Chapter 1

Isolation and identification of γ-HCH degrading bacteria

1.1 Introduction

Gamma-hexachlorocyclohexane (γ-HCH or lindane) is a chlorinated pesticide, which has been extensively used to protect crops. Its indiscriminate and injudicious use in agriculture and long period of persistence in soil is a major concern (Simonich and Hites, 1995; Karanth, 2000; Abhilash et al., 2009a). In this context, bioremediation involving the use of natural microbes to detoxify and degrade environmental contaminants, has received considerable attention as an effective clean up approach for the pollutants (Pieper and Reineke, 2000). A few reports are available on the biodegradation of γ-HCH and other isomers of HCH (Senoo & Wada, 1989; Sahu et al., 1990; Manickam et al., 2006 and 2007). Pesticide degrading bacteria have been isolated from the agricultural and industrial site soil (Sahu et al., 1990; Manickam et al., 2006 and 2007) by enrichment methods, in Winograsdsky column (Winogradsky, 1949; Manonmani et., 2000) or in mineral medium (Sahu et al., 1990; Manickam et al., 2006 and 2007). Bacterial populations capable of degrading γ-HCH or its isomers have been isolated using culture-dependent enrichment procedures. However, dominant HCH degrading bacteria might be lost during enrichment (Lloyd-Jones et al., 1999), as the soil environmental conditions might differ from the enrichment media. Therefore, in present investigation γ-HCH degrading bacteria were isolated both by enrichment and by directly spreading the soil supernatant on Luria agar (LA) supplemented with γ-HCH. Various assays such as pesticide clearance assay (Kiyohara et al., 1982), dehalogenase assay (Phillips et al., 2001; Manichkam et al., 2008) and analytical techniques TLC, HPTLC, GC, etc (Manonmani et al., 2000; Pesce et al., 2004; Manickam et al., 2006) were employed for screening of γ-HCH degrading bacteria. γ-HCH clearance assay was preferred for initial screening of larger number of bacteria, as it is rapid and inexpensive. Subsequently 16S rDNA analysis method (Woese et al., 1975) was used routinely for bacterial identification and phylogenetic analysis. This chapter describes the isolation, preliminary screening and identification of γ-HCH degraders.

1.2 Material and methods

1.2.1 Soil sampling

Soil samples from sugarcane fields having a long history of HCH applications and industrial sites (engaged in γ-HCH manufacturing for more than 20 years) were collected, from Junagadh **(21.419°N 70.4293°E)** and Vadodara **(22.2952°N 73.7238°E)** districts, respectively (Figure 4). Collected soil samples were passed through 2 mm sieve and used for the isolation of γ-HCH degrading bacteria, either by enrichment method or by directly spreading of the soil supernatant on Luria agar supplemented with γ-HCH (10 mg l^{-1}). Remaining portions of the soil samples were stored at 4^0C for further use. Strategy for the isolation of γ-HCH degrading bacteria has been summarized in Figure 5.

1.2.2 Chemicals and reagents

Analytical grade γ-HCH (~99%) and technical HCH were obtained from Shreeram agrochemical Ltd., Gujarat, India. Other chemicals used in the experiments were purchased from either- Sigma Chemical Co, St. Louis, MO, USA or Hi-media, Mumbai, India. Non-radioactive labeling and detection kit were purchased from GE healthcare Ltd, USA. Reagents for molecular analysis were purchased from Bangalore Genie, Bangalore, India or Fermentas, USA. Oligonucleotide primers were custom synthesized by MWG-Biotech AG, Bangalore, India. Other media components and buffers of analytical grade reagents were from Hi-media, Mumbai, India.

Figure 4: Map showing soil sampling locations, Vadodara and Junagadh districts in Gujarat state, India.

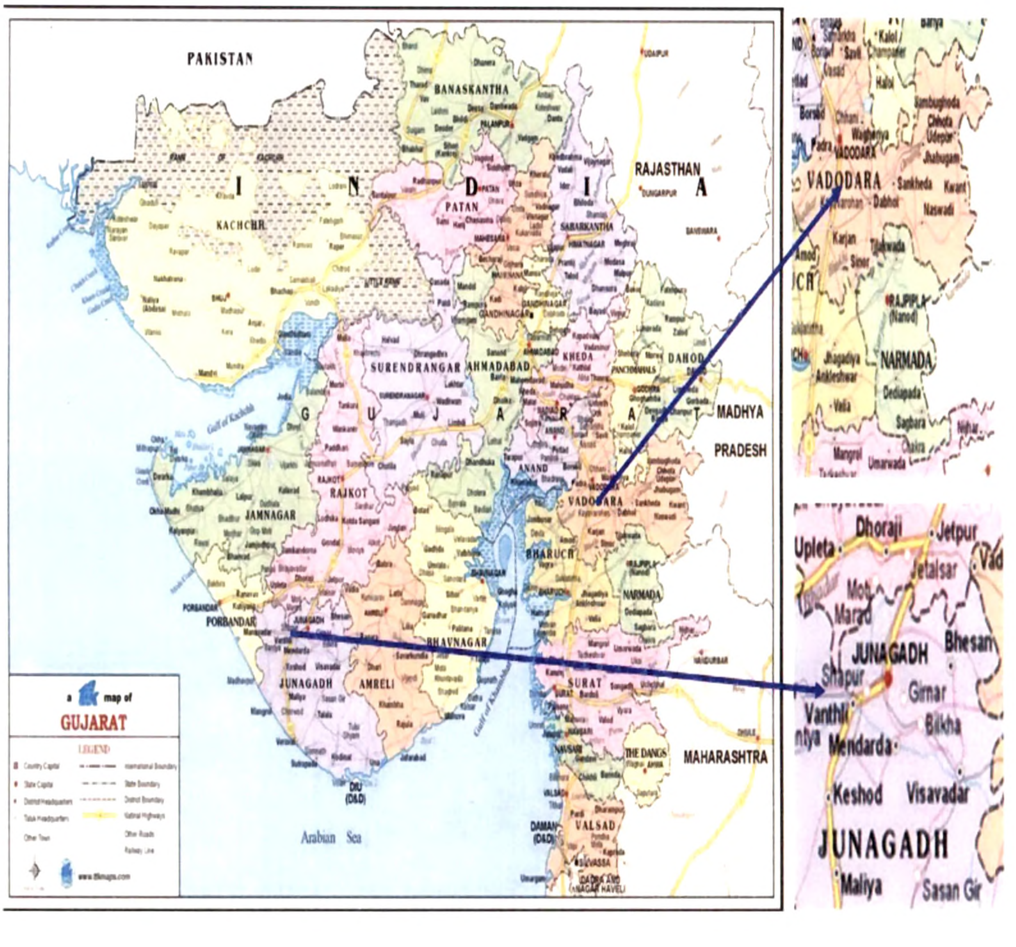
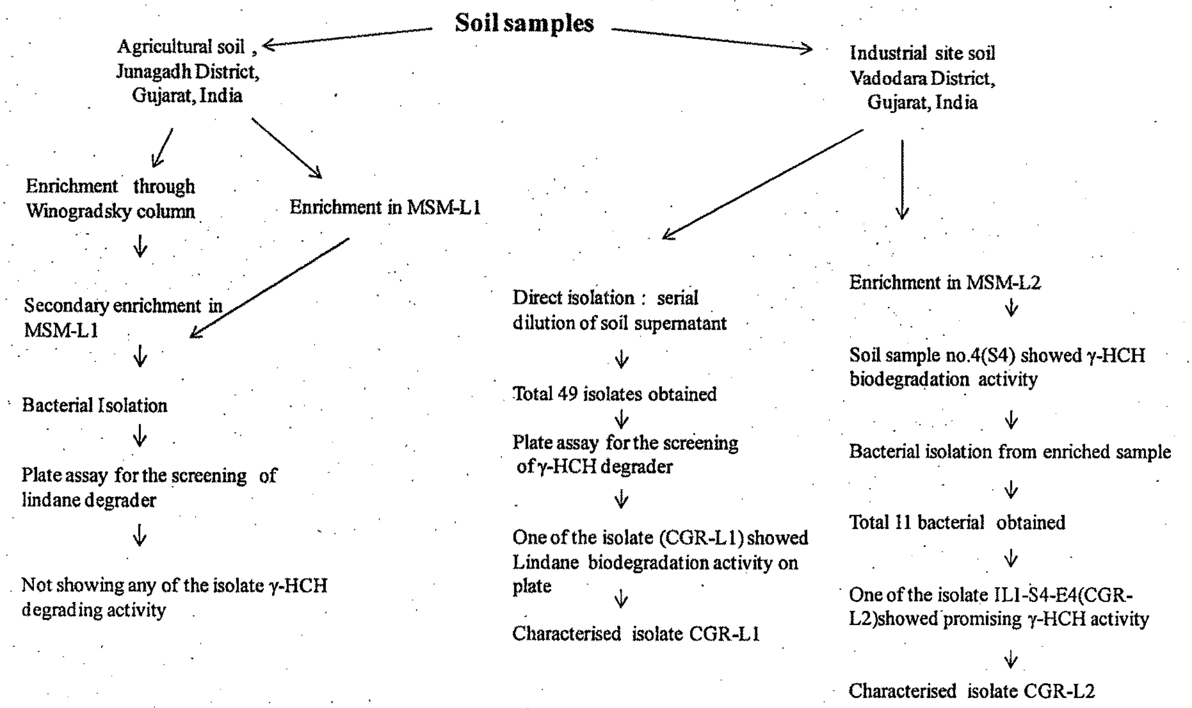

