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APPLICATION OF RESPONSE SURFACE METHODOLOGY FOR BIOREMEDIATION OF GLYPHOSATE CONTAINING SIMULATED EFFLUENT

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ABSTRACT
The purpose of this work was to completely degrade the glyphosate residue from wastewater by using a defined mixed culture of microorganisms by taking into account the potential health risks and toxicity due to possible exposure to glyphosate. Biodegradation process depends on many intrinsic and extrinsic factors for complete and successful mineralization/degradation of the compound. The parameters for complete glyphosate-degradation were optimized using response surface methodology (RSM) with the developed consortium. A central composite rotatable design (CCRD) with three variables i.e., inoculum concentration (50-1500 µg protein/ml), temperature (25-35^{0}C) and pH (4-8) each at levels -1.682, -1, 0, 1 and 1.682 was chosen. Three different concentrations of glyphosate (5, 10 and 20 ppm) were used for the degradation studies with incubation time of 72 hours, which was maintained as constant. The optimized conditions for the degradation of glyphosate were inoculum concentration of 50 µg protein/ml, 898.19 µg protein/ml and 1466.86 µg protein/ml at incubation temperature of 35^{0}C, 25.5^{0}C and 28.39^{0}C, respectively, for 5, 10 and 20 ppm glyphosate concentrations at different pH levels 4.00, 8.0 and 6.38. The experimental values (complete degradation at 10 and 20 ppm and 60% degradation at 5 ppm) were found to be in agreement with the predicted ones. The test results showed the mineralization of glyphosate by the bacterial consortium with the formation of aminomethylphosphonic acid (molecular mass 109), glycine (molecular mass 75) and sarcosine (molecular mass 89) as determined by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography-Mass Spectroscopy (LC-MS) analyses.

KEYWORDS: Glyphosate, Central composite rotatable design (CCRD), Microbial consortium, Degradation/Mineralization.

INTRODUCTION

Broad-spectrum herbicide glyphosate commonly sold in the commercial formulation ‘roundup’ has been frequently used both on crops and non-crops areas world wide since it was introduced in the 1970’s (Borggaard and Gimsing, 2008; The case for a GM- free sustainable world, 2003; Vink *et al.,* 2012). Glyphosate is a member of the amino acid herbicide family and its mode of action is it kills plants by inhibiting the activity of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP) of the shikimic acid pathway, which is necessary for the formation of the aromatic amino acids tyrosine, tryptophan and phenylalanine. These amino acids are important in the synthesis of proteins that link primary and secondary metabolism. A blockage of the shikimic acid pathway leads to a depletion of the free pool of aromatic amino acids in higher plants. EPSPs are present in the chloroplast of most plant species (Duke, 1998; Carlisle and Trevors, 1998). Perennial weeds, overwintering rhizomes and tubers in the plants are controlled by immediate translocation of glyphosate into the plants. It is registered for preplant or postharvest treatment in crops and on noncrop land (Omafra staff, 2002).

Glyphosate being used indiscriminately by agriculturalists results in the residue accumulation in food and water commodities. Indian Prevention of Food Adulteration act (PFA) has set the maximum residue limit (MRL) of glyphosate concentration for food crops at 1.0 ppm (Confederation of Indian Industry, 2006). U. S. Environmental Protection Agency, 1993 has set the limit of detection as 1 ppb (Acquavella, *et al*., 2004; United States Environmental Protection Agency, 1993). The half life of glyphosate in soil is 47 days (Wauchope *et al*., 1992). In many countries, toxicology studies have been conducted to enable evaluation of the potential health risk (Williams *et al.,* 2000). Some negative short-term and long-term health effects have been discovered due to glyphosate usage. Additionally, the glyphosate-containing product ‘Roundup’ has been known to be used in suicide cases in Japan and consumption of it results in symptoms such as intestinal pain, vomiting, excess fluid in the lungs, pneumonia, clouding of consciousness and destruction of red blood cells (Cox, 2000). Short term exposure to glyphosate can cause breathing difficulties, loss of muscle control and convulsions. Some glyphosate-containing products belong to toxicity category I and II (acutely toxic), which are toxic to animals and humans with

symptoms including eye and skin irritation, headache, nausea, numbness, elevated blood pressure and heart palpitations (Cox, 2000). Although glyphosate is an acid, it is commonly used as the isopropylamine or trimethylsulfonium salts and is usually distributed as water-soluble concentrates and powders.

It is highly soluble in water thereby making its entry into the food system and water sources through soil. Its removal from water and soil is therefore a priority and needs to be addressed urgently. Microorganisms have been shown to degrade glyphosate and the mechanism of microbial attack has been described. The conventional method of optimization of degradation parameters involves varying one parameter at a time keeping other parameters constant. This is time consuming and does not bring about the simultaneous effect of interaction of various parameters (Hamsaveni *et al*., 2001). 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 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 variables in the response (Triveni *et al*., 2001).

The objective of the present work was to study the degradation of glyphosate by microbial consortium. The study was aimed at optimizing the degradation conditions by RSM and to study kinetics of glyphosate degradation.

MATERIALS AND METHOD

Chemicals

The analytical standard of glyphosate (99%) was obtained from Monsanto Co (St. Louis, Missouri, USA). All other chemicals used in this study were of analytical grade and procured from standard companies. The media were procured from Hi-Media laboratories Pvt. Ltd., Mumbai, India.

Microbial consortium

The microbial consortium developed for degrading Hexachlorocyclohexane (HCH) and Dichlorodiphenyltrichloroethane (DDT), consisting of ten bacterial isolates: 7 *Pseudomonas* species, 1 *Burkholderia*, 1 *Vibrio alginoleticus*, 1 *Flavobacterium* (Murthy and Manonmani, 2007) (Fig. 1) was used in this study. The individual bacterial isolates were grown in nutrient broth for 72 hours under aerobic conditions (180 rpm). The cells were harvested by centrifugation at 10,000 rpm for 15 minutes at 4^0C. The cells were washed well in minimal medium and resuspended in minimal medium (Deepthi *et al*., 2007) and mixed at equal OD 600. Ten ppm of glyphosate (filter sterilized) was added to these cells. The cells were acclimated by the daily addition of 10 ppm of glyphosate for 96 h then the cells were harvested (10,000 rpm, 15 minutes, 4^0C), washed well in minimal medium, resuspended in known volume of minimal medium and used as inoculum in this study.

