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Degradation of DDT by a Defined Microbial Consortium: Optimization of Degradation Conditions by Response Surface Methodology

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Abstract: In our laboratory, a defined microbial consortium was developed and used for the degradation of DDT. The DDT degradation by the consortium was studied for different time intervals. The degradation was maximum in 72 h. DDT-degradation parameters were optimized using Response Surface Methodology (RSM) using this consortium. A Central Composite Rotatable Design (CCRD)with three variables chosen for the study were inoculum concentration (50-1500 μg protein mL^{-1} i.e., the cells of consortium), temperature (25-35°C) and pH (4-8) each at 5 levels -1.682, -1, 0, 1 and 1.682. Incubation time of 72 h was maintained as constant. Degradation of different concentrations of DDT (5, 10, 20, 30 and 35 ppm) was studied by using this consortium. The optimized conditions were inoculum concentration 1500 μg protein mL^{-1} and temperature 25°C but pH varied for different concentrations of DDT. Validation of the model was done and experimental values were found to be in agreement with the predicted ones. These conditions can be applied in the degradation of DDT in industrial effluents and other contaminated water bodies.

Key words: 1, 1, 1-trichloro-2, 2-bis (*p*-chlorophenyl) ethane (DDT), response surface methodology, Central Composite Rotatable Design (CCRD), microbial consortium, ANOVA, degradation, optimization, validation

INTRODUCTION

1, 1, 1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT) is one of the most widely used organochlorine pesticides. It is a broad-spectrum pesticide, used extensively in public health programmes and agriculture. It is one of the most persistent and recalcitrant pesticides with a long residual action. It is used for the control of insect parasites causing malaria, plague, dengue etc. It is only recently that the agricultural use of DDT has been banned in India. However, it is still used in public health programmes, as no efficient alternative to DDT is available. Thus, our environment is heavily polluted due to the extensive use of DDT from the past five decades. DDT and its degradation products have been detected in water, soil and air (Deo *et al*., 1994; Goel, 1986). Since DDT residues are lipophillic, they tend to accumulate in the fatty tissues of the ingesting organisms along the food chain. Almost all the foodstuffs including processed foods have been shown to contain high levels of DDT residues (Appaiah, 1988; Kannan *et al*., 1992; Lal *et al*., 1989). High levels of DDT and its metabolites have been detected in human adipose tissues, blood plasma, liver, brain and placenta and even in breast milk (Dale *et al*., 1985; Kunhi *et al*., 1995; Siddiqui *et al*., 1981; Tanabe *et al*., 1990). Exposure to DDT in humans can cause nausea, vomiting, dizziness, headache, confusion, excitedness, loss of muscle control and tremors (Smith, 1991). DDT residues in water and soil are of concern as

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their uptake can lead to the accumulation of primary products. Their removal from water and soil is therefore a priority. An effort was made in our laboratory to remediate this pesticide-spiked effluent by using microorganisms. A defined microbial consortium was found to degrade about 70% of the added 5 ppm substrate in 72 h. Hence, an attempt was made to optimize the DDT-degradation parameters to obtain maximum degradation.

The conventional method of optimization involves varying one parameter at a time and keeping the others constant (Hamsaveni *et al*., 2001). This traditional approach is time consuming and incapable of detecting the true optimum, due especially to the interactions among factors, as compared to factorial design (Prapulla *et al*., 1992; Cochran and Cox, 1992). Response Surface Methodology (RSM) is a statistical method that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equation. It usually uses an experimental design such as Central Composite Rotatable Design (CCRD) to fit a first or second order polynomial by least squares technique. An equation is used to describe how the test variables affect the response, determine the interrelationship among the test variables and describe combined effects of all the test variables in the response (Triveni *et al*., 2001).

Application of RSM requires the identification of the major factors. Our preliminary study of this system has indicated that the major variables affecting the degradative ability of the organism are pH, temperature, substrate concentration, inoculum level and incubation time.

The purpose of the present research was to optimize conditions for the degradation of DDT and study the effect of pH, temperature and inoculum level at different DDT concentrations by the microbial consortium.

MATERIALS AND METHODS

DDT 98% Purity-Sigma Aldrich, Mo, USA.

All other chemicals used in this study were of analytical grade and procured from standard companies.

