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Research

OPTIMIZATION OF ENDOSULFAN BIODEGRADATION USING INDIGENOUS BACTERIAL ISOLATE *BACILLUS ARYABHATTI* THROUGH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Endosulfan, a wide-spectrum organochlorine insecticide, is characterized by its toxicity to invertebrates, especially arthropods and medium persistence in the environment. It is considered a serious environmental pollutant and hazardous to human health. A bacterium (GBA) capable of metabolizing endosulfan was isolated from endosulfan contaminated agriculture field of Uttarakhand, India. The organism was characterized as sp. of *Bacillus*. 16S rDNA sequencing of GBA showed 99% similarity with *Bacillus aryabhatti* in the phylogenetic tree. Response Surface Methodology (RSM) was applied to optimize the significant variables for endosulfan degradation. R^2 value were 0.9994 and 0.9957 for α and β-endosulfan respectively indicating that approximately 99% of responses 0.9669 were covered by the model. This experimental result explained that optimum degradation (83.7% and 79.8%) of endosulfan isomers (α and β) was observed by *Bacillus aryabhatii* at 30°C, pH 7 and 120 rpm in 20days.

INTRODUCTION

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine3-oxide), an organochlorine insecticide, is a mixture of α and β-isomers of endosulfan. It is a contact and stomach poison and used to control chewing and sucking insects, such as *Colorado beetle*, *Flea beetle*, cabbage worm aphids and leafhopper (Goebel, 1982). This insecticide is used to control insect pests on a wide range of crops, including cereals, cotton, coffee, fruits, oil seeds and vegetables. In India, it is mainly used in rice, cotton and tea plantations. Presence of the relatively reactive cyclic sulphite diester group in endosulfan makes it moderately persistent as compared to other organochlorine compounds (Singh and Singh, 2011). Endosulfan accumulates in soil and water and becomes extremely toxic to aquatic fauna and may provoke chronic symptoms like testicular and prostate cancer breast cancer and sexual abnormality genotoxicity and neurotoxicity in numerous mammalian species (Sunderam, *et al.,* 1992). Endosulfan and it's products have been detected in atmosphere, soils, sediments, surface water and foods, with an estimated half-life of 3-6 months (Awasthi, *et al.,* 2000). Therefore, the biodegradation and detoxification of endosulfan is of utmost importance.

Different micro-organisms isolated from different sources, have shown the capability to degrade both the isomers of endosulfan (Kullman and Matsumura, 1996; Singh and Singh, 2011). Biodegradation of endosulfan can occur either by oxidation to form the toxic intermediate (endosulfan sulfate) or by hydrolysis to form less toxic endosulfan diol (Hussain, *et al.*, 2007).

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The optimization of growth conditions for endosulfan degradation is of primary importance in the development of the bioprocess. Optimization studies involving one-factor at a time approach tend to overlook the effects of interacting factors and might lead to misinterpretations of the results. Thus statistical planned experiments minimize the error in determining the effect of different parameters and the results achieved are more economical (Yasser, *et al.*, 2007). Response Surface Methodology is an empirical modulization technique derives for the evaluation of the relationship of a set of controlled experimental factors and observed results. It is the most widely used statistical technique for bioprocess optimization. It requires a prior knowledge of the processes to achieve statistical model and involves three major steps: estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model (Box and Draper, 1959).

Till date optimization studies involving only one-factor have been conducted and very few studies have used composite design (response surface methodology) for performing bioremediation studies in case of pesticides. Thus this study was planned to determine the effect of various physical factors on endosulfan biodegradation based on response surface methodology.

MATERIALS AND METHODS

Chemicals and reagents

Technical grade endosulfan (>99.4% purity) was procured from Department of Chemistry of the University (GBPUA&T, Pantnagar, Uttrakhand, INDIA). Stock solution of endosulfan was prepared at a concentration of 1 mg/ml in hexane and filter sterilized. All the chemicals used during the investigation were of analytical grade and high quality. The Non Sulphur Medium (NSM) was used for isolation and cultivation of endosulfan degrading microorganisms (Kumar and Philip, 2006).