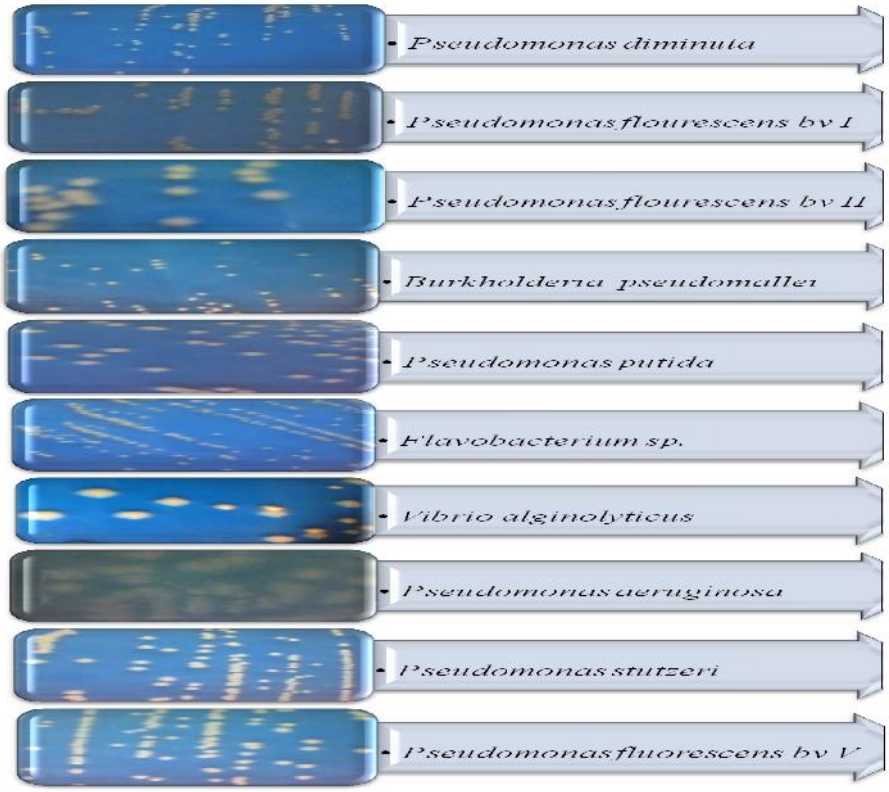

FIGURE 1. Microbial consortium used in this study

Experimental design for response surface methodology

The minimal medium (at required pH) containing known quantity of the pesticide was inoculated with the pre-exposed inoculum and incubated at appropriate temperature for 72 hours and analysed for residual glyphosate (Deepthi *et al*., 2007).

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), temperature (25-35^{0}C) and pH (4-8) each at 5 levels - 1.682, -1, 0, 1 and 1.682 (Table 1). The central points and their concentration ranges were fixed based on previous experiments as well as the information provided in the literature. Incubation time of 72 hours was maintained for all the experiments. Glyphosate degradation was studied at three different initial concentrations (5, 10 and 20 ppm).

TABLE 1: Variables and their levels for CCRD.

	Symbols	-1.682	-1	0	1	1.682	Mean	Std. Deviation
Innoculum Conc (v/v)	X1	50	343.97	775	1206.03	1500	775	431.03
Temperature (^{o}C)	X2	25	27.03	30	32.97	35	30	2.97
pH	X3	4	4.81	6	7.19	8	6	1.19

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 (run 15-20) at the centre 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.

TABLE 2: Treatment Schedule for five-factor CCRD and response in terms of residual concentration.

Exp No.	Inoculum Conc (v/v)	Temp. (^{o}C)	pH	5 ppm		10 ppm		20 ppm	
	X1	X2	X3	Exp	Pre	Exp	Pre	Exp	Pre
1	-1	-1	-1	4.32	4.08	5.02	6.11	8.33	9.33
2	1	-1	-1	3.89	3.85	9.39	10.36	6.44	7.05
3	-1	1	-1	3.50	3.65	6.13	6.23	6.18	6.56
4	1	1	-1	4.00	3.65	4.79	5.66	7.36	7.86
5	-1	-1	1	3.50	3.75	3.00	2.80	3.92	4.24
6	1	-1	1	3.90	3.65	2.93	3.51	2.75	3.19
7	-1	1	1	4.06	4.01	9.08	8.79	4.15	4.36
8	1	1	1	4.00	4.13	5.09	4.67	7.07	6.89
9	-1.682	0	0	4.56	4.45	7.90	7.80	1.03	0.29
10	1.682	0	0	4.10	4.36	8.77	7.91	0.92	0.50
11	0	-1.682	0	3.80	3.92	4.64	3.51	8.29	7.27
12	0	1.682	0	3.95	3.97	4.42	4.59	8.20	8.05
13	0	0	-1.682	3.00	3.23	9.11	7.63	14.00	12.92
14	0	0	1.682	3.45	3.36	3.50	4.02	7.91	7.83
15	0	0	0	4.55	4.50	4.04	4.14	4.98	5.41
16	0	0	0	4.67	4.50	4.15	4.14	5.41	5.41
17	0	0	0	4.44	4.50	4.08	4.14	6.05	5.41
18	0	0	0	4.36	4.50	4.07	4.14	5.11	5.41
19	0	0	0	4.46	4.50	4.08	4.14	5.65	5.41
20	0	0	0	4.53	4.50	4.25	4.14	5.03	5.41

Experiments were done according to the experimental plan for different initial concentrations of glyphosate (Table 2). Samples were removed after 72 hours of incubation at required temperature, extracted and analysed for residual glyphosate by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

For statistical analysis a second order polynomial equation was used to fit the experimental data given in Table 1. The model proposed for the response (Y_i) was,

$$Y_i = a_o + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + \varepsilon \quad (1)$$

where Y_i (i = 1 to 5) is the predicted response for residual glyphosate for initial concentration 5, 10 and 20 ppm, a_o is the value of the fitted response at the centre point of the design, a_i, a_{ii}, a_{ij} *being* the linear, quadratic and cross product terms, respectively and ε is the random error. The coefficients of Equation 1 were obtained using MATLAB 7.0 software (The MathWorks Inc., Natick, MA, USA) based on the data provided in Table 2 and are presented in Table 3.

