IJPSR (2020), Volume 11, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 28 June 2019; received in revised form, 04 November 2019; accepted, 08 February 2020; published 01 May 2020

OPTIMIZATION OF AN ANTI-CANCER METABOLITE PRODUCTION BY *PENICILLIUM RUBENS* JGIPR9 *VIA* RESPONSE SURFACE METHODOLOGY

P. Venkatachalam and V. K. Nadumane *

Department of Biotechnology, School of Sciences, JAIN (Deemed-to-be University), #18/3, 9th main, 3rd block, Jayanagar, Bengaluru - 560011, Karnataka, India.

Keywords:

P. rubens, Anti-cancer, Single-factor screening, Response surface methodology, Central composite design

Correspondence to Author:
V. K. Nadumane

Director and Professor, Department of Biotechnology, School of Sciences, JAIN (Deemed-to-be University), #18/3, 9th main, 3rd block, Jayanagar, Bengaluru - 560011, Karnataka, India.

E-mail: kn.varalakshmi@jainuniversity.ac.in

ABSTRACT: There are many pharmaceutically important compounds from *Penicillium* sp. that made the fungal genera one of the most important sources for the production of bioactive metabolites. As per the results of our previous study, a bioactive metabolite, P5, from *Penicillium rubens* JGIPR9 had highly promising cytotoxic and anti-cancer potentials. This prompted us to undertake the current study, so as to optimize the culture conditions of this fungal isolate and thereby to enhance its growth, *i.e.*, biomass (response 1) and production of the anti-cancer metabolite P5 (response 2). Initially, the single factor method was adopted to screen the individual culture conditions that would enhance the responses under the study. The best factors being $CdCl_2$, glucose, amino acid concentrate, and beef extract were subjected to Central Composite Design (CCD) to identify the optimal conditions that enhance the responses. The biomass dry weight ranged from 11.9-24.1 g/L and that of P5 concentration ranged from 257-774 mg/L. The optimal conditions were verified experimentally and 3.24-fold and 12.21-fold increase were observed for responses 1 and 2 respectively in comparison to the basal culture conditions.

INTRODUCTION: Filamentous fungi are historically very important sources of pharmacologically relevant secondary metabolites having diverse structures with important biological activities [1, 2]. The discovery of pharmaceutically important products through the screening of a large number of metabolites is quite beneficial to biotechnological industries [3]. *Penicillium* is considered to be a very important genus for the production of valuable products of pharmaceutical use [4].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(5).2299-09

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(5).2299-09

A number of bioactive secondary metabolites with diverse chemical structures from micro-organisms have been reported as lead molecules for the development of pharmaceutical drugs. But the main constraint here is the low yield.

Physiochemical and nutritional conditions greatly influence the growth as well as the production of metabolites from micro-organisms. Optimization of culture conditions is one approach to reach the adequate levels of the required metabolite which involves multivariable parameters. The one-factor at a time approach is tedious but it gives us information about the important factors that can possibly influence growth conditions and then the selected factors are chosen to optimize culture conditions by response surface methodology (RSM).

RSM is a statistical design that provides information between one or more measured dependent responses based on the number of input independent factors. RSM is a very useful approach for the modeling and analysis of problems in which the main objective is to optimize the response of interest that is influenced by several factors [5]. RSM is advantageous as it includes a smaller number of experiments, its suitability for multiple factor experiments, search for relativity between the independent factors and more importantly finding the most suitable conditions and forecast response [6]. Central composite design (CCD) is one of the widely used statistical methods that is based on a multivariate nonlinear model for the optimization of process variables and more importantly in studying the interactions between variables that affect the process. It also determines the regression model equations and operating conditions from the appropriate experiments [7].

In the current study, a bioactive anti-cancer fraction, P5 was identified from a newly isolated strain of *Penicillium rubens* as to contain Indole-2, 3-(4,4-dimethyl-3-thiosemicarbazone). Its highly promising anti-cancer properties prompted us to undertake this study to statistically optimize the culture conditions so as to enhance the production of this compound and thereby to obtain its higher yield for future application studies.

MATERIALS AND METHODS:

Isolation and Identification of the Fungus: The fungus used in the current study was isolated from the garden soil obtained from Madurai district, Tamil Nadu by serial dilution method [8]. The isolate was subjected to molecular methods (18s rRNA sequencing) for identification.

Inoculum Preparation and Culture Conditions: Czepak Dox Yeast Agar (CDYA) plates were used to maintain the pure culture, incubated for 8 days at room temperature (RT at 24-28 °C). The fungal spore suspension having 2.3×10^6 cells/ml was maintained as the primary inoculum and used for optimization studies.

Extraction and Identification of the Anti-Cancer Compound: The metabolites from the fungal isolate were extracted by methanol. The extract was first tested for its cytotoxic potentials on HeLa, HepG2, and MCF-7 cancer cell lines by MTT assay [9]. Based on the promising results, it was subjected to bioactivity guided fractionation by thin-layer chromatography (TLC) and the bioactive (anti-cancer) fraction was identified. The yield of this compound was determined by checking the λ_{max}. Statistical significance ($p \leq 0.05$) was calculated using GraphPad Prism version 6.0.

λ_{max} of the Bioactive Metabolite: The wavelength maxima (λ_{max}) for the TLC purified bioactive fraction was determined by using a UV/Vis spectrophotometer (Shimadzu, Japan) and thereafter by plotting wavelength versus optical density. A standard curve was prepared by recording the absorbance at wavelength maxima for the purified bioactive metabolite at varying concentrations, which thereby was used to determine the concentration of the metabolite obtained during the optimization experiments.

Determination of the Dry Weight of Biomass and Extraction of the Metabolite: 1 ml of the primary inoculum was transferred to 100 ml Czepak Dox Yeast Broth (CDYB) and was incubated at RT in stationary condition for mycelial growth. Complete mycelial mat formation was observed only after the 10^{th} day of incubation. 12-day old culture was used for further studies. The mycelial mat was recovered by filtering the culture media through Whatman filter paper no. 1 and was kept overnight in a hot-air oven for drying. The dry weight was recorded and the metabolites were extracted from the dried mat by homogenizing it with methanol using pestle and mortar.

