

**Aerobic degradation of highly chlorinated PCBs by a marine bacterium,
Pseudomonas CH07**

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Summary

Aerobic degradation of PCBs though have been reported to be limited to less chlorinated biphenyls (PCBs), we report here of a marine mercury-resistant bacterium, *Pseudomonas* CH07 (NRRL B-30604) which was capable of degrading a variety of highly chlorinated congeners of PCBs from the technical mixture Clophen A-50. Of the two most toxic coplanar PCBs present in Clophen A-50, one coplanar pentachloro congener CB-126 and one toxic sterically hindered heptachloro congener CB-181 were found to be degraded completely and the other coplanar tetrachloro congener CB-77 was degraded by more than 40% within 40 hours by this microorganism. Apparently absence of *bphC* in this bacterium led to proposition of different mechanism of PCBs degradation.

KEY WORDS: *Pseudomonas* CH07, PCBs, Clophen A-50, CB-126, CB-181, aerobic degradation

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Introduction

Polychlorinated biphenyls (PCBs) are a class of compounds where the aromatic biphenyl rings carry one to ten chlorine atoms and, theoretically, 209 different PCB congeners can thus be produced. Due to their non-flammability, chemical stability, high boiling point and electrical insulating properties, PCBs have been used for hundreds of industrial and commercial purposes (Pieper, 2005). PCBs are considered potential carcinogens because some mixtures have been shown to increase the development of hepatic tumors in rats (Safe, 1984; Kimbrough, 1987; Piper, 2005). PCB contaminations of drinking water, sediments, wastewater, foods, and aquatic organisms have been documented (Boon et al., 1985). Estuaries and the marine sediments are the ultimate global sinks for worldwide accumulations of PCBs (Berkaw et al., 1996). The toxicity of different congeners of PCBs varies according to the chlorine substitution at different positions of the biphenyl ring and the physical effects of PCBs vary from mammals, to birds, to humans. It has been observed that DNA strand-breaks occur in various species of marine organisms due to interaction with polychlorinated biphenyl contaminants having coplanarity (Dunn et al., 1987; Everaarts et al., 1994).

The PCBs have impacted our environment through accidents, spills in industrial facilities and mismanagement of storage or disposal areas (Nagayama, 1976; Chen et al., 1980). The Environmental Protection Agency (EPA) regulates the disposal of PCBs through the Toxic Substance Control Act (TSCA) which requires that the PCBs be disposed in accordance with the methods regulated by the EPA. Incineration is the standard method of destruction for PCBs and the only one approved for the removal of PCBs from the soil and sediment (Blake, 1994). Regulation provides scope to alternate methods like biodegradation/bioremediation that could demonstrate the destruction of PCBs equivalent to incineration. The microbial degradation of PCBs has been extensively studied in recent years. The genetic organization of biphenyl catabolic genes has been elucidated in various groups of microorganisms, their structures have been analyzed with respect to their evolutionary relationships, and new information on mobile elements has become available. Key enzymes, specifically biphenyl 2,3-dioxygenases, have been intensively characterized, structure/sequence relationships have been determined and enzymes optimized for PCB transformation. However, due to the complex metabolic network responsible for PCBs degradation, optimizing degradation by single bacterial species is necessarily limited (Pieper, 2005).

The bacterium CH07 has shown the potential to clean up several other toxic chemicals including high concentrations of phenol, Cd, Pb, and Hg. Such multiple resistances make this bacterium a worthy candidate for use in bioremediation of mixed wastes (De et al., 2003). This bacterium was checked for its potential to degrade different congeners of PCBs from the technical mixture Clophen A-50 and was found to degrade many highly chlorinated congeners convincingly within just forty hours.

Materials and methods

Isolation of microorganism:

Several mercury-resistant *Pseudomonas aeruginosa* like organisms (PALO) were isolated using Cetrimide agar medium amended with 50 ppm Hg (as HgCl₂) from water samples collected from a contaminated coastal site in the east coast of India. A bacterium designated as *Pseudomonas* CH07 was found to degrade several congeners of PCBs containing 5 or more- chlorine atoms on the biphenyl ring from the technical mixture of PCBs, Clophen A-50 (Beyer, Germany).