PAKISTAN
BANASKANTHA
RAJASTHAN
I N D I A
KACHCHH
PATAN
SABARKANTHA
SURENDRANGAR
AHMADABAD
KHEDA
PANCHMAHALS
DAHOD
MADHYA PRADESH
G U J A R A T
JAMNAGAR
RAJKOT
ANAND
VADODARA
BHARUCH
NARMADA
PORBANDAR
BHAVNAGAR
JUNAGADH
AMRELI
SURAT
THE DANGS
MAHARASHTRA
VALSAD
DIU (D&D)
DAMAN (D&D)
Arabian Sea
a map of
GUJARAT
LEGEND
VADODARA
Dabhoi
Karjan
RAJPIPLA (Nanod)
NARMADA
Dediapada
Sagbara
Ankleshwar
Dhoraji
Jetpur
Upleta
Jetalsar
JUNAGADH
Bhesan
Girnar
Vanthli
Mendarda
Bilkha
Keshod
Visavadar
JUNAGADH
Maliya
Sasan Gir

Figure 5: Flow chart of procedure followed for the enrichment and isolation of γ-HCH degraders

why such a distance placed sites were selected. There are sugar cane field near Vadodara also ?



1.2.3 Media used for isolation, screening and analysis of γ-HCH degrading bacteria

MSM-L1 g l^{-1} (Sahu et al., 1990)

$(NH_4)_2HPO_4$	0.50
$MgSO_4.7H_2O$	0.20
$FeSO_4.7H_2O$	0.01
K_2HPO_4	0.10
$Ca\ (NO_3)_2$	0.01

pH was adjusted at pH 7.0

MSM-L2 g l^{-1} (Senoo and Wada., 1989)

KH_2PO_4	1.00
K_2HPO_4	1.00
NH_4NO_3	1.00
$MgSO_4.7H_20$	0.20
$Fe_2\ (SO_4)_3$	0.005
$Na_2MoO_4.2H_2O$	0.005
$MnSO_4.4H_2O$,	0.005

pH was adjusted at pH 7.0

Luria Broth (LB) g l^{-1} (HiMedia, Laboratories, Bombay, India)

Casein enzymic hydrolysate	10.000
Yeast extract	5.000
NaCl	5.000

Final pH (at 25°C) 7.0±0.2

1.2.4 Isolation of γ-HCH degrading bacteria from agricultural soil samples

Soil samples from Sugarcane field (Junagadh district) were used for enrichment of γ-HCH degrading bacteria in two ways. Firstly, collected soil samples were directly used for enrichment of the γ-HCH decomposing bacteria in a MSM-L1, using γ-HCH as a sole carbon source (Senoo & Wada, 1989; Sahu et al., 1990). For this, 10 g of soil–slurry mixture was added to 50 ml of sterile MSM-L1 and stirred for 1 h. The flask was then left static for 10min and 1ml suspension was inoculated in the MSM-L1 containing γ-HCH (10 mg l^{-1}). γ-HCH was dissolved in acetone and filter sterilized using a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) before use. Sub-culturing was done at every 15 days intervals by transferring 1% inoculum in a fresh MSM-L1 containing γ-HCH (10 mg l^{-1}). After 5 successive sub-cultures, serial dilutions (10^{-2} to 10^{-6}) of the suspension were spread onto MSM-L1 agar incubated at 28^{0}C for 3-10 days. Isolated colonies obtained were further sub-cultured on fresh media as before until a pure bacterial culture was obtained. Have you used one or more type of medium?

In a second procedure, enrichment culture for γ-HCH degradation was isolated in two steps, primary enrichment in Wonogradsky column, followed by secondary enrichment in MSM-L1 (containing 10 mg l^{-1} of γ-HCH) using γ-HCH as a sole carbon source.

Primary enrichment in Winograsdky column

For primary enrichment, a 1L glass measuring cylinder (30 cm in height and 5 cm in diameter) was used for preparing the Winograsdky column. Shredded paper, grass, leaves, saw dust, etc were used as supplementary carbon source. Minimal salt medium containing benzene and phenol were also added in the column (each 50 μl l^{-1}). Soil from the sugarcane field supplemented with 10 mg l^{-1} concentration of γ-HCH and pond water was used to fill up the column.

Secondary enrichment in MSM-L1

Soil sample was collected from column after 30 days and used for the secondary enrichment in the MSM-L1 (containing 10 mg l^{-1} of γ-HCH). The procedure followed for secondary enrichment and isolation of γ-HCH degrading bacteria was the same as described earlier in first approach above.

1.2.5 Isolation of γ-HCH degrading bacteria from Industrial site soil Samples

Isolation of γ-HCH degrading bacteria from Industrial site soil was performed by direct spreading of the soil supernatant on Luria agar (supplemented with γ-HCH) and by enrichment in mineral medium.

1.2.5.a Isolation of bacteria from soil samples without any enrichment

Five different soil samples were collected, serially diluted in water on the same day (10^{-2} to 10^{-7}) and plated onto 1/3 Luria agar containing 10 mg l^{-1} γ-HCH, pH 7.0-7.2. γ-HCH was dissolved in acetone and filter-sterilized using a 0.22 μm membrane filter, before use (Millipore Corp., Bedford, MA). Plates were incubated at 28^{0}C for 2-6 days and isolated colonies were further sub-cultured on fresh media as described earlier until a pure bacterial culture was obtained. All the isolates were grown in the 1/3Luria broth (containing γ-HCH 10 mg L^{-1}) for 1-3 days depending on their growth and then stored at -80^0C as 15% glycerol stocks for further use.

1.2.5.b Enrichment and isolation of γ-HCH degrading bacteria from industrial site soil

Procedure for the Enrichment of γ-HCH degrading bacteria has been followed as described in 1.2.4 except filter-sterilized MSM-L2 was used in a place of MSM-L1. After 5 successive sub-cultures, different dilutions (10^{-2} to 10^{-6}) of the suspension were spread onto plates in duplicate containing MSM-L2 agar γ-HCH (10 mg l^{-1}), and incubated at 28^0C for 6-10 days. Different dilutions were spread in one set on MSM-L2 agar plates, overlaid with γ-HCH after 6 days of growth, and further incubated for 3-6 days. Appearance of zone of clearance around the colonies was monitored. In another set, different dilutions were used to isolate total cultivable bacteria in the enrichment culture. Isolated colonies were further sub-cultured in fresh Luria agar plate containing γ-HCH till a pure bacterial culture was obtained. Further all the isolates were maintained on the MSM-L2 agar plate containing γ-HCH. All the isolates were grown in the 1/3 Luria broth (in presence of 10 mg l^{-1}, g- HCH) for 1-3 days and were preserved as glycerol stock at -80^0C.

1.2.6 Preliminary screening for putative γ-HCH degrading bacteria

Preliminary screening for γ-HCH biodegrader was done based on γ-HCH clearance assay on Luria agar plate. Luria agar plates were spread with 1% γ-HCH in diethyl ether (Kiyohara et al., 1982). A colony of isolated pure cultures was spotted on above plates.

All the plates were incubated at 28^0C for 8-10 days and the formation of clearance zone surrounding the colonies was used as an indicator of γ-HCH degradation. Isolates showing positive results in γ-HCH clearance assay were further characterized on the basis of Gram staining and morphological characteristics like shape, margin, elevation, texture, diameter and opacity on Luria agar.

1.2.7 Identification of γ-HCH biodegrader by Biolog system

A GN2 MicroPlate (Biolog, Hayward, Calif.) was used to characterise γ-HCH biodegrader based on substrate utilization profile (as per manufacturer's protocol). Isolates showing positive results in γ-HCH clearance assay, a γ-HCH degrader were streaked on Luria agar (LA) containing 10 mg l^{-1} of γ-HCH and incubated for 1-3 days at 30°C. Cells were suspended in normal saline (0.15%) and inoculated in the GN2 MicroPlate. After incubation for 72 hr, the resulting pattern was read using the Biolog automated Micro-Station.

what was incubation time as per Biolog? Is it 72 h?