The mixture was then centrifuged at 8000 rpm for 10 min at 4 °C and the supernatant was collected. The absorbance of the extract was recorded at the wavelength maxima of the bioactive metabolite to determine its concentration. The biomass was expressed as g/L and the metabolite concentration was as mg/L throughout the optimization studies unless and otherwise mentioned.

Effect of Varying Culture Conditions: CDYB was considered as the basal media (control conditions) for the optimization studies. At first, the addition of a single factor in the basal media was screened for higher biomass and bioactive metabolite production. A list of different nutrients

used in the single factor system along with the basal media for biomass production (response 1) and concentration of bioactive metabolite (response 2) are listed in **Table 1**. Culture conditions such as incubation period (day 9-14), pH (3-11) and temperature (4 °C, RT [24-28 °C], 40 °C and 60 °C) were also considered for enhancing biomass production and quantity of bioactive metabolite. The extraction of metabolite and determination of biomass weight were performed as previously mentioned. All experiments were conducted in triplicates.

TABLE 1: NUTRIENTS USED IN THE SINGLE FACTOR SYSTEM

Nutrient source	Concentration	Varying components
Carbon	1%	Glycerol, Starch, Glucose, Lactose, Maltose, Mannitol, Sucrose, Dextrose, Galactose, Fructose
Nitrogen	1%	Malt extract, Beef extract, Urea, Ammonium sulphate, Ammonium ortho phosphate, Sodium nitrate
Amino acids	0.5X, 1X, 1.5X	(MEM Amino acids solution – 100X, Himedia)
Vitamins	0.001 mg/ml	B1 (Thiamine hydrochloride), B2 (Riboflavin), B3 (Nicotinic acid), B5 (Pantothenic acid), B6 (Pyridoxal 5'- phosphate), B9 (Folic acid), B12 (Cobalamin)
Metal ions	0.05%	Copper sulphate ($CuSO_4$), Nickel chloride ($NiCl_2$), Calcium chloride ($CaCl_2$), Zinc sulphate ($ZnSO_4$), Lead acetate ($Pb(C_2H_3O_2)_2$), Barium chloride ($BaCl_2$), Magnesium sulphate ($MgSO_4$), Ferrous sulphate, Manganese sulphate ($MnSO_4$), Cadmium chloride ($CdCl_2$)

Optimization by Response Surface Methodology: Based on the initial single factor screening results, six factors were initially shortlisted based on statistical significance observed (GraphPad Prism 6.0) and later on, four factors were employed for the Central Composite Design (CCD). The experimental data obtained were analyzed using Design-Expert software version 11.0.5 (Free trial) from Stat-Ease Inc. The four factors were studied at five different levels (-α, -1, 0, +1 and +α) and the total number of experiments were run based on the following equation.

$$N = 2^n + 2n + n_c$$

Where N is the total number of experiments in the design, n is the number of factors used in the design. 2^n represents the number of factorial runs, 2n represents the number of axial runs and n_c represents the number of center runs (number of replicates at the center point).

A second-order polynomial equation was used to fit the experimental results obtained by the response surface model.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 AB + \beta_6 AC + \beta_7 AD + \beta_8 BC + \beta_9 BD + \beta_{10} CD + \beta_{11} A^2 + \beta_{12} B^2 + \beta_{13} C^2 + \beta_{14} D^2$$

Where Y is the response (predicted or observed). A, B, C and D are the coded factors. β_0 is the intercept term. β_1, β_2, β_3 and β_4 are the linear coefficients. β_5, β_6, β_7, β_8, β_9, and β_{10} are the cross-product coefficients. β_{11}, β_{12}, β_{13} and β_{14} are the quadratic coefficients. The levels used to study the response pattern and the optimum combination of the factors is summarized in **Table 2**.

RESULTS:

Identification of the Promising Isolate: The fungal isolate with promising anti-cancer properties was identified as *Penicillium rubens* by 18s rRNA sequencing (Biokart India Pvt. Ltd., Bengaluru) [10]. The sequence was submitted to Genbank and the accession number obtained was MH816935 (*Penicillium rubens* JGIPR9).

λ_{max} of the metabolite: The λ_{max} was found to be at 282 nm for TLC purified P5 fraction as shown in **Fig. 1**. The concentration of P5 in all optimization studies were determined by recording the optical density (OD) at 282 nm and from the standard curve prepared.

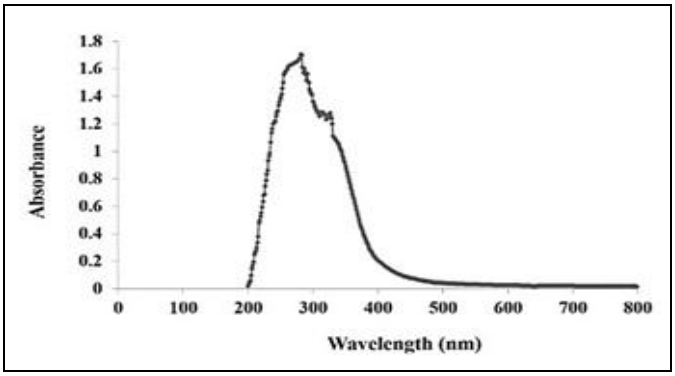

FIG. 1: WAVELENGTH MAXIMA OF P5 FRACTION AT 282 nm

Effect of Single Factor System for Increased Response: CDYB was used as the basal media for all the optimization experiments because minimal media (M9) that was initially used did not support the desired fungal growth. The best response observed for fungal growth (incubation period) was on day 12 with 7.7 g/L of biomass and 44.68 mg/L of P5 concentration. Incubation of the fungus at different pH yielded the best result at pH 7 with 12.0 g/L of biomass and 460.5 mg/L of P5 concentration as compared to the control (pH 5.8) with only 6.7 g/L of biomass and 118.3 mg/L of P5 concentration as shown in **Fig. 2A** and **B**. With respect to varying temperature conditions, *Penicillium* culture incubated at RT (24-28 °C) supported the best growth conditions. Therefore, the above mentioned three parameters were chosen as the best growth conditions for *Penicillium* and were kept constant throughout the single factor screening process involving nutrients as well as RSM. The response was found to be statistically significant.

