Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454

Vol. 23 | No. 3 | pp.1517-1526 | 2024

Original Research Paper

https://doi.org/10.46488/NEPT.2024.v23i03.021

Open Access Journal

Enhanced Phenanthrene Biodegradation by *Bacillus brevis* Using Response Surface Methodology

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 22-11-2023
Revised: 10-01-2024
Accepted: 21-01-2024

Key Words:
Polycyclic aromatic hydrocarbons
Phenanthrene
Response surface methodology
Box-Behnken design
Biodegradation

ABSTRACT

The current investigation assessed the capability of a well-adapted and enriched bacterial strain known as *Bacillus brevis* for the biodegradation of phenanthrene. To enhance the removal efficiency of phenanthrene, employed Response Surface Methodology (RSM) in conjunction with a Box-Behnken design (BBD) model. The experiments were designed to explore the impact of pH (6.0 to 9.0), temperature (20 to 40°C), initial phenanthrene concentration (50 and 100 ppm), and incubation time (7 to 21 days) on biodegradation of phenanthrene. The highest level of phenanthrene biodegradation, approximately 55.0%, was achieved by *Bacillus brevis* when the optimal conditions were met as pH of 7.0, temperature 30°C, and initial phenanthrene concentration (70 ppm) after 21 days of incubation time. This study underscores the significance of employing statistical tools like RSM to enhance the microbial degradation of contaminants.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a class of chemical compounds characterized by the fusion of at least two aromatic rings, and they are classified as persistent organic pollutants. PAHs are ubiquitous organic contaminants with a natural presence in fossil fuels like petroleum and coal. They come into contact with the environment through incomplete combustion of organic materials such as waste incineration, the combustion of wood and vegetation, petroleum refining, volcanic eruptions, and forest fires (Barbosa et al. 2023, Cao et al. 2023). In 1983, the United States Environmental Protection Agency (USEPA) designated 16 PAHs as priority pollutants due to their prevalence in high concentrations, increased exposure levels, persistent characteristics, and toxicity (Dai et al. 2022). Because of their stubborn characteristics, environmentalists are greatly concerned about their long-term persistence (Urana et al. 2021). Prolonged exposure to PAHs via the consumption of contaminated food and inhalation of polluted air can have detrimental impacts on human health (Domingo et al. 2020, Marquès et al. 2020). The level of contamination and the current environmental circumstances will determine how the PAH decontamination strategy is implemented. Bioremediation employs a wide variety of microorganisms, and their selection depends on biotic and abiotic variables (Patel et al. 2020).

Phenanthrene (Phe) stands out among PAHs as a subject of frequent research interest. This is due to its possession of both K and bay regions, recognized as fundamental structures associated with carcinogenicity and mutagenicity in many high molecular weight PAHs (Gu et al. 2023). Its pronounced hydrophilic nature and excellent water solubility render it easily detectable in aqueous environments (Ghosal et al. 2016). So, Phe stands out as the ideal choice as a model for laboratory research on PAHs.

Historically, researchers employed a one-factor-at-a-time approach to optimize multi-variate systems, which proved inadequate in revealing the interplay of factors and their effects (Swati et al. 2019). To circumvent this issue, we utilized Response Surface Methodology (RSM), a widely recognized statistical multivariable experimental approach. RSM serves as a potent tool for the Design of Experiments (DOE) aimed at optimizing processes through modeling techniques. This methodology comprises a set of statistical

and mathematical techniques employed to establish a series of experimental designs, enabling the determination of optimal conditions based on input variables to predict the corresponding output variables (Massoudinejad et al. 2016, Khuri 2017). This study involved the selection of four key factors (pH, temperature, initial Phe concentration, and incubation time) to optimize a single response, which is the percentage degradation of Phe by using the Box-Behnken design.

MATERIALS AND METHODS

Chemicals and Solvents

From Sigma Aldrich Chemicals in Germany, all PAHs (phenanthrene, acenaphthene, anthracene, fluoranthene, and pyrene) with 99.0% purity analytical standard were purchased. All chemicals were used without further purification.

