CHARACTERIZATION OF 1, 2-DCA DEGRADING *Ancylobacter aquaticus* STRAINS ISOLATED IN SOUTH AFRICA

BY

Thiloshini Pillay

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor, I have approved this dissertation for submission.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from May 2007 to April 2010, under the supervision of Professor B. Pillay and the co-supervision of Dr. A. O. Olaniran.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

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Publication 2

Publication 3

Etc.

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THILOSHINI PILLAY

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ABSTRACT

1,2-Dichloroethane (1,2-DCA), a highly toxic and recalcitrant compound, is produced anthropogenically in larger quantities than any other chlorinated compound. It is regarded as a mutagen and carcinogen, thus making it a priority target molecule for biological degradation. In addition, the intermediates of 1,2-DCA degradation are highly reactive and toxic, due to the electrophilic nature of the carbonyl groups in these compounds. Aerobic biodegradation of 1,2-DCA, resulting in complete mineralization, has previously been reported in *Xanthobacter autotrophicus* GJ10 and some *Ancylobacter aquaticus* strains. *X. autotrophicus* GJ10 has been found to possess chloroacetaldehyde (CAA) dehydrogenase and haloacid (HA) dehalogenase enzymes, both of which play a crucial role in 1,2-DCA degradation. Five strains of *Ancylobacter aquaticus* capable of utilizing 1,2-DCA as a sole carbon and energy source have recently been isolated in our laboratory. The degradation potential and specific dehalogenase activities of these bacterial isolates against 1,2-DCA and other halogenated compounds as a carbon source were investigated and compared to previously characterized organisms, viz., *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* strains AD25 and AD27. Furthermore, this study proposed to detect the presence of the CAA dehydrogenase (*aldB*) and HA dehalogenase (*dhlB*) encoding genes in these isolates. Growth of all strains in the presence of 1,2-DCA as a carbon source was monitored over an 84 h period, in minimal medium supplemented with either vitamins or yeast extract. Dehalogenase activities were measured colorimetrically by monitoring halide release by crude cell extracts of the isolates. In order to detect the presence of *dhl*B and *ald*B genes, genomic DNA of the isolates was digested with individual restriction endonucleases, viz., *Eco*RI, *Pst*I, *Hind*III and *Bam*HI, and then subjected to Southern hybridization experiments. All isolates demonstrated significant growth rates in both vitamin and yeast extract supplemented media, with the former having a greater overall growth effect. *Ancylobacter aquaticus* DH5 demonstrated the highest growth rate of $0.147.h^{-1}$ in the presence of vitamins while *Ancylobacter aquaticus* DH12 displayed the highest growth rate of $0.118.h^{-1}$ with yeast extract. Optimum haloalkane dehalogenase activities of these bacterial isolates were

confirmed at pH 8, similar to the activity in *X. autotrophicus* GJ10, while haloacid dehalogenase activity had a broader pH range. Hydrolytic dehalogenase activity of the bacterial isolates using a range of halogenated aliphatic compounds was also determined. Results demonstrated a wide substrate range with activity being observed on 1,3-dibromopropane, 1,2-dibromoethane and 1,3-dichoropropene, for all isolates. Southern Hybridization experiments confirmed the presence of both *aldB* and *dhlB* genes in *X. autotrophicus* GJ10. The *dhl*B probe produced a positive signal for an *Eco*RI fragment in *Ancylobacter aquaticus* DH12 while the *ald*B probe hybridized and produced a single positive signal on similar sized *Pst*I fragments for all organisms except *A. aquaticus* AD25 which produced two positive signals. The results in this study demonstrate the potential application of the newly isolated strains of *Ancylobacter aquaticus*. in future bioremediation strategies. The detection of the genes involved in 1,2-DCA degradation further support the use of these isolates and/or their enzymes for the degradation of 1,2-DCA as well as other halogenated compounds. Future work need to determine sequence similarity of these genes detected in *A. aquaticus* strains to the genes in *Xanthobacter autotrophicus* GJ10 and other previously reported genes. It may also be important to investigate the activity of the enzymes under various environmental conditions and to determine enzyme structure and the catalytic sites, so as to gain knowledge of their degradation potential on site. Characterization of enzymes at both the molecular and protein levels may be necessary and beneficial for implementation in strategies involving bioremediation for the biological degradation of a wide range of halogenated aliphatic hydrocarbons.

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The extraordinary population increase and industrial development during the last century has resulted in the production of a range of synthetic chemicals for which society has become unprepared, increasing conventional solid and liquid waste pollutants to critical levels. Xenobiotics are chemically synthesized organic compounds, most of which do not occur in nature and are foreign to living organisms. Many harmful synthetic organic compounds, which are slowly degradable, have been identified. These include halogenated aromatics, halogenated aliphatics and several pesticides (Ojo, 2007).