The effect of a single factor as an extra source of nutrients was evaluated for the desired responses. Carbon sources are mainly responsible for driving endergonic reactions within the cell which in-turn plays a very important role in the production of metabolites. Among the different carbon sources used for the study, glycerol, starch and glucose promoted biomass production (11.7 - 12.1 g/L) which was statistically significant but glycerol failed to increase the P5 concentration with only 90 mg/L in comparison to that of starch and glucose with 165.7 and 135.9 mg/L of P5 concentration. The basal media (control) supported a biomass growth of 8.5 g/L with 121.2 mg/L of P5 concentration. The effect of starch and glucose were found to be statistically significant. The effect of the other carbon sources (lactose, maltose, mannitol, sucrose, dextrose and fructose) inhibited both biomass and P5 concentrations with biomass ranging from 4.6-9.7 g/L and P5 from 64.7-87.2 mg/L as shown in **Fig. 2C**.

Both organic and inorganic nitrogen sources were used in the initial screening process for increased response. With respect to biomass, malt extract, beef extract and sodium nitrate influenced the growth with 10.2, 12.8 and 11.3 g/L respectively in comparison to 8.5 g/L in control. But for P5 concentration, ammonium sulphate addition resulted in maximum yield with 163.43 mg/L followed by beef extract with 129.4 mg/L as compared to the 121.2 mg/L in control conditions. The results were found to be statistically significant. The rest of the nitrogen sources were not very effective in increasing biomass as well as P5 production as shown in **Fig. 2D**.

Amino acids, the building blocks of proteins, are very essential for all the metabolic activities that occur within the cell and hence play an important role in the growth of an organism. A concentrated solution of amino acids (100X) was used at varying concentrations to check for their effect on both biomass and bioactive metabolite production. Amino acids used at a concentration of 1.0 and 1.5X were found to be very effective in increasing the P5 concentration recorded as 242.1 mg/L and 369.2 mg/L respectively in comparison to 140.7 mg/L of P5 produced in control conditions. The amino acids had a moderate effect on biomass production with 11.1 g/L for 1X and 10.6 g/L for 1.5X amino acid concentration in comparison to the control with 9.3 g/L dry weight of biomass. The results were found to be statistically significant as shown in **Fig. 2E**.

Vitamins are also very essential nutrients important for the metabolism of any organism and it is required in very less quantities. Among the water-soluble vitamins used, vitamin B2 and B3 addition gave highly significant results for P5 production resulting in 280.3 mg/L and 297.0 mg/L respectively in comparison to 140.7 mg/L in control conditions. All vitamins had lesser effects on biomass production with vitamin B3 addition resulting in the highest dry weight of 10.8 g/L in comparison to the 9.3 g/L in control as shown in **Fig. 2F**.

Just like vitamins, metal ions (trace elements) also play a very important role in metabolism, mainly as co-factors for enzymes. The addition of metal ions such as $CuSO_4$ and $CdCl_2$ to the basal media resulted in a P5 yield of 229.2 mg/L and 255.6 mg/L respectively, in comparison to the control with 140.7 mg/L. The P5 concentration for $CaCl_2$, Pb $(C_2H_3O_2)_2$, $BaCl_2$ and $FeSO_4$ were in the range 149.4-194.2 mg/L. $NiCl_2$, $ZnSO_4$ and $MnSO_4$ additions were found to inhibit P5 production. Only

$CuSO_4$ addition resulted in 12.4 g/L dry weight of biomass in comparison to the 9.3 g/L produced in control conditions. The remaining metal ions failed to exert a positive effect on biomass production with the dry weight lesser than that of the control as shown in **Fig. 2G**.

