Analyzing Alternative Nutrient Supplements and Optimization of Production Parameters for Violacein using Central Composite Design

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Abstract- Violacein is a medicinally important dye that is produced by the bacterium Chromobacterium violaceum. Production of violacein requires the presence of ideal nutritional conditions. In the present submerged fermentation method an initial systematic optimization of pH, inoculum age and inoculum level was done. Agricultural substrates were screened for selection of carbon source and a nitrogen supplement was identified. The critical parameters were further optimized by central composite design. As per the design the critical values of the four independent variables under investigation are as follows: wheat bran- 4.82g/L, NaCl- 4.82 g/L, yeast extract- 3.41g/L and inoculum level of 4.77% (v/v). An R2 value 0.97877 indicates 97.8% fitness of the model.

Agricultural substrates, Central composite design, Chromobacterium violaceum, Dye production, Optimization studies, Submerged fermentation, Violacein.

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1. INTRODUCTION

Violacein is considered to be a most widely used bacterial pigment produced by *Chromobacterium violaceum*. The pigment is used in medicine, cosmetics and textiles. It has also been explored for its various biological properties such as an antioxidant, immunomodulator, antitumor and anti parasitic activities [1]. Violacein is also produced by diverse genera of bacterial strains which include *Janthinobacterium lividium* [2],[3], *Collimonas* species [4], *Duganella* species [5] and *Pseudoaltheromonas* [6],[7],[8].

Several parameters were identified that can influence the yield of Violacein by bacterial strains. The parameters that were studied include aeration and agitation[6] inoculum's size [5] tryptophan [9], usage of agricultural wastes and carbon source [10]etc. The *Chromobacterium violaceum* strain from MTCC Chandigarh, MTCC No: 8071 is cultivated on nutrient agar and blood agar [11] and has been studied for pigment production by using various bioprocess techniques.

In the present investigation *Chromobacterium violaceum* MTCC No: 8071 standard strain was used and optimizations of parameters for production of violacein by submerged fermentation were carried out using nutrient broth. Systematic optimization of preliminary parameters was followed by study of the effect of wastes obtained during grain and cereal polishing, groundnut oil extraction unit, dairy farm and corn starch on dye production.

Growth of *Chromobacterium violaceum* was also examined followed by the optimization of production parameters using Central Composite Design. The aim of the study is to produce the dye using economically viable supplements and obtaining the yield on par with nutrient broth. Hence yeast extract along with agricultural wastes were added during optimization process.

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2. MATERIALS AND METHODS

2.1 Growth and maintenance of *Chromobacterium violaceum*:

A bacterial strain of *Chromobacterium violaceum* MTCC No: 8071 obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India was used throughout the study. The culture is maintained in nutrient agar slants. It is sub cultured once in a fortnight and stored at 4℃ until futher use.

2.2 Preparation of seed culture:

Seed culture of *Chromobacterium violaceum* was prepared in nutrient broth. To a slant of *Chromobacterium violaceum* 10ml water was added to disperse the bacterium. 1ml of the cell suspension is taken and added to 100m of sterilized nutrient broth and left for 24hr at 28℃ and 150rpm. The 24h aged culture is used as seed for rest of the studies.

2.3 Preliminary studies:

Optimization of production parameters like effect of pH, inoculum level and inoculum age were carried out using nutrient broth as the production medium. Samples were analyzed for violacein production every 24h by centrifuging the broth and obtaining optical density of supernatant at 560nm. These are carried out in 250ml Erlenmeyer flasks having 50ml working volume, having nutrient broth, maintained at pH 7 and incubated at 150rpm at 28°C in an orbital shaker. Samples were withdrawn at every 24h time interval and analysed for product formation.

2.4 Effect of pH

The effect of the pH on production of violacein was studied by varying the pH of nutrient broth between 3-11. To the sterilized nutrient broth 1% inoculum was added which is from 24h aged seed culture and then kept at 28℃ and 150rpm.

2.5 Effect of inoculum level

Keeping production nutrient broth, the production medium at the pH obtained from above investigation, inoculum level was studied by varying it as follows: 1%, 3%, 5%, 7% and 9%. The concentration of nutrients was maintained vigilantly as increase in the volume of added seed culture can lead to dilution of broth.

2.6 Effect of inoculums age

To the sterilized nutrient broth maintained at pH 7, 1ml of inoculum from seed culture medium was withdrawn at intervals of 12, 24, 36 and 48hours time interval and added to the production medium. The dye production was observed by analyzing the production media at every 24h time interval.

