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Medium Optimization for Herbicidal Compound Production from *Streptomyces anulatus* using Response Surface Methodology

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Abstract

Response surface methodology and central composite design were used to optimize a medium for the production of biomass of *Streptomyces anulatus* and herbicidal metabolites. The four variables involved in growth of the organism were the temperature, pH, galactose and serine concentrations. The optimized medium for the production of biomass contained, galactose -75 mg/ml and serine - 7.5 mg/ml and the temperature - 43°C and pH- 8. This medium resulted in increase in the level of biomass production (1.65g/l) compared to the yield in the normal production medium prior to optimized level (1.01 g/ml) after 120 h of fermentation, whereas the value predicted by the quadratic model was 1.03 g/l.

Keywords: Biomass production, *Streptomyces anulatus*, response surface methodology, optimization, central composite design.

INTRODUCTION

In recent years actinobacteria appears as a promising source of novel metabolites with different properties. In particular, the mycelial gram positive soil bacteria, *Streptomyces*, produce a wide variety of useful compounds. Nutrition plays a vital role in the arrival and increasing the concentration of secondary metabolites, as the choice of limiting nutrients can have specific metabolic and regulatory effects in their production (Doull and Vining, 1990).

To achieve an increase in the product yield, it is essential to formulate a proper production medium for an efficient fermentation process. There is always a relationship between the media composition and the compound biosynthesis (Elibol and Mavituna, 1998). Media components and their optimum levels are critical to the secondary metabolites production by the microorganisms and thus much effort was directed towards optimizing production rates and the products (Wang *et al*., 2011). Secondary metabolites production in a microbial system can be improved by optimization of physical parameters and nutritional constituents of proper production medium (Greasham, 1983).

Classically, optimization experiments are usually performed by one factor at a time method (Singh and Kaur, 2012) which is highly laborious and more time consuming than the statistical methods (Adinarayana *et al*., 2003). On the other hand, statistical optimization method has eliminated the drawbacks of classical methods and proved to be powerful tool for the production of the target metabolites (Deepak *et al*., 2008), which is also used to evaluate the relative significance of several variables simultaneously (Li *et al*., 2008). The present article deals with the effects of the physiological parameters on the biomass production and optimization for obtaining a higher yield of the biomass of *Streptomyces anulatus*

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MATERIALS AND METHODS

Strain and Culture Condition

The herbicidal compound producing *S. anulatus* was isolated from the paddy field soil in Tiruchirappalli district, Tamilnadu, India and extensively studied for their herbicidal secondary metabolite production (Priya Dharsini, 2014). The strain was maintained over the surface of starch casein agar slants for further research.

Inoculum preparation

A loopful of culture was spread on a starch casein agar plate and incubated for 7-14 days at 30 °C. After maturation, 5 ml distilled water was added to each agar plate which was aided to scrap and release the spores. The spore suspension was centrifuged at 4,000 rpm for 10 mins, washed and resuspended in 1 ml of distilled water. 500 µl of spore suspension was used as the inoculum for each shake-flask (500 ml) containing 100 ml of starch casein broth.

Optimization by Central Composite Design

Levels of the significant parameters and the interaction effects between various parameters influence the biomass production were analyzed and optimized by the Central Composite Design (CCD) under Response Surface Methodology(RSM). In this study, the independent variables such as temperature, pH, galactose and serine were chosen based on the one-factor-at-a-time (OFAT) method and studied at five different levels ($-\alpha, -1, 0, 1, \alpha$) and the experimental plan consisted of 30 trials. All the experiments were done in duplicate and the average of biomass production obtained was taken as the response (Y). The second

order polynomial coefficients were calculated and analyzed using the Design Expert package version 7.1.5. The general form of the second degree polynomial equation is:

$$Yi = \beta 0 + \Sigma \beta iXi + \Sigma \beta ii X2i + \Sigma \beta ijXiXj$$

Where, Yi is the predicted response, XiXj are the input variables which influence the response variable Y; β0 is the offset term; βi is the i^{th} linear coefficient; âii is the i^{th} quadratic coefficient and βij is the ij^{th} 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 (F), correlation coefficient R and determination coefficient R^2 which measures the goodness 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 package version 7.1.5.

