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ORIGINAL ARTICLE

Statistical Modelling and Optimization of Culture Condition for Enhanced Pigment Production by Streptomyces Sp. JUA14 with Anticancer Activity

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ABSTRACT
Microbial pigments possess substantial applications in nutraceutical, pharmaceutical and food industry.Actinobacteria have been proved to be potential sources of bioactive compounds/pigments with therapeutic significance. The current study was aimed to optimise the culture condition by employing statistical designs to enhance the growth and bioactive pigment production from a newly isolated Streptomyces sp. with potential anticancer activity. Plackett-Burman (PB) Design for screening of the medium constituents was employed and optimization was performed by constructing a quadratic polynomial response surface model using a central composite design and the optimal values of the significant factors for enhanced growth and pigment yield were determined. The pigment was tested for its anticancer activity against cancer cell lines by MTT assay.Based on the response surface methodology, it could be concluded that dextrose (6.84 g/L), glycerol (12.68 g/L), egg albumin (4.91 g/L) and temperature (29°C) were required for the maximum production of pigment (850 mg/L) by the Streptomyces sp. Optimization of the medium with the above tested features showed a significant increase in the pigment yield. The study optimized the factors that influenced growth and pigment yield using RSM from the Streptomyces sp.JUA14. It is evident that the use of statistical process condition optimization approach, response surface methodology has helped to identify the most significant conditions to enhance the cell mass and pigment yield that could be used in cancer therapy.
Keywords: *Statistical optimization, Streptomyces sp.JUA14, Plackett-Burman design, Central composite design*

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INTRODUCTION

Bacterial pigments are gaining increased scientific attention owing to their tremendous applications in food, textile, pharmaceuticals, cosmetics, nutraceuticals and medicinal field. Microorganisms produce various pigments like carotenoids, alkaloids, melanins, flavins, quinones etc. Actinobacteria have been proved to be potential sources of bioactive compounds and earlier reports show that these microorganisms are the richest source of secondary metabolites [1]. Since the discovery of actinomycin, these bacteria have known to produce wide range of natural pigments and commercially important bioactive compounds[2]. They hold a significant locus as targets in screening programs due to their diversity andconfirmed ability to produce novel metabolites and other molecules of pharmaceutical importance.

Cancer is the second leading cause of death worldwide after cardiac diseases and the current conventional therapy lacks efficacy, impelling researchers to investigate the natural compounds as an effective drug for the treatment of cancer. In this reverence, actinobacteria are biotechnologically and pharmaceutically most valuable prokaryotes. They are responsible for the production of about 50% of the discovered bioactive secondary metabolites, remarkably antibiotics, anticancer compounds and enzymes[3].A single actinobacterial genus Streptomyces have served as a source for the vast majority of

these bioactive compounds. A wide range of pigmented cytotoxic compounds with high activity and efficacy are derived from marine Streptomyces[4-6].

It is essential to improve the performance of the system and thus increase the yield in order to meet the rising demands in the pharmaceutical industry. The media composition influences the growth and production of bioactive metabolite in an organism; thus, optimizing the media components and cultural conditions is the prime task in a biological process [7].The conventional practice that involves optimizing one variable individually are time consuming and also does not evaluate the combined effects of all the factors in the process. These restrictions can be overcome by the use of Response Surface Methodology (RSM), an empirical statistical tool that can identify and quantify the various interactions between different parameters[8].RSM is a potent statistical tool used in constructing a mathematical model that perfectly describes the overall process and has been successfully applied to optimize the culture parameters by evaluating the interactive effect of process variables[9].

In the current study, Plackett-Burman design was applied in the first step of optimization to determine the likely effects of medium components on growth of a newly isolated Actinobacteria, identified as *Streptomyces* sp. JUA14, showed to produce a pigmented bioactive compound with anticancer activity. The Plackett-Burman statistical method is a design in which'n' variables are studied in 'n+1' experimental runs. These experimental designs are available in multiples of four and serve as excellent screening methods. Moreover, the design is orthogonal in nature, inferring that the effect of each variable worked out is pure in nature and not confounded with interaction among variables.

The second step involved the optimization of factors having significant effects using the central composite design to fit a second-order polynomial by a least square's technique. An equation is used to describe the test variables and describe the combined effect of all the test variables in the response and the response of each variable was recognized by the regression analysis. Based on the regression analysis, media component was optimized for the large-scale production of the biomass and pigment[10]. This study reports the growth enhancement and production of high levels of an anticancer pigment by *Streptomyces* sp. JUA14

MATERIAL AND METHODS

Actinobacterial Strain maintenance

The organism used in the study was isolated from the soil sample from Madikeri, Karnataka, Indiaon starch casein media by serial dilution method and pure cultures of the organism was maintained on ISP2 agar slantsat 4° C [13]. The isolate was subjected to morphological, cultural and molecular methods (16s rRNA sequencing) for identification.

Culture Conditions

Pure cultures of Streptomyces sp. were maintained on ISP2 media. A plate full of the lawn culture was scraped and inoculated into 100ml of ISP2 broth in 250ml Erlenmeyer flask and incubated at 30ºC and 120rpm agitation speed for a period of 5 days. Optimization studieswere carried out in 250 ml conical flasks each containing 100 ml of production medium as per the experimental design. Five percent of seed inoculum was added to the sterile media and incubated at 120rpm in a temperature controlled rotary shaker for 15 days. After incubation the cells were harvested by centrifugation at 10000rpm for 10 mins. The growth of the Actinomycetes was estimated by determining the dry weight of the cell biomass.

