Chapter 3

Characterization of γ-HCH degrading isolate *Sphingobium* sp. CGR-L2

3.1 Introduction

Hexachlorocyclohexane (HCH) is a cyclic saturated chlorinated hydrocarbon insecticide that consists primarily of the isomers α- (60–70 %), β- (5–12 %), γ- (10–15 %) and δ-HCH (6–10 %) (Kutz et al., 1991). Prior to the 1990s, two forms of HCH, technical HCH (a mixture of all isomers) and purified γ-HCH (the only isomer with insecticidal properties) were used extensively. Although the use of technical HCH and γ-HCH has been banned in many countries, residues of HCH isomers are still detected from almost all components of the environment (Willem & Wollent, 2005). γ-HCH and other isomers degraded under both aerobic and anaerobic conditions, but it is generally mineralized only under aerobic conditions (Phillips et al., 2005). A small number γ-HCH-degrading aerobic bacteria have been isolated and characterized (Phillips et al. 2005; Lal et al. 2006; Boltner et al. 2005). Furthermore, identification of genes (*lin*A, *lin*B, *linC*, *lin*D, etc) and enzymes involved in the degradation of γ-HCH, is important to understand the role of catalytic enzymes toward the degradation of recalcitrant γ-HCH.

We have characterized γ-HCH degrading *Sphingobium* strain CGR-L2 that was isolated from the industrial site, for its biodegradation potentially. CGR-L2 utilizes γ-HCH as a sole source of carbon and energy under aerobic conditions. Effect of various parameters like inoculum size, supplementary carbon source, pH etc. has been observed in the MSM-L2. Further, microcosm study was performed with technical HCH-spiked soils, which showed that *Sphingobium* strains CGR-L2 could effectively degrade γ- and β-HCH. *lin* genes for HCH degradation.

3.2 Material and methods

3.2.1 Soil analysis, Dehalogenase and antibiotic sensitivity assays

Soil analysis, protein extraction and assays for dehalogenase and antibiotic sensitivity were performed as described in materials and methods 2.2.1, 2.2.2, 2.2.3 and 2.2.4 (Chapter 2), respectively.

3.2.2 Growth kinetics of CGR-L2 in MSM-L2 in presence of γ-HCH

Growth kinetic study was performed in the minimal salt medium (MSM-L2) without glucose. Two days pre-grown cells in 1/3LB in the presence of γ-HCH (10 mg l^{-1}) were harvested by centrifugation (13,200×g for 5 min), washed with sterile water, and resuspended in fresh MSM-L2 to give a final OD_{600nm} of 0.02. In all the experiments filtered sterilized γ-HCH was used at a concentration of 10 mg l^{-1}. Both control (without culture) and test flasks were incubated at 30^0C with shaking at 200 rpm. Optical density and residual concentration of γ-HCH in the medium were analysed up to 9 days in MSM-L2. A flask containing only γ-HCH in MSM-L2 without the inoculum was kept as a control. All the experiments were performed in triplicate.

3.2.3 Analysis of the γ-HCH degradation

Degradation of γ-HCH was analysed as described in material and methods 2.2.6 (Chapter 2).

3.2.4 Dechlorination assay for Cl^- (chloride) estimation from γ-HCH degradation

Chloride released by the degradation of γ-HCH was measured by colorimetric assay (Bergmann and Sanik., 1957 and Sahu et al., 1990). Briefly, flask (250 ml) containing 50 ml of de-ionized water with γ-HCH (10 mg l^{-1}) was inoculated with CGR-L2 culture (0.05 OD_{600nm}). Control was similar in composition, but without any bacterial inoculum. Samples were analyzed in triplicate for the Cl^- content at 24, 72 and 144 hours after incubation. 2 ml of inoculated and uninoculated samples were treated with 0.2 ml of 0.25 M ferric ammonium sulfate in 9M nitric acid and 0.2 ml of a saturated solution of mercuric thiocyanate in ethyl alcohol. The color developed after 10 min was measured at 460 nm by a spectrophotometer (Beckman Coulter, Inc., Brea, CA)) against a blank that contained reagents only. The amount of Cl^- was computed using a standard curve prepared using NaCl.

3.2.5 Effect of various parameters on γ-HCH biodegradation by isolate CGR-L2 in MSM-L2

3.2.5.a Effect of inoculum size

Different microbial cell densities corresponding to 0.02, 0.05, and 0.1 OD_{600nm}, were inoculated in conical flasks containing 100 ml MSM-L2 (without carbon source), amended with γ-HCH (10 mg l^{-1}). The pH of the medium was adjusted to 7.0 and the flasks were incubated at 30^0C and 200 rpm. Samples were collected after 24, 48 and 72 hours of incubation, extracted with n-Hexane and then with ethyl acetate as reported (Manickam et al., 2006), and analyzed for residual γ-HCH concentration using TLC or GC-ECD.

3.2.5.b Carbon utilization profile of *Sphingobium* sp. CGR-L2

A GN2 Micro Plate (Biolog, Hayward, Calif.) was used to characterize strain CGR-L2 based on substrate utilization profiling in presence and absence of pesticide. A single colony of CGR-L2 was streaked on Luria agar and incubated at 30^0C for 72 hours. Cells were suspended in sterile water with final 1.0 OD_{600nm}, in the presence (test) of γ-HCH (final concentration 10 μg ml^{-1}) or absence (control) of γ-HCH, and inoculated onto the GN2 Micro Plate. After incubation for 24, 48 and 72 hours, the resulting developed color was read at 750 nm using the Biolog automated Micro-Station instrument (Biolog MicroStation™ system Hayward, CA).

3.2.5.c Effect of supplementary carbon source (glucose)

γ-HCH was added in different conical flasks containing 100 mL of MSM-L2 to a final γ-HCH concentration of 10 mg l^{-1}. Dextrose was added as a supplementary carbon at concentrations of 0, 1, 5, and 10 g l^{-1} and the pH of the medium was maintained at 7.0. An inoculum of bacterial cells corresponding to final OD_{600nm} of 0.05 were added to the above flasks and kept in an orbital shaker at 30^0C at 200 rpm. Samples were collected at 18 hour and 24 hour, extracted with n-Hexane and then with ethyl acetate as reported earlier and analyzed for residual γ-HCH concentration by using TLC/GC-ECD.

3.2.5.d Effect of pH

The pH of the MSM-L2 was adjusted to 5, 6, 7, 8, and 9 with the addition of nitric acid (HNO_3)/sodium hydroxide (NaOH) solutions. MSM-L2 was prepared as described above with a γ-HCH (10 mg l^{-1}) along with 5gL^{-1} dextrose as a supplementary carbon.

Predetermined final concentration of bacterial cells at 0.05 OD_{600nm} was inoculated. The inoculated flasks were kept in an orbital shaker at 30^0C and 200 rpm. Blank control flasks were kept simultaneously. The samples were collected from the control and test flasks after 12 hours and 18 hours, extracted with n-Hexane and then with ethyl acetate as reported earlier and analyzed for residual γ-HCH concentration by using TLC /GC-ECD.

3.2.5.e Effect of temperature

Predetermined final concentration of bacterial cells at 0.05 OD_{600nm} was taken in 50 ml of MSM-L2 media amended with γ-HCH (10 mg l^{-1}) and 0.5% dextrose. The inoculated flasks were kept on an orbital shaker at 25, 30, 37 and 42 ^{0}C and 200 rpm for 12 hours. Blank controls were also kept simultaneously at different temperatures. The samples were collected, extracted with n-Hexane and then by ethyl acetate for analyzing the residual γ-HCH concentration.

3.2.6 Optimization of γ-HCH biodegradation

Growth kinetics was studied in the MSM-L2 added with 10 mg l^{-1} γ-HCH at optimized above described parameters *i.e.* 0.05 OD_{600nm} inoculum size, pH 8.0, 5 g l^{-1} glucose, at 30^0C and 200 rpm. Optical density and residual concentration of γ-HCH in the medium were analyzed at 0, 6, 9 and 12 hours. Optical density was observed at OD_{600nm} to monitor the growth, followed by γ-HCH residual concentration analysis, as described earlier.

3.2.7 GC-MS analysis for identification of metabolites produced during growth of *Sphingobium* sp. CGR-L2

A GC-MS (Shimadzu QP 2010 gas chromatograph, Shimadzu Scientific Instruments, Columbia, USA) analysis was performed for the metabolites produced by the isolate *Sphingonbium* strain CGR-L2 during γ-HCH degradation. The samples were collected, extracted with n-Hexane, ethyl acetate for analyzing the metabolites as described earlier in material and methods 2.2.8 (Chapter 2). A 1 µl solution of the sample was injected for the analysis. Metabolites were separated on column RTX59 (30 M x 0.25 mm ID x 0.25 µM film) packed with 5% phenyl and 95% dimethyl poly siloxane material and helium was used as the carrier gas (at 2 ml min^{-1}). The column temperature was held at 100^0C for 7 min, and then increased to 250^0C at a rate of 5^0C min^{-1}.

3.2.8 Analysis of technical HCH degradation by CGR-L2

Two day pre grown cells in 1/3 diluted LB in presence of technical HCH (20 mg l^{-1}) were harvested by centrifugation (13,200×g for 5 min), washed with sterile water , and resuspended in fresh MSM-L2 (with glucose 0.5%) to give a final at OD_{600nm} of 0.05 in 200 ml in 1L flask. In all the experiments, filter-sterilized technical HCH was used at a concentration of 20 mg l^{-1}. Both control (without culture) and test flasks were incubated at 30^0C with shaking at 200 rpm. Residual concentrations of HCH isomers in the medium were analyzed by collecting samples at 24, 48 and 96 hours of growth. A flask containing only technical grade HCH in MSM-L2 without the inoculum was kept as control.

3.2.9 Identification of γ-HCH tolerance level of CGR-L2

γ-HCH tolerance level of the isolate CGR-L2 was checked as reported previously (Endo et al., 2006). Isolate CGR-L2 was grown in 1/3 LB medium to the OD_{600nm} 0.1 at 30^0C, γ-HCH added in the flask containing medium with final concentration of 10, 25, 35, 50, 100, 250, 500 and 1000 mg l^{-1}. OD_{600nm} was observed with time interval up to 48 hours. Flask with no γ-HCH was taken as control.

3.2.10 PCR amplification of *lin* genes

The oligonucleotide primers were designed based on sequences reported for *lin* genes (*lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *lin*F and *lin*R) from *S. paucimobilis* UT26 (Nagata et al., 2007) and were used to amplify the homologous DNA fragments from the CGR-L2 (Table 11). PCR conditions for each *lin* gene were optimized and the PCR reaction mixture was set as described in materials and methods 2.2.9 (Chapter 2). The size of the amplified product based on the genes were analysed by electrophoresis on 0.8% agarose gel. The amplified DNA was purified by gel extraction column (GE healthcare Ltd, UK) and was sequenced. Genomic DNA was extracted as mentioned in material methods 1.2.7.a. Presence of *lin*A, *lin*B and *lin*C genes in the isolates from enriched culture other than ILI-I4 (CGR-L2) was performed as described below.

3.2.11 Southern-blot analysis

Nonradioactive nucleic acid labeling of *lin* genes was performed as described by manufacturer (GE healthcare Ltd, UK). Briefly, the *lin* genes present in plasmids (obtained as gift from Prof. Yuji Nagata, Sendai, Japan) were digested with appropriate

restriction enzymes and the insert DNA was separated on 0.8% agarose gel followed by elution using Gen Elute^{TM} Gel Extraction Kit (GE healthcare Ltd, UK). Around 100 ng of each *lin* gene (*lin*A, *lin*B, or *lin*C) insert DNA was labeled separately, using non-radioactively method (Gene Images Alk Phos Direct Labeling and Detection System, GE healthcare Ltd, UK). The resultant DNA probes were used to hybridize and identify the homologous genes from strain CGR-L2. 5 μg of genomic DNA from CGR-L2 was digested individually with restriction enzymes *Eco*RI and *Hind*III, *Pst* I, *Xho*I, *Sal*I, *Hind*III, *Eco*RI or *Bam*H1. Purified fragment of *lin*A, *lin*B or *lin*C were used as a positive control. All the samples were electrophoresed on 0.8% agarose gel and blotted onto nylon membrane (Hybond N^+) (Amersham, Buckinghamshire, England). DNA was fixed on the membranes by UV cross-linking ($12x10^4$ μJ/cm^2) using UV cross-linker (Spectrolinker, Spectronics Corporation, USA).

3.2.12 Analysis of biodegradation of Hydroquinone

Two day pre grown cells in 1/3 LB in presence of γ-HCH (10 mg l^{-1}) were harvested by centrifugation (13,200×g for 5 min), washed with sterile water, and resuspended in fresh MSM-L2 (with glucose 0.5%) to give a final OD_{600nm} of 0.1. Instead of γ-HCH, Hydroquinone (50 mg l^{-1}) was used as substrate. A flask containing Hydroquinone in MSM without culture was used as control. Both control and test flasks were incubated at 30^0C and 200 rpm. Culture was collected at different time intervals (0, 6 and 12 hours) and centrifuged at 13,200×g for 5 min. Supernatant obtained was extracted with ethyl acetate and organic phase was collected. It was further used to analyse residual hydroquinone level in the medium by thin layer chromatography (Miyauchi et al., 1998).

3.2.13 Bioremediation -Technical HCH degradation in soil

3.2.13.a Microcosm studies

Microcosm studies were performed to assess the HCH degradation potential of the *Sphingobium* sp. CGR-L2 for bioremediation. Sterilized organic farm soil amended with technical HCH (0.2 mg g^{-1} of soil) was used for the study. Technical HCH amended soil samples were inoculated with bacterial cells $2x10^7$ cfu gm^{-1}. Blank control (with HCH and without bacterial cells) was set with similar operating parameters. Microcosms were prepared in triplicate, and their performance was monitored regularly under aerobic conditions.