1.2.8 Identification of γ-HCH degrading bacteria based on 16S rRNA gene (16S rDNA)

1.2.8.a Genomic DNA extraction and PCR amplification of 16S rDNA

The total DNA of the bacteria was isolated, according to the procedure as described (Sambrook and Russell, 2001). PCR amplification of partial 16S rDNA was carried out using the *Eubacteria*-specific primers - forward primer 27F (5'-AGAGTTTGATCCTGGCTCA – 3') corresponding to *E. coli* positions 8 to 26 and reverse primer 1089R (5' – GCTCGTTGCGGGACTTATCC – 3') corresponding to *E.*

coli positions 1088 to 1107 or reverse primer 1521R (5'-AAGGAGGTGATCCAGCCGCA - 3') corresponding to *E. coli* positions 1541 to 1522. PCR amplification was carried out using reaction mixtures (final volume 30 μl) containing 25 ng of sample DNA, 3U of Taq DNA polymerase (Bangalore Genie, India) 1X buffer, deoxynucleoside triphosphates at a concentration of 0.25mM and each primer at a concentration of 5pM. Reaction mixtures without any DNA were used as the negative control. The thermal cycle used was as follows: initial denaturation at 94°C for 3 minutes followed by 30 cycles of 94°C, 30 s; 58°C, 30 s and 72°C, 1 min; in a Minicycler (MJ Research, Waltham, MA, USA). The last step included final elongation at 72°C for 5 minutes. Amplified PCR products were analyzed by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

1.2.8.b Sequencing, identification and phylogenetic analysis

The amplified ~1.1 Kb or ~1.5Kb amplified product of 16S rDNA was gel purified (GE Healthcare, Buckinghamshire UK) and partially sequenced at MWG-Biotech (MWG-Biotech AG, Bangalore, India). Sequencing reaction was performed using *Eubacteria*-specific primer used for the PCR amplification. Homology search of the sequence information was performed using BLAST search program (Altschul *et al*, 1990) with the GenBank database maintained by the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) for an initial determination of the nearest phylogenetic neighbour sequences. The 16S rDNA nucleotide sequences determined in the present study were deposited into the GenBank database.

1.3 Results and discussion

1.3.1 Isolation and characterization of γ-HCH degrading bacteria from the agriculture soil samples

Twelve different bacterial isolates obtained after enrichment in MSM-L1 were designated as ASI 1-12 (Agriculture Soil Isolate). Whereas, after primary enrichment in Winogradsky column and further secondary enrichment in MSM-L1, 10 bacterial isolates [WEI-1 to 10, Winogradsky Enrichment Isolate] were obtained (Table 7). In preliminary screening on γ-HCH clearance assays none of the isolates showed a clearing zone. Further, γ-HCH biodegradation potential of the isolates were analysed in MSM-L1 media (observing their growth). Residual concentration of γ-HCH was determined by thin layer chromatography. None of the isolates showed the ability to degrade γ-HCH in the above assays. Simultaneously, to study microbial biodiversity in the soil samples (from agriculture soil and agriculture soil after enrichment in Winogradsky column), 16S rDNA library with more than 200 clones in pBlueScript KS+ were obtained, and grouped by ARDRA (Amplified rDNA Restriction Analysis). As none of these isolates (obtained from agriculture soil directly or after enrichment) were able to degrade γ-HCH, further sequencing of the 16S rDNA clones was not done.

1.3.2 Isolation, identification and confirmation of γ-HCH degrading bacteria from industrial site soil samples

Forty nine different colonies with distinct morphologies isolated after serial dilution of γ-HCH pesticide contaminated soil samples were named as ILI-D1 to 49 (where ILI stands for Isolates from Lindane (γ-HCH) Industry, D-stands Direct (without enrichment)

Table 7: Bacterial isolates obtained from different soil samples in present study. Isolates CGR-L1 and CGR-L2 (indicated in bold letter) were observed to be degrading γ-HCH in further analysis.

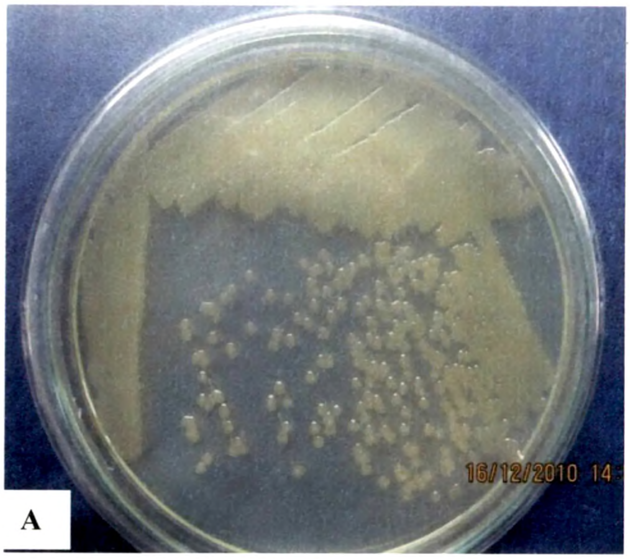
Place	**Crops grown in agricultural fields**	**Enrichment or direct dilution method**	**Primary enrichment**	**Secondary enrichment**	**Isolate name**
Agricultural Soil, Junagadh District,Gujrat,India	Sugarcane, Vegetables, cottons etc.,	enrichment	MSM-L1	-	ALI-1 to 12
Agricultural Soil, Junagadh District, Gujrat, India	Sugarcane, Vegetables, cottons etc.,	enrichment	Winogradsky column	MSM-L1	WLI-1 to 10
Industrial site soil samples, Vadodara District, Gujarat, India	-	Direct (serial dilution of soil supernatant)			
Soil sample 1					S1-ILI-D1 t0 10
Soil sample 2					S2-ILI- D11to18
Soil sample 3					S3-ILI-D19 to30 **(S3-ILI-D19 renamed as CGR-L1)**
Soil sample 4					S4-ILI-D31 to 39
Soil sample 5					S5-ILI-D40 to49
Industrial site soil samples, Vadodara District, Gujarat, India	-	enrichment	MSM-L2	-	S4-ILI-I1 to 11 **(ILI-S4-I4 renamed as CGR-L2)**

(Table-7). In the primary screening, one of the isolates ILI- D19 from soil sample no. 3, showed clearing zone around the colony after 8 days of inoculation (Figure 6). This isolate was renamed as CGR-L1 (CGR stands for the Centre for the Genome Research and L stands for the Lindane (γ-HCH). Isolate CGR-L1 was found to be gram-negative rod-shaped bacterium. Its colonies were mucoid, translucent and off white, circular in shape with entire margin and convex. Diameters of colonies were found to be ≤ 2mm after 24 hours of growth on Luria agar plate. Using BIOLOG system bacterium was identified as *Shewanella algae* with a 100% probability, based on different substrate utilization (Table 8). Further molecular technique was used to identify the isolate CGR-L1, for that ~1.1 kb of partial 16S rDNA was PCR amplified from the genomic DNA of the isolate CGR-L1 (Figure 7). Using purified PCR amplicons, a partial sequence of 16S rDNA (697bp) was obtained and the sequence has been deposited in GenBank with *accession no.* **HM063958**. A standard nucleotide-nucleotide BLAST search (Altschul et al., 1990) against the GenBank database using this sequence confirmed that strain CGR-L1 belongs to genus *Shewanella*, showing ~98% identity with respect to the 16S rDNA sequence of well studied *Shewanella* sp. TS 29 (GenBank *accession* no. **EU073095**) and *Shewanella* sp. ANA-3 (GenBank *accession* no. **CP000469**) (Figure 8). Both isolates have been reported for their ability to detoxify arsenic contamination (Saltikov et al., 2005; Cai et al., 2006) and are well characterized for metal resistance (Valls & Lorenzo, 2002). However, their involvement in biodegradation of γ-HCH has not been reported. Dechlorination of tetrachloromethane by *Shewanella putrefaciens* 200 (Picardal et al., 1995) presumably are co-metabolic processes that are mediated by electron carriers (c-type cytochromes) of the respiratory electron-transport chains. However, Petrovskis et

Figure 6: Morphological characteristics and γ-HCH-clearance assay for isolate CGR-L1.