Identification:

The isolate was characterized biochemically and was identified by 16S rDNA sequence analysis. Overnight grown cultures were used for extraction of the genomic DNA and the extraction was done using the Nucleospin Extract (Macherey Nagel, Germany) and the quality of the product was checked on 0.8% TAE agarose gel. The sequencing primers used were (forward primer 8-27: 5'AGAGTTTGATCCTGG CTCAG 3' (Weisberg et al., 1991) and reverse primer 1492: 5'GGTTACCTTACGACTT 3' (Reysenbach et al., 1992). Sequencing (Sanger et al., 1977) was done using ABI sequencing instrument (ABI PRISOM model no. 3700). The sequence was submitted online, checked for the match using FASTA program (<http://www.ebl.ac.uk>) and was deposited with the gene bank (accession numbers: DQ377451- DQ377452).

Growth pattern of CH07:

The growth of the isolate was determined by monitoring its OD following its inoculation into SWNB. In brief, 50 µl of a 24 h old culture of CH07 was inoculated into two 250 ml flasks containing SWNB (100 ml) with Clophen A-50 added to them to a final concentration of 100 ppm. A control without any PCBs was included. The flasks were incubated on a rotary shaker (200 rpm) at room temperature (ca. 28 ± 2 °C) for 120 h.

The absorbance (OD₆₆₀) of culture was measured every 12 h. Cell numbers were determined by plating aliquot sample after proper dilution with sterile 50% seawater. Log values of cell numbers were plotted to draw growth curves (Figure 1).

Culture media and degradation experiment:

A defined seawater nutrient broth (SWNB) medium containing beef extract 3 g L⁻¹, peptic digest of animal tissue 5 g L⁻¹ was used. One liter of medium contained 500ml seawater and 500 ml distilled water and the final pH was adjusted to 7 using 0.1 N NaOH. Required amount of stock solution (10,000 ppm in hexane) of Clophen A-50 (Bayer, Lot no. 16572) was added into the sterilized medium in sterile condition to achieve a final concentration of 100 ppm. Immediately after adding the stock solution of PCBs, the hexane was evaporated out by gently swirling the flasks in sterile condition. Sterilized glycerol was added to the medium in a 1:1 ratio of stock solution: glycerol. Forty (40) µl of 24 h old broth culture of CH07 was added in two replicates of 20 ml of test medium (SWNB+ClophenA-50) and normal SWNB (without any addition of Clophen A-50). Controls in duplicate were also maintained without the addition of the organism in one set and with killed bacterial cells in another set at room temperature (ca. 28°C). At various predecided intervals of time, the samples were taken out aseptically and prepared for GC analysis. The comparison of degradation of PCBs was done with the control without added bacteria and test condition with the live bacterial cells. Possibility of absorption of PCBs onto the cells was checked by comparing the control with killed bacterial cells and test condition with live bacterial cells.

Analysis of PCBs

The PCBs were extracted following standardized method. The samples were analyzed by gas chromatography (Varian GC-3380) coupled with an electron capture detector (ECD) and an autosampler 8200. A capillary column VA-5 (30 m x 0.25 mm) was employed with ECD for peak detection. Argon with 5% methane was used as the carrier gas. The injector temperature was fixed at 250°C. The analysis of PCBs was calibrated using the standards for individual congeners of PCBs.

Identification of breakdown pathway:

The isolate was streaked on M9 (Sambrook et al., 1989) agar plate adding biphenyl crystal on the lid as the sole source of carbon and sealed with parafilm. As a control, the

M9 agar plate was supplemented with yeast extract as carbon source. After 24 h of incubation in the dark, the colonies were sprayed with 2,3 dihydroxybiphenyl (2,3-OHBP) an intermediate in the biphenyl degradation pathway to see if there was any appearance of yellow metacleavage product (2-hydroxy-6-oxo-6phenylhexa-2, 4-dienoic acid; HOPDA) which would appear because of the production of 2, 3 dihydroxibiphenyl dioxygenase by the *bphC* gene.

Results

Identification of the microorganism:

The microorganism CH07 was purified and was identified from the biochemical test as a *Pseudomonas aeruginosa* (Table 1) which was further confirmed by 16S rDNA analysis. The culture is deposited as NRRL B-30604 as an integral part of the US patent no. 6544773 (Sarkar et al., 2003).

Growth Pattern:

It was clearly evident that there was no appreciable change or effect of PCBs on the growth of CH07 (Figure 1). The bacterium had attained the exponential phase within 12 hours of growth in the presence of PCBs in the growth medium akin to the growth in the control flask.