3.2.13.b Analysis of residual concentration of technical HCH in microcosm study

After 7, 14, 28 days incubation, soil (10gm) was air dried and extracted with 25ml of n-Hexane: Acetone (9:1), then with 25 ml of ethyl acetate. The extracts were filtered through 0.22 micron nylon membrane and reduced in rotary evaporator (Janke and Kunkel, model RVO5-ST, IKA Laboratories, Staufen, Germany) and finally dissolved in the 10 ml of ethyl acetate. Further 2 ml chilled water was added in the extract and vortexed for 30 sec, centrifuged at 13,200×g for 5 min and organic phase collected. Further organic extracts were dehydrated passing through anhydrous $MgSO_4$ column. Samples were filtered through 0.2 micron filter and concentrated on speedvac (Savant Instruments, Inc. New York, USA) and finally dissolved in the 0.1 ml of the ethyl acetate and residual concentration was analysed by TLC/GC-ECD.

3.2.13.c Growth of CGR-L2 bacteria in microcosm study

The number of cultivable CGR-L2 cells in soil was determined by plating. After incubation of 7, 14, 28 days, soil (1gm) was mixed with 9 ml of sterile saline solution, vortexed for 2 min, serially diluted and plated on LB agar (containing 10 mg l^{-1} γ-HCH, 10 μg ml^{-1} streptomycin). After 3-4 days of incubation on LB agar plates at 30^0C, CGR-L2 colonies were identified mostly on the basis of their yellow pigment. These plates were overlayed with 1% γ-HCH in diethyl ether and zone of clearance was observed next day. On the basis of colonies showing zone of clearance, cfu gm^{-1} was analysed. Colonies showing clearance were further identified via PCR amplification and sequencing of 16S rDNA and *lin*A as earlier mentioned.

3.3 Results and discussion

After enrichment in MSM-L2 efficiently γ-HCH degrading isolate CGR-L2 was identified with full 16S rRNA gene sequence as *Sphingobium* sp. Isolate CGR-L2 was characterized further to observe its biodegradation potential.

3.3.1 Soil analysis, dehalogenase and antibiotics assay

Soil sample no. 4 from the industrial site, used for the isolation of CGR-L2, was analysed for physico-chemical characteristics, like pH, organic carbon content and micronutrient (Table 12). pH of the soil was found to be as 7.32. Since, we had used MSM-L2 with neutral pH during enrichment and also maintained pH 7.0 for all further experiments.

The results of antibiotic sensitivity assays of CGR-L2 are summarised in Table 12. Isolate *Sphingobium* sp. strain CGR-L2 was found to be resistant to streptomycin (10 mg l^{-1}). Consequently; streptomycin was used in further experiments, to prevent other bacterial contamination.

In the dehalogenase assay, after an incubation of 24 hours the colour of the solution changed from orange to yellow with drop in pH from 8.0 to ~6.19 in the test assay, because of the chloride release (HCl formation) as per the assay protocol, whereas the control showed no change (Figure 22B). Previously similar results were observed in γ-HCH degrading isolates *Sphingomonas* sp. NM05 (Phillips et al., 2001) and *Rhodanobacter lindaniclasticus* (Manickam et al., 2008).

Figure 22:

A. SDS-PAGE analysis of cell free extract from isolate *Sphingobium* sp. strain CGR-L2

Lane 1 ; Cell free extract from isolate CGR-L2 grown in presence of γ-HCH in 1/3 LB

Lane 2 ; Cell free extract from isolate CGR-L2 grown in absence of γ-HCH in 1/3 LB

Lane 3 ; Protein marker (PMWM, medium range, Bangalore Genei, Bangalore, India)

The band corresponding to the possible *lin*A gene product (~16.5 kDa) is indicated by an arrowhead.

B. (1) Colorimetric assay for γ-HCH dechlorination using cell-free extracts of strain CGR-L2. Microtitre plate was incubated for 24 hr at 30^0C (Experiment was performed in triplicate).

Lane 1. Sonication buffer used to prepare cell-free extract

Lane 2. Heat-denatured cell-free extract with HCH.

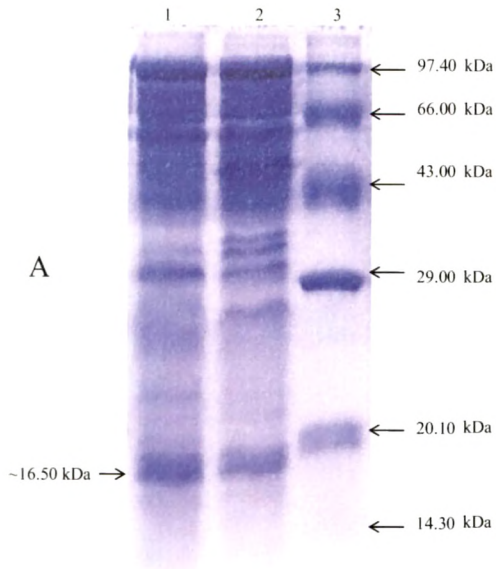
Lane 3. Only HCH without cell extract

Lane 4. Cell-free extract with HCH

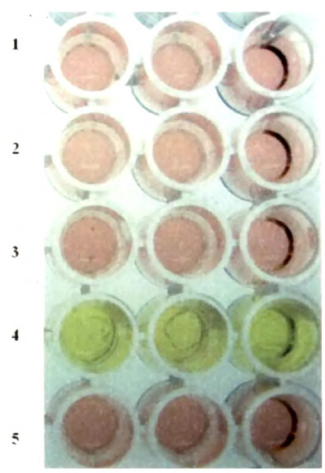
Lane 5. Cell-free extract but without HCH

(2) Change in pH recorded in each well corresponding to Figure A.

C. Estimation of free chloride release by *Sphingobium* sp. CGR-L2



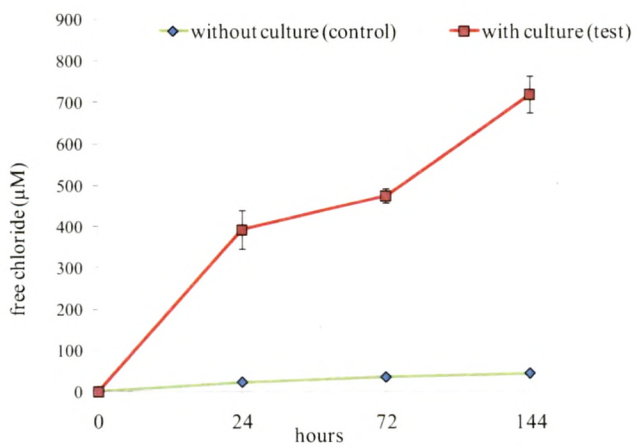
A



B (1)

	pH		
1	8.0	8.0	8.0
2	7.64	7.61	7.55
3	7.95	7.96	7.91
4	6.21	6.25	6.11
5	7.75	7.72	7.72

B (2)



C

In SDS-PAGE analysis, a ~16.5 kDa desired size of the *lin*A protein was found to be present in both the samples test and control (Figure 22A). This suggests that *lin*A is constitutively expressed in the isolate *Sphingobium* sp. CGR-L2. Constitutive expression of *lin*A in the HCH degrading *Sphingomonas* sp. has been also shown previously (Thomas et al., 1996).

3.3.2 Growth curve and γ-HCH degradation analysis

Growth studies were performed in the MSM-L2 in the presence of the γ-HCH (10 mg l^{-1}) as described in materials and methods. The growth of CGR-L2 increased slowly but steadily with time (Figure 23A), however no significant decrease was observed in pH of the medium. On the other hand, a significant decrease in residual concentration of γ-HCH was observed (~76.45% and ~8.95% residual concentration of γ-HCH after 1 and 3 days, respectively). Within 3 days of incubation in the MSM-L2, isolate CGR-L2 was able to degrade up to ~91% of the γ-HCH compared to the control (without culture) (Figure 23B). γ-PCCH, one of the metabolite of γ-HCH degradation was observed in the TLC analysis (Figure 23C). Further, we also observed that after 3 days color of medium turned brown in the test culture flasks, which may be due to the production of phenolics metabolites.

It was observed that the isolate CGR-L2 grew very slowly, when γ-HCH was used as the sole source of carbon. In earlier reports also poor growth was seen in the mineral salts medium, when γ-HCH was used as the sole carbon source (Manickam et al., 2008, Endo et al., 2006). As can be seen from the Figure 23, the optical density of the isolate CGR-L2 increases till 7 days, which is accompanied with the disappearance of the parent

compound, suggesting that isolate CGR-L2 utilized γ-HCH as the sole source of carbon. Also it suggests that by increasing the concentration of γ-HCH beyond 10 mg l^{-1} might have further led to an increase in growth. However, the solubility of γ-HCH decreases at the concentrations above 10 mg l^{-1}. Therefore, further experiments were performed with 10 mg l^{-1} γ-HCH concentration.

3.3.3 Free chloride analysis

To prevent MSM interference in the assay, free chloride release during the γ-HCH degradation was analysed in the distilled water (Sahu et al., 1990). It was observed that isolate CGR-L2 released chloride ~391.66, ~474.66 and ~719.33 μM after 24, 72 and 144 hours respectively (Figure 22C). It is also evident that *Sphingobium* sp. isolate CGR-L2 released significant amounts of chloride in the dechlorination assay. Release of free chloride is supported by previous observation in *Pseudomonas* sp. (Sahu et al., 1990), that *Sphingobium* sp. CGR-L2 degrades γ-HCH.

Figure 23: Growth kinetics and γ-HCH biodegradation analysis for *Sphingobium* sp. strain CGR-L2 (Test in presence of γ-HCH and control in absence of γ-HCH).

A. Growth kinetics in MSM-L2 without glucose

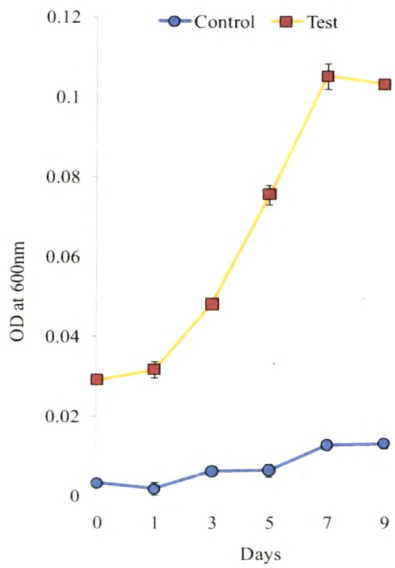
B. Residual concentration of γ-HCH

C. Thin layer chromatography showing reduction in residual concentration of γ-HCH with time

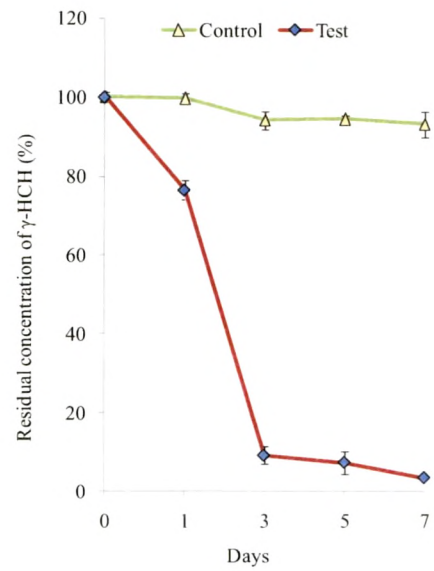
1. Control 0 day
2. Control 3 day
3. Control 7 day
4. Test 1 day
5. Test 3 day
6. Test 5 day
7. Test 7 day

F. 23

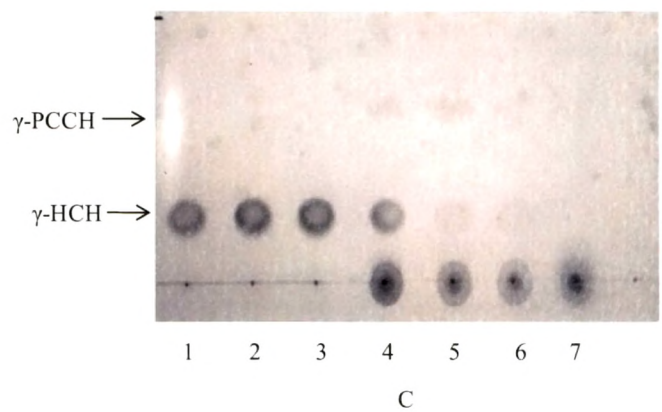
Why spots remain at the point of spotting in test?