2.7 Effect of carbon source

Keeping nutrient broth as control, effect of various plant sources that are estimated to have the carbon and nitrogen content and economically available were chosen for the study. The materials chosen include rice bran, wheat bran, corn flour, green gram husk, black gram husk, ground nut fodder and whey. These materials were supplemented to the organism as the only source of carbon by varying their concentrations in the range 2%, 4%, 6%, 8% and 10%. Nutrient broth preparation was done as per the standard specifications, i.e, by keeping its concentration at 1.3%(w/v). The flasks were inoculated with 24h seed culture at 3% inoculum level and maintained at 28℃ and 150rpm. Samples were withdrawn to estimate violacein production at every 24h time interval.

2.8 Effect of yeast and malt extract

Complex nitrogen sources of organic nature were added to the selected carbon source and the materials chosen include yeast extract and malt extract which are protein hydrolysates that have amino acids including tryptophan that contribute to violacein production.

2.9 Optimization of production medium

From the preliminary studies variables that contribute to pigment production were identified to be wheat bran as carbon source and yeast extract supplement. To keep the production medium composition comparable to that of nutrient broth, NaCl was added as the mineral supplement. Hence the variables chosen for optimization of production medium using Central Composite Design (CCD) are, concentrations of wheat bran, yeast extract, NaCl and inoculum level. Usage of response surface methodology helps to overcome the drawbacks of traditional methods of optimization [12] which include lack of clarity about the interactions between variables and requirement for large number of experiments.

The central composite designs are design formed from the two-level factorials by the addition of just enough points to estimate curvature and interaction effects [13]. The design was generated using statistical software to estimate the response between the four dependent variables wheat bran, yeast extract, and inoculums level and NaCl.

A CCD design of 28 runs for four variables having four centre points is generated by the software. The range of dependent variables is given in table 1. The design consists of coded variables in the range of $-\alpha, -1, 0, +1$ and$+\alpha$ levels. The uncoded and coded for the variables are represented in Table 2. The following second order polynomial equation was adopted to study the effects of variables to the response.

$$Y = \beta_{0+} \beta_1 x_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_{4+} \beta_{34} X_3 X_4$$

Where y is predicted response , βo is the offset term, β_i indicates linear effect, β_{ii} indicates squared effect and βij reveals interaction effect. The experiments were carried out in 250ml Erlenmeyer flasks with 50ml working volume and the results of 48hrs were used for statistical analysis.

TABLE 1
Range of independent variables chosen for CCD design for production of violacein by *Chromobacterium violaceum*

Independent factors	Coded factor level				
	-2	-1	0	+1	+2
Wheat bran (g/L)	1	2.5	4	5.5	7
Inoculum level (%)	1	3	5	7	9
NaCl (g/L)	1	3	5	7	9
Yeast Extract (g/l)	1	2	3	4	5

2.10 Extraction and Estimation of violacein

The pigment produced by *Chromobacterium violaceum* was estimated as per the protocol of [14].

3. RESULTS AND DISCUSSIONS

3.1 Effect of pH, Inoculum level and Inoculum age

The effect of pH was studied by varying it between 3 to 11, in nutrient broth. Figure 1 indicates the violacein yield interms of optical density and highest yield was obtained between pH 7 to 9. At acidic pH i.e., between pH 3 to 6, the yield was low and even at alkaline pH it has decreased indicating the unfavourable environment for the bacterium. Riveros et al [15](1989) and DeMoss and Evans [16](1959) indicated that pH 7 is ideal for production of violacein by *Chromobacterium violaceum*.

Inoculum level has a significant effect on violacein production [5],[17](Wang et al. 2009, Aranda et al. 2011). The effect of inoculum level was studied by varying its concentration between 1 to 9%. A significant increase in dye production with parallel increase in biomass was noticed until 3% (v/v) figure 3. Addition of inoculum levels beyond 3% did not further contribute much to dye production which may be due to limitations with respect to other parameters. Hence 3% inoculum level is considered to be optimal for violacein production.

The effect of inoculum age was studied by adding inoculum from seed culture. A 3% (v/v) inoculum level was added at 12h, 24h, 36h and 48h into nutrient broth taken in various flasks. Samples were withdrawn every 12h and violacein production was estimated. The results of which are shown in figure 2. A 24h and 36h seed inoculum has resulted in better yield of violacein as compared to others. 12h inoculum has resulted in less yield as it may be due inability of the bacterium to reach stationary phase in the seed culture at which they had maximum growth and optimal production of violacein [11]. Leaving the bacterium beyond 36h would makes the *Chromobacterium violaceum* to enter decline phase and these cells would not contribute to violacein production.