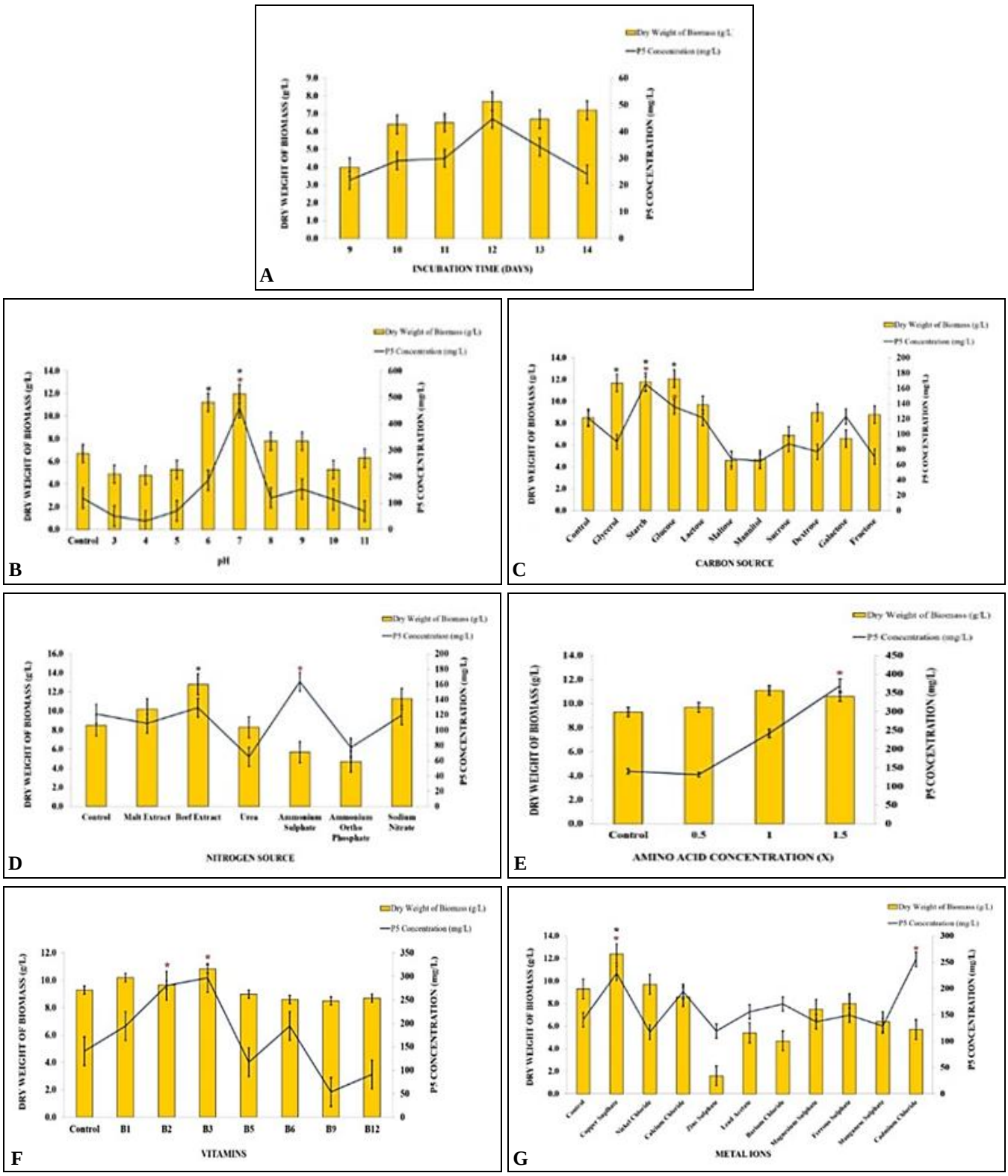

FIG. 2: SINGLE FACTOR CONDITIONS ALONG WITH BASAL MEDIA FOR MAXIMIZED P5 PRODUCTION. (A) INCUBATION TIME (B) VARYING pH (C) CARBON SOURCE (D) NITROGEN SOURCE (E) AMINO ACID CONCENTRATION (F) VITAMINS AND (G) METAL IONS. * P<0.05 INDICATES THE LEVELS OF SIGNIFICANCE IN COMPARISON TO THE CONTROL [* IN BLACK COLOUR INDICATES SIGNIFICANCE FOR DRY WEIGHT OF BIOMASS PRODUCTION AND * IN BROWN COLOUR INDICATES SIGNIFICANCE FOR P5 CONCENTRATION]

Optimization by Response Surface Methodology: Based on the single factor screening results, starch and glucose (carbon source), beef extract and ammonium sulphate (nitrogen source), 1.5X amino acid concentration, vitamins B2 and B3, metal ions $CuSO_4$ and $CdCl_2$ were shortlisted. But when a combination of factors was tested for the best responses at their central values, the combination comprising of $CdCl_2$, glucose, 1.5X amino acid concentration and beef extract yielded the best results for both biomass and P5 concentrations. Therefore, CCD was employed with these factors for enhanced responses and the matrix design with the coded values are summarized in **Table 2**.

TABLE 2: EXPERIMENTAL RANGE ALONG WITH THE CODED VALUES OF THE FOUR FACTORS USED IN CCD

Factors	Levels				
	-α	**-1**	**0**	**1**	**+α**
$CdCl_2$ (%)	0.01	0.03	0.05	0.07	0.09
Glucose (g/L)	4	7	10	13	16
Amino acid (X)	0.5	0.75	1	1.25	1.5
Beef extract (g/L)	2	6	10	14	18

*α = 2.00

A total of 30 experiments were carried out and the two responses under the present study, biomass and P5 concentration, observed due to the interaction of four factors ($CdCl_2$, glucose, 1.5X amino acid concentration and beef extract) are being listed in **Table 3** along with the predicted response generated by the software. The highest biomass was observed for the experimental run 2, with 23.9 g/L along with 637 mg/L of P5 concentration. The highest P5 concentration was observed for experimental run 8 with 774 mg/L along with 23.2 g/L of biomass. A positive correlation (R = 0.725) between the responses indicates the influence of biomass on P5 production. The data obtained was subjected to multiple regression analysis to obtain an empirical model that relates the response measured to the independent factors under the study. The second order regression equation in terms of coded factors for the responses dry weight of biomass (Eq. A) and P5 concentration (Eq. B) as suggested by the software are as follows:

$$\text{Dry weight} = 20.87 - 0.31A + 2.32B - 0.18C + 2.12D - 0.47AB - 0.77AC + 0.46AD + 0.14BC - 0.62BD + 0.15CD - 0.87A^2 - 0.41B^2 - 0.54C^2 - 0.23D^2 \quad \text{……. Eq. A}$$

$$\text{P5 concentration} = 460.50 + 1.33A + 30.50B - 14.50C + 110.17D + 2AB + 6.88AC + 22.5AD + 23.88BC + 18BD + 6.63CD - 13.15A^2 - 9.15B^2 - 18.77C^2 + 30.10D^2 \quad \text{……. Eq. B}$$

TABLE 3: CCD MATRIX OF FACTORS ALONG WITH THE OBSERVED AND PREDICTED VALUES

Run	Factors				Response 1		Response 2	
					Dry weight of Biomass (g/L)		P5 Concentration (mg/L)	
	A: $CdCl_2$ (%)	B: Glucose (g/L)	C: Amino acid (X)	D: Beef extract (g/L)	Observed	Predicted	Observed	Predicted
1	0.05	10	1.5	10	18.52	18.33	354.62	356.42
2	0.07	13	0.75	14	23.94	22.98	637.35	611.17
3	0.05	10	1	10	20.25	20.87	462.79	460.50
4	0.05	10	1	10	20.99	20.87	484.62	460.50
5	0.05	10	1	10	21.15	20.87	468.40	460.50
6	0.07	7	1.25	14	19.76	18.92	512.78	508.17
7	0.05	10	1	10	21.37	20.87	436.64	460.50
8	0.05	10	1	18	23.27	24.18	774.02	801.25
9	0.07	7	1.25	6	11.98	12.21	257.98	265.58
10	0.03	13	1.25	14	23.53	23.83	597.33	591.50
11	0.07	13	0.75	6	18.94	19.38	317.00	323.08
12	0.03	7	1.25	14	19.76	19.21	457.88	450.75
13	0.05	10	0.5	10	18.69	19.06	389.24	414.42
14	0.03	13	0.75	6	19.81	20.32	399.56	375.17
15	0.05	4	1	10	14.10	14.59	362.19	362.92
16	0.05	10	1	10	20.91	20.87	464.55	460.50
17	0.05	10	1	10	20.80	20.87	449.74	460.50
18	0.05	10	1	2	16.42	15.71	360.21	360.58
19	0.03	13	1.25	6	21.44	21.48	369.60	366.92
20	0.07	7	0.75	6	15.30	14.70	364.44	341.83
21	0.07	7	0.75	14	20.94	20.81	556.10	557.92