Mass production, Extraction and purification of herbicide compound

S. anulatus was cultured in a production media formulated using RSM. The optimized composition of the production media composed of galactose -75 mg/ml, serine - 7.5 mg/ml and pH- 8 at 43°C. The culture was separated using centrifugation at 10,000 g/min for 15 mins. The supernatant was extracted with ethyl acetate which yielded brown extract. Two gram of extract was passed through the hexane balanced silica gel column (60-120 mesh) to trap the target herbicidal compound. The loaded column was eluted with hexane and ethyl acetate (2:1 v/v) to liberate the adsorbed product and it was collected and evaporated under reduce pressure to obtain the purified product.

Assessment of herbicidal activity

Herbicidal activity was determined by moist chamber technique by using crop seeds and weeds (Dhanasekaran *et al.*, 2012). In a sterile Petri dish, Whatman no.1 filter paper fully moistened with the *S. anulatus* herbicidal metabolite (test) was placed, while distilled water serves as a control. The seeds of *Echinochola crusgallis* were placed on the filter paper and incubated in a growth cabinet at 28°C and finally the herbicidal activity was observed after 5 days of incubation.

RESULTS AND DISCUSSION

Central composite design

The variables such as temperature, pH, carbon and nitrogen were optimized by RSM using the CCD experimental plan. The levels, together with coded and actual values of the selected variables for biomass production are presented in Table 1. The biomass productions at each trial were fed to the Design Expert software. Based on the central composite design, the experimental levels of biomass production were determined and compared with the corresponding predicted levels (Table 2). The maximum experimental value for biomass production was 1.65 g/l, while the predicted response based on RSM was estimated to be 1.03 g/l (Parekh *et al.*, 2000). The close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The quality of the model can also be checked using various criteria.

The coefficient of determination in terms of predicted R^2 is 0.758, is in close agreement with adjusted R^2 of 0 ·632 which confirms the experimental and predicted levels of biomass production. The strength of the model and the predicted response were revealed more effectively by the closeness of R^2 value to 1 ·0 and this statistical analysis allowed us to determine the influence of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise. Thus, adequate precision of 8.177 for biomass production and also by Fisher's "F" test and Student's test was estimated. Analysis of variance for biomass production showed that the fitted second order response surface model is significant with F-test = 3.36 (p = < 0.0001) and ANOVA as given in Table 3.

The contour plots based on the interactions between the variables showed an increase in biomass production as the concentration of each variable increased to optimum level. The contour plot in Fig. 1 exhibits the behavior of biomass production (mg/l) with respect to changes in the pH and temperature in the selected range. The optimal values of the selected variables were obtained when moving along the major and minor axis of the ellipse. Neutral pH was effective for biomass production, i.e. in the range of 6.5 to 7.2. High biomass production was observed with corresponding temperature in the range of 40.5 to 42.5°C, while incubation time was constant. The interaction between sucrose and glucose was studied in a previous work by Elibol and Mavituna, (1998). It has been reported that in the complex medium, sucrose which was not metabolized by *S. coelicolor* played a crucial role.

The shapes of contours indicated the mutual interaction effects between the test factors. If the shape of the contour is elliptical, the mutual interaction between the two factors is significant; otherwise, if it is circular, the mutual interaction effect is non-significant. From the elliptical contour in Fig. 4, it can be seen that the mutual interaction effect between carbon source (galactose) and pH was significant. It can also be concluded that the effect of the temperature with pH was much more significant than with nitrogen source (serine), which is an insignificant parameter.

Fig 1. 2D contour plot showing the effect of pH and temperature on biomass production.

