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Co-Metabolic Degradation of Dichloro Diphenyl Trichloroethane by a Defined Microbial Consortium*

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Abstract: Persistent organic pollutants such as Dichloro diphenyl trichloroethane (DDT) and related compounds are of particular environmental concern because of their toxicity, high persistence, resistance to degradation and liability to bioaccumulation. In our Laboratory, we have developed a defined microbial consortium capable of degrading DDT. The microbial consortium consisted of ten bacterial isolates of which seven were species of *Pseudomonas* and other three were species of *Flavobacterium*, *Vibrio* and *Burkholderia*. Out of twelve co-substrates used to study the enhancement in DDT-degradation, yeast extract was found to be the best showing 74% degradation at 0.0174 μg mL^{-1} day^{-1} followed by glucose and beef extract (55.98%) at 0.0109 and 0.0111 μg mL^{-1} day^{-1}, respectively. Glycerol and tryptone soya broth showed inhibitory effects with 14.92 and 10.52% degradation with 0.0023 and 0.0015 μg mL^{-1} day^{-1}, respectively. Growth was best with glycerol followed by peptone. Growth of the consortium was not found to have profound influence on the degradation of DDT.

Key words: Dichloro diphenyl trichloroethane, microbial consortium, co-substrates, degradation

INTRODUCTION

Pesticides have been widely used to protect and improve the quality and the quantity of food commodities, building materials, clothing, animal health and to combat certain disease transmitting insect vectors. Among the pesticides, chlorinated hydrocarbons such as hexachlorocylohexane (HCH), Dichloro diphenyl trichloroethane (DDT), endosulfan etc. have had their major share since their introduction in 1940s. However, indiscriminate use has caused serious concern about toxic effects of these compounds on non-target organisms and halogenation has been implicated as a reason for persistence and toxicity of these compounds (Neilson *et al*., 1985). These compounds enter the soil, water and food through several routes e.g., landfill, dumping of industrial wastes, by run-off from treated plant surfaces, spillage during application, use of contaminated manure, drift from aerial and ground applications, erosion of contaminated soil by wind and water into the aquatic system, accidents in transport of insecticides etc. (Johri *et al*., 1996). The residues of these compounds thus enter the human and animal body via food chain (Smith, 1991). Birds were found to either die or severely affected by DDT use (Cooper, 1991; Fry, 1995). DDT-breakdown products such as DDE were found to thin egg shells, thus significantly reducing the number of chicks that hatched. Although the use of DDT was banned in the western countries way back in 1970s, India continues to use DDT for public health programmes for the control of vectors causing malaria, typhus, dengue etc. Thus with continued production of DDT, the environmental contamination (soil, water, air) continues and it is imperative

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to develop technologies to degrade these recalcitrant and persistent pollutants. Studies on the degradation of HCH and DDT have been carried out using microbial isolates (Bidlan and Manonmani, 2002; Murthy and Manonmani, 2007). In the present study, we describe the co-metabolic degradation of DDT by microbial consortium in view to enhance the degradation of DDT.

MATERIALS AND METHODS

Materials

DDT (99% pure) was procured from Sigma-Aldrich chemical company, Mo, USA. The co-substrates used were purchased from Hi-Media laboratories, Mumbai, India. The solvents used in extraction of DDT and solvents used for GC were obtained from E. Merck, India. Other chemicals used in media preparations were purchased from standard chemical companies. All the chemicals were of either AR or HPLC grade.

Methods

Microbial Consortium

The defined microbial consortium used in this study was the HCH-degrading consortium developed in our laboratory by long-term enrichment technique (Murthy and Manonmani, 2007). The individual members of the consortium were grown separately in nutrient broth for 72 h under shaking conditions, mixed together at equal OD_{600} and were induced with the daily addition of 10 ppm of DDT for 7 days. This induced microbial consortium was used in the co-metabolic degradation of DDT.

Basal Medium

The basal medium, M_4 used for biodegradation studies consisted of (per liter of distilled water) 0.675 g KH_2PO_4, 5.455 g Na_2HPO_4 and 0.25 g NH_4NO_3. The pH of the medium was 7.5. Medium was sterilized at 121°C for 20 min.

Degradation of DDT

DDT as acetone solution (10 mg mL^{-1} stock solution) was added at required level to sterile, dry 250 mL Erlenmeyer flasks inside the laminar hood. Acetone was allowed to evaporate and 50 mL of M_4 medium was added to these flasks. The DDT pre-exposed microbial consortium was inoculated at 500 μg protein mL^{-1} level. The flasks were incubated in a rotary shaker (180 rpm) at ambient temperature (26-28°C). Samples were collected at regular intervals and used for the analysis of residual DDT.