Enrichment of Bacterial Strain and Biodegradation Study

The nutrient broth (NB) medium containing ($g.L^{-1}$) glucose10.0, peptone 5.0, yeast extract 3.0, and NaCl 5.0 were used for the proliferation and culturing of Phe degrading microorganisms. Bacterial strain (*Bacillus brevis*) was taken from the laboratory and acclimatized with the same enrichment media, which was used for further study. 1 mL bacterial inoculum was inoculated into a 250 mL conical flask containing 100 mL sterilized Nutrient broth (NB) media amended with 0.01% Phe. It was shaken for five days at 28°C and 120 rpm in a shaker cum-incubator. After five days, enrichment was made by serially subculturing in the same medium using 10% inoculum from the previous culture. The enrichment action was repeated for 3 months and the next three months with a 0.01% mixture of five PAHs (phenanthrene, acenaphthene, anthracene, fluoranthene, and pyrene). The Phe biodegradation study was carried out in 100 mL sterilized nutrient broth (NB) in 250 ml Erlenmeyer flasks. Variables were incubated at 120 rpm as given by the model.

Process Optimization for Phenanthrene Degradation

The optimization process for achieving maximum Phe degradation, a dependent response, involved selecting various independent variables such as pH, temperature, initial Phe concentration, and incubation time. This optimization was carried out using the Box-Behnken design (BBD) within the Stat-Ease Design Expert software. The BBD serves as an excellent design tool for assessing the presence of a lack of fit when a sufficient number of experimental values are available. The experimental region extended from –1 to +1 in terms of coded variables (Table 1). Four variables studied were pH (6.0-9.0), temperature (20-40°C), initial Phe concentration (50-100 ppm), and incubation time (7-21 days) to obtain the response, i.e., biodegradation (*Y*). The experimental design was implemented following the determination of the range for each variable (maximum and minimum), and the model designed a total of twenty-nine experiments. The experimental results yielded quantitative data, which was utilized to establish a regression model equation and identify the optimal operational parameters for achieving the highest desired response. RSM enables the quantitative representation of individual process parameters in Eq. (1):

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad \ldots(1)$$

where *Y* represents the predicted response, while b_0 stands for the response function, X_i and X_j denote the coded experimental variables, respectively. A second-order polynomial regression model was employed to fit experimental data and derive the Phe biodegradation equation. This model included four linear (X_i), four quadratic (X^2_i), six interaction (X_iX_j), and a constant (b_0). Utilizing the software, an Analysis of Variance (ANOVA) was performed ($p < 0.05$), and the coefficient of regression (R) was determined to assess the significance and goodness of fit of the model (Al Farraj et al. 2019). The quadratic equation Eq. (2) was used to assess the second-order polynomial mathematical connection between the response variable *Y* and four factors, i.e., pH, temperature, initial Phe concentration, and incubation period.

Table 1: Experimental factors and their levels used in the BBD design.

Coded factor	Actual factor	Units	Actual level			Coded level		
			Low	Middle	High	Low	Middle	High
A	pH		6	7.5	9	-1	0	1
B	Temperature	°C	20	30	40	-1	0	1
C	Initial Phe concentration	Ppm	50	75	100	-1	0	1
D	Incubation time	days	7	14	21	-1	0	1

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad \ldots(2)$$

Extraction and Quantification

The ultrasonic extraction method was used for quantification of PAHs. The cleanup process involved the use of a chromatographic column (35.5 × 1.5cm) by using hexane as an elution solvent, and glass wool was fixed at the tip of the column. The samples were concentrated on a rotary evaporator (JSGW) twice with hexane and filtrated through 0.45 µm syringe filters. High-performance liquid chromatography (HPLC Water 600) equipped with a UV detector at 254 nm was used for the analysis of the samples. The PAH concentration was determined by assessing the peak areas of the sample chromatogram and comparing them to the peak area of the standard chromatogram. Biodegradation efficiency was calculated as:

$$\text{Biodegradation efficiency (\%)} = \frac{(C_0 - C_e)}{C_0} \times 100 \quad \ldots(3)$$

C_0 is the initial concentration of Phe (ppm); C_e is the final/residual concentration of Phe.

