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ARTICLE

Substantially Enhanced Degradation of Hexachlorocyclohexane Isomers by a Microbial Consortium on Acclimation

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Widespread contamination of the environment, globally, has been caused by extensive and indiscriminate use of hexachlorocyclohexane (HCH) as an insecticide since the 1940s, threatening the biota including humans, and there is an urgent need to eliminate it, preferably through bioremediation technologies. A γ-HCH-degrading microbial consortium was isolated by enrichment of a soil sample from a sugar cane field having a long history of technical grade HCH application. On acclimation the degrading ability improved substantially. The consortium, which took 10 days to degrade 25 μg mL^{-1} of γ-HCH, initially could mineralize even 300 μg mL^{-1} of the substrate within 108 h on acclimation. With 300 μg mL^{-1} substrate, the rate of degradation, as calculated for the early exponential phase, was 216 μg mL^{-1} day^{-1}, the highest reported so far. An amount of 400 μg mL^{-1} of γ-HCH, however, was mineralized partially with only 78% Cl^- release. No apparent accumulation of intermediary metabolites was observed up to 300 μg mL^{-1} substrate, indicating a fast rate of mineralization. Aeration, mesophilic temperatures (20−35 °C), and near neutral pH (6.0−8.0) were favorable conditions for degradation. The presence of glucose at 1000 μg mL^{-1} retarded the degradation, whereas cellulose and sawdust at 1600 μg mL^{-1} and glucose at 100 μg mL^{-1} did not show any marked effect. The consortium also mineralized α-, β-, and δ-HCH efficiently. The consortium consisted of nine bacterial strains and a fungal strain, and individually they were able to degrade 10 μg mL^{-1} of γ-HCH. This mixed culture holds high potential for deployment in bioremediation of HCH-contaminated soils, waste dumpsites, and water bodies.

KEYWORDS: Hexachlorocyclohexane; microbial consortium; acclimation; enhanced mineralization; environmental factors

INTRODUCTION

Hexachlorocyclohexane (HCH) has been used extensively and, many times, indiscriminately all over the world for the past six decades for insect pest control in agriculture, food and seed storage, wood preservation, and public health programs. Although most of the Western countries stopped, as early as 1970s, the use of the technical grade HCH (tech-HCH) containing a mixture mainly of α- (60−70%), β- (5−12%), γ- (10−12%), δ- (6−10%), and ε- (3−4%) isomers (*1*) of which only γ-HCH (lindane) is insecticidal (*2*), the use of this and lindane continued in most developing countries including India until the end of the 1990s. Illegal use of these, however, is continuing in various under-developed nations due to economic reasons, adding more contaminated sites (*2*).

Being highly stable, these compounds persist in the environment in quantities ranging from traces to grams per kilogram near dumping sites, agricultural soils, and other contaminated places (*2*−*6*). Residues of HCH isomers have been detected even in Arctic and Antarctic regions, where they have never been produced or used, due to their migration via various routes (*7*). Besides γ-HCH, residues of all other constituents of tech-HCH are also still present in many agricultural soils where they had been previously applied, and they find their way into agricultural products including food grains, oilseeds, fruits and vegetables, etc. (*4*, *8*, *9*). Uptake and bioaccumulation of HCH residues by organisms of all levels of the food chain and their deleterious effects on animal models have been studied and documented well, although there are not many direct experimental data available on their effect on humans (*7*, *10*). However, serious health problems such as cancer, birth defects, and fertility disorders have been implied to be caused by long exposure to HCH isomers, as has been shown in the case of other organochlorine pesticides (*7*, *10*).

It has been well recognized that there is an urgent need to eliminate these compounds from the environment. Natural degradation of HCH residues from soil is a very slow process, which may take from months to several years depending upon the nature and conditions of the soil. In most cases the degradation is

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Table 1. Degradation of γ-HCH (10 μg mL^{-1}) by the Individual Members of the Microbial Consortium[a]

culture	chloride released (%) after			
	3 h	6 h	9 h	24 h
Pseudomonas putida CFR1021	44 ± 0.82	71 ± 0.89	88 ± 1.30	100 ± 1.12
Pseudomonas fluorescens CFR1022	49 ± 1.14	87 ± 1.32	100 ± 0.88	
Pseudomonas aeruginosa CFR1023	38 ± 0.98	58 ± 0.68	82 ± 1.58	100 ± 0.48
Pseudomonas aeruginosa CFR1024	55 ± 1.56	100 ± 1.16		
Burkholderia cepacia CFR1025	30 ± 1.86	66 ± 1.08	71 ± 1.65	98 ± 1.85
Burkholderia cepacia CFR1026	28 ± 2.16	54± 1.62	64 ± 1.72	77 ± 1.33
Pseudomonas stutzeri CFR1027	31 ± 1.06	64 ± 1.02	78 ± 1.39	82 ± 0.52
Vibrio alginolyticus CFR1028	47 ± 1.62	80 ± 0.91	95 ± 1.09	100 ± 0.92
Acinetobacter lwoffii CFR1029	25 ± 0.89	45 ± 1.29	78 ± 1.27	99 ± 1.46
Fusarium sp. CFR225	5 ± 0.89	11 ± 1.10	18 ± 0.73	33 ± 0.62

[a] Each strain was grown in 25 mL of diluted Luria broth (1:50) containing 10 μg mL^{-1} γ-HCH for 24 h, harvested, suspended in 25 mL of M4 medium containing the same amount of γ-HCH, and shaken overnight for induction. The cells were harvested, washed, and added as inoculum to each flask containing 25 mL of the same medium.

incomplete, resulting in the accumulation of more toxic metabolites. In recent years, the need to develop processes for faster and complete mineralization of the residues to CO_2 and chlorine has been emphasized. One of the strategies adopted has been bioremediation through cell augmentation or gene augmentation using microorganisms possessing degrading potentials, and a few studies have indicated the possibility of successful application of such processes (*11*−*13*). Isolation of natural microbial communities through suitable enrichment techniques and their improvement in the laboratory by various techniques are necessary steps for obtaining potent cultures for bioremediation processes.