A. Colony morphology on 1/3 Luria agar plate after 24 hours

B. Isolate sp. CGR-L1 showing clearance of γ-HCH on 1/3 Luria agar plate. Luria agar plate was overlaid with 1% γ-HCH dissolved in diethyl ether; culture colonies were spotted on the medium and were incubated for 8-10 days at 30^0C.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A

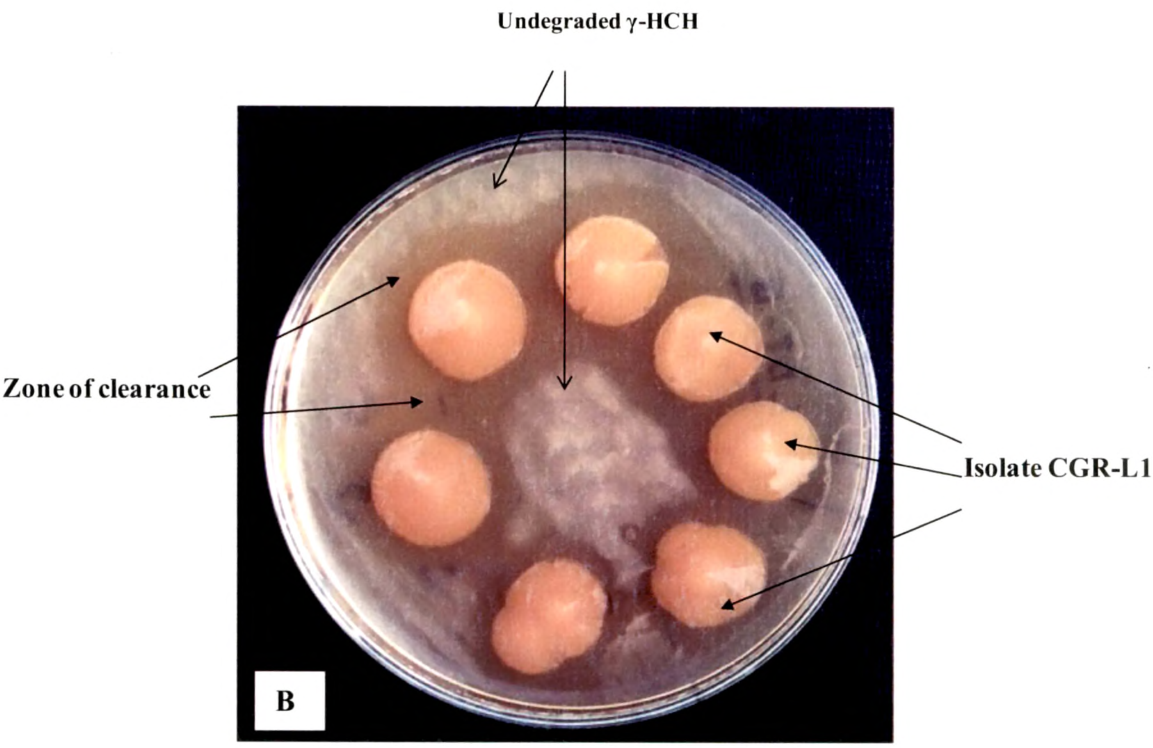

Undegraded γ-HCH
Zone of clearance
Isolate CGR-L1
B

Figure 7: PCR amplification of partial 16S rDNA sequence of the bacterial isolates from the soil samples

A. Amplification of 16S rDNA (1.1Kb) from *Shewanella* sp. CGR-L1 isolated from soil sample no. 3

   Lane 1; 1.1Kb PCR amplified product

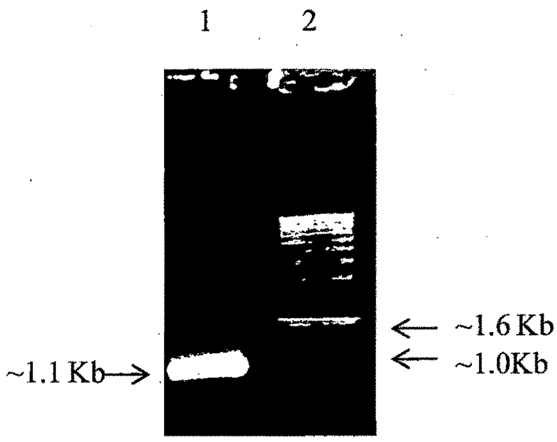
   Lane 7; 1 Kb ladder

B. Amplification of 16S rDNA (1.5Kb) sequence from the bacterial isolates from the enrichment culture of soil sample no. 4

   Lane 1 to 6; Isolate ILI-S4-I1 to 6

   Lane 7; 1 Kb ladder

   Lane 8 to 11; Isolate ILI-S4-I7 to 11



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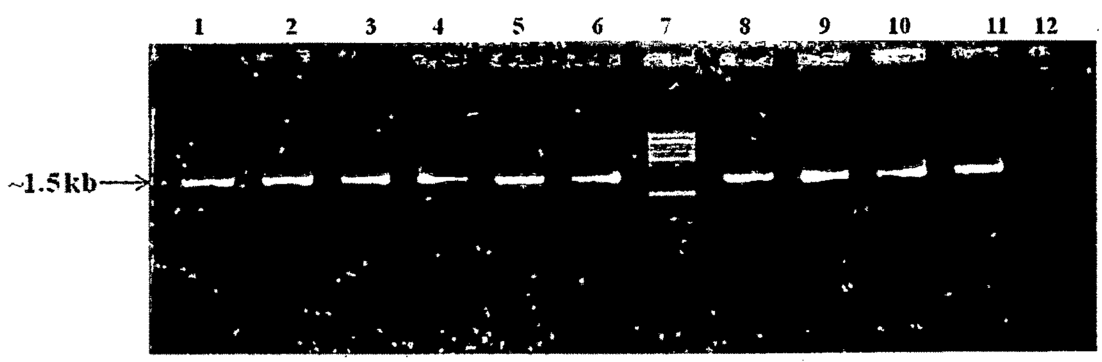
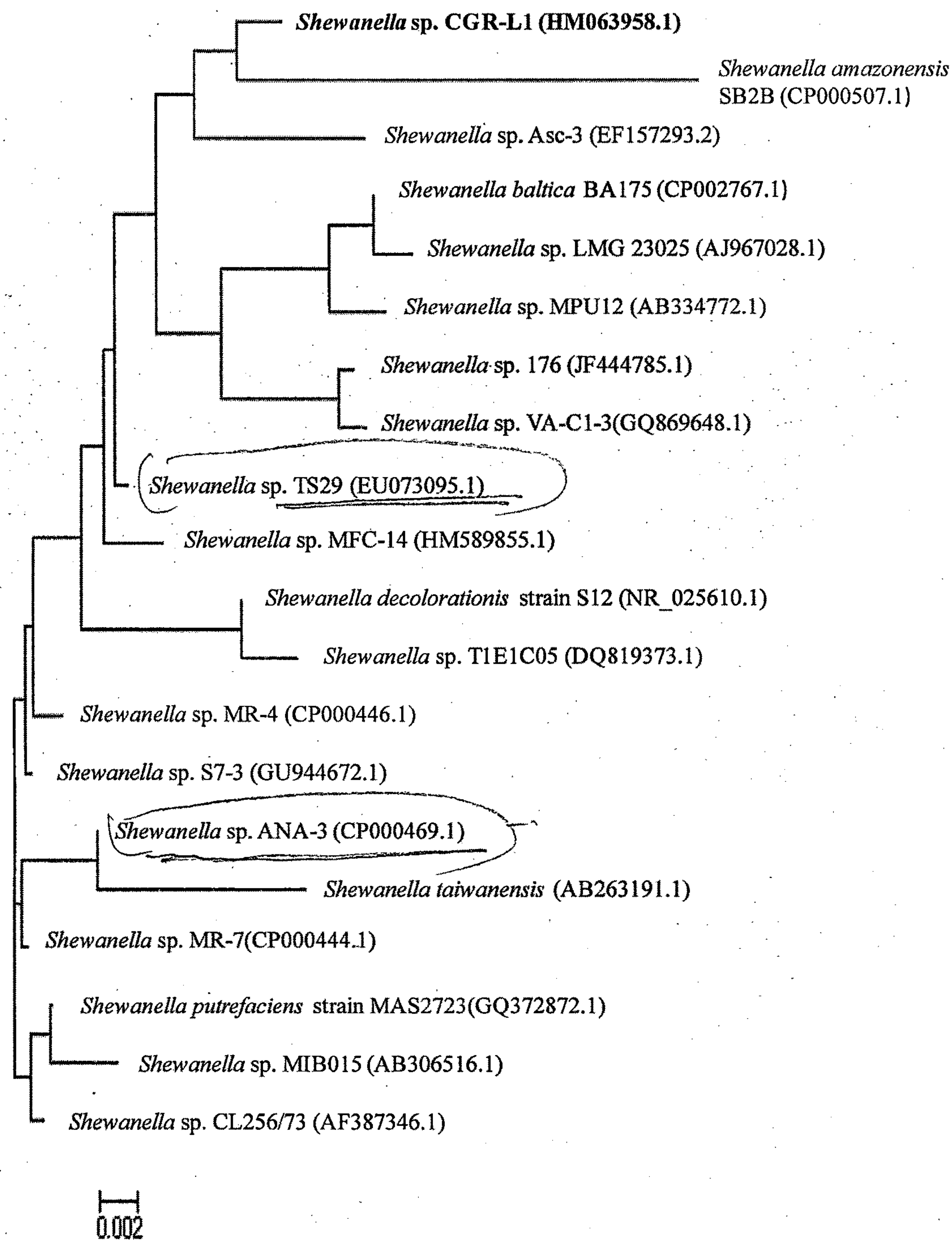


B

Figure 8. Phylogenetic relatedness of *Shewanella* sp. CGR-L1 based on 16S rRNA gene sequences. The position of strain CGR-L1 (bold letter with accession number) is shown with respect to other species of the genus *Shewanella*. Multiple sequence alignment of 16S rRNA gene sequences was performed by using CLUSTAL_X. The phylogenetic tree was generated by the MEGA version 4.0. GenBank accession numbers are shown in parentheses. The scale bar indicates 0.002 estimated substitutions per nucleotide.



