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ENHANCEMENT OF BIOACTIVE CRUDE COMPOUND PRODUCTION BY STATISTICAL OPTIMIZATION OF FERMENTATION PARAMETERS FROM *STREPTOMYCES SP.* NLKPB45 ISOLATED FROM MARINE SOIL SEDIMENT

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ABSTRACT: Actino bacterium *Streptomyces sp*. NLKPB45, which was isolated from mangrove soil sediments of Nellore coastal regions of Andhra Pradesh, India, was used in shake-flask fermentation to explore the different carbon and nitrogen substrates for antimicrobial crude compound. Careful experimentation of ranging each variable in the fermentation medium found that glucose and peptone as preferred carbon and nitrogen sources at 1% (w/v) concentration each, at pH of 7 and 28 °C incubation temperature for 8 days of incubation time. Response surface methodology-based statistical optimization of the process variables with their interconnection was analyzed using a central composite design. A second-order quadratic polynomial equation yielded a complacent fit for experimental with respect to crude compound concentration. Analysis of variance statistics showed that significant-high R2 value for model and adjusted and predicted; R2 values were also shown decent agreement with observed and predicted results. The results have confirmed very good interaction among five process variables at optimized values of 7.51318 pH, 28.734 °C incubation temperature, 8.18409 days incubation time, 1.05185 g/L glucose and 1.0483 g/L peptone yielded 4.5873 g/L of crude compound concentration, which is about 20% increase due to statistical optimization by *Streptomyces sp*. NLKPB45

INTRODUCTION: Aquatic eco-system had been unbolted in modern vistas to spot novel organisms that would become a hefty source of clinically important bioactive secondary metabolites.

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The growth of action mycetes and synthesis of bioactive compounds are influenced by power of strain, nutrients in medium and process conditions because metabolic activities of microorganisms are affected by their surrounding environment [11, 12, 13]. Conventional optimization of microbial fermentation has studied One-Factor-At at-Time (OFAT), which was painstaking and tedious.

In addition, these non-statistical methods did not attempt the interactive effects of the selected variables and misled to erroneous conclusions, so it

is critical to maintaining the optimum process conditions which would yield the highest bioactive metabolite quantities with functional characteristics retained [14]. The other statistical methods, such as Full-factorial design and Taguchi design, did not identify second-order effects and estimate interactive effects of process variables, respectively [12]. Response Surface Methodology (RSM) was known to be a better statistical tool in systemic materializing the process variables for optimizing the many bioactive metabolite productions in submerged and solid-state fermentation [15, 16, 17].

Central Composite Design (CCD) of RSM provides the minimum number of replicates that execute the interactive knowledge of major variables and their viable interactions amid them [18, 19, 20]. Hence, the present study has been conducted to optimize the individualistic process factors using OFAT and CCD program of RSM which estimates the optimal values of process variables and their interactive effects of individual parameters for the bioactive metabolite crude concentration by *Streptomyces sp*. NLKPB45 isolated from coastal marine sediments.

MATERIALS AND METHODS:

Chemicals and Reagents: The chemicals and bio chemicals such as Casein powder, Starch, Agar, Glucose, Fructose, Lactose, Xylose, Sucrose, Peptone, Sodium Nitrate, Sodium Nitrite, Urea, Ammonium Chloride and reagents Seawater, Ethyl Acetate, Dimethyl Sulphoxide of analytical grade were commercially procured from M/s. Merck, India.

Isolation and Screening of Marine Actino-mycetes for Bioactive Metabolite Production: Mangrove soil sediment samples were collected from the territory of Krishna Patnam port at SPSR Nellore district, Andhra Pradesh, India, using a sterile spatula into labelled aseptic jars. Further, one gram of this soil sample was mixed with 10 ml of sterile distilled water, and suspension was serially diluted. Potential actinomycetes were primarily screened using spread starch casein agar plate method for their antagonistic activity by cross streak technique against bacterial pathogen (as zone of inhibition assay). In their secondary screening, filtrate of Actinomycetes culture was compared for antibacterial activities against streptomycin antibiotic, as per our previous method [21].

Production of Antimicrobial Compounds by Submerged Fermentation: After careful screening and molecular characterization, the potential actinomycetes *Streptomyces sp*., NLKPB45 was subcultured in 50 ml of Starch Casein broth medium, and the same was used as inoculum for submerged fermentation studies. All the fermentations were performed in 250 ml Erlenmeyer flask containing 100 ml fermentation medium. 1 ml cultured suspension was inoculated into sterile media flasks, and inoculated flasks were incubated at 28 °C on a rotary shaking incubator at 250 rpm.

Fermentation Variables Screening By OFAT Approach: To evaluate the growth of *Streptomyces sp*., NLKPB45, and release of crude bioactive metabolite extract, the factors affecting fermentation were screened using the One-Factor-At a-Time approach was attempted. Various carbon substrates like Glucose, Fructose, Lactose, Xylose, and Sucrose were studied, each at 1% (w/v) concentration; and different nitrogen sources such as Peptone, Sodium Nitrate, Sodium Nitrite, Urea, Ammonium Chloride were also used each at 1% (w/v) concentration.