To study the co-metabolic degradation of DDT, different co-substrates were supplemented at 0.5% level. The rest of the protocols were same as that used in degradation studies.

All the experiments were done in triplicates.

Analytical Methods

Extraction of Residual DDT

The sample removed after required period of incubation was acidified to pH 2.0. Residual DDT was extracted thrice from the acidified culture broth with equal volumes of dichloromethane in a separating funnel. Sample and solvent were shaken vigorously for 5 min and the two layers were allowed to separate out. The solvent layers were pooled, passed through anhydrous sodium sulphate and then through florisil. The solvent was allowed to evaporate and the residue was resuspended in a known volume of acetone for further analysis. The recovery of DDT by this method was 95±2%.

Growth

Growth of bacterial strains was determined by estimating the total protein in the biomass by modified method of Lowry *et al.* (1951). Cells were harvested from a suitable quantity of culture broth, washed with minimal medium, 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. 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. After 10 min, 0.5 mL of Lowry's D (Folin-Ciocalteau reagent (1:2) was added and mixed well. The colour was read at 660nm 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).

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). One microlitre of the extract suspension was injected in to a BP-5 capillary column (30×0.25 mm ID) set at 180°C and programmed as: 180°C for 10 min and a rise at the rate of 2°C min^{-1} up to 220°C and maintained there for 2 min. Injector was maintained at 250°C while electron capture detector (Ni^{63}) was maintained at 280°C. Pure nitrogen gas was used as the carrier at the rate of 1 mL min^{-1}. Under these conditions, the retention time for standard DDT was 28.16 min. Quantification of DDT in the sample was carried out using the area under the peak and the standard under same conditions.

RESULTS AND DISCUSSION

The key to the assessment of the fate of organic chemicals in the environment is the realistic evaluation of their susceptibility to mineralization. The major factor determining the susceptibility of an organic compound to microbial attack is the length of the time it has been exposed, which can enforce the microorganisms to initiate mineralization. Then care must be taken in establishing a degrading population. With this in view, an organochlorine pesticide (HCH) degrading microbial consortium was acclimated with DDT by enrichment technique in shake flasks. After seven days of continuous pre-exposure to DDT, the microbial consortium was observed to possess the capacity to degrade 10 ppm of DDT under aerobic conditions. All the ten members of the microbial consortium survived during acclimation of DDT indicating that the presence of DDT as a sole carbon source did not cause deleterious effect on the survivability of the members of the consortial population. The problem of foreignness was not observed with these members because of their exposure to a new recalcitrant compound and at 10 ppm level DDT was observed to be non-toxic. This acclimated microbial consortium was used for studies on the degradation of DDT.

Degradation of 10 ppm of DDT

The microbial consortium was found to degrade 10 ppm of DDT upto 65% by 10 days (data not shown). Only 10% of DDT was found to be degraded by 24 h of incubation (Table 2). The degradation increased slowly with time and by 10 days of incubation, 65% of DDT was found to be degraded. With increase in incubation time, there was no substantial improvement in the degradation. The rate of degradation was 0.0034 μg mL^{-1} day^{-1}. All the organisms were found to survive by the end of ten days of incubation period (Table 1) which indicated that all the isolates had the machinery to degrade DDT, which might be a synergistic action. There was practically very little degradation in abiotic controls (data not shown). Only 1.8% degradation was observed after 10 days of incubation.

Table 1: Growth of the individual isolates of the consortium during degradation of 10 ppm of DDT

	Growth (log CFU)									
	Isolate No.									
Incubation time (day)	1	2	3	4	5	6	7	8	9	10
0	2.6×10^{7}	2.1×10^{7}	2.5×10^{7}	4.6×10^{6}	1.8×10^{8}	3.2×10^{7}	2.1×10^{7}	2.2×10^{7}	1.9×10^{7}	2.4×10^{7}
2	0.5×10^{8}	0.1×10^{8}	1.1×10^{8}	2.6×10^{7}	3.4×10^{8}	3.1×10^{8}	3.1×10^{7}	1.8×10^{8}	3.6×10^{7}	1.9×10^{8}
4	0.7×10^{8}	0.3×10^{8}	0.6×10^{8}	1.1×10^{7}	4.2×10^{7}	1.4×10^{6}	1.1×10^{7}	3.1×10^{8}	1.8×10^{8}	1.4×10^{7}
6	1.4×10^{7}	0.4×10^{7}	0.4×10^{6}	4.1×10^{7}	3.6×10^{6}	1.8×10^{5}	3.2×10^{6}	4.2×10^{6}	3.4×10^{6}	0.9×10^{6}
8	2.1×10^{6}	1.2×10^{6}	1.3×10^{5}	1.6×10^{6}	1.8×10^{6}	1.1×10^{5}	1.4×10^{5}	1.4×10^{4}	1.2×10^{5}	1.8×10^{5}
10	1.8×10^{6}	1.1×10^{5}	1.1×10^{4}	1.1×10^{6}	1.1×10^{5}	0.82×10^{5}	1.2×10^{5}	1.1×10^{4}	0.8×10^{5}	0.8×10^{5}

