Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 2159-2166
Received: 09-01-2018
Accepted: 10-02-2018

Megha Shukla
Research Scholar, Centre for Post-Graduate Studies, Jain University, Karnataka, India

Varalakshmi Kilingar Nadumane
Professor, Department of Biotechnology, Jain University, Karnataka, India

Correspondence
Megha Shukla
Research Scholar, Centre for Post-Graduate Studies, Jain University, Karnataka, India

Statistical optimization of culture conditions by response surface methodology for the enhanced production of a cytotoxic pigment from *Pseudomonas stutzeri* JGI 52

Megha Shukla and Varalakshmi Kilingar Nadumane

Abstract
A pigment (PY3) with cytotoxic properties was isolated from a bacterial strain, *Pseudomonas stutzeri* JGI 52. To enhance the production of this promising pigment, various media components and parameters viz., carbon and nitrogen source, mineral components, temperature, pH and incubation time were optimized in the current study. Maximum amount of pigment was produced at temperature 37°C, pH 7.5 and incubation time 72 h. Based on the conventional method of optimization, lactose, beef extract, $MgSO_4$and pH were identified as the most important contributors towards pigment production. To investigate the combined interactive effects of relevant process variables for enhancing pigment production, statistical optimization through Response surface methodology (RSM) and central composite design (CCD) were employed. The optimum culture conditions for maximum pigment yield were 0.52 % lactose, 1.01 % beef extract, 1.003% $MgSO_4$ and 7.5 pH and 37 °C temperature, which resulted in 2.5 fold higher pigment yield than the controls.

Keywords: cytotoxicity, pigment, enhanced production, *Pseudomonas stutzeri*, statistical optimization, RSM

Introduction
Microbial pigments have gained significant research focus due to their diverse application potential in fields like food industry, textile industry and therapeutics. They gained attention in the field of therapeutics as many of these pigments were found to have cytotoxic, immunomodulation, immunosuppressive, anti-inflammatory, antiproliferative and antitumor activities (Bennett and Bently, 2000, Williamson *et al*; 2007, Azuma *et al*; 2000, Han *et al*; 2001, Montaner and Perez-Thomas, 2001) [1, 5]. While screening pigments from novel bacterial isolates, we found that the yellow pigment from one of the bacterial isolate, identified as *Pseudomonas stutzeri* JGI 52, was having high antiproliferative effects on *in vitro* cancer cell lines (unpublished data). As we felt that it is important to increase the production of this promising pigment from the bacterial isolate, in the current study an attempt was made to statistically optimize the media components employing Response surface methodology (RSM). RSM is a combination of statistical and mathematical approaches and it usually involves an experimental design such as Central Composite Design (CCD) that provides a systematic plan for designing experiments, evaluating effect of target factors and standardizing optimum conditions of different variables for obtaining desired and improved response (Rani *et al*; 2011) [6]. This process consists of a second order polynomial equation that is used to describe the test variables and the combined effect of all the test variables in the response (Mayers and Montgomery, 1995) [7]. CCD considerably diminishes the number of experiments and the interaction among all the possible variables can be evaluated with limited number of experiments (Plackett and Burman, 1946) [8]. In the present study, combination of conventional method for initial screening and CCD for evaluating the interactive effect of variables by statistical or mathematical approach was applied to select the optimum media components for obtaining maximum yield of the cytotoxic yellow pigment from *P. stutzeri* JGI 52.

Materials and Methods
Isolation and initial screening of the cytotoxic properties by MTT Assay
Few pigmented colonies of bacteria were isolated from soil samples collected from various locations in and around Bangalore, India. Samples were serially diluted and plated on to nutrient agar for isolating pigmented bacteria.

Pigments from pure cultures were screened on He La (cervical cancer) cell lines for their cytotoxic properties by performing the MTT Assay as per standard protocols (Mosmann, 1983) [9]. The experiments were performed in triplicates and the cell viability was expressed as a percentage of the OD_{540} values of treated cells as comparison to that of the control cells.

Molecular identification of the promising isolate by 16S rDNA sequence analysis

Genomic DNA was isolated from the bacterial isolate using bacterial genomic DNA isolation kit (Chromous Biotech) for identification of the organism using 16S rDNA sequence analysis. Universal 16S rDNA forward 5'-AGAGTTTGATCMTGGCTCAG -3' and reverse5'-TACGGYTACCTTGTTACGACTT -3' primers were used for amplification of the DNA using ABI 3500 XL Genetic Analyzer. The obtained sequence was subjected to NCBI BLAST.

