Isolation and Acclimation of a Microbial Consortium for Improved Aerobic Degradation of α-Hexachlorocyclohexane

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A microbial consortium that can utilize α-hexachlorocyclohexane (α-HCH) as a sole source of carbon and energy was isolated from soil and sewage through a novel technique involving an initial enrichment in a glass column reactor followed by a shake flask enrichment. This consortium took 14 days to completely mineralize 5 and 10 μg mL^{-1} α-HCH in mineral salts medium in shake flasks. The degradative ability of this consortium improved very markedly on acclimation by successive and repeated passages through media containing increasing concentrations of α-HCH. The acclimated consortium could degrade 100 μg mL^{-1} of α-HCH within 72 h at a degradation rate of 58 μg mL^{-1} day^{-1} with concomitant release of stoichiometric amounts of chloride. Accumulation of any intermediary metabolites was not detected in the culture broth as tested by TLC and GC, implying complete mineralization of the substrate. The acclimated consortium contained eight bacterial strains and a fungus. The individual strains and the different permutations and combinations of them, however, were able to utilize only 10 μg mL^{-1} of α-HCH. Mesophilic temperatures (20−30 °C) and near-neutral pH (6.0−8.0) were most favorable for α-HCH degradation. Among the auxiliary carbon sources tested, ethanol, benzoate, and glucose (at higher concentrations) retarded the degradation of α-HCH, whereas the addition of cellulose, sawdust, and low concentrations of glucose (<200 μg mL^{-1}) and acetone enhanced the rate of degradation.

Keywords: *α-Hexachlorocyclohexane; microbial consortium; acclimation; biodegradation; cosubstrate*

INTRODUCTION

Hexachlorocyclohexane (HCH), commonly known as benzene hexachloride (BHC), has been one of the most extensively used broad spectrum organochlorine pesticides, which amounts to nearly 47% of the total pesticides used in India (David, 1992). Commercial formulations of BHC contain a mixture of α-, β-, γ-, δ-, and ϵ-isomers (65−70, 5−20, 10−15, 6−10, and 3−4%, respectively), among which the γ-isomer is the only effective insecticide (Bachmann et al., 1988b). All of these isomers persist for several years and cause environmental concern. The residues of technical grade BHC have been detected in soil, water, and air (Deo et al., 1994). Highest concentrations of HCH residues were detected in the adipose tissue and breast milk of the Indian population (Tanabe et al., 1990). Almost all foodstuffs including processed foods in India have been shown to contain high levels of HCH (Kannan et al., 1992). It has been well established that these chemical residues reach the human body in a bioconcentrated form through the food chain from contaminated soil, water, animal feed, poultry feed, etc. (Deo et al., 1994; Kunhi, 1995). It is, hence, imperative to develop bioremediation technologies to clean up contaminated soil, water, and waste dump sites.

Degradation of HCH under anaerobic conditions in water-logged soils and lake sediments has been reported by several earlier workers (Deo et al., 1994; Haider, 1979; Jagnow et al., 1977; Kunhi, 1995; MacRae, 1989; Ohisa and Yamaguchi, 1979; Raghu and MacRae, 1966; Sethunathan et al., 1969; Yoshida and Castro, 1970). In all of these cases accumulation of different intermediary metabolites, most of them chlorinated, has been observed, implying inefficient degradation. In recent years, however, isolation of a few strains of *Sphingomonas paucimobilis*, *Psuedomonas versicularis*, and *Pseudomonas* spp. or mixed cultures from soil that can degrade different HCH isomers under aerobic conditions has been reported (Bachmann et al., 1988a,b; Bhuyan et al., 1992, 1993; Huntjens et al., 1988; Imai et al., 1989; Kunhi, 1995; Sahu et al., 1990, 1992, 1993, 1995; Senoo and Wada, 1989; Thomas et al., 1996). All of these axenic cultures, except the γ-HCH-degrading *P. vesicularis* (Thomas et al., 1996), however, are capable of degrading only low concentrations of HCH.

Microbial mixed cultures are considered to be potentially more efficient in the biodegradation of recalcitrant compounds (Slater and Lovatt, 1984). Isolation of natural microbial communities through suitable enrichment techniques and their improvement in the laboratory by adaptation to higher concentrations of the compound of interest are necessary steps for obtaining potent microbial cultures for bioremediation processes. Efforts have been made in our laboratory to isolate efficient degraders of organochlorine pesticides including HCH isomers and other toxic chemicals. In this paper, we describe the development of a microbial consortium that can degrade α-HCH and acclimation of the culture with

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increasing concentrations of the substrate to achieve complete mineralization of α-HCH up to 100 mg L^{-1}. It is well-known that various environmental factors can markedly influence the activity of microorganisms. As the present microbial consortium is meant for application in the bioremediation of HCH-contaminated soils, wastewaters, etc., this aspect was also considered, and data are presented on the effects of temperature, pH, and auxiliary carbon sources on the degradation of α-HCH.

MATERIALS AND METHODS

Chemicals. α-HCH (99% pure) was procured from Sigma Chemical Co., St. Louis, MO. Technical grade HCH was obtained from Hindustan Insecticides Ltd., Mumbai, India. Agar−agar used for making solid medium and potato dextrose agar were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Fresh 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 this study were of analytical grade and were purchased from standard manufacturers.

Cultures. Microbial consortia, both unacclimated and acclimated, used in this study were developed in the laboratory as described later in this section. The names of individual microbial strains isolated from the consortium were as listed in Table 2.

Media. The basal mineral medium (M4 medium) used for growing the microbial consortium consisted of (per liter of distilled water) KH_2PO_4, 0.675 g; Na_2HPO_4, 5.455 g; NH_4NO_3, 0.25 g; $MgSO_4{\cdot}7H_2O$, 0.20 g; $Ca(NO_3)_2$, 0.10 g; and trace mineral solution, 1.00 mL [containing (mg per mL of distilled water) $FeSO_4{\cdot}7H_2O$, 1.00; $CuCl_2{\cdot}2H_2O$, 0.25; $MnSO_4{\cdot}H_2O$, 1.00; $Na_2MoO_4{\cdot}2H_2O$, 0.25; and concentrated H_2SO_4, 5.00 μL]. The pH of the medium was 7.5. The medium was sterilized by autoclaving at 121 °C for 20 min. Stock solutions of $MgSO_4{\cdot}2H_2O$ and $Ca(NO_3)_2$ were separately autoclaved similarly, and required quantities were added to the medium after cooling.

Nutrient agar (NA) containing (g L^{-1}) peptone, 5.0; beef extract, 3.0; sodium chloride, 5.0; and agar−agar, 20.0, was used for isolating the bacterial members of the consortium. PDA was used for isolating the fungal culture. Both media were sterilized by autoclaving at 121 °C for 20 min.

Development and Acclimation of the Microbial Consortium. The α-HCH-degrading microbial consortium was developed in the laboratory by a long-term enrichment technique. This involved a two-step enrichment; first, in a semicontinuous column reactor and then in shake flasks. A cylindrical glass column (5 × 50 cm) was filled with wood charcoal cubes (1−2 cm) and basal mineral medium containing acetone, benzene, and phenol (added after filter sterilization) as carbon sources (each 50 μL L^{-1}). To this was added a cloth-filtered aqueous suspension of a mixture of sewage and HCH-contaminated soil samples from sugar cane and paddy fields. The column reactor was maintained semicontinuously, by withdrawal and recirculation of the medium, three or four times a day to enable aeration, and partial replacement of the medium once in two to three days. After two weeks the concentrations of acetone, benzene, and phenol were gradually reduced at the rate of 10 μL L^{-1} $week^{-1}$ and feeding of mineral medium containing technical HCH (5 μg mL^{-1}, the concentration of which was gradually increased and brought to 10 μg mL^{-1}) was begun after 4 weeks. This operation was continued for a total time of 6 months, during which period broth samples were collected every week and further enriched in shake flasks.