Since Matsumura et al. (*14*) first reported the aerobic degradation of γ-HCH by a *Pseudomonas* strain, the literature has become replete with information on bacteria capable of degrading HCH isomers aerobically (*5*, *8*, *12*, *15*−*23*). Many of the bacteria isolated from HCH-contaminated soils from divergent geographical locations of the world have been found to be members of the family Sphingomonadaceae, and the genetics and pathways of degradation of γ-HCH in *Sphingomonas paucimobilis* UT26, *Sphingobium francense* SpC, *Sphingobium indicum* B90A, *Sphingobium ummariense*, and other strains have been well established through extensive molecular studies (*8*, *15*, *17*, *22*−*25*). Interestingly, they all share similar genomic configurations and catabolic pathways. A few fungal strains also have shown the ability to degrade HCH isomers (*26*, *27*). Most of these bacterial and fungal strains, however, are capable of degrading only some of the HCH isomers that too at low concentrations and take long periods of time. Hence, there has been a continuous search for more efficient microbial cultures for use in bioremediation technologies. As in many countries, in recent years, it is lindane that is generally used as against the previous practice of using tech-HCH it is likely that more of this isomer will be present in the newly contaminated sites. Also, it has been shown that γ-HCH is the major isomer detected in soil and marine environments (*7*, *10*). Hence, an ideal culture should be able to degrade, within a reasonably short time, high concentrations of lindane as well as other major isomers of HCH.

Microbial mixed cultures are considered to be potentially more efficient in the biodegradation of recalcitrant compounds (*28*−*30*). However, there are only a few reports on such an approach for HCH degradation under aerobic conditions (*21*, *31*, *32*). Attempts have been made in our laboratory to develop potent microbial mixed cultures for the degradation of a variety of toxic and hazardous chemicals including organochlorine pesticides. In this paper, we present data on the isolation of a γ-HCH-degrading microbial consortium, its acclimation to increasing concentrations of the substrate to improve the degrading ability, the effects of environmental factors such as aeration, temperature, pH, and auxiliary carbon sources on the degradation, and its ability to degrade other isomers of HCH.

MATERIALS AND METHODS

Chemicals. α-, β-, and δ-isomers of HCH (98−99% purity) were purchased from Riedel de Hae'n-Fluka, and γ-HCH isomer (99% purity) was purchased from Sigma-Aldrich. Luria broth agar (LBA), tryptic soy agar (TSA), chocolate agar, MacConkey agar, mannitol salt agar (MSA), and potato dextrose agar (PDA) were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Sawdust of teak wood (*Tectona grandis*) was collected from a local timber mill, and cellulose powder was obtained from E. Merck (India) Ltd. All other chemicals used in the study were of analytical grade and were purchased from standard manufacturers.

Microbial Cultures and Media. Microbial consortia, both unacclimated and acclimated, used in this study were developed in the laboratory as described later. The individual microbial strains isolated from the consortium and used in this study are listed in **Table 1**.

The basal mineral medium (M4) used for growing the microbial consortium was the same as described earlier (*21*). LBA, TSA, chocolate agar, MacConkey agar, and MSA were used for isolating and purifying the bacterial members of the consortium. PDA was used for isolating the fungal culture. Diluted LBA (1:50) containing γ-HCH (10 μg mL^{-1}) was used for preparing inocula of the individual strains. All of the media were sterilized by autoclaving at 121 °C.

Isolation, Acclimation, and Maintenance of the Microbial Consortia. The microbial consortium that degraded γ-HCH was developed by shake flask enrichment of HCH-contaminated soil, collected from a sugar cane field in the outskirts of Mysore City, Karnataka State, where tech-HCH had been sprayed regularly for several years. An aqueous suspension of the soil sample was inoculated into M4 medium (25 mL in 250 mL conical flasks) containing 10 μg mL^{-1} of γ-HCH as the sole carbon source and incubated at 30 °C on a rotary shaker (150 rpm). The culture was transferred at weekly intervals to fresh medium. After five transfers, a mixed microbial population was established, as evidenced by growth and the ability to degrade 10 μg mL^{-1} of γ-HCH. This was the culture used as unacclimated consortium. This was maintained on LBA plates/slopes. This mixed culture was gradually acclimated to increasing concentrations from 10 through 300 μg mL^{-1} of γ-HCH. 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 the substrate, in terms of 100% Cl^- release, was ensured before going to the next higher concentration. The microbial consortium thus developed was maintained on M4 agar plates/slopes containing 25 μg mL^{-1} of γ-HCH.

Separation of Individual Strains of the Consortium. The acclimated consortium was grown on LBA, TSA, chocolate agar, MacConkey agar, MSA, and PDA plates by streaking appropriately diluted samples of 48-h-old cultures, grown with 25 μg mL^{-1} of γ-HCH, and were purified by repeated plating. Nine morphologically distinct bacterial strains and a single fungal strain were thus isolated. All of the bacterial strains isolated on different media were found to grow well on LBA, and the fungal isolate grew well on PDA. The bacteria were identified using the Microbact Gram

Negative Identification System from Medvet Science Pty Ltd., Adelaide, Australia. The fungal strain was identified according to the method of Muller and von Arx (*33*). Bacterial strains and the fungal strain were maintained on agar slopes of diluted LB (1:50) and PDA slopes, respectively, both containing 25 μg mL^{-1} of γ-HCH.

Degradation Studies. All of the experiments on degradation of HCH isomers were carried out in triplicates. The required quantity of the HCH isomer, as an acetone solution, was dispensed into the bottom of sterile Erlenmeyer flasks (250 mL capacity), and acetone was evaporated off at room temperature, keeping the flasks open in a laminar hood. Sterile M4 medium (25 mL) was added to each flask and was inoculated with the consortium or the individual culture as required. [Inoculum was prepared by growing the consortium or the axenic culture in dilute LB (1:50) containing the respective HCH isomer (25 μg mL^{-1} of α-, γ-, or δ-isomer or 10 μg mL^{-1} of β-isomer) for 24 h followed by induction of the culture by incubating the harvested cells in M4 medium containing the respective HCH isomer as the sole source of carbon and energy at the same concentrations, for another 24 h. The cells were then harvested, washed, and resuspended in the required quantity of M4 for use as inoculum.] All of the flasks, those used for inoculum development as well as the experimental flasks, were incubated on a rotary shaker (150 rpm) at 30 °C, unless otherwise indicated. Three flasks each were removed at required intervals for analyses such as growth, inorganic chloride, residual substrate(s), and intermediary metabolites. Uninoculated flasks were maintained as control.

To study the effect of aeration on degradation, the unacclimated consortium was inoculated into M4 medium (25 mL in a 250 mL Erlenmeyer flask) containing 25 μg mL^{-1} of γ-HCH. The inoculum was prepared as described above, but without induction. The cells washed with sterile M4 medium were added at levels equivalent to 7 μg of cell protein mL^{-1}. One set of flasks was incubated at 30 °C on a rotary shaker (150 rpm), whereas the other set was incubated under stationary condition, at the same temperature.

