ISOLATION OF LOCAL BACTERIAL CAPABLE OF DEGRADING HALOGENATED COMPOUNDS AND ANALYSIS OF PUTATIVE HALOACID PERMEASE GENE

NG HONG JING

A thesis submitted in fulfillment of the requirement for the award of the degree of Master of Science (Bioscience)

Faculty of Science
Universiti Teknologi Malaysia

FEBRUARY 2007

For Science

ACKNOWLEDGMENTS

I am deeply indebted to my advisor, Dr. Fahrul Zaman Huyop for his guidance and continuous encouragement throughout this project moving. He has been a mentor for my professional life and has helped make my stay at UTM an enjoyable one.

I would also like to express my sincere gratitude to my lab mates, lab assistants and fellow graduate student for their advice, encouragement, humor and friendship. Their professionalism made it a pleasure to work with them and without their help, it would be impossible for me to overcome the problems that occurred during this project.

My sincere appreciation especially extends to Ms. Wong Yun Yun for her valuable advices in statistical analysis. In addition, I would like to thanks all my colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. I am grateful to my entire family member as well.

ABSTRACT

3-chloropropionic acid and 2,2-dichloropropionic acid are synthetic halogenated compounds used in herbicide. A bacterium isolated from a soil sample and characterised as *Rhodococcus sp.* by 16S rRNA analysis, was able to degrade and utilised 3-chloropropionate as the sole source of carbon and energy. This was supported by the ability of the bacterium to grow on 20 mM 3-chloropropionate with a doubling time of 11.72 hours. The utilisation of 3-chloropropionate was also confirmed by detection of 3-chloropropionate depletion in the medium using HPLC. Cell free extract of *Rhodococcus sp.* had an enzyme specific activity of 0.013 μmol Cl^-/min/mg protein towards 3-chloropropionate. Another bacterium isolated from the same soil sample and identified as *Methylobacterium sp.* by 16S rRNA analysis was found to be able to degrade 2,2-dichloropropionate. The bacterium grew in 20mM 2,2-dichloropropionate minimal medium with a doubling time of 20.32 hours. Degradation of 2,2-dichloropropionate was further confirmed by detection of 2,2-dichloropropionate depletion in growth medium by HPLC. Cell free extract prepared from the cell showed 0.039 μmol Cl^-/min/mg protein specific activity towards 2,2-dichloropropionate. A putative haloacid permease gene (*dehrP*) from *Rhizobium sp*. was subcloned into Novagen pET 43.1a plasmid. The newly constructed plasmid was designated as pHJ. The cloned gene was sequenced and analysed using various online analysis tools. DehrP has a calculated molecular weight of 45 kDa and an isoelectric point of 9.78. The nucleotide sequence of *dehrP* showed significant homology (86%) with the putative mono-chloropropionic acid permease from *Agrobacterium sp*. NHG3 and 62 % homology with the haloacid specific transferase from *Burkholderia sp*.

ABSTRAK

Asid 3-kloropropionik dan asid 2,2-dikloropropionik ialah sebatian halogen sintetik yang terkandung dalam racun rumpai. Bakteria yang disaring dari sampel tanah dikenal pasti sebagai *Rhodococcus sp.* melalui kaedah analisa 16S rRNA mampu mengurai 3-kloropropionat sebagai sumber karbon dan tenaga. Ini disokong oleh kemampuan bakteria untuk tumbuh dalam 20 mM media 3-kloropropionat dengan masa penggandaan 11.72 jam. Penguraian 3-kloropropionat juga disokong oleh analisa kultur menggunakan HPLC. Ekstrak dari *Rhodococcus sp.* menunjukkan aktiviti enzim spesifik sebanyak 0.013 μmol Cl^-/min/mg protein terhadap 3-kloropropionat. Bakteria lain yang disaring dari sampel tanah yang sama telah dikenalpasti sebagai *Methylobacterium sp.* dengan menggunakan analisa 16S rRNA. Bakteria tersebut mempunyai keupayaan untuk mengurai 2,2-dikloropropionat. Bakteria itu tumbuh dalam 20 mM media minimal 2,2-dikloropropionat dengan masa penggandaan dua sebanyak 20.32 jam. Kebolehan *Methylobacterium sp.* untuk mengurai 2,2-dikloropropionat juga disokong oleh analisa media kultur dengan menggunakan HPLC. Ekstrak sel menunjukkan aktiviti spesifik enzim sebanyak 0.039 μmol Cl^-/min/mg protein terhadap 2,2-dikloropropionat. Gen putatif haloasid permease dari *Rhizobium sp*. telah disubklon ke dalam plasmid pET 43.1a Novagen. Plasmid itu dinamakan pHJ. Penjujukan gen yang telah diklonkan itu telah dikenalpasti dan dianalisa dengan menggunakan pelbagai perisian komputer secara terus menerus. Protein DehrP mempunyai pengiraan berat molekul 45 kDa dan tahap isoelektrik 9.78. Jujukan nukleotida gen *dehrP* telah menunjukkan persamaan homolog yang ketara (86%) dengan gen putatif asid monokloropropionik permease dari *Agrobacterium sp.* NHG3 dan 62% jujukan homologi dengan gen haloasid spesifik transferase dari *Burkholderia sp*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,2-DCP	-	2,2-dichloropropionic acid
3-CP	-	3-chloropropionic acid
A	-	Absorbance
BLAST	-	Basic local alignment search tool
DDBJ	-	DNA Data Bank of Japan
DNA	-	Deoxyribonucleic acid
E.coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetraaceticacid, $(HOOCCH_2)_2N(CH_2)_2N(CH_2COOH)_2$
EMBL	-	European Molecular Biology Laboratory
EtBr	-	Ethidium Bromide
H	-	Hour
kb	-	Kilo base
kDA	-	Kilo Dalton
min	-	Minutes
NCBI	-	National Center for Biotechnology Information
OD	-	Optical Density
PCR	-	Polymerase chain reaction
RDP	-	Ribosomal database project
RNA	-	Ribonucleic acid
rDNA	-	Ribosomal DNA
rRNA	-	Ribosomal RNA
s	-	Second
TAE	-	Tris-Acetate-EDTA

TMHMM	-	Transmembrane helices Markov model
UV	-	Ultraviole

DNA BASES:

A	-	Adenine
C	-	Cytosine
G	-	Guanine
T	-	Thymine
N	-	Any base; A or C or G or T
M	-	Amino; represented by either A or C
Y	-	Pyrimidine; represented by either C or T
W	-	Molecule with weak interaction; represented by either A or T
R	-	Purine; represented by either G or A

LIST OF APPENDICES

CHAPTER I

INTRODUCTION

1.1 Xenobiotics as Pollutants

Environmental pollution, caused by increased levels of industrial chemicals, manufacturing wastes and biocides in water and on land, poses a considerable problem for society. Consequently, this has led to major research programs, which have studied the fate of these chemicals in the natural environment (Ejlertsson *et al.,* 1996; Golovleva *et al.,* 1990; Higson, 1991**;** Javorekova *et al.,* 2001; Rozgaj, 1994) and in bioreactor (Baumann *et al.,* 2005; Daugulis and MacCracken, 2003).

Chemical compounds found in the environment can arbitrarily be classified into groups, according to those which are natural products and those that are foreign to the biosphere (Hutzinger and Veerkamp, 1981). Xenobiotic (Greek, xenos "foreign"; bios "life") compounds may be defined as those having structural moieties or groups, which are not found in natural products, and normally arise as a direct result of man's industrial activities.

Transient or permanent contamination by xenobiotic disrupts the normal functioning of the biosphere, which is typically controlled by equilibrated cycles of organic and inorganic matter by plants, animals, and microorganisms. Modern

agriculture methods result in widespread release of novel chemical particularly herbicides and pesticides. Such biocides are directly and deliberately applied into the environment for crop protection, while other industrial lipopholic chemicals are locked for tens of years in closed systems, are susceptible to biomagnifications through food chains, resulting in increased environmental distribution by certain organisms (Atlas, 1996). Table 1.1 provides some general information on world and U.S. pesticide expenditures in year 2000. Table 1.2 shows the most commonly used conventional pesticide active ingredients. Most of them contain halogenated compound.

Pollutants might enter the environment by numerous routes such as illegal dumping from industrial waste, effluent leakages, leaching and from chemical evaporation. The quantity of release is not always sufficient to gauge pollution problems, and properties such as toxicity, carcinogenicity, biomagnifications and persistence must all be considered to appreciate fully the extent of environmental damage.