Pigment extraction and quantification

As it was observed that the bacterial isolate was producing higher quantity of the pigment in nutrient agar (NA) plates rather than in nutrient broth (results not shown), we opted to culture the isolate on NA plates and extract the pigment using methanol as the solvent (Sasidharan *et al*; 2013) [10]. The bacterial biomass was scraped from the surface of the NA plates and the pigment was extracted using methanol. The extract was subjected to centrifugation at 8000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to get the dry pigment. The pigment was fractionated by thin layer chromatography (TLC) using different solvent combinations and the fractions separated were collected by preparative TLC. The yellow coloured band which was directly visible under the normal light, was taken as the pure pigment fraction. This was collected, dissolved in methanol and the λmax was determined by subjecting it to spectral scanning (200 to 700 nm) in a UV-VIS spectrophotometer (Schimadzu 1800, Japan). The solvent was evaporated and the pigment dry weight was determined.

Inoculum preparation

To optimize the culture conditions of the bacterial isolate, a standard inoculum size was used. Inoculum was prepared by transferring a loopful of the bacterial culture from stock culture to 100 ml of sterile distilled water. Bacterial cell suspension with an OD of 0.8 (660 nm) was selected as the standard inoculum size, which contained 4.5×10^7cells/ml.

Effect of incubation period, pH and temperature on pigment production

Effects of incubation time, pH and temperature we reanalyzed by culturing the bacteria on nutrient agar plates with different incubation periods (24. 48, 72 and 96 h), different pH (6, 6.5, 7, 7.5 and 8) and at different temperature conditions (Room Temperature [30±2], 37 and 50°C). After incubation, pigment was extracted with methanol and the absorbance was recorded at 452 nm (λ max) using methanol as the blank.

Media composition for optimization of the pigment production

The effect of different carbon sources (dextrose, fructose, lactose, maltose and sucrose) and different nitrogen sources (urea, casein, peptone, beef extract, yeast extract, tyrosine, $NaNO_3$, NH_4Cl and $[NH_4]_2SO_4$)were analyzed by adding these components at 1% concentration to nutrient agar and allowing the bacteria to grow for 72 h. Pigment production was estimated as previously mentioned. The effect of metal ions on pigment production was analysed after adding$FeCl_3$, $CaCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and KH_2PO_4 at three different concentration (0.01%, 0.1% and 1%).Plates were then incubated for 72h at 37°C to analyze the effect of various carbon and nitrogen sources and metal ions on pigment yield. After 72h, pigment yield was estimated as absorbance at 452 nm and was expressed as mg/ml.

Optimization by response surface methodology

Lactose (A), beef extract (B), $MgSO_4$ (C) and pH (D) were further optimized by central composite design(CCD) for enhanced pigment production as dependent response variables by keeping 37°C temperature and 72 h incubation period as constants. Each of the independent variables were analysed at five different levels (-2,-1, 0, +1, +2) with a total of 30 experiments. By default, the higher levels of the factors were coded as +2 and +1 and the lower levels of the factors were coded as -2 and -1. Yield of the pigment corresponding to the combined effect of the above factors was estimated with respect to concentration of lactose, beef extract, $MgSO_4$ (0.5-1.5%) and pH (6.5-8.5). The plans of CCD in coded levels of the four independent variables were indicated in Table 1. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 1: Levels of variables chosen for experimental design

Factors	Symbols	Levels of coded values				
		-2	**-1**	**0**	**+1**	**+2**
Lactose (%)	A	0.5	0.75	1	1.25	1.50
Beef extract (%)	B	0.5	0.75	1	1.25	1.50
$MgSO_4$ (%)	C	0.5	0.75	1	1.25	1.50
pH	D	6.5	7.0	7.5	8.0	8.5

The following second order polynomial equation was adopted to study the effects of variables on the response

$Y= \beta_0 + \beta_1 A+ \beta_2 B +\beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC+ \beta_{14} AD + \beta_{23} BC + \beta_{24} BD+ \beta_{34} CD \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2$

Where Y is the response (yield of the pigment, mg/ml), β_0 is the constant term, β_1, β_2, β_3 and β_4 the coefficients of linear terms and β_{11}, β_{22}, β_{33}, β_{44} are the coefficients of quadratic terms and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are the coefficients of cross product terms. A,B,C and D represent the factors lactose, beef extract, $MgSO_4$ and pH respectively. The experimental design, regression analysis, analysis of variance(ANOVA) were calculated, 3-D and contour plots were drawn by using Design expert software version 9.0.3.1 (Stat-ease, USA).

Results

Initial screening for cytotoxic properties and identification of promising isolate

Bacteria were isolated from different soil sources and from these, four pigmented colonies were chosen for the study. When the pigments from these four isolates were tested for their cytotoxicity to He La cells by MTT assay, pigment from the isolate 4 (IS4) demonstrated promising results (Fig. 1). The yellow pigment extracted from IS4 when treated at a concentration of 10 µg/ml, reduced the viability of He La cells to 37.2% after 72 h. This Isolate 4 was identified by 16S

rDNA sequence analysis as *Pseudomonas stutzeri* JGI 52 andthe sequence was deposited under GenBank with the accession number KM86644.

