SUMMARY

The pollution of soil and ground water with hexachlorocyclohexane (HCH)-a chlorinated insecticide has caused serious environmental problems due to extensive use from 1950 till late 1980 in agriculture and health sector to control the vector borne diseases. Industrial synthesis of HCH isomers is carried out by chlorinating the benzene under ultra violet (UV) light and this process generates four major isomers of HCH which are known as α-, β-, γ- and δ-HCH. Due to recalcitrant nature the isomers bio accumulate, undergo biomagnification in food chain thus impart toxicity to human and other organisms. The toxicity manifestations include endocrine disruption, blood disorders and cancer. The production in large quantities and the indiscriminate use of HCH, led to the contaminated sites around the world that require remedial measures.

Biodegradation of HCH

Several studies have reported biodegradation of HCH isomers by microorganisms and utilization as sole source of carbon and energy. Under anaerobic conditions the γ-HCH has been shown to form tetra-, tri-, mono-, chlorobenzenes and benzene. Under methanogenic and flooded soil conditions extensive degradation of HCH isomers has been reported. Recently several pure culture bacteria with the ability to biodegrade all four major isomers of HCH were isolated and characterized. Most of the HCH-degrading aerobic bacteria were found to be members of the family Sphingomonadaceae. While many of the bacteria has been studied for degradation kinetics of HCH isomers, only two different strains have been extensively and intensively studied for 1) degradation of major HCH isomers, 2) degradation in contaminated soil, 3) genes and enzymes involved in the degradation of γ- and β-isomers, 4) identification of metabolites, 5) presence of plasmid, and 6) genomic organization of the strain and characterization of the bacteria by polyphasic approach.

Outcomes from the thesis investigation

In the present study, the isolation of a bacterium that is able to biodegrade α-, β-, γ- and δ-HCH, its identification, characterization of genes linA, linB, linC, linD, and
linE involved in upper degradation pathway of γ-HCH is reported. Three metabolites, γ-pentachlorocyclohexene, 1,2,4-trichlorobenzene and chlorohydroquinone from γ-HCH degradation and δ-pentachlorocyclohexene formed during the δ-HCH degradation were identified and characterized. The efficiency of the bacteria to degrade all the four isomers in presence of three surfactants was evaluated. Microbial diversity and detection of the dehydrodechlorinase (linA) gene which initiate the HCH degradation was studied in three different contaminated soils. The observations made in the aforesaid studies are presented.

**Isolation and characterization of HCH degrading bacterium**

A microtitre based colorimetric method was adopted for the selection of bacteria with the ability to biodegrade HCH isomers. The change in pH was used to detect the biochemical activity of dechlorination enzymes. The assay on substrates for the detection of dehalogenase activity is based on the pH decrease in a weakly buffered medium having phenol red as an indicator dye. Change is caused by protons released during the enzymatic reaction using HCH as substrate. From a HCH contaminated soil sample, 14 visibly distinguishable bacteria were obtained. After a single enrichment of these cultures on 0.34 mmol l⁻¹ HCH, the crude cell free extract was used for dehalogenation activity on all four isomers and few reported HCH metabolites. Cell free extract from one of the cultures exhibited rapid dechlorination i.e., turning the phenol red indicator dye to orange yellow. The color change was rapid on all four HCH-isomers and the substrate preference for the enzymes were in the order γ>, δ>, α> and β. The other 13 bacterial extracts and reference control wells did not show any color change. The dechlorination activity was absent on 1,2,4-trichlobenzene and 2,5-dichlorophenol which are the reported dead end products of γ-HCH degradation pathway but chlorohydroquinone underwent dechlorination. The dehalogenation activity was not detected on compounds such as hexachlorobenzene, pentachlorophenol and 2,4,5-trichlorophenol. Other abiotic controls in the assay remained unchanged.

**Assay for HCH clearance on solid minimal medium plate**

To confirm the dehalogenase positive bacterium further for its ability to biodegrade HCH, the bacterium was streaked on minimal medium agar plate and
sprayed with an ethereal solution of \(\gamma\)-HCH. A clearance zone appeared after 4 days of incubation and the zone of clearance increased on further incubation. This method further confirmed the ability of the isolated bacterium for degradation of HCH.

**Identification of the bacterium**

Isolated bacterium was identified by biochemical characteristics as well as by its 16S rRNA sequencing. Colonies on tryptic soy agar (TSA) were pale-yellow pigmented, about 1-3 mm in diameter, circular and smooth. Catalase and oxidase positive and was able to grow between 20 and 42°C. The 16S rRNA gene was PCR-amplified to yield 1484 bp-product, and sequencing gave 1406 unambiguous bases. The sequence similarity search in Genbank showed that the bacterium is a member of the genus *Sphingomonas* with highest similarity to a chlorophenol degrading *Sphingomonas subantarctica* sp nov. The bacterium was designated as strain NM05.