3.2 Effect of carbon sources on violacein production

According to the studies on violacein production of Sivaranjani [18], wheat bran followed by deoiled rice bran was recommended as the best substrates for violacein production. In the present investigation seven different substrates were added and compared with that of dye production in nutrient broth. Figure 4 indicates that wheat bran would be a better substrate as compared to others when added at a concentration of 6% (w/v). The order of suitability of the substrates chosen in the study for violacein production are wheat bran > Rice bran > corn flour > groundnut fodder> green gram husk > Black gram husk > whey.

3.3 Effect of yeast extract and malt extract:

Nutrient broth comprises of 0.5% peptone, 0.3% yeast extract/ beef extract and 0.5% NaCl. As the aim of the study is to search of alternatives to the nutrient broth components, supplementation of yeast extract and malt extract to wheat bran was investigated. Yeast extract must contribute to addition of vitamins, carbohydrates, nitrogen and salts. While malt extract consists of peptone, polypeptides, nitrogen bases and few minerals [19].

A yeast extract was observed to be a better nitrogen supplement to malt extract figure 5. When concentration is increased from 1 to 5% an initial increase in OD at 560nm was observed indicating the proportional increase in production. Supplementation beyond 3% (w/v) has not resulted in much increase in violacein production owing to limitation carbon source.