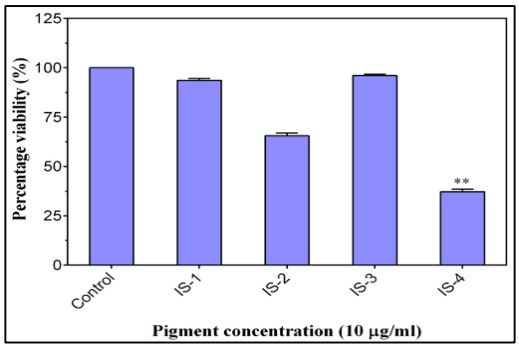

Fig 1: Effect of 10 µg/ml concentrations of pigment extracts from different bacterial isolates on the percentage viability of He La cells when treated for 72 h. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

Characteristic maxima and yield of the pigment

Characteristic maxima (λ-max) of the pigment extract was determined by spectrum scanning (range- 200 to 700 nm) using a UV-VIS spectrophotometer and we found that maximum absorbance was at 452 nm.

Evaluation of physical parameters for obtaining maximum pigment yield

When *P. stutzeri*was incubated at different temperatures (30±2, 37 and 50°C), pigment production was highest at 37°C (1.31 mg/ml) and least at 50°C (Fig. 2). Stankovic *et al*. reported highest biomass and pigment yields (3.92 mg/g of cell dry weight) at 30°C from their isolate *Streptomyces* sp. JS520 (Stankovic *et al*; 2012) [11]. They reported that growth of *Streptomyces* sp. JS520 and undecylprodigiosin production could not be detected at temperatures lower than 10°C and temperatures higher than 42°C. In another study, it wasreported that *Serretiamarcescens* SU-10 produced more prodigiosin at 28°C and the rate was reduced as the temperature was increased (Antony *et al*; 2011) [12]. Williams and Quadri in their study found that prodigiosin was produced at 27°C and when temperature was increased to 38°C, there was no prodigiosin production (Williams and Quadri, 1980) [13]. Based on all these, it can be stated that temperature greatly influences the production of pigments from microorganisms.

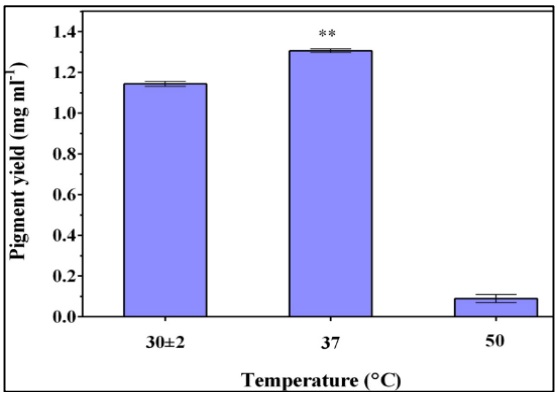

Fig 2: Effect of different temperature on pigment production. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

When *P. stutzeri*was cultured at different pH, maximum pigment production of 1.12 mg/ mlwas observed at pH 7.5 (Fig. 3).There were no remarkable variations in pigment production between pH 6-8. Pigment production was in negligible quantities below pH 6 and above pH 8.According to a report by Stankovic *et al*. pH affects undecylprodigiosin accumulation in Streptomyces sp. JS520 (Stankovic *et al*; 2012) [11].According to their report, when medium pH was 7highest level of undecylprodigiosin production was achieved (65.58 mg/l).They also reported that the productivity of undecylprodigiosin was the highest (4.5 mg/g of cell dry weight) when pH of the medium was 8.

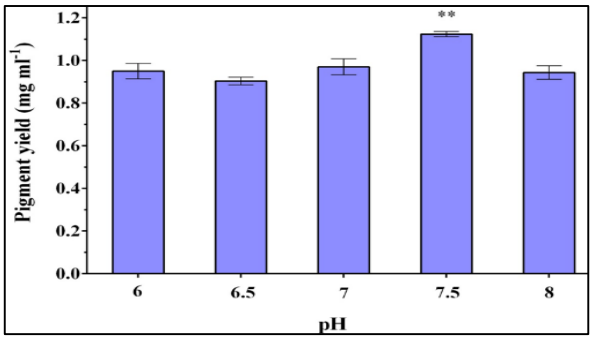

Fig 3: Effect of different pH on pigment production.Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

When pigment production wasanalyzed over a period of time (24, 48, 72 and 96 h), it was found that 72 h of incubation at 37°C resulted in highest pigment production (Fig. 4).As the incubation time increased from 24 to 72 h, pigment yield was found to increase from 0.96 to 2.06 mg/ml. After 72 h there was no significant increase in pigment production.