Table 1.1: World and U.S. pesticide expenditures by pesticide type, year 2000 (Kiely *et al.,* 2004)

Year	**World Market**		**U.S. Market**		**U.S. Percentage of World Market**
Type	Mil $	%	Mil $	%	%
Herbicides	14,319	44	6,365	57	44
Insecticides	9,102	28	3,129	28	34
Fungicides	6,384	19	860	8	13
Other	2,964	9	811	7	27
Total	32,769	100	11,165	100	34

Note:

- Totals may not add due to rounding. Table does not cover wood preservatives and specialty biocides.
- "Herbicides" include herbicides and plant growth regulators.
- "Insecticides" and "fungicides" exclude sulfur and petroleum oil.
- "Other" includes nematicides, fumigants, rodenticides, molluscicides, aquatic and fish/bird pesticides, other miscellaneous conventional pesticides, plus other chemicals used as pesticides (e.g., sulfur and petroleum oil).

Table 1.2: Most commonly used pesticide active ingredients in agricultural market (Ranked by range in millions of pounds of active ingredient) (Kiely *et al.*, 2004)

Active Ingredient	2001		1999		1997		1987	
	Rank	Range	Rank	Range	Rank	Range	Rank	Range
Glyphosate	1	85-90	2	67-73	5	34-38	17	6-8
Atrazine	2	74-80	1	74-80	1	75-82	1	71-76
Metam Sodium	3	57-62	3	60-64	3	53-58	15	5-8
Acetochlor	4	30-35	4	30-35	7	31-36	NA	NA
2,4-D	5	28-33	6	28-33	8	29-33	5	29-33
Malathion	6	20-25	7	28-32	NA	NA	NA	NA
Methyl Bromide	7	20-25	5	28-33	4	38-45	NA	NA
Dichloropropene	8	20-25	11	17-20	6	32-37	4	30-35
Metolachlor-s	9	20-24	12	16-19	NA	NA	NA	NA
Metolachlor	10	15-22	8	26-30	2	63-69	3	45-50
Pendimethalin	11	15-19	10	17-22	9	24-28	10	10-13
Trifluralin	12	12-16	9	18-23	10	21-25	6	25-30
Chlorothalonil	13	8-11	13	9-11	15	7-10	19	5-7
Copper Hydroxide	14	8-10	15	8-10	13	10-13	19	5-7
Chlorpyrifos	15	8-10	16	8-10	14	9-13	14	6-9
Alachlor	16	6-9	17	7-10	12	13-16	2	55-60
Propanil	17	6-9	18	7-10	22	6-8	13	7-10
Chloropicrin	18	5-9	14	8-10	25	5-6	NA	NA
Dimethenamid	19	6-8	20	6-8	20	6-9	NA	NA
Mancozeb	20	6-8	21	6-8	17	7-10	21	4-6
Ethephon	21	5-8	24	5-6	NA	NA	NA	NA
EPTC	22	5-8	19	7-9	18	7-10	8	17-21
Simazine	23	5-7	NA	NA	NA	NA	NA	NA
Dicamba	24	5-7	22	6-8	16	7-10	23	4-6
Sulfosate	25	3-7	NA	NA	NA	NA	NA	NA

1.2 Halogenated Compounds in the Biosphere

Halo-aliphatic compounds such as 2,2-dichloropropionic acid and trichloroacetic acid are commonly used as herbicides. Halo-aromatic compounds can also be considered important for this purpose. 2,4,-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T), having generated much interest with respect to their degradation, and consequently have initiated many researchers to study their fate in the environment (Ghosal *et al.,* 1985; Han & New, 1994) and the mechanism of degradation at molecular level (Farhana *et al.,* 1998; Kitagawa *et al.,* 2002). Nevertheless, halo-organic compounds such as polychlorinated phenols, polychlorinated biphenyls (PCB's), chlorinated benzoates and various insecticides, e.g. methoxychlor, aldrin, lindane and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), also contribute to environmental contamination (Ritter *et al.,* 1995).

This leads us to believe that synthetic halo-organic compounds are undoubtedly a problem of major concern as listed in Table 1.3. However, naturally occurring halogenated compounds are not uncommon. This is demonstrated by the relative abundances of halogens as inorganic salts or minerals in soil and fresh water environments: CL > F > Br > I, with the reversal of F and Br in sea water, where organic fluorine is more limited, and associated with certain plants (Fetzner, 1998). Gribble (1994) have listed more than 2000 halo-organic compounds as natural products, which are released into biosphere by various marine organisms, higher plants and ferns, insects, bacteria, fungi, and mammals. In addition, some bromo and iodo chloralkane analogues have been reported as occurring naturally (Gschwend *et al.,* 1985), and due to their chemical and analytical simplicity can be regarded as models for biodegradation studies of more highly halogenated alkanes.

Table 1.3: Annual industrial production volumes and the use of industrially important chlorinated hydrocarbons (Fetzner, 1998)

Hydrocarbon	**U.S production (x 10^3 tones)**	**Year**	**Some of its major uses**
Monochloromethane	390	1992	Production of silicones, tetramethyllead, methycellulose
Dichloromethane	162	1992	Degreasing agent; paint remover, extraction technology
Trichloromethane	229	1991	Production of monochlorodifluorimethane
Tetrachloromethane	143	1991	Act as solvent. production of trichloromonofluoromethane
Monochloroethane	67	1990	Production of tetramethyllead, ethycellulose
1,1-dichloroethane	220-250	1985	Feedstock for production of 1,1,1-trichloroethane
1,2-dichloroethane	7230	1992	Production of vinyl chloride, ethylenediamines
1,1,1-Trichloroethane	327	1992	Dry cleaning; capor degreasing
1,1,2-Trichloroethane	200-220	1984	Intermediate for production of 1,1,1- trichloroethane
Monochloroethene (vinyl chloride)	6000	1992	Production of PVC
1,1-dichloroethene (vinylidane chloride)	150-200	1986	Basic material for poly(vinylidane chloride)
Trichloroethene	110	1984	Dry cleaning, metal degreasing
Tetrachloroethene	110	1992	Dry cleaning, metal degreasing
2-chloro-1,3-butadiene (chloroprene)	648	1983	Basic material for poly(chloroprene) rubber

1.3 Usage of Halogenated Compound in Malaysia as Pesticide

Organochlorine pesticides are known for their environmental persistence and global concerns. The half-life of most organochlorine pesticides can range from several years to more than 10 years (Ritter *et al.,* 1995). There are also toxic due to their high capacities for bioaccumulation that poses a threat to the ecosystem and human health. However, in Malaysia, the legislation, monitoring and control of halogenated compound usage are still lacking. Not much information concerning halogenated compound in Malaysia had been gathered.

Some countries promote monitoring programs and stepwise evaluation process that seek to identify and minimise risk caused by accumulation of pesticide in general and halogenated compound in particular to human, wildlife and the environment. In the United States of America, environmental pollution in aquatic ecosystems is still monitored today. Some highly toxic organochloride pesticides are still detected due to their persistency in the environment even after almost 30 years of banning. Contamination in aquatic ecosystems that causes various harmful effects on human and wildlife have been well studied especially in North America, Japan and many parts of Europe.

In Malaysia, the input pathways of organochlorine compound into the river environment originate from domestic sewage discharge, industry wastewater, runoff from non-point sources such as from agricultural area and direct dumping of wastes into the river (Leong *et al.,* 2002). Owning to the persistency and bioaccumulation of organochlorine pesticides; a different group of pesticide namely organophosphate is being widely used in agriculture to replace the previous group of pesticide because organophosphate pesticides are less toxic and easily degradable. The transformation of organophosphate group in the environment takes place by conversion of the phosphorothioate (P=S) group to their oxon (P=O) analogues. Both of these compounds have strong inhibition of acetylcholonesterase activity, an enzyme that is involved in neural function. The long-term application of these pesticides will pose a chronic risk to health (Leong *et al.,* 2002).

In view of the hazardous characteristics of these compounds, a number of them had been listed by the UNEP as persistent organic pollutants (POPs). In Malaysia, the usage of organochlorine pesticides listed as POPs are either prohibited or restricted. For example, aldrin, dieldrin, DDT and chlordane were all restricted in usage but after 1998, their usage had been discontinued. Heptachlor, hexachlorobenzene, mirex, toxaphene and endrin are never registered for use. Even though the usage of most POPs-listed organochlorine pesticides are prohibited, studies on rivers and sediments throughout Malaysia demonstrated that most of these compounds are present in the aquatic environment (Cheah and Lum, 1994; Leong *et al.,* 2002). However, detailed information is not available since not much study in this area was conducted in Malaysia. In most of these studies, the sources of the contamination were unknown. Table 1.4 shows some status of common pesticide in Malaysia.

