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Studies On DDT Degradation By Bacterial Strains

Thesis
Submitted to the

University of Mysore

For the award of the degree of

Doctor of Philosophy

In

Microbiology

By

Rajkumar Bidlan

Department of Food Microbiology
Central Food Technological Research Institute
Mysore – 570 013, India

October 2003

Rajkumar Bidlan
Department of Food Microbiology
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DECLARATION

I hereby declare that the thesis entitled **"*Studies on DDT Degradation by Bacterial Strains*",** submitted to the **University of Mysore**, for the award of the degree of **DOCTOR OF PHILOSOPHY** in **Microbiology,** is the result of research work carried out by me under the guidance of *Dr. Haravey Krishnan Manonmani*, Scientist, Department of Food Microbiology, Central Food Technological Research Institute, Mysore - 570 013, India, during the period of 1998- 2003.

I further declare that the results presented in this thesis have not been submitted for the award of any other Degree or Fellowship.

(RAJKUMAR BIDLAN)

Date: 20TH October 2003.

Place: Mysore.

20th October 2003

Dr. H.K. Manonmani
Scientist,
Department of Food Microbiology

CERTIFICATE

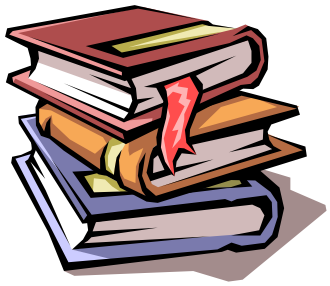
This is to certify that the Ph.D thesis entitled **"Studies on DDT Degradation by Bacterial Strains",** submitted by *Rajkumar Bidlan*, for the award of the degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY,** to the **University of Mysore** is the result of the research work carried out by him at the Department of Food Microbiology, Central Food Technological Research Institute, Mysore under my guidance during the period 1998-2003. This has not been submitted earlier, either partially or fully, for any other degree or fellowship.

(H.K.MANONMANI)
Guide

With the Blessings of Goddess Durga
and My Family,

I Remember

Zeidler, Muller and Rachel Carson,
whose work paved way for this work.



Hi Dad,

Hope your soul there up

is happy to see this bound volume

Sharing the Thanking Gesture

Foremost I would like to thank the Almighty, Goddess Durga and my family members for their blessings throughout my journey till this point and hope to get it throughout my life.

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RAJKUMAR BIDLAN

Studies On DDT Degradation By Bacterial Strains

Synopsis

Dichlorodiphenyltrichloroethane (DDT) is the first synthetic compound used against pests. Though it was first synthesised by Zeidler, the noble prize for its activity against insects especially against body lice and in control of typhus fever was awarded to Dr. Paul Muller. After the discovery that DDT can be used against insects, it was extensively used by almost all the countries for both agriculture as well as health programs. Later, it was discovered that DDT was responsible for many environmental problems *viz*. thinning of the eggshells in many birds especially Falcons, bringing it under the endangered category, being one among the first few hazards noticed. Today it is one of the suspect culprits for hormonal imbalance and even cancer. Its main advantage, when initially used, was its effectiveness on wide variety of insects, leading to a large-scale application on pests. However, the persistency has been now the main cause of concern to the environmentalists. Residues of DDT have been detected in soil, water and also in air. Through food chain they enter the human body causing health hazards. There have been many efforts to remediate DDT- contaminated sites. DDT has also been shown

to undergo transformation and form other compounds that are either dead end metabolites or even more toxic than DDT. Microorganisms have been shown to mineralise many xenobiotic aromatic compounds. Microorganisms can be used to remove DDT from soil and water with very low or no accumulation of intermediary toxic metabolites. Microbial remediation is the most safe and cost effective way of remediation towards a cleaner and safer environment.

With the above concepts in mind, attempts were made to isolate some microbes that could degrade DDT effectively. Four bacterial strains were obtained during long-term enrichment of DDT-contaminated soil and water. Various parameters of DDT degradation were studied. Attempts were also made to decipher the biodegradative pathway. A few catabolic genes were attempted for identification.

The work on Studies of DDT degradation by bacterial isolates has been divided into seven chapters.

The ***first Chapter "Advent, application and effects of DDT-a general introduction",*** discuses about the synthesis of DDT, discovery of its insecticidal properties and applications of DDT as a disinfectant and a pest controller. Different formulations that were used and the industrial use of DDT have also been mentioned. The regulations and the DDT-abuse are described with few of the health hazards related to humans, animals and the

environment. The fate of DDT in various environments has been discussed. The present scenario in the usage of DDT and its possible future form a part of this chapter.

Second Chapter "Bio-remediation-a review of literature" deals with the way pollutants enter the environment and the ways to get rid of these pollutants. Various research groups around the globe used plants or microorganisms to remediate soil or water (bioremediation). Different groups, to remediate the environment contaminated with different pesticides, have adapted different methods, including aerobic, anaerobic and mixed processes/methods using microorganisms. Microbial degradation of DDT in water and soil, factors affecting DDT degradation has been discussed in this chapter. The remediation processes involved and the pathways followed by the microorganisms during the degradation of various pesticides such as 2,4-D, 2,4,5-T, γ-HCH, atrazine, DDT and the enzyme systems involved in these processes have been described from the literature. Different pathways involved in ring cleavage have also been discussed. Treatment of DDT-contaminated sites and water resources and management of DDT-degradation with microorganisms has also been mentioned.

Chapter 3 "Isolation, purification and identification of microbes capable of DDT-degradation", deals with the work carried out in our laboratory to isolate the DDT-degrading microorganisms from contaminated

soil and water by long term enrichment technique, purification of these bacterial isolates, their identification based on microscopic, cultural and biochemical characters and degradation of DDT by microbial consortium and the individual bacterial isolates. Effect of cultural and environmental parameters on DDT-degradation by microbial consortium has been described in this chapter. The consortium obtained during the enrichment with increasing concentrations of DDT, could degrade up to 25ppm DDT. The individual members of this consortium were purified by dilution plating technique and subsequent purification of the individual strains on nutrient agar. Four bacterial isolates obtained were identified as, *Serratia marcescens* strain DT-1P, *Pseudomonas fluorescens* strain DT-2, *Pseudomonas aeruginosa* strain DT-Ct1 and *Pseudomonas aeruginosa* strain DT-Ct2 based on microscopic, cultural and biochemical characters according to Bergey's manual of Determinative Bacteriology. DDT was degraded by the microbial consortium at pH 7.0 – 7.5 and ambient temperature (26-30^{0}C). However, individual isolates could degrade lower concentration of DDT i.e. 5 ppm, partially. The isolates *Serratia marcescens* strain DT-1P, *Pseudomonas fluorescens* strain DT-2, *Pseudomonas aeruginosa* strain DT-Ct1 and *Pseudomonas aeruginosa* strain DT-Ct2 degraded 44, 20, 45 and 25% of the initially added 5 ppm DDT respectively by 48h. Pre-exposure of axenic cultures to 10 ppm DDT improved their

degradation ability. With 72h pre-exposed cells 80, 90, and 5% of the initially added DDT disappeared in case of *Serratia marcescens* strain DT-1P, *Pseudomonas aeruginosa* strain DT-Ct1 and *Pseudomonas aeruginosa* strain DT-Ct2 respectively, whereas 24h pre-exposed cells of *Pseudomonas fluorescens* strain DT-2 could degrade 84% of the initially added substrate by 72h. Influence of nitrogen sources on DDT degradation by axenic cultures showed that nitrogen limiting conditions favoured degradation by the isolates *Serratia marcescens* strain DT-1P, *Pseudomonas fluorescens* strain DT-2 and *Pseudomonas aeruginosa* strain DT-Ct2 where, 100, 65 and 15.2% of added 15 ppm DDT was degraded by 72h, whereas with *Pseudomonas aeruginosa* strain DT-Ct1 45% degradation was obtained in ammonium chloride in the medium. Media supplemented with organic nitrogen also showed better degradation. Consortium, reconstituted by mixing the four isolates in the proportion as present in the original consortium, was found to degrade higher concentration indicating the essentiality of all four bacterial isolates for the degradation of higher concentration of DDT.

Kinetics studies on DDT degradation by these bacterial isolates have been described in ***Chapter 4A, "Kinetics of DDT-degradation by the bacterial cultures"***, making an attempt to understand the rate of degradation at various cultural and environmental conditions such as inoculum level, DDT concentration, pH, Temperature, etc. The degradation of DDT was found to

be efficient at an inoculum level of 200 μg protein/ml for all the isolates. pH 7.0 was found to give better degradation in case of *Serratia marcescens* strain DT-1P , *Pseudomonas aeruginosa* strain DT-Ct1 and pH 6.0 was required for *Pseudomonas fluorescens* strain DT-2, and incubation temperature of 30^0C was found to be optimum for degradation of DDT by *Serratia marcescens* strain DT-1P and *Pseudomonas fluorescens* strain DT-2 and 20^0C was better for *Pseudomonas aeruginosa* strain DT-Ct1 .Degradation of 5, 10 and 15ppm DDT was complete while higher concentrations were degraded partially to 95% and 84% respectively by *Serratia marcescens* strain DT-1P. The rate constants of degradation of DDT by *Serratia marcescens* strain DT-1P at 5, 10, 15, 20 and 25 ppm were 0.028, 0.0241, 0.0202, 0.0153 and $0.0105h^{-1}$ respectively. The isolate *Pseudomonas fluorescens* strain DT-2 was able to degrade 5, 10, 15, 20, 25, 30, 35 and 50 ppm DDT up to 58, 56, 49, 45, 43, 40, 17 and 1.5% respectively even after 168h of incubation. The degradation rates were $0.0134h^{-1}$, $0.0118h^{-1}$, $0.0112h^{-1}$, $0.0107h^{-1}$, $0.006h^{-1}$ and $0.0043h^{-1}$ respectively for each of the concentrations from 5 through 30ppm. *Pseudomonas aeruginosa* strain DT-Ct1 degraded 5ppm DDT completely by 96h with the initial degradation rate of $0.0716h^{-1}$ while 10, 15, 20, 25, 30 and 35 ppm up to 75, 62.5, 58, 54, 38 and 24% by 144h with an initial

degradation rates 0.06, 0.0461, 0.0182, 0.0117, 0.0074 and 0.0036 h^{-1} respectively. .

The interactions between various parameters such as pH, initial DDT concentration, inoculum level, incubation time and incubation temperature have been described in ***Chapter 4B, "Optimisation of conditions for DDT degradation".*** The conditions interacting with each other during the bacterial degradation of DDT have been taken into consideration to define the degradation under any given set of these parameters. Combination of five variables *viz.* pH (4 to 8), initial DDT concentration (5 to 35 ppm), inoculum level (50 to 350μg protein/ml), incubation time up to 144h and temperature (20 to 40^0C), in 35 experiments were carried out at 5, 7, 7, 7 and 3 levels respectively. The coefficients in *Serratia marcescens* DT-1P indicated that pH, initial concentration and temperature influenced more the process of DDT degradation. The other two factors considered *viz*. inoculum level and incubation time also influenced the degradation of DDT by *Serratia marcescens* DT-1P. From our study and observations, it was established that incubation time played an important role in bioremediation of xenobiotics. Therefore it was considered an essential factor for learning the degradation process at other parameters under investigation. Interaction between pH and temperature also influenced DDT degradation by this strain. The *p*-values indicated that interaction between pH-inoculum and initial DDT

concentration-inoculum were the major influencing factors in the degradation process by *Serratia marcescens* DT-1P. In *Pseudomonas fluorescens* DT-2, the coefficients indicated that independently pH and initial DDT concentration were influencing the degradation while in the interactions of the investigated variables; pH-initial concentration and pH-temperature were the ones that influenced DDT degradation by this strain. *Pseudomonas fluorescens* DT-2, under various combinations of the studied parameters, influenced DDT degradation less positively (p-values>0.05). The coefficients, obtained for *Pseudomonas aeruginosa* DT-Ct1, showed that the variables: pH, initial concentration and temperature influenced the degradation of DDT more than the other two parameters, while interactions between pH and temperature had a positive influence in combination than any other interaction. The p-values here showed the association between none of the independent variables could describe the predicted dependent variable ($p>0.05$). *Pseudomonas aeruginosa* DT-Ct2 coefficients described the influence of pH, initial concentration and temperature as independent variables on DDT degradation. Interactions between initial concentration-temperature and incubation time-temperature described the association of temperature with initial concentration and also with incubation time in describing the predicted dependent variable i.e. the predicted residual DDT. The experimental data relating to the degradation of DDT by the individual bacterial strains, obtained

from the experimental design, were subjected to the multivariate analysis and predictions of the microbial behaviour towards degradation were achieved by partial least square regression.

Various carbon sources such as glucose, sucrose, succinate, citrate, acetate, glycerol, yeast extract, peptone and tryptone soya broth were tested for their influence on the enhancement of DDT degradation by these axenic bacteria. Effect of these carbon sources on enhancement/retardation of DDT-degradation has been discussed in ***Chapter 5,*** "***Co-metabolism of DDT by the bacterial isolates".*** DDT was degraded completely by 72h in presence of glycerol, yeast extract, peptone and tryptone soya broth by *Serratia marcescens* DT-1P, All the co-substrates but citrate favoured better degradation of DDT. In citrate degradation was drastically affected and less than 10% of the added 10ppm DDT was degraded in 72h as compared to the controls. In *Pseudomonas fluorescens* DT-2, except for citrate rest of the co-substrates enabled a better degradation of DDT compared to control samples. Degradation of DDT was retarded in presence of all other co-substrates other than citrate, as compared to control samples, in case of *Pseudomonas aeruginosa* DT-Ct1 while all the co-substrates favoured the degradation by *Pseudomonas aeruginosa* DT-Ct2**.**

Chapter 6, "Metabolic pathway of DDT-degradation", deals with the pathway studies of DDT-degradation by the DDT-degrading

bacterial isolates. All the intermediates that are formed during the catabolic degradation of DDT by these bacteria have been compared. The methods of identification and confirmation of the intermediates were HPLC (High performance liquid chromatography), GC (Gas chromatography), GC-MS (Gas chromatography-Mass spectrometry) and NMR (Nuclear magnetic resonance) spectroscopy. The general pathway that could be followed by these four microorganisms during the degradation of DDT under aerobic conditions has been proposed in this chapter. Studies on the existence of DDT-catabolic genes such as dehalogenases, 4-CBA-dehalogenases, 1,2-dioxygenases and 2,3-dioxygenases in these bacteria were studied. PCR primers were designed for these genes using primer 3.0 software as well as manually and genomic DNA was subjected to PCR reactions using these primers. *Serratia marcescens* DT-1P gave amplifications of the expected lengths with the primers for 1,2-dioxygenase, 2,3-dioxygenase. Positive signal for primers of 1,2-dioxygenase, 2,3-dioxygenase, dehalogenases genes were observed with *Pseudomonas fluorescens* DT-2. Primer pairs for dehalogenases showed amplification with genomic DNA of *Pseudomonas aeruginosa* DT-Ct1. The positive results with primers added to the confirmation of the proposed pathways in these bacteria.

Chapter 7, "Application of microbes in soil bioremediation", relates to the application of the bacterial isolates in bioremediation of DDT-

contaminated soil at laboratory level. Studies were carried out in soil spiked with DDT. *Pseudomonas aeruginosa* DT-Ct 1 was found to degrade DDT in soil more efficiently than other isolates. Inoculum level of 10μg dry weight/g soil, pH of 7-7.5, moisture level of 15% and ambient temperature(26^0 – 30^0C) were found to be optimum for the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct1 in soil. The kinetics of DDT degradation in soil by this isolate was carried out at different concentrations of DDT up to 50 ppm level.

The final chapter contains the final inferences and conclusions of the present study and the concluding remarks on the necessity of bioremediation of DDT-contaminated resources such as soil and water.

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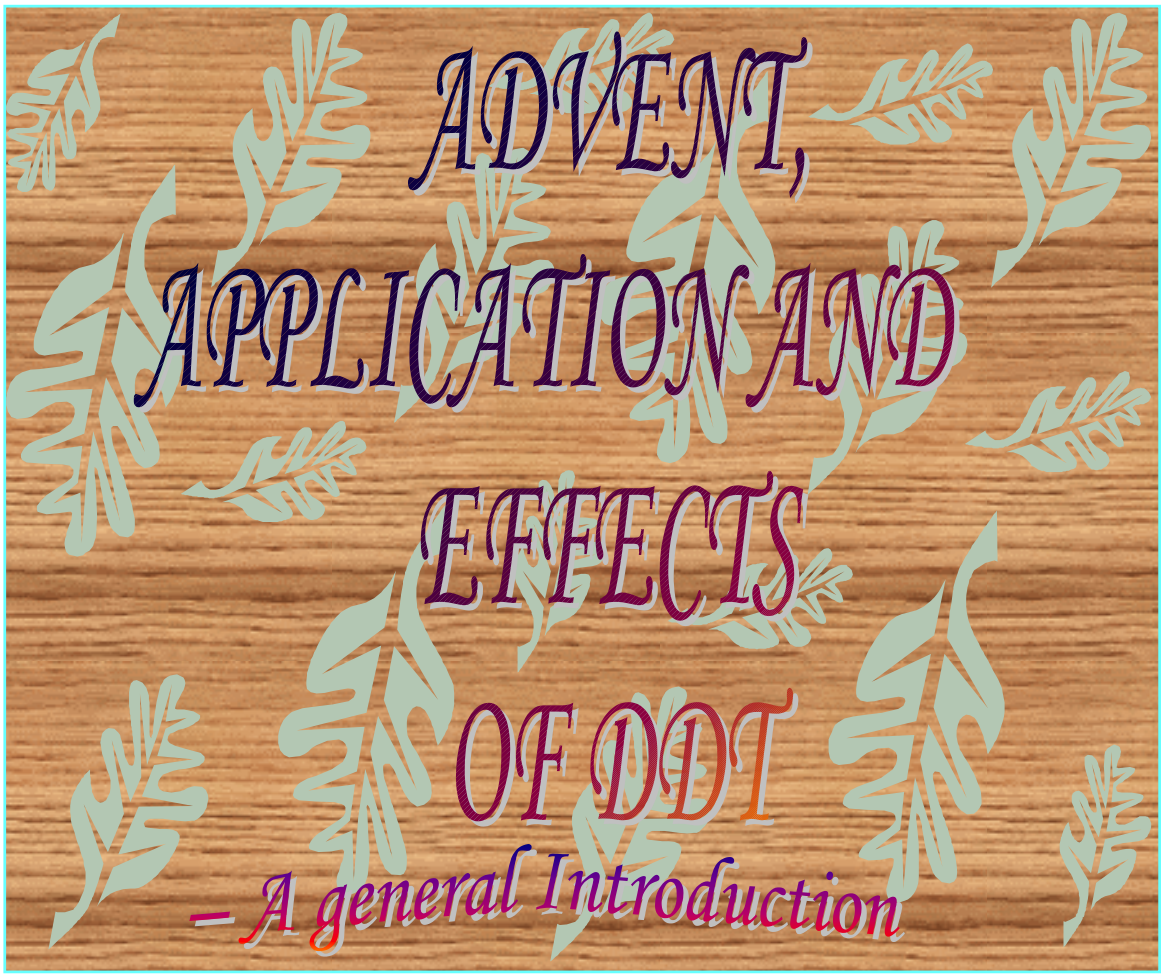
Chapter 1


ADVENT,
APPLICATION AND
EFFECTS
OF DDT
– A general Introduction

"While it is true that we don't know every last risk of using DDT, we know very well what the risk of malaria is- and on balance malaria is far, far more deadly than the worst that one could imagine about DDT".

Amir Attaran

Director, Malaria Project, Washington

1.1 Introduction

In the process of development of agriculture, pesticides have become important protection agents for boosting production. More than 140 pesticides are in use in India and their consumption is approximately 90,000 tons per annum (Gupta, 2001). But their indiscriminate use, apart from being an occupational hazard in the developing world, has been posing a serious threat to human health. There has been a great concern over growing incidence of cancer due to their excessive/long term use (Gupta, 2001; India today, Aug.2003). Some of these agricultural chemicals being poisonous leave behind residues in food and thereby produce ill effects when the concentration exceeds safe tolerance level. While pests and diseases are not a new problem, more intensive farming often provides conditions, which encourage a greater build-up of a particular organism. It is "unnatural" for crops to grow in fields in straight rows and large acreage of one particular host plant offers almost ideal conditions for certain pests and diseases. If weather and other conditions are favourable, the organism may develop at an alarming rate. The same crop grown on the same land year after year, as in glasshouses, or perennial crop such as tree fruits, allows a number of pests and diseases to multiply over a period of years.

India had seen a drastic growth in the food grains during "Green Revolution" that was possible mainly because of the pesticide application. DDT played a heroic role during this revolution not only in India but in many other countries as well. DDT was used extensively in the malaria eradication campaign and since 1970s through today; it stands as a magic saver for more than 20 million children in India. DDT reduced total malaria cases from 75 million to fewer than 5 million in a decade (Encarta online encyclopedia, 2001)

When DDT was introduced in 1940s, then Prime Minister of United Kingdom, Rt. Hon. Winston Churchill in his broadcast on 28th September 1944, stated "we have discovered many preventives against the tropical diseases and often against the onslaught of insects of all kinds, from lice to mosquitoes and

back again. The excellent DDT powder, which has been fully experimented with and is found to yield astonishing results will henceforth be used on a great scale by the British forces in Burma, and the American and Australian forces in the Pacific and in India in all theaters…"

This led the use of DDT in large scale all over the world.

Rachel Carson, in 1962, came out with her work "Silent Spring" that brought to light the non-target life getting affected by DDT. Following this was the ban on DDT use in agriculture in the United States of America, many European countries and the developed nations in 1970s (ATSDR, Atlanta, 1994, Encarta online encyclopedia, 2001). But still DDT was allowed for the health related emergencies due to outbreak of certain vector borne diseases in social and preventive medical practices (ATSDR, Atlanta, 1994). The developing world continued the use of DDT in two major areas of agriculture and tropical disease control programmes. The main reason for its vast usage was its broad spectrum of action on wide variety of pests and the low cost compared to the other existing pesticides.

The indiscriminate use of DDT globally allowed it to enter every part of the environment (Fig.1.1). It had been detected even in the regions where it was never applied (Global distillation), signifying the persistence and distribution of this chemical through various means (Simonich and Hites, 1995; Encarta online encyclopedia, 2001).

Significant levels of pesticides have been detected in the food and vegetable samples from Indian states like Delhi, Uttar Pradesh, Bihar and Andhra Pradesh, Tamil Nadu. Analysis of 13 brands of wheat flour packing in the country's major wheat consuming zones has that many leading brands contain pesticides like DDT (Kannan *et al,* 1992; Gupta, 2001). In the recent past, HCH contaminated water in Agra has been the reason for 19 deaths (Gupta, 2001). Their continuous use has also affected ground water sources through seepage into the soil. As a result, rivers, streams and ponds have become highly polluted with these harmful chemicals, thereby adversely affecting the drinking water sources (Gupta, 2001). Drinking water from ponds in Hasan district of Karnataka, India, was found to

Fig.1.1 Movement of Pesticides in the Environment.
(source Lakshmi *et al*, 2002)

contain 0.02- 0.2ppm of pesticides (Gupta, 2001). People in Delhi, India have been detected with the highest levels of pesticides in their body fat in the world (Gupta, 2001). Table 1.1 and Table1.2 give the figures of few pesticides detected in Indian rivers and the concentrations of DDT in man in India, with the daily intake of DDT in two other countries Australia and Canada. The figures there reflect the excessive intake of DDT by an average Indian citizen compared to these two countries. Table 1.3 details the concentrations of DDT in various edibles in Indian market.

It was estimated that the annual pesticide-poisoning cases globally are 4,30,000 (Gupta, 2001). Of this 10,000 persons dye in developing countries and India contributes to one-thirds of this figure (Gupta, 2001).

Table 1.1: Pesticides detected in few rivers in India (Gupta, 2001)

Source	Pesticide	Detected quantity
Cauvery river, Karnataka	BHC	> 1000ppb
Cauvery river, Karnataka	Methyl parathion	1300ppb
Yamuna river, Delhi	DDT	21.8ppm

Table 1.2 DDT Concentrations in man (Gupta, 2001)

DDT accumulated in an Indian	12.8-31.0ppm
Average daily intake of DDT in India	238.1-224.1μg/person
Average daily intake of DDT in Australia	20.0 μg/person
Average daily intake of DDT in Canada	10.8 μg/person

Table 1.3: DDT Concentration in Few Food Articles in India (Gupta, 2001)

Food article	DDT concentration
Wheat	1.6-17.4ppm
Rice	0.8-16.4ppm
Pulses	2.9-16.9ppm
Groundnut	3.0-19.1ppm
Potatoes	68.5ppm
Bottled milk(Mumbai)	4.8-6.3ppm
Milk (Mumbai, vendors)	97.0ppm
Butter	3.6ppm

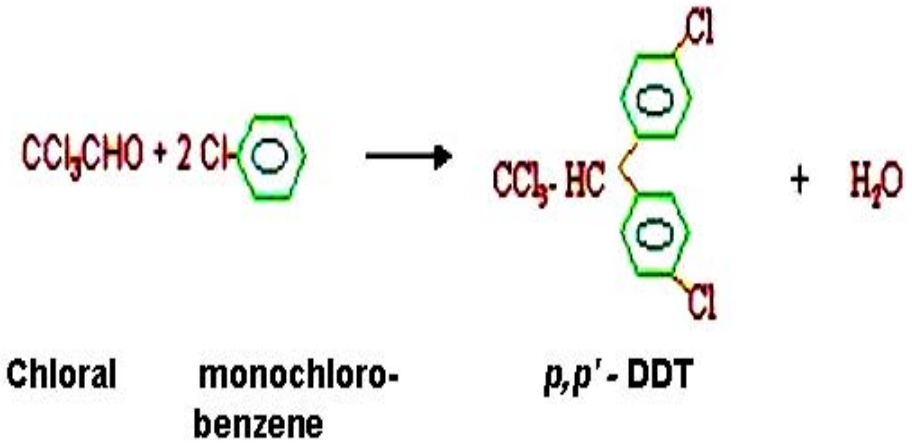
1.2 DDT, the first synthetic pesticide used by man

It is an organochlorine pesticide, Commonly known as dichlorodiphenyltrichloroethane, is chemically 1,1,1- trichloro-2,2-bis(4-chlorophenyl)ethane. It was first synthesised by Zeidler in 1874 on the following lines (West and Campbell, 1950):

DDT was commonly synthesised by the reaction of chloral with monochlorobenzene in the presence of concentrated sulphuric acid as a condensing agent (Fig.1.2). 225 parts of chlorobenzene were mixed with 147 parts of chloral or the corresponding amount of chloral hydrate and then 1000 parts sulphuric acid monohydrate were added. Whilst stirring well, the temperature rose to 60^{o}C, and then sinking slowly to room temperature, the mass then contained solid portions. It was poured into a large excess of water whereupon the product separated in solid form. It was well washed and could be crystallized from ethyl alcohol, forming fine white crystals having a weak fruity odour.

Once the insecticidal effects of DDT were known, it was produced at a larger scale in different ways around the world. Method adapted by the manufacturers have been described later in this chapter (Sec.1.9)

Fig.1.2 Synthesis of DDT as done by Zeidler



1.3 Structure

Grummitt *et al* (1945) confirmed the position of Cl^- atoms in DDT by refluxing with alcoholic potash to give the corresponding dichloroethylene compound and oxidizing the latter with chromic acid to *p,p'*-dichlorobenzophenone.

1.4 Solubility

DDT is practically insoluble in water, readily soluble in most organic solvents. Tables 1.4 and 1.5 describe the solubility in various solvents at different temperatures (Gunther, 1945; Jones, Fluno and Mc Collough, 1946).

1.5 Properties of DDT

Few of the important properties of DDT are:

Form	colourless crystals, technical- waxy solid
Molecular weight	354.5
Vapour pressure	0.025 mPa (109 ^{0}C)
Boiling point	185- 187 ^{0}C/ 0.05 mmHg
Melting point	108.5-109 ^{0}C
Density	0.98-0.99

Table1.4: Solubility of DDT at different temperatures in different solvents

Solvent	Weight % solubility at				
	0^0 C	**7.2^0 C**	**24.0^0 C**	**45.0^0 C**	**48.0^0 C**
Acetone	21.2	27.3	40.3	-	59.0
Benzene	6.8	27.1	44.0	-	57.8
Carbon tetrachloride	9.0	10.5	18.0	34.8	-
Chloroform	18.2	21.9	31.0	47.4	-
Dioxane	8.0	29.0	46.0	-	61.0
Ether	15.0	18.9	27.5	-	-
Ethanol (95%)	0.8	1.0	2.2	-	3.9
Petroleum ether ($30- 60^0$)	1.7	2.4	4.8	-	-
Pyridine	21.0	36.0	51.0	-	62.0

Table.1.5: Solubility of DDT in various solvents at 27^0 - 30 ^{0}C

S. No.	Solvent	Solubility of DDT	
		Per 100 ml of solvent (g)	**Per 100g of solvent (g)**
1	Acetone	58	74
2	Acetophenone	67	65
3	Anisole	70	70
4	Benzene	78	89

S. No.	Solvent	Solubility of DDT	
		Per 100 ml of solvent (g)	Per 100g of solvent (g)
5	Benzyl acetate	45	43
6	Benzyl alcohol	12	11
7	Benzyl ether	41	39
8	Carbon tetrachloride	45	28
9	p- chloroacetophenone	39	33
10	Chlorobenzene	74	67
11	Chloroform	31	-
12	Cyclohexane	15	19
13	Cyclohexanol	10	11
14	Cyclohexanone	116	122
15	o- dichlorobenzene	59	45
16	1,4- dioxane	92	89
17	Ethyl alcohol (95%)	~2	~2
18	Ethyl benzoate	37	54
19	Ethylene dichloride	59	47
20	Ethyl ether	~28	~39
21	Methylene chloride	88	66
22	Propionic acid	16	16
23	Tetrachloroethane	61	38
24	Tetrachloroethylene	38	23
25	Triacetine	10	9
26	1,2,4- trichlorobenzene	44	28

S. No.	Solvent	Solubility of DDT	
		Per 100 ml of solvent (g)	Per 100g of solvent (g)
27	1,1,1-trichloroethane	~52	~39
28	Trichloroethylene	64	44
29	o-xylene	57	66
Aliphatic Petroleum Fractions			
30	Gasoline	10	13
31	Kerosene	8- 10	10- 12
32	Fuel oil 1	8- 11	10- 14
33	Fuel oil 2	7- 10	8- 12
34	Lubricating oil	5	6
35	Refined fly's spray-base kerosene	4	5
36	Transformer oil	4- 6	5- 7
Coal Tar Fractions			
37	Xylene 10^0	53	61
38	Solvent naphtha (Industrial Xylene)	52	60
39	Wire Enamel Solvent	60	64
40	Neutral oil	67	66
41	Special hydrocarbon oil	58	53
Pine Distillation Products			
42	Pine oil	10	11
43	Turpentines, Spirits	17	20

S. No.	Solvent	Solubility of DDT	
		Per 100 ml of solvent (g)	Per 100g of solvent (g)
Miscellaneous			
44	Alox 152 (Methyl esters of oxidized petroleum products)	9	10
45	Alox 800	18	19
46	Aroclor 1242 (Chlorinated biphenyls)	48	35
47	Aroclor 1248	~30	~21
48	Aroclor 1254	12	8
49	Castor oil	7	7
50	Cotton- seed oil	11	12
51	Sesame oil	8	9
52	Linseed oil (raw)	11	12
53	Peanut oil	11	12
54	Iso- propyl cresols	7	7
55	Triton V 1956	9	9
56	Triton X 100	12	11
57	Tween 60	14	13

1.6 DDT as an insecticide

The insecticidal properties of DDT was discovered in the Basle laboratories of the Swiss company of J. R. Geigy S. A. (now Novartis) by **Dr. Paul Muller** in the autumn of 1939 and patent application was made in Switzerland on 7th March 1940 (Swiss Patent 226180/40).

The first proof for DDT to be an insecticide was checking the plague of Colorado beetle in 1941, which threatened the Swiss potato crop at a time when all food crops were of the utmost importance (West and Campbell, 1950). In September 1941, J. R. Geigy of Basle, Switzerland, informed about DDT which in the form of 1% dust had been found extremely effective against the Colorado potato beetle only after the Colorado potato beetle had been successfully controlled in U. S. with lead arsenite (West and Campbell 1950).

1.7 Stability

Domenjoz (1944) reported that little or no decomposition occurred on heating DDT at 150 ^{0}C for 24 hours. West and Campbell (1950) heated DDT very slowly in a boiling tube immersed in a glycerine bath, maintaining at 115^0, 120^0, 125^0, 130^0, 140^0, 145 ^{0}C for about one minute each with fairly rapid current of air passing through. Temperature at which a definite opalescence first appeared in the silver nitrate solution was 140^0-145^{0}C. It undergoes dehydrochlorination in alkaline solution and at temperatures above the melting point to the non-insecticidal DDE. DDT is generally stable to oxidation.

1.8 Decomposition of DDT

Balaban and Sutcliffe (1945) have reported the decomposition point of DDT having the m.p. 108.5-109.5^{0}C, setting point 107.5^{0}C to be 195^{0}C. The m.p. of the recovered material as 106-107.5^{0}C. Various catalysts including anhydrous ferric oxide, anhydrous ferric and aluminium chlorides, iron and other materials such as

Fuller's earth and some mineral products have been reported of eliminating hydrogen chloride from DDT (Fleck and Haller, 1944).

1.9 Production of DDT

In United Kingdom, DDT was manufactured as follows (Callahan, 1944):

a. *Continuous Production of chloral*

1. Direct chlorination of alcohol in presence of ferric chloride catalyst to form chloralalcoholate.
2. Liberation of chloral from chloral alcoholate by water dilution and by sulphuric acid acidulation treatment.
3. Separation of chloral from side- products formed during chlorination and acidulation steps by fractionation.
4. Recovery of by- product, hydrogen chloride and excess chlorine as hydrochloric acid and as sodium hypo chlorite.

b. *Continuous Production of DDT*

1. Reaction of chloral and chlorobenzene with oleum to form DDT, its isomers and polymers.
2. Separation and neutralization of the dissolved DDT from oleum by setting followed by water and alkali washings.
3. Vacuum distillation and recovery of chlorobenzene solvent followed by air stripping of residual chlorobenzene.
4. Cooling and solidification of DDT followed by pulverizing, blending with extenders and final packaging.

This method, well known as Brothman Process, yielded a product containing not less than 70% of *p,p'*- compound having m.p. not less than 104^0C, the setting point not less than 80^0C, acidity (expressed as sulphuric acid) not exceeding 0.3%, the hydrolysable chlorine by treatment with alcoholic

potash lying between 9.5 and 11%, volatile matter, including water, immiscible with petroleum ether, not exceeding 1%, white spirit, insoluble matter not exceeding 1%.

1.10 Chemical composition of technical DDT

According to Haller and co- workers (1945), theoretically, there are 45 possible dichlorodiphenyltrichloroethanes. Table 1.6 gives the composition of technical DDT.

Table 1.6: Composition of dichlorodiphenyltrichloroethane

Compound	Sample 1	Sample 2	Sample 3	Sample 4
***p,p´*-DDT**	(a) 66.7 (b) 72.9	(b) 70.5 (c) 63.5 (d) 64.5 (e) 67.9	(a) 72.7 (b) 76.7	-
o,p´- DDT	19.0	(c) 7.0 (d) 15.3 (e) 20.9	11.9	74.8
p,p´- DDD	0.3	4.0	0.17	-
o,p´- DDD	-	-	0.044	-
1-*p*-chlorophenyl-2-trichloroethanol	0.2	-	-	-
2-trichloro-1-*o*-chlorophenylethyl-*p*-chlorobenzenesulphonate	-	-	0.044	-
Bis(*p*-chlorophenyl)sulphone	0.6	0.1	0.034	-

Compound	Sample 1	Sample 2	Sample 3	Sample 4
α-chloro-α-chlorophenyl acetamide	-	0.01	0.006	-
α-chloro-α-*o*-chlorophenyl acetamide	-	0.007	-	-
Chlorobenzene	-	-	-	2.44
p-dichlorobenzene	-	-	-	0.73
Sodium-*p*-chlorobenzenesulphonate	0.02	-	-	-
Ammonium *p*-chlorobenzenesulphonate	-	-	0.005	-
Inorganic	0.1	0.04	0.01	-
Unidentified and losses	6.5	5.1	10.6	19.4

(a): Isolation from tech-DDT

(b): Recrystallisation from 75% aqueous ethanol previously saturated with *p,p´*-DDT.

(c): Fractional crystallization

(d): Adsorption analysis and fractional crystallization.

(e): Isolation, supplemented with cryscopic analysis on the residue

1.11 Grades of DDT

The term "DDT" is confined to the product obtained by condensing chloral anhydrous, or (as its alcoholate or hydrate) with chlorobenzene in presence of sulphuric acid. According to the US War Production Board, three grades of DDT were recognized (West and Campbell, 1950):

a. Technical DDT: Complex mixture of *p,p'*- DDT (70 %) with *o,p'*- DDT as major impurity. It melts over a wide range of temperatures (hence setting point is used as an indication of purity instead of melting point).
b. Purified or aerosol DDT: Partially refined with higher proportion of DDT. Melting point $\geq 103^{o}$ C.

c. Pure *p,p'*- DDT: Highly purified with a melting point of 108.5- 109^{o} C. Used for biological studies and pharmacological comparisions.

1.12 Trade and other names

DDT was commercially marketed by many trade names. Few of them have been mentioned below (Wasserman et al., 1982; ATSDR, Atlanta, 1994)

1. Anofex
2. Cesarex
3. Chlorophenothane
4. Dedelo
5. *p,p'*-DDT
6. Dichlorodiphenyltrichloroethane
7. Dinocide
8. Didimac
9. Digmar
10. ENT 1506
11. Genitox
12. Guesapon
13. Gexarex
14. Gyron
15. Hildit
16. Ixodex
17. Kopsol
18. Neocid
19. OMS 16
20. Micro DDT 75
21. Pentachlorine
22. Rukseam
23. R50

24. Zerdane

1.13 Applications of DDT

The pioneer work of Muller was against the housefly, and the field trials were taken over by Wiesmann (West and Campbell, 1950)

1. ***DDT in paints and miscellaneous materials*:** 1% suspension of a powder containing 5% DDT on inert fillers was sprayed with Guesarol spray to walls. The insecticide that was adsorbed on wall surfaces continued to kill flies that came into contact with them even up to two months after spraying (West and Campbell, 1950). Dry distemper containing DDT was very good in controlling the flies. The killing was inferred to be due to either abrasion effect on the cuticle allowing dehydration to take place or perhaps by blocking the spiracles, which are entrances to the respiratory system (West and Campbell, 1950). The oil paint and synthetic enamel containing DDT did not prove insecticidal to flies at all, which was presumed to be due to the prevention of contact between DDT and insect due to oil or resin layer. However, DDT-oil bound water paint, a decorative coating with some degree of durability, killed flies even up to six months (West and Campbell, 1950). The insecticidal activity was found to have decreased only slightly after one year. Tests with flies of different ages indicated that older flies were affected rather more quickly by the DDT paint. A 99% death of older flies was recorded even after 3 months of application. In any of these cases, repellent effects, suspected in preliminary tests could not be corroborated. In all the experimental stages, DDT concentration used was up to 5% (West and Campbell, 1950).

   It was observed that a film of coumarone resin containing 1% and 3% DDT gave excellent results in killing flies. In view of these good results,

coumarone resin/ DDT film, called Guesapon EXM, was prepared according to the following formula (West and Campbell, 1950):

Parts by weight	Substance added	
16	DDT	**Solution A**
16	Coumarone resin	
16	Xylol	
0.8	Oleic acid	
0.8	Belloid FR	
1.6	Belloid NW	
0.8	46% caustic soda	**Solution B**
48	Water	

Solution A and Solution B were mixed with vigorous stirring and the mixture was passed through a colloid mill. This, when painted on to boards, gave excellent results. In addition to flies, this was insecticidal towards both common and German cockroaches.

Oil- bound water paint containing 5% DDT was effective against bed bugs (*Cimex lectularius L.*) and resin: DDT mixture was lethal with slow effect. DDT incorporated into linoleum was found effective at a concentration of 2% against house flies, at 4-6% against bed bugs, 2-4% against grain weevils and 6% against cockroaches (West and Campbell, 1950).

2. ***DDT in textiles and papers*****:** DDT is insoluble in water and has no affinity for any of the textile fibres. However, when DDT was dissolved in organic solvents and impregnated on the wool fibre, there appeared to be more retention of the active ingredient which was presumed to be a physico-chemical adsorption or a chemisorption of the active ingredient on the

surface of the wool fibre and this was found to resist removal by washing (West and Campbell, 1950).

This property was used as moth proofing by impregnating it from white spirit solution on woollen fabrics that gave temporary effect compared to accepted water-soluble moth proofing agents (West and Campbell, 1950). But the property of contact poison effect was used later notably against lice and fleas. DDT was then given the priority to be used by army authorities for impregnation of union shirtings and underwear. Impregnation was carried out with 5% DDT in white spirit to retain about 1% DDT on the weight of material. These garments were reported to be still effective in killing lice after 5- 8 washings (West and Campbell, 1950). DDT content as low as 0.1% was still effective. DDT could be impregnated when its level fell very low. These results were extended to blankets, sleeping bags, carpets and furnishings against moths, the cleansing of furnishing fabrics in hotels and public vehicles (buses, ships, airplanes, trains etc.), against all vermin affecting man, the treatment of curtains to kill flies and mosquitoes, the impregnation of bed linen and blankets against many such pests.

The most extensive survey of application of DDT to textiles was made by Goodall *et al* (1946). Hayhurst (1945), working on jute sacks reported that a good measure of control could be obtained by the use of DDT against the rice weevil, the confused flour beetle and spider beetle. However, when the fabric was subjected to 100 ^{o}C, the insecticidal activity diminished. This loss in the insecticidal property could be because of the slight hydrolysis of DDT into chloral and chlorobenzene, in presence of moisture (Goodall *et al.,* 1946).

DDT was used to spray (Guesarol spray) on the papers to protect from insect attack especially against silver fish (*Lepisma saccharina* L.) (West and Campbell, 1950). Another approach in this aspect was to add DDT in the beater in order to get a uniform distribution throughout. 2% DDT in paper proved insecticidal to houseflies (West and Campbell, 1950).

Cotton *et al* (1944) investigated the insect-proof paper bags made out of soft towel paper and kraft paper impregnated with 10% solution of DDT and found them to resist attack from cadelle and lesser grain borer.

3. Other Uses of DDT: DDT was reported to bring a considerable degree of immunity from infestation of the dog-flea, *Ctenocephalus canis*, and dog-louse, *Trichodectes canis*, when incorporated in soap (Campbell *et al*, 1945).

DDT was used mainly to control mosquito-borne malaria and typhus fever; use on crops has generally been replaced by less persistent insecticides (Wasserman *et al*, 1982). It was extensively used during the Second World War among Allied troops and certain civilian populations to control insect typhus and malaria vectors, and was then extensively used as an agricultural insecticide after 1945 (ATSDR, Atlanta, 1994). DDT was banned for use in Sweden in 1970 and in the United States in 1972 (ATSDR, Atlanta, 1994). Many insect pests may have developed resistance to DDT (Wasserman *et al*, 1982). Unless otherwise specified, the toxicological, environmental effects and environmental fate and chemistry data presented here refer to the technical product DDT. Technical grade DDT is actually a mixture of three isomers of DDT, principally the *p,p'*-DDT isomer (ca. 85%), with the *o,p'*-DDT and *o,o'*-DDT isomers typically present in much lesser amounts (ATSDR, Atlanta, 1994).

1.14 FORMULATION

DDT is available in several different forms: aerosols, dustable powders, emulsifiable concentrates, granules and wettable powders (Wasserman, 1982; Meister, 1992). It is reported to be compatible with many other pesticides and incompatible with alkaline substances (Wasserman, 1982).

1.15 Environmental Impact

1.15(i) Atmospheric fate

If released to air, DDT will be subject to direct photo oxidation and reaction with photo- chemically produced hydroxyl radicals (half life of ~ 2 days for the latter) (Encarta online encyclopedia, 2001). Presence of DDT in samples far away from places where DDT is used suggests that photo degradation may be very slow. Fig.1.1 (page 5) shows the movements of pesticides in atmosphere.

1.15(ii) Terrestrial fate

If released to soil, it will adsorb very strongly to the soil and should not appreciably leach to ground water. However, it has been detected in some ground water samples the source of which is unknown. It will be subject to photo oxidation on soil surfaces but will not hydrolyse.

It may significantly biodegrade in flooded soils or under anaerobic conditions provided high populations of the required microorganisms are present. Reports for biodegradation in soil range from 2 years to > 15 years (Encarta online encyclopedia, 2001). Fig.1.3 represents the fate of pesticides in soil environment.

1.15(iii) Aquatic fate

If released to water, it will adsorb very strongly to the sediments, significantly bioconcentrates in fish. It may be subject to biodegradation in water and sediments where high populations of required microorganisms are present, but generally biodegradation in water is poor. Direct photolysis of DDT in aqueous solution is very slow, with a half-life of probably greater than 150 years (Encarta online encyclopedia, 2001). Fig1.4 represents the movement and fate of pesticides in the aquatic environment.

Fig.1.3 Pesticides in Soil Environment. Key: Movement of pesticides or products —►. Agencies affecting the pesticides or products ----►. Rhizosphere- shaded area. Microorganisms- animations.
(source Lakshmi *et al*, 2002)

Fig.1.4 Pesticides in Aquatic Environment.
(source Lakshmi *et al*, 2002)

1.16 TOXICOLOGICAL EFFECTS

- **Acute Toxicity:** DDT is moderately to slightly toxic to studied mammalian species *via* the oral route. Reported oral LD_{50}s range from 113 to 800 mg/kg in rats (Wasserman, 1982; ATSDR, Atlanta, 1994); 150-300 mg/kg in mice (Wasserman, 1982); 300 mg/kg in guinea pigs (ATSDR, Atlanta, 1994)); 400 mg/kg in rabbits (ATSDR, Atlanta, 1994) ; 500-750 mg/kg in dogs (Wasserman, 1982) and greater than 1,000 mg/kg in sheep and goats (Wasserman, 1982). Toxicity will vary according to formulation (Wasserman, 1982). DDT is readily absorbed through the gastrointestinal tract, with increased absorption in the presence of fats (ATSDR, Atlanta, 1994). One-time administration of DDT to rats at doses of 50 mg/kg led to decreased thyroid function and a single dose of 150 mg/kg led to increased blood levels of liver-produced enzymes and changes in the cellular chemistry in the central nervous system of monkeys (Wasserman, 1982). Single doses of 50-160 mg/kg produced tremors in rats, and single doses of 160 mg/kg produced hind leg paralysis in guinea pigs (ATSDR, Atlanta, 1994). Mice suffered convulsions following a one-time oral dose of 200 mg/kg. Single administrations of low doses to developing 10-day old mice are reported to have caused subtle effects on their neurological development (ASTDR, Atlanta, 1994). DDT is slightly to practically non-toxic to test animals *via* the dermal route, with reported dermal LD_{50}s of 2,500-3,000 mg/kg in female rats (Wasserman, 1982; ASTDR, Atlanta, 1994), 1000 in guinea pigs (ATSDR, Atlanta, 1994) and 300 in rabbits (ATSDR, Atlanta, 1994). It is not readily absorbed through the skin unless it is in solution (ATSDR, Atlanta, 1994). It is thought that inhalation exposure to DDT will not result in significant absorption through the lung alveoli but rather that it is probably trapped in mucous secretions and swallowed by exposed individuals following the tracheo-bronchial clearance of secretions by the cilia (ATSDR, Atlanta, 1994). Acute effects likely in humans due to

low to moderate exposure may include nausea, diarrhoea, increased liver enzyme activity, irritation (of the eyes, nose or throat), disturbed gait, malaise and excitability; at higher doses, tremors and convulsions are possible (Van Ert and Sullivan, 1992; ATSDR, Atlanta, 1994). While adults appear to tolerate moderate to high ingested doses of up to 280 mg/kg, a case of fatal poisoning was seen in a child who ingested one ounce of a 5% DDT:kerosene solution (ATSDR, Atlanta, 1994).

- **Chronic Toxicity**: DDT has caused chronic effects on the nervous system, liver, kidneys and immune systems in experimental animals (WHO, Environmental Health Criteria, 1979; ATSDR, Atlanta, 1994). Effects on the nervous system observed in test animals include: tremors in rats at doses of 16-32 mg/kg/day over 26 weeks; tremors in mice at doses of 6.5-13mg/kg/day over 80-140 weeks; changes in cellular chemistry in the central nervous system of monkeys at doses of 10 mg/kg/day over 100 days, and loss of equilibrium in monkeys at doses of 50 mg/kg/day for up to 6 months (ATSDR, Atlanta, 1994). The main effect on the liver seen in animal studies was localized liver damage. This effect was seen in rats given 3.75 mg/kg/day over 36 weeks, rats exposed to 5 mg/kg/day over 2 years and dogs at doses of 80 mg/kg/day over the course of 39 months (ATSDR, Atlanta, 1994). In many cases lower doses produced subtle changes in liver cell physiology, and in some cases higher doses produced more severe effects (ATSDR, Atlanta, 1994). In mice doses of 8.33 mg/kg/day over 28 days caused increased liver weight and increased liver enzyme activity (ATSDR, Atlanta, 1994). Liver enzymes are commonly involved in detoxification of foreign compounds, so it is unclear whether increased liver enzyme activity in itself would constitute an adverse effect. In some species (monkeys and hamsters), doses as high as 8-20 mg/kg/day caused no observed adverse effects over exposure periods as

long as 3.5-7 years (ATSDR, Atlanta, 1994). Kidney effects observed in animal studies include adrenal gland haemorrhage in dogs at doses of 138.5 mg/kg/day over 10 days and adrenal gland damage at 50 mg/kg day over 150 days in dogs (ATSDR, Atlanta, 1994). Kidney damage was also seen in rats at doses of 10 mg/kg/day over 27 months (ATSDR, Atlanta, 1994). Immunological effects observed in test animals include: reduced antibody formation in mice following administration of 13 mg/kg/day for 3-12 weeks and reduced levels of immune cells in rats at doses of 1 mg/kg/day (ATSDR, Atlanta, 1994). No immune system effects were observed in mice at doses of 6.5 mg/kg/day for 3-12 weeks (ATSDR, Atlanta, 1994). Dose levels at which effects were observed in test animals are very much higher than those which may be typically encountered by humans (WHO, Environmental Health Criteria, 1979). The most significant source of exposure to individuals is occupational, occurring only to those who work or worked in the production or formulation of DDT products for export (Sax, 1984). Analysis of U. S. market basket surveys showed approximately a 30-fold decrease in detected levels of DDT and metabolites in foodstuffs from 1969-1974, and another threefold drop from 1975-1981, with a final estimated daily dose of approximately 0.002 mg/person/day (ATSDR, Atlanta, 1994). Based on a standard 70-kg person, this results in a daily intake of approximately 0.00003 mg/kg/day. Due to the persistence of DDT and its metabolites in the environment, very low levels may continue to be detected in foodstuffs grown in some areas of prior use (ATSDR, Atlanta, 1994). It has been suggested that, depending on patterns of international DDT use and trade, it is possible that dietary exposure levels may actually increase over time (ATSDR, Atlanta, 1994). Persons eating fish contaminated with DDT or metabolites may also be exposed *via* bioaccumulation of the compound in fish (ATSDR, Atlanta, 1994). Even though current dietary levels are quite low, past and current exposures may result in measurable body burdens due to its persistence in the body

(ATSDR, Atlanta, 1994). Adverse effects on the liver, kidney and immune system due to DDT exposure have not been demonstrated in humans in any of the studies, which have been conducted (ATSDR, Atlanta, 1994). Few observations made around the world create an atmosphere of worries among the population as DDT has been suspected the causative agent in many abnormal findings.

- **Reproductive Effects:** There is evidence that DDT causes reproductive effects in test animals. No reproductive effects were observed in rats at doses of 38 mg/kg/day administered at days 15-19 of gestation (ATSDR, Atlanta, 1994). In another study in rats, oral doses of 7.5 mg/kg/day for 36 weeks resulted in sterility (ATSDR, Atlanta, 1994). In rabbits, doses of 1 mg/kg/day administered on gestation days 4-7 resulted in decreased foetal weights and 10 mg/kg/day on days 7-9 of gestation resulted in increased abortions (ATSDR, Atlanta, 1994). In mice, doses of 1.67 mg/kg/day resulted in decreased embryo implantation and irregularities in the oestrus cycle over 28 weeks (ATSDR, Atlanta, 1994). It is thought that many of these observed effects might be the result of disruptions in the endocrine system (ATSDR, Atlanta, 1994). Available epidemiological evidence from two studies does not indicate that reproductive effects have occurred in humans as a result of DDT exposure (ATSDR, Atlanta, 1994). No associations between maternal blood levels of DDT and miscarriage or premature rupture of foetal membranes were observed in two separate studies (Ron *et al*, 1988; Leoni *et al*, 1989; ATSDR, Atlanta, 1994). One study did report a significant association between maternal DDT blood levels and miscarriage, but the presence of other organochlorine chemicals (e.g., PCBs) in maternal blood, which might have accounted for the effect making it difficult to attribute the effect to DDT and its metabolites (Wasserman, 1982).

- **Teratogenic Effects:** There is evidence that DDT causes teratogenic effects in test animals as well. In mice, maternal doses of 26 mg/kg/day DDT from gestation through lactation resulted in impaired learning performance in maze tests (ATSDR, Atlanta, 1994). In a two-generational study of rats, 10 mg/kg/day resulted in abnormal tail development (ATSDR, Atlanta, 1994). Epidemiological evidence regarding the occurrence of teratogenic effects as a result of DDT exposure is unavailable (ATSDR, Atlanta, 1994).

- **Mutagenic Effects**: The evidence for mutagenicity and genotoxicity is contradictory. In only 1 out of 11 mutagenicity assays in various cell cultures and organisms DDT showed positive results (ATSDR, Atlanta, 1994).Results of *in vitro* and *in vivo* genotoxocity assays for chromosomal aberrations indicated that DDT was genotoxic in 8 out of 12 cases, and weakly genotoxic in 1 case (ATSDR, Atlanta, 1994). In humans, blood cell cultures of men occupationally exposed to DDT showed an increase in chromosomal damage. In a separate study, significant increases in chromosomal damage were reported in workers who had direct and indirect occupational exposure to DDT (ATSDR, Atlanta, 1994). Thus it appears that DDT may have the potential to cause genotoxic effects in humans, but does not appear to be strongly mutagenic. It is unclear whether these effects may occur at exposure levels likely to be encountered by most people.

- **Carcinogenic Effects**: The evidence regarding the carcinogenicity of DDT is equivocal. It has been shown to cause increased tumour production (mainly in the liver and lung) in test animals such as rats, mice and

hamsters in some studies but not in others (ATSDR, Atlanta, 1994) In rats, liver tumours were induced in three separate studies at doses of 12.5 mg/kg/day over periods of 78 weeks to life, and thyroid tumours were induced at doses of 85 mg/kg/day over 78 weeks (ATSDR, Atlanta, 1994). In mice, lifetime doses of 0.4 mg/kg/day resulted in lung tumours in the second generation and leukaemia in the third generation; liver tumours were induced at oral doses of 0.26 mg/kg/day in two separate studies over several generations. In hamsters, significant increases in adrenal gland tumours were seen at doses of 83 mg/kg/day in females (but not males), and in males (but not females) at doses of 40 mg/kg/day (ATSDR, Atlanta, 1994). In other studies, however, no carcinogenic activity was observed in rats at doses less than 25 mg/kg/day; no carcinogenic activity was seen in mice with doses of 3-23 mg/kg/day over an unspecified period, and in other hamster studies there have been no indications of carcinogenic effects (ATSDR, Atlanta, 1994). The available epidemiological evidence regarding DDT's carcinogenicity in humans, when taken as a whole, does not suggest that DDT and its metabolites are carcinogenic in humans at likely dose levels (ATSDR, Atlanta, 1994). In several epimiological studies, no significant associations were seen between DDT exposure and disease, but in one other study, a weak association was observed (ATSDR, Atlanta, 1994). In this latter study, which found a significant association between long-term, high DDT exposures and pancreatic cancers in chemical workers, there were questions raised as to the reliability of the medical records of a large proportion of the cancer cases (ATSDR, Atlanta, 1994).

- **Organ Toxicity:** Acute human exposure data and animal studies reveal that DDT can affect the nervous system, liver, kidney (ATSDR, Atlanta, 1994). Increased tumour production in the liver and lung has been observed in test animals (ATSDR, Atlanta, 1994). An association with pancreatic

cancer was suggested in humans in one study (ATSDR, Atlanta, 1994).

- **Fate in Humans & Animals:** DDT is very slowly transformed in animal systems (WHO, Environmental Health Criteria, 1979). Initial metabolites in mammalian systems are 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), which are very readily stored in fatty tissues (ATSDR, Atlanta, 1994). These compounds in turn are ultimately transformed into bis(dichlorodiphenyl) acetic acid (DDA) *via* other metabolites at a very slow rate (ATSDR, Atlanta, 1994). DDA, or conjugates of DDA, are readily excreted *via* the urine (ATSDR, Atlanta, 1994). Available data from analysis of human blood and fat tissue samples collected, from the developed nations that banned DDT during 1970s, in the early 1970s showed detectable levels in all samples, but a downward trend in the levels over time (ATSDR, Atlanta, 1994). Later study of blood samples collected in the latter half of the 1970s showed that blood levels were declining further, but DDT or metabolites were still seen in a very high proportion of the samples (ATSDR, Atlanta, 1994). Levels of DDT or metabolites may occur in fatty tissues (e.g. fat cells, the brain, etc.) at levels of up to several hundred times that seen in the blood (ATSDR, Atlanta, 1994). DDT or its metabolites may also be eliminated *via* mother's milk by lactating women (ATSDR, Atlanta, 1994).

1.17 ECOLOGICAL EFFECTS

- **Effects on Birds**: DDT may be slightly toxic to practically non-toxic to birds. Reported dietary LD_{50}s range from greater than 2,240 mg/kg in mallard, 841 mg/kg in Japanese quail and 1,334 mg/kg in pheasant (Hudson *et al*, 1984). Other reported dietary LD_{50}s in such species as bobwhite quail, California quail, red-winged blackbird, cardinal, house sparrow, blue jay, sandhill

crane and clapper rail also indicate slight toxicity both in acute 5-day trials and over longer periods of up to 100 days (WHO, environmental Health Criteria, 1989). In birds, exposure to DDT occurs mainly through the food web through predation on aquatic and/or terrestrial species having body burdens of DDT, such as fish, earthworms and other birds (WHO, environmental Health Criteria, 1989). There has been much concern over chronic exposure of bird species to DDT and effects on reproduction, especially eggshell thinning and embryo deaths (WHO, environmental Health Criteria, 1989). The mechanisms of eggshell thinning are not fully understood. It is thought that this may occur from the major metabolite, DDE, and that predator species of birds are the most sensitive to these effects (WHO, environmental Health Criteria, 1989). Laboratory studies on bird reproduction have demonstrated the potential of DDT and DDE to cause subtle effects on courtship behaviour, delays in pairing and egg laying and decreases in egg weight in ring doves and Bengalese finches (WHO, environmental Health Criteria, 1989). The implications of these for long-term survival and reproduction of wild bird species is unclear. There is evidence that synergism may be possible between DDT's metabolites and organophosphate (cholinesterase-inhibiting) pesticides to produce greater toxicity to the nervous system and higher mortality (WHO, environmental Health Criteria, 1989). Aroclor (polychlorinated biphenyls, or PCBs) may result in additive effects on eggshell thinning (WHO, environmental Health Criteria, 1989).

- **Effects on Aquatic Species:** DDT is very highly toxic to many aquatic invertebrate species. Reported 96-hour LC_{50}s in various aquatic invertebrates (e.g., stoneflies, midges, crayfish, sow bugs) range from 0.18 μg/μL to 7.0 μg/L, and 48-hour LC_{50}s are 4.7 μg/L for daphnids and 15 μg/L for sea shrimp (Johnson and Finlet, 1980). Other reported 96-hour LC_{50}s for

various aquatic invertebrate species are from 1.8 μg/L to 54 μg/L (WHO, environmental Health Criteria, 1989). Early developmental stages are more susceptible than adults to DDT's effects (WHO, environmental Health Criteria, 1989). The reversibility of some effects, as well as the development of some resistance, may be possible in some aquatic invertebrates (Johnson and Finlet, 1980). DDT is very highly toxic to fish species as well. Reported 96-hour LC_{50}s are less than 10 μg/L in coho salmon (4.0 μg/L), rainbow trout (8.7 μg/L), northern pike (2.7 μg/L), black bullhead (4.8 μg/L), bluegill sunfish (8.6 μg/L), largemouth bass (1.5 μg/L), and walleye (2.9 μg/L) (Johnson and Finlet, 1980). The reported 96-hour LC_{50}s in fathead minnow and channel catfish are 21.5 μg/L and 12.2 μg/L respectively (Johnson and Finlet, 1980). Other reported 96-hour LC_{50}s in largemouth bass and guppy were 1.5 μg/L and 56 μg/L respectively (WHO, environmental Health Criteria, 1989). Observed toxicity in coho and chinook salmon was greater in smaller fish than in larger (WHO, environmental Health Criteria, 1989). It is reported that DDT levels of 1 ng/L in Lake Michigan were sufficient to affect the hatching of coho salmon eggs (Matsumura, 1985). DDT may be moderately toxic to some amphibian species and larval stages are probably more susceptible than adults (Hudson *et al*, 1984; WHO, environmental Health Criteria, 1989). In addition to acute toxic effects, DDT may bioaccumulate significantly in fish and other aquatic species, leading to long-term exposure. This occurs mainly through uptake from sediment and water into aquatic flora and fauna, and also fish (WHO, environmental Health Criteria, 1989). Fish uptake of DDT from the water will be size-dependent with smaller fish taking up relatively more than larger fish (WHO, environmental Health Criteria, 1989). A half-time for elimination of DDT from rainbow trout was estimated to be 160 days (WHO, environmental Health Criteria, 1989). The reported bioconcentration factor for DDT is 1,000 to 1,000,000 in various aquatic species (USEPA,

Washington, 1989), and bioaccumulation may occur in some species at very low environmental concentrations (Johnson and Finlet, 1980). Bioaccumulation may also result in exposure to species, which prey on fish or other aquatic organisms (e.g., birds of prey).

- **Effects on Other Animals (Non-target species):** Earthworms are not susceptible to acute effects of DDT and its metabolites at levels higher than those likely to be found in the environment, but they may serve as an exposure source to species that feed on them (WHO, environmental Health Criteria, 1989). DDT is non-toxic to bees; the reported topical LD_{50} for DDT in honeybees is 27 μg/bee (WHO, environmental Health Criteria, 1989). Laboratory studies indicate that bats may be affected by DDT released from stored body fat during long migratory periods (WHO, environmental Health Criteria, 1989).

1.18 ENVIRONMENTAL FATE

- **Breakdown in Soil and Groundwater:** DDT is very highly persistent in the environment, with a reported half-life of between 2-15 years (USEPA, Washington, 1989; Augustijn *et al*, 1994) and is immobile in most soils. Routes of loss and degradation include runoff, volatilization, photolysis and biodegradation (aerobic and anaerobic) (ATSDR, Atlanta, 1994). These processes generally occur only very slowly. Breakdown products in the soil environment are DDE and DDD, which are also highly persistent and have similar chemical and physical properties (WHO, environmental Health Criteria, 1989, Augustijn *et ai*, 1994). Due to its extremely low solubility in water, DDT will be retained to a greater degree by soils and soil fractions with higher proportions of soil organic matter (WHO, environmental Health Criteria, 1989). It may accumulate in the top soil layer in situations where

heavy applications are (or were) made annually; e.g., for apples (Meister, 1992). Generally DDT is tightly sorbed by soil organic matter, but it (along with its metabolites) has been detected in many locations in soil and groundwater where it may be available to organisms (WHO, environmental Health Criteria, 1989, USEPA, Washington, 1989). This is probably due to its high persistence; although it is immobile or only very slightly mobile, over very long periods of time it may be able to eventually leach into groundwater, especially in soils with little soil organic matter. Residues at the surface of the soil are much more likely to be broken down or otherwise dissipated than those below several inches (Matsumura, 1985). Studies in Arizona have shown that volatilization losses may be significant and rapid in soils with very low organic matter content (desert soils) and high irradiance of sunlight, with volatilization losses reported as high as 50% in 5 months (Jorgensen *et al*, 1991). In other soils (Hood River and Medford) this rate may be as low as 17-18% over 5 years (Jorgensen *et al*, 1991). Volatilization loss will vary with the amount of DDT applied, proportion of soil organic matter, proximity to soil-air interface and the amount of sunlight (WHO, environmental Health Criteria, 1989).

- **Breakdown of Chemical in Surface Water:** DDT may reach surface waters primarily by runoff, atmospheric transport, drift, or by direct application (e.g. to control mosquito-borne malaria) (ATSDR, Atlanta, 1994). The reported half-life for DDT in the water environment is 56 days in lake water and approximately 28 days in river water (USEPA, Washington, 1989). The main pathways for loss are volatilization, photodegradation, adsorption to water-borne particulates and sedimentation (ATSDR, Atlanta, 1994) Aquatic organisms, as noted above, also readily take up and store DDT and its metabolites. Field and laboratory studies in the United Kingdom demonstrated that very little breakdown of DDT occurred in

estuary sediments over the course of 46 days (WHO, environmental Health Criteria, 1989). DDT has been widely detected in ambient surface water sampling in the United States at a median level of 1 ng/L (part per trillion) (ATSDR, Atlanta, 1994; Van Ert and Sullivan, 1992).

- **Breakdown of Chemical in Vegetation**: DDT does not appear to be taken up or stored by plants to a great extent. It was not translocated into alfalfa or soybean plants, and only trace amounts of DDT or its metabolites were observed in carrots, radishes and turnips all grown in DDT-treated soils (WHO, environmental Health Criteria, 1989). Some accumulation was reported in grain, maize and rice plants, but little translocation occured and residues were located primarily in the roots (ATSDR, Atlanta, 1994).

1.19 REGULATIONS

DDT is regulated by EPA (Environmental Protection Agency) under Clean Water Act (CWA), Comprehensive Environmental Response, Compensation and Liability Act (CERCLA), Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Food, Drug and Cosmetic Act (FD&CA), Resource Conservation and Recovery Act (RCRA) and Superfund Amendments and Reauthorization Act (SARA). Effluent discharge guidelines and water quality criteria have been set under CWA. DDT is subjected to reporting rules under CWA and SARA. A reportable quantity (RQ) of 1 lb has been established under CERCLA and CWA. It is regulated as hazardous constituent of waste under RCRA. Tolerances of residues of DDT in or on raw agricultural commodities have been established under FD&CA. In 1972, EPA cancelled most DDT registrations under FIFRA. National Institute for Occupational Safety and Health (NIOSH) has recommended that DDT exposure be limited to 0.5mg/m^3 as 10h Time-Weighted Average (TWA). The Occupational Safety and Health Administration (OSHA) has established a

Permissible Exposure Limit (PEL) of 1 mg/m^3 as an 8h TWA. OSHA also regulates DDT under the Hazard Communication Standard and as a chemical hazard in laboratories.

1.20 REGULATORY STATUS

Control actions to ban or severely restrict DDT have been taken by over 38 countries that began in the early 1970s. In at least 26 countries, DDT is completely banned and in 12 others it is severely restricted. In these latter cases, it is permitted for use by government agencies for special programmes usually for involving vector control. DDT is no longer registered for use in the United States, although it is still used in other (primarily tropical) countries. It is in EPA Toxicity Class II, moderately toxic (Meister, 1992). DDT was banned from use in the United States in 1972, and remains banned barring public health emergency (e.g., outbreak of malaria) (ATSDR, Atlanta, 1994). Few countries that implemented total ban include Cuba (1970), Poland (1976), Singapore (1984), Canada (1985), Chile (1985), Liechtenstein (1986), Korea (1986) and Switzerland (1986). It was banned in India for agriculture in April 1997 but is still permitted to be used for health programmes. The environmental protection agencies are seeking complete ban on the use of DDT and there were reports stating the ban would be brought by the year 2007. Presently DDT has been banned globally in agriculture.

But as per this date, Centre for Science and Environment (CSE), a non-government organisation in India, has come out with their observations of DDT in bottled mineral water and then in soft and cool drinks. According to the reports, tests conducted for the presence of 32 major pesticides being used in India, 4 of them were detected in the colas and coke. The pesticides detected were DDT (15 times more than the EU standards), lindane (21 times higher than the EU standards), Chlorphyrifos (42 times more than the EU standards) and malation (87 times higher than the EU standards) (India Today, Aug., 2003).

With the above finding, the statistics of insecticide usage appearing in the major dailies of Andhra Pradesh, India (August 2003 issues of Deccan Chronicle and Hindi Milap), revealed the amount of these insecticides consumed in the state makes it take the first position in insecticide consumption in the country. From all these it becomes evident that the insecticide residues are remaining in major resources of drinking water, where they were never applied. It also reflects a major phenomenon "Global Distillation" wherein the chemicals are carried away from the place of their application to a far of place where it was never taken. The presence of residues of major pesticides in the Arctic and Antarctica circles is the outcome of this phenomenon.

India accounts for the major biomagnification zones of these pesticides mainly because of the growing population and poor economy. Studies revealed the presence of elevated levels DDT-residues in buffalo milk, soil, water and human blood where DDT had been sprayed to control malaria (Battu *et* al, 1989)

A report on Net by Whitestar Software Limited, stated the DDT residues in breast milk of lactating mothers in India was found to be over 4%, which is 2.1 times more than the prescribed WHO limit. Mean levels of total DDT in breast milk was noted as 17.18ppm in Ludhiana and 26.66ppm in Faridkot districts of India as compared to 2.65 ppm in Brazil and 0.321ppm in Australia.

1.21 EPA Pesticide Fact Sheets Online

The registration division of the EPA Office of Pesticides Programs (OPP) has made pesticide fact sheets available on the OPP home page. The web address for the EPA fact sheets is <http://www.epa.gov/pesticides/factsheets/>. The fact sheets contain information on specific chemicals and items involving pesticides. Information on conventional pesticides include:

Active ingredients

Year of initial registration

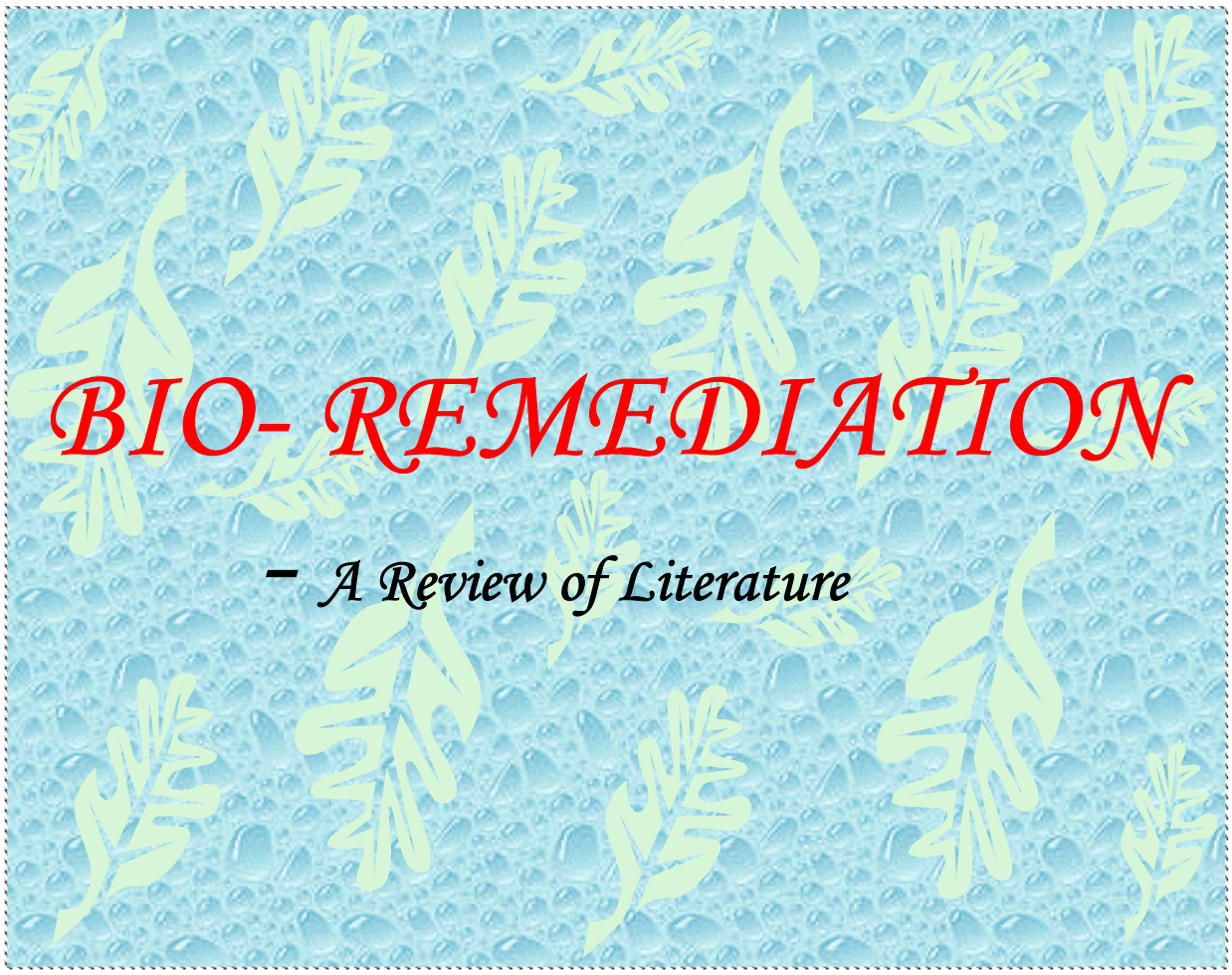
Chemical family

U.S. producer
Application sites
Types of formulations
Types and methods of application
Application rates
Science statements
Chemical characteristics
Toxicology characteristics for both human health and the environment

1.22 Conclusions

Though DDT is still considered as the wonder or miracle for preventing malaria and other vector borne diseases, its persistence in the nature and bioaccumulation resulting in the biomagnification ultimately is considered a health hazard. Though as a young insecticide it was appreciated by all the world nations, middle aged DDT was looked at with many hidden hazards not only to the non-target animals but also to humans. When the Environmental Protection Agencies and other related organisations are seeking a complete ban on its use world wide, the old aged DDT is struggling to survive with only poor nations supporting it. If there are no affordable alternatives, DDT may continue its role in protecting the population against malaria and vector related diseases. Since this compound has come under suspicion as a possible carcinogen and responsible for many of the health problems, strategies to deplete its concentration in the environment should be developed that are easily available at an affordable cost to all.