Fig. 1. Effect on pH on Violacein production

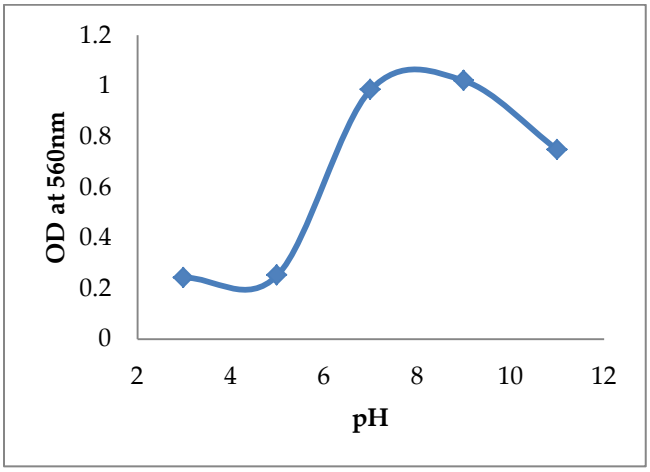


Fig. 2. : Effect of inoculum level on violacein production

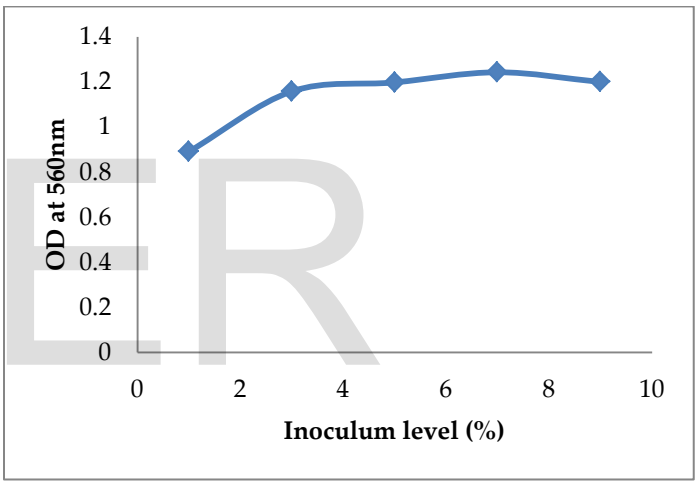


Fig.3. Effect of inoculum age on violacein production

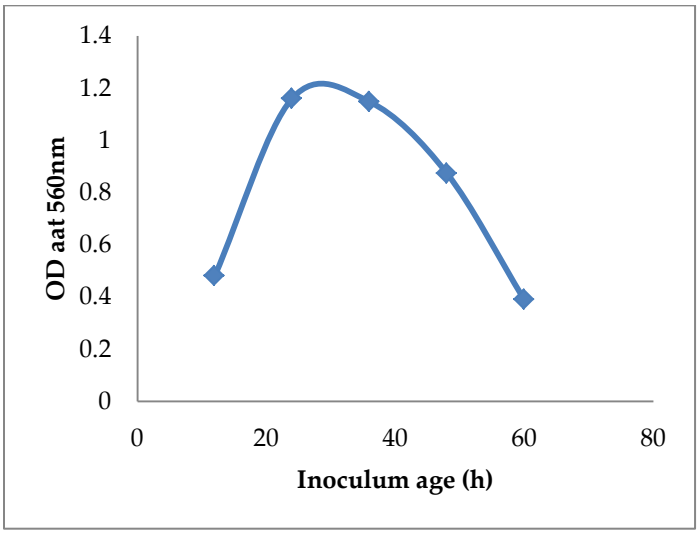


Fig.4. Effect of carbon sources on Violacein production

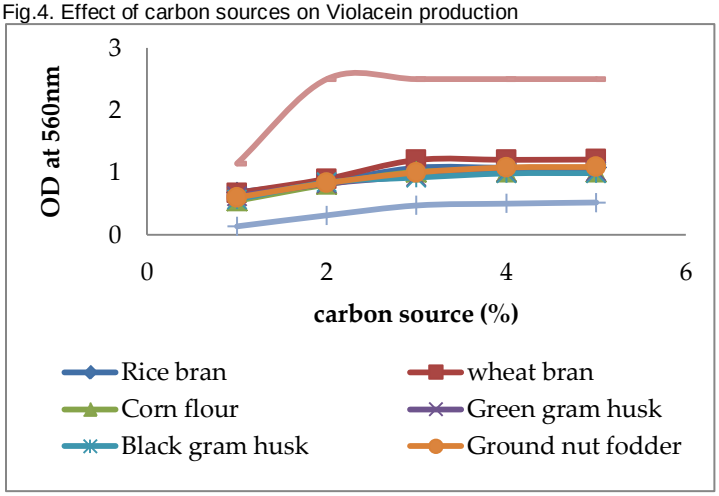
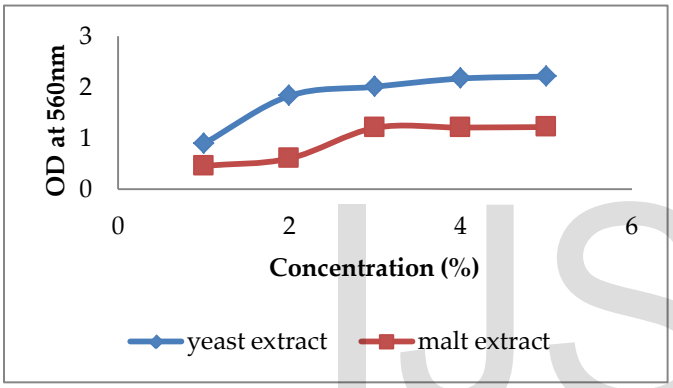


Fig.5. Effect of yeast extract and malt extract on violacein production in the presence of wheat bran as carbon source



3.4 Optimization studies using central composite design

Violacein production in terms of optical density at the end of 48h by submerged fermentation studies carried out using the 28runs CCD design is represented in table 2. On regression analysis of experimental data, the following second order polynomial equation is obtained for violacein production.

Where y represents the response variable and X_1, X_2, X_3, and X_4 are the four independent variables wheat bran, inoculum level, NaCl and yeast extract respectively. From the above equation optimal values of the variables for maximum production would be estimated.

The ANOVA for the second order polynomial model given in table 3 showed an R^2 value of 0.97894 indicating 97.897% fitness of the model. R^2 value is a measure of the linear relationship between the experimental values and the predicted values [20]. The studied t-distribution and the corresponding P-values of the linear and quadratic interactions among variables were above 0.5 with corresponding lower t-values.

$$Y= 1.8137+0.161250X_1 -0.042750X_2 -0.078500X_3 +0.140250X_4 - 0.238063X_1^2 -0.293562X_2^2- 0.375687X_3^2 -0.186188X_4^2 +0.0181500X_1 X_2 + 0.034500X_1 X_3 + 0.016125X_1X_4 – 0.039750X_2X_3 -0.053125X_2X_4 -0.009125X_3X_4$$

TABLE 2.
Central composite design representing the coded values and levels for the four independent variables