To study the effect of pH (from 3.0 to 11.0) on the degradation, the acclimated consortium was inoculated into M4 medium containing 25 μg mL^{-1} of γ-HCH, which was adjusted to different pH values by varying the proportion of KH_2PO_4 and Na_2HPO_4 (pH between 5 and 8) and adjusting the pH below 5.0 and above 8.0 with 1 M HNO_3 and 1 M NaOH solutions, respectively. Induced inoculum was added at 40 μg of cell protein mL^{-1} level. Flasks were incubated at 30 °C on a rotary shaker (150 rpm) for 24 h.

The effect of temperature (from 5 through 60 °C) on degradation of 25 μg mL^{-1} of γ-HCH was studied by incubating the flasks, inoculated with acclimated consortium (at about 50 μg of cell protein mL^{-1} level), at required temperatures under stationary condition for 48 h.

The effect of auxiliary carbon sources such as glucose (at 100 and 1000 μg mL^{-1}), cellulose, or sawdust (at 1600 μg mL^{-1}) on degradation of 50 μg mL^{-1} of γ-HCH by the acclimated consortium was studied by incorporating them into the medium.

Analytical Methods. The growth of the consortium was determined by estimating the total protein in the biomass according to a modified method of Lowry et al. (*34*) as described earlier (*21*) and was expressed as micrograms of cell protein per milliliter of culture.

Residual HCH isomers in the culture broths were determined by gas chromatography (GC), using a Fisons 8800 with a ^{63}Ni electron capture detector (Fisons Instruments, Italy). A known volume of the whole culture broth was extracted three times with equal volumes of an *n*-hexane/acetone mixture (8:1), each time by thoroughly mixing for 5 min. The solvent layers were pooled and purified by passing through a Florisil column. Moisture was removed by filtration through anhydrous Na_2SO_4 granules, and the extract was evaporated to dryness. The residue was redissolved in a known volume of *n*-hexane. A known quantity of this was injected into the GC. The conditions used were as follows: column, 0.25 in. DD and 11 ft in length, packed with 1.5% OV17 plus QF1 on 80−100 mesh chromosorb W; carrier gas, nitrogen, at a flow rate of 40 mL min^{-1}; column temperature, 210 °C; injector temperature, 230 °C; and detector temperature, 300 °C.

To detect the intermediary metabolites accumulated in the medium, the concentrated solvent extract of the culture broth, prepared as above, was subjected to thin layer chromatography (TLC) as well as GC. GC was done as described above. TLC was done on a silica gel G plate using cyclohexane as mobile phase. Spots were visualized by spraying *o*-tolidine and exposing to sunlight. TLC of the concentrated culture broth (aqueous phase) was done by using benzene/ethanol (19:1). Developed plates were sprayed either with Folin−Ciocalteu reagent for phenolic compounds or with *o*-tolidine for chloroaromatics. Chlorophenols, chlorobenzenes, and catechols were used as reference standards.

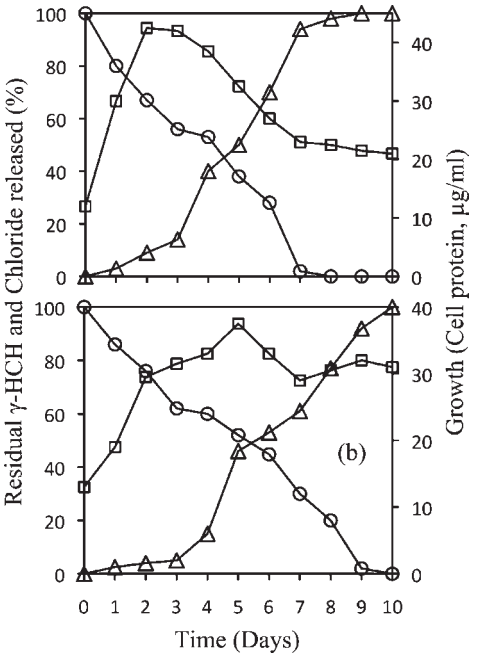

Figure 1. Degradation of (**a**) 10 and (**b**) 25 μg mL^{-1} of γ-HCH by the unacclimated microbial consortium: (○) residual substrate (γ-HCH); (□) growth; (△) chloride released. Details of the experiment are as given under Materials and Methods. All experiments were done in triplicate.

The concentration of Cl^- released was estimated by using a slightly modified procedure of Bergmann and Sanik (*35*) based on the principle of displacement of thiocyanate ion from mercuric thiocyanate by chloride ion, in the presence of ferric ion as previously described (*21*).

Glucose in the medium was estimated according to the method of Miller (*36*), using dinitrosalicylic acid reagent. Optical density at 450 nm (OD_{450}) was quantified using a standard curve prepared with D-glucose.

RESULTS AND DISCUSSION

Isolation and Acclimation of the Microbial Consortium and Degradation of γ-HCH. Over 10 million tons of tech-HCH was used globally during the past 60 years, of which India shared a major bulk (*8*). Many countries including India continue to use lindane. Residues of HCH isomers persist in the environment and bioaccumulate at various levels of the food chain, thus, in turn, becoming a threat to human health. To eliminate these contaminants from the environment, attempts have been made in our laboratory to isolate pesticide-degrading native microorganisms and improve their degrading ability. From soil samples collected from a sugar cane field, having a long history of tech-HCH usage, a microbial consortium was enriched after 5 weekly transfers to fresh medium in shake flasks containing 10 μg mL^{-1} of γ-HCH as the sole source of carbon and energy. This consortium degraded >98% of 10 μg mL^{-1} γ-HCH within 7 days with a concomitant release of 95% of Cl^-, complete disappearance of the substrate occurring within 8 days, and 100% Cl^- being released on day 9 (**Figure 1a**). Growth of the consortium started without any lag and reached maximum after 2 days. An amount of 25 μg mL^{-1} of γ-HCH was mineralized within 10 days (**Figure 1b**). Maximum growths of 42.5 and 37.5 μg of cell protein mL^{-1} were achieved on day 5 by the consortium with 10 and 25 μg mL^{-1} of the substrate, respectively.