Pigment Extraction and Estimation

The cell pellet obtained was washed with deionised water, followed by centrifugation at 10000rpm for 10mins to recover the cells by decanting the supernatant. The recovered cells were extracted using butanol. The sample was vortexed for 10 mins in order to completely bleach the cells and no residual pigment was intact with the cells. The suspension was then centrifuged again and the supernatant containing the pigment was evaporated under reduced pressure in a rotary evaporator and resuspended in methanol and subjected to TLC.

The wavelength maximum (λmax) of the TLC purified pigment was determined using UV/Vis spectrophotometer (Shimadzu, Japan) by plotting wavelength versus optical density. A standard curve was prepared by recording the absorbance at the wavelength maxima for varying concentrations of the purified pigment, which thereby was used to determine the concentration of the pigment obtained during the optimization experiments.

Anticancer activity of pigment

The cells growing in the exponential phase were harvested after trypsinization for HeLa, MCF 7 and HepG2 cells. 100µl of cell suspension in culture medium were plated in 96-well microtitre plate and allowed to grow for 24 h prior treatment. Increasing concentrations of the pigment produced by Streptomyces sp. (50 and 100 µg/ml) dissolved in 10% DMSO were added in quadruples to different

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wells of the microtitre plates. Cytotoxicity was analyzed using MTT assay following the standard protocol [12]. The cells were incubated for 24h, 48h and 72h in the presence of the pigment. After the treatment period, 10µl of MTT prepared at a concentration of 5mg/ml in PBS, was added to each well and incubated in the dark for 3h at 37°C. After incubation 100µl of DMSO was added to each well and the absorbance was read at 540 nm using the ELISA plate reader. Decreased cell viability upon the pigment treatment was calculated and the IC_{50}value was extrapolated from the graph of concentration vs. viability.

Identifying the significant variables using Plackett-Burman Design

In the current study, primary investigation of the factors affecting the growth and the pigment production of the Streptomyces sp. was carried out using Plackett-Burman design. The variables chosen for the study were placed in two widely spaced intervals designate at lower level of -1 and higher level of +1. The experimental design for screening the media components is shown in table 1. The factors chosen were added to ISP2 media which was used as a basal medium. The effect of individual parameters on the growth and pigment production was calculated by the following equation.

$$E= [\sum M_1 - \sum M_2]/N$$

Where, E is the effect of the factor under investigation, M1 and M2 are the responses of the trials at which the factor was at higher and lower levels respectively and N is the number of trials.

Optimization using Central Composite Design

The levels of significant factors and the interactive effects between various media components that influence the growth and pigment production significantly was analysed by Central Composite Design (CCD). Four factors namely glycerol, dextrose, egg albumin and temperature were selected and successfully employed using CCD for the optimization of growth and pigment production. Linear and second order polynomial was fitted to the experimental data to obtain the regression equations. The sequential F test, lack-of-fit and various adequacy measures were employed in selecting the best model. [11]. The experimental plan consisted of 30 runs done in duplicates and the average of the growth and pigment yield obtained was taken as a response Y. The effects of the individual variables were studied in five different levels (-2, -1, 0, 1, 2). The second order polynomial coefficients were calculated and analysed using Design Expert software version 11.1. The general form of second-degree polynomial equation is given as follows.

$$Y_i = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

Where, Y_i is the predicted response, X_iX_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability P, correlation coefficient R and determination coefficient R^2 which measure the significance of fit of regression model. For each variable, the quadratic models were represented as counter plots (3D) and response surface curves were generated using Design Expert software version 11.1

RESULTS

The organism used in the study was a yellow pigment producing actinomycetes identified as *Streptomyces* sp. JUA14 (GenBank Accession number: MG923835) in accordance with morphological, cultural, molecular and physiological methods. ISP2 media was used as a basal media for all the optimization studies. The best response observed for Streptomyces sp. growth in ISP2 media at the controlled conditions of pH 7.0 and 28°C temperature for 15 days produced 6.4g of cell mass and 112 mg/L of pigment yield, hence a pH of 7.0 and an incubation period of 15 days were kept constant for all the optimization experiments. The optical density of the pigment measured using a UV-Visible spectrophotometer showed a λmax at 545nm, which was used to estimate the pigment yield from a standard graph.

Anticancer activity of the pigment.

The pigment extracted from *Streptomyces* sp. when tested for in vitro cytotoxicity using MTT cell viability assay, showed significant inhibition of cell growth and proliferation. The pigment demonstrated significant anticancer activity to HepG2, MCF-7 and HeLa cell lines with low IC_{50} values (**Table 1**). Treatment of pigment at concentrations of 50 and 100 µg/mL resulted in significant cytotoxicity with considerable decrease in the cell viability. The viability of HeLa cells treated with 50 µg/mL, was 50% after 48h which further decreased to 42% after 72h while treating with 100 µg/mL reduced to 42% after 48h and decreased further to 40% after 72h.(**Fig. 1**). HeLa cells were found to be more sensitive to this pigment than MCF and HepG2 cell lines.