RESULTS AND DISCUSSION

Regression Model and Statistical Analysis

Table 2 shows the experimental design and results for Phe degradation in each run. ANOVA was conducted to assess the model's significance and adequacy (Table 3). When Prob > F is less than 0.05, it indicates that the model terms hold significance. The lack of fit displaying a non-significant value confirms the validity of the quadratic model for Phe removal by the bacterial strain *B. brevis*, and these models

Table 2: Box-Behnken design matrix for experimental design along with actual and predicted response.

Std	Coded value of variables				Response	
	pH (A)	Temperature (^oC) (B)	Phenanthrene conc. (ppm) (C)	Incubation time (days) (D)	Actual	Predicted
1	-1	-1	0	0	34.02	33.80
2	+1	-1	0	0	26.54	27.35
3	-1	+1	0	0	35.97	35.65
4	+1	+1	0	0	32.96	33.67
5	0	0	-1	-1	29.92	31.62
6	0	0	+1	-1	28.99	29.81
7	0	0	-1	+1	52.18	51.85
8	0	0	+1	+1	44.93	43.71
9	-1	0	0	-1	32.18	32.46
10	+1	0	0	-1	27.24	26.97
11	-1	0	0	+1	47.4	48.25
12	+1	0	0	+1	45.01	45.31
13	0	-1	-1	0	32.96	33.17
14	0	+1	-1	0	38.36	39.09
15	0	-1	+1	0	30.04	29.96
16	0	+1	+1	0	31.91	32.29
17	-1	0	-1	0	39.97	39.09
18	+1	0	-1	0	35.13	33.77
19	-1	0	+1	0	32.72	33.01
20	+1	0	+1	0	30.09	29.90
21	0	-1	0	-1	28.13	27.04
22	0	+1	0	-1	33.18	31.74
23	0	-1	0	+1	44.34	44.71
24*	0	+1	0	+1	48.17	48.19
25*	0	0	0	0	50.15	50.71
26*	0	0	0	0	50.23	50.71
27*	0	0	0	0	51.25	50.71
28*	0	0	0	0	50.91	50.71
29*	0	0	0	0	51.02	50.71

Std = Standard run order, * Central value

align well with the measured data. The second-order model exhibited strong statistical significance ($p < 0.05$) in the context of Phe degradation, demonstrating the actual relationship between variables and the response. Eq. (4) below represents the model for Phe degradation.

% biodegradation (Y^2) = +50.71 -2.11*A +2.04*B -2.49*C +8.53*D -8.88*A^2 -9.21*B^2 -7.89*C^2 -3.58*D^2 +1.12*A*B +0.55*A*C +0.64*A*D -0.88*B*C -0.31*B*D -1.58 *C*D …(4)

The data exhibited the most favorable fit when described by a second-order polynomial equation, as evidenced by the strong correspondence between the experimental data and the

Table 3: Analysis of variance (ANOVA) for variables.

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	
Model	2139.31	14	152.81	140.72	0.0001***	Significant
A	53.30	1	53.30	49.08	0.0001***	
B	50.10	1	50.10	46.14	0.0001***	
C	74.20	1	74.20	68.33	0.0001***	
D	873.64	1	873.64	804.51	0.0001***	
A^2	511.84	1	511.84	471.34	0.0001***	
B^2	550.73	1	550.73	507.15	0.0001***	
C^2	403.47	1	403.47	371.54	0.0001***	
D^2	83.04	1	83.04	76.47	0.0001***	
AB	5.00	1	5.00	4.60	0.05 ^{NS}	
AC	1.22	1	1.22	1.12	0.3069 ^{NS}	
AD	1.63	1	1.63	1.50	0.2413 ^{NS}	
BC	3.12	1	3.12	2.87	0.1124 ^{NS}	
BD	0.37	1	0.37	0.34	0.5676 ^{NS}	
CD	9.99	1	9.99	9.20	0.009***	
Residual	15.20	14	1.09			
Lack of Fit	14.23	10	1.42	5.86	0.0516	Not significant
Pure Error	0.97	4	0.24			
Cor total	2154.51	28				
R^2	0.9929					
R^2_{adj}	0.9859					
CV	2.71					
Std dev	1.04					