22	0.03	13	0.75	14	22.40	22.07	582.50	573.25
23	0.07	13	1.25	6	17.60	17.43	350.14	342.33
24	0.03	7	1.25	6	13.77	14.35	300.79	298.17
25	0.03	7	0.75	6	13.97	13.74	388.23	401.92
26	0.09	10	1	10	16.19	16.78	396.84	410.58
27	0.01	10	1	10	18.46	18.01	392.39	405.25
28	0.07	13	1.25	14	21.54	21.64	671.70	656.92
29	0.05	16	1	10	24.12	23.89	458.22	484.92
30	0.03	7	0.75	14	18.15	18.00	548.56	528.00

The statistical significance of the dry weight of biomass is being summarized in **Table 4**. The model was found to be significant for both the responses with p-value <0.0001 and the lack of fit is not significant. The R^2 value was 0.9777 with the adj. R^2 value being 0.9569. The F-value of 21.72 for the interaction between $CdCl_2$ and the amino acid was found to be the highest which suggests that these two factors have a major role with respect to an increase in biomass. But glucose as a single factor was found to have a larger effect on biomass production with an F-value of 293.22 which suggests that its interaction with other factors decreased its ability to increase the yield of biomass.

TABLE 4: ANALYSIS OF VARIANCE (ANOVA) FOR THE RESPONSE SURFACE QUADRATIC MODEL FOR THE ESTIMATION OF DRY WEIGHT OF BIOMASS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	291.06	14	20.79	46.99	< 0.0001	significant
A - CdCl2	2.28	1	2.28	5.16	0.0383	
B - Glucose	129.73	1	129.73	293.22	< 0.0001	
C - Amino acid	0.8067	1	0.8067	1.82	0.197	
D - Beef extract	107.53	1	107.53	243.03	< 0.0001	
AB	3.61	1	3.61	8.16	0.012	
AC	9.61	1	9.61	21.72	0.0003	
AD	3.42	1	3.42	7.74	0.014	
BC	0.3025	1	0.3025	0.6837	0.4213	
BD	6.25	1	6.25	14.13	0.0019	
CD	0.36	1	0.36	0.8137	0.3813	
A^2	20.7	1	20.7	46.79	< 0.0001	
B^2	4.53	1	4.53	10.23	0.006	
C^2	8.11	1	8.11	18.33	0.0007	
D^2	1.47	1	1.47	3.32	0.0886	
Residual	6.64	15	0.4424			
Lack of Fit	5.94	10	0.5943	4.29	0.0609	not significant
Pure Error	0.6933	5	0.1387			
Cor Total	297.7	29				

R^2= 0.9777, Adj. R^2 = 0.9569, Pred. R^2 = 0.8817

The statistical significance for P5 concentration is being summarized in **Table 5**. p<0.0001 and the lack of fit being non-significant indicates a significant model. The R^2 value was 0.9831 with the adj. R^2 value being 0.9673. The F-value for factor D (beef extract) was the highest with 649.79 which infers that beef extract has a major role in increasing the P5 concentration. But when it comes to interaction between factors, glucose and amino acid were predicted to have a higher influence on P5 production with the F-value being 20.35. Overall, the models for both responses were found to be good fits.

TABLE 5: ANALYSIS OF VARIANCE (ANOVA) FOR THE RESPONSE SURFACE QUADRATIC MODEL FOR THE ESTIMATION OF P5 CONCENTRATION

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.91E+05	14	27908.56	62.26	< 0.0001	significant
A-CdCl2	42.67	1	42.67	0.0952	0.7619	
B-Glucose	22326	1	22326	49.81	< 0.0001	
C-Amino acid	5046	1	5046	11.26	0.0043	
D-Beef extract	2.91E+05	1	2.91E+05	649.79	< 0.0001	
AB	64	1	64	0.1428	0.7108	
AC	756.25	1	756.25	1.69	0.2136	

AD	8100	1	8100	18.07	0.0007	
BC	9120.25	1	9120.25	20.35	0.0004	
BD	5184	1	5184	11.56	0.004	
CD	702.25	1	702.25	1.57	0.2299	
A^2	4740.01	1	4740.01	10.57	0.0054	
B^2	2294.3	1	2294.3	5.12	0.039	
C^2	9664.3	1	9664.3	21.56	0.0003	
D^2	24857.44	1	24857.44	55.45	< 0.0001	
Residual	6724	15	448.27			
Lack of Fit	5368.5	10	536.85	1.98	0.2334	not significant
Pure Error	1355.5	5	271.1			
Cor Total	3.97E+05	29				