Analysis of PCBs:

It was observed that Clophen A-50 was sufficiently degraded by CH07. Among the different congeners of PCBs present in Clophen A-50, 14 chlorobiphenyls were degraded to varying degrees (Table 2). Of the three most toxic coplanar PCBs (cb-77, cb-126 and cb-169), this organism degraded one of them (cb-126) completely in about 40 hours (Figure 2). The other coplanar PCBs, CB-77 (3, 3', 4, 4'-Tetrachlorobiphenyl) was also degraded substantially within a short period of 40 hours. One heptachlorobiphenyl, CB-181 (2,2',3,4,4',5,6) was degraded completely within 40 hours (Table 3). Two asymmetric di-ortho chlorinated biphenyls viz. 2,2',4,5,5'-pentachlorobiphenyl and 2,3',4,4',6 pentachlorobiphenyl were degraded to 20.19% (20.19±0.6) and 19.66% (19.66±0.5) respectively. One of the congener CB-116 (2,3,4,5,6-pentachlorobiphenyl) with all the chlorines substituted on one of the aromatic rings being highly polar was degraded to only 20.04% (Table 2).

As the control with the dead cells did not show any remarkable decrease of PCBs from the growth medium, it was quite obvious that the PCBs were indeed biodegraded, not absorbed and/or adsorbed by the cells.

Identification of breakdown pathways:

The marine MRB strain CH07 did not grow on M9 medium which had biphenyl crystals as the sole source of carbon whereas it grew normally on the M9 agar plates supplemented with yeast extract as carbon source. The colonies on the latter medium when sprayed with 2,3 dihydroxybiphenyl (2,3-OHBP) were negative for meta-cleavage product (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) indicating the non-functioning or total absence of 2,3 dihydroxybiphenyl dioxygenase by *bphC* gene. Subsequently, there was no production of yellow color meta-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid after spraying the colonies with 2,3 dihydroxybiphenyl.

Discussion

Some PCB congeners are found to be transformed by both anaerobic and aerobic bacteria (Bradley et al., 1996; Kimbara et al., 1999). Degradation of mono- di- and tri-chlorinated biphenyl is relatively common (Bedard et al., 1987). Some strains like *Pseudomonas* LB400 (Gibson et al., 1993), *Alcaligenes eutrophus* H850 (Bedard et al., 1987), *Corynebacterium* MB1 (Williams et al., 1997) and *Acinetobacter* P6 (Kohler et al., 1988) are exceptional PCB degraders. The specificity of the dioxygenases in these organisms differs greatly. Strains like P6 and MB1 are particularly active against double para chlorinated PCBs. Strains H850 and LB400 preferentially express a 3,4-dioxygenase, forming a *cis*-dihydrodiol form 2,5,2',6'- tetrachlorobiphenyl, which is degraded faster by H850 than PCBs with an unchlorinated ring (Bedard et al., 1987). The chlorobenzoic acids are not further degraded by most PCB-degrading bacteria. An exception is the degradation of 2,3'-dichlorobiphenyl by LB400. It is generally believed that biodegradation of PCBs decreases with the increase in chlorine substitution (Furukawa et al., 1979). Generally, for mixtures of lower chlorinated PCBs, sufficient degradation may be achieved using a one-stage procedure involving only aerobes. There is a danger, however, if higher chlorinated PCBs are left intact because they are generally more bioaccumulative than the less chlorinated PCBs (Unterman, 1996). Additionally, there are the highly toxic congeners referred to as "dioxin-like", containing chlorine at the two para positions (4 on the biphenyl ring) and at least two chlorines at the meta positions (3 or 5 position).

The lack of ortho groups allows the atoms in these congeners to line up in a single plane (referred to as coplanar PCBs), that makes them especially toxic.

A combination of anaerobic followed by aerobic biodegradation has been found to maximize the removal of chlorine. Significance of aerobic degradation of PCBs can be explained and understood in the following way. The most critical stage of the two-step, anaerobic to aerobic process is getting the anaerobic bacteria to proliferate at a new site, cometabolizing the higher chlorinated PCBs (greater than 4 chlorines per biphenyl). Because aerobes are only capable of degrading lower chlorinated PCBs (4 or less chlorines per biphenyl), it is important that anaerobes grow well, transforming as many of the higher chlorinated PCBs as possible to lower chlorinated PCBs. Several factors affect the growth of bacteria in presence of toxic PCBs. Briefly, they are the chemical structure and composition of the PCB waste, the initial concentration, the solubility of the PCBs in the media, and the environmental conditions (temperature, pH, salinity, redox potential, available carbon, and presence of co-contaminants).