To analyze the physical factors influencing the fermentation, culture medium pH (at 4.0, 5.0, 6.0, 7.0, 8.0), incubation temperature (at 19, 22, 25, 28, 31, 35 °C) and incubation time (at 4, 5, 6, 7, 8, 9, 10 days) were also varied. After each fermentation study, the broth was collected and extracted for bioactive metabolite, as per our previous method [21]. The extracted antimicrobial metabolite crude concentrations (in g/l) were recorded and analyzed.

Optimization of Metabolite Production Using RSM Experimental Design: Central Composite Design of Response Surface Methodology using Design Expert® software v.11.0 (StatEase® Inc., Minnepolis, USA) was designed to show and interpret the issues in the fermentation process which impacts by a few factors and their interaction to yield optimal concentrations of the antimicrobial metabolites by *Streptomyces sp*., NLKPB455. From the initial OFAT experiments, medium pH (A), medium temperature (B), incubation time (C), concentrations of glucose (D), and peptone (E) were chosen for optimum metabolite production (response).

The range and ideal coded levels of five process variables (A, B, C, D and E) were presented in **Table 1**. In total, 50 experiments were calculated using the following equation, which includes 25 full factorial CCD of five variables comprised of 32 factorial points 10 axial points and 8 replicates as per the following equation (1):

$$N = 2n + 2*n + nc = 25 + 2 * 5 + 8 = 50$$

Where N is the total number of experiments to be performed, n is number of process variables, NC is the number of replicated at center points. The total design matrix of CCD, comparison of experimental versus predicted metabolite crude concentrations (responses) are tabulated in **Table 2**. The results obtained from experiments were exactly fitted into the second-order polynomial model through the RSM.

TABLE 1: CODED AND ACTUAL FACTORS AND THEIR EXPERIMENTAL RANGES

Factors	Code	Coded factors actual levels		
		-1 **(Low)**	***0*** **(Middle)**	***+1*** **(High)**
Medium pH	*A*	4	7	10
Incubation Temperature, ^{0}C	*B*	19	28	37
Incubation time, days	*C*	6	8	10
Glucose concentration, g/L	*D*	0.5	1.0	1.5
Peptone concentration, g/L	*E*	0.5	1.0	1.5

Statistical Analysis using ANOVA: To evaluate the significance of variables for metabolite production by *Streptomyces sp*., NLKPB45 and their impact against the crude compound concentration, the statistical analysis using Analysis of Variance (ANOVA) was done.

Model parameters adequacy in terms of fit and prediction accuracy was checked by estimating the correlation coefficient (R2), adjusted R2, Coefficient of Variance (CV), and Fisher's test (F-test) value. Central points were used to determine the experimental errors, and data reproducibility and randomization of the experimental sequence were also done to reduce the effect of the uncontrolled factors. A quadratic regression equation was obtained with each variable yielding an empirical model which correlates the response (crude concentration) to each of five variables, as per the following equation [2, 14].

$$Y = \beta_0 + \Sigma^{\eta}_{i} = 1\ \beta_1 X_1 + (\Sigma^{\eta} = 1\ \beta_1 X_1)^2 + \Sigma^{n-1}{}_{i=I}\Sigma^{\eta}{}_{I} = I + I\ \beta_{ij}\ X_1 X$$

Where Y is Predicted response, β0 is intercept coefficient, βi is linear coefficient, βij is interaction coefficients, βii is quadratic coefficients, Xi and Xj are coded values of the five additive variables [25].

TABLE 2: EXPERIMENTAL DESIGN USED IN CCD FOR PRODUCTION OF CRUDE COMPOUND BY *STREPTOMYCES SP.*, NLKPB45

Run no.	Medium pH	Incubation Temperature (^{o}C)	Incubation Time (days)	Concentration of		Concentration of crude compound	
				Glucose (g/L)	Peptone (g/L)	Experimental (g/L)	Predicted (g/L)
1	10	37	6	1.5	1.5	2.1 ± 0.39[§]	1.7
2	7	28	3.24317	1	1	0.21 ± 0.09	0.222
3	-0.135243	28	8	1	1	1.21 ± 0.18	1.38
4	10	37	6	0.5	1.5	1.45 ± 0.07	1.57
5	10	19	10	0.5	1.5	1.45 ± 0.11	1.31
6	10	19	6	0.5	0.5	0.97 ± 0.034	0.8464
7	7	28	8	1	1	4.56 ± 0.039	4.58
8	4	19	6	1.5	1.5	0.89 ± 0.102	0.7286
9	10	37	10	1.5	0.5	2.12 ± 0.343	1.77
10	10	19	6	0.5	1.5	1.45 ± 0.029	1.41
11	7	28	8	1	-0.189207	0.23 ± 0.187	0.0939
12	7	28	8	-0.189207	1	0.64 ± 0.114	0.6685
13	4	37	10	1.5	1.5	0.78 ± 0.241	1.00
14	7	28	8	1	1	4.56 ± 0.017	4.58
15	4	37	6	0.5	0.5	0.84 ± 0.102	0.7323
16	14.1352	28	8	1	1	2.09 ± 0.002	2.07