Isolation, Acclimation, Identification and Maintenance of the Consortium

The microbial population capable of degrading DDT was isolated from DDT-contaminated soil in Menasagere village of Mandya district according to Bidlan and Manonmani (2002) by long term enrichment technique in shake flasks (150 rpm). The microbial population that got acclimated was then exposed to 2 ppm DDT for one week. After one week, the concentration was slowly increased to 5 ppm for one week and later the concentration was increased further to 10 and 15 ppm after one week's interval. From the microbial consortium that got established, the individual isolates were isolated by dilution plating technique on nutrient agar. Pure isolates were identified based on morphological, microscopic and bio-chemical characteristics using Bergey's Manual of Determinative Bacteriology.

Inoculum

The consortium grown on peptone-glycerol medium and pre-exposed to 10 ppm DDT for 72 h was used as inoculum in these studies. The microbial cells grown in this medium were harvested by centrifugation at 10, 000 g for 10 min, washed well in M_4 medium and re-suspended in required quantity of M_4 to be used as inoculum. Freshly prepared inoculum was used in all the studies unless otherwise stated.

All experiments on the degradation of DDT were carried out in replicates of five. The data presented here is an average of these five values.

Experimental Design

A CCRD with three variables was used to study the response patterns and to determine the optimum combination of variables. The variables optimized were inoculum concentration (50-1500 μg protein mL^{-1}), temperature (25-35°C) and pH (4-8) each at 5 levels-1.682,-1, 0, 1 and 1.682 (Table 1). Incubation time of 72 h was maintained as constant. DDT degradation was studied for different DDT initial concentrations (5, 10, 20, 30 and 35 ppm).

The CCRD was arranged to allow for fitting an appropriate regression model using multiple regression program. CCRD combines the vertices of hypercubes whose co-ordinates are given by a 2n factorial design to provide for the estimation of curvature of the model. Six replicates (treatments 15-20) at the center of the design were used to allow for the estimation of a pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

Experiment was done according to the experimental plan for different initial concentrations of DDT (Table 2). Samples were removed after 72 h of incubation at required temperature, extracted and analysed for residual DDT by Thin Layer Chromatography (TLC) and Gas Chromatography (GC). All results are an average of five replicates.

Statistical Analysis

A second order polynomial was used to fit the experimental data given in Table 2.

Optimization of the polynomials for the degradation of DDT was performed by a non-linear mathematical optimization procedure of the Quattro Pro Software Package (Quattro Pro Version 4.0, Borland International Inc. USA) and feasible conditions for maximum degradation of DDT were predicted.

Table 1: Variables and their levels for CCRD

	Symbols		Levels				
Variables	Coded	Uncoded	-1.682	-1	0	1	1.682
Inoculum Conc. (v/v)	X1	x1	50	343.97	775	1206.03	1500
Temperature (°C)	X2	x2	25	27.03	30	32.97	35
pH	X3	x3	4	4.81	6	7.19	8

where X1 = (x1-775)/431.03; X2 = (x2-30)/2.97 and X3 = (x3-6)/1.19

Table 2: Treatment schedule for 3-factor CCRD and the response

	Inoculum Conc. (v/v)	Temperature (°C)	pH	Degradation for initial DDT concentration (%)				
S. No.	(X_1)	(X_2)	(X_3)	5 ppm	10 ppm	20 ppm	30 ppm	35 ppm
1	-1	-1	-1	50.00	79.1	80.60	91.85	94.76
2	1	-1	-1	74.34	84.3	85.20	90.13	93.01
3	-1	1	-1	60.94	72.1	82.47	88.64	94.76
4	1	1	-1	54.94	56.8	81.31	84.19	91.55
5	-1	-1	1	73.47	76.3	86.83	90.13	95.48
6	1	-1	1	84.94	89.2	90.20	91.39	95.45
7	-1	1	1	59.17	80.1	83.14	90.13	92.35
8	1	1	1	60.00	79.0	81.10	86.78	91.83
9	-1.682	0	0	59.87	79.1	87.34	93.40	94.83
10	1.682	0	0	63.47	75.6	89.61	92.54	91.59
11	0	-1.682	0	67.60	91.6	90.80	90.83	96.14
12	0	1.682	0	53.02	75.8	80.47	87.15	91.03
13	0	0	-1.682	50.92	72.4	78.60	87.89	93.01
14	0	0	1.682	70.50	82.5	81.81	85.43	93.92
15	0	0	0	70.90	82.8	85.20	91.64	93.92
16	0	0	0	70.20	82.6	85.70	92.14	93.86
17	0	0	0	71.10	83.1	85.55	90.50	93.66
18	0	0	0	70.50	82.9	84.70	90.64	94.03
19	0	0	0	70.90	82.5	85.05	91.47	93.74
20	0	0	0	70.80	82.3	85.45	92.81	93.80

*Average of five replicates

The fitted polynomial equation was expressed as surface plots to visualize the relationship between the response and experimental levels of each factor.