For shake flask enrichment, M4 medium containing α-HCH (10 μg mL^{-1}) was used. Fifty milliliters of medium taken in a 250 mL Erlenmeyer flask to which the required amount of α-HCH was added as acetone solution (50 μL) was incubated at 30 °C on a rotary shaker (150 rpm). The consortium that developed from the broth sample collected from the column after 6 months showed utilization of 10 μg mL^{-1} of α-HCH as indicated by the release of stoichiometric amounts of chloride (Cl^-).

The consortium was then acclimated to higher concentrations of α-HCH from 10 to 100 μg mL^{-1}, by gradually increasing the substrate concentration. The cells harvested from the previous batch were used as inoculum to start a fresh batch with or without an increase of the substrate concentration. This was repeated until complete degradation in terms of release of stoichiometric amounts of Cl^- was obtained.

Maintenance of the Microbial Consortium. The consortium grown on mineral agar plates/slopes containing technical HCH (25 μg mL^{-1}) for one week at 30 °C was preserved at 4 °C. Alternatively, liquid culture grown on mineral salts medium containing 25 μg mL^{-1} α-HCH for 48 h was stored at 4 °C.

Resolution of the Consortium into Individual Strains. The microbial profile of the α-HCH-degrading consortium was analyzed by plating 96-h-old cultures, grown with 25, 50, and 100 μg mL^{-1} of α-HCH, on nutrient agar and PDA by serial dilution technique. Morphologically distinct colony types were picked up, further purified by repeated streaking, and the bacteria were identified according to *Bergey's Manual of Determinative Bacteriology* (Palleroni, 1986) as well as by using the Microbact Identification System from Medvet Science Pty Ltd., Australia. The fungal strain was identified according to the findings of Muller and von Arx (1973).

The microbial profile of the unacclimated consortia was also similarly studied after growing them at different pH values (4.5−8.5). The bacterial colonies were grouped into 10 distinct types on the basis of size, color, and morphology on nutrient agar plates, and their relative predominance was determined by plate counts of serially diluted samples from cultures grown at different medium pH values. Plate counts of the fungal colonies were made using PDA plates.

Degradation of α-HCH. The required quantity of α-HCH as acetone solution (50−100 μL) was dispensed in to sterilized 250 mL Erlenmeyer flasks. After evaporation of the acetone at room temperature by keeping the flasks open in a laminar hood, 25 mL of sterile M4 medium was dispensed into each flask. The flasks were inoculated with 1 mL of the washed cell suspension of the consortium previously grown on M4 medium with α-HCH as carbon source. Flasks were inoculated in triplicate and were incubated on a rotary shaker (150 rpm) at 30 °C unless otherwise stated. Flasks were removed at various intervals and analyzed for growth, inorganic chloride, residual substrate, and intermediary metabolites, if any. Uninoculated flasks served as control.

Individual strains also were grown similarly. The inocula were prepared by growing the cells initially on 1:50 nutrient broth containing 10 μg mL^{-1} of α-HCH and then inducing the washed cell biomass with 10 μg mL^{-1} α-HCH in M4 medium overnight. The induced cells were centrifuged, washed, and used as inoculum (cell biomass was washed with M4 medium).

To study the effect of pH on the degradation of α-HCH (10 μg mL^{-1}) in M4 medium, the pH was adjusted to different values (from 3.0 to 11.0) by varying the proportion of KH_2PO_4 and Na_2HPO_4. pH values <5.0 and >8.0 were adjusted with 1 M HNO_3 and 1 M NaOH solution, respectively. The effect of the incubation temperature on the degradation of α-HCH (10 μg mL^{-1} in M4 medium) was studied from 4 to 60 °C under stationary conditions for 48 h.

The effect of auxiliary carbon sources such as glucose, cellulose powder, sawdust, acetone, sodium benzoate, and ethanol on the degradation of α-HCH by both unacclimated and acclimated consortia was studied. The concentrations of the auxiliary carbon sources were selected arbitrarily and were added as given in the legends to the respective figures.

Analytical Methods. Inorganic chloride (Cl^-) was estimated according to a slightly modified procedure of Bergmann and Sanik (1957). To 2 mL of aliquot of the culture supernatant was added 0.2 mL of 0.25 M ammonium iron(III) sulfate in 9 M nitric acid followed by the addition of 0.2 mL of a saturated solution of mercury(II) thiocyanate in ethanol. The color developed was measured at 460 nm by a spectrophotometer (Shimadzu UV-160A). The amount of chloride was computed using a standard curve prepared using NaCl.

The growth of the consortium was determined by estimating the total protein in the biomass according to a modified method of Lowry, as follows: The cells were harvested, washed with distilled water, and resuspended in 3.4 mL of distilled water; 0.6 mL of 20% NaOH was added, mixed, and digested in a constant-boiling water bath for 5 min; total protein in the cooled sample was determined according to the method of Lowry et al. (1951) using the Folin−Ciocalteu reagent.

Residual α-HCH was determined by gas chromatography (GC). The culture broth was extracted three times with equal volumes of an *n*-hexane/acetone (8:1) mixture, each time by thorough mixing for 15 min. The solvent layers were collected, pooled, and passed through a Florisil column for cleanup. Moisture was removed by adding solid Na_2SO_4 and evaporated to dryness. The residue was redissolved in a convenient volume of *n*-hexane. A known volume of this was injected into Fison's model GC instrument with ^{63}Ni electron capture detector. The conditions used were as follows: column, 0.25 in. DD and 11 ft in length, packed with 1.5% OV-17 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. The amount of α-HCH was computed by using a standard curve prepared under the same conditions.

To detect whether any intermediary metabolites were accumulating 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 silica gel G plate using cyclohexane as mobile phase. Spots were visualized with *o*-tolidine spray and exposure to sunlight. TLC of the concentrated culture broth 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, cholrobenzenes, catechols, etc., were used as reference standards.

Glucose was estimated according to the method of Miller (1959) using dinitrosalicylic acid reagent.

RESULTS

Development and Acclimation of α-HCH-Degrading Consortium. The broth samples collected after six months from the column reactor were enriched in shake flasks for 8 weeks with weekly transfers to fresh medium, and a microbial consortium was established that could degrade α-HCH. With this consortium, 5 and 10 μg mL^{-1} of α-HCH disappeared in 6 days with the release of stoichiometric amounts of Cl$^-$ on day 14; 25 and 50 μg mL^{-1} of α-HCH disappeared by 10 and 14 days with the release of 72 and 80% Cl$^-$, respectively (Figure 1). Growth increased steadily and reached maxima on days 4, 6, and 10 with the substrate concentrations of 5, 10, and 25 μg mL^{-1}, respectively, whereas the chloride release continued to increase until 14 days. More than 95% of the added α-HCH (10 μg mL^{-1}) was recovered from the uninoculated control flasks, and no Cl$^-$ release was detected in these flasks, indicating clearly that autodegradation did not occur under the culture conditions followed.