17	7	28	8	1	1	4.54 ± 0.005	4.58
18	10	19	10	1.5	1.5	1.12 ± 0.012	1.15
19	4	19	10	0.5	1.5	1.45 ± 0.023	1.28
20	4	37	10	0.5	0.5	0.78 ± 0.024	0.7645
21	10	19	6	1.5	1.5	0.93 ± 0.004	1.06
22	7	28	8	1	1	4.58 ± 0.001	4.58
23	7	28	8	1	1	4.57 ± 0.18	4.58
24	7	28	8	1	1	4.58 ± 0.002	4.58
25	4	19	10	0.5	0.5	0.97 ± 0.12	1.09
26	10	37	6	0.5	0.5	0.92 ± 0.105	0.8004
27	4	19	10	1.5	1.5	0.79 ± 0.121	0.6484
28	10	37	10	0.5	0.5	0.84 ± 0.224	1.01
29	10	19	10	0.5	0.5	0.97 ± 0.005	0.9111
30	7	28	8	1	1	4.55 ± 0.004	4.58
31	10	37	10	0.5	1.5	1.45 ± 0.042	1.6
32	10	37	10	1.5	1.5	2.14 ± 0.15	1.93
33	7	49.4057	8	1	1	0.43 ± 0.183	0.5891
34	4	19	6	0.5	1.5	1.45 ± 0.11	1.56
35	7	28	8	1	2.18921	0.42 ± 0.215	0.708
36	10	19	10	1.5	0.5	0.97 ± 0.135	1.2
37	4	37	6	0.5	1.5	1.45 ± 0.102	1.29
38	4	37	6	1.5	0.5	0.84 ± 0.08	0.8244
39	7	28	8	2.18921	1	0.47 ± 0.124	0.5934
40	7	28	8	1	1	4.58 ± 0.002	4.58
41	4	19	10	1.5	0.5	0.97 ± 0.122	0.9001
42	4	37	6	1.5	1.5	0.94 ± 0.004	0.9427
43	4	19	6	0.5	0.5	0.97 ± 0.127	1.2
44	10	19	6	1.5	0.5	0.97 ± 0.312	0.9361
45	7	6.59427	8	1	1	0.23 ± 0.004	0.2228
46	4	19	6	1.5	0.5	0.97 ± 0.122	0.8104
47	4	37	10	1.5	0.5	1.12 ± 0.182	1.05
48	4	37	10	0.5	1.5	1.45 ± 0.21	1.16
49	7	28	12.7568	1	1	0.23 ± 0.131	0.37
50	10	37	6	1.5	0.5	0.93 ± 0.124	1.37

§ - mean ± standard deviation of 5 experiments ($p < 0.005$)

3-D Surface Interaction Plots: The ANOVA data obtained from statistical analysis, the second-order polynomial equation were shown as 3-D surface interaction plots to view the relationships between independent variables and the response (dependent variable) used in the CCD. These response surface and contour plots visualize interactive effects among the dependent variable in the capacity of two independent variables where the third variable is kept constant (Coded terms). So, the developed 3-D plots would accurately represent the interaction among variables in a geometrical sense and provide the correctness of the system behavior data [22].

RESULTS AND DISCUSSION: Selection of Carbon and Nitrogen sources for optimal yield of crude compound in the present study antimicrobial (bioactive metabolite) crude compound was produced using *Streptomyces sp*. NLKPB45 in submerged fermentation using different carbon and nitrogen substrates in the medium. The effect of carbon sources on the crude compound was studied using Fructose, Lactose, Glucose, Xylose, Sucrose, and Starch that were added at 1% (w/v). **Fig. 1A** shows that the addition of Glucose (2.39 g/l), Fructose (2.11 g/l), and Sucrose (2.01 g/l) significantly influenced the crude compound production, after 8 days of incubation at 28 °C.

Our results confirmed that Glucose emerged as an efficient carbon source on crude compound production. Similar findings were also reported in antibiotics production by *Streptomyces sp*. AS4 [5], *S. hygroscopicus* and *S. griseus* in solid state fermentation [24, 25, 26]. To understand the optimum level of Glucose in the fermentation media, Glucose concentration was varied from 0.25 to 1.5 g/l in the flask, and broth was analyzed for crude compound yield after 8 days of fermentation **Fig. 1B**. Surprisingly, the highest crude compound concentration (2.4 g/l) was achieved at 1.0 g/l glucose concentration and confirmed that the

optimum carbon substrate concentration to be maintained at this level. Additionally, different nitrogen substrates (Sodium Nitrite ($NaNO_2$), Peptone, Sodium Nitrate ($NaNO_3$), Urea and Ammonium Chloride (NH_4Cl)) were also added to media at 1% (w/v) to study their influence on bioactive metabolite production. Amon these, Peptone yielded high value (3.19 g/l) of crude compound concentration **Fig. 1C**. Recently, have also reported that there was the critical effect of nitrogen sources on the growth of *Streptomyces sp*. bacteria and antibiotics production [5].

To study the influence of nitrogen source concentration on the crude compound increased levels (0.25 - 1.5 g/l) of peptone was also added to the media and found that the highest concentration of crude was also obtained at 1.0 g/l (**Fig. 1d**).