Period of Incubation Vs Degradation of DDT

To study the effect of incubation period on the degradation of DDT by the microbial consortium, samples were removed at 24h interval from 0h through 120h. Samples were then extracted and analysed for residual DDT as described later.

Analytical Methods

Amount of inoculum added as protein was determined by estimating the total protein in the biomass by a modified method of Lowry *et al.* according to Bidlan and Manonmani (2002).

Residual DDT was estimated by GC. The acidified culture broth was extracted thrice with equal volumes of dichloromethane and the solvent layers were pooled after passing through anhydrous sodium sulphate and florisil. The solvent layer was concentrated by evaporating completely at room temperature and resuspending in a known volume of acetone. A known quantity of this was injected into GC (Chemito 1000) with ^{63}Ni electron capture detector. The conditions used were; capillary column BP 5 (30×0.25 mm ID), column temperature was programmed for 180°C/10 min/2°C min^{-1}/220°C/2 min, injector temperature was maintained at 250°C and detector was maintained at 280°C. Flow rate of the carrier nitrogen gas was 1 mL min^{-1}. Under these conditions, the retention time of DDT was 28.13 min (Bidlan and Manonmani, 2002).

RESULTS AND DISCUSSION

Identification of the DDT-degrading Isolates

The composition of the microbial consortium was determined by identifying the individual members using the microscopic, morphological and biochemical characters and identified according to Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994). The consortium was found to be made of 10 bacterial isolates, containing seven different strains of *Pseudomonas*, one each of *Flavobacterium*, *Vibrio* and *Burkholderia* sp.

Diagnostic Checking of Models

Response was measured in terms of % degradation of DDT. The co-efficients for the actual functional relatives for predicting response are presented in Table 3. The insignificant terms were omitted based on student's t-ratio (Deavin *et al.*, 1977). The responses under different combinations as defined in the design (Table 1 and 2) were analysed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is represented in Table 4. It is evident that the regression terms were found to be significant and residual was not

Table 3: Estimated coefficient for the fitted second order polynomial representing the relationship between the response and process variables

	Initial DDT concentration				
Variables	5 ppm	10 ppm	20 ppm	30 ppm	35 ppm
a0	70.594	82.771	85.303	91.542	93.827
a1	3.426b	-0.307ns	0.629c	-0.711c	-0.802a
a2	-5.660a	-4.940a	-2.356a	-1.461b	-1.230a
a3	4.155b	3.609a	1.251b	-0.038ns	0.187ns
a11	-1.232ns	-2.354a	0.944b	0.452ns	-0.167ns
a22	-3.306b	-0.109ns	-0.059ns	-0.955b	-0.035ns
a33	-3.057b	-2.319a	-1.979a	-1.778a	-0.077ns
a12	-5.123b	-4.313a	-1.396b	-0.918c	-0.244ns
a13	-0.755ns	2.738b	-0.264ns	0.510ns	0.551b
a23	-3.848c	3.513b	-1.346b	0.567ns	-0.661b

[a]Significant at 0.1%, [b]Significant at 1.0%, [c]Significant at 5.0%, [-]Not significant even at 5% level

Table 4: Analysis of variance for the fitted second order polynomial model and lack of fit for biodegradation as per CCRD

		Sum of squares				
Source of variation	df	5 ppm	10 ppm	20 ppm	30 ppm	35 ppm
Regression	10	1438.855[a]	964.520[a]	208.701[a]	109.032[a]	36.815[a]
Residual	10	118.758[ns]	30.409[ns]	8.993[ns]	12.488[ns]	1.531[ns]
Total	20	1557.613	994.930	217.694	121.520	38.346
Coefficient of Determination (R_2)		0.961	0.969	0.959	0.947	0.960

[a] Significant at 5% level, [ns] Not significant

significant. The values of coefficients of determination (R^2) also suggest that the model is a good fit. The R^2 is proportion of variability in response values explained or accounted for by the model (Sreedharan *et al*., 1999; Manonmani *et al*., 2000).