The above consortium was acclimated to increasing concentrations of α-HCH by repeated transfers, at least two to three times, to fresh medium containing the same concentration of α-HCH and then going to higher concentrations. The total cell biomass obtained was pelleted and washed with sterile 0.1% (v/v) Tween 80 solution for dislodging the adsorbed Cl$^-$ from the cells followed by a wash with M4 medium and used as the inoculum for the next culture. After a serial passage through different concentrations of α-HCH, namely, 10, 25, 50, and 100 μg mL^{-1}, an acclimated microbial consortium was obtained. With this consortium, when

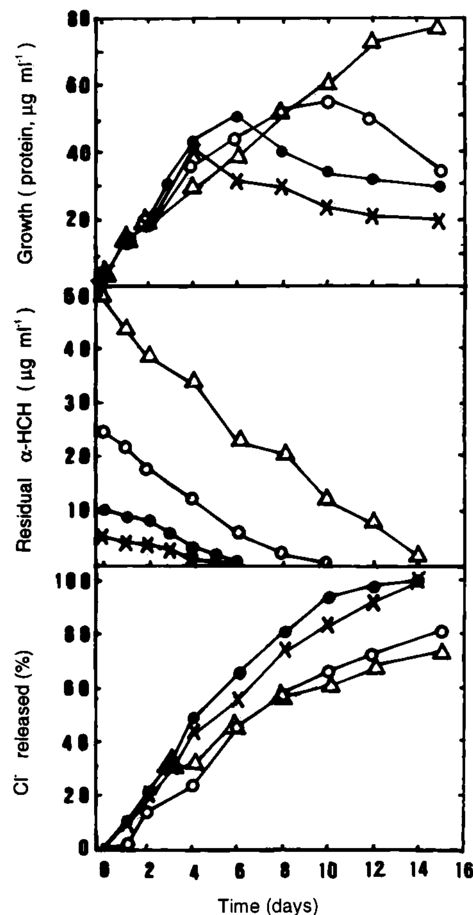

Figure 1. Degradation of different concentrations of α-HCH by the unacclimated microbial consortium. One milliliter of washed cell suspension (∼5 μg of protein) was added to 25 mL of M4 medium containing 5 (×), 10 (●), 25 (○), or 50 (△) μg mL^{-1} of α-HCH and incubated at 30 °C on a rotary shaker (150 rpm).

the α-HCH concentration was 25 μg mL^{-1}, degradation was complete by 48 h, the growth reaching a maximum by 24 h (Figure 2). Degradation of 50 μg mL^{-1} of α-HCH by the culture previously grown with 25 μg mL^{-1} of α-HCH was complete by 72 h (Figure 2), whereas the unacclimated culture was not able to completely mineralize the same amount of substrate even after 15 days (Figure 1). The consortium acclimated to 50 μg mL^{-1} was used for the degradation of 100 μg mL^{-1} of α-HCH. Complete degradation of 100 μg mL^{-1} of α-HCH was observed by 72 h, with growth increasing steadily up to 72 h (Figure 2). The rates of disappearance of the substrate showed an increasing trend with increase in concentration, maybe because the cells from the previous batch that had been acclimated were used as inoculum (Figure 2). The rate of degradation of 100 μg mL^{-1} α-HCH was computed to be ∼58 μg mL^{-1} day^{-1} at the exponential phase. No degradation of α-HCH was observed in the control flasks of all the above experiments (data not shown).

No intermediary metabolite was detected either in the solvent extract or in the aqueous phase of the culture broths from the flasks containing even 100 μg mL^{-1}, both in TLC and in GC.

Resolution of Microbial Consortia into Individual Strains. Ten distinct types of bacteria and a single type of fungus with the colony characteristics as described in Table 1 were observed in the unacclimated

Table 1. Microbial Profile and Relative Counts of Different Microbial Types in the Unacclimated Consortium When Grown on M4 Medium Containing 10 μg mL^{-1} of α-HCH and 0.2% (v/v) Acetone at Different Medium pH Values[a]

	bacterial types[b] and number (cfu/mL)										total bacterial counts	fungal counts
pH	I	II	III	IV	V	VI	VII	VIII	IX	X	(cfu × 108)	(cfu × 10^6)
4.5	2.2×10^{8a}	1.0×10^{7ns}	$1.0 \times 10^{7\,ns}$								2.4	69^{a}
6.6		2.0×10^{7ns}	1.8×10^{7ns}	1.2×10^{8a}	7.5×10^{8}						10.7	184^{b}
6.5		2.0×10^{7ns}				1.3×10^{8a}	3.5×10^{8a}	3.0×10^{7a}	2.0×10^{7a}		5.5	1^{c}
7.5	2.0×10^{7a}			3.0×10^{8b}		8.5×10^{8b}	8.8×10^{8b}	1.1×10^{8b}	3.1×10^{8b}	2.3×10^{8a}	24.3	9^{d}
8.5	7.0×10^{7b}	1.0×10^{7ns}		8.0×10^{8c}		3.5×10^{8c}	6.1×10^{8c}			1.5×10^{8b}	12.7	$16^{b,c}$

[a] 25 mL of culture in 250 mL conical flasks was incubated at 30 °C for 10 days on a rotary shaker (150 rpm) before serially diluting and plating on nutrient agar for bacteria and on PDA for fungal colonies. Statistical analysis was carried out using the ananlysis of variance technique. The means were then separated using Duncan's New Multiple Range test ($P < 0.05$). Standard errors: I, ±27.21 (df = 9); II, ±31.21 (df =12); III, ±40.37 (df = 6); IV, ±37.23 (df =9); VI, ±33.69 (df = 9); VII, ±34.76 (df = 6); VIII, ±40.71 (df = 6); IX, ±34.19 (df = 6); X, ±30.15 (df = 6); fungus, ±21.67 (df = 15) (df, degree of freedom); ns, not significant. In each column values with similar letters do not differ significantly at the 5% level. [b] Colony characteristics of bacterial types: I, thin, perfectly circular, with slightly opaque center, surrounding region being more or less transparent, slightly fluorescent colonies; II, large opaque colorless with rough surface; III, small thick colony with bluish green fluorescent rim; IV, large, uneven edges, bluish fluorescent with slightly yellowish brown center; V, tiny flourescent bluish colonies; VI, very tiny colonies shining against light with slight blue fluorescence; VII, small round almost transparent fluorescent shining against light (larger than type VI); VIII, large, round, umbonate, fluorescent, slightly yellowish transluscent colonies; IX, round, fluorescent, yellowish green, umbonate transluscent colonies, size between types IV and VIII; X, white, nonfluorescent (smaller than type II) round colonies; fungus, white colonies on PDA with pink color on the reverse side of the plate, fairly fast growing; septate, multicellular, sickle shaped, and other types of spores were observed.

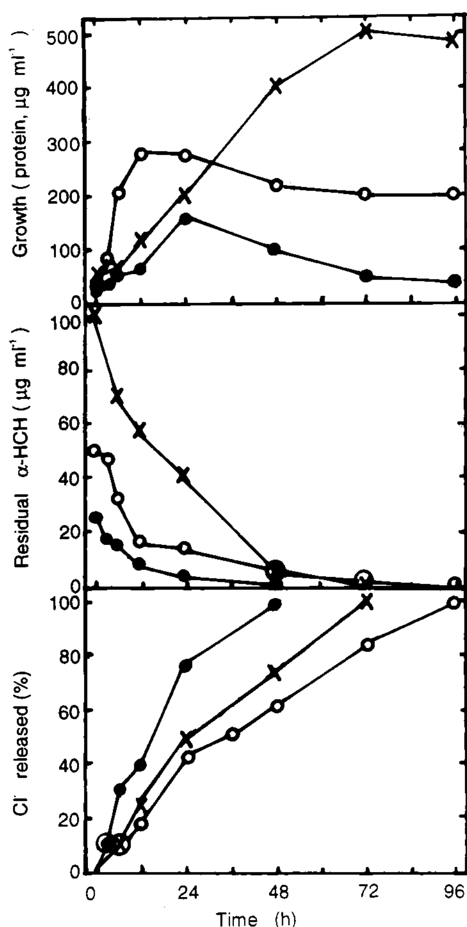

Figure 2. Degradation of 25 (●), 50 (○), and 100 (×) μg mL^{-1} of α-HCH by the acclimated microbial consortium. Culture conditions were as described in Figure 1.

consortium. The relative counts of these different types of bacteria and the fungus as a function of pH of the growth media are also shown in Table 1. It can be seen that among the 10 types of bacteria, 7 types were most predominant at pH 7.5, which was the optimal pH for degradation of α-HCH. Although the count of the fungus at this pH was low, its presence in the consortium was essential for a faster degradation of the α-HCH as discussed later.