TABLE 3: Estimated coefficients of the fitted second order polynomial representing the relationship between the response and the process variable

	5 ppm		10 ppm		20 ppm	
	Estimated coefficients	t-value	Estimated coefficients	t-value	Estimated coefficients	t-value
a_0	4.50 [a]	46.42	4.14 [a]	11.31	5.41 [a]	17.11
a_1	-0.03 [ns]	-0.41	0.03 [ns]	0.13	0.06 [ns]	0.30
a_2	0.02 [ns]	0.23	0.32 [ns]	1.33	0.23 [ns]	1.10
a_3	0.04 [ns]	0.58	-1.07 [a]	-4.42	-1.51 [a]	-7.22
a_{11}	-0.03 [ns]	-0.53	1.31 [a]	5.55	-1.77 [a]	-8.68
a_{22}	-0.19 [a]	-3.10	-0.03 [ns]	-0.13	0.80 [a]	3.91
a_{33}	-0.42 [a]	-6.78	0.60 [b]	2.52	1.76 [a]	8.61
a_{12}	0.06 [ns]	0.68	-1.20 [a]	-3.79	0.90 [a]	3.27
a_{13}	0.03 [ns]	0.39	-0.89 [b]	-2.79	0.31 [ns]	1.12
a_{23}	0.17 [b]	2.04	1.47 [a]	4.62	0.72 [b]	2.64

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

The t-values of the estimate were compared with the tabular value and the terms having t-values lower than the tabular values were omitted (Khuri and Cornell, 1987). All the statistically insignificant coefficients were omitted before predicting the response. All the three responses under different combinations as defined in the design (Table 2) were analysed using the analysis of variance (ANOVA) appropriate to the experimental design (Table 4), which indicated that the sum of squares due to regression (first and second order terms) was significant. The lack of fit was found to be significant in a few cases. However, the high values of coefficient of determination, R^2 (Table 4) suggested that the model is a good fit. The R^2 is the proportion of variability in response values explained or accounted for, by the model.

Minimization of fitted polynomials for the responses for 5, 10 and 20 ppm was performed by a non-linear mathematical maximization procedure of the Quattro Pro software package (Nandini and Rastogi, 2010). The fitted polynomial equation was expressed as surface plots using MATLAB 7.0 software (The MathWorks Inc., Natick, MA, USA) in order to visualize the relationship between the response and experimental levels of each of the factors.

TABLE 4: Analysis of variance for the fitted polynomial model as per CCRD.

	df	Sum of squares (SS)		
		5 ppm	10 ppm	20 ppm
Regression				
First order terms	3	0.03 [ns]	17.18 [a]	32.09 [a]
Second order terms	6	3.20 [a]	63.68 [a]	120.27 [a]
Total	9	3.24	80.86	152.36
Residual				
Lack of fit	5	0.47 [a]	5.59 [ns]	3.94 [ns]
Pure error	5	0.10	2.46	2.06
Total error	10	0.56	8.06	6.00
Grand Total	19	3.80	88.92	158.37
Coefficient of Determination (R^2)		0.85	0.91	0.96

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

Degradation of glyphosate

To study the effect of incubation period on the degradation of glyphosate, different concentrations of the pesticide in minimal medium was inoculated by microbial consortium. Samples (whole flasks) were removed at 3 hours interval from 0 hour through 120 hours. Samples were then extracted and analysed for residual glyphosate by TLC and GC.

To study the pathway of glyphosate degradation, all the pre-exposed and washed inoculum were inoculated to 50 ml of sterilized phosphate buffer (pH 7.2) in 250 ml of Erlenmeyer flasks containing 20 ppm of glyphosate. Other conditions used were as obtained from optimized results of RSM studies. The inoculated flasks were then incubated at 30^0C on a rotary shaker (180 rpm) for different intervals of time. Samples (whole flasks each time) were drawn at regular intervals, after every 24 hours up to 10 days. These samples were centrifuged at 10,000 rpm at 4^0C for 10 minutes and the supernatant was pooled and analysed by TLC, HPLC and LC-MS.

Kinetic studies

The degradation of glyphosate was assumed to follow first order kinetics. The experimental data were fitted to the following Equation 2.

$$\ln\left(\frac{C_t - C_f}{C_i - C_f}\right) = -kt \qquad (2)$$

where, C_i, C_f, and C_t are the concentrations of glyphosate at initial, final and any time t, respectively and k is the rate constant.

Determination of protein

The growth of microbial consortium was determined by estimating total protein in the biomass by modified method of Lowry et al., (1951) and Murthy and Manonmani, (2007).

Detection of glyphosate

TLC was done using Silica gel 60 of 20 x 20 cm ready to use precoated aluminium plates. Known volume of the residual extract of glyphosate (acetone solution) was spotted on to the plates. Plates were developed in isopropanol: 5% ammonia in 1:1 ratio. After the solvent has reached 3/4th of the plates, the plates were removed and air dried, then sprayed with 0.2% of ninhydrin. The plates were kept at 37^{0}C for 10 minutes. Glyphosate and its by-products give a dark purplish colour. Spots were marked with a needle and area measured. The concentration was delineated using a standard curve. The Rf values were: glyphosate 0.6; glycine 0.38; sarcosine 0.43 and AMPA 0.71 (Fig. 2).

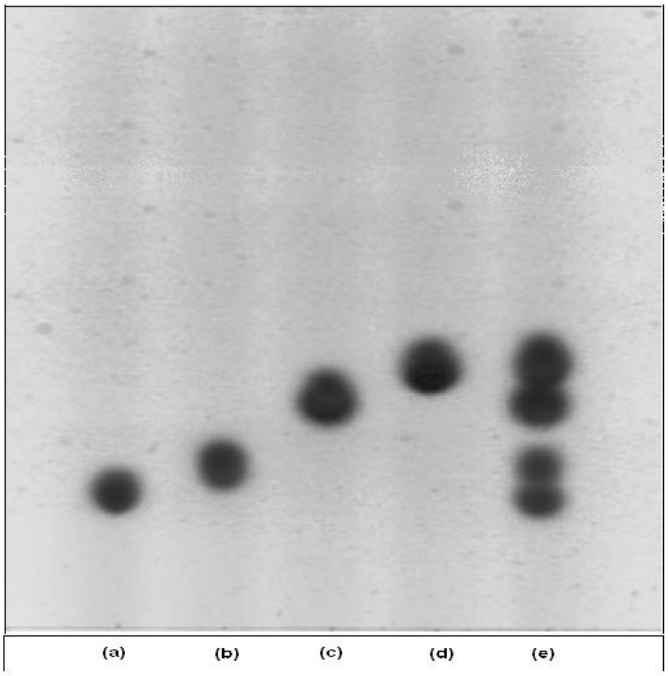

FIGURE 2. TLC pattern of glyphosate and its metabolites. (a) standard glycine; (b) standard sarcosine; (c) standard glyphosate; (d) standard AMPA; (e) microbial degraded glyphosate by-products