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Table 1: IC_{50} value of the pigment against cancer cell lines

	HeLa	*HepG2*	*MCF7*
24 hrs	*12.60*	*16.71*	*23.07*
48 hrs	*5.34*	*8.20*	*11.27*
72 hrs	*3.79*	*5.13*	*4.75*

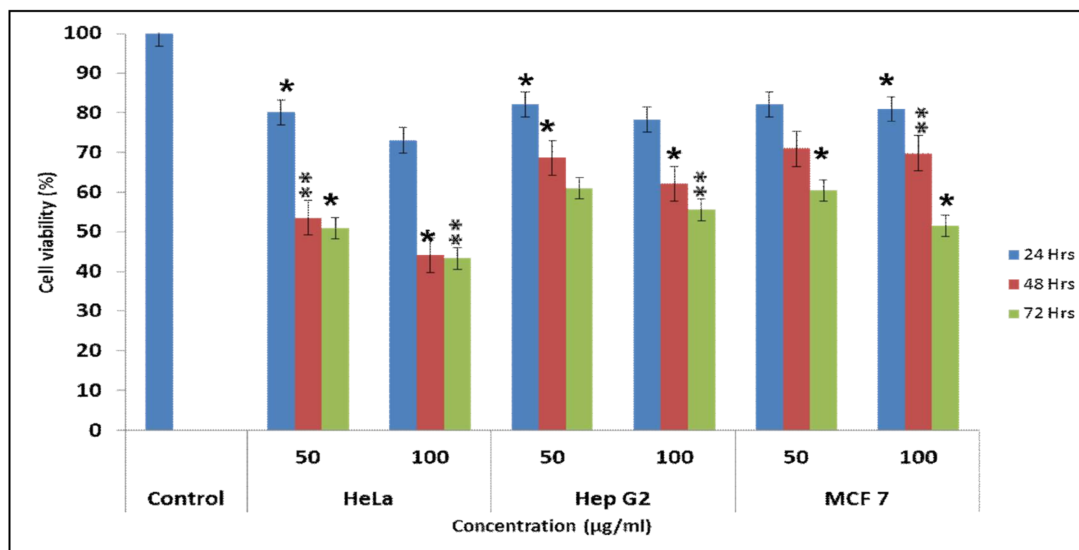

Fig. 1 Cell viability of cancer cell lines treated with the pigment at time intervals of 24, 48 and 72 h. ("*" indicates P value <0.05 and "**" indicates P value <0.001)

Screening of significant variables using Plackett-Burman Design

The design was applied with 11 different factors which included carbon, nitrogen sources, trace elements minerals and growth conditions as shown in **table 2.**

Table 2: Screening for factors affecting the production of pigment by the Streptomyces sp. using the Plackett-Burman design

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2
Std	Run	A:Dextrose	B:Malt Extract	C:Yeast Extract	D:Sucrose	E:Starch	F:Egg albumin	G:MgSO4	H:Glycerol	J:Glycine	K:Temperature	L:pH	R1	R2
		g/100ml	g/100ml	g/100ml	g/100ml	g/100ml	g/100ml	g/100ml	g/100ml	g/100ml	degrees		g/100 ml	mg/100 ml
10	1	0.2	1.5	0.6	1.5	0.5	0.25	0.03	1.5	0.25	31	7	0.3	12.03
5	2	0.2	0.5	0.6	0.5	1.5	0.75	0.03	1.5	0.75	31	5	0.4	10.36
2	3	0.2	1.5	0.6	0.5	1.5	0.75	0.07	0.5	0.25	27	7	1.5	24.32
12	4	0.2	0.5	0.2	0.5	0.5	0.25	0.03	0.5	0.25	27	5	1.6	32.65
1	5	0.6	1.5	0.2	1.5	1.5	0.75	0.03	0.5	0.25	31	5	1.2	10.65
8	6	0.6	1.5	0.2	0.5	0.5	0.75	0.03	1.5	0.75	27	7	0.5	9.45
6	7	0.2	0.5	0.2	1.5	0.5	0.75	0.07	0.5	0.75	31	7	1.3	13.54
9	8	0.6	1.5	0.6	0.5	0.5	0.25	0.07	0.5	0.75	31	5	0.6	10.64
4	9	0.2	1.5	0.2	1.5	1.5	0.25	0.07	1.5	0.75	27	5	1.2	9.89

All the experiments were performed in triplicates and the average of the results were presented as the response cell mass and pigment yield. The main effect of each of the variables on cell mass and pigment yield is represented in the Pareto chart (**Fig. 2 and 3**). The pareto chart of the standardized effect shows that temperature as a physical factor, dextrose and glycerol as carbon sources and egg albumin as a nitrogen source have shown significant effect on the growth and pigment production of Streptomyces sp. as compared to that of the other factors such as starch. sucrose, malt extract, yeast extract, magnesium sulphate, glycine and pH whose cell mass ranged between 3 to 6 g/L and pigment yield ranging between98 to 130 mg/L. The significance of the medium can be evaluated by the p value of each factor shown in **table 3.** P value less than 0.05 is generally considered to be statistically significant of the factors.

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The Model F-value of 13.37 implies the model is significant. There is only a 0.22% chance that an F-value this large could occur due to noise. The Predicted R^2 of 0.6600 is in reasonable agreement with the Adjusted R^2 of 0.8182; i.e. the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable and the ratio of 10.166 indicates an adequate signal.

