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Response Surface Methodological Approach for Optimizing Process Variables for Biodegradation of 2, 4, 6-Trinitrotoluene using Acinetobacter Noscomialis

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ABSTRACT

The aim of present paper was to study the biodegradation of 2,4,6- trinitrotoluene (TNT) in waste water using free cultures of a bacterial strain Acinetobacter noscomialis isolated from a TNT contaminated site. Three operating variables i.e. pH, initial TNT concentration and time for degradation were studied using response surface methodology (RSM), involving Box-Behnken design. Experiments were performed in the batch mode to determine maximum degradation of 2, 4, 6- trinitrotoluene (TNT). Very high regression coefficient between the variables and the response (R2 =0.9909) indicates excellent evaluation of experimental data by second-order polynomial regression model. Results indicated that 50–60 mg/L initial TNT concentration, 8-9 pH and a time period of 48-72 hrs were optimal for degradation of 2,4,6- trinitrotoluene by free cultures of Acinetobacter noscomialis and 99% of degradation was observed at optimal conditions. It was confirmed that the studied bacterial strain has a good potential for the degradation of 2,4,6- trinitrotoluene within a short time period of 72 Hrs.

Key words: Optimization, Box-Behnken Design, Wastewater, Explosive contaminated site, Bacterial strain

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INTRODUCTION

Contamination of soil and water resources by explosives occurs during manufacturing, processing, storage, test firing and due to inappropriate waste disposal practices. TNT is the most widely used nitro-organic military explosive due to its low melting point, high blasting power, and relative security of handling and thermal stability. TNT has been detected in soil and ground water bodies in and around the manufacturing facilities [1] [2].

Research and development in the field of bioremediation has gained much importance in recent years due to more stringent environmental laws. Since explosives are toxic, carcinogenic and mutagenic in nature, it is a major environmental concern and has received a great deal of attention. TNT exposure in humans occurs through inhalation, ingestion and dermal routes. Numerous symptoms of poisoning in humans following inhalation or dermal absorption of mononitrotoluene, dinitrotoluene, and trinitrotoluene are observed a few days after exposure. Headache, loss of appetite, dizziness, nausea, insomnia, numbness of various parts of the skin and diarrhoea and changes in the haemogram are the result of exposure. A particularly striking symptom is cyanosis, a bluish-red discoloration of lips, fingernails and skin due to oxygen deficiency. That is caused by reduced metabolites of TNT which are blamed for increased methemoglobin formation and haemolysis. The metabolites of TNT cause liver damaging effects [3]. Due to the toxic effects exerted by TNT, extensive research is being carried out for treating this contaminant in the soil and water. Various treatment technologies have been developed with varying degree of success. Technologies using the bioremediation methods for explosives contaminated soils, such as soil-slurry reactors, composting and land farming, have been developed [4] as bioremediation is an eco-friendly technology utilizing microorganisms and plants to transform hazardous materials into more benign substances [5]. A few Microorganisms have demonstrated their potential in degradation of organic compounds worldwide [6] [7].

The present paper evaluates the potential of bacterium *Acinetobacter noscamialis* isolated from an explosive contaminated site for biotransformation of 2, 4, 6- trinitrotoluene in free culture shake flask study to optimize process variables like pH, initial TNT concentration and time period of degradation. Maximum degradation

capacity was studied adopting a full range of response surface methodology using Box-Behnken model to analyse the efficiency of the system under different conditions. This model was adopted as an experimental design model to study the effect of interactions of combined parameters like pH, initial TNT concentration (mg/L) and time period for degradation of the aqueous solution while permitting reduction in the actual number of combination experiments to be performed. The experimental data was analyzed with a second order polynomial model.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The bacterial strain was isolated from a TNT contaminated site. Identification was carried out by adopting the polyphasic taxonomic approach. It was identified as *Acinetobacter noscomialis* and the culture was lyophilized. This work was carried out in collaboration with Institute of Microbial Technology (IMTECH), Chandigarh, India. Lyophilized cultures were revived in Tryptic Soy Broth (TSB) and were maintained on agar slants at 4°C prior to use during the study which is a standard method [13]. The bacterial strain was maintained on nutrient agar plates through fortnightly sub-culturing.