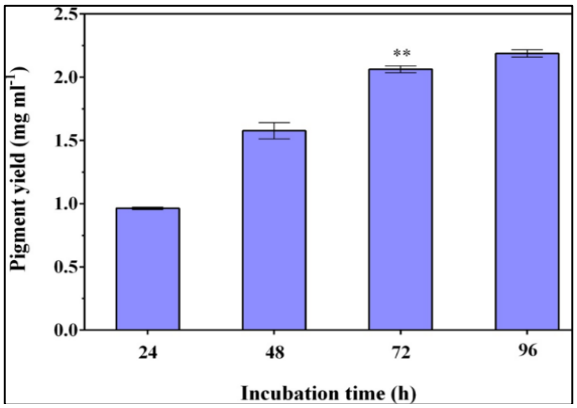

Fig 4: Effect of incubation time on pigment production. Vertical bars indicate SE of the mean for n = 3 *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

Enhanced pigment production by supplementation of media with different carbon sources

When different carbon sources (1%) were screened for their effect on pigment production, maximum pigment production was seen with lactoseaddition (1.72 mg/ml) to the basic media (Fig. 5).Media supplimented with dextrose, fructose, maltose and sucrose (1%) did not alter the production of the pigment. There was a 1.5 fold increase in pigment production in media added with lactose as compared to the control.In a previous study Subhasree *et al.* reported that media supplemented with fructose as a carbon source resulted in higher production of red and yellow pigmentsby *Monascus purpureus* (Subhasree

et al; 2011)[14]. Chang *et al*. reported the maximum production of prodigiosin (3mg/ml) in a media supplemented with dextrose (Chang *et al*; 2000) [15]. In one more study by Sundaramoorthy *et al*., it was reported that *Serratia marcescens* produces more prodigiosin in a medium supplemented with maltose (Sundaramoorthy *et al*; 2009) [16]. Oller reported the repressive effect of glucose and sorbitolon prodigiosin synthesis (Oller, 2005) [17].

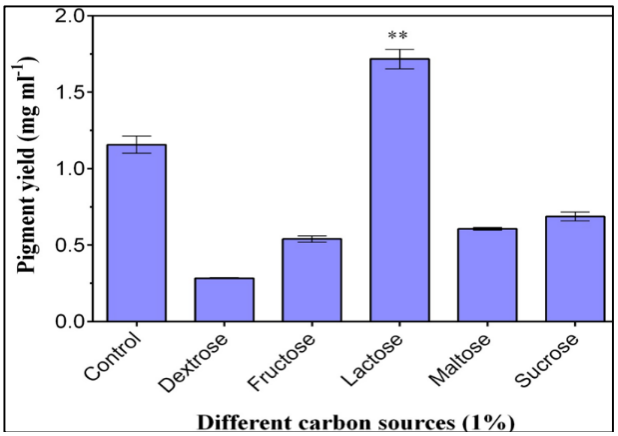

Fig 5: Effect of various carbon sources on pigment production. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

Effect of different nitrogen source on pigment production

When *P. stutzeri* was cultured in a media supplemented with various organic and inorganic nitrogen sources (1%), beef extract was found to be the best source of nitrogen for maximum yield of the pigment (2.13 mg/ml) followed by peptone (Fig. 6). Urea and tyrosine also contributed towards pigment production but not significantly. Media supplemented with casein, yeast extract, tyrosine, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$ (1%) resulted ina reduction of pigment production as compared to the control. In one study, Palanichamy *et al*. reported that sodium caseinate and peptone were suitable substrates for pigment production with sodium casinate providing highest biomass yield of 0.058 gm/ml (Palanichamy *et al*; 2011) [18]. Ammonium chloride was reported as a better inorganic nitrogen source for pigment production as per earlier studies (Chen and Johns, 1993, Juzlova *et al*; 1996) [19, 20].

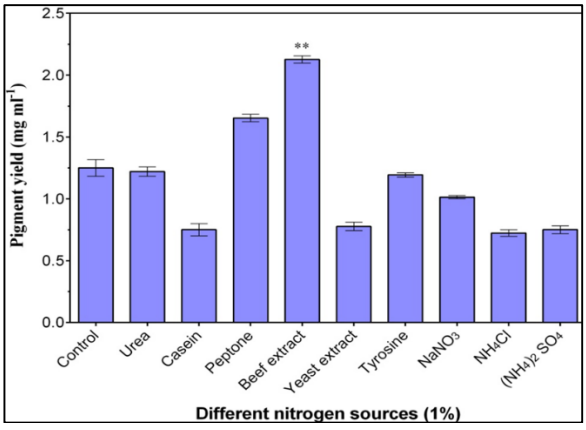

Fig 6: Effect of various nitrogen sources on pigment production. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

Effect of metal ions on pigment production

When effect of different metal ions like $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and KH_2PO_4 at 0.01%, 0.10% and 1% concentrations on the pigment yield was analyzed, it was found that media incorporated with 1% $MgSO_4$resulted in highest pigment production of 1.85 mg/ml (Fig. 7). No pigment production was observed in a media supplemented with 1% $FeCl_3$, 1% $MnCl_2$, 0.1% $ZnSO_4$, 1% $ZnSO_4$ and 1% KH_2PO_4. Earlier in study it was reported that among all the metal salts tested KH_2PO_4 gave the highest biomassyield from *Fusarium moniliforme* KUMBF1201, with the maximum pigment production of 1.524±0.02 nm in PDB medium (Pradeep, 2013) [21].