Response Surface Plotting

The effect of inoculum concentration, pH and temperature on responses such as degradation of DDT are reported (Table 3) by the coefficients of second order polynomials. The response surfaces (keeping the third variable at an optimum level) based on these coefficients are given in Fig. 1-3. In general, the exploration of the response surfaces indicated a complex interaction between the variables.

Effect of Inoculum Concentration and Temperature on DDT Degradation

The variation of inoculum concentration and temperature is shown in Fig. 1, while the pH was kept at respective optimum conditions as indicated in Table 5. At low level of temperature (25°C, coded level-1.682), the percentage degradation of DDT was found to increase rapidly with an increase in inoculum concentration for 5 and 10 ppm initial DDT concentrations, whereas for concentrations above 10 ppm, it increased with a lower rate. At higher levels of temperature (35°C, coded level + 1.682), the percentage degradation did not change significantly. At lower levels of inoculum concentration, (50 μg protein mL^{-1}, coded level -1.682) the percentage degradation remained almost same with an increase in temperature. At higher level of inoculum concentration (1500 μg protein mL^{-1}, coded level +1.682) the percentage degradation was found to decrease with an increase in temperature for all concentrations of DDT. However, the decrease was more prominent up to 10 ppm concentration. For all the initial concentrations of DDT, the maximum degradation was obtained for inoculum concentration 1500 μg protein mL^{-1} (coded level +1.682) and at temperature 25°C (coded value -1.682) (Table 5).

Effect of Inoculum Concentration and pH on DDT Degradation

The effect of inoculum concentration and pH on the variation of DDT degradation is shown in Fig. 2, while the temperature was kept at optimum. At lower level of inoculum concentration (50 μg protein mL^{-1}, coded level-1.682), the percentage degradation was found to increase with an increase in pH at 5 ppm initial DDT concentration, whereas at 10 ppm initial DDT concentration, the percentage degradation decreased with an increase in pH. For initial concentrations higher than 20 ppm, the increase in percentage degradation with pH was not much significant. At higher concentrations of inoculum concentration 1500 μg protein mL^{-1} (coded level +1.682), the percentage degradation was found to increase with pH at 5 ppm initial concentration of DDT. Beyond 5 ppm, there was no marked difference in the percentage degradation. For all the inoculum concentrations (50-1500 μg protein mL^{-1}) the percentage degradation was found to increase even for all the initial concentrations of DDT (5-35 ppm). The maximum predicted percentage degradation of 94.69, 96.37, 97.97, 92.44 and 98.19 for 5, 10, 20, 30 and 35 ppm initial concentrations of DDT, respectively was obtained at inoculum concentration 1500 μg protein mL^{-1} (coded level +1.682) and at different pH levels 7.82 (coded value 1.53), 6.59 (coded value 0.497), 6.92 (coded value 0.776), 7.06 (coded value 0.892) and 8.00 (coded value 1.682) respectively (Table 5).