Chemicals

2,4,6-trinitrotoluene was obtained from a manufacturing facility in pure form (99.9%). All other chemicals used throughout the study were of analytical grade and were obtained from standard manufactures.

Batch Studies

A stock solution of TNT (1000mg/L) was prepared and desired concentrations (10 ppm, 55ppm and 100ppm) were obtained by further dilutions. For TNT degradation studies 7 days old secondary cultures of above mentioned strain were used. Erlenmeyer flasks having 100ml pre-autoclaved simulated TNT aqueous solution in varying concentrations (10–100mg/L) were inoculated with 24 hrs bacterial cultures which has obtained log phase growth, each 1ml inside laminar flow system under hygienic conditions was prepared by adding a loop full of secondary culture of *A. noscomialis* in TSB and was kept at 20°C and 120 rpm for 16 hrs to obtain an optical density of 1.5 at 600 nm [8]. Experiments were performed at pH 3–9 for a time period of 0-72 hrs keeping other parameters constant to examine the effect of pH and time period on TNT degradation. pH of aqueous TNT solution was adjusted using 0.1M HCl and 0.1M NaOH. Experiments were performed at an ambient temperature of 20± 2°C.

Batch studies were performed to determine the equilibrium-time required for maximum TNT degradation. Erlenmeyer flasks containing 100 ml TNT solution were inoculated with *A. noscomialis* and were shaken in an incubator shaker at 120 rpm for the maximum predetermined time period of 72 hrs. Samples were withdrawn at fixed time intervals from the flasks, optical density was checked using UV-visible spectrophotometer at 600 nm and then sonicated for 1hr. after adding the acetonitrile. After that all samples were centrifuged (10000 rpm) for 15 minutes and filtered supernatant was analyzed with the help of HPLC for residual TNT concentration in the aqueous solution. All the experiments were performed in triplicates and their mean values are reported here. The maximum deviation was found to be ±1.98%. One control was maintained without bacterial culture and with pure 100mg/L TNT and other was maintained with pure bacterial culture without TNT. The following formula was used to calculate the percentage degradation:

$$\text{Degradation}\,(\%) = \frac{C_0 - C_e}{C_0} \times 100 \qquad (1)$$

Where C_0 is the initial conc. of TNT (mg/L) and C_e is the residual TNT conc. (mg/L) at different time interval

Monitoring of Growth of Bacteria in TNT Wastewater

The optical densities (OD) of different combinations were checked at a specific predetermined time interval using UV-Visible spectrophotometer at 600 nm.

Analysis of TNT

Samples inoculated with *A. noscomialis* were analyzed for residual TNT concentration by high-performance liquid charomatography (HPLC) with photo diode array as detector. Standard EPA method 8330, was followed. Analytical separation was carried out using Flexar HPLC of Perkin Elmer Inc. C18 Column, (3um, 150 x 4.6mm) was used as stationary phase and the mobile phase was methanol: water (50:50 v/v) mixture at a flow rate 1ml/min.

Optimization of Process Parameters using RSM Approach

Response surface methodology is an approach that combines various statistical and mathematical techniques, and is useful for developing, improving and optimizing a process [9] [10]. In the present study, Box–Behnken model for three variables (TNT concentration, pH and degradation time period), each with two levels (the minimum and the maximum), was used for degradation of TNT by free cultures of *A. noscomialis* as experimental design model. The model has the advantage that it permits the use of relatively few combinations of variables for determining the complex response function [9] [11] [12] [14]. A total of 17 experiments are required to be performed to calculate 10 coefficients of second-order polynomial equation [14]. In the experimental design model, TNT concentration (10–