Table 3: Analysis of variance (ANOVA) for Plackett-Burman design

Source	Sum of Squares	DF	Mean Square	F-value	P-value	
Model	16.39	4	4.10	13.37	0.0022	SIGNIFICANT
A-Dextrose	3.32	1	3.32	10.83	0.0133	
F-Egg albumin	1.88	1	1.88	6.14	0.0423	
H-Glycerol	2.13	1	2.13	6.96	0.0335	
K-Temperature	9.06	1	9.06	29.57	0.0010	
Residual	2.14	7	0.3063			
Cor Total	18.53	11				

Std. Dev.	0.5535	**R^2**	0.8843
Mean	2.29	**Adjusted R^2**	0.8182
C.V. %	24.13	**Predicted R^2**	0.6600
		Adeq Precision	10.1661

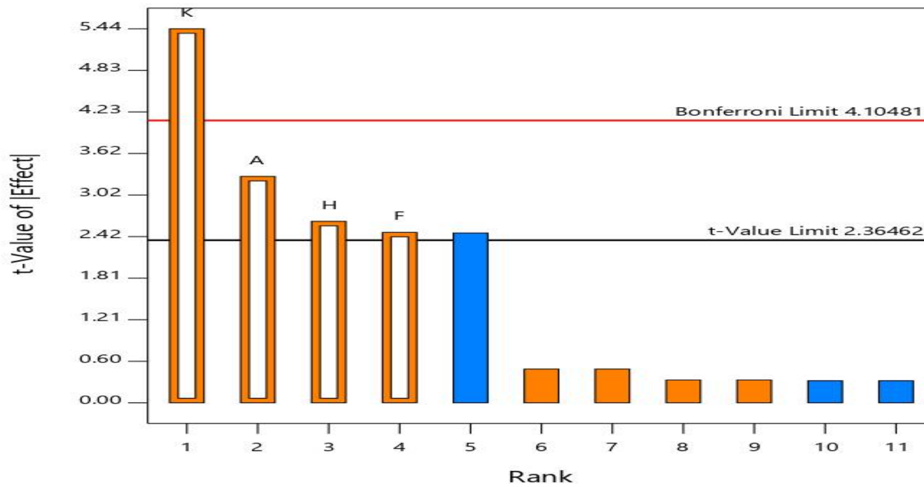

Fig. 2 Pareto chart showing the effect of variable K- temperature, A- Dextrose, H- Glycerol and F- Egg albumin on the cell mass

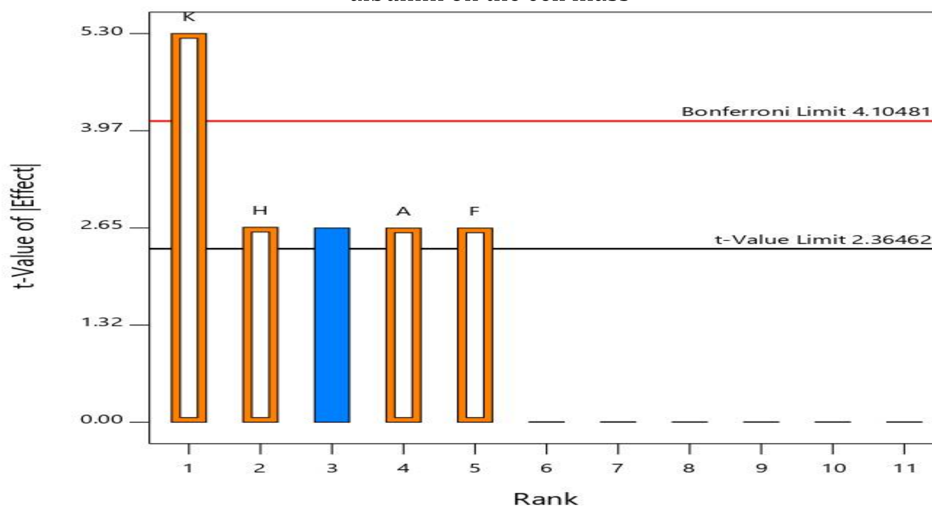

Fig. 3 Pareto chart showing the effect of variable K- temperature, A- Dextrose, H- Glycerol and F- Egg albumin on the pigment production

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Optimization using Central Composite Design

Preliminary results from the Plackett-Burman design showed that Dextrose, Egg albumin, Glycerol and Temperature influenced the growth and pigment production. The optimal level of these parameters for maximum growth and pigment production was determined using Central Composite Design and the level of these factors was fixed based on the results of the Plackett-Burman Design. The effect of four parameters at five levels each and their interactions on the growth and pigment production was determined by performing 30 experimental runsand the results are tabulated in **table4**. All the experiments were performed in 250ml Erlenmeyer flasks containing 100mL of the growth media. The cell massand the pigment yield are represented byquadratic model as a function of Dextrose (A), Glycerol (B), Egg albumin (C) and Temperature (D) and the coded equation from the set of experiments is given below.

Cell mass = 15.4414 - 0.4532A + 0.300389B + 0.524611C + 0.496947D - 0.212976AB + 0.325476AC + 0.6423AD - 0.167079BC - 0.388083BD + 0.0505832CD - 1.22508A^2 - 0.969001B^2 - 0.869001C^2 - 1.5565D^2

Pigment yield = 43.4427 + 2.62778A - 1.05822B - 3.99178C - 4.26053D - 1.01356AB - 2.88644AC - 4.81666AD + 1.59671BC + 1.23733BD + 3.83767CD + 2.69653A^2 + 2.56605B^2 + 3.21605C^2 + 6.37855D^2

The statistical significance of the polynomial equation was analysed using P test and Analysis of Variance (ANOVA) for the response surface quadratic model is represented in **tables5 and 6**. F-values and p-values are important in defining the significance of each coefficient. The resultant coefficient term is more significant when value of F-values and p-values are larger and smaller, respectively [14]. The cell mass quadratic model F-value of 136.01 implies the model is significant and P-values less than 0.0500 indicate model terms are significant. In this case A, C, D, AB, AC, AD, BC, A^2, B^2, C^2, D^2 are significant model terms and values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 1.02 implies that it is not significant relative to the pure error. The P-value co-efficient for AB, BC and AC was 0.0084, 0.0303 and 0.0003 respectively, followed by<0.0001 for AD and BD,confirmed a significant interaction between the variables. Similarly, the pigment yield quadratic model F-value of 303.51 implies the model is significant and the P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms and values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 0.82 implies that it is not significant relative to the pure error. The P-value co-efficient for AB was 0.0004 and for all other variables was less than 0.0001 confirming significance between the variables. The highest F-value for factor D (Temperature) with 881.72 and 1497.35, infers that the temperature has a significant role in increasing the cell mass and pigment yield respectively.