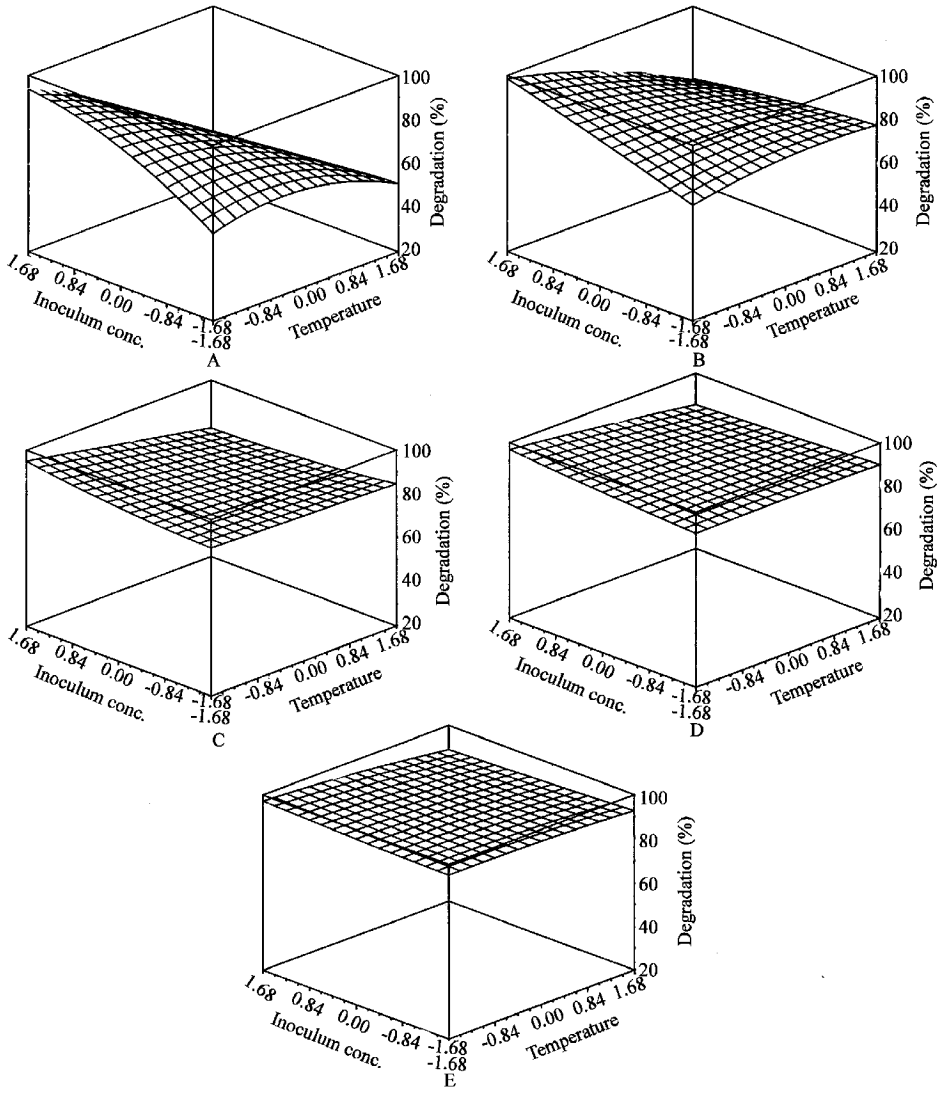

Fig. 1: Effect of inoculum concentration and temperature on DDT degradation. A: 5 ppm; B: 10 ppm; C: 20 ppm; D: 30 ppm; E: 35 ppm

Effect of pH and Temperature on DDT Degradation

The effect of pH and temperature on DDT degradation is shown in Fig. 3, while the inoculum concentration was kept at optimum level. At low level of temperature (25°C, coded value -1.682), the percentage degradation was found to increase with an increase in pH for 5 ppm level initial concentration, whereas for initial concentration from 10 to 20 ppm, the percentage degradation was found to increase and then decrease with an increase in pH. Further increase in initial concentration beyond 20 ppm, the variation in percentage degradation was not significant. At higher level of temperature (35°C, coded value +1.682), the percentage degradation did not change significantly for all initial concentrations of DDT with an increase in pH, except for 10 ppm. At 10 ppm level of DDT, the percentage degradation was found to increase with an increase in pH. At lower level of pH