Chapter 2



We have subjected enormous numbers of people to contact with these poisons, without their consent and often without their knowledge. It is the public that is being asked to assume the risks that the insect controllers calculate. The public must decide whether it wishes to continue on the present road, and it can only do so when in full possession of the facts.

Rachel Carson (1962)

2.1 Introduction

The use of xenobiotics continues to be controversial because they are indispensable yet pose a problem of harmful persistence in the environment. Aromatic compounds have been known as major pollutants. Environmental organisations like United States' Environmental Protection Agency (USEPA) and Environment and Forests, India, have designated many of these compounds as toxic, hence they need to be removed from the contaminated sites and sources (Mishra *et al*, 2001). There are many compounds that have been used by man for his benefit but led to the contamination of the environment. They include both organic and inorganic contaminants. Organic contaminants include many industrially derived compounds. Some of these compounds such as agricultural pesticides are intentionally applied to the soil, water and our house surroundings. However, others, such as polynuclear aromatic hydrocarbons (PNH), polychlorinated biphenyls (PCB), and polychlorinated dibenzo-*p*- dioxins and furans, enter the soil accidentally*. Analysis of organic contaminants requires specialised techniques and equipments are costly. That is the reason why it becomes more expensive to first identify the contaminant and then to treat it further to get rid of it. On the other hand, inorganic contaminants enter the soil, water and atmosphere as a result of human activities. The heavy metals arsenic, cadmium, chromium, cobalt, lead, mercury, molybdenum, nickel, selenium and zinc are the most hazardous of these substances*. Though plants and animals require these metals in very minute quantities, they may enter soil in large quantities and pose health risks. The inorganic contaminants enter the soil as a result of mining, metallurgy, use of fossil fuels, and application of soil amendments (such as triple superphosphate, urea, potassium chloride, agricultural lime, cow manure, sewage sludge: in all these applications Cd, Co, Cr, Cu, Ni, Pb and Zn enter the soil in different concentrations)*.

Under the organic contaminants Pesticides and non- pesticide compounds can be found. They include organochlorines, organophosphorus compounds,

neutral herbicides, phenoxy acid herbicides and carbamate herbicides. Production of organochlorine pesticides, except endosulfan, has been banned in most parts of *the developed world*. But still they are being used in many parts of the developing nations. Non-pesticide contaminants are polychlorinated biphenyles, naphthalene, phthalate esters, phenol and pentachlorophenol, haloethers, chlorinated benzenes, BTEX (Benzene, Toluene, Ethylene, Xylene), TCE (Trichloroethylene), PCE (Pentachloroethylene), nitrosamines, cresols, heterocyclic nitrogenous compounds, explosives, etc. are again of significant agricultural and environmental concern* (Ryan *et al*, 1991). Fig.2.1 gives the outline of how the chemicals get into the environment as pollutants.

The use of pesticides has become an indispensable tool in agriculture for the control of pests and in public health programmes for the eradication of vector borne diseases. It is estimated that nearly four million tonnes of pesticides were applied to the world crops annually for pest control. Less than 1% of the applied pesticide generally reached the target pests (Pimentel, 1983). Therefore most of the pesticide remained unused and entered into the ecosystem (Fig.2.2). These excessive pesticide residues accumulate in the biosphere and create ecological stress. Soil and water are the ultimate sinks for the excessive pesticides. According to a report by Sheryl Gay Stolberg, (The New York Times, Aug. 29, 1999), “A child dies of malaria every 12 seconds and that could go up dramatically if DDT, an important control tool, is lost”. In another report published in “USA Today” (Nov.29, 2000), India can afford to spray 70% of malarious areas with DDT and if it uses the next cheapest alternative, only 23% of the area could be sprayed. Similarly, the use of other chemical pesticides as well cannot be discontinued despite their persistence in nature, their tendency towards bioaccumulation and toxicity to non-target organisms including human beings as well as the toxicological and environmental problems of its residues (Singh *et al*, 1989). DDT

* http://sis.arg.gc.ca/cansis/publications/health/chapter09.html

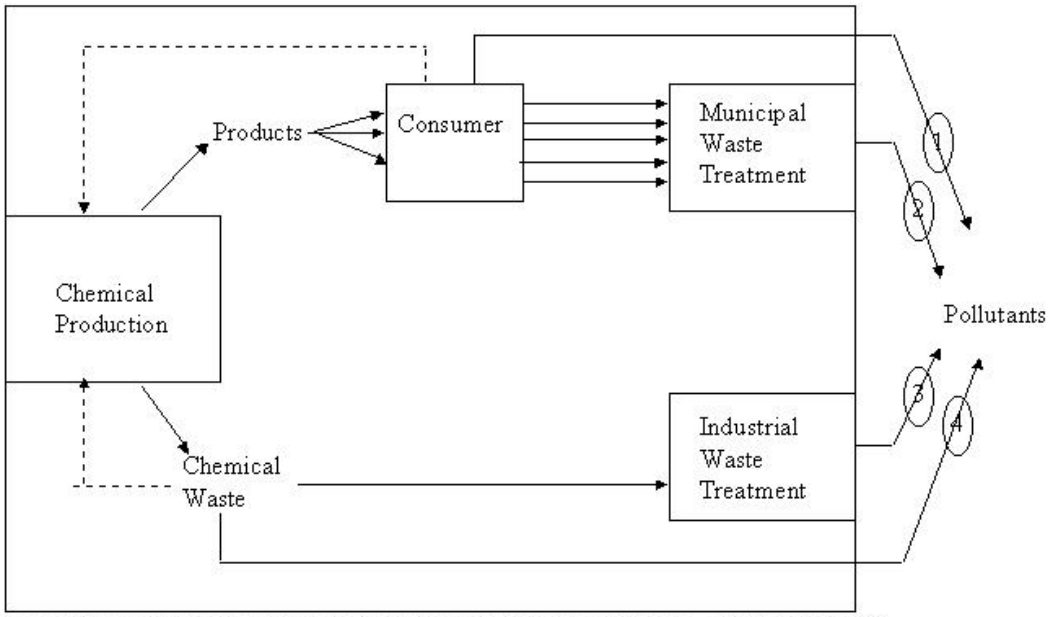


Fig.2.1 Release of Organic Chemicals into the Environment (from, Leisinger, 1983).

1. Chemicals whose use leads to their entry into the environment, eg. Aerosol pollutants, pesticides, fertilizers.
2. Chemicals entering the environment in the effluent of municipal sewage treatment systems, eg., hard detergents, solvents.
3. Chemicals resistant to biological degradation in industrial waste treatment wsystems, eg., chlorobenzenes, aminonaphthol sulphonic acids.
4. Direct discharge, losses, spills and accidents leading to the entry of chemicals from production sites into the environment

Fig.2.2 Entry and Movement of Pesticides in Ecosystem
(Source: Lakshmi *et al*, 2002)

was used profusely mainly because of its long lasting effect on the pests. These long lasting effects of DDT can be attributed to its persistent nature. Though it has been given the tag of recalcitrant compound, there are organisms that made it appear a persistent chemical because of their ability to act and transform it under favourable conditions into other degradable metabolites. In the 1940s and 50s the work done by the researchers and their interpretations of results reflected the harmless nature of DDT to humans and many non-target animals. But in 1962, the work of Rachel entitled “Silent Spring” brought to light the merciless chemical responsible for the drastic decline in the number of American Falcons. Later the studies revealed that DDT was responsible for the thinning of Falcons’ eggshell rendering them less supportive for the production of future generation. Therefore search for remedies and techniques for decontamination and detoxification of pesticide-contaminated environments has become imperative. Special attention has been given to the remediation of contaminated soil and aquifers worldwide by CERCLA and SARA after the Love Canal case, in Niagara Falls, NY, USA (It is assumed that 22,000 tonnes of chemical waste had been disposed in this region that include PCBs, dioxins and other pesticides by Hooker Chemical Company during 1940s and 50s. This was reflected as abnormalities like miscarriages and birth defects in the residents of the area) (Iwamoto and Nasu, 2001). Most of the chemicals put in to the environment usually undergo various breakdown processes. These transformations of the applied chemicals can be attributed to various factors such as chemical and physical nature of the compound that include stability of the compound, ability to bind to the soil, sediments or any other support matrix, half-life, solubility, volatility, vapour pressure, ability of the organisms to degrade or accumulate, its biocompatibility, and its effect on the surrounding environment, etc. The effect of air, light, heat, moisture, and biota on the compound, play a very important role on the type of transformation it undergoes. It will always be the combined effect of all these major factors that determines the transformation process in the environment Table 2.1 describes the persistence of some chlorinated organic compounds in soil.

Table 2.1 Persistence of Some Organochlorines in Soil

Compound	Approximate duration of Persistence (years)
PCBs	>30
Chlordane	>12
HCH	>10
DDT	>10
Aldrin	>9
Heptachlor	>9
Toxaphene	>6
Dicamba	2

(Source: Manonmani and Kunhi, 1999)

Many workers then started to look for various methods to lessen pesticide residue concentration in the environment. Similarly residues of many other chemicals that pose environmental threat have been studied for degradation/removal from the environment by researchers in various ways. Remediation includes photochemical remediation, phytoremediation, chemical remediation and microbial remediation.

Photochemical remediation: Though there are sufficient evidences to suggest that photodecomposition of pesticides occur under field conditions, the probable photolysis of the chemicals would only be on the surface soil or on the plant surface other than in atmosphere (Lakshmi *et al*, 2002). When the pesticides are applied to the soil or water bodies, they tend to get transported to subsurface sites impenetrable by sunlight. Furthermore, most pesticides are applied to established crops where the foliar cover can drastically reduce the intensity of light

reaching the soil (Hill and Wright, 1978), it rarely occurs under natural conditions and would be very slow (Fig.1.3).

Chemical remediation: Pesticides are also susceptible to various chemical reactions in presence of metals and metal ions such as iron, aluminium, etc. The alkalinity of the medium also plays an important role in destabilising many pesticides. It needs to be supplied continuously to the contaminant for a slow decomposition and in case to improve the process speed; conditions have to be altered that accounts for a large investment.

Bioremediation: Bioremediation can broadly be classified as phytoremediation and microbial remediation.

Phytoremediation: Phytoremediation is the use of vegetation for *in situ* treatment of contaminated soils, sediments and water. It is applicable at sites containing organic, nutrient, or metal pollutants that can be accessed by the roots of plants and sequestered, degraded, immobilised or metabolised in place. In the last few years, a great understanding has been achieved regarding the uptake and metabolism of xenobiotic chemicals by plants especially chlorinated solvents, petrochemicals, some pesticides and explosives (Anderson *et al*, 1993; Schnoor *et al*, 1995; Newman *et al*, 1997; Hughes *et al*, 1997; Burken and Schnoor, 1998; Thompson *et al*, 1998; Raskin and Ensley, 2000; Terry and Banuelos, 2000). Inorganics and metals (nutrients, selenium, arsenic, lead, cadmium, Nickle and zinc) have been successfully remediated using plants.

Plants have become popular because of their cost effectiveness, aesthetic advantages and long-term applicability (Schnoor *et al*, 1995). Applications include hazardous waste sites where other methods of treatments are expensive or impracticable, low-level contaminated sites only. Limitations of phytoremediation include the presence of hazardous metabolites in the food chain. The time required to clean up below action-level is long and toxicity encountered in establishing and maintaining vegetation at the contaminated sites are among few other limitations here. A few plants have been shown to have the capacity to

withdraw high concentrations of organic chemicals without showing toxicity effects (Briggs *et* al, 1982; Burken and Schnoor, 1998). These plants have the capacity to take up and transform the contaminants to less toxic metabolites (in some cases) (Newman *et al*, 1997; Schalk *et al*, 1997; Ohkawa *et al*, 1999; Werck-Reichart *et al,* 2000). Plants can also stimulate the degradation of organic chemicals in the rhizosphere by release of root exudates, enzymes and the build up organic carbon in the soil (Shimp *et al*, 1993; Burken and Schnoor, 1996).

Microbial Degradation: Microbial degradation involves the use of microbes to detoxify and degrade environmental contaminants. This has received increased attention as an effective biotechnological approach to clean up a polluted environment (Iwamoto and Nasu, 2001). Microbial remediation of soils and ground water can be done by excavating the materials or pumping the groundwater and treating them on-site or off-site. When the contamination of the subsurface is limited to the soil in the unsaturated zone, the need for oxygen and water restricts the use of *in-situ* bioremediation to near surface contamination (Ritter and Scarborough, 1995). Two basic forms of bioremediation had been practiced: microbiological approach and the microbial ecology approach (Protrowski, 1991). The microbiological approach involves augmentation of a contaminated site with one or more species of contaminant-specific degrading microorganisms. The idea behind this approach is that the rate of degradation of contaminant would be appreciably enhanced because the density of the contaminant-specific degraders is increased artificially. Two methods have been used to achieve augmentation of contaminated site with species of contaminant-specific degrading microorganisms. The first one involves the use of pre-packed, contaminant-specific degraders that have been selected because of their ability to degrade that contaminant. Such microorganisms are usually obtained from the contaminated sites by subjecting the native microflora to stress conditions of elevated concentrations of the contaminant for a long time.

The second method involves selection, culture and application of site-specific strains that exhibit desirable degradative qualities. This approach is to sample the contaminated soil or water at a site and analyse the samples for microbial strains. The strains that exhibit desirable traits for degrading specific contaminant are cultured and applied to the contaminated sites in high densities along with nutrients identified as being important for high activity rates of the microbe.

Ex-situ bioremediation is the treatment that removes contaminants at a separate treatment facility. The treatment is undertaken away from the contaminated site. After remediation, the remediated soil is brought back to the site and refilled. Carberry *et al* (1991) described a controlled land farming technique to bio-remediate petroleum contaminated soil. The contaminated soil was placed in a green house on a plastic sheet to a depth of 18 inches. The soil was periodically stirred for aeration and nutrients and microorganisms were also added periodically. pH could also be controlled in such a case with lot of ease. Water can also be added to maintain the moisture content of the soil. USEPA-SITE Program had undertaken the development of processes along with different companies, applicable to biodegradable insecticides and other contaminants (Ritter and Scarborough, 1995). Slurry-phase bioreactor in which soil is mixed with water to form a slurry, biodegradation has been shown to be effective in treating highly contaminated soils and sludge with contaminant concentrations of 2,500-2,50,000ppm (Ritter and Scarborough, 1995). The decontamination of explosive-contaminated soils by composting was effected in pilot plant studies wherein the concentrations of explosives like RDX, HMX and PETN, were reduced to below detection limits within 3 weeks of composting (Doyle *et al*, 1992)

Though there are different strategies adapted worldwide to get rid of these contaminants, the best strategy appears to be only bioremediation. This is the best way to treat pollution because it leaves less or no toxic metabolites in the environment compared to other strategies wherein the intermediary metabolites

may remain in the environment that might pose more danger than the parent compound. Moreover, all the other strategies are more expensive and energy consuming. Bioremediation, on the other hand works out to be cheaper and more effective strategy to eliminate these toxic chemicals from the environment. Phytoremediation involves the cultivation of plants having an affinity towards the contaminant and that take up the maximum amount of the contaminant from the surroundings and then these flora can be taken for incineration as further treatment of the accumulated chemical. Another strategy here is that the plant itself might take the charge to mineralise the chemical into simpler forms that can be used elsewhere in the metabolism. Phytodegradation of DDT by aquatic plant *Elodea Canadensis* and a terrestrial plant *Pueraria thunbergiana* was reported by Garrison *et al* (2000). The major disadvantage here appears to be the length of treatment: the site needs to be left only for such plants till the plant absorbs the complete or maximum quantity of the toxicant. This depends upon the ability of the plants to take up the chemical. Moreover, the plant needs to grow up to maximum state to absorb the chemicals from the site through roots that may take a longer time factor. With this scenario, microbial remediation appears to be the only way out for treatment of contaminated resources in short time and with more efficiency and safety, with less investment. Many reports are available on the microbial degradation (microbial bioremediation) of environmental pollutants.

2.2 Bioremediation and microbes

DDT is one of the most extensively studied insecticides, mainly for its broad insecticidal spectrum. The primary metabolic mechanism that was studied was the reductive dechlorination of DDT, with the formation of DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane or dichlorodiphenyldiichloroethane) (Kallman and Andrews, 1963; Barker and Morrison, 1964). This degradation was later determined to be microbial and *Proteus vulgaris* was isolated (Barker and Morrison, 1965) which could degrade DDT mainly to DDD. Several minor metabolites like DDMU (1-

chloro-2,2-bis(4-chlorophenyl)ethylene), DDM (bis(4-chlorophenyl)methane) were also formed (Barker and Morrison, 1965; Barker *et al*, 1965). Numerous microorganisms have been isolated from animal and plant sources that could degrade DDT to DDD. These include *Escherichia coli* and *Aerobacter aerogenes* isolated from rat faeces (Mendel and Walton, 1966; Mendel *et al*, 1967), anaerobic bacteria from stable fly gut (Stenerson, 1965) rumen fluid microflora (Miskus *et al*, 1965), rat intestinal microflora (Braunberg and Beck, 1968), *E. coli* (Langlois, 1967) and plant pathogens (Johnson *et al* 1967). The formation of DDD from DDT is also a common reaction among soil microorganisms (Guenzi and Beard, 1967). Chacko *et al* (1966) isolated numerous actinomycetes (*Nocardia* sp., *Streptomyces aureofaciens, Streptomyces cinnamoneus, Streptomyces viridochromogenes*) from soil, which readily degraded DDT to DDD. These organisms however, required another carbon source to facilitate degradation. Soil fungi not only produced DDD and small amounts of dicofol (4,4'-dichloro-a-(trichloromethyl) benzhydrol), but some variants could produce DDA (bis(4-chlorophenyl)acetic acid) or DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) exclusively (Matsumura and Boush, 1968). Wedemeyer (1967) reported dehalogenation of DDT to various metabolites under anaerobic conditions by *Aerobacter aerogenes*. DDD was obtained under both aerobic as well as anaerobic conditions when DDT was incubated with *Aerobacter aerogenes* (Mendel *et al*, 1967; Wedemeyer, 1967). *Escherichia coli* dechlorinated 50% of DDT to DDE when grown in various broths or skimmed milk (Langlois, 1967). Under aerobic conditions the major product of DDT metabolism, in *Bacillus cereus, B. coagulans, B. subtilis,* was DDD while DDMU (1-chloro-2,2-bis(4-chlorophenyl)ethylene), DDMS (1-chloro-2,2-bis(4-chlorophenyl)ethane), DDNU (2,2-bis4-chlorophenyl)ethane), DDOH (2,2-bis(4-chlorophenyl)ethanol), DDA and DBP (4,4'-dichlorobenzo phenone) were in trace amounts and were found under anaerobic conditions (Langlois *et al*, 1970). *Hydrogenomonas* sp. yielded DDD, DDMS, DDMU, DBH (4,4'-dichlorobenzhydrol), DDM (bis(4-chlorophenyl)methane) and DDA (Focht and Alexander, 1970). DDD was further

degraded through dechlorination, dehydrochlorination and decarboxylation to DBP or to a more reduced form, DDM. 4% of initial DDT was 75% transformed by *Phanerochaete chrysosporium* (Bumpus and Aust, 1987). Bumpus *et al* (1985) showed the involvement of lignin peroxidase in the degradation of DDT. There are many reports on the biodegradation of HCH (1,2,3,4,5,6-hexachlorocyclohexane), another pesticide commonly used. *Clostridium sphenoides*, *Clostridium butyricum* have been implicated to be involved in anaerobic degradation of HCH (Sethunathan *et al*, 1983; Mac Rae, 1989). Bhuyan *et al* (1992) reported aerobic degradation of γ-HCH (lindane) after their experiments of acclimatisation in flooded and non-flooded rice fields. *Pseudomonas* sp. has been isolated from rhizosphere of HCH-treated sugarcane plant for the degradation of all major isomers of HCH in aerobic conditions (Sahu *et al*, 1990). Bhuyan *et al* (1993) reported that all the HCH isomers could be degraded by *Sphingomonas paucimobilis*. Manonmani *et al* (1995) reported different levels of HCH degradation for each of its four major isomers by a defined microbial consortium. Degradation metabolites have been described for HCH by various researchers (Tu, 1976; Bachmann *et* al, 1988a, 1988b; Senoo and Wada, 1989; Deo *et al*, 1994; Sahu *et al*, 1995; Thomas *et* al, 1996; Nagata *et al,* 1999; Kumari *et al*, 2002). Mirex (dodecachlorooctohydro-1,3,4-methano-2H-cyclobuta(cd)pentalene), another organochlorine insecticide has been shown to get degraded to unknown metabolites in sewage sludge in anaerobic conditions in the dark (Androde and Wheeler, 1974). Kepone (1, 1a, 3, 3a, 4, 5, 5a, 6-decachlorooctahydro-1, 3, 4-methano-2H- cyclobuta(co)pentalene-2-one) derivatives have been described in soil by Borsetti and Roech, 1978). Orndorff and Colwell (1980) described a mixed culture and *Pseudomonas aeruginosa* to be involved in dechlorination of kepone under aerobic conditions. Endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,6,8,8a-octahydro-1,4-endo-endo-5,8-dimethano naphthalene) was observed to be degraded in flooded soils by *Aspergillus niger, Aspergillus flavus, Penicillium notatum, Penicillium chrysogenum, A. fusarium* sp*., Trichoderma* sp., *Streptomyces* sp., *Micromonospora* sp., *Bacillus* sp., and *Pseudomonas* sp. (Korte *et* al, 1962; Tu *et*

al, 1968). Heptachlor (1,4,5,6,7,8,8-heptachloro-3a,5,7,7a-tetrahydro-4,7-methano diene), another organochlorine, gets oxidised in soil to epoxide (Barthel *et al*, 1966; Wilkinson *et al*, 1964). Well-known insecticide endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 2, 4,3-benzodiexathiopin-3-oxide) degrades to endosulfan sulphate and endosulfan diol by soil bacteria and fungi (Goebel *et al*, 1982). Complete degradation of endosulfan using a mixed culture of soil organisms has been reported by Miles and Moy (1979) and Awasthi *et al* (2000). Few reports on the degradation of monochloro- and dichlorotoluenes by *P. cepacia* and other species of *Pseudomonas* using them as sole carbon source was described by Pierce *et al* (1983). Vandenbergh *et al* (1981) isolated *P. cepacia* from a landfill area used as a disposal site for chlorinated organic wastes, which could metabolise 2,6-dichlorotoluene. Another group of environmental pollutants, chlorobenzenes, is used as solvents, fumigants and intermediates in the production of pesticides and dyes. Several microorganisms have been shown to degrade different chlorobenzenes (Bartholomew and Pfaender, 1983; Bouwer and McCarty, 1983; deBont *et* al, 1986; Schraa *et al,* 1986; Spain and Nishino, 1987; Ramanand *et* al, 1993 and Brunsbach and Reineke, 1994). Several organisms have been shown to degrade chlorobenzoates, a group that interested many researchers for studying the catabolic mechanisms of haloorganics (Knackmuss, 1981; Reineke, 1984; Reineke and Knackmuss, 1988; Chaudhry and Chapalmadugu, 1991; Ajithkumar, 1995). Most of the reported organisms can utilise only low concentrations of chlorobenzoates. *Pseudomonas* sp. B13, one of the most extensively studied organisms, can degrade up to 2 grams per litre of 3-chlorobenzoate (3-CBA) (Dorn *et al*, 1974). *Arthrobacter* sp. SB8 could degrade up to 10 grams per litre of 4-CBA (4-Chlorobenzoic acid). *Pseudomonas aeruginosa* 3mT could degrade up to 8 and 12 grams per litre of 3-CBA and 4-CBA respectively (Ajithkumar, 1995; Babu *et al*, 1995). The degradation of dicamba (3,6-dichloro-2-methoxy benzoate), a chlorobenzoate herbicide, has been studied under aerobic and anaerobic conditions (Smith, 1974; Krueger *et al*, 1991). Eight different bacteria had been described that could degrade dicamba

(Krueger *et al*, 1989). *Alcaligenes eutrophus* JMP134 has been extensively studied for the degradation of 2,4-D (2,4-Dichlorophenoxy acetic acid) (Don and Pemberton, 1981). The degradation of 2,4-D was found to be six times faster under aerobic conditions than under anaerobic conditions in fresh water sediments (Delaune and Salinas, 1985). Michel *et al* (1995) reported the microbial degradation of 2,4-D at composting temperatures of 60^0C. Effect of dissolved oxygen concentration (Shaler and Klecka, 1986), effect of organic and inorganic amendments such as rice straw and compost (Duah-Yentumi and Kuwatsuka, 1982), effect of temperature and soil moisture (Parker and Doxtader, 1983; Ou, 1984), effect of sorption (Ogram *et al*, 1985), effect of soil type (Smith, 1985), concentration of 2,4-D (Subba Rao *et al*, 1982; Hoover *et al*, 1986, Kim and Maier, 1986; Wiggins *et al*, 1987) on the microbial degradation of 2,4-D have been reported. Most of the earlier information concerning 2,4,5-T (2,4,5-trichlorophenoxy acetic acid), another common herbicide, has been using reductive sediments (Krans *et al*, 1984; Suflita *et al*, 1984; Mikesell and Boyd, 1985). Kellog *et al* (1981) described a mixed microbial population that could degrade 2,4,5-T. A pure culture of *P.cepacia* AC1100 was isolated by Kilbane *et al* (1982), which could utilise 2,4,5-T as sole carbon source. Chatterjee *et al* (1982) and Gangadhara (1991) reported the bioremediation of 2,4,5-T-contaminated soils with *P. cepacia* AC1100 and elimination of inhibition of seed germination. A mixture of *Pseudomonas* sp., *Alcaligenes* sp., *Flavobacterium* sp., and *Acinetobacter calcoaceticus* were found to degrade MCPA (2-methyl-4-chlorophenoxy acetic acid), another herbicide (Lappin *et al*, 1985). Degradation of MCPA in soil and the effects of various soil amendments have also been reported by Duah-Yentumi and Kawatsuka (1982). Pentachlorophenol (PCP), a multipurpose pesticide that tends to accumulate in the environment, has been shown to be acted upon by *Arthrobacter* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Rhodococcus chlorophenolicus* (MacRae, 1989; Chaudhry and Chapalamadugu, 1991). Among fungi, *Phanerochaete chrysosporium* has been reported to degrade PCP. In field studies, *Phanerochaete chrysosporium* and *P.*

sortida removed 88-91% PCP from soil containing 250-400 ppm of this contaminant. The PCP-contaminated soil with 2% sterile peat and 3% wood chips over grown with *Phanerochaete chrysosporium*, demonstrated low levels of leaching of these compounds to the underlying soil and low rates of volatilisation to the atmosphere (Fungal Treatment Bulletin, 1993). Co-immobilisation of *Phanerochaete chrysosporium* mycelium and activated carbon degraded PCP more efficiently than similar mixtures that were not immobilised (Lin *et al*., 1991). Other chlorinated phenols, other than PCP, that have been used extensively as wood preservatives, herbicides, fungicides and tanneries are generated as breakdown products of 2,4-D, 2,4,5-T and PCP, etc. Some PCP degrading strains also could degrade lower chlorinated phenols (Kilbane *et al,* 1982; Krans *et al*, 1984). Some were isolated by enrichment techniques with respective substrates like 4-chlorophenol degrading *Pseudomonas* sp. and 3,4-dichlorophenol degrading transconjugant of *Alcaligenes* sp..A7-2 (Schwien and Schmidt, 1982). Haloanilines include 4-chloro-, 3-chloro-, 2-chloro-, 3,4-dichloro- and 2,4,5-trichloroanilines. Degradation of *o-, m-* and *p-* chloroanilines by *Pseudomonas* sp. (Zeyer and Kearney, 1982) *Moraxella* sp. (Zeyer *et al*, 1985) and *p*-chloroaniline by wood rot fungus, *Tramates versicolor* (Hoff *et al*, 1985), 3,4-dichloroaniline by *Pseudomonas putida* (You and Bartha, 1982), 2,3-dichloroaniline and 3,5-dichloroaniline by adapted and non-adapted fresh water sediment slurries (Liu and Jones, 1995) have been reported. Polychlorinated biphenyls have also been reported to undergo bacterial degradation (Ahmed and Focht, 1973; Ballshmitter *et* al, 1977; Furukawa *et* al, 1978; Eaton, 1985; Brown *et al*, 1987). Chlorinated dioxins, chlorodibenzo-*p*-dioxins were reported to undergo bio-transformation (Klecka and Gibson, 1980; Phillipi *et al*, 1982; Quensen and Matsumura, 1983; Wilkes *et al*, 1996). Table2.2 describes the chemicals and the microbes that had been used for bio-transformation work by researchers.

Much work has been carried out on the biodegradation of DDT by microorganisms under various conditions. The metabolites formed and the factors influencing the degradation of DDT in water and in soil environments has been described in the Tables 2.3-2.6.

Table 2.2: Microorganisms responsible for Pesticide Degradation

Pesticide	*Microorganism*	Reference
Chlorophenoxy acids		
2,4-D	*Alcaligenes eutrophus*	Don and Pemberton (1981)
	Alcaligenes xylosoxidans	Gunulan and Fournieer (1993)
	Flavobacterium sp. 50001	Chaudhry and Huang(1988)
	Pseudomonas putida	Lillis *et al* (1983)
	Pseudomonas cepacia	Kilbane *et al* (1982)
	Comamonas sp.	Bulinski and Nakatsu (1998)
2,4,5-T	*Pseudomonas cepacia*	Karns *et al* (1982)
DPA	*Flavobacterium* sp.	Horvath *et al* (1990)
Mecoprop	*Sphingomonas herbicidivorans* MH	Zipper *et al* (1966)
Mecocarp	*Alcaligenes denitrificans*	Tett *et al* (1997)
Organochlorines		
DDT	*Aerobacter aerogenes*	Wedemeyer (1966)
	Alcaligenes eutrophus A5	Nadeau *et al* (1994)
	Agrobacterium tumefaciens	Johnson *et al* (1967)

DDT	*Arthrobacter* sp.	Patil *et al* (1967)
	Bacillus cereus	Johnson *et al* (1967)
	Bacillus cooagulans	Langlois *et al* (1970)
	Bacillus megaterium	Plimmer *et al* (1968)
	Bacillus subtilis	Johnson *et al* (1967)
	Clostridium pasteurianum	Johnson *et al* (1967)
	Clostridium michiganense	Johnson *et al* (1967)
	Enterobacter aerogenes	Langlois *et al* (1970)
	Erwinia amylovora	Johnson *et al (1967)*
	Escherichia coli	Langlois *et al* (1970)
	Hydrogenomonas sp.	Focht and Alexander (1970)
	Klebsiella pneumoniae	Wedemeyer (1966)
	Kurthia zapfii	Johnson *et al* (1967)
	Micrococcus sp.	Plimmer *et al* (1968)
	Nocardiai sp.	Chacko *et al* (1996)
	Pseudomonas aeruginosa DT-Ct1	Bidlan and Manonmani (2002)
	Pseudomonas aeruginosa DT-Ct2	Bidlan and Manonmani (2002)
	Pseudomonas fluorescens DT-2	Bidlan and Manonmani (2002)
	Serratia marcescens	Mendel and Walton (1966)
	Serratia marcescens DT-1P	Bidlan and Manonmani (2002)
	Streptomyces annomoneus	Chacko *et al* (1996)
	Streptomyces aureofacians	Chacko *et al* (1996)
	Streptomyces viridochromogens	Chacko *et al* (1996)

DDT	*Xanthomonas* sp.	Johnson *et al* (1967)
	Phanerochaete chrysosporium (fungus)	Bumpus and Aust (1987)
	Trichoderma viride (fungus)	Matsumura and Boush (1968)
γ-HCH	*Aerobacter aerogenes*	Mecksongsee and Guthrie (1965)
	Bacillus cereus	Mecksongsee and Guthrie (1965)
	Bacillus megaaterium	Mecksongsee and Guthrie (1965)
	Citrobacter freundii	Jagnow *et al* (1977)
	Clostridium rectum	Jagnow *et al* (1977)
	Escherichia coli	Francis *et al* (1975)
	Pseudomonas fluorescens	Mecksongsee and Guthrie (1965)
	Pseudomonas putida	Benzet and Matsumara (1973)
	Pseudomonas paucimobilis	Bachmann *et al* (988)
	Pseudomonas sp.	Sahu *et al* (1990)
	Anabaena sp. (Cyanobacteria)	Kurtiz and Wolk (1995)
	Nostocellipssosun (Cyanobacterium)	Kurtiz and Wolk (1995)
	Phaenrochaete chrysosporium (fungus)	Mougin *et al* (1996)
	Trametesversicolor (fungus)	Singh and Kuhad (1999a)
	Phanerochaete sordida (fungus)	Singh and Kuhad (1999b)

γ-HCH	*Cyathus bulleri (Fungus)*	Singh and Kuhad (1999b)
Organophosphates		
Parathio	*Flavobacterium* sp	Sethunathan and Yoshida (1973)
	Pseudomonas aeruginosa	Gibson and Brown (1974)
	Pseudomonas diminuta	Serdar *et al* (1982)
	Pseudomonas melophthara	Boush and Matsumura (1967)
	Pseudomonas stutzeri	Doughton and Hsieh (1967)
Phorate	*Rhizobium japonium*	Mich and Dahm (1970)
	Rhizobium melioloti	Mich and Dahm (1970)
	Streptomyces lividans	Steiert *et al* (1989)
	Bacillus megaterium	La Partourel and Wright (1976)
Carbamates		
Carbary	*Pseudomonas cepacia*	Venkateswarlu *et al* (1980)
	Pseudomonas melophthora	Bousch and Matsumura (1967)
	Pseudomonas aeruginosa	Chapalamadugu and Chaudhry (1993)
	Gliocladium roseum (Fungi)	Liu and Bollog (1971)
	Aspergillus flavus (Fungi)	Bollog and Liu (1972)
	Aspergillus terreus (Fungi)	Bollog and Liu (1972)
	Culcitalna sp. (Fungi)	Sikka *et al* (1975)
	Halosphaeria sp. (Fungi)	Sikka *et al* (1975)

Carbary	*Fusarium solani* (Fungi)	Bollog and Liu (1972)
	Rhizopus sp. (Fungi)	Bollog and Liu (1972)
	Penicillium sp. (Fungi)	Bollog and Liu (1972)
Carbofuran	*Achromobacter* sp. WMIII	Karns *et al* (1986)
	Arthrobacter sp.	Ramanand *et al* (1988)
	Flavobacterium sp.	Chaudhry anf Ali (1988)
	Pseudomonas cepacia	Venkateswarlu *et al* (1980)
	Pseudomonas stutzeri	Mohapatra and Awasthi (1977)
	Bacillus pumilis	Mohapatra and Awasthi (1977)
s-Triazines	*Pseudomonas* sp.	Cook and Hutter (1981)
	Klebsiella pheumoniae	Cook and Hutter (1981)
	Rhodococcus corallinus	Cook and Hutter (1981)
	Rhizobium sp. PATR	Bauguard *et al I(1997)*
	Phanerochaete chrysosporium (Fungus)	Mougine *et al* (1994)

Source (Part of the table): Singh *et al*, (1999)

Table 2.3 Microbial Degradation of DDT Residues

Organism/conditions	Reference	Year/ remarks
Microbes growing at the expense of growth substrate	Bollag and Liu	1990
Microbial degradation	Fries *et al*.	1969
Indigenous micro flora in Rodents	Barker *et al.*	1965
Indigenous micro flora in Rodents	Mendel and Walton	1966
E.coli, E.aerogenes, E.cloacae, Klebsiella pneumoniae, P.aeruginosa, P.putida, Bacillus sp., Hydrogenomonas sp., Saccharomyces cervisiae, Phanerochaete chrysosporium, Trichoderma viridae	Johnsen Lal and Saxena Subba Rao and Alexander Sharma *et al* Beunink and Rehm	1976 1982 1985 1987 1988
E.coli, Enterobacter aerogenes	Langlois *et al*	1970
Aerobacter aerogenes	Wedemeyer	1967
Hydrogenomonas sp	Pfaender and Alexander	1972

Organism/conditions	Reference	Year/ remarks
Fungi	Subba Rao and Alexander	1985
Bacterium	Masse *et al.*	1989
Arthrobacter	Pfaender and Alexander	1972
P. aeruginosa 640X	Golovleva and Skryabin	1981
Genetically modified *Pseudomonas* BS-827	Golovleva *et al.*	1982
Phanerochaete chrysosporium	Bumpus and Aust Fernando *et al.* Aust Shah *et al.* Bumpus *et al.*	1987, 1989, 1990, 1992, 1993.
Gleophyllum traveum	Fernando *et al.*	1989
Phanerochaete cordylines	Walter	1992
Phanerochaete chrysosporium	Bumpus *et al.*	1993
Fungi	Kohler *et al.*	1988
Hydrogenomonas	Bartha	1990, Aerobic conditions, DDT analogue
Hydrogenomonas	Focth and Alexander	1970
Alcaligenes eutrophus A5	Nadeau *et al.*	1994, aerobic degradation of DDT
Bacteria	Parsons *et al.*	1995

Table 2.4 Soil Studies on DDT Degradation With the Native Organisms in the Soil

Reference	Year	Remarks
Burge	1971	No information on complete degradation
Castro and Yoshida	1971, 1974	-do-
Sethunathan *et al.*	1983	^{14}C-DDT studies
Nair *et al.*	1992	-do-
Boul	1996	-do-
Guenzi and Beard	1967, 1968	DDT to DDE
Mitra and Raghu	1988	-do-
Xu *et al.*	1994	-do-
Agarwal *et al.*	1994	DDE to DDMU
Nair *et al.* Boul	1992 1996	Flooded soil studies
Scheunert *et al.*	1987	Flooded soil flushed with Nitrogen
Zayed *et al.* Boul.	1994 1996	Non- flooded soil with similar results as flooded soil.

Table 2.5 Soil Studies on DDT Degradation With the External Inoculum Added to the Soil

Organism	Reference	Year, remarks
Enterobacter aerogenes	Kearney *et al.*	1969
Phanerochaete chrysosporium	Barr and Aust	1994, DDT to DDE
Phanerochaete chrysosporium	Fernando *et al*	1989, added to soil with corn cob mixture, degraded ^{14}C- DDT in 60 days by 39 %
Phanerochaete chrysosporium	Katayama and Matsumura	1991, added to the soil with UV exposure
Alcaligenes eutrophus	Nadeau *et al.*	1994
Pseudomonas aeruginosa	Manonmani *et al.*	IP # 226/DEL/2000

Table 2.6 Factors affecting DDT Degradation

Reference	Year	Remarks
Guenzi and Beard, Burge	1968 1971	Readily available carbon sources enhanced DDT conversion to DDD under anaerobic conditions
Mitra and Raghu	1988	Green manuring tends to decrease the persistency of DDT
Guenzi and Beard Castro and Yoshida Farmer *et al.*	1967 1971 1974	Flooding resulted in faster degradation of DDT
Boul Xu *et al.* Nair *et al.*	1996 1994 1992	Flooding showed slower degradation
Boul	1995	Slower degradation in clay surface and organic matter
Keller and Rickabaugh Parfitt *et al.*	1992 1995	Reported treatment of DDT- contaminated soil. Soil with surfactants released DDT from its surface, enhancing aqueous solubility

2.3 Paths Followed by Microorganisms During Degradation

The environment and the microflora in it play an important role in the depletion of many hazardous chemicals. As described above (Table 2.2-Table 2.6), many microorganisms have been studied for their ability to degrade the environmental pollutants. The available literature on microbial degradation of xenobiotics indicates that many studies have mainly considered two aspects: (1) the fundamental basis of biodegradation activities, the evolution and transformation of such activities among microbes. (2) Bioremediation techniques to detoxify severely pesticide-contaminated environments (Tiedje *et al*, 1987; Reineke and Knackmuss, 1988; Marks *et al*, 1989; Cork and Krueger, 1991; Deo *et al*, 1994). For bioremediation technologies to work, all the physiological, microbiological and biochemical aspects involved in the pollutant transformation, have to be considered (Singh *et al,* 1999). There are three major aspects of microbial degradation. Study of the intermediary metabolites involved, hydroxylating enzymes involved and the ring-fission and further breakdown and elucidation of the genetic regulation of catabolic pathways. The pathways followed by few of the strains during the degradation have been studied. Some of the major compounds that stand on the top of the priority list have been reviewed here. Degradation mechanisms are well understood under aerobic conditions (Singh *et al*, 1999). Anaerobiosis also had been paid attention to in the recent past (Commandeur and Parsons, 1990). In anaerobic conditions, nitrate, sulphate or carbonate might function as alternate electron acceptor while in aerobic conditions; oxygen is both the terminal electron acceptor and a reactor in the initial reactions (Singh *et al*, 1999). The primary processes that are involved in the bioconversions of xenobiotics are: (1) Mineralisation, wherein complete degradation results and the compound if finally converted to carbon dioxide or methane. (2) Detoxification, wherein pesticides are converted into non-toxic metabolites. (3) Co-metabolism, where, some microorganisms can bring about degradation of pesticide while growing on other compounds and (4) Activation, wherein, non-toxic molecules might convert to toxic ones by the microorganisms.

Hydrolases and oxygenases are the two most important classes of enzymes involved in the conversion of pesticides (Singh *et al*, 1999). Hydrolases include halidohydrolases (dehalogenate many halogenated aliphatic and aromatic compounds), esterases (Attack phosphodiester bonds of organophosphates) and amidases (degrades propanil). Oxygenases require molecular oxygen as substrates and are less stable than hydrolases (Singh *et al*, 1999). Oxygenases are more complex enzymes and can be classified further into monooxygenases and dioxygenases. Monooxygenases require reduced pyridine nucleotide as cofactors while dioxygenases do not require a reduced compound as cofactor. Table 2.7 shows list of few enzymes responsible for biodegradation of pesticides.

Wedemeyer (1967) studied DDT metabolic pathway by incubation of proposed intermediates with organisms and examining the products formed. The metabolism of DDT in *Aerobacter aerogenes* goes in order

DDT → DDD → DDMU → DDNU → DDOH → DDA → DBP

and direct conversion of DDT to DDE (Wedemeyer, 1967). The drawback was that the products beyond DDNU could not be detected after incubating any of the preceding metabolites with the organism. But later, it was reported that DDA transformed to DBP *via* DBH (Wedemeyer, 1967). *Pseudomonas* has been shown to metabolise DDT in the sequence that involved ring cleavage to PCPA (*p*-chloro phenyl acetic acid) (Focht and Alexander, 1970). Subba Rao and Alexander (1985) reported the formation of DBH, DBP, benzhydrol, benzophenone, *p*-chlorophenyl ethanol and *p*-chlorophenyl glycoaldehyde. DDE, dicofol and DBP were formed during DDT degradation by *Phanerochaete chrysosporium* (Bumpus and Aust, 1987). A review of biodegradation of DDT and its residues has been given by Aislabie *et al* (1997). Few of the proposed pathways for some of the xenobiotics have been depicted in Fig.2.3-Fig.2.6 (source: Singh *et al*, 1999) wherein different chemicals like 2,4-D, 2,4,5-T, lindane, and atrazine have been given. Fig.2.7- Fig.2.9 describe the pathways proposed for DDT by various researchers (source: Aislabie *et al*, 1997).

Table 2.7: Some Enzymes responsible for Biodegradation of Pesticides

Enzyme	Target Pesticide	Reference
Esterases	Organophosphates	Dumans *et al* (1989)
	Phenyl carbamates	Karns and Tomasek (1991)
Lyases	Organophosphates	Munneke (1977)
Phosphatases	Organophosphates	Bourquin (1977)
	Dithioates	Munneke (1977)
Acyl amidases	Phenylamide	Wallnofer and Engelhardt (1989)
Oxygenases	Chlorinated phenols	Bollog *et al* (1968)
	2,4-D	Tiedje and Alexander (1964)
	HCH	Karanth *et al* (1984)
	DDT	Ahmed *et al* (1991)
Hydrolases	Carbamates	Chapalamadugu and Chaudhry (1993)
	2,4-D	Tiedje and Alexander (1964)
	Chlorinated phenols	Bollog *et al* (1968)
	s-Triazines	Cook and Hutter (1981)
Dehydrogenases	HCH	Franken *et al* (1991)
	DDT	Nadeau *et al* (1994)
Cytochrome P-450	DDT	Castro *et al* (1985)
Dehalogenases	DDT	Bourquin (1977)
	HCH	Karanth *et al* (1984)

Source: Singh *et al* (1999)

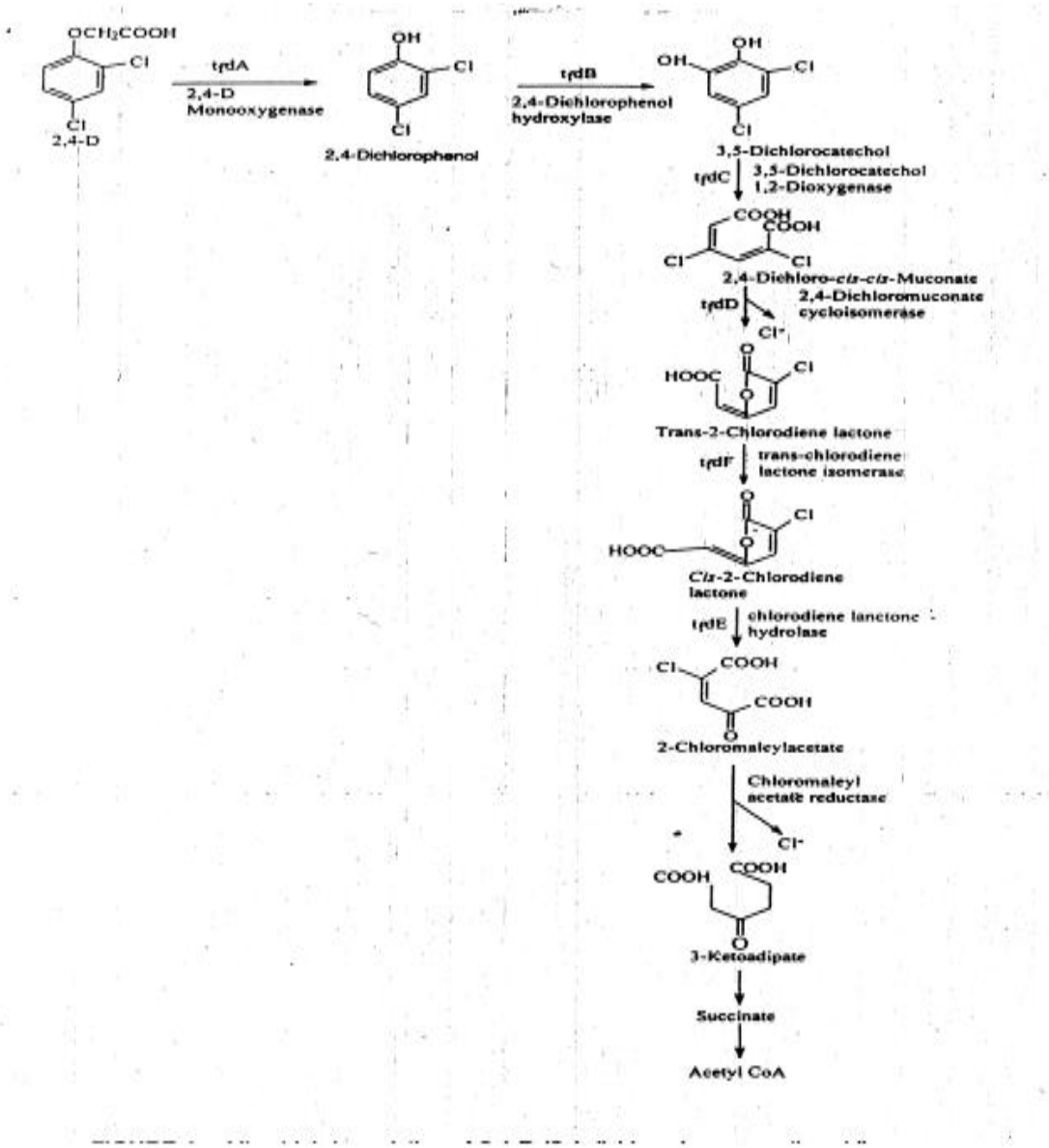


Fig.2.3 Microbial Degradation of 2,4-D

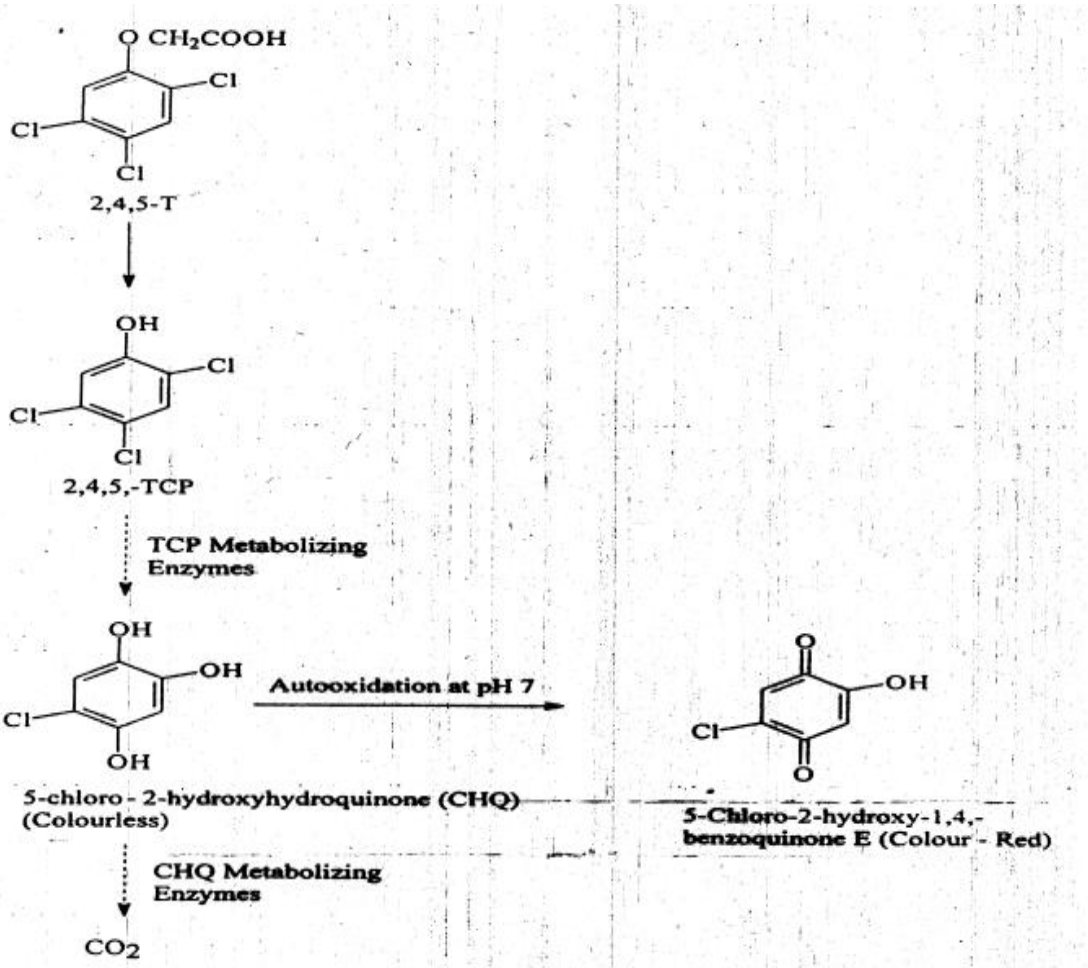


Fig.2.4 Microbial Degradation of 2,4,5-T

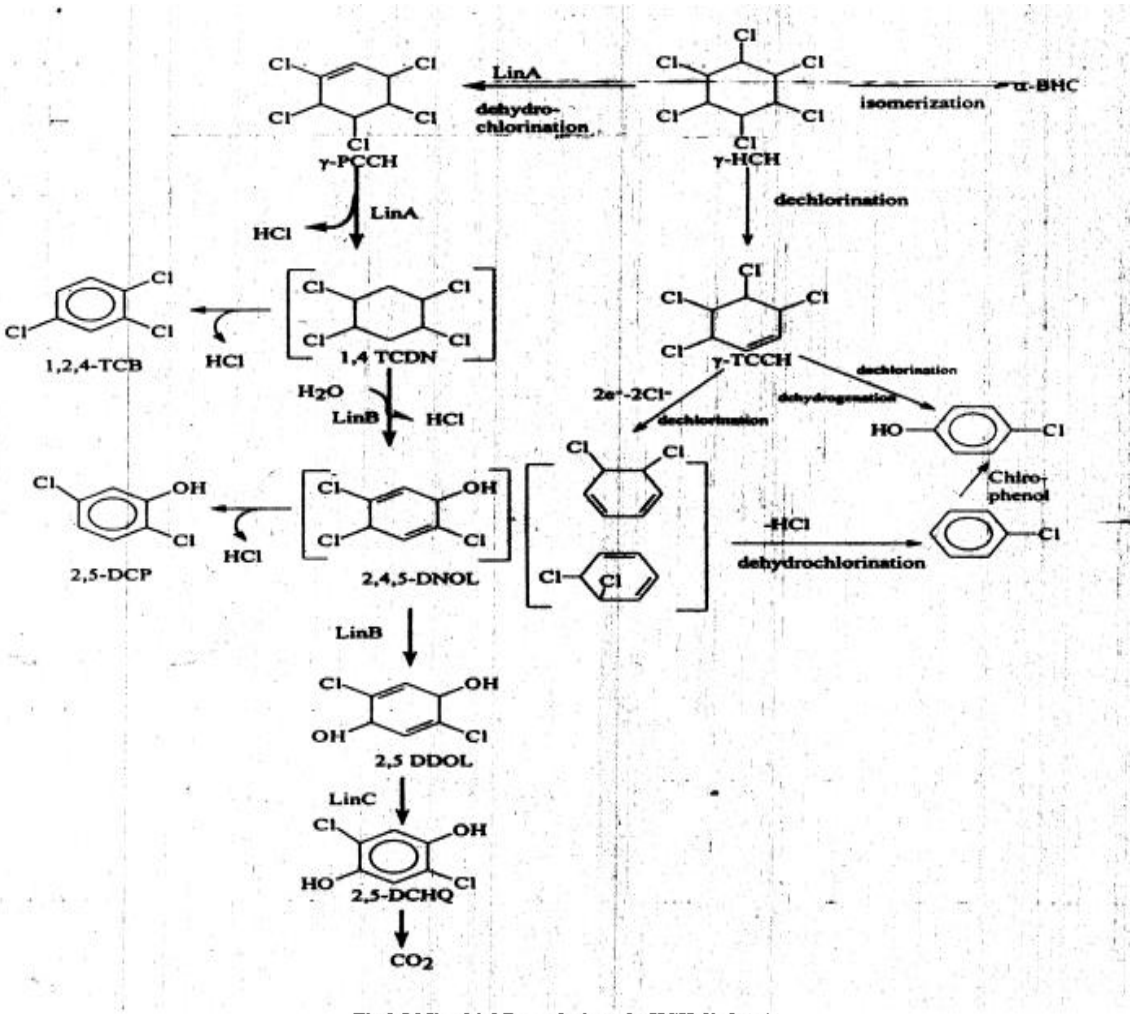


Fig.2.5 Microbial Degradation of γ-HCH (lindane)

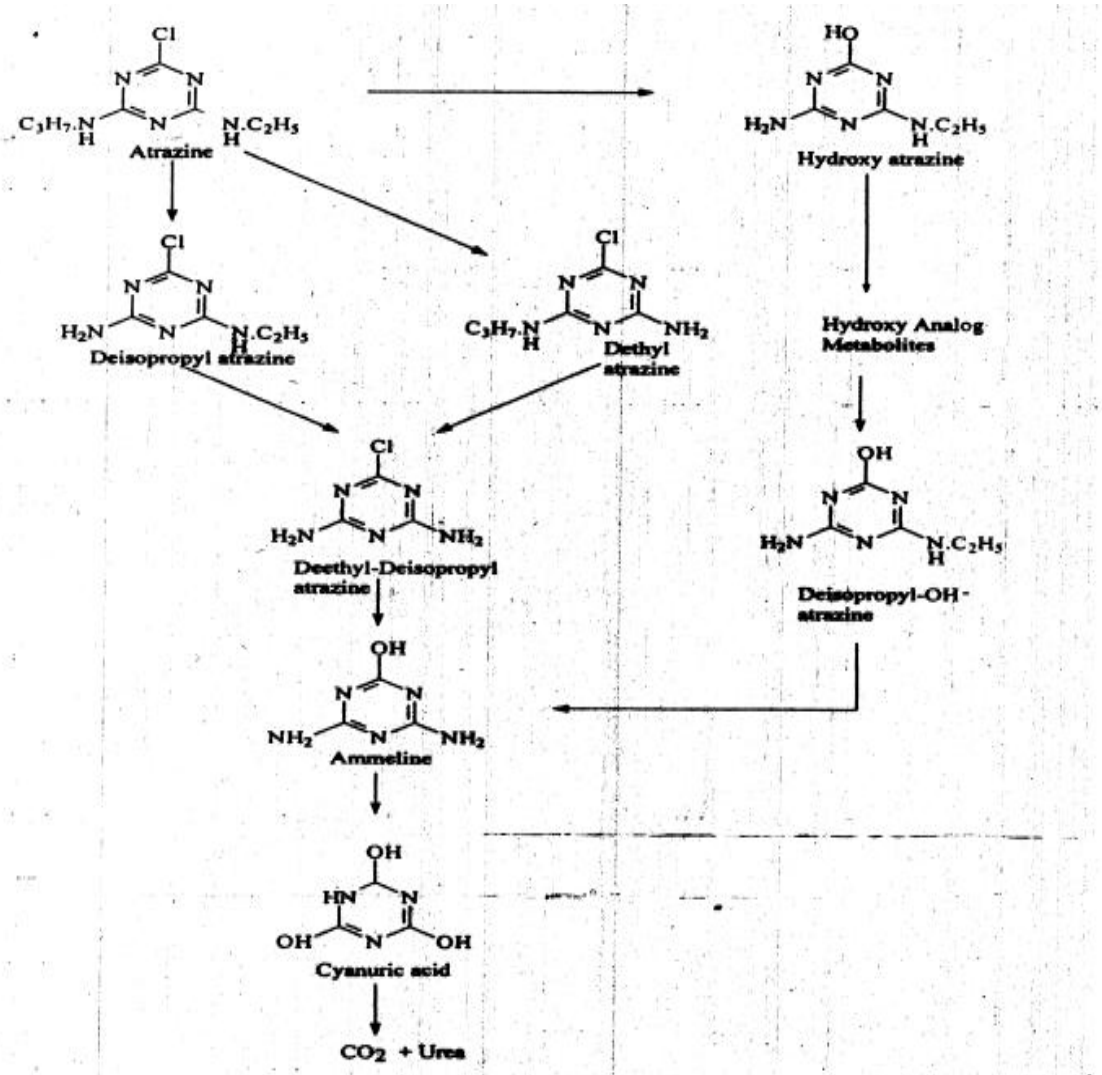


Fig.2.6 Microbial degradation of atrazines

Fig. 2.7 Proposed Pathway for Bacterial Degradation of DDT *via* Reductive Dechlorination.
(Source: Wedemeyer, 1967; Langloise *et al*, 1970; Pfander and Alexander, 1972)

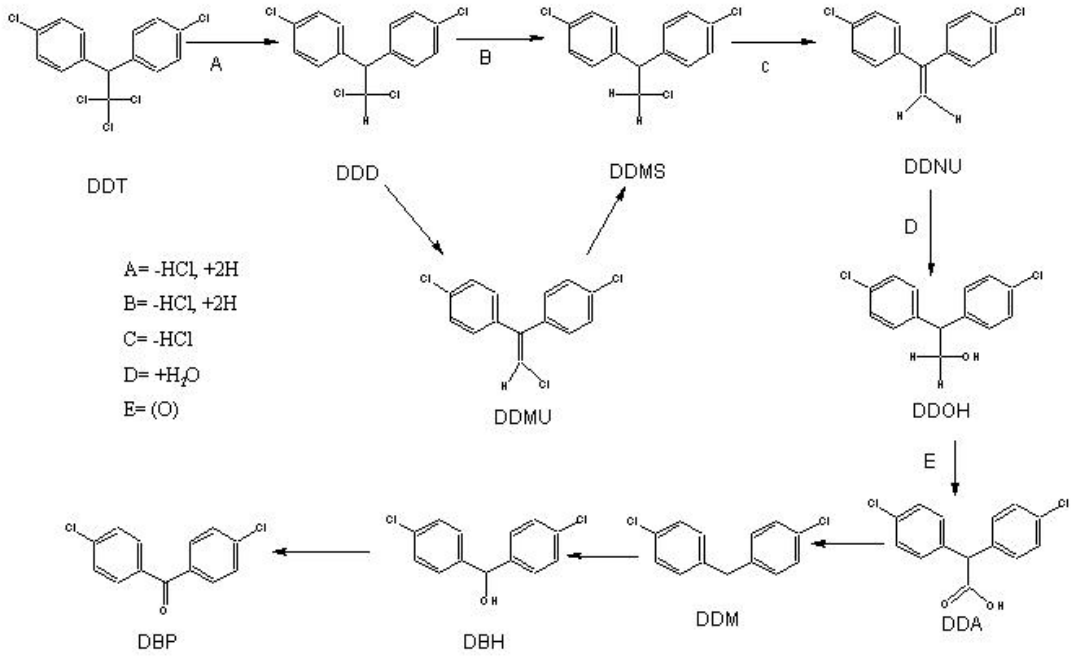


Fig.2.8 Proposed Pathway for DDT Degradation by *Alcaligenes eutrophus* A5

(Source: Nadeau *et al*, 1994, 1998)

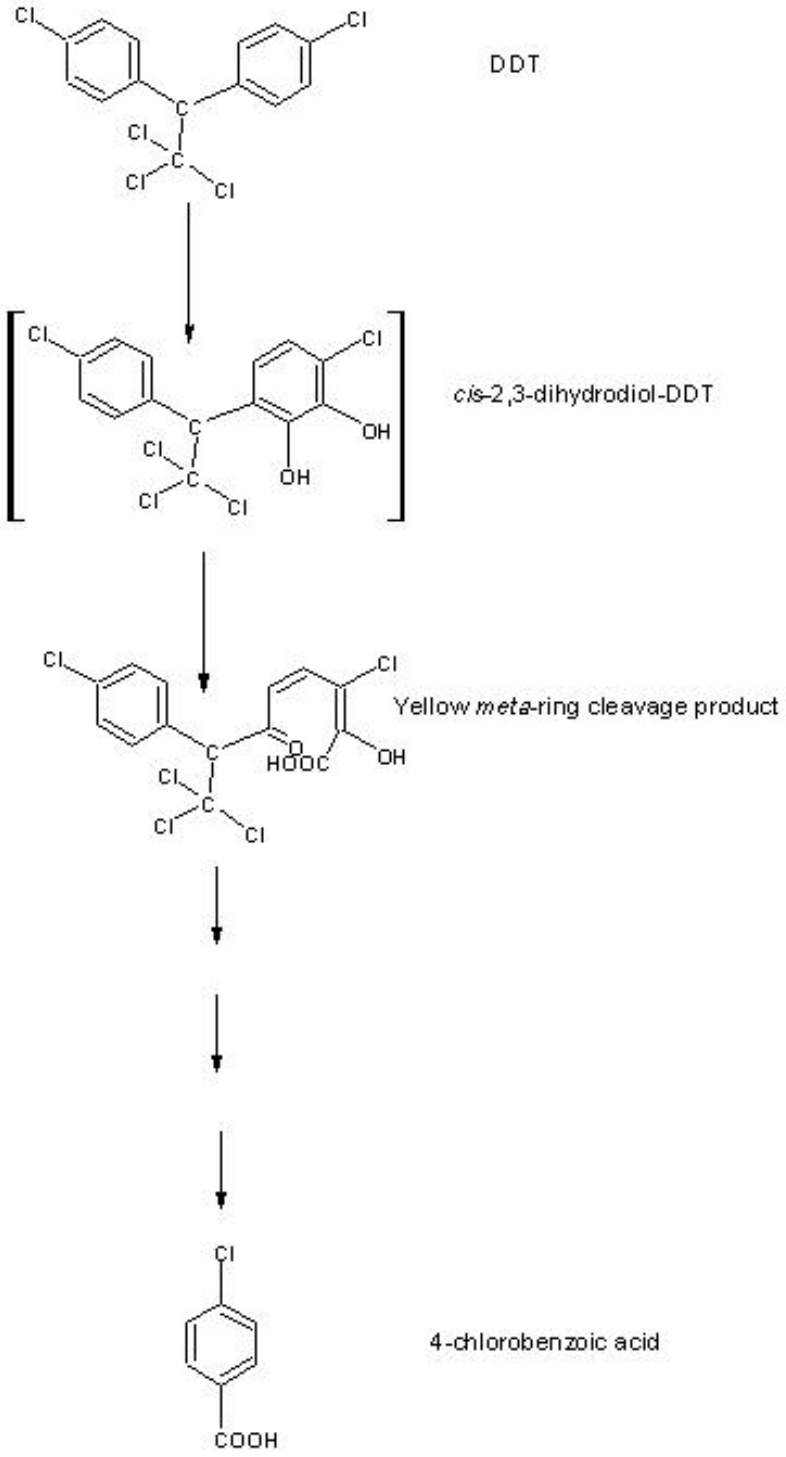
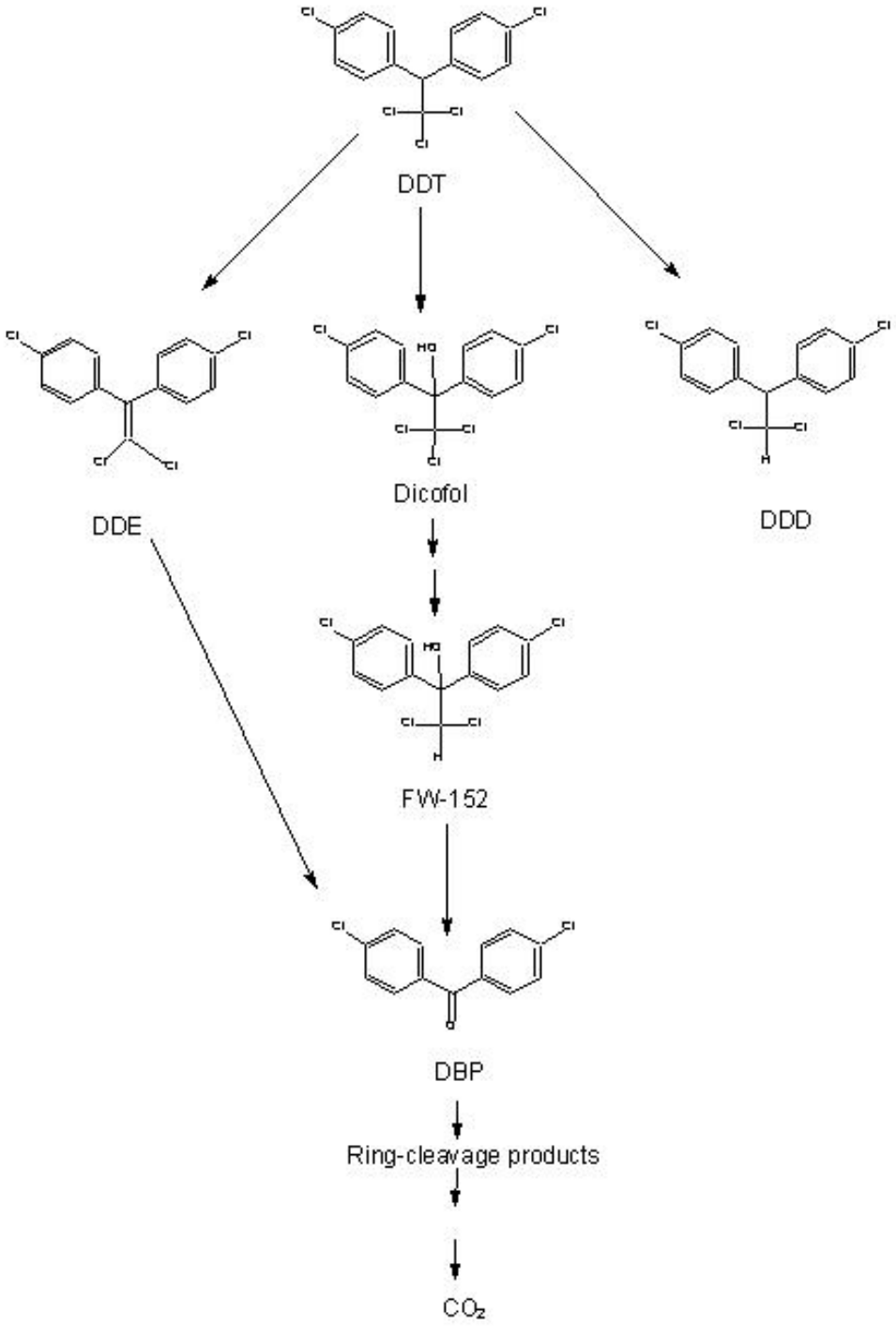


Fig2.9 Proposed Pathway for DDTr Degradation by *Phanerochaete chrysosporium*
(Source: Bumpus and Aust, 1987)



Ring Cleavage

Dihydroxylation of the benzene nucleus with the formation of a dihydrodiol is the primary step before the ring-fission occurs in an aromatic compound, because of the enzymatic mechanisms involved in the microbial degradation. The enzymes catalysing the hydroxylation of the aromatic ring may incorporate one or both the atoms of oxygen depending upon the substrate molecule. In the presence of suitable electron donating compounds the excess atom of oxygen molecules are reduced to water. Catechol and protocatechuic acid have been shown to be the ring fission products in many of the aromatic compounds. Fig.2.10 represents the reaction.

Ring cleavage also depends on the type of functional group attached to the ring, the behaviour of the enzymes, etc. Hayashi and Hashimoto (1950) identified a product *viz.*, *cis, cis*-muconic acid formed from catechol by the action of an enzyme catechol 1,2-dioxygenase. The enzyme catalysed the incorporation of molecular oxygen into the catechol molecule. The reaction is presented in Fig.2.11. Dagley and Stopher (1959) reported an alternative enzymatic cleavage of catechol. The enzyme catechol 2,3-dioxygenase catalyses the reaction resulting in the ring-cleavage of catechol at the *meta*-position (extradiol cleavage) forming 2-hydroxymuconic semialdehyde as the product. Fig.2.12 represents the reaction.

Another *meta*-cleavage enzyme, catechol 1,6-oxygenase that was active on both chloro- and methyl-substituted catechol was reported by Horvath (1970). Co-metabolism of 3-methyl catechol, 4-chlorocatechol and 3,5-dichlorocatechol by *Achromobacter* sp. was shown to result in the accumulation of 2-hydroxy-3-methyl-muconic semialdehyde, 4-chloro-2-hydroxymuconic semialdehyde and 3,5-dichloro-2-hydroxymuconic semialdehyde, respectively. Fig.2.13 represents the reaction. A similar reaction as the formation of *cis-cis*-muconic acid from catechol was observed by Stanier and Ingraham (1954) when protocatechuic acid was used as a substrate. The enzyme protocatechuic acid 3,4-dioxygenase cleaved the acid between the hydroxyl groups to form β-carboxy-*cis,cis*-muconic acid.

Fig.2.14 represents the reaction.

The formation of α-hydroxy-γ-carboxy muconic semialdehyde produced by the oxidation of protocatechuic acid by the action of protocatechuic acid 4,5-dioxygenase has been reported by Dagley *et al* (1960). The enzyme was induced in *Pseudomonas* sp. when it was grown with *p*-cresol. Thus the ring cleavage can occur either through *ortho*- (intradiol cleavage) or *meta*-mode (extradiol cleavage).