HPLC was done using amino acid analyser (Shimadzu, Japan, Model no: RF-10A$_{XL}$) with a fluorescence detector. A cation exchange analytical column was used. All glyphosate standards and the residual glyphosate extract were prepared in distilled water and were hydrolysed at 36^{0}C (Clegg and Ripley, 1996) with sodium hypochlorite to form glycine. The glycine was then reacted with *o*-phthalaldehyde (OPA) in the presence of mercaptoethanol at 55^{0}C to produce a highly fluorescent isoindole, which was detected fluorometrically (excitation 330 nm, emission 465 nm). All glyphosate standards were prepared in distilled water and serially diluted in potassium dihydrogen phosphate buffer to the concentration range required for analysis. A volume of 10 µl of standard / sample was injected into the liquid chromatograph, which had a mobile phase of potassium dihydrogen phosphate buffer (0.005 M) with a flow rate of 1.0 ml/minutes. The total chromatographic run was for 30 minutes with isocratic mobile phase. Peak areas of the standards were plotted against the concentration of glyphosate, and the resulting standard curve was used to interpolate glyphosate concentration in the food samples. The approximate retention times of the derivatized glyphosate and its metabolites were: glyphosate 6.89, glycine 8.00, sarcosine 11.00 and amino methyl phosphonic acid (AMPA) 14.39 (Fig. 3).

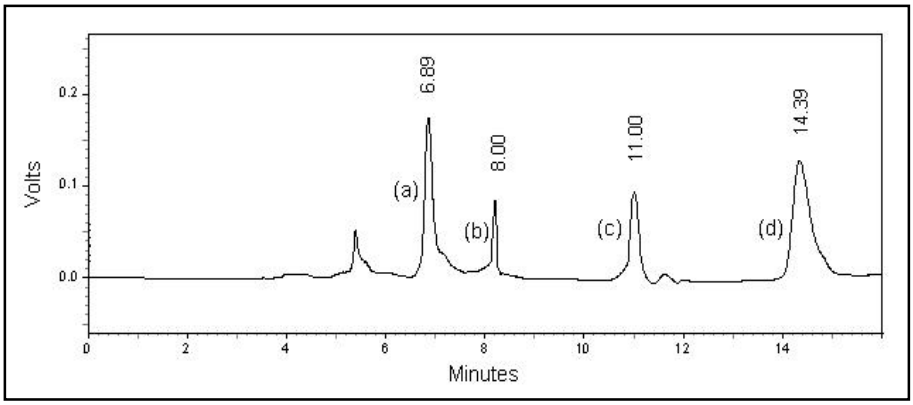

FIGURE 3. HPLC profile of the derivatized glyphosate and its metabolites. (a) glyphosate; (b) glycine; (c) sarcosine; (d) AMPA

RESULTS

Acclimation microbial consortium with glyphosate

DDT-degrading microbial consortium consisting of seven strains of *Pseudomonas*, one each of *Flavobacterium, Burkholderia and Vibrio* (Deepthi *et al*., 2007) were acclimated with 10 ppm of glyphosate. This consortium that had developed the capability to degrade glyphosate was used in degradation studies.

Diagnostic checking of models

Response was measured in terms of residual concentrations of glyphosate. The coefficients for the actual functional relation for predicting response are presented in Table 3. The insignificant terms were omitted based on student's t-ratio (Deavin *et al*., 1977). The response under different combinations as defined in the design (Table 1 and 2) were analysed using 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 the residual content was not significant ($p < 0.05$). The values of coefficient of determination (from 0.85 to 0.96) also suggest that the model is a good fit. The R^2 proportion of variability in response values explained or accounted for by the model (Sreedharan *et al*., 1999; Manonmani *et al*., 2008).

Optimization

The optimum conditions for glyphosate degradation are presented in Table 5. In order to deduce the workable optimum conditions, non-linear mathematical optimization technique was adopted. The second order polynomial equations (Equation 1) for the initial concentration of glyphosate (Y_1 to Y_5, based on the coefficients provided in Table 3) were minimized (Table 5). This technique drastically reduces the amount of time and effort required for the investigation of multifactor multiresponse system. It also provides comprehensive and informative insight into the system, which leads to fast process optimization.

TABLE 5: Feasible optimum conditions and predicted and experimental value of response at optimum conditions.

	Inoculum Concentration	Temperature	pH
5 ppm			
Coded Value	-1.682	1.682	-1.682
Uncoded	50.000	35.000	4.000
	Predicted Value		Experimental Value
Residual Glyphosate (Y)	2.105		2.000±.003
10 ppm			
Coded Value	0.286	-1.490	1.682
Uncoded	898.196	25.571	8.000
	Predicted Value		Experimental Value
Residual Glyphosate (Y)	0.00		0.0±.0.00
20 ppm			
Coded Value	1.605	-0.541	0.327
Uncoded	1466.869	28.393	6.389
	Predicted Value		Experimental Value
Residual Glyphosate (Y)	0.00		0.0±.0.00

Response surface plotting

The effect of inoculum concentration, pH and temperature on responses such as degradation of glyphosate (in terms of residual glyphosate) are reported (Table 3) by the coefficients of second order polynomials. The response surfaces (keeping third variable at optimum level) based on these coefficients are given in Figs. 4–6. In general, the exploration of the response surfaces indicated a complex interaction between the variables.