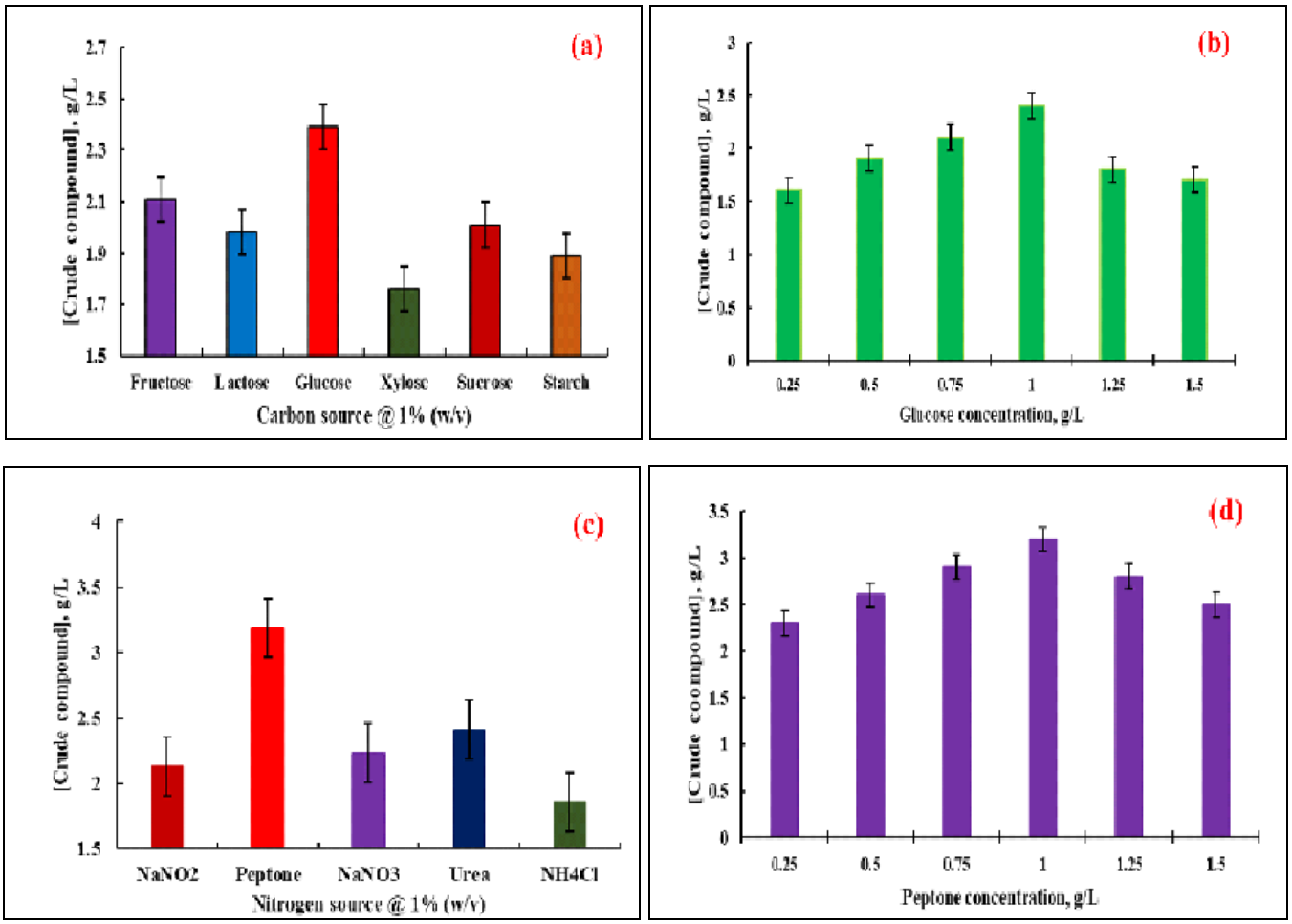


FIG. 1: EFFECT OF (A) SIMPLE AND COMPLEX CARBON SOURCE TYPE, (B) GLUCOSE LEVEL, (C) ORGANIC AND INORGANIC NITROGEN SOURCE TYPE, (D) PEPTONE LEVEL ON THE PRODUCTION OF CRUDE COMPOUND BY *STREPTOMYCES SP.* NLKPB45

Selection of Fermentation Conditions using OFAT: Optimization of fermentation conditions like medium pH, incubation time, and temperature are necessary to improve the synthesis of antimicrobial compounds [27, 28, 29, 17, 5].

Optimum pH, incubation time, and incubation temperature for crude compound production by *Streptomyces sp*. NLKPB45 were estimated in an increased range of conditions (pH: 4 - 10; time: 4 - 10 days; temperature: 160 - 37 °C) and are presented in **Fig. 2A, C**. Highest crude compound was observed at an incubation temperature 28 °C (3.2 g/l), incubation time 8 days (3.6 g/l), and pH 7.0 (4.2 g/l). These results were in accordance with the fact that extreme fermentation conditions were highly unfavorable for crude compound production.