The accuracy of the model and the polynomial function infitting the data is expressed by R^2. The R^2value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts The coefficient of determination between measured data and simulated results (R^2) andadjusted R^2 were 0.9922 and 0.9849, respectively for cell mass and 0.9965 and 0.9932 for pigment yield. The high R^2value suggested the reliability of the proposed quadratic model for cell mass and pigment production.The R^2value showed a measure of variability in the observed response values, which could be described by the experimental factorsand their interactions. The adjusted R^2 corrects the R^2 value for the sample size and number of terms in the model. If the sample size is not very large and there are many terms in the model, then adjusted R^2 may be noticeably smaller than predicted R^2.

The purpose of statistical analysis is to determine the experimental factors,which generate signals that are large in comparison to noise. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable and a ratio of 39.372 for quadratic cell mass modeland 69.001 for quadratic pigment yield model indicates an adequate signal. This model proves to be used to navigate the design space. The sum of squares for the cell mass and pigment yield quadratic model was 143.93 and 3176.19 also indicates that the model is significant.

Three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two attheir centre point levels. The graphs represented to focusthe roles played by various factors (**Fig. 4 and 5)**. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified.

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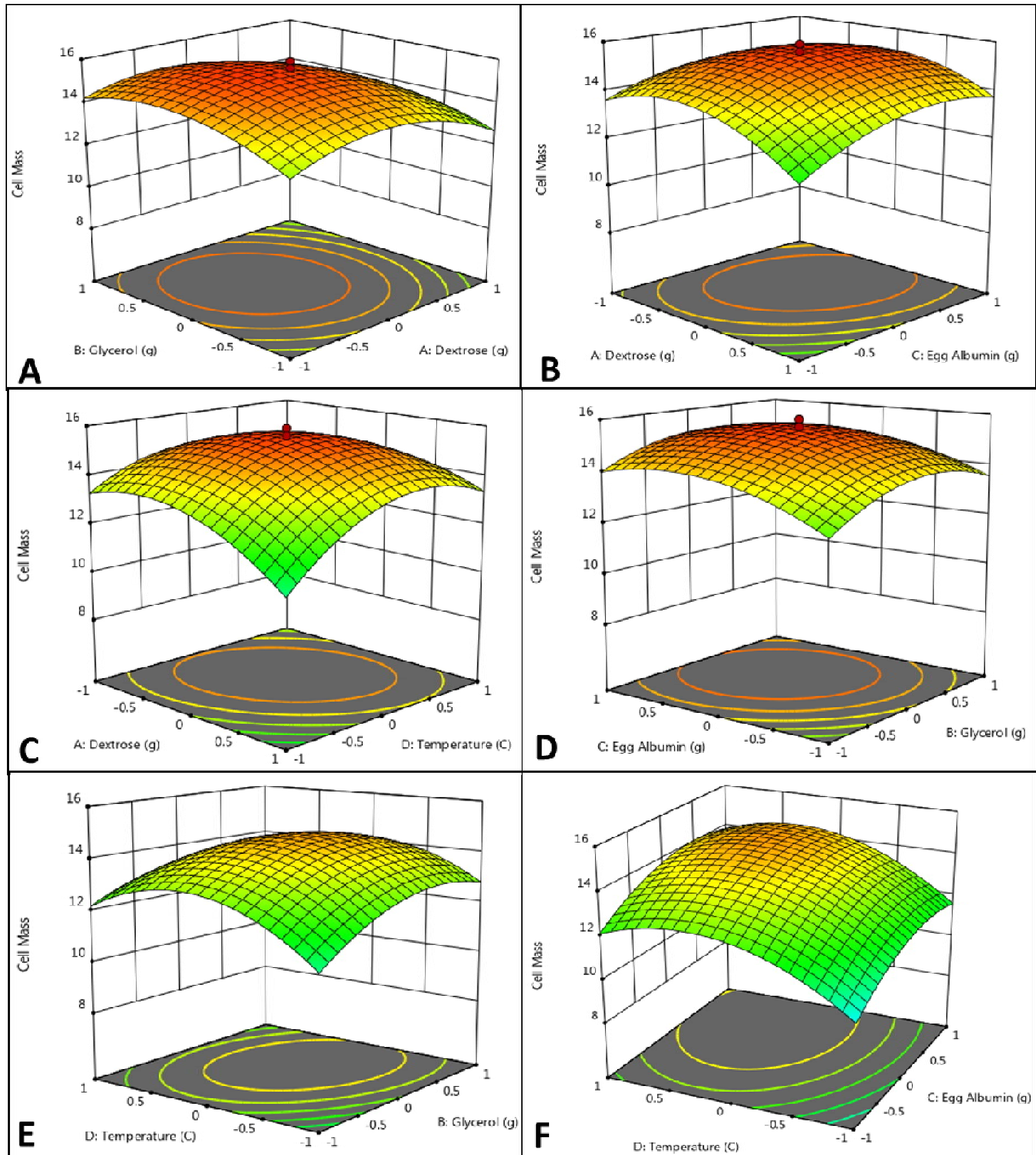