Std dev = Standard deviation, * Significance (P< 0.001), NS = Not significant, DF = Degree of freedom, CV = Coefficient of variation

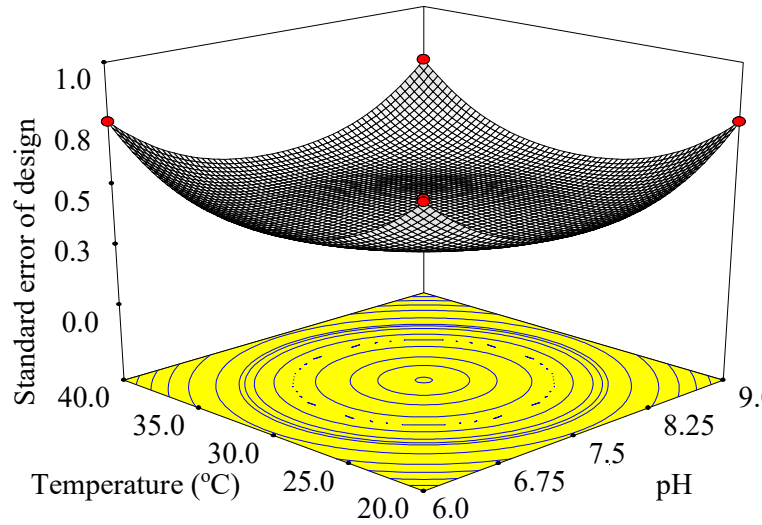

Fig. 1: 3D graph of the standard error design of the model with temperature and pH for biodegradation of Phenanthrene.

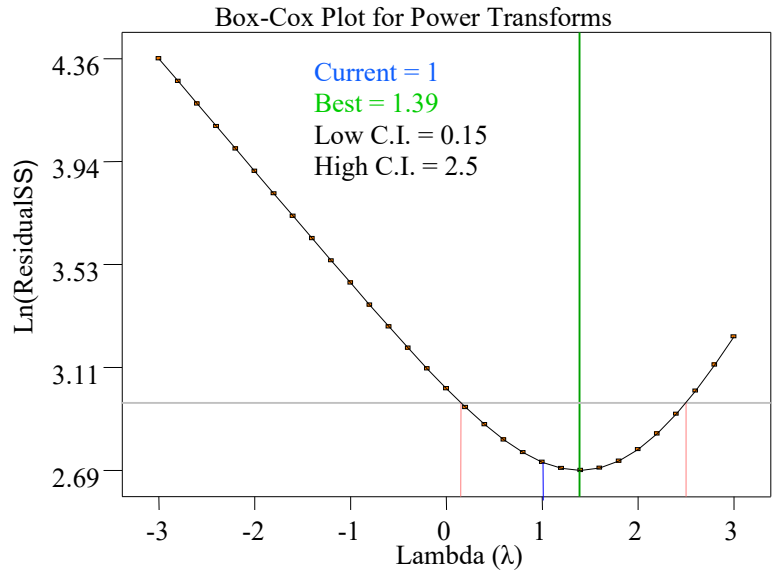

Fig. 2: Box-Cox plot of model transformation for biodegradation of Phenanthrene.

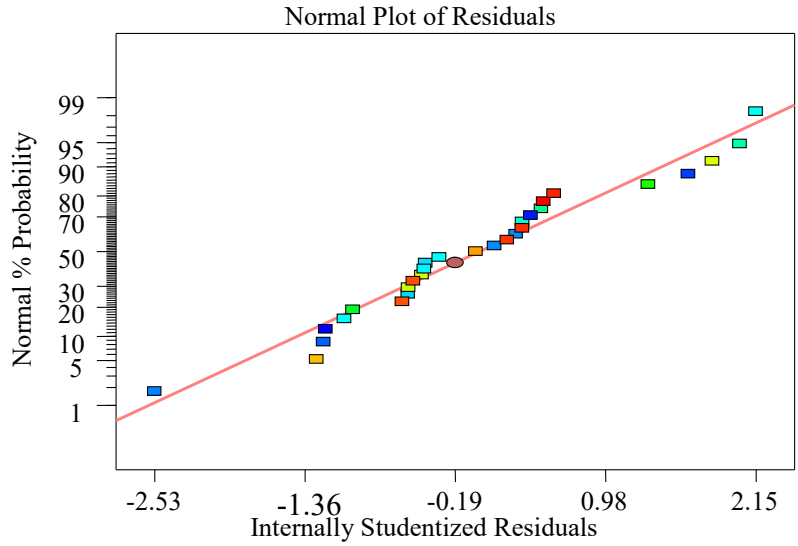

Fig. 3: Normal plot of studentized residuals versus normal percentage probability for biodegradation of Phenanthrene.

