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Aerobic degradation of technical hexachlorocyclohexane by a defined microbial consortium

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Abstract

Organochlorine pesticides including hexachlorocyclohexane (HCH) and dichlorodiphenyltrichloroethane (DDT) are largely used in developing countries like India for public health and agricultural purposes. Even though the agricultural use of technical mixture (tech-HCH) is banned, countries like India are still using γ-HCH for economic purposes. Thus, in addition to already contaminated sites, new sites are being contaminated with γ-HCH and its stereoisomers. In the environment, these isomers have a half-life of 8–10 years. In our laboratory, we developed a microbial consortium capable of degrading tech-HCH. Conditions such as induction, inoculum level, concentration of the substrate, pH of degradation and interaction between isomers were optimized for tech-HCH degradation. Up to 25 ppm tech-HCH was degraded at an inoculum level of 100 μg protein/mL, pH 7.5 at ambient temperature (26–28 °C). The degradation of HCH-isomers was in the order of γ > α > β > δ. The rate of degradation was also determined.

Keywords: Bioremediation; tech-HCH; Microbial consortium; Aerobic degradation; Pre-exposure

1. Introduction

Organochlorine pesticides are largely used in developing countries for public health and agricultural purposes. Currently, their use largely is being phased out because of their toxicity, environmental persistence and accumulation in the food chain. Among these organochlorine pesticides, hexachlorocyclohexane was widely used for both agriculture and medical purposes. Even though, technical mixture containing eight stereoisomers is banned, some countries like India are still using γ-HCH for economic reasons. Thus in addition to already contaminated sites, new sites are continuously being contaminated by γ-HCH and its stereoisomers. [1,2]. Although only lindane is insecticidal, HCHs as a group are toxic and considered as potential carcinogens [3]. During the production of lindane (γ-isomer) up to 85% of the final product consists of mainly α, β and δ-isomers [4]. For the supply of γ-isomer, the other stereoisomers are separated from γ-HCH and dumped as waste at different spots on the production sites causing serious soil pollution [5]. HCH continues to pose a serious toxicological problem at industrial sites where post-production of lindane along with unsound disposal practices has led to serious contamination. In addition, many countries still allow HCH production and use, despite localized limitations, HCH contamination continues to be global issue. These compounds have moderate volatility and can be transported by air to remote locations [6]. Concentration in air ranged from 3.6 to 1021 pg m^{-3} α-HCH and 1 to 580 pg m^{-3} γ-HCH. Mean concentration in surface water ranged from 0.09 to 40 ng m^{-3} and 0.04 to 61 ng m^{-3} for α- and γ-HCH, respectively [3]. Lakes showed a range of 19.9–51.4 pg m^{-3} during summer months and much lower concentrations during autumn and winter months [7]. α- and γ-isomers ranged from 2 to 25 pg m^{-3} in all ground level samples [8]. Microbial degradation of chlorinated pesticides such as HCH is usually carried out by using either pure or mixed culture systems. The main goal of the laboratory studies is to predict the biodegradation rates in the environment. But it is very difficult to extrapolate the results obtained in the laboratory systems to predict their fate in the environment [9]. The microbial degradation of HCH isomers in liquid cultures has been studied using pure microbial cultures such as *Clostridium rectum*, *Pandoraea* [10,11], mixed native soil microbial population (undefined consortium) [12,13],

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Phanerochaete chrysosporium [14,15] and sewage sludge under aerobic and anaerobic conditions [16–18]. The degradation of α and γ-isomers was almost complete and β and δ-isomers showed more resistance to degradation. At this stage it is imperative to develop technologies where all isomers of tech-HCH are degraded completely. In this communication we describe the degradation of tech-HCH in shake flasks using a microbial consortium.

2. Materials and methods

2.1. Substrate

α-, β-,γ- and δ-HCH isomers (99% pure) were procured from Sigma–Aldrich Chemical Company, St. Louis, MO, USA; technical grade HCH was obtained from Hindustan insecticides, Mumbai, India. Other chemicals and the reagents used in this study were of analytical grade and were purchased from standard chemical companies.

2.2. Microbial consortium

The microbial consortium capable of degrading tech-HCH was developed in our laboratory by long term enrichment of HCH contaminated soil and sewage according to Manonmani et al. [19]. The tech-HCH degrading consortium that got enriched was acclimated with increase in concentration of tech-HCH from 5 to 25 ppm. The consortium thus obtained was maintained as liquid culture in minimal medium containing 10 ppm of tech-HCH and in minimal agar medium containing 10 ppm of tech-HCH.

2.3. Cultural conditions

The minimal medium used in degradation studies contained KH_2PO_4, 0.76 g/L; Na_2HPO_4, 5.455 g/L and NH_4NO_3, 0.25 g/L, in a liter of distilled water. The pH of the medium was 7.2–7.5 [15].

Degradation experiments in liquid cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL minimal medium. Required quantity of tech-HCH was dissolved in 50 μL acetone and added to the bottom of the sterile, dry 250 mL capacity Erlenmeyer flasks in laminar hood and acetone was allowed to be evaporated. Then 50 mL of minimal medium was added and inoculated with tech-HCH degrading consortium at required protein level [20]. The flasks were then incubated at ambient temperature (26–28 °C) in a rotary shaker. Samples (whole flasks) were taken out at regular intervals and analysed for growth (protein and cfu), and residual HCH-isomers.

