Chapter 2

Characterization of γ-HCH degrading bacterium *Shewanella* sp. CGR-L1

2.1 Introduction

Shewanella sp. is reported as facultative bacterium, capable of surviving and proliferating in both aerobic and anaerobic conditions. Species of *Shewanella* are commonly found growing anaerobically in an environment contaminated by heavy metals such as iron, lead, and perhaps even uranium (Caccavo et al., 1992). A species of *Shewanella*, *S. oneidensis* MR-1 is able to transfer electrons to solid metal oxides and shows remarkable anaerobic versatility (Fredrickson et al., 2008). Cellular respiration for these bacteria is not restricted to heavy metals though; the bacteria can use more than ten different electron acceptors such as sulfates, nitrates and chromates, when grown anaerobically. In the present study, *Shewanella* sp. CGR-L1 was found to grow aerobically. So far, aerobic γ-HCH degradation has not been reported, which makes it interesting to further characterize this isolate for its ability to degrade γ-HCH under aerobic conditions. Other than plate clearing assay, a colorimetric assay that detects dehalogenase activity is used for the isolation and further confirmation of γ-HCH degrader (Holloway et al., 1998; Phillips et al., 2001; Manickam et al., 2008). Genes such as *lin*A, *lin*B, *lin*C, *lin*D etc. (Yuji et al., 2007; Suar et al., 2006) encoding enzymes involved in γ-HCH biodegradation have been identified and cloned from different strains of *Sphingobium japonicum* (formerly *Sphingomonas paucimobilis* UT26; Nagata et al., 1999), *Sphingobium francense* (formerly *Rhodanobactor lindaniclasticus*, Thomas et al., 1996), *Sphingobium indicum* (formerly *S. paucimobilis* B90; Kumari et al., 2002) and a *Microbacterium* sp. (Manickam et al., 2006). We also used molecular approach to characterize isolate *Shewanella* sp. CGR-L1. It is reported that genes for the γ-HCH biodegradation are located on plasmid (Cérémonie et al., 2006). Two copies of *lin*A were

observed in *S. indicum* B90, (Kumari et al., 2002). We reported, here isolation a species of *Shewanella* by using serial dilution method from soil supernatant, which not only grows very well aerobically in presence of γ-HCH, but is also able to degrade γ-HCH under aerobic conditions.

2.2 Materials and Methods

2.2.1 Soil analysis

Physico-chemical characteristics of the soil like pH, organic carbon content and micronutrient were analysed from soil testing laboratory, GSFC, Gujarat, India.

2.2.2 Protein Extraction from *Shewanella* sp. CGR-L1

CGR-L1 showed a good clearing zone around the colony and was further characterized. 24 hr grown culture of CGR-L1 (with γ-HCH, 10 mg l^{-1}) were used to extract total protein. Biomass was centrifuged at 13,200 g for 5 minute at 4°C, culture supernatant was discarded, and then biomass was resuspended in sonication buffer (50mM Tris-sulphate (pH 7.5), 1mM EDTA and 1mM DTT). This step was repeated twice. Biomass was resuspended in sonication buffer containing 1 mM PMSF. The cells were lysed by sonication in an Ultrasonicator (Brown et al., 2006). The extract (total protein) was clarified by centrifuging at 13200 g for 15 min at 4°C. This step was repeated twice. Protein concentration was estimated by Bradford method (Bradford, 1976). The protein samples (25 μg) were electrophoresed on 12 % SDS-polyacrylamide gels, followed by Coomasie Blue stain for 2 hours to overnight.

2.2.3 Dehalogenase activity assay for the isolated *Shewanella* sp. CGR-L1

Dehalogenase activity of HCH degradative bacteria was assayed using a 96-well microtitre plate. The assay is based on a decrease in the pH of a weakly buffered medium containing phenol red as an indicator dye (Phillips et al., 2001). Stock of the substrate (γ-

HCH) was prepared at a concentration 10 mg ml^{-1}. The assay buffer contained 0.5 mM HEPES pH 8.2, 10 mM sodium sulphate and 0.5 mM EDTA. Phenol red, dissolved in acetone, was added to the buffer prior to addition to the 96-well microtitre plate, to give a final concentration of 20 μg ml^{-1}. Each well contained 194 μl of buffer and 1 μl substrate stock solution, to which 6 μl cell-free extract (protein 10μg) was added. Plates were covered to prevent volatilization of substrates or evaporation of reaction mixture, and were incubated at 28^0C, for 24 hours. Dechlorination activity was visually observed after 24 h by change in the colour of reaction mixture. Blanks included wells containing only sonication buffer instead of cell-free extract, wells where the cell-free extract had been denatured by boiling for 15 min, and wells where cell-free extract was applied but in the absence of γ-HCH.

2.2.4 Antibiotic assay

Isolate CGR-L1 tested for sensitivity to antimicrobial agents by means of disc diffusion method. The following antibiotics (all from Hi-media, Mumbai, India) were used. The abbreviations and concentrations (μg/disc) of the respective antibiotics are given in bracket: Ampicillin (A 10), Chloramphenicol (C 30), Co-trimoxazole (Co 25), Gentamicin (G 10), Carbenicillin (Cb 100) Cefotaxime (Ce 30) Norfloxacin (Nx 10), Oxacillin (Ox 5).

2.2.5 Growth kinetics study of *Shewanella* sp. CGR-L1

Growth kinetic study was performed in the minimal salt medium (MSM-L2) with glucose. Overnight pre-grown cells in 1/3LB 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 mineral medium to give a final OD_{600nm} of 0.02 in 200ml in 1L flask. In all the experiments filtered sterilized γ-HCH was used with concentration of 10 mg l^{-1}. Both control (without inoculum) and test flasks were incubated at 28°C with shaking at 200 rpm. Optical density and residual concentration of γ-HCH in the medium were analyzed at intervals of 24hr, 48hr and 96hr in MSM-L2. A flask containing only γ-HCH in MSM-L2 without the inoculum was kept as control.

Have you kept flasks inoculated but not have γ HCH as the control ?

2.2.6 Analytic techniques

A 250-ml Erlenmeyer flask with γ-HCH to the final concentration of 10 mg l^{-1} was inoculated with CGR-L1 in 1/3LB and incubated for 24 hr. The cells were harvested by centrifugation (13,200×g for 10 min), washed with sterile water and resuspended in fresh MSM-L2. The inoculum was added to the flask containing 10 mg l^{-1} of HCH to follow the biodegradation by inoculation of isolate CGR-L1 in the medium to give a final OD_{600nm} of 0.02 in 100 ml, but without any inoculum served as controls. The test and control flasks were incubated at 28°C with shaking at 200 rpm for 4 days. At time intervals of 0, 24, 48 and 96 hr, one flask each was used for extraction with an equal volume of n-hexane on a rotary shaker for 1 h. This step was repeated with ethyle acetate. The extracts (organic phase) were filtered through 0.22 μm nylon membrane and concentrated in rotary evaporator (Janke and Kunkel, Model RVO5-ST, IKA Laboratories, Staufen, Germany) and dissolved in 10 ml of n-hexane. 2 ml of chilled water was then added to the extract and vortexed for 30 sec, centrifuged at 12470 g for 5 min and the organic phase was collected. The organic extract was then dehydrated by

passing it through anhydrous magnesium sulphate column. Samples were filtered through 0.2 micron filter, concentrated using a SpeedVac instrument (Savant Instruments, Inc. New York, USA) and finally dissolved in the hexane. For HPTLC, a 10µl sample was spotted on the plates (silica gel 60 20×20 cm, 0.20 mm thickness, Merck, Darmstadt, Germany) by HPTLC (CAMAG Scientific Inc, Wilmington, N.C., USA) , and the chromatograms were developed in cyclohexane and visualized by spraying o-toludine, followed by UV exposure. The chlorinated compounds were detected as dark brown spots on HPTLC plate and quantitated depending on their residual concentration by Quantity one (Quantity One, Bio-Rad, Hemel Hempstead, UK).