**Genetic analysis and sequencing of lin genes**

To elucidated the DNA sequences polymerase chain reaction (PCR) technique was used to amplify *lin* genes using the strain NM05 genomic DNA as a template and homologous sequence primers from *Sphingobium indicum* strain B90 amplification products obtained were of 471, 891, 753, 1041 and 966 bp for *linA*, *linB*, *linC*, *linD* and *linE* genes, respectively. We have also used the plasmids harbouring *linA*, *linB*, *linC*, *linD* and *linE* gene constructed from the *Sphingobium japonicum* strain UT26 as positive controls which yielded same size of amplicons. Nucleotide sequences of PCR products when compared in the GenBank database revealed 97–100% identity to the respective *lin* genes present in other HCH degrading bacteria. PCR products when used as gene probes against the genomic DNA of strain NM05 produced cross-hybridizing fragments for all the five *lin* genes tested. The Southern hybridization results confirmed the presence of upper pathway genes in the isolated strain. The genomic DNA NM05 strain obtained by growing them in liquid LB medium, when used for Southern hybridization did not show cross hybridizing DNA bands with any of the *lin* probes tested. Also, DNA from LB-grown cells when used as a template, no PCR product was amplified for *lin* genes. These results are presumably due to the loss of genes when grown on rich medium such LB in this case.
Degradation of HCH by Sphingomonas sp NM05 and metabolites formation

Strain NM05 was tested for its ability to degrade α-, β-, and γ- and δ-HCH separately containing 0.34 mol l-1ml (100 μg ml 1) in minimal medium. The initial inoculum of 0.1 OD increased during the incubation and rapid disappearance of HCH isomers were noted. In 5 days 90% of α-, β-, and δ-HCH and 60% of β-HCH was obtained as quantified by gas chromatography. Thus the degradation rate of β-isomer was found to be slower than the other isomers. Gamma-HCH degradation by strain NM05, by dehydrochlorination resulted in the formation of various metabolites including the three metabolites identified such as γ-Pentachlorocyclohexene (γ-PCCH), 1,2,4-Trichlorobenzene (TCB) and chlorohydroquinone (CHQ). Similarly, NM05 when grown on δ-HCH degraded it via dehydrodechlorination to form δ-pentachlorocyclohexene (δ-PCCH).

Diversity of bacteria from HCH contaminated sites

To understand the microbial communities prevalent in chlorinated pesticides contaminated sites we have studied bacterial diversity using RFLP method. Soil samples collected from three different geographic locations in India (Lucknow, Nagpur, Cochin) were analyzed for chemical and physical analysis. Two soils were of alluvium (Cochin, Lucknow) and the third soil was black clay (Nagpur). The carbon/nitrogen/organic contents were around 7:1:10 ratios in all the samples. All the three soils investigated in this study had residues of any or all of the chlorinated pesticides DDT, endosulfan and HCH. Among the three, soil from Hindustan Insecticides Limited had all three pesticides and also at higher concentrations.

Bacterial community profiling was obtained via total soil DNA-based PCR amplifications of 16S rRNA genes using universal Eubacterial primers. Clone libraries of the 16S rRNA gene PCR amplicons were subjected to restriction fragment length polymorphism (RFLP). The DNA band on agarose gels were clustered based on their RFLP patterns. Bacterial community profiles were obtained by sequencing one of the representative clones and the phylogenetic profiles were generated accordingly. The community structure from each soil revealed that the unculturable bacteria were also present in almost equal ratio compared to the culturable bacteria. In HIL soil, the
unculturable bacteria dominated with 17 phylotypes, only major culturable bacteria belonged to *Desulfuromonas michiganensis* AF 357915.2, *Acetonema longum* AJ 010964.1 and a *Flavobacteriaceae* bacterium TDMA-34 AB 264128.1, were of culturable and similar to degraders of chlorophenols and organophosphorous chemicals. Interestingly two uncultured clones showed sequences similar to benzene mineralizing consortium. A maximum number of 20 different phylotypes were observed in IPL samples which were dominated by the unculturable bacterial communities. The sample which had relatively lower level of pesticide contamination exhibited mostly the culturable bacteria. Many gram-positive bacteria such as *Clostridium* were present in this IPL soil. Significantly, a high number of bacterial species involved in the biodegradation of monoaromatic compounds such as pentachlorophenol, benzene, toluene, trichlorophenol, 2,4-dichlorophenoxy acetic acid (2,4-D), PCBs and HCH were observed.