The acclimated consortium consistently showed the presence of eight distinct bacterial types and the fungus. The bacterial members, *Pseudomonas stutzeri* CFR 1007, *Pseudomonas fluorescens* CFR 1002, *Pseudomonas pseudomallei* CFR 1004, *Pseudomonas aeruginosa* CFR 1006, *Pseudomonas fluorescens* CFR 1008, *Acinetobacter haemolyticus* CFR 1009, *Pseudomonas diminuta* CFR 1003, and *Pseudomonas putida* CFR 1005, observed in the acclimated consortium (Table 2) corresponded with types I, III, IV, V, VI, VII, VIII, and IX, respectively, of the unacclimated consortium (Table 1), and their relative predominance was 19.6, 4.9, 8.8, 16.2, 17.7, 18.6, 3.4, and 10.8% of the total bacterial counts, respectively. Fungus occurred at 2–3% of the total microbial counts.

Degradation of α-HCH by Pure Cultures. All of the individual strains of bacteria from the consortium were found to utilize up to 10 μg mL^{-1} α-HCH as the sole source of carbon and energy, whereas the fungal strain degraded it only partially (Table 2). All of these pure cultures and even different combinations of them failed to completely degrade 25 μg mL^{-1} of α-HCH even after induction (Table 3). However, the consortium reconstituted by inclusion of all nine cultures could degrade α-HCH (Table 3) but needed acclimation for a long period to attain the ability to degrade higher concentrations. On the other hand, the acclimated consortium maintained as liquid culture in M4 medium containing α-HCH (25 μg mL^{-1}) could adapt itself easily to higher concentrations of α-HCH (data not shown).

Effect of Temperature and pH. The acclimated consortium was able to grow and degrade α-HCH over a fairly wide range of temperature, although the mesophilic temperatures were more favorable, optimum being 30 °C (Figure 3). Although the growth was not very good at lower temperatures, the degradation of α-HCH, as evidenced by Cl^- release, was better. About 30% Cl^- release was observed even at 4 °C. The degradation rate dropped rapidly at temperatures >30 °C, the Cl^- release being <50% at 35 °C. Nevertheless, the culture was still active and showed the release of 40% Cl^- even at 40 °C. The culture was active in degrading α-HCH over a wide range of pH (Figure 4). However, near-neutral pH (6.0–8.0) was found to be the most favorable for growth and effective degradation. No

Table 2. Degradation of 10 μg mL^{-1} of α-HCH by Individual Microbial Strains[a]

culture[b]	Cl$^-$ released (%) after			
	3 h	6 h	8 h	24 h
Pseudomonas fluorescens CFR1002	46 ± 2.39[c]	75 ± 1.37	79 ± 0.81	100 ± 0.41
Pseudomonas diminuta CFR1003	45 ± 1.88	55 ± 1.44	73 ± 1.65	100 ± 0.46
Burkholderia pseudomallei CFR1004	41 ± 1.58	96 ± 0.89	95 ± 0.91	100 ± 0.00
Pseudomonas putida CFR1005	34 ± 1.37	77 ± 1.16	100 ± 0.21	100 ± 0.00
Pseudomonas aeruginosa CFR1006	31 ± 1.08	64 ± 1.27	79 ± 1.34	100 ± 0.00
Pseudomonas stutzeri CFR1007	31 ± 1.32	64 ± 0.90	70 ± 1.07	100 ± 0.22
Pseudomonas fluorescens CFR1008	31 ± 1.79	51 ± 1.27	70 ± 1.19	100 ± 0.49
Acinetobacter haemolyticus CFR1009	33 ± 0.83	73 ± 1.52	80 ± 1.41	100 ± 0.00
Fusarium sp. CFR217				25 ± 1.33

[a] Each strain was grown in 25 mL of diluted nutrient broth (1:50) containing 10 μg mL^{-1} α-HCH for 24 h, harvested, suspended in 25 mL of M4 medium containing α-HCH (10 μg mL^{-1}), and shaken overnight for induction. These induced cells were used as inoculum. [b] Cultures 1−8 corresponded with the types III, VIII, IV, IX, V, I, VI, and VII isolated from the unacclimated consortium (Table 1). [c] ± standard deviation.

Table 3. Degradation of α-HCH (25 μg mL^{-1}) in M4 Medium by Different Combinations of Microbial Strains[a]

strain combination[b]	% Cl$^-$ released after		
	24 h	48 h	72 h
A	25.16 ± 0.035[c]	25.16 ± 0.029	26.27 ± 0.025
B	23.56 ± 0.031	26.25 ± 0.011	21.42 ± 0.602
C	25.70 ± 0.691	32.67 ± 0.721	26.43 ± 0.711
A + B	32.81 ± 0.662	25.16 ± 0.572	29.17 ± 0.395
B + C	27.89 ± 0.569	33.90 ± 0.473	26.89 ± 0.458
A + C	25.89 ± 0.219	32.67 ± 0.357	28.24 ± 0.453
A + B + C	26.25 ± 0.398	26.25 ± 0.289	44.21 ± 0.452
Fusarium sp. alone	21.90 ± 0.398	35.16 ± 0.289	37.74 ± 0.452
A + B + C + *Fusarium* sp.	35.82 ± 0.526	43.75 ± 0.429	74.79 ± 0.127

[a] The cells obtained by growing the strains in diluted nutrient broth (1:50) containing α-HCH (10 μg mL^{-1}) were used as inoculum. [b] A, *Pseudomonas fluorescens* CFR1002 + *Pseudomonas diminuta* CFR1003 + *Burkholderia pseudomallei* CFR1004; B, *Pseudomonas putida* CFR 1005 + *Pseudomonas aeruginosa* CFR 1006; C, *Pseudomonas stutzeri* CFR1007 + *Pseudomonas fluorescens* CFR1008 + *Acinetobacter haemolyticus* CFR1009. [c] ± standard deviation.

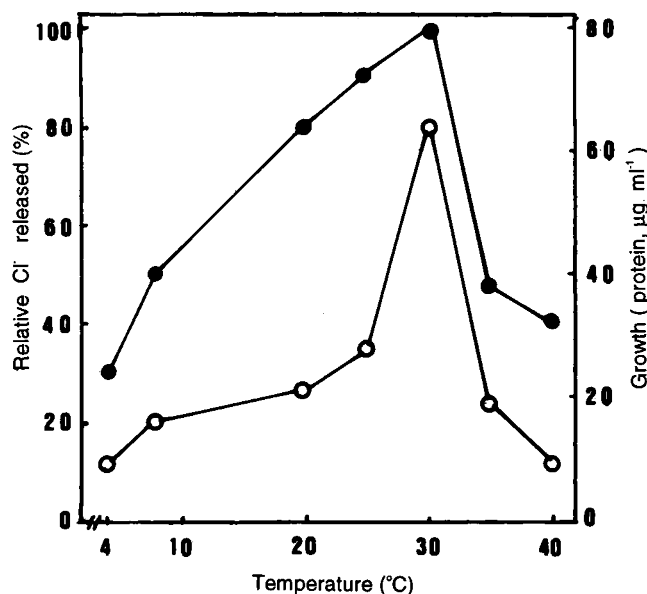

Figure 3. Effect of incubation temperature on degradation of α-HCH (10 μg mL^{-1} in M4 medium) by the acclimated microbial consortium: (○) growth and (●) Cl$^-$ released. The flasks were incubated at temperatures of 4−40 °C under stationary condition for 48 h.