Control
Test
OD at 600nm
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
3
5
7
9
Days

A


Control
Test
Residual concentration of γ-HCH (%)
120
100
80
60
40
20
0
0
1
3
5
7
Days

B


γ-PCCH
γ-HCH
1
2
3
4
5
6
7

C

3.3.4 Effect of various parameters on γ-HCH biodegradation by isolate CGR-L2

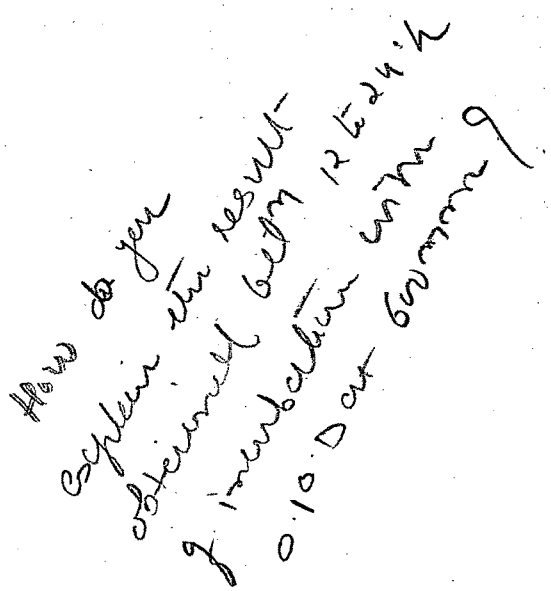
3.3.4.a Effect of Inoculum size

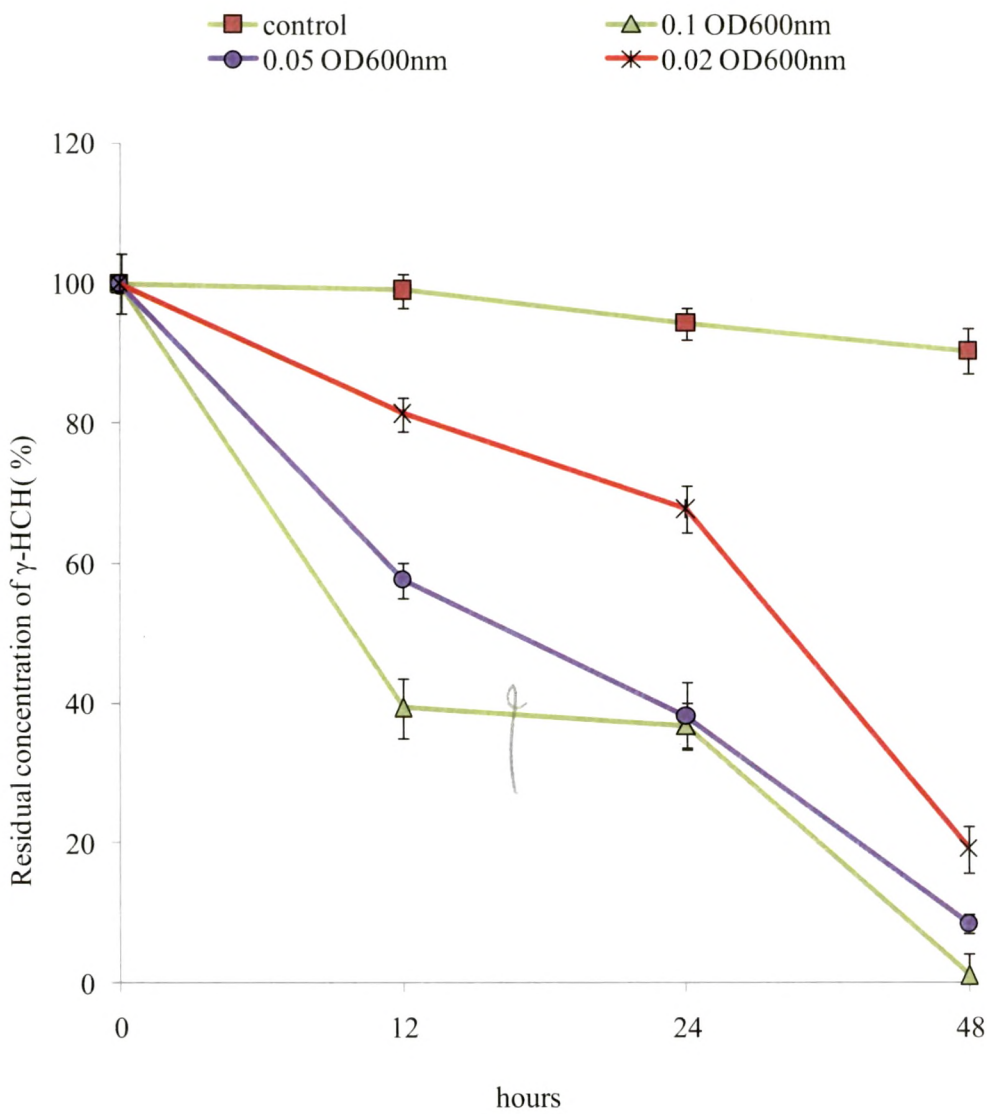
During growth assay it was observed that growth did not increase in the medium up to 3rd days as such, but γ-HCH residual concentration decreased with time (Figure 24). Therefore, in further experiments to analyze biodegradation potential of the CGR-L2, we monitored the residual concentrations of γ-HCH. The effect of inoculum size on the biodegradation potential of isolate CGR-L2 was studied. The isolate CGR-L2 was inoculated at different initial cell densities (OD_{600nm} of 0.01, 0.05 and 0.2) in the MSM-L2 medium and the residual concentration of γ-HCH was monitored at different time intervals as indicated (Figure 24). It is evident from that with the increased in inoculum size, increase in biodegradation rate of γ-HCH by isolate CGR-L2 was observed. Previously similar results have been also reported in case of *S. indicum* B90 (Johri et al. 2000) and *Pseudomonas aeruginosa* ITRC-5 (Kumar et al., 2006). When the inoculum size of 0.05 and 0.1 at OD_{600nm} were used, almost complete degradation of γ-HCH by isolate CGR-L2 was observed within 48 hours of growth. Therefore, in the further experiments the inoculum size with initial cell density of 0.05 OD_{600nm} was used in the MSM-L2.

3.3.4.b Carbon utilization profile of *Sphingobium* sp. CGR-L2

γ-HCH degradation efficiency is affected by supplementary carbon source (Phillips et al., 2005). BIOLOG system was used to analyse the carbon source utilisation by the isolate CGR-L2. Results are shown in Table 8. This system the isolate CGR-L2 was identified as *Sphingobium* sp.

Figure 24: Effect of initial inoculum on biodegradation of γ-HCH by isolate *Sphingobium* sp. CGR-L2. Figure showing residual amount of γ-HCH at different time intervals in control (with culture) and test (without culture)




control
0.1 OD600nm
0.05 OD600nm
0.02 OD600nm
120
100
80
60
40
20
0
Residual concentration of γ-HCH(%)
0
12
24
48
hours

The carbon source utilization studies were performed in the presence and absence of γ-HCH. Increase in color development means as increase in the metabolic activity of the bacterium. In these assays it was observed that D-glucose, L-arabinose, D-cellbiose and trehalose were good sources of carbon compared to other carbon sources like Dextrin, D-fructose, L-Fucose, Maltose and D-Mannose for the isolate CGR-L2. Whereas, other carbon sources like D-Mannitol, D-Raffinose, Sucrose and Xylitol were observed to be either the poor sources of carbon for the isolate CGR-L2. It was evident that the metabolic activity of isolate CGR-L2 (as monitored by change in color development at OD_{740nm}) was maximum in the presence of glucose, indicating that glucose was preferred carbon source for isolate CGR-L2 (Figure 25). Further, no change was observed in metabolic activity of isolate CGR-L2 in presence (Test) and absence of γ-HCH (Control) when glucose was used as the sole carbon source. These results also indicated that γ-HCH showed no interference in metabolic activity of the CGR-L2 in presence of glucose. Therefore, in further experiments glucose was used in MSM-L2, to observe effect of glucose as a supplementary carbon source on biodegradation of γ-HCH.

3.3.4.c Effect of supplementary carbon source (glucose)

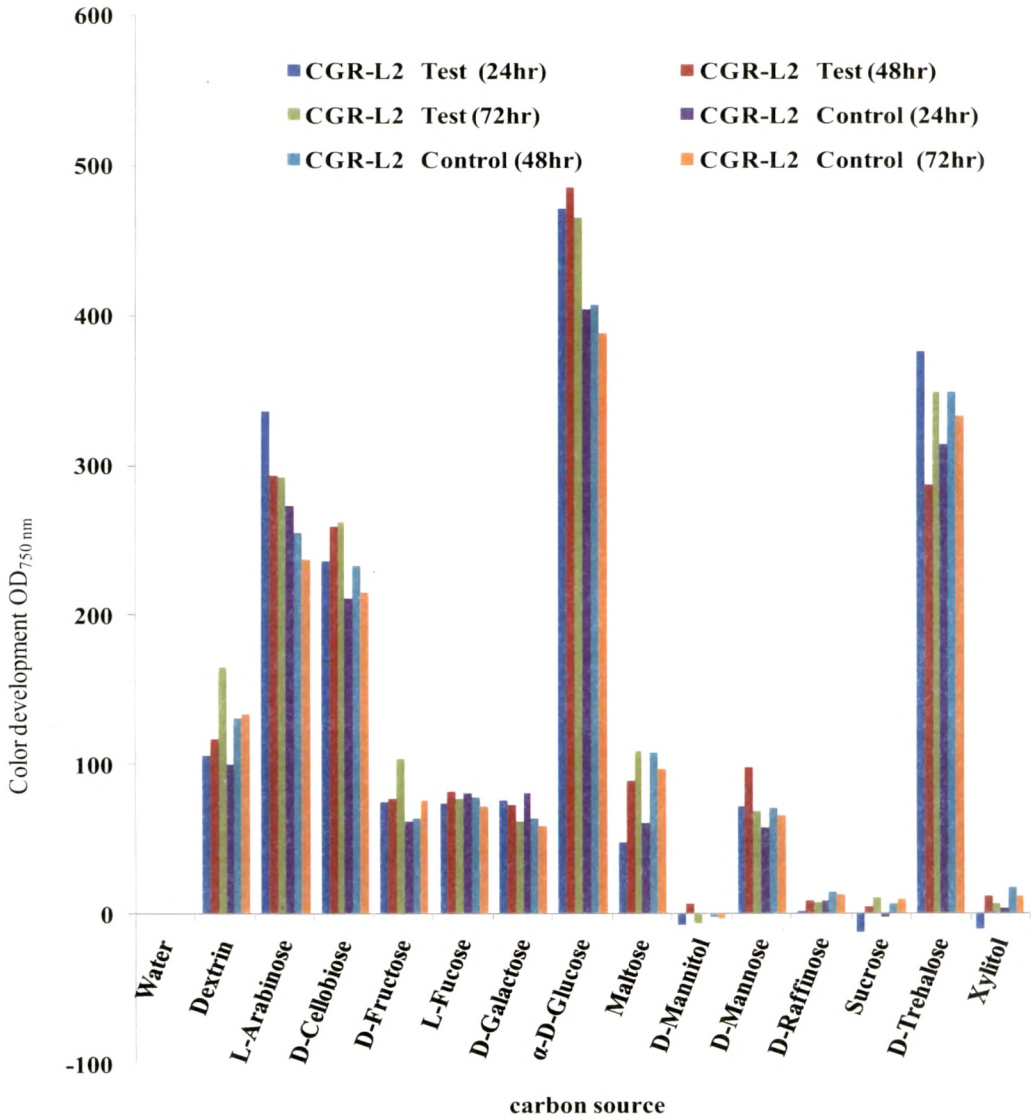
Different concentrations of glucose ranging from 0, 0.1, 0.5 and 1.0 % in the MSM-L2 were used to monitor the effect of supplementary carbon source on degradation of γ-HCH (Figure 26). At glucose concentrations 0, 0.1, 0.5 and 1.0 % in the MSM-L2; residual concentration of γ-HCH was ~61.23%, ~35.77%, ~1.29 % and ~1.24 % respectively, in comparision to control, after 18 hours. Whereas after 36 hours, γ-HCH residues were not observed in any of glucose added samples.

From the figure it is evident that there was a drastic difference in the residual concentration of γ-HCH in the presence of 0.5% glucose in the medium after 18 hours. After 18 hours, the residual concentration of γ-HCH was ~61.23% in glucose free medium whereas in presence of 0.5% of glucose the residual concentration was only ~1.29 %. Not only that, by addition of 0.1% glucose in the medium residual concentration of γ-HCH reduced to about ~43% compared to glucose free medium.

The effect of glucose on biodegradation of γ-HCH or other isomers has been shown to be concentration dependent. (Phillips et al., 2005). Glucose at higher concentrations (1.6mg/ml) retarded the degradation of α-HCH, whereas the low concentrations of glucose (<0.2μgml^{-1}) enhanced the rate of degradation (Manonmani et al., 2000). In another study, glucose was shown to be required for growth by the organism or consortium, which in absence of glucose did not degrade γ-HCH (Ohisa et al., 1980). However, it has been reported that in presence of glucose 10 mg ml^{-1}, degradation of γ-HCH was reduced by the mixed culture (Maule et al., 1987).

Overall glucose affects HCH biodegradation efficiency of the culture. Here, no significant difference was observed in the growth of CGR-L2 when glucose was provided in the medium. This might be due to production of metabolites which inhibited the growth of culture as reported (Endo et al., 2006). However, in presence of glucose, γ-HCH biodegradation efficiency of the isolate CGR-L2 was enhanced in the MSM-L2, and therefore, we used 0.5% glucose in the subsequent experiments.

Figure 25: Effect of carbon source on growth of *Sphingobium* sp. strain CGR-L2. Well containing glucose showed highest color development in both presence of γ-HCH (test) and absence of γ-HCH (control).


