Discussion

HCH degradation by *Sphingomonas* strain NM05

The studies embodied in this thesis present different aspects of HCH biodegradation using the isolated *Sphingomonas* sp NM05 from a contaminated site. The study also includes understanding of microbial diversity at three sites contaminated with HCH from different regions of India. The poor solubility of HCH due to its hydrophobic nature was a major concern and has aroused scientific interests to use both chemical and biological agents for enhancing the bioavailability of HCH. Thus the effect of three biosurfactants on HCH dissolution and biodegradation was evaluated under different conditions. Earlier studies with HCH-contaminated soils suggested that degradation was faster under anoxic conditions and that microbial degradation was the primary route of HCH disappearance from soil (MacRae *et al.* 1967). Microbial degradation of the four common isomers has since been observed under oxic conditions, both in soil (Bachmann *et al.* 1988b; Doelman *et al.*1985; Sahu *et al.* 1993) and in pure cultures of microorganisms (Bhuyan *et al.* 1993; Thomas *et al.* 1996). Accordingly the previous reports deal with isolation and characterization of microorganisms able to metabolize HCH by enrichment method. Usually the enrichment studies were performed for few weeks to several months or years under laboratory culture conditions to obtain bacteria which use HCH as sole source of carbon and energy. This study reports a bacterium, screened based on dehalogenase enzyme assay for the ability to biodegrade all the four major isomers of HCH.

A *Sphingomonas* sp., designated as strain NM05, has been isolated and identified as hexachlorocyclohexane-degrading alphaproteobacterium from a HCH contaminated industrial site. Isolation of microorganisms capable of degrading a specific xenobiotic compound via culture enrichment is often a long and tedious process, in which the slow or poor degrader was lost. Isolation of the bacteria based on degradative enzyme activity like the one used in the present study, leads to direct screening and identification of useful bacteria. Previously, Phillips *et al.* (2001) used the colorimetric assay against the known γ-HCH degraders *S. paucimobilis* UT26 and *S.*
francenses. However we employed this technique to screen bacteria possessing favorable degradation activity based on many dehalogenation enzymes expected to be present in chlorinated compounds degrading bacterium. We optimized the previously developed colorimetric microplate method for activity testing with haloalkane dehalogenase linB by two different research groups (Philips et al. 2001, Marvanov et al. 2001). In the experimental design, we used other chlorinated substrate such as hexachlorobenzene, pentachlorophenol and many other substituted chlorinated metabolite reported from the biodegradation pathway. Understandably, this method can be applied to screen large number of bacteria possessing dechlorination activity from diverse contaminated samples. Also since this method can be employed using microtitre plates in a high throughput fashion to score for the positive cells having dechlorination capabilities from a mutants or engineered bacterium.

The observation of γ-HCH clearance zone around the colonies strain NM05 further confirmed the HCH degradation ability. The clearance zone formation indicates that the dehalogenase enzyme was extracellular. Thomas et al. (1992) have confirmed that the culture supernatant of a γ-HCH degrading bacterium had the dechlorination activity and observed that 90% of the added chloride production into the medium was measured, indicating the extracellular nature of the dehalogenase. Such a property can also be used to screen HCH degradative bacteria from environmental samples and also be used to detect clones carrying dechlorination genes constructed from a genomic library. However the linA and linB genes both coding for different types of dehalogenases, were not detected in the extracellular fraction, indicating that LinA and LinB are not secreted extracellularly. From Sphingobium japonicum UT26, these two genes were found to be localized in periplasmic space of the bacterium as studied by immune-electron microscopy (Nagata et al. 1999). Since the elimination of halogens from halogenated xenobiotic molecules is a key step in their degradation these compounds may enter the periplasm through non-specific porins in the outer membrane. Since dehalogenases degrade complex halogenated molecules into simpler ones for utilization and possibly for detoxification, the localization of dehalogenases in the periplasmic space seems reasonable (Nagata et al. 1999). In general, the plate based substrate clearance method can be widely applicable for the isolation of bacteria able to
degrade other nonvolatile, water-insoluble, biologically inert, and solid compounds such as high molecular polyaromatic hydrocarbons. Also this spraying method can be adopted for the detection of auxotrophic mutants of such bacteria.