Cl$^-$ release was observed in any of the control flasks maintained at pH 3.0−8.0, whereas some Cl$^-$ was released at pH >9.0. These Cl$^-$ values were subtracted from that of the experimental values to obtain the net release of Cl$^-$.

Effect of Acetone as a Cosubstrate. Addition of acetone, as single dose of 2 mL L^{-1} or as four split doses, to the culture with unacclimated consortium showed an increase in growth that was more than twice that of the control (Figure 5A). The Cl$^-$ release pattern was more or less similar in the control and in the culture supplemented with acetone in incremental doses. Detectable levels of Cl$^-$ were observed in these on day 1, whereas Cl$^-$ was detected in the medium only on day 3 when acetone was added as a single large dose. The maximum Cl$^-$ release on day 10 was less (87%) in the latter as against 95% in the former two cases (Figure 5A).

Effect of Ethyl Alcohol as a Cosubstrate. Supplementation of the medium with ethanol as a single dose of 1 mL L^{-1} resulted in increased growth, almost twice as much as the control (Figure 5B). Interestingly, however, the degradation of α-HCH (10 μg mL^{-1}) was adversely affected, the maximum Cl$^-$ release being only ~50% on day 10 as against 95% that of the control. Detectable concentrations of Cl$^-$ were also observed only on day 6 (Figure 5B).

Effect of Sodium Benzoate as a Cosubstrate. Growth of the consortium started after a lag of one day in the benzoate-supplemented medium (1.9 mg mL^{-1} as a single dose) and showed a gradual increase up to day 7 (Figure 5C). As in the case of ethanol-supplemented culture, the degradation of α-HCH was retarded, releasing a maximum of ~50% Cl$^-$. There was also a long lag of ~4 days before the Cl$^-$ release started (Figure 5C).

Effect of Glucose as a Cosubstrate. When glucose was added to the medium, marked retardation of the degradation of α-HCH (10 μg mL^{-1}) by the unacclimated consortium was observed, although the growth of the consortium was almost 4 times as high as that of the control (Figure 6). In the case of control ~97% of Cl$^-$ was released on day 10, whereas in the case of glucose supplementation as split doses it was 52%, and with glucose as a single initial large dose, it was a meagre 38%. The appearance of Cl$^-$ in the medium also was delayed in the glucose-supplemented cultures (Figure 6). On the contrary, when glucose was added at lower concentrations (100 μg L^{-1} as a single dose or 25 μg L^{-1} as four split doses), improved degradation of α-HCH (25 μg mL^{-1}) occurred (Figure 7A). On day 14, a maximum of ~67% of the Cl$^-$ was released in the control culture, whereas ~92% Cl$^-$ was released in the medium supplemented with glucose.

The acclimated consortium, however, presented a slightly different picture (Figure 8A). When glucose was

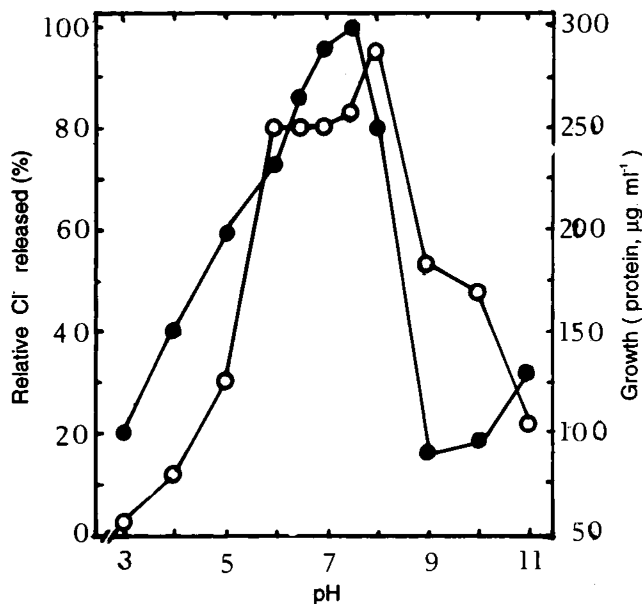

Figure 4. Effect of medium pH on the degradation of α-HCH (10 μg mL^{-1} in M4 medium, 25 mL in 250 mL conical flasks): (○) growth and (●) relative Cl$^-$ released. The pH of the medium was adjusted by altering the ratio of KH_2PO_4 and Na_2HPO_4 (pH 5.0−8.0) or by the addition of 1 N HNO_3 or 1 N NaOH (to obtain pH <5.0 and >8.0, respectively). The flasks were incubated on a rotary shaker (150 rpm) at 30 °C for 24 h. The Cl$^-$ values plotted are the net values obtained after deduction of the autoreleased Cl$^-$ values of uninoculated control flasks of different pH values.

added at 1600 μg mL^{-1} as a single dose or as split doses, the rate of disappearance of α-HCH was similar to that in the control, the degradation being complete in the control and in the glucose-incrementally fed cultures by 96 h. The rates of Cl$^-$ release were also almost similar in these, whereas it was slightly lower in the culture supplemented with glucose as a single large dose. Increased growth was observed in the glucose-supplemented culture (Figure 8A). However, the additional growth in the acclimated consortium was not as much as that in the unacclimated consortium with the same amount of glucose (Figure 6).

Effect of Cellulose and Sawdust as Cosubstrates. Improved degradation of α-HCH (25 μg mL^{-1}) was observed in both cellulose and sawdust (1000 μg mL^{-1}) supplemented cultures, inoculated with the unacclimated consortium (Figure 7B,C). In both cases, α-HCH disappeared on day 8, whereas the complete disappearance of the compound occurred on day 10 in control flasks. A total of 95−97% of Cl$^-$ was released on day 14 in the supplemented flasks, whereas only 75−80% Cl$^-$ release was observed in the control. In the supplemented flasks, biomass buildup also was more, twice as much as the control.