To study the necessity of induction/pre-exposure on the degradation of HCH, the individual members of the microbial consortium were grown individually in nutrient broth for 72 h. The harvested and washed cells were pooled together at equal OD_{600} and were inoculated with all four isomers of HCH, i.e. α, β, γ, and δ-isomers individually and also with tech-HCH in minimal medium. The flasks were then incubated in a rotary shaker at ambient temperature (26–28 °C) for 72 h with the addition of respective substrates at every 24 h duration. The cells were then harvested, washed well in sterile minimal medium and used in degradation studies as described above. Samples (whole flasks) were taken out at regular intervals and analysed for growth (protein and cfu), and residual substrate.

To study the interaction of different isomers of HCH during degradation, all isomers were mixed at 10 ppm level in all permutations and combinations. Microbial consortium induced with tech-HCH was inoculated to these flasks as described above. The samples (whole flasks) collected at regular intervals were analysed for growth (protein and cfu), and residual substrate.

The effect of pH was studied at pH levels 4.0–9.0. Acetate buffer was used for pH values between 4.0 and 5.0, phosphate buffer for pH values between 6.0 and 8.0 and Carbonate–bicarbonate buffer for pH 9.0.

The effect of inoculum level was studied by inoculating the flasks with the microbial consortium from 5 to 100 μg protein/mL.

2.4. Analytical

The survival of individual members of the HCH degrading microbial consortium was done by estimating the colony forming units (Cfu) [17].

The sample (whole flask) collected at regular intervals was acidified to pH 2.0 with 1N HNO_3. The residual HCH was extracted three times with equal volumes of dichloromethane. The solvent fractions were pooled, passed over a bed of anhydrous sodium sulphate, concentrated and purified by passing through florisil column. The fraction containing residual substrate was evaporated to dryness, resuspended in a known volume of acetone and used for quantification by TLC and GC.

Thin layer chromatography (TLC) was done using silica gel G TLC plates. Residual substrate samples dissolved in required quantity of acetone were spotted on TLC plates and these plates were developed in cyclohexane. The residual tech-HCH spots were identified after spraying the air-dried developed plates with *O*-tolidine in acetone. The residual substrate spots were delineated by marking with a needle and the area was measured. The concentration of residual substrate was computed from a standard plot of log concentrations versus square root of the area prepared for standard tech-HCH.

The acetone layer containing residual substrate after appropriate dilution was injected into gas chromatograph (Fison's model) equipped with ^{63}Ni detector and SS column (200 cm × 2 mm) packed with 1.5% OV 17 plus 1.95 QF1 on chromosorb W 80/100 mesh. The column, injector and detector were maintained at 230, 230 and 320 °C, respectively with a flow rate of carrier gas nitrogen at 50 mL/min. Under these conditions, the retention time of HCH-isomers was α-HCH, 3.34 min; γ-HCH, 3.98 min; β-HCH, 4.42 min; δ-HCH, 5.1 min. The recovery of HCH isomers ranged from 92 to 95% from mineral salts medium. All the data presented in this study are based on triplicate estimations.

2.5. Kinetics of degradation of HCH

Degradation of HCH was studied using first order kinetics. Kinetic equation has been given in Appendix A.

3. Results

3.1. The microbial consortium

The defined microbial consortium used in the degradation of tech-HCH was developed by the long-term enrichment of the contaminated soil and sewage samples [19,21]. This microbial consortium was acclimated with increasing concentration of tech-HCH from 5 to 25 ppm. The consortium that got established at 25 ppm level was used in our studies. The community structure of the consortium was identified by dilution plating technique. The consortium was found to be made of ten bacterial isolates consisting of seven *Pseudomonas* spp.; one species each of *Burkholderia*, *Flavobacterium* and *Vibrio* (Table 1).

3.2. Degradation of tech-HCH by the microbial consortium

3.2.1. Pre-exposure and degradation of tech-HCH

The adaptation of the developed consortium with different isomers of HCH and also tech-HCH was done to understand the choice of the inducer that could be used for complete mineralization of tech-HCH. Induction of the tech-HCH degrading consortium with α-isomer resulted in the complete degradation of α-isomer of tech-HCH by 72 h of incubation. β-, γ- and δ-isomers were still present at 2.035, 6.019 and 24.13% levels by the end of 72 h of incubation. However, the rate of degradation of tech-HCH isomers inoculated with α-isomer induced microbial consortium was 0.0786, 0.051, 0.0389 and 0.0197 μg h^{-1}, respectively for α-, β-, γ- and δ-isomers, indicating that the degradation of α-isomer was more compared to other isomers. δ-isomers was degraded least (Table 2). Induction with β-HCH showed 89.97% degradation of β-isomer. α-, γ- and δ-isomers showed 20.1, 26.06 and 59.95% residual substrate levels by the end of 72 h of incubation. The rate of degradation of α-,β-, γ- and δ-isomers was 0.0252, 0.0364, 0.0209 and 0.0151 h^{-1} (Table 2). γ-HCH induced consortium, when inoculated to tech-HCH showed complete disappearance of γ-isomer by 72 h with a rate of degradation of 0.0699 μg h^{-1}. Of the other isomers of tech-HCH, 14.01% of α-isomer, 26.01% of β-isomer and 28.01% of δ-isomer were still found to be remaining by 72 h with rate of degradation respectively of 0.0288, 0.018 and 0.0045 μg h^{-1} (Table 2). All the isomers of HCH were found to be still remaining after 72 h of incubation when inoculum induced with δ-HCH was inoculated to tech-HCH. Degradation of 77.99, 45.98, 97.93 and 85.82% of α-,β-, γ- and δ-isomers was observed with 0.022, 0.0092, 0.047 and 0.0285 degradation rates, respectively (Table 2), when tech-HCH induced inoculum was used 5.03, 23.37, 2.07 and 40.05% α-,β-, γ- and δ-isomers were still remaining by 72 h incubation. The rate of degradation of these isomers was 0.0381, 0.0173, 0.0476 and 0.0136 μg h^{-1}, respectively for α-,β-, γ- and δ-isomers (Table 2). However, the tech-HCH induced consortium when inoculated to individual isomers, was able to degrade them completely. α- and γ-isomers were degraded completely with 6.03 and 22.14% of β- and δ-HCH remaining by 120 h of incubation. There was no initial lag in degradation with any inducer used. In all the cases, the HCH-isomer used for induction was degraded completely and other isomers were partially degraded.