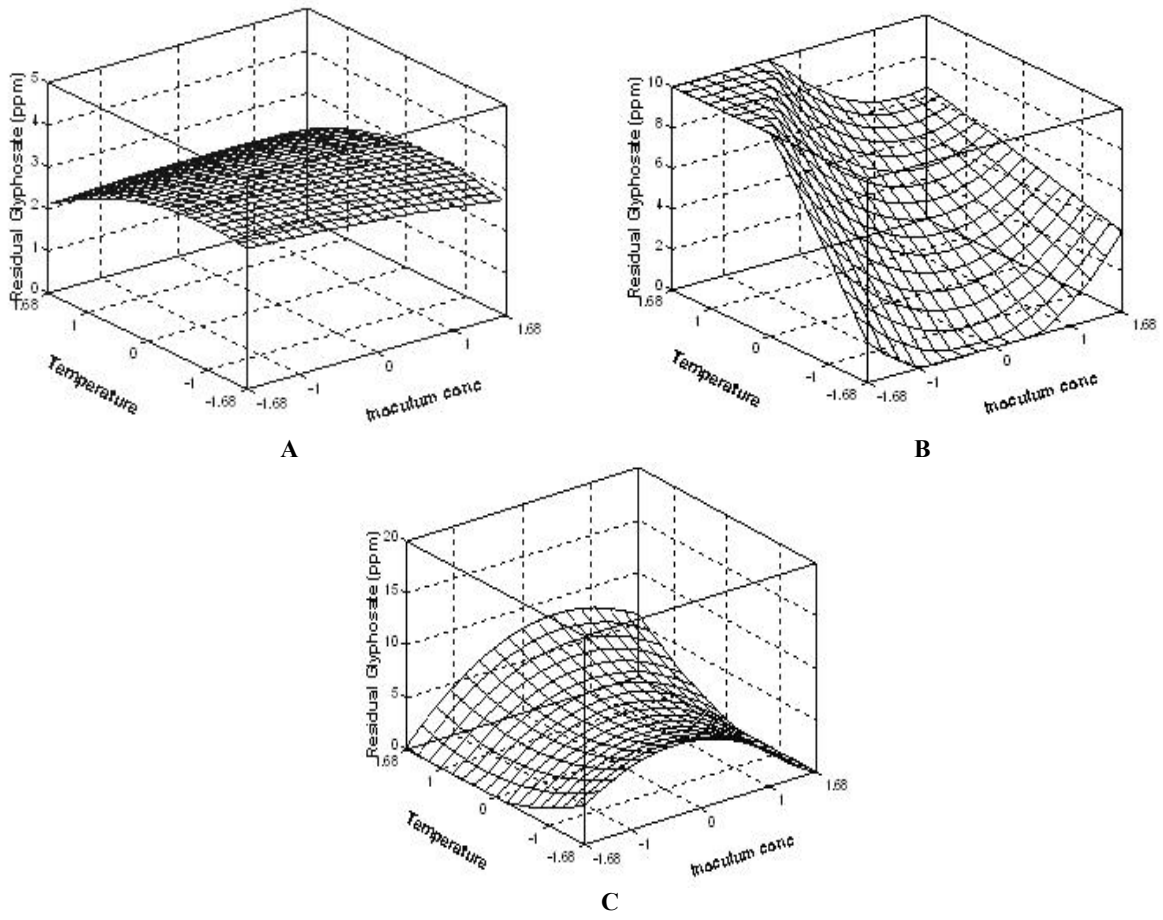

FIGURE 4. Effect of inoculum concentration and temperature on glyphosate degradation. (a) 5 ppm; (b) 10 ppm; (c) 20 ppm

a. Effect of inoculum concentration and temperature on glyphosate degradation

The effect of variation of inoculum concentration and temperature on glyphosate degradation is shown in Fig. 4, while pH was maintained at respective optimum conditions as indicated in Table 5. At low temperature (25^0C), the degradation of glyphosate was found to be low and then increased at higher temperature (35^0C). Only 40% of the added 5 ppm remained after the degradation period. The degradation showed a slow decrease at low level of inoculum concentration (50 µg protein/ml) and remained almost constant till higher inoculum level (1500 µg protein/ml) when the initial glyphosate concentration was 5 ppm. However for concentration at 10 ppm, the glyphosate degradation decreased rapidly upto 30 to 32^0C and no degradation was observed thereafter. At low inoculum concentration (50 µg protein/ml) the degradation was around 90% with complete degradation i.e., zero residual concentration of glyphosate with increase in inoculum level (upto 1200 µg protein/ml). However at the highest inoculum concentration (1500 µg protein/ml) the degradation dropped down. The decrease in degradation at higher inoculum level was probably due to dominance of certain microbes in the consortium or due to the death of important degrading species in the consortium. For higher glyphosate concentration 20 ppm, the degradation rate remained almost constant at all temperatures showing maximum degradation with slow increase in residual glyphosate at low temperatures. i.e. the rate of degradation was low at low temperatures and complete degradation was observed at around 28^0C. The degradation decreased with increase in inoculum concentration upto 1200 µg protein/ml and reached 100% degradation at 1500 µg protein/ml.

TABLE 6: Kinetic equations for the biodegradation of glyphosate by microbial consortium.

Concentration of Glyphosate (ppm)	Degradation Constant (h^{-1})	R^2
20	0.033	0.9202
50	0.028	0.9620
75	0.016	0.9931

b. Effect of inoculum concentration and pH

The effect of inoculum concentration and pH on glyphosate degradation is shown in Fig. 5, while the temperature was kept at optimum. There was no change in degradation of glyphosate at all the inoculum concentrations used i.e. degradation remained almost constant. However the degradation decreased with pH upto 7 and increased at higher pH level. At 10 ppm glyphosate concentration, the degradation decreased upto inoculum concentration of 1000 µg protein/ml and remained almost constant. The degradation was low at low pH levels and increased with increase in pH upto pH 7.19 and remained almost constant at higher pH values. For higher glyphosate concentrations i.e. 20 ppm the decrease in degradation rate was observed upto around 1200 µg protein/ml and increased with further increase in inoculum level. The degradation increased with pH and complete degradation was observed from pH 6 which remained constant even at higher pH levels.