**Screening of linA gene variants from soils**

Occurrence of *linA* coding for the dehydrodechlorinase in γ-HCH degradation pathway was evaluated using the metagenomic soil DNA from the three contaminated sites. Most of the bacteria reported for HCH degradation had highly identical *linA* genes in them, although from few strains there were two types of *lin* genes designated as *linA1/*linA2. To understand the degradation potential of three sites, we have PCR-amplified *linA* genes from 11 different samples of the three sites. An expected 471bp PCR product was obtained from 8 samples of HIL and IPL sites. The DNA sample of Nagpur soil did not yield any amplification even after repeated efforts. DNA sequences of the eight *linA* genes obtained from soil samples showed minimum 92% and maximum 98% similarity *linA* gene reported from 11 HCH degrading bacteria.

The cluster analysis of *linA* amino acid sequences revealed maximum 12 amino acid differences in one of the variants. The *linA* variants were found to be highly similar to *linA* gene sequences of strain UT26. Although it would be interesting to study the expression patterns these modified *linA* for dehydrodechlorinase activity using γ-HCH as substrate based on the modification of the ratio of strongly basic, strongly acidic, hydrophobic, and strongly polar amino acids in its structure.
**Effect of Biosurfactants on Solubility of HCH isomers**

Chlorinated pesticides HCH, DDT Endosulfan are extremely hydrophobic, with low octanol-water partition coefficients. The recalcitrance is a function of the synthetic nature of the molecules and thus diverse organisms have not evolved to biodegrade them. Due to hydrophobicity, the HCH isomers result in intimate binding to the soil organic matter, making remediation difficult and expensive. Therefore, we have investigated the effect of three biosurfactants rhamnolipid, sophorolipid and trehalolipid for their dissolution capacity under liquid and soil slurry conditions. Increased solubilization of HCH was obtained when the surfactant concentration was in the range of 40-60 µg/ml. Higher concentration of surfactant beyond 60 µg/ml did not show any further increase in dissolution of HCH isomers. We have used equal concentrations of HCH isomers at 200 ppm (0.68 µM), which was several fold (α-HCH, 10; β-HCH, 20; γ-HCH, 5; and δ-HCH, 10 folds) higher than the reported aqueous solubility of isomers (10, 5, 17, 10 ppm of α-, β-, γ-, and δ-HCH respectively). Addition of surfactants in minimal medium led to approximately 3-9 fold increase in the solubilization of HCH isomers. Interestingly the sophorolipid showed highest solubility for α-HCH which was 40.4% and 35.6% higher than rhamnolipid and trehalose respectively.

**Effect of Biosurfactants on Biodegradation of HCH isomers in Liquid Culture by strain NM05**

Effect of surfactants on the biodegradation of all four HCH isomers was studied. In the absence of surfactant, biodegradation of HCH isomers by *Sphingomonas* sp strain NM05 HCH was found low. After 10 days of incubation, approximately 60% of 200 ppm (0.68 µM) degradation of HCH isomers was achieved without surfactants. However addition of surfactant resulted in degradation of 95 % of the HCH provided to strain NM05. An enhancement of 35% of degradation in presence of surfactant was highly significant. It is important to note that this increase in degradation was also achieved in 4 days when compared to the 10 days of degradation in the absence of surfactant. The enhanced degradation was achieved in the presence of 40 µg/ml concentration of surfactant. It can be was observed that HCH dissolution was increased
several fold in presence of surfactants. Among the three, sophorolipid was found to show the highest solubilization and biodegradation of HCH isomers.

**Biodegradation of HCH isomers in Soil in presence of Surfactants**

Many surfactants have potential for use in improving remediation in sites where biodegradation is affected by rate-limited desorption and dissolution of the contaminant. Therefore, three biosurfactants were evaluated for desorption of the HCH pesticides from the soils. In the absence of surfactant, in soil, the HCH degradation by *Sphinogomonas* sp NM05 was comparable in all the flasks. After 30 days of incubation without surfactant, the residual of HCH isomers were in the range of $\alpha$-57-63, $\beta$-88-100, $\gamma$-28-34, and $\delta$-44-52 $\mu$g /ml as analyzed by gas chromatography. The degradation efficiency in the presence of strain NM05 was around 80-95% as compared to the control where bacteria and surfactant was not added. However, the results from surfactant amended medium showed significant enhancement in degradation of HCH by all the three biosurfactant studied. Among these, rhamnolipid and sophorolipid showed 95% degradation for all the isomers except for $\beta$-isomer, for which the degradation was 10% less than other HCH isomers. The results of these studies such as dissolution, degradation in liquid culture and soil showed that surfactants greatly enhanced the degradation of HCH by improving bioavailability. The observations in this study also indicate that sophorolipid may be the most suitable biosurfactant as its addition showed the highest solubilization capacity leading to enhanced degradation in liquid culture and under soil conditions.