Table 1
List of isolates of microbial consortium

Sl. No.	Bacterial isolate	No.
1	*Pseudomonas fluorescens* biovar II	T_1
2	*Pseudomonas diminuta*	T_2
3	*Pseudomonas fluorescens* biovar I	T_3
4	*Burkholderia pseudomallei*	T_4
5	*Pseudomonas putida*	T_5
6	*Flavobacterium* sp.	T_6
7	*Vibrio alginolyticus*	T_7
8	*Pseudomonas aeruginosa*	T_8
9	*Pseudomonas stutzeri*	T_9
10	*Pseudomonas fluorescens* biovar V	T_{10}

Table 2
Kinetics of degradation of tech-HCH (microbial consortium induced with α-, β-, γ-, δ- and tech-HCH)

HCH isomer used for induction	Rate of degradation (μg h^{-1})			
	α	β	γ	δ
α	0.0786	0.051	0.0389	0.0197
β	0.0252	0.0364	0.0209	0.0151
γ	0.0288	0.018	0.0699	0.0045
δ	0.022	0.0092	0.0047	0.0285
tech-HCH	0.0381	0.0173	0.0476	0.0136

3.2.2. Inoculum level and degradation of tech-HCH

The degradation of HCH isomers increased with increase in inoculum level, from 25 μg protein/mL (Fig. 1). The time required for complete degradation decreased with increase in inoculum size. Complete degradation of all isomers was observed at 100 μg protein/mL level which did not improve

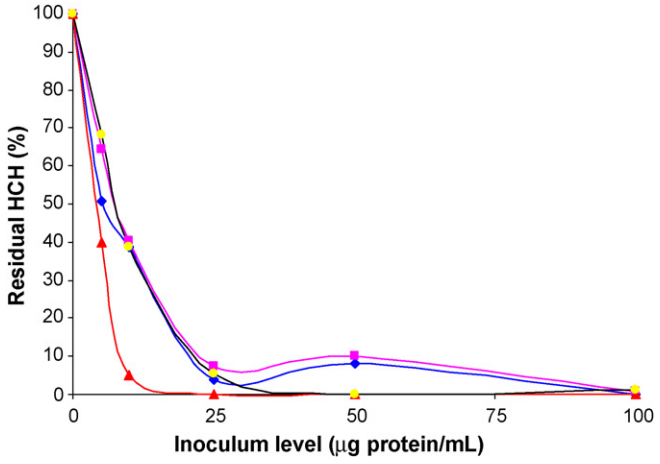

Fig. 1. Degradation of tech-HCH at different inoculum levels. (♦) α-isomer, (■) β-isomer, (▲) γ-isomer, (●) δ-isomer.

Table 3
Kinetics of degradation of tech-HCH at different inoculum levels

Inoculum level (μg protein/mL)	Rate of degradation (μg h^{-1})			
	α	β	γ	δ
5	0.0054	0.0038	0.0088	0.0036
10	0.0094	0.0086	0.0235	0.0079
25	0.0203	0.017	0.0254	0.0211
50	0.0299	0.0303	0.0386	0.0285
100	0.0314	0.026	0.0439	0.0352

Table 4
Kinetics of degradation of tech-HCH at different concentrations

Concentration of tech-HCH (ppm)	Rate of degradation (μg h^{-1})			
	α	β	γ	δ
5	0.037	0.0828	0.0998	0.0774
10	0.0297	0.0178	0.035	0.0209
25	0.0381	0.0221	0.0473	0.0124

much with further increase in inoculum level (Table 3). The rate of degradation of different isomers of technical mixture at 100 μg protein/mL was 0.0314, 0.026, 0.0439 and 0.0352 μg h^{-1}, respectively for α-, β-, γ- and δ-isomers. In all the levels, the degradation pattern was γ > α > β > δ.