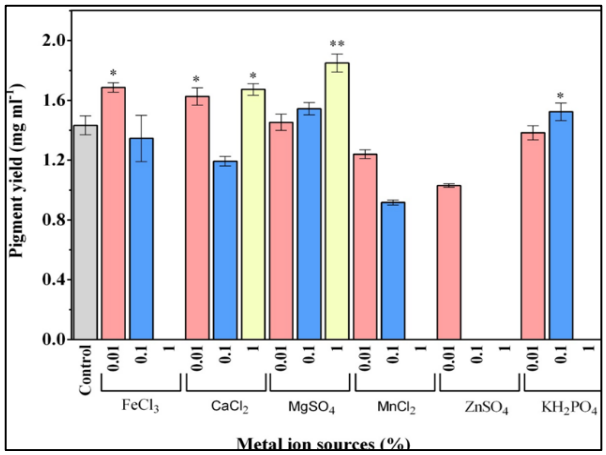

Fig 7: Effect of various metal ion salts on pigment production. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control

Optimization of culture conditions by Central Composite Design (CCD)

The interactive effect of the independent variables, viz., Lactose (A), beef extract (B), $MgSO_4$ (C) and pH (D) on the yield of the cytotoxic pigment from *P. stutzeri* was analyzed by CCD and optimum level of each variables was determined. An experiment with 30 runs was performed according to experimental design with an incubation period of 72 hrs.

The response quadratic equation from the above set of experiments can be written as

$Y= +2.67- 0.29A - 0.061B- 0.043C - 0.13D - 0.045AB – 0.019AC – 0.020AD - 0.055BC + 0.079BD - 0.012CD - 0.075A^2 - 0.16B^2 - 0.27C^2 - 0.43D^2$

Analysis of variance (ANOVA)

Analysis of variance (ANOVA) and t test were performed using a response surface quadratic model (Table 2). The Model F-value of 197.21 implies that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob> F" less than 0.05 indicate that the model terms are significant. The p-value coefficient of A, B, C, D, AB, BC, BD, A^2, B^2, C^2, D^2 were<0.0001, 0.0001, 0.0024, <0.0001, 0.0075, 0.0018, <0.0001, <0.0001, <0.0001, <0.0001 and <0.0001 respectively. In this case A, B, C, D (coefficient of linear terms of lactose, beef extract, $MgSO_4$and pH), A^2, B^2, C^2, D^2 (quadratic terms of lactose, beef extract, $MgSO_4$ and pH) and AB, BC and BD (coefficient of cross product terms of A, B, C, D) are significant model terms and had a significant effect on pigment yield. Values greater than 0.1000 indicate about the model terms being not significant.

Table 2: Analysis of variance for response surface quadratic model obtained from experimental designs

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	9.374	14	0.670	197.212	<0.0001
A	2.053	1	2.053	604.816	<0.0001*
B	0.089	1	0.089	26.161	0.0001*
C	0.045	1	0.045	13.274	0.0024*
D	0.400	1	0.400	117.943	<0.0001*
AB	0.032	1	0.032	9.543	0.0075*
AC	5.625E-03	1	5.625E-03	1.657	0.2175
AD	6.400E-03	1	6.400E-03	1.885	0.1899
BC	0.048	1	0.048	14.256	0.0018*
BD	0.099	1	0.099	29.227	<0.0001*
CD	2.500E-03	1	2.500E-03	0.736	0.4043
A^2	0.154	1	0.154	45.445	< 0.0001*
B^2	0.735	1	0.735	216.634	< 0.0001*
C^2	2.055	1	2.055	605.441	< 0.0001*
D^2	5.160	1	5.160	1519.996	< 0.0001*
Residual	0.051	15	3.395E-03		
Lack of Fit	0.051	10	5.093E-03		
Pure Error	0.000	5	0.000		
Cor Total	9.424	29			

*Significance at p>0.05, R^2=0.9946

Coefficient for all the four factors, i. e, lactose, beef extract, $MgSO_4$ and pH on response is negative, based on the regression analysis of experimental data which indicates that lower values of theseparametes led to higher yield of the pigment. The coefficient of determination (R^2) for pigment yield was calculated as 0.9946. The value of R^2 is always between 0.0 and 1.0, R^2 value close to 1.0 implies that model predicts better response and it is accurate. The yield of the pigment obtained from the designed experiments of the independent variables along with the predicted values are as per Table 3

.