1.4 Pollution Caused by Halogenated Compound in Malaysia

The monitoring of several different pesticides in the Selangor River, Malaysia had been done under UNU Project on EDC Pollution in the East Asian Coastal Hydrosphere (Leong *et al.,* 2002). According to the author, Selangor River was chosen as a water sampling area for the UNU hydrosphere project for many reasons. Firstly, it is a main source of water supply to many agricultural activities such as vegetable farms, oil palm and rubber plantation. Furthermore it is also used to support aquaculture activities such as the cultivation of fresh water fishes for human consumption. Further down stream of this river is an ecotourism area for the observation and study of fireflies which is somehow dwindling with time and human interference. Selangor River is also a source of water for most of the residents living along and will soon be a major source of water to part of Kuala Lumpur city after building of a dam is being carried out at the upper stream of the river.

Table 1.4: Common pesticides in Malaysia

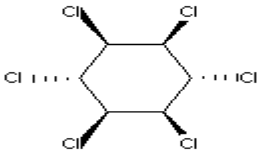
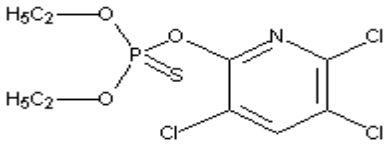
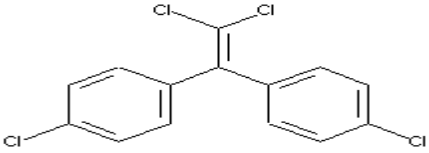
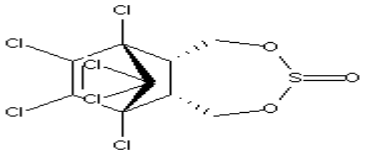
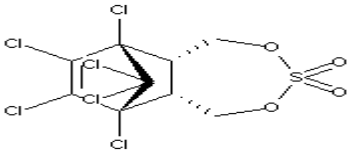
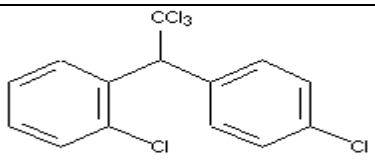
CAS Number	Chemical	Registered for Use	Banned or Restricted
93-76-5	2,4,5-T	No	
309-00-2	Aldrin		Banned
1336-36-3, 11097-69-1	Aroclor		
1332-21-4, 12001-28-4, 77536-66-4, 77536-67-5, 77536-68-6	Asbestos		
485-31-4	Binapacryl		Banned
2939-80-2, 2425-06-1	Captafol (cis isomer)		Banned
57-74-9, 12789-03-6, 5103-71-9, 5103-74-2	Chlordane		Banned
6164-98-3	Chlordimeform		Banned
510-15-6	Chlorobenzilate	No	
50-29-3, 789-02-6	DDT		Banned
60-57-1	Dieldrin		Banned
x88-85-7	Dinoseb and dinoseb salts		Banned
106-93-4	Ethylene dibromide		Banned
107-06-2	Ethylene dichloride		Banned
75-21-8	Ethylene oxide		Banned
640-19-7	Fluoroacetamide		Banned
133-07-3	Folpet	No	Banned
76-44-8	Heptachlor		Banned
118-74-1	Hexachlorobenzene	No	
608-73-1, 319-86-8	Hexachlorocyclohexane		Banned
58-89-9	Lindane	Yes	

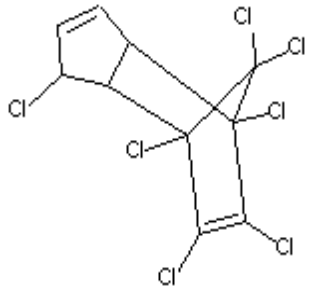
x7439-97-6	Mercury and mercury compounds		Banned
10265-92-6	Methamidophos	Yes	Restricted
298-00-0	Methyl parathion	No	
6923-22-4	Monocrotophos	Yes	Restricted
56-38-2	Parathion	No	
87-86-5	PCP		Banned
13171-21-6	Phosphamidon	No	
61788-33-8	Polychlorinated terphenyls		
8001-35-2	Toxaphene		Banned

* The only data available for Malaysia is the information on banned and severely restricted chemicals provided by the United Nations Prior Informed Consent (PIC) Circulars. (PIC Circular XII, 2000; PIC Circular XIV, 2001)

Pesticides that were listed in Table 1.5 are halogenated pesticides, for example lindane, chlorpyrifos, DDE, endosulfan, endosulfan sulfate, o,p'-DDT and heptachlor whereas fenitrothion, malathion and diazinon are organophosphate. All of the halogenated pesticides listed here could be detected in Selangor River. Lindane is most heavily used by the plantation operators and farmers around the Selangor District. The presences of halogenated pesticides in river waters may impair the river beneficial uses and their biological resources. In view of that, knowledge in degradation of halogenated pesticide is important to protect the environment.

Table 1.5: Halogenated pesticides detected in Selangor River (Leong *et al.,* 2002)

Structure Drawing	**Compound Name**	**IUPAC Name**
	Lindane	1,2,3,4,5,6-hexachlorocyclohexane
	chloropyrifos	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
	p,p'-DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene
	endosulfan	6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide
	endosulfan sulfate	6,7,8,9,10,10-hexachloro-6,9-methano-2,4,3-benzodioxathiepin-3
	o,p'-DDT	2-(2-chlorophenyl)-2(4-chlorophenyl)-1,1,1-trichloroethane

	heptachlor	1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoinden

1.5 Microbial Degradation of Halogenated Compound

Microorganisms are the primary agents of biological recycling, and have evolved an extensive range of enzymes, pathways and control mechanisms in order to degrade and utilise pollutants as an energy sources (Madigan *et al.,* 2000, Talaro and Talaro, 2002). No single organism possesses the mechanisms for the biodegradation of every compound, and evidence from laboratory based studies clearly shows that the involvement of microorganisms in the transformation of halogenated xenobiotics is an important factor in determining the fate of these compounds in the environment (Talaro and Talaro, 2002). Microorganisms are not solely responsible for degradation of organic compounds, as many other organisms do participate albeit to a smaller extent. Photochemical decomposition has been suggested as a significant route by which some compounds are degraded (Zabik *et al.,* 1976; Konstantinou *et al.,* 1999; Hirahara *et al.,* 2001).

All organic compounds in existence are thought to be thermodynamically unstable to varying degrees, and in principle, can be mineralised by microorganisms to generate CO_2 and energy for microbial growth. In the kinetic sense however, most organic compounds are perfectly stable, and under physiological conditions in the absence of catalysis, they will not be degraded or mineralised at significant rates. Such catalysis using enzymes are common in aerobic soil microorganisms seeking to obtain energy for growth.

The general requirement for biodegradation may be summarised as follow:

1. **Accessibility of a compound to microorganisms.**

Organic compounds may be adsorbed to particular matter in the soil (e.g. clays), thereby preventing potential microbial attack. Similarly, chemical complexing to other molecules leads to the same consequence.

2. **Entry of a compound into an organism.**

The lack of penetration, coupled with the absence of suitable extra-cellular enzymes may result in a compound being resistant to biological attack. Specific transport mechanisms have evolved to facilitate the entry of naturally occurring compounds. However, on initial exposure to many unnatural halogenated molecules the uptake mechanisms are unlikely to function.

3. **Induction of catabolic enzymes.**

The compound must induce the synthesis of, and act as substrate for degradative enzymes (assuming that the enzymes are not produced constitutively). Xenobiotic halo-organic compounds are quite frequently unable to be utilised as substrates for microbial growth, as they are too far removed from the main stream of catabolic pathways. Consequently, a xenobiotic compound is unable to be converted to an intermediate that can be mineralised further in an existing biochemical pathway. Halogen substitution sufficiently alters a molecule's structure so as to reduce the rate of its transformation, and possible prevent its metabolism. In the instance, the fate of xenobiotics is to some extent determined by the degree of structural analogy between the synthetic compound and a natural compound for which catabolic function exist. Such structural analogies include comparable reactivities, together with analogous size and polarity of functional groups (Hughes, 1988).