This consortium was acclimated to higher concentrations of γ-HCH as follows: The cell biomass of the consortium grown on

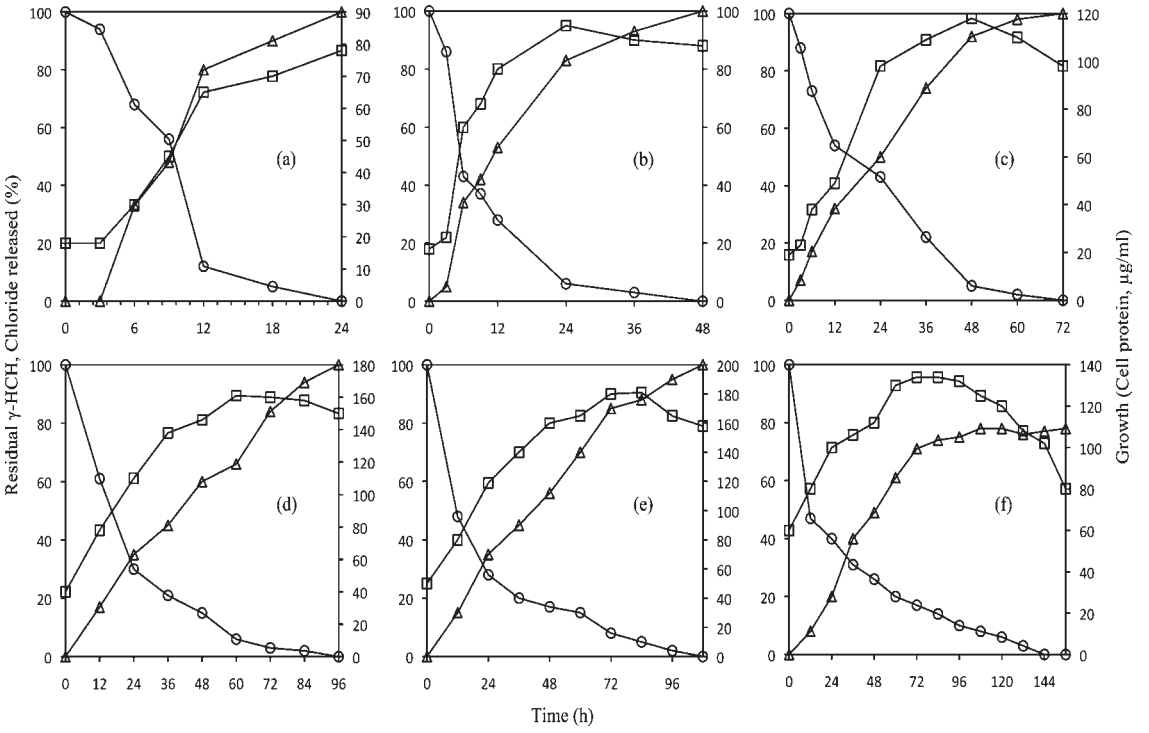

Figure 2. Degradation of (**a**) 25, (**b**) 50, (**c**) 100, (**d**) 200, (**e**) 300, and (**f**) 400 μg mL^{-1} of γ-HCH by the acclimated microbial consortium. Details of acclimation and the experiment are as given under Materials and Methods. Symbols used are as in **Figure 1**.

25 μg mL^{-1} of γ-HCH was harvested and washed thoroughly with sterile 0.1% (v/v) Tween 80 (for dislodging the adsorbed chlorine from the cells, if any), followed by a wash with sterile M4 medium. This was used as the inoculum for the consecutive culture either for the same or higher concentrations of γ-HCH, which ranged from 25 through 300 μg mL^{-1}, until the complete mineralization of the added substrate was obtained. (Growth with the same concentration of the substrate was repeated two to three times, as required, to ensure complete mineralization.) The acclimated consortium was able to degrade γ-HCH up to 400 μg mL^{-1}. Complete degradation of 25, 50, 100, 200, and 300 μg mL^{-1} of γ-HCH occurred within 24, 48, 72, 96, and 108 h, respectively with the concomitant release of stoichiometric amounts of Cl^- when the biomass from the previous culture was used as inoculum for the successive culture (added at almost equal levels of 20 μg mL^{-1} of cell protein) (**Figure 2a**−**e**). Growth picked up after a short lag of 3 h and reached a maximum level of about 78 and 95 μg of cell protein mL^{-1} in the case of 25 or 50 μg mL^{-1} of substrate, respectively, at 24 h. With γ-HCH at 100, 200, and 300 μg mL^{-1} no apparent lag occurred, and maximal growths of 118, 161, and 180 μg of cell protein mL^{-1} were attained at 48, 60, and 72 h, respectively. In the case of 400 μg mL^{-1} of γ-HCH, although the substrate disappeared completely within 144 h, only 78% Cl^- was released at 108 h, which did not increase even after 156 h of incubation (**Figure 2f**). From an inoculum of 40 μg of cell protein mL^{-1} the growth reached a maximum of only 134 μg of cell protein mL^{-1} at 72 h, which remained stationary up to 96 h and started receding quickly thereafter (**Figure 2f**). No degradation was observed in the uninoculated control flasks as indicated by the full recovery of the added substrate, lack of Cl^- release, and absence of any metabolites (data not plotted in the figures).

Earlier, we have isolated a microbial consortium, through a novel technique, which could degrade up to 100 μg mL^{-1} of α-HCH (*21*). In that case the traditional shake flask enrichment failed to yield a degrading culture. However, through a long-term enrichment technique involving two steps, first in a semicontinuous glass column reactor, which initially contained acetone, benzene, and phenol as carbon sources, which was gradually tapered off and replaced with α-HCH (for about 6 months), and then in shake flasks with α-HCH alone (for 2 months), a microbial consortium that could utilize all of the HCH isomers as sole source of carbon and energy was obtained. The samples taken for enrichment were a mixture of soils and sewages from various locations within and in the outskirts of Mysore. The present consortium, however, was isolated easily, from a soil sample from a sugar cane field with a long history of tech-HCH usage, by the simple shake flask enrichment, within a short period of 5 weeks. The prolonged exposure to a fairly high concentration of HCH residues that built up over a period of time might have helped the natural selection of a microbial community that degraded HCH concertedly, evolving the pathways through possible gene exchanges (*11*, *29*, *30*, *37*), unlike in the former case when there was no apparent selection force in the natural environment. Similarly, Senoo and Wada (*38*) were able to isolate a strain of *Pseudomonas paucimobilis* (now called *Sphingobium japonicum*) from an upland field, where γ-HCH was supplied every year for 12 years. Thomas et al. (*39*) isolated a strain of *Rhodanobacter lindaniclasticus* (formerly *Pseudomonas* sp.) by enrichment of mixed soils from wood treatment sites contaminated with high concentrations of γ-HCH. They, however, failed to isolate HCH degraders from agricultural soils, containing very low concentrations of HCH residues. These results indicate that natural selection of HCH-degrading microflora occurs only when the substrate is continuously present for a long time and in fairly high concentrations.