Table 3: Central composite design matrix for the experimental design and predicted pigment yield

Run Order	Lactose (%) (A)	Beef extract (%) (B)	MgSO4 (%) (C)	pH (D)	Pigment Yield (mg/ml)	
					Actual	Predicted
1	-1	-1	-1	-1	2.22	2.18
2	0	0	0	0	2.67	2.67
3	-1	-1	1	1	1.91	1.86
4	1	-1	1	-1	1.76	1.77
5	-1	-1	1	-1	2.31	2.26
6	0	0	2	0	1.45	1.49
7	0	-2	0	0	2.1	2.14
8	2	0	0	0	1.87	1.79
9	1	-1	-1	1	1.31	1.33
10	1	1	-1	-1	1.48	1.50
11	-2	0	0	0	2.81	2.96
12	1	1	1	1	1.11	1.13
13	0	0	0	0	2.67	2.67
14	0	0	0	2	0.65	0.68
15	0	0	0	0	2.67	2.67
16	1	1	-1	1	1.37	1.38
17	-1	1	-1	-1	2.14	2.10
18	0	0	0	-2	1.16	1.19
19	0	0	0	0	2.67	2.67
20	1	-1	-1	-1	1.75	1.76
21	-1	1	-1	1	2.1	2.06
22	0	0	0	0	2.67	2.67
23	-1	1	1	1	1.92	1.88
24	1	1	1	-1	1.28	1.29
25	0	2	0	0	1.87	1.89
26	-1	-1	-1	1	1.87	1.83
27	1	-1	1	1	1.28	1.29
28	0	0	-2	0	1.64	1.66
29	-1	1	1	-1	2.01	1.96
30	0	0	0	0	2.67	2.67

Analysis of response surface

The interactive effects of independent variables on the yield of the pigment were analysed by plotting the 3D response surface curve graph and the interactions between the variables were shown in Fig. 8. As per this analysis, coefficients of the cross product termsie, AB, BC and BD were significant.

Response surface plot of AB (Fig. 8a) shows that decrease in concentration level of lactose resulted in increase in pigment yield whereas a slight variation in concentration of beef extract from control point leads to decrease in pigment yield. According to response plot BC (Fig. 8b) simultaneous variation from control point of beef extract and $MgSO_4$decreases pigment yield. The response surface plot BD (Fig. 8c) demonstrated that beef extract and pH deviation around centre point leads to low pigment yield.

