Results

4.1 Soil collection and analysis and pesticide residue measurements

Soil samples were collected from three major industrial sites manufacturing chlorinated pesticides from different locations (Figure 7). Soil properties such as soil type, pH, carbon and nitrogen content, cation exchange capacity and organic matter are summarized in Table 1. The soil pH, ranging from 5.85-6.80 suggests that it was slightly acidic in nature, may be due to the chlorinated hydrocarbons and presence of organic acids in the soils. The values of cation exchange capacity are in accordance with the pH. Soil texture was found to be sandy loam (HIL), alluvium with sandy loam (IPL) and clay (NGP). Carbon / Nitrogen / Organic contents were around 7:1:10 in all the samples (Table 1).

Residue levels of hexachlorocyclohexane (HCH), 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) and endosulfan (ES) found in the samples are given in Table 2. HCH-isomers were predominant in all three soils studied here and among all isomers, the concentrations of α-, and γ-HCH were high at 664 and 662 µg g⁻¹ soil respectively. Surprisingly, the highly persistent β-HCH was not found in the samples from NGP. The DDT-residues (DDT, DDD and DDE) was found in the HIL sample as the company is still manufacturing DDT for the health care program. While the soil from IPL, Lucknow had a high concentration of β- HCH, the α- HCH concentrations were relatively less and no γ- and δ-HCH were detected in this sample. Also the β-isomer measured in this sample was found highest among all the samples. In the Nagpur (NGP) soil only the γ-isomer was found as they do not manufacture γ-technical HCH but only the formulation is done. Among the endosulfan, only α-ES was found in both HIL and NGP soil samples but not β-ES. Technical endosulfan mixture contains 70:30, α and β isomers respectively.

4.2 Colorimetric dehalogenase activity and clearance zone assay

A single enrichment using 0.34 mM γ-HCH as the sole carbon source from HCH contaminated soil samples (Figure 8), resulted in isolation of 14 distinct colonies from HCH contaminated mixed samples (Figure 7B). Phenol red indicator dye turned to
yellow in only one microtitre well out of 14 experimental samples tested due to chloride release (HCl formation) and lowering of the pH. In reaction mixtures with a promising HCH degrader, the color change was immediate for γ-HCH (2 min) followed by δ-HCH (5 min.) (Figure 9A) then α-HCH (Figure 9B) and β-HCH (15 min.) of incubation at room temperature 28±2°C (Figure 9C) Substrate specificity of the dehalogenase enzyme in the strain NM05 exhibited preference in the order of γ>, δ>, α>, and β> (Figure 9A,B,C). In the other 13 bacterial extracts and reference control wells the dye color remained red indicating the inability to dechlorinate any of the HCH isomers.

4.3 HCH-Clearance assay

To confirm the HCH degradation ability of the dehalogenase enzyme positive bacteria, agar plate based substrate clearance was monitored. The pure culture was incubated for 5 days on γ-HCH sprayed MM2 agar plate. The colonies utilized solid γ-HCH to form colonies which were surrounded with transparent, clear zones (Figure 10). Gamma-HCH had disappeared from cleared areas and on further incubation the zone of clearance increased suggesting the extensive utilization of the substrate provided. Substrate clearance assay was clear indication of the HCH degradation potential of the bacterium and thus taken for further investigation.

4.4 Identification of the bacterium

The bacterium positive for dehalogenase enzyme and γ-HCH clearance was designated as strain NM05. Colonies of strain NM05 on TSA were pale-yellow pigmented and about 1-3 mm in diameter, circular, smooth, glistening, opaque and convex with an entire margin. Catalase and oxidase are produced. Growth temperature ranges from 20°C and 42°C, optimum growth temperature is 30°C. The amplified16S rRNA gene sequence consisting of 1406 base pair (Figure 11) was compared with those of type strains of species of the genera retrieved from the GenBank database and revealed that the strain NM05 is a member of the genus Sphingomonas. The highest identity of 97% was found for a Sphingomonas haloaromaticamans reported to degrade chlorophenol (Nohynek et al 1996). The 16S rRNA gene nucleotide sequence identity of 95-96% was observed for several other Sphingomonas sp which was isolated from the HCH contaminated soil (refer following section). The strain NM05 = MTCC 8061,
was deposited at Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