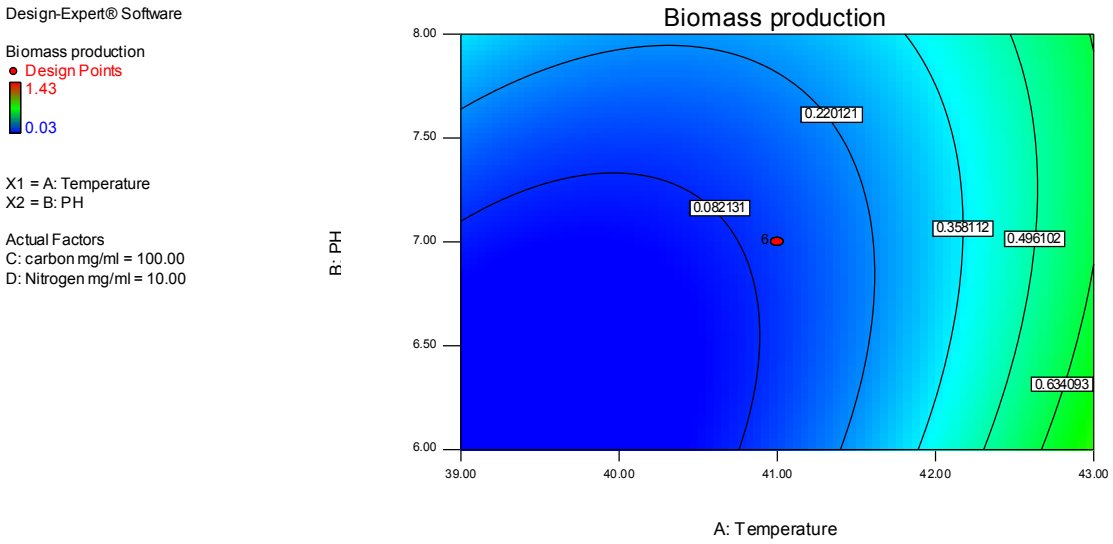

Fig 2. 2D contour plot showing the effect of carbon and temperature on biomass production.

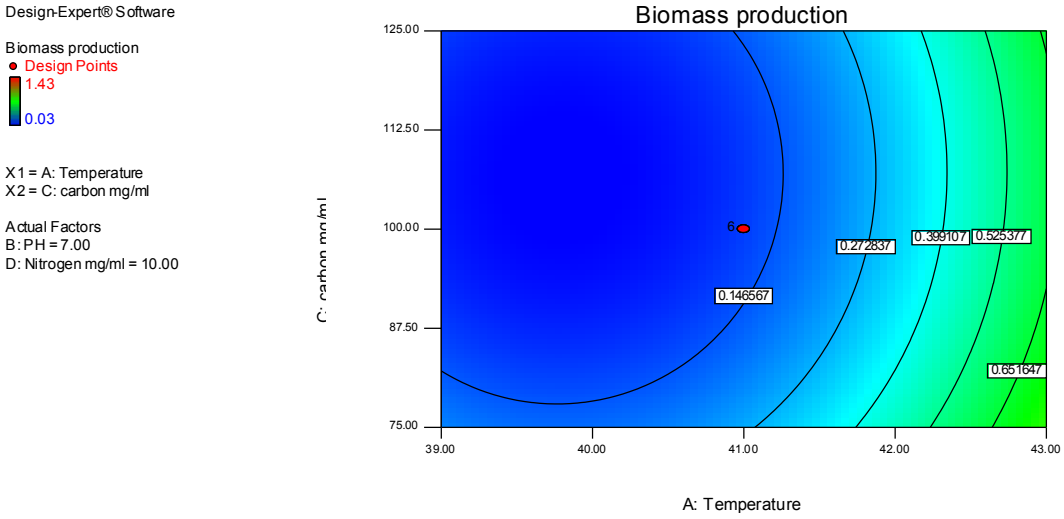

Fig 3. 2D contour plot showing the effect of nitrogen and temperature on biomass production.

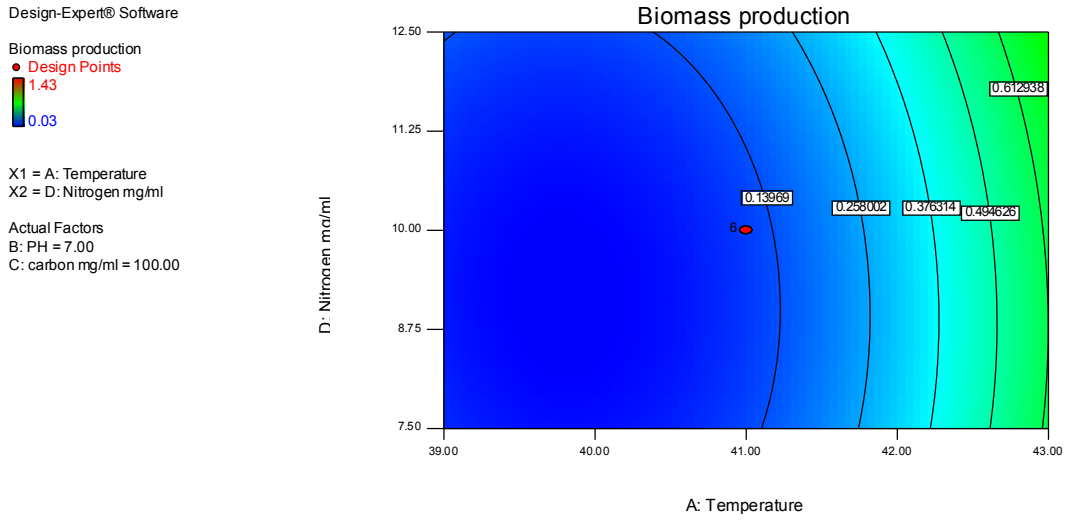

Fig 4.2D contour plot showing the effect of carbon and pH on biomass production.

