Exploitation of native strains of *pseudomonas* for decontamination of HCH contaminated soils

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Abstract

Organochlorine insecticides have been extensively used in tropical countries for the control of agricultural and medical pests. Hexachlorocyclohexane (HCH) is a major organochlorine pesticide, produced and used in India. In fact, it has accounted for about 45% of the total pesticides used in India during 1988 - 89. Technical mixture of HCH contains four main isomers viz. alpha, beta, gamma and delta (Alpha, Beta, Gamma, Delta) out of which only gamma-HCH has the insecticidal properties. Gamma-HCH is volatile and does not persist in the environment for long, but the remaining isomers have a high persistence. Due to their persistence and tendency to bio-accumulate in non-target organisms, technical mixture of HCH has been banned worldwide. However, HCH contaminated sites are even now common and widespread due to the excessive and indiscriminate use prior to the ban.

Previous studies have shown that mineralisation of chemical pollutants is usually not at an environmental friendly rate thus making their dissipation almost impossible.

Bioremediation of the contaminated sites by micro-organisms is a challenging field of community research. Keeping this objective in view, an attempt has been made towards the biodegradation of HCH by isolated and subsequently acclimatised strains of genus *Pseudomonas*. The four isomers of HCH (alpha, beta, gamma and delta) belonging to the family of broad spectrum, persistent, organochlorine pesticides, were found to be degraded at a fairly rapid rate by the isolated bacteria under aerobic conditions.

Keywords

Bioremediation, HCH, acclimatisation, contaminants, *Pseudomonas*.

Introduction

The introduction of high yielding varieties not only increased the agricultural production but also transformed the agricultural environment leading to numerous pest problems due to their inability to withstand pest infestation. All this led to the increased use of pesticides in agricultural practices. Organochlorines, like HCH, due to their high efficacy, availability and economic viability became popular among the farmers in no time after their introduction in 1948 in the Indian market. The need for increasing the agricultural production grew, due to the population explosion. In the agriculture sector, it is calculated that even after the effective use of pesticides, pests continue to cause annual losses of about 21 billion dollars on a global scale. Such losses would have been many times more if persistent pesticides had not been in use (Observer, 1997). Long stability and residual activity of organochlorines, which were the desirable features turned out to be harmful, as these pesticides have entered practically every component of the ecosystem (Agarwal, 1997).

Although the use of organochlorines like HCH and DDT has been banned in most of the developed and developing countries yet gamma-HCH, due to its less persistence and high insecticidal properties has been permitted for use.

Moreover, in view of the absence of any acceptable replacement, HCH and DDT, due to their low cost and immediate benefits, continue to be officially used in vital health programmes like malaria, filariasis and kala azar etc. in India (Pesticide information 1992-93). Even where the use of only gamma-isomer containing HCH is permitted, other isomers like alpha, beta and delta are generated by chemical isomerisation until their configurational equilibrium is achieved leading to the contamination of the environment by these undesirable isomers. In the present study an attempt has been made to isolate the bacteria from the HCH contaminated site and acclimatise them to degrade all the isomers of HCH at a fairly rapid rate.

Materials and methods

The nutrient agar and nutrient broth were used as the growth media and were prepared as per Difco manual (1953). All the isomers of the insecticide hexachlorocyclohexane were of purity greater than 99%. All the other chemicals and solvents used were of analytical grade and the ingredients of growth medium were for bacteriological purpose.

Isolation of micro-organisms from soil

5 g soil sample was taken from the surroundings of the Hindustan Insecticides Limited New Delhi. The site was chosen on the basis of its prolonged exposure to the dust of HCH. Soil was transferred to Erlenmeyer flask containing 50 ml of distilled water and stirred vigorously for half an hour. The suspension was subjected to serial dilution and 10^{-6} dilution was used as inoculum. 1 ml was added to 50-ml nutrient both in Erlenmeyer flask and incubated in BOD at 33 ± 20ºC. After three days, the prepared liquid culture was serially diluted and plated on petri dishes containing nutrient agar (25 ml) with 5 µg/ml of HCH. The petri plates were incubated inverted in BOD and colonies appearing after three days were again streaked on fresh petri dishes. After 3-4 transfers, the isolated colonies were pure and were used for testing their ability to degrade HCH.

Identification of bacterial strains

The soil isolates were identified as *Pseudomonas morsprunorum* and *Pseudomonas alcaligenes* according to the biochemical criteria shown in Table 1.