$R^2 = 0.9831$, Adj. $R^2 = 0.9673$, Pred. $R^2 = 0.9173$

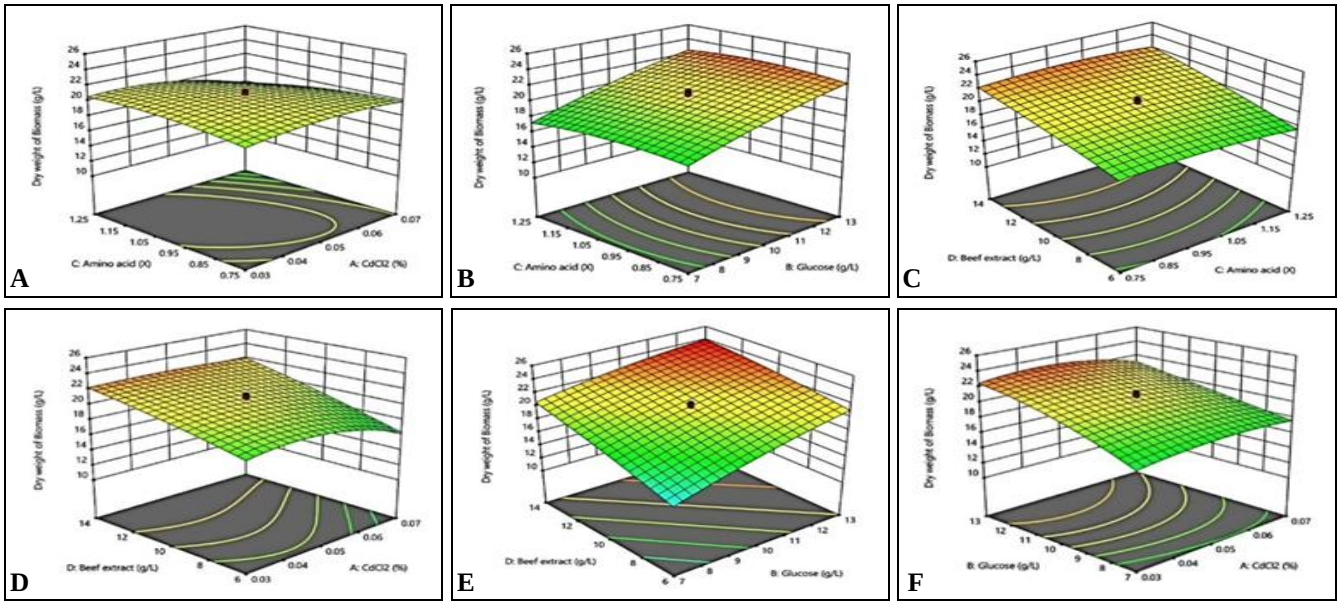

FIG. 3: 3D RESPONSE SURFACE PLOTS SHOWING THE EFFECT OF INTERACTION BETWEEN TWO FACTORS ON DRY WEIGHT OF BIOMASS WHILE KEEPING THE OTHER TWO FACTORS AT THE CENTRAL POINT. THE INTERACTION BETWEEN FACTORS ARE (A) $CdCl_2$ AND AMINO ACID (B) GLUCOSE AND AMINO ACID (C) AMINO ACID AND BEEF EXTRACT (D) $CdCl_2$ AND BEEF EXTRACT (E) GLUCOSE AND BEEF EXTRACT AND (F) $CdCl_2$ AND GLUCOSE

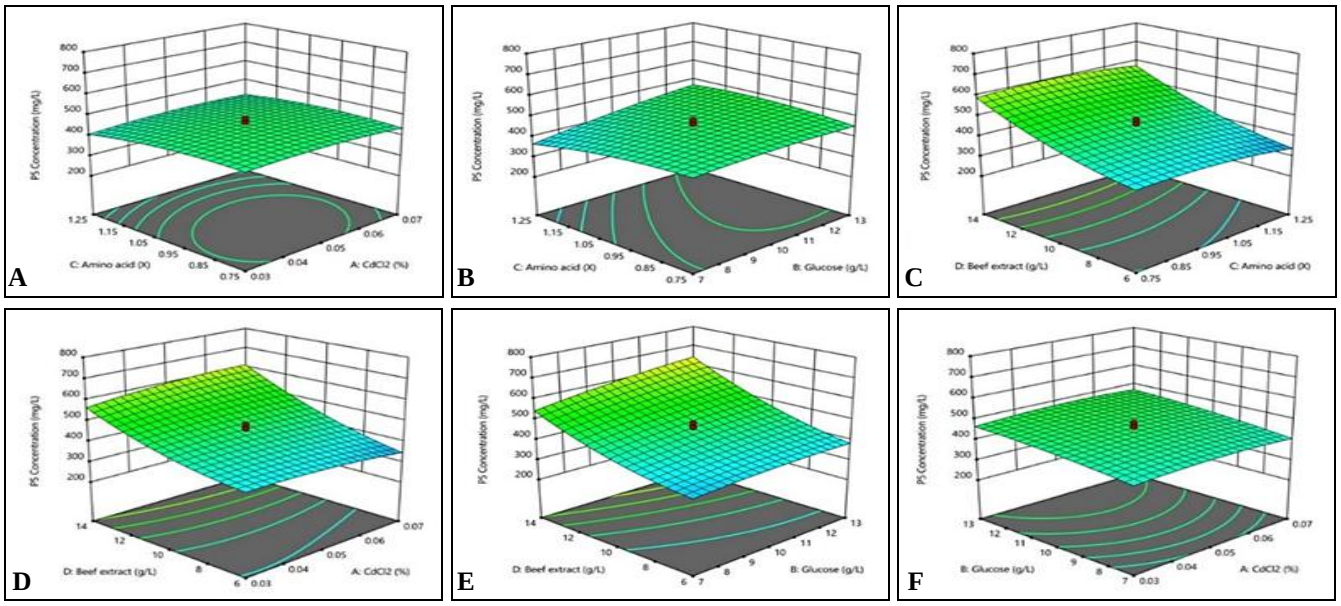

FIG. 4: 3D RESPONSE SURFACE PLOTS SHOWING THE EFFECT OF INTERACTION BETWEEN TWO FACTORS ONTHE P5 CONCENTRATION WHILE KEEPING THE OTHER TWO FACTORS AT THE CENTRAL POINT. THE INTERACTION BETWEEN FACTORS ARE (A) $CdCl_2$ AND AMINO ACID (B) GLUCOSE AND AMINO ACID (C) AMINO ACID AND BEEF EXTRACT (D) $CdCl_2$ AND BEEF EXTRACT (E) GLUCOSE AND BEEF EXTRACT AND (F) $CdCl_2$ AND GLUCOSE

The interactive effects between factors for the responses have been represented by 3D response surface plots as shown in **Fig. 3** and **Fig. 4**. The interaction between an amino acid and $CdCl_2$ had a moderate effect on both the responses as shown in **Fig. 3A** and **4A**.

The interaction between amino acid and glucose indicated that an increasing concentration of glucose greatly influenced the increase in biomass as shown in **Fig. 3B**, whereas both factors equally influenced P5 productions shown in **Fig. 4B**. The interaction between beef extract and amino acid interprets that increasing concentration of beef extract had a major role both in terms of biomass production as shown in **Fig. 3C**, as well as P5 concentration as shown in **Fig. 4C**. A similar effect was observed due to interactions of beef extract and $CdCl_2$, where beef extract played a major role with respect to both the responses as shown in **Fig. 3D** and **4D**.