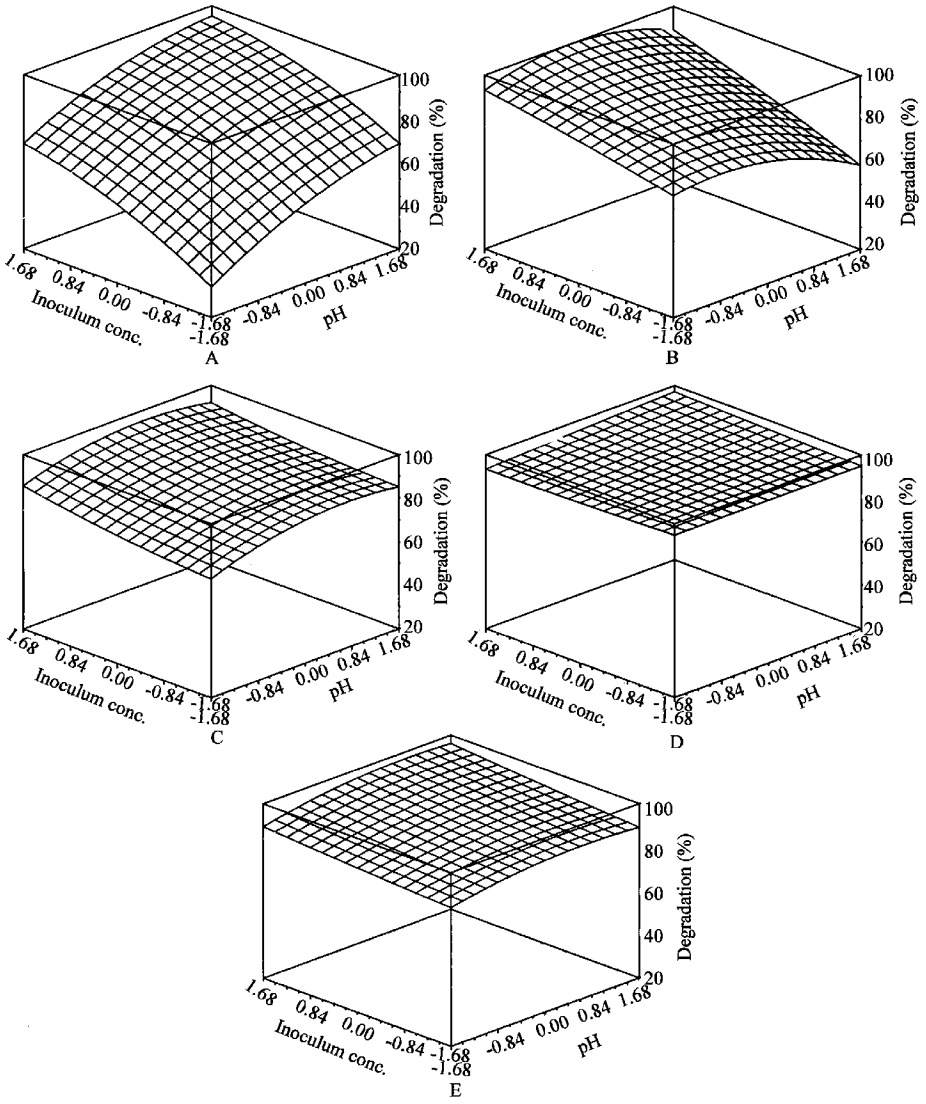

Fig. 2: Effect of inoculum concentration and pH on DDT degradation. A: 5 ppm; B: 10 ppm; C: 20 ppm; D: 30 ppm; E: 35 ppm

(4, coded value-1.682), the percentage degradation was found to increase with an increase in temperature for all the initial DDT concentrations. The increase was curvilinear for 5 ppm initial DDT concentration and beyond 5 ppm, it was linear and slope reduced with an increase in initial DDT concentration. At higher levels of pH (8, coded value +1.682), the percentage degradation was found to increase with an increase in temperature for initial DDT concentrations. For 5 ppm initial DDT concentration, the variation in percentage degradation was very prominent, whereas, for increasing DDT concentrations up to 35 ppm, the slope was significantly found to reduce. The maximum percentage predicted degradation of 94.69, 96.37, 97.97, 92.44 and 98.19 was obtained at 25°C (coded value-1.682) for 5, 10, 20, 30 and 35 ppm initial DDT concentrations, respectively and at different pH levels 7.82 (coded value 1.53), 6.59 (coded value 0.497), 6.92 (coded value 0.776), 7.06 (coded value 0.892) and 8.00 (coded value 1.682), respectively (Table 5).