Screening and isolation of endosulfan degrading microorganisms

Soil used for the isolation of endosulfan-degrading bacteria was collected from the endosulfan contaminated agriculture fields of Uttarakhand, India. Rhizospheric or subsurface soil samples were collected at a depth of 15 cm to 30 cm in sterile polythene bags and used for the isolation of pesticide degrading bacteria by enrichment culture technique. Soil samples (5 g) in triplicate were taken in 250 mL erlenmeyer flasks containing 50 mL of the NSM broth and 20 mg/L endosulfan. Flasks were incubated at 30°C with continuous shaking (150 rpm). After 10 days, 5 mL of broth from each flask was inoculated to 50 mL of fresh medium supplemented with 20 mg/L endosulfan. This process was repeated three times. After three consecutive transfers, 0.2 mL of culture broth was pour plated, and pure bacterial cultures were obtained. Endosulfan degrading bacteria were screened on the basis of their ability to grow in NSM medium supplemented with endosulfan at the rate of 20, 50, 70, 100, 130 and 150 mg/L.

Characterization of selected bacteria

Morphological and biochemical characterization of the selected bacteria was done on the basis of cell arrangement, colony morphology and biochemical tests according to Bergey's Manual of Systematic Bacteriology (Bergey's Manual of Determinative Bacteriology, 1994). Molecular characterization of endosulfan degrading bacteria was conducted by Chromous Biotech, Bangalore, India using partial sequencing of 16S rDNA. The universal eubacterial 16S rRNA gene-specific primers used were (F) 5'-AGAGTTTGATCCTGGCTCAG-3' and (R) 5'-TACCTTGTTACGACTT-3'. Amplified gene product(s) were sequenced by dideoxy method using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with an automated sequencing system. Obtained 16S rRNA sequences were searched out for their homology using BLAST (Basic Local Alignment Search Tool) through National Center for Biotechnology Information (NCBI) website www.ncbi.nlm.nih.gov/blast (Altschul, *et al.*, 1990). Multiple sequence alignment and construction of phylogenetic tree (based on the neighbour-joining method) were performed by MEGA 5.1 software (Saitou and Nei, 1987).

Optimization of culture condition for endosulfan biodegradation

Response surface methodology (RSM) was explored to optimize the degradation conditions of endosulfan using strain GBA. The optimal levels of the significant factors and the interactions of these variables was analyzed. The central composite design consisting of 15 experimental runs with three replicates at the center point was used to optimize the independent variables which significantly influenced the endosulfan biodegradation with *Bacillus* Spp. Three critical factors and their optimal ranges selected for biodegradation of endosulfan in this experiment were: temperature (150°C, 300°C and 400°C), pH (5,7 and 9) and shaking (100 rpm, 120 rpm and 140 rpm). The dependent variable was degradation of 50 ppm

endosulfan in 50 ml of mimimal medium for 20 day. Analysis of variance (ANOVA) was conducted to determine the significance of model and regression coefficients. The quality of polynomial equation was judged by determination coefficient (R^2), and its statistical significance was checked by Fischer's F-test. The significance of regression coefficients was tested by Student's t-test. The response contour plots of the model predicted responses were utilized to assess the interactive relationships between the significant variables.

Analytical procedures

Residual endosulfan was extracted by adding 5 mL of culture broth/5 g of soil/ to 20 ml acetone in a flask and shaken for 1 h. It was then filtered using Buchner funnel and obtained residue was washed thoroughly with 10 ml of acetone and filtered again. Filtrate was collected in a round bottom flask and evaporated to dryness in rotary flash evaporator at 50°C. The left over (residue) was dissolved in n-hexane and filtered by passing through a 0.2 µ membrane filter. Average recovery of α and β endosulfan was 93.8% and 91.5%, respectively. Samples were analysed using a high-performance Gas Chromatography system (Chemito, Ceres 800 plus, Packed column 10% SE 30, 63Ni ECD). Nitrogen was used as a carrier and make-up gas. Temperature of oven, injector and detector were programmed at 180°C, 260°C and 300°C respectively.

RESULTS AND DISCUSSION

In the present study, a bacterial isolate GBA able to utilize endosulfan was isolated from the pesticide contaminated soil of agricultural fields of Uttarakhand, India. The organism was aerobic, gram positive and rod shaped. Colonies grown on nutrient agar plates were rough, opaque and white. Analysis of the 16S rDNA gene sequences demonstrated that GBA was among *Bacillus aryabhatti* species and was allotted with an accession number KF450813 after retrieving the sequence from NCBI database.