3.2.3. Initial substrate concentration and degradation of tech-HCH

At the initial substrate level of 5 ppm, all the four isomers were found to be degraded completely by 72 h (Fig. 2a). γ-isomer was degraded faster at 0.0998 μg h^{-1} followed by α-, β- and δ-isomers, which were degraded at 0.037, 0.0828 and 0.0774 μg h^{-1}. There was no initial lag in the degradation of any isomer of the technical mixture. At a slightly higher concentration of the substrate, tech-HCH, the time required for the degradation was more. Only 50% of the α-isomer of technical mixture was degraded by 24 h of incubations at a rate of 0.0297 μg h^{-1} (Table 4). But during the same period of time 12, 61, 36%, respectively of β-, γ- and δ-isomers were degraded (Fig. 2b) at a rate of 0.0178, 0.035 and 0.0209 μg h^{-1}, respectively. However, all the isomers of tech-HCH were degraded completely by 96 h of incubation time. The time required for the degradation of 25 ppm of tech-HCH got increased further (Fig. 2c). By 120 h both α-and γ-isomers disappeared completely at a rate of 0.0381 and 0.0473 μg h^{-1}. β- and δ-isomers were degraded partially with 4.03 and 22.14%, respectively, of the initially present substrate, still remaining by 120 h (Fig. 2c).

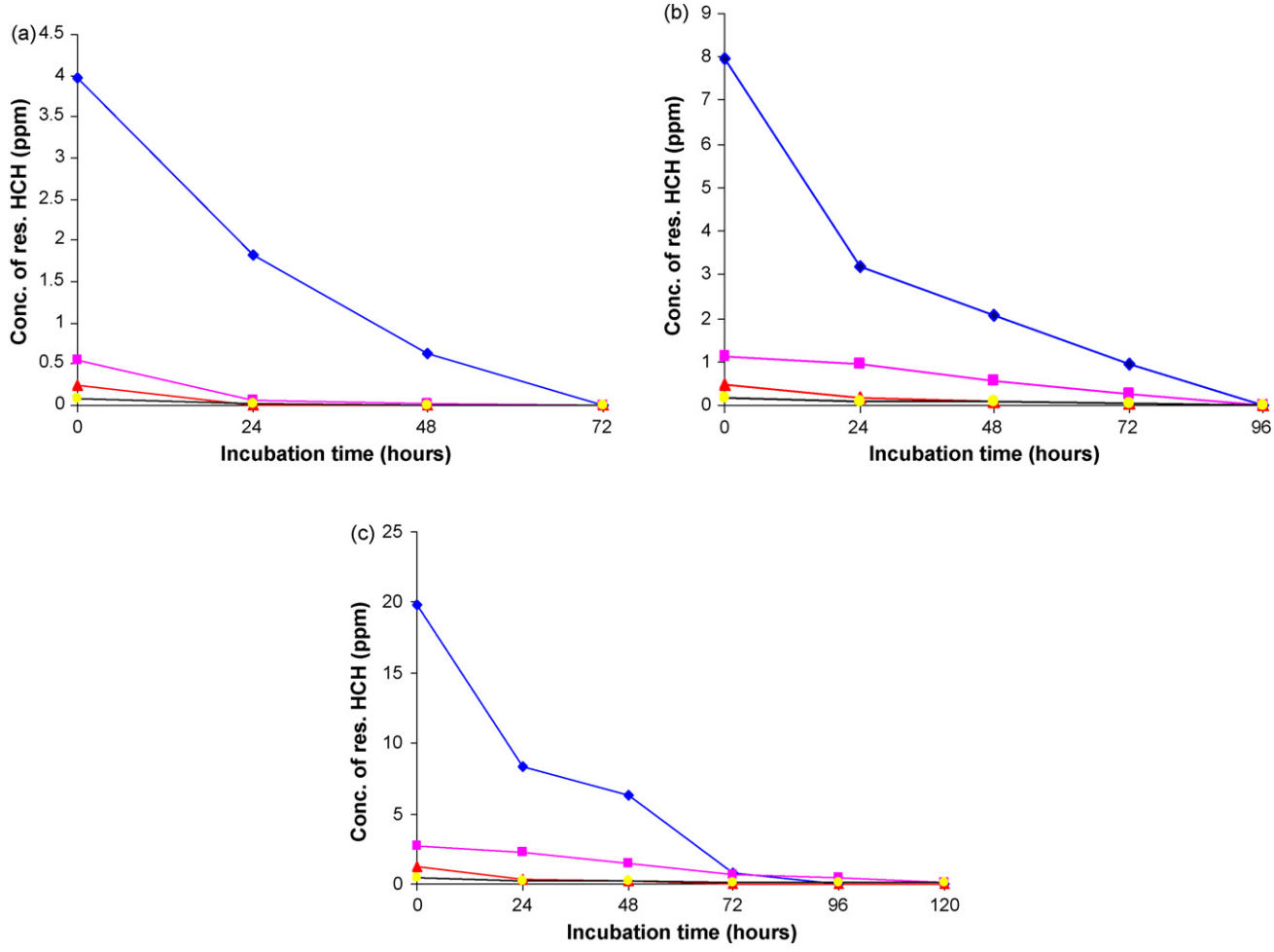

Fig. 2. Degradation of tech-HCH at different initial concentrations: (a) 5 ppm, (b) 10 ppm and (c) 25 ppm. (♦) α-isomer; (■) β-isomer; (▲) γ-isomer; (●) δ-isomer.