Table 2: Growth of the isolates and degradation of DDT by microbial consortium in presence of different co-substrates

	Growth (OD_{600})			Degradation (%)*		
Co-substrates	24 h	48 h	72 h	24 h	48 h	72 h
Glycerol	0.1830	0.2540	0.3356	5.41±0.080	10.69±0.025	14.92±0.028
Glucose	0.0336	0.0672	0.0872	21.40±0.0250	38.50±0.031	55.98±0.012
Sucrose	0.0254	0.0591	0.0662	15.61±0.009	28.62±0.018	39.55±0.028
Lactose	0.0539	0.0926	0.1720	18.36±0.030	31.58±0.018	47.77±0.011
Citrate	0.0350	0.0649	0.0520	15.32±0.015	29.65±0.026	37.28±0.021
Succinate	0.0160	0.0354	0.0380	15.95±0.017	30.54±0.010	37.28±0.024
Tween 80	0.0316	0.0687	0.0534	20.64±0.026	30.55±0.011	48.42±0.029
Yeast extract	0.0957	0.1590	0.2166	25.52±0.008	51.36±0.022	74.56±0.011
Tryptone soya broth	0.0560	0.1190	0.1822	3.21±0.032	7.24±0.045	10.52±0.027
Tryptone	0.1500	0.2610	0.3048	17.52±0.035	36.24±0.017	47.77±0.023
Peptone	0.1680	0.2460	0.3242	11.84±0.040	24.37±0.280	34.34±0.020
Beef extract	0.0824	0.1950	0.3174	19.54±0.028	40.89±0.016	55.98±0.032
Control	0.0041	0.0084	0.0066	10.64±0.056	20.25±0.029	32.15±0.022

*Average of three replicates

The biodegradation of any compound will be a success if enzymes are synthesized in response to the presence of a recognisable substrate and the genetic capability of the microorganism(s) in action. As most of these enzymes are inducible enzymes, the degradation capability of any organism(s) depends on the extent of induction of these degrading enzymes i.e., the requisite quantity of the requisite enzyme. The degradation of 65% of 10 ppm DDT by 240 h of incubation without further improvement indicated that sufficient quantities of the enzymes might not have been produced to carry out complete degradation which could be due to insufficient quantity of microbial cells or there could have been inhibition of the enzyme activity by the intermediates formed during degradation. If the xenobiotic substrate, DDT is incapable of causing induction of the requisite quantity of the enzyme(s), due to fact that microbial cells would act as resting cells and DDT-acting as non-growth substrate, the increase in biodegradation could be achieved by the addition of a growth substrate during the degradation of a non-growth substrate, DDT. With this in view, enhancement in DDT degradation was tried with the addition of few co-substrates.

Co-metabolic Degradation of DDT

Among twelve different co-substrates used along with 10 ppm of DDT, yeast extract was found to be the best helping with 74.56% degradation by 72 h (Table 3) at the rate of 0.0174 μg mL^{-1} day^{-1}. The degradation was 2.319 times more than the control (without co-substrate). This was followed by beef extract and glucose which showed 55.98% degradation at 0.0110 μg mL^{-1} day^{-1} (Table 3). This was observed to be 1.74 times more than the control. Co-substrates such as glycerol and TSB showed inhibitory effects towards DDT-degradation. The degradation was found to decrease by 0.464 and 0.327 times, respectively compared to control, with the rate of degradation being 0.0023 and 0.0015 μg mL^{-1} day^{-1}, respectively. However, the growth of the consortium in the presence of these