2.2.7 GC-MS analysis for identification of metabolites

More than 10 Luria agar plates spread with 1% γ-HCH prepared in diethyl ether were spotted with the isolated bacterial culture, as mentioned in primary screening procedure. Extraction of γ-HCH biodegradation metabolites was carried out from the clearing zone around the growing culture on Luria agar plates (Kaur et al., 2006). A part of zone of clearance around the bacterial growth was cut into pieces of approximately 0.2-1 cm^2 size and was extracted using an equal volume of ethyl acetate on a rotary shaker for 1 hour. This step was repeated twice. Further extraction and purification was followed as mentioned in the materials methods 2.2.6. GC-MS analysis of metabolites produced by *Shewanella* strain CGR-L1 from γ-HCH was performed by using a Shimadzu QP 2010 Gas Chromatograph, Tokyo, Japan. 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 polysiloxane material and helium was used as a carrier gas (@ 2 ml min^{-1}). The column temperature

was maintained at 100°C for 7 min, and then increased to 250°C at a rate of 5°C min^{-1}. A 0.4 µl solution of the sample was injected.

2.2.8 Identification of metabolites produced in cell-free extract assay by GC-MS

Cell free extract assay has been reported in the study of biodegradation of pesticides and further identification of metabolites. (Turnbull et al., 2001; Datta et al., 2000; Phillips et al., 2001 and Ohisa et al., 1980). Here, the same protocol as reported for dehalogenase assay was followed, except that the reaction was set in 2 ml in duplicate, one with phenol red and one without phenol red. Change in the color was observed after 24 hours in the tube containing phenol red. Further, vial without phenol red was used for the identification of metabolites produced during the assay. Total assay sample was extracted with equal volume of n-hexane and further with ethyle acetate and processed as described in material methods 2.2.6. Finally sample was dissolved in the ethyl acetate and used for the GC-MS analysis. GC-MS analysis of metabolites produced by *Shewanella* strain CGR-L1 from γ-HCH (during cell free extract assay) was performed by using a Perkin Elmer GC-MS; model Autosystem XL GC+. Metabolites were separated on column PE-05 and helium was used as a carrier gas (1 ml min^{-1}). The column temperature was maintained at 70°C for 5 min, and then increased to 250°C at a rate of 10°C min^{-1}. A 1.0 µl solution of the sample was injected.

2.2.9 PCR amplification of the *lin*A

Genomic DNA was extracted as mentioned in material methods 1.2.7.a. Protocol for the extraction of large plasmid was followed (Heringa et al., 2007). The oligonucleotide

primers were designed based on *lin*A and *lin*B sequence from *S. paucimobilis* UT26 (Manickam et al., 2006b; Nagata et al., 1999b) (Table 11). Designed primer were synthesized were used to amplify the homologous DNA fragments from the CGR-L1. Plasmid pIMA2 (obtained from Prof. Yuji Nagata, Japan) bearing insert gene for *lin*A served as positive control in PCR. The PCR reaction mixture (50µl) contained 1x reaction buffer, 50ng of genomic DNA as template, 0.25 mM deoxynucleoside triphosphates, 0.5pmol of each of the primer and 3U of Taq DNA polymerase (Bangalore Genei, Bangalore, India). The PCR was performed with initial denaturation for 5 minutes at 94^0C, followed by 30 cycles; each cycle comprising of denaturation (95^0C, 30 s) annealing (55^0C for *lin*A and 50^0C for *lin*B, 1min) and polymerization (72^0C, 30 s), with an additional 10 min of polymerization after the last cycle. The size of the amplified product was analysed by electrophoresis on 0.8% agarose gel. The amplified DNA was purified by gel extraction column (GE healthcare Ltd, UK) and was sequenced. Homology search of the sequence information was performed using BLAST (Altschul et al., 1990) function of GenBank.

2.2.10 Plasmid curing from isolate CGR-L1

Flasks were inoculated with 0.02 final OD_{600nm} inoculum of CGR-L1 in 1/3LB with 6µM concentration of Ethidium bromide (Bouanchaud etal., 1969), incubated on rotary shaker at RT for 24hr. Then incubation the cultures were plated on LA with different dilution and incubated at 30°C for 24 hr. Randomly 50 colony were inoculated in LB tube individually from plate and next day total DNA was extracted. Extracted DNA was

Table 11: Primers used in the present study for the amplification of *lin* genes

Gene	Primer Sequence 5' to 3'	Primer designation	Length (bp)	Reference	Reference
*lin*A	GGCCGCGATTCAGGACCTCTACT	*lin*A Fw	23	D90355	Imai et al., 1991
	CGGCCAGCGGGGTGAAATAGT	*lin*A Rev	21		
*lin*B	ATGAGCCTCGGCGCAAAG	*lin*B Fw	18	D14594	Nagata et al., 1993
	TTATGCTGGGCGCAATCGC	*lin*B Rev	19		
*lin*C	ATGTCTGATTTGAGCGGC	*lin*C Fw	18	D14595	Nagata et al., 1994
	TCAGATCGCGGTAAAGCC	*lin*C Rev	18		
*lin*D	ATGAGCGCTGATACAGAA	*lin*D Fw	18	D89733	Miyauchi et al., 1998
	TTAGGCGTTGCTCAGGAG	*lin*D Rev	18		
*lin*E	ATGATGCAACTGCCCGAA	*lin*E Fw	18	AB021867	Miyauchi et al., 1999
	CTCAAATGACGATCGGATC	*lin*E Rev	19		
*lin*R	GTGAATATAGATGACCTGGA	*lin*R Fw	20	AB021863	Miyauchi et al., 2002
	TCACATCCGCGCGGACA	*lin*R Rev	17		
*lin*F	ATGCAATTCGTTTATGATCCC	*lin*F Fw	21	AP010804	Nagata et al., 2010
	TTATGCGGTGATCGGCGG	*lin*F Rev	18		
*lin*G	ATGTCCGGCGGGTTCGG	*lin*G Fw	17	AP010804	Nagata et al., 2010
	TCAGGCCTTCTCCTCCGT	*lin*G Rev	18		

loaded on a 0.8% agarose gel and plasmid elimination was checked. Plasmid cured culture was further analysed by γ-HCH clearance assay.

2.2.11 Southern analysis of *lin* genes

The truncated *lin*A and *lin*B genes cloned previously (Nagata et al., 1999b) from *S. japonicum* UT26 were present in plasmids pIMA2 and pYNA4, respectively. The ~500-bp and ~900-bp of the coding regions of *lin*A and *lin*B were released from these plasmids, respectively, by digesting with *Eco*RI and *Hin*dIII, and were purified using a gel band purification kit (GE Healthcare, Buckinghamshire, UK). Purified fragment of *lin*A and *lin*B were used as probe in southern analysis further. Southern analysis protocol was followed as reported (Sambrook and Russell, 2001). Total DNA (also contains natural plasmid) from *Shewanella* Sp. CGR-L1 was digested individually with *Bam*HI, *Hind*III and *Sal*I restriction enzymes. Southern blot analyses were carried out using non-radioactive labelling and detection kit (GE Health care, Buckinghamshire UK).