4. **Aerobic or anaerobic environments.**

Anoxic/aerobic environments can accumulate some compounds which would otherwise be degraded under different condition (Alexander, 1981).

Three main biodegradative mechanisms have been reported for halo-organo compounds. Ultimately, complete degradation or mineralisation of a compound is required, so as to prevent or reduce its persistence in the environment. These involve complete breakdown of an organic compound into an inorganic state, and subsequently the conversion of the carbon-skeleton into intermediary metabolites.

A second mechanism constitutes partial degradation, which is clearly demonstrated by halo-aromatic compounds. For example, PCB's are composed of

aromatic ring pairs which may possess one unsubstituted and a chloro-substituted ring. A single organism can sometimes use the unsubstituted nucleus as the growth substrate, while the halo-substituted ring will be excreted into the culture fluid as an organic end-product.

The third mechanism type is co-metabolism (Alexander, 1981; Baggi *et al*., 2005) as the microbial action that modifies the structure of a chemical, without deriving energy from the catabolism for microbial growth. The population involved in co-metabolism is assumed to grow on other substrate while performing the transformation and the lack of increase in population biomass is reflected by the inability of the co-metabolising microorganisms to utilise the chemical for biosynthetic purposes.

Co-metabolism of halo-organic compounds does not result in complete mineralisation to inorganic halide, CO_2 and H_2O, but it does seemingly reduce toxicity in the environment, which indicates the ecological importance of this phenomenon. It has been suggested that co-metabolism may account for the degradation of many pesticides which do not sustain microbial growth (Alexander, 1981). Co-metabolism and degradation of compound such as 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (DDT) and related molecules have been studied extensively (Bumpus and Aust, 1987; Aislabie *et al.,* 1997; Quensen *et al.,* 1998). In some cases, although complete mineralisation did not occur, the toxic effect of the insecticide in the environment was reduced. The chemical alteration were however unable to guarantee its complete detoxification.

In view of the large number of synthetic chemicals that are apparently co-metabolised, establishing a physiological explanation is quite important. The most likely hypothesis is related to enzyme specificity. Many enzymes present in microbial cells catalyse reactions involving several different but chemically related substrates and are enzymes of broad substrate specificities. Products that are formed from such chemically related compounds may not subsequently be altered by these enzymes, and consequently accumulated, preventing further mineralisation and generation of energy for microbial growth. This suggests that the toxicity of a compound is somewhat reduced by co-metabolism but not eliminated.

Very often the term recalcitrance is used for the many xenobiotics that endure for long periods in natural ecosystems in chemically unchanged states, owing to the inability of microorganisms to degrade them. Halogenated compound, especially polychlorinated types are generally degraded slowly and regarded as recalcitrant molecules. This is a phenomenon discussed by many authors since considerations about the fates of these recalcitrant compounds by biological, chemical and physical agents are extremely important in understanding persisting toxological effects in the biosphere (Barriault *et al.,* 1998; Kim and Picapdal 2001; Araoz and Viale, 2004; Xu *et al.,* 2004,).

1.6 Microbial Degradation of Halogenated Compound in Mixed Population

Xenobiotics are supplied as growth-limiting carbon sources in chemostat enrichments, and it is not uncommon to isolate mixed populations. Situations arise where primary utilisers obtain energy directly from the growth-limiting carbon-source and the secondary utilisers benefit from the breakdown products of the primary utilisers. The struggle for existence within a growth-limiting system is therefore dependent on competition, a phenomenon that may explain the enormous diversity of the microbial forms and species in the case of evolution. Competition however is not the only interaction possible between organisms, particularly in the case of xenobiotics, where various other forms have been observed. The interactions listed in Table 1.6 should be considered as simplified definitions, as it is likely that many microbial interactions in nature are mixtures of two or more of these extremes that contribute to the establishment of integrated microbial communities (Hughes, 1988).

.

Table 1.6: Various form of microbial interactions (Talaro and Talaro, 2002)

Interaction	Definition
Neutralism	Lack of interaction
Competition	A race for nutrients and space
Mutualism	Organisms live in an obligatory but mutually beneficial relationship.
Commensalisms	The commensal benefits; other member not harmed.
Parasitism	Parasite is dependent and benefits, host harmed.
Synergism	Members cooperate and share nutrients.
Antagonism	Some members are inhibited or destroyed by others.

In halogenated substrate degradation, mutualism and commensalisms are the 2 main interacting systems to be considered, where a dependent community population benefits from the presence of a second population. For example, mutualism was observed in a soil microbial community isolated by Jensen (1957a) growing on trichloroacetate. The community consisted of a primary trichloroacetate -utilising bacterium, designated strain 3-Cl, and two *Streptomyces* species. The latter organisms provided Vitamin B_{12} which was essential for bacterium strain 3-Cl (Figure 1.1). In this case the *Streptomyces sp*. did not appear to benefit directly from the presence of the bacterium strain 3-Cl. On the basis of these observations a stable community was formed which was capable of metabolising trichloroacetate.

A complex microbial community, utilising 2,2-dichloropropionate (Dalapon) as the growth-limiting substrate was isolated and studied by Senior *et al.* (1976), who suggested that commensalisms was responsible for the success of the stable community. Seven organisms were originally characterised, three of which were primary utilisers able to grow on 2,2-dichloropropionate in pure culture, while the remainder were secondary utilisers, unable to utilise the herbicide as a carbon and energy source (Figure 1.2). The community was very stable and was maintained in continuous-flow culture over 18,000 h (25 months). Initially, the three primary organisms, *Pseudomonas sp*. strain P1; Bacterium strain P2 and *Trichoderma viride* strain P4, co-existed under conditions where they were competing for the limiting carbon-source, indicating that they were interacting in such a way so as to stabilise the community, and thus exhibit a form of concerted metabolism.

However, the mixed culture showed a change in structure which was possibly very significant in natural environments. After prolonged continuous cultivation the carbon-limited nature of the culture conditions thus exerted a strong selective pressure for evolving the capabilities of one of the secondary organisms, PP1, to utilise the growth-limiting carbon source (Figure 1.2). From subsequent studies, it was revealed that elevated dehalogenase activity of this new primary utiliser, PP3 evolved under varying growth conditions (Weightman *et al.,* 1979).

Senior *et al.* (1976) showed that a community structure produces a far more stable catabolising population because environmental fluctuations appeared to have

little effect on substrate metabolism. They disclosed that changes in pH of the culture caused marked changes in the dominant 2,2-dichloropropionate utilising population, reflecting their varying capabilities to grow at different pH values. As a multi specific unit however, all the primary population were retained within the growth vessel, whatever the pH of the culture medium.

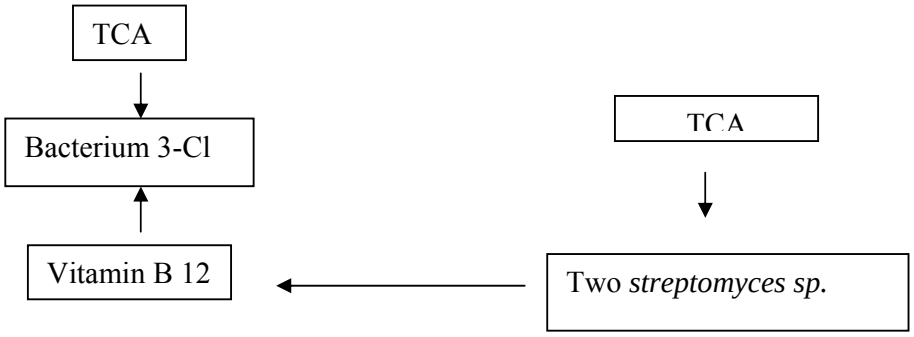


Figure 1.1: A three-member microbial community growing on TCA (Jensen, 1957a)