In the *ortho*-pathway, β-oxoadipic acid enol lactone was found to be the common intermediate formed after the action of catechol 1,2-dioxygenase and protocatechuic acid 3,4-dioxygenase. Hydrolysis of the lactone ring of this compound gives β-oxoadipic acid that further gives acetyl coenzyme A and succinic acid (Ornston and Stanier, 1966). The reaction is represented in Fig.2.15. Since β-oxoadipic acid is a common intermediate in both *ortho*- and *meta*-pathways, cells metabolising catechol would carry the enzymes responsible for both the pathways *viz.* β-carboxy-*cis,cis*-muconate and γ-carboxy mucono lactone (Fig.2.15). Gibson (1968) found that the cells induced with protocatechuic acid did not possess the enzymes that could catalyse the transformation of *cis,cis*-muconate and muconolactone. It was found that these enzymes were induced by *cis,cis*-muconic acid.

In *meta*-pathway, 2-oxo-4-hydroxy valeric acid was produced from α-hydroxy muconic semialdehyde (Dagley and Gibson, 1965). Pyruvic acid and acetaldehyde were the next following products (Fig.2.16).

Since microorganisms do not follow a rigid set of rules, there would be few exceptions in these pathways. From the established mechanisms of the ring cleavage, a model (Fig.2.17) can be generated for the degradation of phenol by both the pathways.

Fig.2.10 Formation of Catechol and Protocatechuic Acid

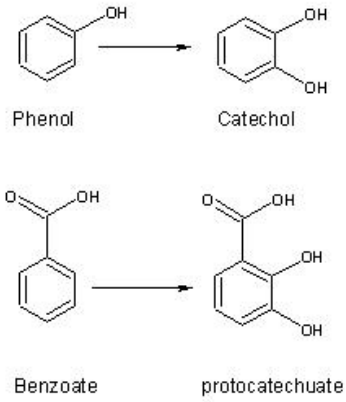


Fig.2.11 Action of Catechol 1,2-Dioxygenase

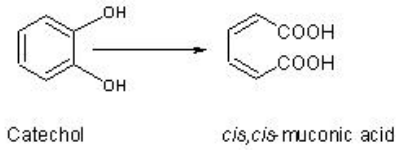


Fig.2.12 Action of CAtechol 2,3-Dioxygenase

Catechol 2-Hydroxymuconic semialdehyde

Fig.2.13 Action of 1,6-Oxygenase on Different Substituted Catechols

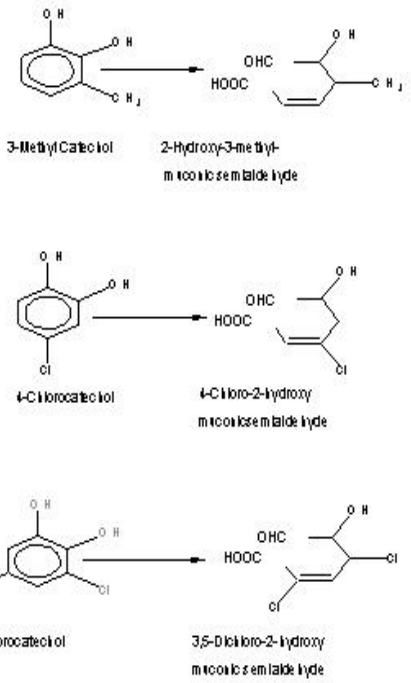
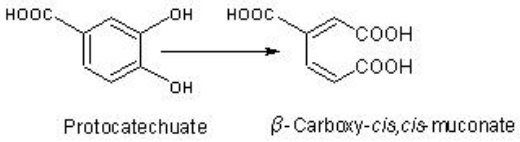


Fig.2.14 β-Carboxy-*cis,cis*-muconate Production from Procatechuic acid



Protocatechuate β-Carboxy-*cis,cis*-muconate

Fig.2.15 *ortho*-cleavage Pathway of Catechol and Protocatechuic acid

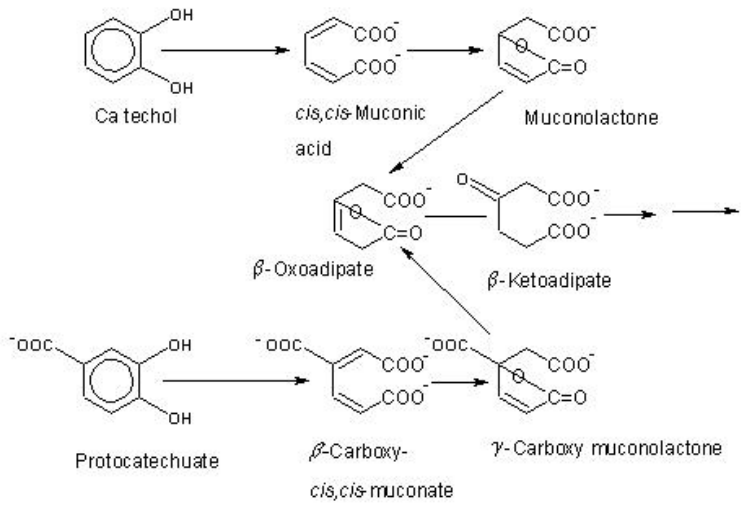


Fig.2.16 *meta*-cleavage Pathway of Catechol

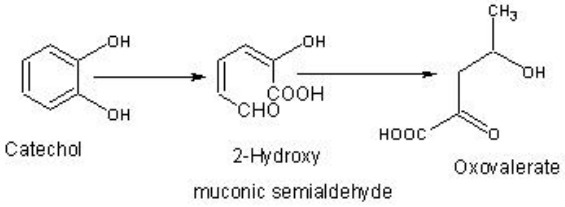
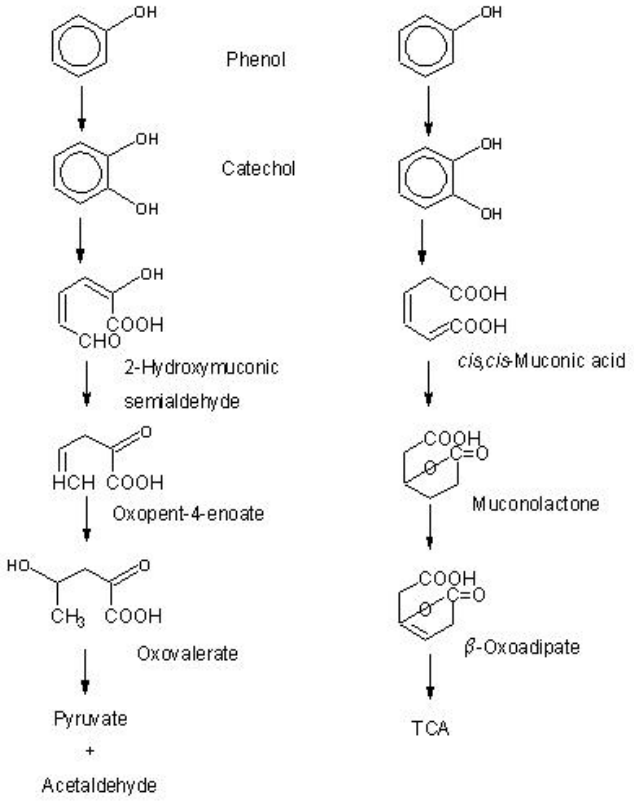


Fig.2.17 Phenol Catabolism Through *ortho*- and *meta*- Pathway



2.4 Treatment of DDT-contaminated sites and water resources

DDT being present in almost every place now because of its extensive use in the past supported by its persistence nature with comparatively longer half-life, it becomes imperative to look into its removal from the environment. There are different ways of treating the contaminated sites and water. Major classification can be given broadly as Chemical treatment, Photochemical Treatment and Biological treatment. DDT may undergo breakdown by photochemical reactions in the environment. This process transforms a major part of the surface DDT. DDT being labile to alkaline pH, it is the major strategy that usually DDT undergoes during the Chemical treatments. Other physicochemical strategies involve the osmosis, adsorption on activated charcoal, etc. (India Today, Aug. 2003). But neither the transformed products nor the separated or adsorbed DDT is completely detoxified. Here they are accumulated and need further treatments. It is apparent to imagine the kind of hazard it poses to the environment, with the chemistry of DDT and its breakdown products known. Moreover, it would be very expensive to carry out the chemical and physico-chemical treatment strategy under controlled conditions with the added disadvantage of not mineralising DDT. Not all the DDT is present on the surface of the contaminated sites or water leading to a very low photochemical degradation of DDT. Moreover, the greater proportion of the breakdown products here again is DDD and DDE. DDD was earlier used as pesticide to a limited extent. DDE is only found in the environment as a result of contamination or breakdown of DDT (http://www.eco-usa.net/toxics/ddt.shtml)

2.5 Managing DDT Biodegradation With Microbes

A process for the microbial Degradation of DDT which comprised of treating DDT with certain non-pathogenic, hydrocarbon utilising strain of *Nocardia rubra* (B1002)ATCC 21508, *Nocardia globerula* (B1 1039) ATCC 21505, *Candida lipolytica* (B1 2002) ATCC 20255 and *Penicillium* sp. (B1 3005) ATCC 20369, until

the DDT had been substantially degraded was proposed by Bioteknika International, Inc., Springfield, in 1974 and the US patent issued was in 1976 Sept. The mixed population of these organisms could degrade 16-67% by weight of the originally present in the dirty effluent. The process was effective in degrading DDT as it might have been present as a pollutant or contaminant in water, in industrial effluents, in various land areas such as industrial sites and the like or in varied laboratory or commercial installations (US patent No. US3979283).

Zeneca Corp., Stoney Creek, Canada, had a patent in 1997, on compost decontamination of DDT-contaminated soil (US patent No. US5660612) and another patent on anaerobic/aerobic decontamination of DDT-contaminated soil by repeated anaerobic/aerobic treatments (US patent No. US5660613).

Microbial degradation technologies aim to render harmless those substances which, generally mixes together in large numbers as a waste, cannot be utilised profitably. The concentrations of the single solubilised substances are usually very low. Sterilisation followed by degradation with pure cultures is generally impeded by the quantity and quality of the delivered wastes. Therefore a mixed population already present or enriched by chance accomplishes the decomposition (Schonborn, 1987). A technology for using soil bacteria to convert chlorinated pesticides into less toxic by-products has been developed by Canadian scientists at AstraZeneca; Xenorem technology uses enzymes called dehalogenases (New Scientist, May, 1999). In a yearlong test at a former pesticide factory in Tampa, FL, the process reduced DDT levels in soil by at least 95%, as well as DDD, DDE and other chlorinated pesticides to below the Environmental Protection Agency's safety limits (This Week, 9 May 1998, p 16).

Stauffer Management Company, a subsidiary of AstraZeneca, reportedly is altering the process so that it can break down other persistent organic pollutants such as PCBs and TNT. Frank Peter, AstraZeneca's director of environmental services, says the new on-site bioremediation technology removes contaminants at half or two thirds of the cost the incineration (New Scientist, May 1999; PTCN).

2.6 Conclusion

Bacterial bioremediation is picking up in the present world as it is safer, easy, faster and requires less expenditure. The cells can be grown in less time and both *ex-situ* and *in-situ* remediation techniques can be applied with ease. The field augmentation with the required microbe is one of the hottest areas of biotechnology with respect to bioremediation of the environment. The major area of concern here is the bacterial interactions with the native microflora and the environmental factors. This can also be taken care by selecting the microbe from the same contaminated site that has to be remediated. Hopefully the future is waiting for the bioremediation technologies to give the biota a safe, cleaner and healthier environment for the coming generations.

Chapter 3


Isolation, Purification and Identification of Microbes capable of DDT- Degradation

Even though the pattern of our relationship to the environment has undergone a profound transformation, most people still do not see the new pattern.... The sights and sounds of this change are spread over an area too large for us to hold in our field of awareness.

Gore (1992)

3.1 Introduction

Xenobiotics are man- made compounds with chemical structures to which microorganisms have not been exposed in the course of evolution (Hutzinger and Veerkamp, 1981). The concept of microbial degradation of xenobiotics is of interest to know how microorganisms develop new metabolic sequences enabling them to degrade chemicals that were not present in the biosphere before the advent of industrial chemistry. Many workers have tried degradation of xenobiotics in the past using microorganisms. These workers were able to isolate organisms that could degrade one such xenobiotic, DDT. There are reports showing the presence of DDT residues (DDTr) in soil several years of its use was ceased (Aislabie *et al*, 1997). DDT- metabolising microorganisms have been isolated from a range of habitats including animal faeces, sewage, marine and fresh water sediments, activated sludge and soil (Johnsen, 1976; Lal and Saxena, 1982; Rochkind- Dubinsky *et al*, 1987). Different bacteria, fungi and yeasts have been isolated and shown to harbour the ability to degrade many of the xenobiotics. Wedemeyer (1967) showed that DDT could be degraded by *Enterobacter aerogenes* under anaerobic conditions. Bollog and Liu (1990) showed the co-metabolic degradation of DDT by microorganisms. Many microbes have been shown to act on DDT under various conditions. Indigenous micro flora in rodents, *Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas putida, Bacillus* sp., *Hydrgenomonas sp., Alcaligenes eutrophus, Arthrobacter, Sacchromyces cervisiae, Phanerochaete chrysosporium, Trichoderma viridae, Gleophyllum traveum,* and *Phanerochaete cordylines* have been shown to degrade DDT residues by different workers (Fries *et al,* 1969; Barker *et al* 1965; Mendel and Walton, 1966; Subba Rao and Alexander, 1985; Sharma *et al,* 1987;, Beunink and Rehm, 1988; Langlois *et al,* 1970; Pfaender and Alexander, 1972; Masse *et al,* 1989; Golovleva and Skryabin, 1981; Golovleva *et al,* 1982; Bumpus and Aust, 1987; Fernando *et al,* 1989; Aust, 1990; Shah *et al,* 1992; Walter, 1992; Bumpus

et al, 1993; Kohler *et al,* 1988; Bartha (1990), Focht and Alexander, 1970; Nadeau *et al,* 1994 and Parsons *et al,* 1995). The degradation concentration used by these workers was very low and time required for degradation was long. Most of the times it will be more appropriate to use the microbes isolated from the same site as of application. In our laboratory we have developed a consortium capable of degrading DDT and its metabolites in a short time and more efficiently.

3.2 Materials

***3.2.1 Solvents**:* Acetone, dichloromethane and cyclohexane were of analytical and HPLC grade and were procured from E-Merck (India) Ltd.

***3.2.2 Chemicals**:* DDT, 98% pure, was purchased from Sigma- Aldrich Chemical Company, Mo, USA. *o*- tolidine, glucose, sucrose, acetate (sodium salt), succinate (sodium salt), glycerol, peptone, yeast extract, Tryptone soya broth (TSB), *Pseudomonas* agar, *Serratia* differential agar and N, N- dimethyl-*p*- phenylene diamine were purchased from HiMedia, Mumbai, India. All other chemicals used were of analytical grade and were purchased from standard manufacturers.

3.3 Media

3.3.1a. Minimal Medium (MM)

Ingredients	**Grams per litre distilled water**
KH_2PO_4	0.675
Na_2HPO_4	5.455
NH_4NO_3	0.25
$Ca(NO_3)_2$	0.1
$MgSO_4.\ 7H_2O$	0.2
Trace Mineral Solution (3.3.1b)	1 mL

3.3.1b Trace Mineral solution contained

Ingredients	Grams per litre distilled water
$FeSO_4$. $7H_2O$	1.0
$MnSO_4$. H_2O	1.0
$CuCl_2$. $2H_2O$	0.25
Na_2MoO_4. $2H_2O$	0.25
H_3BO_4	0.1
Conc. H_2SO_4	5.0 mL

Minimal medium was prepared in double distilled water and pH of the medium was 7.5. Stocks of $Ca(NO_3)_2$ and $MgSO_4$. $7H_2O$ were autoclaved separately and added after cooling to the sterile MM maintained at room temperature. Medium was autoclaved at 121^0 C, 15 lbs for 20 minutes.

3.3.1c Minimal agar

Minimal agar was prepared by adding 2% agar to MM (3.3.1a).

3.3.2a Nutrient Broth (NB)

Ingredients	Grams per Litre of distilled water
Peptone	5.0
Beef extract	3.0
NaCl	5.0

pH was adjusted to 7.0 with 1.0 N NaOH. Medium was autoclaved at 15 lbs, 121^0 C for 20 minutes.

3.3.2b Nutrient agar

Nutrient agar was prepared by adding 20 g of agar- agar to 1L of NB (3.3.2a).

3.3.3 Tryptone Soya Broth (TSB)

Required amount of dehydrated TSB was added to required quantity of distilled water. The medium was then autoclaved at 121^{0}C, 15 lbs for 20 minutes.

3.3.4 Peptone- glycerol medium

Ingredients	**Grams per Litre of distilled water**
Peptone	5.0
Glycerol	5.0
KH_2PO_4	3.0
K_2HPO_4	7.0

pH of the medium was 7.5. Medium was autoclaved at 121 ^{o}C, 15 lbs for 20 minutes.

3.3.5 Pseudomonas agar

a: Pyocyanin agar

Pyocyanin agar powder was procured from HiMedia Laboratories, Mumbai, India that contained the following required concentrations of ingredients. Marked quantity of this powder was dissolved in 1L distilled water.

Ingredients	**Grams per Litre of distilled water**
Peptone	20.0
$MgCl_2$	1.4
K_2SO_4	10.0
Agar- agar	15.0

Final pH was 7.0. Medium was autoclaved at 121 ^{o}C, 15 lbs for 20 minutes.

b*: Fluorescein agar*

Fluorescein agar powder was procured from HiMedia, Mumbai, India that contained the following required concentrations of ingredients. Marked quantity of this powder was dissolved in 1L distilled water.

Ingredients	**Grams per Litre of distilled water**
Tryptone	10.0
Proteose peptone	10.0
K_2SO_4	1.5
$MgSO_4$	1.5
Agar	15.0

Final pH of the medium was 7.0. Medium was autoclaved at 121 ^{o}C, 15 lbs for 20 minutes.

3.3.6 Serratia differential agar

Serratia differential agar powder was procured from HiMedia, Mumbai, India that contained the following required concentrations of ingredients. Marked quantity of this powder was dissolved in 1L distilled water.

Ingredients	**Grams per Litre of distilled water**
Yeast extract	10.00
L- ornithine	10.00
L- arabinose	10.00
Sodium chloride	5.00
Triclosan (Irgasan)	0.01
Bromothymol blue	0.02
Phenol red	0.01
Agar	4.0

Final pH of the medium was 7.0. Medium was autoclaved at 121 ^{o}C, 15 lbs for 15 minutes.

3.3.7 Gram Stain

Ingredients	Quantity
Crystal Violet	2.0 g
Ethyl alcohol (95%)	20.0 mL
Ammonium oxalate	0.8 g
Distilled water	80.0 mL

Crystal violet and ammonium oxalate were dissolved respectively in ethyl alcohol and distilled water and the two solutions were then mixed. The prepared stain was filtered and stored in a clean and dry glass stoppered bottle.

3.3.8 Lugol's iodine (Mordant)

Ingredients	Quantity
Iodine	1.0 g
KI	2.0 g
Distilled water	300.0 mL

3.3.9 Safranin (Counter stain)

Ingredients	Quantity
Safranin	2.5 g
Ethyl alcohol (95%)	100.0 mL

10 mL of the above stock solution was mixed with 90 mL of distilled water for use as counter stain.

3.3.10 Hugh Leifson medium

Ingredients	Grams per Litre of distilled water
Peptone	2.0
NaCl	5.0
Glucose	10.0
K_2HPO4	0.3
Bromothymol blue	0.05
Agar	2.0

Final pH of the medium: 7.2 ± 0.2. The medium was autoclaved at 121 ^{o}C and 15 lbs for 20 minutes.

3.3.11 Kohn two tube medium No.1

Ingredients	Grams per Litre of distilled water
Beef extract	2.0
Peptone	15.0
Yeast extract	2.0
Dextrose	1.0
Mannitol	10.0
Phenol red	0.05
Agar	15.0

Final pH of the medium: 7.2 ± 0.2. Prior to use, 25 mL of 40% membrane filtered urea solution was added to the molten and tempered sterile medium, mixed well and 1 inch butt and slants were prepared using sterile glass tubes.

3.3.12 MR- VP medium- buffered glucose broth

Ingredients	Grams per Litre of distilled water
Buffered Peptone	7.0
Dextrose	5.0
K_2HPO_4	5.0

Final pH of the medium: 7.2 ± 0.2. The medium was autoclaved at 121 ^oC and 15 lbs for 20 minutes.

3.3.13 Nitrate broth

Ingredients	Grams per Litre of distilled water
Beef extract	3.0
Peptone	5.0
KNO_3	1.0

Final pH of the medium: 7.2 ± 0.2. The medium was autoclaved at 121 ^oC and 15 lbs for 20 minutes.

3.3.14 Simmons citrate medium

Ingredients	Grams per Litre of distilled water
$MgSO_4.7H_2O$	0.2
Ammonium dihydrogen phosphate	1.0
K_2HPO_4	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

Final pH of the medium: 6.8 ± 0.2. The medium was autoclaved at 121 ^{o}C and 15 lbs for 20 minutes.

3.3.15 Tryptone broth and medium

Ingredients	Grams per Litre of distilled water
Tryptone	10.0
NaCl	5.0

Final pH of the medium: 7.2 ± 0.2. The medium was autoclaved at 121 ^{o}C and 15 lbs for 20 minutes.

3.3.16 Kovac's reagent

Ingredients	Grams per Litre of distilled water
p-Dimethyl amino benzaldehyde	5.0 g
Amyl alcohol	75.0 mL
Conc. HCl	25.0 mL

Dissolved *p*-Dimethyl amino benzaldehyde in the amyl alcohol and then slowly added HCl.

3.3.17 **S*ugar solutions***

Requisite quantities of the stock solutions (10%) each of the following sugars were individually prepared in distilled water, membrane filtered (0.22 mμ filters) and stored in sterile screw- capped tubes at –20^{0} C. The sugars used were glucose, lactose, arabinose, raffinose. Rhamnose, sucrose, mannitol, inositol, sorbitol, adonitol, salicin and xylose.

3.3.18 Sugar fermentation basal medium

Ingredients	Grams per Litre of distilled water
Peptone	10.0
NaCl	5.0
Beef extract	3.0
Bromocresol blue	0.04

Final pH: 7.2 $\pm$ 0.2. The medium was autoclaved at 121^0 C, 15 lbs for 20 min.

The basal medium was substituted with required quantity of sugar solutions (3.3.16) separately.

3.3.19 Lowry's A

2 % Na_2CO_3 in 0.1 N NaOH.

3.3.20 Lowry's B

0.5 g% $CuSO_4$ in 1 g% sodium- potassium tartarate.

3.3.21 Lowry's C

50 mL Lowry's A + 1 mL Lowry's B

3.3.22 Lowry's D

1: 2 of Folin- Ciocalteau reagent (diluted with distilled water)

3.3.23 Triple sugar iron agar

Ingredients	Grams per Litre of distilled water
Peptic digest of animal tissue	10.0
Casein enzymatic	10.0

hydrolysate	
Beef extract	3.0
Yeast extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	10.0
NaCl	5.0
$FeSO_4$	0.2
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

The final pH of the medium was 7.4±0.2. The medium was autoclaved for 15 minutes at 121^0C.

3.3.24 Lysine decarboxylase broth

Ingredients	**Grams per Litre of distilled water**
Pancreatic digest of animal tissue	5.0
Yeast extract	3.0
Dextrose	1.0
L-Lysine hydrochloride	5.0
Bromocresol purple	0.02

The final pH of the medium was 6.8±0.2. The medium was autoclaved at 15 lbs for 15 minutes.

3.3.25Ornithine decarboxylase broth

L-Ornithine monohydrochloride	5.0 g/L
Yeast extract	3.0 g/L

Glucose	1.0 g/L
Bromocresol purple	15 mg/L

The pH of the broth was 6.8±0.2. 5mL of the medium was dispensed in to tubes and autoclaved at 121^0 C, 15 lbs for 15 min.

3.4 Methods

3.4.1 Microbial Consortium

The microbial consortium capable of degrading DDT was developed by shake flask enrichment of DDT- contaminated soil for nearly 2 years. An aqueous suspension of the soil sample was inoculated to minimal medium containing phenol (5 ppm) and DDT (2 ppm). The culture that was getting established was regularly transferred, at monthly intervals, to fresh medium. During each transfer, phenol concentration was maintained constant. Later (after 10- 15 transfers) phenol addition was reduced gradually and only DDT at 2 μg/ mL level was maintained for nearly 6 months. Finally a mixed culture, capable of degrading 2μg DDT/mL, was established. DDT concentration was gradually increased to 5 μg/ mL level. This acclimated microbial consortium was maintained on minimal agar (3.3.1c) containing 5 μg DDT/ mL and 1/ 50 nutrient broth (3.3.2a) and as liquid culture in minimal medium (3.3.1a) containing 5 μg DDT/ mL at 4^0 C. This consortium was gradually acclimated to increasing concentrations of DDT, 5 μg/ mL through 25 μg/ mL. The cells harvested from the previous batch were used as inoculum to start a fresh batch with or without increasing the substrate concentration. At each step, complete degradation of DDT in terms of 100% chloride release and absence of residual substrate, was assured before going to the next higher concentration. The consortium thus developed was used for the degradation studies.

3.4.2 Resolution of the consortium into individual strains

The individual microbial strains of the acclimated consortium were resolved on nutrient agar (3.3.2b) plates by plating appropriately diluted samples of 48 h old cultures, grown with 25 μg DDT/ mL. Morphologically distinct bacterial strains were isolated, purified by repeated plating on nutrient agar (3.3.2b).

3.4.3 Tests for bacterial identification

Cultural characters

3.4.3a Morphological and colony Characters

Morphological and colony characters such as colony colour, shape, size, and margin type were studied in the nutrient agar (3.3.2b) grown colonies.

3.4.3b Microscopic characters

a. Light Microscopy

Pure cultures were inoculated to NB (3.3.2a) and incubated for 12-18 h at room temperature under shaking conditions (180 rpm). A loop full of each of these cultures was placed on to a clean glass slide and a smear was made. These cells were heat fixed by passing over a gentle flame. A drop of safranin was added to it and left for 1 min. Extra stain was drained after placing the cover slip. These slides were then observed under the light microscope for the cell type, size, shape, etc.

b. Electron Microscopy

Pure cultures were inoculated to nutrient broth (3.3.2a) and incubated for 12- 18 h at room temperature under shaking conditions (180 rpm). These cultures were then taken for the preparation of samples (specimen) for electron microscopy. 1.5 mL of the culture broth was taken in a sterile micro centrifuge tube and centrifuged at 8000 rpm for 10 min at 4 ^{o}C. The cell pellet was resuspended in phosphate buffer (0.1 M, pH 7.0). Cells were then fixed with 2.5 % gluteraldehyde solution for 2-4 h (kept

undisturbed at room temperature). Cell pellet was obtained by centrifugation and the gluteraldehyde was discarded. Fixed cells were then dehydrated by stepwise treatment to increasing concentrations of ethanol. The final treatment was with absolute alcohol. These cells were then lyophilised and taken for electron microscopy. Scanning Electron Microscopy (SEM) was done using Leo Electronic Microscopic Ltd. (Cambridge, UK).

3.4.4 Biochemical Characters

3.4.4.1 Gram's staining

The axenic cultures were grown in nutrient broth (3.3.2a) for 12- 18 h. The smear was prepared on a clean glass slide, air-dried and fixed by gentle heat. This was stained with crystal violet (3.3.7) for 1 min followed by washing off excess stain with water. Then Lugol's iodine solution (3.3.8) was added and allowed to react for 1 min. After washing off excess iodine with water, the smear was treated with 95% ethanol for 30 sec. So as to remove excess of crystal violet. Finally, the smear was counter stained with safranin (3.3.9) allowing to react for 30 sec., washed with water, dried and examined under a compound microscope. Gram-positive cells appeared violet while Gram-negative cells appeared pink.

3.4.4.2 Motility

Hanging drop method

18 h old culture, grown in nutrient broth (3.3.2a), was used for this test. A drop of the culture broth was placed on a clean cover slip and then inverted a cavity slide on to it taking care of the drop not getting ruptured/ spread between the slide and the cover slip. The slide was then carefully inverted and was then observed under the microscope. The movements of the cells in each culture were recorded as positive or negative. The types of movements were also recorded in case of positive observations.

3.4.4.3 Catalase Production

Test cultures were grown freshly on nutrient agar (3.3.2b), onto which were added few drops of 3% (v/v) hydrogen peroxide. Culture tubes were observed for the liberation of nascent oxygen in the form of bubbles. This indicated the production of catalase.

3.4.4.4 Oxidase Production

A speck of freshly grown culture (12-15 h old) was smeared on the edge of a piece of filter paper, onto which was added a drop of the reagent - 1% aqueous solution of N,N- dimethyl-p- phenylene diamine. The edge of the culture smear was observed for colour change in 30 sec. Violet colouration indicated positive reaction for oxidase. Otherwise it was recorded as negative.

3.4.4.5 Test for motility and oxidative/ fermentative reactions

Stab inoculation of the test cultures individually were performed in the sterile tubes of Hugh Leifson medium (3.3.10). Each culture was inoculated in duplicate. To one set of the inoculated tubes, few drops of sterile liquid paraffin were added to overlay the agar medium (anaerobic condition). The other set of tubes was kept without any overlay. Both the sets of tubes were incubated at 37°C for 24-48 h. Incubated tubes were observed for the spreading of the culture from the line of inoculation, which indicated the motile nature of the culture. Acid production from glucose was indicated by colour change of the medium from light green to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicated fermentative nature.

3.4.4.6 Methyl Red and Voges-Proskauer (MR-VP) reaction

These two tests were performed using MR-VP broth (3.3.12). Test cultures individually were inoculated into the broth medium and incubated for 24 h at 37°C. The culture broth was divided into two parts; one part was used for MR reaction and the other for VP reaction.

3.4.4.6.1 Methyl red test

Methyl red indicator was prepared by dissolving 0.1 g methyl red in 300 mL of 95% ethanol and later made upto 500 mL with distilled water. The MR reagent in 5-6 drops was added to one part of the culture broth. Development of pink colour indicated positive reaction.

3.4.4.6.2 Voges-Proskauer test

VP test reagent consists of 2 solutions

Solution A		**Solution B**	
α-napthol	5.0 g	Potassium hydroxide	40.0 g
Absolute alcohol	100.0 mL	Creatine	0.5 g
		Distilled water	100.0 mL

To the second part of culture broth, 0.6 mL of solution A and 0.2 mL of solution B were added, mixed well and tubes kept unplugged so as to facilitate aerobic environment. Formation of eosin pink colour indicated positive reaction.

3.4.4.7 Urease production

This test was carried out using Kohn two-tube medium No.1 (3.3.11). Individual test cultures were inoculated into the prepared slants by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated

for 24-48 h at 37°C. Positive urease reaction (i.e. alkaline) was indicated by a deep cerise (cherry red/ bright red) colour of the whole medium.

3.4.4.8 Indole production

Individual test cultures were inoculated into prepared tryptone broth (3.3.15) tubes and incubated for 24 h at 37°C. To each of the incubated tubes was added 0.2-0.3 mL of Kovac's reagent (3.3.16). The formation of a dark red colour in the surface layer of the culture broth indicated a positive test for indole.

3.4.4.9 Nitrate reduction

Individual test cultures were inoculated into the prepared nitrate broth (3.3.13) and incubated for 24 h at 37°C. The culture broth was tested for nitrate reduction using the following reagent, which consisted of two solutions:

Solution 1		**Solution 2**	
Sulphanilic acid	8. 0 g	α-napthol	5. 0 g
5N Acetic acid	1000. 0 mL	5N Acetic acid	1000. 0 mL

To 5 mL of the 24 h-old culture broth, was added 2 drops each of solution (1) and (2). Development of orange/brick red colour was indicative of nitrate reduction to nitrite.

3.4.4.10 Acid production from sugars

To the individual tubes of 3 mL each of sugar fermentation basal medium (3.3.18) was added 0.3 mL each of the individual 10% membrane filtered sugar solutions namely glucose, lactose, arabinose, raffinose. Rhamnose, sucrose, mannitol, inositol, sorbitol, adonitol, salicin and xylose (in separate tubes). These tubes were then inoculated with the individual test cultures, mixed well and incubated at 37°C for a period of 5 d.

Incubated tubes were observed at 24 h intervals for acid production by the colour change in the medium i.e. from purple to yellow.

3.4.4.11 Starch hydrolysis

A loop full of 20 h-old broth culture of individual organisms was spotted onto portions of pre-poured plates of plate count agar (HiMedia Laboratories, Mumbai, India) containing 1% soluble starch and incubated at 37°C for 24-48 h. Incubated plates were exposed in a glass chamber saturated with iodine vapours, to read for the positive or negative action on starch.

3.4.4.12 Citrate utilization test

Freshly prepared slants of Simmon's citrate agar (3.3.14) were inoculated with individual test organisms and incubated for 24-48h at 37°C. The formation of deep blue colour in the incubated slants indicate positive reaction for citrate utilization.

3.4.4.13 Gelatin hydrolysis

Nutrient agar (3.3.2b) was prepared with 8% gelatin and plates were pre-poured. A loop full of 20 h-old broth cultures of individual organisms was spotted onto portions of the prepared plates and incubated for 24-48 h at 37°C. Incubated plates were examined for gelatin hydrolysis by pouring a mixture of 1.5% mercuric chloride and 10% hydrochloric acid solution over the individual cultures. The formation of a clear transparent zone around the growth area indicated positive reaction for gelatin hydrolysis.

3.4.4.14 Casein hydrolysis

Sterile 10% reconstituted skim milk in 10 mL quantities taken in test tubes was inoculated with individual cultures. Inoculated tubes were incubated for 24-48 h at 37°C. The incubated tubes were examined for the

coagulation of milk and lyses of coagulated milk protein as indication of hydrolysis.

3.4.4.15 Ammonia from arginine

The prepared arginine dihydrolase broth (HiMedia Laboratories, Mumbai, India) tubes were inoculated with a loop full of 20 h-old culture, mixed well and incubated at 37°C for 24-48 h. Incubated tubes were observed for colour change in the medium due to ammonia production, as reflected by the indicator bromothymol blue (light green to dark blue).

3.4.4.16 Ornithine

The cultures were inoculated into the ornithine decarboxylase broth (3.3.25) and incubated at 30^0C for 24-48 h. These tubes were then observed for the change in colour from yellow to purple.

3.4.4.17 Production of H_2S

The slants of triple sugar iron agar (3.3.23) were prepared with a butt of about 1 inch long. The organisms were inoculated and incubated at 30^0C for 24-48 h and observed for the formation of black precipitate in the butt region.

3.4.4.18 TDA

7.0 mL of triple sugar iron agar (3.3.23) was sterilised and slants were prepared with 1 inch butt. The cultures were inoculated and incubated for 24-48 h. The change in colour in both butt and slant was observed. Gas production was observed with the formation of cracks in the butt region.

3.4.4.19 Lysine

5 mL of the lysine decarboxylase broth (3.3.24) was dispensed in to test tube and inoculated. The tubes were incubated at 30^0C for 24-48 h and observed for colour change, initially to yellow and then to purple.

3.4.4.20 ONPG (Ortho Nitro phenol Galactosidase)

ONPG discs from HiMedia Laboratories (Mumbai, India) were used to detect β-galactosidase. The culture were inoculated into saline along with ONPG discs and incubated at 35^0C for 24-48 h. The tubes were observed for the formation of yellow colour.

3.4.4.21 Pigment Production

The pigment production by the individual strains was studied in nutrient agar (3.3.2b) and also in specific media such as *Pseudomonas* agar (Pyocyanin agar (3.3.5a), Fluorescene agar (3.3.5b)) and *Serratia* differential agar medium (3.3.6). The individual strains were streaked on the agar plates and incubated at room temperature for 24-48 h. These plates were then observed for the pigment production.

3.4.4.22 Antibiotic Sensitivity of individual strains

The antibiotic sensitivity test were conducted for the individual strains by spreading the overnight nutrient broth (3.3.2a)- grown cells on to nutrient agar (3.3.2b) plates to get a uniform bacterial lawn. On these spread cultures, 6-8 antibiotic discs (HiMedia Laboratories, Mumbai, India) were placed. The plates were incubated at room temperature, in dark for 24/ 48 h. Clear zones around a disc represented the sensitivity of the culture for that particular antibiotic and resistance where the growth was observed without clearance zone.

3.4.5 Growing of Individual Strains

All the individual strains of the DDT- degrading consortium were grown separately in peptone-glycerol medium (3.3.4) for 48 h and then mixed in the same proportion as was observed in the original consortium ($3x10^3$: $2x10^3$: 2.5x10 : 2.5x10 for DT-1P : DT-2 : DT-Ct1 : DT-Ct2). This reconstituted consortium was also studied for the degradation of DDT.

3.4.6 Inoculum development

All the individual strains of the DDT-degrading consortium were grown on NB (3.3.2a) for 48 h, separately. These were then harvested by centrifugation at 8000 rpm (radius=20cm) for 20 min., washed in minimal medium (3.3.1a) and divided in to two parts. One part was subjected to the DDT (10 ppm) pre-exposure for 24, 48 and 72h. After the pre-exposures, all the four strains were harvested separately by centrifugation and washed in MM (3.3.1a) and resuspended in known volume of MM (3.3.1a). Cell density was tested for each strain and mixed in the required proportion as was there in the consortium with the optimum required pre-exposed individual cells. The individual strains and different combinations of these were used as inocula for degradation studies.

3.4.7 Degradation of DDT

All experiments on the degradation of DDT were carried out in triplicates. Required quantity of DDT, as acetone solution (50- 100μl), was dispersed into sterile, dry, 250 mL Erlenmeyer flasks in UV- sterilized laminar airflow hood. Acetone was allowed to evaporate. 25 mL of sterile minimal medium (3.3.1a) was added to each flask and inoculated with cell suspension of the consortium. The flasks were incubated in a rotary shaker (180 rpm) at ambient temperature (26- 30^o C). Samples (whole flasks) were removed at regular intervals for the determination of growth, inorganic chloride, residual DDT and intermediary metabolites, if any. Flasks

containing only DDT and minimal medium (without microorganisms) served as abiotic control. Flasks containing MM (3.3.1a) and microorganisms (without DDT) were also maintained as biotic control.

3.4.8 pH and degradation of DDT

To study the effect of pH on the degradation of DDT, the inoculum developed under *sec. 3.4.6* was used. pH values between 4.0 to 8.0 were studied to know the effect of pH on DDT degradation. Acetate, and phosphate buffers at 0.1M strength were used for pH values 4.0- 6.0 and 7.0- 8.0 respectively. DDT (10 ppm), as acetone solution, was added to the bottom of sterile, dry 250 mL capacity Erlenmeyer flasks in UV- sterilized laminar hood. Acetone was allowed to evaporate. 25 mL of the medium (of required pH) was added to the flasks. These flasks were inoculated with acclimated DDT- degrading consortium for 72 h and incubated at 180 rpm on a rotary shaker at ambient temperature (26–30^{o}C). Flasks at each pH value containing same quantity of DDT without inoculum were maintained as abiotic controls. After 72h of incubation period, samples were analysed for growth, inorganic chloride released and residual DDT.

3.4.9 Temperature and DDT degradation

To study the effect of temperature on the degradation of DDT three temperatures were selected *viz.* 20 ^{o}C, 30 ^{o}C and 40 ^{o}C. 10 ppm of DDT (as acetone solution) was added to sterile 250 mL Erlenmeyer flasks inside a UV- sterilized laminar hood. Acetone was allowed to evaporate completely and 25 mL of MM (3.3.1a) was poured into the flasks. These flasks were inoculated with acclimated DDT-degrading consortium for 72 h and incubated at 20, 30 and 40 ^{o}C with shaking (180 rpm). Flasks at each temperature, containing same quantity of DDT without inoculum, were maintained as abiotic controls. After 72h of incubation period, samples were analysed for growth, inorganic chloride released and residual DDT.

3.4.10 Influence of Nitrogen Sources on DDT Degradation by Individual Strains

To study the influence of supplied nitrogen on the degradation of 15ppm DDT by the individual strains, the cultures, grown for 48 h with a required pre exposure to each culture, were inoculated to MM (0.1M phosphate buffer, pH 7) containing nitrogen equivalent of 0.250g ammonium nitrate in 1L. Various nitrogen sources were tested for their effect on DDT degradation. These were ammonium acetate, sodium nitrate, calcium nitrate, ammonium sulphate, ammonium chloride, ammonium nitrate and ammonium ferric sulphate. Other complex nitrogen sources like Tryptone, yeast extract, peptone, Casaminoacids and beef extract were used at 0.5g% level. Control with no nitrogen source was also studied. The flasks were incubated at 180rpm on a rotary shaker for 72 h at room temperature (26-30^{0}C). Samples were extracted and tested for the residual DDT.

3.4.11 Combination studies

DDT degrading strains from the DDT-degrading consortium were mixed in different combinations. These strains were inoculated to 30ppm DDT in shake flasks and incubated at room temperature (26-30^{0}C). The samples were drawn at regular intervals and analysed for the residual DDT.

3.4.12 Extraction of the residual DDT

Residual DDT was extracted thrice from the aqueous samples with equal volumes of dichloromethane in a separating funnel. Sample and the solvent were taken in a separating funnel and shaken vigorously for 5 min and the two layers were allowed to separate out. Then the solvent layers were pooled and passed through anhydrous sodium sulphate and then through activated florisil. The solvent was allowed to evaporate and the

residue was resuspended in a known volume of acetone for further analysis.

3.4.13 Analytical

3.4.13a Inorganic chloride

Inorganic chloride was estimated according to a modified HNO_3 – $AgNO_3$ method of Frier (1974). A total of 1 mL culture broth was centrifuged and the supernatant placed in a test tube. The cells were washed in 0.1 N NaOH (50 μl) and 950 μl mineral medium. The supernatants were pooled with the above supernatant. A total of 1 mL each of 0.15 N HNO_3 and 0.1 N $AgNO_3$ were added to the supernatant with mixing at each step. The contents were allowed to stand at room temperature for 20 min and turbidity measured at 600_{nm} using spectrophotometer (Shimadzu UV- 160A, Japan). The amount of chloride was computed from a standard curve prepared for NaCl in a similar way.

3.4.13b Growth

Growth of the consortium was determined by estimating total protein in the biomass by modified method of Lowry *et al* as follows: Cells were harvested from a suitable quantity of culture broth, washed with minimal medium (3.3.1a), suspended in 3.4 mL distilled water and 0.6 mL of 20% NaOH. This was mixed and digested in a constant boiling water bath for 10 min. Total protein, in cooled sample of this hydrolysate, was estimated by using Folin-Ciocalteau reagent (3.3.22). A total of 0.5 mL of the hydrolysate was taken in a clean test tube. To this was added 5.0 mL of Lowry's C (3.3.21). After 10 min 0.5 mL of Lowry's D [Folin-Ciocalteau reagent (1:2)(3.3.22)) was added and mixed well. The colour was read at 660_{nm} after 20.0 min of standing at room temperature, using a spectrophotometer (Shimadzu UV- 160A, Japan). Total amount of protein was computed using the standard curve prepared with BSA (Bovine serum Albumin).

Growth was also measured in terms of colony forming units (cfu) as descrtibed by Sahu *et al* (1996) using appropriately diluted broth.

3.4.13c Residual DDT

Residual DDT was estimated by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

3.4.13c(i) I Thin Layer Chromatography

Thin layer chromatography (TLC) was done on silica gel G 60- 100 mesh uniformly spread over a 20 x 20 cm^2 glass plate. The thickness of the gel was set at 300 μm. These plates were left to dry at room temperature after spreading (using Camag automatic spreader, Germany) then activated at 100^o C for 1 h. Known volume of the residual extract of DDT (acetone solution) was spotted on to these plates. Spotted plates were developed in cyclohexane, air- dried and the residue was detected by spraying *o*-tolidine (2% solution in acetone) followed by exposure to bright sunlight. The chloro-compounds give peacock green/ blue colour with this chromogen. Spots were delineated by marking with a needle and area measured. Quantity of DDT in each spot was estimated from a standard graph prepared for √area *vs* log (DDT concentration).

3.4.13c(ii) Gas Chromatography

Concentrated residual substrate was resuspended in a known volume of HPLC grade acetone and gas chromatography was done using Chemito 1000 series gas chromatograph (Nasik, India) gas chromatograph. 1μl of the extract suspension was injected in to a BP-5 capillary column (30m x 0.25 mm ID) set at 180^{o}C and programmed as: 180^{o}C for 10 min and a rise @ 2^{o}C/ min up to 220^{o}C and maintained there for 2 min. Injector was maintained at 250^{o}C while electron capture detector (Ni^{63}) was maintained at 280^{o}C. Pure nitrogen gas was used as the carrier @ 1 mL

min^{-1}. Under these conditions, the standard retention time for DDT was 28.16 min. Quantification of DDT in the sample was done using the area under the peak with and comparing with the standard under same conditions.

3.5 Results

3.5.1 Isolation and acclimation of DDT-degrading microbial consortium

After two years of enrichment of DDT-contaminated soil, a microbial consortium got established in shake flasks capable of using DDT as sole source of carbon. The consortium was acclimated slowly to increasing concentrations of DDT up to 25 ppm.

3.5.2 Degradation of different concentrations of DDT by the microbial consortium

The above-established consortium was inoculated to 5 ppm DDT. The added DDT completely disappeared by 48 h (Fig.3.1). This consortium was then used as inoculum for the next higher concentration of DDT, 10 ppm. Degradation of 10 ppm DDT was complete in 72 h of incubation (Fig.3.1). Similarly, the cell mass from previous batch was taken for studying the degradation of next higher concentrations *viz.* 15 and 25 ppm DDT. These concentrations were completely degraded by 96 and 144 h respectively (Fig.3.1). In all cases the stoichiometric release of chloride was observed (Fig.3.2). Increase in the total protein level was also observed at these concentrations (Fig.3.3). When higher concentrations of DDT were used, degradation was partial even after 240 h of incubation (Fig.3.4).

Fig.3.1 Degradation of DDT by the Bacterial Consortium

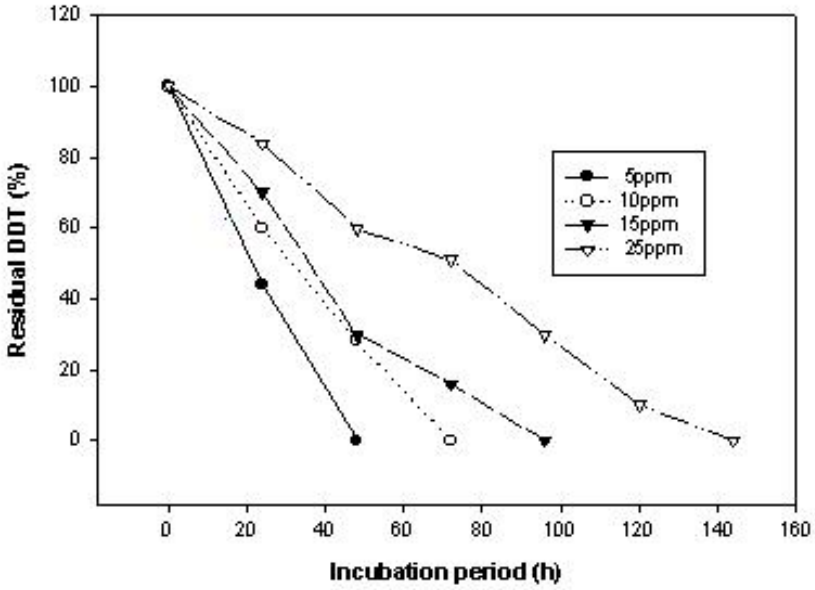


Fig.3.2 Chloride Release During the Degradation of DDT by the Bacterial Consortium

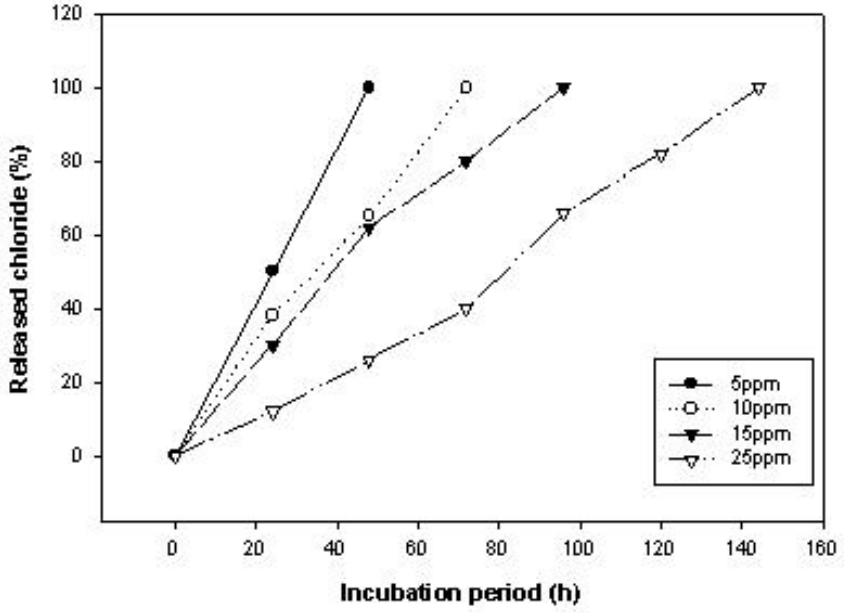


Fig.3.3 Growth during DDT degradation by the bacterial consortium

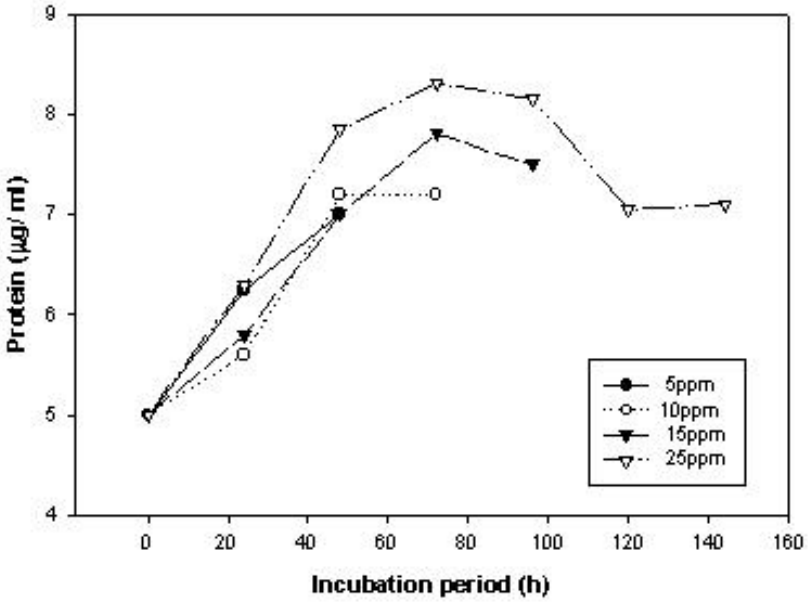
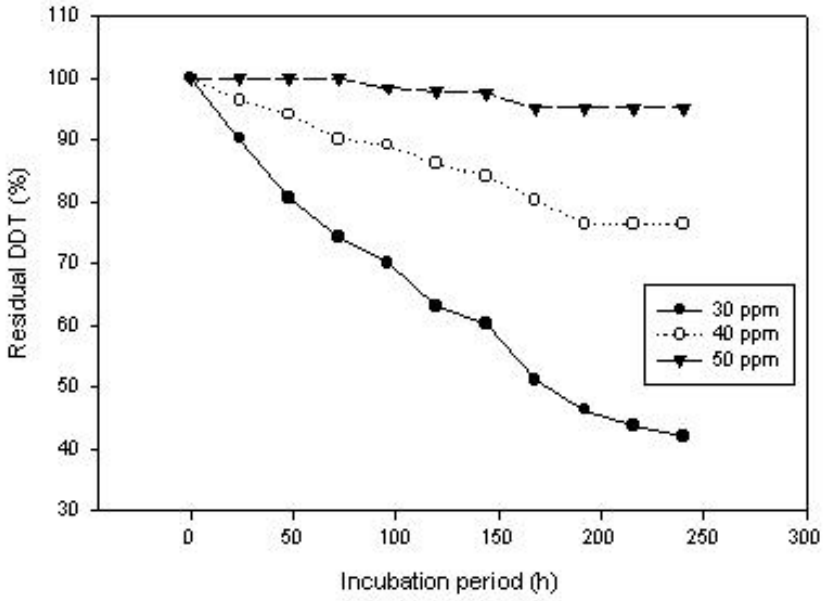


Fig.3.4 Degradation of Higher Concentrations of DDT by the Bacterial Consortium



3.5.3 Effect of pH on degradation of DDT by the microbial consortium

To study the effect of different pHs on the degradation of DDT by the established microbial consortium, the acclimated consortium was inoculated to 10 ppm DDT. Different pHs were set from 4 to 8 and the flasks incubated for 72 h at room temperature at 180 rpm. Results are shown in Fig.3.5. It is clear from the figure that pH 7 was the best for DDT degradation by the consortium. In other pH levels the degradation was partial and a greater percentage of added DDT (92%, 80%, 66% and 40% respectively at pH 4, 5, 6 and 8) was observed even after 72 h of incubation.

3.5.4 Effect of temperature on degradation of DDT by the microbial consortium

To study the effect of different temperatures on the degradation of DDT by the established microbial consortium, the acclimated consortium was inoculated to 10 ppm DDT. Different temperatures (20^0C, 30^0C and 40^0C) were maintained for each set of the flasks incubated for 72 h at 180 rpm. Results are shown in Fig.3.6. It was observed that 10ppm DDT could be degraded by the end of 72 h only in case of 30^0C. In other two temperatures, the residual DDT was 45% and 63% respectively for 20^0 and 40^0C.

3.5.5 Resolution of the microbial consortium and identification of the individual members

The acclimated consortium capable of degrading 25 ppm DDT was appropriately diluted and plated on nutrient agar. Four types of bacteria were isolated and purified. All these bacterial cultures were identified based on cultural, morphological, microscopic and biochemical characters as *Serratia marcescens* strain DT-1P, *Pseudomonas fluorescens* strain DT-2,

Fig.3.5 Degradation of 10ppm DDT at Different pH by the Microbial Consortium (After 72h)

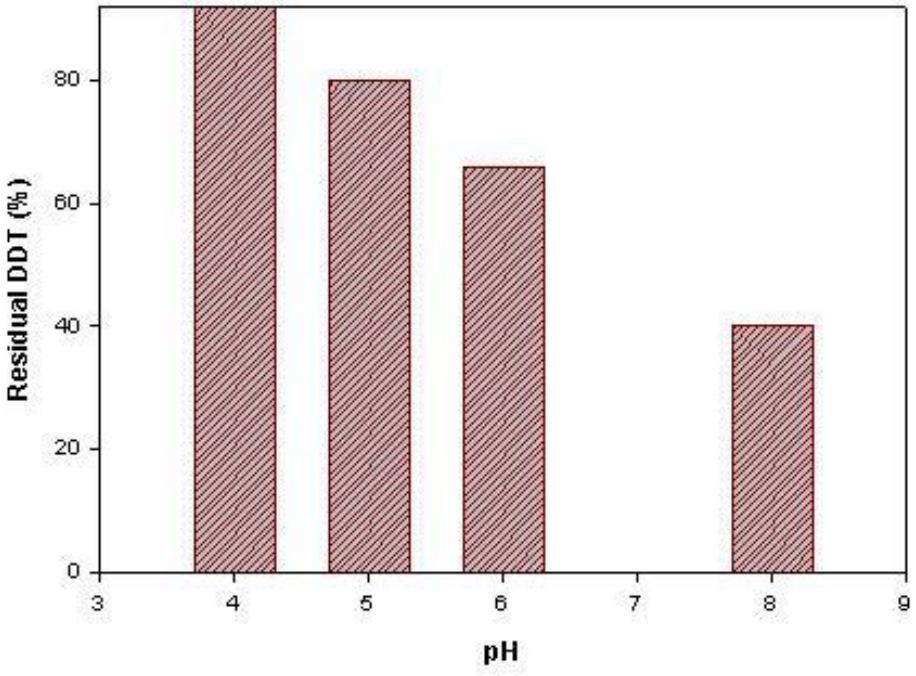
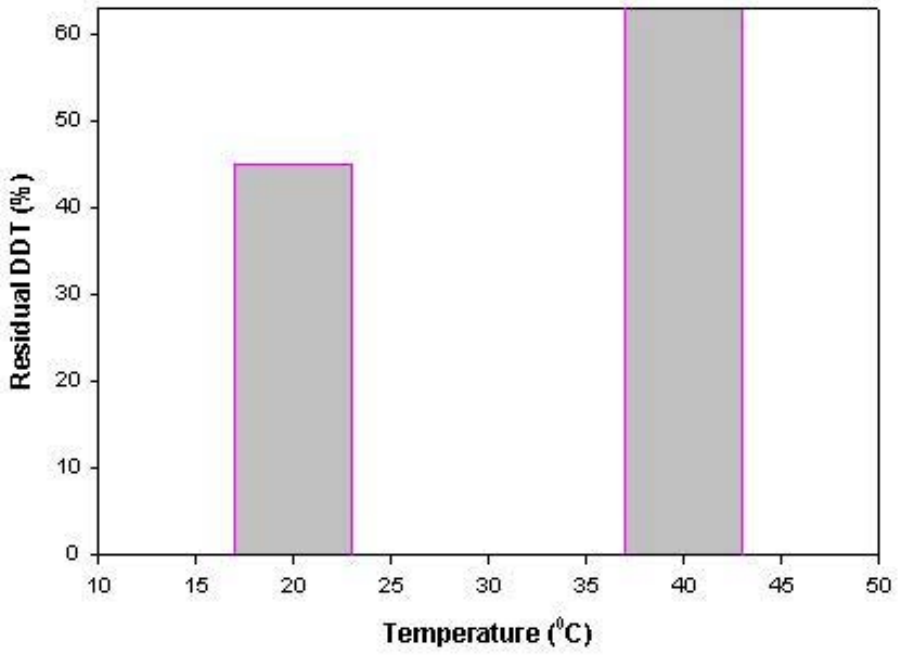


Fig.3.6 Degradation of 10ppm DDT at Different Temperatures by the Microbial Consortium



Pseudomonas aeruginosa strain DT-Ct1 and *Pseudomonas aeruginosa* strain DT-Ct2, according to the Bergey's manual of determinative Bacteriology. The characteristics of these isolates are given in Table 3.1.

Table 3.1 Results of Various Tests for the Identification of Individual Members of the DDT-Degrading Microbial Consortium

S.No.	Test	Inference			
		DT-1P	DT-2	DT-Ct1	DT-Ct2
1.	Gram staining	Gr.-ve	Gr.-ve	Gr.-ve	Gr.-ve
2.	Oxidase	+	+	+	+
3.	Catalase	+	+	+	+
4.	Motility	+	+	+	+
5.	Nitrate	+	-	+	-
6.	Lysine	+	+	+	+
7.	Ornithine	-	-	-	-
8.	H_2S	-	-	-	-
9.	Glucose	+	+	+	+
10.	Mannitol	+	+	-	-
11.	Xylose	+	+	+	-
12.	ONPG	+	-	-	-
13.	Indole	-	-	-	-
14.	Urease	-	+	+	-
15.	MR				
16.	VP	+	-	-	-
17.	Starch	+	+	+	+
18.	Casein				
19	Citrate	+	+	+	-
20.	TDA	+	-	-	-

21.	Gelatin	+	+	-	-
22.	Malonate	-	+	+	-
23.	Inositol	+	-	-	-
24.	Sorbitol	+	-	-	-
25	Rhamnose	-	+	+	-
26	Sucrose	+	-	-	-
27	Lactose	-	+	-	-
28	Arabinose	-	+	+	-
29	Adonotol	+	-	-	+
30.	Raffinose	-	-	-	-
31.	Salicin	+	+	-	-
32.	Arginine	+	+	+	+
33.	Pigment	Orange-Deep red	Lt.Fluorescent yellow	Sap green	Brown

Other characters of the individual strains are as follows:

DT-1P

It is an orange-red coloured gram-negative bacterium. On the nutrient agar the colonies appear reddish with a fluorescent rim and umbonate in shape (Plate 1(a)). In glycerol- peptone agar the colonies appear dark pink to reddish maroon. On the *Serratia* differential agar the colonies appeared red-maroon. Under the light microscope, the cells were cocci, usually in pairs and occasionally in chains of six-eight.

The Scanning Electron Microscopy (SEM) showed cells with depressions in the middle of the cells. Cells measured 0.57- 0.81 μm in length and 0.32- 0.49μm in width (Plate 2(a)).

DT-2

These gram-negative colonies appeared slightly lemon yellow in colour on nutrient agar plates (Plate 1(b)). Fluorescence was observed in the centre of the colonies. Pyocyanin agar showed light green where as fluorescene agar showed yellow pigmentation. On glycerol- peptone agar it showed light fluorescent yellow. The cells appeared long-bacilloid. Single or in chains of three under the light microscope.

SEM: The cells are long rods measuring 1.39- 1.61μm in length and 0.42- 0.45μm in width (Plate 2(b)).

DT-Ct-1

It is a fast spreading gram-negative bacterial culture. Initially showed sap green colour with the pigment diffusing into the medium that turns brown at a later stage on nutrient agar (Plate 3 (a)) and fluorescene agar whereas on pyocyanin agar, medium remained green with the pigment diffusing into it even at later stages. On glycerol- peptone agar, colonies showed fluorescent green- yellow pigment in the medium. In all agar media the colonies were shiny on the upper surface. The cells were small, cocco-bacilli, single or in pairs when observed under the light microscope.

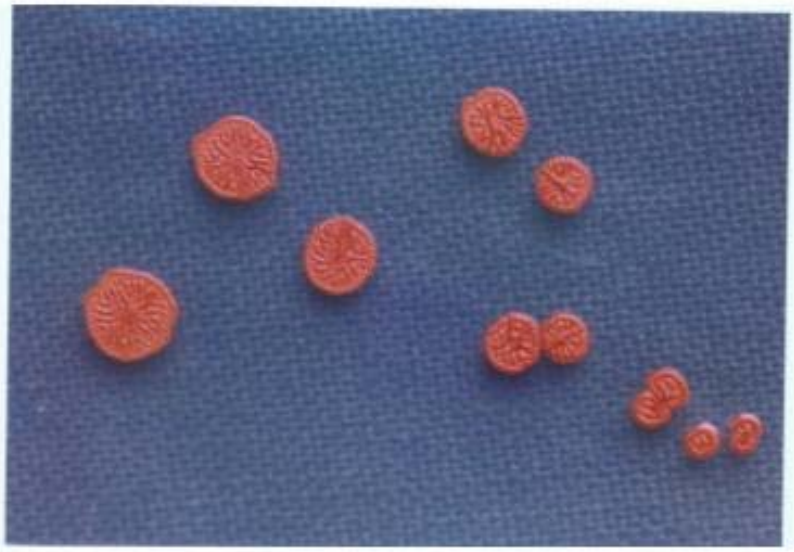
SEM: These are small rods measuring 0.81- 1.43μm in length and 0.31- 0.48μm in width (Plate 4(a)).

DT-Ct-2

These were highly spreading, fast growing gram negative colonies on agar medium. On Nutrient agar (Plate 3(b)) and Pyocyanin agar it produces deep green-brown pigment that diffuses in to the medium. On fluorescene agar and glycerol- peptone agar slightly brownish pigment with greenish tinge was produced. The surface was metallic sheen with little

Plate 1: (a) *Serratia marcescens* DT-1P and (b) *Pseudomonas fluorescens* DT-2 as seen on Nutrient agar plates (48h old)

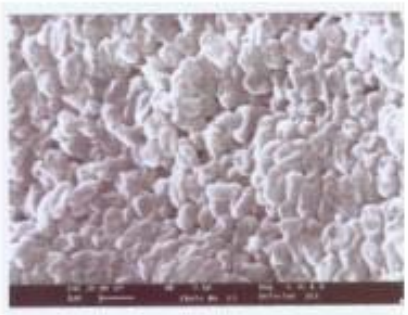
(a) *Serratia marcescens* DT-1P



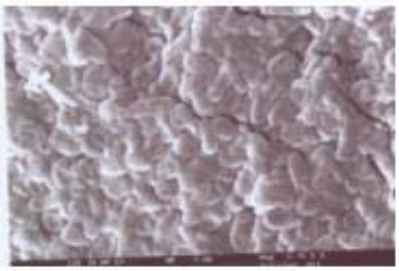
(b) *Pseudomonas fluorescens* DT-2

Plate 2: (a) *Serratia marcescens* DT-1P and (b) *Pseudomonas fluorescens* DT-2 as seen in SEM

(a) *Serratia marcescens* DT-1P



(b) *Pseudomonas fluorescens* DT-2



roughness. Under the light microscope, the cells were cocci, single or in short chains of 5-6 cells.

SEM: These cells are small rods with 0.79- 1.27μm length and 0.47-0.55μm in width (Plate 4(b)).

3.5.6 Antibiotic Sensitivity of the Individual Bacterial Strains

The antibiotic sensitivity of each of the individual strains was carried out using the antibiotic discs. The results of the tests are shown in the Table 3.2.

3.5.7 *Degradation of DDT by axenic bacterial isolates of the consortium*

The bacterial isolates grown on peptone- glycerol medium, when inoculated to 5 ppm DDT, the degradation was partial. Only 44%, 20%, 45% and 25% was degraded by DT-1P, DT-2, DT-Ct1 and DT-Ct2 respectively by 48 h (Fig.3.7). The Cl^- released was stoichiometrically equal to the substrate utilized. Neither any more Cl^- release nor decrease in residual substrate was observed with further incubation.

3.5.8 *Pre- exposure and DDT degradation*

The peptone- glycerol grown cells were pre- exposed to 10 ppm of DDT for 24, 48 and 72 h. Degradation of DDT increased with increase in pre- exposure time in case of DT-1P, DT-Ct1 and DT-Ct2 wherein 80%, 90% and 5% of the initially added substrate disappeared after 72 h of incubation with 72 h pre- exposed inoculum. A total of 84% of the substrate disappeared by 72 h in case of DT-2 with 24 h pre- exposed cells. There was no improvement in the degradation after 72 h pre-exposure of DT-1P, DT-Ct1 and DT-Ct2 with DDT. While there was a drop in the degradation rate by DT-2 with 48 h pre-exposed cells compared to 24 h pre-exposure.

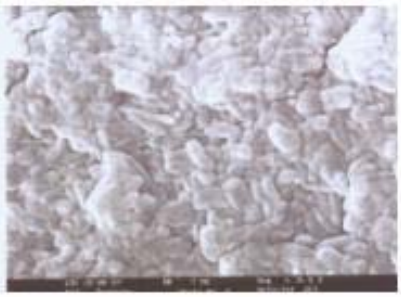
Plate 3: (a) *Pseudomonas aeruginosa* DT-Ct1 and (b) *Pseudomonas aeruginosa* DT-Ct2 as seen on Nutrient agar plates (48h old)

(a) *Pseudomonas aeruginosa* DT-Ct1

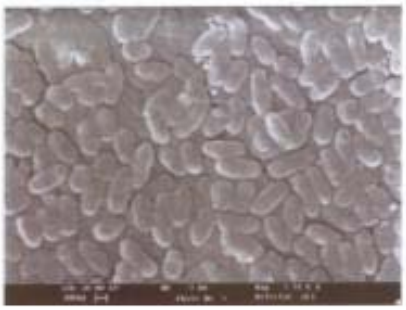
(b) *Pseudomonas aeruginosa* DT-Ct2

Plate 4: (a) *Pseudomonas aeruginosa* DT-Ct1 and (b) *Pseudomonas aeruginosa* DT-Ct2 as seen in SEM

(a) *Pseudomonas aeruginosa* DT-Ct1



(b) *Pseudomonas aeruginosa* DT-Ct2



But it also degraded the initially added DDT in the same time as in the case of 24 h pre-exposed cells. DT-Ct2 was very slow in degrading DDT and the amount of DDT degraded by it was very less compared to the other three strains. Only 5 % of the initially added DDT was observed to disappear by the end of 72 h in this case. DT-1P and DT-Ct1 showed increase in the degradation rate with increase in pre-exposure time from 24 h through 72 h but further there was no more improvement in the degradation efficiencies of these with increased pre-exposure time. Therefore it was concluded that DT-1P, DT-Ct1 and Dt-Ct2 required 72 h of pre-exposure while DT-2 needed 24 h of pre-exposure to DDT prior to the actual degradation.

Table 3.2 Antibiotic Sensitivity of the Individual Strains From the DDT-Degrading Consortium

S.No.	Antibiotic	DT-1P	DT-2	DT-Ct1	DT-Ct2
1.	Penicillin G	R	R	R	R
2.	Ampicillin	R	R	R	R
3.	Streptomycin	S	S	R	R
4.	Kanamycin	S	S	S	S
5.	Bacitracin	R	R	R	R
6.	Nitrofurantoin	R	R	R	R
7.	Carbenicillin	S	S	S	S
8.	Erythromycin	R	R	R	R
9.	Neomycin	S	S	S	S
10.	Rifamycin	S	S	S	S
11.	Novobiocin	R	R	S	S
12.	Oxytetracycline	S	S	S	S
13.	Tetracycline	S	S	S	S
14.	Trimethoprim	R	R	S	S
15.	Nalydixic acid	S	S	S	S

16.	Chloramphinicol	S	S	S	S
17.	Norfloxacin	S	S	S	S
18	Rifampicin	S	S	-	S

R= Resistant; S= Sensitive

3.5.9 Influence of Nitrogen on DDT Degradation

All the four strains *viz Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *pseudomonas aeruginosa* DT-Ct2 were studied for the influence of different nitrogen sources on DDT degradation. All the strains were inoculated to 15ppm DDT in MM (0.1 M phosphate buffer, pH 7) containing nitrogen equivalent of ammonium nitrate in 1L. After 72 h of incubation at room temperature (26-30^{0}C), flasks were analysed for the residual DDT. Table 3.3 gives the details of degradation of DDT in all these media. Degradation of the supplied DDT was found to be maximum in the samples where no nitrogen was supplemented in case of *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2 wherein 100%, 65% and 15.20% of the added DDT was degraded by the end of 72 h of incubation. *Pseudomonas aeruginosa* DT-Ct1 could degrade only 30% in samples without nitrogen while the maximum disappearance in its case was observed with ammonium chloride (45% degradation), a supplement of inorganic nitrogen and peptone (organic nitrogen) where 47% degradation was achieved. Ammonium ferric sulphate and Tryptone favoured maximum degradation in case of *Serratia marcescens* DT-1P as inorganic and organic nitrogen sources respectively. 56 and 57% degradation was attained by *Pseudomonas fluorescens* DT-2 with ammonium ferric sulphate

Fig. 3.7 Degradation of 5ppm DDT by Individual Strains of the DDT-Degrading Consortium

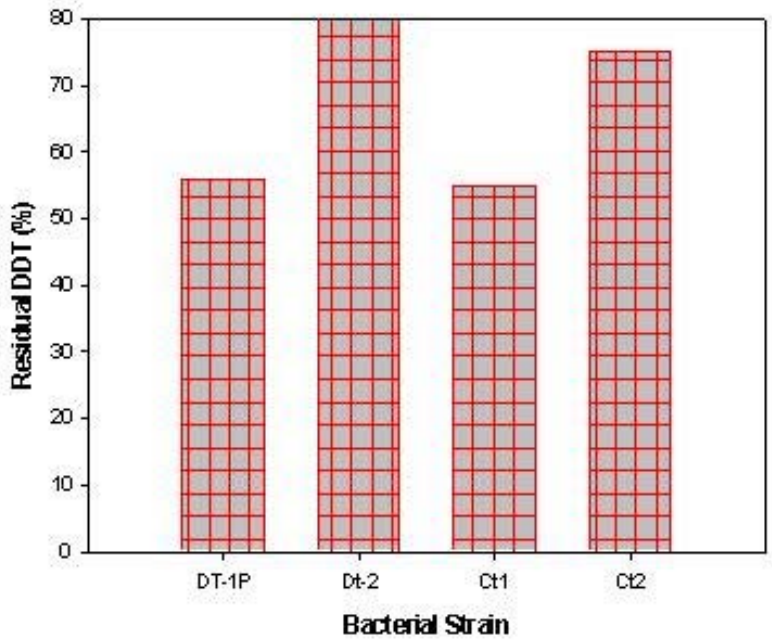


Table 3.3 Degradation of 15ppm *p,p'*-DDT in different nitrogen sources by individual isolates of DDT- degrading consortium under shake flask conditions

S.No.	Nitrogen source	% Degradation after 72 h of incubation			
		DT-1P	DT-2	DT-Ct-1	DT-Ct-2
1.	Ammonium acetate	30.40	44.63	3.33	11.38
2.	Sodium nitrate	0.20	33.5	23.61	-
3.	Calcium nitrate	39.20	-	33.33	5.88
4.	Ammonium sulphate	28.33	12.75	26.67	0.00
5.	Ammonium chloride	-	-	45.83	8.63
6.	Ammonium nitrate	36.67	-	38.88	5.88
7.	Ammonium ferric sulphate	40.20	55.75	40.00	4.50
8.	Tryptone	100.00	39.15	31.67	0.25
9.	Yeast extract	32.73	57.13	13.33	4.50
10.	Peptone	56.67	32.13	47.22	-
11.	Casaminoacids	4.52	44.63	31.67	14.13
12.	Beef extract	0.00	-	40.28	0.25
13.	Without nitrogen source	100.00	65.35	30.55	15.20

(inorganic nitrogen) and yeast extract (organic nitrogen) respectively. *Pseudomonas aeruginosa* DT-Ct1 could degrade 46% and 47% of the added DDT with ammonium chloride (inorganic nitrogen) and peptone (organic nitrogen) supplemented respectively. Ammonium acetate (inorganic nitrogen) and Casaminoacids (organic nitrogen) could help *Pseudomonas aeruginosa* DT-Ct2 in degradation of 11% and 14% of the supplied DDT.