600
500
400
300
200
100
0
-100
Color development $OD_{750\,nm}$
CGR-L2 Test (24hr)
CGR-L2 Test (48hr)
CGR-L2 Test (72hr)
CGR-L2 Control (24hr)
CGR-L2 Control (48hr)
CGR-L2 Control (72hr)
Water
Dextrin
L-Arabinose
D-Cellobiose
D-Fructose
L-Fucose
D-Galactose
α-D-Glucose
Maltose
D-Mannitol
D-Mannose
D-Raffinose
Sucrose
D-Trehalose
Xylitol
carbon source

concentrations

Figure 26: Effect of glucose on biodegradation of γ-HCH by isolate *Sphingobium* sp. CGR-L2

A. Residual concentration of γ-HCH at different concentrations of glucose

B. TLC showing residual γ-HCH at different concentrations of glucose

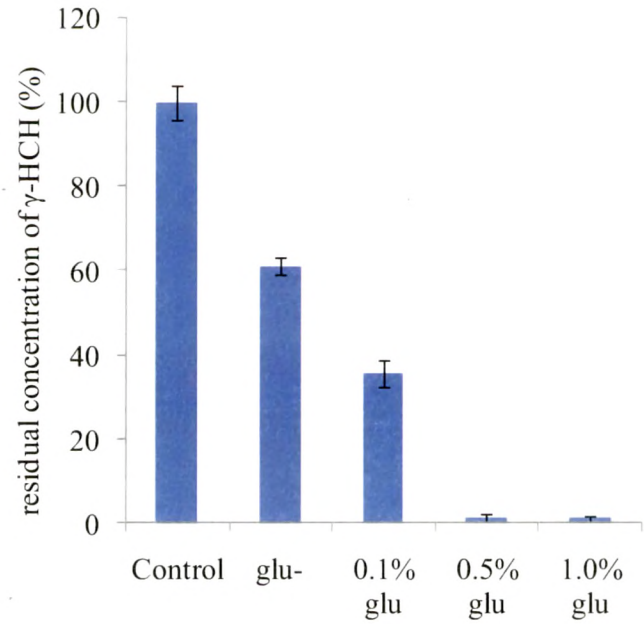
Lane 1. MSM-L2 without glucose

Lane 2. MSM-l2 with 0.1% glucose

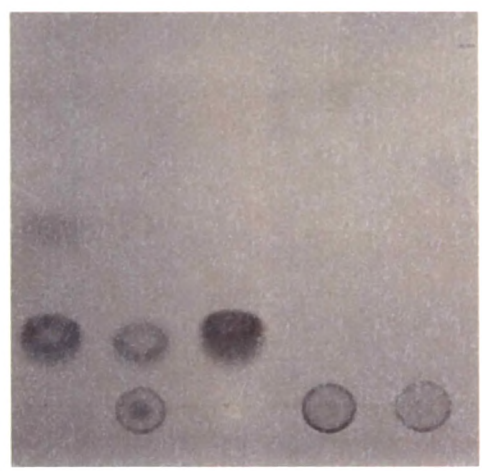
Lane 3. Control without culture

Lane 4. MSM-L2 with 0.5%

Lane 5. MSM-L2 with 1%


120
100
80
60
40
20
0
residual concentration of γ-HCH (%)
Control
glu-
0.1% glu
0.5% glu
1.0% glu

A



1 2 3 4 5

B

3.3.4.d Effect of pH

Effect of pH on biodegradation of γ-HCH was analysed after 12 hours and 18 hours. We observed comparable difference after 12 hours only (Figure 27A). After 12 hours at pH 5, 6, 7, 8 and 9, residual concentration of γ-HCH observed as ~69.57%,~ 10.36%, ~0.86%, ~0.002% and ~3.74 % in comparison to control respectively. We did not observe any change in the degradation of γ-HCH in control flasks. As pH of the soil sample, from where isolate CGR-L2 was isolated, was found to be 7.2, isolate CGR-L2 showed good activity at pH 7, 8 and 9. But the biodegradation activity was observed at all the pH. We observed biodegradation of γ-HCH at pH 5 also, but it was slower than at pH 6 to 9. In previous studies, the optimum pH for the degradation of HCH isomers in different microorganisms have been observed between pH range from 6.0 to 8.0 (Kumar et al., 2006; Manonmani et al., 2000; Siddique et al., 2002).

3.3.4.e Effect of temperature

In order to study the effect of temperature on γ-HCH biodegradation isolated CGR-L2 was grown in MSM-L2 at temperatures 25^{0}C, 30^{0}C, 37^{0}C and 42^{0}C. At temperature of 25^{0}C, 30^{0}C, 37^{0}C and 42^{0}C, residual concentration of γ-HCH was found as ~19.23, ~2.48, ~32.36 and ~59.51 respectively after 12 hours. As can be observed from the Figure 27B, 30^{0}C was found to be the optimum temperature for γ-HCH degradation by isolate CGR-L2 in MSM-L2.

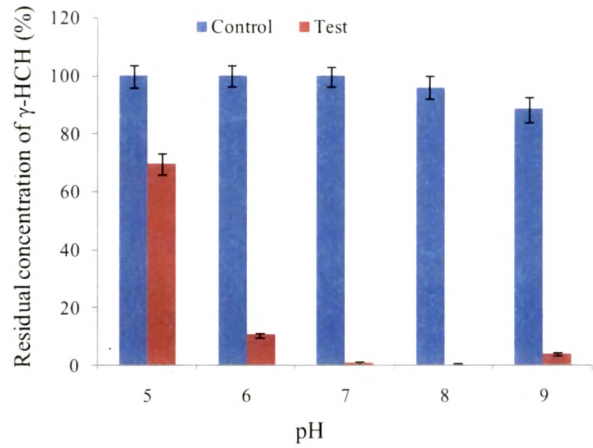
How pathway & steps of assay could be established?

Figure 27: Effect of pH and temperature on biodegradation of γ-HCH by isolate *Sphingobium* sp. CGR-L2

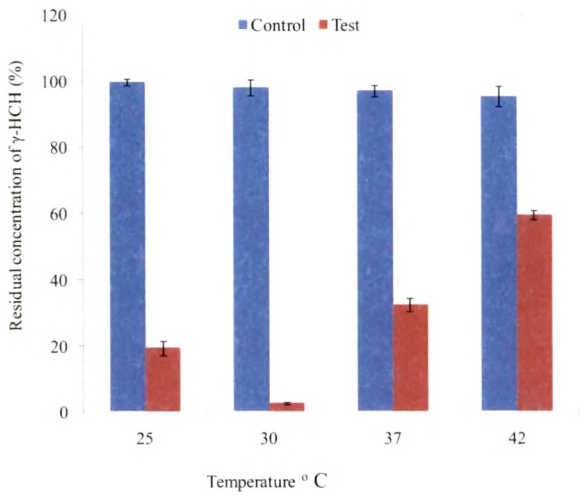
A. Residual concentration of γ-HCH at different pH after 12 hours of growth

B. Residual concentration of γ-HCH at different temperature after 12 hours

A


Control
Test
Residual concentration of γ-HCH (%)
120
100
80
60
40
20
0
5
6
7
8
9
pH

B


Control
Test
Residual concentration of γ-HCH (%)
120
100
80
60
40
20
0
25
30
37
42
Temperature ° C

A previous study has also reported that 30^{0}C temperature was the most favorable for the biodegradation of α-HCH in soil slurry by the mixed native microbial population (Bachmann et al. 1988). Maximum growth and degradation activity of γ-HCH by the fungus *Pleurotus sajor-caju* was also observed at 30^{0}C (Arisoy and Kolankaya, 1997). The degradation of the α-HCH isomer by a microbial consortium under a wide range of temperatures (4-40^{0}C) in liquid culture medium was analysed and 30^{0}C was found to be the optimum for α-HCH degradation (Manonmani et al., 2000). For *Pandoraea* sp. an incubation temperature of 30^{0}C was the optimum for degradation of γ-HCH (57.7%) in liquid culture and soil slurry (51.9%) (Siddique et al., 2002). All these reports suggest that 30^{0}C could be the optimum temperature for the activity of the enzymes that are involved in the HCH biodegradation.

3.3.5 Optimization of γ-HCH biodegradation and GC-MS analysis for identification of metabolites

From the above experiments, inoculum size and carbon source were found to be the major parameters in the experiments. After optimization of different parameters, the biodegradation potential of the *Sphingobium* sp. CGR-L2 was analysed under optimized conditions (Figure 28 and 29). Under these conditions, it was observed that ~87 % of γ-HCH was degraded within 9 hours by isolate CGR-L2. When the degradation metabolites were analysed only γ-PCCH was detected as γ-HCH degradation product, whereas other metabolites such as 2, 5-DCP and 2, 5-DCHQ were not detected. γ-PCCH that was observed during the γ-HCH biodegradation showed a retention time at 16.64 min, whereas the parent compound had a retention time of 20.22 min (Figure 30).

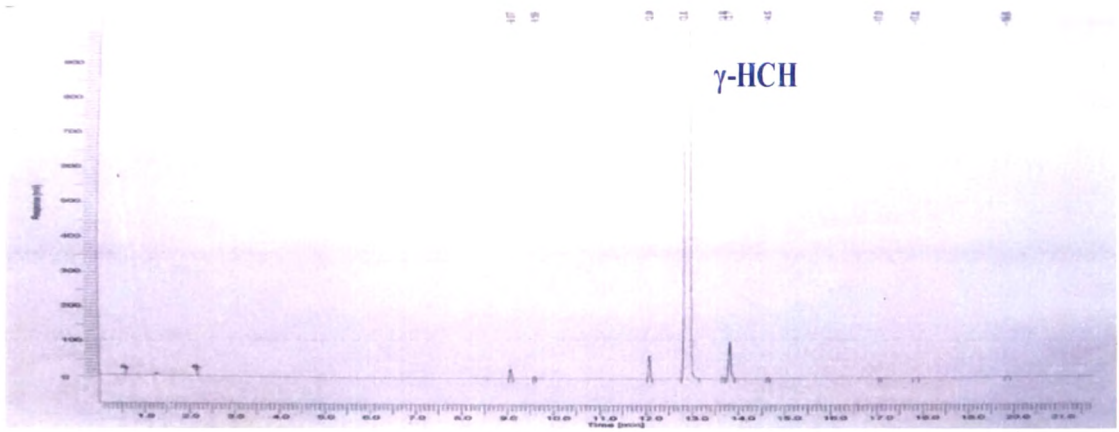
Figure 28: GC-analysis of γ-HCH biodegradation by *Sphingobium* sp. CGR-L2 in optimized parameters (*i.e.* 0.05 OD_{600nm} inoculum size, pH 8.0, 5 g l^{-1} glucose, at 30^0C, pH 8.0 and 200 rpm).

A. At zero hour

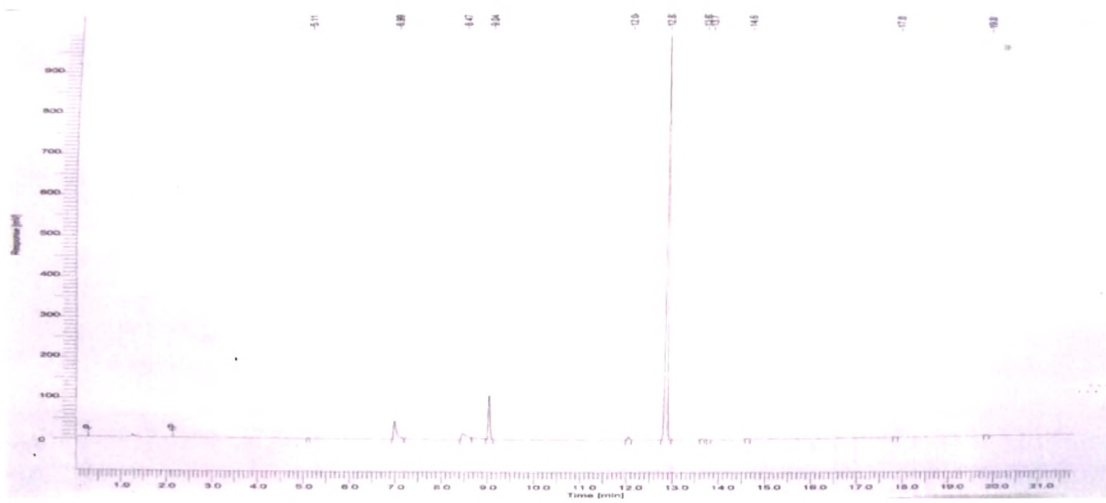
B. After 6 hours

C. After 9 hours

A



B



C

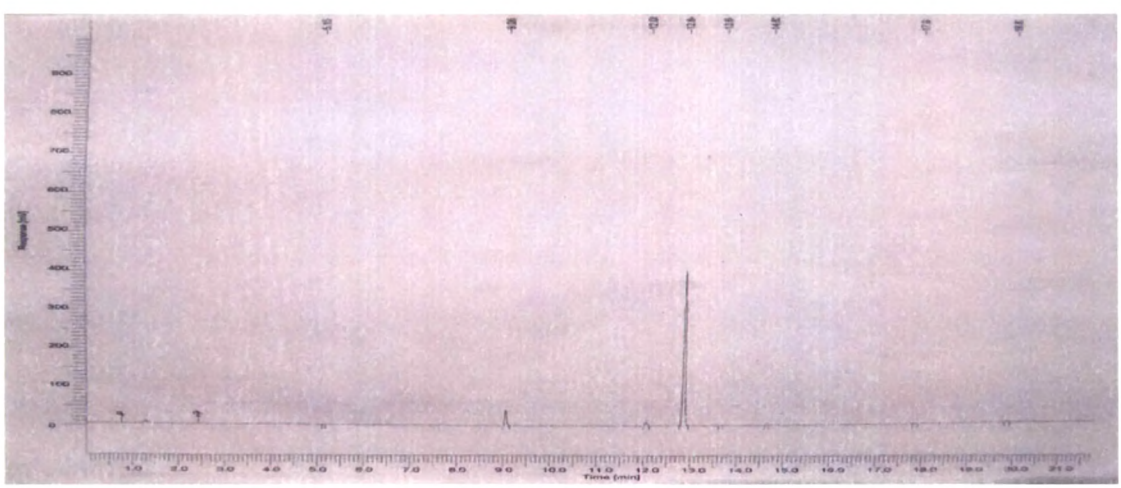


Figure 29: Analysis of growth and γ-HCH residual concentration for *Sphingobium* sp. CGR-L2 under optimized parameters (*i.e.* 0.05 OD_{600nm} inoculum size, pH 8.0, 5 g l^{-1} glucose, at 30^0C , pH 8.0 and 200 rpm).