2.2.12 Tolerance of γ-HCH by *Shewanella* sp. CGR-L1

Tolerance of isolate CGR-L1 to γ-HCH was studied by the method described by Gupta et al., (1994). Stock solutions of γ-HCH were prepared in acetone and then added to MSM-L2 in varying concentrations from 10, 20, 30, 40 and 50 mg l^{-1} All the flasks were then inoculated with culture to final OD_{600nm} 0.02 in medium and incubated at 28°C for 96 h. OD_{600nm} at different time interval observed with spectrophotometer (Beckman Coulter, Inc., Brea, CA).

2.3 Results and Discussion

2.3.1 Soil analysis

As mentioned in the chapter no. 1, one of the bacteria isolated from supernatant of soil sample No. 3 showed biodegradation activity in the γ-HCH clearance assay. Therefore, the physical characteristics like pH, organic carbon content and micronutrient were analysed for the soil sample no.3 (Table 12). The results of antibiotic sensitivity assays of CGR-L1 are summarised in Table 11. Isolate *Shewanella* sp. strain CGR-L1 was found to be resistant to ampicillin (10 mg l^{-1}). Consequently, ampicillin was used at required concentration in the further all the experiments, to prevent other bacterial contamination.

2.3.2 Dehalogenase assay for the isolate *Shewanella* sp. CGR-L1

For performing dehalogenase assay, cell-free extract was prepared from *Shewanella* sp. CGR-L1 grown in 1/3 LB with 10 mg L-1 γ-HCH, as described in M&M. After an incubation of 24 h, the color of the solution changed from orange to yellow with drop in pH from 8.0 to 6.65 in the test assay, whereas the control remained unchanged (Figure 14). We concluded that the enzymatic activity of the protein component released Cl^- from γ-HCH, which formed HCl in the weak buffer and, in turn, changed the pH of the solution.

2.3.3 Growth kinetics of *Shewanella* sp. CGR-L1

Optical density of the isolate increased with time and increased to ~0.658, with corresponding decrease in the pH of the medium from 7.0 to 5.2 (Figure 15). This demonstrated that γ-HCH was degraded by the isolate CGR-L1 which was further

Figure 14: Colorimetric determination for γ-HCH dechlorination using cell-free extracts of *Shewanella* sp. strain CGR-L1. Microtitre plate was incubated for 24 hours at 30^0C (Experiment was performed in triplicates).

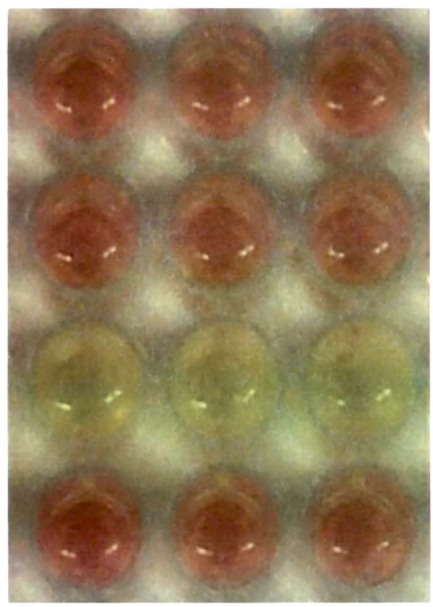
A. Lane 1. Sonication buffer used to prepare cell-free extract

Lane 2. Cell-free extract but without HCH

Lane 3. Cell-free extract with HCH

Lane 4. Heat-denatured cell-free extract with HCH.

B. Change in pH recorded in each well corresponding to Figure A.



A

	pH		
1	8.0	8.0	8.0
2	[illegible]4	[illegible]	[illegible]5
3	6.65	6.65	6.59
4	[illegible]52	[illegible]53	[illegible]56

B

Figure 15: Growth kinetics and biodegradation of γ-HCH by isolate *Shewanella* sp. CGR-L1

A. Residual concentration of γ-HCH observed on HPTLC

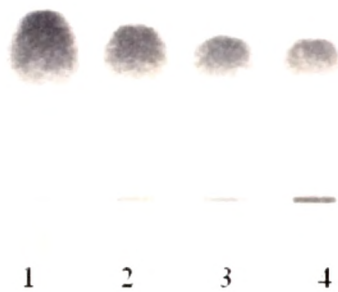
Lane 1; 0hr

Lane 2; 24hr

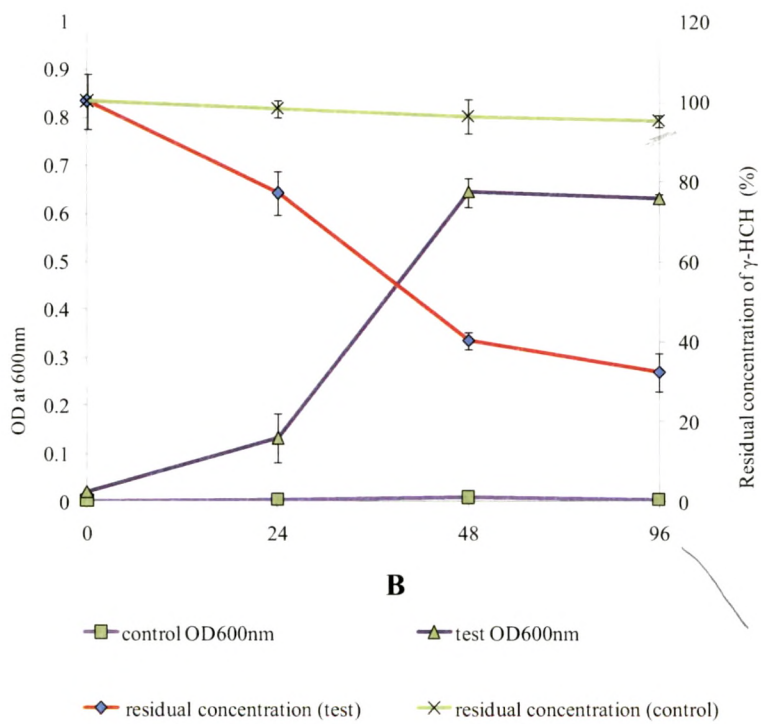
Lane 3; 48hr

Lane 4; 96hr

B. Growth kinetics of isolate *Shewanella* sp. CGR-L1 in presence of γ-HCH and residual concentration of γ-HCH at respective time intervals is shown here.


1
2
3
4

A


OD at 600nm
Residual concentration of γ-HCH (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
20
40
60
80
100
120
0
24
48
96
control OD600nm
test OD600nm
residual concentration (test)
residual concentration (control)

B

Table 12: Soil analysis and antibiotic sensitivity assay for the isolate CGR-L1 and CGR-L2

A. Physico-chemical analysis of the soil samples used for the isolation of γ-HCH degrading bacteria

B. Isolate *Shewanella* sp. CGR-L1 showing sensitivity with different antibiotics after 24 hours of growth on Luria agar plate

C. Isolate *Sphingobium* sp. CGR-L2 showing sensitivity with different antibiotics after 24 hours of growth on Luria agar plate

A.

	Soil Sample no. 3	Soil Sample no. 4
pH	6.60	7.3
Total Nitrogen/Organic carbon (OC)	1.48	1.48
Phosphorus (P2O5, Kg/Ac)	9.0	6.0
Potash (K2O, Kg/Ac)	58.0	70.0
Laed ppm(S, ppm)	18.60	11.30
Zn (ppm)	2.78	7.3
Fe(ppm)	52	14.48
Mn (ppm)	7.80	9.0
Cu (ppm)	12.40	2.96
Isolate obtained	***Shewanella* sp. CGR-L1**	***Sphingobium* sp. CGR-L2**

B.