Shewanella sp. CGR-L1 (HM063958.1)
Shewanella amazonensis SB2B (CP000507.1)
Shewanella sp. Asc-3 (EF157293.2)
Shewanella baltica BA175 (CP002767.1)
Shewanella sp. LMG 23025 (AJ967028.1)
Shewanella sp. MPU12 (AB334772.1)
Shewanella sp. 176 (JF444785.1)
Shewanella sp. VA-C1-3(GQ869648.1)
Shewanella sp. TS29 (EU073095.1)
Shewanella sp. MFC-14 (HM589855.1)
Shewanella decolorationis strain S12 (NR_025610.1)
Shewanella sp. T1E1C05 (DQ819373.1)
Shewanella sp. MR-4 (CP000446.1)
Shewanella sp. S7-3 (GU944672.1)
Shewanella sp. ANA-3 (CP000469.1)
Shewanella taiwanensis (AB263191.1)
Shewanella sp. MR-7(CP000444.1)
Shewanella putrefaciens strain MAS2723(GQ372872.1)
Shewanella sp. MIB015 (AB306516.1)
Shewanella sp. CL256/73 (AF387346.1)
0.002

Table 8: Analysis of the carbohydrate utilization profiles by *Shewanella* sp. CGR-L1 and *Sphingobium* sp. CGR-L2 based on the GN2 Micro Plate (BIOLOG)

	CGR-L1	CGR-L2		CGR-L1	CGR-L2
Water	-	-	p-Hydroxy Phenylacetic acid	-	-
α-Cyclodextrin	+	-	Itaconic acid	-	-
Dextrin	+	+	α-Keto Butyric acid	+	+
Glycogen	-	-	α-Keto Glutaric acid	+	+
Tween 40	+	-	α-Keto Valeric acid	+	-
Tween 80	+	-	D,L-Lactic acid	+	-
N-Acetyl-DGalactosamine	+	-	Malonic acid	-	-
N-Acetyl-DGlucosamine	+	-	Propionic acid	+	-
Adonitol	-	-	Quinic acid	-	-
L-Arabinose	+	+	D-Saccharic acid	-	-
D-Arabitol	-	-	Sebacic acid	-	-
D-Cellobiose	-	+	Succinic acid	+	+
i-Erythritol	-	-	Bromosuccinic acid	+	+
D-Fructose	-	+	Succinamic acid	-	+
L-Fucose	-	+	Glucuronamide	-	-
D-Galactose	-	+	L-Alaninamide	-	+
Gentiobiose	-	+	D-Alanine	-	-
α-D-Glucose	+	+	L-Alanine	+	+
m-Inositol	-	-	L-Alanylglycine	+	+
α-D-Lactose	-	-	L-Asparagine	-	-
Lactulose	-	-	L-Aspartic Acid	-	-
Maltose	+	+	L-Glutamic Acid	-	+
D-Mannitol	-	-	Glycyl-Laspartic Acid	+	-
D-Mannose	-	+	Glycyl-LGlutamicAcid	+	-
D-Melibiose	-	-	L-Histidine	-	-
β-Methyl-D-Glucoside	-	-	Hydroxy-LPro*line*	-	-
D-Psicose	-	-	L-Leucine	+	-
D-Raffinose	-	-	L-Ornithine	-	-
L-Rhamnose	-	-	LPhenylalanine	-	-
D-Sorbitol	-	-	L-Pro*line*	+	+
Sucrose	-	-	L-Pyroglutamic Acid	-	-
D-Trehalose	-	+	D-Serine	-	-
Turanose	-	+	L-Serine	+	-
Xylitol	-	-	L-Threonine	+	+
Pyruvic Acid Methyl Ester	+	+	D,L-Carnitine	-	-
Succinic Acid Mono-Methyl-Ester	+	+	γ-Amino Butyric Acid	-	-
Acetic acid	+	-	Urocanic Acid	-	-
Cis-Aconitic acid	-	-	Inosine	+	-
Citric acid	-	-	Uridine	+	-
Formic acid	+	-	Thymidine	+	-
D-Galactonic acid lactone	-	-	Phenyethylamine	-	-
D-Galacturonic acid	-	-	Putrescine	-	-
D-Gluconic acid	-	+	2-Aminoethanol	-	-
D-Glucosaminic acid	-	-	2,3-Butanediol	-	-
D-Glucuronic acid	-	-	Glycerol	+	-
α-Hydroxybutyric acid	+	-	D,L-α-Glycerol Phosphate	-	-
β-Hydroxybutyric acid	+	+	α-D-Glucose-1-Phosphate	-	+
γ-Hydroxybutyric acid	+	-	D-Glucose-6-Phosphate	+	+

al., (1995) suggested that menaquinone or a menaquinone oxidase was involved in tetrachloromethane transformation by *S. putrefaciens* strain MR-1. Thus, *Shewanella* genus is metabolically versatile and is able to use a diverse range of organic substrates and metals as terminal electron acceptors for growth and survival. The molecular characterization and the biodegradation potential of *Shewanella* sp. CGR-L1 isolate is described in chapter no. 2.

1.3.3 Isolation and confirmation of γ-HCH degrading bacteria from enrichment culture of industrial soil

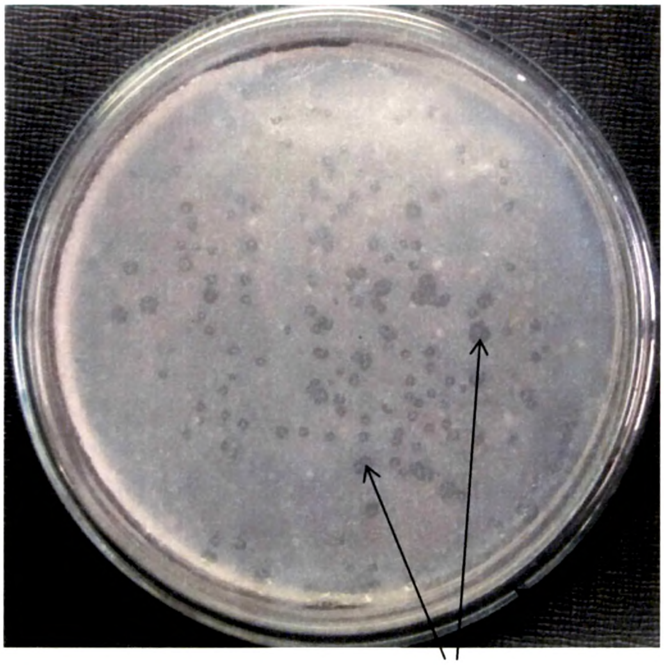
Different enrichment cultures were obtained from industrial site soil samples after successive sub culturing. Clearing zone was observed around the colonies on the plates from soil sample no. 4, after being overlaid with 1% γ-HCH (Figure 9A). From this sample, a total of eleven morphologically different bacteria ILI-S4-I1 to I11 (where ILI stands for Isolates from Lindane industry, S-stands Soil sample number and I stand for Isolate) were isolated (Figure 10). Abundance of the isolates was calculated based on their cfu ml^{-1} in the enrichment culture (Figure 11). Abundance of ILI-S4-I1 was the highest among the enrichment culture that is $8.4x10^6$ cfu ml^{-1}, whereas the isolates no. ILI-S4-I1 and I11 were lowest in number with $0.1x10^6$ cfu ml^{-1}. 16S rDNA (1.5Kb) was PCR amplified from the genomic DNA of each isolate as described in the materials and methods. After partial sequencing of 16S rDNA, all the isolates were identified based on sequence homology on the NCBI (http://www.ncbi.nlm.nih.gov/BLAST)). 4 out of 11 isolates belonged to *Pseudomonas* genus. Other isolates were identified as *Sphingobium* sp., *Rhodococcus* sp., *Staphylococcus* sp., *Azospirillum* sp., *Sphingopyxis* sp. and *Bosea*

Figure 9: γ-HCH clearance assay for *Sphingobium* sp. strain CGR-L2

A. Dilution plate of enrichment culture from soil sample no. 4 showing zone of clearance around the few colonies on the MSM-L2 agar plates after overlaid with 1% γ-HCH.