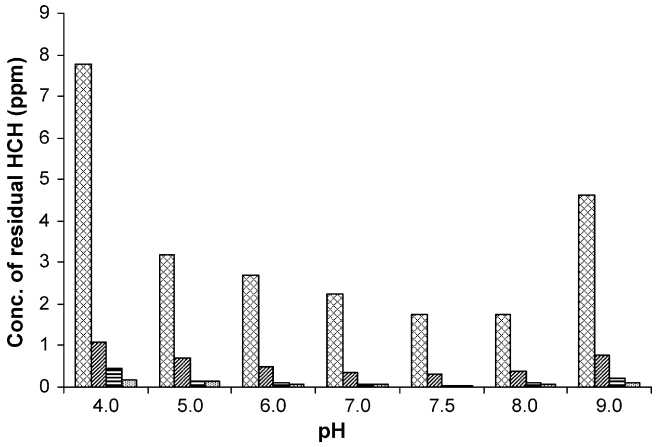

Fig. 3. Effect of pH on the degradation of HCH. ▨ = α-isomer, ▨ = β-isomer, ▤ = γ-isomer, ▨ = δ-isomer; initial HCH level, 10 ppm; incubation time, 72 h.

With increase in concentration of the substrate the degradation rate of each isomer decreased. With increase in substate level, the rate of degradation of α-, β-, γ- and δ-isomers decreased by around 20, 76, 60 and 80%, respectively. However, at all the concentrations of tech-HCH used the degradation pattern of the four isomers was γ > α > β > δ. At any of these substrate loads, the growth of different members of the consortium showed a very marginal increase in colony forming units (Cfu) (Table 5), i.e. the microbial cells were behaving as resting cells. In abiotic controls there was practically no degradation of the substrate (data not shown).

3.2.4. Effect of pH on the biodegradation of HCH-isomers

The microbial consortium was found to efficiently degrade all isomers of HCH at pH values near neutral. The degradation increased with increase in pH towards neutral range. At pH 4.0, practically there was no degradation (Fig. 3). The degradation improved with increase in pH. Even the easily degradable γ-isomer was not degraded at pH 4.0. At higher pH ranges, the degradation improved in the same order of γ > α > β > δ. At pH 7.5, the γ-isomer disappeared completely by 72 h of incubation (Fig. 3). At still higher pH levels, the degradation again decreased. The rate of degradation of each isomer at different pH levels is given in Table 6. The pH of the medium had a substantial effect on the survivability of the members of the consortial community. At low pH levels, there was decrease in the microbial population. The survivability of the consortium also improved with increase in pH towards neutrality. In abiotic controls, the recovery of HCH-isomers at pH 9.0 was less indicating some chemical hydrolysis of these isomers in the liquid cultures.

3.2.5. Interactions of different isomers of HCH

The different isomers of HCH of technical mixture were found to influence the degradation of the other isomer. To find out the interaction between different isomers, inoculations were made at all permutations and combinations of the different isomers (Table 7). When the isomers were used individually, up to 82, 84, 100 and 80% degradation, respectively of α-,β-, γ- and δ-isomers was observed. Individually, β-isomer was degraded

Table 5
Bacterial profile of HCH degrading consortium grown at different concentration of tech-HCH