With acclimated consortium also, supplementation of the medium with cellulose powder or sawdust (1000 μmL^{-1}) slightly improved the rates of disappearance of α-HCH (50 μg mL^{-1}) (Figure 8B,C). In both cases, α-HCH disappeared from the medium after 72 h, whereas this disappearance occurred in control flasks after 96 h. However, the rate of Cl$^-$ release was marginally higher in the control flasks than in the supplemented flasks. However, in all sets of flasks, 100% Cl$^-$ release was observed at 96 h. The growth of the consortium was gradual in the control flasks, whereas in the supplemented flasks there was an initial

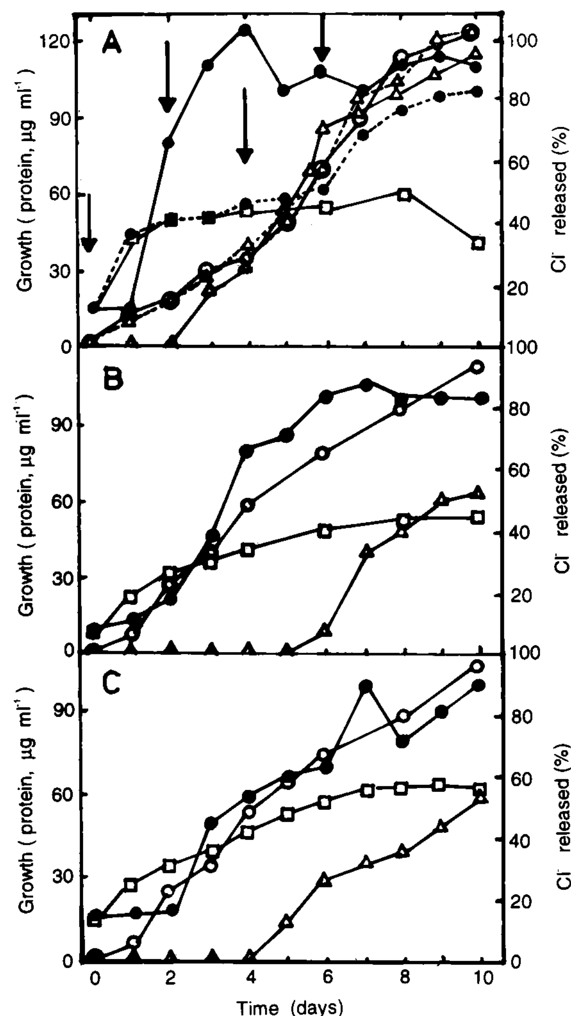

Figure 5. Effect of acetone (A), ethanol (B), and sodium benzoate (C) added as auxiliary carbon sources on the growth and release of Cl$^-$ from 10 μg mL^{-1} α-HCH by the unacclimated microbial consortium: (●) growth with cosubstrate (single dose); (- -●- -) growth with cosubstrate (split doses); (□) growth without cosubstrate (control); (△) Cl$^-$ released with cosubstrate (single dose) (- -△- -) Cl$^-$ released with cosubstrate (split doses); (○) Cl$^-$ released without cosubstrate (control); ↓ indicates the time of addition of the cosubstrates. Acetone was added to the medium as an initial single dose of 2 mL L^{-1} or as four split doses of 500 μL L^{-1} on days 0, 2, 4, and 6. Ethanol (1 mL L^{-1}) and benzoate (1.9 mg mL^{-1}) were added as single doses initially. Other conditions were as in Figure 1.

surge of growth up to 24 h. Glucose was detected in the medium supplemented with these cellulosic materials throughout the culture period in flasks inoculated with unacclimated as well as acclimated cultures (Figures 7B,C and 8B,C).

DISCUSSION

By a long-term two-step enrichment technique we were able to develop a microbial consortium that can mineralize α-HCH efficiently. A glass column packed with charcoal cubes has the advantage of (i) absorbing the added substrate and releasing it in low concentrations and (ii) the ability to adsorb microbial cells helping them to flock together, enabling them to exchange their genetic material (Ehrhardt and Rehm, 1985; Slater and Lovatt, 1984). Samples of contaminated soil and sewage may contain microbes that can degrade HCH, but their relative population may be very small (Slater and

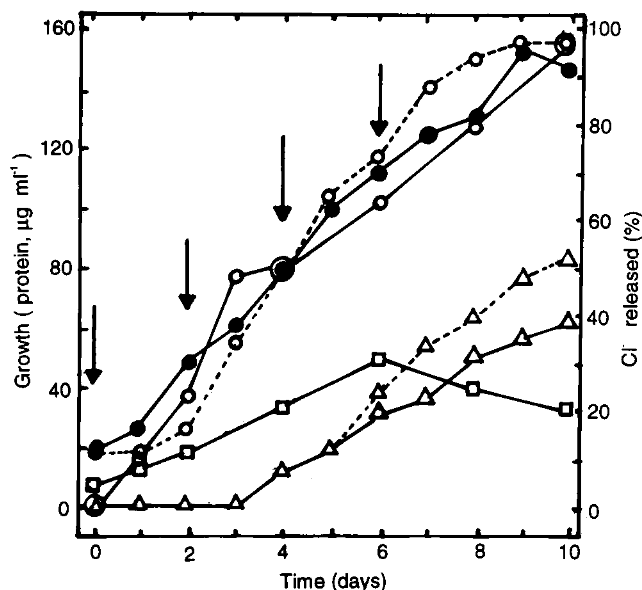

Figure 6. Glucose as an auxiliary carbon source at 1.6 mg mL^{-1} as a single initial dose or 400 μg mL^{-1} as four split doses on days 0, 2, 4, and 6 improves the growth of the unacclimated consortium but retards the release of Cl^- from 10 μg mL^{-1} of α-HCH. Symbols used are the same as in Figure 5 except (- -○- -) for growth with cosubstrate (split doses).

Lovatt, 1984). Hence, we thought an initial supply of easily utilizable carbon sources such as acetone, phenol, and benzene would help in building up the biomass, which would later be exposed to HCH. It has been known that HCH isomers are catabolized through the formation of aromatic derivatives, namely, chlorophenols or chlorobenzenes or both (Bachmann et al., 1988a; Imai et al., 1991; Kunhi, 1995; Senoo and Wada, 1989). Hence, it was thought that benzene and phenol could enrich microbial populations that might contain the HCH degraders.

The presence of chlorine makes the benzene ring refractile to enzyme attack (Hartmann et al., 1979). It has been shown that isolation of chloroaromatics-degrading cultures is rather difficult and involves long-term enrichment in bioreactors (Reineke and Knackmuss, 1988; Slater and Lovatt, 1984). Isolation of bacteria capable of degrading HCH isomers aerobically has been reported only in recent years, implying that natural selection and adaptation took long periods of continuous exposure. Senoo and Wada (1989) were able to isolate a strain of *Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* from an upland field, where γ-HCH was applied every year for more than 3 years. Similarly, Sahu et al. (1992, 1995) reported isolation of *Pseudomonas* sp. that degrade α-, β-, γ-, and δ-isomers of HCH from the rhizosphere of HCH-treated sugar cane plants. Aerobic mineralization of α-HCH in heavily contaminated soil by native microorganisms was reported by Bachmann et al. (1988a,b). Subsequently, a strain of *P. vesicularis* that could degrade α-HCH was isolated from this soil inoculum (Huntjens et al., 1988). Enrichment for over 2 years of soil samples collected from sites where γ-HCH had been applied every 2 years for ~20 years for agricultural purposes failed to yield a degradative microbial community even with the addition of cometabolites such as glucose or succinate, whereas mixed soil samples from wood treatment sites in which the γ-HCH concentrations were higher than in the agricultural soil when enriched similarly yielded two bacterial strains, one of which, *P. vesicularis* (or *Xanthomonas* as identified by two different methods), showed the ability to degrade γ-HCH (Thomas et al., 1996). From all of these reports, it is evident that exposure of natural microflora to fairly high concentrations of the substrate is required for selection and adaptation to attain the degradative ability. A similar observation was made in the present study, too. It took about 6 months before a microbial consortium that could degrade α-HCH became established in the column reactor.