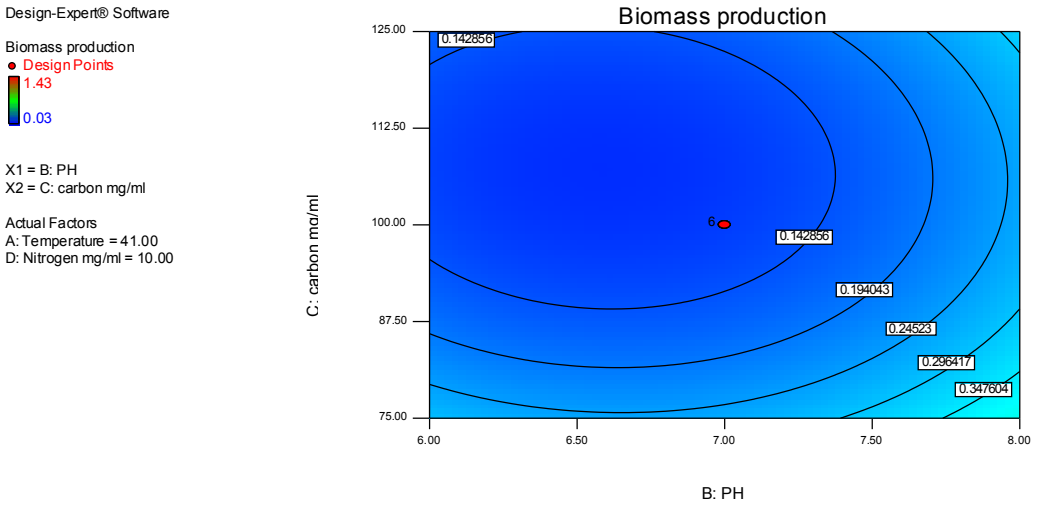

Fig 5. Residual diagnostic plots of quadratic model for *S. anulatus* (a) Observed versus predicted response plot, (b) Normal probability plot of the studentized residuals, (c) Internally studentized residuals versus predicted response plot, (d)) Internally studentized residuals versus run number