A multitude of halogenated organic compounds are known to exist in the biosphere, occurring both naturally and synthetically (Fetzner, 1998). Halogenated compounds are an important class of chemicals, as these compounds are widely used on an industrial-scale as pharmaceuticals, pesticides, intermediates in organic synthesis and solvents (Van Pee and Unversucht, 2003). Unfortunately, many halogenated compounds are inadvertently released into the environment resulting in the contamination of soil, underground waters and surface waters (Song *et al*., 2003). Halogenated compounds are usually more persistent than non-halogenated hydrocarbons (Swanson, 1999). Therefore, toxicity, bioconcentration and the ubiquitous distribution of halogenated compounds in the biosphere have caused public concern over the possible effects on the quality of life (Fetzner and Lingens, 1994). Increasing concentrations of xenobiotic compounds has prompted the need for understanding the impact of toxic compounds on microbial populations, the possible catabolic degradation pathways of xenobiotics and upgrade in bioremediation processes (Ojo, 2007).

Since microorganisms are ubiquitous and nature's own original recyclers, they have the ability to adapt to xenobiotic compounds as growth and energy substrates (Jain *et al*., 2005; Ojo, 2007). In some cases, microorganisms convert these toxic organic compounds

to harmless products, often carbon dioxide and water (Jain *et al*., 2005). The recalcitrance of halogenated hydrocarbons to biodegradation is mainly due to the lack of enzymes in microbial populations that can perform critical steps in a catabolic pathway (Janssen *et al*., 2005). Thus, there is a requirement for the isolation and characterization of novel microorganisms and enzymes with enhanced capabilities to degrade such toxic compounds. It is also imperative to understand the role of such microbial catabolic genes and their characterization for degradation of a particular organic compound. This will afford better knowledge regarding the diversity of enzymes involved in the degradation of halogenated compounds and can eventually be applied in bioremediation processes for the biological removal of recalcitrant chemical compounds (Ojo, 2007).

1.2 CHLORINATED ALIPHATIC HYDROCARBONS (CAHs)

1.2.1 Properties and uses of CAHs

Chlorinated aliphatic hydrocarbons (CAHs) are an important class of chemicals that are produced in large amounts. They are widely used in pharmaceuticals, herbicides, flame retardants, soil fumigants, degreasing agents, pesticides, as well as solvents in the dry-cleaning process (Fetzner, 1998; Van Pee and Unversucht, 2003). The most common chlorinated compounds, including some physical and chemical properties of the common CAHs in the environment are listed in Table 1.1. With the increase in the number of substituted chlorine atoms, molecular weight and density generally increases, and vapour pressure and aqueous solubility generally decreases (EPA, 2000). It is these properties that make chlorinated compounds highly recalcitrant and thus difficult to degrade biologically.

1.2.2 Presence and persistence of CAHs in the environment

Chlorinated compounds are inadvertently or neglectfully released into the environment as a result of improper disposal and accidental or deliberate spillages, notably to the soil and

groundwater (Van der Zaan *et al*., 2009). When released, the compounds interact physically and chemically, forming non-aqueous phases, adsorbing strongly with soil organics and minerals and dissolving into groundwater as well as deeper in the soil. Many of these compounds tend to persist and bioaccumulate in the environment because of their resistance to both chemical and biological attack. The shorter-chained chlorinated compounds tend to accumulate in environments with reductive conditions with their half-life usually exceeding several decades (de Wildeman and Verstraete, 2003). This results in considerable environmental pollution and human health problems due to their carcinogenic and/or genotoxic properties (Squillace *et al*., 1999). The electron-withdrawing effects of the halogen substituents make the halogen-substituted carbon atom susceptible to reactions with nucleophilic groups in biomolecules. This may lead to covalent modifications that inhibits or alters the biological function in some way. Research has, therefore, progressively focused on biological methods for the degradation and elimination of these pollutants (Van den Wijngaard *et al*., 1993).