Continuous exposure to increasing concentrations of the substrate markedly improved the degrading ability. When complete mineralization of 10 μg mL^{-1} of α-HCH by the unacclimated consortium took 14 days, 100 μg mL^{-1} of the substrate was degraded within 72 h (Figures 1 and 2). It was also interesting to observe that degradation of 100 μg mL^{-1} α-HCH was faster (72 h) with the release of 100% Cl^- than that of 50 μg mL^{-1}, which took 96 h for complete Cl^- release. This was because the inoculum used for the former culture was the one that was already acclimated by virtue of its growth with 50 μg mL^{-1} α-HCH. A similar observation was made by Bhuyan et al. (1992, 1993), who found that pretreatment with HCH resulted in accelerated aerobic degradation of γ-HCH in flooded and nonflooded soils, the rate of degradation increasing after every successive treatment. Rapid degradation of γ-HCH in upland soil was also observed after multiple applications of the compound (Wada et al., 1989).

The acclimated consortium showed good α-HCH-degrading ability, the rate being ~58 μg mL^{-1} day^{-1} from an initial concentration of 100 μg mL^{-1}, at the early exponential phase. This is much faster than what has been reported so far. Even the potent mixed native microflora reported by Bachmann et al. (1988b) took 18 days to degrade 400 μg of α-HCH g^{-1} soil slurry, with a degradation rate of only 23 μg g^{-1} day^{-1} under aerobic conditions.

The degradation of α-HCH by the consortium seems to be complete as no intermediary metabolites accumulated at any stage of the culture period and also as 100% Cl^- release was observed. HCH degradation generally occurs via the formation of chlorobenzenes, chlorophenols, or both (Bachmann et al., 1988a; Imai et al., 1991; Kunhi, 1995; Senoo and Wada, 1989), and the release of the last chlorine atom is expected almost at the end of the pathway (Rieneke and Knackmuss, 1988; Schlomann, 1994). Hence, 100% Cl^- release would mean complete mineralization.

Eight distinct types of bacteria and a fungal strain were isolated from the consortium, and all of the individual bacterial strains had the ability to mineralize α-HCH, although at a low concentration of 10 μg mL^{-1} (Table 2). It is, however, difficult to explain how so many bacterial strains possessing the α-HCH-degrading ability became enriched. The long-term enrichment method adopted might have been responsible for this. Several individual members of a microbial community that was able to degrade the herbicide dalapon (2,2′-dichloropropionic acid) were shown to degrade dalapon, but at lower rates than that shown by the consortium (Bull, 1980). Several such examples were also quoted by Slater and Lovatt (1984) in their review. The possibility of exchange of genetic material coding for the pathway of degradation among members of the microbial com-

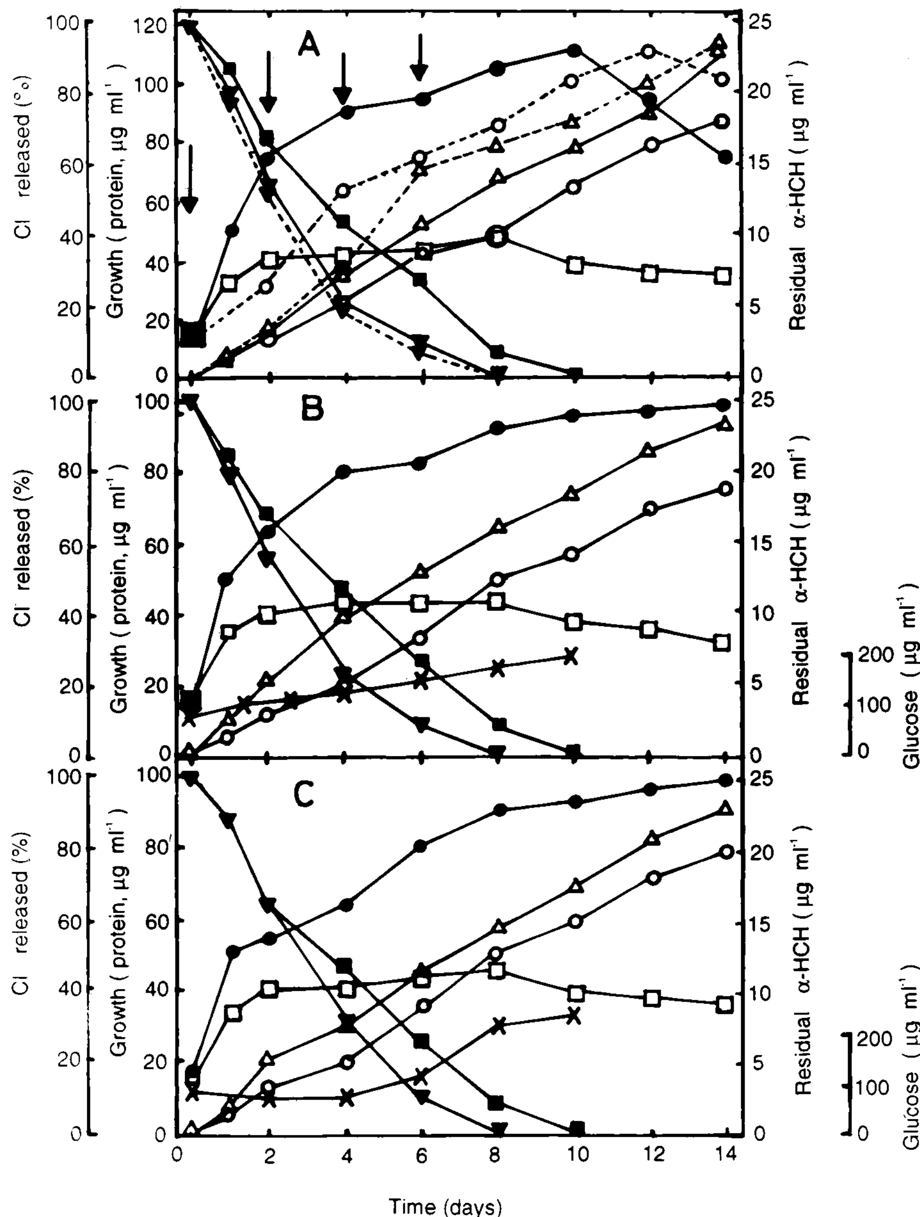

Figure 7. Low concentrations of glucose (100 μg mL^{-1}) (as an initial single dose or as four split doses of 25 μg mL^{-1} on days 0, 2, 4, and 6) (A), cellulose powder (1000 μg mL^{-1} as a single initial dose) (B), or sawdust (1000 μg mL^{-1} as a single initial dose) (C) as cosubstrate improves the degradation of 25 μg mL^{-1} of α-HCH by the unacclimated microbial consortium. Symbols used are the same as in Figures 5 and 6 except (▼) for residual α-HCH (with single dose of cosubstrate), (- -▼- -) for residual α-HCH with split doses of cosubstrate, (■) for residual α-HCH without cosubstrate (control), and (×) for glucose in the medium.

munity during the enrichment in the column in the present study cannot be ruled out (Slater and Lovatt, 1984).

Individual strains as well as different combinations of them did not show any improvement in the degrading ability even after adaptation to 10 μg mL^{-1} α-HCH through several passages. However, a reconstituted consortium containing all eight of the bacteria and the fungal strain showed the degrading ability, which improved further on acclimation (Table 3). There must be some kind of physiological synergism among the strains. Mixed microbial communities are known to be more efficient in degrading xenobiotic compounds than the individual members alone (Slater and Lovatt, 1984). For example, orcinol metabolism by *P. stutzeri* is substrate inhibited at concentrations >450 mg L^{-1}, but the activity of a three-membered bacterial community containing *P. stutzeri*, *Brevebacterium linens*, and *Curtobacterium* sp. was uninhibited by orcinol concentrations 33% higher and the rate of degradation was 20% greater than the combined rates of the individual populations (Bull and Brown, 1979). However, a thorough understanding of the biochemical pathways of each strain is required for a clear understanding of this phenomenon in the present case.