model's predictions. The correlation coefficient (R^2) values stood at 0.9929, implying that the data displayed limited variation and a high degree of uniformity and homogeneity. The adjusted R^2 of 0.9859 was in reasonable concordance with the R^2 value, which approached 1.0, signifying the model's superior fitness to the experimental data. An R^2 value near 1.0 is desirable, and it is essential for the adjusted R^2 to exhibit reasonable agreement (Dalvand & Ghiasvand 2019).

The standard error has a minimum value of 0.422207 around the centroid, and the maximum prediction variance is 0.578 at the design points. These observations suggest that the current model is suitable for exploring the design space in the present study (Fig. 1). The model exhibits a minimum confidence interval value of 0.15 and a maximum value of 2.5. When examining the natural logarithm (Ln) of the residual sum of squares (SS) against λ is one, it reveals a minimum within a steeply declining region, indicating an optimal value of 1.39 (Fig. 2). The diagnostic details articulated by Design-Expert can best be digested by viewing plots of normal probability versus studentized residual. The complete set of data points for the normal probability and studentized residual is almost linear and clustered around the

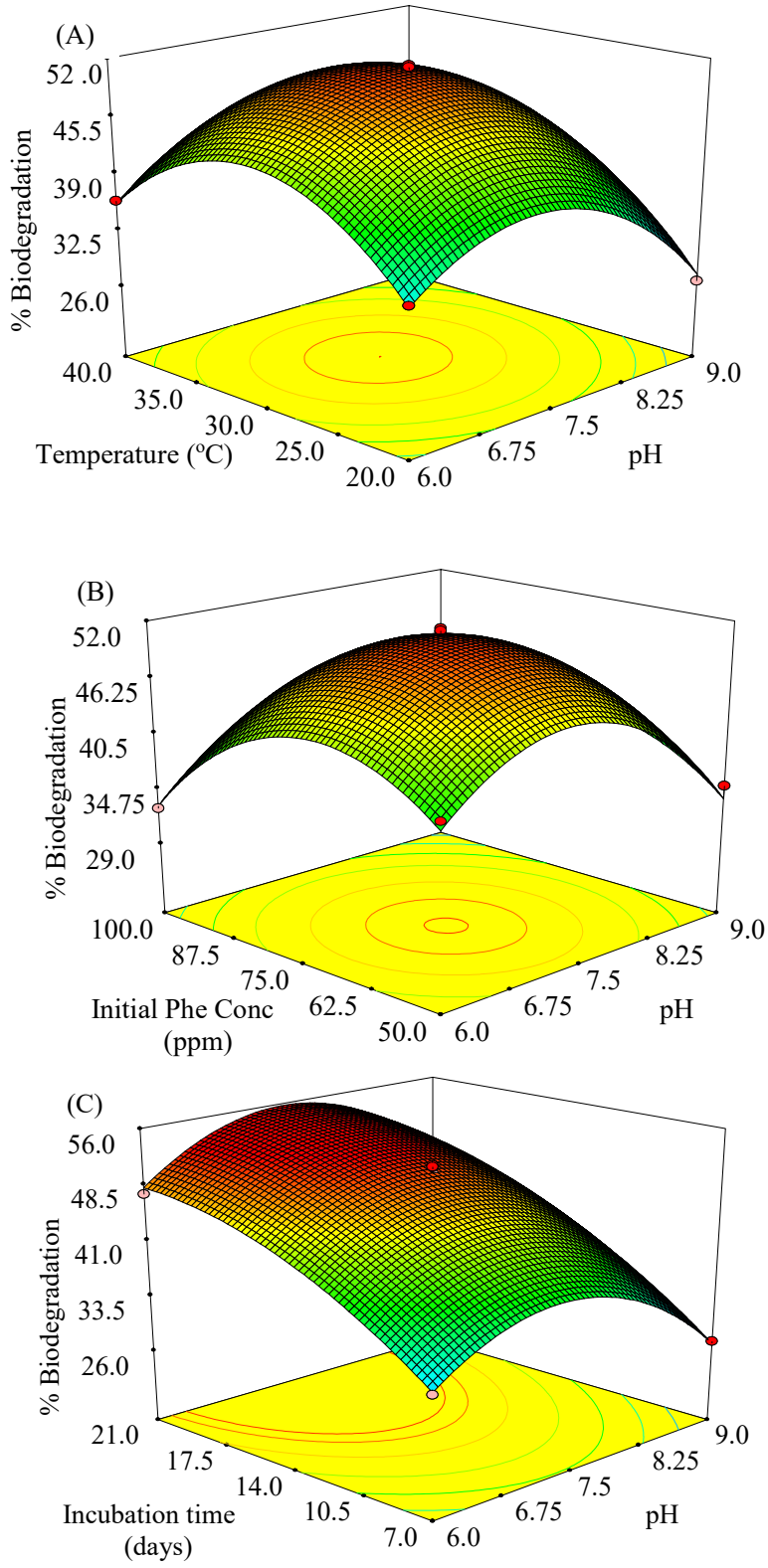

Figure Cont....