Antibiotic	Concentration (µg/disc)	Sensitivity
Ampicillin (A)	10	Resistance
Chloramphenicol (C)	30	Sensitive
Co-trimoxazole (Co)	25	Sensitive
Gentamicin (G)	10	Sensitive
Carbenicillin (Cb)	100	Resistance
Cefotaxime (Ce)	30	Sensitive
Norfloxacin (Nx)	10	Sensitive
Oxacillin (Ox)	5	Resistance

C.

Antibiotic	Concentration(µg/disc)	Sensitivity
Ciprofloxacin (Cf)	10	Sensitive
Co-trimoxazole (Co)	25	Sensitive
Kanamycin (K)	30	Sensitive
Streptomycin (S)	10	Resistance
Tetracycline (T).	30	Sensitive
Carbenicillin (Cb)	100	Sensitive
Amicacin (Ak)	10	Sensitive
Nitofurantoin (Nf)	300	Sensitive

confirmed by the HPTLC. After four days of incubation in the MSM-L2 with 1% glucose, isolate was able to degrade up to ~68% of the γ-HCH in comparison to the control. It is reported that genus *Shewanella* is metabolically versatile and is able to use a diverse range of organic substrates and metals as terminal electron acceptors for growth and survival (Picardal et al., 1993 and 1995; Petrovskis and Adriaens, 1995; Fredrickson et al., 2008). This is the first report on biodegradation of γ-HCH by bacterium from *Shewanella* sp. It was observed that the presence of γ-HCH in culture medium did not inhibit the growth of *Shewanella sp.* CGR-L1. Also, no significant difference was found in bacterial growth in MSM-L2 with and without the γ-HCH, similar results have been reported previously (Benimeli et al., 2007).

2.3.4 Identification of the metabolites produced during the γ-HCH biodegradation by *Shewanella* sp. CGR-L1

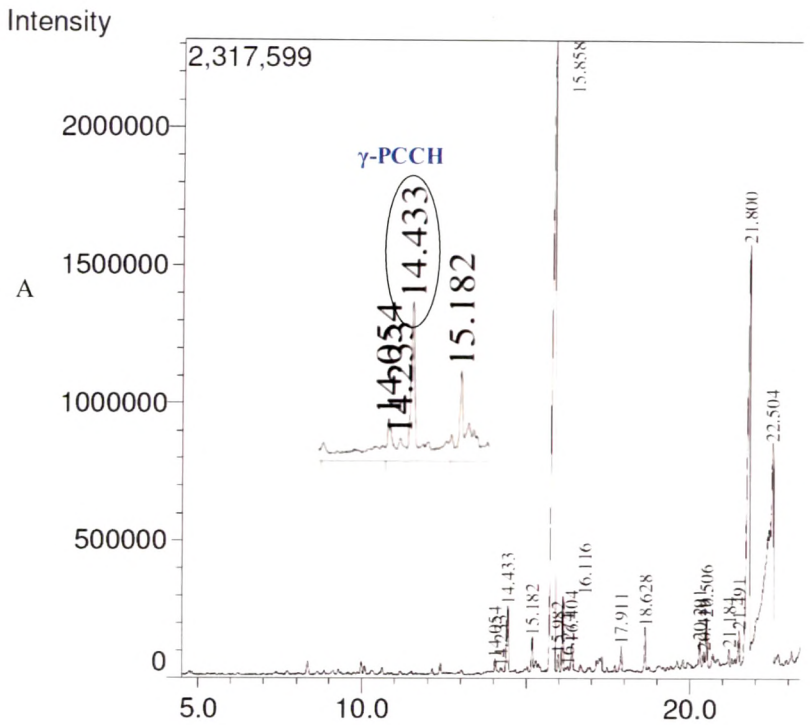
Initially to detect metabolites produced during growth, agar plate method was used (Kaur et al., 2006), where the production of only γ-PCCH (Rt-14.43 min) was observed by GC-MS analysis (figure 16). Further in cell free extract assay, after GC-MS analysis, two metabolites 1, 2, 4-TCB (Rt-12.66 min) and γ-PCCH (Rt-17.19min). Genes involved in its degradation pathway of γ-HCH have been studied previously in detail in *Sphingomonas* sp. (Thomas et al., 1996; Nagata et al., 1999; Kumari et al., 2002) and *Microbacterium* sp. (Manickam et al., 2006). Degradation of γ-HCH by *Sphingomonas japonicum* UT26 (Nagata et al., 1999) led to the production of γ-pentachlorocyclohexene (γ-PCCH), which was subsequently degraded to 2, 5-dichloro-2, 5-cyclohexadiene-1, 4-diol (2, 5-DDOL). Conversion of γ-PCCH to 2, 5-DDOL involves formation of unstable

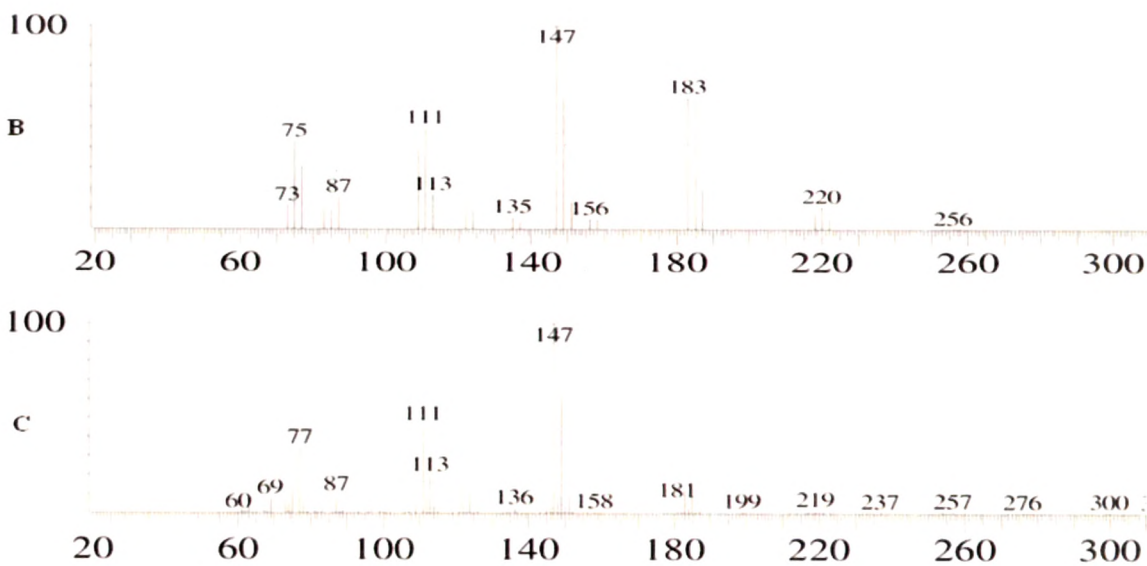
Figure 16: GC-MS analysis of γ-HCH degradation by *Shewanella* sp. strain CGR-L1 (analyzed from the clearance zone around culture of agar plate). A part of agar on a plate showing zone of clearance around the bacterial growth was used to extract γ-HCH degadation metabolite in GC-MS. One of the metabolite γ-PCCH observed during the analysis confirmed by comparing spectra of library data.