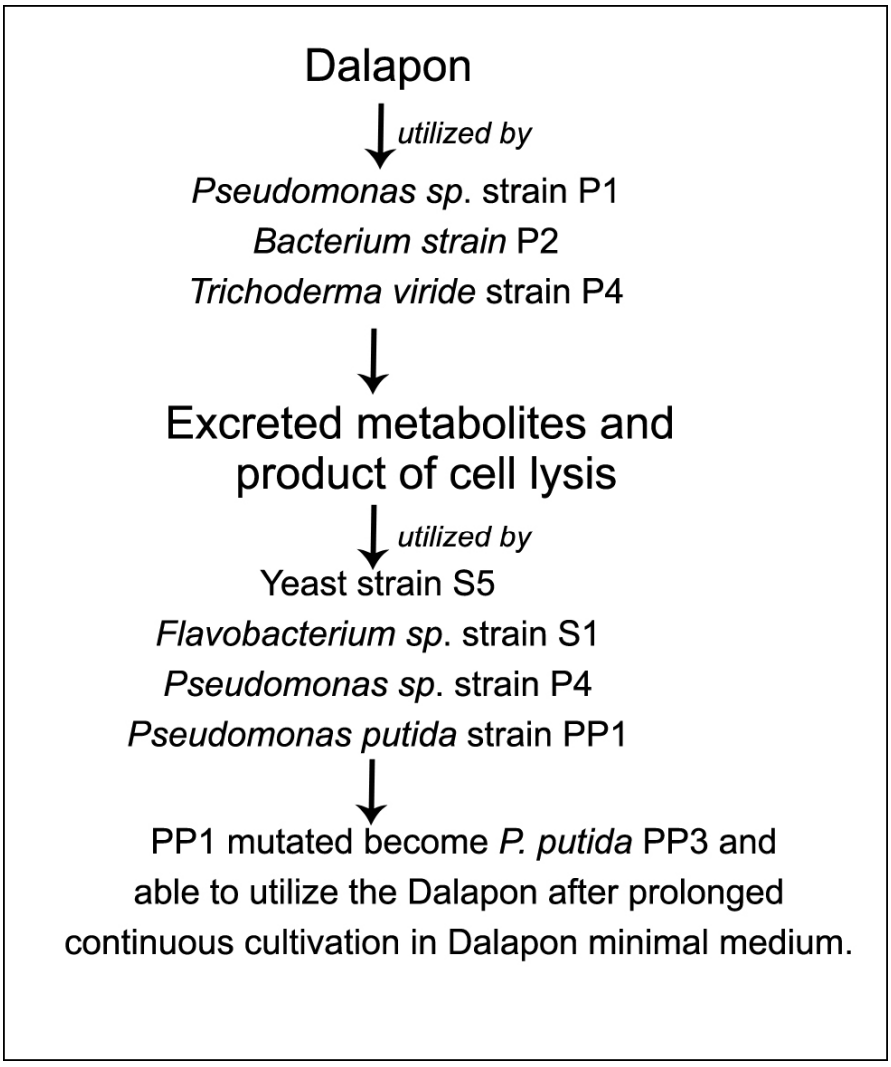


Figure 1.2: A seven member microbial community growing on 2,2-dichloropropionate (Dalapon) continuous culture (Senior *et al*., 1976)

1.7 The Chemistry of Halogenated Compound

The relationship between chemical structure and biological activity is very complex and may be one contributory factor as to why certain halogenated compounds remain recalcitrant. As discussed by Slater *et al*. (1995), the removal of halogens, particularly fluorine and chlorine, from organic molecules has fascinated chemists and microbiologists for many years since such mechanisms relieve inhibitory effect and provide alternative carbon and energy sources for growth. Generally, the greater the number of halogens per organic molecule, the more difficult it is to be degraded by microbes either alone or in consort as part of a microbial community (Commandeur and Parsons, 1990).

Halogenated compounds have been shown to function as ideal pesticides. Leasure (1964) notes how chlorinated substrates are best suited for herbicidal activity over the brominated, fluorinated and iodinated compounds. Within the propionic acid and butyric acid homologous, α-chlorination results in the highest herbicidal activity, trichloromethane, monochloroacetate (MCA) and dichloroacetate (DCA) do not however result in activity, and in combination with α-chlorinated compounds may weaken the desired effect hoped to be achieved. Increased chain length also seems to reduce activity of even highly biocidal α-substituted compounds, this is demonstrated by a weakly active 2,2-dichloropentanoic acid and an inactive 2,2-dichlorohexanoic acid.

An additional factor which may render halo-organo compounds desirable for biocidal action is the acidic properties associated with halogen when it is bound to the parent molecule. The relative strength of an acid is indicated by acid-dissociation constant (K_a). The stronger the acid, its dissociation constant are greater. Due to a wide range of spanning, K_a is often expressed on a logarithmic scale (Figure 1.3). Therefore, stronger acids would have smaller values of pK_a (Wade, 2003; Solomons, 1994).

$$pK_a = - \log K_a$$

Figure 1.3: Calculation of pK_a

The more highly substituted analogous aliphatic and aromatic compounds proving the stronger acids, which are thought to be attributable to the strong electronegativity of halogen substituents. Acidity of halo-organo compounds is also affected by substitution position, and as indicated by p*Ka* values, the further the halogen substituent is placed away from the –COOH group, the weaker the acid (Figure 1.4). This is a phenomenon termed "inductive effect". The inductive effect is obvious if one or more strongly electron-withdrawing groups are present on the carbon atom. The magnitude of a substituent effect depends on its distance from the carboxylic acid, whereas more distant substituents have smaller effects on acidity, showing that inductive effects decrease rapidly with distance (Wade, 2003).

In a halogenated carboxylic acid, the halogen atom is bonded to a sp^3 hybrid carbon atom (Wade, 2003; Solomons, 1994). The arrangement of groups around the carbon atom is generally tetrahedral (Solomons, 1994). The halogen atoms are more electronegative than carbon; the carbon-halogen bond is polarised with a partial positive charge on carbon and a partial negative charge on the halogen, as shown in Figure 1.5 (Wade, 2003; Solomons, 1994).

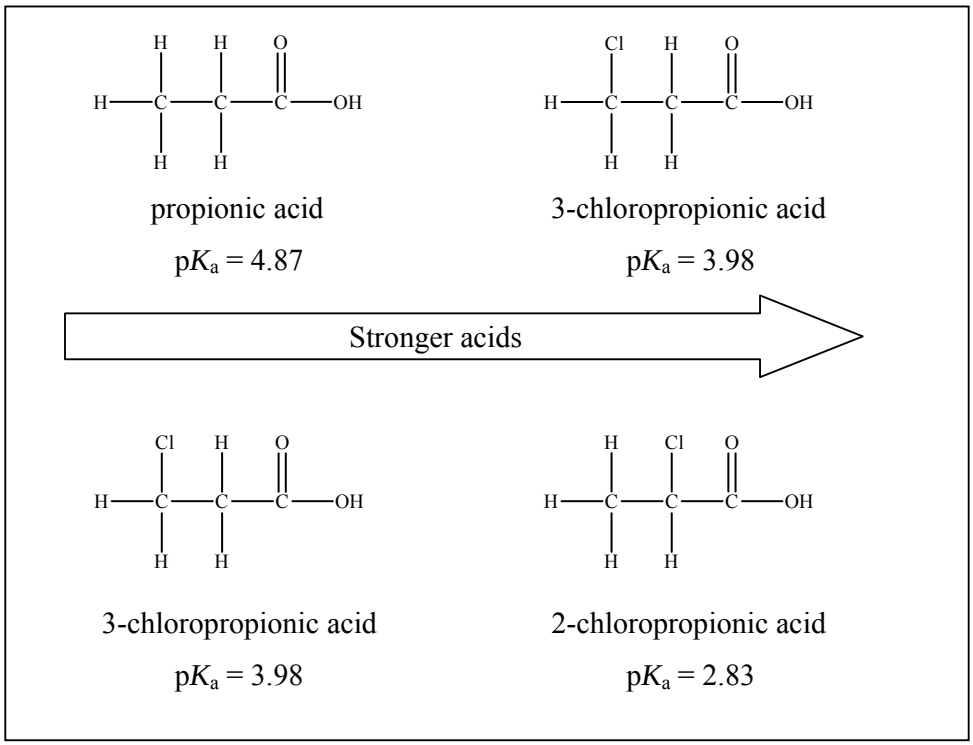


Figure 1.4: Substituent effects on acidity

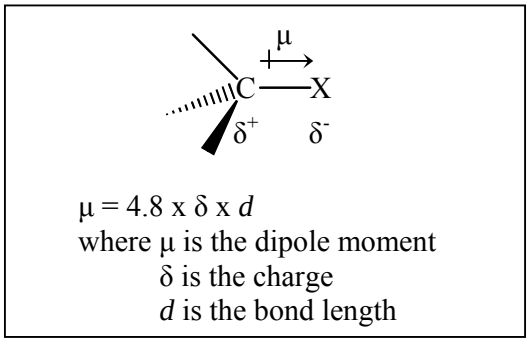


Figure 1.5: Polarised carbon-halogen bond

As go down the periodic table, the size of the halogen atom increases. Due to the increases in size, the electron affinity of the atom decreases (Umland and Bellama, 1999) thus causes the electronegativity of the halogen decrease as going down the periodic table. When the atomic radii of halogen atoms become larger, the carbon-halogen bond lengths increase. These two effects oppose each other, with the larger halogens having longer bonds but weaker electronegativities (Table 1.7). Therefore, the bond dipole moments decrease as go down the periodic table (Wade, 2003; Solomons, 1994).