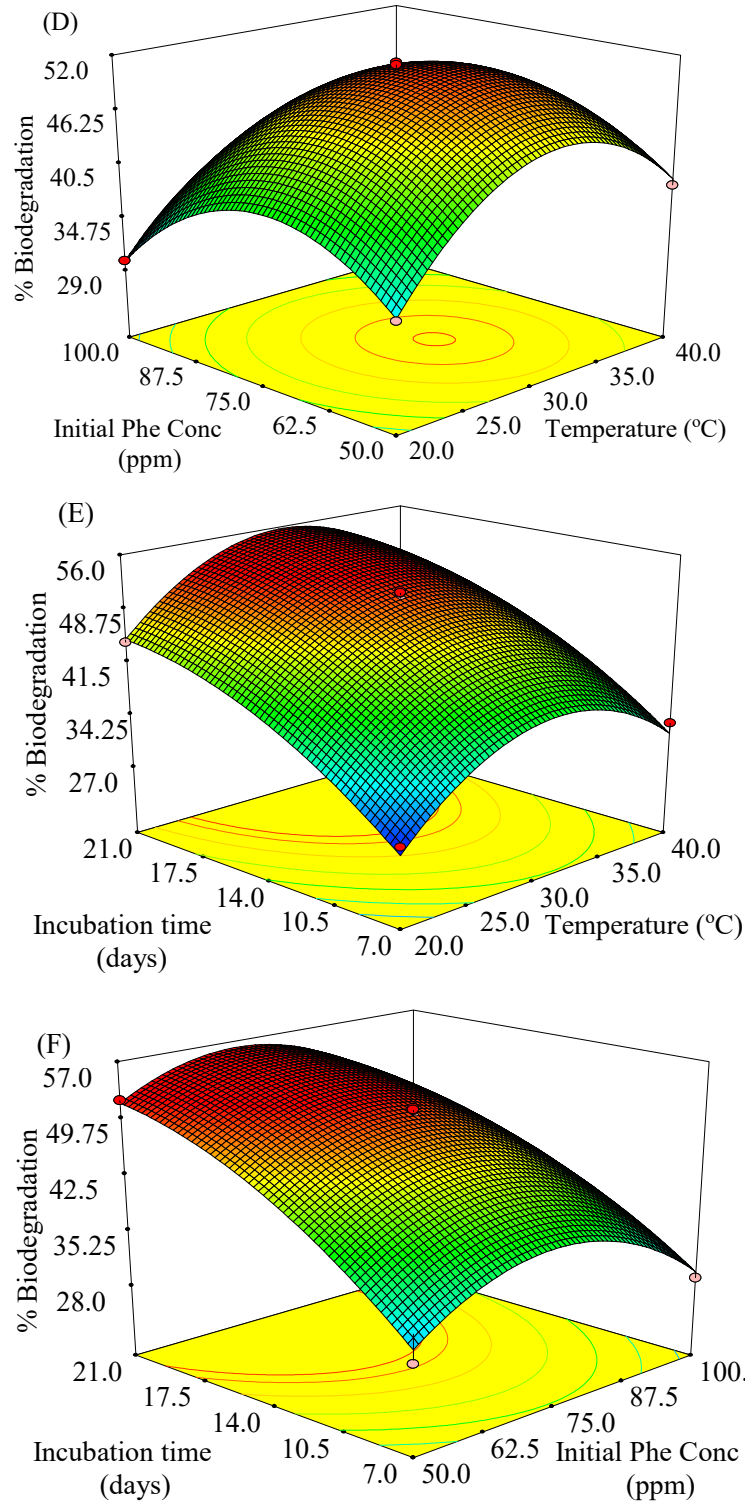

Fig. 4: 3D-surface plot showing the interactive of (A) pH and temperature (B) of pH and initial Phenanthrene Conc (C) incubation time and pH (D) temperature and initial Phenanthrene Conc (E) incubation time and temperature (F) incubation time and initial Phenanthrene Conc on biodegradation of Phenanthrene.

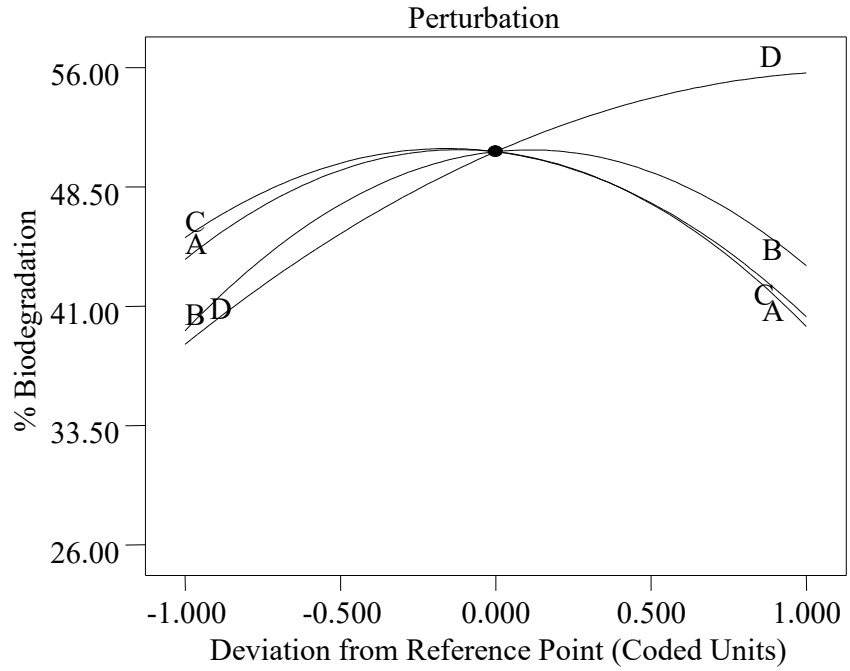

Fig. 5: Perturbation plots representing the individual variable effect on biodegradation of Phenanthrene.

zero line, which represents the model's suitability and shows no signs of any problems in our data (Fig. 3).

The statistical analysis of the positive linear coefficient for incubation time was observed to be the paramount factor impacting biological degradation significantly (Eq. 5). Up to a point, the negative quadratic coefficient for each variable showed the existence of maxima. Beyond that, the variable as a whole had an inhibiting effect on response. Out of the total of 14 effects, 9 (A, B, C, D, A^2, B^2, C^2, D^2, and CD) were significant. In the current investigation, the remaining five factors exhibited no significant influence on the response. Nevertheless, none of these factors displayed a noteworthy impact on the ultimate biodegradation percentage, as indicated by probability (p) values exceeding 0.05 for all five effects (Table 3).

Effects of Variables on Biodegradation of Phenanthrene

To ascertain the optimal levels of each variable for achieving maximum results, we generated 3D plots by graphing the response. Based on the interaction between two variables and the fixed level of another variable for Phe biodegradation, the response surface was selected.