**HCH degradation and metabolite characterization**

Degradation of all the four isomers of HCH including the persistent but less soluble β-and δ-HCH were also dechlorinated by the strain NM05 as in the previously reported isolates (Nagata et al. 2005; Sharma et al. 2006; Ito et al. 2007). Both the strains B90 and UT26 were able to grow with HCH concentration 0.17 mol l\(^{-1}\) in liquid cultures. But NM05 could grow on double the concentration which is 0.34 mol l\(^{-1}\) for all the isomers. The higher degradative ability of our isolates may be attributed to its adaptation to high concentrations of HCH from where the sample was collected. In fact, the soil from where the bacterium was isolated had HCH concentrations of 50 mg/g soil.

We identified γ-PCCH, 1,2,4-TCB and CHQ (Figure 14 A) as metabolites of γ-HCH in the strain NM05. Among them 1,2,4-TCB is reported to be dead-end product and thus accumulates in the culture medium (Nagata et al. 1999). Presence of lin genes and formation of less chlorinated metabolites from γ-HCH was an indication that a pathway for the degradation of γ-HCH is operative in the strain NM05. The metabolites also confirm that degradation was catalyzed by the dehalogenase activity based on which the degradative bacterium was isolated. We have also characterized the 5 upper pathway genes involved in biotransforming the HCH from six chlorinated state to single chloride state of chlorohydroquinone (CHQ). The first reaction is dehydrochlorination of γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via γ-pentachlorocyclohexene (γ-PCCH). The second reaction is hydrolytic dechlorination of 1,4-TCDN to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). The third reaction is reductive dechlorination of 2,5-dichlorohydroquinone (2,5-DCHQ), which is produced from 2,5-DDOL by a dehydrogenase (LinC) to hydroquinone (HQ) via chlorohydroquinone (CHQ) Because 2,5-DCHQ is mineralized by strain UT26, the pathway via 2,5-DCHQ is considered to be the assimilation pathway which is very similar to γ-HCH in strain UT26.
Hydroquinone produced from 2,5-DCHQ by LinD is ring-cleaved by a novel type of dioxygenase (LinE) to \(\gamma\)-hydroxymuconic semialdehyde (\(\gamma\)-HMSA) CHQ is directly ring-cleaved by LinE to form acylchloride possibly as reported in UT26 (Nagata et al. 1999). Since the entire upper pathway \(lin\) genes from NM05 showed DNA sequence similarity above 92% to the corresponding \(lin\) genes of other reported strains, we did not characterize the lower pathway genes (\(lin\)GH, \(lin\)J and \(lin\)I) from our isolate. The existence of multiple copies, stability and the location of \(lin\) genes (Kumari et al. 2002), distribution (Nagata et al. 2006), role of large plasmids (Cérémonie et al. 2006) and \(IS6100\) elements (Dogra et al. 2004) from strain NM05 are focus of future investigations.

Before 1980s, it was largely believed that HCH degradation occurs only under anaerobic conditions (Jagnow et al. 1977, Ohisa et al. 1980, Tu 1976). The predominant bacteria studied include \textit{Clostridium rectum} (Ohisa et al. 1978), \textit{Clostridium sphenoides} (MacRae et al. 1969), \textit{Clostridium butyricum}, \textit{Clostridium pasteurianum}, \textit{Citrobacter freundii} (Jagnow et al. 1977), \textit{Desulfovibrio gigas}, \textit{Desulfovibrio africanus}, \textit{Desulfococcus multivorans} (Boyle et al. 1999), and a \textit{Dehalobacter} sp. (van Doesburg, et al 2005). Recently several aerobic bacteria with the ability to biodegrade HCH under aerobic conditions were isolated and characterized (Lal et al. 2010). Though several soil microorganisms capable of degrading HCH were reported, indisputably the most aerobes isolated were members of family Sphingomonadaceae (Lal et al. 2005; Lal et al. 2008). Though we have investigated a soil samples from geographically far away from several other soils, the bacterium we have obtained also was a \textit{Sphingomonas} species. A number of other recent bacteria for HCH-degradation were also \textit{Sphingomonas} strains and their phylogenetic diversity highlights the importance of this genus in the degradation of HCH (Mohn et al. 2006b, Böltner et al. 2005). The microarray and DGGE finger printing also supported the presence of \textit{Sphingomonas} members in different HCH contaminated soils (Neufeld et al. 2006). Interestingly, the strains originating from geographically different regions have similar genotypes for \(\gamma\)-HCH degradation pathway (Nagata et al. 1999, Thomas et al. 2000, Kumari et al. 2002). With the existing information, it is believed that the horizontal gene transfer due to plasmids, transposon and other conjugative elements resulted in isolation of similar
lin genotypes mostly from *Sphingomonas* species worldwide (Lal et al. 2006). Alternatively, avoiding the traditional culturing method, a metagenomic library constructed (Rondon et al. 2000) from HCH contaminated environmental DNA (Boubakri et al. 2006, Mohn et al. 2006b) may result in the identification of novel genotypes. In the light of the present studies, it will be remarkable if an alternate and distinct HCH degradation pathway can be identified from bacteria occupying diverse environmental niches.