Table 1.1: Physiochemical properties of chlorinated ethanes and ethenes (Ma and Wang, 2009)

	Molecular formula	Molecular weight	Solubility (mg/L)	Octanol–water partitioning coefficient (log K_{ow})	Henry's Law Constant (atm-m^3/mole)
PCE	C_2Cl_4	165.83	206	3.40	0.0177
TCE	$CHCCl_3$	131.39	1280	2.42	0.00985
1,1-DCE	$CHCCl_2$	96.94	2420	2.13	0.0261
1,2-DCE (cis)	$CHCCl_2$	96.94	6410	1.86	0.00408
1,2-DCE(trans)	$CHCCl_2$	96.94	4520	2.09	0.00938
1,1,2,2-TeCA	$C_2H_2Cl_4$	167.85	2830	2.39	0.000367
1,1,1-TCA	CH_3CCl_3	133.41	1290	2.49	0.0172
1,1,2-TCA	CH_3CCl_3	133.41	4590	1.89	0.000824
1,1-DCA	CH_3CHCl_2	98.96	5040	1.79	0.00562
1,2-DCA	CH_3CHCl_2	98.96	8600	1.48	0.00118

1.2.3 Biodegradation of CAHs

Microorganisms signify the richest collection of molecular and chemical diversity in nature, as they consist of the most diverse forms of life. Microorganisms have adapted to very diverse environments over millennia and have developed a wide range of metabolic pathways (Jain *et al*., 2005). Regardless of the toxicity and persistence of halogenated hydrocarbons, many microorganisms have evolved mechanisms that have enabled the degradation of these compounds and the use of the by-products as sole sources of carbon and energy (Table 1.2) (Van Hylckama Vlieg and Janssen, 2001).

The redox reactions result in the flow of electrons from the substrate to a terminal electron acceptor (e.g. an oxidant such as O_2) and the release of energy that is used to support cell synthesis (Tiedje, 1993). On the other hand, the chlorinated compound can serve as the electron acceptor in a process known as halorespiration. This type of anoxic decomposition of simple organic substrates (e.g. lactic acid) is linked to the use of chlorinated compounds as electron acceptors resulting in their reductive dehalogenation (Field and Sierra-Alvarez, 2004). The application of microorganisms to clean-up sites contaminated with chlorinated compounds represents a possible solution to the removal of such compounds from the environment (Widada *et al*., 2002).

The most critical reaction for microbial degradation of chlorinated or halogenated compounds is dehalogenation. Dehalogenation is generally the first step in most pathways, initiated by specialized enzymes known as dehalogenases. These enzymes can break the carbon-halogen covalent bond of chlorinated hydrocarbons under both aerobic and anaerobic conditions (Fetzner and Lingens, 1994; Song *et al*., 2004).

Table 1.2:Examples of microorganisms involved in the biodegradation of chlorinated aliphatic hydrocarbons (Chaudhry and Chapalamadugu, 1991)

COMPOUND	MICROORGANISM	REFERENCES
2-Chloropropionic acid	*Pseudomonas* sp.	(Hardmen *et al*., 1986)
2-Monochloroacetic acid	*Alcaligenes* sp.	
1,2-Dichloroethane	Anaerobes	(Bouwer and McCarty, 1983)
	Xanthobacter autotrophicus GJ10	(Janssen *et al*., 1985)
	Methane-utilizing bacteria	(Yokata *et al*., 1986)
	Pseudomonas fluorescens	(Vandenbergh and Kunka., 1988)
	Ancylobacter aquaticus sp.	(Van den Winjaard *et al*., 1992)
1,1,1-TCE	Anaerobes	(Bouwer and McCarty, 1983)
	Methanotrophs	(Vogel and McCarty, 1987)
Perchloroethane	*Methanosarcina sp.*	(Fathepure *et al*., 1988)
1-Chlorobutane	Aerobes and anaerobes	(Wubbolts and Timmis, 1990)
1,2-Dichloropropane	*Pseudomonas fluorescens*	(Vandenbergh and Kunka, 1988)
1,3-Dichloropropane	Aerobes	(Yokata *et al*., 1986)
1,9- Dichlorononane	Anaerobe	(Yokata *et al*., 1986)
TCE	Methane utilizing bacteria	(Fogel *et al*., 1986)
	Pseudomonas putida	(Nelson *et al*., 1988)
	Methane-oxidizing bacteria	(Little *et al*., 1988)
	Pseudomonas fluorescens	(Vandenbergh and Kunka, 1988)
3-Chloro-4-hydroxybenzaldehyde	Anaerobes	(Neilson *et al*., 1988)

1.3 DEHALOGENASES: TYPES AND MECHANISMS

Dehalogenation is the key reaction performed by dehalogenase enymes during microbial degradation of halogenated compounds. During this step, the halogen substituent responsible for the toxic and xenobiotic character of the compound is often replaced by hydrogen or a hydroxyl group (Fetzner and Lingens, 1994).

Numerous factors dictate which of these enzyme systems are employed for degradation *in vivo*. Some of these factors include the chemical features of the carbon-halogen bond (e.g. aliphatic, olefinic, aromatic, and number of halogens per carbon) and the presence of compatible metabolic pathways allowing the dehalogenated intermediates to be utilized by the microorganism (Swanson, 1999). Seven mechanisms of dehalogenation proposed by Fetzner and Lingens, (1994) are discussed below.