Environmental factors such as temperature, pH, redox condition, and the presence of cosubstrates are known to influence the activity of microorganisms. Bachmann et al. (1988a) have reported that temperatures in the range of 20−30 °C were most favorable for the degradation of α-HCH in a soil slurry by a natural microbial population under aerobic conditions. The optimal temperature for the degradation of γ-HCH in paddy soil under anaerobic conditions was shown to be 28 °C (Ohisa and Yamaguchi, 1979). Bhuyan et al. (1993) have reported enhanced degradation of γ-HCH in acclimated soil at temperatures of 20, 25, and 35 °C. In the present study degradation of α-HCH was ob-

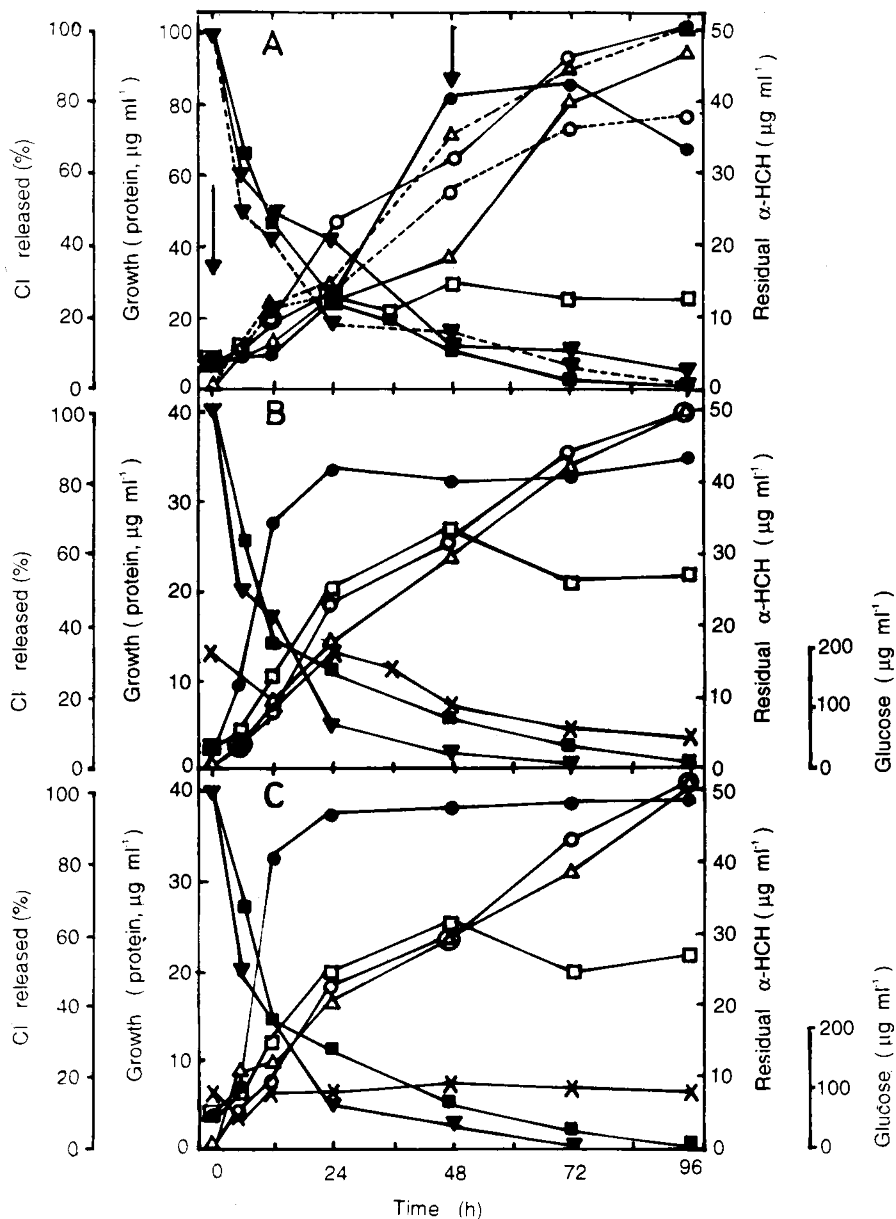

Figure 8. Effect of cosubstrate, glucose (1.6 mg mL^{-1} as a single initial dose or 400 μg mL^{-1} as split doses on days 0 and 2) (A), cellulose powder (1000 μg mL^{-1} as single dose) (B), and sawdust (1000 μg mL^{-1} as single dose) (C) on the degradation of 50 μg mL^{-1} of α-HCH by the acclimated microbial consortium. Symbols used are the same as in Figures 5–7.

served within a temperature range of 10–40 °C, the optimum being 30 °C (Figure 3).

Another major factor that can influence the biodegradability of any toxic compound in the natural environment is the likely presence of organic matter and other easily utilizable chemical compounds. Acetone was found to slightly promote the degradation of α-HCH, whereas ethanol and benzoate caused retardation of degradation, resulting in the delayed release of Cl^-. Glucose at higher concentrations (1.6 mg mL^{-1}) showed suppression of the degradation rate when an unacclimated consortium was used (Figure 6), whereas the effect was not so pronounced with an acclimated consortium (Figure 8A). Generally, retardation may occur when the cosubstrate is utilized as a preferred growth substrate. Delayed degradation of γ-HCH in an acetate-amended medium was thought to be due to the preferential utilization of acetate as a growth substrate (Sahu et al., 1993). In the present case the same thing might have happened when an unacclimated consortium was used (Figure 6). However, when the consortium was acclimated with α-HCH by repeated passages, the microbes might have lost the ability to utilize glucose efficiently as a growth substrate, resulting in better utilization of α-HCH even in the presence of glucose. This is evident from the fact that the additional growth due to glucose is not as pronounced in the acclimated consortium as in the unacclimated one (Figures 6, 7A, 8A).

Lower concentrations of glucose exerted a promotive effect (Figure 7A). A similar effect was observed when cellulose powder or sawdust was used as a cosubstrate. These materials seem to promote the degradation rate by releasing glucose in small amounts, through the enzymatic activity of some of the microbes of the consortium. Glucose was detected in the cellulose powder and sawdust-supplemented medium at concentrations of not more than 210 μg mL^{-1} throughout the culture period, clearly indicating a slow release of glucose from the added cellulosic material. Bachmann

et al. (1988a) have reported the inhibitory effect of glucose and acetate, the latter being more pronounced, on the aerobic transformation of α-HCH in a soil slurry by native microorganisms. On the other hand, Jagnow et al. (1977) have observed promotion of dechlorination of γ-HCH under anaerobic conditions in the presence of several cosubstrates, among which glucose was far more the active stimulator. Contrasting effects of acetate on the degradation of γ- and β-HCH by *Pseudomonas* sp. were shown by Sahu et al. (1993). Acetate promoted the degradation of β-HCH, whereas it distinctly retarded the degradation of γ-HCH.

CONCLUSIONS

A microbial consortium capable of aerobically degrading fairly high concentrations of α-HCH was developed. When acclimated with other isomers of HCH such as β-, γ-, and δ-HCH and technical HCH, the consortium showed the ability to degrade them also efficiently (data will be published elsewhere). Moreover, some preliminary studies done in the laboratory on the application of the consortium on contaminated soil have indicated efficient elimination of soil-borne HCH residues. With the higher degrading ability over fairly wide ranges of pH and temperature and in the presence of various organic compounds, the consortium is ideally suited for bioremediation of HCH-contaminated soils, waste dump sites, water bodies, and industry effluents.

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