A. Total ion chromatogram of γ-HCH degradation

B. Mass spectra of γ-PCCH (Rt 14.33) produced during the γ-HCH biodegradation by isolate *Shewanella* sp. strain CGR-L1

C. Standard γ-PCCH (library data)


Intensity
2,317,599
A
γ-PCCH
14.433
15.182
14.954
15.858
21.800
22.504
16.116
17.911
18.628
2000000
1500000
1000000
500000
0
5.0
10.0
20.0


100
B
147
183
111
75
73
87
113
135
156
220
256
20
60
100
140
180
220
260
300

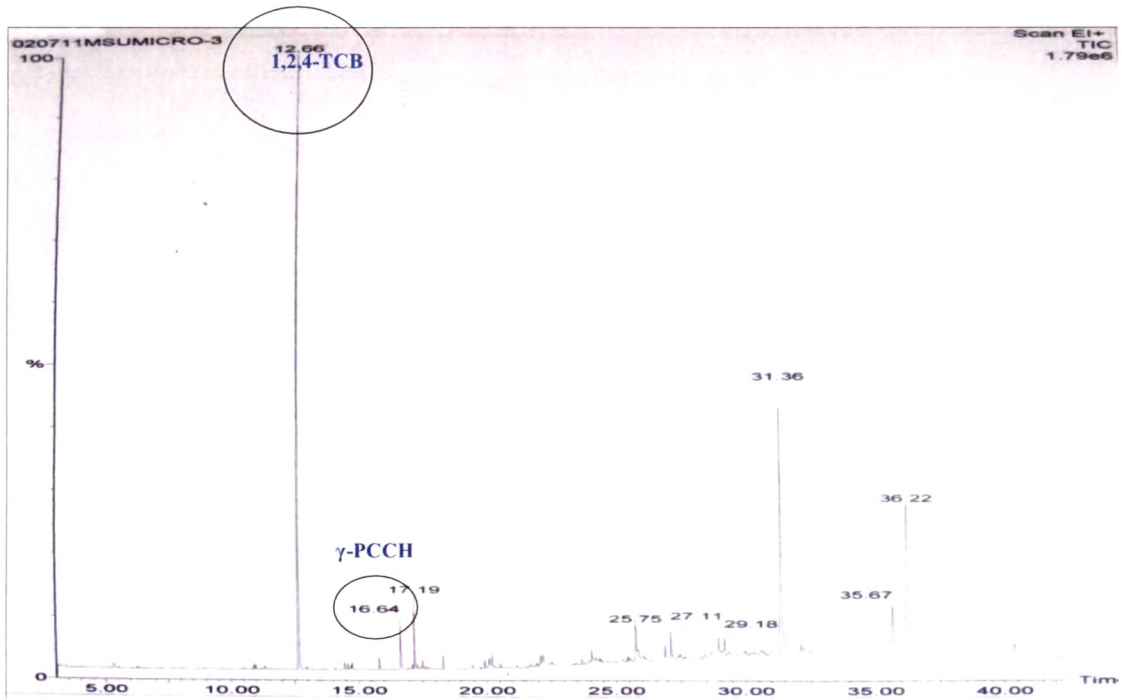
100
C
147
111
77
113
60
69
87
136
158
181
199
219
237
257
276
300
20
60
100
140
180
220
260
300

intermediates 1, 3, 4, 6-tetrachloro-1, 4-cyclohexadiene (1, 4-TCDN) and 2, 4, 5-trichloro-2, 5-cyclohexadiene-1-ol (2, 4, 5-DNOL) which can also be metabolized to form dead end products 1,2,4-trichlorobenzene (1,2,4-TCB) and 2, 5-dichlorophenol (2, 5-DCP), respectively that were detected at extremely low concentrations in *S. japonicum* UT26. GC–MS analysis of cell free extract of *Shewanella* sp. CGR-L1 showed a spectrum that matched with γ-PCCH and 1, 2, 4-TCB (Figure 17). The isolate CGR-L1 produced γ-PCCH and 1, 2, 4-TCB, which is likely product of γ-HCH degradation by *linA*. *Escherichia coli* isolated from rat feaces are known to produce γ-PCCH from the γ-HCH and even in that case no other metabolites formed further (Francis et al., 1975). It has been reported that γ-PCCH is 1000 times less toxic than γ-HCH (Yule et al., 1967 and Francis et al., 1975). In *Pseudomonas fluorescens* it was found that EC50 for 1, 2, 4-TCB was higher than BHC, which suggest that 1, 2, 4-TCB is less toxic than γ-HCH (Boyd et al., 1998). It has been also reported that 1,2,4 –TCB is biodegraded by the bacteria (Adrian et al., 2007), and possibly such bacteria might be present in the soil which can degrade 1,2,4- TCB. It is reported that growth of *S. japonicum* UT26 in rich medium was inhibited by γ-HCH at final concentration of 25μg/mL in the culture due to the production of toxic compounds like 2,5-dichlorophenol (2,5-DCP) and 2,5-dichlorohydroquinone (2,5-DCHQ)(Endo et al., 2006). However, isolate CGR-L1 does not appear to produce any toxic compounds like 2, 5-DCP and 2, 5-DCHQ, hence it is able survive and grow even at 50μg ml^{-1} concentration. Taken together, it can be concluded that isolate CGR-L1 is novel for biodegradation of γ-HCH and it does not produce any toxic metabolite which could inhibit the growth of the isolate.

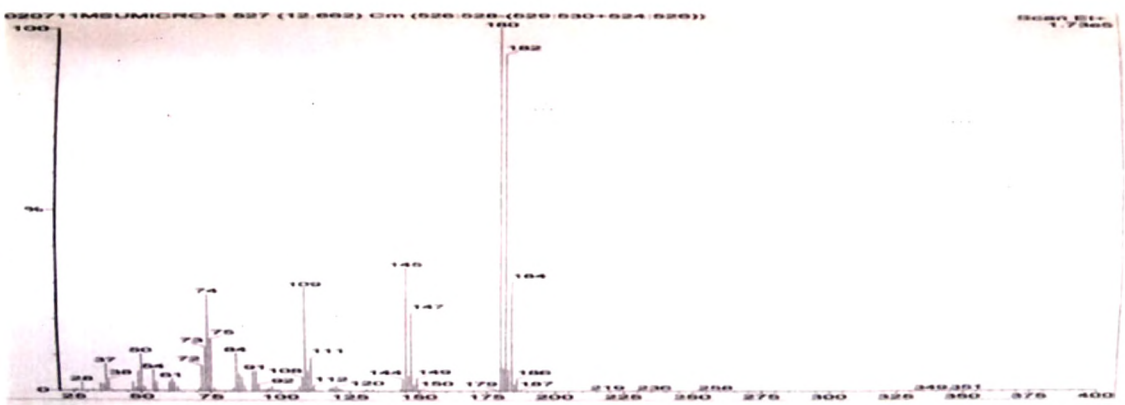
Figure 17: GC–MS analysis for the identification of metabolites produced during γ-HCH degradation using cell free extract of *Shewanella* sp. CGR-L2.

A. Total ion chromatogram of γ-HCH shows two peak of metabolites with 1,2,4-TCB (Rt 12.66) and γ-PCCH (Rt 16.64)
B. Mass spectra of metabolites produced during γ-HCH degradation showing 1,2,4-TCB (Rt 12.66 min)
C. Mass spectra of 1,2,4-TCB standard

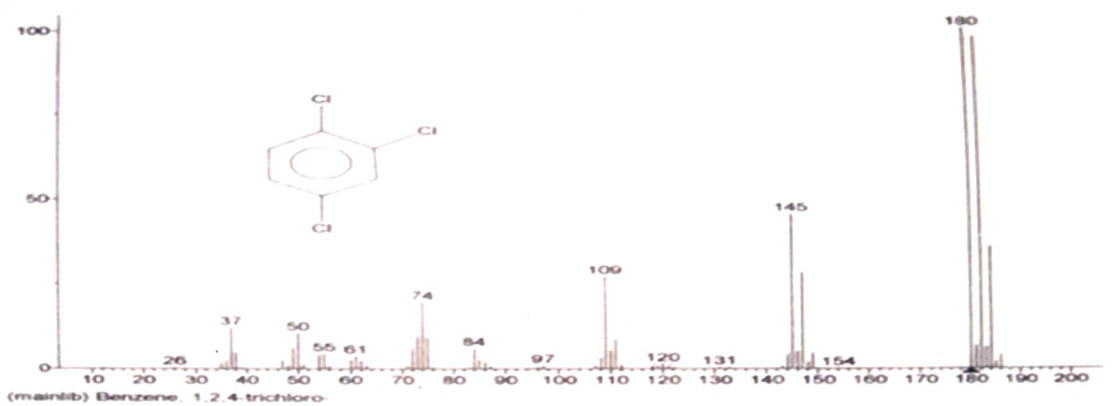
A


020711MSUMICRO-3
Scan EI+
TIC
1.79e6
12.66
1,2,4-TCB
γ-PCCH
16.64
17.19
25.75
27.11
29.18
31.36
35.67
36.22
Time