4.5 Phylogenetic relationship of Sphingomonas strain NM05 with other HCH degrading bacteria

Phylogenetic relationship of HCH degrading Sphingomonas sp NM05 was determined against 18 other bacteria which were characterized for HCH degradation (Figure 12). The 16S rRNA gene sequences of two bacterial genera such as Sphingobium and Sphingomonas were only used for the analysis. The results indicate that NM05 share a close relationship to the reported HCH degrading Sphingomonas reported to have been isolated from soil samples obtained from three HCH-contaminated sites near Bilbao in northern Spain. Although, there were many other Sphingomonas species were reported from the sample, only 3 Sphingomonas sp, namely Sphingomonas sp α4-2, Sphingomonas sp α4-5 and Sphingomonas sp α1-2 showed close phylogenetic relationship with strain NM05. However, many other Sphingomonas sp isolated from same sample shared close rooting to the well characterized HCH degrading bacteria such as Sphingobium indicum, Sphingobium japonicum, Sphingobium francense and Sphingobium chinhatense. It is interesting to note that the strain NM05 which was isolated from India was found phylogenetically close to cultures from Spain indicate that though these places were geographically distant but the bacteria may have had evolutionarily a common ancestor.

4.6 Growth on HCH-isomers and metabolite characterization

The strain NM05 utilized 90% of α- γ- and δ-HCH, and around 60% of the β-HCH at the exposed concentration of individual isomers 0.34 M (100 µg/ml) (Figure 13). Accordingly, the growth was more in γ-HCH followed by α- and δ-HCH isomers after 5 days of incubation at 28ºC. The HCH degradation was set up with strain NM05 without any additional carbon source. Degradation of both γ- and δ-HCH was comparatively faster than other two isomers. GC-mass spectra of the degradation products by NM05 grown on γ-HCH revealed the formation of intermediates 1,2,3,4,5-pentachlorocyclohexene (γ-PCH, Rt 8.13 min) 1,2,4-trichlorobenzene (1,2,4-TCB, Rt 5.57 min) and chlorohydroquinone (CHQ, Rt 7.14 min) compounds while the substrate
γ-HCH showed Rt. 11.49 min (Figure 14A). The formation of γ-PCCH from δ-HCH as a substrate by strain NM05 was identified (Figure 14B). The retention time and mass spectra profiles of these three metabolites were compared with the authentic γ-PCCH, 1,2,4-TCB and CHQ and found matching fragmentation profiles (Figure 15). In previous studies, under the aerobic degradation pathway of γ-HCH it was shown that γ-HCH undergoes two initial dehydrochlorination reactions to produce the putative product 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,3,4,6-TCDN) via γ-pentachlorocyclohexene (γ-PCCH) as an intermediate. Subsequently 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) is generated by two rounds of hydrolytic dechlorinations via a second putative metabolite 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). 2,5-DDOL is then converted by a dehydrogenation reaction to 2,5-dichlorohydroquinone (2,5-DCHQ). Further 2,5-DCHQ is converted to chlorohydroquinone (CHQ) as identified based on GC-mass spectrometry. We could not detect the formation of putative unstable metabolites 2,5-DDOL and 2,4,5-DNOL but could characterize the major upstream pathway metabolites that accumulated (Figure 16).

The mass spectrum of the compound showed a strong molecular ion peak at m/z 181 which correspond to [M+ -HCl₂] and other distinct peaks at m/z 185, m/z 146 as a resultant ions due to dechlorination. The same fragmentation profile was observed in the mass spectrum obtained for authentic γ-PCCH. From these results the compound formed from δ-HCH was identified as γ-PCCH. The metabolites from α- and β-HCH could not be obtained in detectable quantities.

4.7 Southern hybridization of lin genes

Southern hybridization of genomic DNA from strain NM05 revealed the hybridizing DNA fragments for all the lin gene probes tested The gene probes were prepared from PCR products using NM05 DNA as template and later sequenced. The five genes used for Southern hybridizations were dehydrochlorinase (linA), haloalkane dehalogenase (linB), dehydrogenase (linC), a reductive dechlorinase (linD) and a ring cleavage oxygenase linE. For the lin genes, the genomic DNA of strain NM05 produced strong positive hybridization signals against the lin genes tested individually
(Figure 17). The genes present in this strain appear to have high similarity DNA sequences to their corresponding $linA$, $linB$, $linC$, $linD$, and $linE$ genes involved in $\gamma$-HCH degradation from a $S. japonicum$ and $S. indicum$ (Nagata et al 1999 and Kumari et al 2002). For a few restriction digests, more than one hybridizing bands was observed. Though these multiple bands originated owing to the internal sites for the enzymes (Kumari et al 2002, Atul et al 2007), it also implies the existence of multiple copies based on the previous reports.