The interactions between beef extract and glucose revealed that both the factors, with an increase in their concentration because an increase in biomass Production as shown in **Fig. 3E**, but in terms of P5 concentration, beef extract highly influenced the response as shown in **Fig. 4E**. The interaction between glucose and $CdCl_2$ resulted in glucose playing a major role in biomass production as shown in **Fig. 3F**, whereas the same for P5 concentration was not significant as shown in **Fig. 4F**.

Validation of Optimized Conditions: From the CCD matrix, the optimum values of factors for increased biomass production were 0.05% of $CdCl_2$, 16 g/L of glucose, 1X of amino acid concentrate and 10 g/L of beef extract. The optimum values of factors for increased P5 concentration were 0.05% of $CdCl_2$, 10 g/L of glucose, 1X of amino acid concentrate and 18 g/L of beef extract. The validation experiments were carried out in triplicates. The observed and predicted values closely match, which indicates that the generated model adequately predicts the response.

When the basal media was set up parallel to the validation experiment, the observed dry weight of biomass was 7.5 g/L and that of P5 concentration was 64 mg/L while that under optimized conditions were 24.3 g/L and 781.3 mg/L respectively. These values correspond to 3.24-fold and 12.21-fold increase respectively for biomass and P5 concentrations as shown in **Fig. 5**. Therefore, the optimized values were considered to be most suitable for increasing biomass and P5 concentrations.

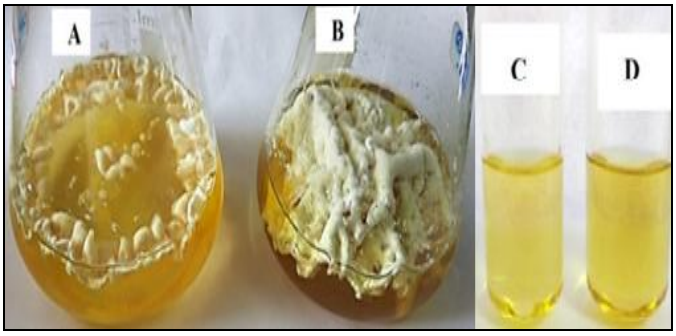

FIG. 5: PICTORIAL REPRESENTATION OF (A) BIOMASS PRODUCED UNDER CONTROL CONDITIONS (B) BIOMASS PRODUCED UNDER OPTIMIZED CONDITIONS (C) P5 CONCENTRATION UNDER CONTROL CONDITIONS AND (D) P5 CONCENTRATION UNDER OPTIMIZED CONDITIONS FROM *P. RUBENS* JGIPR9

DISCUSSION: Nature is an exhaustive resource of medicinal agents for thousands of years. Many drugs have originated from natural sources, including microorganisms which are prolific sources of structurally diverse bioactive metabolites. Some of the important products for the pharmaceutical industry like penicillin, mevastatin and lovastatin were produced by *Penicillium* sp. [11].

Penicillium rubens JGIPR9 was isolated from garden soil and was found to have promising anti-cancer properties. Since the quantity of the anti-cancer compound P5 was produced at lower quantities, we aimed to enhance the production of P5 in the present study through RSM.

Initially, the culture conditions such as incubation time, pH and temperature that would be suitable for optimal growth were evaluated. The best response was observed on the 12^{th} day of incubation at RT (24-28°C), at neutral pH 7.0.

These optimal culture conditions were maintained throughout the optimization process. Optimum growth conditions for different *Penicillium* spp. have been reported earlier as at temperatures varying from 25°C-30°C, incubation time ranging from 12 to 15 days and at neutral pH [12-14].

The single-factor system was first employed to screen for the factors that enhanced P5 production. Amongst the various nutrients/factors, starch, glucose, beef extract, ammonium sulphate, 1.5X concentration of the amino acid mix, B2 and B3 vitamins along with $CuSO_4$ and $CdCl_2$ gave an increased response both in terms of biomass and P5 production.

Certain combinations of factors were initially chosen for CCD which showed a marginal effect on the required response (data not shown). Therefore, the most favorable combination of four factors that included glucose, beef extract, 1.5 X amino acid concentration and $CdCl_2$ yielded the best results in terms of both the responses under study. There are many reports of microbial cultures that are accustomed to utilizing varying carbon and nitrogen sources for their optimal growth along with metabolite production [15-17].

The metal ion $CdCl_2$ which is considered toxic under certain conditions was influential in bringing about a positive response in our study. Several fungal species are tolerant of heavy metals by certain inherent physiological characteristics or by adapting to temporary alteration in their developmental pattern [18, 19]. Cadmium is considered to be one of the most widely distributed industrial and environmental pollutants. It exhibits carcinogenic, phytotoxic and ecotoxic effects due to its ability to inactivate enzymes containing sulphydryl groups and to inhibit oxidative phosphorylation. Micro-organisms exhibit a mechanism of resistance towards certain metal ions up to a certain limit which ultimately results in the detoxification of the metal [20]. The tolerance/survival of *Trichoderma harzianum* in the presence of cadmium ions at 1, 2 and 3 mM concentrations was reported earlier [21]. In the present study, we used a 0.05% concentration of cadmium that corresponds to 2.78 mM concentration. Since the fungus understudy has the capacity to grow in the presence of cadmium along with causing an increase in the production of P5, it could even serve an important role in the bioremediation of heavy metals.

It was reported that *Penicillium chrysogenum* XJ-1 could serve as a potential candidate for bioremediation of cadmium [22]. There are reports about *Penicillium* sp. exhibiting tolerance towards cadmium metal ions [23]. The effect of the above-mentioned factors on the responses was found to be significant with the predicted responses provided by the software. The dry weight of biomass ranged from 11.9-24.1 g/L and P5 concentration ranged from 257-774 mg/L which was found to be higher than what was observed in the single factor screening system.