B


180
182
184
145
147
109
74
111
186

C


Cl
Cl
Cl
180
145
109
74
37
50
84
(mainlib) Benzene, 1,2,4-trichloro-

2.3.5 PCR amplification of *lin*A and its sequence identity

To check for the presence of the gene for γ-HCH degradation in the isolate CGR-L1, PCR based detection technique was used. F*lin*A and R*lin*A primers were used to amplify the *lin*A gene from the genomic DNA of the isolate and an expected amplification product of ~420 bp was obtained (Figure 18A) and it was further sequenced. The sequence obtained from strain CGR-L1 was compared with GenBank (http://www.ncbi.nlm.nih.gov/blast) database, and it showed 97% identity to *lin*A of *Sphingobium japonicum* UT26 (GenBank accession no. **D90355.2**) and *lin*A2 of *Sphingobium indicum* B90 (GenBank accession no. **FJ608814.1**), well studied for γ-HCH degradation. Phylogenetic analysis of *lin*A from strain CGR-L1 showed a close relationshiop to the *lin*A of *Xanthomonas* sp. ICH12 (**DQ910545.1**) and *Sphingomonas* sp. NM05 (**DQ767898.1**) (Figure 18B). The *lin*A sequence from the bacterial isolate CGR-L1 was deposited in NCBI (accession no. **HM063959**). GC-MS analysis in cell free extract assay mentioned in materials and methods, we found two peaks of metabolites i.e. PCCH and 1, 2, 4-trichlorobenze, confirmed by spectra. This data supported that CGR-L1 degrades γ-HCH to γ-PCCH and 1, 2, 4-trichlorobenzene, as *lin*A product is involved in this step. It is reported that 1, 2, 4-trichlorobenzene is the end product in some of the isolates. PCR analysis was carried out to check the presence of *lin*B gene which is required for subsequent conventional biodegradation of the γ-HCH; however, *lin*B was not detected in this analysis indicating that *lin*B gene may not present in this organism. Thus, *Shewanella* sp. isolate CGR-L1 appears to be an important species that can be utilized for the degradation of γ-HCH in the environment.

Figure 18: PCR amplification and phylogenetic analysis of *lin*A gene from isolate *Shewanella* sp. CGR-L1

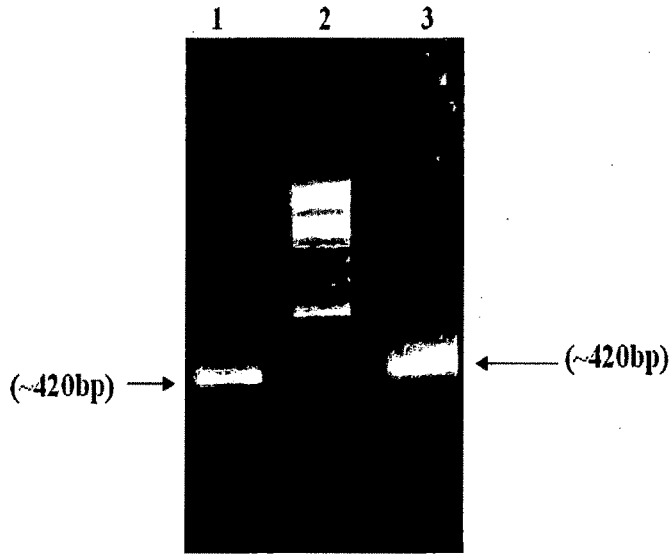
A. PCR amplification of *lin*A gene from isolate *Shewanella* sp. CGR-L1 using gene-specific primers.

   Lane 1; Amplification of *lin*A gene (420bp) from plasmid pIMA2 as positive control

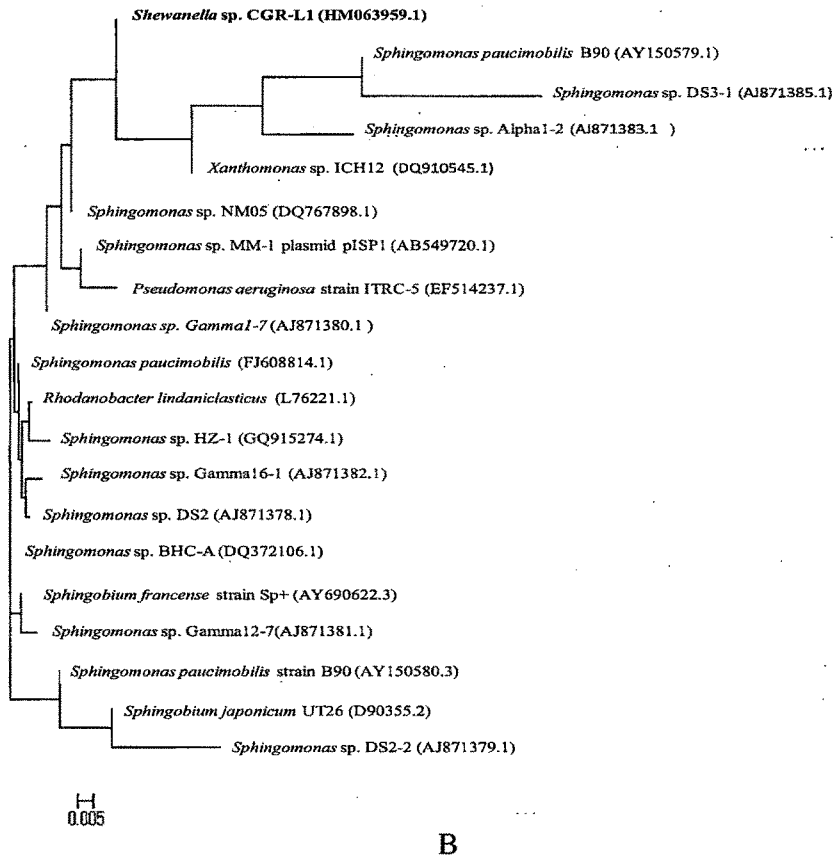
   Lane 2; 1 Kb ladder

   Lane 3; Amplification of *lin*A (420bp) gene from genomic DNA of *Shewanella* sp. strain CGR-L1

B. Phylogenetic tree of *lin*A gene of *Shewanella* sp. CGR-L1 (indicated in bold letter). Multiple sequence alignment of *lin*A 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 0.005 estimated substitutions per nucleotide.