3.5.10 Combination Studies

Degradation of higher concentration (30ppm) of DDT was taken up to test the efficiency of different combinations of the members of the DDT-degrading consortium (combined in the same ratio as present in the original consortium). Table 3.4 describes the results of this study. It appears obvious that the presence of all the four strains is necessary for the faster and efficient degradation of higher concentrations of DDT. Though the combination of DT-1P, DT-Ct1 and DT-Ct2 (134 in the table 3.4) started with 23% reduction in the substrate level in the first 24 h, it appeared to lag behind the combination of DT-1P and DT-Ct1 (13 in the table 3.4) by the end of 48 h of incubation. But at the end of 72 h of incubation it overtook the total amount of DDT degraded by any combination studied till then. There was a sudden depletion in the DDT level in case of 1234 (all the strains reconstituted with the respective densities of each as in consortium) at the end of 96 h of incubation wherein 83% of the initially added 30ppm DDT had disappeared. At the end of 120 h of incubation there was 13% of the 30ppm DDT remaining in case of the combination of all the four constituent strains of the DDT-degrading consortium. Next best combination was DT-1P, DT-Ct1 and DT-Ct2 (134 in Table 3.4) (83% degradation) followed by DT-1P, DT-2 and DT-Ct1 (123 in Table 3.4) (81.5% degradation) and DT-1P, DT-2 and Dt-Ct2 (124 in Table 3.4) (80% degradation). Though DT-Ct2

was not a good degrader independently, it appeared to contribute to the degradation when used in combination.

Table 3.4. Combination Studies of 4 bacterial Isolates on DDT degradation (30ppm)

	Percent Residual DDT in the combinations								
	12	**13**	**14**	**24**	**34**	**134**	**123**	**124**	**1234**
0h	100	100	100	100	100	100	100	100	100
24h	98	92.51	98.9	84.6	84.7	***77.4***	83.7	87.5	98.2
48h	65.8	***63.7***	79.6	68.2	65.9	69.4	72.7	76	73.4
72h	58.7	47.7	67.7	61.1	39.4	***44.8***	48.1	66.1	50.4
96h	57.8	36.9	61.9	42.1	31.7	27.5	47.5	40.5	***17.1***
120h	35.5	25	44.1	21.7	27.8	17.2	18.5	19.8	***12.9***

12=DT-1P + DT-2

13=DT-1P + DT-Ct1

14=DT-1P + DT-Ct2

24=DT-2 + DT-Ct2

34=DT-Ct1+ DT-Ct2

123=DT-1P + DT-2 + DT-Ct1

124=DT-1P + DT-2 + DT-Ct2

134=DT-1P + DT-Ct1 + DT-Ct2

1234=DT-1P + DT-2 + DT-Ct1 + DT-Ct2

3.6 Discussion

Microbial degradation studies have been conducted with pure cultures isolated by selective enrichment techniques (Robra, 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 present microbial consortium is one such potent degrader of DDT that was isolated from DDT-contaminated soil, by long-term shake flask-enrichment technique. 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 *et al*, 1980). The DDT-degrading consortium, established in our laboratory by the enrichment and continuous DDT-stress, when acclimated with increasing concentrations of DDT could degrade 5, 10, 15, and 25ppm DDT by 48, 72, 96 and 144 h respectively.

Isolation of microbes from sewage has advantages that it provides heavy inoculum of multivariant organisms during acclimation. Domestic sewage with dog food extract was used in the isolation of a microbial community (Moos *et al*, 1980). Variation in the sewage character was found to influence the biodegradation exhibited by the community (Moos *et al*, 1980). It can be assumed that, continuous exposure of this consortium to increasing concentrations of DDT improved its DDT-degrading ability. Similar observations have been made in our study with HCH- degrading consortium, where acclimation improved the degradative ability of HCH- degrading consortium (Manonmani *et al*, 2000). The acclimated consortium showed a higher rate of HCH- degradation, with higher concentrations of HCH. Bhuyan *et al* (1992) and Wada *et al* (1989) have also made similar observations, where ϒ- HCH degradation improved after every successive application of the compound. However, in our studies, acclimation to still higher concentrations and

time did not show any further improvement in DDT-degradation. During acclimation in our study, succession of microbial members had taken place resulting ultimately in the survival of four members at the end of acclimation period. Aislabie *et al* (1997) have reported that it is difficult to isolate microbes from the environment that can attack a compound co-metabolically. A technique known as analogue enrichment was adapted by Bartha (1990) in which a structural analogue was substituted for the compound of interest. Focht and Alexander (1970a), applied diphenyl methane, a structural analogue of DDT to sewage samples and isolated a DDT-metaboliser, *Hydrogenomonas* sp. Similar technique was adapted using 4-chlorobiphenyl, another structural analogue of DDT to isolate DDT degrading bacteria (Masse *et* al, 1989; Nadeau *et* al, 1994; Parsons *et* al, 1995). Among the four members of DDT- degrading microbial community, *Serratia marcescens* DT-1P and *Pseudomonas aeruginosa* DT-Ct1 degraded more quantity of 5ppm DDT than the other two strains *viz. Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2. All the four members of the consortium were gram-negative bacteria. Many gram negative and gram-positive bacteria have been reported to have metabolic capabilities of attacking DDT. *Alcaligenes eutrophus* A5 (Nadeau *et al*, 1994), *Hydrogenomonas* sp. (Focht and Alexander, 1971; Pfaender and Alexander, 1972), *Pseudomonas putida* (Subba Rao and Alexander, 1985) and fungi such as *Aspergillus niger, Penicillium brefeldianum* (Subba Rao and Alexander, 1977) and *Phanerochaete chrysosporium* (Bumpus and Aust, 1987) have been reported to degrade DDT. 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 (Robra, 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 *et al* (1993) isolated two strains of bacteria, *Bacillus* sp. B75 and an unidentified gram-variable rod B116, which degraded DDT at extremely low level of 10pg/mL. In our studies the consortium was able to degrade 5ppm to 25ppm DDT by 144h and degradation of higher concentrations was partial. The four strains purified were tested for the effect of various nitrogen sources and the results indicated that the nitrogen limiting conditions were more favourable towards DDT-degradation by at least three of the strains while the utilisation of organic nitrogen sources by the organisms helped in more efficient degradation. The effect of various carbon sources effecting DDT degradation has been discussed in detail in chapter 5.

As the degradation of xenobiotics such as DDT requires more than one enzyme, single organisms having all the required degradative potential are rare. The pathways can be formed in a microbial community through the concerted action of the individual members within it in a greater way in the larger genetic pool of the community. 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 for the requirements of a community with those of individual members. Studies on the microbial degradation of DDT will be useful for the development of methods for the remediation of the contaminated sites. The *in- situ* biodegradation may be limited because of a complex set of environmental conditions. To enhance the biodegradation, these engineered microorganisms may have to be introduced into the contaminated soil or water. But, individual members with the capacity to degrade these compounds, by their ability to access a large library of outside information which give them the adaptive capacity (Reanney, 1976). This capacity can be achieved by continuous exposure to the compound of interest that might cause mutations leading to the evolution of

new pathways or by the mechanism of the gene transfer (mainly *via* conjugation) (Williams, 1978; 1982). In our studies individual members of the consortium had less capacity to degrade DDT than the consortium itself. This could be due to any of the reasons mentioned above. The low degree of degradation by the individual members may be due to non-optimised conditions provided for the degradation. Knowledge of the various optimised parameters would facilitate an easy and more effective translation of the laboratory results to the fields. Although the bioremediation of DDT- contaminated sites is difficult, all avenues for research have not been closed.

The observations in our laboratory indicate the efficiency of a mixed population of organisms for the purpose of bioremediation. These populations develop in nature as one of the best combinations that can take part in maintaining the ecological balance. The major factor here could be the period of exposure of the natural population to the chemicals as well as the concentration of these chemicals. Other factors (environmental, chemical and physical) also play a vital role in the development of such populations of microorganisms. When all the factors are taken into consideration and the favourable conditions supplied, the chances of such beneficial microbes flourishing and improving the environment increases leading to a safer place to live.

Chapter 4a

Kinetics of DDT-Degradation by the Bacterial Cultures

Models are, for the most part, caricatures of reality, but if they are good, then, like good caricatures, they portray, though in a distorted manner, some of the features of the real world.

Kac (1969)

4a.1 Introduction

The DDT-degrading consortium was developed and the individual members constituting this consortium were purified. They were initially screened for their ability to degrade DDT (Fig.3.7) and the influence of various nitrogen sources on DDT degradation was studied. It would be appreciable to follow the DDT degradation under given conditions. This could be achieved by means of mathematical expression of the degradation rate under the set conditions. This expression is usually called a kinetic model for the process. When the kinetics of a particular reaction or process is known, we can ascertain the progress of the process at a particular set of conditions. As far the aromatics and organic pesticides are concerned, the biodegradation-kinetics has been worked out for the degradation based on the Monad's principle. Most of the workers have taken growth into consideration for getting to the kinetics of degradation of the pesticides or other xenobiotic organic compounds. A consortium with six different strains, under aerobic conditions, has been studied for the degradation kinetics of 2,4,6-TCP (2,4,6-trichlorophenol) (Kharoune *et al*, 2002). A microbial consortium was developed to degrade benzene under oxygen limiting conditions (Yerushalmi *et al.,* 2002). Monad parameters were compared with the kinetics of 3-CBA metabolism in soil by Focht and Shelton (1987). Kinetics of high concentration of iso-propanol biodegradation has been worked out by Bustard *et al* (2002) by a mixed microbial culture using the growth kinetics. Chang *et al* (2001) described the biodegradation of phenanthrene in soil by a polycyclic aromatic hydrocarbon-adapted consortium using the first order kinetics. Cort and Bielefeldt (2001) gave the kinetic model for surfactant inhibition of pentachlorophenol biodegradation by *Sphingomonas chlorophenolica* sp. strain RA2. They have taken into consideration the Monad growth kinetics and the Michaelis- Menten equations of kinetics to describe the model. Ely *et al* (1995) described the kinetics of cometabolic biodegradation of TCE (trichloethylene) to be dependant on various factors of inhibition, inactivation and recovery of the enzyme in presence of the substrate.

For DDT, there are hardly any reports detailing the kinetics of its degradation under microbial activity. In our work, we looked in to the degradation of DDT by the axenic cultures in the shake flasks and in soil. A kinetic expression for each set has been developed at the laboratory scale studies. . For obvious reasons *Pseudomonas aeruginosa* DT-Ct2, which did not show good response towards DDT-degradation when challenged alone as carbon source, was not considered for this study. The cultures used in these studies were *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1

4a.2 Materials and Methods

4a.2.1 Chemicals

p,p´-DDT of 98 % purity was procured from Sigma Aldrich Chemical Co., Mo, USA. Peptone special and analytical grade glycerol were obtained from HiMedia Cemical Laboratories, Mumbai, India. Other chemicals used in the studies were of analytical grade and were procured from standard chemical companies.

4a.2.2 Cultures

The bacterial strains were isolated from contaminated waste-water after a long term enrichment with DDT in shake flasks (3.4.1). These strains were studied separately for DDT degradation under shaking conditions. Studies with respect to the degradation kinetics were taken up after other preliminary studies in laboratory to identify the efficiency of each strain to degrade DDT as a sole source of carbon. *Pseudomonas aeruginosa* DT-Ct2 was not much active in DDT alone and therefore it was left out for the kinetic study under only DDT supply as carbon source. As it was observed that not much significant growth was there in any of these cultures in presence of only DDT as the carbon source, resting cells were used for most part of these studies.

4a.2.3 Culture conditions

Serratia marcescens DT-1P, *Pseudomonas fluorescens* DT-2 and Pseudomonas *aeruginosa* DT-Ct1 were grown on peptone- glycerol media (3.3.4) containing 10ppm DDT. Cultures were allowed to shake at 180 rpm for 72 h at ambient temperature (26-30^{o}C). Cells were then harvested by centrifugation and subjected to acclimation with 10ppm DDT for 72 h at room temperature shaking at 180 rpm. These cells were then harvested and used as inoculum for various experiments after thorough washing with minimal medium (3.3.1a). Minimal medium (3.3.1a) was used for the degradation studies.

4a.2.4 Degradation studies in shake flasks

4a.2.4a Degradation and Inoculum Level

DDT Degradation at various inoculum levels was carried out by inoculating 72 h pre- exposed cells of *Serratia marcescens* DT-1P and *Pseudomonas aeruginosa* DT-Ct1 (3.5.7), 24 h pre-exposed cells of *Pseudomonas fluorescens* DT-2 (3.5.7) to 10ppm DDT in a sterile 250 mL Erlenmeyer flasks containing 25 mL minimal medium. Cells were inoculated at various levels viz. 10, 20, 50, 100, 200, 250, 300, 500 and 8000μg protein/mL and incubated at room temperature (26-30^{0}C) on a rotary shaker at 180 rpm for different hours. Samples (whole flasks) were drawn and analysed for the residual DDT levels.

4a.2.4b Degradation at Different pH

DDT degradation at various pH was carried out with 10 ppm DDT inoculated with set inoculum level of pre-exposed cells of *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1 separately in sterile 250 mL Erlenmeyer flasks containing 50mL 0.1 M buffer set at required pH. Different pH viz. 4, 5, 6, 7 and 8 were set using acetates for lower pHs and phosphates for higher

pHs. Flasks were incubated at room temperature (26-30^{0}C) on a rotary shaker set at 180 rpm for different periods and analysed for residual DDT levels.

4a.2.4c Degradation at Different Temperatures

DDT degradation at various temperatures was studied with pre-exposed cells inoculated at the set inoculum level to 50mL of 10 ppm DDT in sterile 250 mL Erlenmeyer flasks containing minimal medium (3.3.1a) set at the optimum pH and incubating at various temperatures of 20, 30 and 40^o C respectively. Sample flasks were drawn at different intervals of time and analysed for residual DDT.

4a.2.4d Degradation of Different Concentrations of DDT

DDT degradation studies were conducted with different concentrations of DDT (5 ppm through 50 ppm). All the required optimum parameters of inoculum level, pH, and temperature were taken into consideration while studying the degradation of different concentrations of DDT by *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1 in shake flasks. Required amount of DDT as acetone solution was added to sterile 250 mL Erlenmeyer flasks and acetone was allowed to evaporate. 50 mL of minimal medium set at required pH was added to these tubes and pre-exposed (acclimated) cells of *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1 were inoculated at the optimum protein level. All the flasks were incubated at 180 rpm at the set temperature and samples removed at regular intervals for the analysis of residual DDT.

4a.2.5 Analytical methods

4a.2.5.1 Extraction and analysis of residual DDT

The samples (whole flasks), removed at different periods of incubation, were extracted thrice with equal volumes of dichloromethane. The three solvent extracts were pooled and passed through column containing sodium sulphate (anhydrous) and activated florisil. These fractions were concentrated at room temperature to dryness and resuspended in a known volume of HPLC grade acetone.

4a.2.5.2 Quantifications of Residual DDT

Residual DDT was estimated by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

4a.2.5.2 a Thin Layer Chromatography

Thin layer chromatography (TLC) was done on silica gel G 60-100 mesh uniformly spread over a 20 x 20 cm^2 glass plate. The thickness of the gel was set at 300 μm. These plates were left to dry at room temperature after spreading (using Camag automatic TLC plate spreader, Germany) then activated at 100^oC for 1 h. A known volume of the resuspended residual extract of DDT (acetone solution) was spotted on to these plates. Spotted plates were developed in cyclohexane, air-dried and the residue was detected by spraying *o*-tolidine (2% solution in acetone) followed by exposure to bright sunlight. The chloro-compounds give peacock green/ peacock-blue colour with this chromogen. Spots were delineated by marking with a needle and area measured. Quantity of DDT in each spot was estimated from a standard graph prepared for √area *vs* log (DDT concentration).

4a.2.5.2b Gas Chromatography

Concentrated residual substrate was resuspended in a known volume of HPLC grade acetone and gas chromatography was done using Chemito 1000 series gas chromatograph (Nasik, India). 1μl of the extract suspension was injected in to a BP-5 capillary column (30m x 0.25 mm ID) set at 180^{o}C and programmed as: 180^{o}C for 10 min and a rise @ 2^{o}C/ min up to 220^{o}C and maintained there for 2 min. Injector was maintained at 250^{o}C while electron capture detector (Ni^{63}) was maintained at 280^{o}C. Pure nitrogen gas was used as the carrier @ 1 mL min^{-1}. Under these conditions, the standard retention time for DDT was 28.16 min. Quantification of DDT in the sample was done using the area under the peak with and comparing it with the standard under same conditions.

4a.2.6 Kinetics of DDT degradation

During the degradation of DDT by bacterial strains, the carbon supply will not be sufficient enough to promote growth. The carbon that is obtained during the mineralisation of DDT could only be used for just sustaining the bacterial population inoculated initially. Therefore growth could not be taken up as a parameter for studying the degradation kinetics. Since the whole cells would behave as an enzyme system that acts on supplied DDT and transform it, kinetics by these bacteria was carried out using the enzyme principles. Degradation of DDT was studied using first order kinetics with whole cell biocatalysts with kinetic equation as derived below:

The rate of depletion of substrate is given by

$\frac{-dc}{dt} = k\left(C_t - C_f\right)$, Where –dc/dt represents the rate of decrease in concentration of DDT at time 't', k is the degradation rate constant and C_t stands for the concentration of DDT at time t whereas C_f is the final DDT concentration.

Rearranging the terms, we get

$$\frac{dc}{C_t - C_f} = -kdt$$

Integrating both sides

$$\int_{c_i}^{c_f} \frac{dc}{C_t - C_f} = -\int_0^t kdt$$

i.e.

$$\left[\ln\left(C_t - C_f\right)\right]_{c_i}^{c_f} = -k\left(t - 0\right)$$

$\Rightarrow [\ln(C_t - C_f) - \ln(C_i - C_f)] = -kt$

$\Rightarrow \ln\frac{C_t - C_f}{C_i - C_f} = -kt$, Where C_i is the initial DDT concentration.

When a graph is plotted with $-\ln[(C_t - C_f)/(C_i - C_f)]$ *vs* time, the slope of the curve would give the value of k, which is the degradation rate constant.

4a.3 Results

4a.3.1 Inoculum Level

Experiments conducted with varying levels of inoculum to degrade 10 ppm DDT were carried out at 10, 20, 50, 100, 200, 250, 300, 500 and 800μg protein/ mL of the medium and incubated at ambient temperature for 72 h. Analysis of residual DDT after extraction in dichloromethane for each set in triplicate (values show the average of the three) has been depicted in Fig.4a.1. Degradation percentage was observed to increase with increase in inoculum level. After 200μg protein/mL there was no substantial increase in degradation in any of the strains. As the studies here concerned with higher concentrations of DDT as well, 350μg protein/mL, a fixed concentration that was little higher than the minimum efficient concentration required to achieve maximum degradation (i.e. 200μg protein/mL), was

considered for the optimisation of pH and temperature levels. The same concentration of protein was used for the degradation of different concentrations of DDT. The main aim was to obtain degradation with constant low inoculum level even at higher substrate concentrations. Degradation was observed to be very less even with increasing cell population in case of *Pseudomonas aeruginosa* DT-Ct2 (Fig.4a.1) and was hence not considered for the kinetics studies, but is an essential component in the efficient degradation of DDT in mixed population (sec. 3. 5.10)

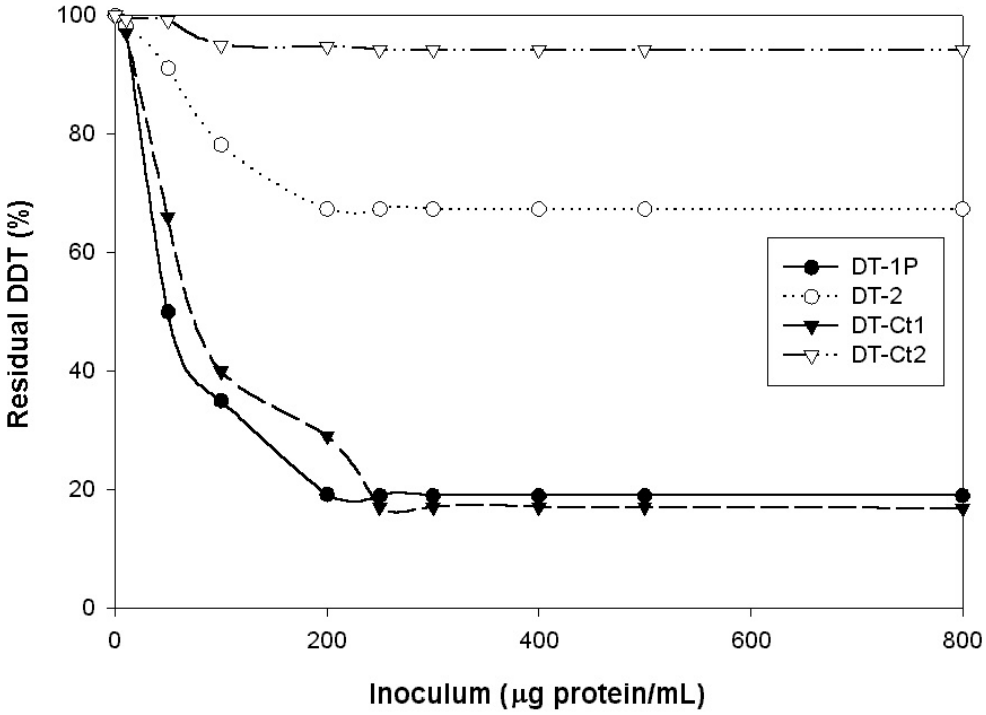
4a.3.2 pH and DDT Degradation

Pre- exposed cells (350μg protein/mL) were inoculated to flasks with 10 ppm DDT and basal buffered media set at required pH. The pHs tested for DDT degradation were 4.0, 5.0, 6.0, 7.0 and 8.0. All the flasks were incubated at ambient temperatures (26-30^{0}C) at 180 rpm for different time intervals. Samples were extracted for the analysis of residual DDT at various intervals of time. Fig.4a.2, Fig.4a.5 and Fig.4a.8 depict the degradation pattern by the three bacterial strains *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1 respectively at different intervals of time in different pH.

4a.3.2a Degradation by Serratia marcescens DT-1P

Degradation in *Serratia marcescens* DT-1P at different pH has been shown in Fig.4a.2. Degradation at pH 4, 5, 6 and 8 was very less and a steady state was reached by the end of 72 h. Thereafter there was no decrease in the DDT levels. 20%, 23%, 33% and 23% degradation was observed at pH 4, 5, 6 and 8 respectively (Fig.4a.2). At pH 7, degradation was complete and there was a gradual decrease in the substrate level at this pH. Fig.4a.3 shows the kinetics of DDT degradation at various pHs and the linear fitting to each set of degradation is shown. The curves fit well with

Fig.4a.1 Effect of inoculum level on 10ppm DDT degradation by the four strains (after 72 h of Incubation), (pH 7.0-7.5)



the data points with R^2 of 0.9324, 0.9106, 0.9489, 0.9414 and 0.8347 respectively for pH 4, 5, 6, 7 and 8. The degradation rate constants were calculated as $0.0536h^{-1}$, $0.0465h^{-1}$, $0.0618h^{-1}$, $0.15471h^{-1}$ and $0.0338h^{-1}$ for pH 4, 5, 6, 7 and 8 respectively using MS-Excel 2000 software. These rate constants were plotted against the pH. Microsoft excel 2000 was used to fit the curve in this data. The best fit was obtained that followed a third degree polynomial trend (Fig.4a.4). The equation satisfying this curve can be given as

Degradation rate constant at a given pH

$$^{1P}K_{pH} = -0.0197x^3 + 0.1664x^2 - 0.3934x + 0.3052; (R^2 = 0.8169)$$

Where, $^{1P}K_{pH}$ is the rate constant of DDT degradation by DT-1P at different pH and x is the pH.

The maximum degradation rate constant for *Serratia marcescens* DT-1P was at pH7 (4a.4), the maximum degradation observed was also observed at pH 7 (Fig.4a.2). Therefore, it was concluded that pH 7 was optimum for the degradation studies as it gave maximum rate and percentage of degradation.

4a.3.2b Degradation by Pseudomonas fluorescens DT-2

As seen in Fig.4a.5, 40.2%, 41%, 59.1%, 46.7% and 20% degradation was observed at each of the pH 4, 5, 6, 7 and 8 respectively. At pH 4 and pH 8 there was no apparent degradation till 24 and 36 h respectively, degradation at other pHs was observed from the very initial stages of sampling (Fig.4a.5). Though there was no degradation at pH 4 in the beginning, the final concentration of DDT, at the end of 144 h of incubation showed a close comparison with pH 5. pH 7showed a gradual decline in the DDT levels as pH 6, but the latter was observed to be more

Fig.4a.2 Degradation of 10 ppm DDT at different pH by *Serratia marcescens* DT-1P

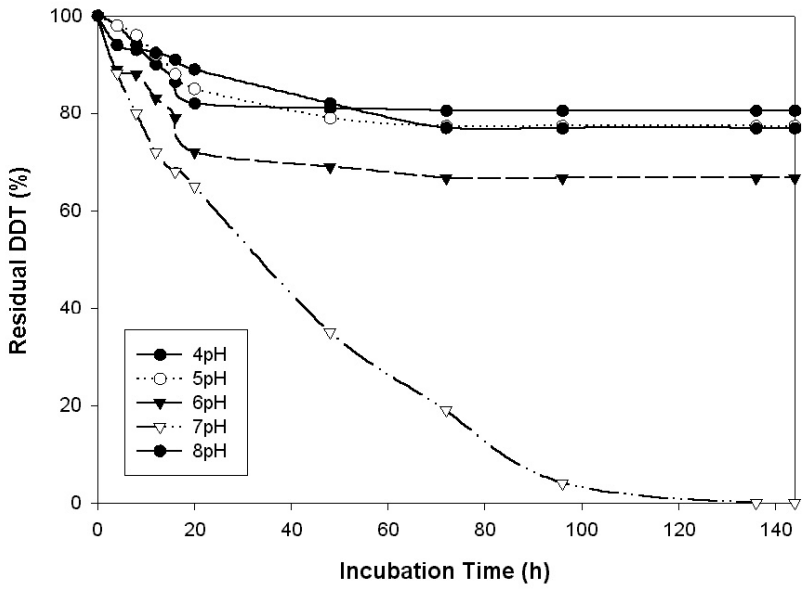


Fig.4a.3 Degradation trend of 10ppm DDT in different pH by *Serratia marcescens* DT-1P

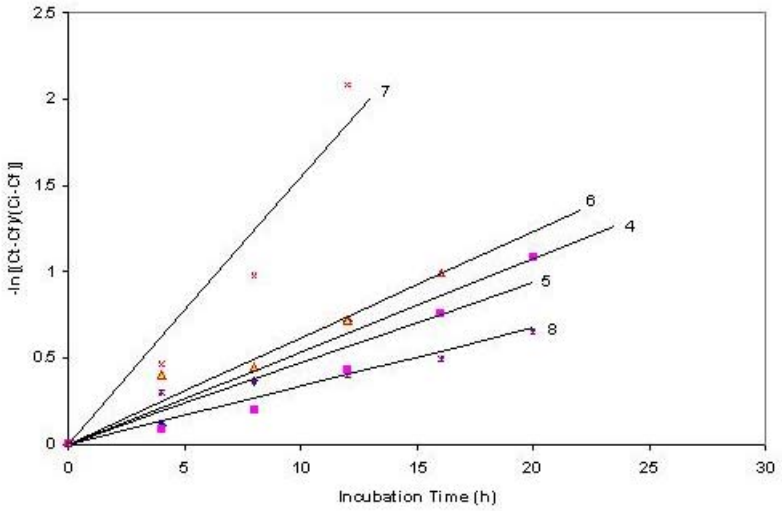
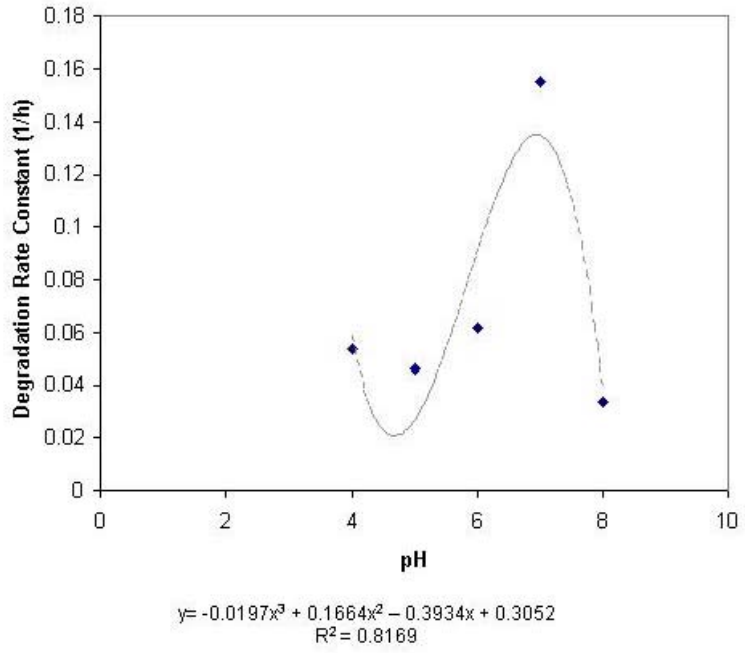


Fig.4a.4 Degradation rate of 10ppm DDT at different pH by *Serratia marcescens* DT-1P



$y = -0.0197x^3 + 0.1664x^2 - 0.3934x + 0.3052$
$R^2 = 0.8169$

Fig.4a.5 Degradation of 10ppm DDT at different pH by *Pseudomonas fluorescens* DT-2

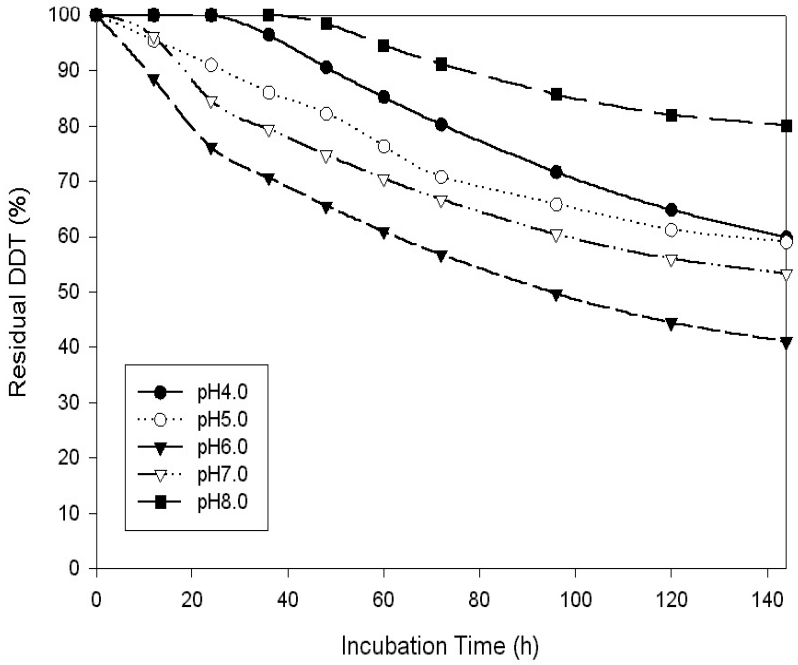
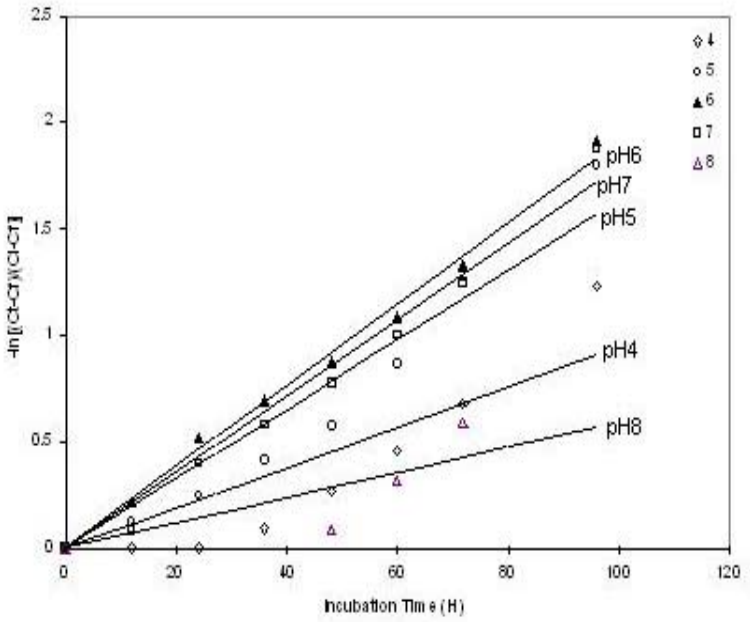


Fig.4a.6 Degradation trend of 10ppm DDT in different pH by *Pseudomonas fluorescens* DT-2



favourable for efficient degradation (Fig.4a.5). Fig. 4.6 shows the kinetics of DDT degradation of at different pH by *Pseudomonas fluorescens* DT-2. The linear curves here fitted (using MS Excel 2000) the data points well with the respective R^2 values of 0.7942 (good fit), 0.9347, 0.9932, 0.978 and 0.6774 (fair fit) for pHs 4, 5, 6, 7 and 8. The calculated degradation rate constants at these pHs, using Microsoft Excel 2000, were 0.0094h^{-1}, 0.0163h^{-1}, 0.0191 h^{-1}, 0.0179 h^{-1} and 0.0059 h^{-1} respectively for pHs 4, 5, 6, 7 and 8. When the degradation rate constants were plotted against the pH, the best fit followed a quadratic trend (Fig.4a.7) depicting the following equation:

Degradation rate constant at a given pH

$$\boxed{{}^{DT\text{-}2}k_{pH} = -0.003x^2 + 0.0353x - 0.0846;\ (R^2=0.9611)}$$

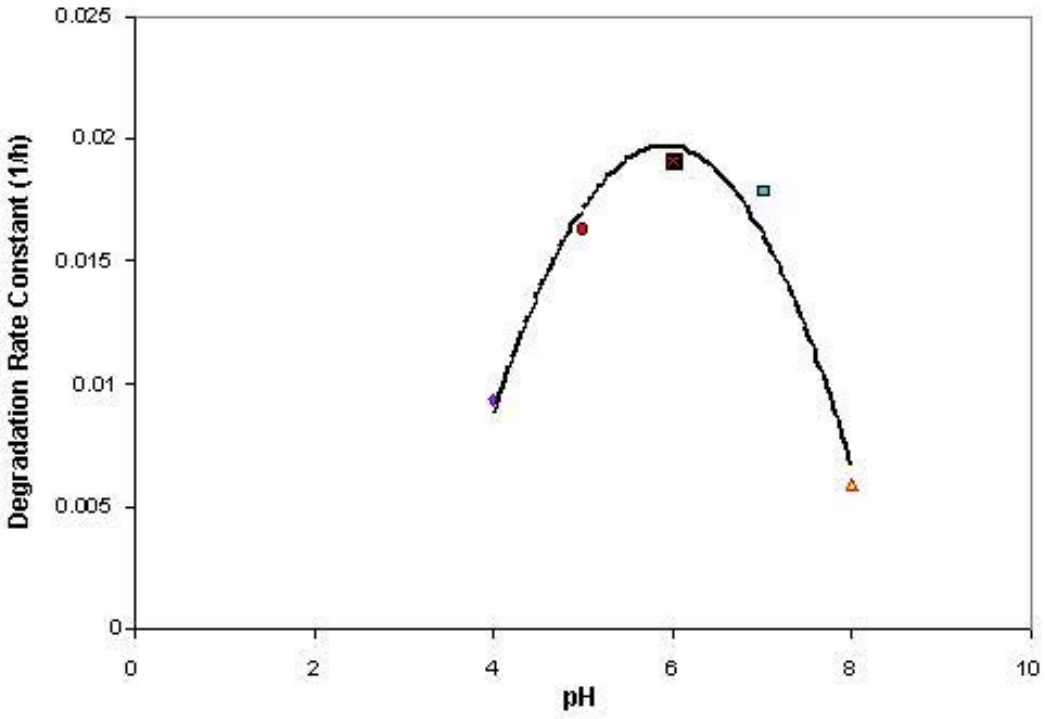
Where, ${}^{DT\text{-}2}k_{pH}$ = Degradation Rate constant of DT-2 at Different pH and x = pH.

The optimum pH that was considered for further degradation studies was 6 (Fig.4a.7).

4a.3.2c Degradation by Pseudomonas aeruginosa DT-Ct1

Pseudomonas aeruginosa DT-Ct1 degraded the added 10ppm DDT well in all pH except at pH 4 where the degradation was only up to 27% of the initially added concentration (Fig.4a.8). 66.6%, 79%, 96.3% and 85.3% depletion in the DDT concentration was observed at pH 5, 6, 7 and 8 respectively in the same period of time (Fig.4a.8). The kinetic graph for the degradation of 10ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 is shown in Fig.4a.9. The MS-Excel-2000-supported linear curve fitting for these data points showed a good fit with R^2 values of 0.8093, 0.9323, 0.9446, 0.9749 and 0.9285 for pH 4, 5, 6, 7 and 8 respectively. The slopes of these curves gave the rate constants as 0.0108h^{-1}, 0.0157h^{-1}, 0.019 h^{-1}, 0.0247 h^{-1} and

Fig.4a.7 Degradation Rate of 10ppm DDT in Different pH by *Pseudomonas fluorescens* DT-2



$y = -0.003x^2 + 0.0353x - 0.0846$

$R^2 = 0.9611$

Fig.4a.8 Degradation of 10ppm DDT in different pH by *Pseudomonas aeruginosa* DT-Ct1

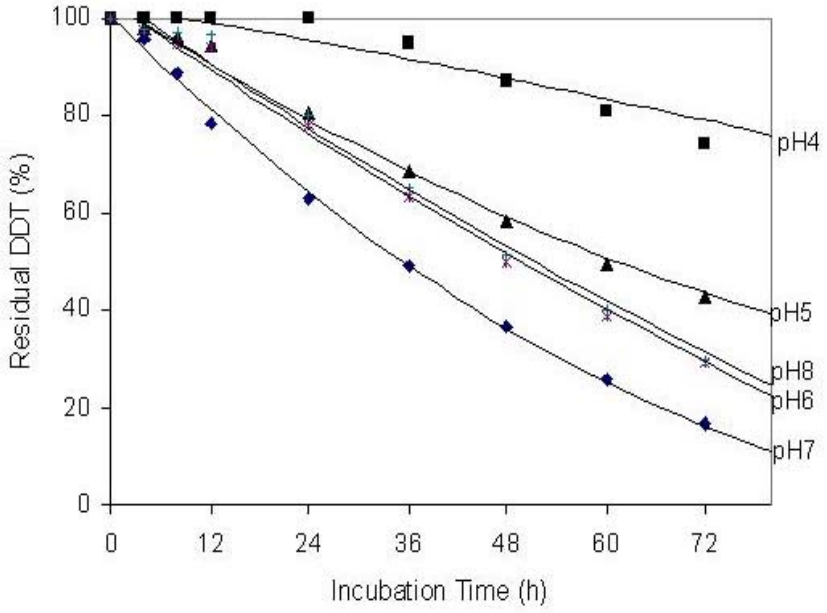
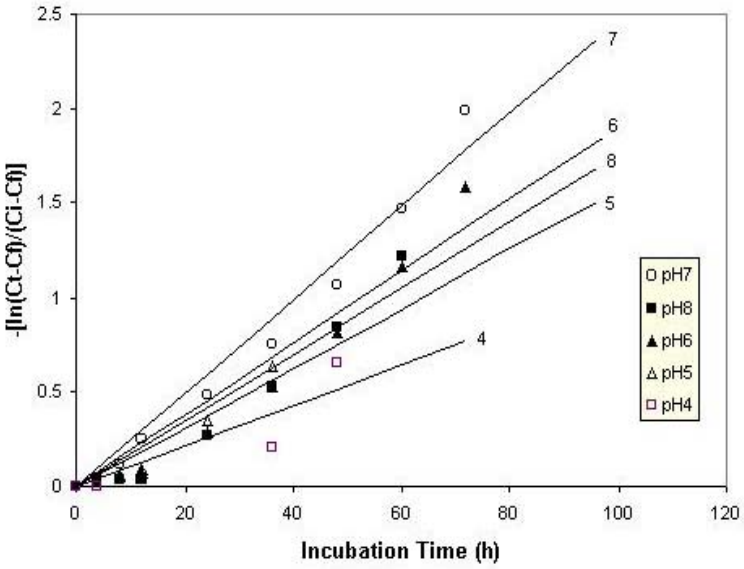


Fig.4a.9 Degradation trend of 10ppm DDT in different pH by *Pseudomonas aeruginosa* DT-Ct1



0.0175 h^{-1} respectively for pH 4, 5, 6, 7 and 8. Microsoft excel 2000 gave a fit for these rate constants against pH (Fig.4a.10) in terms of the equation:

Degradation rate constant at a given pH

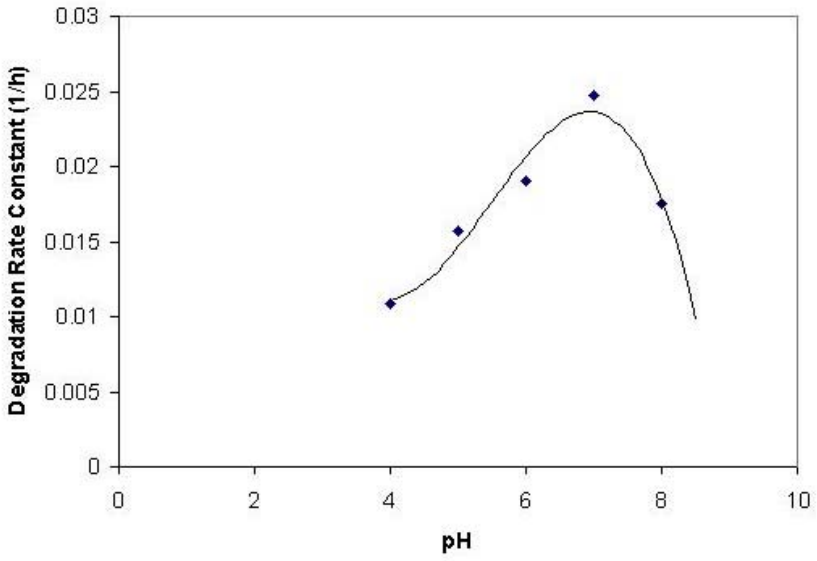
$$^{Ct1}K_{pH} = - 0.0009x^3 + 0.0154x^2 – 0.0776x + 0.1353;\ (R^2= 0.9479)$$

Where $^{Ct1}k_{pH}$ = DDT-Degradation Rate Constant at a given pH and x= pH
Maximum degradation was observed at pH 7 (Fig.4a.8). Maximum degradation rate constant was also observed at pH 7 (Fig.4a.10). Therefore, the optimum pH for further degradation studies was considered to be 7

4a.3.3 Temperature and DDT Degradation

Degradation kinetics at three different temperatures *viz* 20^0C, 30^0C and 40^0C was carried out using the optimum level of inoculum set in the beginning (4a.3.1) (i.e. 350μg protein/mL) and at the optimum pH obtained in 4a.3.2. The optimum pH for *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1 were 7, 6 and 7 respectively. After the incubation for various time intervals at these three different temperatures, residual DDT was analysed. Results are given in Fig.4a.11, Fig.4a.14 and Fig.4a.17 respectively for *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1.

Fig.4a.10 Degradation rate of 10ppm DDT in different pH by *Pseudomonas aeruginosa* DT-Ct1

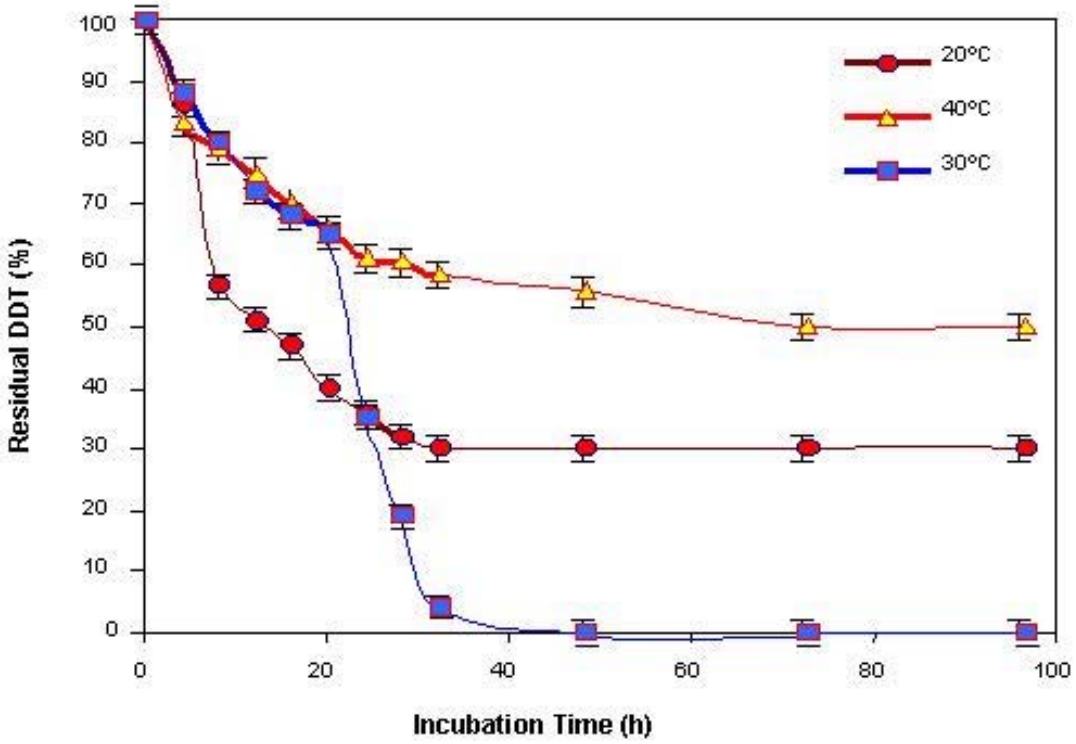


$Y = -0.0009x^3 + 0.0154x^2 - 0.0776x + 0.1353$
$R^2 = 0.9479$

4a.3.3a Degradation by Serratia marcescens DT-1P

Fig.4a.11 gives the degradation of 10ppm DDT at different temperatures of 20^0C, 30^0C and 40^0C by Serratia marcescens DT-1P at various time intervals of incubation. Degradation was effective at temperature 30^0C wherein 100% of the added DDT disappeared by the end of 48 h of incubation. At temperatures 20^0C and 40^0C the amount of degradation attained was 70% and 44.43% respectively after the same period of incubation. A total of 74.2% and 50% degradation could be achieved after 120 h of incubation at 20^0 and 40^0C respectively. At 30^0 and 40^0C the degradation was comparable with each one showing 65% residual DDT by the end of 20 h while 20^0C showed greater favour towards degradation in the first 20 h with only 40% residual DDT seen at that time. After 32 h of incubation, degradation apparently ceased in case of 20^0 and 40^0C while it took a steep incline in 30^0C after 20 h and at the end of 32 h only 4% residual DDT was observed. The kinetics of DDT degradation at these three temperatures by Serratia marcescens DT-1P has been shown in Fig.4a.12 (up to 20 h) with the linear curve fitting done using MS-Excel-2000 with R^2 values for 20^0, 30^0 and 40^0C curves are 0.9632, 0.9565 and 0.9541 respectively. The slopes for these linear curves, passing through the origin, gave the rate constant at each temperature. The rate constants $0.0843h^{-1}$, $0.0239h^{-1}$ and $0.0588h^{-1}$ respectively for 20^0, 30^0 and 40^0C have been described by means of Fig.4a.13a wherein the rates are for up to first 20 h of incubation. The rates for each of these temperatures between 20-32h of incubation were calculated as $0.101h^{-1}$ (for 20^0C), $0.233h^{-1}$ (for 30^0C) and $0.055h^{-1}$ (for 40^0C). This shows the rate of degradation at 30^0C to be very high compared to other two temperatures between 20 and 32h of incubation while there is only a marginal increment at 20^0C and it shows marginal decrease at 40^0C. Fig.4a.13b shows the degradation rates at

Fig.4a.11 Degradation of 10ppm DDT at different temperatures by *Serratia marcescens* DT-1P



three temperatures in the overall incubation period. The maximum rate was between 25-30^{0}C and hence 30 ^{0}C was considered optimum for further studies. The equation describing the overall rate constant at different temperatures was calculated to be:

Degradation rate constant

$$\boxed{{}^{1P}k_T = -0.0002x^2 + 0.0096x - 0.0319;\ (R^2=1)}$$

Where, ${}^{1P}k_T$= Degradation Rate constant at different temperatures and x=Temperature.

4a.3.3b Degradation by Pseudomonas fluorescens DT-2

The overall degradation of the added 10ppm DDT by *Pseudomonas fluorescens* DT-2 was comparable at temperatures 20^{0}C and 30^{0}C with 45% DDT disappearing by the end of 144h (Fig.4a.14). At 40^{0}C only 17% of the added DDT was degraded. Fig.4a.15 shows the degradation kinetics of 10ppm DDT by *Pseudomonas fluorescens* DT-2 with the linear fit for each of the three temperatures (R^2= 0.9726, 0.9372 and 0.9375 for 20^0, 30^0 and 40^{0}C respectively). The rates, as calculated by MS-Excel 2000 supported software, at these temperatures were 0.0252h^{-1}, 0.0299h^{-1} and 0.0231h^{-1} for 20^0, 30^0 and 40^{0}C respectively. When these rates were fitted against temperatures using MS-Excel 2000 software (Fig.4a.16), the best fit was obtained for the curve satisfying the quadratic equation:

Degradation Rate Constant at Different Temperatures

$$\boxed{{}^{DT\text{-}2}k_T = -6(10^{-5})\,x^2 + .0033x - 0.0187;\ (R^2=1)}$$

Fig.4a.12 Degradation trend of 10ppm DDT at different temperatures by *Serratia marcescens* DT-1P

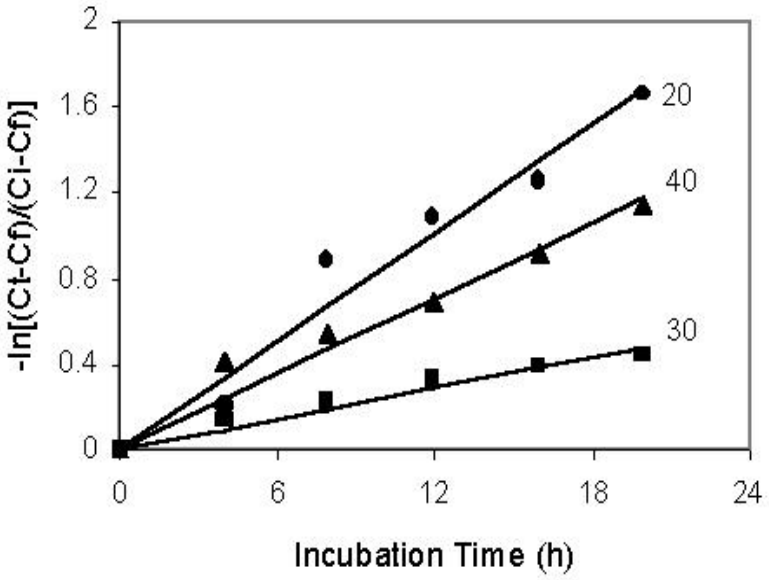


Fig.4a.13a Degradation rate of 10ppm DDT at different temperatures by *Serratia marcescens* DT-1P

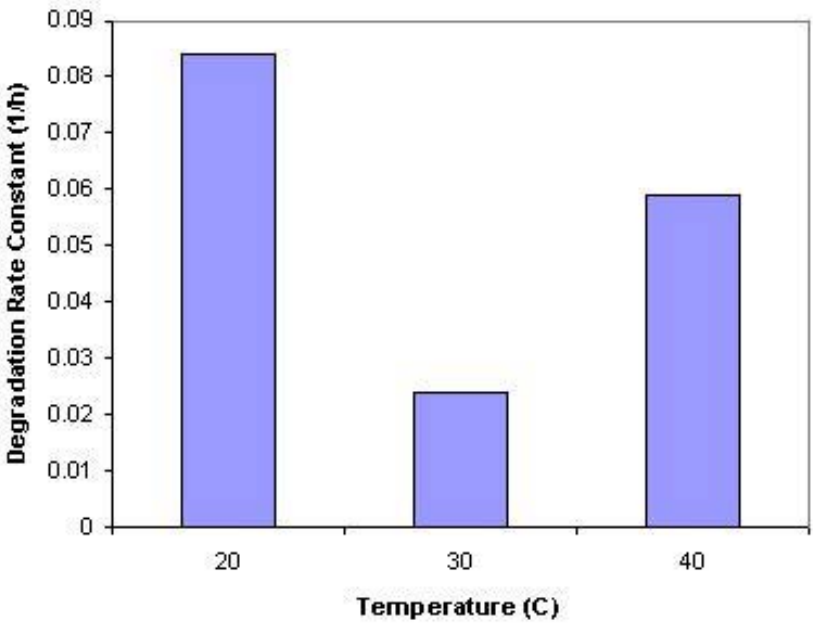
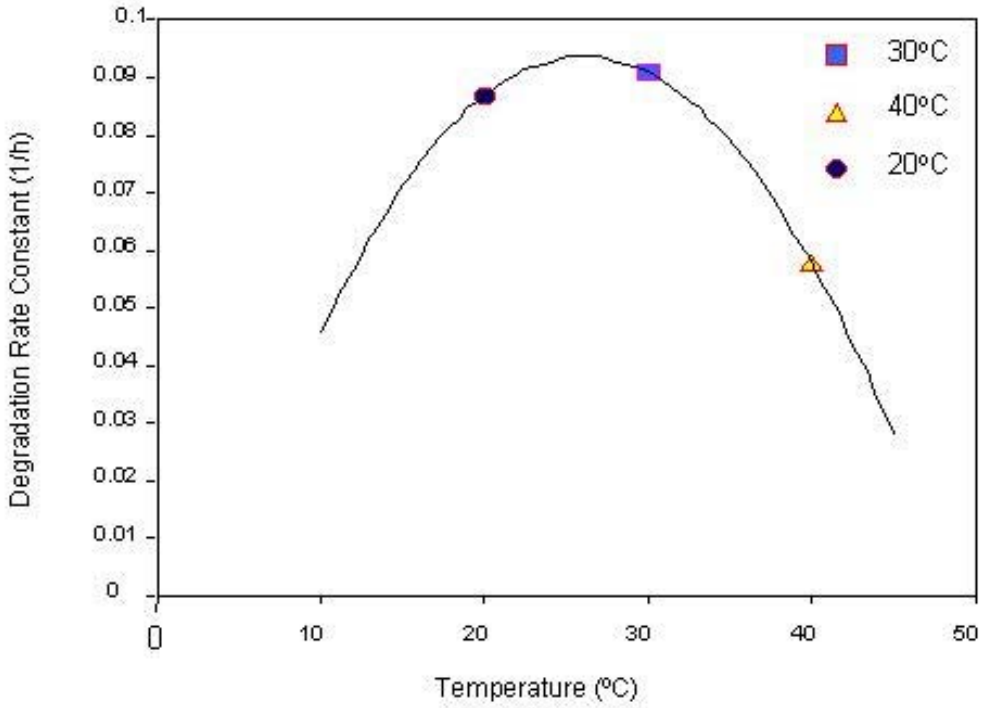


Fig.4a.13b Degradation rate of 10ppm DDT at different temperatures by *Serratia marcescens* DT-1P



$y = -0.0002x^2 + 0.0096x - 0.0319$

$R^2=1$

Fig.4a.14 Degradation of 10ppm DDT at different temperatures by *Pseudomonas fluorescens* DT-2

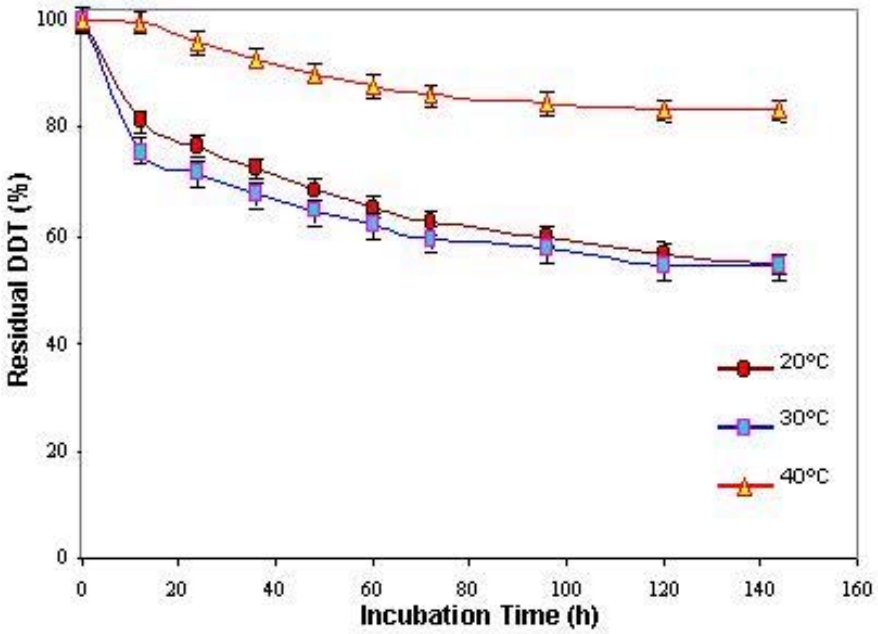


Fig.4a.15 Degradation Trend of 10ppm DDT at Different Temperatures by *Pseudomonas fluorescens* DT-2

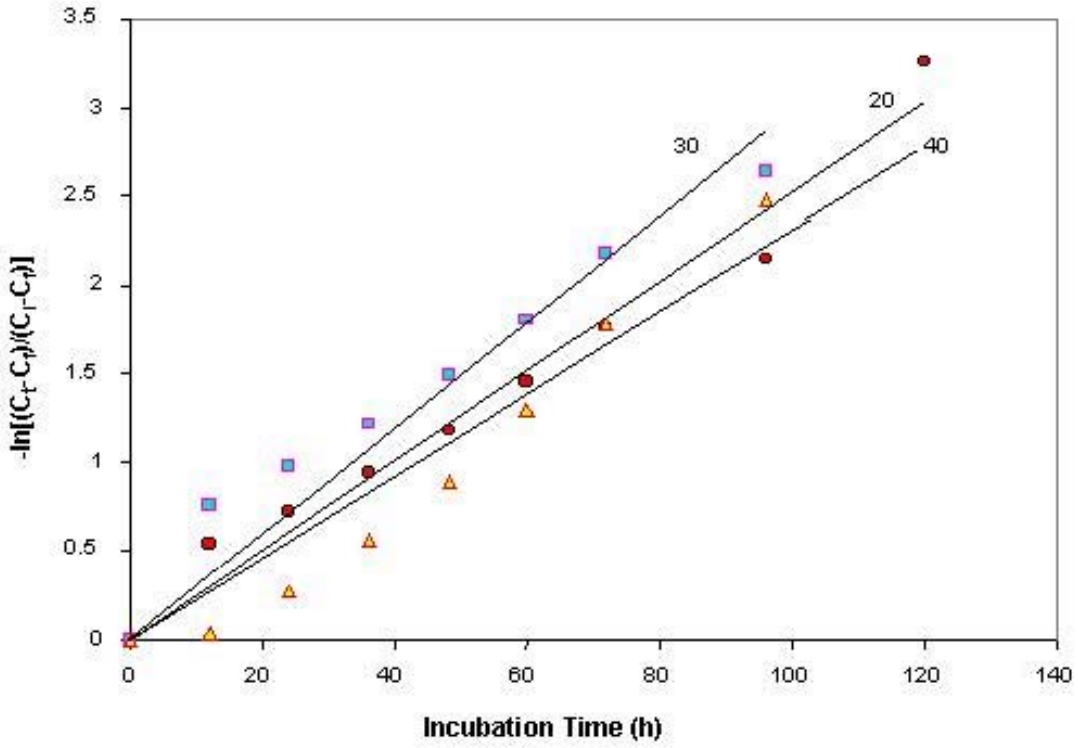
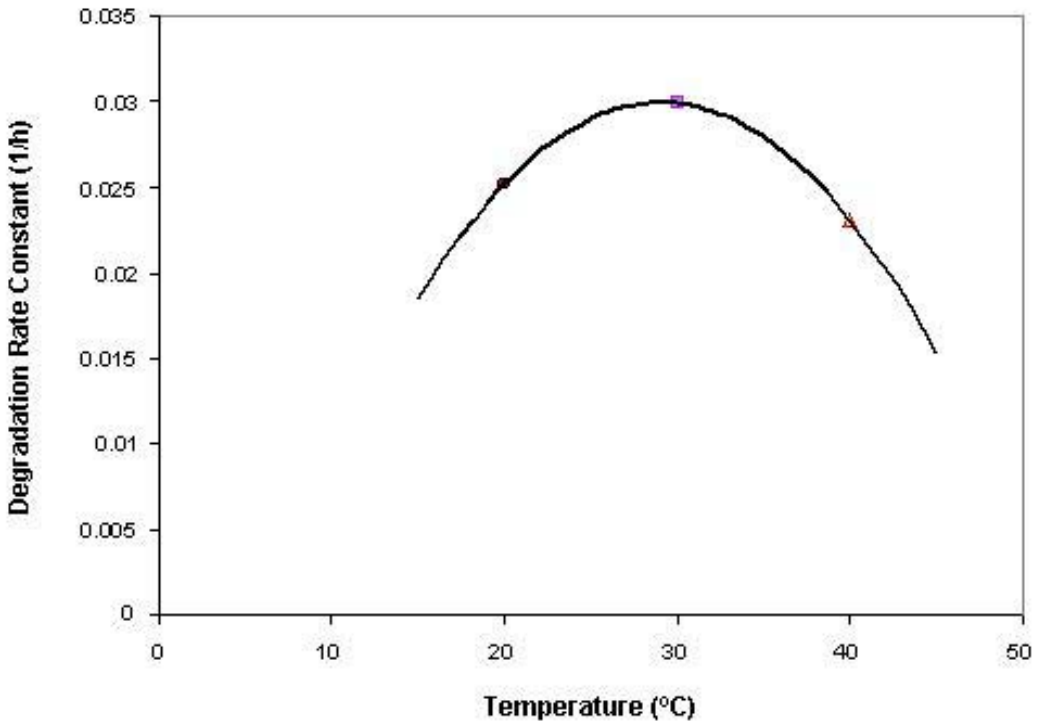


Fig.4a.16 Degradation Rate of 10ppm DDT at Different Temperatures by *Pseudomonas fluorescens* DT-2



$y = -6x10^{-5} x^2 + 0.0033x - 0.0187$

$R^2 = 1$

Where $^{DT-2}k_T$ =Degradation rate constant at different temperatures by *Pseudomonas fluorescens* DT-2 and x= Temperature.

Though the degradation was comparable at 20^0 and 30^0C, the rate constant was higher at 30^0C. Hence 30^0C was considered as optimum temperature condition for further studies.

4a.3.3c Degradation by Pseudomonas aeruginosa DT-Ct1

Pseudomonas aeruginosa DT-Ct1 could degrade 100% of the supplied 10ppm DDT by the end 96 h of incubation at 20^0C while it could degrade up to 79% and 52% at 30^0 and 40^0C respectively in the same period (Fig.4a.17). The degradation rate constants were calculated as $0.0236h^{-1}$, $0.0182h^{-1-}$ and $0.0154h^{-1}$ respectively for 20, 30 and 40^0C, by plotting the $-\ln[(C_t - C_f)/C_i - C_f)]$ *vs* Time (Fig.4a.18) and calculating the slope for the linear fit for these three sets of data points (R^2= 0.9499, 0.9354 and 0.8864 for 20^0, 30^0 and 40^0C respectively) using MS-Excel 2000 software. The rate constants thus obtained were plotted against temperature (Fig.4a.19) and MS-excel 2000 software was used to get the best fitting curve and was obtained as a curve satisfying the linear equation as:

Degradation rate constant

$$\boxed{^{Ct1}k_T = -4(10^{-4})x + 0.0314;\ (R^2 = 0.9676)}$$

Where $^{Ct1}k_T$= degradation rate constant by *Pseudomonas aeruginosa* DT-CT1 at different temperatures and x= Temperature.

Major part of the microbial degradation had to be carried out at ambient temperature (26-30^0C), in the tropical regions, for less expensive investment, though the temperature at 20^0C was optimum and maximum degradation was observed at this temperature, 30^0C was also favourable

Fig.4a.17 Degradation of 10ppm DDT at different temperatures by *Pseudomonas aeruginosa* DT-Ct1

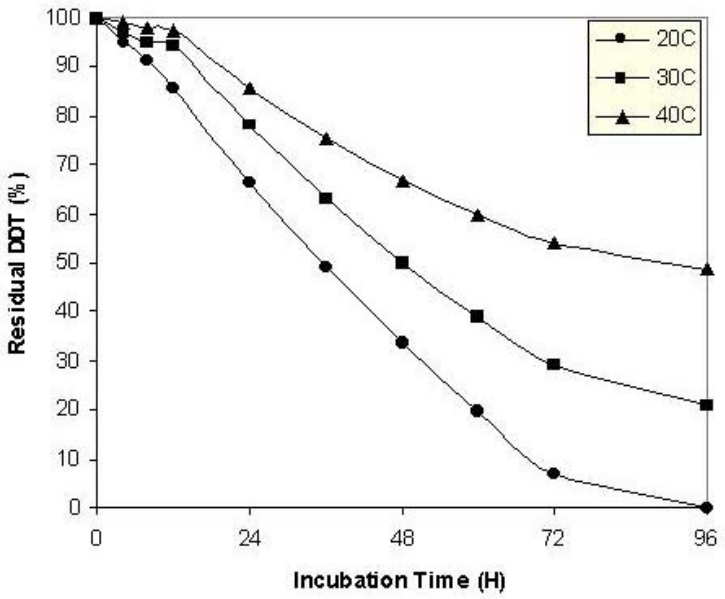


Fig.4a.18 Degradation trend of 10ppm DDT at different temperatures by *Pseudomonas aeruginosa* DT-Ct1

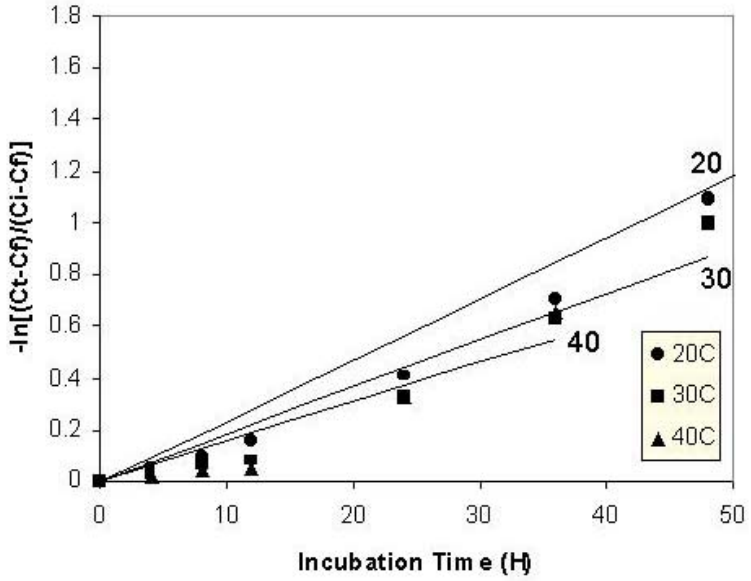
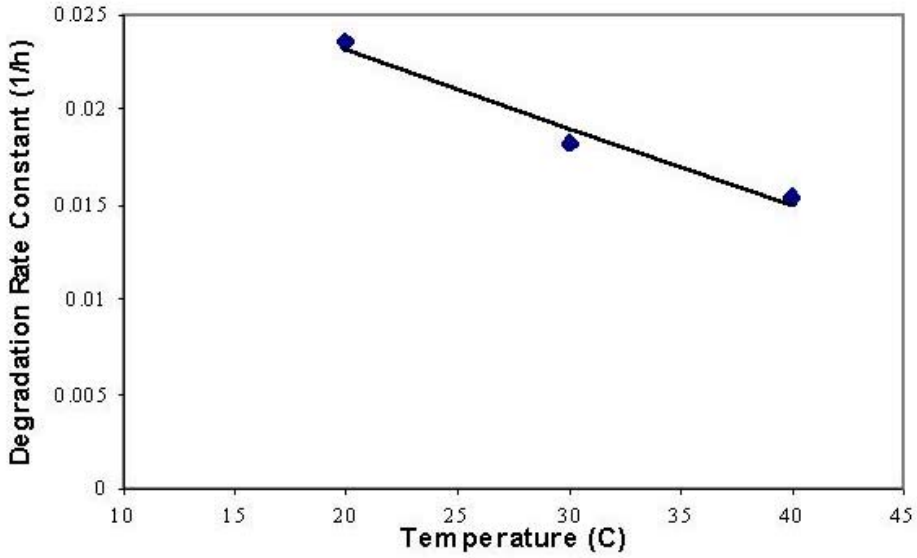


Fig.4a.19 Degradation rate of 10ppm DDT at different temperatures by *Pseudomonas aeruginosa* DT-Ct1



Y= -0.0004x + 0.0314
R^2 = 0.9676

with slight decreased rate of degradation but with an added advantage of being inexpensive as ambient temperature ranges between 26-30^{0}C and therefore it was chosen for the further degradation studies.

4a.3.4 Degradation of Different Concentrations of DDT

Degradation of different concentrations of DDT was studied with each of the three strains with the set optimum inoculum level, pH and temperature.