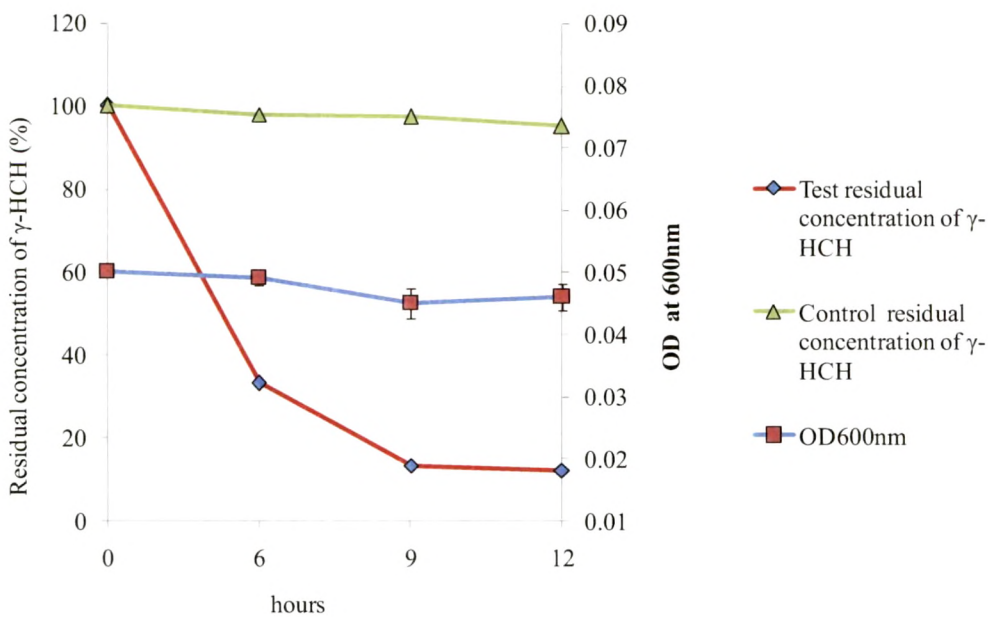
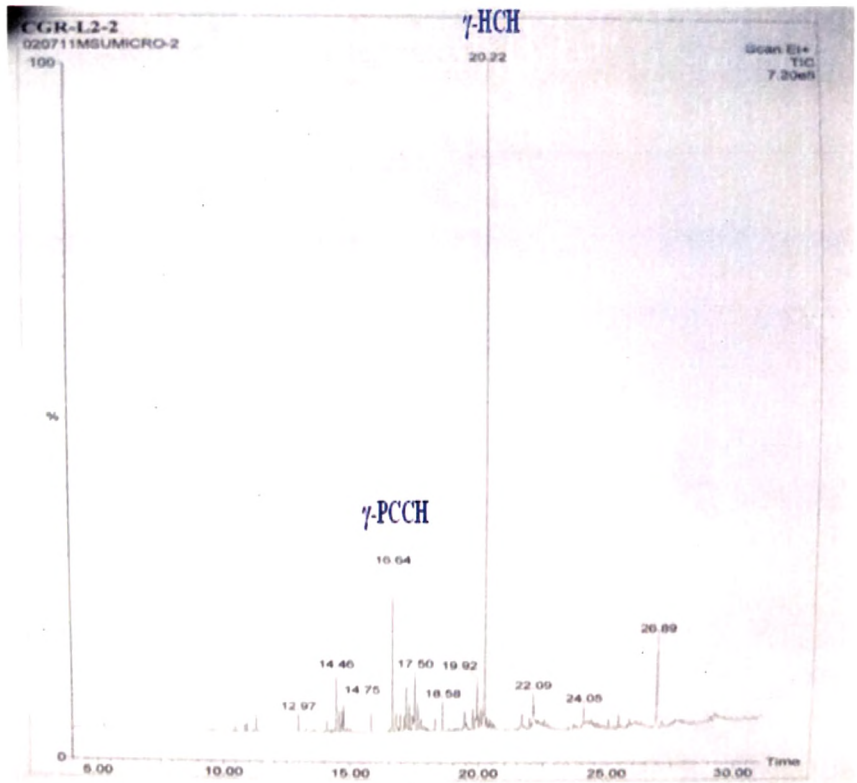

120
100
80
60
40
20
0
Residual concentration of γ-HCH (%)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
OD at 600nm
0
6
9
12
hours
Test residual concentration of γ-HCH
Control residual concentration of γ-HCH
OD600nm

Figure 30: GC-MS analysis of the metabolites producing during biodegradation of γ-HCH by *Sphingobium* sp. CGR-L2 after 6 hours in the MSM-L2.

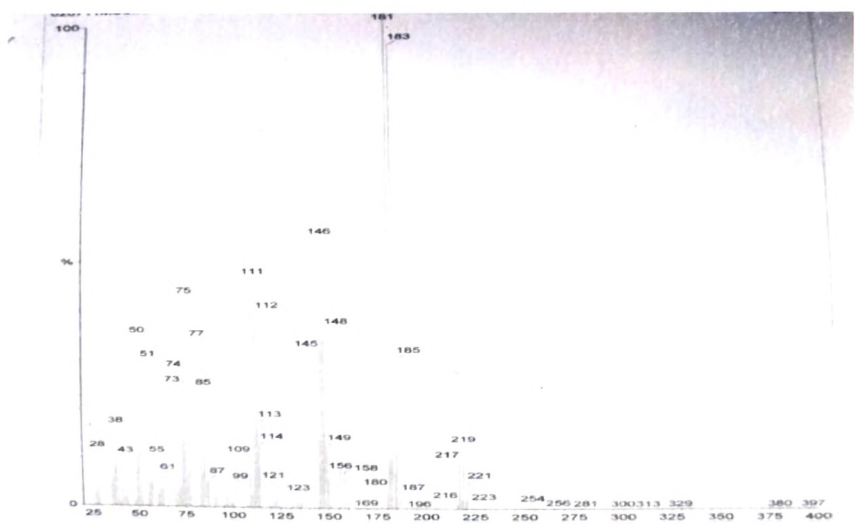
A. Total ion chromatogram of γ-HCH degradation by *Sphingobium* sp. CGR-L2 after 6 hours in the MSM-L2

B. Mass spectra of γ-PCCH of metabolite produced during γ-HCH degradation

A


CGR-L2-2
020711MSUMICRO-2
100
%
0
γ-HCH
20.22
γ-PCCH
16.64
14.46
14.75
12.97
17.50
19.92
18.58
22.09
24.05
26.89
5.00
10.00
15.00
20.00
25.00
30.00
Time

B


100
%
0
181
183
146
111
75
112
148
50
77
145
185
51
74
73
85
113
38
114
149
219
28
43
55
109
217
61
87
99
121
156
158
221
123
180
187
216
223
169
196
254
256
281
300
313
329
380
397
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400

3.3.6 Analysis of technical HCH degradation by CGR-L2

Different microorganisms having varied biodegradation potential with different HCH isomers have been reported previously (Kumari et al., 2002). Here the degradation of different HCH isomers was analysed by monitoring the residual concentrations of all the isomers after growing CGR-L2 in MSM-L2. We observed residual concentration of α-HCH, ~81.08%, ~74.56% and ~72.12% after 24, 48 and 96 hours respectively. Residual concentration of β-HCH was found to be ~46.95, ~34.53 and ~37.6 after 24, 48 and 96 hours, respectively. Residual concentration of γ-HCH was found to be ~23.46%, ~15.69% and~18.33% after 24, 48 and 96 hours, respectively. Residual concentrations of δ-HCH observed after 24, 48 and 96 hours were ~93.47%, ~94.40% and ~87.65%, respectively (Figure 31 and 32).

Isolate CGR-L2 was isolated in an enrichment medium containing γ-HCH only, but it was found to also degrade β-HCH as well as α-HCH, whereas, in case of δ-HCH, no significant difference was observed from the control (Figure 31 and 32). Isolate CGR-L2 showed almost 100% similarity for 16S rDNA with *Sphingobium ummariense* RL-3^T. *Sphingobium ummariense* RL-3^T was shown to degrade all the isomers that is α-HCH, β-HCH, γ-HCH and δ-HCH (Singh and Lal., 2009), whereas isolate CGR-L2 degraded only γ-HCH, β-HCH and poorly α-HCH isomer, indicating that this isolate was different from the *Sphingobium ummariense* RL-3^T.

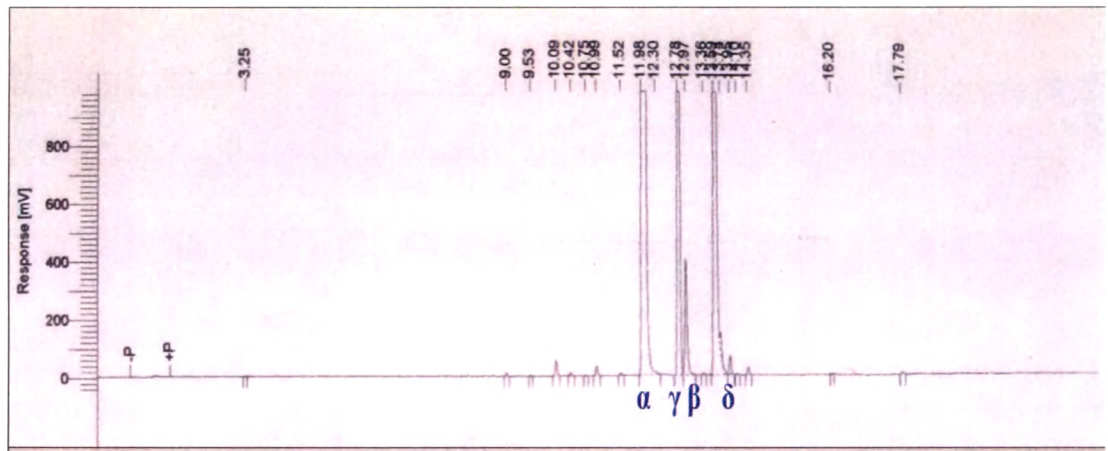
Figure 31: GC analysis of biodegradation of technical HCH by isolate *Sphingobium* sp. CGR-L2 in MSM-L2 medium.Showing peaks of α-HCH (Rt 11.9 min), γ-HCH (Rt 12.8 min), β-HCH (Rt 12.9 min) and δ-HCH (13.5 min) during analysis in GC. Reduction in area of peack of γ-HCH (Rt 12.8 min) and β-HCH (Rt 12.9 min) was observed during analysis in GC in time-dependent manner.

I. Zero time point

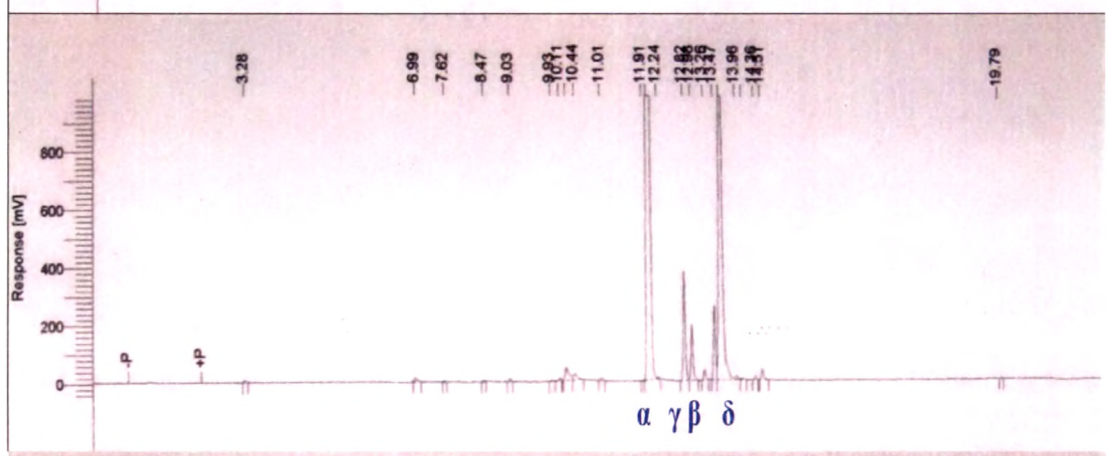
II. After 24 hours

III. After 96 hours

I


Response [mV]
800
600
400
200
0
-P
+P
3.25
9.00
9.53
10.09
10.42
11.52
11.98
12.30
16.20
17.79
α γ β δ

II


Response [mV]
800
600
400
200
0
-P
+P
3.28
6.99
7.62
8.47
9.03
10.44
11.01
11.91
12.24
19.79
α γ β δ

III

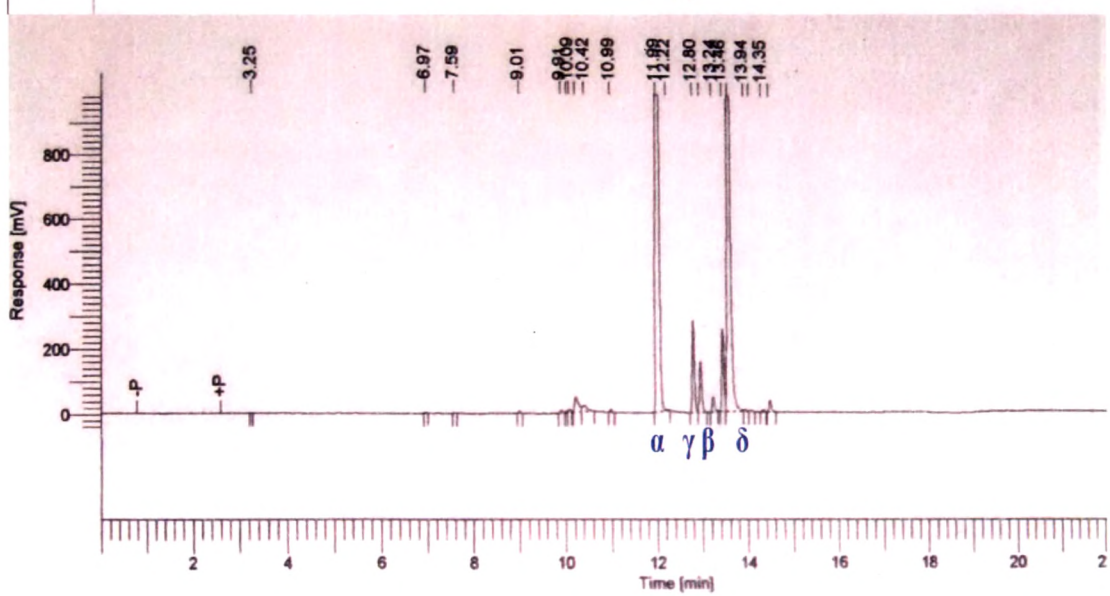

Response [mV]
800
600
400
200
0
-P
+P
3.25
6.97
7.59
9.01
10.42
10.99
12.80
13.94
14.35
α γ β δ
2
4
6
8
10
12
14
16
18
20
Time [min]

Figure 32: Reduction in residual concentration of HCH isomers in a time-dependent manner in MSM-L2, in presence and absence of Sphingobium sp. CGR-L2.