**Bacterial Diversity in HCH contaminated soils**

Interestingly, one of the clusters (rooted early) has two uncultured clones showing the similarity to the results indicate that the bacterial communities may adopt differently under high selection pressure due to the presence of varieties of hydrocarbons. All the three soils reflect the dominant composition of non-culturabe fractions of soil microbiota suggesting that their cultivation is still a challenge to the researchers. The type of bacteria present in the soil samples was drastically different from each other for example the representative organisms belonging to benzene mineralizing consortium which were characterized (HIL) from a marine source (Phelps et al. 1998) and *Saltmarsh* clones (IPL). We could also obtain a pure culture isolate capable of metabolizing alpha-endosulfan into its non-toxic metabolites by a *Pseudomonas* species (Bajaj et al. 2010). For endosulfan degradation, this finding is novel as previously a *Mycobacterium* and *Arthrobacter* were reported (Sutherland et al. 2002) for the degradation of β-endosulfan only. A recent study using 16S rRNA gene sequences as probes in microarray based detection revealed distinct phytoprotypes mainly *Sphingomonas* sp in HCH-contaminated samples (Neufeld et al. 2006). The results also revealed that HCH has influence on soil parameters and microbial community composition. Mohn et al. (2006) have successfully isolated several HCH-degrading organisms from the HCH polluted soil from Bilbao, Spain that were members of the genus *Sphingomonas*. Their study based on denaturing gradient gel electrophoresis (DGGE) experiments detected single predominant band corresponding to a unique *Sphingomonas* phylotype from the soil sample contaminated with α-HCH. Another interesting study using the HCH-contaminated soil from dumping site in Chemnitz,
Germany also revealed the existence of HCH-degrading Sphingomonas strains (Boltner et al. 2005).

Our studies provide results on amplification of \textit{lin}A gene directly from environmental DNA. The sequences of all 8 PCR products for \textit{lin}A were highly identical to those HCH degradation isolates from diverse environments. These findings strongly suggest that the gene might have originated from culturable organisms as all the HCH degrading strains followed same degradation pathway. Recently many research groups reported the isolation of strains of HCH degrading \textit{Sphingomonas} species harboring highly conserved \textit{lin} genes in the complete pathway as in strains UT26 (Nagata et al. 1999), B90 (Kumari et al. 2002), NM05 (DQ767898), ITRC-5 (Singh et al. 2007). Though there are no reports dealing with the amplification of the other 5 \textit{lin} genes (\textit{linBCDEF}) from environmental culture or metagenomic DNA. The abundance of microbial communities occurring in diverse contaminated soils is required to understand the response of indigenous communities to the pollution. The approaches taken in this study represent attempts to simultaneously select the functional genes important for the catabolism of chlorinated pesticides pollution. The data obtained here shows a successful attempt to provide bacterial community structure and the prevalence of \textit{lin}A gene at specific Indian environments.

In this study the microbial diversity of three geographically different industrial sites contaminated with wide varieties of pollutants have been established. The results based on the 16S rRNA sequencing suggest that the microbial communities changed based on their habitat. In total ~64 % of the total phylotypes obtained were representing the unculturable microbial communities.

\textit{lin}A gene variants

Most significantly we have determined the presence of many variant forms of \textit{lin}A gene coding for a dehydrodechlorinase from two different soil metagenomic DNA. \textit{lin}A gene is involved in conversion of \(\gamma\)-hexachlorocyclohexane into 1,2,4-trichloronbenzene via \(\gamma\)-pentachlorocyclohexene, which is a persistent chlorinated pollutant present many places in world wide. The \textit{lin}A genes isolated and characterized from various parts of the world shared more than 98 % nucleotide homology with
changes in very few amino acid positions (Boltner et al. 2005). Our results along with previous reports suggest that linA gene is still evolving under different substrate pressures and it can be reasoned that these differences may leads to conformational changes in catalytic pockets between the linA variants (Nagata et al. 2007). Therefore cloning, expression and kinetic analysis of linA protein in heterologous hosts is desirable and will pave way to select the suitable linA form for the application enzymatic bioremediation.