Similar observations were also found in the production of the antibiotic by *Streptomyces galbus* 30, *Streptomyces SP*-AZ-SH-2931, *Streptomyces sp*. JAJ06 32, 33, *Streptomyces sparsus* VSM-30 (Ushakiranmayi *et al.*, 2017), *Streptomyces sp*. AS4 [5].

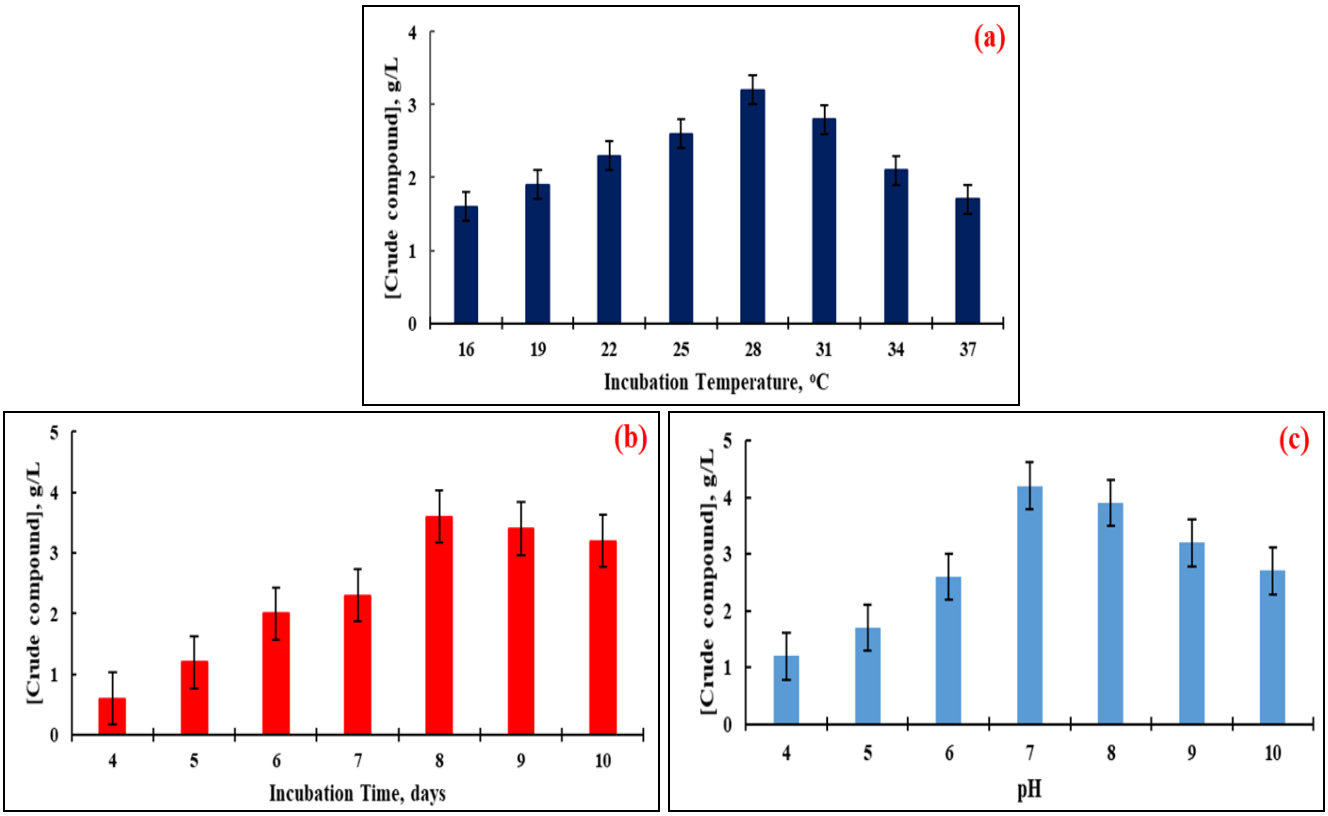


FIG. 2: EFFECT OF DIFFERENT (A) INCUBATION TEMPERATURE, (B) INCUBATION TIME, (C) MEDIUM PH ON THE PRODUCTION OF CRUDE COMPOUND BY *STREPTOMYCES SP*. NLKPB45.

RSM Optimization Studies and Model Adequacy for the Bioactive Metabolite Production: Many researchers worked on antimicrobial compounds research have attempted RSM as a statistical method to recognize, manipulate and optimize the influencing medium constituents and fermentation conditions and reported increased bioactive metabolites by *Streptomyces sp.* [34, 35, 36, 37, 17, 5]. A total of 50 experiments were conducted as per CCD **Table 2,** and crude compound concentrations were estimated to study the impact of five variables.

The suggested quadratic model analyses the sum of squares (SoS), degrees of freedom (df), mean square, F- and p- values were tabulated in **Table 3**. The model is found to be significant with moderate SoS (92.64), d_f (20), low mean square (4.63), high F- value (105.77), the least p-value (<0.0001) for the response (Crude compound concentration).