4a.3.4a Degradation by Serratia marcescens DT-1P

Degradation of different concentrations of DDT was carried out from 5ppm through 50ppm in shake flasks at pH 7 and temperature 30^{0}C with 350 μg protein/mL at various intervals of time. Samples in the form of whole flasks were extracted and analysed for the residual DDT by TLC and GC. The results given here are the comparable average values in each case. 5ppm, 10ppm and 15ppm DDT were degraded completely by 72h, 96h and 120h respectively (Fig.4a.20). 20ppm and 25ppm could be degraded up to 95 and 84% respectively (Fig.4a.20). 50ppm showed negligible quantity of degradation wherein only 4% DDT was degraded by the end of 24 h and the concentration remained constant thereafter (Fig.4a.20). The kinetic data points for all these concentrations (except 50ppm) are shown in Fig.4a.21. Microsoft Excel 2000 was used get the linear fit with R^2 values of 0.9877, 0.9862, 0.9741, 0.9561 and 0.9572 respectively for 5, 10, 15, 20 and 25ppm. The rate constants were also calculated by taking the slopes of these curves and they were found to be 0.028h^{-1}, 0.0241h^{-1}, 0.0202h^{-1}, 0.0153h^{-1} and 0.01051h^{-1} respectively for the concentrations 5, 10, 15, 20 and 25ppm respectively. These rate constants were plotted against the initial DDT concentrations and MS-Excel 2000- supported best curve fit was

Fig.4a.20 Degradation of Different DDT Concentrations at optimum conditions by *Serratia marcescens* DT-1P

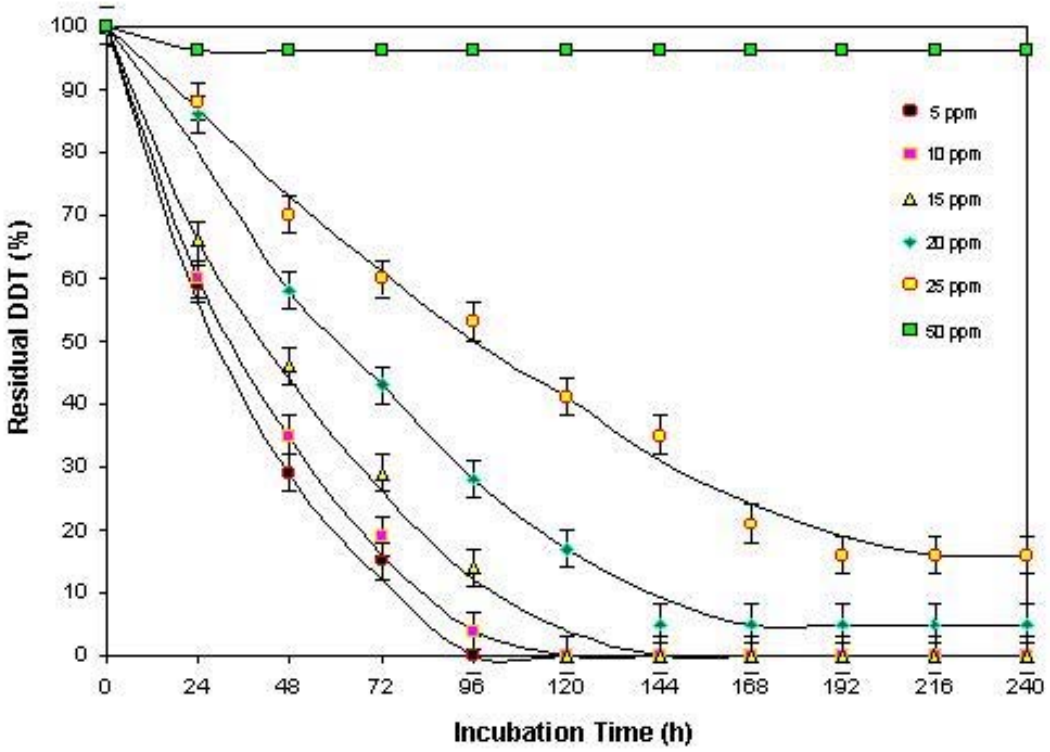


Fig.4a.21 Degradation Trend of Different DDT Concentrations at optimum conditions by *Serratia marcescens* DT-1P

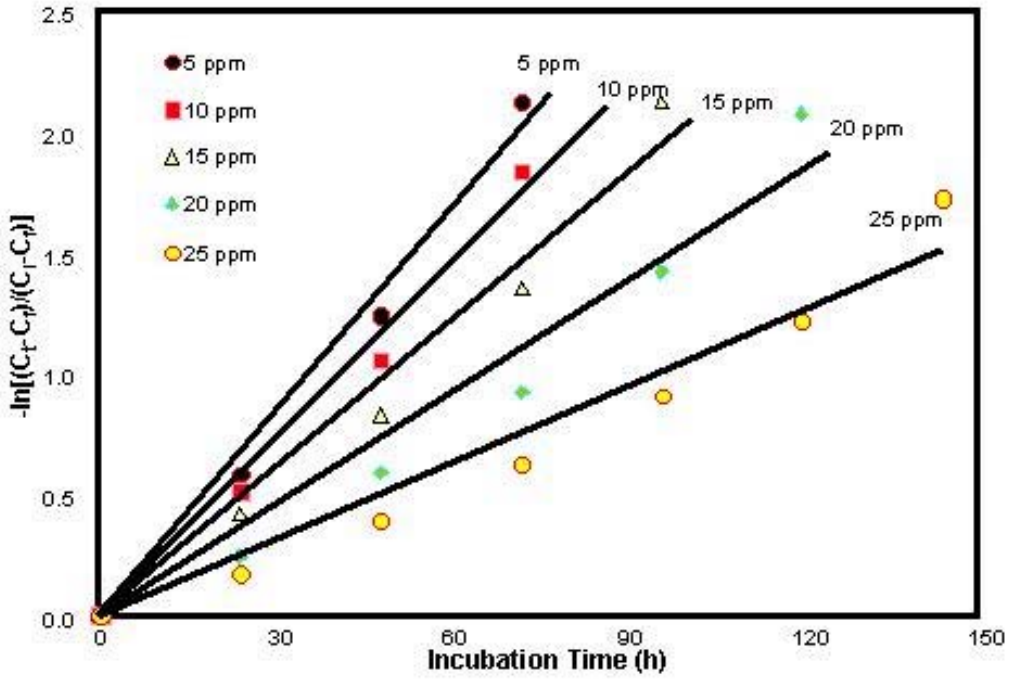
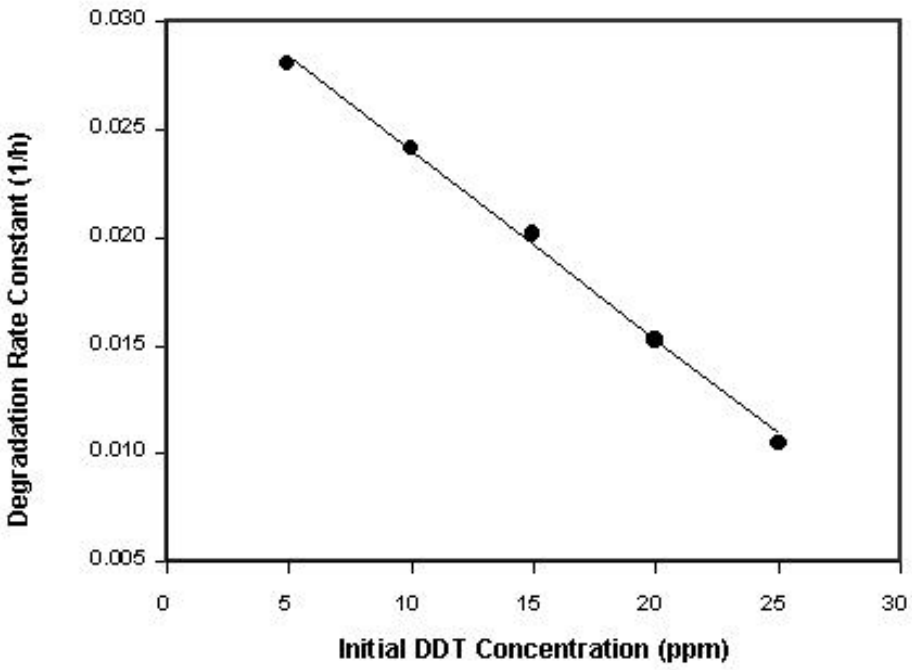


Fig.4a.22 Degradation Rate of Different DDT Concentrations at optimum conditions by *Serratia marcescens* DT-1P



$y = -0.0009x + 0.0328$
$R^2 = 0.9968$

obtained that followed a linear trend (Fig.4a.22) satisfying the following equation:

Degradation Rate Constant

$$\boxed{{}^{1P}k_C = -0.0009x + 0.0328;\ (R^2 = 0.9968)}$$

Where ${}^{1P}k_C$ is the degradation rate constant of *Serratia marcescens* DT-1P and x is the initial DDT concentration.

4a.3.4b Degradation by Pseudomonas fluorescens DT-2

Degradation of different concentrations of DDT, 5ppm through 50ppm, was conducted and the results are depicted in Fig.4a.23. The degradation of all the concentrations was incomplete even after 168h of incubation. 5ppm, 10ppm, 15ppm, 20ppm, 25ppm, 30ppm, 35ppm and 50ppm DDT could be degraded up to 58%, 56%, 49%, 45%, 43%, 40%, 17% and 1.5% respectively. These were used for the kinetic calculations and the data was fit to a linear curve using MS-Excel 2000 as shown in Fig.4a.24 with R^2 values calculated to be 0.9792, 0.9772, 0.878, 0.8774, 0.8702 and 0.853 respectively for 5, 10, 15, 20, 25 and 30ppm. The degradation rates, as calculated using the MS-Excel 2000 were $0.0134h^{-1}$, $0.0118h^{-1}$, $0.0112h^{-1}$, $0.0107h^{-1}$, $0.006h^{-1}$ and $0.0043h^{-1}$ respectively for each of the concentrations from 5 through 30ppm. The rate constants when plotted against concentrations the curve satisfying the best linear fit (Fig.4a.25) satisfied the following equation:

Degradation Rate Constant

$$\boxed{{}^{DT\text{-}2}k_C = -0.0004x + 0.0157;\ (R^2 = 0.9745)}$$

Fig.4a.23 Degradation of different DDT concentrations by *Pseudomonas fluorescens* DT-2 at optimum conditions

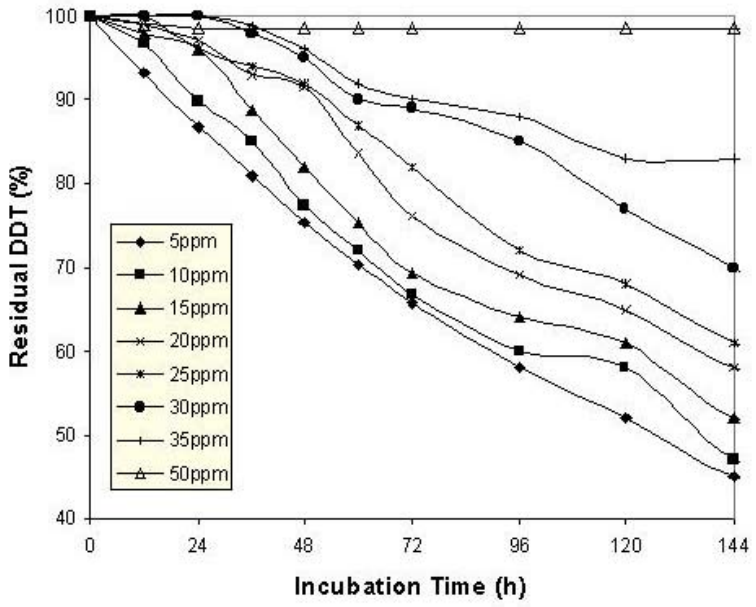


Fig.4a.24 Degradation trend of different DDT concentrations at optimum conditions by *Pseudomonas fluorescens* DT-2

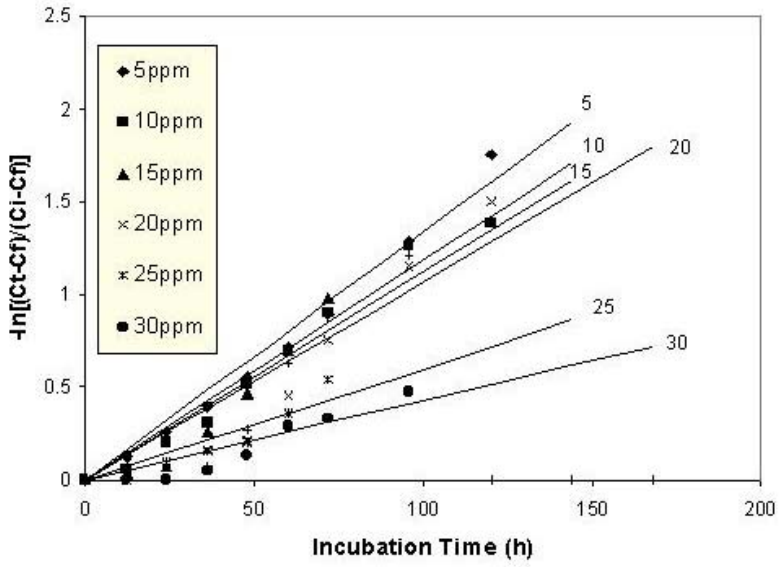
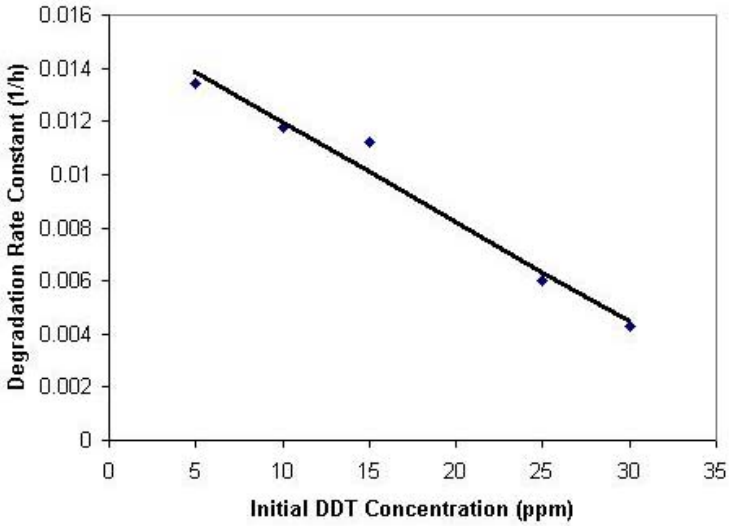


Fig.4a.25 Degradation rate of different DDT concentrations at optimum conditions by *Pseudomonas fluorescens* DT-2



y= - 0.0004x + 0.0157
R^2 = 0.9745

Where $^{DT\text{-}2}k_C$ is the degradation rate constant for *Pseudomonas fluorescens* DT-2 while x is the initial DDT Concentration.

4a.3.4c Degradation by Pseudomonas aeruginosa DT-Ct1

Pseudomonas aeruginosa DT-Ct1 when inoculated to different concentrations of DDT showed the decrease in substrate concentrations as depicted in the Fig.4a.26. The initially added 5ppm of DDT was completely degraded by the end of 96h whereas the initial DDT concentrations of 10ppm, 15ppm, 20ppm, 25ppm, 30ppm and 35ppm of were degraded only up to 75%, 62.5%, 58%, 54%, 38% and 24% by the end of 144 h. There was no more detectable degradation after this time up to 240h of incubation. The kinetic data points were used in MS-Excel 2000 for obtaining the rate constants for each of these concentrations and the rate constants were calculated as $0.0716h^{-1}$ (R^2=0.954), $0.06h^{-1}$ (R^2=0.9894), $0.0461h^{-1}$ (R^2=0.9615), $0.0182h^{-1}$ (R^2=0.7599), $0.0117h^{-1}$ (R^2=0.5986), $0.0074h^{-1}$ (R^2=0.4548) and $0.0036h^{-1}$ (R^2=0.3229) respectively for 5, 10, 15, 20, 25, 30 and 35ppm initial DDT concentration (Fig.4a.27). These rate constants were then used to obtain the equation for rate constants at various DDT concentrations by plotting against the concentrations (Fig.4a.28). The simplest equation fitting best to the observed rate constants was obtained using MS-Excel 2000 and is given by:

Degradation Rate Constant

$$\boxed{^{Ct1}k_C = -0.0024x + 0.0828;\ R^2 = 0.9908}$$

Where $^{Ct1}k_C$ is the degradation rate constant for *Pseudomonas aeruginosa* DT-Ct1 and x is the initial DDT concentration

Fig.4a.26 Degradation of different concentrations of DDT by DT-Ct1 at optimum conditions

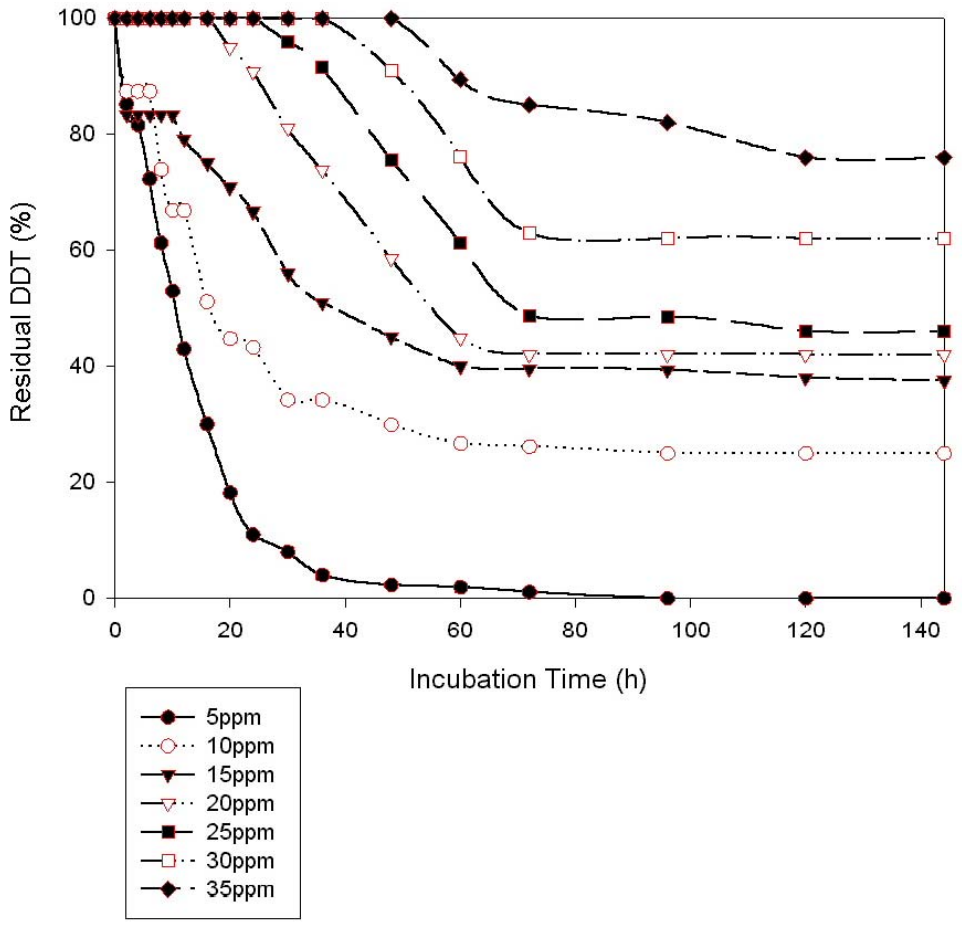


Fig.4a.27 Degradation Trend of Different DDT Concentrations at optimum conditions by *Pseudomonas aeruginosa* DT-Ct1

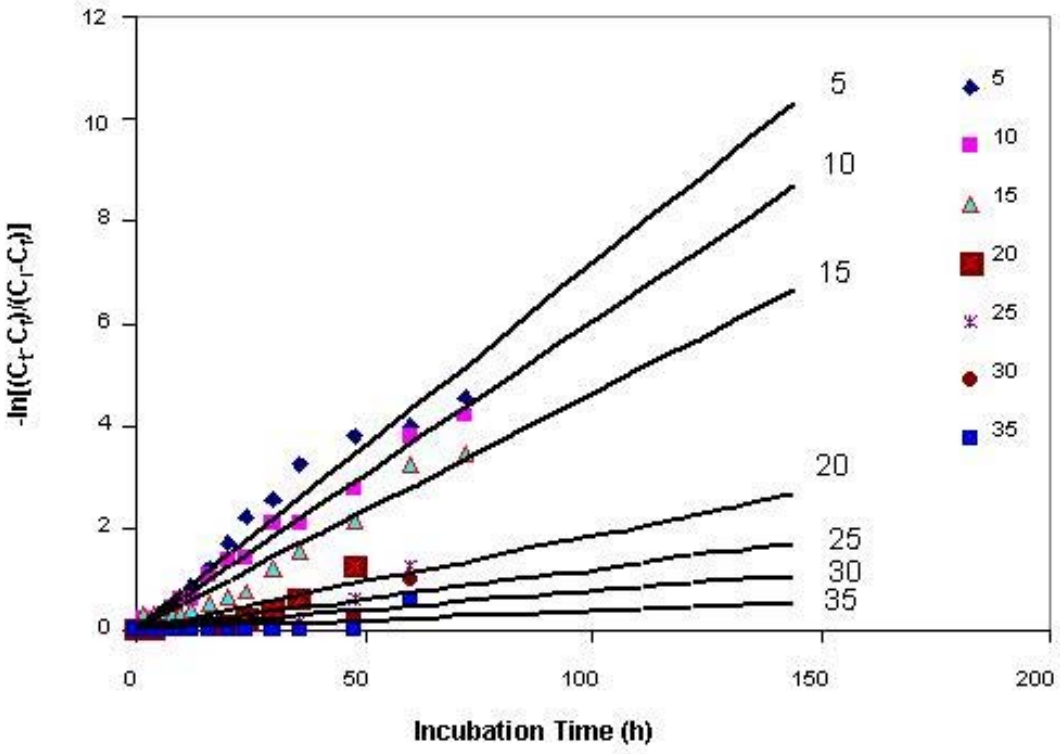
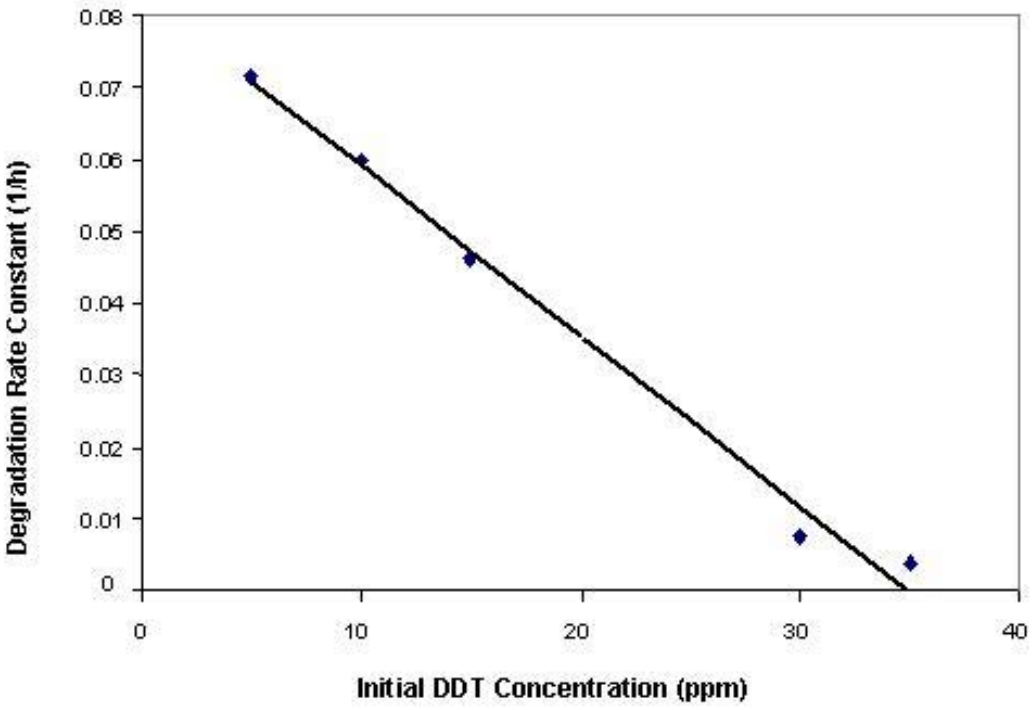


Fig.4a.28 Degradation Trend of Higher DDT Concentrations at optimum conditions by *Pseudomonas aeruginosa* DT-Ct1



$y = -0.0024x + 0.0828$

$R^2 = 0.9908$

4a.4 Discussion

Many workers have described kinetics of xenobiotic degradation in different systems (Focht and Shelton, 1987; Ely *et al,* 1995; Chang *et al,* 2001; Cort and Bielefeldt, 2001; Kharoune *et al*, 2002; Yerushalmi *et al.,* 2002; Bustard *et al,* 2002). The major consideration in these studies was the growth of the microorganisms that was accounted for the depletion of the xenobiotic concentration in the medium (Tros *et al*, 1996). Thus, most of the times it was the Monad's growth kinetics that was taken as the basis for this. The consideration of growth as a factor determining the depletion of the carbon source in the medium can be visualised only when the amount of carbon supplied to the microorganisms is sufficient enough to bring about such noticeable change in cell number. The carbon-limited nature of the xenobiotics will ensure a strong and selective pressure for the organisms capable of attacking these chemicals (Moos *et al,* 1980). But when the carbon is not enough to generate enough number of noticeable duplications, growth cannot describe the degradation of the xenobiotics. The cells, in this case, appear as if there is no metabolism taking place inside them and thus seem to be in resting state. During such events the cells are said to be resting (resting cells). In any case of resting cells, though the cell number does not increase markedly, the enzymes present in them are sufficient enough to act on the xenobiotic compound ultimately bringing a decline in the concentration of such polluting chemicals (Schonborn, 1987). In our studies also, the amount of carbon supplied by the added DDT was not sufficient to give a good biomass yield, but of course there was a decrease in DDT concentration with the passage of incubation time, thus indicating the involvement of enzymes already present in the cells. Therefore the enzyme kinetics was considered for working out DDT degradation. The cultures used were the ones isolated from the contaminated soil and water through long term enrichment. The consortium thus developed was capable of degrading up to 25ppm DDT completely (sec.3.5.2). The isolated strains were used to find the effect of each strain independently on DDT degradation and the equation model that satisfies the degradation process. When

these strains were tested for their ability to degrade DDT with increasing inoculum sizes, it was found that the degradation of studied 10ppm DDT improved with the increase in cell density up to 200 μg protein/mL of the medium and thereafter remained non-influential on the degradation. The degradation of different DDT concentrations (5ppm through 50ppm) was, therefore carried with slightly higher inoculum size than this. This could eliminate unnecessary bioaugmentation during the remediation process. Earlier reports reveal bioaugmentation as one of the necessary steps during the bioremediation of contaminated sites or water (Ritter and Scarborough, 1995). Here the environment is populated with high cell densities of the culture(s) specific for the xenobiotic degradation. Of course, there would be interactions between the native microbes and the inoculated culture(s) in the environment (Schonborn, 1987); this type of study can also help in understanding the synergistic or the antagonistic influence from the other organisms present in the vicinity of inoculated culture thereby giving a thorough understanding of the action on the pollutant. Moreover, if the augmentation is more than required, it may disturb the natural ecology at the site of bioremediation. Therefore if the inoculum added is just sufficient enough to act on the xenobiotic of our interest with no or least affect on the environment, would prove of great advantage and applicability in the process of cleaning the contaminated sites'/water bodies'/effluents'.

Enzymes usually are active at specific or in particular range of pH and temperature. Here we assume that the set of enzymes responsible for the degradation of DDT, at least the first or a couple of initial steps in the transformation of DDT to other metabolites, as a single unit acting on the substrate. Considering it as a single substrate-enzyme reaction, we carried out the degradation kinetics as a preliminary tool in understanding the degradation process of DDT by individual cultures capable of degrading DDT. There are no reports on the degradation kinetics of DDT so far to our knowledge. As the enzymes responsible for most of the xenobiotic degradation have low specificity (Grady, 1985), they can act on more than one or similar kind of substrates.

Therefore it can be predicted that the enzymes are present in the cells but need to be exposed to the substrate to be degraded (pre-exposure/acclimation). The residual substrate was related to the substrate concentration required for the induction of enzymes responsible for degradation of that chemical (Tros *et al*, 1996). The maximum degradation, in our studies with different pH levels and temperatures were observed to be pH 6 for *Pseudomonas fluorescens* DT-2 while it was 7 for the other two strains and temperature optimum was observed to be 20^0C for *Pseudomonas aeruginosa* DT-Ct1 and 30^0C for *Serratia marcescens* and *Pseudomonas fluorescens* DT-2. This would be of advantageous in the treatment of industrial effluents and water bodies as the temperature in tropical countries ranges from 20-30^0C. Neutral and near neutral pH is always advantageous for the treatment of the contaminated effluents as no additional chemical requirements need to be met for releasing the treated neutral effluent into the environment. This may go a long way in cleaner and healthier rivers and other water bodies further maintaining the ecological balance at low and comfortable investment of available resources. The acceptance of the substrate at different pH like 6-7 and temperatures such as 20-30^0C by at least one of the members of the consortium might prove helpful in the treatment of effluents in such pH and temperature ranges when treated with the consortium.

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 (Robra, 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 *et al* (1993) isolated two strains of bacteria, *Bacillus* sp. B75 and an unidentified gram-variable rod B116, which degraded DDT at extremely low level of 10pg/mL. In our studies the bacterial isolates were able to degrade low concentrations of DDT more effectively in short period of time and the maximum threshold varied depending on the culture i.e. *Serration marcescens* DT-1P could degrade up to 15ppm DDT above which the degradation observed was partial. Similarly, *Pseudomonas aeruginosa* DT-Ct1 could degrade 5ppm completely whereas *Pseudomonas fluorescens* DT-2 partially degraded even 5ppm of the added initial DDT. This reflects the differential behaviours of the microorganisms obtained from the common consortium. This could also be related to the enzymes present in these organisms for the degradation of the substrate and its metabolites. The effectiveness of consortium is evident from the fact that the individual strains (except *Serratia* that degraded up to 15ppm DDT) were unable to degrade the substrate at even low concentrations while the consortium was able to degrade higher concentrations of the same substrate in less time (sec.3.5) This is a reflection of the synergistic effect of mixed cell population wherein the enzymes responsible for the initial breakdown of the substrate might be present in one or more of the constituent members while the other enzymes for further breakdown could be active from the other or same members. The total cascade of the enzyme machinery may come into play when all these individual members are allowed to work simultaneously/in harmony in the treatment plants. Similar observations have been reported by Horvath (1972), Grady (1985), wherein the initial reaction carried out by a non-specific enzyme appear to stop if the enzyme specific for the metabolite degradation are not present. The transformation product was transformed by a second species until, finally, a product resulted that was a normal metabolite and could be utilised completely as a carbon and energy source. They concluded that complete pathways of degradation were more likely to be operational through the combined effects of different types of organisms within a community than to be present within a single

species. The depression of some inductive enzymes may offer new opportunities for degradation of the chemical pollutant by the mixed population. The models developed through the curve fitting could be used to predict the degradation rate constants of the available substrate concentrations and other conditions of pH and temperatures. The degradation rate constants at the low concentrations of DDT was observed to be higher and decreased with concentration. This could be due to the toxicity of the metabolite formed in the initial reactions and also could be due to the non-availability of the further specific degradative enzymes. In few of the strains the initially required enzyme may also get inhibited because of the substrate. Moreover, the microbial degradation kinetics of xenobiotics is a complicated process that could change with a slight variation in the set conditions. The microorganisms do show variations from time to time due to many factors and should be taken into consideration while dealing with them. Therefore the knowledge of kinetics would bring an understanding of the bioremediation process of xenobiotic compounds under various conditions that prevail prior to the treatment. Great care must be exercised in exploiting the results from the kinetic data to the field conditions.

Chapter 4B

Optimisation of Conditions for DDT Degradation

As our understanding of state-of-the-art models increases, they too may become popular in the future.

Bishop and Kinner (1987)

4B.1 Introduction

Once the cultures were isolated and identified, they were tested for the conditions necessary for the optimum degradation of DDT and kinetics of biodegradation was worked out. While conducting the kinetic experiments only one variable (parameter) was varied maintaining other parameters constant at optimum. In the previous chapter, the effect of inoculum level, pH, temperature and initial DDT concentration was discussed. There the effect of inoculum, pH and temperature were studied only at an initial DDT concentration of 10ppm. Based on these results and the feasibility of conditions under safe and inexpensive form in the environment, different initial concentrations of DDT were studied for degradation. The results of the kinetics could predict the degradation rate constants at the worked out conditions at set parameters. When all these parameters are considered together, their interactions would influence the degradation process. For such interactions the optimisation of conditions for the prediction of required response (degradation) needs to be worked out. The best way of understanding the interactions among various parameters and achieving the optimised conditions for any process could be through the response surface methodology (RSM). For this the preliminary studies about the consortium's preference of temperature and pH was done and then the range of the parameters selected for optimisation. Also the previous chapter studies established the range of various parameters. It is a well-established fact that the biodegradation process is cell-density dependant. The contaminant concentration also forms one of the major factors in its biodegradation in the environment. As observed in the preliminary studies, incubation time and pH also play a vital role in reducing the contaminant concentration. With all these major factors taken into consideration, an attempt was made to obtain a regression equation model to describe the degradation of DDT under the influence of these factors. Response surface methodology (RSM) is one of the accepted methods to get to such models wherein the conditions set for are satisfied with the optimum expected response. RSM has been done mainly for the engineering aspects as a basic tool for the

optimisation and validation studies. In recent years, microbial modelling has attracted the attention of researchers to predict the microbial response. Most reports are concentrated in the maximum response, either in terms of product yield or microbial growth. Till date there are no reports on the adaptation of RSM for the optimisation of degradation conditions for DDT or any other pesticide by microorganisms. One of such attempt was made in our laboratory to develop a regression model that could predict the degradation process under a given set of conditions.

4B.2 Materials and Methods

4B.2.1 Chemicals

DDT used in this study was 98% pure and procured from Sigma-Aldrich Chemical Company, Mo, USA. Peptone and Glycerol were procured from HiMedia Laboratories, Mumbai, India. Solvents were of analytical and HPLC grade, purchased from eMerck, India. Other chemicals used were of analytical grade and purchased from standard chemical companies.

4B.2.2 cultures and inocula development

Cultures used for the optimisation process were three strains, *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct1, of the four that were isolated from the DDT-degrading consortium in our laboratory after a long enrichment. These cultures were grown in Peptone-glycerol medium (3.3.4) for 72 h at room temperature and 180 rpm. *Serratia marcescens* and *Pseudomonas aeruginosa* DT-Ct1 were pre-exposed to 10ppm DDT for 72 h while *Pseudomonas fluorescens* DT-2 was pre-exposed for 24 h. After the pre-exposure, the cultures were harvested by centrifugation at 8000 rpm (r=20cm) and washed well in MM (3.3.1a) and used as inoculum for the optimisation studies.

4B.2.3 Degradation Experiments

DDT degradation was carried out by incubating the required quantity of the pre-exposed cells of each of the DDT-degrading cultures to the given DDT concentrations, according to the experimental design (Table 4B.2) and incubating at the required temperature as per the design for specified period.

4B.2.4 Analytical

4B.2.4.1 Extraction and analysis of residual DDT

The samples (whole flasks), removed at required period of incubation, were extracted thrice with equal volumes of dichloromethane. The three solvent extracts were pooled and passed through column containing sodium sulphate (anhydrous) and activated florisil. These fractions were concentrated at room temperature and resuspended in a known volume of acetone. The residual DDT redissolved in known volume of acetone and used for TLC and GC.

4B.2.4.2 Quantifications of Residual DDT

Residual DDT was estimated by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

4B.2.4.2 a Thin Layer Chromatography

Thin layer chromatography (TLC) was done on silica gel G 60- 100 mesh uniformly spread over a 20 x 20 cm^2 glass plate. The thickness of the gel was set at 300 μm. These plates were left to dry at room temperature after spreading (using Camag automatic spreader, Germany) then activated at 100^o C for 1 h. Known volume of the residual extract of DDT (acetone solution) was spotted on to these plates. Spotted plates were developed in cyclohexane, air- dried and the residue was detected by spraying *o*-tolidine (2% solution in acetone) followed by exposure to bright sunlight. The

chloro-compounds give peacock green/ blue colour with this chromogen. Spots were delineated by marking with a needle and area measured. Quantity of DDT in each spot was estimated from a standard graph prepared for √area *vs* log (DDT concentration).

4B.2.4.2b Gas Chromatography

Concentrated residual substrate was resuspended in a known volume of HPLC grade acetone and gas chromatography was done using Chemito 1000 series gas chromatograph (Nasik, India) gas chromatograph. 1μl of the extract suspension was injected in to a BP-5 capillary column (30m x 0.25 mm ID) set at 180^{o}C and programmed as: 180^{o}C for 10 min and a rise @ 2^{o}C/ min up to 220^{o}C and maintained there for 2 min. Injector was maintained at 250^{o}C while electron capture detector (Ni^{63}) was maintained at 280^{o}C. Pure nitrogen gas was used as the carrier @ 1 mL min^{-1}. Under these conditions, the standard retention time for DDT was 28.16 min. Quantification of DDT in the sample was done using the area under the peak with and the standard under same conditions.

4B.2.5 Experimental design and statistics

The RSM was used to optimise conditions for DDT degradation under various parameters and their interactions. The parameters considered were pH, DDT concentration, inoculum level, incubation time and incubation temperature. The parameter ranges were pH (4-8), DDT concentration (5-35ppm), inoculum level (50-350μg protein/mL), incubation time (0-144h), and incubation temperature (20-40^{0}C). The experimental design was based on the uniform shell designing proposed by Doehlert (1970). The experimental matrix displayed a uniform distribution of the points within the experimental domain and allowed a number of distinct levels of each variable. Maximal number levels were assigned to the most important factors. The variables investigated were pH (x1, 5 levels), DDT

concentration (x2, seven levels), inoculum concentration (x3, seven levels), incubation time (x4, seven levels), and incubation temperature (x5, three levels). The total number of experiments was 35 with four repetitions at the centre point (0,0,0,0,0).

Multivariate model of the data was developed using Corel Word Perfect Suite 8 (1997, COREL CORPORATION, Dublin, Ireland). Analyses were carried out using MS-Excel 2000 version 5.0 (Microsoft Corporation, USA). For the validation of the model, the five significant parameters were selected based on the contour plots to get maximum degradation (i.e. minimum residual DDT). Predicted values were compared with the experimental values at the stationary points derived from the model.

4B.3 Results and Discussion

Table 4B.1 gives the description of the five variables investigated and their respective levels. Table 4B.2 shows the combinations of the five variables in 35 experiments. There were four replicates at the central coding conditions (0,0,0,0,0), i.e., pH at 6; DDT concentration at 20ppm, inoculum at 200μg protein/mL, incubation time at 72h and incubation temperature at 20^0C. They were used to evaluate the reproducibility of the experiment.

Data analysis

The equation model sets the predicted variable to be a function of one or more independent variables and various unknown coefficients. Regression statistics (Tables 4B.3a-d) describes various terms like the multiple correlation coefficient, R and the coefficient of determination, R^2, adjusted R^2 and standard error. R and R^2 are both measures of how well the regression model describes the data. R-values near 1 indicate that the equation is a good description of the relation between the independent and dependent variables. The adjusted R^2, R^2adj., is also a measure of how well the regression model describes the data, but takes into account the number of independent variables that reflect the degrees of

freedom. Larger R^2adj-.values (nearer to 1) indicate that the equation is a good description of the relation between the independent and dependent variables. Standard errors are estimates of the uncertainties in the estimates of the regression coefficients (analogous to the standard error of mean)

Table 4B.1 Various variables investigated for RSM

Variable	Value	Levels
X_1	pH	5
X_2	DDT Conc.	7
X_3	Inoculum	7
X_4	Incubation Time	7
X_5	Temperature	3

Table 4B.2 Experimental design matrix

Run	pH	DDT Conc. (ppm)	Inoculum (μg protein/mL)	Incub. Time (h)	Temperature (^{0}C)
1	6	20	200	72	30
2	8	20	200	72	30
3	7	35	200	72	30
4	7	25	350	72	30
5	7	25	250	144	30
6	7	25	250	96	40
7	4	20	200	72	30
8	5	5	200	72	30
9	5	15	50	72	30
10	5	15	150	0	30
11	5	15	150	48	20
12	7	5	200	72	30
13	7	15	50	72	30
14	7	15	150	0	30
15	7	15	150	48	20
16	6	30	50	72	30
17	6	30	150	0	30
18	6	30	150	48	20
19	6	20	300	0	30
20	6	20	300	48	20
21	6	20	200	120	20
22	5	35	200	72	30
23	5	25	350	72	30
24	5	25	250	144	30
25	5	25	250	96	40
26	6	10	350	72	30
27	6	10	250	144	30
28	6	10	250	96	40
29	6	20	100	144	30
30	6	20	100	96	40
31	6	20	200	24	40

32	6	20	200	72	30
33	6	20	200	72	30
34	6	20	200	72	30
35	6	20	200	72	30

Table 4B.3 Regression statistics

a. *Serratia marcescens* DT-1P

Multiple R	0.93
R Square	0.86
Adjusted R Square	0.67
Standard Error	18.88
Observations	35

b. *Pseudomonas fluorescens* DT-2

Multiple R	0.90
R Square	0.81
Adjusted R Square	0.55
Standard Error	15.41
Observations	35

c. *Pseudomonas aeruginosa* DT-Ct1

Multiple R	0.83
R Square	0.69
Adjusted R Square	0.24
Standard Error	23.31
Observations	35

d. *Pseudomonas aeruginosa* DT-Ct2

Multiple R	0.96
R Square	0.93
Adjusted R Square	0.83
Standard Error	11.99
Observations	35

The multiple coefficient of correlation, R-values, and the R^2 -values in all the four organisms indicate, the regression describes the data well, while the R^2adj. In case of *Pseudomonas aeruginosa* DT-Ct2 infers that the equation is a good description of the relationship between the independent variables considered with the degradation response achieved. The R^2adj. is not near to unity in other three

strains, hence chances of the relationship description between independent and dependent variables are fair in case of *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2 while in *Pseudomonas aeruginosa* DT-Ct1 it can be considered as a poor description of the relationship between the dependent and independent variables with the investigated observations size. This response is expected in any biological system.

Analysis of variance (ANOVA) table (Table 4B.4a-d) includes few terms such as: residuals, standardised residuals, lack of fit, pure error, t-statistics and p-value. Residuals are the difference between the predicted and observed values for the dependent variables. Standardised residuals are the residuals divided by the standard error of the estimate. A larger standard residual indicates that the point is far from the regression. Lack of fit describes whether the order of the model tentatively assumed is correct. Pure error refers to the errors occurring purely from experiments. t-statistics tests the null hypothesis that the independent variable is zero, that is , the independent variable does not contribute to predicting the dependent variable. 't' is the ratio of the regression coefficient to its standard error. Large t-values indicate that the independent variable can be used to predict the dependent variable (i.e., that the coefficient is not zero). p-value is the probability of being wrong in concluding that there is an association between the dependent and independent variables (i.e., the probability of falsely rejecting the null hypothesis). The smaller the p-value, the greater the probability that there is an association. Traditionally, it is accepted that the independent variable can be used to predict the dependent variable when $p<0.05$. Another term used in the analysis is the mean square that provides two estimates of the population variances. Comparing these variance estimates is the basis of analysis of variance. The mean square regression is a measure of the variation of the regression from the mean of the dependent variable. Residual mean square is a measure of the variation of the residuals about the regression plane. Degrees of freedom (df) represent the number of observations and the variables in the regression equation. The regression degrees of freedom is a measure of the number of independent

variables. The residual degrees of freedom is a measure of the number of observations less the number of terms in the equation. Sum of squares are measures of variability of the dependent variable. The sum of squares due to regression measures the difference of the regression plane from the mean of the dependent variable. The residual sum of squares is a measure of the size of the residuals that are the differences between the observed values of the dependent variable and the values predicted by the regression model. Alpha (α) is the acceptable probability of incorrectly concluding that the model is correct. This is also the confidence level for the final conclusion of the acceptance of the regression model with the expected error in predicting the dependent variable from the independent variable. In the ANOVA tables we find the F-value to be large indicating the regression equation to be significant (F-values are 4.39, 3.05, 1.52 and 9.39 respectively for *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 respectively. With the values obtained, it appears that the independent variables contribute to the prediction of dependent variable mainly with *Pseudomonas aeruginosa* DT-Ct2 followed by *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2 while it might create many unexplained variability during the predictions of the dependent variable and may be, there is no association between the variables and hence they are just randomly distributed in case of *Pseudomonas aeruginosa* DT-Ct1. The lack of fit is significant at 5% level of significance for *Serratia marcescens* DT-1P and is significant at 1% level of significance for the other three strains. The values of standard residuals in each of the observations in all the four organisms are very small. Hence the points in the experimental data are not far from the regression equation describing the path. Therefore the equation according to the standard residuals can also be accepted as a good description of the dependent variable from the independent variables.

The coefficients in *Serratia marcescens* DT-1P described in Table 4B.6a, indicate that pH, initial concentration and temperature affect more the process of DDT degradation. The other two factors considered *viz*. inoculum level

and incubation time are also affecting the degradation of DDT by *Serratia marcescens*. From the present study, it is clear that incubation time plays an important role in bioremediation of xenobiotics. Therefore it has been considered an essential factor for learning the degradation process at other parameters under investigation.

Table 4B.4

a. Analysis of variance in *Serratia marcescens* DT-1P

	df	Sum of Squares	Mean Square	F	Significance F
Model	20	31275.48	1563.77	4.39	0.003404
Residual	14	4989.46	356.39		
Lack of fit	10	4889.70	488.97	19.60	
Pure error	4	99.77	24.94		
Total	34	36264.94			

b. Analysis of variance in *Pseudomonas fluorescens* DT-2

	df	Sum of Squares	Mean Square	F	Significance F
Model	20	14492.69	724.63	3.05	0.02
Residual	14	3326.42	237.60		
Lack of fit	10	3285.92	328.59	32.46	
Pure error	4	40.50	10.12		
Total	34	17819.11			

c. Analysis of variance in *Pseudomonas aeruginosa* DT-Ct1

	df	*Sum of quares*	*Mean Square*	*F*	*Significance F*
Model	20	16555.87	827.79	1.52	0.21
Residual	14	7610.12	543.58		
Lack of fit	10	7585.31	758.53	122.32	
Pure error	4	24.80	6.20		
Total	34	24165.98			

d. Analysis of variance in *Pseudomonas aeruginosa* DT-Ct2

	df	Sum of Squares	Mean Square	F	Significance F
Model	20	26995.41	1349.77	9.39	5.04E-05
Residual	14	2012.01	143.71		
Lack of fit	10	2005.58	200.56	124.74	
Pure error	4	6.43	1.61		
Total	34	29007.42			

Table 4B.5a *Serratia marcescens* DT-1P

Observations	Residuals	Stdzd Residuals
1	-4.81	-0.25
2	10.76	0.57
3	6.89	0.36
4	-13.09	-0.69
5	-13.29	-0.70
6	13.17	0.69
7	-10.89	-0.58
8	16.11	0.85
9	6.09	0.32
10	13.29	0.70
11	-13.17	-0.69
12	-8.53	-0.45
13	-0.79	-0.04
14	0.97	0.05
15	-6.69	-0.35
16	14.00	0.74
17	3.28	0.17
18	-16.97	-0.89
19	8.04	0.43
20	11.25	0.59
21	25.58	1.36
22	0.08	0.004
23	16.52	0.88
24	-0.97	-0.05
25	6.69	0.35
26	-22.73	-1.20
27	-3.27	-0.17
28	16.97	0.89
29	-8.04	-0.43
30	-11.25	-0.59
31	-25.58	-1.36
32	-1.31	-0.07
33	-5.01	-0.27
34	3.08	0.16
35	-6.35	-0.34

Table 4B.5b *Pseudomonas fluorescens* DT-2

Observations	Residuals	Stdzd Residuals
1	-0.54	-0.04
2	-3.42	-0.22
3	7.87	0.51
4	9.53	0.61
5	-16.48	-1.07
6	-1.90	-0.12
7	5.88	0.38
8	-19.88	-1.29
9	-9.16	-0.59
10	16.48	1.07
11	1.90	0.12
12	-11.03	-0.72
13	-4.12	-0.27
14	14.09	0.91
15	6.42	0.42
16	-0.74	-0.05
17	-10.29	-0.67
18	-14.59	-0.95
19	-6.30	-0.41
20	-7.71	-0.50
21	13.97	0.91
22	12.45	0.81
23	-2.60	-0.17
24	-14.09	-0.91
25	-6.42	-0.42
26	7.09	0.46
27	10.29	0.67
28	14.59	0.95
29	6.30	0.41
30	7.71	0.50
31	-13.97	-0.91
32	3.30	0.21
33	-0.61	-0.04
34	5.34	0.35
35	0.62	0.04

Table 4B.5c *Pseudomonas aeruginosa* DT-Ct1

Observations	*Residuals*	*Stdzd Residuals*
1	3.36	0.14
2	2.57	0.11
3	-2.64	-0.11
4	7.79	0.33
5	-25.02	-1.07
6	13.58	0.58
7	-0.31	-0.01
8	-6.70	-0.29
9	-7.97	-0.34
10	25.02	1.07
11	-13.58	-0.58
12	-24.19	-1.04
13	-6.97	-0.30
14	0.00	0.00
15	30.07	1.29
16	0.36	0.02
17	1.34	0.06
18	-28.27	-1.21
19	-5.56	-0.24
20	-9.02	-0.39
21	20.80	0.89
22	24.88	1.07
23	1.96	0.08
24	0.00	0.00
25	-30.07	-1.29
26	4.83	0.21
27	-1.34	-0.06
28	28.27	1.21
29	5.56	0.24
30	9.02	0.39
31	-20.80	-0.89
32	-1.03	-0.04
33	3.36	0.14
34	1.02	0.04
35	-0.32	-0.01

Table 4B.5d *Pseudomonas aeruginosa* DT-Ct2

Observations	Residuals	Stdzd Residuals
1	-0.68	-0.05
2	-5.05	-0.42
3	0.89	0.07
4	-3.59	-0.29
5	-1.98	-0.16
6	10.76	0.89
7	5.38	0.45
8	6.15	0.51
9	0.93	0.08
10	1.99	0.16
11	-10.76	-0.89
12	-8.82	-0.73
13	1.61	0.13
14	9.70	0.81
15	1.19	0.09
16	5.32	0.44
17	-7.06	-0.59
18	-2.92	-0.24
19	13.85	1.16
20	-5.99	-0.49
21	18.48	1.54
22	5.76	0.48
23	3.44	0.29
24	-9.70	-0.81
25	-1.19	-0.09
26	-7.71	-0.64
27	7.06	0.58
28	2.92	0.24
29	-13.84	-1.15
30	5.99	0.49
31	-18.47	-1.54
32	-0.67	-0.06
33	-1.12	-0.09
34	0.20	0.02
35	-2.05	-0.17

Interaction between pH and temperature also has influence on DDT degradation by this strain. The *p*- values indicate the interaction between pH-inoculum and initial DDT concentration-inoculum are the major influencing factors in the degradation process by *Serratia marcescens* DT-1P.

Table 4B.6a *Serratia marcescens*

	Coefficients	Standard Error	t Statistic	P-value
Intercept	334.37	252.85	1.32	0.19
X_1	-101.14	55.22	-1.83	0.08
X_2	1.97	5.53	0.36	0.72
X_3	-0.49	0.61	-0.81	0.42
X_4	-0.76	1.18	-0.64	0.53
X_5	4.40	8.35	0.53	0.60
X_1^2	6.94	3.92	1.77	0.09
X_1X_2	-0.25	0.59	-0.43	0.67
X_1X_3	0.16	0.07	2.15	0.04
X_1X_4	-0.26	0.14	-1.81	0.08
X_1X_5	0.14	1.03	0.13	0.90
X_2^2	0.02	0.05	0.49	0.62
X_2X_3	-0.02	0.01	-2.89	0.01
X_2X_4	0.02	0.02	1.25	0.22
X_2X_5	0.07	0.12	0.60	0.55
X_3^2	0.00	0.00	1.53	0.14
X_3X_4	0.00	0.00	-0.68	0.50
X_3X_5	-0.01	0.01	-1.08	0.29
X_4^2	0.01	0.00	4.45	0.00
X_4X_5	0.02	0.02	0.82	0.42
X_5^2	-0.09	0.08	-1.04	0.31

Table 4B.6b for *Pseudomonas fluorescens* DT-2, shows the coefficients that indicate the independently pH and initial DDT concentration are affecting the degradation while in the interactions of the investigated variables, pH-initial concentration and pH-temperature are the ones that influence DDT degradation by this strain. *Pseudomonas fluorescens* DT-2, under various combinations of the studied parameters, effect DDT degradation less positively (*p*-values>0.05).

Table 4B.6b *Pseudomonas fluorescens*

	Coefficients	Standard Error	t Statistic	P-value
Intercept	252.43	206.45	1.22	0.23
X_1	-90.79	45.09	-2.01	0.05
X_2	13.33	4.52	2.95	0.01
X_3	-0.27	0.50	-0.54	0.60
X_4	-0.27	0.96	-0.28	0.78
X_5	0.00	6.81	-0.0004	1.00
X_1^2	7.24	3.20	2.26	0.03
X_1X_2	-0.80	0.48	-1.67	0.10
X_1X_3	-0.03	0.06	-0.54	0.60
X_1X_4	0.03	0.12	0.28	0.78
X_1X_5	0.73	0.84	0.87	0.39
X_2^2	-0.05	0.04	-1.34	0.19
X_2X_3	-0.01	0.01	-1.46	0.15
X_2X_4	-0.02	0.01	-1.17	0.25
X_2X_5	-0.09	0.10	-0.90	0.37
X_3^2	0.00	0.00	1.66	0.11
X_3X_4	0.00	0.00	-1.22	0.23
X_3X_5	0.01	0.01	1.61	0.12
X_4^2	0.00	0.00	1.09	0.28
X_4X_5	0.00	0.02	0.25	0.80
X_5^2	-0.10	0.07	-1.47	0.15

The coefficients in Table 4B.6c, described for *Pseudomonas aeruginosa* DT-Ct1, show that the variables pH, initial concentration and temperature influence the degradation of DDT more than the other two parameters. While interactions between pH and temperature has a positive effect in combination than any other interaction. The *p*-values here show the association between none of the independent variables could describe the predicted dependent variable ($p>0.05$).

Table 4B.6d gives the *Pseudomonas aeruginosa* DT-Ct2 coefficients that describe the influence of pH, initial concentration and temperature as independent variables on DDT degradation. Interactions between initial concentration-temperature and incubation time-temperature describe the association of temperature with initial concentration and also with incubation time in describing the predicted dependent variable i.e. the predicted residual DDT.

Table 4B.6c *Pseudomonas aeruginosa* DT-Ct1

	Coefficients	Standard Error	t Statistic	P-value
Intercept	346.36	312.27	1.11	0.27
X_1	-105.26	68.20	-1.54	0.13
X_2	8.81	6.83	1.29	0.21
X_3	0.57	0.75	0.76	0.45
X_4	-0.74	1.46	-0.51	0.61
X_5	-7.57	10.31	-0.73	0.47
X_1^2	8.62	4.85	1.78	0.08
X_1X_2	-0.23	0.73	-0.31	0.76
X_1X_3	-0.10	0.09	-1.13	0.27
X_1X_4	-0.08	0.17	-0.48	0.63
X_1X_5	1.10	1.27	0.87	0.39
X_2^2	-0.03	0.06	-0.53	0.59
X_2X_3	0.00	0.01	0.49	0.63
X_2X_4	-0.01	0.02	-0.50	0.62
X_2X_5	-0.18	0.15	-1.19	0.24
X_3^2	0.00	0.00	-0.15	0.88
X_3X_4	0.00	0.00	-1.17	0.25
X_3X_5	0.00	0.01	0.31	0.76
X_4^2	0.01	0.00	2.65	0.01
X_4X_5	0.02	0.03	0.85	0.4
X_5^2	0.04	0.10	0.40	0.69

Table 4B.6d *Pseudomonas aeruginosa* DT-Ct2

	Coefficients	Standard Error	t Statistic	P-value
Intercept	206.91	160.56	1.29	0.21
X_1	-127.02	35.07	-3.62	0.00
X_2	4.80	3.51	1.37	0.18
X_3	0.01	0.39	0.03	0.98
X_4	0.37	0.75	0.49	0.62
X_5	10.87	5.30	2.05	0.05
X_1^2	10.46	2.49	4.19	0.00
X_1X_2	0.23	0.37	0.60	0.55
X_1X_3	-0.02	0.05	-0.51	0.61
X_1X_4	-0.01	0.09	-0.07	0.94
X_1X_5	-0.07	0.65	-0.11	0.91
X_2^2	0.00	0.03	-0.15	0.88
X_2X_3	0.01	0.00	1.15	0.26
X_2X_4	-0.01	0.01	-1.07	0.29
X_2X_5	-0.17	0.08	-2.27	0.03
X_3^2	0.00	0.00	0.72	0.48
X_3X_4	0.00	0.00	-1.20	0.24
X_3X_5	0.00	0.01	-0.49	0.63
X_4^2	0.00	0.00	4.07	0.00
X_4X_5	-0.03	0.01	-2.55	0.02
X_5^2	-0.05	0.05	-0.95	0.35

The experimental data relating to the degradation of DDT by the individual bacterial strains, obtained from the experimental design, was subjected to the multivariate analysis and predictions of the microbial behaviour towards degradation was achieved by partial least square regression. The general equation model used during the studies was

Equation – Modelling:

$$Y = \beta_o + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1} \beta_{ii} X_i^2 + \sum \sum_{\substack{i=1 \\ j=i+1}} \beta_{ij} X_i X_j$$

Y = Response (Degradation)
β_0= Constant co-efficient (intercept)
β_i= Linear Co-efficient
β_{ii}= Quadratic co-efficient
β_{ij}= Second order interaction co-efficient
X = Variable investigated

With the above set of experiments conducted with *Serratia marcescens* DT-1P, the polynomial equation based on the coded values obtained from the analysis of multiple regressions was:

$$\textit{Residual DDT (\%)} = 334.3673 - 101.145x_1 + 1.9698x_2 - 0.4999x_3 - 0.7569x_4 + 4.3993x_5 + 6.9418x_1^2 - 0.2531x_1x_2 + 0.1581x_1x_3 - 0.2563x_1x_4 + 0.1364x_1x_5 + 0.0247x_2^2 - 0.0199x_2x_3 + 0.0205x_2x_4 + 0.0714x_2x_5 + 0.0007x_3^2 - 0.0010x_3x_4 - 0.0119x_3x_5 + 0.0077x_4^2 + 0.0177x_4x_5 - 0.0874x_5^2$$

Where Residual DDT (%) is the response (Y) while x_1, x_2, x_3, x_4 and x_5 are the variables investigated. The ANOVA (Analysis of variance) for the above models are shown in Table 4B.3. Table 4B.4 describes the regression statistics. The table indicates that the regression is significant in each of the cases and gives the indication that the five variables considered contribute significantly towards the

degradation according to the regression and any of the variability with unknown cause would be smaller than what could be expected from the random sampling variability of the degradation about its mean.

Fig.4b.1-3 and 4b.13-19 describe the interactions between few selected variables in *Serratia marcescens* DT-1P.

Experiments with *Pseudomonas fluorescens* DT-2 gave a regression equation as:

$$\text{Residual DDT (\%)} = 252.4329 - 90.7851x_1 + 13.32678x_2 - 0.26546x_3 - 0.27211x_4 - 0.00297x_5 + 7.2417x_1^2 - 0.80495x_1x_2 - 0.03214x_1x_3 + 0.032938x_1x_4 + 0.732455x_1x_5 - 0.05462x_2^2 - 0.0082x_2x_3 - 0.01575x_2x_4 - 0.08739x_2x_5 + 0.000637x_3^2 - 0.00149x_3x_4 + 0.014553x_3x_5 + 0.00155x_4^2 + 0.004416x_4x_5 - 0.10131x_5^2$$

Fig.4b.4-6 and 4b.20-26 describe the interactions between few selected variables in *Pseudomonas fluorescens* DT-2.

Experiments with *Pseudomonas aeruginosa* DT-Ct1 gave the following regression expression:

$$\text{Residual DDT (\%)} = 346.359 - 105.26x_1 + 8.80989x_2 + 0.5674x_3 - 0.7418x_4 - 7.5692x_5 + 8.62005x_1^2 - 0.2263x_1x_2 - 0.1023x_1x_3 - 0.0835x_1x_4 + 1.10215x_1x_5 - 0.0328x_2^2 + 0.00413x_2x_3 - 0.0102x_2x_4 - 0.1759x_2x_5 - 0.00009x_3^2 - 0.0022x_3x_4 + 0.0042x_3x_5 + 0.00571x_4^2 + 0.02254x_4x_5 + 0.04208x_5^2$$

Fig.4b.7-9 and 4b.27-33 describe the interactions between few selected variables in *Pseudomonas aeruginosa* DT-Ct1.

Experiments with *Pseudomonas aeruginosa* DT-Ct2 resulted in the following regression equation:

$$\text{Residual DDT (\%)} = 206.9065 - 127.017x_1 + 4.7965x_2 + 0.010804x_3 + 0.370466x_4 + 10.86693x_5 + 10.45831x_1^2 + 0.225961x_1x_2 - 0.02367x_1x_3 - 0.00634x_1x_4 - 0.07267x_1x_5 - 0.00471x_2^2 + 0.005043x_2x_3 - 0.01118x_2x_4 - 0.17131x_2x_5 + 0.000215x_3^2 - 0.00115x_3x_4 - 0.00344x_3x_5 + 0.0045x_4^2 - 0.00473x_4x_5 - 0.05074x_5^2$$

Fig.4b.10-12 and 4b.34-40. describe the interactions between few selected variables in *Pseudomonas aeruginosa* DT-Ct2.

It is important to emphasise that derived models should be used to predict the responses within the range of influencing factors studied for which the experimental data were collected (Buchanan *et al*, 1993). The above-derived models for the four strains were also studied for their validation under the practical conditions of various combinations to obtain the maximum response for the degradation of DDT. The RMSD (values 5.5, 6.2, 8.57 and 6.32 were obtained for *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 respectively with the percentage error of 5-7. Though the variations are wide in most of the biological systems because of the variations shown in the populations from time to time, this error can be considered obvious. Thus the experimental data obtained under the tested conditions could be considered to reflect the predictions from the models derived for each of the strains for DDT degradation.

There are no reports on the RSM of xenobiotics degradation so far to predict the behaviour of the degrading organisms. This is the first attempt to understand the interactions in terms of pH, initial concentration of the xenobiotic, inoculum level, incubation time and the incubation temperature towards the bioremediation and the models derived. Reports on inhibitory effects of various substances deal mainly with the enzyme kinetics and the models proposed for the same (Beltrame *et al*, 1988; Ely *et al*, 1995;Kim *et al*, 2002a,b). With our work it becomes an apparent insight on the degradation of DDT under the given conditions wherein the above five parameters are taken into account. It would also be an added advantage in understanding the other interactions that might influence the biodegradation process in the effluent/waste water treatment containing DDT residues and its metabolites. The above model can also be adapted and extended to other strains/organisms as well as other xenobiotics and pollutants.

Fig.4b.1 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Serratia marcescens* DT-1P at pH 7.5, inoculum 50 µg protein/mL and 20^{0}C

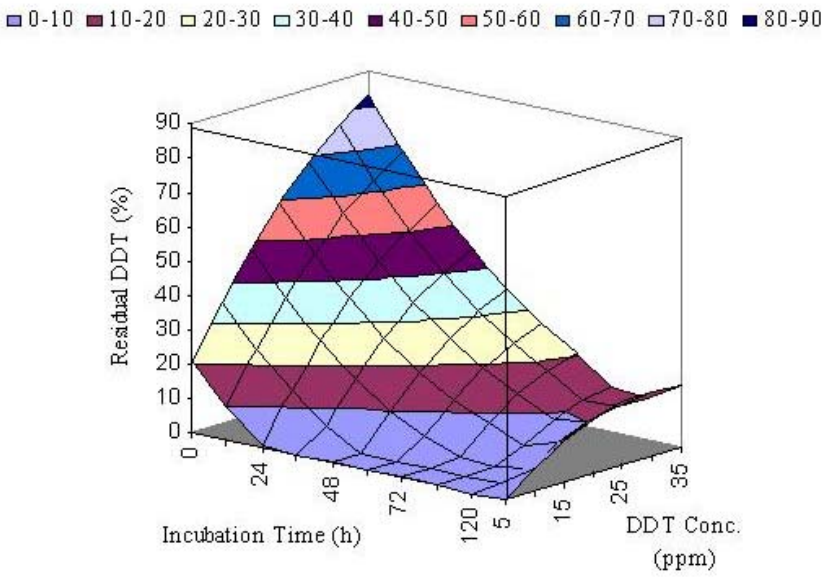


Fig.4b.2 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Serratia marcescens* DT-1P at pH 7.5, inoculum 50 µg protein/mL and 30°C

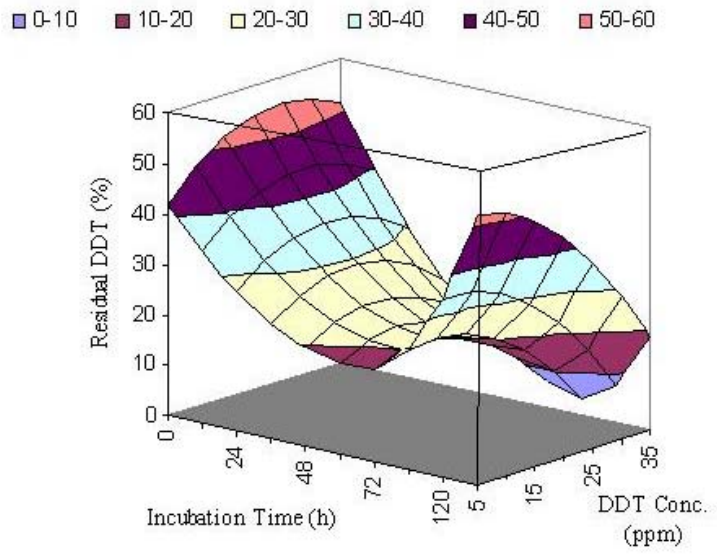


Fig.4b.3 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Serratia marcescens* DT-1P at pH 7.5, inoculum 80 µg protein/mL and 40^{0}C

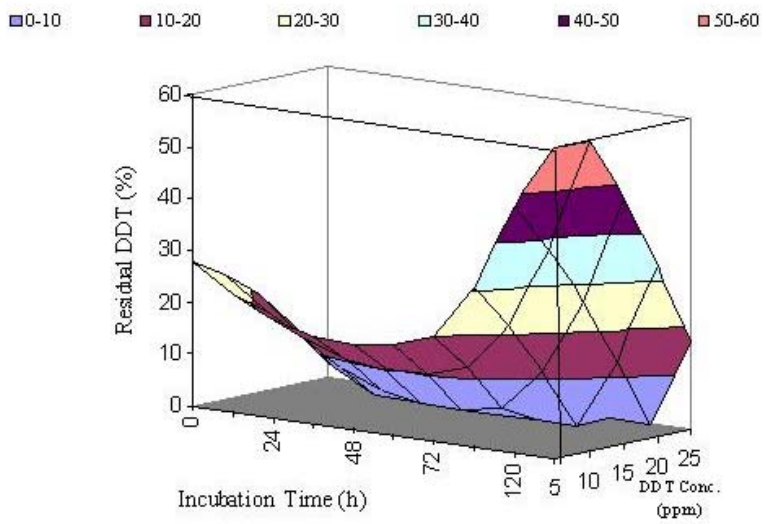


Fig.4b.4 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas fluorescens* DT-2 at pH 6, inoculum 150 µg protein/mL and 20^{0}C

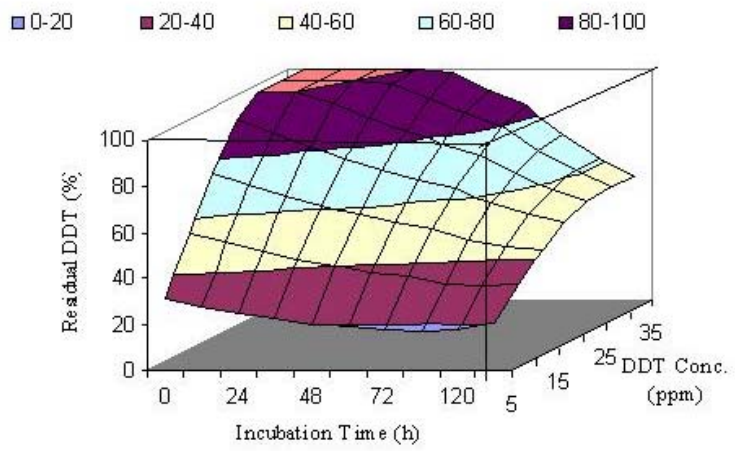


Fig.4b.5 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas fluorescens* DT-2 at pH 6, inoculum 150 µg protein/mL and 30^{0}C

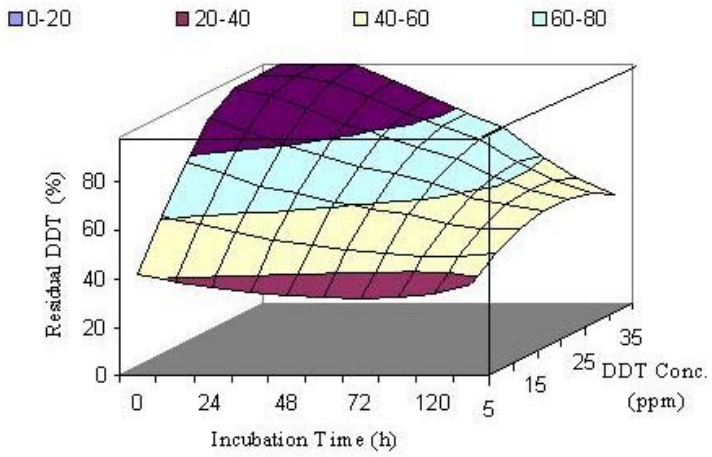


Fig.4b.6 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas fluorescens* DT-2 at pH 6, inoculum 150 µg protein/mL and 40^{0}C

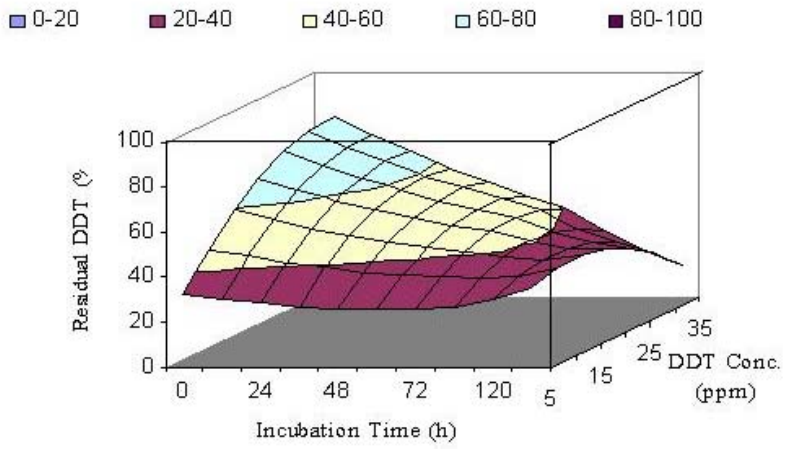


Fig.4b.7 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct1 at pH 7, inoculum 150 µg protein/mL and 20^{0}C

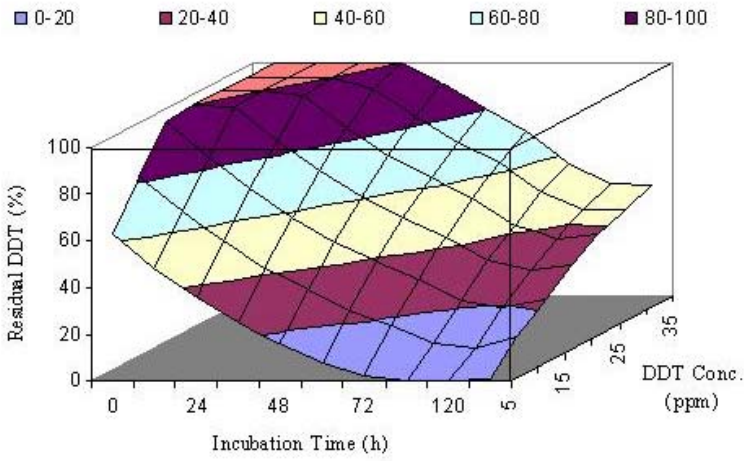


Fig.4b.8 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct1 at pH 7, inoculum 250 µg protein/mL and 30^{0}C

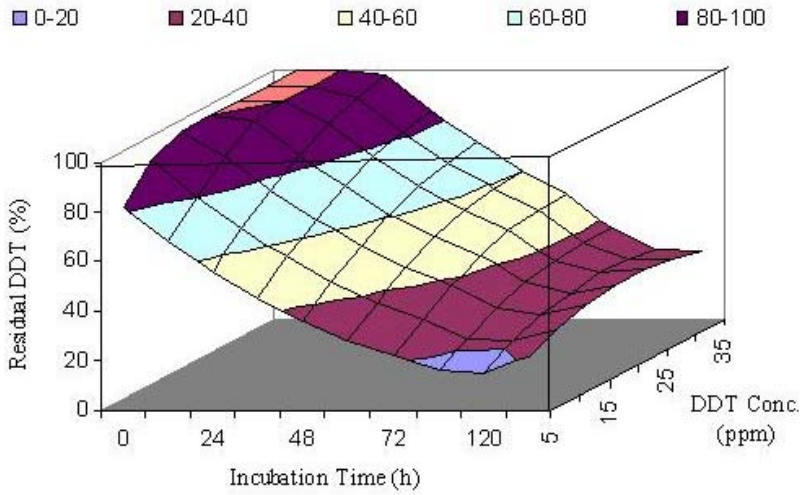


Fig.4b.9 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct1 at pH 7, inoculum 250 µg protein/mL and 40^{0}C

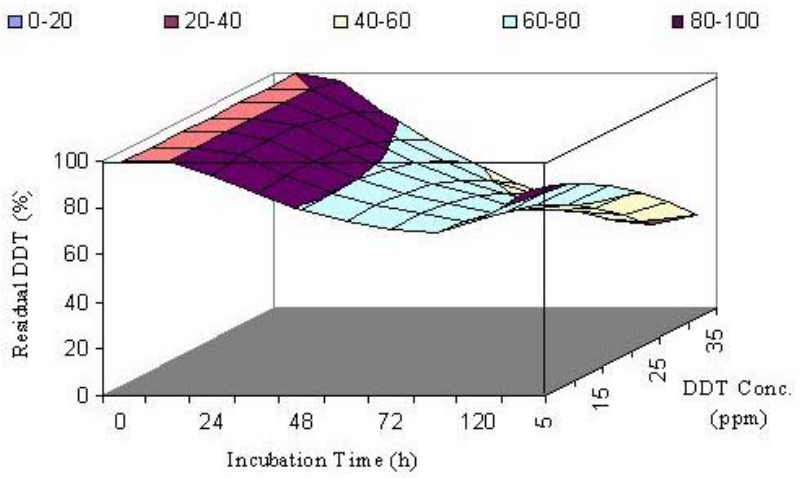


Fig.4b.10 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct2 at pH 6, inoculum 175 µg protein/mL and 20^{0}C