Optimization of endosulfan degradation conditions for *Bacillus aryabhatti*

Response surface methodology (RSM) is a powerful statistical technique for investigating the interactive effects between several factors at different levels and has been successfully employed to optimize removal of pollutants (Pang, *et al.*, 2011). The actual responses are fitted to a polynomial model in a range of optimal responses. The model then determines the relationship between the responses and the variables and calculates the optimal responses and variables (Abdollahi, *et al.*, 2012). Box-Behnken design was employed to study the interactions between the significant factors and also to determine their optimal levels. Three variables (pH (A), temperature (B) and Shaking speed (C)) were used. The experimental design was processed by response surface regression procedure of Design Expert 9.0.10 software, and results were obtained by fitting with the quadratic model equation:

% Biodegradation of α-endosulfan by *Bacillus aryabhatti*=

$$+80.4+3.44*A+5.22*B+0.68*C-0.11*AB-2.82E-015*AC-2.46E$$
$$-014*BC-14.1*A^2-7.4*B^2-0.91C^2$$

% Biodegradation of β-endosulfan by *Bacillus aryabhatti*=

$$+79.40+3.83*A+4.83*B+0.83*C-3.23E-014*$$
$$AB-2.37-014*AC+7.66E-016*BC-14.5*A^2-8.20*B^2-2.20*C^2$$

Where (Y) is the predicted α/β-endosulfan degradation (%) by strain *Bacillus aryabhatti* and A, B and C are the values for pH, temperature, and Shaking speed respectively.

The results of the second order response surface model fitting in the form of ANOVA are given in Tables 1 and 2. The goodness of the fit of the model was checked by the determination coefficient (R^2). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response. Determination coefficient (R^2) are 0.9994 and 0.9957 for α and β-endosulfan respectively indicating that approximately 99% of responses were covered by the model, demonstrating that predicted values of the model were in good agreement with the experimental values. In addition, the value of the adjusted determination coefficient (Adj R^2=0.99) is also very high adding to high significance of the model. This specifies that regression model provides an excellent correlation between the independent variables (factors) and the response.

It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F-value (=S^2r/S^2e), is a statistical measurement of how well the factors describe the variation in the data about its mean. The greater the F-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test (Fmodel=1257.48; 284.06) for α and β-endosulfan respectively. The model for endosulfan biodegradation is highly significant ($p<0.0001$),

Table 1. ANOVA for the fitted quadratic polynomial model of α-endosulfan degradation by *Bacillus aryabhatii.*

Source	SS	DF	Mean Square	F- Value	P- value	
Model	1452.17	9	161.35	1257.48	0.0001	significant
A-pH	94.90	1	94.90	739.58	0.0001	
B-Temperature	218.20	1	218.20	1700.49	0.0001	
C-Shaking speed	3.68	1	3.68	28.69	0.0011	
AB	0.050	1	0.050	0.39	0.5527	
AC	0.000	1	0.000	0.000	1.0000	
BC	0.000	1	0.000	0.000	1.0000	
A^2	835.45	1	835.45	6511.01	0.0001	
B^2	231.79	1	231.79	1806.39	0.0001	
C^2	3.46	1	3.46	26.95	0.0013	
Residual	0.90	7	0.13			
Lack of Fit	0.050	3	0.017	0.078	0.9684	*not significant*
Pure Error	0.85	4	0.21			
Cor Total	1453.07	16				

*R^2=0.9994, Std. Dev=0.36, C.V%=0.51. DF degrees of freedom, SS sum of squares. *P level less than 0.05 indicates that the model terms are significant

Table 2. ANOVA for the fitted quadratic polynomial model of β-endosulfan degradation by *Bacillus aryabhatii.*

Source	SS	DF	Mean Square	F- value	P-value	
Model	1590.25	9	176.69	284.06	0.0001	significant
A-pH	117.56	1	117.56	188.99	0.0001	
B-Temperature	186.89	1	186.89	300.45	0.0001	
C-Shaking speed	5.56	1	5.56	8.93	0.0203	
AB	0.000	1	0.000	0.000	1.0000	
AC	0.000	1	0.000	0.000	1.0000	
BC	0.000	1	0.000	0.000	1.0000	
A^2	889.75	1	889.75	1430.39	0.0001	
B^2	283.35	1	283.35	455.52	0.0001	
C^2	20.44	1	20.44	32.86	0.0007	
Residual	4.35	7	0.62			
Lack of Fit	0.000	3	0.000	0.000	1.0000	not significant
Pure Error	4.35	4	1.09			
Cor Total	1594.60	16				