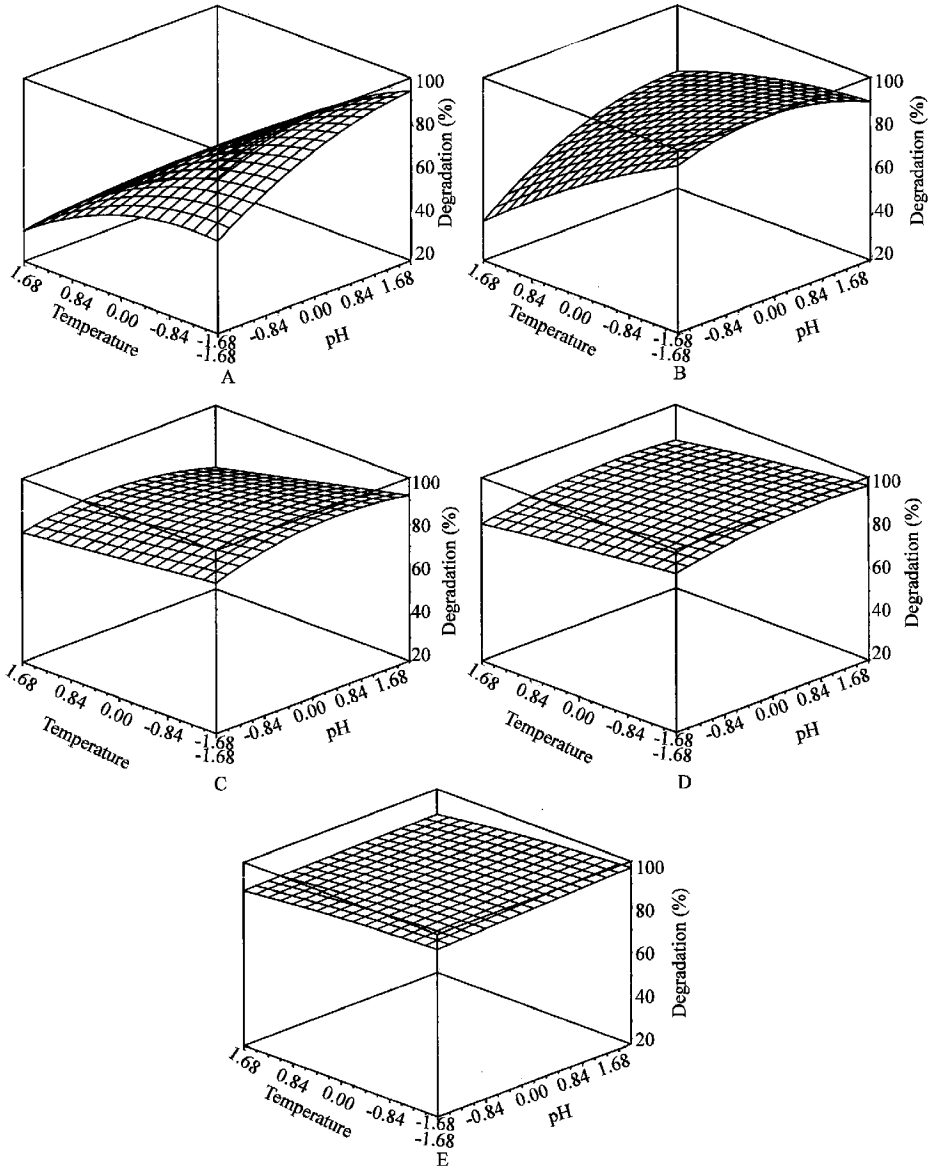

Fig. 3: Effect of pH and temperature on DDT degradation. A: 5 ppm; B: 10 ppm; C: 20 ppm; D: 30 ppm; E: 35 ppm

Optimisation

The optimum conditions for DDT-degradation are presented in Table 5. In order to deduce the workable optimum conditions, non-linear mathematical optimization technique was adopted. This technique drastically reduces the amount of time effort required for the investigation of multifactor, multiresponse system. It also provides comprehensive and informative insight of the system, which leads to fast process optimization.

Table 5: Feasible optimum conditions and predicted and experimental value of response at optimum condition

Initial DDT concentration	Inoculum Conc. (v/v) (X1)	Temperature (°C) (X2)	pH (X3)	Degradation (%)	
				Predicted	Experimental
5 ppm	1.682 (1500)	-1.682 (25)	1.530 (7.82)	94.69	95.50±0.30
10 ppm	1.682 (1500)	-1.682 (25)	0.497 (6.59)	96.37	96.55±0.25
20 ppm	1.682 (1500)	-1.682 (25)	0.776 (6.92)	97.97	98.35±0.60
30 ppm	1.682 (1500)	-1.682 (25)	0.892 (7.06)	92.44	93.35±0.80
35 ppm	1.682 (1500)	-1.682 (25)	1.682 (8.00)	98.19	98.00±0.50

* Average of five replicates

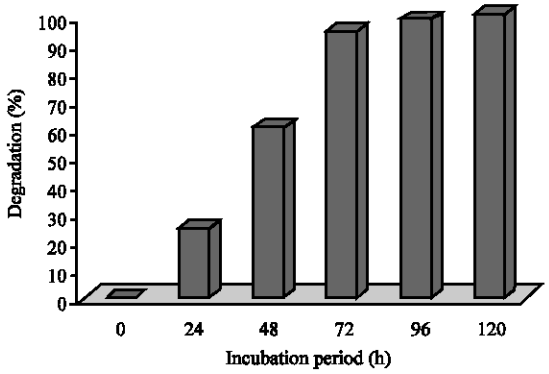

Fig. 4: Period of incubation v/s DDT degradation

Verification of Results

The suitability of the model equation for predicting the optimum response values were tested using the feasible optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to validate experimentally and predict the value of the response using model equations. The experimental values were found to agree with the predicted ones (Table 5). The temperature and inoculum concentration remained the same for all the concentrations of DDT but pH varied for each concentration.