Fig. 4 illustrates the quadratic surface response. The interactive effects of pH and temperature, with another variable being at the fixed level on biodegradation of Phe by *B. brevis,* is reported in Fig. 4A. Initially increase in pH resulted in the enhancement of biodegradation to values in the vicinity of 7.0. Still, beyond that, a decreasing trend was observed. The level of biodegradation observed was 33.8%, 50.7%, and 27.35% at the lowest (pH 6 and 20°C), middle (pH 7.5 and 30°C), and higher levels (pH 9 and 40°C) of variables by *B. brevis*. The Phe removal rates varied with pH, with the highest recorded at 88.33% when the pH was 7.0 and the lowest observed at 82.07% when the pH was 4.0, as reported by Liu et al. (2019). The optimum temperature observed for biodegradation of Phe was 30°C for *B. brevis* holding Phe concentration at 75ppm and 14 days of incubation time. Fig. 4B shows the interactive effects of pH and initial Phe concentration. The increase in initial Phe concentration from 50 to 70 ppm resulted in increased biodegradation, i.e., 39.09 to 51.03% by *B. brevis* with a change in pH 6 to 7.3. Beyond these, the reduction in the rate of biodegradation was observed with an increase in both variables. Phe biodegradation dropped above 100 mgL^{-1} and was less than 10% at 400 mgL^{-1}(Mai et al. 2021). At higher PAH concentrations, the substrate functions as the limiting factor because of their toxicity to microbes and slower biodegradation rate (Yuan et al. 2002). Fig. 4C depicts the interactive effects of incubation time and pH on the biodegradation of Phe by *B. brevis,* keeping the temperature (30°C) and initial Phe concentration (75 ppm). The negative value of the quadratic coefficient (Eq 5) and three-dimensional plots indicated that an increase in duration beyond optimum did not affect the

biodegradation significantly as the Phe degradation became slower or nearly constant. The similar response curvature for lower and higher ranges of temperature and initial Phe concentration for biodegradation showed the same effects (Fig. 4D). An increase in biodegradation with the increase in temperature from 20°C to 30°C and a decrease with an increase in temperature from 30°C to 40°C was observed. As the temperature rose, the solubility of Phe increased, leading to enhanced Phe bioavailability. However, excessively high temperatures could hinder microbial activity, consequently reducing Phe degradation (Liu et al. 2019). Fig. 4E shows the interactive effects of temperature and incubation time. The 3D figure clearly showed that the biodegradation rate was initially high, but after 18 days of incubation, biodegradation did not increase proportionally with increasing incubation period. The increase in duration to 18 days and temperature (30°C) increased the degradation efficiency to 55.7% after that, the degradation is about constant. Fig. 4F shows the interactive effects of the incubation time and initial Phe concentration. The strains presumably degraded Phe at slower rates during the initial incubation time. At the end of the incubation period, the degradation trend reversed, with the highest Phe degradation observed at 53% on the 18th day of incubation.

The interactive effects of the four process variables on the biodegradation of Phe are presented in (Fig. 5). The perturbation plots of biodegradation potential versus all four tested variables suggest that the variable contributed to the biodegradation activity, representing outline views of the response surface. The parabolic curves of pH (A), temperature (B), and initial Phe concentration (C) reflected that the initial rate of biodegradation increased with an increase in variables, and beyond, an optimum reduction in degradation was observed with further increases in the variables.

CONCLUSION

By RSM using Box-Behnken design, this study showed the significant effects of pH, temperature, initial Phe concentration, and incubation time on the biodegradation of Phe by *B. brevis*. A "Prob>F" value below 0.05 signifies that model terms substantially influence Phe's biodegradation. The model explains that the determination coefficient value $R^2 = 0.9929$ indicates that the data is more uniform and less variable in the experimental data. Maximum biodegradation of Phe was observed at almost 55.0% by *B. brevis*at pH 7.0, temperature 30°C, and initial Phe concentration (70 ppm) after 21 days of incubation time. The mathematical evaluation, significant model, and RSM approach proved advantageous.

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