1.3.1 Hydrolytic dehalogenation

The largest groups of dehalogenases, described thus far, are the hydrolytic dehalogenases. They catalyze the cleavage of carbon-halogen bonds through a nucleophilic substitution by water to yield alcohol (Van Pee and Unversucht, 2003), as indicated in Fig. 1.1. There are at least two distinct subgroups based on substrate range within this group, viz., haloalkane dehalogenases and haloacid dehalogenases. Haloalkane dehalogenases are enzymes that catalyze the net hydrolytic conversion of a chloroalkane or a bromoalkane to the corresponding alcohol and hydrogen halide (Janssen, 2004). The haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 was the first dehalogenase for which the crystalline structure was determined, but other structures have since become available (de Jong and Dijkstra, 2003). Haloacid dehalogenases catalyze the hydrolysis of halogenated carboxylic acids, such as 2-chloroacetate, which is an intermediate in the degradation of 1,2-DCA (de Jong and Dijkstra, 2003). L-2-Haloacid dehalogenases have been isolated from various bacterial strains including *Pseudomonas* YL, *Pseudomonas putida* 109, and *X. autotrophicus* GJ10 (Kurihara *et al*., 2000).

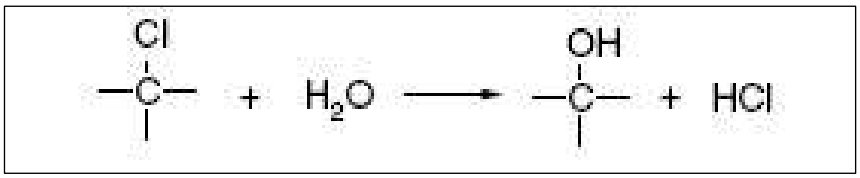

Figure 1.1: Hydrolytic cleavage of the carbon-chlorine by hydrolytic dehalogenases (Van Pee and Unversucht, 2003)

1.3.2 Oxygenolytic dehalogenation

Oxidative dehalogenation reactions occur in the biodegradation of haloaliphatic and haloaromatic compounds. The enzymes involved in these reactions are monooxygenases and dioxygenases. Methane monooxygenase from *Methylococcus capsulatus* and *Methylococcus trichosporium* have broad substrate specificity for oxygenolytic reactions (Van Pee and Unversucht, 2003). Dioxygenases consist of a two-component system found in various microorganisms such as the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp. CBS3, and 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS. The 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp. CBS3 consists of a monomeric flavin mononucleotide- and [2Fe-2S]-containing reductase component (35 kDa), which transfers electrons from the co-substrate NADH to the terminal oxygenase component (Fetzner and Lingens, 1994).

1.3.3 Thiolytic dehalogenation

Glutathione S-transferase is the dehalogenating enzyme involved in the degradation of dichloromethane. Methylotrophic bacteria, such as *Methylophilus* sp., *Methylobacterium* sp. and *Hyphomicrobium* sp., isolated with dichloromethane as growth substrate, produces an inducible glutathione S-transferase which catalyze the formation of an unstable S-chloromethyl glutathione intermediate (Van Pee and Unversucht, 2003). The nucleophilic substitution by glutathione is seen below in Figure 1.2. The chloromethyl thioether then undergoes rapid hydrolysis in an aqueous environment (Fetzner and

Lingens, 1994). The final products are glutathione, chloride, and formaldehyde (Van Pee and Unversucht, 2003).

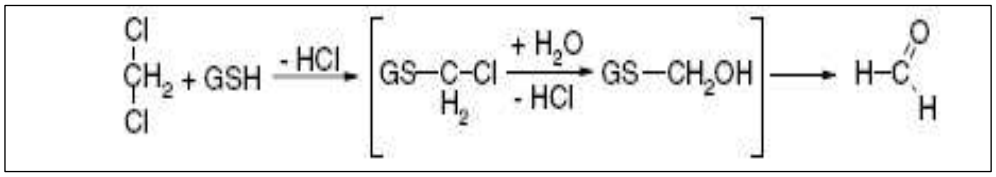

Figure 1.2: Thiolytic dehalogenation with glutathione S-transferase (Van Pee and Unversucht, 2003)

1.3.4 Reductive dehalogenation

Reductive dehalogenation is a two-electron transfer reaction which involves the release of the halogen as a halogenated ion and its replacement by hydrogen (Fig. 1.3). Aerobic microorganisms often fail to metabolize the longer-chained halogenated compounds. For highly chlorinated biphenyls, hexachlorobenzene, and tetrachloroethylene, anaerobic reductive dehalogenation is the only known biodegradation mechanism (Fetzner and Lingens, 1994). *Sphingomonas chlorophenolica* is involved in the aerobic reductive dehalogenation of pentachlorophenol. Tetrachlorohydroquinone is the intermediate produced after pentachlorophenol hydroxylase acts on the pentachlorophenol. The next step is dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase which is also a member of the glutathione S-transferase family (Van Pee and Unversucht, 2003).