B. Isolate sp.CGR-L2 showing clearance of γ-HCH on 1/3 Luria agar plate. Luria agar plate was overlaid with 1 % γ-HCH in diethyl ether; culture colonies were spotted on the medium and were incubated for 4-6 days at 30^0C

A


zone of clearance around the colonies

B

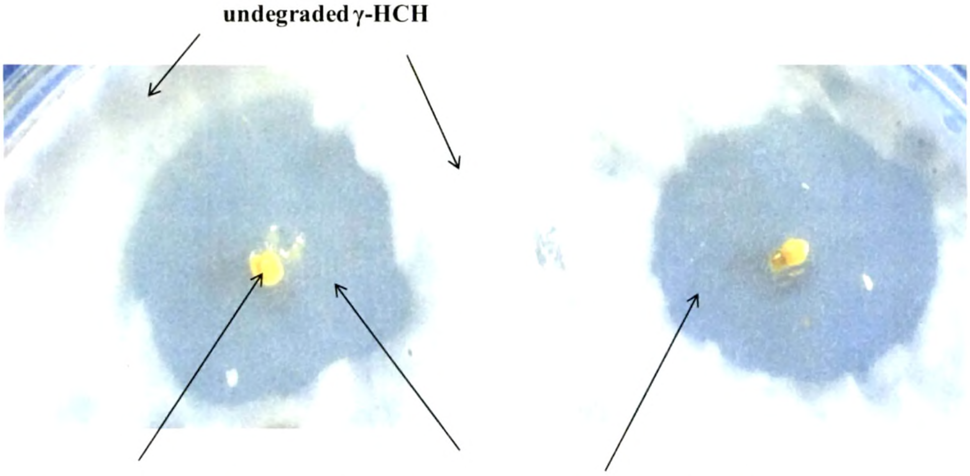

undegraded γ-HCH
Growing culture *Sphingobium* sp. CGR-L2
zone of clearance around the culture

Figure 10: Bacterial isolates obtained from γ-HCH degrading enrichment culture in MSM-L2 (presence γ-HCH) of soil Sample no. 4

Label	Identified by 16S rDNA sequence
I1	*Pseudomonas* sp. S4-ILI-I1
I2	*Pseudomonas* sp. S4-ILI-I2
I3	*Pseudomonas* sp. S4-ILI-I3
I4	***Sphingobium* sp. S4-ILI-I4(CGR-L2)**
I5	*Rhodococcus* sp. S4-ILI-I5
I6	*Staphylococcus* sp. S4-ILI-I6
I7	*Azospirillum* sp. S4-ILI-I7
I8	*Pseudomonas* sp. S4-ILI-I8
I9	*Sphingopyxis* sp. S4-ILI-I9
I10	*Sphingopyxis* sp. S4-ILI-I10
I11	*Bosea* sp. S4-ILI-I11

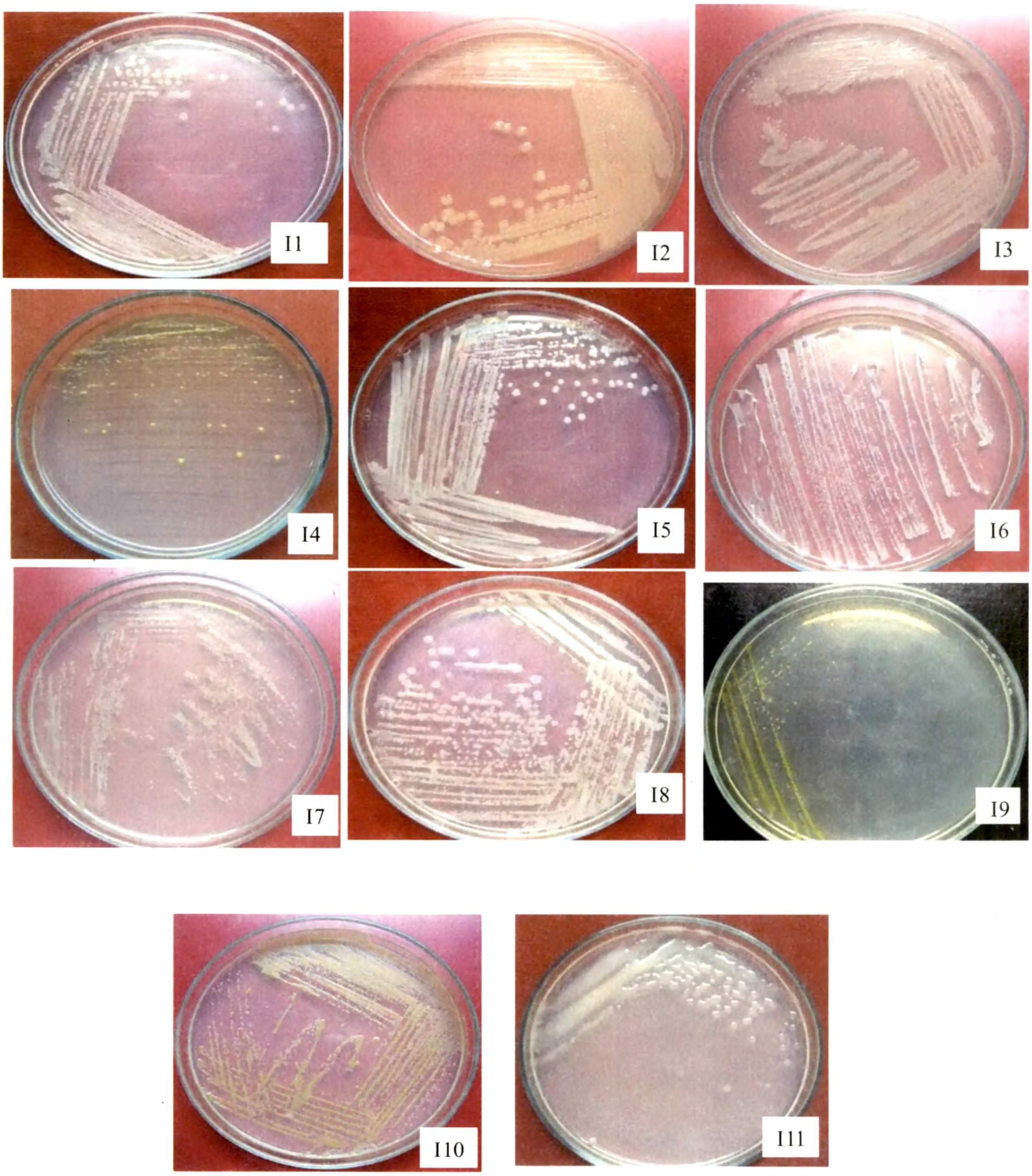
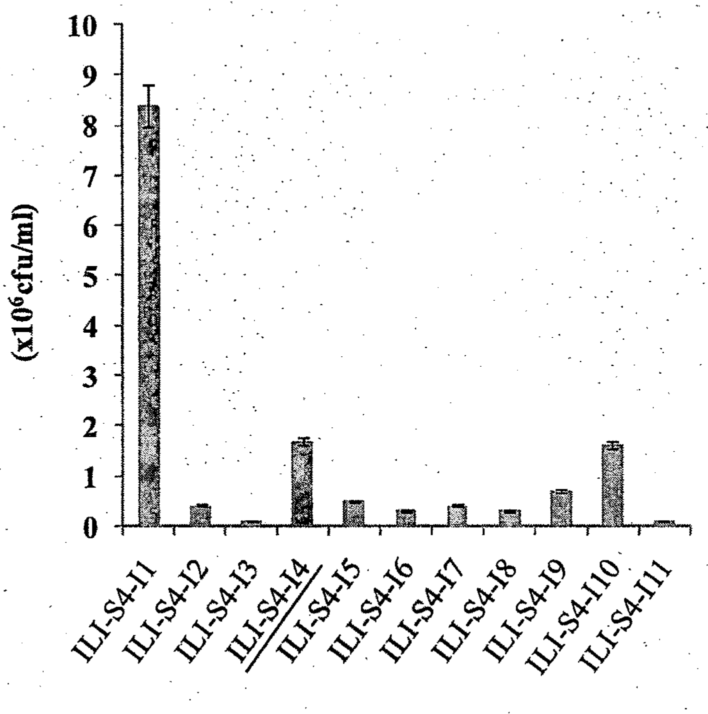