Table 3: Rate of degradation of DDT with different co-substrates

Substrates	μg mL^{-1} day^{-1}
Glycerol	0.0023
Glucose	0.0109
Sucrose	0.0070
Lactose	0.0087
Citrate	0.0068
Succinate	0.0069
Tween 80	0.0088
Yeast extract	0.0174
Tryptone soya broth	0.0015
Tryptone	0.0091
Peptone	0.0058
Beef extract	0.0111
Control	0.0034

co-substrates did not show any relation to degradation (Table 2). That is, the growth of the consortium was highest in glycerol, which showed inhibitory effects towards degradation of DDT. Glucose showed less growth (around four times less than that in glycerol) but helped in better degradation of DDT (56%). Nitrogenous substances supported better growth of the consortium compared to simple sugars and organic acids. It was clear that the presence of a growth substrate i.e., co-metabolite assisted in the better transformation or degradation of DDT i.e., the compound showing resistance to degradation. This kind of situation has been used to make a distinction between co-metabolism and gratuitous metabolism by Dalton and Stirling (1982), wherein substantial amount of degradation would not be observed with pre-grown cells because these cells could not extract energy from the subsequent metabolism of it to drive the first requiring step. Only when a growth substrate or other transformable compound was present that could yield energy to drive the initial reaction would transformation occur and this has been defined as co-metabolism (Dalton and Stirling, 1982).

When the composition of the microbial consortium was studied, all the cultures showed increase in their numbers during co-metabolic degradation whereas without co-substrates, isolate numbers 3 and 8 slightly decreased in number by 72 h (Table 1). These members of the consortium might have been necessary for synergistic complete degradation. However, the necessity of these isolates for completion of degradation of DDT needs to be worked out in detail. In the presence of co-metabolite, the increase in cell number of all members could help in concerted attack by many microbes within the community that can lead to complete degradation i.e., the co-metabolic metabolite by one or few species of the community could be degraded by another species of organism within the community (Bull, 1980). This clearly indicates significant coordinative interaction within the community wherein, degradation can be completed accurately by the mixed-culture system. Perhaps, difficulty is associated in proving the existence of specific interactions based on effects in microbial communities. In our studies, in the microbial consortium, all the species were found to be present. There were no dominants, associates or incidentals. It was not clear which organism(s) were primary utilizers and which were secondary utilizers. The community as a whole might be playing an important role in biodegradation.

Yeast extract at 1% level assisted in co-metabolic degradation. Concentration lesser to this showed lesser degradation. An increase in yeast extract concentration did not show any further increase in degradation (Fig. 1).

Co-metabolic degradation of DDT by microbial consortium was maximum at 72 h of incubation period (Fig. 2). Degradation was 94% by 72 h and reached 100% only by 120 h i.e., disappearance of DDT after 94% of degradation was slow.

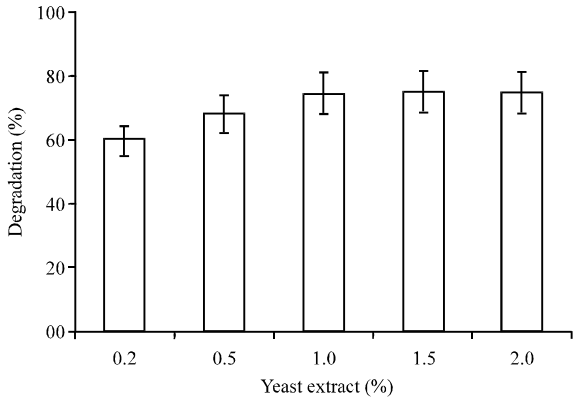

Fig. 1: Effect of different concentrations of yeast extract on DDT-degradation

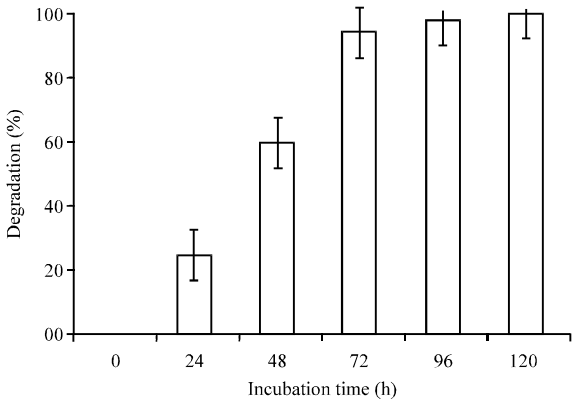

Fig. 2: Effect of incubation period on the degradation of DDT

CONCLUSION

All microorganisms cannot utilize the xenobiotic compound as the sole source of carbon and energy. In such cases, co-metabolism enhances the degradation of the compound. Effect of different co-substrates on the degradation of DDT by the microbial consortium was studied. The consortium showed enhanced degradation with yeast extract at a level of 1%.

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