The strength of the carbon-halogen bond is the important factor in determine the acidity (Wade, 2003; Solomons, 1994). A more electronegative element gives a stronger carbon-halogen bond molecule. The stronger the bond results in the weaker the acid (Solomons, 1994). The acidic properties of such halo-organo compounds may also be one factor which contributes to the prevention of microbial degradation, as acidic environments do not characteristically support extensive growth of microorganisms and are as a result associated with a chemical's persistence.

Table 1.7: Electronegativity, bond length and dipole moment

	I	<	Br	<	Cl	<	F
electronegativity:	2.7		3.0		3.2		4.0
	C – F	<	C – Cl	<	C – Br	<	C – I
bond length :	1.38 Å	<	1.78 Å	<	1.94 Å	<	2.14 Å
	C – I	<	C – Br	<	C – F	<	C – Cl
dipole moment :	1.29 D	<	1.48 D	<	1.51 D	<	1.56 D

1.8 The Biochemistry of Dehalogenation

It was well accepted that the utilisation of halogenated substrate by microorganisms require enzymes of broad substrate specificities that can adapt to catalyse conversion of many substrates with the same basic structure. Halogenated substituents therefore, are very often removed by fortuitous reactions, the enzymes responsible being primarily associated with the metabolism of unsubstituted substrate analogues. Alternatively, specific dehalogenase enzymes are responsible for catalysing the removal of the halogen substituent from the substrate preferably at an early stage in catabolism.

The effect of halogen substitutions on the biodegradation of halo-aromatics have been discussed by Reineke (1984). Aerobic catabolism of halo-aromatics appears to closely resemble the pathways described for unsubstituted analogous. In addition, the biodegradation of halo-alkanes such as dichloromethane by bacteria is also well documented. The best investigated dichloromethane degrader at the molecular level is the pink pigmented *Methylobacterium dichloromethanicum* DM4 (Galli and Leisinger, 1985). Degradation of dichloromethane depends solely on the reaction catalysed by dichloromethane dehalogenase / glutathione *S*-transferase, a glutathione-dependent enzyme converting dichloromethane to formaldehyde and two molecules of hydrochloric acid. The postulated conversion pathway of dichloromethane to formaldehyde is shown in Figure 1.6. The latter compound can be oxidised to CO_2 for energy or assimilated into cellular organic molecules.

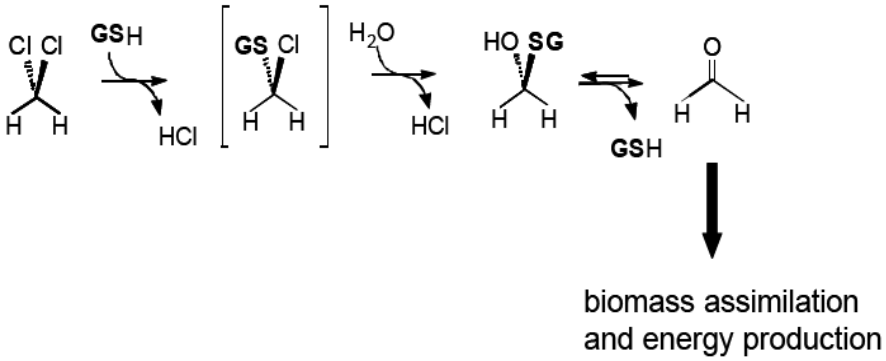


Figure 1.6: Schematic representation of the proposed reaction mechanism of DCM dehalogenase (Kayser, 2001)

1.8.1 Dehalogenation of Haloalkanoic Acids

Halo-alkanoates dehalogenases are responsible for the dehalogenation of both mono- and di-halogenated substrates yielding hydroxyl- and keto- acids respectively as products (Jensen, 1963). Early work by Jensen (1957a, 1957b and 1960) demonstrated that soil microorganisms able to metabolise chlorinated alkanoates (mainly acetates and propionates) as sole source of carbon and energy can be readily isolated. However, others have studied a broader range of substrates which fall within the scope of this study, and have suggested that a greater number of different bacterial species with dehalogenating capabilities can be isolated from environment which have previously been exposed to the halo-alkanoates. To date, only α-halo-alkanoates dehalogenases/halidohydrolases, responsible for dehalogenation reaction at the α-substituted position have been well characterised.

1.8.1.1 Dehalogenation of α-Halocarboxylic Acids (2,2-dichloropropionate)

Since 2,2-dichloropropionate is an active ingredient in herbicides, many reports has focused on the isolation of 2,2-dichloropropionate degrading bacteria. Jensen (1957a), using soil perfusion and enrichment technique, isolated five strains of *Pseudomonas sp*. which able to degrade 2,2-dichloropropionate and other halogenated substrate such as dichloroacetate and 2-chloropropionate.

Magee and Colmer (1959) isolated from soil six 2,2-dichloropropionate degrading bacteria with very similar properties and tentatively assigned them to either of both the genera *Agrobacterium* and *Alcaligens*. Using an enrichment technique, Hirsch and Alexander (1960) isolated five strains of *Norcadia* and three strains of *Pseudomonas* from soil which had been incubated with 2,2-dichloropropionate. All eight organisms readily decomposed the latter compound at a concentration of 0.1% (w/v) in basal medium, liberating free chloride ion.

Senior *et al*. (1976) used a continuous-flow culture enrichment procedure to isolate from soil a microbial community which could grow on 2,2-dichloropropionate as the sole source of carbon and energy. After three weeks of growth in the chemostat, seven different microorganisms were isolated from the community. These could be divided into primary and secondary 2,2-dichloropropionate utilisers (as describe in section 1.6 and Figure 1.2).

The dehalogenase system of *Pseudomonas putida* strain PP3, that was originally isolated from chemostat culture following selection on 2,2-dichloropropionate (Senior *et al.,* 1976) is now well studied. Strain PP3 produced two dehalogenases, DehI and DehII (Weightman *et al.,* 1979), which were encoded by genes of the group I and group II *deh* families, respectively (Hill *et al.,* 1999).

Similar to *P.putida* strain PP3, a *Rhizobium sp*. isolated by Allison (1981), produced two dehalogenase activities when grown in the presence of 2,2-dicholorpopionate. However, further investigation indicated that *Rhizobium sp.* produced three dehalogenases, collectively known as DehD, DehL and DehE. DehD and DehL are stereospecific for D-2-chloropropinate and L-2-chloropropionate respectively, whereas DehE can act on both D,L-2-chloropropionate and 2,2-dichloropropionate (Cairns *et al*., 1996; Stringfellow *et al*.,1997).

.

In addition, four bacterial strains identified as *Agrobacterium tumefaciens* RS4, *Agrobacterium tumefaciens* RS5, *Comamonas acidovarans* and *Alcaligenes xylosoxidans* were isolated from 2,2-dichlropropionate polluted soils (Schwarze *et al*., 1997). All bacterial strains expressed a single dehalogenase. Upon further biochemical characterisation, two different D,L-specific 2-haloalkanoic acid dehalogenase were described. The dehalogenases of these strains have been shown to be inducible and catalyse halide hydrolysis with the inversion of the product configuration.

Dehalogenation of 2,2-dichloropropionic acid is catalysed by hydrolytic dehalogenases (Janssen *et al*., 2001). There are two distinct evolutionary families of these enzymes, the group I and group II dehalogenases. Group I dehalogenases dechlorinate D-2-chloropropionate, whereas all group II dehalogenases dechlorinate

L-2-chloropropionate. As described by Hill *et al.* (1999), group I dehalogenases do not share any obvious feature with group II dehalogenase in terms of nucleotide or deduced amino acid sequences, suggesting that they are not evolutionarily related. They also seem to be functionally distinct.

Group II dehalogenase enzymes catalyse the hydrolytic dehalogenation of L-2-haloalkanoic acids to yield the corresponding D-2-hydroxyalkanoic acids (van der Ploeg *et al*., 1991). They belong to the Haloacid Dehalogenase (HAD) superfamily of aspartate-nucleophile hydrolases, class (subfamily) I.