I1
I2
I3
I4
I5
I6
I7
I8
I9
I10
I11

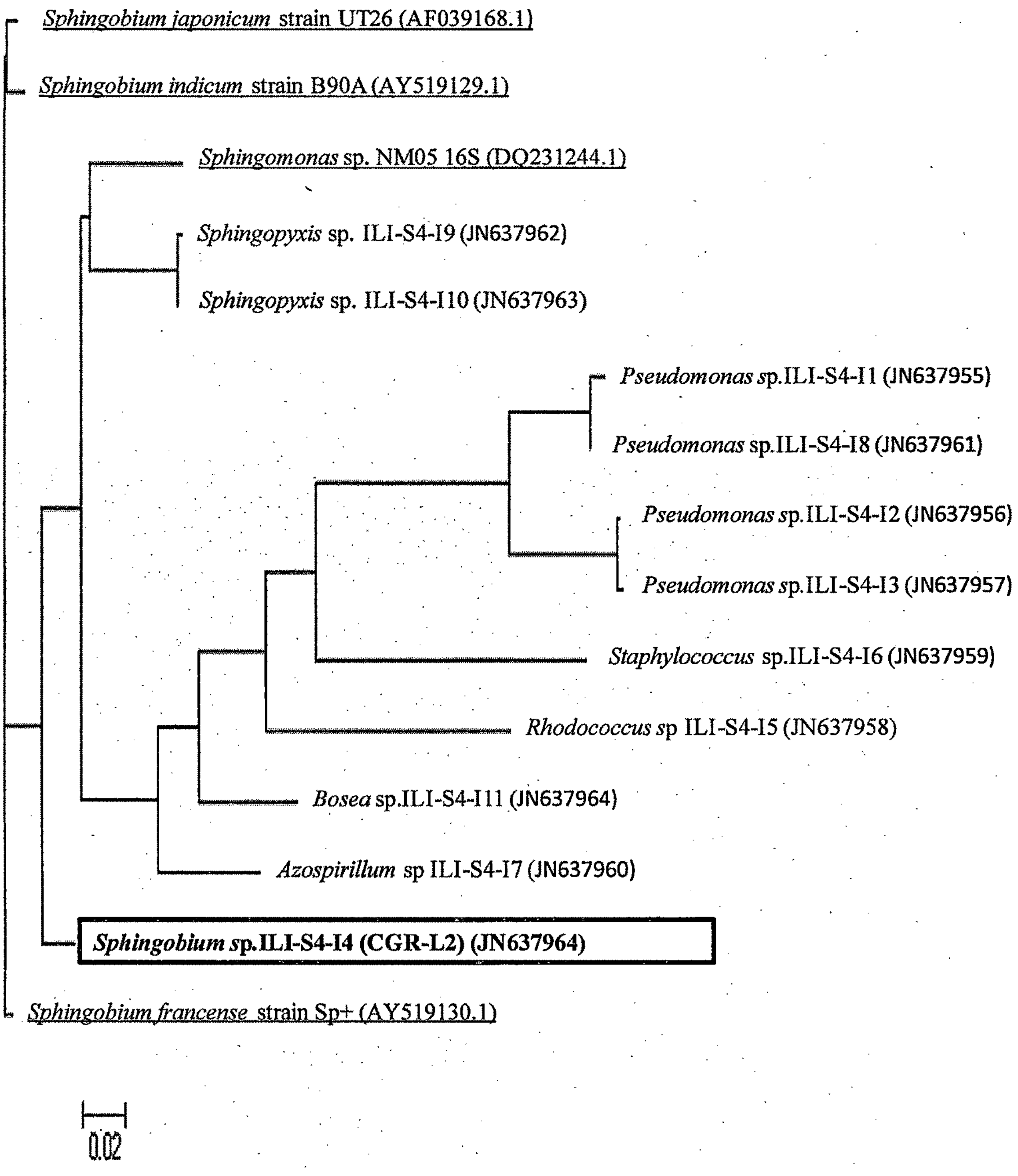
Figure 11: Abundance of each bacterium in enrichment culture of soil sample no. 4 observed as cfu ml^{-1}. Isolate **I4** (indicated with undeline) in the graph was confirmed as γ-HCH degrader in further analysis.



10
9
8
7
6
5
4
3
2
1
0
(x10^{6}cfu/ml)
ILI-S4-I1
ILI-S4-I2
ILI-S4-I3
ILI-S4-I4
ILI-S4-I5
ILI-S4-I6
ILI-S4-I7
ILI-S4-I8
ILI-S4-I9
ILI-S4-I10
ILI-S4-I11

sp. Phylogenetic tree of all these isolates was generated based on sequence obtained (Figure 12). The percent identities of the isolates with their closest relatives are shown in Table 9. Closest relatives of most of these genera have been reported previously from pesticide contaminated sites and are involved in biodegradation of γ-HCH or other halogenated compounds (Senoo & Wada, 1989; Kumar et al., 2005; Singh et al., 2007). Among these isolates, ILI-S4-I4 identified as *Sphingobium* sp. showed clearing zone around the colony γ-HCH clearance assay (Figure 9B). In the enrichment culture isolate ILI-S4-I4 was the second most abundant bacteria with $1.7x10^6$ cfu ml^{-1}. Further, this isolate I4 was renamed as CGR-L2. Isolate CGR-L2 was found to be gram-negative rod-shaped bacteria. Its colonies were mucoid, translucent and yellow pigmented, circular, pin-point with entire margin and convex. Diameters of colonies were found to be ≤ 1mm after 72 hours on Luria agar plate. Interestingly, homology search with this isolate showed 100% identity with reported γ-HCH degrading isolate *Sphingobium ummariense* RL-3^T (EF207155.1), 99% with *Sphingomonas* sp. SO1a (AB453305.1) and 96 % with *Sphingobium japonicum* strain UT26 (AF039168.1) (Figure 13). Most of the HCH-degrading bacteria that have been isolated belong to *Sphingomonads* (Boltner et al. 2005, Lal et al., 2010) due to their ability to degrade chlorinated compounds in the environment. Importantly, the whole genome sequence of isolate *S. japanicum* UT26 is available (Nagata et al., 2010), which makes it easy to analyse γ-HCH degrading ability at molecular level. In BIOLOG assay also isolate CGR-L2 identified as *Sphingomonas* sp. (Table 8). The isolate *Sphingobium sp.* CGR-L2 was further characterized (chapter no. 3). The 16S rDNA nucleotide sequences determined in these studies have been deposited into the GenBank database with accession number as mentioned in Table 10.

Figure 12: Phylogenetic tree constructed by using bacterial partial 16S rRNA gene sequences of the cultivable isolates obtained from the γ-HCH degrading enrichment culture of the soil sample no. 4. Name of the bacteria and their accession number in parenthesis represents the identified isolates. Reference sequences of the bacterial isolates (indicated with underlined and their accession numbers), reported to be involved in γ-HCH biodegradation were used to compare their relatedness. The isolate CGR-L2 that was further confirmed as γ-HCH degrader, is indicated in square with bold letters. Multiple sequence alignment of 16S rRNA gene sequences was performed by using CLUSTAL_X. The phylogenetic tree was generated by the Mega4 version 4.0. GenBank accession numbers are shown in parentheses. The scale bar represents the expected number of substitutions per nucleotide position.



Sphingobium japonicum strain UT26 (AF039168.1)
Sphingobium indicum strain B90A (AY519129.1)
Sphingomonas sp. NM05 16S (DQ231244.1)
Sphingopyxis sp. ILI-S4-I9 (JN637962)
Sphingopyxis sp. ILI-S4-I10 (JN637963)
Pseudomonas sp.ILI-S4-I1 (JN637955)
Pseudomonas sp.ILI-S4-I8 (JN637961)
Pseudomonas sp.ILI-S4-I2 (JN637956)
Pseudomonas sp.ILI-S4-I3 (JN637957)
Staphylococcus sp.ILI-S4-I6 (JN637959)
Rhodococcus sp ILI-S4-I5 (JN637958)
Bosea sp.ILI-S4-I11 (JN637964)
Azospirillum sp ILI-S4-I7 (JN637960)
Sphingobium sp.ILI-S4-I4 (CGR-L2) (JN637964)
Sphingobium francense strain Sp+ (AY519130.1)
0.02

Figure 13. Phylogenetic analysis of the isolate strain CGR-L2. The position of strain CGR-L2 is shown with respect to other species of the genus *Sphingobium* (indicated in square). Reference sequences of the bacterial isolates (indicated with underlined and their accession numbers), reported to be involved in γ-HCH biodegradation were used to compare their relatedness. *Sphingobium ummariense* sp. RL-3^T found to be as closely related to strain CGR-L2 (indicated in bold letters), on the basis of sequence similarity. Multiple sequence alignment of 16S rRNA gene sequences was performed by using CLUSTAL_X. The phylogenetic tree was generated by the Mega version 4.0. GenBank accession numbers are shown in parentheses. The scale bar indicates 0.01 estimated substitutions per nucleotide