1
2
3
(~420bp)
(~420bp)

A


Shewanella sp. CGR-L1 (HM063959.1)
Sphingomonas paucimobilis B90 (AY150579.1)
Sphingomonas sp. DS3-1 (AJ871385.1)
Sphingomonas sp. Alpha1-2 (AJ871383.1)
Xanthomonas sp. ICH12 (DQ910545.1)
Sphingomonas sp. NM05 (DQ767898.1)
Sphingomonas sp. MM-1 plasmid pISP1 (AB549720.1)
Pseudomonas aeruginosa strain ITRC-5 (EF514237.1)
Sphingomonas sp. Gamma1-7 (AJ871380.1)
Sphingomonas paucimobilis (FJ608814.1)
Rhodanobacter lindaniclasticus (L76221.1)
Sphingomonas sp. HZ-1 (GQ915274.1)
Sphingomonas sp. Gamma16-1 (AJ871382.1)
Sphingomonas sp. DS2 (AJ871378.1)
Sphingomonas sp. BHC-A (DQ372106.1)
Sphingobium francense strain Sp+ (AY690622.3)
Sphingomonas sp. Gamma12-7(AJ871381.1)
Sphingomonas paucimobilis strain B90 (AY150580.3)
Sphingobium japonicum UT26 (D90355.2)
Sphingomonas sp. DS2-2 (AJ871379.1)
0.005

B

2.3.6 Localization of catabolic properties and plasmid curing from *Shewanella* sp.CGR-L1

Genes for the degradative pathway for numerous chemical compounds has been found to be located on extrachromosomal replicons. This was the case for the γ-HCH degradation determinant in two *Pseudomonas* strains (Ceremonie et al., 2006). Total DNA extracted from the isolate CGR-L1 showed the presence of one plasmid of ~2.5 kb (Figure 19). To examine chromosomal location of *Lin*A gene and its copy number in the genome Southern blot analysis was carried out. Total DNA extracted (also contains natural plasmid) from the isolate was digested individually with *Bam*HI, *Hind*III and *Sal*I restriction enzymes. These enzymes do not cut anywhere in *Lin*A (Kumari et al., 2002). Non-radioactively labeled *Lin*A partial coding region from previously published sequence of *Sphingomonas japonicum UT26* was used as probe for hybridization. A 4.3-kb fragment from the chromosomal DNA gave hybridization signal when the DNA was digested with *Bam*HI (Figure 20). Further confirmation was done by PCR using *lin*A primers with eluted plasmid also, but no amplification was observed. The protocol reported previously (Heringa et al., 2007) was followed for extraction of large plasmid from the isolate *Shewanella* sp. CGR-L1. However, no high molecular weight plasmid was observed on agarose gel, but a small plamid around 2.5 kb was seen on agarose gel. Further, the plasmid was cured from the isolate *Shewanella* sp. CGR-L1 by 6mM Ethidium bromide and degradation of γ-HCH in plasmid cured strain was compared with the parent strain. No difference was observed in the γ-HCH clearing zone in the plate assay, suggesting that this plasmid is not involved in biodegradation of γ-HCH (Figure 19). These results indicate that the γ-HCH degradation function in the bacterial isolate CGR-L1 is encoded

by the chromosomal DNA. Further hybridization of genomic DNA digested with *Hind*III and *Sal*I gave four hybridizing bands in each case, indicating presence of more than one copy of *lin*A in the genome of CGR-L1, as no restrictions sites were present in *lin*A sequence of *Shewanella* sp. CGR-L1 (Figure 20). It indicates that this new γ-HCH biodegrading bacterial strain contained 4 copies of the *lin*A genes in the genome. Previously a maximum two copies of the *lin*A gene was reported in *Sphingomonas* sp. (Kumari et al., 2002).

2.3.7 Tolerence of γ-HCH by isolate CGR-L1

Isolate CGR-L1 was able to grow even up to 50ug ml^{-1} of γ-HCH in the MSM-L2 (Figure 21). Other reported isolate like *Sphingomonas japonicum* UT26 was able to tolerate up to 25 mg l^{-1} concentration only, since the production of toxic metabolites like 2,5-dichlorohydroquinone and chrolohydroquinone inhibited the growth of bacteria (Endo et al., 2006). As discussed earlier CGR-L1 isolate producing metabolites like γ-PCCH and 1, 2, 4-TCB during biodegradation of γ-HCH, and further genes for the γ-HCH biodegradation pathways do not appear to be present. Therefore it does not produce any toxic metabolites which may inhibit its growth.

Figure 19: Analysis of role of plasmid in γ-HCH biodegradation by *Shewanella* sp. CGR-L1

A. Detection of plasmid DNA in the isolate *Shewanella* sp. strain CGR-L1

Lane 1; Total DNA from isolate CGR-L1 showing presence of plasmid (~2.5 kb)

Lane 2; 1 Kb ladder

B. Curing of plasmid from *Shewanella* sp. strain CGR-L1 confirmed by Agarose gel electrophoresis

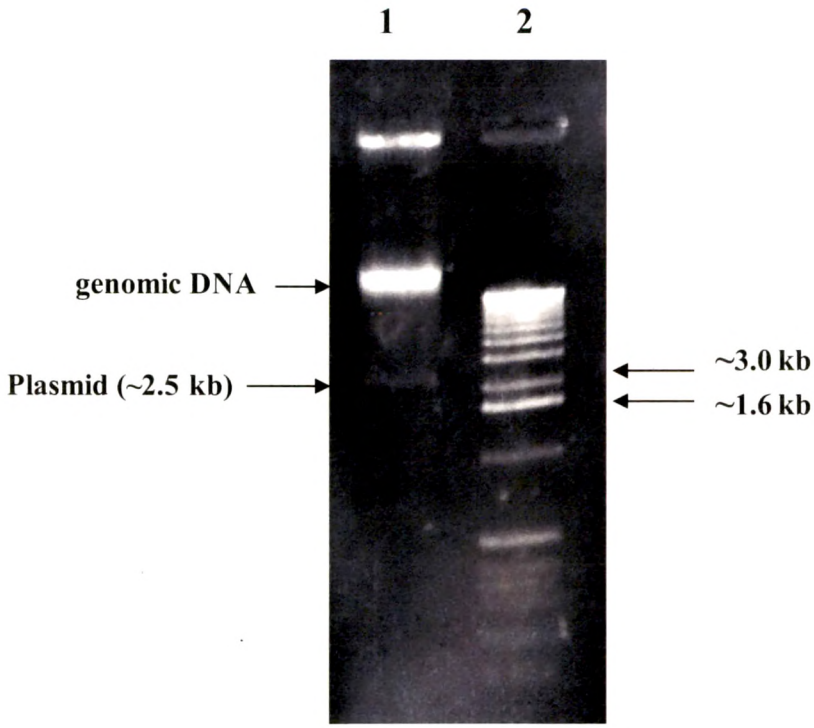
Lane1 and 2; DNA extracted from untreated culture

Lane 3 and 4; DNA extracted after plasmid curing indicating the absence of plasmid

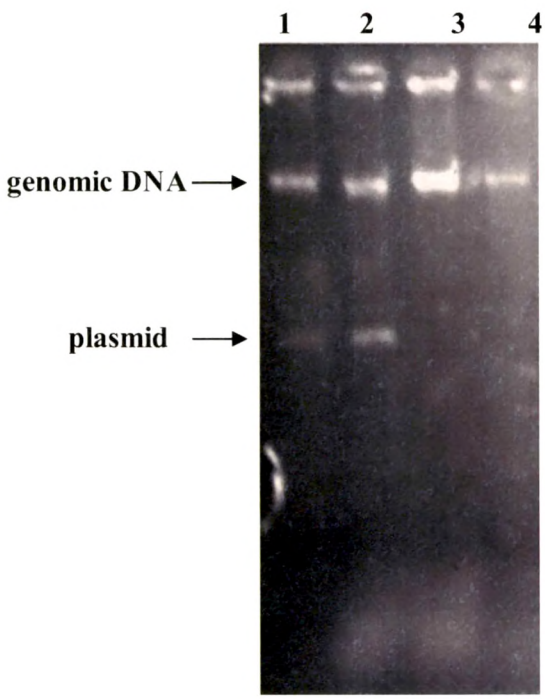
C. Plate assay of the growing culture showing clearance zone in γ-HCH overlayed plates.

a. Before plasmid curing

b. After plasmid curing


1
2
genomic DNA
Plasmid (~2.5 kb)
~3.0 kb
~1.6 kb

A


1
2
3
4
genomic DNA
plasmid

B

a b

C

Figure 20: Southern blot hybridization of genomic DNA extracted from *Shewanella* sp. strain CGR-L1 and hybridised with non-radioactively labeled *lin*A gene probe. Genomic DNA of *Shewanella* sp. strain CGR-L1 was digested with restriction enzymes, Lanes 1. *Bam*HI , 2. *Hin*dIII and 3. *Sal*I; and Lane 4. 1 Kb ladder. Resticition enzymes *Hind*III and *Sal*I gave four hybridizing bands (signal) in each case, indicating presence of more than one copy of *lin*A in the genome of CGR-L1, as none of these restriction sites were present in *lin*A sequence of *Shewanella* sp.