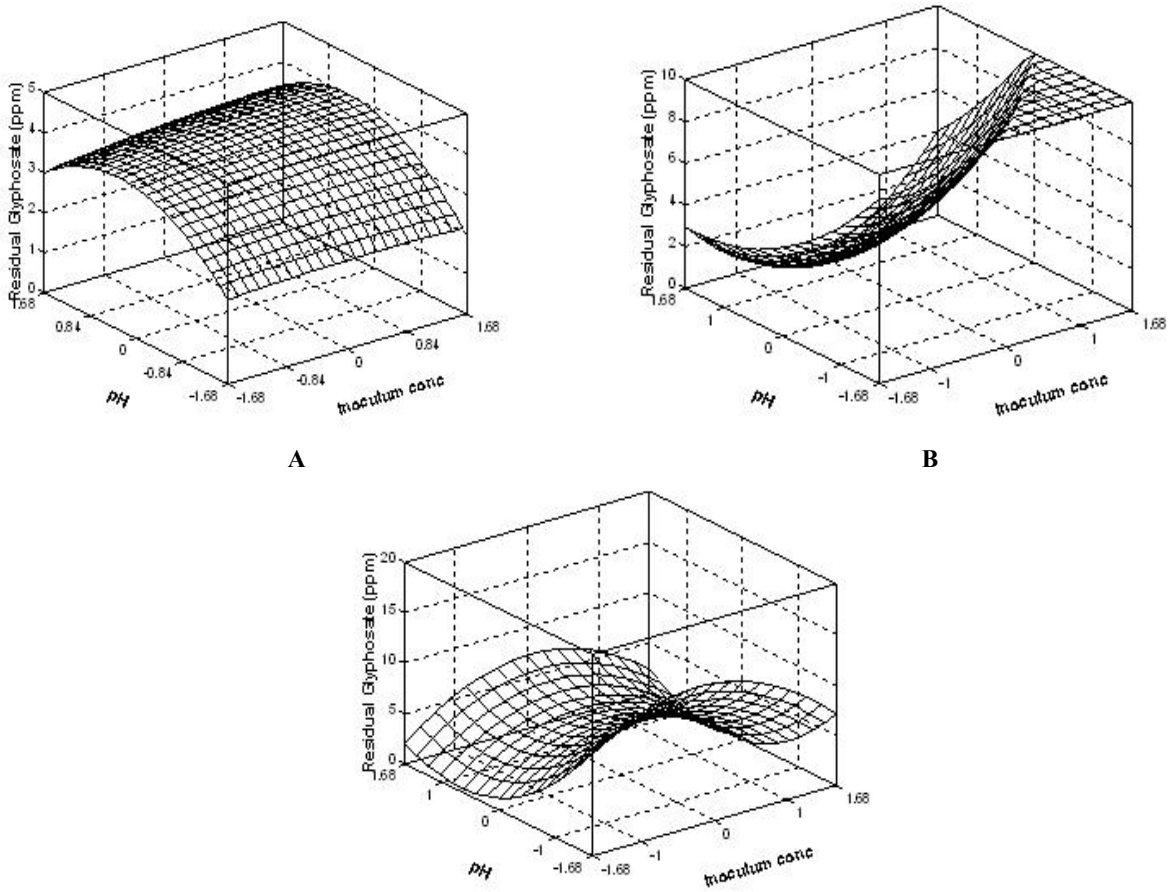

FIGURE 5. Effect of pH and inoculum concentration on glyphosate degradation. (a) 5 ppm; (b) 10 ppm (c) 20 ppm

c. Effect of pH and temperature on glyphosate degradation

The effect of pH and temperature on glyphosate degradation is shown in Fig. 6, while the inoculum level was kept at optimum concentration. At low temperature (25^0C) and low glyphosate concentration of 5 ppm, the degradation showed a very slight decreasing trend. i.e. only 10 % enhancement in degradation was observed with increase in temperature. At low pH values, the degradation was only 60% and the degradation decreased with increase in pH i.e. at around 6.25 pH maximum glyphosate concentration of 64% was observed to be remaining. At higher pH values the degradation again showed a increasing trend. At 10 ppm, the glyphosate degradation was low upto 27^0C and then increased gradually with increase in temperature. 60% degradation was observed at 35^0C. Similarly the degradation increased with pH. At low pH values there was practically no degradation and 10 ppm of glyphosate was found to be remaining even after the degradation period. At higher pH values the complete degradation was observed. At 20 ppm of glyphosate concentration, at low temperatures, the degradation of glyphosate was maximum and decreased with increase in temperature. At low pH values the degradation was only upto 70% i.e. 30% of glyphosate residue was found to remain even after degradation period was completed. With increase in pH, the degradation was complete reaching 100%.