A significant value (252.22) of lack of fit (LoF) indicates that there is only a 0.01% chance that LoF occurred due to noise. Further, fit statistics (in **Table 3** also indicated a very good fit of model R2 (0.9865), and the predicted R2 (0.9515) was also in reasonable agreement with adjusted R2 (0.9772), confirmed that their difference is less than 0. 238, 39, 17, 12, 40, 5. An adequate precision (33.06, which is much greater than 4) is also a highly needed signal model to navigate the design space. The second-order polynomial model regressed by only taking significant variables, shown in the following equation (3):

[Crude compound] = + 4.58 + 0.1434A + 0.0770B + 0.0311C - 0.0158D + 0.1291E + 0.1056AB + 0.0431AC + 0.1200AD + 0.0519AE + 0.035BC + 0.1206BD + 0.05BE + 0.0494CD - 0.0425CE - 0.1106DE - 0.5041A^2 - 0.7374B^2 - 0.7569C^2 - 0.6977D^2 - 0.7383E^2

TABLE 3: QUADRATIC MODEL'S FITTING AND ANALYSIS OF ANOVA (FIT STATISTICS) RESULTS FOR CRUDE COMPOUND PRODUCTION BY *STREPTOMYCES SP*. NLKPB45

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	92.64	20	4.63	105.77	*< 0.0001*	*significant*
A-pH	0.8912	1	0.8912	20.35	< 0.0001	
B-Temperature, ^{0}C	0.2569	1	0.2569	5.87	0.0219	

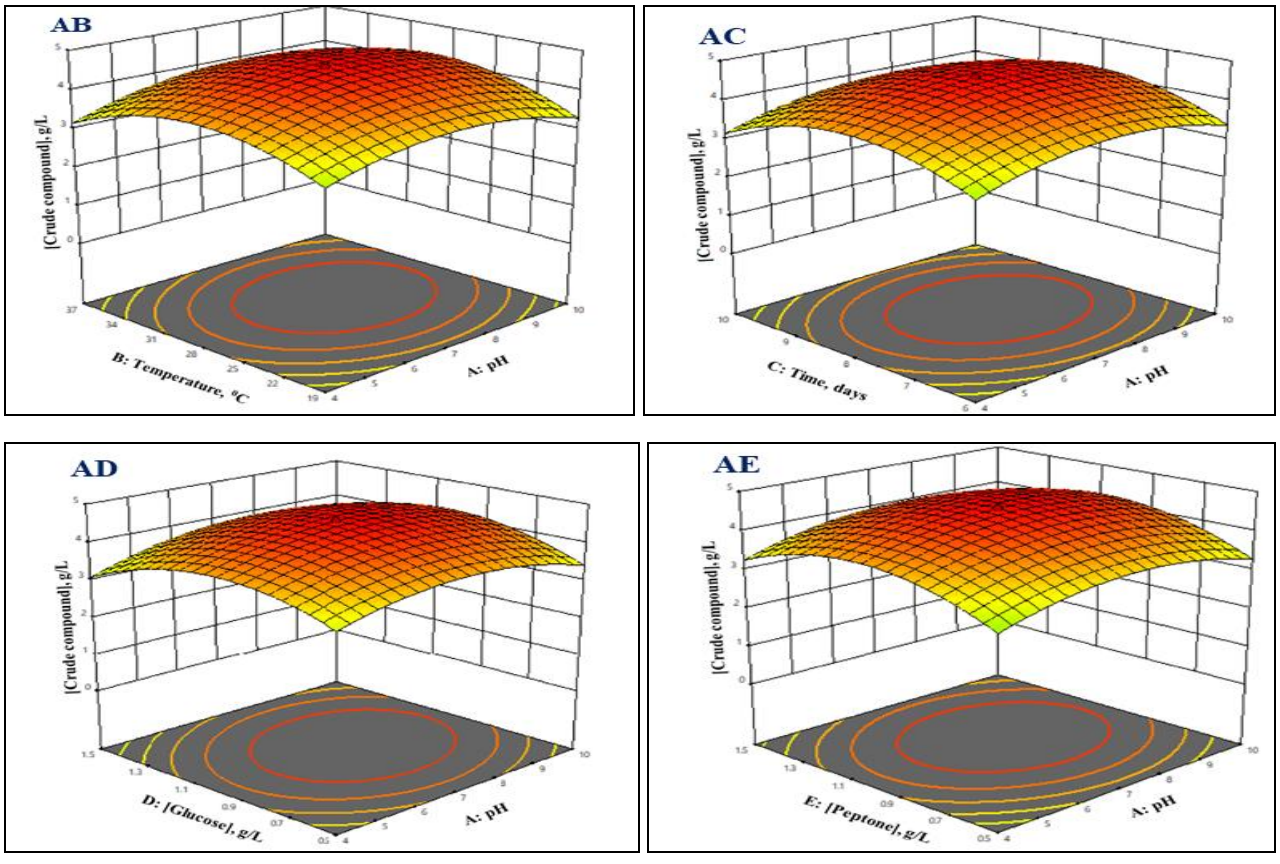
C-Time, days	0.0419	1	0.0419	0.9574	0.3359	
D-[Glucose], g/L	0.0108	1	0.0108	0.2469	0.6230	
E-[Peptone], g/L	0.7219	1	0.7219	16.49	0.0003	
AB	0.3570	1	0.3570	8.15	0.0079	
AC	0.0595	1	0.0595	1.36	0.2532	
AD	0.4608	1	0.4608	10.52	0.0030	
AE	0.0861	1	0.0861	1.97	0.1714	
BC	0.0392	1	0.0392	0.8952	0.3519	
BD	0.4656	1	0.4656	10.63	0.0028	
BE	0.0800	1	0.0800	1.83	0.1869	
CD	0.0780	1	0.0780	1.78	0.1923	
CE	0.0578	1	0.0578	1.32	0.2600	
DE	0.3916	1	0.3916	8.94	0.0056	
Residual	1.27	29	0.0438			
Lack of Fit	1.27	22	0.0577	252.22	*< 0.0001*	*significant*
Pure Error	0.0016	7	0.0002			