Group II dehalogenases share a conserved nucleophilic aspartate residue that is located close to the N terminus and is involved in formation of the covalent intermediate. The Asp-10 residue is the active-site nucleophile, attacking the α-carbon of the substrate, forming an ester intermediate which is subsequently hydrolysed by a water molecule (Kurihara *et al.,* 1995; Liu *et al.,* 1995). The aspartate is positioned by interaction with a conserved lysine. An arginine, an asparagine and a phenylalanine residue are also conserved and are involved in binding of the halogen/halide.

Group I dehalogenase enzymes have yet to be fully characterised. Although no structural information is available as yet, biochemical studies have indicated that group I haloacid dehalogenases (Ridder *et al*., 1999) do not use a covalent mechanism for catalysis. Nardi-Dei *et al.* (1999) proposed that the dehalogenase directly activates a water molecule to attack the α-carbon of 2-haloalkanoic acid, thereby displacing the halogen atom. It has not yet been established which amino acids are involved in the dehalogenation, although a number of candidates have been highlighted by mutagenesis experiments.

Almost all of the α-halocarboxylic acids dehalogenases isolated and described in literature was now assigned to either the group I or group II dehalogenase. However, the DehL from *Rhizobium sp.* (Cairns *et al.,* 1996) showed the same stereospecificity as group II dehalogenase (dechlorination of L but not D-2-chloropropionate) was not a member of this family. From amino acid sequences of various L-2-haloacid dehalogenase compared, DehL from *Rhizobium sp*. was suggested to constitute a separate homology group of L-specific enzymes.

1.8.1.2 Dehalogenation of β-halocarboxylic Acids (3-chloropropionate)

Microbial degradation of halogenated aliphatic compounds has been studied to an increasing extent within the last five decades. However, most reports mainly described aerobic and anaerobic bacterial degradation of various haloalkanes, α-halocarboxylic acids and aromatic halogenated compounds. Except for an early study by Bollag and Alexander (1971) and Yokata *et al*. (1986) which deals with bacterial dehalogenation of β-chlorinated aliphatic acids (3-chloropropionate) and a recent study by McGrath and Harfoot (1997), which describe utilisation of 3-chloropropionate by a group of phototrophic organisms under anaerobic condition, all investigations concerning bacterial degradation of haloacids have focused on saturated α -halogenated acids especially 2,2-dichloropropionate (Section 1.8.2.1).

Using an enrichment technique, Bollag and Alexander (1971) isolated from soil a strain of *Micrococcus denitrificans* capable of utilising 3-chloropropionate as a sole source of carbon and energy. Bacterium suspensions derived from cultures grown in a medium containing 3-chloropropionate readily metabolised this compound without a lag phase. Crude cell-free extracts of 3-chloropropionate-grown bacteria liberated chloride ion from many β-substituted acids, but aliphatic acids halogenated only in the α-position were not dechlorinated.

Yokata *et al*. (1986) described seven bacterial species isolated using a soil enrichment technique which were capable of utilising β-chlorinated butyrates and propionates as sole source of carbon and energy. Two of the organisms were identified as *Corynebacteria sp*. Cell free extract of *Corynebacteria sp*. did not liberate chloride ions from 3-chloropropionates although resting cells growns on 1-chlorobutane were reported to dehalogenate 3-chloropropionate.

McGrath and Harfoot (1997) demonstrated that 3-chloropropionate can be degraded under anaerobic condition by a group of phototrophic organisms from the purple nonsulfur bacteria group. Under illuminated anaerobic conditions, strains from the genera *Rhodospirillum* and *Rhodopseudomonas* were shown to grow

phototrophically on chlorinated and brominated acetic and propionic acids by the reductive removal of the halogens and subsequent utilisation of the acids for growth. In all cases, the degradation of each of the substrates was accompanied by the release of the associated halogen, as halide, and the formation of the corresponding nonhalogenated acid. Thus, the dehalogenation of haloacetic acid yielded acetic acid and the dehalogenation of the halopropionic acids yielded propionic acid. Both of these acids are readily photometabolised by each of the strains. Nevertheless, all strains have slow growth rates with generation times of between 36 to 72 hours when growing on 2mM of these substrates.

Very few reports have been published on 3-chloropropionate degradation and dehalogenases from these organisms were not characterised and the pathways were not elucidated. An *Alcaligenes sp*. that is able to degrade α -chlorinated aliphatic acids (2-chlorobutyrate, 2-chloropropionate) as well as on the β -chlorinated four-carbon aliphatic acids (trans-3-chlorocrotonate, cis-3-chlorocrotonate, and 3-chlorobutyrate) as sole carbon and energy sources may give some idea about the pathway of 3-chloropropionate degradation. However, the 3-chloropropionate proved not to be a substrate for growth for this bacterium (Staub and Kohler, 1989).

As described by Staub and Kohler (1989), the proposed pathways for catabolism of trans-3 chlorocrotonate and 3 chlorobutyrate by *Alcaligenes sp*. strain CC1 was showed in Figure 1.7. The dechlorination of β-chlorinated four-carbon fatty acids by *Alcaligenes sp.* strain CC1 is dependent on a prior reaction of the aliphatic acids with CoA, which presumably results in the formation of the corresponding CoA esters. This esterification is followed by the removal of the chlorine substitute, allowing the four-carbon acids to be further catabolised using a β –oxidation pathway.

The proposed reaction for dehalogenation of 3-chloropropionate is by hydrolytic dehalogenation similar to Group II dehalogenase enzyme described in section 1.8.1.1. During nucleophilic substitution, the hydroxide ion derived from water (van Pée and Unversucht, 2003) attacks the electrophilic carbon atom that attached to the chloride group. The electron pairs from the electron-rich nucleophile moved to the electron-poor carbon atom of the electrophile. Since carbon can

accommodate only eight electrons in its valence shell, the carbon-chlorine bond must begin to break as the carbon-oxygen bond begins to form. The chloride ion leaves with the pair of electrons that once bonded it to the carbon atom (Figure 1.8).

Besides, there is a possibility that the removal of chloride from 3-chloropropionic acid was catalysed by an enzyme system involved in the β-oxidation of fatty acids (Yokota *et al.,* 1986). Thus, it will resulting in formation of α,β-unsaturated acids, which were also known as acrylic acids. The formation of acrylic acid was showed in Figure 1.9.

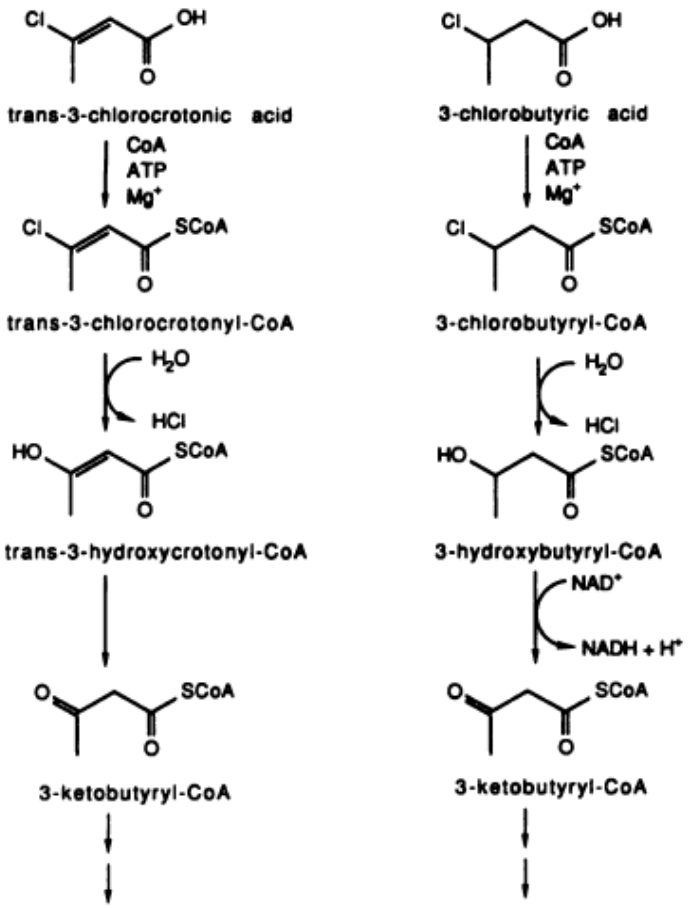


Figure 1.7: Proposed pathways for catabolism of trans-3 chlorocrotonate and 3 chlorobutyrate by *Alcaligenes sp*. strain CC1 (Staub and Kohler, 1988)