Substrate concentration (ppm)	Period of incubation (h)	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
5	0	$7.2 \times 10^{14} \pm 0.11$	$4.5 \times 10^{14} \pm 0.22$	$6.2 \times 10^{14} \pm 0.2$	$4.4 \times 10^{14} \pm 0.38$	$2.8 \times 10^{13} \pm 0.24$	$2.5 \times 10^{14} \pm 0.11$	$8.6 \times 10^{14} \pm 0.32$	$3.0 \times 10^{15} \pm 0.12$	$1.9 \times 10^{14} \pm 0.03$	$4.0 \times 10^{14} \pm 0.14$
	24	$1.2 \times 10^{16} \pm 0.32$	$6.8 \times 10^{14} \pm 0.05$	$8.4 \times 10^{15} \pm 0.03$	$3.2 \times 10^{15} \pm 0.41$	$6.4 \times 10^{15} \pm 0.31$	$3.0 \times 10^{16} \pm 0.21$	$4.8 \times 10^{16} \pm 0.20$	$8.4 \times 10^{16} \pm 0.03$	$6.4 \times 10^{16} \pm 0.21$	$4.4 \times 10^{15} \pm 0.31$
	48	$2.1 \times 10^{15} \pm 0.05$	$4.0 \times 10^{11} \pm 0.04$	$3.4 \times 10^{14} \pm 0.13$	$1.1 \times 10^{14} \pm 0.02$	$6.4 \times 10^{10} \pm 0.94$	$9.0 \times 10^{13} \pm 0.01$	$3.41 \times 10^{14} \pm 0.03$	$2.81 \times 10^{14} \pm 0.15$	$4.8 \times 10^{14} \pm 0.01$	$3.4 \times 10^{13} \pm 0.08$
	72	$1.4 \times 10^{11} \pm 0.09$	$2.5 \times 10^{8} \pm 0.03$	$2.5 \times 10^{10} \pm 0.07$	$2.8 \times 10^{11} \pm 0.09$	$3.6 \times 10^{9} \pm 0.04$	$1.81 \times 10^{10} \pm 0.03$	$2.6 \times 10^{10} \pm 0.04$	$3.0 \times 10^{13} \pm 0.09$	$2.8 \times 10^{10} \pm 0.03$	$3.8 \times 10^{10} \pm 0.04$
10	0	$8.2 \times 10^{14} \pm 0.03$	$9.1 \times 10^{14} \pm 0.02$	$6.4 \times 10^{15} \pm 0.04$	$2.9 \times 10^{15} \pm 0.09$	$2.06 \times 10^{15} \pm 0.08$	$2.5 \times 10^{14} \pm 0.08$	$3.14 \times 10^{14} \pm 0.09$	$2.9 \times 10^{14} \pm 0.07$	$2.8 \times 10^{14} \pm 0.04$	$4.1 \times 10^{15} \pm 0.01$
	24	$3.4 \times 10^{16} \pm 0.09$	$6.4 \times 10^{16} \pm 0.09$	$4.0 \times 10^{16} \pm 0.02$	$4.81 \times 10^{16} \pm 0.03$	$9.04 \times 10^{17} \pm 0.04$	$4.52 \times 10^{16} \pm 0.09$	$4.8 \times 10^{16} \pm 0.08$	$1.31 \times 10^{16} \pm 0.08$	$1.6 \times 10^{16} \pm 0.07$	$6.8 \times 10^{17} \pm 0.06$
	48	$2.2 \times 10^{16} \pm 0.05$	$3.8 \times 10^{14} \pm 0.06$	$2.1 \times 10^{15} \pm 0.08$	$1.2 \times 10^{15} \pm 0.04$	$1.64 \times 10^{14} \pm 0.04$	$4.3 \times 10^{14} \pm 0.07$	$3.0 \times 10^{14} \pm 0.04$	$5.14 \times 10^{12} \pm 0.07$	$2.9 \times 10^{13} \pm 0.1$	$2.1 \times 10^{14} \pm 0.12$
	72	$2.1 \times 10^{14} \pm 0.10$	$4.8 \times 10^{12} \pm 0.09$	$1.0 \times 10^{10} \pm 0.07$	$2.3 \times 10^{14} \pm 0.05$	$1.0 \times 10^{10} \pm 0.02$	$3.4 \times 10^{13} \pm 0.09$	$8.0 \times 10^{13} \pm 0.03$	$1.28 \times 10^{10} \pm 0.1$	$3.7 \times 10^{9} \pm 0.1$	$8.4 \times 10^{13} \pm 0.10$
	96	$2.68 \times 10^{10} \pm 0.04$	$2.02 \times 10^{8} \pm 0.10$	$3.2 \times 10^{8} \pm 0.03$	$1.21 \times 10^{12} \pm 0.06$	$1.21 \times 10^{10} \pm 0.09$	$2.2 \times 10^{8} \pm 0.01$	$1.0 \times 10^{10} \pm 0.01$	$4.6 \times 10^{9} \pm 0.1$	$3.0 \times 10^{8} \pm 0.09$	$3.4 \times 10^{9} \pm 0.07$
25	0	$9.4 \times 10^{14} \pm 0.12$	$1.8 \times 10^{15} \pm 0.034$	$4.2 \times 10^{13} \pm 0.41$	$1.21 \times 10^{12} \pm 0.31$	$6.4 \times 10^{4} \pm 0.25$	$5.8 \times 10^{14} \pm 0.11$	$1.76 \times 10^{16} \pm 0.2$	$2.6 \times 10^{15} \pm 0.2$	$9.1 \times 10^{14} \pm 0.38$	$4.2 \times 10^{15} \pm 0.24$
	24	$6.91 \times 10^{16} \pm 0.09$	2.8×10^{16} (± 0.02	$2.4 \times 10^{8} \pm 0.04$	$1.6 \times 10^{15} \pm 0.03$	$8.1 \times 10^{14} \pm 0.04$	$6.1 \times 10^{15} \pm 0.01$	$1.02 \times 10^{16} \pm 0.02$	$6.4 \times 10^{16} \pm 0.04$	$6.4 \times 10^{15} \pm 0.09$	$6.8 \times 10^{15} \pm 0.07$
	48	$1.52 \times 10^{16} \pm 0.08$	$3.6 \times 10^{15} \pm 0.04$	$3.6 \times 10^{4} \pm 0.07$	$2.1 \times 10^{15} \pm 0.03$	$6.8 \times 10^{15} \pm 0.01$	$3.8 \times 10^{14} \pm 0.04$	$4.1 \times 10^{14} \pm 0.1$	$1.1 \times 10^{16} \pm 0.09$	$1.41 \times 10^{15} \pm 0.02$	$4.1 \times 10^{15} \pm 0.02$
	72	$1.41 \times 10^{16} \pm 0.08$	$2.02 \times 10^{16} \pm 0.08$	$4.1 \times 10^{1} \pm 0.05$	$0.4 \times 10^{15} \pm 0.07$	$3.4 \times 10^{15} \pm 0.04$	$4.1 \times 10^{14} \pm 0.05$	$2.8 \times 10^{14} \pm 0.08$	$3.41 \times 10^{14} \pm 0.03$	$3.4 \times 10^{15} \pm 0.06$	$1.81 \times 10^{15} \pm 0.10$
	96	$3.2 \times 10^{14} \pm 0.04$	$6.1 \times 10^{15} \pm 0.05$	0	$1.4 \times 10^{14} \pm 0.08$	$1.4 \times 10^{12} \pm 0.04$	$2.4 \times 10^{12} \pm 0.04$	$3.52 \times 10^{12} \pm 0.07$	$5.1 \times 10^{12} \pm 0.08$	$1.1 \times 10^{14} \pm 0.01$	$6.1 \times 10^{12} \pm 0.10$
	102	$1.01 \times 10^{10} \pm 0.04$	$4.8 \times 10^{14} \pm 0.02$	0	$0.8 \times 10^{12} \pm 0.04$	$0.1 \times 10^{10} \pm 0.03$	$0.1 \times 10^{10} \pm 0.01$	$1.1 \times 10^{12} \pm 0.08$	$1.0 \times 10^{10} \pm 0.07$	$2.1 \times 10^{10} \pm 0.02$	$2.1 \times 10^{10} \pm 0.05$