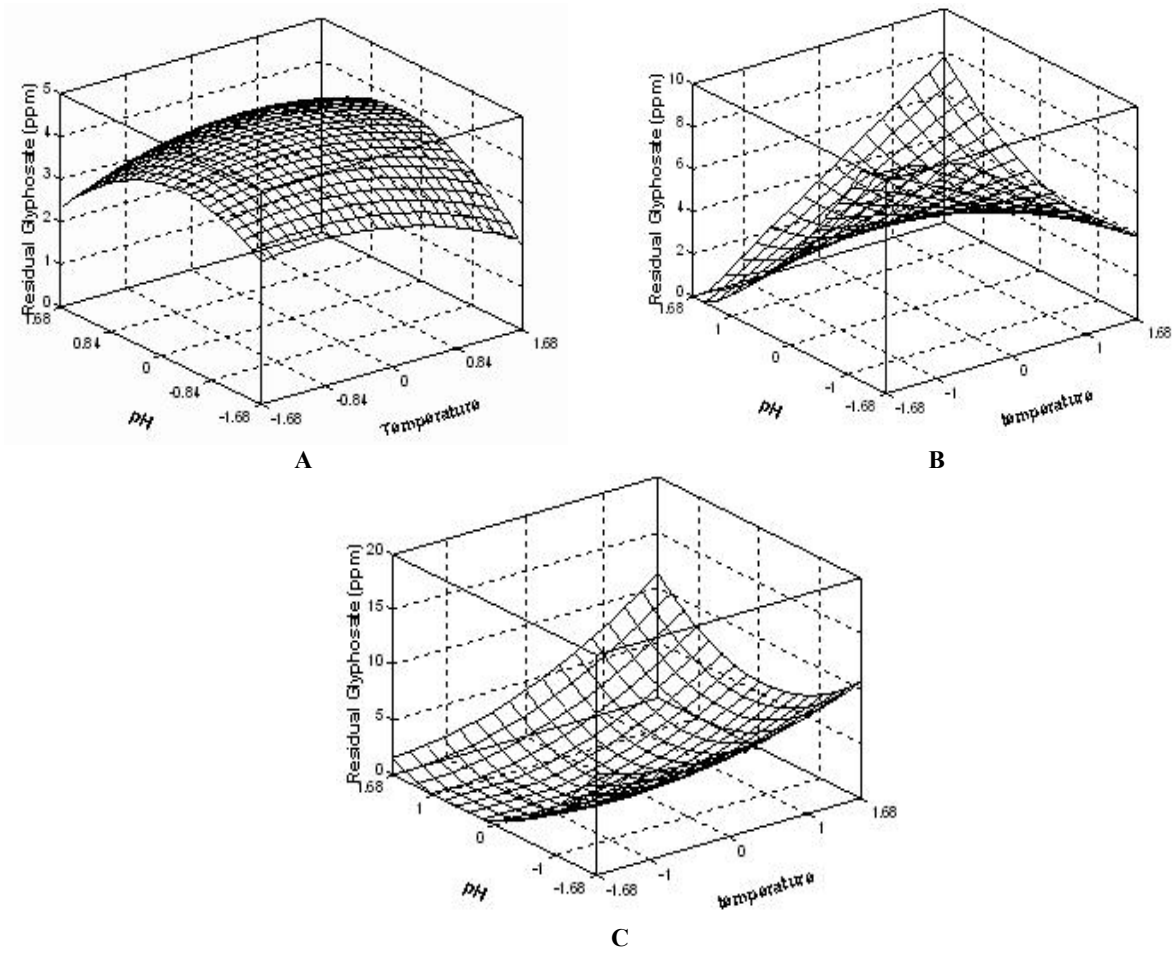

FIGURE 6. Effect of pH and temperature on glyphosate degradation. (a) 5 ppm; (b) 10 ppm; (c) 20 ppm

Verification of results

The suitability of the model equation for producing the optimum response values were tested using the feasible optimum conditions. This set of conditions was determined to be optimum by RSM optimization approach, which was 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). Degradation of glyphosate was validated at inoculum concentration of 50 µg protein/ml (coded value -1.682), 898.196 µg protein/ml (coded value 0.286) and 1466.869 µg protein/ml (coded value 1.605). The incubation temperature used was 35^0C (coded value 1.682), 25.5^0C (coded value -1.490) and 28.39^0C (coded value -0.541), respectively, for 5, 10 and 20 ppm of glyphosate concentrations. The different pH levels used were 4.0 (coded value -1.682), 8.0 (coded value 1.682) and 6.389 (coded value 0.327).

Period of incubation and degradation of glyphosate

In general, the degradation of glyphosate increased with incubation time. The time required for degradation of different concentrations of glyphosate was time dependent. Five ppm of glyphosate was degraded completely by 72 hours (Fig. 7). There was slight initial lag. No degradation was observed by 3 hours of incubation period. After 3 hours of lag, the degradation of glyphosate started at a rate of around 0.006 µg/ml for all the higher concentrations studied. Similarly the degradation of 20 ppm of glyphosate increased steadily by 96 hours of incubation and the rate increased to 3.194 µg/ml. However at 40, 50, 75 and 100 ppm glyphosate initial concentrations, the degradation rates were around 0.006 after 3 hours lag and by 96 hours of incubation, the degradation rates were 2.96, 2.74, 1.507 and 3.957 µg/ml, respectively. That is, the degradation increased with incubation time (Fig. 8). The time required for the degradation of higher concentrations of glyphosate increased with increase in concentration of glyphosate. The degradation of 20, 40 and 50 ppm glyphosate was completely degraded by 120 hours of incubation and degradation of higher concentrations of glyphosate needed more time for complete degradation (Fig. 7). The degradation constants for 20, 40 and 50 ppm of glyphosate were between 0.03 and 0.2 (Table 6) with an R^2 value of ~0.92.