4.8 PCR amplification and sequencing of $lin$ genes from NM05

Based on previously reported (Kumari et al 2002) $lin$ gene sequences, PCR primers were used to amplify initial five genes involved in the biodegradation of $\gamma$-HCH (Table 3). Although the verification for the presence of $lin$ genes were confirmed by Southern hybridization, These genes were obtained from strain NM05 by polymerase chain reaction (PCR), to understand its sequence composition (Table 4). The corresponding primer sets for each gene amplified DNA products with expected sizes of 471, 891, 753, 1041 and 966-bp for $linA$, $linB$, $linC$, $linD$, and $linE$ genes respectively (Figure 18). Same size of amplicons was observed in the controls when plasmids having inserts for all these five genes were used which were constructed from strain UT26 (Nagata et al, 1999). The PCR amplified products were sequenced and compared to the GenBank database (Altschul et al. 1997). The gene sequences from strain NM05 had 93-98% homology to the respective corresponding $lin$ genes of $S. japonicum$ UT26, $S. indicum$ B90 and to many other HCH degraders (Table 5).

4.9 Effect of Biosurfactants on Solubility of HCH isomers

An enhanced solubility of four HCH isomers at different concentration of biosurfactants rhamnolipid, sophorolipid and trehalose was observed (Figure 19). HCH isomers at 200 ppm (0.68 μM) were used, which was several fold ($\alpha$-HCH, 10; $\beta$-HCH, 20; $\gamma$-HCH, 5; and $\delta$-HCH, 10 folds) higher than the reported aqueous solubility of isomers (10, 5, 17, 10 ppm of $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-HCH respectively) (www.atsdr.cdc.gov). The solubilization capacity was calculated (Table 6) based on the data obtained from GC quantification remaining HCH (Figure 20). Increase in the solubilization of HCH isomers in presence of surfactants was observed in the range of
40-60 μg/ml concentration of surfactants. However, the higher concentration range of 80-100 μg/ml of surfactant did not display an increase in solubility. While rhamnolipid and sophorolipid showed enhanced solubility of HCH at 40 μg/ml, the trehalose sugar exhibited maximum solubility at 60 μg/ml. At each specific surfactant concentration, almost all the HCH isomers showed increased solubility. The critical micelle concentration (CMC) of three surfactants varies slightly for each isomer. Among the three, sophorolipid apparently showed higher solubility for α- and δ-HCH. However, for more persistent β-isomer, rhamnolipid could solubilize it two fold more than the other two surfactants (Table 6). Three concentration values (60, 80 and 100 μg/ml) above the CMC did not showed enhancement in solubility except for sophorolipid which showed increased solubility of β-HCH at the concentration of 60 μg/ml. These solubility concentration values suggest approximately 3-9 fold increase in solubilization of HCH isomers. Interestingly the sophorolipid showed 9 folds increase in solubility for α-HCH which was 40.4% and 35.6% higher than rhamnolipid and trehalose respectively.

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

The effect of surfactants on the biodegradation of HCH isomers by strain NM05 are shown in Figure 21. The optimum CMC value of surfactant concentration 40 μg/ml was used derived from solubilization experiments. The experimental results based on the gas chromatographic analysis were further used for the quantification. After 10 days of incubation, around 60% degradation of all the HCH isomers was achieved in the absence of surfactant. However, in the presence of surfactant the strain NM05 showed enhanced (~95%) degradation for most of the HCH isomers (Figure 21). The degradation rate of HCH isomers was slow, as expected for a substrate with low water solubility in the absence of biosurfactant; however a 10-20 fold increase was observed in the presence of biosurfactant. An increase in the growth of strain NM05 was observed following the addition of surfactants, indicating the higher substrate availability and utilization with respect to control setup which did not receive any surfactant. For all the isomers, nearly 70% degradation was achieved in just 2-4 days in
the presence of surfactant, in contrast to 10 days in absence of surfactant. More than 95% degradation of all the isomers was observed in 8-10 days. Three distinct observations were made by the addition of surfactant, 1) Trehalose enhanced the HCH isomers degradation in equal proportion, 2) In presence of rhamnolipid, 30% of residual β-isomer was detected indicating the inability of surfactant to solubilize β-isomer to same extent as other three isomers, and 3) Sopohorolipid exhibited varied kinetics as there was slow degradation of β- and δ-isomers, however it showed maximum enhancement in degradation of HCH 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 in the presence of all surfactants by strain NM05 was enhanced, indicating that the solubilization and bioavailability (Table 7) of HCH isomers was significantly increased by use of surfactants (Figure 21). However 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. Among all, sophorolipid showed efficient and enhanced degradation kinetics. 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 bioavailability of HCH was more for the strain NM05 to utilize it as sole source of carbon and energy.

