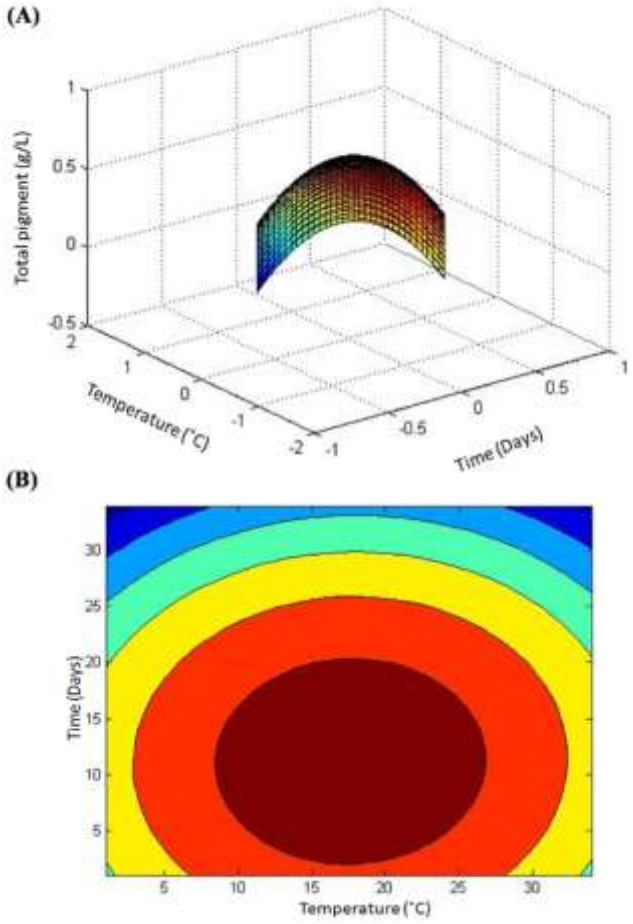

Figure 1: Plots showing the effect of temperature and incubation time on pigment yield at constant pH (A) The response surface plot (B) Contour plot

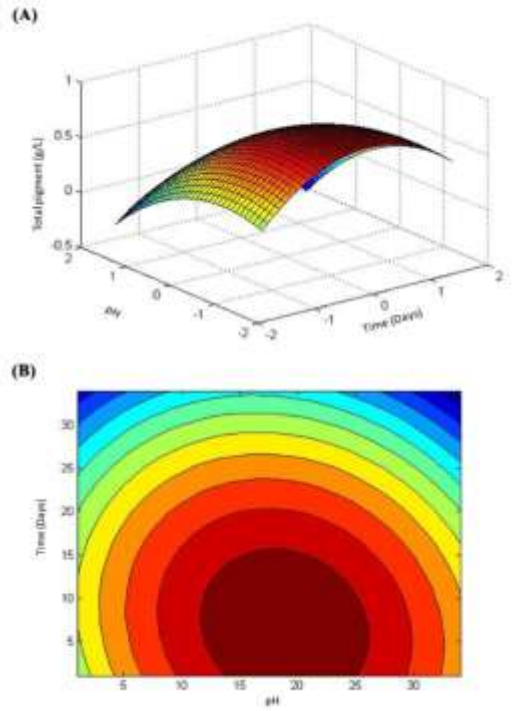

Figure 2: Plots showing the effect of pH and incubation time on pigment yield at constant temperature (A) Response surface plot (B) Contour plot

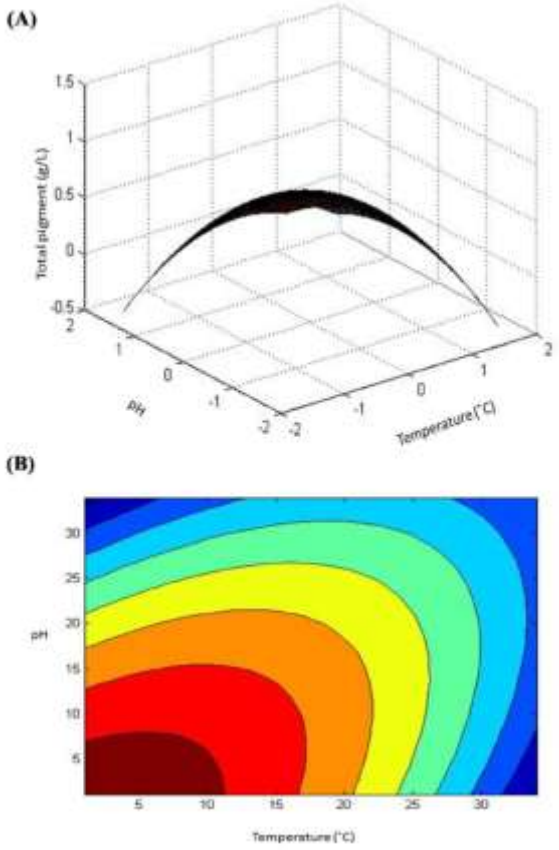

Figure 3: Plots showing the effect of pH and temperature on pigment yield at constant incubation time (A) Response surface plot (B) Contour plot