S.No	Wheat bran	Inoculum level	NaCl	Yeast extract	Wheat bran (g/L)	Inoculum level (%)	NaCl(g/L)	Yeast extract (g/L)	OD at 560nm
1	-1	-1	-1	-1	2.50	3.00	3.00	2.00	0.450
2	-1	-1	-1	+1	2.50	3.00	3.00	4.00	0.810
3	-1	-1	+1	-1	2.50	3.00	7.00	2.00	0.312
4	-1	-1	+1	+1	2.50	3.00	7.00	4.00	0.590
5	-1	+1	-1	-1	2.50	7.00	3.00	2.00	0.540
6	-1	+1	-1	+1	2.50	7.00	3.00	4.00	0.720
7	-1	+1	+1	-1	2.50	7.00	7.00	2.00	0.330
8	-1	+1	+1	+1	2.50	7.00	7.00	4.00	0.416
9	+1	-1	-1	-1	5.50	3.00	3.00	2.00	0.760
10	+1	-1	-1	+1	5.50	3.00	3.00	4.00	1.020
11	+1	-1	+1	-1	5.50	3.00	7.00	2.00	0.650
12	+1	-1	+1	+1	5.50	3.00	7.00	4.00	1.201
13	+1	+1	-1	-1	5.50	7.00	3.00	2.00	0.744
14	+1	+1	-1	+1	5.50	7.00	3.00	4.00	1.050
15	+1	+1	+1	-1	5.50	7.00	7.00	2.00	0.679
16	+1	+1	+1	+1	5.50	7.00	7.00	4.00	0.715
17	-2	0	0	0	1.00	5.00	5.00	3.00	0.625
18	+2	0	0	0	7.00	5.00	5.00	3.00	1.230
19	0	-2	0	0	4.00	1.00	5.00	3.00	0.810
20	0	+2	0	0	4.00	9.00	5.00	3.00	0.601
21	0	0	-2	0	4.00	5.00	1.00	3.00	0.550
22	0	0	+2	0	4.00	5.00	9.00	3.00	0.204
23	0	0	0	-2	4.00	5.00	5.00	1.00	0.810
24	0	0	0	+2	4.00	5.00	5.00	5.00	1.460
25	0	0	0	0	4.00	5.00	5.00	3.00	1.887
26	0	0	0	0	4.00	5.00	5.00	3.00	1.702
27	0	0	0	0	4.00	5.00	5.00	3.00	1.856
28	0	0	0	0	4.00	5.00	5.00	3.00	1.810

TABLE. 3
Multiple regression analysis for chosen variables

	Coeff.	Std.Err.	t(13)	p
Mean/Interc.	1.813750	0.050280	36.0727	0.000000
(1)Wheat bran(g/L)(L)	0.322500	0.041054	7.8555	0.000003
Wheat bran(g/L)(Q)	-0.476125	0.041054	-11.5976	0.000000
(2)Inoculum level (%)(L)	-0.085500	0.041054	-2.0826	0.057598
Inoculum level (%)(Q)	-0.587125	0.041054	-14.3013	0.000000
(3)NaCl(g/L)(L)	-0.157000	0.041054	-3.8242	0.002108
NaCl(g/L)(Q)	-0.751375	0.041054	-18.3022	0.000000
(4)Yeast extract (g/L)(L)	0.280500	0.041054	6.8325	0.000012
Yeast extract (g/L)(Q)	-0.372375	0.041054	-9.0704	0.000001
1L by 2L	-0.037000	0.050280	-0.7359	0.474877
1L by 3L	0.069000	0.050280	1.3723	0.193184
1L by 4L	0.032250	0.050280	0.6414	0.532407

2L by 3L	-0.079500	0.050280	-1.5811	0.137863
2L by 4L	-0.106250	0.050280	-2.1131	0.054496
3L by 4L	-0.018250	0.050280	-0.3630	0.722465