Anaerobic reductive dehalogenation, also known as halorespiration, is coupled to energy metabolism. Halogenated compounds serve as a terminal electron acceptor during the oxidation of an electron rich compound such as hydrogen or an organic substrate (Fig. 1.4). Reductive dehalogenases, have been isolated from a number of bacteria viz., *Dehalococcoides ethenogenes* or *Desulfitobacterium dehalogenans* (Van Pee and Unversucht, 2003).

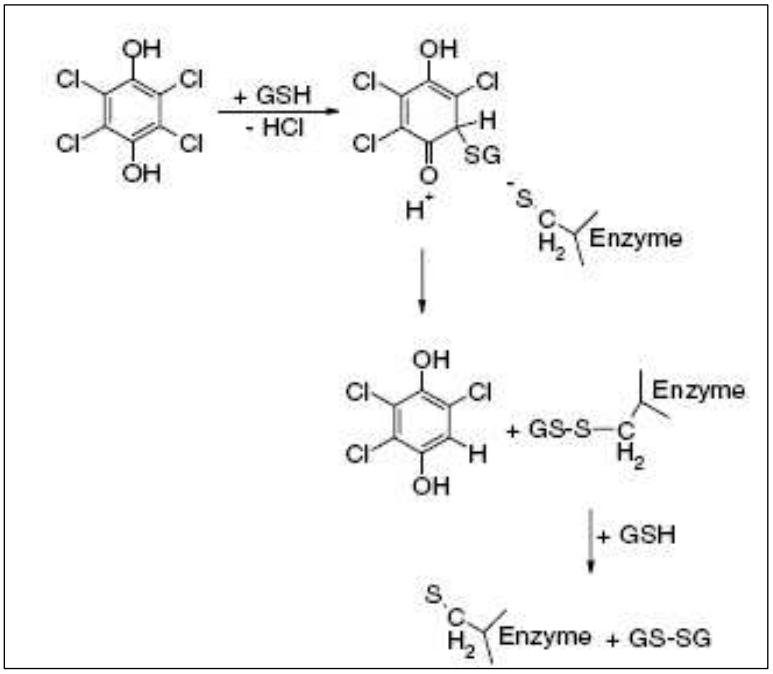

Figure 1.3: Reductive dehalogenation of tetrachlorohydroquinone under aerobic conditions catalyzed by a glutathione transferase (Van Pee and Unversucht, 2003)

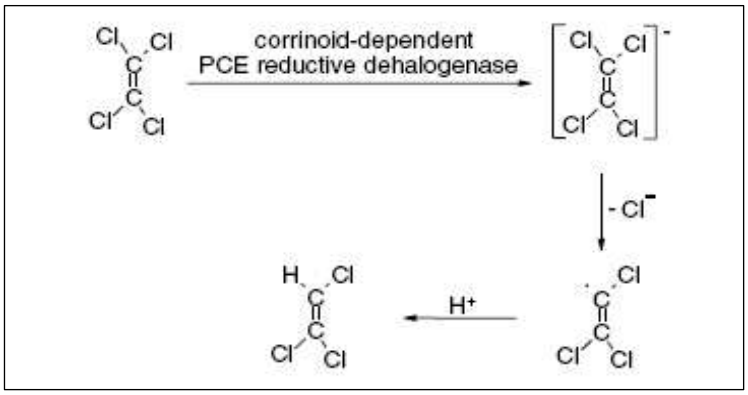

Figure 1.4: Reductive dehalogenation of PCE under anaerobic conditions (Van Pee and Unversucht,2003)

1.3.5 Haloalcohol dehalogenation

This reaction proceeds via a nucleophilic displacement of a halogen by a vicinal hydroxyl function in halohydrins to yield epoxides (Fig. 1.5). The enzyme that catalyze these reactions are known as haloalcohol dehalogenases, also known as, halohydrin dehalogenases or halohydrin hydrogen halide lyases which have been isolated from *Arthrobacter* sp. and other bacteria, such as *Pseudomonas* sp. and *Corynebacterium* sp. (Fetzner and Lingens, 1994; Van Pee and Unversucht, 2003).