A. Residual concentration of α-HCH

B. Residual concentration of β-HCH

C. Residual concentration of γ-HCH

D. Residual concentration of δ-HCH

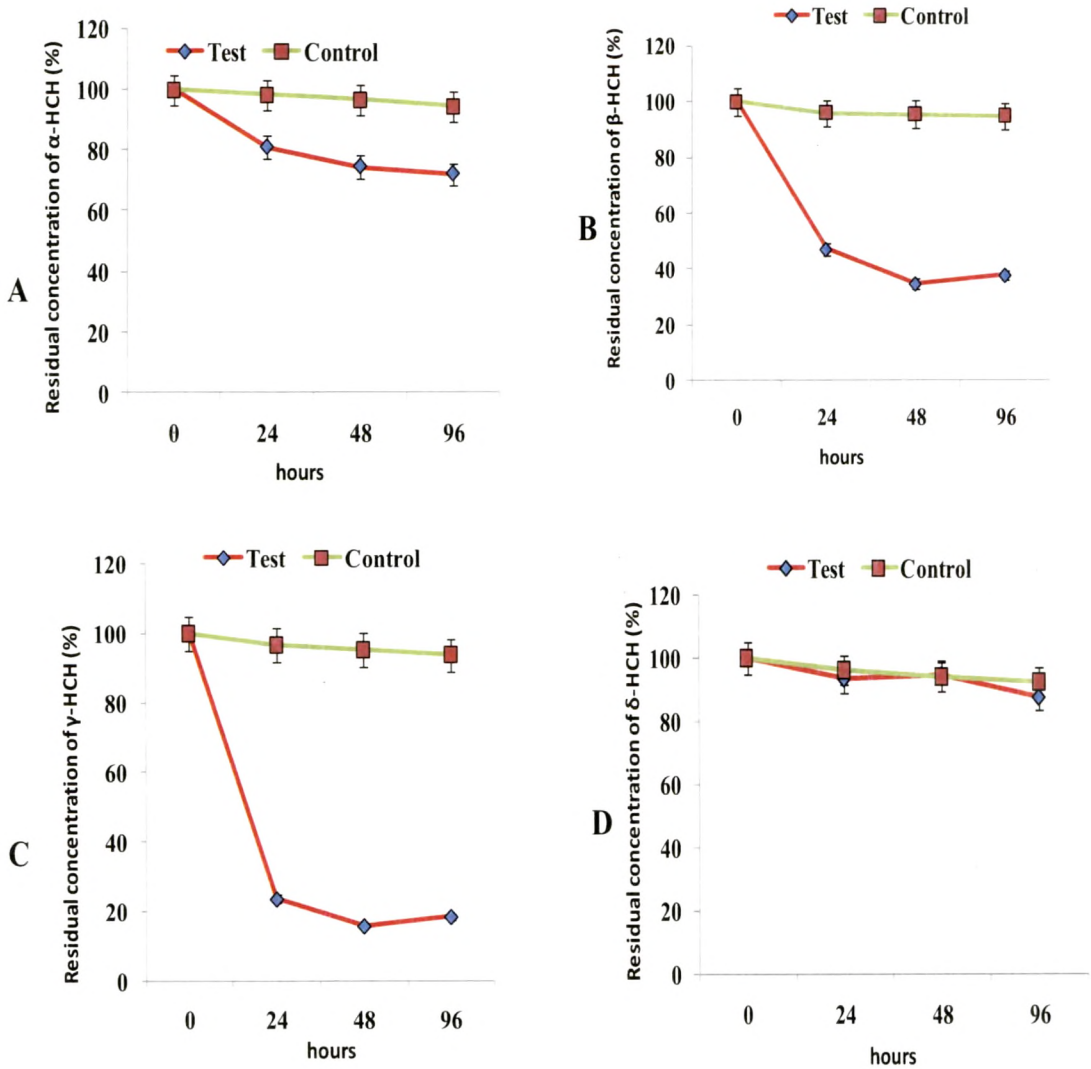
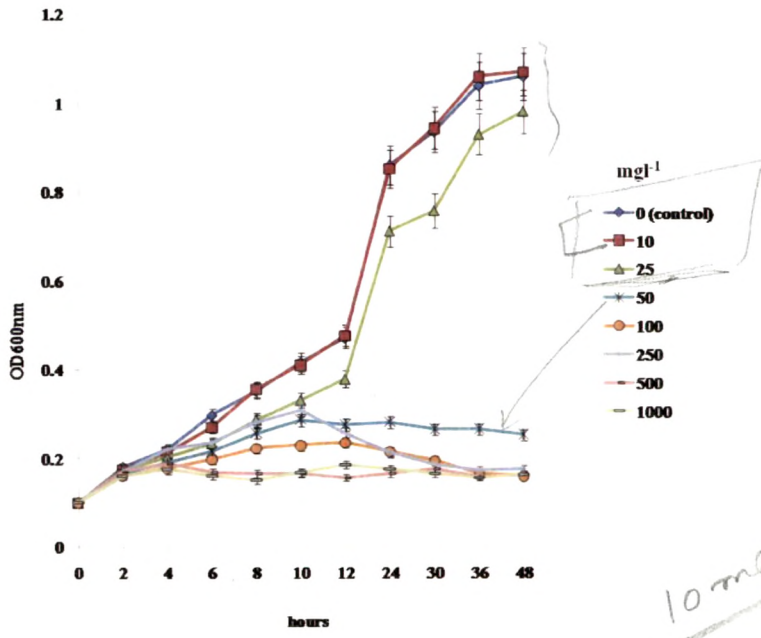

A
Residual concentration of α-HCH (%)
Test
Control
120
100
80
60
40
20
0
0
24
48
96
hours
B
Residual concentration of β-HCH (%)
Test
Control
120
100
80
60
40
20
0
0
24
48
96
hours
C
Residual concentration of γ-HCH (%)
Test
Control
120
100
80
60
40
20
0
0
24
48
96
hours
D
Residual concentration of δ-HCH (%)
Test
Control
120
100
80
60
40
20
0
0
24
48
96
hours

Figure 33: Growth kinetics of *Sphingobium* sp. CGR-L2 in different concentration of γ-HCH, from 0 to 1000mg l^{-1}


1.2
1
0.8
0.6
0.4
0.2
0
OD600nm
0
2
4
6
8
10
12
24
30
36
48
hours
mgl-1
0 (control)
10
25
50
100
250
500
1000

10 mg/L

3.3.7 Identification of γ-HCH tolerance level of CGR-L2

We observed drastic change in biodegradation of γ-HCH by CGR-L2 in the presence of glucose, but there was no effect on growth. Therefore we further analysed the γ-HCH tolerance level of the isolate γ-HCH; tolerance study was carried out in the 1/3 LB as reported earlier (Ryo et al., 2006)(Figure 33). Culture OD_{600nm} after 48 hours reached at ~1.06, ~1.07, 0.98 in flask containing concentration of 0, 10 and 25 mg l^{-1} γ-HCH. Growth was not observed in the flask containing 50, 100, 250 mg l^{-1} of γ-HCH. At 50 mg l^{-1}, produced phenolics metabolites in excess could inhibit the growth of CGR-L2 (Endo et al., 2006).

Further, we monitored the biodegradation of γ-HCH at increasing initial concentration of γ-HCH (10, 25, 50, 100 and 250 mg l^{-1}). After 48h, it was observed that in the flask containing 50, 100 and 250 mg l^{-1} residual concentration of γ-HCH was ~10.96%, ~18.11% and ~39.73%, respectively, whereas in the flasks with 10 and 25 mg l^{-1} initial γ-HCH concentrations the residual concentration of γ-HCH was undetectable (Figure 34). Although growth was not observed at the concentration of γ-HCH with 50 mg l^{-1} to 250 mg l^{-1}, biodegradation of γ-HCH was seen even at these concentrations of γ-HCH. Further during growth an increase in the brown colour of medium was seen in a concentration dependent manner due to production of phenolics metabolites that might have inhibited the growth of the culture. At γ-HCH concentration of 500 and 1000 mg l^{-1} no degradation and growth was observed.

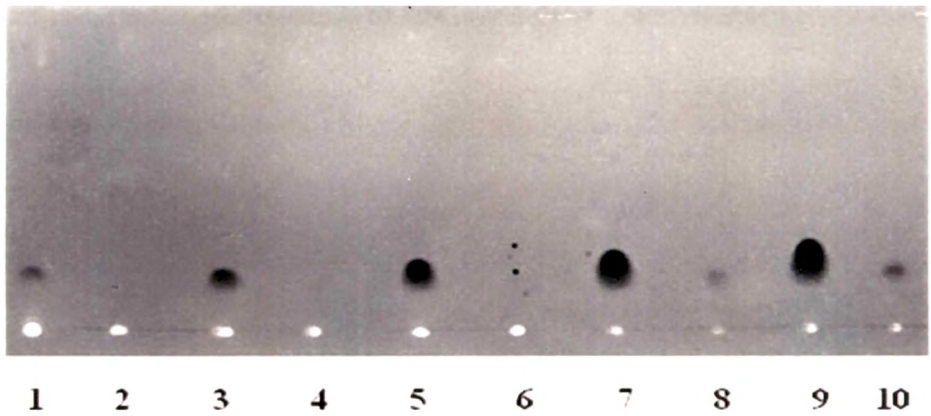
Figure 34: Biodegradation of γ-HCH by isolate *Sphingobium* sp. CGR-L2 at different initial concentrations of γ-HCH.

A. Analysis of residual concentration of γ-HCH during growth in 1/3 LB by TLC

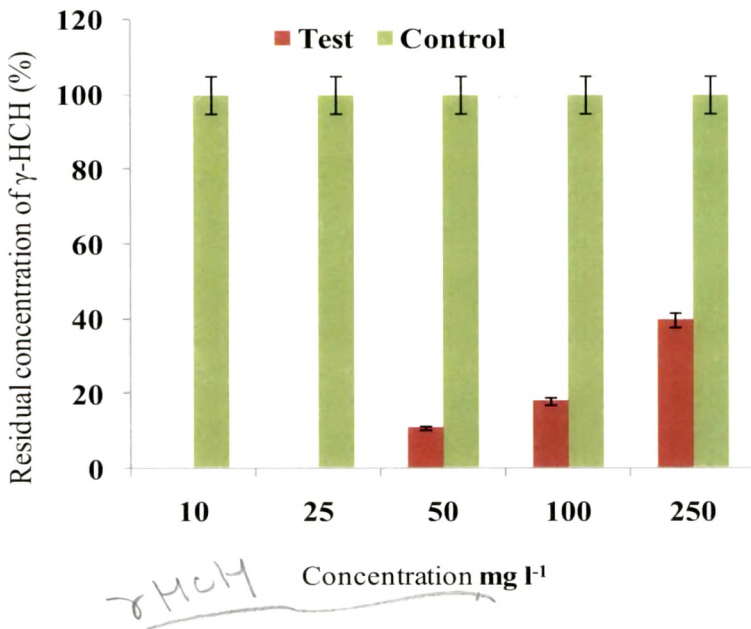
Lane 1 control (10mg/L)
Lane 2 test (10mg/L)
Lane 3 control (25mg/ml)
Lane 4 test (25mg/L)
Lane 5 control (50mg/L)
Lane 6 Test (50mg/L)
Lane 7 control (100mg/L)
Lane 8 test (100mg/L)
Lane 9 control (250mg/L)
Lane 10 test (250mg/L)

B. Graph showing residual concetration of γ-HCH after 48 hours analysed through TLC

A


1
2
3
4
5
6
7
8
9
10

B


Test
Control
Residual concentration of γ-HCH (%)
120
100
80
60
40
20
0
10
25
50
100
250
Concentration mg l-1
γ HCH

3.3.8 PCR amplification of *lin* genes

PCR based detection technique was used to check the presence of genes involved in γ-HCH degradation. A pair of primers for all the reported different genes *lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *linf*, *lin*G and *Lin*R (Table 11) were synthesized and used to amplify the respective genes from the genomic DNA of the isolate CGR-L2 and an expected amplification product were obtained from gene *lin*A, B, C, D, E and R (Figure 35) (Table 13A), but expected size of amplification product was not observed from gene *lin* F and G. Amplified PCR fragments were further purified and partial sequences were obtained. The sequences obtained from *lin* genes were compared using BLAST search against the Genbank (http://www.ncbi.nlm.nih.gov/blast) database. On the basis of sequence identity, closest match in the GenBank database was identified as depicted in the Table 13B. The new sequences of *lin* genes from CGR-L2 bacterial isolate were deposited in NCBI with accession numbers mentioned in Table 10.

We could not get amplification in genes like *lin*F and *lin*G from isolate CGR-L2 which is reported by isolate *S. japanicum* U26, which may be possibly due to variability in the sequence of these genes from the reported sequence or could be due to the absence of these genes. When hydroquinone as a substrate, we found that hydroquinone was also degraded by the isolate CGR-L2. This data showed that isolate CGR-L2 carries functional *lin* genes at least from *lin*A to *lin*E (Miyauchi et al., 1999). Our isolate is closely related to the reported isolate *Sphingobium unmareiae* RT-3^T. Gene *lin*E is not reported in the isolate *Sphingobium unmareiae* RT-3^T (Singh and Lal, 2009), whereas we observed the presence of *lin*E in our isolate CGR-L2, which suggest that isolate CGR-L2 is different

Figure 35: PCR amplification of *lin* genes from *Sphingobium* sp. strain CGR-L2 genomic DNA using gene specific primers

A.

Lane 1; 1 Kb ladder

Lane 2; *lin*B (~888bp)

Lane 3; *lin*A (~420bp)

B.

Lane 1; *lin*D (~1038bp)

Lane 2; 1 Kb ladder

Lane 3; *lin*C (~750bp)

C.