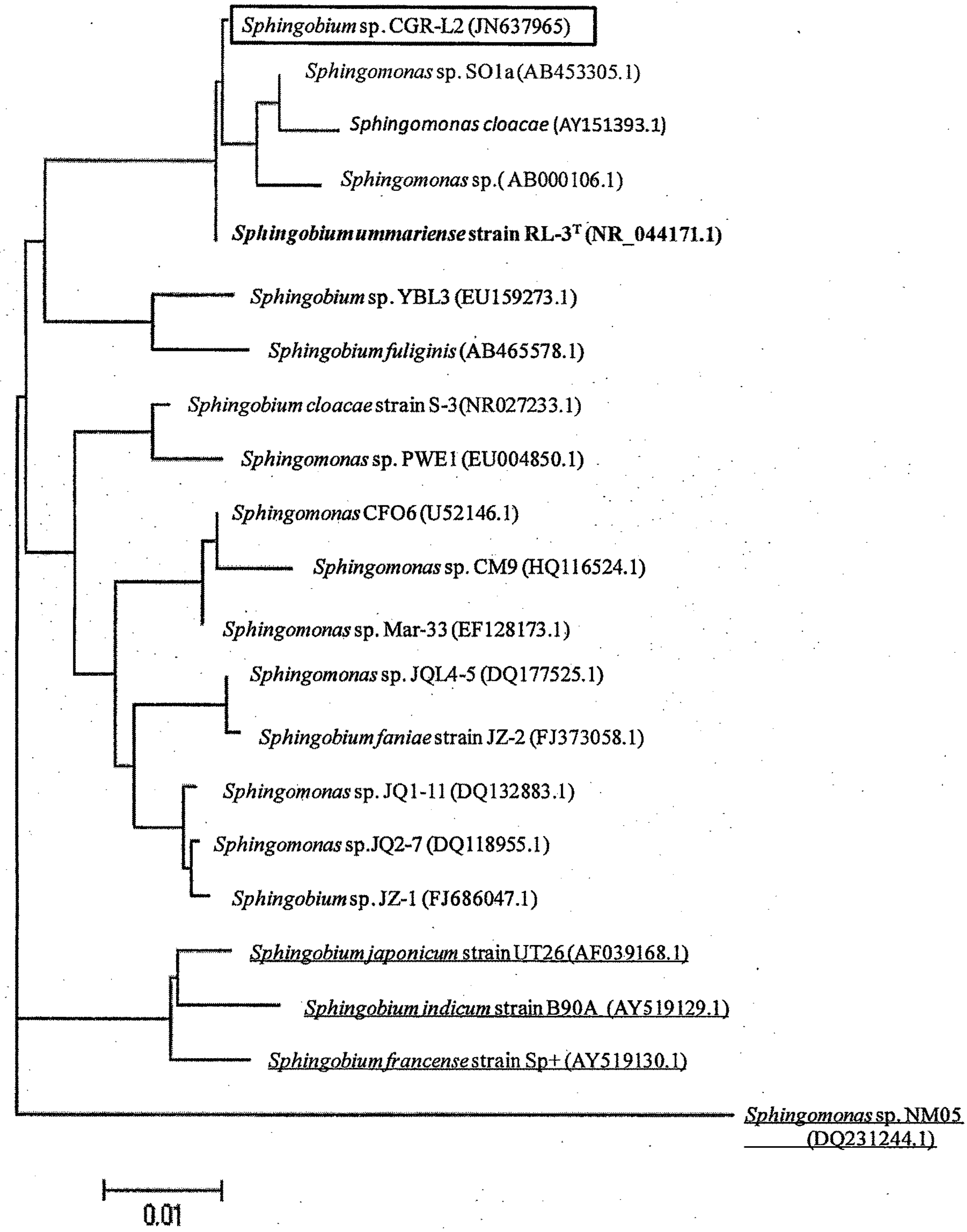

Sphingobium sp. CGR-L2 (JN637965)
Sphingomonas sp. SO1a (AB453305.1)
Sphingomonas cloacae (AY151393.1)
Sphingomonas sp.(AB000106.1)
Sphingobium ummariense strain RL-3T (NR_044171.1)
Sphingobium sp. YBL3 (EU159273.1)
Sphingobium fuliginis (AB465578.1)
Sphingobium cloacae strain S-3 (NR027233.1)
Sphingomonas sp. PWE1 (EU004850.1)
Sphingomonas CFO6 (U52146.1)
Sphingomonas sp. CM9 (HQ116524.1)
Sphingomonas sp. Mar-33 (EF128173.1)
Sphingomonas sp. JQL4-5 (DQ177525.1)
Sphingobium faniae strain JZ-2 (FJ373058.1)
Sphingomonas sp. JQ1-11 (DQ132883.1)
Sphingomonas sp.JQ2-7 (DQ118955.1)
Sphingobium sp. JZ-1 (FJ686047.1)
Sphingobium japonicum strain UT26 (AF039168.1)
Sphingobium indicum strain B90A (AY519129.1)
Sphingobium francense strain Sp+ (AY519130.1)
Sphingomonas sp. NM05 (DQ231244.1)
0.01

Table 9: Identification of bacteria on the basis of their 16S rDNA sequence identity on NCBI (BLAST), obtained from enrichment culture of soil no. 4, showing close relative with percentage identity.

Isolate Name	Total sequence (bp)	Identified by 16S rDNA sequence	Close reported isolate and their accession number		Maximum identity (%)
ILI-I1	500	*Pseudomonas* sp.	*Pseudomonas stutzeri* strain ISA12	HQ189755.1	99
ILI-I2	632	*Pseudomonas* sp.	*Pseudomonas* sp. bD39(2011)	JF772535.1	100
ILI-I3	628	*Pseudomonas* sp.	*Pseudomonas azelaica* strain HBP1	FJ227303.1	99
ILI-I4	**1386**	***Sphingobium* sp.**	***Sphingobium ummariense* RL-3^T**	**EF207155.1**	**100**
ILI-I5	725	*Rhodococcus* sp.	*Rhodococcus qingshengii* strain ZJB-09153 1	HQ439600.1	100
IILI-I6	746	*Staphylococcus* sp.	*Staphylococcus pasteuri* strain SNA59	HQ220117.1	100
ILI-I7	616	*Azospirillum* sp.	*Azospirillum* sp. TSO9	AB545621.1	99
ILI-I8	629	*Pseudomonas* sp.	*Pseudomonas* sp. M060706-6	EU589405.1	100
ILI-I9	742	*Sphingopyxis* sp.	*Sphingopyxis* sp. SM105	EF424407.1	100
ILI-I10	649	*Sphingopyxis* sp.	*Sphingopyxis* sp. UI2	EF424391.2	100
ILI-I11	522	*Bosea* sp.	*Bosea* sp. dv3	FJ774000.1	100

Table 10: Accession numbers of the sequences deposited in the GenBank database (NCBI)

A. Accession numbers for the 16S rRNA gene (16S rDNA) for the bacterial isolates that were used in present study

B. Accession number of the *lin* genes analysed in the γ-HCH degrading bacterial isolates *Shewanella* sp. CGR-L1 and *Sphingobium* sp. CGR-L1

A.

Isolate Name	Identified by 16S rDNA sequence on NCBI data bank	Accession number
CGR-L1	***Shewanella* sp.CGR-L1**	**HM063958.1**
ILI-I1	*Pseudomonas* sp.	JN637955
LI-I2	*Pseudomonas* sp.	JN637956
ILI-I3	*Pseudomonas* sp.	JN637957
ILI-I4 (CGR-L2)	***Sphingobium* sp.**	**JN637965**
ILI-I5	*Rhodococcus* sp.	JN637958
IILI-I6	*Staphylococcus* sp.	JN637959
ILI-I7	*Azospirillum* sp.	JN637960
ILI-I8	*Pseudomonas* sp.	JN637961
ILI-I9	*Sphingopyxis* sp.	JN637962
ILI-I10	*Sphingopyxis* sp.	JN637963
ILI-I11	*Bosea* sp.	JN637964

B.

Name of the gene	Bacterium source	Accession number
*lin*A	*Shewanella* sp. CGR-L1	HM063959.1
*lin*A	*Sphingobium* sp. CGR-L2	N638439
*lin*B	*Sphingobium* sp. CGR-L2	N638440
*lin*C	*Sphingobium* sp. CGR-L2	N638441
*lin*D	*Sphingobium* sp. CGR-L2	N638442
*lin*E	*Sphingobium* sp. CGR-L2	N638443
*lin*R	*Sphingobium* sp. CGR-L2	N638444