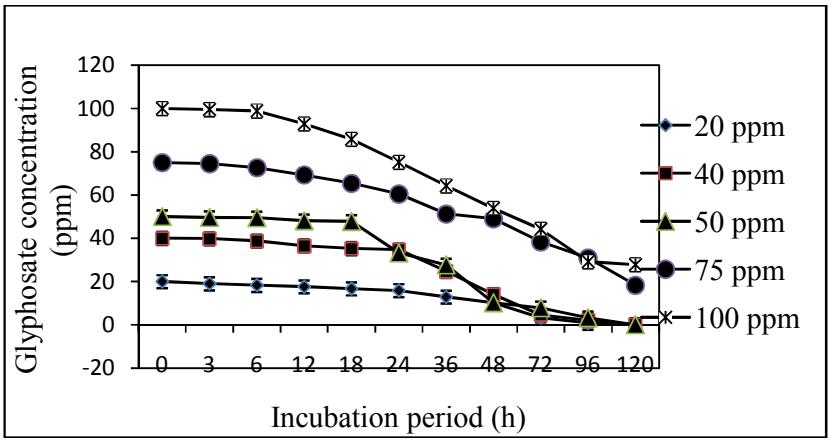

FIGURE 7. Incubation time Vs degradation of glyphosate

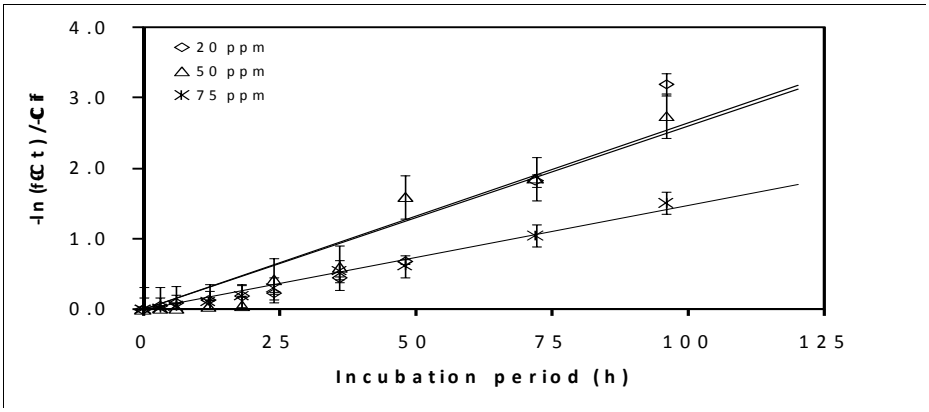

FIGURE 8. Kinetics of glyphosate degradation

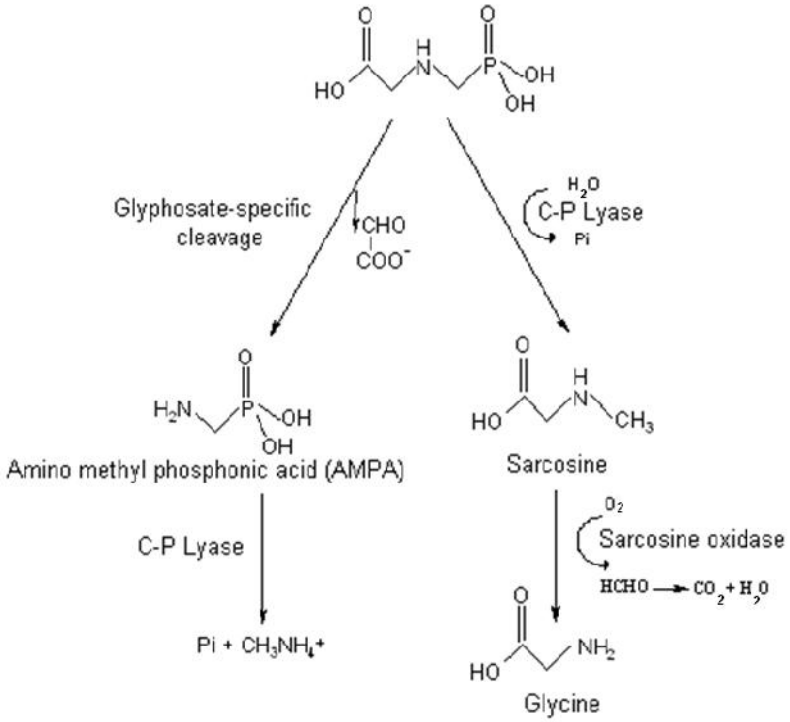

FIGURE 9. Proposed pathway of microbial degradation of glyphosate

Pathway of glyphosate degradation

The pathway of glyphosate biodegradation by the defined bacterial consortium was evaluated by analysing the products formed at higher initial substrate concentrations. Accumulation of AMPA (molecular mass 109), Glycine (molecular mass 75), and Sarcosine (molecular mass 89) were observed by LC-MS analysis (data not shown). The probable pathway of glyphosate degradation by the microbial consortium is given in Fig. 9.

DISCUSSION

DDT-degrading microorganisms were acclimated with 10 ppm of glyphosate for 72 hours with the addition of the pesticide every 24 hours. The glyphosate degrading consortium that got acclimated was used for studying glyphosate degradation. Microbial degradation studies have been conducted with pure cultures selected by enrichment technique (Karl-Heinz, 1986). Most control tests of biodegradation are based upon the enrichment culture technique, where by the initial population contains different varieties of microorganisms tolerant to a given environment with possibly different metabolic pathways (Ludzack and Ettinger, 1963; Wagner, 1973). The microbial consortium developed by us is one such xenobiotic degrader that has developed glyphosate degradation upon acclimation. The acclimation of microbial community to glyphosate would have led to the interactions among microbial community. The individual defined microbial consortium got adapted to glyphosate. This indicated that the bacterial isolates of the microbial consortium are very versatile and have the capacity to degrade many xenobiotic compounds. The carbon limited nature of xenobiotics will ensure a strong and selective pressure for the organisms capable of attacking these recalcitrant chemicals. Hence, under such competitive environment, adaptation favours the development of complex microbial community allowing the required time for all the adaptable members to get established, either through induction from outside or through mutations. The acclimation and enrichment techniques have been employed with continuous culture of microorganisms by applying the compound to be degraded continuously, initially at low concentrations and subsequently by either addition of the same concentration or by increasing the xenobiotic concentration in a systematic manner (Moos, 1980). In a similar trial HCH and DDT degrading consortium have been isolated and acclimated to get good degrading microbial community (Murthy and Manonmani, 2007; Manonmani *et al*., 2008). Bhuyan *et al*., (1992) and Wada *et al*., (1989) showed that γ-HCH degradation improved after every successive application of the compound.

The maximum and minimum biodegradable concentration is an important factor for obtaining complete degradation of a xenobiotic compound. Some biodegradative strains when inoculated into environmental samples are unable to metabolize the pollutant. Among the reasons cited for such behaviour the presence of very low concentration of the xenobiotic compound limits induction. For some compounds there is a threshold concentration below which the biodegradation rate is negligible. An explanation of biodegradation of organic compound at concentrations below threshold level is that the microorganisms are simultaneously using higher concentrations of other compounds for maintenance and growth (Karl-Heinz, 1986). The degradation of glyphosate has been given by Zelenkova and Vinokurova, (2008). The *in situ* biodegradation of a contaminant is a function of the catabolic activity of bacteria and bioavailability of the contaminant to bacteria. Many bacteria require additional substrate to degrade the compound (Bidlan and Manonmani, 2002). The minimum and maximum substrate concentrations required to degrade a xenobiotic compound are very important factors to obtain maximum degradation. In our studies we obtained complete degradation of glyphosate from 5 ppm to 50 ppm. The lower concentrations were degraded very fast in a shorter time. Fifty ppm was also degraded completely, but it took slightly longer time.

Environmental factors such as pH and temperature, cultural factors such as inoculation concentration, substrate concentration, incubation temperature etc. may affect degradative abilities. The abilities vary from one organism to another and one substrate to another. Bidlan and Manonmani, (2002) have reported degradation of 10 ppm of DDT by 72 hours at 30^0C.

In our studies the degradation of different concentrations of glyphosate was pH, temperature and inoculum dependent. Optimum degradation of glyphosate was at inoculum concentration of 50 µg protein/ml (coded value -1.682), 898.196 µg protein/ml (coded value 0.286) and 1466.869 µg protein/ml (coded value1.605), at incubation temperature of 35^0C (coded value 1.682), 25.5^0C (coded value -1.490) and 28.39^0C (coded value -0.541), respectively, for 5, 10 and 20 ppm glyphosate concentrations at different pH levels 4.00 (coded value -1.682), 8.0 (coded value 1.682) and 6.389 (coded value 0.327). Glyphosate was degraded with the formation of aminomethylphosphonic acid (AMPA) (Molecular mass 109), glycine (Molecular mass 75) and sarcosine (Molecular mass 89) as observed by LC-MS analysis. Similar observations have been made by Zelenkova and Vinokurova, (2008) as products of microbiological utilization of glyphosate. But Schuette, 1998 obtained AMPA and Sarcosine as products of plant metabolism. In conclusion the acclimated defined bacterial consortium was found to develop the abilities to degrade glyphosate, a weedicide against herbs. Results indicated that glyphosate concentrations of 5, 10 and 20 ppm could be degraded completely at optimised temperature and pH. With the knowledge of various optimized parameters, such as inoculum level, pesticide concentration, temperature and pH of the effluent, effective and easy degradation of the pesticide glyphosate can be translated to large scale, from laboratory to field. An understanding of the biochemical pathway of degradation would indicate the synergism involved among the microbial community during the biodegradation of glyphosate.

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