Table 6
Kinetics of degradation of tech-HCH at different pH levels

pH	Rate of degradation ($(g\,h^{-1})$)			
	α	β	γ	δ
4.0	0.0004	0.0003	0.001	0
5.0	0.0108	0.0059	0.0146	0.0034
6.0	0.0131	0.0092	0.0181	0.0077
7.0	0.0152	0.013	0.0216	0.0112
7.5	0.0165	0.0134	0.0293	0.0126
8.0	0.0161	0.011	0.0216	0.0105
9.0	0.0064	0.0041	0.0119	0.0042

better compared to α-isomer which is shown by the higher rate of degradation. The degradation rate of these isomers was 0.0237, 0.0271, 0.0243 and 0.0198 $\mu g\,h^{-1}$ respectively. When two isomers were used in different combinations, it was observed that, in all the combinations, γ-isomer was degraded completely, with degradation rates ranging from 0.0333 to 0.0349 $\mu g\,h^{-1}$. The degradation of γ-isomer in presence of any other isomer was not effected. The degradation rate was always higher with whatever combination used. However, the degradation of other isomers α-, β- and δ was influenced by the presence of other isomer, the degradation slowed down. β-isomer showed more resistance compared to other isomers. The degradation of β-isomer was least in presence of γ-isomer and better degradation was observed in presence of α-isomer. This is reflected by the rate of degradation where in the rate of degradation was 0.0125 in presence of α-isomer. In the presence of γ-isomer, the rate of degradation was very low (0.0077 $\mu g\,h^{-1}$). But when β- and δ-isomer were together the rates of degradation of both the isomers was very low, indicating their high recalcitrance. δ-isomer, another highly recalcitrant isomer, did not show much interference of other co-isomers during degradation. When the isomers were taken in a mixture of three, γ-isomer was found to be influenced a little, where there was slight delay in its complete degradation. The degradation of β-isomer showed very low degradation rate in any combination used. α-isomer was also not affected during degradation. But the degradation of δ-isomer varied with the presence of the substrate type. The degradation rate of δ-isomer was low, i.e. 0.0069 $\mu g\,h^{-1}$ when used with β- and γ-isomers. But in presence of α- and β-isomers the rate was nearly double the combination of β- and γ-isomers. However, the degradation time was still more when all the four isomers were present together. Thus, the rate of degradation of each isomer depends on the type of other isomer present.

Table 7
Interaction between different isomers of HCH

Isomer combination	Rate of degradation ($\mu g\,h^{-1}$)			
	α	β	γ	δ
α	0.0237	–	–	–
β	–	0.0271	–	–
γ	–	–	0.0243	–
δ	–	–	–	0.0198
αβ	0.0186	0.0125	–	–
αγ	0.0186	–	0.0349	–
αδ	0.0157	–	–	0.0122
βγ	–	0.0077	0.0333	–
βδ	–	0.013	–	0.011
γδ	–	–	0.0345	0.0132
αβγ	0.0124	0.0081	0.0254	–
αβδ	0.012	0.0073	–	0.0108
βγδ	–	0.0087	0.029	.0069
αβγδ	0.0297	0.0178	0.035	0.021

4. Discussion

The defined microbial consortium used in the degradation of tech-HCH was developed by the long-term enrichment of the contaminated soil and sewage samples [19,21]. This microbial consortium was acclimated with increasing concentrations of tech-HCH from 5 to 25 ppm. The consortium that got established at 25 ppm level was used in our studies.

The initial inoculum used in acclimation is obtained from diverse sources such as HCH contaminated soil and sewage. The advantage of sewage is that it provides sufficient inoculum during acclimation. As the test compound is used as a sole source of carbon and energy the organisms having the machinery for the degradation of the compound would survive and therefore would be able to accomplish the mineralization process. Moss [22] employed an acclimation and enrichment procedure that uses a continuous culture of microorganism growing at very low specific growth rates. The compound being tested was applied continuously at low concentrations and the concentration was increased in a systematic manner. Similar acclimation technique has been used by Bidlan and Manonmani [21] to isolate DDT degrading microorganisms. Acclimation also would help in evading toxicity prior to the actual degradation and this is an essential part for further degradation studies. Acclimation would result in altered composition of the microbial populations involved in the early stages of degradation.

Pre-exposure helped in obtaining faster degradation without any lag. The degradation appeared to have started as soon as inoculum and substrate were together. There was no initial lag in degradation with the inoculum induced with any of the isomers of HCH. In all these cases, the HCH-isomer used for induction was degraded completely and other isomers were partially degraded. This probably could be due to presence of required enzyme that got induced with the particular isomer. The partial degradation could be due to the multiple functions of the enzyme which was able to degrade the substrates only to certain extent. The partial degradation could be due to the enzymes, which might not have got induced by the other isomer used, or there could be inhibition of pathway enzymes by the intermediatory metabolites formed during the degradation of non-inducer substrates. Also the complete degradation could be achieved with increase in incubation time. It has been reported that the degradation of α- and γ-isomers follow the same pathway and the degradation of β-HCH has been deciphered to only one or two steps of biodegradation [23]. To our knowledge no reports are available on the degradation pathway of δ-HCH degradation. In our studies also the degradation of these isomers might be following a different pathway (data not shown). As tech-HCH is a mixture of all isomers, degradation of different isomers is

a complex phenomenon, as these enzymes might face many inhibitory/stimulating effects by the intermediates formed by different isomers present together. Failure to achieve results with consistent complete mineralization, on the other hand, would also suggest that complete biodegradation is not possible in short time or that it is dependent on co-metabolism. Thus, the tech-HCH induced inoculum was used in further studies.