Also, upon solving the regression equation, the optimal values predicted by the software were verified experimentally for the responses which were found to be significant with the observed (experimental) values. The optimal value derived for biomass was 24.1 g/L, and that of P5 concentration was 774.02 mg/L which were 3.24-fold and 12.21-fold respectively higher than that observed under unoptimized conditions.

CONCLUSION: In the current study, the optimum conditions required for the responses understudy, as determined by CCD matrix, was found to have a positive influence on enhancing the biomass and P5 production, yielding in a much higher (12.2-fold for P5 concentration, which is the one response in requirement) response when compared to the single factor approach, proving the statistical approach to be highly significant.

ACKNOWLEDGEMENT: This research was financially supported by the Department of Science and Technology – Innovation in Science Pursuit for Inspired Research (DST – INSPIRE) Fellowship (grant no. IF130879). The authors are grateful to Prof. Leela Iyengar, Adjunct Professor, JAIN (Deemed-to-be University) for her valuable suggestions towards the research work conducted. The authors are grateful to JAIN (Deemed-to-be University), Bengaluru for providing infrastructural facilities.

CONFLICTS OF INTEREST: We declare that we have no conflicts of interest.

REFERENCES:

1. Pandey PK, Singh S, Yadav RNS, Singh AK and Singh MCK: Fungal endophytes: promising tools for pharmaceutical science. Int J Pharm Sci Rev Res 2014; 25(2): 128-38.
2. Kornienko A, Evidente A, Vurro M, Mathieu V, Cimmino A, Evidente M, van Otterlo WAL, Dasari R, Lefranc F and

Kiss R: Towards a cancer drug of fungal origin. Med Res Rev 2015; 35(5): 937-67.
3. Newman DJ and Cragg GM: Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016; 79(3): 629-61.
4. Nicoletti R and Trincone A: Bioactive compounds produced by strains of Penicillium and Talaromyces of marine origin. Mar Drugs 2016; 14(2): 37.
5. Montgomery DC: Design and analysis of experiments. Wiley, Ninth Edition 2017.
6. Katapodis P, Christakopoulou V and Christakopoulos P: Optimization of xylanase production by *Thermomyces lanuginosus* in tomato seed meal using response surface methodology. World J Microb Biotec 2006; 22(5): 501-6.
7. Chattoraj S, Sadhukhan B and Mondal NK: Predictability by BoxeBehnken model for carbaryl adsorption by soils of Indian origin. J Environ Sci Health B 2013; 48(8): 626-36.
8. Aneja KR: Cultivation techniques for isolation and enumeration of microorganisms. In: Experiments in Microbiology, Plant Pathology and Biotechnology. New Age International Publishers, New Delhi, Ed 4, 2003: 154-88.
9. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65(1-2): 55-63.
10. Venkatachalam P and Nadumane VK: Modulation of Bax and Bcl-2 genes by secondary metabolites produced by *Penicillium rubens* JGIPR9 causes the apoptosis of cancer cell lines. Mycology 2019; 1-13. doi:10.1080/21501203.2019.1707315.
11. Youssef DTA and Alahdal AM: Cytotoxic and antimicrobial compounds from the marine-derived fungus, Penicillium species. Molecules 2018; 23(2): 394.
12. Chandra P and Arora DS: Optimization of antioxidant potential of *Penicillium granulatum* Bainier by statistical approaches. ISRN Microbiol 2012; Article ID: 452024. doi.org/10.5402/2012/452024.
13. Patel G, Patil MD, Soni S, Khobragade TP, Chisti Y and Banerjee UC: Production of mycophenolic acid by *Penicillium brevicompactum* - A comparison of two methods of optimization. Biotechnol Rep 2016; 11: 77-85.
14. Tannous J, Atoui A, Khoury AE, Francis Z, Oswald IP, Puel O and Lteif R: A study on the physicochemical parameters for *Penicillium expansum* growth and patulin production: effect of temperature, pH and water activity. Food Sci Nutr 2016; 4(4): 611-22.
15. Jain P and Gupta S: Effect of carbon and nitrogen sources on antimicrobial metabolite production by endophytic fungus Penicillum sp. against human pathogens. J Pharm Res 2012; 5(8): 4325-8.
16. Itoo ZA and Reshi ZA: Effect of different nitrogen and carbon sources and concentrations on the mycelial growth of ectomycorrhizal fungi under *in-vitro* conditions. Scand J For Res 2014; 29(7): 619-28.
17. Hamad HO, Alma MH, Ismael HM and Goceri A: The effect of some sugars on the growth of *Aspergillus niger*. KSU J Nat Sci 2014; 17(4): 7-11.
18. Cooke RC and Whipps JM: Constraints, limitations and extreme environments. In: R.C. Cooke and J.M. Whipps (eds.), Ecophysiology of Fungi. Blackwell Scientific Publications, London 1993: 85-110.
19. Oladipo OG, Awotoye OO, Olayinka A, Bezuidenhout CC and Maboeta MS: Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. Braz J Microbiol 2018; 49(1): 29-37.
20. Anahid S, Yaghmaei S and Ghobadinejad Z: Heavy metal tolerance of fungi. Sci Iran 2011; 18(3): 502-8.
21. Lima AF, Moura GF, Lima MAB, Souza PM, da Silva CAA, Takaki GMC and Nascimento AE: Role of the morphology and polyphosphate in *Trichoderma harzianum* related to cadmium removal. Molecules 2011; 16(3): 2486-00.
22. Xu X, Xia L, Zhu W, Zhang Z, Huang Q and Chen W: Role of *Penicillium chrysogenum* XJ-1 in the detoxification and bioremediation of cadmium. Front Microbiol 2015; 6.
23. Manguilimotan LC and Bitacura JG: Biosorption of cadmium by filamentous fungi isolated from coastal water and sediments. J Toxicol 2018; Article ID: 7170510. doi.org/10.1155/2018/7170510.

How to cite this article:
Venkatachalam P and Nadumane VK: Optimization of an anti-cancer metabolite production by *Penicillium rubens* JGIPR9 *via* response surface methodology. Int J Pharm Sci & Res 2020; 11(5): 2299-09. doi: 10.13040/IJPSR.0975-8232.11(5).2299-09.