Fit Statistics			
Standard Deviation	0.2093	R^2	0.9865
Mean	1.600	Adjusted R^2	0.9772
C.V.%	13.06	Predicted R^2	0.9515
		Adequate Precision	33.0606

Analysis of Three-Dimensional Surface Plots: Fig. 3 shows the 3-D response surface graphs for the crude compound concentration generated by the predicted central composite design quadratic model.

Each plot in **Fig. 3** assisted in illustrating the pairwise interaction (AB, AC, AD, AE, BC, BD, BE, CD, CE, and DE) of all the five factors, while the rest was held at the middle level.

The analysis of these plots had shown that all the five parameters recorded a positive effect at the beginning and reached a maximum crude concentration, and a further increase in variables levels has shown a negative effect.



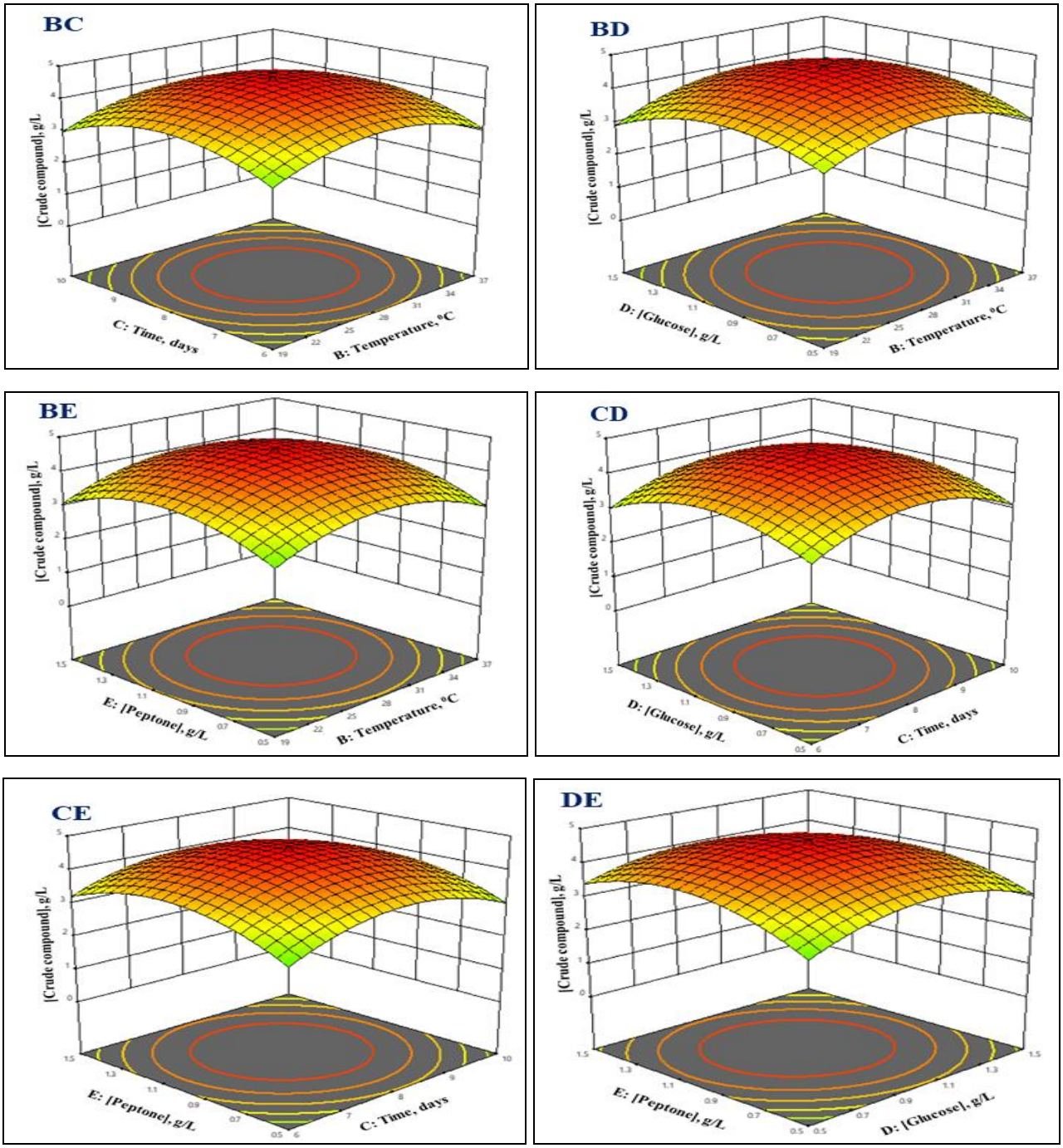


FIG. 3: 3-D SURFACE RESPONSE PLOTS SHOWING THE INTERACTIVE (AB, AC, AD, AE, BC, BD, BE, CD, CE AND DE) EFFECTS OF SELECTIVE VARIABLES ON THE PRODUCTION OF THE CRUDE COMPOUND CONCENTRATION (IN G/L) BY *STREPTOMYCES SP*. NLKPB45