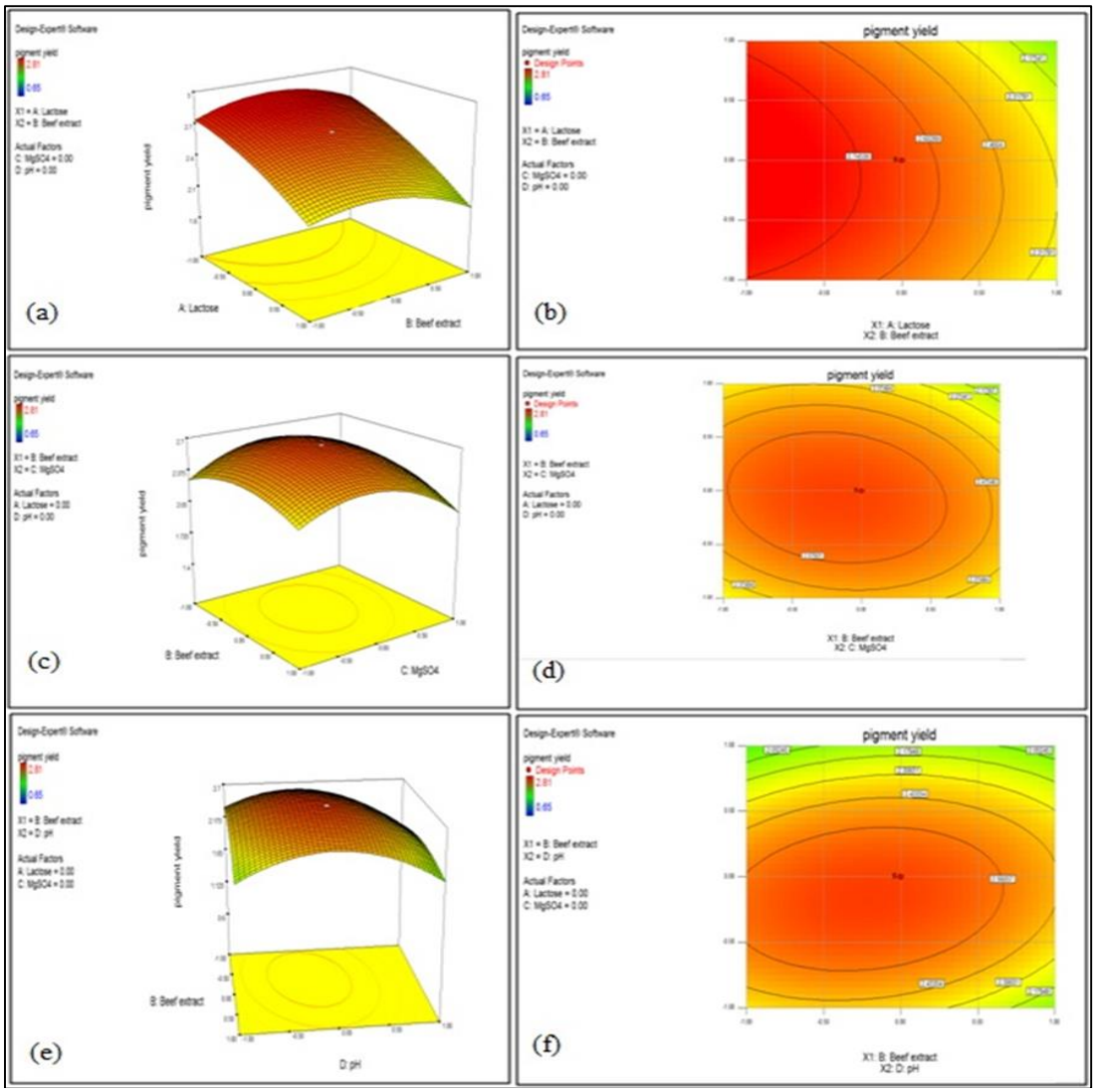

Fig 8: 3D Response surface plots and corresponding contour graph for pigment yield by *P. stutzeri* showing interaction between (a, b) lactose and beef extract (c, d) beef extract and $MgSO_4$ (e, f) $MgSO_4$ and pH

Model verification experiments

To determine the fitness of the model, experiments for obtaining the yield of the pigment were designed using selected optimal conditions. The optimum values of the tested variables were 0.52% lactose, 1.01% beef extract, 1.003% $MgSO_4$and pH 7.5. The maximum experimental response for pigment production was 2.89 mg/ml and this was closer to the predicted value of 2.95 mg/ml (Table 4).

Table 4: Validaton of the model showing pigment yield at optimum level of all parameters

Factors	Optimal Conditions	Predicted Activity	Observed Activity
Lactose (%)	0.52	2.95mg/ml	2.89mg/ml
Beef extract (%)	1.01		
$MgSO_4$ (%)	1.003		
pH	7.5		

Discussion and Conclusion

In our current study, the yellow pigmented bacterial isolate exhibiting highest cytotoxicity to the cervical cancer cell line He La was identified as *Pseudomonas stutzeri* JGI 52 by 16S RNA sequence analysis. The sequence was submitted to GENBANK with the Accession number KM386644.The yellow pigment fraction (PY3) from this isolate was having anticancer significance. Though there are reports of pigments with anticancer potentials from other bacterial species, like *Serratia marcescens*, *Chromobacterium violaceum*, and *Psuedomonas aeruginosa*, till now there are no reports about the yellow pigment from *P. stutzeri* having anticancer activities. As far as our knowledge goes this is the first report of an anticancer yellow pigment from *P. stutzeri*, and therefore its production needs to be optimized. Hence we made an attempt to optimize theproduction of this pigment by varying its culture conditions. It was found that the production

of yellow pigment from *P. stutzeri* was highly dependent on various physical and culture parameters.

During the past decades, Response surface methodology (RSM) has been extensively applied in the optimization of media components and physicochemical parameters for enhancing the yield of economically important microbial compounds. Chen *et al*. (2013) [22] conducted a statistical experimental design for enhancing the production of the pigment prodigiosin from *Serratiamarcescence* C3, and reported an increase in its production from 2.3 g/L to 7.07 g/L when the concentration of two trace compound $FeSO_4.H_2O$ and $MnSO_4.4H_2O$ were optimized using the statistical optimization methodology (Chen *et al*; 2013) [22]. In one more study, there was a report about the increase in production of flexirubin pigment (7.23 folds) by *Chrysebacterium artocarpi* CECT8497 using response surface methodology (Venil *et al*; 2015) [23]. In the present work also we used Response surface methodology and central composite design for screening the significant parameters to determine the optimal conditions for enhancing the pigment yield by developing a process to find out the combined interactive effect and analysis of different variables. The adequacy of the quadratic model was confirmed by ANOVA, and this model had satisfactorily fitted the variation observed in pigment production by *P.stutzeri* with the designed level of physical and chemical parameters. The optimum culture condition for obtaining higher pigment production was determined and the corresponding values of significant variables were found as 0.52% lactose, 1.01% beef extract, 1.003% $MgSO_4$ and 7.55 pH. This resulted in a pigment yield of 2.89 mg/ml (which is 2.5 fold higher than control conditions).At optimum physical and culture parameters, in just 30 runs it was possible to get 2.5 fold increase in pigment yield, which was significant. Thus it can be concluded that this statistical design offers a valid and efficient approach for enhancing pigment production, which in this case is having promising anticancer properties.