Fig. 4 Response surface curve showing the effect of factors on cell mass generated for the pair-wise combination of the factors keeping the other two at their zero levels, (A) Glycerol vs Dextrose, (B) Dextrose vs Egg albumin, (C) Dextrose vs Temperature, (D) Egg albumin vs Glycerol, (E) Temperature vs Glycerol and (F) Temperature vs Egg albumin

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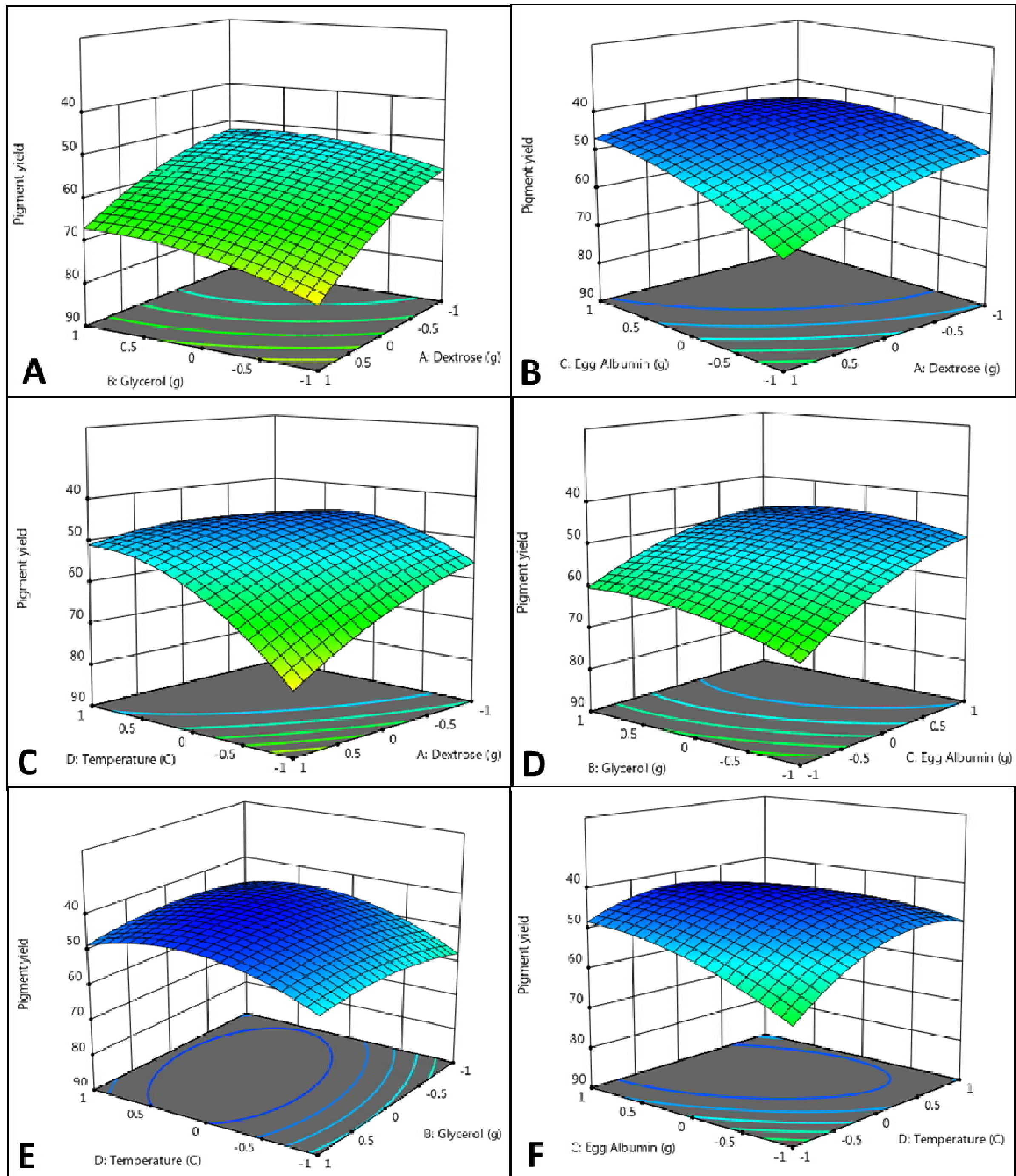

Fig. 5 Response surface curve showing the effect of factors on pigment production generated for the pair-wise combination of the factors keeping the other two at their zero levels, (A) Glycerol vs Dextrose, (B) Dextrose vs Egg albumin, (C) Dextrose vs Temperature, (D) Egg albumin vs Glycerol, (E) Temperature vs Glycerol and (F) Temperature vs Egg albumin

Three-dimensional plots of two factors versus cell mass and pigment production were drawn, by keeping the other variables at its optimal level and the corresponding contour plot was obtained. The interaction between dextrose and glycerol showed a significant increase in the biomass yield and pigment yield. The interaction between egg albumin and temperature indicated that an increasing concentration of egg albumin greatly influenced the pigment yield whereas both the factors equally influenced the cell mass. An interaction between egg albumin and dextrose showed a significant increase in both cell mass and pigment yield with their increasing concentrations respectively. A similar effect was observed due to the interaction between temperature and glycerol. The interaction between egg albumin and glycerol showed

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a moderate effect on both the responses and a similar effect were also observed for the interaction between dextrose and temperature. From the bump of three-dimensional plot or the central point of its corresponding contour plot; the optimal composition of medium components was identified. The optimal concentrations as obtained from the maximum point of the model was calculated for dextrose, glycerol, egg albumin and temperature and was found to be 6.84 g/L, 12.68 g/L, 4.91 g/L and 29°C respectively.