Numerical Optimization Analysis: Using numerical optimization technique of CCD, comparison of predicted and experimental crude compound concentration was reported by Pareto plot **Fig. 4A**.

It could be evident from **Fig 4B,** the optimal crude compound concentration of 4.5873 g/L was observed at 7.51318 pH, 28.734 °C incubation temperature, 8.18409 days incubation time 1.05185 g/L glucose, 1.0483 g/L peptones. In order to validate the statistical model, experiments (in triplicate) were conducted and verified that the experimental value (4.58 g/L) was very close to the model-predicted value (4.58733 g/L).

This optimized medium and its conditions have increased crude compound concentration upto 20% higher than the unoptimized medium and conditions.

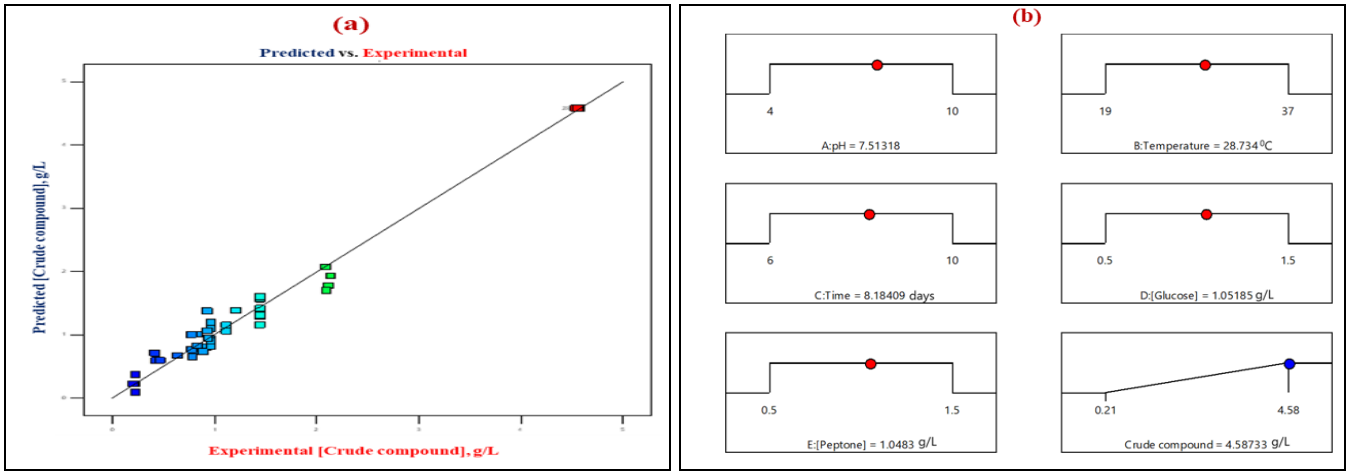


FIG. 4: (A) OBSERVED PARETO PLOT SHOWING PREDICTED VERSUS EXPERIMENTAL RESPONSES (B) SUMMARY OF CRITERIA SET FOR OPTIMIZATION RUN. RAMPS SHOWING PREDICTED VARIABLES LEVELS WITHIN THEIR RANGES AND OPTIMIZED CRUDE COMPOUND CONCENTRATION OBTAINED FROM *STREPTOMYCES SP.* NLKPB45

CONCLUSION: A novel *Streptomyces sp*. NLKPB45 was grown on different carbon and nitrogen sources to understand the better medium constituents for antimicrobial compound production. Further, using non-statistical OFAT method, critical experimental conditions were studied by performing shake-flask fermentation at 28 °C for 8 days. In addition, CCD (of RSM) based experimental study was conducted to determine the significant process variables *viz.*, medium pH, incubation time and temperature, concentrations of glucose and peptone, and their interactions, in an efficient way. Statistical parameters of the obtained quadratic model were adequate, precise, and in good agreement with the experimental data in near concurrence with the predicted values. High values of model R2, predicted R2, adjusted R2 values, and adequate precision, and low value of CV % confirmed the accurate fit between observed and modeled data. The numerical optimization with optimal levels of five variables enhanced the crude compound concentration by up to 20% by *Streptomyces sp*. NLKPB45 strain isolated from marine sediments in coastal Bay of Bengal of Andhra Pradesh. The optimized medium constituents and conditions identified from this study will be attempted in large-scale fermentations for efficient antimicrobial compounds from this new bacterium *Streptomyces sp*. NLKPB45.

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