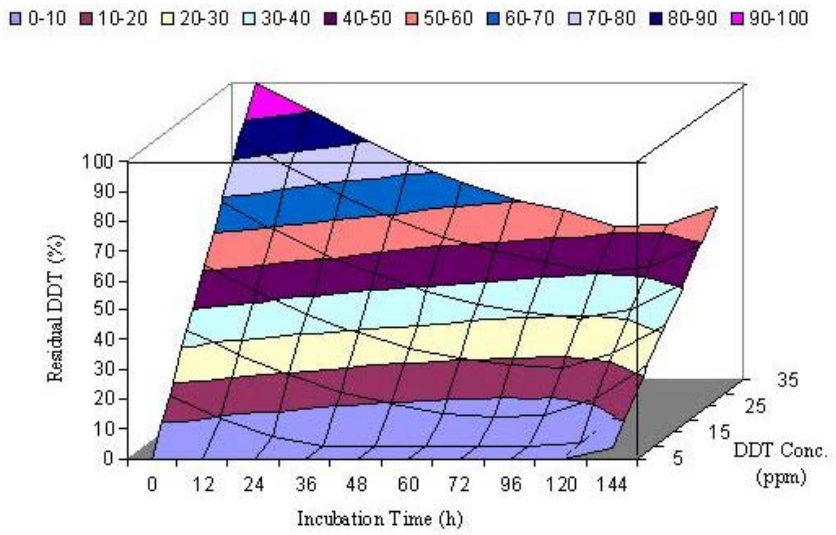


Fig.4b.11 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct2 at pH 6, inoculum 175 µg protein/mL and 30^{0}C

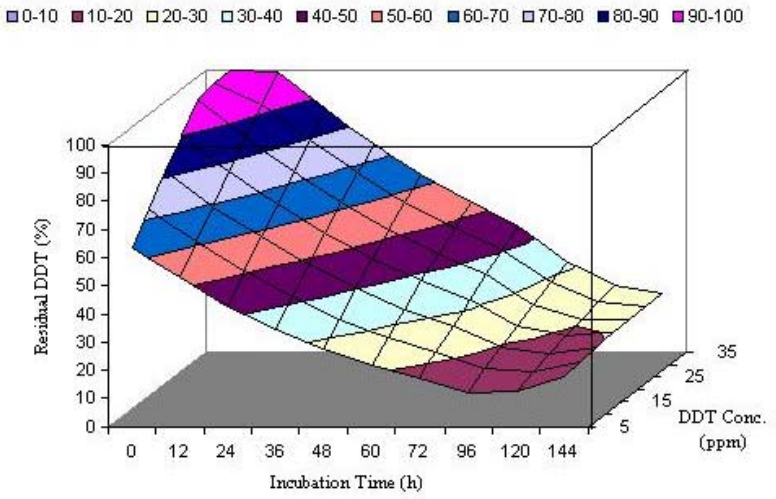


Fig.4b.12 Interaction between incubation time and initial DDT concentration during the degradation of DDT by *Pseudomonas aeruginosa* DT-Ct2 at pH 6, inoculum 175 µg protein/mL and 40^{0}C

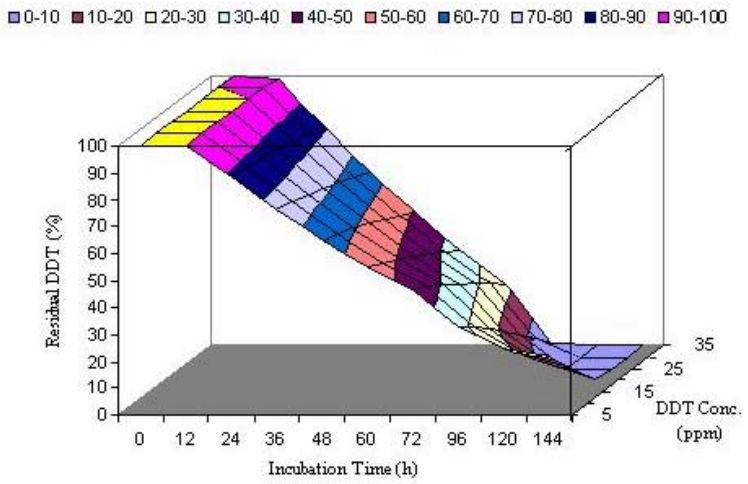


Fig.4b.13 Interaction between incubation time and pH during the degradation of 5ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

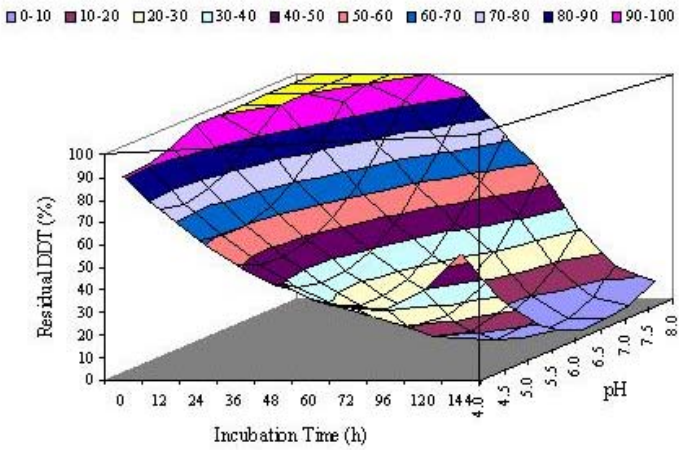


Fig.4b.14 Interaction between incubation time and pH during the degradation of 10ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

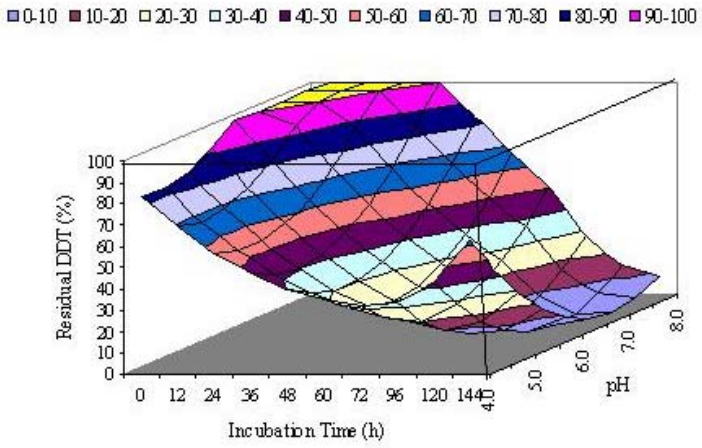


Fig.4b.15 Interaction between incubation time and pH during the degradation of 15ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

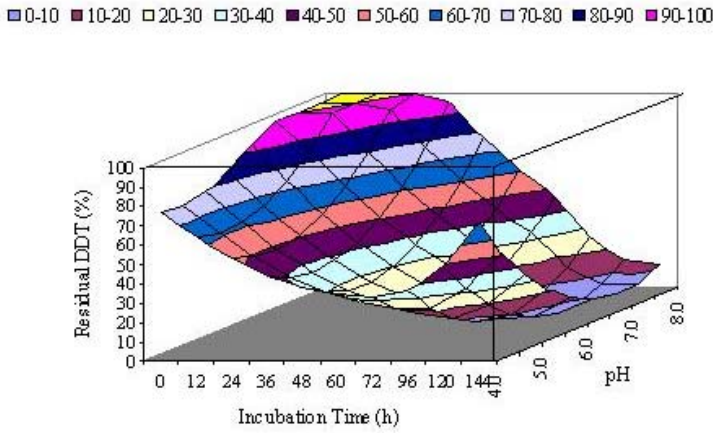


Fig.4b.16 Interaction between incubation time and pH during the degradation of 20ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

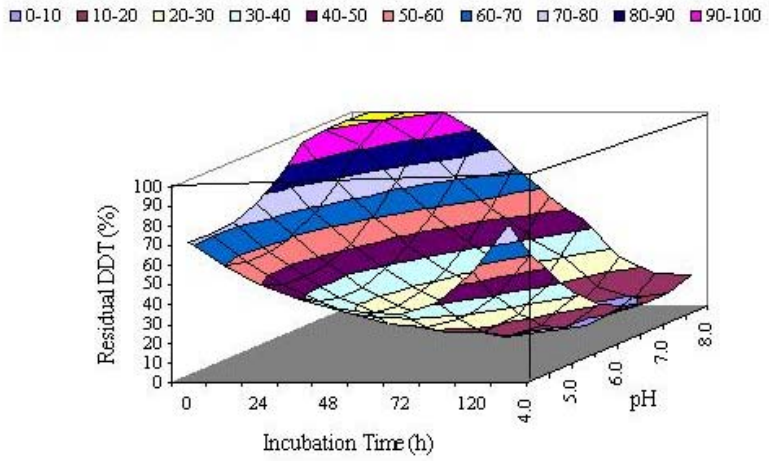


Fig.4b.17 Interaction between incubation time and pH during the degradation of 25ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

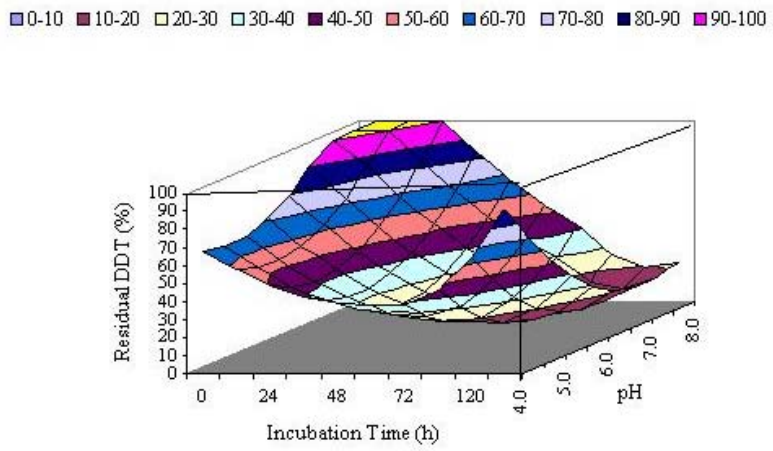


Fig.4b.18 Interaction between incubation time and pH during the degradation of 30ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

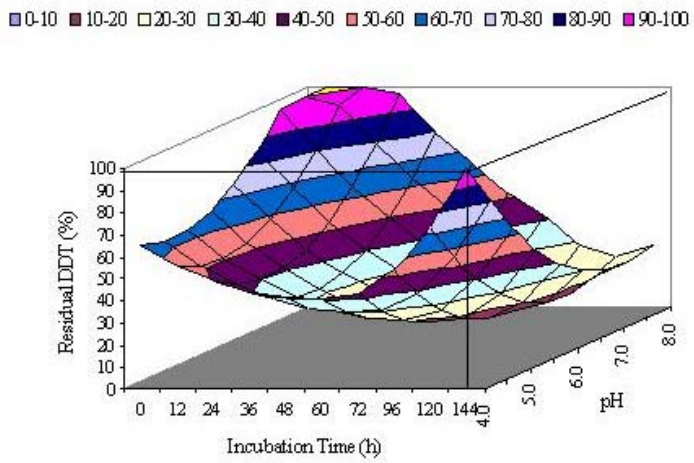


Fig.4b.19 Interaction between incubation time and pH during the degradation of 35ppm DDT by *Serratia marcescens* DT-1P (inoculum 250µg protein/mL and 30^{0}C)

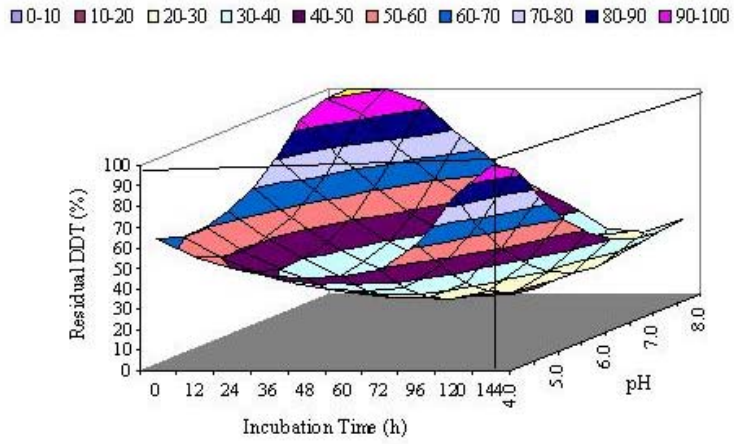


Fig.4b.20 Interaction between incubation time and pH during the degradation of 5ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

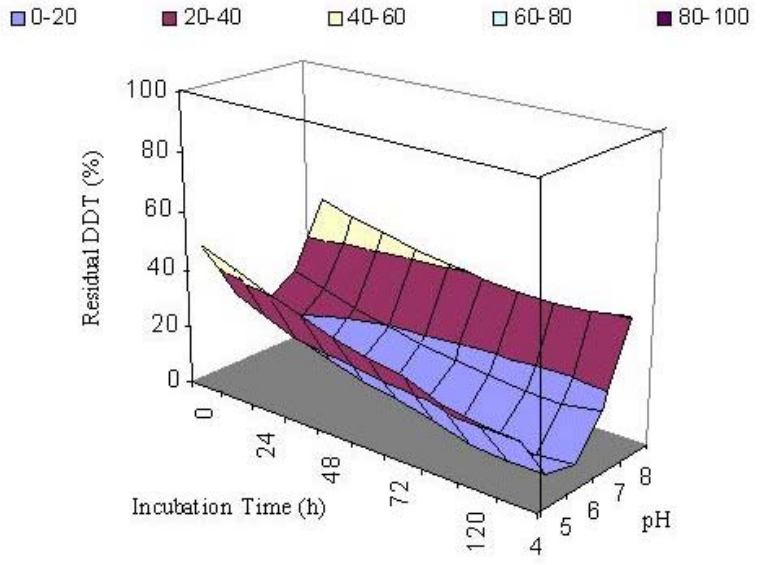


Fig.4b.21 Interaction between incubation time and pH during the degradation of 10ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

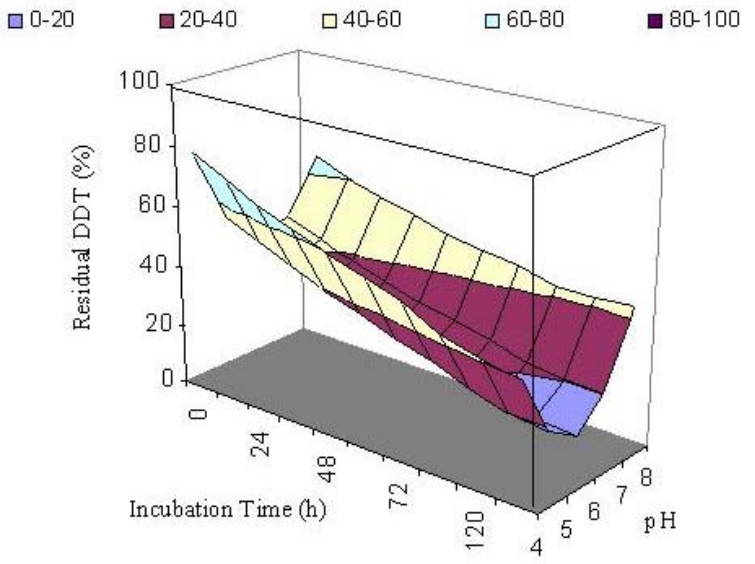


Fig.4b.22 Interaction between incubation time and pH during the degradation of 15ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

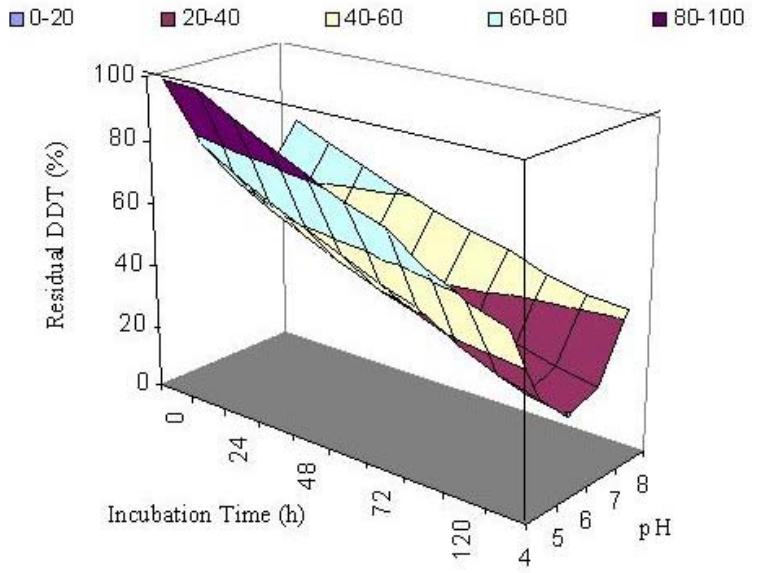


Fig.4b.23 Interaction between incubation time and pH during the degradation of 20ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

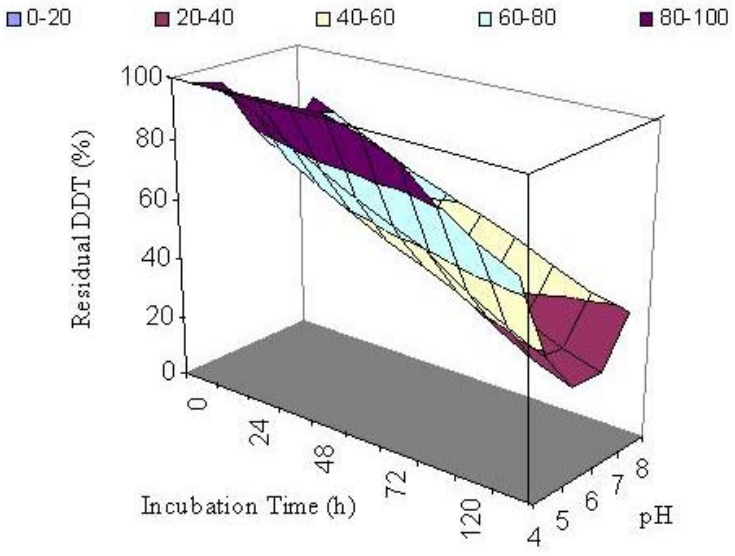


Fig.4b.24 Interaction between incubation time and pH during the degradation of 25ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

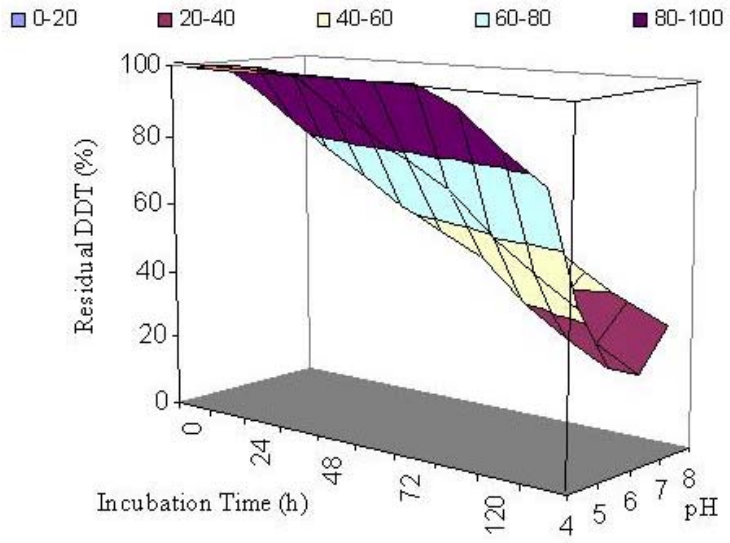


Fig.4b.25 Interaction between incubation time and pH during the degradation of 30ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

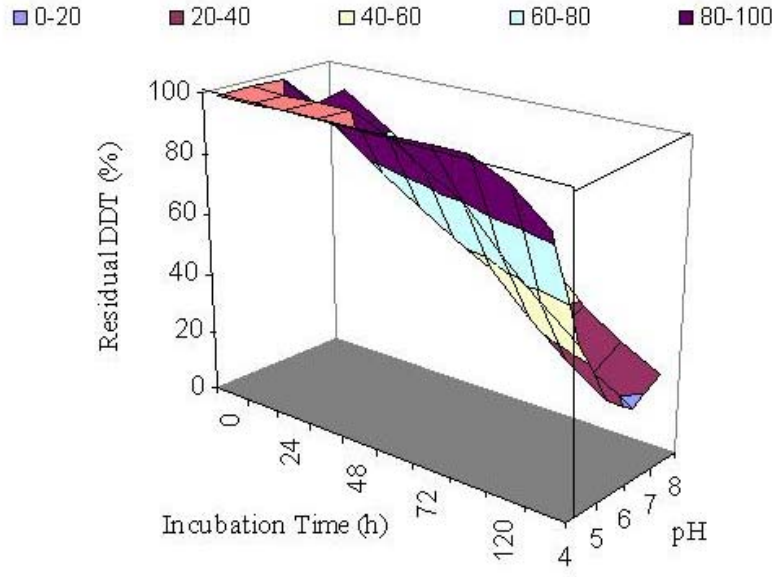


Fig.4b.26 Interaction between incubation time and pH during the degradation of 35ppm DDT by *Pseudomonas fluorescens* DT-2 (inoculum 200µg protein/mL and 20^{0}C)

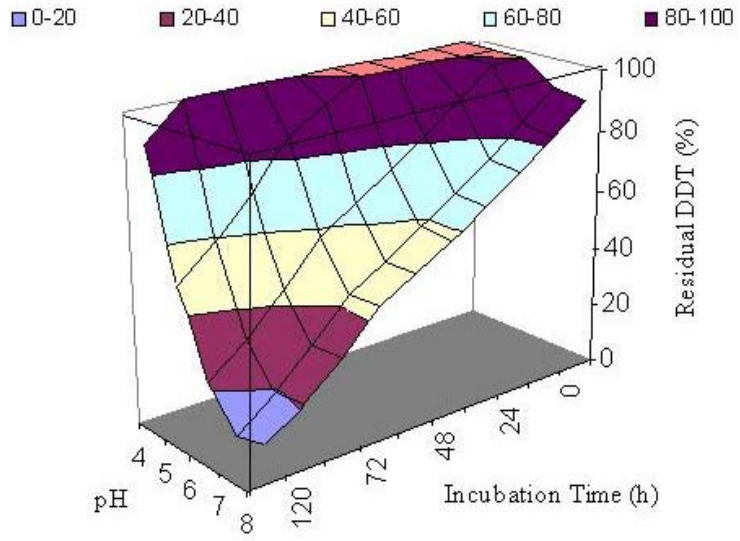


Fig.4b.27 Interaction between incubation time and pH during the degradation of 5ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

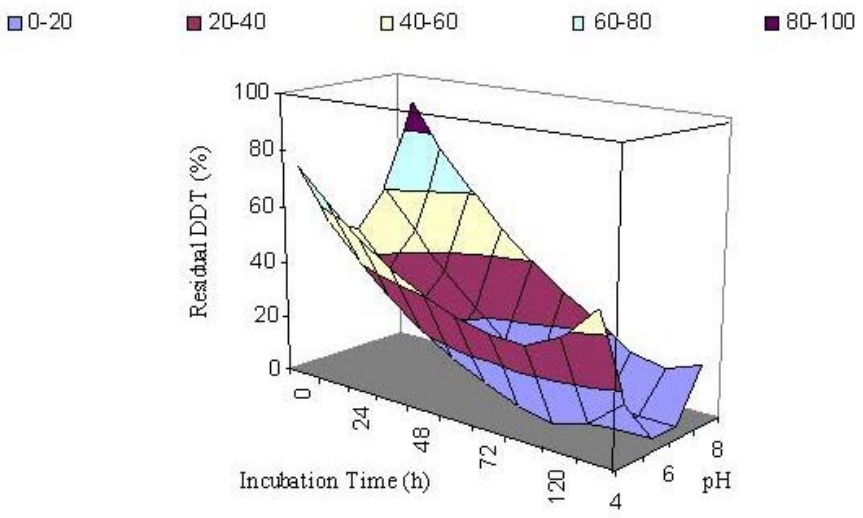


Fig.4b.28 Interaction between incubation time and pH during the degradation of 10ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

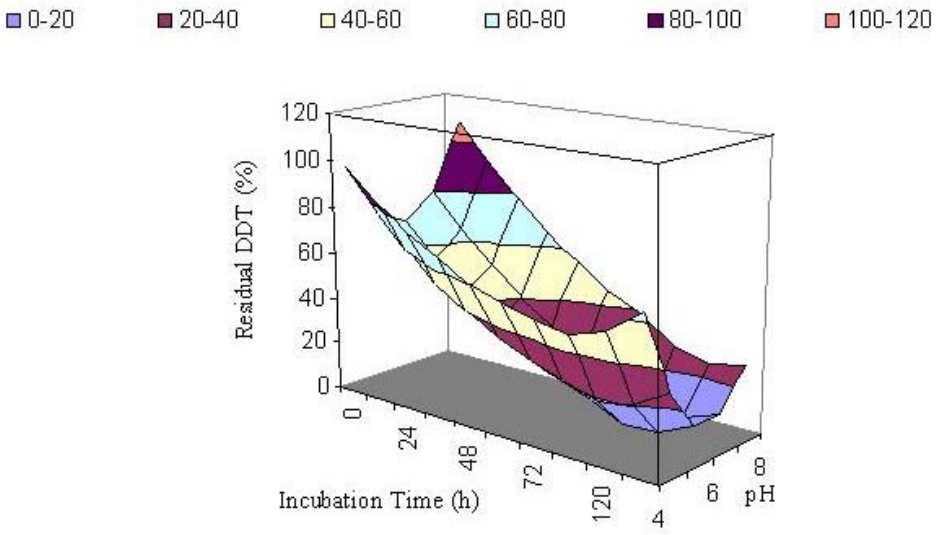


Fig.4b.29 Interaction between incubation time and pH during the degradation of 15ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

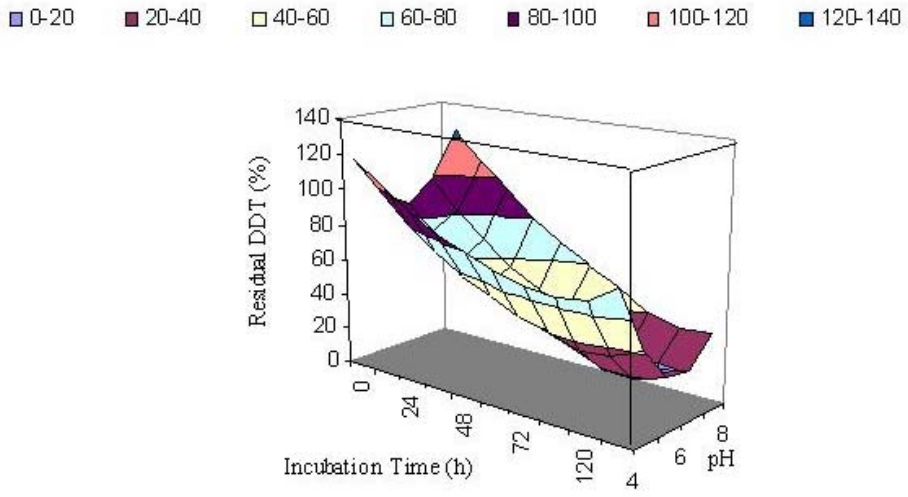


Fig.4b.30 Interaction between incubation time and pH during the degradation of 20ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

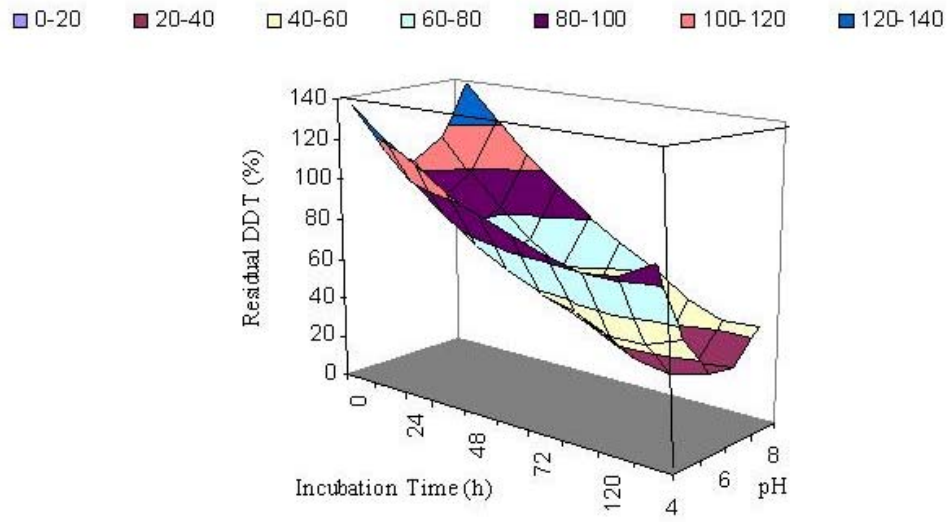


Fig.4b.31 Interaction between incubation time and pH during the degradation of 25ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

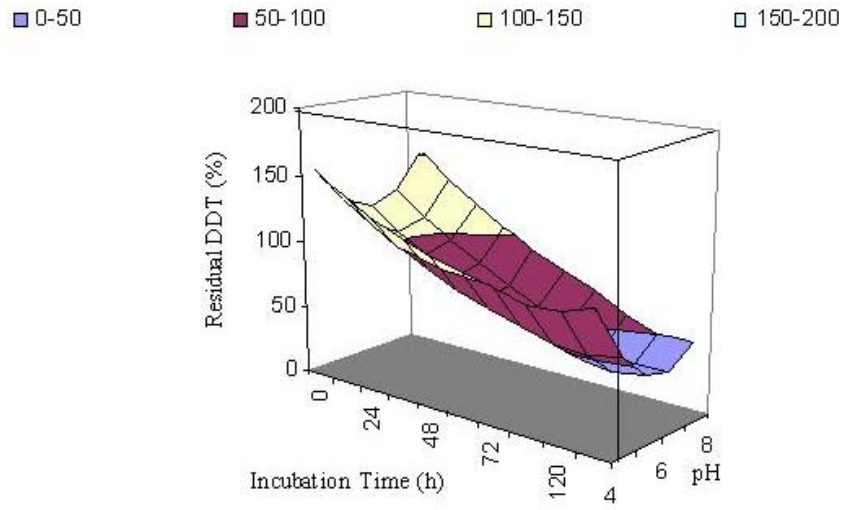


Fig.4b.32 Interaction between incubation time and pH during the degradation of 30ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

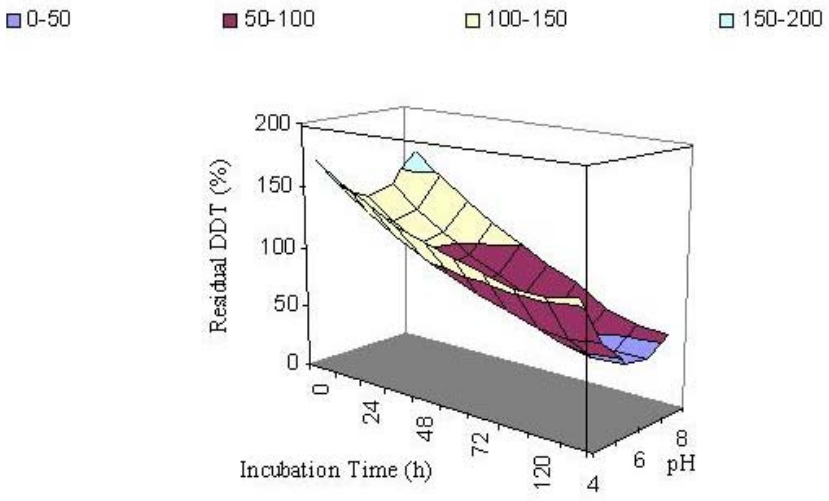


Fig.4b.33 Interaction between incubation time and pH during the degradation of 35ppm DDT by *Pseudomonas aeruginosa* DT-Ct1 (inoculum 150µg protein/mL and 20^{0}C)

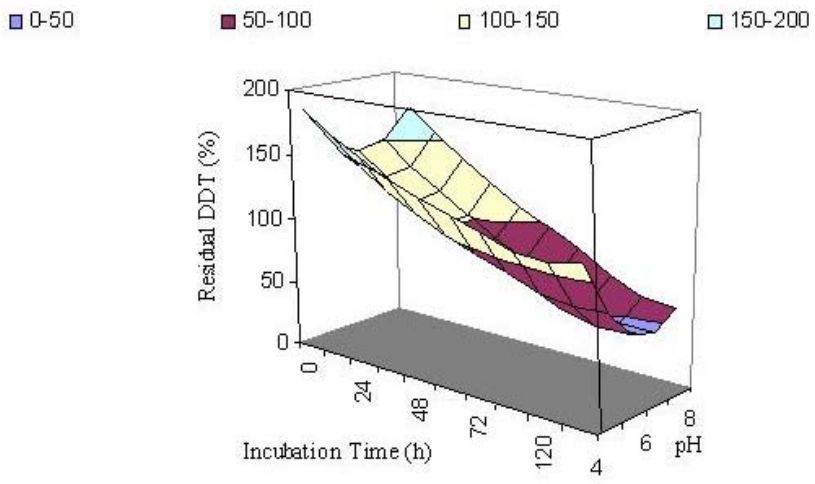


Fig.4b.34 Interaction between incubation time and pH during the degradation of 5ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)

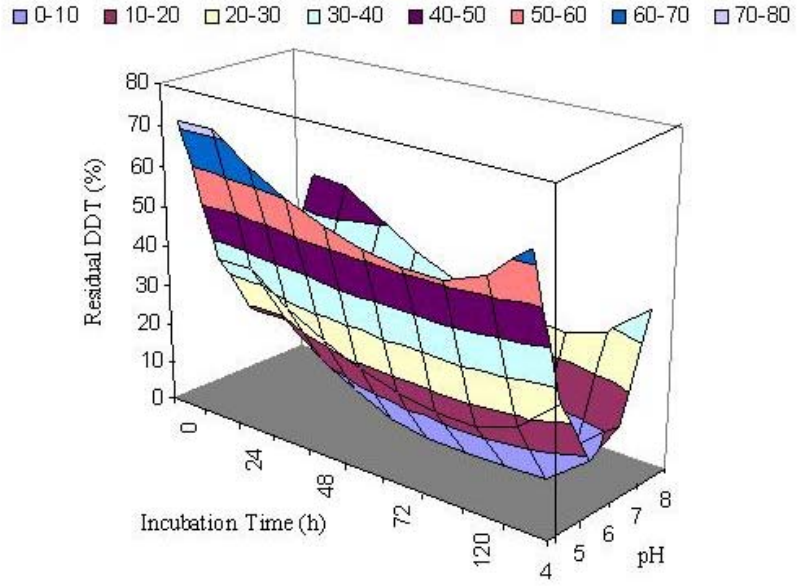


Fig.4b.35 Interaction between incubation time and pH during the degradation of 10ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)

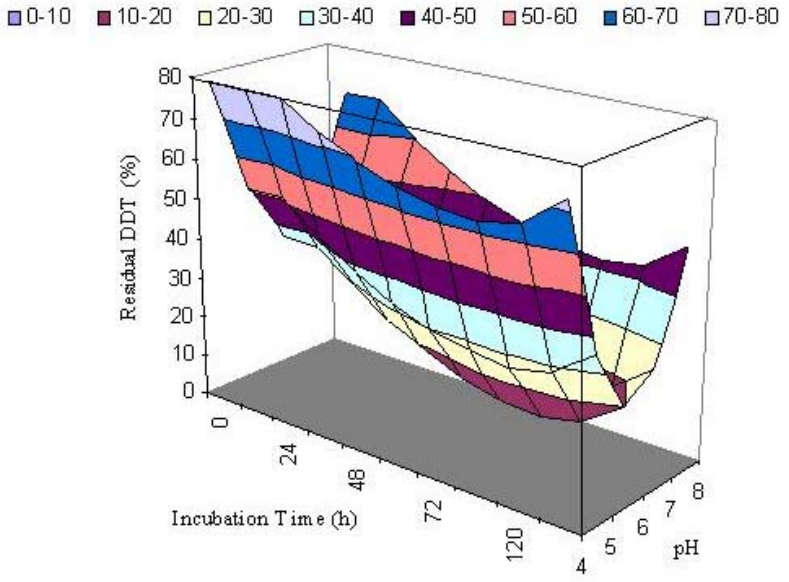


Fig.4b.36 Interaction between incubation time and pH during the degradation of 15ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)

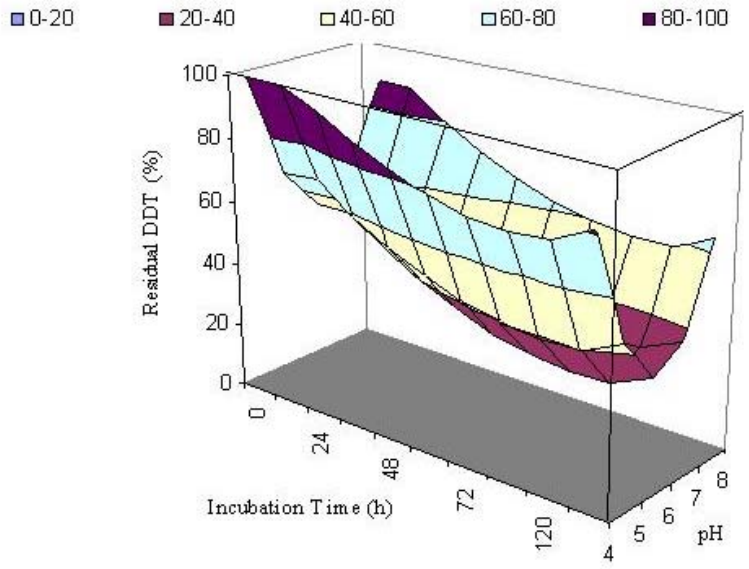


Fig.4b.37 Interaction between incubation time and pH during the degradation of 20ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)

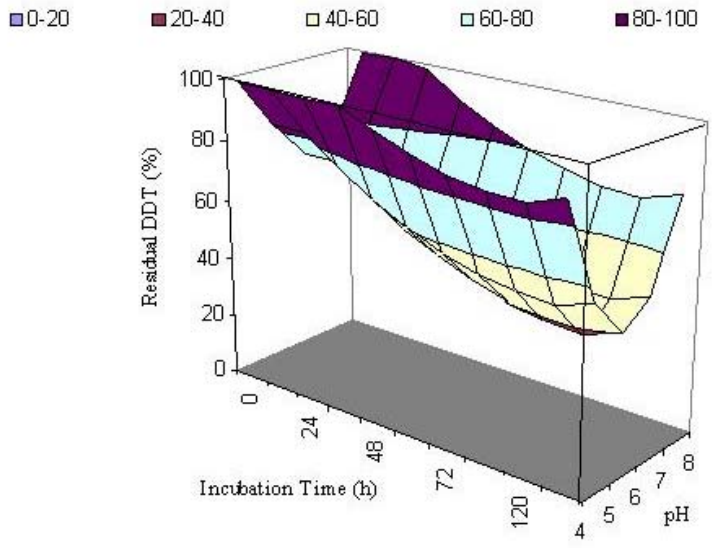


Fig.4b.38 Interaction between incubation time and pH during the degradation of 25ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175ug protein/mL and 20^{0}C)

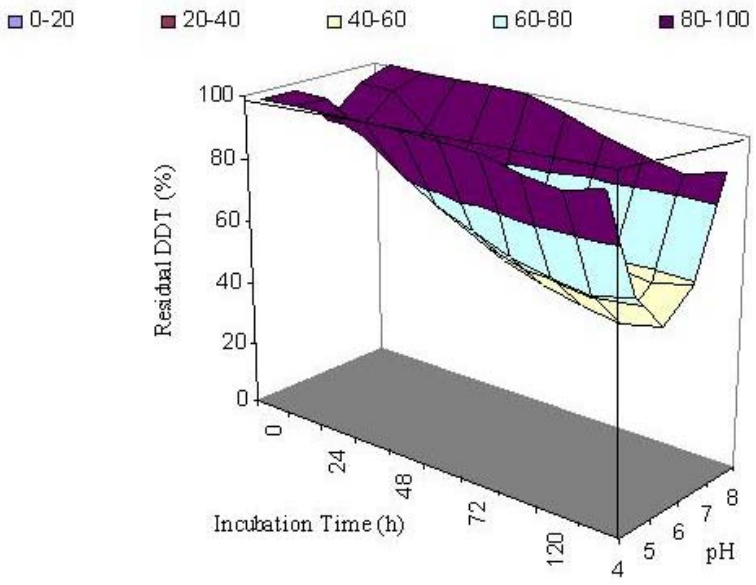


Fig.4b.39 Interaction between incubation time and pH during the degradation of 30ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)

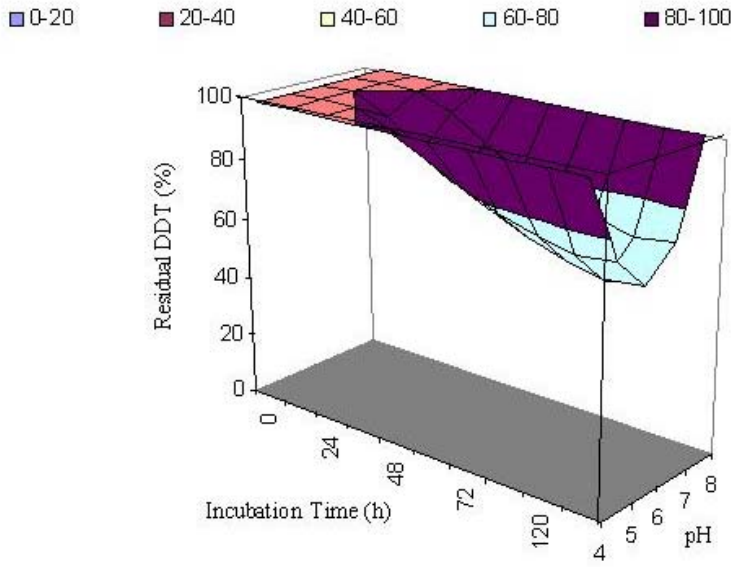
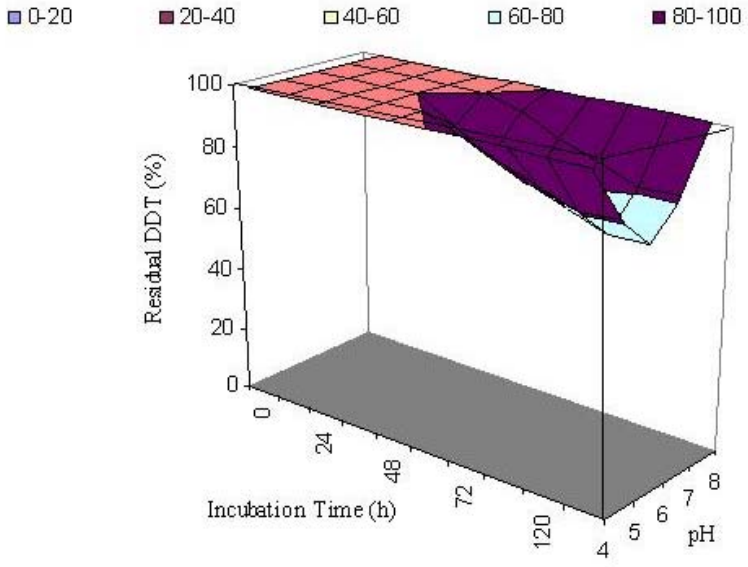


Fig.4b.40 Interaction between incubation time and pH during the degradation of 35ppm DDT by *Pseudomonas aeruginosa* DT-Ct2 (inoculum 175µg protein/mL and 20^{0}C)



Chapter 5

CO-METABOLISM OF DDT BY THE BACTERIAL ISOLATES

Our notions of law and harmony are commonly confined to those instances, which we detect; but the harmony, which results from a far greater number seemingly conflicting, but really concurring laws, which we have not detected, is still more wonderful.

Thoreau (1954)

5.1 Introduction

A broad- spectrum insecticide, DDT, is readily converted to 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene (DDE) and 1,1- dichloro-2,2-bis(4-chlorophenyl)ethane (DDD) by the process that involves the removal of only a single chloride (Subba Rao and Alexander, 1985). Further degradation may or may not occur because of more recalcitrance of these dechlorinated metabolites (Aislabie *et al*, 1999). However, extensive degradation of DDT may occur during its co-metabolism by bacteria that are capable of growing at the expense of non-chlorinated compounds or analogues (Focht and Alexander, 1970a, b; 1971). Studies on the products of microbial metabolism of non-chlorinated analogs (Focht and Alexander, 1970a, b; Subba Rao and Alexander, 1977a, b) have demonstrated the potential for the extensive microbial metabolism of DDT in nature. The presence of such microorganisms has been demonstrated in fresh water, sewage and marine environments (Pfaender and Alexander, 1972; Juengst and Alexander, 1976; Patil *et al*, 1972). Wang and Loh (2002) have described a model for the cometabolic transformation of 4-chlorophenol. Verce *et al* (2001) could isolate *Pseudomonas aeruginosa* strain DL1 that was able to cometabolise venyl chloride in presence of ethene. Involvement of certain fungi in the cometabolism of DDT has also been described by Anderson and Lichtenstein (1971). Subba Rao and Alexander (1985) did an extensive study on the cometabolic degradation of DDT and its metabolites by both bacteria as well as various fungi. Ahuja *et al* (2001) conducted studies on the cometabolism of DDE, one of the more persistent metabolites of DDT, by *Alcaligenes denitrificans*.

In the present study, the co- metabolism of DDT was studied by measuring the degradative activity of microbial cells capable of growing on other simple and/ or complex carbon sources.

5. 2 Materials and Methods

5.2.1 Chemicals

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 98% pure, was purchased from Sigma-Aldrich Chemical Company, Mo, USA. *o*-Tolidine, glucose, sucrose, sodium acetate, sodium succinate, sodium citrate, glycerol, peptone, beef extract, yeast extract, tryptone, TSB (tryptone soya broth), nutrient agar were purchased from Hi Media Chemical Laboratories, Mumbai, India. Wheat bran, rice straw etc. were obtained from local market. Solvents: acetone, dichloromethane and cyclohexane were of analytical/HPLC grade and were procured from E- Merck (India) Ltd. Silica gel was procured from Sisco Research Laboratories, India. All other chemicals and reagents used in this study were of analytical grade and were purchased from standard chemical companies.

5.2.2. Cultures and media

Microbial strains used in this study were the four bacteria isolated and further developed in the laboratory as described in earlier chapters (3.4.1).

5.2.2.1 Bacterial Inoculum

The individual bacterial strains were grown in peptone- glycerol medium (3.3.4) and induced with 10 ppm DDT as described earlier for the efficient degradation by individual strains (3.5.7). The respective axenic cultures, pre-exposed to DDT, were used as inocula.

5.2.2.2 Basal medium

The basal mineral medium, MM (M_4) used for studying the co-metabolic degradation of DDT by the bacterial strains has been described in 3.3.1a. Other carbon sources used in the study were prepared separately as 5% stock solutions in distilled water. The MM and stock solutions were

sterilized separately by autoclaving at 121 ^{o}C for 15 minutes. The cool stock solutions were added individually at 0.5% level to MM.

5.2.2.3 Co-metabolic degradation of DDT

The effect of different carbon sources on the degradation of DDT was studied by supplementing the carbon sources at 0.5% level. DDT at 10μg/ml (10 ppm) level, as acetone solution, was added to sterile, dry 250 mL Erlenmeyer flasks inside a laminar hood. Acetone was allowed to evaporate and 50 ml of MM (3.3.1a) containing 0.5% of sterile carbon source was added to these flasks. The DDT- pre-exposed cells of the four bacterial strains, washed well in MM, were inoculated separately at 100μg protein level. The flasks were incubated in a rotary shaker (180 rpm) at ambient temperature (26- 28^{o}C) for 72 h.
All the experiments were done in triplicates.

5.2.3 Analytical

5.2.3.1 Extraction of residual DDT

Residual DDT was extracted thrice from the aqueous samples with equal volumes of dichloromethane in a separating funnel. Sample and the solvent were taken in a separating funnel and shaken vigorously for 5 min and the two layers were allowed to separate out. Then the solvent layers were pooled and passed through anhydrous sodium sulphate and then through activated florisil. The solvent was allowed to evaporate and the residue was resuspended in a known volume of acetone for further analysis.

5.2.3.2 Growth

Growth of the bacterial strains was determined by estimating total protein in the biomass by modified method of Lowry *et al* (Bidlan and

Manonmani, 2002) as follows: Cells were harvested from a suitable quantity of culture broth, washed with minimal medium (3.3.1a), suspended in 3.4 ml distilled water and 0.6 ml of 20% NaOH. This was mixed and digested in a constant boiling water bath for 10 min. Total protein, in cooled sample of this hydrolysate, was estimated by using Folin- Ciocalteau reagent (3.3.22). A total of 0.5 ml of the hydrolysate was taken in a clean test tube. To this was added 5.0 ml of Lowry's C (3.3.21). After 10 min 0.5 ml of Lowry's D [Folin- Ciocalteau reagent (1:2)(3.3.22)) was added and mixed well. The colour was read at 660_{nm} after 20.0 min of standing at room temperature, using a spectrophotometer (Shimadzu UV- 160A, Japan). Total amount of protein was computed using the standard curve prepared with BSA (Bovine serum Albumin).

5.2.3.3 Residual DDT

Residual DDT was estimated by Thin Layer Chromatography (TLC) and Gas Chromatography (GC)

5.2.3.3 a Thin Layer Chromatography

Thin layer chromatography (TLC) was done on silica gel G 60- 100 mesh uniformly spread over a 20 x 20 cm^2 glass plate. The thickness of the gel was set at 300 μm. These plates were left to dry at room temperature after spreading (using Camag automatic spreader, Germany) then activated at 100^o C for 1 h. Known volume of the residual extract of DDT (acetone solution) was spotted on to these plates. Spotted plates were developed in cyclohexane, air- dried and the residue was detected by spraying *o*-tolidine (2% solution in acetone) followed by exposure to bright sunlight. The chloro- compounds give peacock green/ blue colour with this chromogen. Spots were delineated by marking with a needle and area measured.

Quantity of DDT in each spot was estimated from a standard graph prepared for √area *vs* log (DDT concentration).

5.2.3.3 b Gas Chromatography

Concentrated residual substrate, after passing through activated florisil, was resuspended in a known volume of HPLC grade acetone and gas chromatography was done using Chemito 1000 series gas chromatograph (Nasik, India). 1μl of the extract suspension was injected in to a BP-5 capillary column (30m x 0.25mm ID) set at 180^{o} C and programmed as: 180^{o} C for 10 min and a rise @ 2^{o} C/ min up to 220^{o} C and maintained there for 2 min. Injector was maintained at 250^{o} C while electron capture detector (Ni^{63}) was maintained at 280^{o} C. Pure nitrogen gas was used as the carrier @ 1 ml min^{-1}. Under these conditions, the standard retention time for DDT was 28.16 min. Quantification of DDT in the sample was done using the area under the peak with and the standard under same conditions.

5.3 Results

5.3.2 Growth and degradation of DDT in presence of different carbon sources

5.3.2.1 Glucose

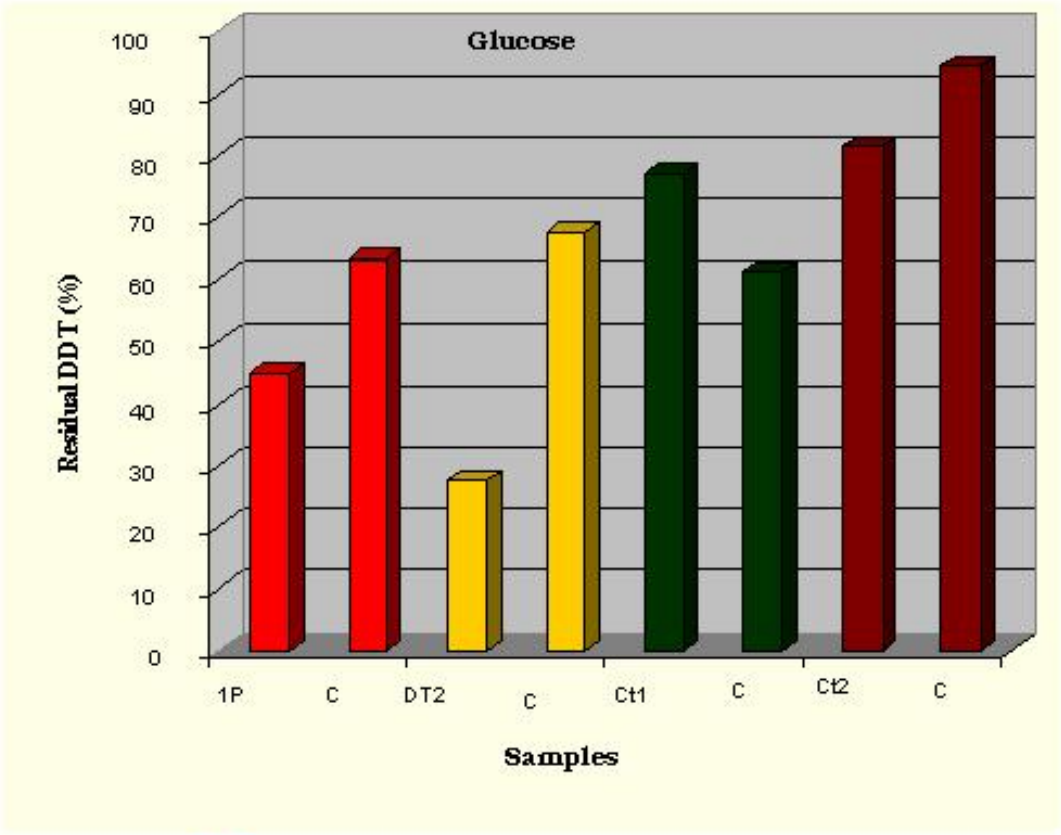
In presence of glucose, maximum level of degradation of 10 ppm DDT was observed in *Pseudomonas fluorescens* DT-2 by the end of 72 h of incubation under shaking conditions, wherein 72.3% degradation was achieved (Fig.5.1). *Serratia marcescens* DT-1P, *Pseudomonas aeruginosa strains* DT-Ct1 and DT-Ct2 degraded 23.8%, 68.1% and 74.1% less DDT compared to *Pseudomonas fluorescens* DT-2, in the same period of incubation (Fig.5.1). However, *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2 showed better degradation than the respective controls (Fig.5.1) while degradation in case

of *Pseudomonas aeruginosa* DT-Ct1 was less than the control. Though there was good growth observed in *Pseudomonas aeruginosa* DT-Ct1 (Table 5.3), degradation was less than the control. Almost similar pattern in growth with *Pseudomonas fluorescens* DT-2 (Table 5.2) was observed but with comparatively higher degree of degradation of the initially added 10ppm DDT in same time of incubation. Compared to the respective controls, degradation percentages in *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2 were 50.26, 121.24 and 218.03 respectively towards positive influence while negative influence was clear in *Pseudomonas aeruginosa* DT-Ct1 with 40.56% less degradation with respect to the control (Fig.5.1).

5.3.2.2 Sucrose

Pseudomonas fluorescens DT-2 degraded the maximum percent of the initially added 10ppm DDT by 72 h of shaking, wherein 77.8 % degradation was observed. Under the same conditions of incubation, *Serratia marcescens* DT-1P, *Pseudomonas aeruginosa strains* DT-Ct1 and DT-Ct2 degraded 16.3%, 84.4% and 73.3% less DDT than that DT-2 (Fig.5.2). Maximum amount of growth was observed in *Serratia marcescens* DT-1P (Table 5.1) while the other three strains of *Pseudomonas* DT-2, DT-Ct1 and DT-Ct2 showed comparable increase in the protein (Table 5.2; Table 5.3 & Table 5.4). Though the growth was more than other three strains in *Serratia marcescens* DT-1P, the degradation with respect to the control was 77.53% more after 72 h of incubation (Fig. 5.2). Although total soluble cellular protein increase was similar in case of *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2, the level of enhancement observed in case of latter was almost double that of the former with 253.74% and 138.07% respectively with respect to the individual control samples (Fig.5.2). But still the total DDT degradation after

Fig. 5.1 Co metabolism of DDT in Presence of Glucose by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

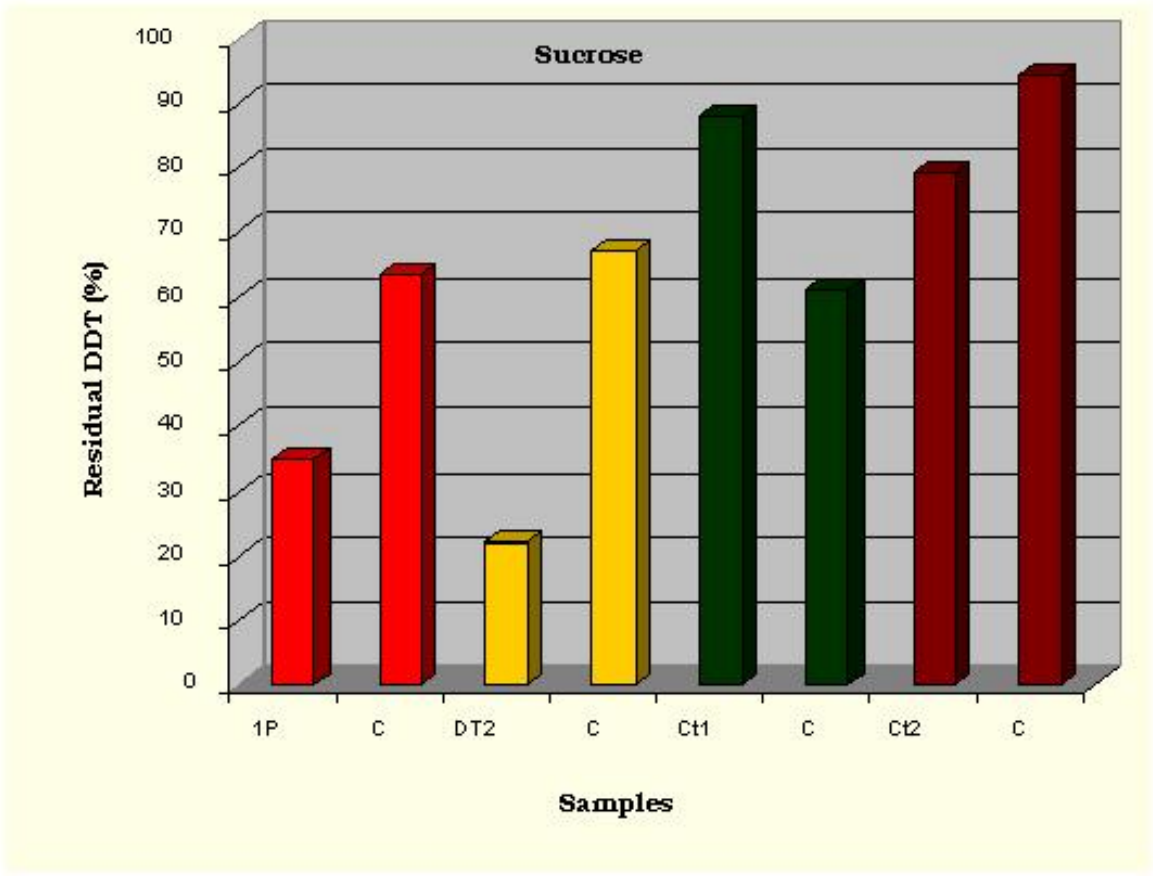
DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

C= Control (Without auxiliary carbon source)

Fig. 5.2 Co metabolism of DDT in Presence of Sucrose by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

C= Control (Without auxiliary carbon source)

the 72 h of incubation was far less than DT-2 in case of DT-Ct2 wherein the degradations achieved were 77.8% and 20.8% respectively (Fig.5.2). Comparable growth was seen in *Pseudomonas aeruginosa* DT-Ct1 (Table 5.3) but the degradation was 68.85% less than that achieved in control flasks (38.88% of the added DDT) (Fig.5.2).

5.3.2.3 Succinate

Sodium salt of succinate helped *Pseudomonas fluorescens* DT-2, to degrade 44.6 % (Fig.5.3) of the initially added 10 ppm DDT, which was maximum among all the four strains. *Serratia marcescens* DT-1P degraded 2.7% less DDT than DT-2. *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 degraded 42.7% and 62.3% less DDT compared to DT-2 (Fig.5.3). Protein wise growth was comparable in *Serratia marcescens* DT-1P (Table 5.1), *Pseudomonas aeruginosa* strains DT-Ct1 (Table 5.3) and DT-Ct2 (Table 5.4). Growth was one-third in case of *Pseudomonas fluorescens* DT-2 when compared with the other three strains. Still the maximum degradation was observed with this strain (Fig.5.3). Almost equal amount of the initially added DDT was degraded by *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2 (43.4% and 44.6% respectively). The highest positive influence was observed in *Pseudomonas aeruginosa* DT-Ct2 with 185.71% more DDT getting eliminated compared to the control of the same individual organism. 18.35% and 36.47% enhancement was achieved in case of *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2 respectively whereas 34.24% decline was observed in *Pseudomonas aeruginosa* DT-Ct1 (Fig.5.3).

Table 5.1: Growth of *Serratia marcescens* DT-1P during Co-metabolism

S.No.	Carbon Source	Total cell protein (μg/mL) At 0 h	Total cell protein (μg/mL) At 72 h
1	Glucose	30	0891
2	Sucrose	30	1206
3	Succinate	30	1085
4	Citrate	30	0871
5	Acetate	30	1060
6	Glycerol	30	1124
7	Yeast extract	30	1096
8	Peptone	30	1104
9	TSB	30	1724
10	Control	30	0510

Table 5.2: Growth of *Pseudomonas fluorescens* DT-2 during Co-metabolism

S.No.	Carbon Source	Total cell protein (μg/mL) At 0 h	Total cell protein (μg/mL) At 72 h
1	Glucose	30	0995
2	Sucrose	30	0943
3	Succinate	30	0309
4	Citrate	30	0527
5	Acetate	30	1124
6	Glycerol	30	1626
7	Yeast extract	30	1257
8	Peptone	30	1246
9	TSB	30	1460
10	Control	30	0041

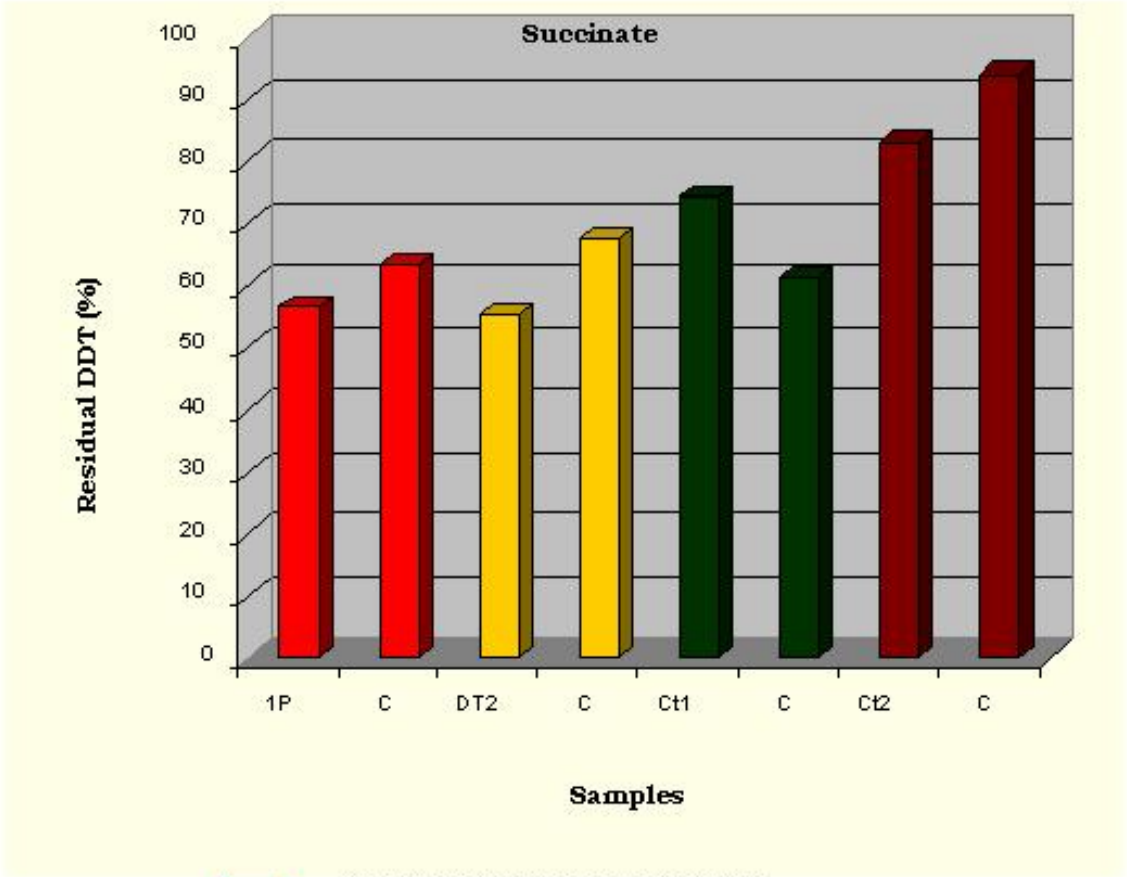
Table 5.3: Growth of *Pseudomonas aeruginosa* DT-Ct1during Co-metabolism

S.No.	Carbon Source	Total cell protein (μg/mL) At 0 h	Total cell protein (μg/mL) At 72 h
1	Glucose	30	1002
2	Sucrose	30	0995
3	Succinate	30	1032
4	Citrate	30	0998
5	Acetate	30	1103
6	Glycerol	30	1114
7	Yeast extract	30	1101
8	Peptone	30	1125
9	TSB	30	1120
10	Control	30	0045

Table 5.4: Growth of *Pseudomonas aeruginosa* DT-Ct2 during Co-metabolism

S.No.	Carbon Source	Total cell protein (μg/mL) At 0 h	Total cell protein (μg/mL) At 72 h
1	Glucose	30	0849
2	Sucrose	30	0952
3	Succinate	30	1002
4	Citrate	30	1101
5	Acetate	30	1111
6	Glycerol	30	1240
7	Yeast extract	30	1253
8	Peptone	30	1211
9	TSB	30	1195
10	Control	30	038.0

Fig. 5.3 Co metabolism of DDT in Presence of succinate by the four Strains.



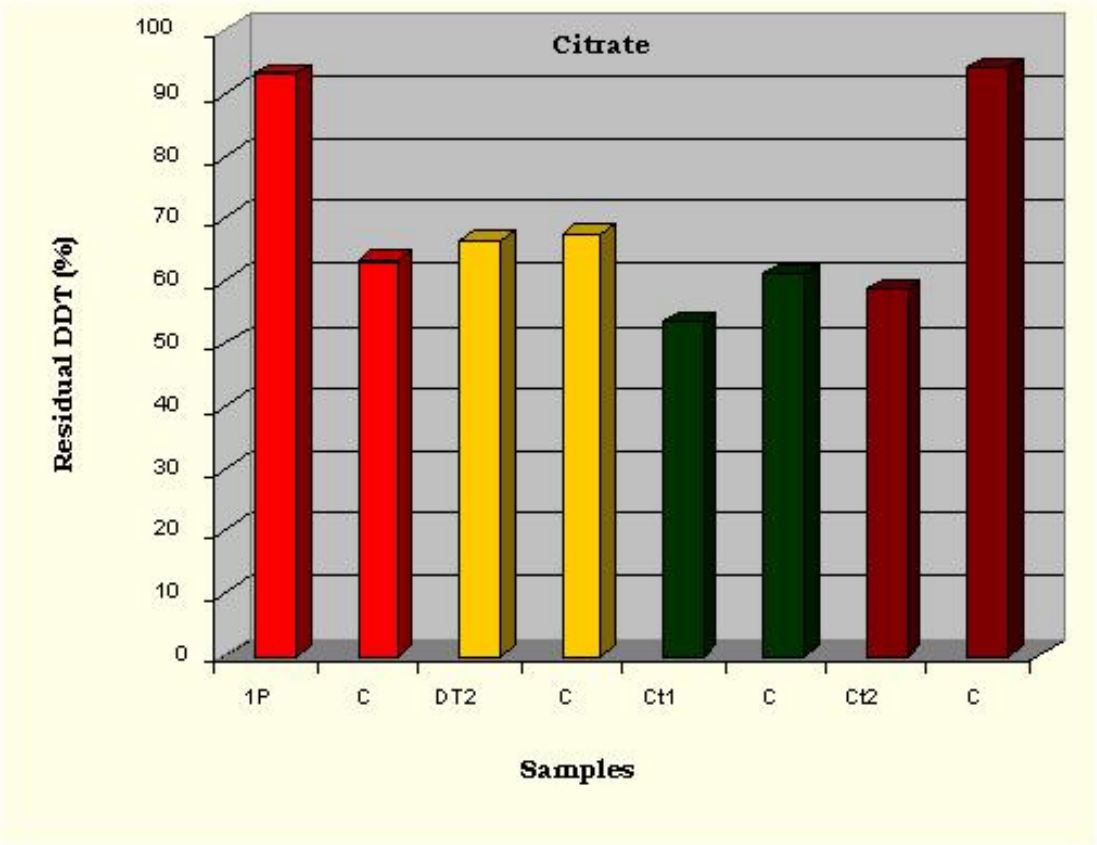
5.3.2.4 Citrate

Pseudomonas aeruginosa DT-Ct1 showed the maximum degradation in presence sodium citrate with 46.4% of initially added 10 ppm DDT disappearing by the end of 72 h of incubation (Fig.5.4). This was followed by *Pseudomonas aeruginosa* DT-Ct2 with 41.3% degradation of DDT in the same period (Fig.5.4), which was 602.38% of its control samples while the DT-Ct1 could attain 19.34% boost with the addition of citrate in the medium (Fig.5.4). In case of *Pseudomonas fluorescens* DT-2, there was a marginal improvement in the degradation with only 2.5% (Fig.5.4) increase from its control flasks while *Serratia marcescens* DT-1P falling down by 81.46% in its activity under controlled conditions (Fig.5.4). Growth wise *Pseudomonas fluorescens* DT-2 was least responsive (Table 5.2). *Pseudomonas aeruginosa* DT-Ct1 (Table 5.3) and *Pseudomonas aeruginosa* DT-Ct2 (Table 5.4) were comparable while *Serratia marcescens* DT-1P being intermediary among these (Table 5.1).