Fig. 6 Representation of profiles for Predicted variables and desirability

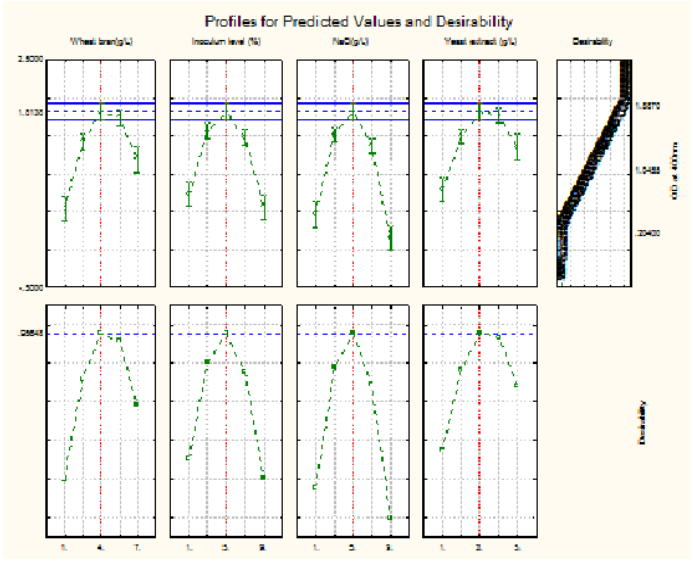
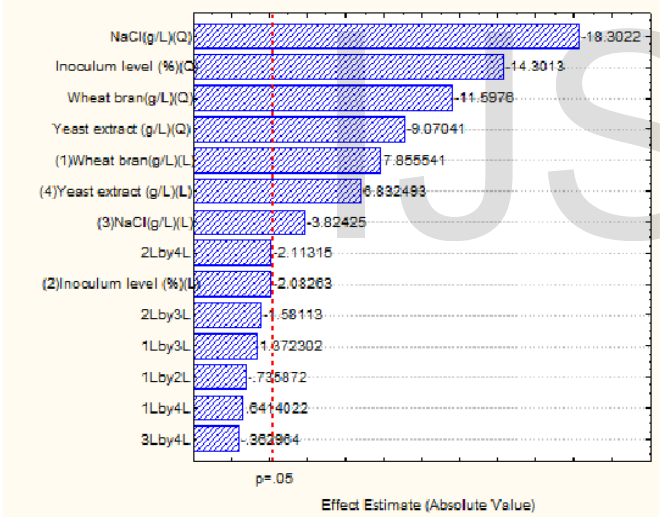


Fig. 7. Pereto chart of standardised effects for the Central Composite Design



Perito charts also indicate the significance of values under investigation (figure 6). All the four values had a P-values less than 0.5 indicating their direct impact on violacein production also reveals that the linear interaction among the variables are not that significant. (Figure 7) indicates the profiles predicted values and their desirability. The range of the variables chosen and the independent nature is revealed.

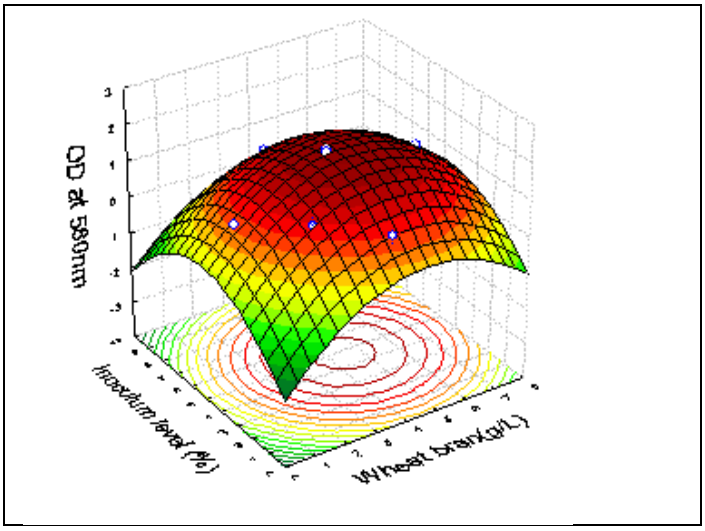
The interaction among the variables was described by the 3D response surface plots. These were obtained by plotting the response (i.e. optical density of violacein) on Z-axis against two variables, keeping the other two variables constant. Figure 8a to 8f represents the 3D graphs. Figure 8a shows that an increase in wheat bran with parallel increase in inoculum level keeping yeast extract and NaCl constant at 3g/L and 5g/L showed an increase in violacein OD up to certain level, while further increase in the concentrations of the independent variables did not contribute much to product formation due to limitations with respect to the other two variables. Figures 8b and 8c indicate the effect of wheat bran versus NaCl and wheat bran versus yeast extract respectively. Both exhibit the same pattern as figure 8, indicating that an initial increase invariables had contributed to violacein production upto certain limit.

Figure 8d and 8e indicate the effect of Inoculum level versus NaCl and Inoculum level versus yeast extract. NaCl being an essential mineral supplement has contributed to violacein production with increase in inoculum level. While further increase does not result in product formation rather had a detrimental effect due to the toxic effect of NaCl at high concentration and parallel uptake of more nutrients by organism for growth of added inoculum than its utilization for product formation. Figure 8e indicates that inoculum level and yeast extract contributes to product formation. While limitations of other two factors i.e, wheat bran and mineral supplement have resulted in diversion of nutrients in yeast extract for the growth of high volumes of inoculum added than for product formation.