Bioremediation technology (BT) has countless advantages and is a highly attractive alternative for dealing with PCBs due to the high costs of transportation, incineration and other procedures of remediation that currently exist. BT can be used to treat low concentration of contaminants; prevent physical and chemical treatment; maintain alteration of the contaminated area to a minimum; eliminate long term responsibility; destroy organic contaminants, does not generate secondary waste and can be combined with other treatment technologies for highly complex mixed waste. BT is cost effective (Churchill et al., 1995; McGraph, 1995; Sturman, 1995). PCBs do present a challenge to bioremediation, as they are highly stable. Nevertheless, the availability and evolution of potential cost saving possibilities makes BT worthy of consideration when assessing/choosing treatment options.

The marine bacterium, CH07 (*Pseudomonas aeruginosa*) is capable of degrading PCBs of different configurations. This is the first conclusive demonstration of an aerobic microbial process involving a marine bacterium that degrades highly chlorinated PCBs contained in Clophen A-50. The extent of degradation of different congeners of PCBs in presence of other chlorobiphenyls and with varying degree of polarity and stereochemical asymmetry is a clear indication bacterial strains such as CH07, isolated from marine environments can be used effectively for their detoxification. Most importantly, highly chlorinated congeners, CB-180 and CB-181 were found to be degraded

sufficiently. However, inability of the organism to grow on biphenyl as the sole carbon source indicates that the organism was unable to use biphenyl as sole carbon and energy source. Absence of *bphC* product 2,3-dihydroxybiphenyl dioxygenase indicates the absence of complete *bph* operon and also possibility different pathways of PCBs degradation which warrants further research.

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Table 1. Biochemical characteristics of CH07

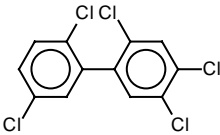
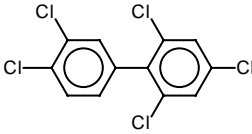
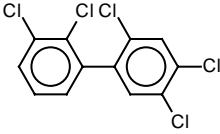
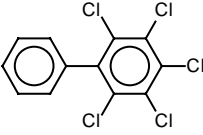
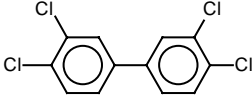
Test	Response	Test	Response
Gram stain	-ve	Nitrate reduction	++
Shape	Very small rod	H ₂ S	- ^a
Motility	+ ^b	Indole	-
Pigment	Fluorescent green	Urease	-
Oxidase	Slow reaction	<i>Growth on</i>	
OF	No reaction	Citrate	+
Gelatinase	+	Sucrose	Alkaline
Catalase	++ ^c	Mannitol	Acidic
Lipase	-	Rhamnose	Acidic
Starch hydrolysis	+	Arabinose	Alkaline
Arginine	+	Streptomycin	++
Omithin	-	Tetracycline	++
VP	-	Demechlocyclin	++
MR	-	Kanamycin	++

^anegative response (no growth), ^bpositive response (slight or moderate growth) ^cvery good response (good growth).

Table 2. Molecular formulae of PCBs in Clophen A-50 and their percent (mean±SD) degradation by CH07