The microbial consortium developed in the laboratory was capable of degrading all isomers of HCH and the biodegradation could occur in a particular environment. With all the optimized conditions, the biodegradation of tech-HCH becomes a highly system specific event. These optimized results can be adapted well in the treatment of industrial effluent or water bodies contaminated with HCH.

α- and γ-isomers have been reported to be degraded rapidly under aerobic and anaerobic conditions. α- and γ-isomers were degraded first in 12 h of incubation [19,24,25], where as β-isomer was found to remain undegraded under similar environmental conditions [12,26]. The degradation of different isomers of HCH has been shown to be dependent on many features mainly the type of microorganism used, aeration, the adaptability of these microorganisms to the pollutants [27], type of carbon source to cultivate them [28], pre-exposure of the used organism to the pollutant [21], etc. The recalcitrancy of the isomers also has been shown to play a key role in the degradation of different isomers of HCH [12,29]. In our studies also, the time required for the highly recalcitrant β- and δ-isomers was more compared to the other two isomers. The degradation of tech-HCH by the microbial consortium appears to be gratuitous metabolism where in, the substrate, i.e. tech-HCH is used as a sole source of carbon and energy and no other co-substrates are being supplemented. This is evident from the survival of all members of consortial community during degradation. However, no substantial growth was observed, i.e. the cells behaved as resting cells as the amount of carbon supplied by the substrate is not sufficient to support good growth of the consortium. But the cell count was being maintained during degradation. The initiation of degradation might be by the enzyme system that was already induced during pre exposure cycle, and hence degradation did not show any lag.

The pH of the medium had a substantial effect on the survivability of the members of the consortial community. At low pH levels, there was decrease in the microbial population. The survivability of the consortium also improved with increase in pH towards neutrality. In abiotic controls, the recovery of HCH-isomers at pH 9.0 was less indicating some chemical hydrolysis of these isomers in the liquid cultures. Similar observations were made by Manonmani et al. [19] with α-HCH. During the degradation of α- and γ-isomers of HCH by *Pandoraea* species [30], the degradation was observed to take place over a wide range of pH from 4.0 to 8.0. Degradation was found to decrease at pH 9.0. Similarly growth was very less at pH 4.0 which improved with increase in pH and reached high at pH 9.0.

The degradation of each isomer was influenced by the presence of other isomer. The HCH isomers, i.e. non-growth substrates have γ-isomer, the more easily degradable and β- and δ-isomer very highly recalcitrant. These two isomers thus show resistance toward degradation. It could be that because of recalcitrance, the structure may prevent it fitting into enzyme within the cell when it is likely to accumulate or the transformation product of one substrate may become toxic than the original substrate that might result in slower rate of degradation and also the degradative pathway of each isomer may be different which would be influenced by many factors. However, the different isomers present in the mixture will not associate in their co-metabolic degradation.

5. Conclusion

The use of microbes to clean up polluted environments, bioremediation is rapidly changing and expanding the area of environmental biotechnology. Although much work is being done to remediate the polluted environment, our limited understanding of the biological contribution and their impact on the ecosystem has been an obstacle to make the technology more reliable and safer. In our studies a defined microbial consortium was able to degrade HCH (technical grade containing all four major isomers) up to 25 ppm level under shaking conditions at ambient temperature and neutral pH. The inhibition of degradation by the presence of other isomer was marginal. With the process optimization at large scale trials coupled with process molecular microbiological techniques can make the bioremediation process more reliable and safer technology.

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Appendix A

A.1. Kinetic equation

The rate of depletion of substrate is given by $-\mathrm{d}c/\mathrm{d}t = k(C_\mathrm{t} - C_\mathrm{f})$ where $-\mathrm{d}c/\mathrm{d}t$ represents the rate of decrease in concentration of HCH at time 't', k is the degradation rate constant and C_t stands for the concentration of HCH at time t whereas C_f is the final HCH concentration.

Rearranging the terms, we get

$$\frac{\mathrm{d}c}{C_\mathrm{t} - C_\mathrm{f}} = -k\mathrm{d}t$$

Integrating both sides

$$\int_{C_\mathrm{i}}^{C_\mathrm{f}} \frac{\mathrm{d}c}{C_\mathrm{t} - C_\mathrm{f}} = -\int_0^t k\mathrm{d}t$$

i.e.

$$[\ln(C_\mathrm{t} - C_\mathrm{f})]_{C_\mathrm{i}}^{C_\mathrm{f}} = -k(t - 0)$$

$$\Rightarrow [\ln(C_\mathrm{t} - C_\mathrm{f}) - \ln(C_\mathrm{i} - C_\mathrm{f})] = -kt$$

$$\ln \frac{C_t - C_f}{C_i - C_f} = -kt$$

where C_i is the initial HCH concentration.

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

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