The consortium could initially degrade 10 and 25 μg mL^{-1} of γ-HCH within 8 and 10 days, respectively (**Figure 1**). Exposure to increasing concentrations of the substrate improved the degrading ability remarkably. The degradation became faster and faster when successively inoculated with the cells harvested from the previous cultures (**Figure 2b**−**e**). Similarly, we have shown earlier a significant enhancement in the rate of degradation of α-HCH by a microbial consortium on acclimation to increasing concentrations

from an initial 5 to 100 μg mL^{-1} (*21*). The acclimated consortium could mineralize 100 μg mL^{-1} of α-HCH within 72 h, whereas the unacclimated consortium took 14 days to degrade even 10 μg mL^{-1} of substrate. Bhuyan et al. (*40*) also have reported accelerated degradation of γ-HCH in flooded and nonflooded soils after pretreatment of the soils with HCH, the rate of degradation increasing after every successive treatment. A strain of *S. paucimobilis* also has exhibited better degradation of α-, β-, γ-, and δ-HCH after adaptation, as compared to normal culture (*41*). In the present study, not only was a potent microbial consortium readily enriched, but all of the members of the consortium (except the fungus) individually showed the ability to degrade γ-HCH efficiently (**Table 1**). None of the individual strains, however, showed any improvement in the degrading ability, even after adaptation to γ-HCH, whereas the reconstituted consortium on acclimation readily acquired the ability to degrade higher concentrations. Some kind of physiological synergism among the strains might have been acquired in natural condition as a functional entity that was retained under laboratory conditions and which readily responded to the acclimation process (*29*). A combined metabolic activity and higher interactive properties can, generally, be observed in microbial consortia, due to the genetic divergence leading to metabolic complementation (*30*). The mechanism of acclimation here can be hypothesized as an adaptation of microbes in the presence of HCH as a sole carbon source by genetic alterations that may be occurring through DNA sequence changes, recombination by plasmid exchanges among different strains, etc. Gradual increase in the substrate concentration during acclimation may result in enhanced induction of various enzymes of the pathways. In a microbial consortium the net result of such a process may be synergistic through complementation of the enzymes in different member strains.

To the best of our knowledge, complete mineralization of 300 μg mL^{-1} of γ-HCH within 108 h is the first report on any HCH-degrading microbial system hitherto studied. The rates of degradation at the early exponential phase were calculated to be 216 and 140 μg mL^{-1} day^{-1} when the substrate concentrations were 300 and 200 μg mL^{-1}, respectively. This is much higher than what has been reported so far. Earlier, we have reported a degradation of ~58 μg mL^{-1} day^{-1} from 100 μg mL^{-1} of α-HCH (*21*). Complete mineralization of 400 μg mL^{-1} of γ-HCH, however, did not occur. Addition of more inoculum or further acclimation may, probably, enable the culture to degrade this and higher concentrations, but has to be verified. An optimum inoculum level of cells equivalent of 100 μg of protein mL^{-1} was reported for the degradation of 25 μg mL^{-1} of tech-HCH by a microbial consortium (*32*), whereas in the present study inoculum levels of only about 20 μg of cell protein mL^{-1} were used up to a substrate concentration of 300 μg mL^{-1} and only 40 μg mL^{-1} in the case of 400 μg mL^{-1} substrate, which are much lower than the former (**Figure 2**). Increased rates of degradation of α-, β-, γ-, and δ-isomers of HCH by a *S. paucimobilis* strain upon increase of the inoculum level from 10^2 to 10^8 cells mL^{-1} have been reported by Johri et al. (*41*), by which degradation of 5 μg mL^{-1} of the substrate was achieved within 3 days as against 12 days.

It was found that the biomass buildup at the expense of γ-HCH was not proportional to the concentrations used, in the case of both unacclimated and acclimated consortia (**Figures 1** and **2a**−**e**), although the substrate was completely mineralized. Growth was meager on α-, β-, and δ-HCH also (**Figure 6**). This could be because of partial inhibition of growth by one or more of the intermediary metabolites formed at excess levels due to the higher activity of one or more of the catabolic enzymes; that is, the toxic metabolite may not be converted at the same rate at which it is formed. A similar observation was made by Endo et al. (*42*) in the case of the γ-HCH-utilizing *S. japonicum* UT26. They found

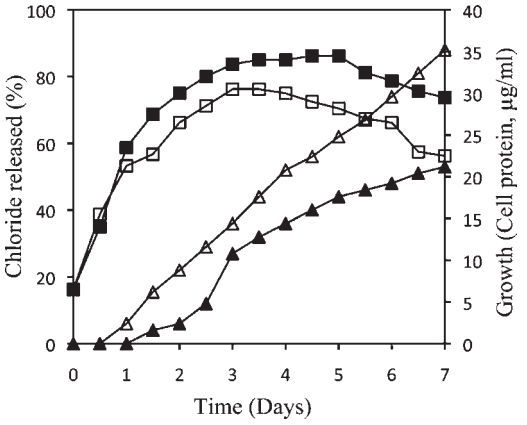

Figure 3. Growth and chloride release from 25 μg mL^{-1} of γ-HCH by the unacclimated microbial consortium under shaken (150 rpm) and stationary conditions: (■) growth under stationary condition; (▲) Cl^- release under stationary condition; other symbols as in **Figures 1**and **2**. Details of the experiment are as given under Materials and Methods.

that the poor growth of the strain was due to the inhibition by two metabolites, namely, 2,5-dichlorophenol (2,5-DCP) and 2,5-dichlorohydroquinone (2,5-DCHQ), particularly the former, which was accumulated as a dead-end product. Although in our study no apparent accumulation of any metabolites was observed during or at the end of complete disappearance of the substrate, even at 300 μg mL^{-1} levels, a transient accumulation of any toxic metabolite at some stage at low concentrations cannot be ruled out. Another observation that the biomass formation was slightly greater under stationary condition as compared to the shaken condition, whereas the degradation of the substrate was faster under aerated (shaken) condition, as evidenced by higher Cl^- release of 88 against 53% at stationery condition, also may support this argument (**Figure 3**). Aerated condition may be more favorable for the pathway, thus enabling a faster degradation, generating intermediary metabolites at faster rates, thus affecting the growth, whereas slower catabolism with low release of intermediates at stationary condition may allow a better growth. A rapid decline in growth after 96 h in flasks with 400 μg mL^{-1} γ-HCH (**Figure 2f**), in which accumulation of chlorinated metabolites was observed, also points to this possibility. It should, however, be confirmed by stringent analysis of the intermediary metabolites and the catabolic pathways.

No intermediary metabolite was detected, as already mentioned, either in the solvent extract or in the aqueous phase of the culture broths, from the flasks containing up to 300 μg mL^{-1} of γ-HCH, either by TLC or by GC analysis. The degradation can be taken as complete, because of this fact as well as due to the release of 100% Cl^-. However, the samples from 400 μg mL^{-1} of substrate showed extra peaks in GC and blue spots in *o*-tolidine-sprayed TLC plates, indicating the accumulation of chlorinated intermediary metabolites. HCH degradation generally occurs through the formation of chlorobenzenes, chlorophenols, or both, and the release of the last chlorine atom is expected almost at the end of the pathway (*8*, *16*, *17*). Detection of chlorinated intermediary metabolites points to the possibility of a similar pathway(s) in the present culture, which, however, has to be substantiated by further studies.