5.3.2.5 Acetate

Maximum degradation was observed in *Pseudomonas fluorescens* DT-2 when inoculated in to the medium containing sodium salt of acetate. The degradation by the end of 72 h of incubation was 77.8 % of the initially added 10ppm DDT (Fig.5.5). *Serratia marcescens* DT-1P degraded 44.2% less than *Pseudomonas fluorescens* DT-2 while the two strains of *Pseudomonas aeruginosa* DT-Ct1 and DT-Ct2 degraded 84.4 % and 17.2 % less when compared to it. (Fig.5.5). Increment in total soluble proteins was highest in *Pseudomonas fluorescens* DT-2 (Table 5.2) followed by *Pseudomonas aeruginosa* DT-Ct2 (Table 5.4), *Serratia marcescens* DT-1P (Table 5.1) and *Pseudomonas aeruginosa* DT-Ct1 (Table 5.3). Both positive as well as negative influences were observed in presence of acetate in the

Fig. 5.4 Co metabolism of DDT in Presence of citrate by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

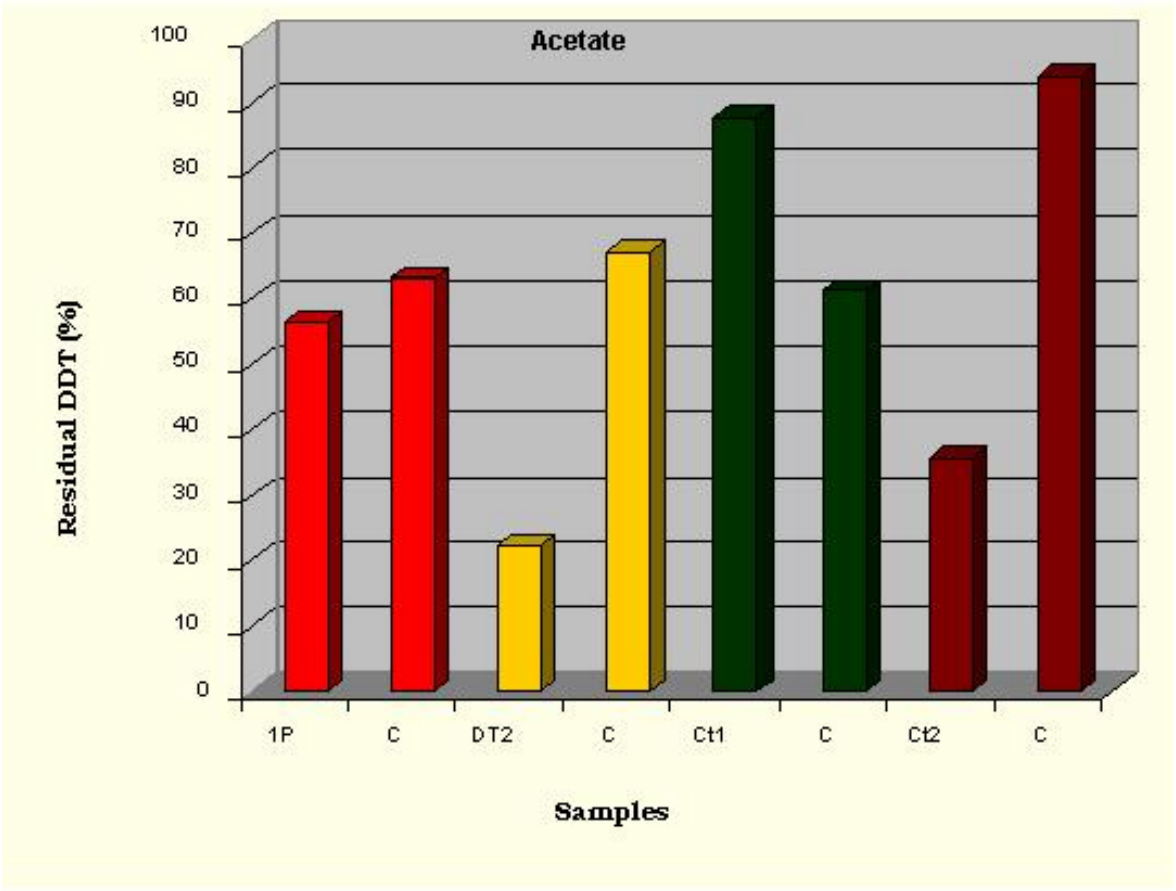
DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

C= Control (Without auxiliary carbon source)

Fig. 5.5 Cometabolism of DDT in Presence of acetate by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

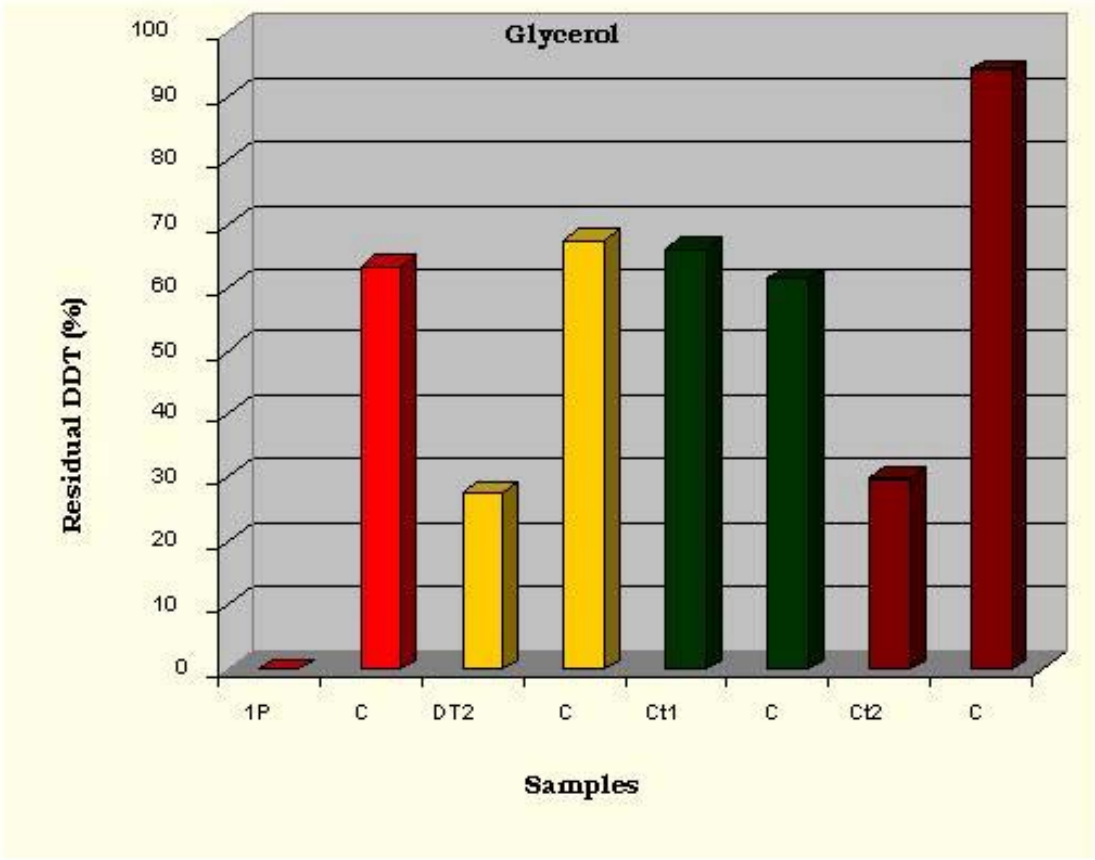
C= Control (Without auxiliary carbon source)

medium. *Pseudomonas aeruginosa* DT-Ct2 was highly supported by the presence of acetate in the medium where it could be 995.24% active on the added 10ppm DDT than its normal activity in absence of acetate (Fig.5.5) yet the total quantity of the added DDT eliminated by it was 64.4% (Fig.5.5). *Pseudomonas fluorescens* DT-2 had an increment in its action on DDT by improving to 135.07% of the control samples with ~78% degradation (Fig.5.5). *Serratia marcescens* DT-1P showed an enhancement to 18.35% that of the control (Fig.5.5) in degradation of the added 10ppm DDT. *Pseudomonas aeruginosa* DT-Ct1 had a retarding action in presence of acetate wherein the degradation of DDT was 68.85% less than that of the control flasks (Fig.5.5).

5.3.2.6 Glycerol

When glycerol was used as the auxiliary carbon source, degradation of 10 ppm DDT was best in *Serratia marcescens* DT-1P wherein 100% degradation was achieved by the end of 72 h of shaking incubation Fig.5.6). *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 degraded 27.7 %, 65.9 % and 30.0 % less as compared to *Serratia marcescens* DT-1P (Fig.5.6). Though the degradation in *Serratia marcescens* DT-1P was 100% the enhancement was accountable to 172.7% that of the control (Fig.5.6) while there was an improvement in case of *Pseudomonas aeruginosa* DT-Ct2 with 1090.47% that of the control, the amount of DDT degradation was only 70.0% of the added DDT (Fig.5.6). In both these cases total soluble protein was comparable (Table 5.1 & Table 5.4). Though the growth was highest in *Pseudomonas fluorescens* DT-2, the improvement was 121.24% of the control with 72.3% of the added 10ppm DDT getting transformed by the end of 72 h of incubation. Degradation in *Pseudomonas aeruginosa* DT-Ct1 was reduced to 12.24% of the control (Fig.5.6).

Fig. 5.6 Cometabolism of DDT in Presence of glycerol by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

C= Control (Without auxiliary carbon source)

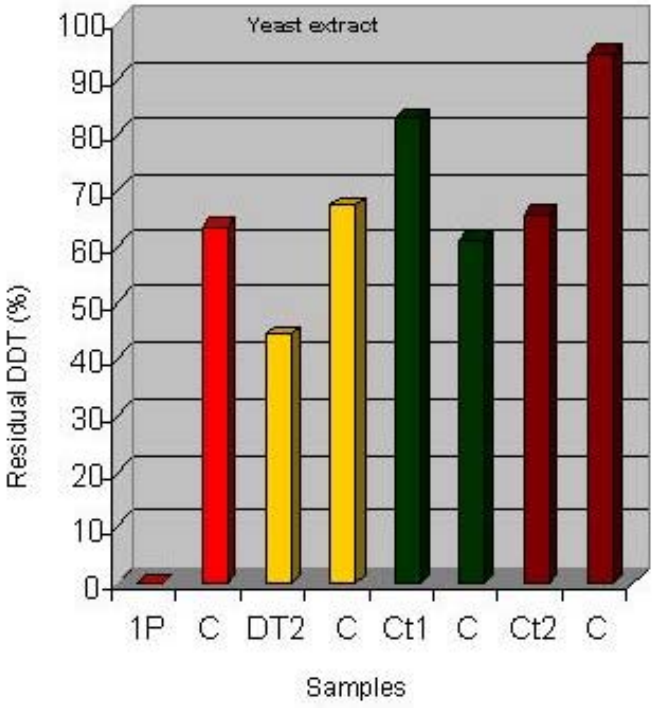
5.3.2.7 Yeast Extract

In yeast extract, 100% degradation of the initially added 10 ppm DDT was observed in *Serratia marcescens* DT-1P (Fig.5.7), by the end of 72 h of incubation. *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 achieved 44.3%, 82.9% and 65.7% less degradation when compared to *Serratia marcescens* DT-1P during the same period of incubation (Fig.5.7). An enhanced rate was seen with 172.7% elimination compared to the control flasks in *Serratia marcescens* DT-1P that accounted for 100% elimination of the parent DDT in the medium and the total soluble proteins were 36 times that of the initially inoculated cells (Table 5.1). *Pseudomonas fluorescens* DT-2 showed 42 times of the initially (at 0 h) provided proteins at the end of 72 h incubation period (Table 5.2) with the elimination of 55.7% of added DDT (Fig.5.7) that was 70.44% more than the control flasks (32.68%) (Fig.5.7). There was a slowed degradation of DDT in case of *Pseudomonas aeruginosa* DT-Ct1 wherein the elimination was 56.09% less than the control flasks (Fig.5.7) though the growth in terms of proteins (Table 5.3) was comparable to that of *Serratia marcescens* DT-1P (Table 5.1). However, *Pseudomonas aeruginosa* DT-Ct2 had an increase in activity by 483.33% more than the control flasks (Fig.5.7) but the total degradation attained was 34.3% of the added 10ppm DDT along with the maximum increment in terms of protein generated (Table 5.4) comparable to that of *Pseudomonas fluorescens* DT-2 (Table 5.2) by the end of 72 h.

5.3.2.8 Peptone

Serratia marcescens DT-1P could degrade 100% of the initially added 10 ppm DDT within 72 h (Fig.5.8) of incubation in presence of peptone. With *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa*

Fig. 5.7 Cometabolism of DDT in Presence of yeast extract by the four Strains.



1P=*Serratia marcescens* DT-1P

DT-2=*Pseudomonas fluorescens* DT-2

Ct1= *Pseudomonas aeruginosa* DT-Ct1

Ct2=*Pseudomonas aeruginosa* DT-Ct2

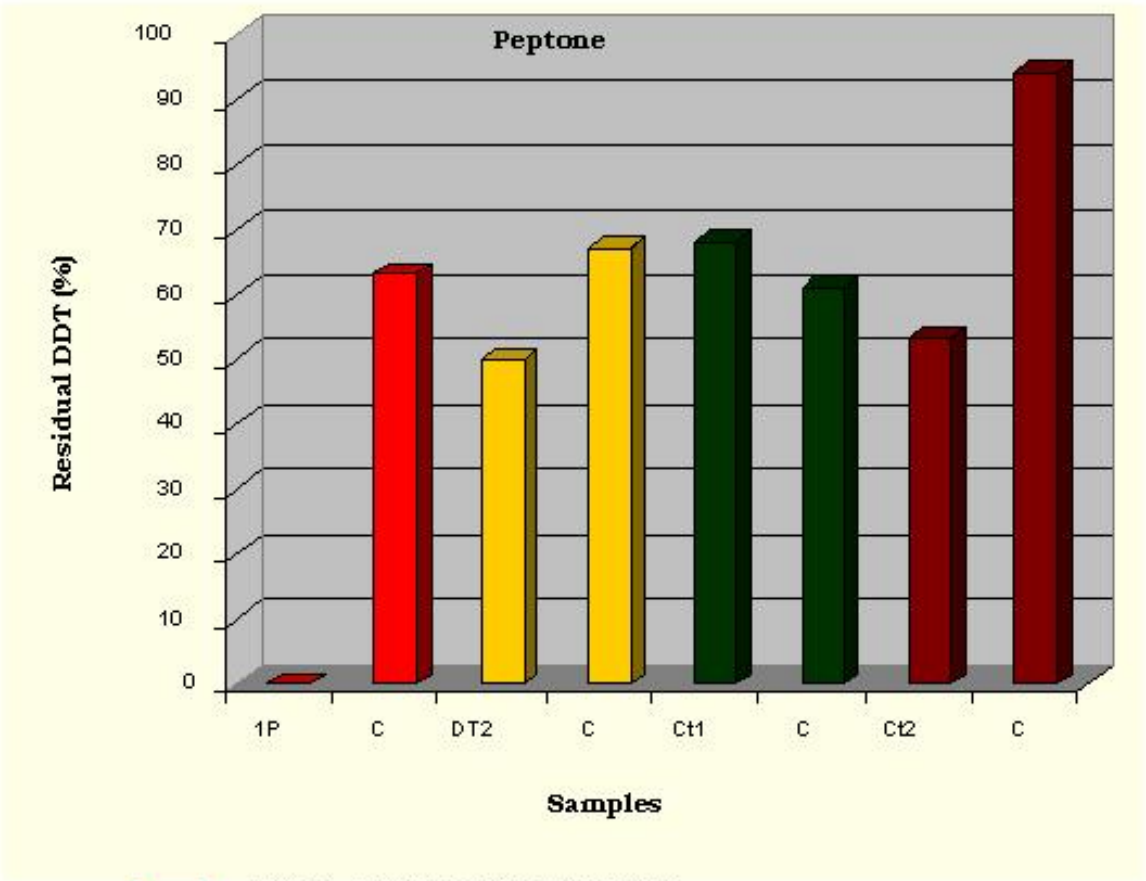
C=Control (Withoutauxiliary carbon source)

DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 49.8%, 68.3% and 53.4% less degradation was achieved as compared to *Serratia marcescens* DT-1P during the same period of incubation (Fig.5.8). There was again 172.7% enhancement in *Serratia marcescens* DT-1P with respect to the control. Final protein here (at the end of 72 h incubation) was ~37 times more than that added at the initial stage (at 0 h) (Table 5.1). *Pseudomonas fluorescens* DT-2 was able to transform 53.46% more DDT than in the control flasks (Fig.5.8) with 41.5 times of total soluble protein available, by the end of 72 h of incubation, with respect to the initially added 30μg soluble protein per mL. The negative influence observed in *Pseudomonas aeruginosa* DT-Ct1 resulted in 18.42% less degradation than the control flasks (Fig.5.8). Growth was 37.5 times to the initially added total soluble proteins (Table 5.3). *Pseudomonas aeruginosa* DT-Ct2 showed 692.52% enhanced degradation than the control samples but with only 46.6% degradation in the initially added 10ppm DDT (Fig.5.8). Increase in protein was ~40 times that of the initially inoculated cells (Table 5.4).

5.3.2.9 Tryptone soya broth

100 percent degradation was observed with *Serratia marcescens* DT-1P inoculum when incubated with Tryptone soya broth as compared to *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2 wherein the degradation was 44.3%, 70.8 % and 45.0 % less respectively than the former. Protein wise growth was very high in both *Serratia marcescens* DT-1P (Table 5.1) and *Pseudomonas fluorescens* DT-2 (Table 5.2) while it was comparable in the two strains of *Pseudomonas aeruginosa* DT-Ct1 (Table 5.3) and DT-Ct2 (Table 5.4). Protein increase observed was 56.46%, 47.66%, 36.33% and 38.83% respectively in case of *Serratia marcescens* DT-1P (Table 5.1), *Pseudomonas fluorescens* DT-2 (Table 5.2), *Pseudomonas aeruginosa* DT-

Fig. 5.8 Cometabolism of DDT in Presence of peptone by the four Strains.



DT-1P= *Serratia marcescens* DT-1P

DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

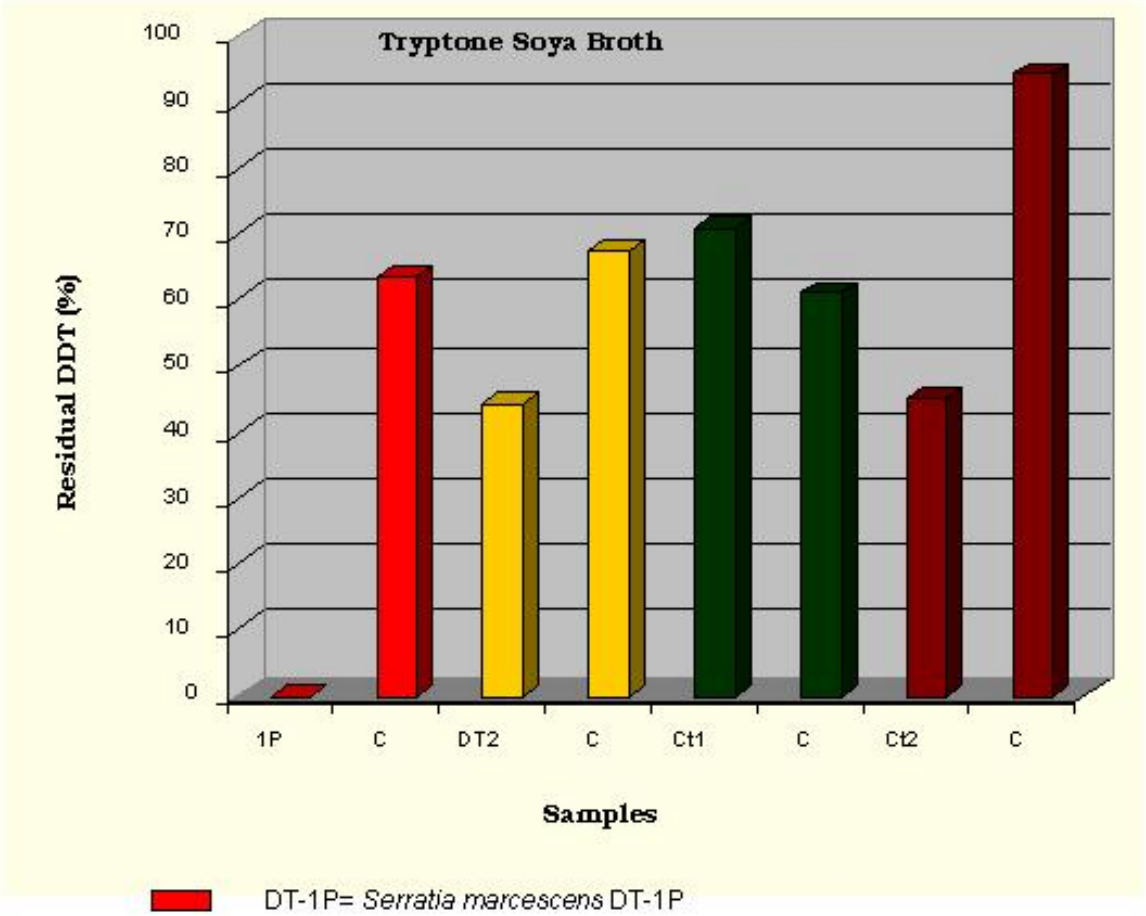
C= Control (Without auxiliary carbon source)

Ct1 (Table 5.3) and *Pseudomonas aeruginosa* DT-Ct2 (Table 5.4). The initially added 10 ppm DDT was efficiently degraded in *Pseudomonas aeruginosa* DT-Ct2 (with 835.37% more degradation than the control flasks) (Fig.5.9), *Serratia marcescens* DT-1P (with 172.7% more degradation than the control flasks) (Fig.5.9) and *Pseudomonas fluorescens* DT-2 (with 70.44% more degradation than control flasks) (Fig.5.9) while it declined by 24.89% than the control flasks in *Pseudomonas aeruginosa* DT-Ct1 (Fig.5.9). The total degradation was ~55% of the initially added DDT in both *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2 while it was ~30% in *Pseudomonas aeruginosa* DT-Ct1 (Fig.5.9).

5.4 Discussion

Cometabolism has been defined in a number of ways (Alexander, 1973; Foster, 1962, Jensen, 1963; Horvath, 1972). It can been defined as the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound (Dalton and Stirling, 1982). It is a known fact that the bioremediation works best as cometabolism (Grady, 1985). A stimulation of DDT metabolism by the addition of nutrients has been shown to occur in the soil (Ko and Lockwood 1968; Parr *et al.* 1970). The enhanced rate of DDT–degradation by the addition of some carbon sources could be ascribed to the frequency of DDT–co-metabolising cells and the stimulation of metabolizing enzymes by the additional carbon sources. It could be possible that the degradative enzymes either be constitutive or induced by non–insecticidal compounds (Pfaender and Alexander 1973). Pfaender and Alexander (1972) have shown evidence for the presence of degradation enzymes in cultures grown in the absence of added DDT, which shows the possibility of involvement of constitutive enzyme(s) in the degradation of DDT. Possible differential effects of additional carbon sources on DDE degradation by *Alcaligenes denitrificans* has been

Fig. 5.9 Cometabolism of DDT in Presence of Tryptone soya broth by the four strains



DT-1P= *Serratia marcescens* DT-1P

DT2= Pseudomonas fluorescens DT-2

Ct1= Pseudomonas aeruginosa DT-Ct1

Ct2= Pseudomonas aeruginosa DT-Ct2

C= Control (Without auxiliary carbon source)

reported by Ahuja *et al.* (2001) wherein addition of glucose enhanced the degradation while addition of sodium acetate and sodium succinate had an inhibitory effect on the degradation of DDE. In all the three carbon sources bacterial growth had been shown to be better. Influence of more favorable carbon sources, on the degradation of environmental pollutants, has also been shown in other studies (Hartline and Gunsalus 1971; Holtel *et al* 1994; Samson *et al* 1998). Decrease in the degradation capability in presence of certain auxiliary carbon source, such as sodium salt of citrate in case of *Serratia marcescens* DT-1P (6.8% as compared to 36.7% degradation in control, of the added 10 ppm DDT) (Fig.5.10), 12.11% degradation by *Pseudomonas aeruginosa* DT-Ct1 in presence of both sucrose (Fig.5.12) and sodium salt of acetate (Fig.5.12), 25.55% in Sodium salt of succinate (Fig.5.12), 34.12% degradation in glycerol (Fig.5.12) as compared to 38.9% in control flasks (Fig.5.12), could be due to the availability of the easily degradable carbon sources wherein the organisms might try to save the energy and time for the synthesis of required enzymes (or enzyme systems) necessary for the degradation of DDT. It could also be possible that the metabolites in these cases were either more toxic than the original substrate (DDT) to the organisms or they have a structure that prevents them from being acted upon by the other enzyme(s) (Grady, 1985). When considering the definition of co-metabolism, Dalton and Stirling (1982) differentiated it from gratuitous metabolism wherein the enzyme required to transform the substrate in to a non-metabolisable metabolite with its accumulation in the medium, is present within the cell. Once the metabolite is formed, degradation (transformation) would cease. On the other hand, when the substrate/metabolite requires to be transported in to the cell, the energy required for it would be generated by the other transformable growth substrate, is considered as co-metabolism. When the organism comes across a source that can help it in carrying out the necessary life processes and helps by providing the reducing power for the biosynthetic reactions by donating electron (Gardy, 1985), the organism tends to save the energy required for the

Fig. 5.10: Co- metabolism of DDT by DT- 1P

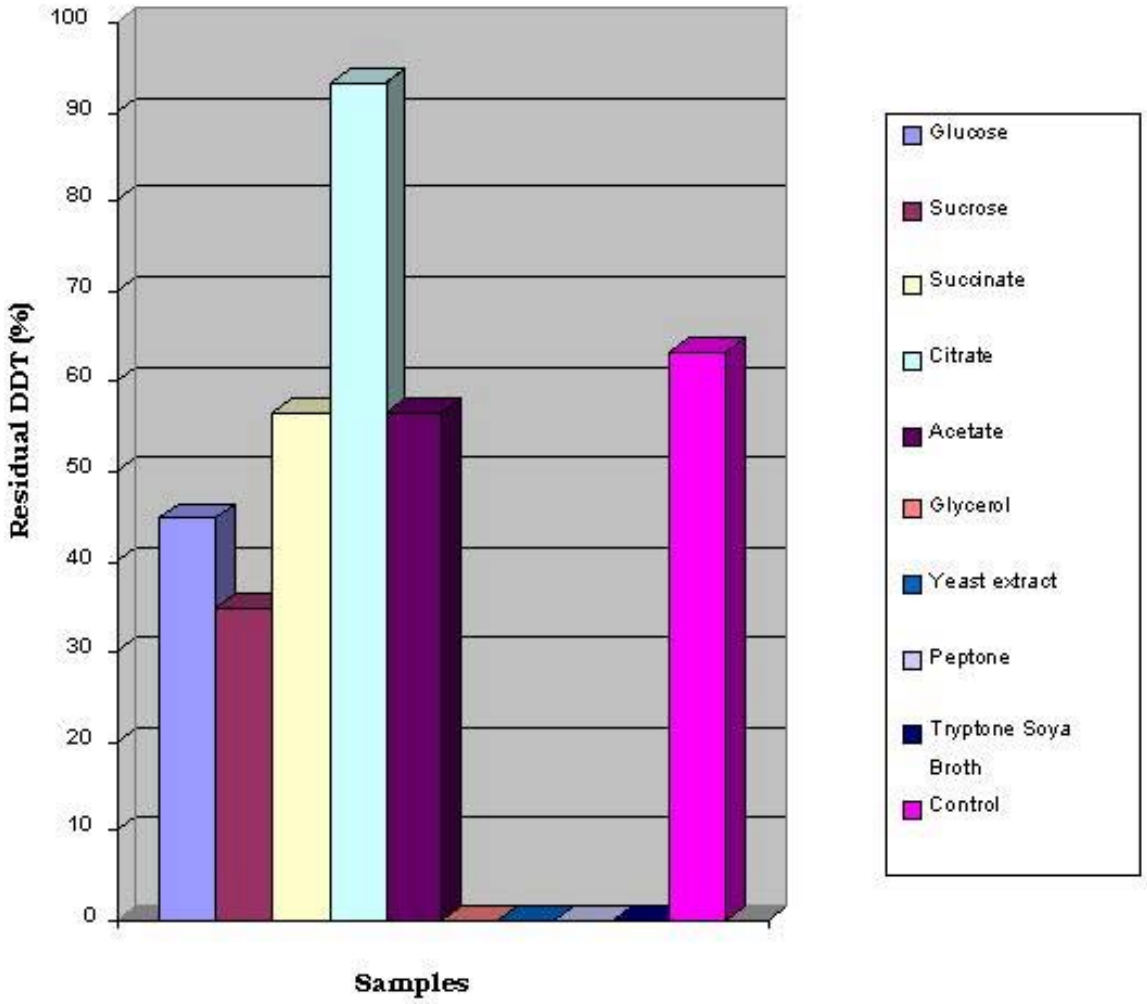


Fig.5.11: Co-metabolism of DDT by DT-2

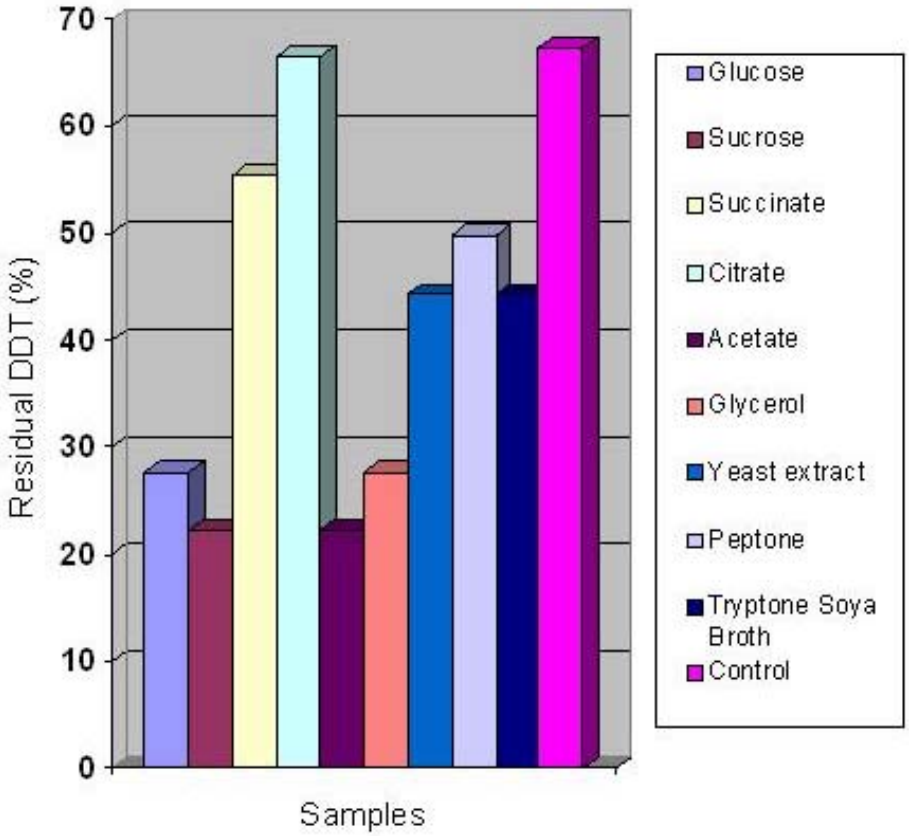


Fig. 5.12: Co- metabolism of DDT by DT-Ct-1

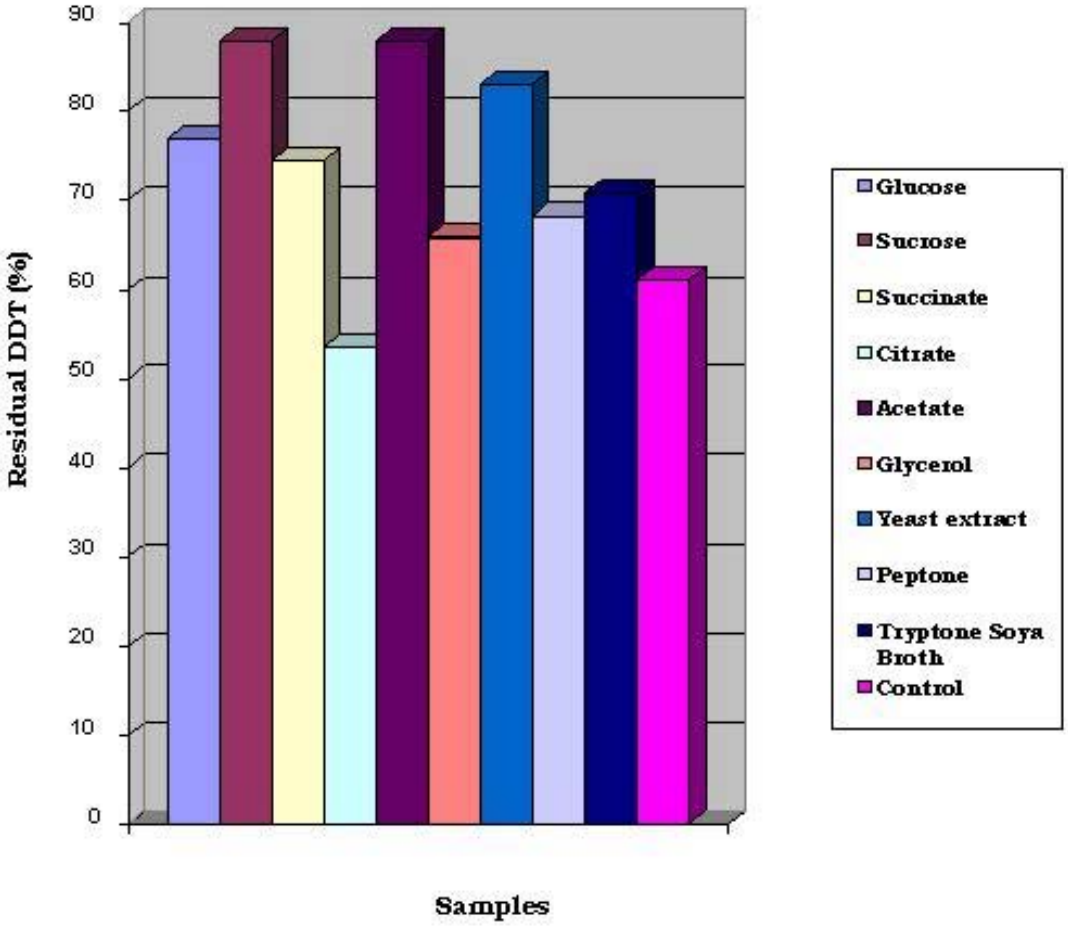
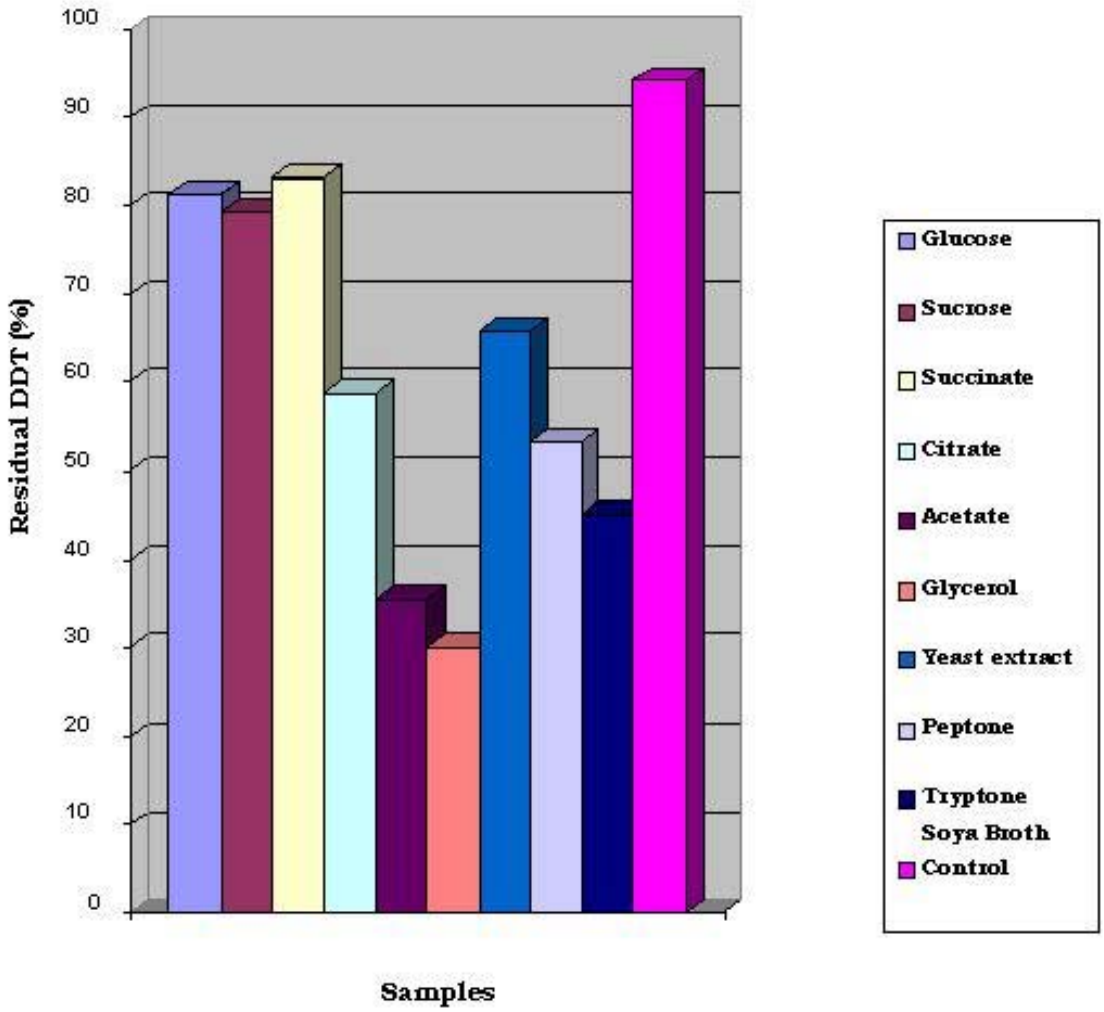


Fig. 5.13: Co- metabolism of DDT by DT-Ct-2



synthesis of induced enzymes, thus leading to the accumulation or no degradation of the xenobiotics as could be the case with reduced degree of degradation observed in presence of citrate for *Serratia marcescens* DT-1P (Fig.5.10) and *Pseudomonas fluorescens* DT-2 (Fig.5.11) and also with all other carbon sources used (except citrate) in case of *Pseudomonas aeruginosa* DT-Ct1 (Fig.5.12).

Most of the organisms tend to transform the xenobiotics into an intermediate that is easily degradable by the most commonly occurring enzyme systems. Most such intermediates are directed towards the tricarboxylic acid (TCA) cycle and in to the electron transport chain. May be this is one of the reasons where we find very less degradation in case of sodium citrate, that could directly enter the TCA cycle, rendering DDT undetected to a greater extent at least in case of the two strains viz. *Serratia marcescens* DT-1P (Fig.5.4) and *Pseudomonas fluorescens* DT-2 (Fig.5.4). Other two strains of *Pseudomonas* could degrade DDT in presence of citrate (Fig.5.4), indicating one of the possibilities that similar enzymes from different sources show different degree of substrate and, probably functional group specificities.

Degradation is also dependent on the biomass available to act on the xenobiotics. When the organisms are provided with high nutrient medium along with the xenobiotics, the population increases due to the rich medium and finally they act on the target compound. One reason can be the synthesis of enzymes with low specificity that can bind to the substrate as well, converting it to a possibly easily degradable form. It could also be possible that the substrate (DDT) gets tagged to the rich nutrient molecules, without affecting the charge on the active site of the enzyme, leading to its bioconversion in that functional group form.

As evident from the Fig.5.10, Fig.5.11 and Fig.5.13, *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2, could degrade DDT at higher rate in presence of auxiliary carbon sources than controls where no additional carbon source was added, it can be accepted that co-metabolism is faster. In nature, the pollutant is always present along with the other

carbon and energy sources that the organisms can grow on and simultaneously act on the xenobiotics thereby eliminating them from the environment. Also, there will always be the native microbial community present at the site of bioremediation (effluent or water bodies or soil or waste dump sites) that may show synergistic or a negative effect on the activity of the inoculated bacterial strain. Most successful trials for the perfect bioremediation of contaminated sites and resources are the use of consortium or a community that can adapt itself well with the existing native communities. In such instances the probability of bioremediation *via* co-metabolism would be high.

Chapter 6

There will come soft rains and the smell of the ground,
And swallows circling with their shimmering sound;
....
.....
Not one would mind, neither bird nor tree,
If mankind perished utterly.

Teasdale (1937)

6.1 Introduction

DDT is one of the most extensively studied insecticides, mainly for its broad insecticidal spectrum. The primary metabolic mechanism that was studied was the reductive dechlorination of DDT, with the formation of DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane or dichlorodiphenyldiichloroethane) (Kallman and Andrews, 1963; Barker and Morrison, 1964). This degradation was later determined to be microbial and *Proteus vulgaris* was isolated (Barker and Morrison, 1965) which could degrade DDT mainly to DDD. Several minor metabolites like DDMU (1-chloro-2,2-bis(4-chlorophenyl)ethylene), DDM (bis(4-chlorophenyl)methane) were also formed (Barker and Morrison, 1965; Barker *et al*, 1965). Numerous microorganisms have been isolated from animal and plant sources that could degrade DDT to DDD. These include *Escherichia coli* and *Aerobacter aerogenes* isolated from rat faeces (Mendel and Walton, 1966; Mendel *et al*, 1967), anaerobic bacteria from stable fly gut (Stenerson, 1965) rumen fluid microflora (Miskus *et al*, 1965), rat intestinal microflora (Braunberg and Beck, 1968), *E. coli* (Langlois, 1967) and plant pathogens (Johnson *et al* 1967).

The formation of DDD from DDT is also a common reaction among soil microorganisms (Guenzi and Beard, 1967). Chacko *et al* (1966) isolated numerous actinomycetes (*Nocardia* sp., *streptomyces aureofaciens, Streptomyces cinnamoneus, Streptomyces viridochromogenes*) from soil, which readily degraded DDT to DDD. These organisms however, required another carbon source to facilitate degradation. Soil fungi not only produced DDD and small amounts of dicofol (, but some variants could produce DDA (bis(4-chlorophenyl)aceticacid) or DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) exclusively (Matsumura and Boush, 1968). Wedemeyer (1967) reported dehalogenation of DDT to various metabolites under anaerobic conditions by *Aerobacter aerogenes*. DDD was obtained under both aerobic as well as anaerobic conditions when DDT was incubated with *Aerobacter aerogenes* (Mendel *et al*, 1967; Wedemeyer, 1967). *Escherichia coli* dechlorinated 50% of DDT to DDE when grown in various broths

or skimmed milk (Langlois, 1967). Under aerobic conditions the major product of DDT metabolism, in *Bacillus cereus, B. Coagulans, B. subtilis,* was DDD while DDMU (1-chloro-2,2-bis(4-chlorophenyl)ethylene), DDMS (1-chloro-2,2-bis(4-chlorophenyl)ethane), DDNU (2,2-bis4-chlorophenyl)ethane), DDOH (2,2-bis(4-chlorophenyl)ethanol), DDA and DBP (4,4'-dichlorobenzo phenone) were in trace amounts and were found under anaerobic conditions (Langlois *et al*, 1970). *Hydrogenomonas* sp. yielded DDD, DDMS, DDMU, DBH (4,4'-dichlorobenzhydrol), DDM (bis(4-chlorophenyl)methane) and DDA (Focht and Alexander, 1970). DDD was further degraded through dechlorination, dehydrochlorination and decarboxylation to DBP or to a more reduced form, DDM. 4% of initial DDT was 75% transformed by *Phanerochaete chrysosporium* (Bumpus and Aust, 1987). Bumpus *et al* (1985) showed the involvement of lignin peroxidase in the degradation of DDT. Wedemeyer (1967) studied DDT metabolic pathway by incubation of proposed intermediates with organisms and examining the products formed. The metabolism of DDT in *Aerobacter aerogenes* goes in order

DDT → DDD → DDMU → DDNU → DDOH → DDA → DBP and direct conversion of DDT to DDE (Wedemeyer, 1967). The drawback was that the products beyond DDNU could not be detected after incubating any of the preceding metabolites with the organism. But later, it was reported that DDA transformed to DBP *via* DBH (Wedemeyer, 1967). *Pseudomonas* has been shown to metabolise DDT in the sequence that involved ring cleavage to PCPA (*p*-chloro phenyl acetic acid) (Focht and Alexander, 1970). Subba Rao and Alexander (1985) reported the formation of DBH, DBP, benzhydrol, benzophenone, *p*-chlorophenyl ethanol and *p*-chlorophenyl glycoaldehyde. DDE, dicofol and DBP were formed during DDT degradation by *Phanerochaete chrysosporium* (Bumpus

and Aust, 1987). A review of bidegradation of DDT and its residues has been given by Aislabie *et al* (1997). Reports on the involvement of enzymes in the degradation of DDT indicate the presence of enzymes like dioxygenases (Nadeau *et al*, 1994, 1998), dehydrogenases (Bourquin (1977) oxygenases (Ahmed *et al,* 1991). Only a few enzymes have been described in DDT degradative pathway (Singh *et al,* 1999).

The recent work of Nadeau *et al* (1994, 1998) described the aerobic degradation of DDT by *Alcaligenes eutrophus* A5. They found the degradation to take place *via* the formation of DDT-2,3-dihydrodiol and a final yellow coloured ring fission product. The ring fission product then transformed to 4-chlorobenzoate. In their study the microorganism was grown on biphenyls. In our laboratory studies were undertaken to deciphor the DDT degradation pathway by the identification of intermediary metabolites.

6.2 Materials and Methods

6.2.1 Chemicals

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), DDD, DDE, DDNU, DDOH, mono-, di-, tri-, tetra- chlorobenzoates, mono chloro- catechols, hydroquinone, nitrophenol, phenol, etc., all 98-99% purity, were purchased from Sigma-Aldrich Chemical Company, Mo, USA. *o*-Tolidine, peptone and glycerol were purchased from HiMedia, Mumbai, India. Solvents like acetone, DMF (dimethyl formamide), ethyl acetate, diethyl ether, cyclohexane were of analytical and HPLC grade and were procured from E- Merck (India) Ltd. HPLC purified oligonucleotides (Primers) were procured from MWG company, Germany.

Agarose (Gelling temperature 40-42^{0}C) was purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Ethedium bromide was procured from Sigma Chemical Co., US. Other chemicals were of analytical/HPLC grade and were purchased from standard chemical companies.

6.2.2 Cultures and media

Peptone-glycerol (3.3.4) media was used for growing the inoculum. Cultures taken for the pathway studies were *Serratia marcescens, Pseudomonas fluorescens* and *pseudomonas aeruginosa.* Cultures were grown in this medium for 72 h on a rotary shaker at ambient temperature (26-30^{0}C). These peptone-glycerol grown cells were then pre-exposed to 25ppm DDT for 48h respectively, followed by 30ppm for 48h. The pre-exposed cells were harvested, washed well in sterilised minimal medium (3.3.1a) and then used as inoculum for further studies (as resting cells).

6.2.3 Methods

6.2.3a Inoculation, Incubation and Sampling

All the pre-exposed and well-washed inocula from the two cultures were used when freshly harvested. To 200mL, 0.1M sterilised phosphate buffer (pH 7.2) in 1L sterile Erlenmeyer flasks was added 0.02 mM DDT as DMF solution inside a UV-sterilised laminar hood. The cells were inoculated at an OD_{600} of 5.0. The inoculated flasks were then incubated at room temperature (26-30^{0}C) on a rotary shaker set at 180rpm for different intervals of time. Samples (whole flask each time) were drawn at regular intervals, first after every three hours up to 12 h, followed by sampling at every 12 h up to 72 h and then every 24 h up to 240 h. The flasks were extracted thrice in equal volumes of diethyl ether. The extracted solvent layers were pooled, for each culture separately. These solvent extracts were allowed to pass through anhydrous sodium sulphate to remove any aqueous matter in it. Further purification was done by passing the solvent extracts through

florisil columns. The solvent was allowed to evaporate at room temperature to concentrate the residual DDT and its metabolites. Residual metabolites were dispensed in a known volume of HPLC grade acetone and used for the detection of metabolites by TLC, GC, HPLC and GC-MS and NMR. The aqueous phase from the extracts was also used for detecting the polar intermediates.

6.2.3b Analytical

(i) Thin Layer Chromatography

Thin layer chromatography (TLC) was done on silica gel G 60- 100 mesh uniformly spread over a 20 x 20 cm^2 glass plate. The thickness of the gel was set at 300 μm. These plates were left to dry at room temperature after spreading (using Camag automatic spreader, Germany) then activated at 100^o C for 1 h. Known volume of the residual extract of DDT (acetone solution) was spotted on to these plates. Spotted plates were developed in cyclohexane, air- dried and the residue was detected by spraying o-tolidine (2% solution in acetone) followed by exposure to bright sunlight. The chloro- compounds give peacock green/ blue colour with this chromogen. Spots were delineated by marking with a needle and area measured. Quantity of DDT in each spot was estimated from a standard graph prepared for √area vs log (DDT concentration).

(ii) Gas Chromatography

Concentrated residual substrate (air dried completely at room temperature), after passing through activated florisil, was resuspended in a known volume of HPLC grade acetone and gas chromatography was done using Chemito 1000 series gas chromatograph (Nasik, India). 1μl of the extract suspension was injected in to a BP-5 capillary column (30m x 0.25mm ID) set at 180^o C and programmed as: 180^o C for 10 min and a rise @ 2^{o}C/min up to 220^o C and maintained there for 2 min. Injector was maintained at 250^o C while electron capture detector (Ni^{63}) was maintained at 280^o C. Pure nitrogen gas was used as the carrier @ 1 ml min^{-1}. Under these conditions, the standard retention time for

DDT was 28.16 min. Quantification of DDT in the sample was done using the area under the peak with and the standard under same conditions.

(iii) HPLC of aqueous phase

The aqueous phase of each of the extracted samples was concentrated to a minimum quantity by flash evaporation at 35^0C (Brun, Germany) and was used for the HPLC (LC-10A/AAA, Shimadzu, Japan) fitted with C-18 Column and methanol:water:acetic acid at 40:60:1 as the mobile phase at a flow rate of 1 mL/min. Detection was done by a UV detector at 235 and 250nm.

(iv) Preparative HPLC of solvent extract

Florisil-passed solvent extracts were used for the HPLC (LC-18, Shimadzu, Japan) with C-18 column (20mm x 250mm) and mobile phase acetonitrile: water (7:3) at a flow rate of 10mL/min. UV detector at 210nm was used for detecting the peaks. Fractions collected for each peak were concentrated and used for GC, GC-MS and NMR analysis.

(v) GC-MS (gas chromatography- Mass spectrometry)

The extracts from the samples were used in the GC-MS (GC-17A gas chromatograph; QP-5000 mass spectrometer, Shimadzu, Japan) analysis. The conditions used were temperature programme from 50^0C-250^0C with increase at the rate of 10^0C per minute after an initial temperature stand of 50^0C for 2 minutes in OV-1 capillary column. Helium gas carried the sample injected at 250^0C @ 1mL/min. The GC detector was maintained at 250^0C. The GC peaks and the corresponding mass spectra were used to interpret the intermediary metabolites by comparing the standard GC and MS patterns for various compounds from the library as well as from the available authentic compounds after injecting into the instrument at the same conditions for the generation of the spectra.

(vi) ^{1}H-NMR

Proton-nuclear magnetic resonance was done using the solvent extracts in $CDCl_3$ solvent in a DRX500 NMR instrument (Bruker, Germany). Analysis was carried out at 400MHz with 5000 scans per sample. The inbuilt software integrated the data generated.

6.2.4 Designing the primers

Primers were designed using the online software Primer3.0 from internet, after obtaining the gene sequences of the enzymes of interest from NCBI site. The sequences from various organisms were aligned using the online software help Dialign 2.0. These dialign results were then used for generating the primers, manually as well as using the software Primer 3.0.

6.2.5 Isolation of Genomic DNA

Genomic DNA was isolated using the method described below:

Suspended a 30 h old culture (after centrifugation) in 500μL lysozyme solution (containing 2mg/mL lysozyme and 50mg/mL heat treated RNase) and incubated at 37^0C for around 30 minutes or till the cells became translucent. To this was added 250μL of 2% SDS (sodium dodecyl sulphate) and vortexed gently to mix until the viscosity of the solution decreased noticeably. 250μL of neutral chloroform solution was added to this and vortexed for 30 seconds, spun for 2 minutes in micro centrifuge and the supernatant was removed leaving the white interface behind. This was repeated twice or till no or very little interface was seen. To this 0.1 volume of 3M sodium acetate (pH 4.8) was added and mixed followed by the addition of 1 volume of isopropanol and mixing. Contents were incubated for 5 minutes at room temperature and spun for 2 minutes in the micro centrifuge. Supernatant was poured and spun briefly, all the liquid was removed using pipette. The pellet was redissolved in 500μL of TE buffer and kept at room temperature for 5 minutes, centrifuged and the supernatant was removed. The pellet was

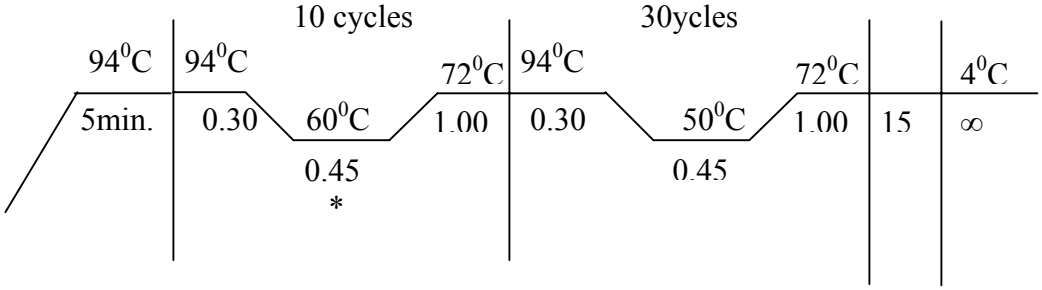
dispersed by vortxing in 300μL of 0.3 M sodium acetate, 10mM $MgCl_2$. 700μL ethanol was added, mixed well and kept at room temperature for 1 hour. The supernatant was removed completely by centrifugation. The pellet was dissolved in 500μL of TE buffer.

6.2.6 Polymerase Chain Reaction

Polymerase chain reaction was carried out with the isolated genomic DNA (6.2.5) and the designed primers (6.2.4) for different genes with Perkin Elmer, US PCR unit as follows:

Sterile deionised water	18.98μL
Taq. Buffer	2.5μL
dNTPs	0.5μL
Taq Polymerase	0.3μL
Forward Pimer	1.0μL
Reverse Primer	1.0μL

A touchdown PCR was done programmed as follows



6.3 Results and Discussion

Degradation Intermediates were obtained by inoculating DDT-induced cells of three of the DDT-degrading strains of the DDT-degrading consortium. The three strains were *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* Dt-Ct1. Since the DDT concentration was high, the chances of intermediates accumulating and their detectable concentration were

more. Two intermediates were observed in TLC. One of these appeared as very faint yellow band near the origin with a low R_f value on the TLC plates and could not be detected. A preparative HPLC was carried out with the solvent extracts in C18 column at 210nm with a flow rate of the mobile phase at 10mL/min. All the fractions corresponding to various peaks were collected. The peaks corresponding to the pure and mixed standards were tentatively identified and the purified fractions then were used for the GC, GC-MS and ^{1}H-NMR analysis. The analysis with GC and GC-MS confirmed the identity of the compounds tentatively identified by HPLC. All the results of preparative HPLC and GC-MS are given in Table 6.1a,b.

Table 6.1a Compounds detected in HPLC of the solvent extracts

Compound Identified	*Serratia marcescens*	*Pseudomonas fluorescens*	*Pseudomonas aeruginosa*
DDT	✓	✓	✓
DDA	✓	✓	✓
DDOH	✓	-	-
2-CBP	✓	✓	✓
4-CP	✓	-	✓
DCBP	✓	✓	-
4-CBA	✓	✓	✓
3-CBP	-	✓	-
4-CC	✓	✓	-

DDT=dichloodiphenyltrichloroethane
DDA= Dichlorodiphenyl acetic acid
DDOH= 4,4'-Dichlorodiphenyl ethanol
2-CBP= 2-chlorobenzophenone
4-CP= 4-chlorophenol
DCBP= 4,4'-Dichlorobenzophenone
4-CBA= 4-chlorobenzoate
3-CBP=3-chlorobenzophenone
4-CC= 4-chlorocatechol

Table 6.1b Few compounds identified in MS

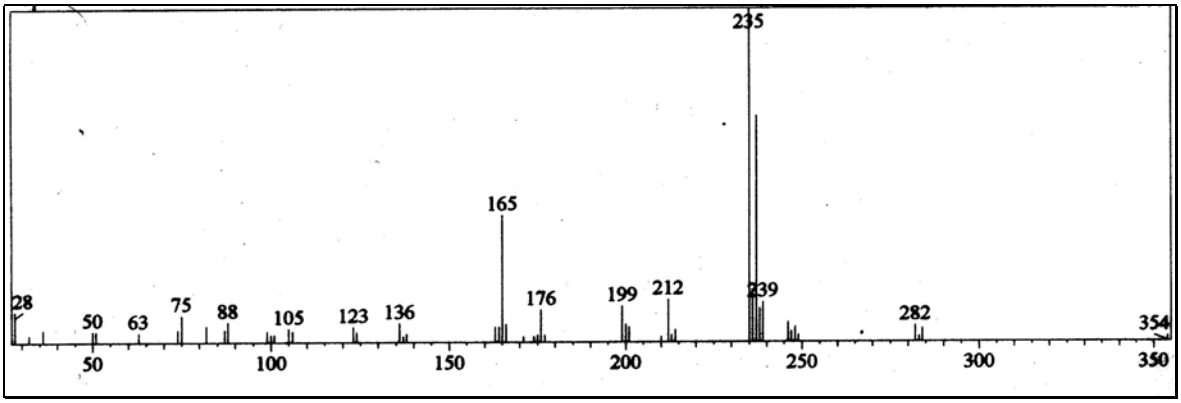
Compound	*Serratia marcescens*	*Pseudomonas*
p,p'-DDT	✓	✓
DDD	✓	✓
DDMU	✓	✓
DDMS	✓	✓
DDE	✓	✓
PCPA	✓	
DDOH		✓
DDA	✓	✓

Fig.6.1 depicts the MS of the intermediates that matched with the standard MS patterns.

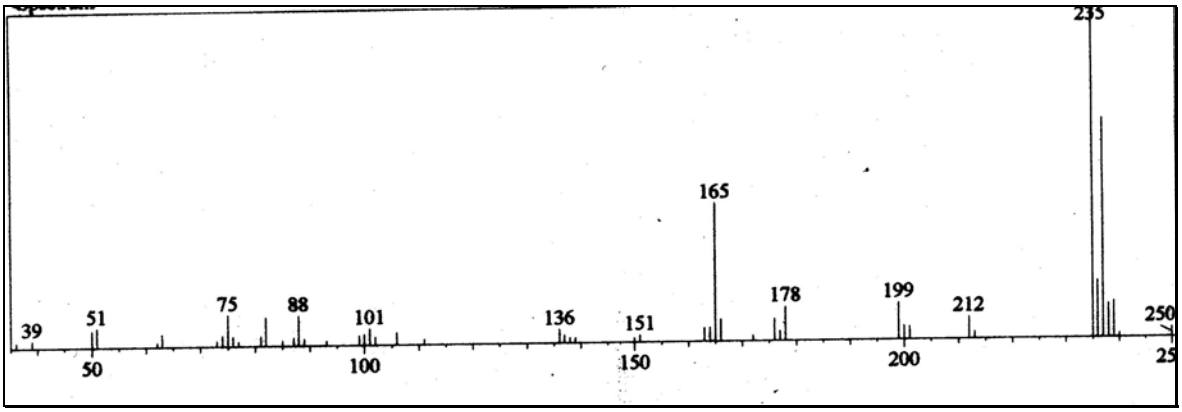
HPLC of the concentrated aqueous phase was carried out in the process of identification of certain water-soluble intermediates. C-18 column was used and a wavelength of 235 and 250nm were used to identify various intermediates. The list of intermediates identified during HPLC of the aqueous phase is given in Table 6.2.

Proton NMR was used to confirm the formation of the early intermediates during the degradation of DDT. Few intermediates confirmed with NMR are listed in Table 6.3. Few of the ^{1}H-NMR peaks are shown in the Fig.6.2.

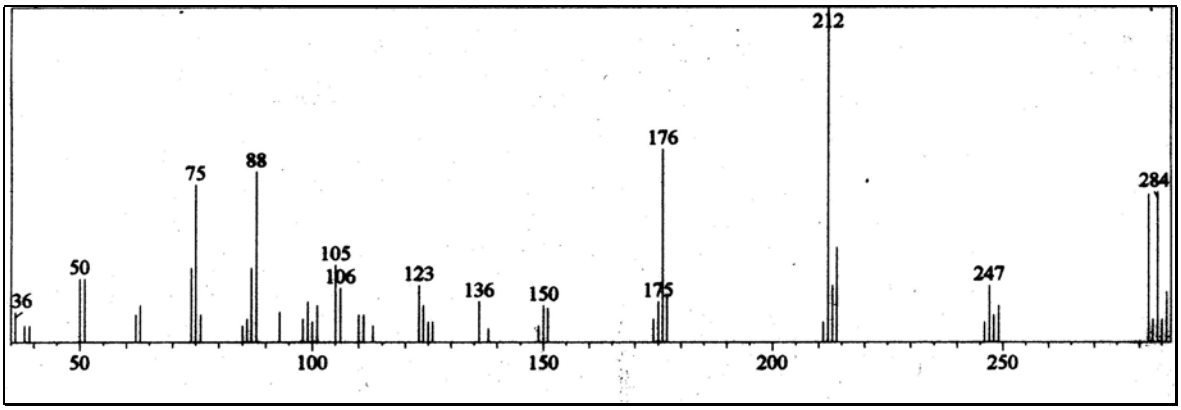
Fig.6.1: MS pattern of few standards and sample-peaks



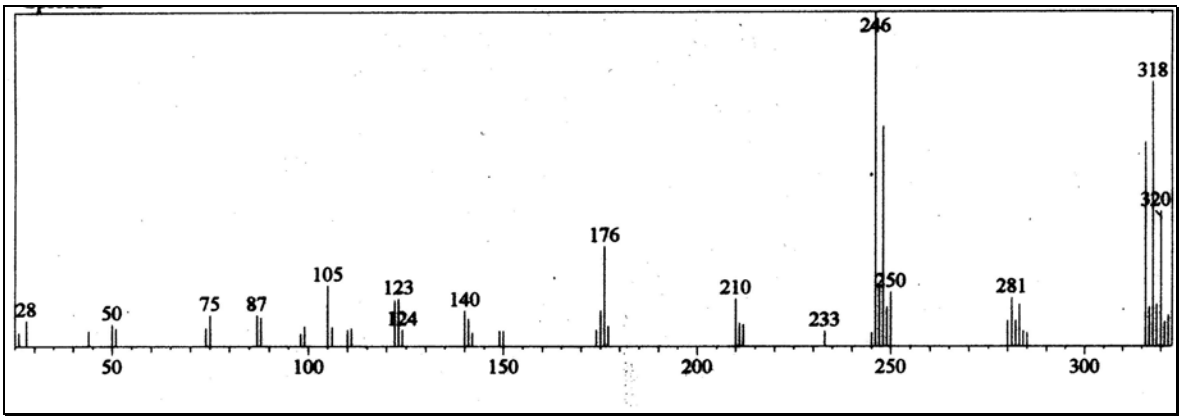
***p,p'*-DDT**



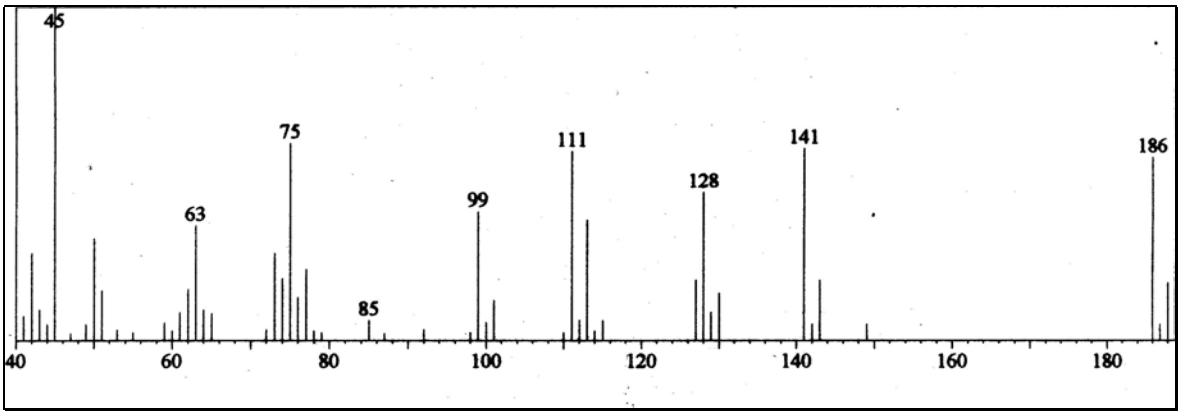
***p,p'*-DDD**



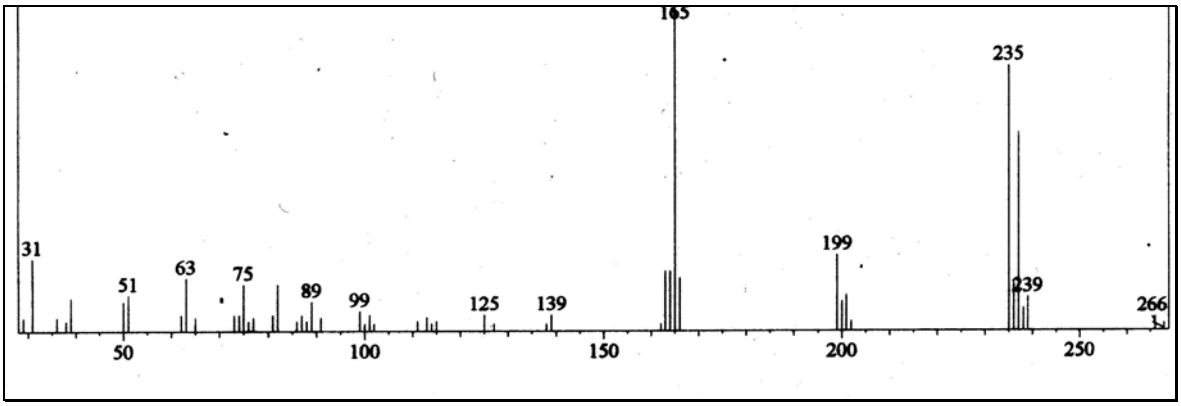
***p,p*D'DMU**



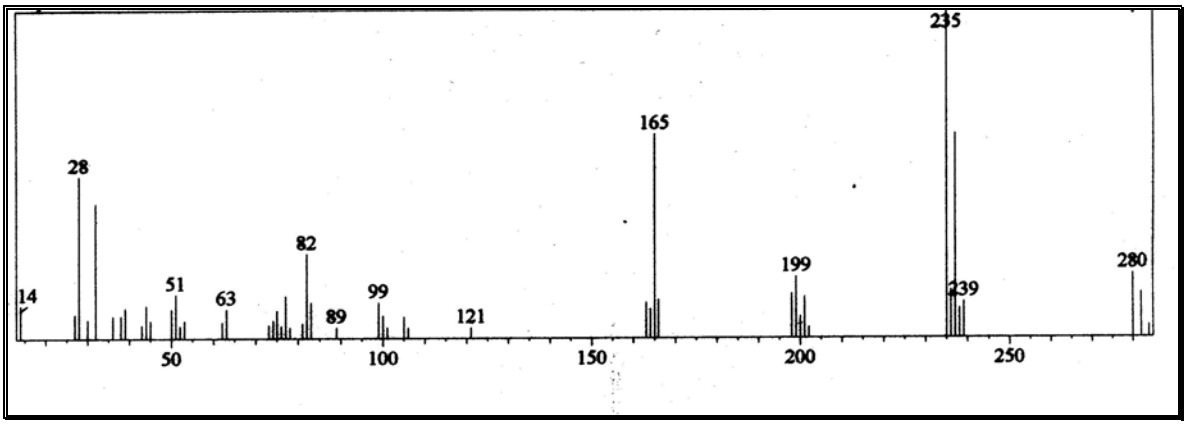
***p,p'*-DDE**


45
75
111
141
186
128
99
63
85
40
60
80
100
120
140
160
180

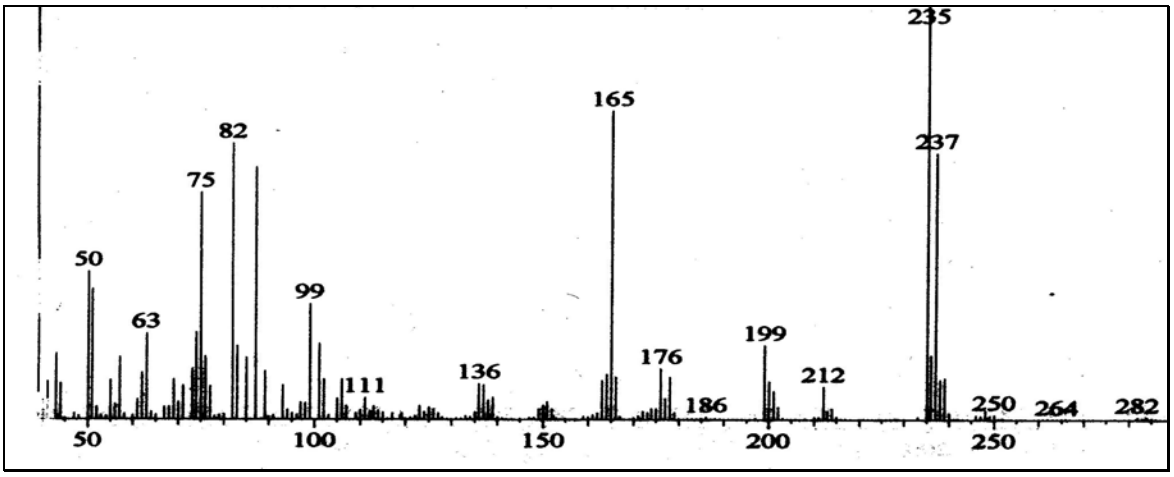
PCPA


165
235
31
199
63
75
51
89
99
125
139
239
266
50
100
150
200
250

***p,p'*-DDOH**

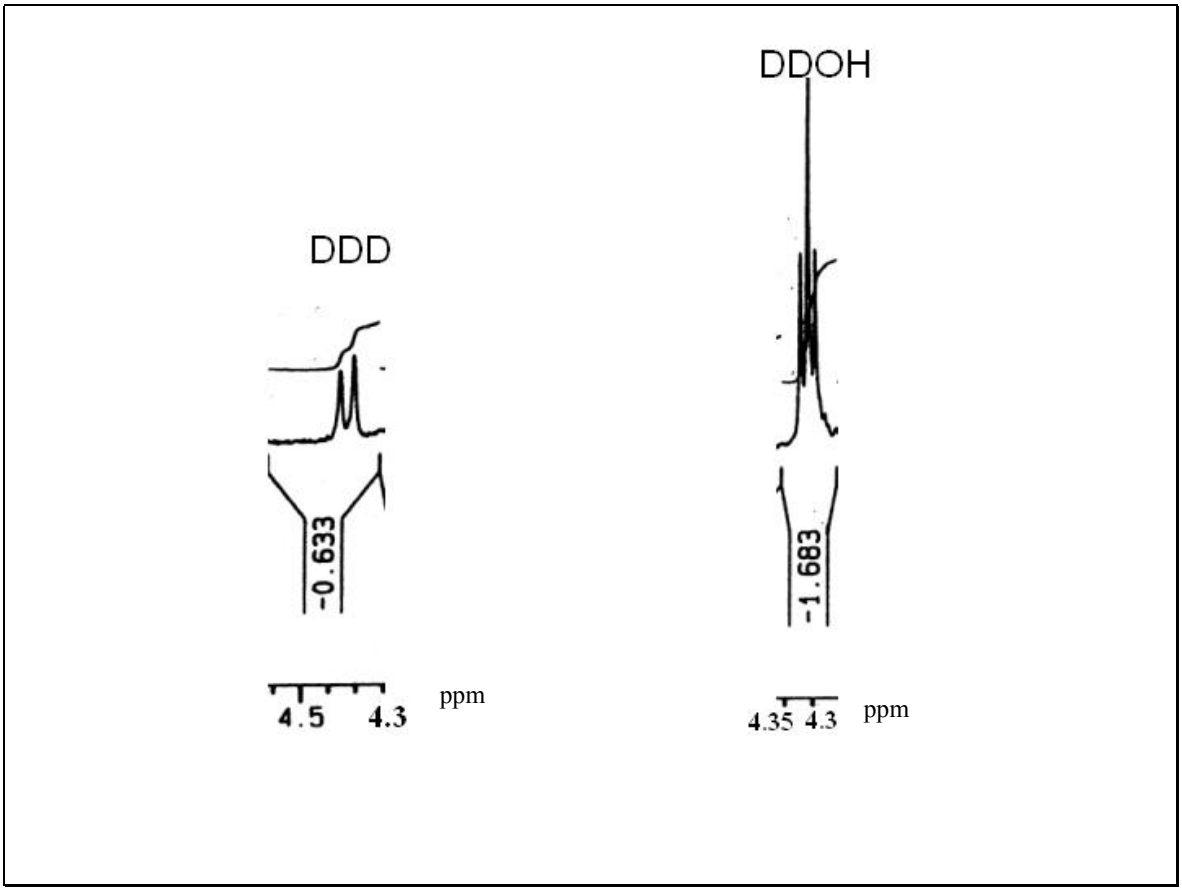


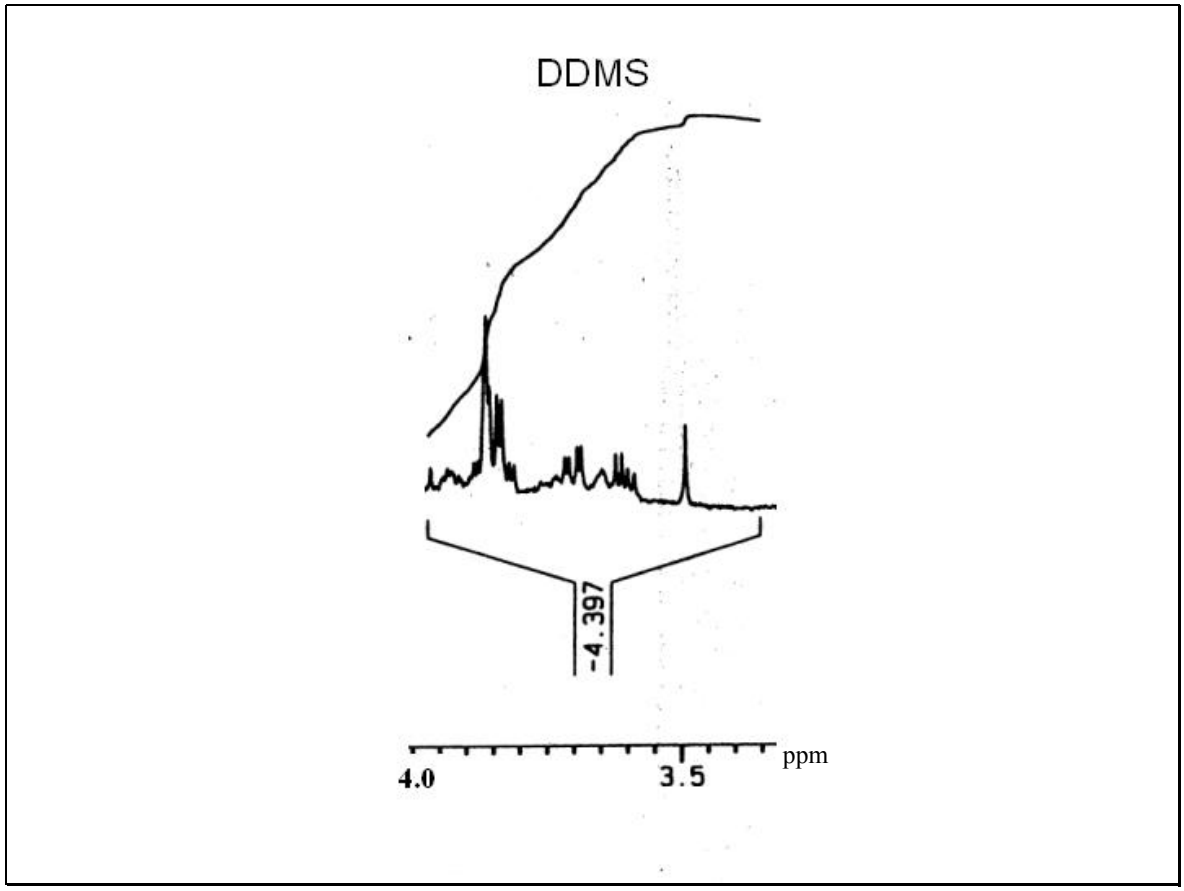
***p,p'*-DDA**

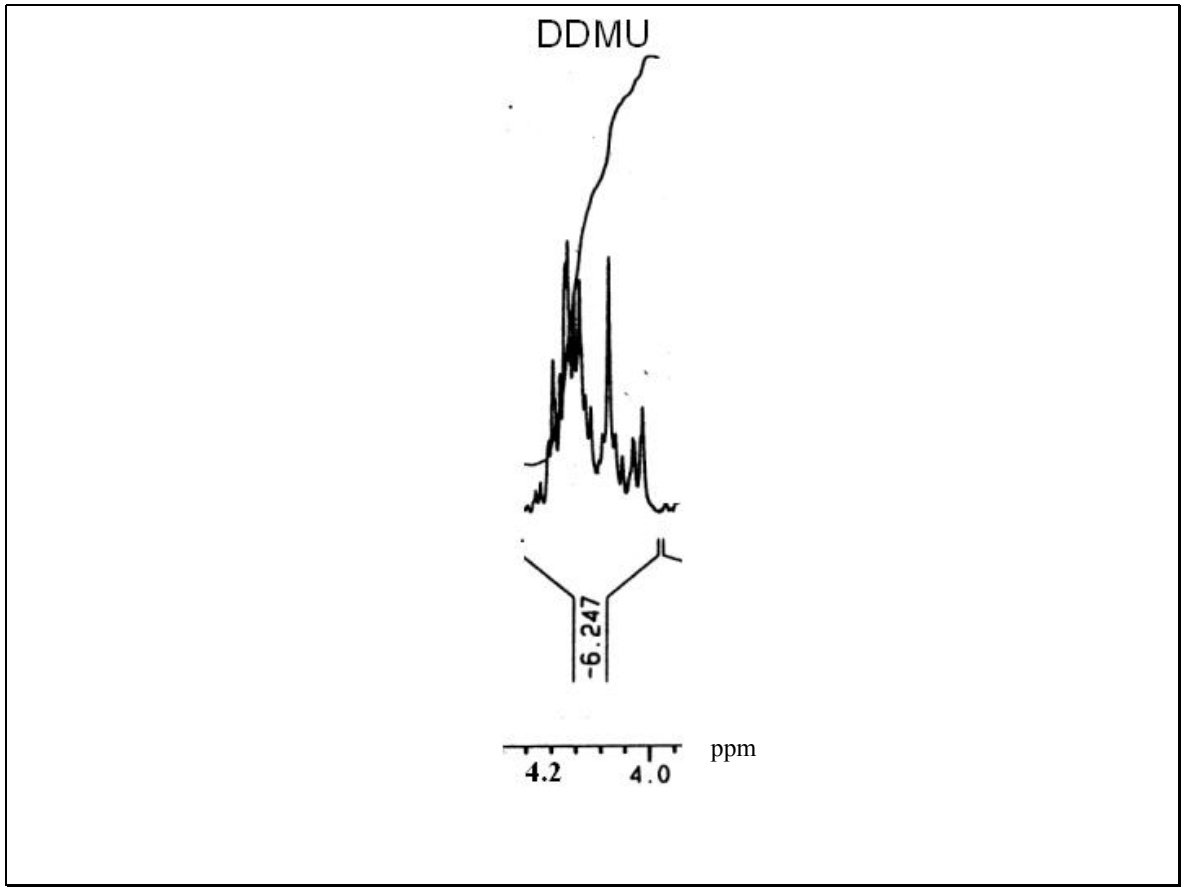


***p,p'*-DDMS**

Fig.6.2: ^{1}H-NMR Spectra for some of the identified intermediates during DDT-degradation by bacterial strains (the aromaticity has not been shown here)