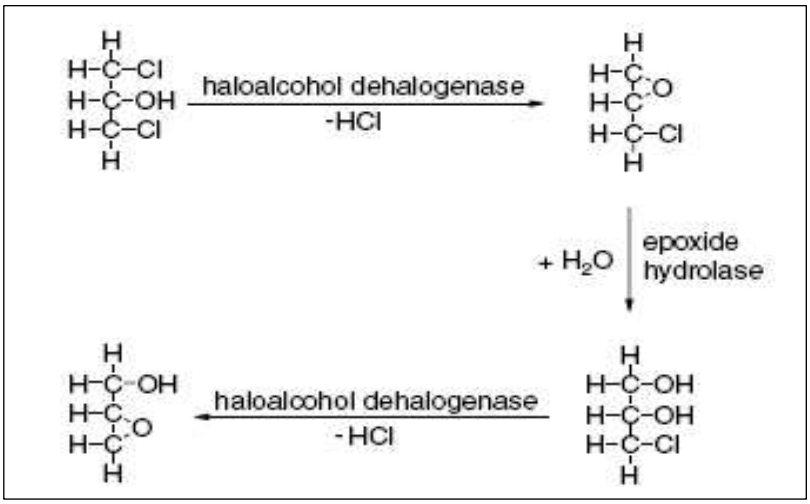

Figure 1.5: Thiolytic nucleophilic substitution of halogen catalyzed haloalcohol dehalogenase (Van Pee and Unversucht, 2003)

1.3.6 Dehydrohalogenation

Dehydrohalogenation, also known as elimination reactions, involve the removal of hydrogen and a chlorine atom from a chlorinated alkane with the subsequent formation of a corresponding chlorinated alkane. Elimination reactions become more effective as the CAHs become more chlorinated (EPA, 2000). Dehydrohalogenase enzymes, found in *Pseudomonas paucimobilis,* are involved in the first two steps in the degradation of lindane (hexachlorocyclohexane), catalyzing the removal of hydrochloric acid and

creating a double bond (Fig. 1.6) (Fetzner and Lingens, 1994; Van Pee and Unversucht, 2003).

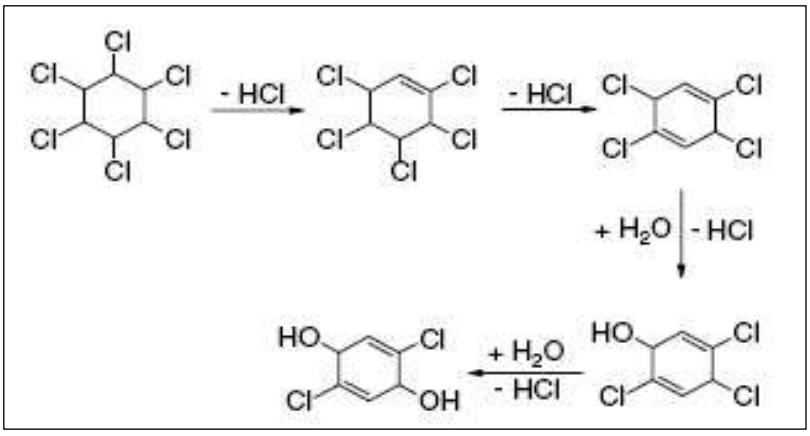

Figure 1.6: Dehalogenation of lindane catalyzed by dehydrohalogenases (Van Pee and Unversucht, 2003)

1.3.7 Hydration

Hydration reactions, catalyzed by hydratase enzymes, involve the addition of a water molecule to an unsaturated bond, resulting in the dehalogenation of vinylic compounds by chemical decomposition of an unstable intermediate (Fetzner and Lingens, 1994). These dehalogenases were detected in *Corynebacterium* sp. (Van Pee and Unversucht, 2003), whereby the dehalogenation reaction proceeds by the addition of a water molecule to the double bond resulting in the formation of an unstable intermediate, from which malonate semialdehyde is formed (Fig. 1.7).