Chlorobiphenyls	Molecular Formula	Retention time (Min)	PCBs at 0 hr (ng/ml)	PCBs at 40 hrs. (ng/ml)	Degradation of PCBs (%)
CB-101 (2,2',4,5,5')	C ₁₂ H ₅ Cl ₅	19.564	18.17	14.50	20.19±0.6
CB-119 (2,3',4,4',6)	C ₁₂ H ₅ Cl ₅	19.886	8.07	6.48	19.66±0.5
CB-97 (2,2',3',4,5)	C ₁₂ H ₅ Cl ₅	20.892	8.17	6.57	19.69±1.2
CB-116 (2,3,4,5,6)	C ₁₂ H ₅ Cl ₅	21.211	10.09	8.06	20.04±0.9
CB-77 (3,3',4,4')	C ₁₂ H ₆ Cl ₄	21.823	53.37	40.42	24.25±1.7
CB-151 2,2',3,5,5',6)	C ₁₂ H ₄ Cl ₆	22.595	2.04	1.28	37.32±0.6
CB-118 (2,3',4,4',5)	C ₁₂ H ₅ Cl ₅	23.400	1.31	0.77	40.72±2.5
CB-105 (2,3,3',4,4')	C ₁₂ H ₅ Cl ₅	24.830	17.54	9.29	46.69±1.3
CB-141 (2,2',3,4,5,5')	C ₁₂ H ₄ Cl ₆	25.449	3.57	1.59	55.38±2.4
CB-138 (2,2',3,4,4',5')	C ₁₂ H ₄ Cl ₆	25.819	1.62	0.71	55.97±1.6
CB-126 (3,3',4,4',5)	C ₁₂ H ₅ Cl ₅	26.658	2.75	00.00	100±0.00
CB-128 (2,2',3,3',4,4')	C ₁₂ H ₄ Cl ₆	27.702	5.02	1.79	64.33±2.3
CB-181 (2,2',3,4,4',5,6)	C ₁₂ H ₃ Cl ₇	29.219	2.87	00.00	100±0.00
CB-180 (2,2',3,4,4',5,5')	C ₁₂ H ₃ Cl ₇	30.484	1.64	0.63	61.33±1.7

Table 3. Structural Characteristics of PCBs degraded by CH07

Chlorobiphenyls	Molecular Formula	Mol. Wt.	Cl (%)	Structures
CB-101 (2,2',4,5,5' - Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-119 (2,3',4,4',6-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-97 (2,2',3',4,5-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-116 (2,3,4,5,6-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-77 (3,3',4,4'-Tetrachloro)	$C_{12}H_6Cl_4$	220	64.54	

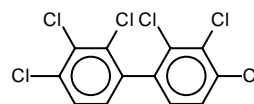
CB-151	$C_{12}H_4Cl_6$	289	73.70	
(2,2',3,5,5',6-Hexachloro)				
CB-118	$C_{12}H_5Cl_5$	254.5	69.74	
(2,3',4,4',5-Pentachloro)				
CB-105	$C_{12}H_5Cl_5$	254.5	69.74	
(2,3,3',4,4'-Pentachloro)				
CB-141	$C_{12}H_4Cl_6$	289	73.70	
(2,2',3,4,5,5'-Hexachloro)				
CB-138	$C_{12}H_4Cl_6$	289	73.70	
(2,2',3,4,4',5'-Hexachloro)				
CB-126	$C_{12}H_5Cl_5$	254.5	69.74	
(3,3',4,4',5-Pentachloro)				

CB-128

$C_{12}H_4Cl_6$

289

73.70



(2,2',3,3',4,4'-

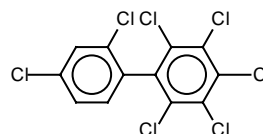
Hexachloro)

CB-181

$C_{12}H_3Cl_7$

323.5

76.81



2,2',3,4,4',5,6-

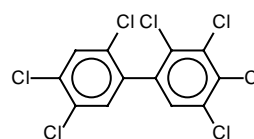
Heptachloro)

CB-180

$C_{12}H_3Cl_7$

323.5

76.81



(2,2',3,4,4',5,5'-

Heptachloro)