Effect of Culture Conditions on Degradation of γ-HCH. Environmental factors such as temperature, pH, aeration, and the presence of other substrates might affect the growth of microbes and their degrading abilities.

The effect of pH on the growth and degradation of 25 μg mL^{-1} of γ-HCH by the acclimated consortium was studied by incubating the culture at 30 °C on a rotary shaker (150 rpm) for 24 h. The

consortium could degrade γ-HCH at a wide range of pH of 3.0−9.0, the optimum being between pH 6.0 and 8.0. Growth was observed at a wide pH range of 4.0−9.0, maximum being between 6.0 and 7.0. Beyond pH 7.0 there was a steep drop in growth. However, Cl^- release was maximal at pH 7.5, almost 98% within 24 h. Our previous study had shown best growth and degradation of α-HCH at pH between 6 and 8 and a decline in bacterial population beyond pH 8.0 (*21*). Siddique et al. (*43*) have reported that the degradation of α- and γ-HCH by *Pandoraea* sp. was optimal at an initial pH of 8.0 in liquid culture and at an initial pH of 9.0 in soil slurry. The tech-HCH-degrading consortium was shown to have an optimal pH of 7.5 (*32*).

The effect of temperature on growth and degradation was studied by incubating the flasks, containing 25 μg mL^{-1} of γ-HCH inoculated with the acclimated culture (30 μg of cell protein mL^{-1}) at the required temperatures, 5−60 °C, at stationery condition. Growth and degradation of the substrate were observed at a wide range of temperatures, Cl^- release being 8 and 18% even at 5 and 60 °C, respectively. Maximum growth occurred at 30 °C, optimal being between 25 and 35 °C, whereas optimum Cl^- release was at 30−35 °C. A sharp decrease in both growth and degradation of the substrate was observed at temperatures above 35 °C. Degradation of α-HCH also by a consortium was occurring under a wide range of temperatures (4−40 °C) in the liquid culture medium, and 30 °C was most favorable (*21*). A sharp decline in cell population was also observed above 35 °C, as observed in this study. Siddique et al. (*43*) have reported an optimal temperature of 30 °C for growth and degradation of α- and γ-HCH by *Pandoraea* sp. in liquid culture as well as soil slurry. Degradation of tech-HCH by a microbial consortium was shown to be optimal at temperatures of 26−28 °C (*32*).

To study the effect of aeration on the degradation of γ-HCH, flasks were incubated under stationary and shaken (150 rpm) conditions. After 7 days of incubation of the unacclimated consortium, 88% of Cl^- was released from 25 μg mL^{-1} of the substrate under shaken condition, whereas under stationary condition it was about 53% (**Figure 3**). Growth, on the contrary, was slightly higher under stationary condition than under shaken condition, the maxima being 34.5 and 30.5 μg of cell protein mL^{-1}, respectively. The probable reason for this phenomenon was already discussed above. The adaptability of the present microbial consortium to a wide range of pH and temperature and the ability to perform well under both aerated and stationary conditions would make it highly suitable for deployment in bioremediation of contaminated sites of different geoclimatic regions.

The presence of easily utilizable carbon sources in the environment is another major factor that may influence the degradation of the target substrate. Amendment of the medium with glucose was tried in two concentrations, namely, 100 and 1000 μg mL^{-1} along with 50 μg mL^{-1} of γ-HCH. Glucose at 1000 μg mL^{-1} showed a marked retardation of γ-HCH degradation, although the growth was higher than in the control, due to the utilization of glucose, as expected (**Figure 4a**,**c**). Even after 72 h of incubation, about 8% of the substrate remained undegraded, whereas almost complete mineralization was observed in the control flasks without glucose at 36 h (**Figure 4a**,**c**). Only 90% Cl^- was released in the case of 1000 μg mL^{-1} of glucose-supplemented culture (**Figure 4a**). The presence of 100 μg mL^{-1} of glucose, however, did not show any adverse effect on the degradation of γ-HCH (**Figure 4b**). The substrate disappearance and Cl^- release patterns were almost similar to that of the control (**Figure 4b**,**c**). The added glucose was completely utilized in both cases (**Figure 4a**,**b**). The maximum biomasses formed in the cultures supplemented with 1000, 100, and 0 μg mL^{-1} of glucose were equivalent to 209, 115, and 80 μg of protein mL^{-1}, respectively, maximum growth occurring at 24, 12, and 48 h, respectively (**Figure 4**).

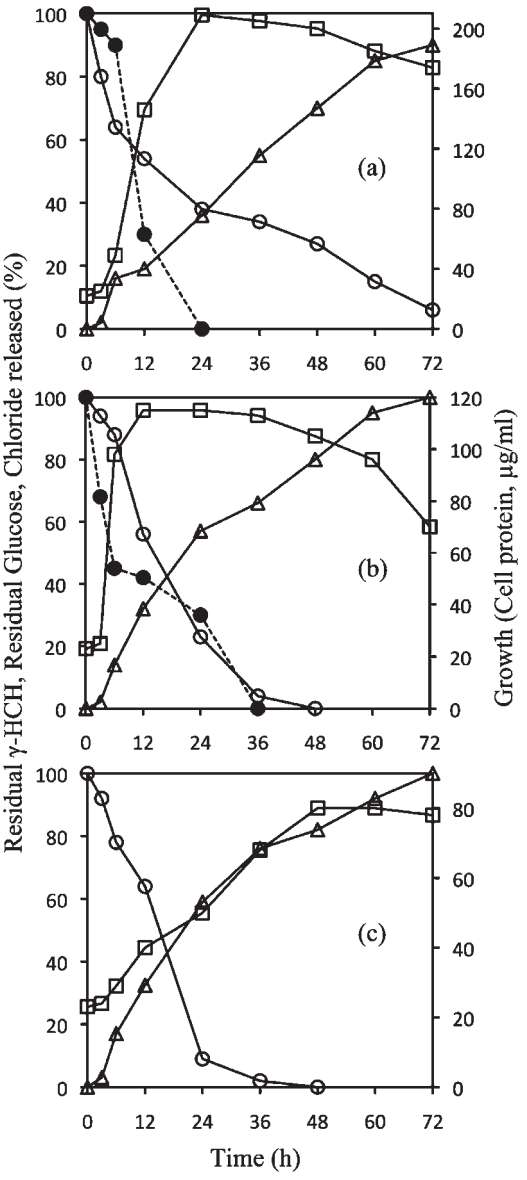

Figure 4. Effect of supplementation of the medium with glucose at (**a**) 1000 and (**b**) 100 μg mL^{-1} on the growth and degradation of 50 μg mL^{-1} of γ-HCH by the acclimated consortium; (**c**) control with no supplementation: (●) residual glucose; other symbols as in **Figures 1**−**3**. Experimental details are as given under Materials and Methods.