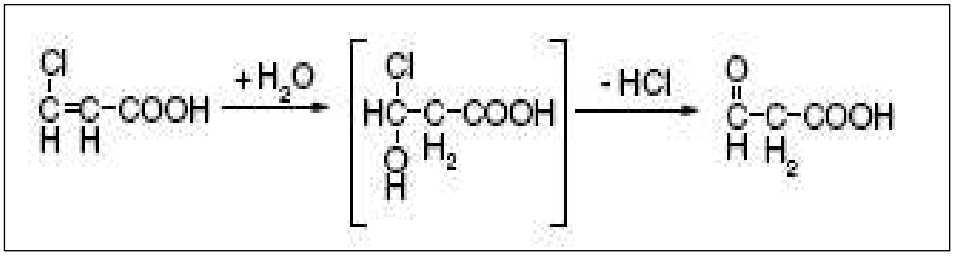

Figure 1.7: Dehalogenation by the addition of a water molecule (Van Pee and Unversucht, 2003)

1.4 1,2-DICHLOROETHANE (1,2-DCA)

1.4.1 Properties and uses of 1,2-dichloroethane

1,2-Dichloroethane is a short chain chlorinated aliphatic compound, produced in larger quantities than any other chlorinated hydrocarbon (Van den Wijngaard *et al*., 1993) with an estimated global production of 1.6×10^7 tons per annum (Field and Sierra-Alvarez, 2004). The characteristics and chemical properties of 1,2-DCA are listed in Table 1.3. (Baden, 2008). 1,2-DCA has a high water solubility coefficient (Table 1.3) and can remain in water phase under average environmental conditions (Dewulf *et al*., 1995). It is predominantly used as an intermediate in the synthesis of vinyl chloride, also a known carcinogen. However, smaller amounts of 1,2-DCA are used in the production of vinylidene chloride, 1,1,1-TCA, TCE, tetrachloroethene, ethylene diamines and in various other chlorinated solvents (Mena-Benetez *et al*., 2008).

Table 1.3: Characteristics and chemical properties of 1,2-DCA (Baden, 2008)

Molecular formula	$C_2H_4Cl_2$
Molar mass	98.97 g/mole
Appearance	Colourless liquid with characteristic odour
Density	1.25 g/cm^3
Melting point	-35.5C (238 K)
Boiling point	83.5- 84.1C (357 K)
Water solubility	8000 mg/l

1.4.2 Impact of 1,2-DCA on the environment

1,2-DCA is frequently detected in the environment and due to its high water solubility and low sorption coefficient, it can rapidly leach into groundwater where degradation rates are low (Mena- Benetez *et al*., 2008). It is extremely hazardous and has an environmental half life of up to 50 years (De Wildeman *et al*., 2003). In fish and mammals, 1,2-DCA can be bioactivated to toxic compounds also resulting in various detrimental effects (Mena- Benetez *et al*., 2008).

1.4.3 Impact of 1,2-DCA on human health

1,2-DCA has been found in at least 570 of 1,585 National Priorities List (NPL) sites recognized by the U.S. Environmental Protection Agency (EPA). Concentrations of 0.005 mM have also been detected in ambient urban and rural air and indoor samples of residences near hazardous waste disposal sites. Human ingestion of 1,2-DCA occurs by drinking water or breathing the air containing this chemical (National Toxicology Program, 2005), resulting in diseases of the liver, kidney, neural and cardiovascular systems following high levels of exposure (Grostern and Edwards, 2006). Thus, 1,2-DCA is listed as a priority pollutant by the EPA due to its contribution in the development of human carcinogenesis (Mena-Benetez *et al*., 2008). It is also recognized to have mutagenic properties due to its conversion to the highly toxic chloroacetaldehyde (CAA) (Richard and Hunter, 1996). CAA is known to bind and react with nucleic acid bases thus yielding cyclic adducts. This cyclic adducts result in potential miscoding of the DNA and ultimately the occurrence of mutagenesis (Jacobsen *et al*., 1989). Hence, due to the harmful effects to humans and animals, 1,2-DCA is a target molecule to degrade chemically and/or biologically.

1.4.4 Microbial degradation of 1,2-DCA

1,2-DCA is subject to both biotic and abiotic transformations in the environment (Janssen *et al*., 1985). Under abiotic conditions, 1,2-DCA is transformed slowly to toxic products such as vinyl chloride (Hunkeler and Aravena, 2000). 1,2-DCA has been given much attention as a model compound, since it can be biologically degraded under several geochemical conditions. The main route of removal of such compounds from the environment is through degradation by microorganisms (Van Hylckama Vlieg *et al*., 2000), as it offers environmentally-friendly and low-cost remediation (Pham *et al*., 2009). The biodegradation of 1,2-DCA has thus been the subject of intensive studies (Grostern and Edwards, 2006; Hage & Hartmans, 1999; Janssen *et al*., 1984; Stucki *et al*., 1983; Torz *et al*., 2007; Van den Wijngaard *et al*., 1992). It has been observed that 1,2-DCA is susceptible to both aerobic and anaerobic biodegradation conditions as shown in Table

1.4 (Grostern and Edwards, 2006). Under anaerobic conditions, ethene is usually the main end product, while aerobic conditions result in the complete mineralization of 1,2-DCA accompanied by the formation of carbon dioxide, inorganic chloride and water (Janssen *et al*., 1985).