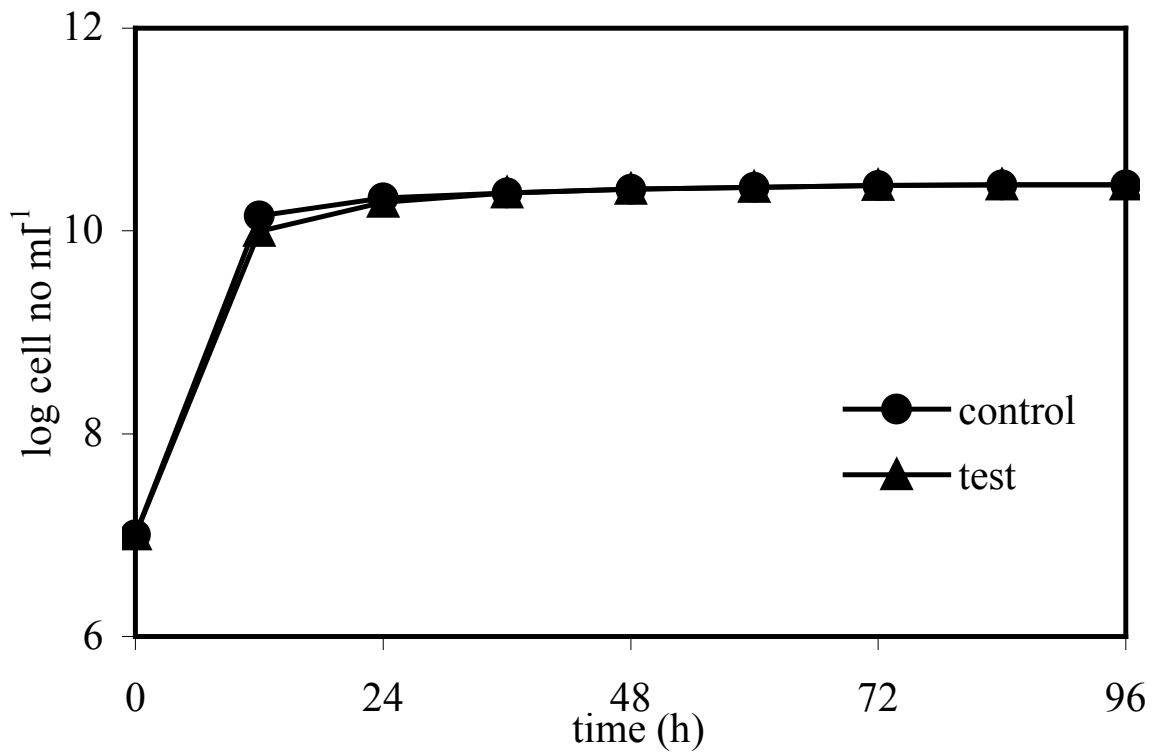
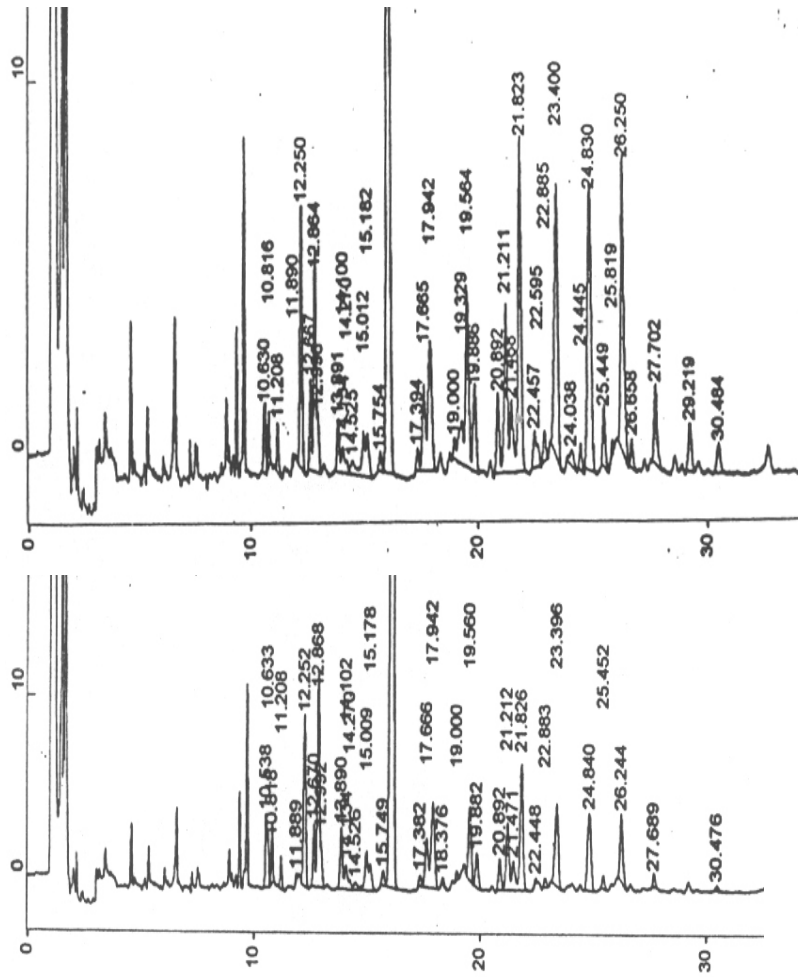


Figure 1. Growth curve of CH07 with Clophen A-50 (100 ppm) and without Clophen A-50 in 50% seawater nutrient broth.



“0” h

“40” h

Figure 2. Gas chromatograms showing PCB degradation (upper panel represents “0” h and lower one represent “40” h) by CH07.