100 mg/L), pH (3–9) and time period (24–72 hrs), were taken as input variables. Percentage removal of TNT from the solution was taken as response of the system. The experimental design matrix derived from the Box–Behnken model is shown in Table 2. Percentage of TNT removal by the free cultures of bacterial strain in different experimental conditions based on the experimental design matrix was estimated, the results of which have also been included in the same table. A second order polynomial model where interaction terms have been fitted to the experimental data obtained from the Box–Behnken model experiment can be stated in the form of the following equation:

$$Y = a_o + \Sigma\, a_i x_i + \Sigma a_{ii} x^2_i + \Sigma\, a_{ij} x_i x_j \quad (2)$$

Where Y is the percent removal of TNT, a_o offset term, a_i first-order main effect, a_{ii} second-order main effect and a_{ij} is the interaction effect. The data were subjected to analysis of variance and the coefficient of regression (R^2) was calculated to find out the goodness of fit of the model.

RESULTS AND DISCUSSION

In the present study, the Box–Behnken model was used to statistically design the experiments and to evaluate the interactive effects of process parameters for optimizing the degradation of TNT by free cultures of a bacterium *A. noscomialis*. The experiments were planned to obtain a quadratic model consisting of 12 trials plus 5-centre points. The range and levels of three independent variables, viz. pH, initial TNT concentration and time period for degradation are presented in Table 1. Table 2 shows the Box-Behnken design matrix for three variables along with observed distinct response pattern in terms of percent TNT removal under different combinations of initial TNT concentration, pH and time period for degradation. Each run was performed in triplicate and mean values for % removal of TNT are presented in Table 2. Statistical significance of the variables and their interaction at various levels of probability based on analysis of variance fitted to second order polynomial equation are depicted in Table 3. Smaller *P* values (<0.05) represent the statistical significance of parameter effect [9] [15].

Table-1 Independent Factors and their Coded Levels used for Optimization

Factors	Range and levels (coded)		
	-1	0	+1
pH, A	3	6	9
Initial TNT Conc. B (mg/L)	10	55	100
Time Period, C (Hrs)	24	48	72

Table- 2 Box-Behnken Design Matrix for three Variables along with Observed Response

Experimental run	Variables			Response % removal of TNT, Y
	pH, A	Initial TNT concentration, B (mg/L)	Time C (Hrs)	
1	9.00	100.00	48.00	97.06
2	6.00	55.00	48.00	77.81
3	6.00	55.00	48.00	77.81
4	3.00	100.00	48.00	71.50
5	9.00	55.00	24.00	94.70
6	6.00	55.00	48.00	80.00
7	3.00	55.00	72.00	75.45
8	6.00	10.00	72.00	82.90
9	3.00	10.00	48.00	54.40
10	9.00	10.00	48.00	85.00
11	6.00	100.00	72.00	90.54
12	6.00	100.00	24.00	86.70
13	9.00	55.00	72.00	99.00
14	3.00	55.00	24.00	54.90
15	6.00	55.00	48.00	77.81
16	6.00	55.00	48.00	77.81
17	6.00	10.00	24.00	55.40

Table- 3 Estimated Parameters of Box-Behnken Model and their Statistical Significance

Variables	Parameters Estimate	Probability Level (p> t for H_o)
Intercept	78.248	0.0001**
A	14.94	0.0001**
B	8.51	0.0001**
C	7.02	0.0001**
AB	-1.26	0.2444^{NS}
AC	-4.06	0.0046*
BC	-5.92	0.0006**
A^2	0.43	0.6664^{NS}
B^2	-1.69	0.1233^{NS}
C^2	2.33	0.0467*