Cellulose powder and sawdust (each at 1600 μg mL^{-1}) did not show any marked influence on the degradation of γ-HCH, except that the disappearance of γ-HCH (50 μg mL^{-1}) was slightly slower (**Figure 5a**,**b**) as compared to the unamended control (**Figure 5c**). The retardation of γ-HCH disappearance was a little more in the cellulose-supplemented flasks, taking about 60 h (**Figure 5a**), than in the sawdust-supplemented flasks, in which complete disappearance occurred at 48 h (**Figure 5b**). Almost complete disappearance of γ-HCH occurred in the control flasks, however, within 36 h (**Figure 5c**). The Cl^- release pattern was not too different in any of the sets except that the release was slightly slower in the supplemented cultures (**Figure 5**). Glucose was observed in the medium, reaching maximum levels of about 40 and 60 μg mL^{-1} in cellulose- and sawdust-supplemented flasks, respectively (**Figure 5a**,**b**). Increased growth was observed in both of the supplemented flasks, as expected due to the utilization of

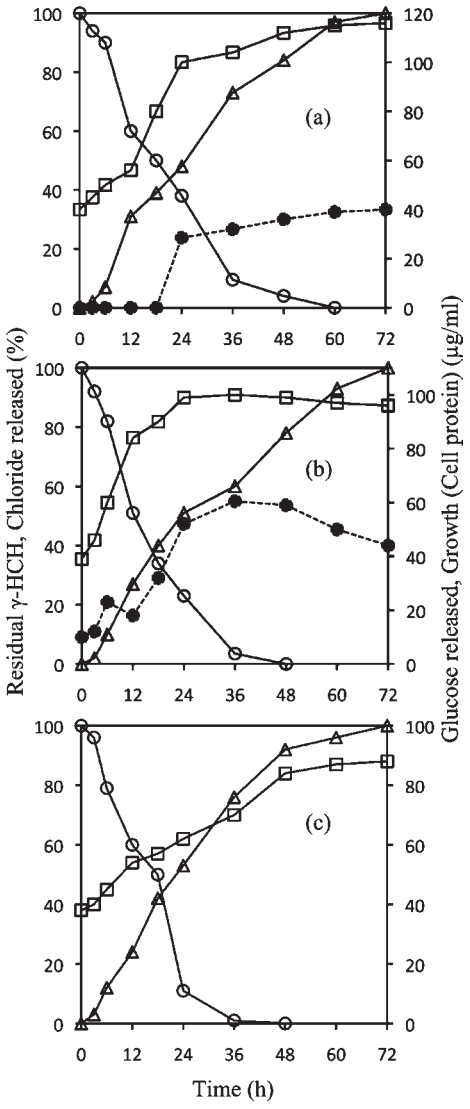

Figure 5. Effect of supplementation of the medium with cellulose powder (**a**) and sawdust (**b**) (both at 1600 μg mL^{-1}) on the growth and degradation of 50 μg mL^{-1} of γ-HCH by the acclimated consortium; (**c**) control with no supplementation: (--●--) glucose released from the auxiliary substrates; other symbols as given in **Figures 1**−**4**. Experimental details are as given under Materials and Methods.

the cosubstrates, reaching maxima of 116 and 100 μg of cell protein mL^{-1} in cellulose- and sawdust-supplemented flasks, respectively, as compared to 87 μg mL^{-1} in the control (**Figure 5**).

Among the auxiliary carbon sources tested only glucose at 1000 μg mL^{-1} retarded the degradation of γ-HCH (**Figure 4**). This may be due to the fact that the target substrate might become diluted in the presence of another utilizable substrate. However, in the presence of 100 μg mL^{-1} of glucose or 1600 μg mL^{-1} of cellulose or sawdust no marked changes in the rate of degradation or Cl^- release were observed, although increased growth was obtained, indicating their efficient utilization. Presumably, all or some members of the consortium possess cellulolytic activity, as a slow glucose release and additional growth were observed in the cellulosics-amended flasks. This is an added advantage as soils would generally have a lot of cellulosic plant materials, which will support the growth of the inoculated culture, without hampering its ability to degrade HCH, as one of the major problems faced in bioremediation technologies is the poor survival of the inoculum in the soil environment (*44*, *45*). Abhilash and Singh (*44*) have demonstrated that amendment of contaminated soil with sugar cane bagasse can accelerate lindane degradation by enhancing microbial activity and preventing pesticide mobility through the soil column by adsorption. Similarly, we have shown earlier that addition of cellulose, sawdust, and low concentrations of glucose (< 200 μg mL^{-1}) and acetone enhanced the rate of degradation of α-HCH, whereas ethanol, benzoate, and glucose (at higher concentrations) retarded the degradation (*21*). An improved cometabolic degradation of β- and δ-HCH in mineral salts medium and soil, amended with glucose (0.1%) and rice straw (0.25%), respectively, by *S. paucimobilis* was reported by Sreedharan et al. (*46*).

Degradation of Other Isomers of HCH by the γ-HCH-Degrading Consortium. The γ-HCH-degrading microbial consortium showed the ability to degrade α-, β-, and δ-isomers of HCH. M4 medium containing α- or δ-HCH (both 25 μg mL^{-1}) or β-HCH (10 μg mL$^{-1)}$ was inoculated with the consortium induced with the respective substrate.

An amount of 25 μg mL^{-1} of α-HCH disappeared completely from the medium within 96 h, with the concomitant release of a stoichiometric amount of Cl^- (**Figure 6a**). There was a lag of about 6 h before the growth and the substrate disappearance started. Growth was meager, and at 24 h a maximum level of only 30.2 μg of cell protein mL^{-1} from the initial 20 μg mL^{-1} was observed, which started declining after 72 h. The Cl^- release initially was rather slow, and after 48 h it accelerated, reaching 100% at 96 h (**Figure 6a**).

An amount of 10 μg mL^{-1} of β-HCH was degraded by the consortium within 48 h with the release of 100% Cl^- (**Figure 6b**). After a lag of about 6 h, the growth started and continued up to 48 h. The biomass buildup in this case was also low, reaching only 30 μg mL^{-1} cell proteins from an initial 22 μg mL^{-1}. The Cl^- release rate was faster up to 12 h, which then slowed, but reached 100% at 48 h.