Table 4: Central composite rotatable design matrix of factors and the corresponding experimental yield.

		Factors				Response 1		Response 2	
Std	Run	A:Dextrose (g/L)	B:Glycerol (g/L)	C:Egg Albumin (g/L)	D:Temperature (C)	Dry weight of cell mass (g/L)		Pigment yield (mg/100mL)	
						Observed	Predicted	Observed	Predicted
3	1	7.5	7.5	4	26	10.5	10.2	61.80	62.94
14	2	12.5	7.5	4	26	8.1	7.8	86.10	85.63
17	3	5	12.5	4	26	10.2	10.3	55.80	55.95
16	4	12.5	12.5	4	26	9	9.1	75.00	75.81
4	5	12.5	7.5	6	26	9.6	9.7	60.10	61.00
24	6	12.5	7.5	6	26	11.4	11.5	52.90	52.73
6	7	7.5	12.5	6	26	12.3	12.3	50.20	50.49
9	8	12.5	12.5	6	26	10.5	10.3	58.00	57.58
22	9	7.5	7.5	4	30	10.5	10.6	53.40	53.90
15	10	12.5	7.5	4	30	10.8	10.7	57.10	57.32
7	11	7.5	12.5	4	30	11.4	11.2	53.40	53.09
28	12	12.5	12.5	4	30	10.6	10.5	52.30	52.46
11	13	7.5	7.5	6	30	11.5	11.4	56.40	56.17
10	14	12.5	7.5	6	30	13.1	12.9	48.20	48.05
8	15	7.5	12.5	6	30	11.1	11.3	61.20	61.75
21	16	12.5	12.5	6	30	11.7	11.9	50.20	49.57
29	17	5	10	5	28	11.5	11.4	49.80	48.97
25	18	15	10	5	28	9.5	9.6	59.40	59.48
19	19	10	5	5	28	10.7	11.0	56.40	55.82
2	20	10	15	5	28	12.3	12.2	51.60	51.59
1	21	10	10	3	28	10.6	10.9	65.10	64.29
26	22	10	10	7	28	13.2	13.0	48.10	48.32
18	23	10	10	5	24	8.1	8.2	78.30	77.48
23	24	10	10	5	32	10.2	10.2	60.20	60.44
30	25	10	10	5	28	15.6	15.4	43.50	43.44
20	26	10	10	5	28	15.2	15.4	43.60	43.44
13	27	10	10	5	28	15.3	15.4	44.90	43.44
27	28	10	10	5	28	15.2	15.4	42.10	43.44
12	29	10	10	5	28	15.9	15.4	43.50	43.44
5	30	10	10	5	28	15.4	15.4	42.90	43.44

Table 5: Analysis of variance (ANOVA) for optimization of cell mass

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	143.93	14	10.28	136.01	< 0.0001	significant
A-Dextrose	4.79	1	4.79	63.42	< 0.0001	
B-Glycerol	2.09	1	2.09	27.65	< 0.0001	
C-Egg Albumin	6.37	1	6.37	84.34	< 0.0001	
D-Temperature	5.80	1	5.80	76.72	< 0.0001	
AB	0.6954	1	0.6954	9.20	0.0084	
AC	1.62	1	1.62	21.48	0.0003	
AD	6.33	1	6.33	83.77	< 0.0001	
BC	0.4323	1	0.4323	5.72	0.0303	
BD	2.29	1	2.29	30.24	< 0.0001	
CD	0.0388	1	0.0388	0.5138	0.4845	
A^2	45.77	1	45.77	605.51	< 0.0001	
B^2	25.83	1	25.83	341.73	< 0.0001	
C^2	20.77	1	20.77	274.83	< 0.0001	
D^2	66.65	1	66.65	881.72	< 0.0001	
Residual	1.13	15	0.0756			
Lack of Fit	0.7605	10	0.0760	1.02	0.5258	not significant
Pure Error	0.3733	5	0.0747			
Cor Total	145.06	29				

Table 6: Analysis of variance (ANOVA) for optimization of pigment yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3176.19	14	226.87	303.51	< 0.0001	significant
A-Dextrose	161.17	1	161.17	215.61	< 0.0001	
B-Glycerol	25.94	1	25.94	34.70	< 0.0001	
C-Egg Albumin	369.08	1	369.08	493.77	< 0.0001	
D-Temperature	426.24	1	426.24	570.23	< 0.0001	
AB	15.75	1	15.75	21.07	0.0004	
AC	127.72	1	127.72	170.87	< 0.0001	
AD	356.10	1	356.10	476.39	< 0.0001	
BC	39.48	1	39.48	52.82	< 0.0001	
BD	23.24	1	23.24	31.09	< 0.0001	
CD	223.53	1	223.53	299.04	< 0.0001	
A^2	221.75	1	221.75	296.65	< 0.0001	
B^2	181.14	1	181.14	242.33	< 0.0001	
C^2	284.53	1	284.53	380.65	< 0.0001	
D^2	1119.25	1	1119.25	1497.35	< 0.0001	
Residual	11.21	15	0.7475			
Lack of Fit	6.96	10	0.6964	0.8196	0.6324	not significant
Pure Error	4.25	5	0.8497			
Cor Total	3187.40	29				

Validation of optimized condition

On substituting levels of the factors into the regression equation, the maximum predictable response for cell mass and pigment production was calculated and experimentally verified. The optimum values of factors were supplemented into the basal media and incubated at the optimal conditions for growth and pigment production. The maximum cell mass and yield of pigment obtained experimentally using the optimized medium was found to be 18.74 g and 850 mg/L, which is in correlation with the predicted value of the RSM regression study (**Table 7**). The growth in basal ISP2 media was set up parallel to the validation experiment and the observed dry weight of the cell mass and the pigment yield was 6.4g and 112 mg/L respectively. On comparing these values to the optimized condition values, it was found that a 2.93 and 7.59-fold increase in the cell mass and pigment yield respectively. Therefore, the optimized values were most suitable for increasing the cell mass and pigment yield.