**Significance (*P< 0.001*), *P< 0.05 and NS= not significant

Very high value of parameter estimate, for the variable A and high value for *B* and *C* showing a high level of significance indicate the importance of these variables in the degradation process. The first order effect of pH (*A*), initial TNT concentration (*B)* and time (*C)* were highly significant ($P < 0.0001$) and had a positive relationship with degradation process, while the variable B^2 (initial TNT concentration) had negative effect on degradation process. The parameters were then fitted into second order polynomial equation as follows:

$$Y = 78.5+14.9\ 4A+8.51\ B+7.02\ C-1.26\ AB-4.06\ AC-5.92\ BC+0.43A^2-1.69\ B^2+2.33C^2 \quad (3)$$

Where Y is the observed response and A, B and C are the coded values of the test variables; pH, initial TNT concentration and time period for degradation respectively. The analysis of Variance (ANOVA) was conducted to test the significance of fit of the second order polynomial equation for the experimental data and the results of which are shown in Table 4. The second order polynomial model taken as a factor (source of variation) gave a highly significant *F*-value ($F = 84.96$, degree of freedom = 9, $P < 0.0001$) and a low value of standard deviation (1.98) between the measured and modelled results shows that the equation adequately represents actual relationship between the response (TNT removal) and significant variables (initial TNT concentration, pH and time). High value of R^2 (0.9909) indicates a high dependence and correlation between the observed and the predicted values of response. The goodness of the model can be checked by the determination coefficient R^2 and the multiple correlation coefficients *R*. The value of adjusted R^2 (0.9793) suggests that the total variation of 97.9% for the TNT degradation to the independent variables and only about 2.1% of the total variation cannot be explained by the model. The closer the values of *R* (multiple correlation coefficient) to 1, better the correlation between the experimental and predicted values [13] [16]. Here, the value of *R* (0.9909) indicates good relation between the experimental and predicted values of the response.

Table- 4 One way ANOVA of Quadric Model for RSM Parameters for Removal of TNT

Source	Sum of Squares	Df	Mean Square	F-value	Prob> F	
Model	3006.21	9	334.02	84.96	< 0.0001	Significant
A	1785.33	1	1785.33	454.12	< 0.0001	
B	579.70	1	579.70	147.45	< 0.0001	
C	394.66	1	394.66	100.39	< 0.0001	
AB	6.35	1	6.35	1.62	0.2444	
AC	66.02	1	66.02	16.79	0.0046	
BC	139.95	1	139.95	35.60	0.0006	
A^2	0.80	1	0.80	0.20	0.6664	
B^2	12.06	1	12.06	3.07	0.1233	
C^2	22.85	1	22.85	5.81	0.0467	
Residual	27.52	7	3.93			
Pure Error	3.84	4	0.96			
Cor Total	3033.73	16				

Stddev= 1.98, R^2 =0.9909, Adj-R^2=0.9793, Pred-R^2 =0.8731, Mean = 78.75, C.V. % = 2.52, PRESS =384.92