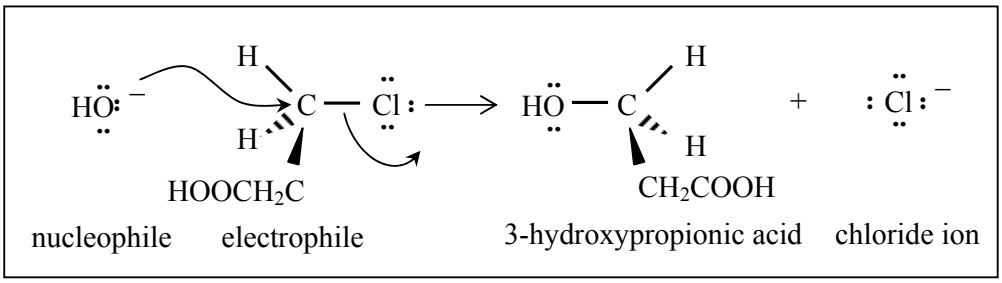


Figure 1.8: Nucleophilic substitution in degradation of 3-chloropropionic acid

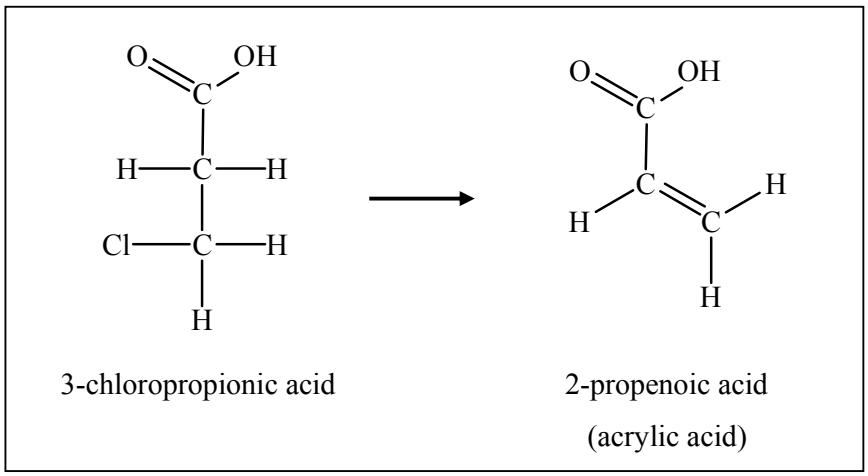


Figure 1.9: The formation of acrylic acid from 3-chloropropionic acid

1.9 The Genetics of Haloalkanoic Acids Dehalogenase

A dechlorination reaction often requires only a single protein that can recognise and convert a xenobiotic substrate. However, regulated expression by means of binding of a halogenated substrate and interaction with the transcription machinery requires a second protein. Therefore, if the synthesis of a dehalogenation enzyme is subject to regulation, the pathway must be more evolved than in the case of constitutive protein expression (Poelarends *et al*., 2000).

A number of regulatory genes that influence dehalogenase expression have been characterised in dehalogenating organisms. The classical halo acid dehalogenase are usually regulated, which is not surprising because they are natural compounds (Janssen *et al*., 2001). In contrast, dehalogenase from some organisms that utilise xenobiotic haloalkanes might be expressed constitutively. For example, Janssen *et al.* (1985) described the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus* strain GJ10. The bacterium constitutively produces a hydrolytic haloalkane dehalogenase (DhaA) that converts dichloroethane to 2-chloroethanol.

In addition, gene transfer is an important process during the evolution of novel catabolic pathways. Acquisition of foreign DNA by horizontal gene transfer requires integration into a replicon that is well maintained in the recipient microorganism. Transposition and gene integration are key mechanisms for the formation of stable new constructs. An classic example was showed by *Pseudomonas putida* PP3 isolated by Senior *et al*. (1975), which grown on 2,2-dichloropropionate and produced dehalogenase I (dehI) and dehalogenase II (dehII).

Thomas *et al.* (1992a, 1992b) showed that the *dehI* gene was carried on a mobile genetic element and gave it the general designation *DEH*, since it was found to vary in size following transposition into different plasmid targets. Thomas *et al.* (1992a) identified a hot spot for insertion of *DEH* into the TOL plasmid pWW0 (Worsey *et al.,* 1975) and one such *DEH* element insertion was cloned and characterised (Thomas *et al.,* 1992b) following its transposition from the

Pseudomonas putida PP3 genome to pWW0 and conjugal transfer of pWW0::*DEH* to another strain of *P. putida*. Thus, the *DEH* element was shown to carry the *dehI* gene immediately adjacent to $dehR_I$, which encoded a σ^{54}-dependent activator (Thomas *et al.,* 1992b; Topping *et al.,* 1995).

1.10 Role of Transport Protein in Transporting Halogenated Compound

Natural organisms capable of metabolising a wide range of haloaromatic and aliphatic substances have been isolated, and ultimately these are important factors in determining the fate of halogenated substances in the environment. Inside the cell, the carbon-halogen bond in halogenated substances is cleaved and this is essential for yielding products that may be readily metabolised by the organism (Hardman and Slater, 1981). Nevertheless, in order for this event to take place, the halogenic compounds must first be translocated into the cell.

Active uptake of halogenated carboxylic acids has been observed in *Pseudomonas putida* PP3 (Slater *et al*., 1985), which utilises halogenated alkanoic acids such as monochloroacetate as its sole carbon and energy sources. *Pseudomonas putida* PP3 evolved the ability to utilise halogenated alkanoic acids as the consequence of an event that occurred during chemostat selection with the 2,2-dichloropropionate as the growth substrate (Senior *et al.,* 1976). This event led to the inducible expression of two dehalogenases and associated membrane transporters that permitted growth on several halogenated alkanoic acids. It is interesting to note that none of these proteins were used by its parental strain *P. putida* PPl. (Senior *et al.,* 1976; Slater *et al.* 1979; Weightman *et al.,* 1979, 1982).

Subsequent analysis showed that two permease proteins were discovered within transposable elements in *Pseudomonas putida* PP3 (Slater *et al.,* 1985). These permeases were found closely associated with the dehalogenases, and

contributed an essential role of enabling strain PP3 grow on D,L-2-chloropropionate compounds. Additionally, the permeases were reported to have broad specificity in transporting both metabolisable substrates and the toxic analog MCA in strain PP3 (Slater *et al*., 1985).

Similar to *Pseudomonas putida* PP3, *Xanthobacter autotrophicus* GJ10 is a microorganism capable of dehalogenating haloalkanoic acids. It was noted that the growth rate of strain GJ10 with monochloroacetate as a substrate was poor compared to that with 2-chloropropionate (Janssen *et al*., 1985), despite the fact that haloalkanoic acid dehalogenase activity was sufficient and that monochloroacetate was proven non-toxic to the cell (van der Ploeg and Janssen, 1995). Therefore, the suggestion was that the reduction in growth rate could be caused by the transport protein of strain G10 having a difference affinity for the substrates being used.

In addition, van der Ploeg and Janssen (1995) also reported that the DNA sequence upstream of the *dhl*B gene encoding the haloalkanoic acid dehalogenase of *Xanthobacter autotropicus* GJ10 contained an open reading frame (ORF), designated *dhlC*. The ORF was subjected to database sequence comparison and encoded for a protein with high similarity to the family of Na^+-dependent symport proteins. This suggests that *dhlC* encodes a protein with an uptake function and further analysis proposed that it is specifically involved in growth with haloalkanoic acids.

1.10.1 The Presence of a Putative Rhizobial *dehrP* Gene in pSC1

Escherichia coli K-12 strain NM522 transformed with plasmid pSC1 was endowed with the novel ability to grow at the expense of 2-chloropropionic acid. However, *Escherichia coli* without the plasmid was unable to grow on 2-chloropropionic acid (Cairns *et al*., 1996). Therefore, it was hypothesised that the

pSC1 plasmid encodes gene for dehalogenase enzyme and a dehalogenase associated permease that enable the *E.coli* to uptake the haloacid into the cell.

1.11 Objectives

I Isolate and characterise a local novel bacteria strain which able to degrade 3-chloropropionate.

II Isolate and characterise a local 2,2-dichloropropionate degrading bacteria as well.

III Analysis of a putative haloacid permease gene (*dehrP*) located upstream of *dehD* in pSC1.