Table 7: Growth conditions for validation experiment with optimum level of all parameters for biomass and pigment yield

Components		*Cell biomass*	*Pigment yield*
Dextrose	***6.84 g/L***	***Optimized condition***	
Glycerol	***12.68 g/L***	***18.74 g***	***850 mg/L***
Egg albumin	***4.91 g/L***		
Maltose	***10 g/L***	***Control condition***	
Yeast extract	***4 g/L***	***6.4 g***	***112 mg/L***
pH	***7***		
Incubation temperature	***29°C***		
Incubation period	***15 Days***		

DISCUSSION

Nature is an extensive resource of medicinal compounds and many drugs have originated from natural sources, including microorganisms which serve as an abundant source of structurally varied bioactive metabolites. Streptomyces species have the ability to synthesize many biologically active secondary metabolites such as antibiotics, herbicides, pesticides, and enzyme inhibitors. Approximately one third of known antibiotics in therapeutics have been isolated from Streptomyces sp. [15].

Recent developments have been made on drug discovery from Actinobacteria by using altering genomes for cryptic pathways and high-throughput screening and fermentation to generate new secondary metabolites related to existing pharmacophores [21]. In the present study, anticancer effect of the pigment on HeLa, HepG2 and MCF7 cancer cell line was studied and the pigment showed cytotoxicity in a dose and time dependent manner. Maximum cytoxicity of the pigment was observed at 72 h of incubation and the IC_{50} value ranging from 3.79 to 23.07 μg/mL indicated a potential cytotoxic activity against cancer cell lines.

Statistical optimization of the medium is an essential and comprehensive tool for planning experiments, building models and assessing the effects of different variables and their optimal concentrations. Optimization of culture conditions is projected to improve the secondary metabolite production. There are many reports of microbial cultures that are adapted to utilize various carbon and nitrogen sources for their optimal growth and secondary metabolite production [16-18]. The study was aimed to optimize the cell mass and pigment production using two different statistical designs by a strain of Streptomyces sp. as the pigment from this had demonstrated pharmaceutical significance with promising anticancer potential against three cancer cell lines. Statistical techniques for experimental design provide with a more precise and sophisticated means of designing the best medium. The most commonly used statistical experimental designs are Plackett-Burman design and Central Composite design. Advantages of these designs include ease and assessment of a many factors on the relative efficiency of the production process.

The Plackett-Burman design is basically used to determine the optimum media component that impacts the growth and pigment production of the Streptomyces sp. However, this design does not estimate the concentration of the media components and the potential interaction between the variables[19]. In this study eleven different factors were tested to determine the suitable media components, of which factors such as dextrose, glycerol, egg albumin and temperature showed a positive significance that influenced the growth and pigment yield of the organism. Considering carbon source plays a major role in enhancing growth and pigment production, the addition of dextrose and glycerol to the media that was devoid of any carbon source showed a significant increase in the yield. The addition of glycerol was reported to decrease the pigment yield [8] however in our study glycerol increased the cell mass and pigment yield. The organism also utilised egg albumin as a nitrogen source as it is a rich source of amino acids, peptides and vitamins. It is proven that amino acids enhance the pigment production by the formation of many precursor molecules, thus egg albumin significantly increased the growth and pigment yield. The ability of Streptomyces to utilise egg albumin as a nitrogen source for its growth and metabolite production have been reported earlier [20] which was also observed in our current study.

Response Surface Methodology used in this study suggested the significance of various factors at different levels. Based on the results of screening experiments by Plackett-Burman design, variables such as dextrose, glycerol, egg albumin and temperature were selected and further optimized using Central Composite Design. This showed a high resemblance between the predicted and experimental values, thereby revealing the accuracy and applicability of RSM to optimize the process for growth and pigment production.

The optimum concentration and interaction between various factors on the growth and pigment production by the Streptomyces sp. was investigated by plotting the response surface curves considering two variables at a given point and keeping the other variables at '0' level. Thus, 6 different 3D response surface graphs were obtained for each cell mass and pigment yield by considering all the possible combinations. The growth and pigment yield increased with the increase in concentrations of all the factors up to an optimum level further to which the responses declined. The optimum concentrations of dextrose, glycerol, egg albumin and temperature were intended by solving the coded equation of the central composite design and response surface graphs and was found to be 6.84 g/L, 12.68 g/L, 4.91 g/L and 29°C respectively. In order to validate the adequacy of the model, confirmation experiments were conducted under predicted optimal conditions and the results have indicated that the model is significant.

CONCLUSION

The study optimized the factors that influenced the growth and pigment yield using RSM. It is evident that the use of statistical process condition optimization approach, response surface methodology has helped to determine the most significant conditions with minimum effort and time. In addition, it has also proved to be useful inenhancingthe growth and pigment yield. The results of our study also indicate the feasibility and applicability of using dextrose and glycerol as carbon source, egg albumin as a nitrogen source and at the optimum temperature for good growth and pigment yield in large scale production during pharmaceutical application of the Streptomyces sp. JUA14.

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CONFLICTS OF INTEREST
The authors declare no conflicts of interest

AVAILABILITY OF DATA AND MATERIAL
The data that support the findings of this study are available on request from the corresponding author

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