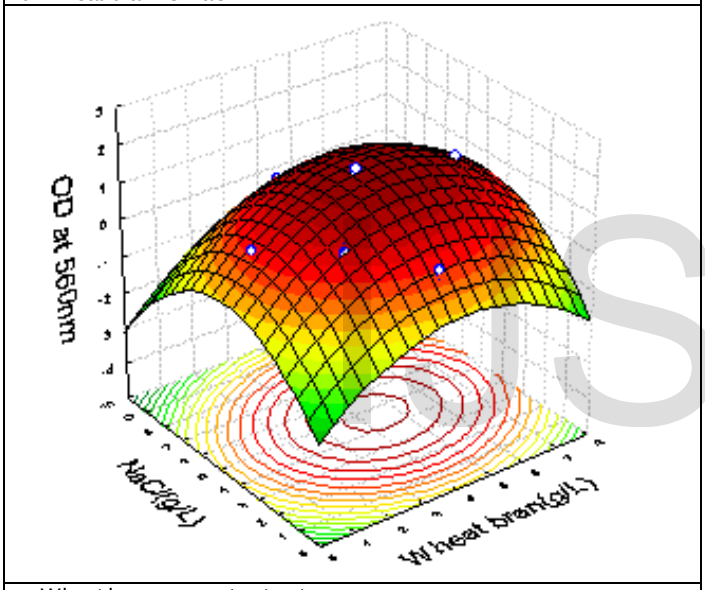
Figure 8f indicates the linear effect of NaCl and yeast extract. Both of them initially had a positive impact. NaCl beyond certain levels becomes toxic to the organism and other limitations would lead to a decrease in violacein production. The critical values as suggested by the statistical design are as follows.: Wheat bran (g/L) -4.526, inoculum level 3% (v/v) -4.77, NaCl (g/L) -4.82 and yeast extract (g/L) -3.41 production that would give an optical density of 1.87. Triplicate of the above composition was performed that has resulted in production of violacein whose average OD value is 2.04. This indicates that optimization of violacein production by CCD had a significant contribution in increasing product yield.

Fig. 8: 3D Response surface graphs of independent variables for violacein production