4.11 Biodegradation of HCH isomers in presence of Surfactants under Soil Conditions

Soil slurry spiked with HCH isomers and strain NM05 as inoculum was incubated for 30 days. The results showed enhanced degradation of HCH isomers as quantified by GC (Figure 22). Slower rate of degradation was observed in the soil slurry as compared to liquid cultures, as degradation was observed only after 30 days. In the absence of surfactant, HCH degradation by *Sphinogomonas* sp NM05 was comparable in all the flasks (Figure 22. A, B, C). After 30 days of incubation without surfactant, the residual HCH isomers were α- 57-63, β- 88-100, γ- 28-34, and δ- 44-52 µg/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 β-isomer, for which the degradation was 10% less than other isomers. Though there was a variation among the percentage degradation by each biosurfactant for each of the isomers, sophorolipid had substantially enhanced HCH degradation among all others (Figure 22), as also observed under liquid culture condition. Under the conditions tested, the degradation efficiency of the surfactants on HCH isomers were in the order of sophorolipid>rhamnolipid>trehaloseas. These results were in agreement with solubilization experiments where sophorolipid dissolved highest concentrations of all the isomers. The higher degradation rate may be attributed to the enhanced bioavailability of the HCH isomers to the strain NM05 which could utilize it as a substrate for its growth.

4.12 Phylogenetic diversity of microbial communities

Comparative analysis based on 16S rRNA gene sequences from soil samples revealed a broad spectrum of bacterial diversity occurring in the three environmental samples studied. The RFLP pattern of randomly selected 125 clones from each of the three sites was analyzed and grouped. A total number of 17 (3 culturable- and 14 unculturable-bacteria), 20 (7 culturable- and 13 unculturable-bacteria), and 18 (10 culturable- and 8 unculturable-bacteria) phylotypes were obtained from HIL, NGP, and IPL sites respectively. Rarefaction curves plotted for each clone library did not reach an asymptote suggesting the diversity present in all the three libraries had not been sampled to the saturation. Further sequencing of 16S rRNA clone library may yield new bacterial families. The clustering pattern for HIL soil, which has high residues of all the pesticides measured, indicates the formation of 4 major clusters (Figure 23). In this case the unculturable bacteria dominated as of 17 phylotypes, only three bacterial species belong 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. The dendrogram presented in Figure 24 shows 5 major clusters obtained from IPL, with
higher number of 20 RFLP types obtained among the three soils studied. Representative clones of all RFLP types here occurred more than one time which occupy 5 zones in the cluster. The unculturable representatives were dominant here also as out of 20 phylo groups, 13 sequences belonged to unculturable and 7 genera were culturable. Many bacteria showed sequence similarity to polychlorinated biphenyl degraders including a cluster which occupied 3 different Saltmarsh clones. The results presented in Figure 25 indicate that majority of bacteria representing the culturable bacterial communities occurring in soil sample from Nagpur (NGP). In this the phylotypes grouped into 5 distinct divisions. Three clusters shared the culturable bacterial genera and one cluster was completely occupied with unculturable bacteria showing high similarity (90-100%) to Acidobacteria. Four Clostridium species assembled into one group and none of the other soil types has so many gram-positive bacterial representations.

4.13 Metagenomic amplification of lin functional genes and their alignment

Using the linA gene primers (Kumari et al. 2002), specific for the dehydrodechlorinase amplification products were successfully obtained from 8 of 11 samples studied. An expected product size of 471 bp DNA was amplified from 4 samples each of HIL and IPL metagenome (Figure 26). Also, the linA gene product of same size was obtained with DNA from Sphingomonas sp NM05 (DQ 767898) consistently when used as control. The strain NM05 was isolated and characterized for its capability to degrade all the four HCH isomers from IPL site through enrichment studies. However amplification from NGP soil for linA gene could not be found. Sequences of the PCR products generated with the linA revealed that all the eight products from the soil showed ≥ 92-98% similarity to their counterpart linA sequences available in the data base. Since linA gene initiates the degradation of γ-HCH we have examined the amino acid compositions in more detail for all the 8 linA genes obtained. Alignment of the translated linA sequences revealed the changes in amino acid substitutions at various positions (Figure 27). When compared with reported linA gene sequences characterized for γ- HCH degradation, the highest number of 12 amino acid substitutions was observed in HIL-3 and 6 changes in HIL-1.
4.14 Nucleotide sequence accession numbers

The 16S rRNA gene sequence of strain NM05 has the GenBank accession number DQ231244 and lin genes were deposited under accession numbers DQ767898–DQ767902. The nucleotide sequences of community 16S rRNA gene sequences of all 55 clones are available under accession numbers EU037905 to EU037959. The sequences for *linA* gene obtained from soil DNA are available from GenBank under accession no EU037789 to EU037795.