Table 1. Identification of HCH degrading soil isolates

Test	*Pseudomonas morsprunorum*	*Pseudomonas alcaligenes*
Gram's Reaction	-	-
Shape	Smooth Rod	Smooth Rod
Endospore	-	-
Motility	+	+
Fluorescence	-	-
Starch hydrolysis	-	+
Nitrate reduction	-	-
Nitrite reduction	-	+
Oxidase test	+	+
Arabinose test	-	-
Lactose test	-	-
Mannose test	±	-
Maltose test	-	+
Xylose test	+	-
Growth at 42^{0}C	+	-

Cultures in experimental Erlenmeyer flasks

One ml of the pure seven days bacterial inoculum was inoculated in Erlenmeyer flask containing 50 ml of nutrient broth and 5 µg/ml or 1 µg/ml concentration of HCH-isomers on an individual basis, where all sets were in triplicate. Uninoculated nutrient broth with same concentration of HCH-isomer was kept as control. Immediately after bacterial inoculation, 2 ml of medium was extracted with 30 ml hexane and analysed for the concentration of HCH at zero time. Periodic analysis of HCH concentration was done on a regular time interval of 2 days for alpha-, gamma- and delta-isomer whereas for beta-HCH, it was 7 days.

GLC

HCH-isomers, extracted by shaking the samples with hexane, were analysed on a gas chromatograph equipped with 63Ni electron capture detector, capillary injector and a capillary column (0.25 mm id and 25 m length).

Results

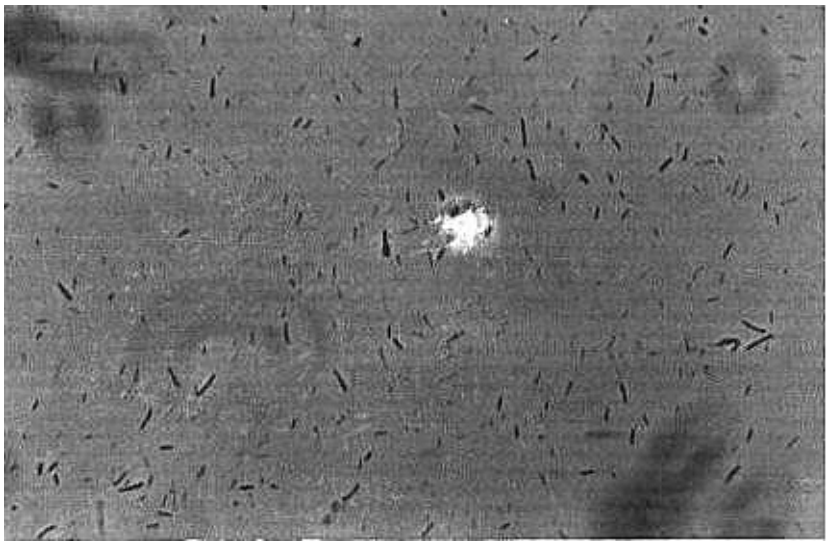
Both the isolated bacterial strains *Pseudomonas morsprunorum* and *Pseudomonas alcaligenes* were capable of degrading all the isomers of HCH viz. alpha, beta, gamma and delta. The degradation pattern observed was gradual. The strains degraded gamma-HCH at the fastest and beta-HCH at the slowest rate.

Degradation efficiency of Pseudomonas morsprunorum

The initial concentration provided for each isomer of HCH was 5 µg/ml in separate flask. The amount of alpha-HCH recovered on the 2nd, 4th, 6th and 8th exposure day was 1.592, 1.356, 0.672 and 0.489 µg/ml respectively. The corresponding percentage degradation was 68.16, 72.88, 86.56 and 90.22 of the initial concentration of alpha-HCH (Table 1). The amount of beta-HCH recovered at the 7th, 14th, 21st and 28th exposure day was 4.770, 4.280, 4.001 and 3.421 µg/ml, respectively. The gamma-HCH recovered on the 2nd, 4th, 6th and 8th exposure day was 2.557, 0.837, 0.251 and 0.056 µg/ml respectively. The delta-HCH recovery from the experimental set up of delta-HCH for the similar period was 2.456, 1.853, 1.357 and 0.282 µg/ml, respectively. The corresponding degradation per cent of all the isomers of HCH at different concentrations are shown in Table 2 and 3.