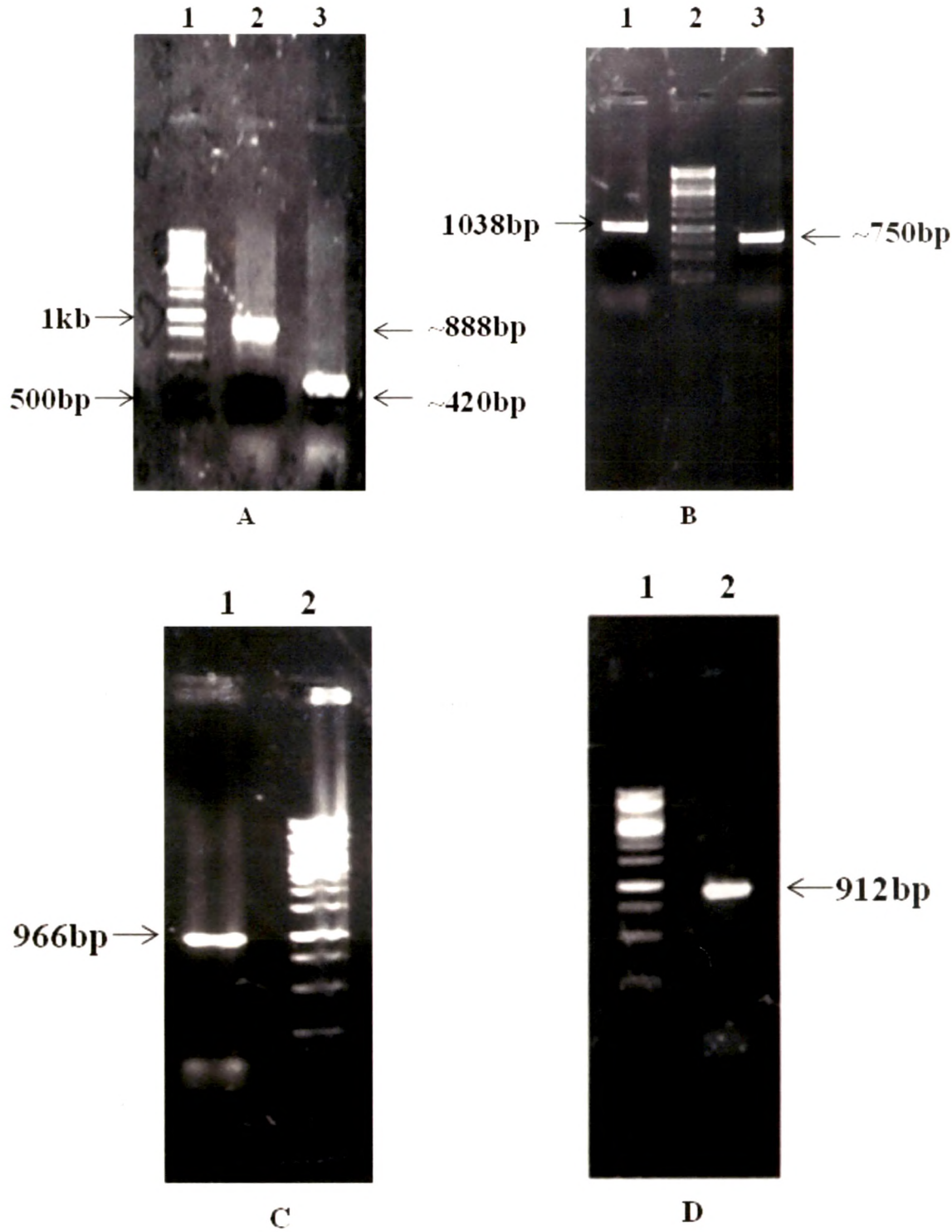
Lane 1; *lin*E (~966bp)

Lane 2; 1 Kb ladder

D.

Lane 1; 1 Kb ladder

Lane 2; *lin*R (~912bp)


1 2 3
1kb
500bp
~888bp
~420bp
A
1 2 3
1038bp
~750bp
B
1 2
966bp
C
1 2
912bp
D

from RT-3^T. It is previously reported that *lin*R is involved in positive regulating the expression of *Lin*D and *lin*E genes (Nagata et al., 2007), which was also present in our isolate CGR-L2.

3.3.9 Confirmation of presence of *lin* genes in *Sphingobium* sp. CGR-L2 by Southern analysis

To examine the presence of genes involved in γ-HCH degradation pathway like *lin*A, *lin*B and *lin*C and its copy number in the genome, Southern blot analysis was carried out. When *lin*A sequence was used as a probe against the blot containing genomic DNA digested with *Hind*III, *Eco*RI, and *Bam*HI, hybridization gave one signal in each, which suggest that probably one copy of *lin*A present in the genome as these restriction sites are not present in the sequence reported for the *lin*A gene (Figure 36A). Previously two copies were reported in *S. indicum* B90 (Kumari et al., 2002) and 4 copies of *lin*A were observed in *Shewanella* sp. CGR-L1.

When *lin*B fragment was used as a probe against the blot containing genomic DNA digested with *Eco*RI and *Hind*III, *Pst* I, *Xho*I, *Hind*III, *Eco*RI or *Bam*H1, after hybridization gave one signal, whereas *Sal*I gave two signal, suggesting that only one copy of *lin*B present in the CGR-L2, as *Sal*I site is present in the *lin*B gene sequence (Figure 36B).

Table 13: PCR amplification of *lin* genes from isolate *Sphingobium* sp. CGR-L2 and its percentage sequence identity with *lin* genes of other well studied γ-HCH degraders

A. PCR amplified *lin* genes from isolate *Sphingobium* sp. CGR-L2

B. Percentage identity of the *lin* gene sequences of *Sphingobium sp.* strain CGR-L2 with *lin* genes of other well studied γ-HCH degraders

A

Gene	Size of amplicon (bp) expected during PCR	Size of amplicon (bp) obtained	Full/Partial gene	Amplification +(present), - (absent)
*lin*A	420	~420	partial	+
*lin*B	888	~888	full	+
*lin*C	750	~750	partial	+
*lin*D	1038	~1038	Full	+
*lin*E	966	~966	Full	+
*lin*R	912	~912	Full	+
*lin*F	1058	-	Full	-
*lin*G	648	-	Full	-

B

	Percentage identity of *lin* genes from *Sphingobium* sp. CGR-L2 with lin genes of closely related reported HCH degrader (%)					
	*lin*A	*lin*B	*lin*C	*lin*D	*lin*E	*lin*R
Sphingobium japonicum strain UT26	99	99	100	99	100	100
Sphingobium indicum strain B90A	96 (*lin*A1) 99 (linA2)	99	99	99	99	100
Sphingomonas sp. NM05	99	99	100	99	99	-
Sphingomonas sp. MM-1 plasmid ISP1	99	100	100	99	100	99
Pseudomonas aeruginosa strain ITRC-5	99	100	100	99	100	99

Figure 36:

A. Southern blot hybridization of genomic DNA of *Sphingobium* sp. strain CGR-L2 probed with non-radioactively labeled ***lin*A.** Genomic DNA of *Sphingobium* sp. strain CGR-L2 was digested with restriction enzymes:

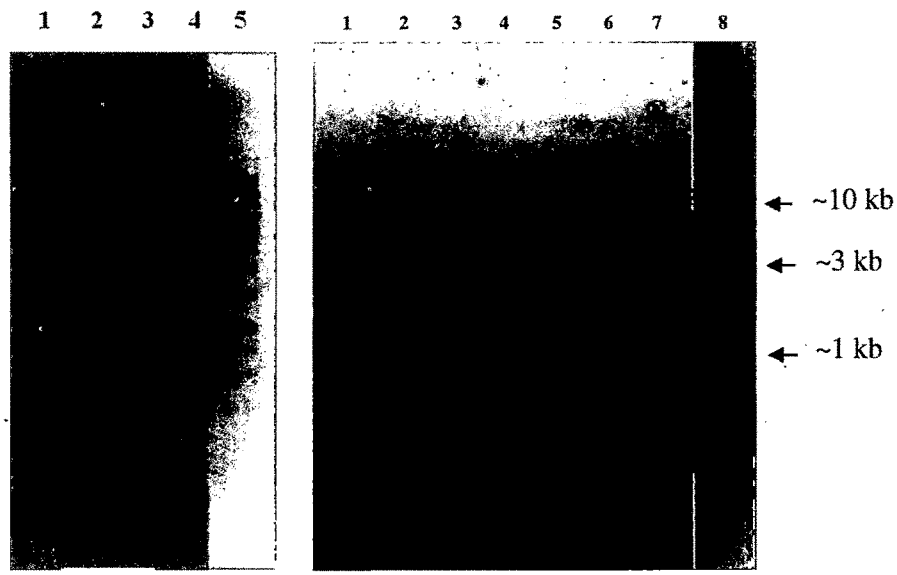
Lane 1; *Hind*III, Lane 2; *Eco*RI, Lane 3; *Bam*HI, Lane 4; Blank and Lane 5; 1 kb ladder

B. Southern blot hybridization of genomic DNA of *Sphingobium* sp. strain CGR-L2 probed with non-radioactively labeled ***lin*B.** Genomic DNA of *Sphingobium* sp. strain CGR-L2 was digested with restriction enzymes:

Lane 1; *Eco*RI and *Hind* III, Lane 2; *Pst* I ,Lane 3;*Sal* I , Lane 4;*Xho* I , Lane 5;*Hind*III, Lane 6; *Eco* RI, Lane 7; *Bam* HI and Lane 8; 1 kb ladder

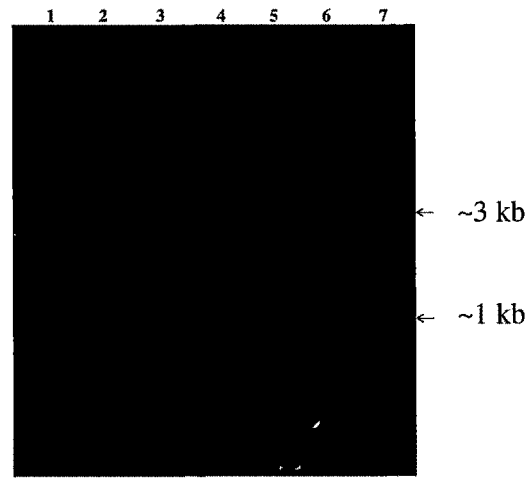
C. Southern blot hybridization of genomic DNA of *Sphingobium* sp. strain CGR-L2 probed with nonradioactively labeled ***lin*C.** Genomic DNA of *Sphingobium* sp. strain CGR-L2 was digested with restriction enzymes :

Lane 1; *Eco*RI and *Hind* III, Lane 2; *Pst* I, Lane 3; *Sal* I , Lane 4; *Xho* I, Lane 5;*Hind*III, Lane 6; *Eco* RI and Lane 7;1 kb ladder



A

B



C

Similarly with *lin*C probe, one signal observed against all the restriction digested samples, which suggest that one copy of *lin*C is present in the genome (Figure 36C). Over all these results indicate that isolate CGR-L2 carries a single copy of *lin*A, *lin*B and *lin* C gene.

3.3.10 Analysis of biodegradation of Hydroquinone

Our lin gene PCR amplification results showed that *lin*A, *lin*B, *lin*C, *lin*D, *lin*E and *lin*R genes were present in the isolate CGR-L2 (Figure 35). However, we could not amplify *lin*F and further *lin*G, may be because these genes were not present or our synthesized primers could not amplify these genes. In γ-HCH degradation pathway (Nagata et al., 2007), we amplified *lin*A to *lin*E gene. To detect functional activity of *lin*E, we used hydroquinone as substrate in the assay. We observed the disappearance of hydroquinone. Residual concentrations of hydroquinone found at 6 and 12 hours were 45.32 % and 3.12% respectively, whereas in the control at 6 and 12 hours were 89.35% and 75.21% respectively (Figure 37). These data suggested that hydroquinone was degraded by the isolate CGR-L2, which indicates *lin*E is functional.

3.3.11 Bioremediation study: Technical HCH biodegradation in soil

We also studied the biodegradation potentiality of isolate CGR-2 in soil with technical HCH in laboratory conditions (Figure 38-39). We observed residual concentration of α-HCH as ~88.32%, ~81.55% and ~80.16 after 7, 14, and 28 days respectively, β-HCH as ~71.14%, ~59.41%, ~41.98% after 7, 14, and 28 days respectively, γ-HCH as ~23.36%, ~14.32% and ~11.15% after 7, 14, and 28 days respectively and δ-HCH as ~73.86%,

Figure 37: Biodegradation of Hydroquinone by isolate CGR-L2 in MSM-L2. Graph showing reduction in residual concentration of hydroquinone at 0, 6 and 12 hours, in both control (without inoculum) and Test (with inoculum).