DDMS
-4.397
4.0
3.5
ppm


DDMU
-6.247
4.2
4.0
ppm

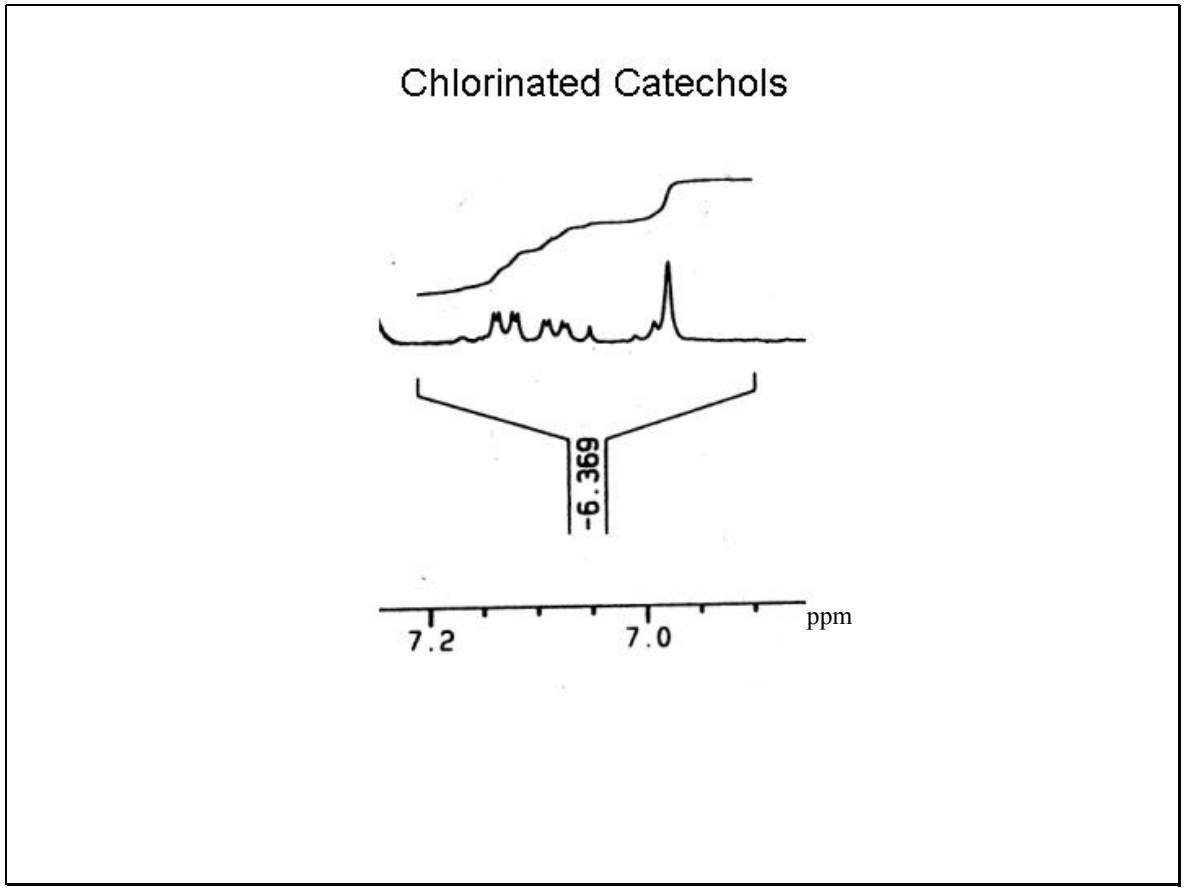

Chlorinated Catechols
-6.369
7.2
7.0
ppm

Table 6.2 Intermediates observed in the aqueous phase of the samples

Compound	*Serratia marcescens*	*Pseudomonas fluorescens*	*Pseudomonas aeruginosa*
4-CBA	✓	✓	-
Catechol	✓	✓	✓
Hydroquinone	✓	✓	✓
4-Nitrophenol	-	✓	-
Unknown	**5**	**5**	**7**

Table 6.3 Intermediates detected in ^{1}H-NMR

Compound	*Serratia marcescens*	*Pseudomonas fluorescens*	*Pseudomonas aeruginosa*
DDT	✓	✓	✓
DDD	✓	✓	
DDMU		✓	✓
DDMS	✓	✓	✓
PCPA	✓		
Chloro-Catechols	✓		

Proposed pathway of DDT degradation

Based on the above findings, the intermediates found during the aerobic degradation of DDT by the individual strains of the DDT-degrading consortium, we

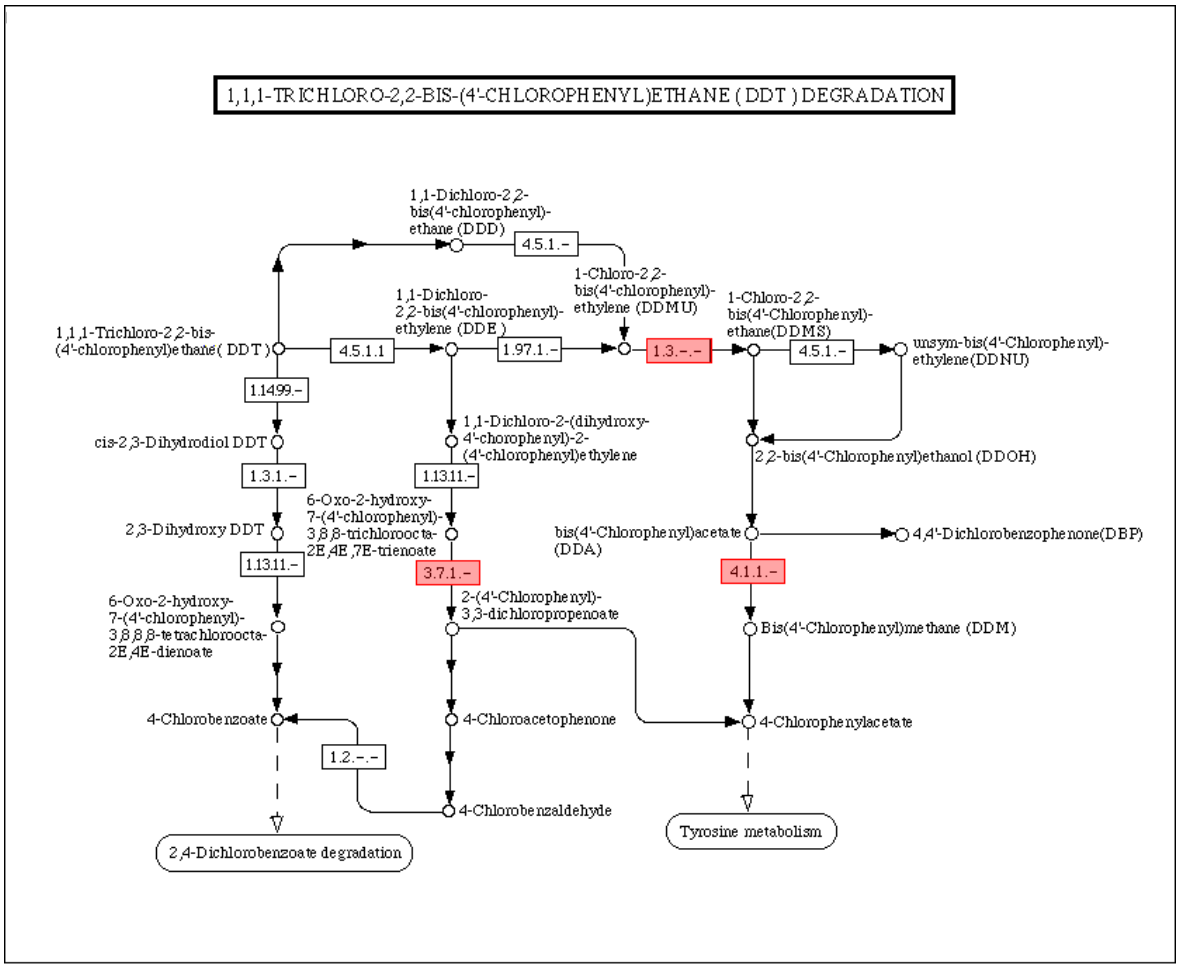
propose a generalised and combined pathway followed by the bacterial species studied by us (Fig.6.4).

Microorganisms usually convert DDT *via* two major pathways: (1) reductive dechlorination, a process favoured under anaerobic conditions, and (2) dehydrochlorination, occurring in the presence of oxygen (Kumar *et al*, 1996). DDT has been reviewed from time to time and degradative pathway proposed (Lal and Saxena, 1982). Microbes such as *Enterobacter aerogenes, Pseudomonas fluorescens, Escherichia coli and Klebsiella pneumoniae* dechlorinated *p,p'*-DDT to DDE, DDD, DDMU, DDMS and DDNU exclusively under anaerobic conditions. The first report describing the aerobic bacterial degradation of DDT *via* 4-CBA was reported by Nadeau *et al* (1994). The initial aerobic degradative step here was oxidation of the phenyl ring at the adjacent *orthro* and *meta* positions to form hydroxy-DDT. This type of attack has been expected from a dioxygenase activity (Kumar *et al*, 1996). Hydroxy-DDT, a transient metabolite, was converted to a dihydrodiol, which further metabolised to 2,3-dihydroxy-DDT by a dehydrogenase activity. 2,3-dihydroxy-DDT would be further metabolised through meta-cleavege to form the yellow ring-fission product that would then be transformed to 4-CBA. This metabolic pathway was based on its oxidation and subsequent ring fission of the oxidation products to 4-CBA. Fig.2.7-2.9 depicts the pathways of DDT by various workers. Fig.6.4 describes our proposed pathway of DDT degradation by *Serratia marcescens*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa.* The compounds formed during the microbial action on DDT in buffered medium indicate the ready conversion of DDT to these metabolites.

The depression of some inductive enzymes may offer new opportunities for degradation of the chemical pollutant by either mixed population or an individual strain. The proposed pathway describes few of the suspected enzymes that could be involved in the degradation of DDT. These enzymes are the ultimate product of the DNA translation of the bacterial strains to utilise DDT as the sole source of carbon. The enzymes stated in the pathway can be observed in the proposed DDT degradation by KEGG on the internet (Fig.6.3).

Four of the key enzymes that are involved in the two major stages of the pathway *viz.* the initial removal of chlorides and then at the lower pathway, the degradation of metabolites through ring cleavage, were considered for further testing. The DNA sequences of these enzymes from various organisms were obtained from the NCBI site on the internet. Thus obtained sequences were aligned using the online software programme dialign 2.0. The results from dialignments were considered for designing the primers. Primer designing was done in two ways: using online software Primer 3.0 and manually. Different primers were designed for each gene sequence so as to get the longest amplicon as well as the various intermediary sized amplicons. The four enzymes considered were dehalogenases (a group of enzymes that removes a halogen from the molecule), 4-CBA-dehalogenase (an enzyme that catalyses the removal of chloride from the substrate 4-CBA), 1,2-dioxygenases (an enzyme that cleaves the ring between the C1 and C2) and 2,3-dioxygenases (an enzyme responsible for the bond cleavage in the ring molecule between C2 and C3). The primers designed are described in Table 6.4.

Fig.6.3: Pathway of DDT degradation as downloaded from NCBI site



EC #	Contig ID:Gene ID		
1.3.-.-	Contig276:3828		
3.7.1.-	Contig309:7574		
4.1.1.-	Contig276:3838	Contig305:6922	Contig313:8385

Fig.64Proposed pathways for DDT degradation by bacteria under aerobic conditions

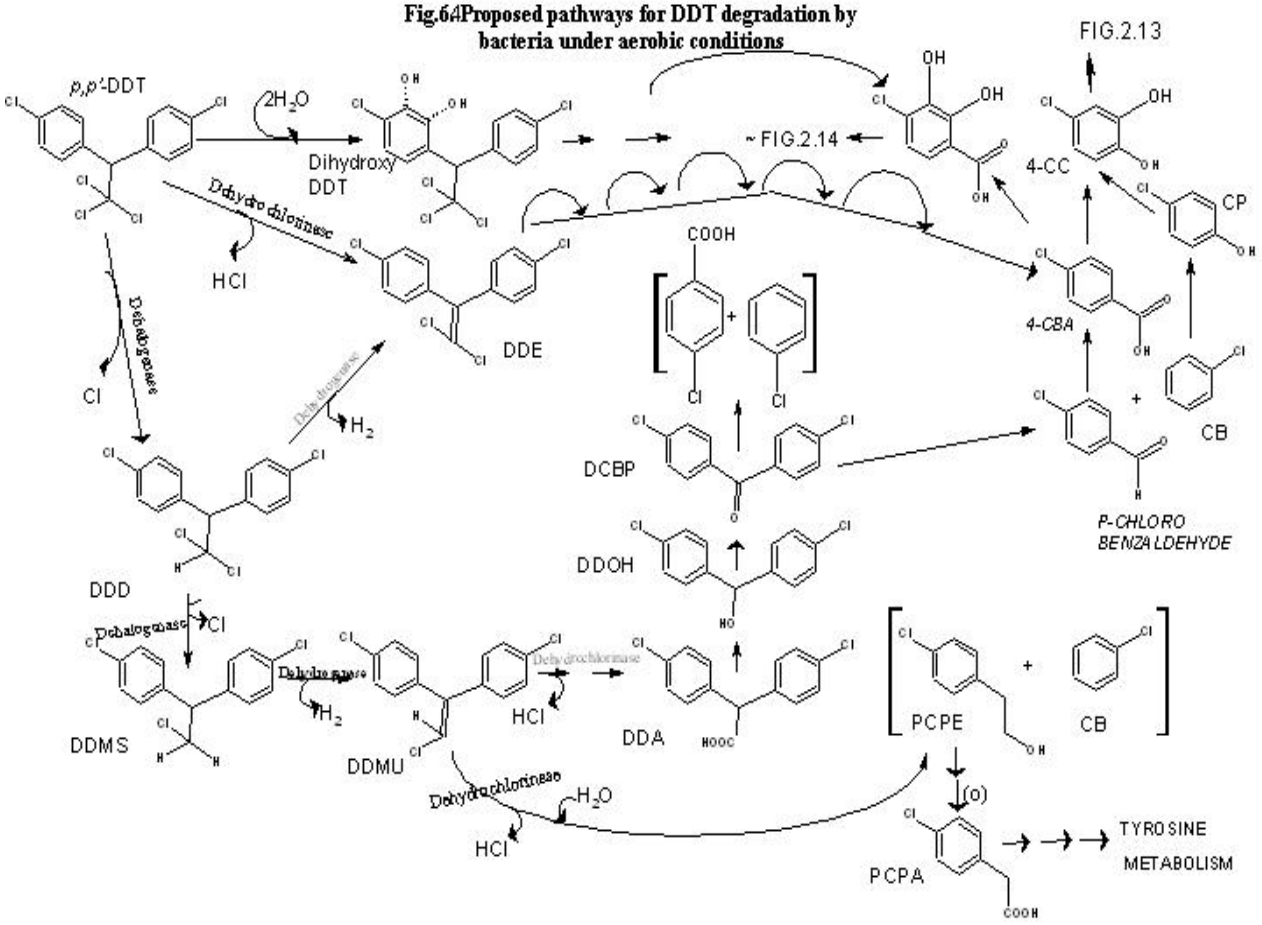


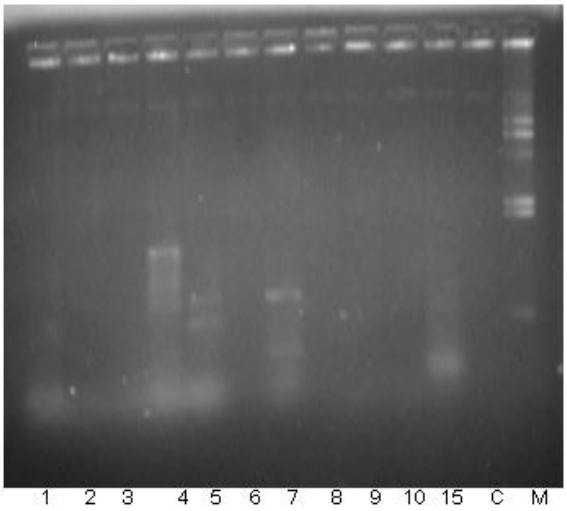
Table 6.4 Different primers designed for the identification of various genes in the DDT degraders

S.No.	Primers used		Expected length (bp)	Gene
1	12D1F 12D1R	AAGCACGATGACCGTGAAAAT TTGGMGTGCCACAGGTCGAC	480	1,2-dioxygenase
2	12D1F 12D2R	AAGCACGATGACCGTGAAAAT AGTCGGAGGACTCGTAGCAA	1149	-do-
3	12D2F 12D1R	CGGCGGCACCCCGCGCACCAT TTGGMGTGCCACAGGTCGAC	180	-do-
4	12D2F 12D2R	CGGCGGCACCCCGCGCACCAT AGTCGGAGGACTCGTAGCAA	849	-do-
5	23D1F 23D1R	GGTGTTMTGCGTATTGGTCATG ACCAAGTTGATCAGCAGTCCAAGTA	861	2,3-dioxygenase
6	23D2F 23D1R	TTTCTWSTTCCTTCTGGTCAT ACCAAGTTGATCAGCAGTCCAAGTA	540	-do-
7	23D1F 23D2R	GGTGTTMTGCGTATTGGTCATG AACAAAAGCAATATCATGAG	630	-do-
8	23D2F 23D2R	TTTCTWSTTCCTTCTGGTCAT AACAAAAGCAATATCATGAG	309	-do-
9	CBA1F CBA1R	TGGTGGTGGTCTTGGT AAGTTTGCTCGTGAACTTGCT	275	4CBA dehalogenase
10	CBA1F CBA2R	TGGTGGTGGTCTTGGT ACCAGMAGGAAGTTCAACTT	467	-do-
11	CBA2F CBA1R	CTTCCTCGTCATCRTAATGCT AAGTTTGCTCGTGAACTTGCT	553	-do-
12	CBA2F CBA2R	CTTCCTCGTCATCRTAATGCT ACCAGMAGGAAGTTCAACTT	745	-do-
13	CBA3F CBA1R	GTGCTGGTTTTTATCTTCGTGAA AAGTTTGCTCGTGAACTTGCT	429	-do-
14	CBA3F CBA2R	GTGCTGGTTTTTATCTTCGTGAA ACCAGMAGGAAGTTCAACTT	621	-do-
15	DHA1F DHA1R	AATCTTTCCACGGCCTTGCGCCA CGAACAGACGGTCCCAAATA	283	Dehalogenase
16	DHA2F DHA1R	CARTTYTGGRTYCATAC CGAACAGACGGTCCCAAATA	154	-do-

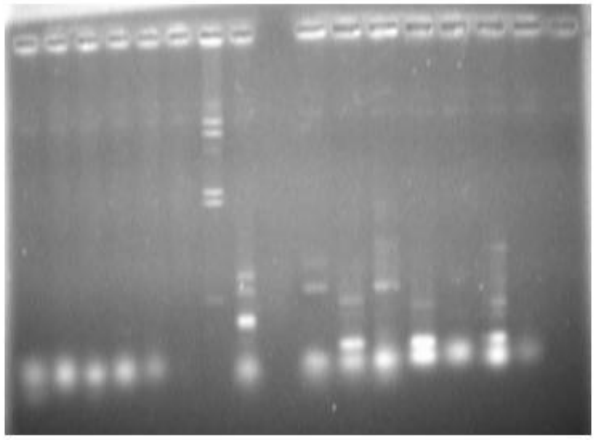
Polymerase chain reaction (PCR) was carried out using the designed primers and the isolated genomic DNA. The results of the PCR are shown in the form of gel photographs in Fig.6.5. It was observed that the primers designed for the gene 1,2-dioxygenase gave magnification in the samples of DNA isolated from *Pseudomonas fluorescens* DT-2 (Fig.6.5, gel#2) and *Pseudomonas aeruginosa* DT-Ct1 (Fig.6.5, gel#4) while no amplification was observed in the case of *Serratia marcescens* DT-1P (Fig.6.5, gel#1) and multiple bands were observed in this case. Even *Pseudomonas aeruginosa* DT-Ct2 (Fig.6.5, gel#5) did not show any amplification for this gene. Amplification for the primers specific for 2,3-dioxygenase were observed with the genomic DNA of *Serratia marcescens* DT-1P (Fig.6.5, gel#1) and *Pseudomonas fluorescens* DT-2 (Fig.6.5, gel#2). *Pseudomonas aeruginosa* DT-Ct1 (Fig.6.5, gel#4) showed amplification of the gene fragment of 4-CBA dehalogenase and no other bacterial genomic DNA studied showed amplification for this gene. Dehalogenases, a general group of halogen removing enzymes, were found to be present in the strains *Pseudomonas fluorescens* DT-2 (Fig.6.5, gel#3) and *Pseudomonas aeruginosa* DT-Ct1 (Fig.6.5, gel#4). Amplified genes when tested with Nested-PCR showed that the selected primers amplified the specific genes under study (Fig.6.6). The results are in conformity with the observed pathway for DDT wherein, DDT is dehalogenated to less chloride-containing intermediates and subsequent ring cleavage by dioxygenases. However, not all the genes were found to be present in a single organism, such as dehalogenases and 1,2-dioxygenases were found to be present in *Pseudomonas* sp. and 2,3-dioxygenases were found to be present in *Serratia marcescens* DT-1P and *Pseudomonas fluorescens* DT-2. The presence of 1,2- and 2,3-dioxygenases system indicates the presence of *ortho*- and *meta*-cleavage pathways in these organisms that could be responsible for the lower degradative stages. This also implies the fact that the complete biodegradative pathway need not be present in a single organism as reported by Schonborn (1987) and Grady (1985) and microbial consortium gets evolved with newer pathways for the same reasons in nature. However in our studies, an attempt was

made to study dehalogenases and dioxygenases. Since the pathway also involves dehydrohalogenases, dehydrogenases, decarboxylases, etc., a study of all these enzymes will give a complete insight in to the total biocatalytic machinery involved in the degradation of DDT and other similar chlorinated/halogenated xenobiotic aromatic pollutants.

Fig.6.5 Amplification of some of the gene sequences of DDT degradative pathway in the genomic DNA from the four bacterial strains

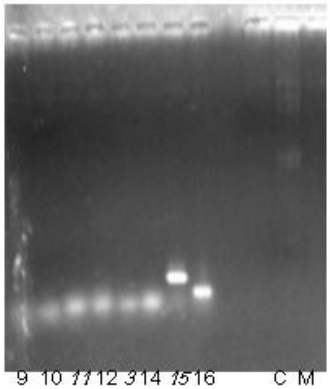


Gel # 1 showing different primers used for testing the amplification of certain gene sequences in *Serratia marcescens* DT 1P. 1- 15 are the samples, C= control and M= marker. Details in the Table 6.4 . (Lanes numbered according to the S. No. in the table 6.4)

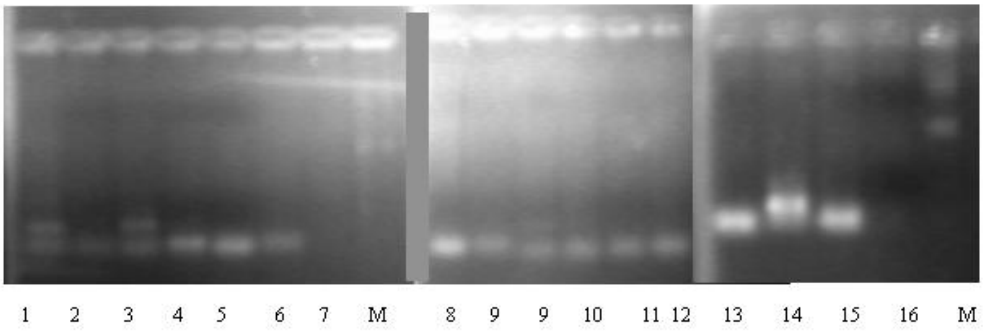


Gel # 2 showing different primers used for the amplification of various sequences/ genes.

First five i.e. first 11- 16 are *S. marcescens* DT1P, C= control, M= marker, 1- 8 are *Pseudomonas fluorescens* DT2,. Details in Table 6.4. (Lanes numbered according to the S.No. in the Table 6.4)



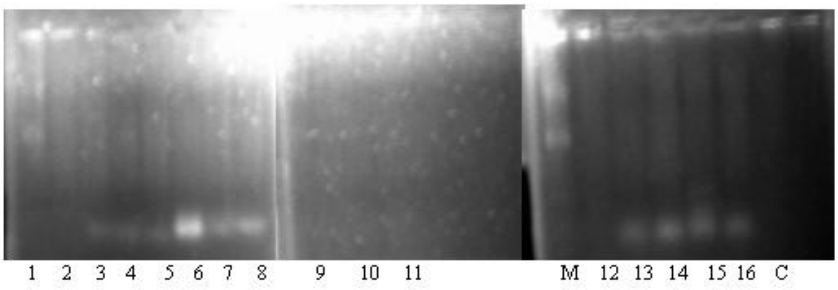
Gel#3 *Pseudomonas fluorescens* DT-2, 9-16 are the samples, C=control and M= marker.
Details in the Table 6.4.
(All the lanes are numbered according to the S. No. in the Table 6.4)



Gel#4 *Pseudomonas aeruginosa* DT-Ct1, 1-16 are the samples, M= marker.

Details in the Table 6.4.

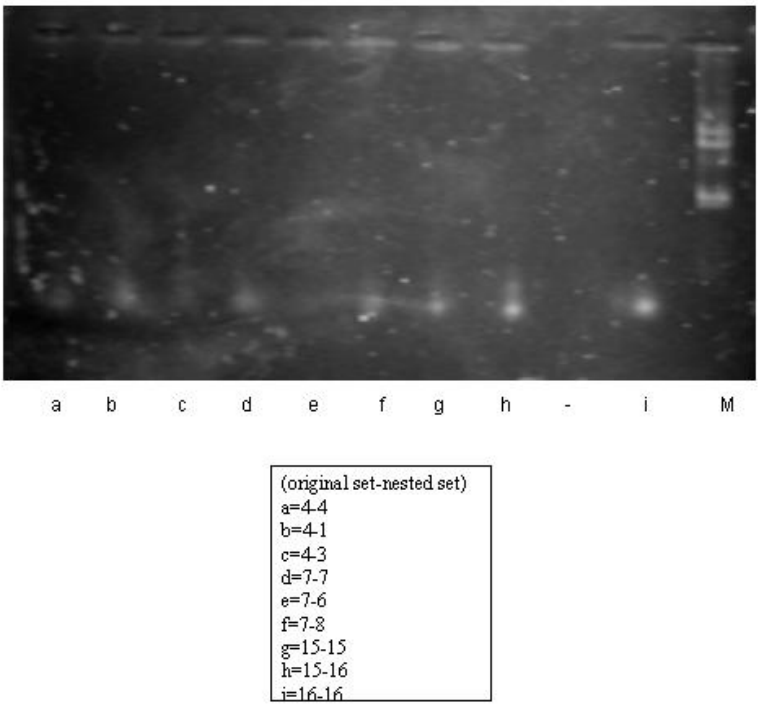
(All the lanes are numbered according to the S. No. in the Table 6.4)



Gel#5 *Pseudomonas aeruginosa* DT-Ct2, 1-16 are the samples, C=control and M= marker.
Details in the Table 6.4.
(All the lanes are numbered according to the S. No. in the Table 6.4)

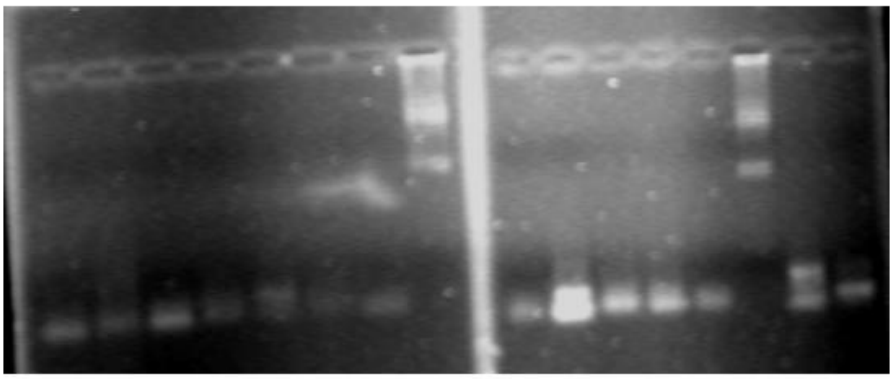
Fig.6.6 Gels showing the amplification in Nested-PCR conducted with the amplicons from the touchdown PCR of the bacterial species

For *Serratia marcescens* DT-1P



M=marker

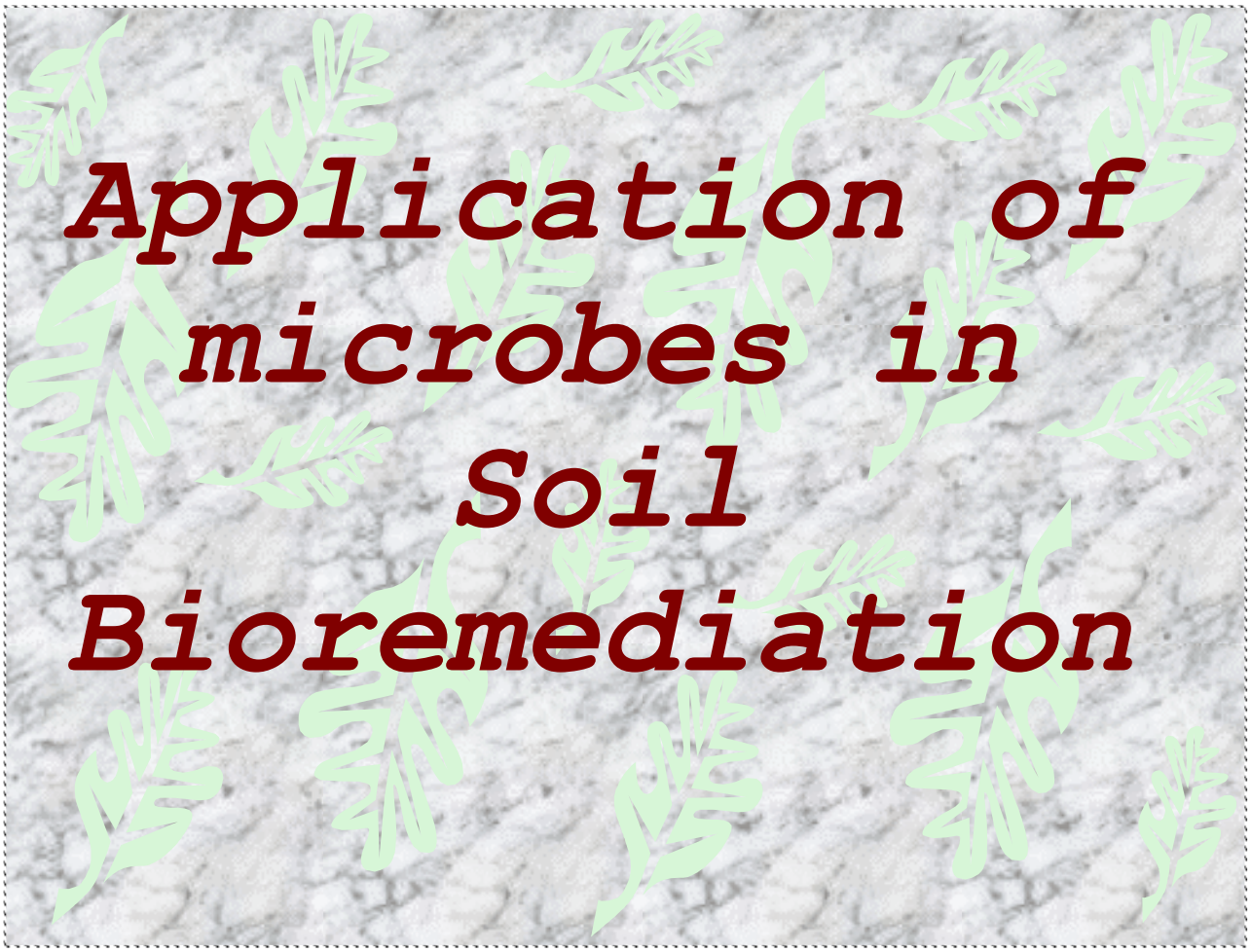
For *Pseudomonas* strains DT-2 and DT-Ct1

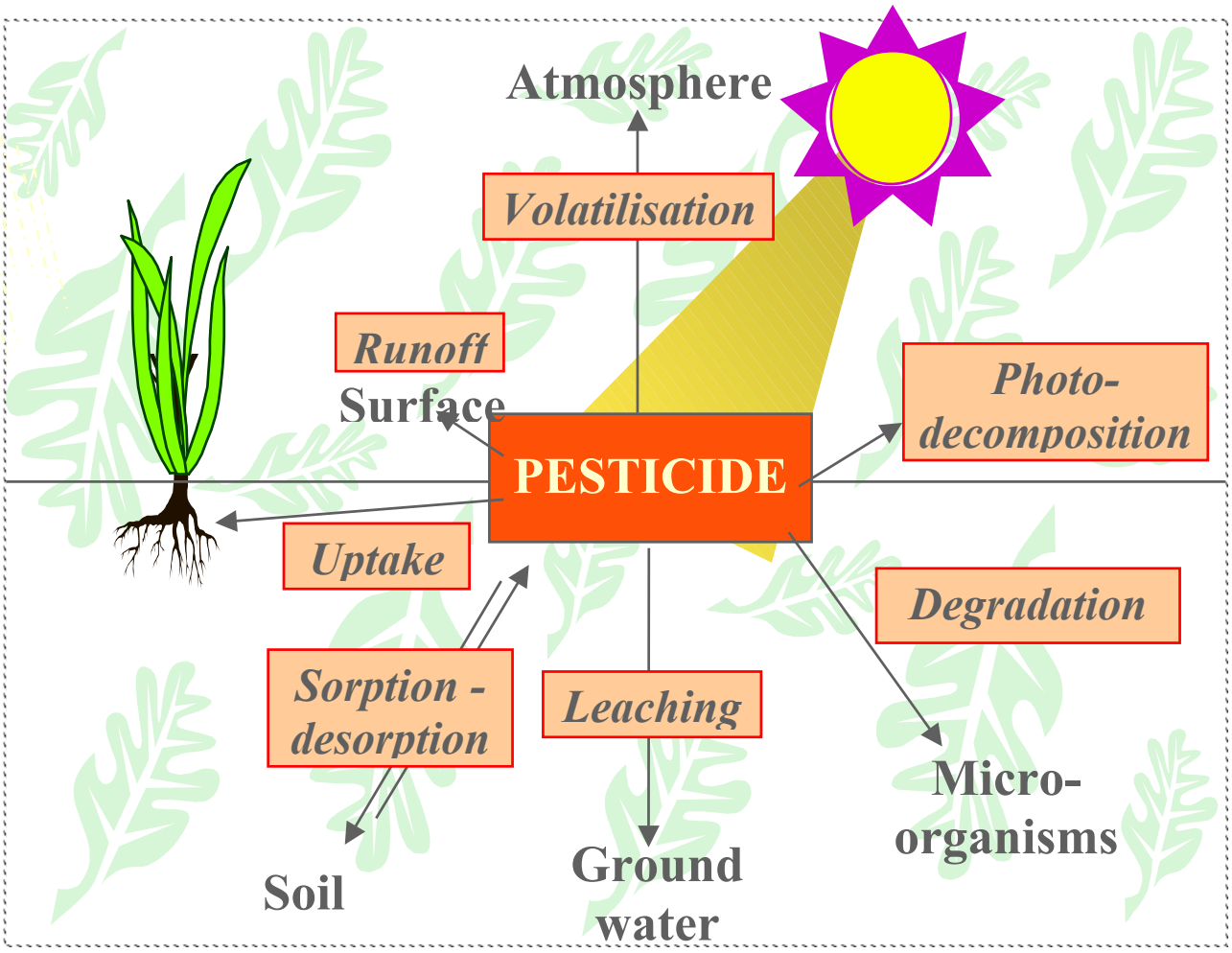


(original set-nested set)
a=1-1
b=1-3
c=5-5
d=5-6
e=5-7
f=5-8
g=6-6
h=6-8
i=7-7
j=7-6
k=7-8
l=8-8
m=15-15 (ct1)
n=15-16 (ct1)

M=marker

Chapter 7




Atmosphere
Volatilisation
Runoff
Surface
PESTICIDE
Photo-
decomposition
Uptake
Degradation
Sorption -
desorption
Leaching
Micro-
organisms
Soil
Ground
water

7.1 Introduction

Pesticides are being used in most parts of the developing world, including India. The use of pesticides, especially the insecticides in public health as well as agriculture has brought these chemicals to alarmingly increasing concentrations in our environment. The concentrations in the soil and water are so high that they can be detected in almost all the food and dairy products. The recent reports from CSE, India (India Today, Aug.2003) gave an indication of the vastness of pesticide pollution. It is the need of hour that the concentrations of these pesticides should be brought down. The use of pesticides would continue in more quantities than those applied earlier as most of the insect species have developed resistance to the existing concentrations. Moreover, the want of more powerful and inexpensive, safe and eco-friendly pesticide appears too far from availability to developing nations. The usage continues in the present scenario. Hence, there is a need of simple, safe and inexpensive strategies to reduce the environmental concentrations of the pesticide chemicals. One of the ways to achieve this is the microbial remediation. Astra Zeneca, a company in Canada has come up with a process of reducing DDT concentrations in the soil using the animal waste (New Scientist, May, 1999). The reports on microbial remediation of soil under aerobic conditions are scanty. In our laboratory preliminary studies were conducted to bioremediate DDT-spiked soils using the DDT degrading cultures. The bacteria isolated after the enrichment of DDT-contaminated soil with DDT, were tried for the soil remediation in a model-contaminated soil with different DDT concentrations. The four strains were tested with 5ppm DDT initially and then the selected strain was used to work out the degradation kinetics.

7.2 Materials and Methods

7.2.1 Chemicals

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 99% pure, was purchased from Sigma-Aldrich Chemical Company, Mo, USA. *o*-Tolidine

was purchased from Hi Media, Mumbai, India. Wheat bran, rice straw etc were obtained from local market. Solvents like acetone, hexane, ethyl acetate, dichloromethane, cyclohexane were of analytical and HPLC grade and were procured from E-Merck (India) Ltd.

7.2.2 Cultures and media

Microbial cultures used in this study were developed in the laboratory as described in chapter 3. The 4 members of this consortium were tested for DDT degradation in soil separately. All the cultures were grown in peptone-glycerol medium (3.3.4) for 48 h and pre-exposed to 10ppm DDT for 72 h before using as inocula.

7.2.3 Soil

Soil for the study was taken from the CFTRI campus, Mysore, India. This soil was sieved to get a uniform particle size and then sterilised by autoclaving for 40 minutes, thrice at a gap of 3 days each. The soil used was red-loamy with 1.0-1.5% organic matter.

7.2.4 Degradation studies

100g sterile soil was taken in plastic cups (15% moisture) and DDT (5 and 10μg.g^{-1} soil) was mixed well for uniform distribution of the substrate. This was then inoculated with induced microbial cultures containing 10^8 cells of each organism separately. The cups were incubated at ambient temperature (26-30^{0}C) for seven days. Required volume of sterile water was added to each cup every alternate day to maintain moisture. Samples were analysed at regular intervals for residual substrate. The samples were extracted in ethyl acetate, hexane: acetone (8:1) and dichloromethane. All the solvent layers were pooled after passing through anhydrous sodium sulphate and activated florisil and allowed to evaporate at room temperature (26-30^{0}C). The dried residue for each sample was

resuspended in a known small volume of HPLC grade acetone and tested for the residual DDT.

Degradation of 10ppm DDT was carried out for various inoculum sizes, temperatures and moisture levels. For each set, sterile soil was spiked with required amount of DDT and mixed thoroughly for uniform distribution and then inoculated with the required quantity of inoculum after adding required quantity of moisture. These were incubated at required temperatures. Sampling was done at known periods of time. The soil was air-dried and used for further analysis. Complete soil from each cup was taken as a single sample. Experiments were carried out in replicates of 10. The results describe only the average in each case.

To study the effects of co-substrates on the degradation of DDT, co-substrates such as rice straw powder and peanut meal were added to soil spiked with DDT at 1% level.

7.2.5 Analytical

The residual DDT was analysed using TLC and GC as described in previous chapters.

The cell count was measured counting colony forming units (cfu) as described by Sahu *et al* (1996).

7.2.6 Kinetics of DDT degradation

Kinetics of DDT degradation by the bacteria was carried out based on the derivation of rate constant as discussed in sec. 4a.2.6.

7.3 Results and discussion

7.3.1 Degradation of DDT by bacterial strains

Initial degradation screening was done with 5 and 10ppm DDT in soil. Degradation of 5 and 10ppm DDT has been shown in Table 7.1. It is clear from the table that only *Pseudomonas aeruginosa* DT-Ct1 could

Table 7.1 Degradation of soil-spiked DDT by four bacterial strains

DDT Conc.	DT-1P			DT-2			DT-Ct1			DT-Ct2		
	Residual DDT (%)											
	0h	***48h***	***96h***	***0h***	***48h***	***96h***	***0h***	***48h***	***96h***	***0h***	***48h***	***96h***
5ppm	99.5	75	62	99.4	87	82	99.6	0	0	99.5	95	84
10ppm	99.4	88	80	99.4	96	91	99.6	30	0	99.4	98	96

degrade the soil-spiked DDT in less time compared to the other three strains. *Serratia marcescens* DT-1P was the other strain that could degrade the applied DDT. *Pseudomonas fluorescens* DT-2 and *Pseudomonas aeruginosa* DT-Ct2 did not show any promise in independent degradation. Therefore the two strains of *Serratia marcescens* DT-1P and *Pseudomonas aeruginosa* DT-Ct1 were selected for further studies. *Serratia marcescens* DT-1P degraded higher concentrations of DDT at a very slow rate and the degradation was partial even after 240 h of incubation (Table 7.2). On the other hand *Pseudomonas aeruginosa* DT-Ct1 was able to degrade concentrations up to 25 ppm in less time (Table. 7.2).

7.3.2 Inoculum and DDT degradation by P. aeruginosa DT-Ct1

Degradation of DDT increased with increasing inoculum size (Table 7.3). At low inoculum level of 0.5μg dry weight of cells per gram soil, the degradation was complete by 120h of incubation at ambient temperature (26-30^{0}C). The time required for degradation decreased with increasing inoculum size. At 10μg dry weight of cells per gram soil, complete degradation was achieved by 48h of incubation. However there was very less quantity of DDT disappearing after 72 h of incubation that remained constant thereafter.

7.3.3 Effect of moisture content of soil on DDT- degradation by DT-Ct1

The effect of moisture content of soil was studied with 3, 5, 10, 15, 20% moisture respectively and also with flooded soil. The degradation of added 10ppm DDT was low in soils with very low moisture contents (3% moisture). The degradation rate increased with increase in moisture content and complete degradation was observed at a moisture level of 15%. Degradation decreased with further increment in moisture content of soil and only 42% degradation was observed in flooded soil (Table 7.4).

Table 7.2 Degradation of different concentrations of DDT *Serratia marcescens* DT-1P and *Pseudomonas aeruginosa* DT-Ct1

DDT Conc (ppm)	Residual DDT (%) after (h)			
	DT-1P		DT-Ct1	
15	0	100.0	0	100.0
	24	96.0	24	31.2
	48	92.0	48	22.7
	72	88.0	72	8.1
	96	88.0	96	0.0
25	0	100.0	0	100.0
	24	98.0	24	62.1
	48	96.0	48	29.1
	72	95.0	72	21.9
	120	95.0	120	15.3
	144	95.0	144	13.4
	168	95.0	168	0.0
50	0	100.0	0	100.0
	24	100.0	24	56.4
	48	98.7	48	46.9
	72	98.2	72	42.6
	120	97.0	120	35.2
	144	97.0	144	22.8

Table 7.3 Effect of inoculum size on the degradation of 15μg DDT/ g soil by Pesudomonas aeruginosa DT-Ct-1

Inoculum size (μg dry weight/ g soil)	Incubation period (H)	Residual DDT(μg/ g soil)	Degradation (%)
0.5	0	15.00	0.00
	24	13.80	8.0
	48	9.58	36.1
	72	8.33	44.5
	96	7.43	50.5
	120	0.00	100.00
1.0	0	15.00	0.00
	24	12.05	19.7
	48	11.25	25.0
	72	8.34	44.4
	96	7.09	52.7
	120	0.00	100.00
2.0	0	15.00	0.00
	24	11.51	23.3
	48	8.16	43.6
	72	5.67	61.5
	96	0.00	100.00
5.0	0	15.00	0.00
	24	7.96	46.9
	48	2.82	81.2
	72	0.00	100.00
10.0	0	15.00	0.00
	24	2.82	81.2
	48	0.00	100.00
Control (uninoculated)	0	15.00	0.00
	24	15.00	0.00
	48	14.49	3.4
	72	12.05	19.60

Table 7.4 Effect of moisture on 10ppm DDT degradation by DT-Ct1 (after 72 h)

Moisture (%)	Residual DDT (%)
3	78
5	36
10	14
15	0
20	10
Flooded	58

7.3.4 Incubation temperature and DDT degradation by DT-Ct1

Degradation of 10ppm DDT studied at different temperatures by *Pseudomonas aeruginosa* DT-Ct1 was complete at 30^0C. At lower and higher temperatures (20 and 40^0C respectively) the degradation was 85 and 58% respectively (Table 7.5).

7.3.5 Initial Concentration of DDT and degradation by DT-Ct1

At low initial concentration of DDT, i.e. 5μg DDT g^{-1} soil, the degradation was rapid. Complete disappearance of the spiked DDT was observed by 24 h of incubation at ambient temperature. At 10 μg DDT g^{-1} soil, the degradation was complete by 72 h while 15 μg DDT g^{-1} soil was degraded by the end of 96 h of incubation (Table 7.6). However, the degradation of 25 and 50 μg DDT g^{-1} soil was partial wherein 14 and 33% DDT was observed even after 144 h of incubation under the same conditions.

7.3.6 Kinetics of DDT degradation in soil by DT-Ct1

Kinetics of DDT degradation was worked out with different DDT concentrations and other parameters maintained at optimum as studied above. Concentrations of 5, 10, 15, 25 and 50 ppm DDT in sterile soil were used for the degradation studies as above and the degradation rate calculated using Microsoft Excel 2000 in a similar fashion as described in Chapter 4a. Fig. 7.1 depicts the linear fitting of the curve passing through the origin. The slopes of the curves for each of the concentrations gave the rate constants at the respective initial DDT concentrations.

Table 7.5 Degradation of 10ppm DDT at different temperatures by DT-Ct-1 (After 72 h)

Temperature (^{0}C)	Residual DDT (%)
20	15
30	0
40	42

Table 7.6 Degradation of different concentrations of DDT in sterile soil by *Pseudomonas aeruginosa* DT-Ct-1

Concentration of DDT added (μg/ g soil)	Incubation period (H)	Residual DDT recovered (μg/ g soil)	Percent degradation
5	0 24	5.03 0.00	0 100.00
10	0 24 48 72	11.22 5.50 2.92 0	0 44.96 70.85 100.00
15	0 24 48 72 96	15.35 5.58 3.40 1.21 0	0 62.83 77.35 91.96 100.00
25	0 24 48 72 120 144	25.33 15.51 7.75 5.48 3.82 3.46	0 37.94 71.01 78.09 84.73 86.17
50	0 24 48 72 120 144	48.46 28.18 23.42 21.28 17.57 16.39	0 43.64 53.16 57.44 64.86 67.22

The rate constants have been described in Table 7.7. When the rates were plotted against the concentrations (Fig.7.2), and regression worked out to fit the data points, it was observed to fit best, satisfying the following equation:

$$K = -1x10^{-5}x^3 + 0.001x^2 – 0.0255x + 0.2159, \ (R^2 = 0.8804)$$

where, k= degradation rate constant

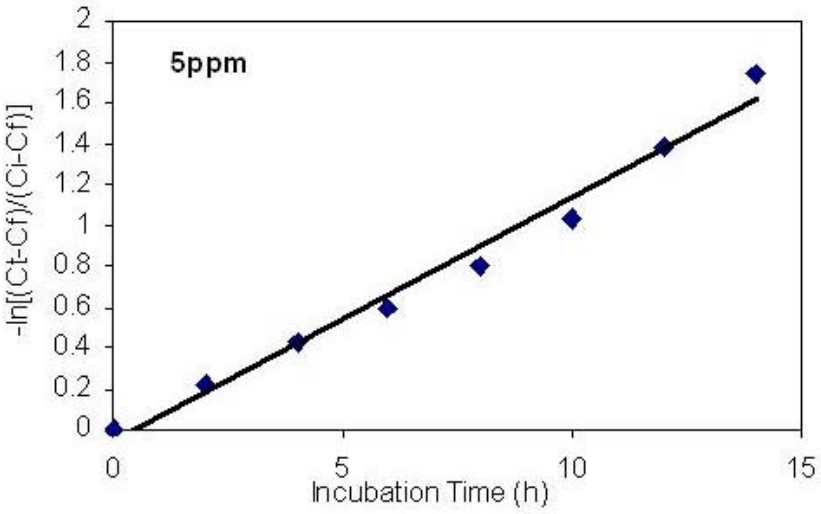
x= initial DDT concentration

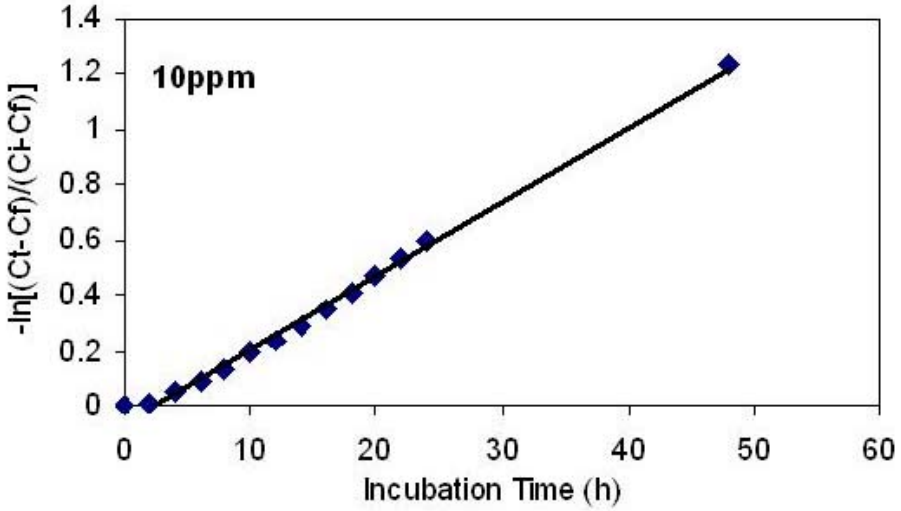
7.3.7 Co-metabolism of DDT

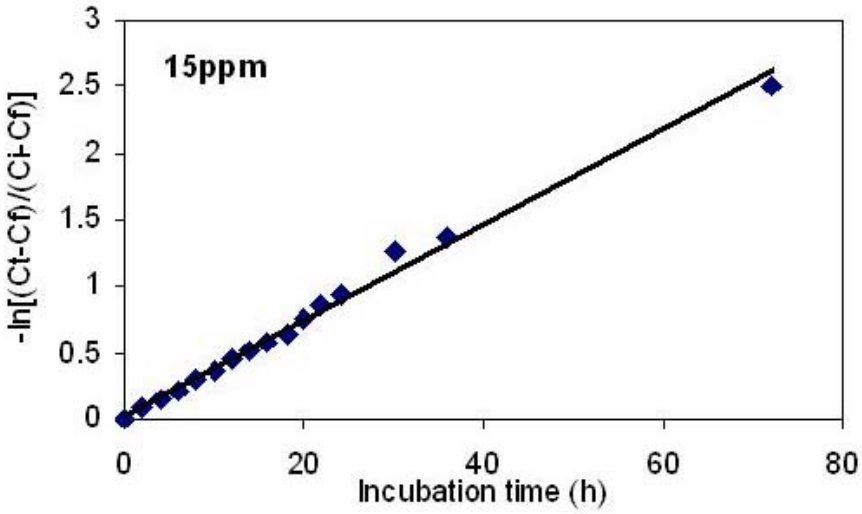
In presence of co-substrates such as rice straw powder and peanut meal, the degradation of added DDT was observed to be less compared samples. The residual DDT found in rice straw as co-substrate was 32% after 120 h of incubation at ambient temperature whereas it was 29.1% in presence of peanut meal in the same period. However, the degradation was complete in control samples, by 96 h itself (Fig.7.3).

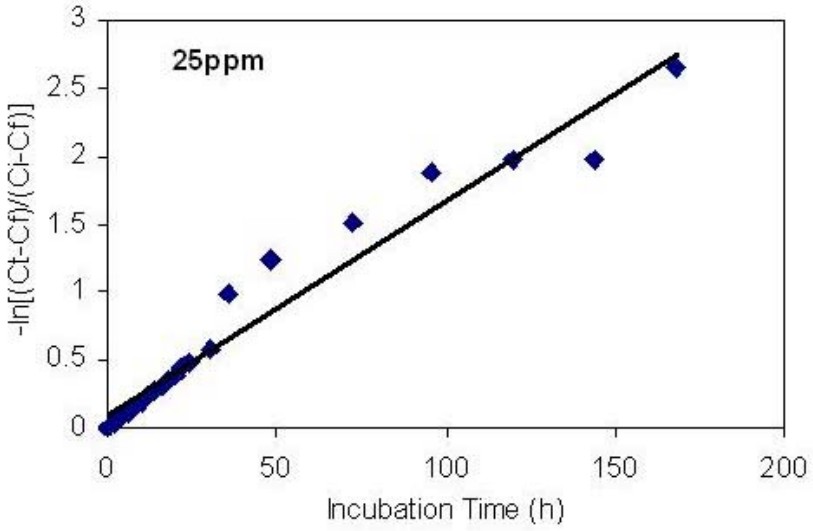
The use of microorganisms to clean up the polluted environments is a rapid expanding area in biotechnology. Limited understanding of biological contributions in both bioremediation and its impact on the ecosystem has been an abstract to make this technology reliable and safer (Iwamoto and Nasu, 2001). The behaviour of the microorganisms in the polluted soil is directly related to many physico-chemical factors such as type of soil, moisture content of the soil, pH, soil texture, presence of organic matter and other co-pollutants, the influence of native micro flora, soil temperature, etc. Biodegradation of DDT was studied in soil in terms of moisture content of the soil, the initial concentration of the pollutant, incubation temperature and presence of co-substrates. During degradation of lower concentrations of DDT, there was no accumulation of any of the intermediary metabolites in case of *Pseudomonas aeruginosa* DT-Ct1. However, with other isolates the intermediary metabolites such as DDD,

Fig. 7.1 Linear fitting of the data points for different DDT concentrations degraded by *Pseudomonas aeruginosa* DT-Ct1 in soil




10ppm
-ln[(Ct-Cf)/(Ci-Cf)]
Incubation Time (h)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
10
20
30
40
50
60


15ppm
-ln[(Ct-Cf)/(Ci-Cf)]
Incubation time (h)
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80


25ppm
-ln[(Ct-Cf)/(Ci-Cf)]
Incubation Time (h)
0
0.5
1
1.5
2
2.5
3
0
50
100
150
200

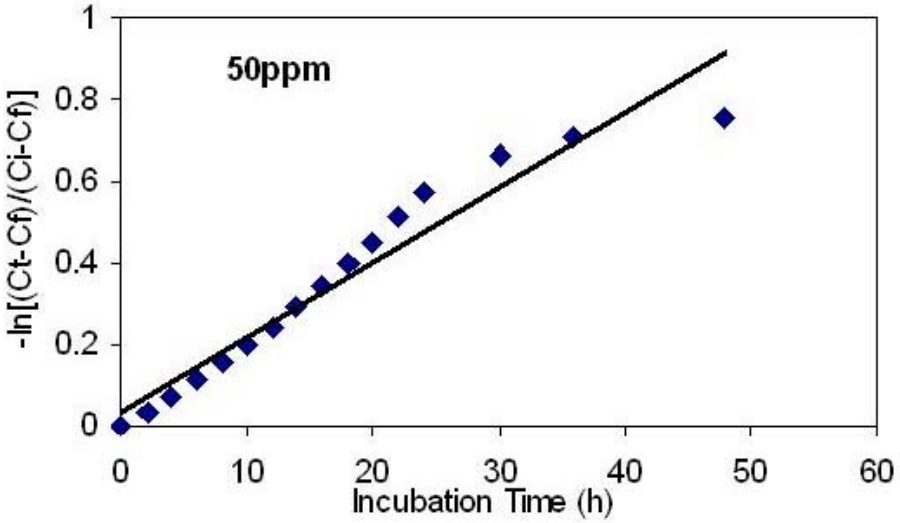
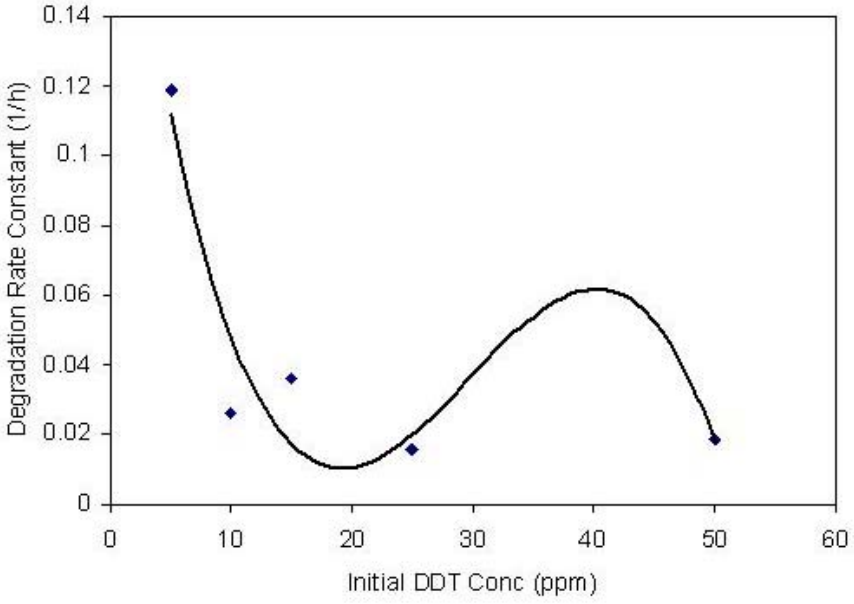

50ppm
-ln[(Ct-Cf)/(Ci-Cf)]
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Incubation Time (h)

Table 7.7 Rate constants of DDT-degradation at various initial DDT concentrations in soil by *Pseudomonas aeruginosa* DT-Ct1

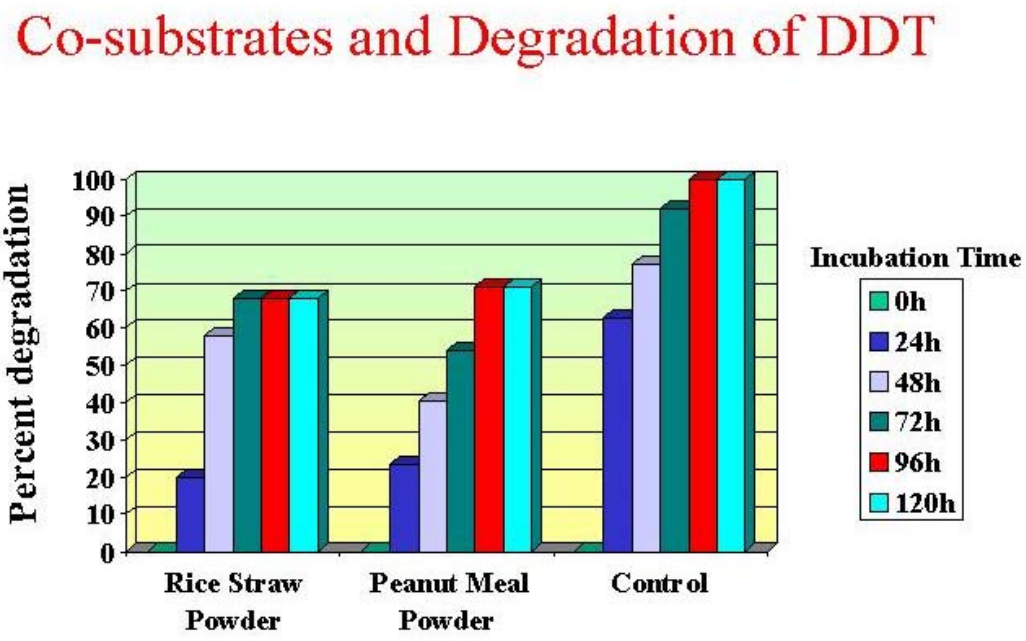
Initial DDT Concentration (ppm)	Rate Constant (h^{-1})	R^2
5	0.1189	0.9815
10	0.0265	0.9952
15	0.0359	0.9923
25	0.0157	0.9508
50	0.0184	0.9388

Fig.7.2 Degradation rate constants at different DDT concentrations in soil by *Pseudomonas aeruginosa* DT-Ct1



$y = -1x10^{-5}x^3 + 0.001x^2 - 0.0255x + 0.2159$
$R^2 = 0.8804$

Fig.7.3


Co-substrates and Degradation of DDT
Percent degradation
100
90
80
70
60
50
40
30
20
10
0
Rice Straw Powder
Peanut Meal Powder
Control
Incubation Time
0h
24h
48h
72h
96h
120h

DDE and DDA were found to accumulate. This could be due to the non-functioning of the complete DDT-degradation machinery or due to the non-availability of the substrates for degradation. Similar observations have been made during the degradation of lindane (Senoo and Wada, 1989) and α-HCH (Singh and Kuhad, 1999; Bachmann *et al*, 1988) where accumulation of metabolites has been reported. Initial concentration of the target pollutant plays a key role in the degradation of the chemical and survivability of the degrading organism. Very low concentrations of the pollutant might be unable to induce the degradative enzymes and very high concentrations might be toxic to the degrading organism. Hence, it is imperative to understand the lowest inducible, degradable concentration and the maximum level that could be acted upon. In our studies, the degradation was tried from 5ppm DDT through 50ppm DDT in soil. The degradation of DDT was complete upto 15ppm of DDT in soil. With increase in DDT concentration, the degradation was slower. The degradation rates were $0.1189h^{-1}$, $0.0265h^{-1}$, $0.0359h^{-1}$, $0.0157h^{-1}$ and $0.0184h^{-1}$ for 5, 10, 15, 25 and 50 ppm DDT respectively. Low solubilisation could be another limiting factor in the degradation of the substrate. Adsorptive surface area and surface characteristics have also been shown to significantly influence the degradation of the pollutant compound and this has been shown to have a linear relationship with substrate concentration and bio-conversion (Bachmann *at al*, 1988. This is important for the application of biotechnological methods to clean up the contaminated soil and in quantifying biodegradation of hydrophobic xenobiotic compounds. Soil inhomogeneity also adds to the complexity of factors. The soil used in our study was sieved before use with the intention of maintaining homogeneity.

When the biodegradative microorganisms are added to the contaminated sites, they face challenge from the native microbial communities *viz.* risk of getting out-competed by native microbial

communities and also of predation by protozoans, etc. (Awasthi *et al*, 2000). The optimum number of microbial cells that need to be added to obtain complete degradation of DDT was studied by using different inoculum sizes. The degradation was low at low inoculum levels and the degradation time got reduced with increase in inoculum size. Complete degradation was observed even at 0.5 μg dry weight of cells but the time required decreased from 120 h to 48 h with inoculum size reaching 10μg dry weight of the cells. The degradation of DDT was also found to be influenced by other factors such as moisture level, pH, and temperature of soil. The degradation was retarded in presence of co-substrates indicating the inhibitory/antagonistic effects of these co-substrates on DDT degradation. Thus these microbial cells can be used effectively in treatment technologies for the remediation of DDT-contaminated sites. However, a deep understanding of other interactive variables such as native micro flora, other pollutant compounds at the remediation sites are essential for effective, complete elimination of the pollutants from the contaminated environments.


Summary and Conclusion

Inferences are movements of thought within the sphere of belief.

Josephson and Josephson (1996)

DDT is the one of the first synthetic pesticides used by modern man. Though it was synthesised in 1874, its insecticidal properties were explained in 1930s. Since then it was used for controlling vector borne diseases and pests. AT a later stage it was found to be an environmental hazard for its toxicity towards many non-target species. It was banned completely in few countries while its use was restricted to public health programmes and emergencies. Eventhough its use was restricted, the developing world still used it for both agriculture and health programmes. The earlier unrestricted use and its persistency in environment is the cause of its existence in many water resources and soil today. The long persistence was considered a blessing is turning to be a curse as DDT is suspected to cause many health problems including cancer. Therefore, it becomes imperative to remove DDT from the environment in a short time and in an inexpensive way. Microbial remediation is one such tool that has come as a rescue weapon in this modern biotechnology era.

In our laboratory we could isolate a consortium from long term enrichment of DDT-contaminated soil and water that degraded upto 25ppm DDT in shake flasks within 144 h. Near neutral pH and mesophilic temperature were found to favour degradation of DDT. The consortium consisted of four bacterial strains, which were identified as *Serratia marcescens* DT-1P, *Pseudomonas fluorescens* DT-2, *Pseudomonas aeruginosa* DT-Ct1 and *Pseudomonas aeruginosa* DT-Ct2. Among these *Serratia marcescens* DT-1P and *Pseudomonas aeruginosa* DT-Ct1 were found to be better DDT degraders. The four strains showed variations in degrading DDT in different nitrogen sources wherein the nitrogen limiting condition was found to promote degradation in case of all but *aeruginosas*. The presence of all the strains was found to be important for better degradation.

Kinetics of DDT degradation with pure cultures showed that degradation at different parameters *viz.* temperature, pH and initial concentrations could be used

to calculate the degradation rate constant using specific equations derived for each strain and parameter.

The interactions between the parameters such as inoculum level, initial DDT concentration, incubation temperature, pH and incubation time were studied and a model was derived for each of the four strains to describe the degradation at any given set of combinations studied, within the range. The models described well the degradation of DDT at laboratory level in shake flasks.

Degradation was studied in presence of various auxiliary carbon sources and found that most of them had a synergistic effect. Individual strains showed different responses to different carbon sources thereby implying the effectiveness of both cometabolism as well as consortia in the bioremediation technologies.

Various metabolites were found to be generated during the course of DDT degradation by the individual strains under shaking conditions. These were identified by HPLC, GC-MS and NMR. The identified intermediates were DDD, DDA, 2-CBP, chlorophenol, DDMU, DDMS, DDOH, PCPA, DDE, chloro catechols, etc. Using the intermediates identified and their appearance during the sampling hours, a pathway has been proposed for DDT degradation under aerobic conditions by bacteria. The genes responsible for the synthesis of a few of the important enzymes helping the vital conversions during the process of mineralisation of DDT were tested for their presence in the bacterial strains of the consortium using PCR. The presence of dehalogenases and dioxygenases had been observed in these members of DDT-degrading consortium.

The soil bioremediation was taken up by individual strains and was found that only *Pseudomonas aeruginosa* DT-Ct1 could degrade DDT among the four effectively. The conditions studied for the degradation included inoculum size, pH, temperature, moisture and different initial concentrations. This strain could degrade up to 25 ppm DDT by 168 h of incubation. Inoculum level of 10μg dry weight of cells per gram of soil, near neutral pH, 15% moisture, and ambient temperature were found to be effective. The kinetics of DDT degradation with respect to initial concentrations was studied and the rate constant equations

derived for each as well as a general equation describing the degradation rate at a given initial DDT concentration has been proposed.

Final Concluding Remarks

Bioremediation is an interdisciplinary technology, involving microbiology, engineering, ecology, geology, chemistry, etc. Microbes are the primary stimulants in the bioremediation of contaminated environments. Investigations in to the microbial degradation of DDT are useful in the development of methods for the remediation of the contaminated environments. Laboratory studies have shown that microbes have the machinery to metabolise DDT and other xenobiotics. However, little is known about the conditions, which favour the degradation of DDT and other pollutants. *In situ* remediation may be limited because of the presence of complex sets of environmental conditions. The knowledge of the biological contribution to the effect of bioremediation and its impact on the eco-system is limited. The microbial communities are considered as a "***Black Box"*** and will make remediation a more reliable and safer technology as and when the information hidden within it is deciphered and used in a constructive way.

Nature has provided with every facility and given complete rights to each form of life to live and survive in the environment. Man has taken this liberty of this right to improve his survival with more comforts and luxuries and while doing so; he not only has created hazards for himself but even for other life forms and nature itself. Instead of looking into the welfare of man if we start monitoring the environment for the safe ecology we can survive with more security, happiness and satisfaction that we need not pollute the environment and nor create dangers to the other lives which are of same significance as ours.

Let us try and give a safe and clean environment to the future generation.

Look in References:

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* Non-detailed References- Originals not seen.