Table 2. Degradation % of alpha-HCH, gamma-HCH and delta-HCH at the initial concentration of 5 µg/ml by *Pseudomonas morsprunorum*

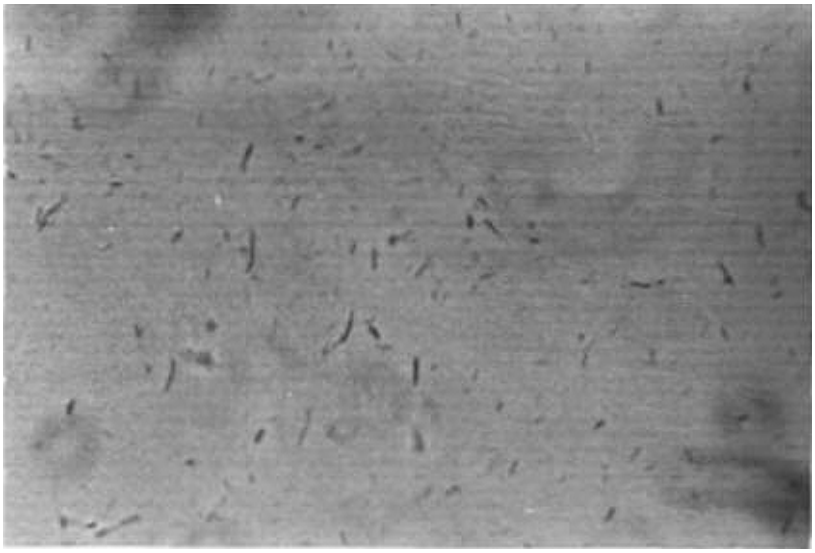
Sample No.	Exposure time (days)	alpha-HCH	gamma-HCH	delta-HCH
1	0	0	0	0
2	2	68.16	48.86	50.88
3	4	72.88	83.26	62.94
4	6	86.56	94.98	72.86
5	8	90.22	98.88	94.36



Pseudomonas morsprunorum

Degradation efficiency of Pseudomonas alcaligenes

At the beginning of the experiment, every flask was provided with one isomer of HCH at 5 µg /ml conc. and inoculated with *Pseudomonas alcaligenes*. The observations were recorded on the 2nd, 4th, 6th and 8th exposure day for alpha, gamma and delta-HCH whereas in case of beta-HCH the exposure time was 7, 14, 21 and 28 days.



Pseudomonas alcaligenes

The amount of alpha-HCH recovered was 4.042, 2.534, 1.773 and 1.412 µg/ml on the 2nd, 4th, 6th and 8th exposure day respectively. The amount of beta-HCH recovered was 4.774, 4.501, 3.624 and 3.526 µg/ml on the 7th, 14th, 21st and 28th exposure day, respectively. The amount recovered for gamma-HCH was 4.051, 1.004, 0.351 and 0.115 µg/ml on the 2nd, 4th, 6th and 8th exposure day, respectively while that of delta-HCH was 2.246, 1.148, 0.772 and 0.521 µg/ml respectively, for the same period.

The degradation % of all of the HCH-isomers at a concentration of 5 µg/ml in the flask inoculated with *Pseudomonas alcaligenes* is shown in Tables 3 and 4.

Table 3. Degradation % of beta-HCH at the initial concentration of 5 µg/ml by *Pseudomonas morsprunorum* and *Pseudomonas alcaligenes*

Sample No.	Exposure time (days)	P. morsprunorum	P. alcaligenes
1	0	0	0
2	7	4.6	4.52
3	14	14.28	9.98
4	21	19.28	27.52
5	28	31.58	29.48

Table 4. Degradation % of alpha-, gamma- and delta-HCH at the initial concentration of 5 µg/ml inoculated with *Pseudomonas alcaligenes*

Sample No.	Exposure time (days)	alpha-HCH	gamma-HCH	delta-HCH
1	0	0	0	0
2	2	19.16	18.86	55.08
3	4	49.32	79.92	77.04
4	6	64.54	92.86	84.56
5	8	71.76	97.70	94.96

Degradation efficiency of both the bacterial strains was also tested at the lower concentration of different isomers of HCH (alpha, beta, gamma and delta). Both strains were equally effective in degrading the lower concentration of 1 µg/ml of HCH. The degradation rates were almost comparable at both concentrations, of 5 µg/ml and 1 µg/ml though in a few cases it was higher at lower concentration. The amounts of different isomers of HCH degraded by these bacterial isolates at the initial concentration of 1µg/ml are shown in Tables 5 to 7.