*R^2=0.9994, Std. Dev=0.36, C.V%=0.51. DF degrees of freedom, SS sum of squares. *P level less than 0.05 indicates that the model terms are significant

indicating that the established quadratic model for endosulfan degradation by *Bacillus aryabhatti* was adequate and reliable in representing the actual relationship between response and variables.

Three-dimensional (3D) response surface plots were used to assign the interaction between three variables. The effect of two relative variables on the degradation efficiency was tested as the others were held constant. The 3D response surface plots were organized based on the quadratic model. The optimal relative variables are located at the coordinates of the central point in the highest level in each figure. Fig. 1 and 2 shows the interactive influence of pH, temperature and shaking speed on degradation efficiency. Optimum degradation (83.7% and 79.8%) of endosulfan isomers (α and β) was observed by *Bacillus aryabhatii* at 30°C, pH 7 and 120 rpm. As compared to the α isomer, percent degradation of β isomer under the optimized condition was lesser. Awasthi, (1997) have also reported greater degradation of α endosulfan as compared to β endosulfan. This may be due to stereoisomerism, where the enzymes released from bacterial system may be active toward one of the stereo isomers (Table 2).

RSM has been widely applied for optimizing processes in different domains such as the chemical process, geotechnical engineering and animal science research (Noureddine, *et al*., 2015). (Noureddine, *et al*., 2015) determined the optimal degradation conditions of hydroquinone, resorcinol and catechol using RSM. In a study conducted by (Chandana, *et al*., 2011) a 23-full factorial Central Composite Design was employed combining with RSM to

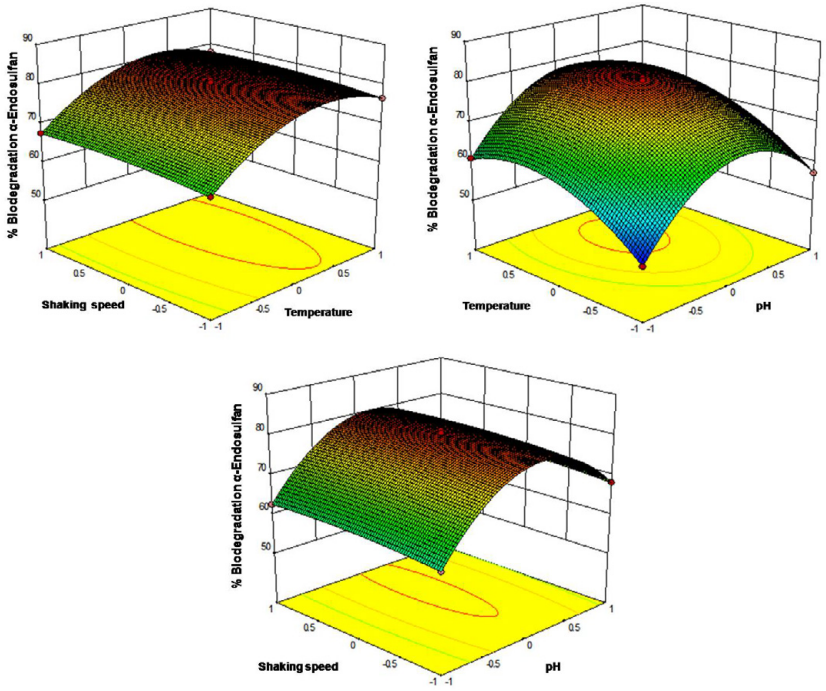

Fig. 1(a-c) Optimization of α-enodsulfan biodegradation by *Bacillus aryabhatii.*