Period of Incubation vs Degradation of DDT

The degradation of DDT by the microbial consortium increased with time. The degradation reached maximum of around 95% by 72h of incubation. Further increase in incubation time did not increase the degradation of the remaining substrate much. By the end of 120 h, 100% degradation of the added substrate was observed (Fig. 4).

DDT-degrading microorganisms were isolated from soil and efforts were made to improve their degradative ability. Microbial degradation studies have been conducted with pure cultures isolated by selective enrichment techniques (Karl-Heinz, 1986). Most control tests of biodegradation are based upon an enrichment culture technique whereby the initial population contains different varieties of microorganisms tolerant to a given environment with possibly different metabolic pathways (Ludzack and Ettinger, 1963; Wagner, 1973; Gilbert and Watson, 1977; Gustafsson, 1978; Alexander, 1980). The microbial consortium is one such potent degrader of DDT.

The acclimation of the microbial community to DDT would have led to the interactions among the community members and only those that could adapt to these stress conditions could survive. The

carbon-limited nature of the xenobiotics will ensure a strong and selective pressure for the organisms capable of attacking these chemicals. Hence under such competitive environment, adaptation favours the development of a complex microbial community, allowing the requiring adequate time for all the adaptable members to get established either through introduction from outside or through mutations. The acclimation and enrichment procedures have been employed with continuous culture of microorganisms by applying the compound to be degraded continuously; initially at low concentrations and subsequently increasing it in a systematic manner once the evidence of biodegradation has been established (Moos, 1980). Studies with HCH-degrading consortium by Manonmani *et al.* (2000) showed that acclimated consortium degraded higher concentrations of HCH. Studies by Bhuyan *et al.* (1992) and Wada *et al.* (1989) showed that γ-HCH degradation improved after every successive application of the compound. The consortium, which was isolated from DDT-contaminated soil by long-term shake-flask enrichment technique with increasing concentrations of DDT, could degrade up to 98% of 35 ppm of DDT by the end of 72 h.

The minimum and maximum biodegradable concentration is an important factor. Some biodegradative strains when inoculated into the environmental samples are unable to metabolise the pollutant. Among the reasons proposed for this observation is that the presence of very low concentrations of the substrate limits the enzyme induction (Aislabie *et al*., 1997). For some chemicals there is a threshold concentration below which the biodegradation rate is negligible. An explanation of biodegradation of organic compounds at concentrations below the threshold level is that the microorganisms are simultaneously using higher concentrations of other compounds for maintenance of energy and growth (Karl-Heinz, 1986). LaPat-Polasko *et al.* (1984) demonstrated that a pure culture of bacteria was capable of using a synthetic compound, methylene chloride, in trace concentration in the presence of acetate as the primary substrate in relatively high concentrations. Katayama and Mastsumura (1991) isolated two strains of bacteria, *Bacillus* sp. B75 and an unidentified gram-variable rod B116, which degraded DDT at extremely low level of 10 pg mL^{-1}. In our studies, the microbial consortium was able to degrade 35 ppm DDT in 72h under favorable cultural and environmental conditions.

Environmental factors such as pH, temperature and other substrates in the environment may affect the growth of microorganisms and their degradative abilities. These conditions vary from one organism to the other. Bidlan and Manonmani (2002) reported that the 10 ppm of DDT could be degraded by the end of 72 h in 30°C. In our studies, different concentrations of DDT were degraded by the end of 72 h in 25°C.

Bidlan and Manonmani (2002) reported that pH 7 was the best for DDT degradation. In our studies, pH between 6-8 was found to enhance the degradation of DDT. The maximum predicted percentage degradation of 96.69, 96.37, 97.97, 92.44 and 98.19 was obtained at 25°C (coded value-1.682) for 5, 10, 20, 30 and 35 ppm initial DDT concentrations respectively and at different pH levels 7.82 (coded value 1.53), 6.59 (coded value 0.497), 6.92 (coded value 0.776), 7.06 (coded value 0.892) and 8.00 (coded value 1.682), respectively.

CONCLUSIONS

The consortium used in the present study was very stable and consistent results were obtained for degradation of DDT. The results indicate that based on the type of effluent containing DDT, conditions can be chosen to get complete degradation of DDT in the effluent. Knowledge of the various optimized parameters would facilitate an easy and more effective translation of the laboratory results to the fields.

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