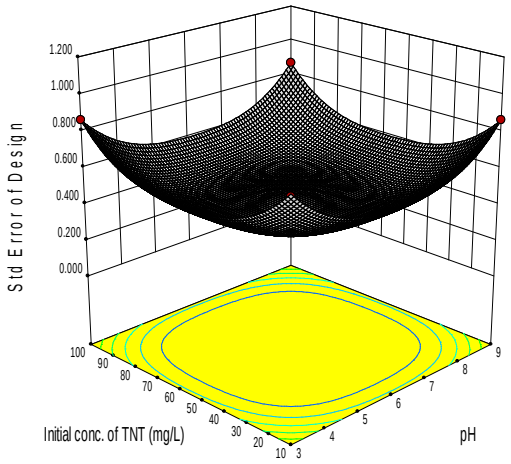

Fig.1 Three Dimensional Standard Error Plot for Degradation of TNT

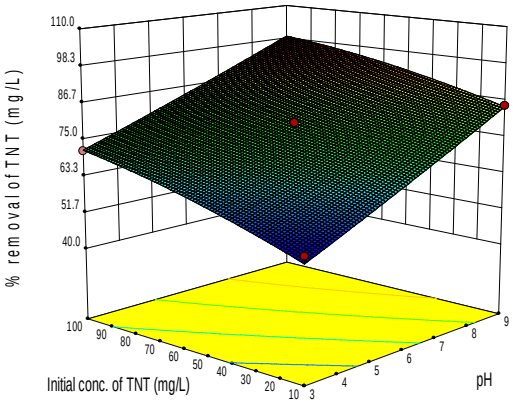

Fig.2 Effect of pH and Initial TNT Concentration. on Degradation of TNT

The three-dimensional response surface plots were used to determine percent TNT removal over interactive variables viz. pH, initial TNT concentration and time period and are presented in (Fig. 2, 3). The standard error values increases at the centroid as well as away from optimization point. The three-dimensional response surface plots are the graphical representations of the regression equation. The best response range can be calculated by analyzing the plots and response can be maximized by tracking efficiency for the optimum values of the variables. The optimum values of the variables can be analyzed by saddle point or by checking the maxima formed by the *X*

and *Y* coordinates. Fig.1 shows the shape of standard error plot fit on the design points and polynomial having circular contours and symmetrical shape which is an ideal condition. The actual magnitude of the plot will be a function of the standard deviation, which depends on the response data [13] [17]. The standard error values increases at the centroid as well as away from optimization point. It is clearly evident, as the pH increases from 6 to 9, an increase in degradation rate was observed.

However, there was a sharp decline in degradation rate as pH was decreased from 6.0 to 3. (Fig.2). The degradation rate increases with increase in the time period (up to 72 Hrs) observed and initial TNT concentration up to 50-60 mg/L and further increase in concentration has showed a decrease in degradation (Fig. 3).

Increased degradation rate may be attributed to inherent surface characteristics of the bacterium which was locally isolated from TNT contaminated soil. Similar trends have been reported by other authors also [9] [18]. The conditions obtained at the saddle point for best responses were pH 9, time period of 72 Hrs. and initial TNT concentration of 55mg/L where up to 99% TNT degradation was achieved under optimum growth conditions.

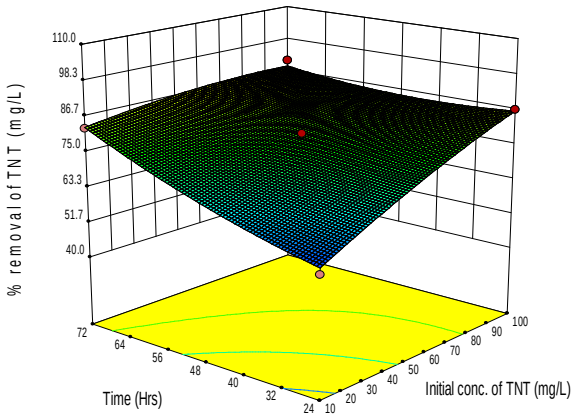

Fig.3 Effect of Initial TNT Concentration and Time Period on Degradation of TNT

CONCLUSION

It is concluded that by applying Box–Behnken design to the optimization experiments, we could investigate the process variables completely and achieved TNT degradation values up to 99%. The use of an experimental design permitted the rapid screening of a large experimental domain for optimization of the % degradation ability of bacterial isolate *A. noscomialis*. On the basis of RSM approach using Box–Behnken model for experimental design and fitness of polynomial equation, optimal conditions for TNT degradation were found to be pH 8–9, initial TNT concentration 50–60 mg/L and a time period of 48-72 hrs wherein 99% TNT removal can be achieved using the *A. noscomialis*. The present strain of *A. noscomialis* that has been isolated from the soil within the premises of TNT manufacturing factory seems to be best suited for removal of TNT. Moreover, the ability of the bacterial strain to degrade TNT indicates its potential application for degradation of TNT. Further pilot scale studies are required with this strain for real field applications and detailed study is desirable to explore the mechanism and secondary metabolites involved.

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