Acknowledgments

The financial support by DST-INSPIRE Fellowship is greatly acknowledged by the authors. The authors are grateful to the management of Jain Group of Institutions for the infrastructural facilities provided to carry out the work

References

1. Bennett JW, Bentley R. Seeing red: the story of prodigiosin. Adv. Appl. Microbiol 2000; 47:1-32.
2. Williamson NR, Fineran PC, Gristwood T, Chawrai SR, Leepaer FJ, Salmond GP. Anticancer and immunosuppressive properties of bacterial prodiginines. Future Microbiol 2007; 2: 605-618.
3. Azuma T, Watanabe N, Yagisawa H, Hirata H, Iwamura M, Kobayashi Y. Induction of apoptosis of activated murine splenic T cells by cycloprodigiosin hydrochloride, a novel immunosuppressant. Immunopharmacology 2000; 46:29-37.
4. Han SB, Park SH, Jeon YJ, Kim YK, Kim HM, Yang KH. Prodigiosin blocks T cell activation by inhibiting interleukin-2Rα expression and delays progression of autoimmune diabetes and collagen- induced arthritis. J. Pharmacol. Exp.Ther 2001; 299:415-425.
5. Montaner B, Perez-Thomas R. Prodigiosin induced apoptosis in human colon cancer cells. Life sciences 2001; 68:2025-2036.
6. Rani UM, Rastogi NK, Appaiah KA. Statistical Optimization of Medium Composition for Bacterial Cellulose Production by Gluconacetobacter Hanenii UAC09 using Coffee Cherry Husk Extract-an Agro Industry Waste. J Microb. Biot. 2011; 21:739-745.
7. Mayers RH, Montgomery DC. Response surface methodology: Process and product optimization using designed experiments, A Wiley International Publication. 1995.
8. Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1946; 33:305-325.
9. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. J Immunol. Methods. 1983; 16:55-63.
10. Sasidharan P, Raja R, Karthik C, Ranandkumar S, Indra AP. Isolation and characterization of yellow pigment producing Exiguobacterium sps. J Biochem. Tech. 2013; 4(4):632-635.
11. Stankovic N, Radulovic V, Petkovic M, Vuckovic I, Jadranin M, Vasiljevic B, Runic JN Streptomyces sp. JS520 produces exceptionally high quantities 5 of undecylprodigiosin with antibacterial, antioxidative, 6 and UV-protective properties. Appl. Microbiol. Biotechnol. 2012; 96(5):1217-1231.
12. Antony VS, Chandana K, Senthilkumar P, Narendra KG. Optimization of prodigiosin production by *Serratia marcescens* SU-10 and evaluation of its bioactivity. Int. Res. J Biotechnol. 2011; 2(5):128-133.
13. Williams RP, Quadri SM. The pigments of Serratia. In The Genus Serratia, Edited by A. Von, Graevenitz. S. J. Rubin., Boca Raton, FL: CRC Press Inc. 1980, 31-75.
14. Subhasree RS, Babu PD, Vidyalakshmi R, Mohan VC. Effect of carbon and nitrogen sources on stimulation of pigment production by *Monascus purpureus* on jackfruit seeds.2011 Int. J Microbiol. Res. 2011; 2(2):184-187.
15. Chang S, Sanada M, Johdo O, Ohta S, Nagamatsu Y, Yoshimoto. A High production of prodigiosin by *Serratiamarcescens* grown on ethanol. Biotechnol. Lett. 2000; 22(22):1761-1765.
16. Sundaramoorthy N, Yogesh P, Dhandapani R. Production of prodigiosin from *Serratiamarcescens* isolated from soil. Indian J Sci. Technol. 2009; 2(10):32-34.
17. Oller AR. Media effects of sugars on pigmentation and antibiotic susceptibility in *Serratiamarcescens*. Sci. &Technol. Transac. Of Missouri Acad. Sci. 2005; 2:243-246.
18. Palanichamy V, Hundet A, Mitra B, Reddy N. Optimization of cultivation parameters for growth and pigment production by *Streptomyces spp.* isolated from marine sediment and rhizospere soil. Int. J. of Pl. An and Env. Sci. 2011; 1:158-170.
19. Chen MH, Johns MR. The effect of pH in nitrogen source on pigment production by *Monascuspurpureus*. Appl. Microbiolo. Biotechnol. 1993; 40:132-138.
20. Juzlova P, Martinkova L, Kren V. Secondary metabolites of the fungus monascus: a review. J.Ind.Microbiol. 1996; 16:163-167.
21. Pradeep FS, Pradeep BV. Optimization of pigment and biomass production from *Fusariummoniliforme*under submerged fermentation conditions. Int. J. Pharm. Pharm. Sci. 2013; 5(3):526-535.
22. Chen WC, Yu WJ, Chang CC, Chang JS, Huang SH, Chang CH. Enhancing production of prodigiosin from

Serratiamarcescens C3 by statistical experimental design and porous carrier addition strategy.Biochem. Eng. J. 2013; 8:93-100.
23. Venil CK, Zakaria ZA, Ahmad WA. Optimization of culture conditions for flexirubin production by *Chryseobacteriumartocarpi* CECT 8497 using response surface methodology.Acta. Biochim.Pol. 2015; 62(2):185-90.