Table 5. Degradation % of alpha-, gamma- and delta-HCH at the initial concentration of 5 µg/ml at the initial concentration of 1 µg/ml inoculated with *Pseudomonas morsprunorum*

Sample No.	Exposure time (days)	alpha-HCH	gamma-HCH	delta-HCH
1	0	0	0	0
2	2	24.70	85.10	17.50
3	4	77.10	93.60	64.20
4	6	83.90	100	75.90
5	8	91.70	ND	83.40

ND - not determined

Table 6. Degradation % of beta-HCH at the initial concentration of 1 µg/ml inoculated with *Pseudomonas morsprunorum* and *Pseudomonas alcaligenes*

Sample No.	Exposure time (days)	P. morsprunorum	P. alcaligenes
1	0	0	0
2	7	12.90	8.20
3	14	31.90	12.50
4	21	43.80	17.50
5	28	50.30	29.10

Table 7. Degradation % of alpha-, gamma- and delta-HCH at the initial concentration of 1 µg/ml inoculated with *Pseudomonas alcaligenes*

Sample No.	Exposure time (days)	Initial conc. of 5 µg /ml		
		alpha-HCH	gamma-HCH	delta-HCH
1	0	0	0	0
2	2	22.80	60.0	20.20
3	4	59.90	100	70.10
4	6	83.10	ND	80.90
5	8	89.20	ND	90.60

ND - not determined

Discussion

The enrichment culture method is used to isolate the micro-organisms. Each particular set of conditions, induces a specific group or micro-organisms to predominate due to it's ability to grow more rapidly than any other organism present in the inoculum.

Lindane (gamma-HCH) is the only isomer in the formulation, which is insecticidal and observed to be less persistent. This resulted in the most of the work being confined to the degradation of gamma-HCH (Heritage and Mac Rae, 1977; Ohisa and Yamaguchi, 1978; Senoo et al., 1996). Earlier, anaerobic conditions were reported to be more favourable for the degradation of HCH-isomers (Siddaramappa and Sethunathan, 1975) but recent reports showed aerobic conditions to be more favourable for HCH degradation (Sahu *et al.*, 1992; Senoo *et al.*, 1996).

Both the bacterial strains reported in this study are aerobic and belong to the genus *Pseudomonas*, which is the most common genus reported in literature for the degradation of aromatic halogenated hydrocarbons. In this study, the initial lag period for the degradation of the HCH-isomers was not observed. These strains have been highly adapted to the HCH environment for more than two years and the initial lag in degradation becomes shorter with successive application of the pesticide due to the enrichment of the pesticide degrading micro-organisms (Sethunathan *et al.*, 1976). Both the strains were capable of degrading the HCH even when initial concentration provided was low (1 µg/ml). These observations are similar to the results reported by Matsumura (1982). The report stated that higher concentration of HCH can force the microbes to degrade and utilise the molecules but once the bacterial strains adopt to degrade HCH, they can continue to chew up even if the concentration is low. No metabolite could be detected during the degradation. So it can be concluded that either metabolites were not formed or they were more volatile than the parent compound and escape in the environment soon after their formation.

The beta-isomer of HCH is the most recalcitrant of all the isomers due to its stable, strainless spatial configuration, hence it is very less responsive to the microbial degradation. In the present study also, beta-HCH was the slowest isomer to be degraded but P. *morsprunorum* could degrade it to the extent of 31.58 % on the 28th day of exposure. The degradation observed for *Pseudomonas alcaligenes* was 29.48 %,slightly lower than that observed for *Pseudomonas morsprunorum*. Degradation of HCH has been shown by Ohisa *et al.* (1980) to take place rapidly in the early growth phase of bacteria, but slowly in the resting stage e.g. spore formation. This may explain as to why degradation of HCH-isomers is very fast at some point of time and slow at the other in the present investigation even though the observations were made at similar intervals.

The identification of microorganisms with an ability to degrade common environmental contaminants is an important step in increasing the understanding of pollution problems and the possible solution. Further, elucidation of the metabolic pathway involved in this degradation is under way which will help us to manipulate or maximise them for use in the waste treatment and bioremediation of contaminated sites.

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