**HCH solubilization**

This study demonstrates the effect of three structurally different biosurfactants on HCH biodegradation. A rhamnolipid reported to exhibit relatively high surface activity (Mata-Sandoval et al. 2000; Mata-Sandoval et al. 2002; Zhang et al. 1997; Desai and Banat 1997), a sophorolipid with moderate activity (Gobbert et al. 1984; Hommel et al. 1987) and a trehalose-containing lipid with mild activity (Rapp et al. 1979; Sifour et al. 2007) were employed to study solubilization and consequent enhancement of biodegradation of a highly persistent chlorinated pesticide. The critical micelle concentration (CMC) was found to be in the range of 40-60 µg of biosurfactant rendering 3-9 fold enhanced solubilization of HCH pesticide in liquid medium. The CMC values were derived based on previous studies on other hydrophobic compounds (Mata-Sandoval et al. 1999). It was observed that sophorolipid dissolved 90 µg/mL of α-HCH which is nearly 2 times higher than rhamnolipid (53.7 µg/mL) and trehalose-containing lipid (58.1 µg/mL) surfactants. Similarly, rhamnolipid showed increased solubility for β- and δ-HCH unlike the other two surfactants. These results also suggest that each surfactant exhibited a specific affinity to HCH isomers, possibly due to partitioning of substrate for micelles formation. Despite the difference in solubilization capacity among the surfactants, approximately 3-9 folds (Table 6) increase in dissolution of the HCH isomers was obtained for all the surfactants. However, it should be noted that no biosurfactant under investigation clearly showed higher solubility (Table 6) for all the HCH isomers. This may be due to their structural properties, aqueous phase thermodynamics and spatial arrangement of the chloride molecules in HCH isomers.
Surfactant mediated enhanced degradation of HCH isomers in liquid culture by *Sphingomonas* sp strain NM05

Increased solubilization of isomers, in presence of surfactant concurrently enhanced (30-50%) biodegradation by *Sphingomonas* sp strain NM05. The rate of biodegradation also increased as the time reduces from 10 days to 2 days for the same extent of degradation. However, it is important to note that this enhancement was achieved in 2 days of incubation in contrast to the time frame of 10 days in the absence of surfactant. The degradation of all the isomers showed enhancement, however, α-, β- and δ-HCH degradation had initial lag phase till 6 days in presence of rhamnolipid and sophorolipid respectively. In contrast, a uniform degradation pattern by the strain NM05 was observed for all four isomers in presence of trehalose surfactant (Figure 21).

These results suggest that among all the three surfactants used, sophorolipid displayed higher solubilization and degradation capability for all the HCH isomers. It is also noteworthy that during the batch culture studies using strain NM05, there was an increase of about 0.17 OD$_{600}$ in total bacterial biomass when sophorolipid was added than either of the other surfactant. This response was due to the enhanced growth in presence of surfactant and consequently enhanced degradation in 8 days (Figure 21). The presence of surfactant may either facilitate the bioavailability of the pesticide or there is a possibility of stimulating the metabolic machinery whereby both HCH and biosurfactant served as a substrate for the growth of the bacteria or it may affect the bioaccessibility by increasing dissolution of HCH (Semple *et al.* 2004). However, with the data obtained it is not possible to conclude as to which biosurfactant, under culture conditions, may be preferentially beneficial to the strain NM05 for its degradation capabilities. Though, recently HCH degradation has been extensively studied, from different parts of the world (Frister 2006; Thomas *et al.* 1996; Nagata *et al.* 1999; Kumari *et al.* 2002), the role of biosurfactant in its degradation kinetics has not been evaluated.

**HCH degradation in soil slurry by Sphingomonas sp strain NM05 with surfactants**

Enhanced biodegradation of HCH-spiked soil slurry incubated with surfactant is also attributable to the accessibility of the HCH and the efficiency of surfactant in the
decreasing order of sophorolipid, rhamnolipid and trehalose-containing lipid. Three distinct observations were made after the addition of surfactant, 1). Enhanced degradation of HCH isomers in equal proportion in trehalose biosurfactant amended culture medium. 2). In presence of rhamnolipid, 30% of residual β-isomer was detected indicating the inability of surfactant to affect its solubilization to the same extent of three isomers, 3. Presence of Sopohorolipid exhibited preferential degradation of α- and γ- over β- and δ-isomers, however, it showed maximum enhancement in degradation of HCH isomers among all the three surfactants after 8-10 days of incubation. This pattern of degradation may be attributed to the differential solubilization properties of the individual surfactants toward HCH isomers in mineral medium. Interestingly, the biodegradation behavior by strain NM05 in presence of all of the surfactants was enhanced, indicating that the solubilization and availability (Table 6) of HCH isomers was significantly increased by the use of biosurfactant (Figure 21). It is noteworthy that the calculated increase of about 30-40% in degradation of all the isomers in 2-4 days is significantly higher with any of the biosurfactant added. All these results suggest that sophorolipid was more efficient in solubilization of all the HCH-isomers except β-HCH. Thus in presence of sophorolipid the strain NM05 could efficiently utilize HCH as sole source of carbon and energy.