An amount of 25 μg mL^{-1} of δ-HCH was efficiently degraded by the consortium with the release of 100% Cl^- within 48 h, although the growth was rather slow (**Figure 6c**). There was not only a lag of about 18 h before the growth started but also the cell population declined until 12 h, from an initial 26 μg of cell protein mL^{-1} to 21 μg mL^{-1}, which then picked up and reached a maximum of 33 μg mL^{-1} at 48 h. Cl^- release was very slow up to 6 h, which then was accelerated until 24 h, which again became slow (**Figure 6c**).

The ability of the consortium to efficiently degrade α-, β-, and δ-HCH also, at reasonably high concentrations, is a very positive attribute from the point of view of its expected use in bioremediation of HCH-contaminated sites, which invariably will have most of these isomeric forms. Several microorganisms capable of degrading all four major isomers of HCH have been reported, and in a recent review Lal et al. (*8*) have presented an extensive list of microbes utilizing different isomers.

Isolation and Degradation of γ-HCH by Individual Strains of the Consortium. The acclimated consortium grown on 25 μg mL^{-1} of γ-HCH was appropriately diluted and plated on LBA, TSA, chocolate agar, MacConkey agar, MSA, and PDA. All of the bacterial strains isolated on different media grew well on LBA, and further purification was done using this medium. Nine distinct types of bacteria and a single type of fungus were thus isolated and purified. These were tentatively identified and were designated the names listed in **Table 1**. However, their identity needed to be confirmed by 16S rRNA isolation and sequencing.

The ability of these axenic cultures to degrade γ-HCH was tested by growing them with 10 and 25 μg mL^{-1} of the substrate. All of the bacterial strains degraded 10 μg mL^{-1} of γ-HCH with varying efficiency within 6−24 h with 100% Cl^- release, except *Burkholderia cepacia* CFR1026 and *Pseudomonas stutzeri* CFR1027, which released only 77 and 82% Cl^-, respectively,

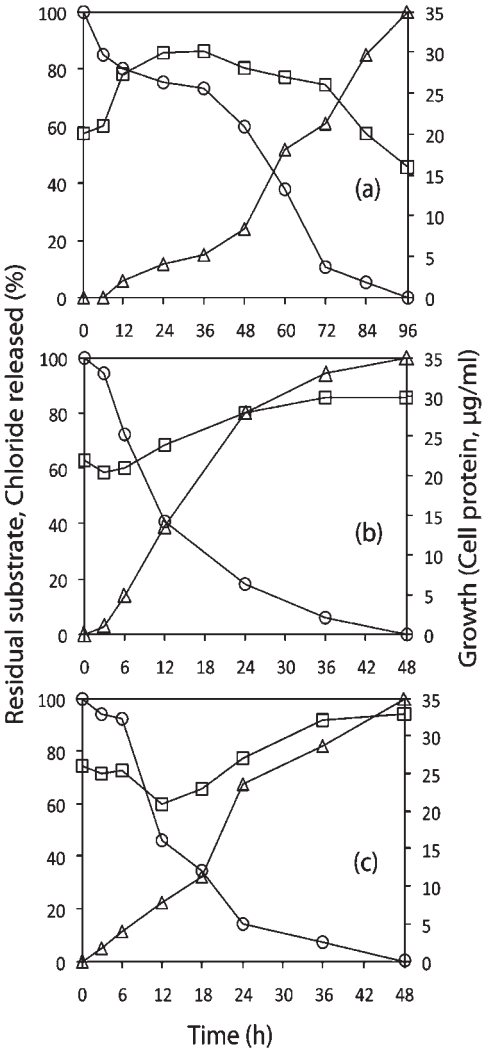

Figure 6. Degradation of (**a**) α-HCH (25 μg mL^{-1}), (**b**) β-HCH (10 μg mL^{-1}), and (**c**) δ-HCH (25 μg mL^{-1}) by the γ-HCH-degrading microbial consortium: (○) residual substrate (**a**) α-HCH, (**b**) β-HCH, and (**c**) δ-HCH; other symbols as given in **Figures 1**−**5**. Experimental details are as given under Materials and Methods.

within 24 h (**Table 1**). The fungal strain, *Fusarium* sp. CFR225, however, degraded it only partially, the Cl^- release being only 33% after 24 h, and prolonged incubation did not improve the degradation. *Pseudomonas aeruginosa* CFR1024 and *Pseudomonas fluorescens* seem to be particularly potent as they mineralized the substrate within 6 and 9 h, respectively. It is possible that on proper acclimation they may be able to degrade higher concentrations. *B. cepacia*, *Vibrio alginolyticus*, and *Acinetobacter lwoffii* seem to be reported for the first time as HCH degraders. At 25 μg mL^{-1} of the substrate only partial degradation was shown by all of the strains (data not shown). The ability of the individual strains to degrade other isomers, however, was not tested.

All of the bacteria that constitute this consortium are Gram negative. Most of the HCH-degrading aerobic bacterial strains reported until now have been Gram-negative, among which the majority was *Sphingomonas* spp. (now called *Sphingobium* spp.) (*5*, *8*, *12*, *15*−*17*, *22*−*25*). A few others such as *Rhodanobacter lindaniclasticus* (*47*), *Pandoraea* sp. (*43*), *Xanthomonas* sp. strain ICH12 (*19*), and eight of a nine-membered α-HCH-degrading consortium (*21*) were also reported. Only very few Gram-positive strains such as *Microbacteruim* sp. ITRC1 (*18*) and *Bacillus* sp. MTCC 9235 [a strain of a three-membered consortium degrading mixtures of lindane, carbofuran, and methyl parathion (*31*)] have been shown to degrade HCH.

Fusarium sp. CFR225 was an inseparable member of the present consortium as this could be isolated from the consortium, like all of the bacterial members, after several passages through γ-HCH-containing medium, indicating its role in the degradation, although individually it could degrade the substrate only partially (**Table 1**). The α-HCH-mineralizing consortium also had a *Fusarium* strain that degraded α-HCH also only partially (*21*). Degradation of various HCH isomers by several white rot fungi has been reported, albeit at low concentrations (*26*, *27*).

In conclusion, it can be said that this microbial consortium, possessing the ability to mineralize up to 300 μg mL^{-1} of γ-HCH completely and at a very high rate, with all its positive attributes, such as the ability to degrade all of the major isomers of HCH at reasonably high concentrations and its tolerance to wide ranges of pH and temperature and the presence of natural organic substances as cosubstrates, can be a favorite candidate for deployment in treatment technologies for bioremediation of HCH-contaminated soils, water bodies, or industrial effluents.

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