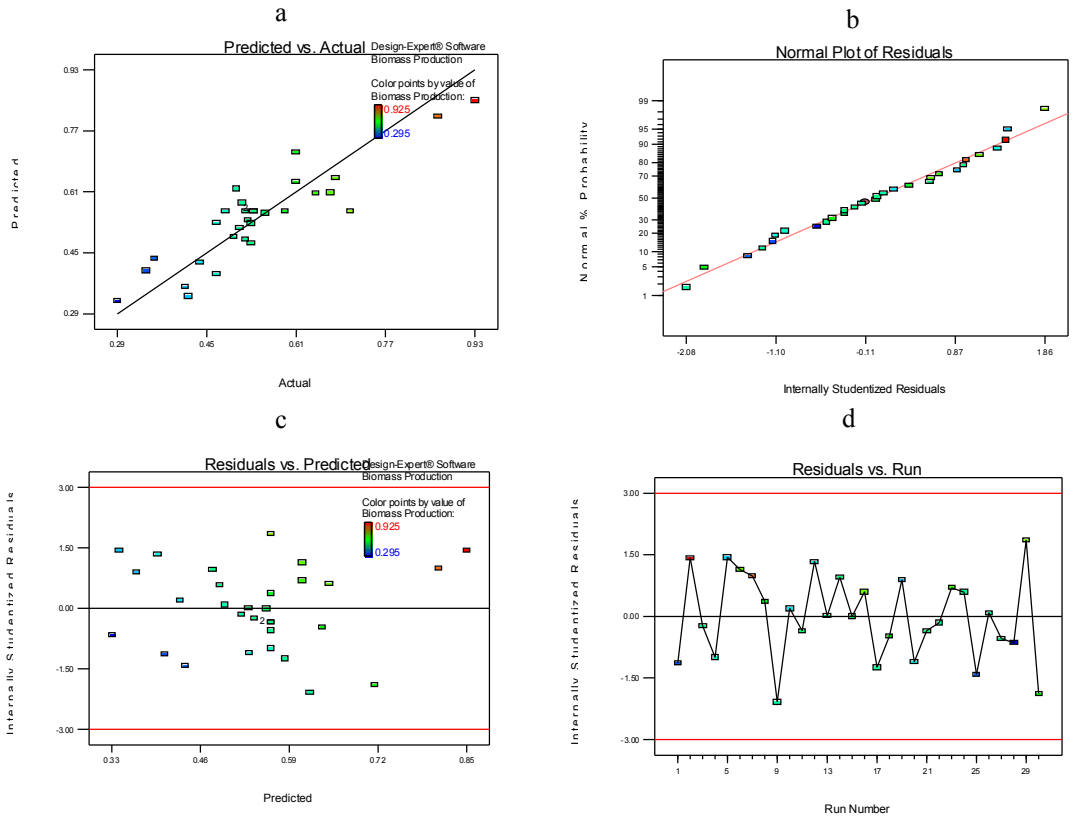

Fig 6. Compound from *S. anulatus* tested for herbicidal activity using microtitre assay

Table 1. Range of independent variables for response surface method

	Actual Values				
Independent Variables	-α	-1	0	+1	+α
A. Temperature	37	39	41	43	45
B. pH	5	6	7	8	9
C. Galactose mg/ml	50	75	100	125	150
D. Serine mg/ml	5	7.5	10	12.5	15

Table 2. Coded experimental design and results for the response surface of maximum biomass production of *S. anulatus as* a function of temperature, pH, galactose and serine

Runs		Type	Coded Values				Biomass Production	
Number	Order		A. Temp(°C)	B. pH	C. Galactose	D. Serine	Experimental	Predicted
7	1	Factorial	43.00	6.00	125.00	12.50	0.36	0.875
19	2	Axial	37.00	7.00	100.00	10.0	1.65	1.03
29	3	Factorial	39.00	8.00	125.00	12.5	0.53	1.205
14	4	Center	41.00	7.00	100.00	10.0	0.13	0.81
4	5	Axial	41.00	7.00	100.00	15.0	0.09	0.8
28	6	Axial	41.00	9.00	100.00	10.00	0.39	0.315
12	7	Factorial	39.00	8.00	75.00	7.50	0.62	0.995
1	8	Center	41.00	7.00	100.00	10.0	0.03	0.475
21	9	Factorial	39.00	8.00	125.00	7.50	0.079	1.18
23	10	Factorial	43.00	6.00	75.00	12.50	1.25	0.76
16	11	Center	41.00	7.00	100.00	10.00	0.11	0.46
22	12	Axial	41.00	7.00	150.00	10.00	0.23	0.525
27	13	Factorial	39.00	6.00	75.00	12.50	0.09	1.405
10	14	Factorial	39.00	6.00	125.00	7.50	0.17	1.07
6	15	Factorial	43.00	8.00	125.00	12.50	0.9	1.245
8	16	Axial	45.00	7.00	100.00	10.00	1.1	1.145
17	17	Factorial	39.00	8.00	75.00	12.50	0.64	1.315
18	18	Factorial	43.00	8.00	75.00	7.50	0.99	1.06
30	19	Axial	41.00	5.00	100.00	10.00	0.11	0.285
13	20	Factorial	43.00	8.00	75.00	12.50	0.87	1.57
5	21	Center	41.00	7.00	100.00	10.00	0.16	0.755
2	22	Factorial	43.00	8.00	125.00	7.50	1.08	0.8
26	23	Axial	41.00	7.00	50.00	10.00	0.39	0.525
24	24	Axial	41.00	7.00	100.00	5.00	0.19	0.605
15	25	Factorial	39.00	6.00	125.00	12.50	0.09	0.985
20	26	Factorial	43.00	6.00	75.00	7.50	1.25	0.44
25	27	Center	41.00	7.00	100.00	10.00	0.13	0.75
3	28	Factorial	43.00	6.00	125.00	7.50	0.45	0.695
11	29	Center	41.00	7.00	100.00	10.00	0.12	0.575
9	30	Factorial	39.00	6.00	75.00	7.50	0.1	1.265