Organochlorine pesticides are usually inaccessible to the microbes due to their hydrophobic nature. In aqueous phase, the accessibility of the pesticide is more as compared to the pesticide under soil conditions due to other organic and inorganic matters which may hinder with the micelle formation. Usually biosurfactants enhance the micelles formation; however, under soil conditions various intrinsic factors may affect the interaction of the biosurfactant, target pesticide and the microorganism. Considering the above factors, in the present study, efforts were made to use a soil which has low high humic contents or other organic matters that may affect surfactant and chemical interactions. Also to minimize such effects uniformly sieved and autoclaved soil was used for the degradation studies.

Chlorinated pesticides are poorly soluble in water, and hence their availability is highly limited to microorganisms. Particularly, aqueous solubility of HCH (10-20 mg/L), DDT (0.025mg/L) and endosulfan (0.33 mg/L) are too little, thus making them
more persistent and recalcitrant towards microbial assimilation. This has led to increase in their residues in polluted sites, especially in respect to HCH in countries like Germany, Spain, Poland, India and China (Frister 2006). With age, there is a decrease in bioaccessibility and bioavailability of toxic pollutants at the contaminated sites due to their adsorption in soil matrix, which can be overcome by application of biosurfactants. These surfactants desorb and dissolve hydrophobic compounds, like pesticides, alkanes or chloroaromatic compounds. To the best of our knowledge this report is perhaps the first attempt to explore enhanced degradation of HCH isomers in the presence of three biosurfactants to develop protocols for bioremediation of chlorinated toxic pollutants in the environment. In conclusion, the results obtained in this study reveal that biosurfactants enhances HCH biodegradation using *Sphingomonas* sp NM05 possibly by desorption and dissolution of the contaminant and thereby enhancing its bioaccessibility for degradation.

**Conclusions**

The studies presented that a bacterium, screened based on dehalogenase-enzyme based method, is able to degrade hexachlorocyclohexane (HCH) as sole source of carbon and energy aerobically. The degradation pathway of γ- and δ-HCH as substrates revealed extensive dechlorination of the compounds leading to formation of less chlorinated metabolites. The upstream pathway *lin* genes involved in degradation were characterized. The genes and enzymes of γ-HCH degradation pathway were found highly similar to *lin* genes present in other HCH biodegraders. The bacterium was identified as a member of *Sphingomonas* family and its 16S rRNA gene nucleotide sequence shows similarity to chlorophenol and other hydrocarbon degrading strains. The evaluation of the diverse microbial communities present in HCH and other pesticides contaminated sites revealed the occurrence of many bacteria useful in biodegradation in consortium with a large number of unculturable bacterial species. The observation in the study show the effectiveness of surfactants in improving biodegradation of HCH isomers by the solubilizing power of the surfactant and increasing the bioavailability of micellar contaminant.
**Future Perspectives**

A major problem in \( \gamma \)-HCH degradation is persistence of the upper pathway metabolites. In all the sphingomonads and even from the other HCH degraders so far studied, HCH is not converted to complete degradation. \( \gamma \)-HCH is degraded to TCDN which would undergo dehydrochlorination spontaneously to be converted to 1,2,4-TCB and further for 2,5-dichlorophenol. These two metabolites are a dead-end product as it was also observed in our studies using strain NM05 and the results suggest the possibility of several patch work of genetic events to evolve enzymatic machinery for the degradation of HCH. Overall, since the two products accumulate in HCH degradation, the pathway is considered to be an inefficient pathway. However, to overcome these issues, genes for 1,2,4-trichlorbenzene degradation may be inserted to a suitable HCH degrading bacterium and make the degradation pathway of HCH a complete one.

Another understanding is that there may be the variant forms of linA and other lin genes existing in nature which can efficiently metabolize HCH isomers. We could obtain few linA variants from pesticides contaminated soils show difference in few amino acid positions. Such variant forms need to be thoroughly studied on different isomers. From their catalytic reactions the metabolites should be characterized in all respects to learn the specificities of enzymes as why 1,2,4-TCB and 2,5-DCP are not undergoing further transformation and funneled into to the main pathway. These characteristics are also influenced by the orientation of the chlorine atoms on each HCH isomer.