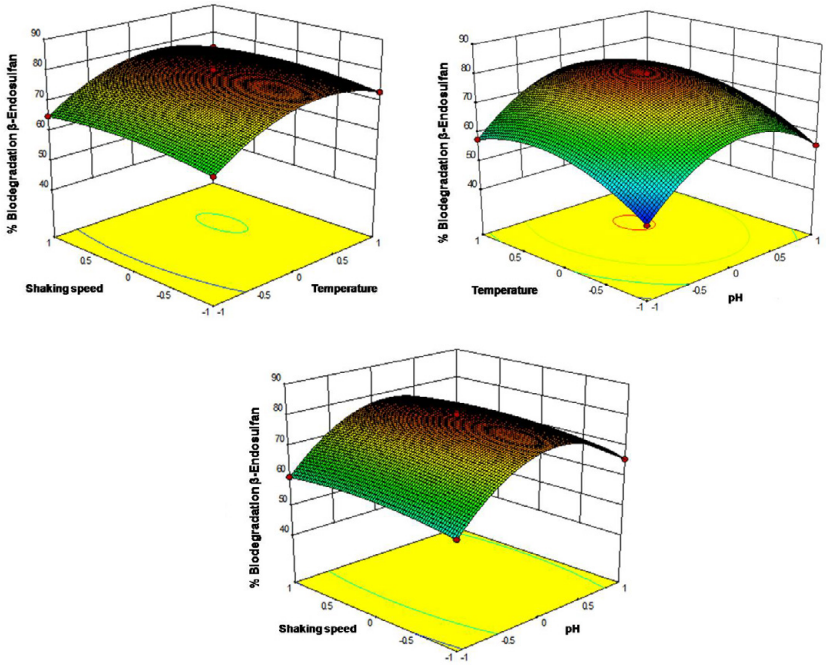

Fig. 2(a-c) Optimization of β-enodsulfan biodegradation by *Bacillus aryabhatii.*

optimize the process parameters for the degradation of phenol by *P. aeruginosa* (NCIM 2074). A second order polynomial regression model was found to properly interpret the experimental data with R^2 value of 0.9669 and F-value of 32.5 based on which the maximum degradation of phenol was estimated up to 80.45% within the range examined. Valipour (2016) have reported a study on annual precipitation forecast by coding in MATLAB software environment based on a non-linear autoregressive neural network (NARNN), non-linear input-output (NIO) and NARNN with exogenous input (NARNNX) for optimization. Annual data of 27 precipitation gauge stations of Iran and their average were used. Results showed that the accuracy of the NARNNX was better than that of the NARNN and NIO, based on r values. The r values were <0.73 only for five stations in the optimized NARNN and <0.74 only for those stations with optimized NIO. It was concluded that NARNNX (R value >0.90) could be applied successfully for precipitation forecasting for the 20 next years and optimized ANNs are applicable not

only for the case study but also for all humid regions with various precipitation ranges.

The capacity of three different models (multivariate fractional polynomial (MFP), robust regression, and Bayesian regression) for evapotranspiration prediction was investigated by (Khoshravesh, 2015). All models were found to have a closer agreement with the calculated values for FAO-PM (R^2>0.95 and RMSE<12.07 mm month^{-1}). MFP showed high regression coefficient values and the less prediction errors than other two models and thus was most accurate model for prediction of evapotranspiration.

11 mass transfer-based models were used to estimate reference crop evapotranspiration in 31 provinces of Iran (Valipour, 2015). The results showed that the Penman model estimates reference crop evapotranspiration better than other models. The best weather conditions to use mass transfer-based equations were reported to be 8°C to 18°C, <25.5°C, <15°C, >55% for mean, maximum, and minimum temperature, and relative humidity, respectively.

CONCLUSION

Endosulfan degradation by *Bacillus aryabhatii* was studied under different parameter and RSM was used to optimize the degradation conditions. A Box-Behnken design was employed to identify the significant variables that influence the degradation of endosulfan isomers. The variables included pH, temperature an shaking speed. The quadratic mathematical model was suggested for enoduslfan degradation. *Bacillus aryabhatii* has the ability to maximum degradation of endosulfan isomers in minimal medium after 20 days of experiment. The results obtained from RSM were clearly explained that optimum values of significant variables had a significant effect and promotes an increase in percentage of enoduslfan degradation. The ANOVA confirmed the high validity of the model by using excellent evidences such as very low P-value (<0.0001), non-significant lack of fit, and R^2 (0.99). The optimization results shows that the maximum degradation (83.7% and 79.8%) of endosulfan isomers (α and β) was observed by *Bacillus aryabhatii* at 30°C, pH 7 and 120 rpm in 20 days.

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