Table 3. ANOVA for response surface quadratic model

Source	Sum of square	Degree of freedom	Mean square	F value	P value Probability > F
Model	4.16	14	0.30	3.36	0.0131 Significant
A - Temperature	1.91	1	1.91	21.59	0.0003
B - pH	0.10	1	0.10	1.16	0.2986
C – Galactose mg/ml	0.067	1	0.067	0.76	0.3968
D – Serine mg/ml	0.041	1	0.041	0.46	0.5068
AB	0.27	1	0.27	3.08	0.0995
AC	1.501E-004	1	1.501E-004	1.696E-003	0.9677
AD	1.785E-003	1	1.785E-003	0.020	0.8889
BC	1.388E-003	1	1.388E-003	0.016	0.9020
BD	0.022	1	0.022	0.25	0.6277
CD	0.12	1	0.12	1.41	0.2541
A2	1.46	1	1.46	16.50	0.0010
B2	0.18	1	0.18	2.02	0.1757
C2	0.25	1	0.25	2.84	0.1126
D2	0.078	1	0.078	0.88	0.3636
Lack of Fit	1.32	10	0.13	67.67	0.0001 Significant

Table 4. R-Squared, Adj R-Squared, Pred R-Squared and Adeq Precision value of the model

Std. Dev	0.30	R- Squared	0.7582
Mean	0.48	Adj R - Squared	0.6325
C.V%	61.76	Pred R - Squared	0.3852
PRESS	7.60	Adeq Precision	8.177

Similarly, the contour plot in Fig.2 describes biomass production with respect to changes in carbon and temperature, with remaining variables constant. The contour plots in Fig. 3 and 4 shows the effect on biomass production with respect to changes in carbon and nitrogen source. From the experiment, the optimized conditions for the mass production of *S. anulatus* are 43°C temperature and pH- 8, and0.00 galactose -75 mg/ ml and serine - 7.5 mg/ml. A similar trend of maximum accumulation during the stationary phase of growth has previously been reported (Pilet *et al*., 1995).

A number of numerical solutions were suggested by Design Expert software within the experimental range of parameters for maximum biomass production. The suggested solutions revealed that the physico chemical parameters were optimized at points located at the center of the plot. The desirability (0.857) of the solution for the maximum production of biomass was also at the center of the plot, which represents 1.8 mg of biomass / ml of optimized media or *S. annulatus.* In the present work, it was not possible to determine a model for actinobacterial growth, but relationship between actinobacterial growth and biomass production was observed.

The quadratic effect of pH was the most significant effect, followed by the quadratic effect of temperature. When the biomass production was inspected as a response of temperature and initial pH as variables, enhancement in production at the center point was observed. Optimization of biomass production from *S. anulatus* using RSM indicated that interaction between temperature and galactose (carbon source) was significant with P value. Results also revealed that in the range studied, the four variables observed have significant effects on biomass production.

Diagnostic plots were drawn to judge the model adequacy and clarify the signs of any problems in the experimental data. Plot of observed response (biomass production) versus predicted response is shown in Fig. 5a. In the present study, predicted values were in agreement with observed ones in the range of the operating variables. The normal probability plot of the studentized residuals was used to check for normality

of residuals (Fig.5b). A linear pattern observed in this plot suggests that there was no sign of any problem in the experimental data. Figure 5c represents a plot of studentized residuals versus predicted values to check for constant error. Residuals displayed randomness in scattering and suggested that the variance of the original observation was constant (Fig. 5d).

Mass production, Extraction, purification and assessment of herbicidal compound

The herbicide compound producing organism was inoculated into a 1000 ml conical flask containing 500 ml of starch casein broth. The culture was incubated on a rotary shaker (4000 rpm) at 30°C for 7-10 days. All the fractions from column chromatography were subjected to micro titer plate assay in order to evaluate the herbicidal activity. The third and fourth fraction of *S. anulatus* exhibited tremendous inhibition whereas moderate inhibition was observed in fifth fraction which is showed in the Fig. 6.

A perusal of the literature proved that there is no report on the production of herbicidal compound by *S. anulatus* ***in the engineered*** media composition. The results strongly support the use of RSM for medium optimization which resulted not only in higher concentration than unoptimized medium but also in a reduced amount of the medium constituents. The chosen method of optimization of medium composition was efficient, relatively simple, time and material saving.

CONCLUSION

This work has demonstrated that CCD and regression analysis methods are effective in determining the optimized temperature, pH, carbon source (galactose), nitrogen source (serine) for the biomass yield of *Streptomyces anulatus*. The medium was optimized at the temperature 43°C, pH- 8, and in the presence of galactose -75 mg/ml and serine - 7.5 mg/ml. These results suggest that the *Streptomyces anulatus* strain possesses interesting herbicidal property for industrial application in the weed management process.

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