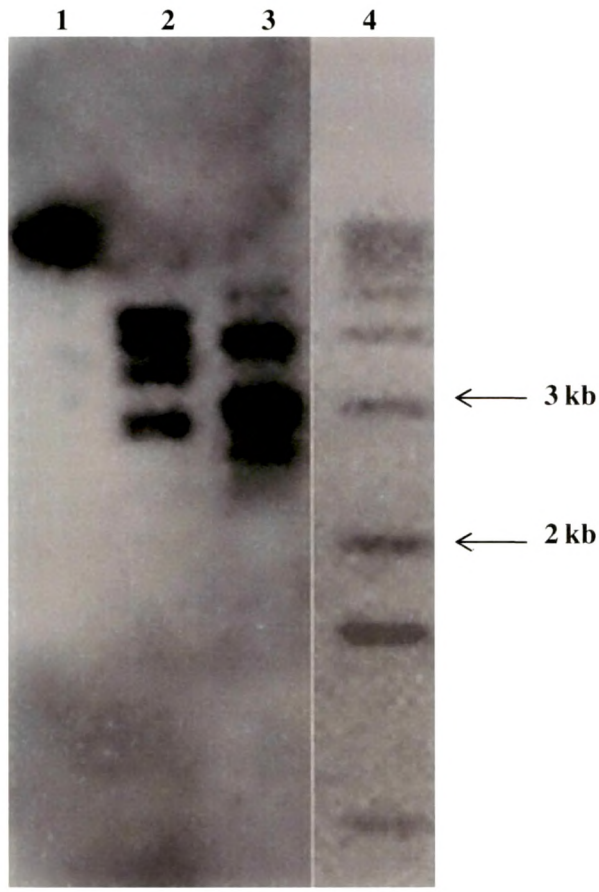
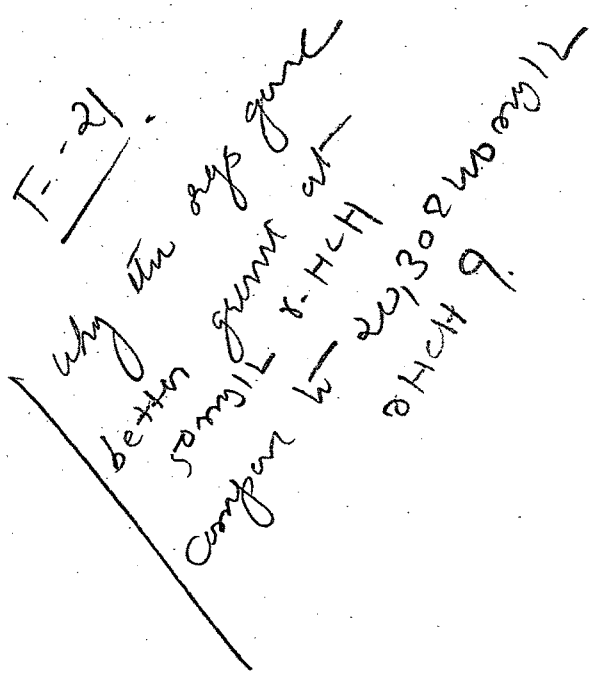
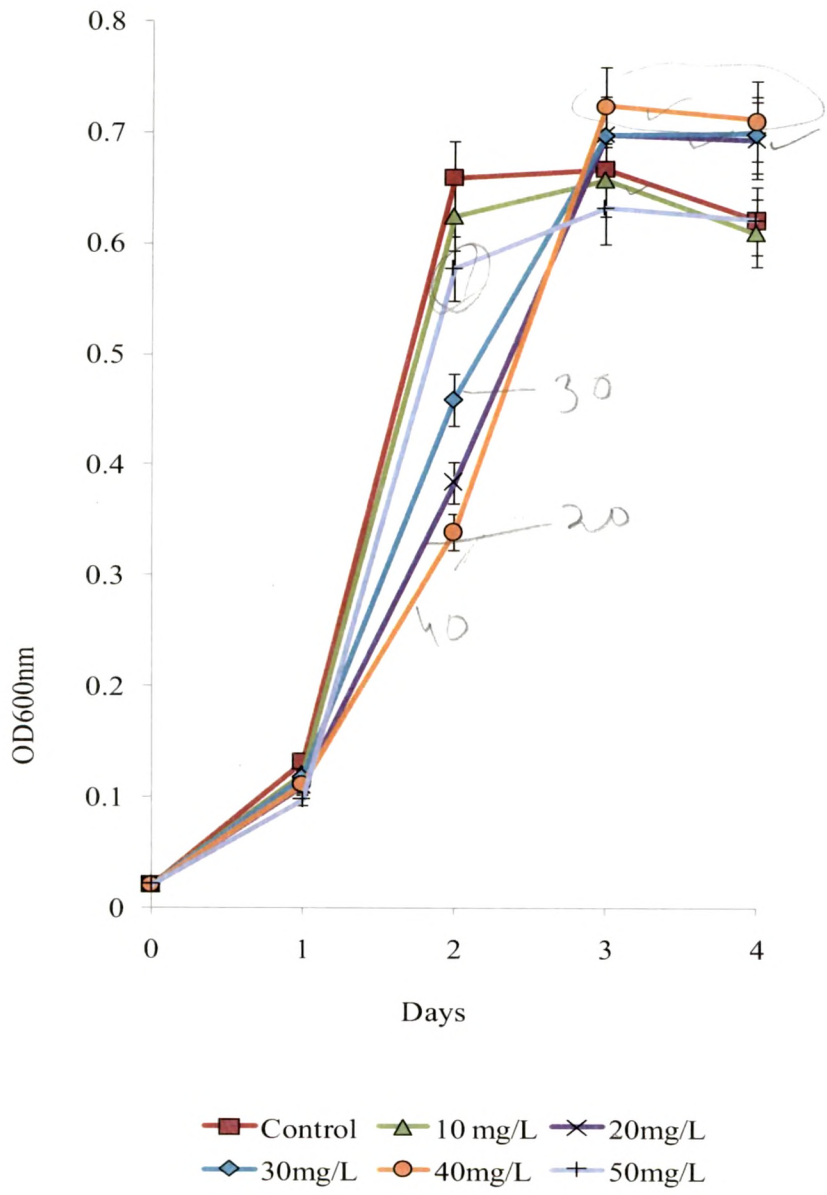


Figure 21: Growth of *Shewanella* sp. CGR-L1 in MSM-L1 (supplemented with glucose) in presence of different concentrations (10 to 50 mg l^{-1}) of γ-HCH




0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600nm
0
1
2
3
4
Days
Control
10 mg/L
20mg/L
30mg/L
40mg/L
50mg/L