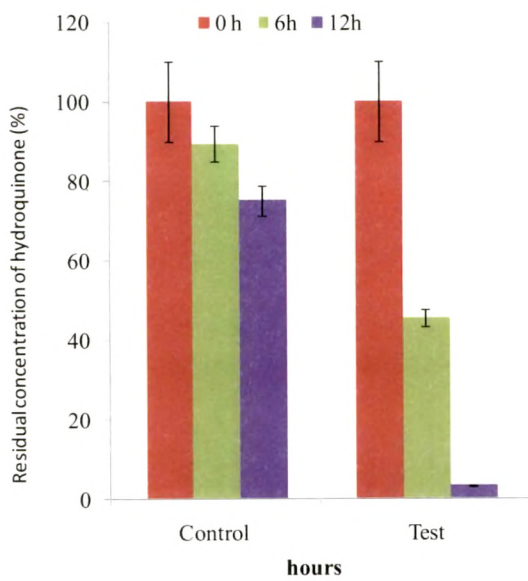

0 h
6h
12h
120
100
80
60
40
20
0
Residual concentration of hydroquinone (%)
Control
Test
hours

Figure 38: Microcosm (bioremediation) study using the isolate CGR-L2

A. Growth of CGR-L2 during microcosm study checked as cfu gm^{-1} of soil

B. Amplification of *lin*A and 16S rDNA from colony showing clearing zone during cfu count

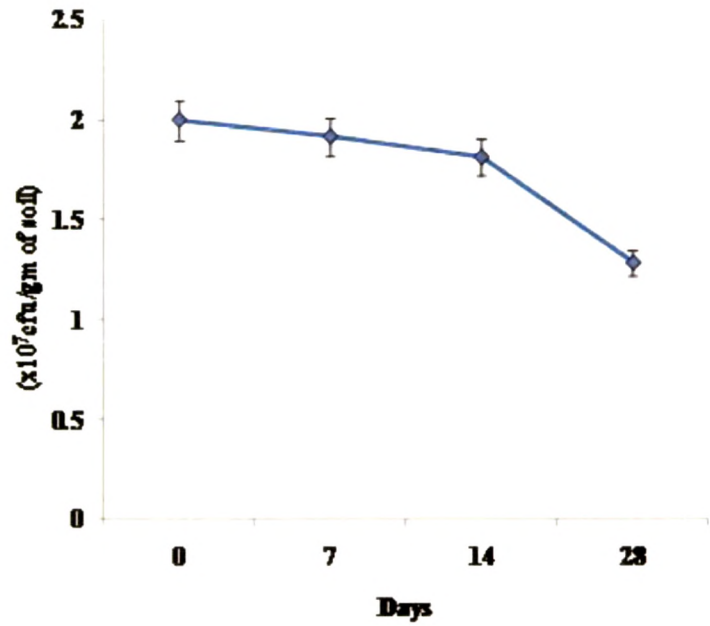
Lane 1. 16S rDNA amplification

Lane 2. 100 bp ladder

Lane 3. *lin*A amplification from colony showing clearing zone

Lane 4. *lin*A amplification from colony showing clearing zone

A


2.5
2
1.5
1
0.5
0
(x10⁷cfu/gm of soil)
0
7
14
28
Days

B

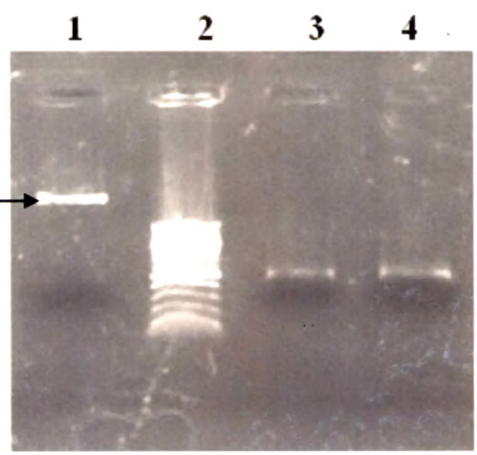

1
2
3
4
16s rDNA (~1.5kb)
linA

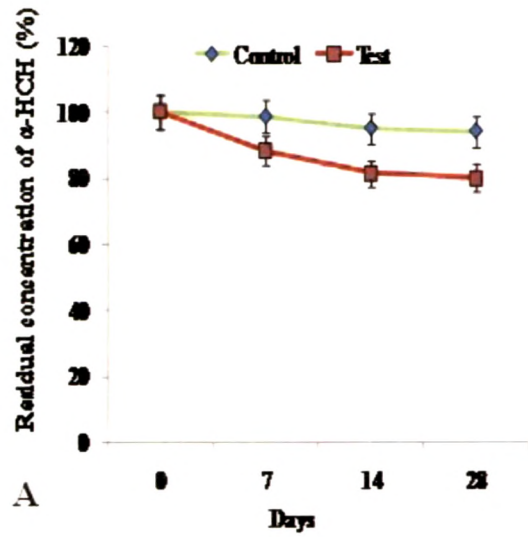
Figure 39: Analysis of technical HCH biodegradation by *Sphingobium* sp. CGR-L2 during microcosm study (control = without inoculum and test = with inoculum).

A. Residual concentration of α-HCH

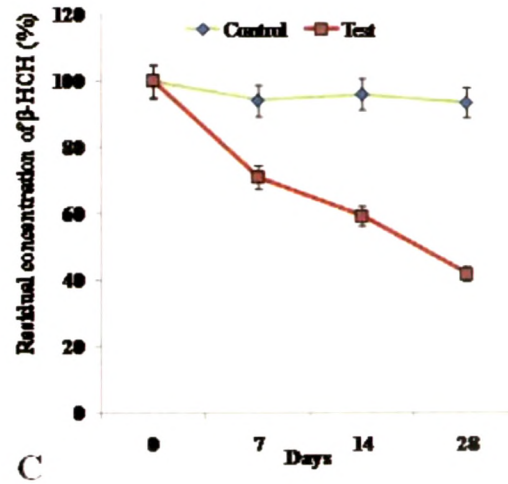
B. Residual concentration of β-HCH

C. Residual concentration of γ-HCH

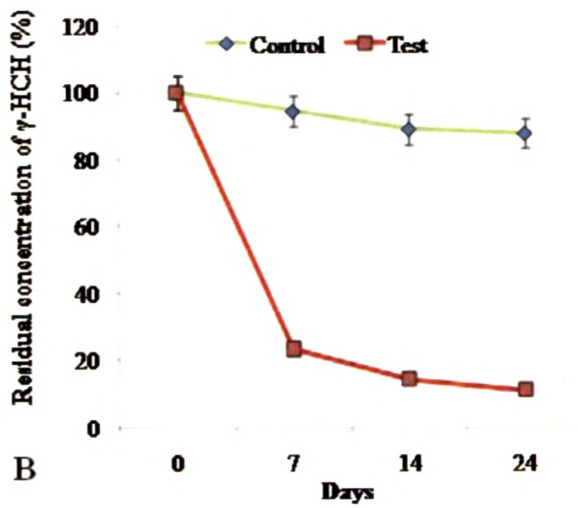
D. Residual concentration of δ-HCH


Residual concentration of α-HCH (%)
Control
Test
120
100
80
60
40
20
0
0
7
14
28
Days

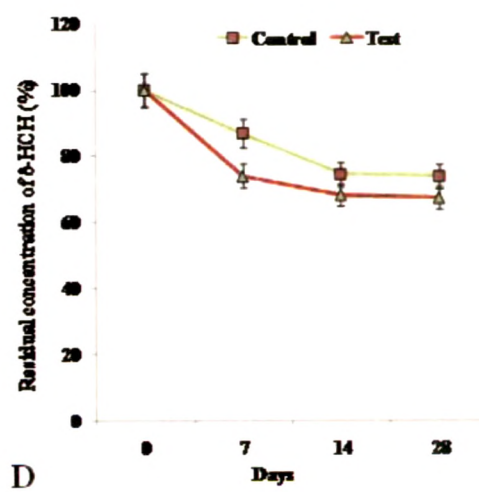
A


Residual concentration of β-HCH (%)
Control
Test
120
100
80
60
40
20
0
0
7
14
28
Days

C


Residual concentration of γ-HCH (%)
Control
Test
120
100
80
60
40
20
0
0
7
14
24
Days

B


Residual concentration of δ-HCH (%)
Control
Test
120
100
80
60
40
20
0
0
7
14
28
Days

D

68.16% and 67.28%, after 7, 14, and 28 days, respectively. Whereas, in controls no significant changes were observed in the residual concentrations of α, β and γ-HCH.

In microcosm study, the cell densities of CGR-L2 were estimated by cfu count. For cfu count, colonies showing zone of clearance were selected as described in Materials and Methods. Further confirmation of CGR-L2 isolate was done by amplification of linA and partial sequencing of 16S rDNA, from the selected colonies (Figure 38). In the soil, cell densities (cfu gm^{-1}) of CGR-L2 were observed to be ~$1.81x10^7$, ~$1.71x10^7$ and ~$1.51x10^7$ at OD_{600nm} after 7, 14 and 28 days, respectively.

In bioremediation studies, CGR-L2 isolate was found to degrade γ-HCH and β-HCH upto ~90% and ~58%, respectively as compared to control, after 28 days. *S. indicum* B90 has been reported to degrade all the isomers of HCH, whereas *S. japanicum* UT26 was shown to degrade α, γ- and δ-HCH isomers only, not β-HCH (Kumari et al., 2002). Biodegradation of α-HCH in microcosm study was expected based on its biodegradation in shake flasks, but we could not observe biodegradation of α-HCH in bioremediation study. During bioremediation study, we observed that cfu count decreased, which might be due to production of toxic metabolites (Endo et al., 2006).

3.3.12 PCR amplification of the *lin* genes from other bacterial isolates of enriched culture

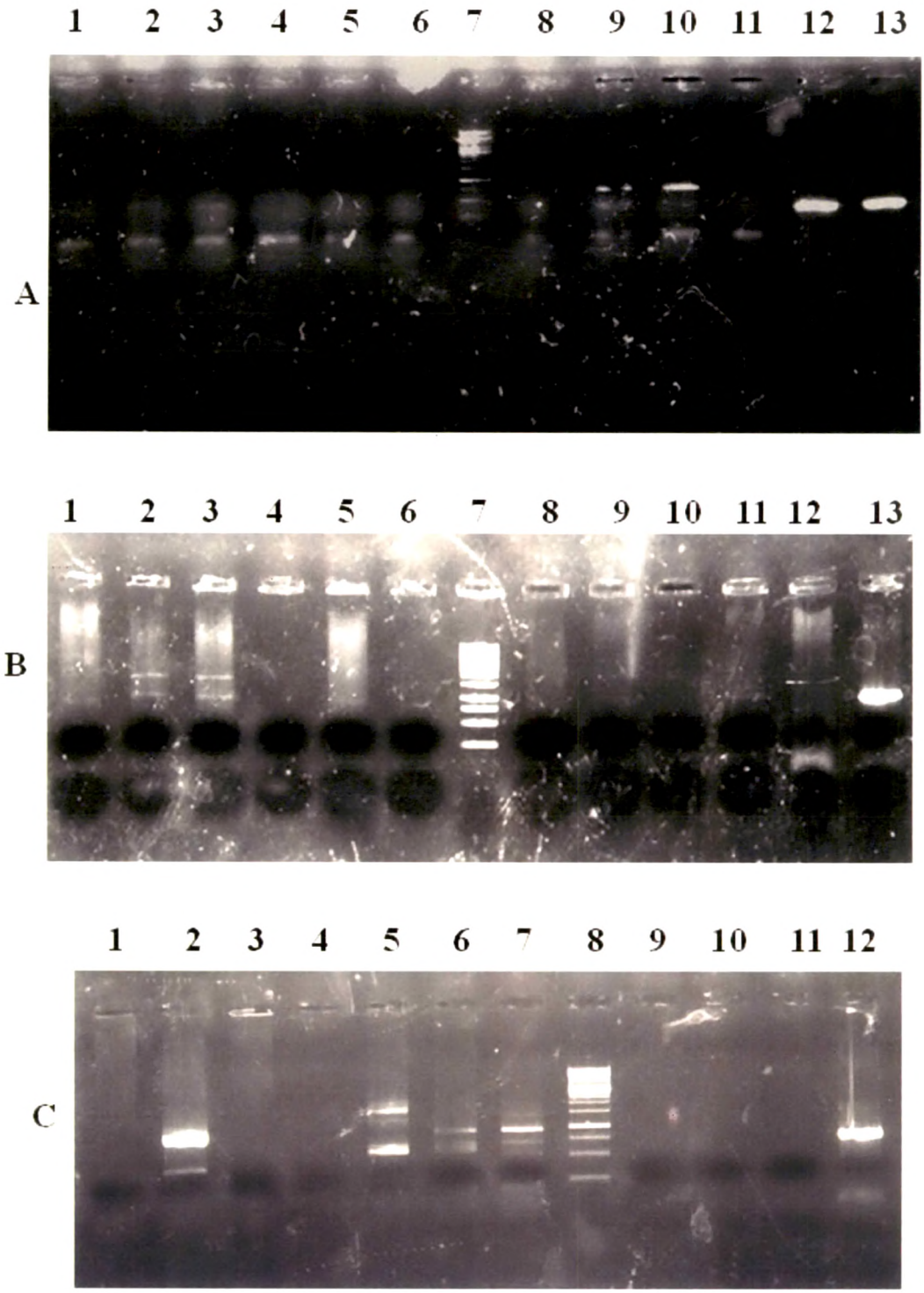
We amplified *lin*A, *lin*B and *lin*C involved in the γ-HCH pathway from all the isolates of enriched sample. We observed interesting pattern of these genes in all the isolates (Figure

40). We observed random amplification of either *lin*A or *lin*B or *lin*C or their combinations in the bacterial isolates from enriched culture. Amplification of *lin* genes with proper size of amplification from the isolate of consortia suggest that, they must be involved in biodegradation of γ-HCH, either in one or more step of pathway. As described in the chapter 1 that homologues of most of them were isolated from pesticide contaminated site and are involved in biodegradation of γ-HCH or other halogenated compounds (Senoo & Wada, 1989; Kumar et al., 2005; Verma et al., 2006; Singh et al., 2007; Sharma et al., 2010). However, clearing zone around the culture in the plate assay was not found. It is possible that genes in the isolates might not be functional or degrading γ-HCH.

Figure 40: Amplification of *lin*A (A), *lin*B (B) and *lin*C (C) genes from bacterial isolate of enrichment culture. Genomic DNA of CGR-L2 was used as positive control for the amplification of each gene.

Lane	Name of the isolate	(A) *lin*A	(B) *lin*B	Name of the Isolate	(C) *lin*C
1	ILI-I1	-	+	ILI-I1	-
2	ILI-I2	+	+	ILI-I2	+
3	ILI-I3	+	+	ILI-I3	-
4	ILI-I5	-	-	ILI-I5	-
5	ILI-I6	-	+	ILI-I6	-
6	ILI-I7	-	-	ILI-I7	+
7	1 kb ladder			ILI-I8	+
8	ILI-I8	-	-	1 kb ladder	
9	ILI-I9	+	+	ILI-I9	-
10	ILI-I10	+	-	ILI-I10	-
11	ILI-I11	-	+	ILI-I11	-
12	CGR-L1	+	-	ILI-I4 (CGR-L2)	+
13	ILI-I4 (CGR-L2)	+	+		

Note : + obtained amplification , - not obtained amplification


1 2 3 4 5 6 7 8 9 10 11 12 13
A
1 2 3 4 5 6 7 8 9 10 11 12 13
B
1 2 3 4 5 6 7 8 9 10 11 12
C