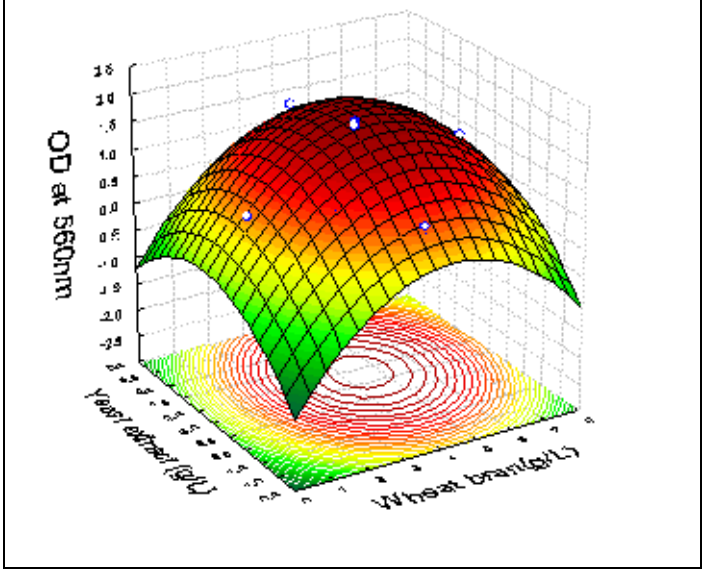
a: Wheat bran vs inoculums level



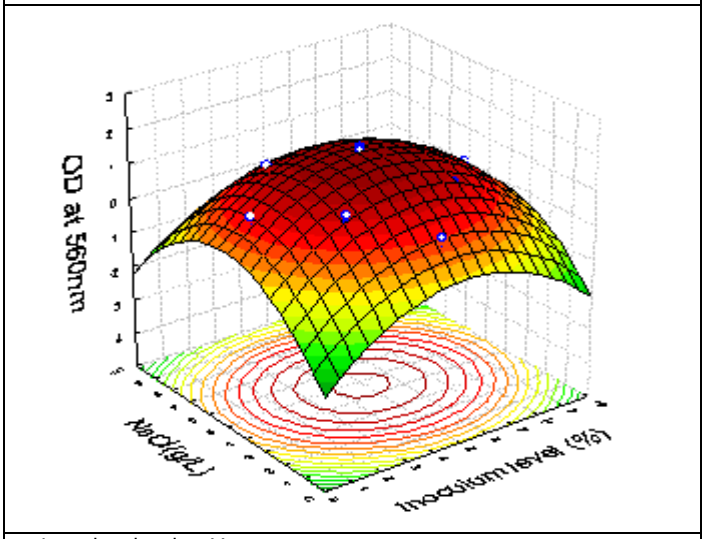
b: Wheat bran vs NaCl



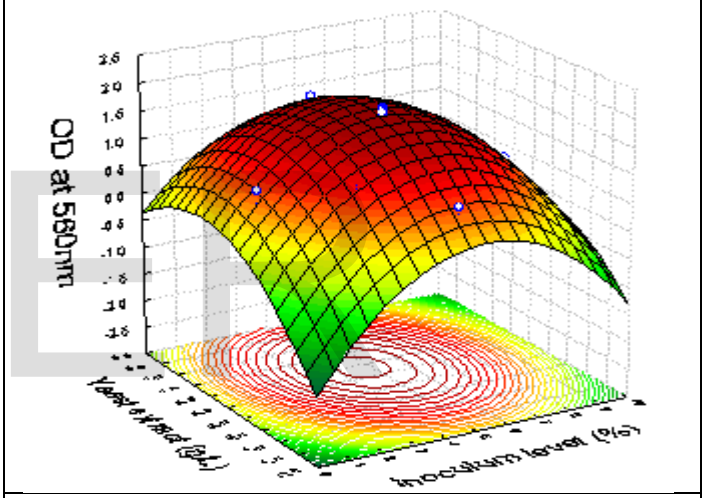
c: Wheat bran vs yeast extract



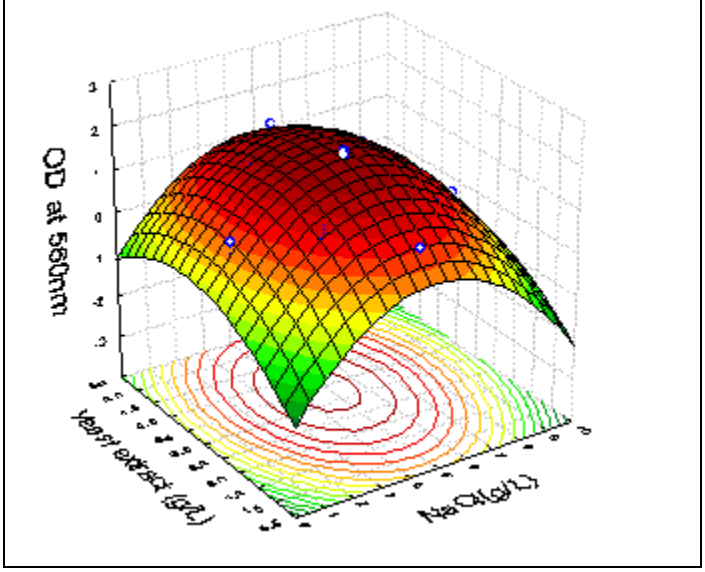
d: Inoculum level vs NaCl



e: Inoculum level vs Yeast extract



f: NaCl vs yeast extract



4. CONCLUSION

Product of violacein by *Chromobacterium violaceum* involved systematic optimization as well as optimization of critical independent variables by central composite design has helped to the preliminary studies. Optimized physical parameters and concluded that pH of 4 and inoculum age of 24h are optimal for product formation among the various substrates screened wheat bran contributed significantly to violacein production.

Central composite design for optimization of important independent variables has revealed that when the production medium is prepared by using 4.526g/L of wheat bran, 4.82g/l of NaCl, 3.41g/L of yeast extract and by the addition of 4.77 (v/v) inoculum level of 24h culture the violacein production has significantly increased to an OD value of 2.04, which is significantly nearer to the expensive and most widely used nutrient broth. An R^2 value of 0.97877 indicates 97.8% fitness of the model.

ACKNOWLEDGEMENTS

We express our sincere thanks to the management of ANITS for providing the infrastructure and facilities. We thank UGC for providing financial support through UGC-MRP- BIOT-2013-9237.

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