Table 1.4: Organisms capable of using 1,2-DCA as energy source either by aerobic or anaerobic metabolism (Adapted from Dinglasan-Panlilio *et al*., 2006)

Condition	Organism	Substrate utilized	Reference
Aerobic metabolism (1,2-DCA is the electron donor)	*Pseudomonas* DE1	1,2-dichloroethane, 2-chloroethanol, chloroacetic acid	Stucki *et al*. (1983)
	Xanthobacter autotrophicus GJ10	1,2-dichloroethane, 2-chloroethanol, chloroacetic acid, 1,3-dichloropropane, 1-chlorobutane	Janssen *et al*. (1984)
	Pseudomonas sp. strain DCA1	1, 2-dichloroethane	Hage & Hartmans (1999)
	Ancylobacter aquaticus AD20, AD25, AD27	1,2-dichloroethane, 2-chloroethanol, 2-chloroethylvinylether	Van den Wijngaard *et al*. (1992)
	Ancylobacter aquaticus DH12,DH5,DH2,UV6,UV5	1,2-dichloroethane, 2-chloroethanol,	Govender (2009)
Anaerobic metabolism (1,2-DCA is the electron acceptor)	*Dehalococcoides ethenogenes* strains 195 and BAV-1	chlorinated ethenes, 1,2-DCA	Maymo-Gatell *et al*. (1999), He *et al*. (2003)
	Desulfitobacterium dichloroeliminans DCA 1	1,2-dichloroethane, 1,2-dichloropropane, 1,2-dichlorobutane, D-2,3-dichlorobutane, L-2,3-dichlorobutane	De Wildeman *et al*. (2003)

1.4.4.1 Anaerobic conditions

Anaerobic biodegradation is an attractive alternative for *in situ* bioremediation as there is no need for the introduction of oxygen into the subsurface. Studies on anaerobic conditions during the last decade have focused on chlorinated ethenes, however, recent work (Grostern and Edwards, 2006) have shown that microorganisms have the ability to anaerobically degrade 1,2-DCA. Under these conditions, reductive dihaloelimination is the mechanism by which the organisms utilize 1,2-DCA forming ethene. *Dehalococcoides* sp. strains 195 and BAV1 and *Desulfitobacterium dichloroeliminans* strain DCA1 can utilize 1,2-DCA (via dichloroelimination) as an electron acceptor (Dinglasan-Panlilio *et al*., 2006; Grostern and Edwards, 2006).

1.4.4.2 Aerobic conditions

Aerobic biodegradation, in contrast to anaerobic conditions, of 1,2-DCA has been well documented (Dinglasan-Panlilio *et al*., 2006; Hage & Hartmans, 1999; Janssen *et al*., 1984; Stucki *et al*., 1983; Van den Wijngaard *et al*., 1992). These conditions result in its complete mineralization to harmless substances, such as water and CO_2. Aerobic biodegradation could eliminate the need for potentially expensive and complex dual treatment systems (Davis *et al*., 2009).

Stucki *et al.* (1983) was the first to demonstrate aerobic degradation of 1,2-DCA using *Pseudomonas* sp. strain DE2. This bacterium was able to grow on 1,2-DCA as the sole source of carbon and energy with a growth rate of 0.08 h^{-1} at 30°C. Subsequently, other organisms were found to show activity on 1,2-DCA viz., *Xanthobacter autotrophicus* GJ10 (Janssen *et al*., 1985), *Ancylobacter aquaticus* strains (Van den Wijngaard *et al*., 1992), *Xanthobacter flavus* (Song *et al*., 2004) and certain strains of *Mycobacterium* sp. (Jesenská *et al*., 2002).

Due to the increasing number of contaminated sites, the enzymes possessed by strains that were capable of aerobically utilizing 1,2-DCA became of great interest (Copley,

1998; Fetzner, 1998). Hydrolytic dehalogenases are an important group of enzymes that play a pivotal role in the aerobic biodegradation of 1,2-DCA. The first step in 1,2-DCA metabolism (Fig 1.8) involves the conversion of 1,2-DCA to 2-chloroethanol catalyzed by the enzyme haloalkane dehalogenase (hydrolytic dehalogenase). This is followed by two sequential oxidations by alcohol and aldehyde dehydrogenase to yield chloroacetate. Finally, chloroacetate is converted to glycolate by the enzyme haloacetate dehalogenase (Janssen *et al*., 1985; Stucki *et al*., 1983). This mechanism of degradation has thus far been seen for *Xanthobacter autotrophicus* GJ10 and some *Ancylobacter aquaticus* species.

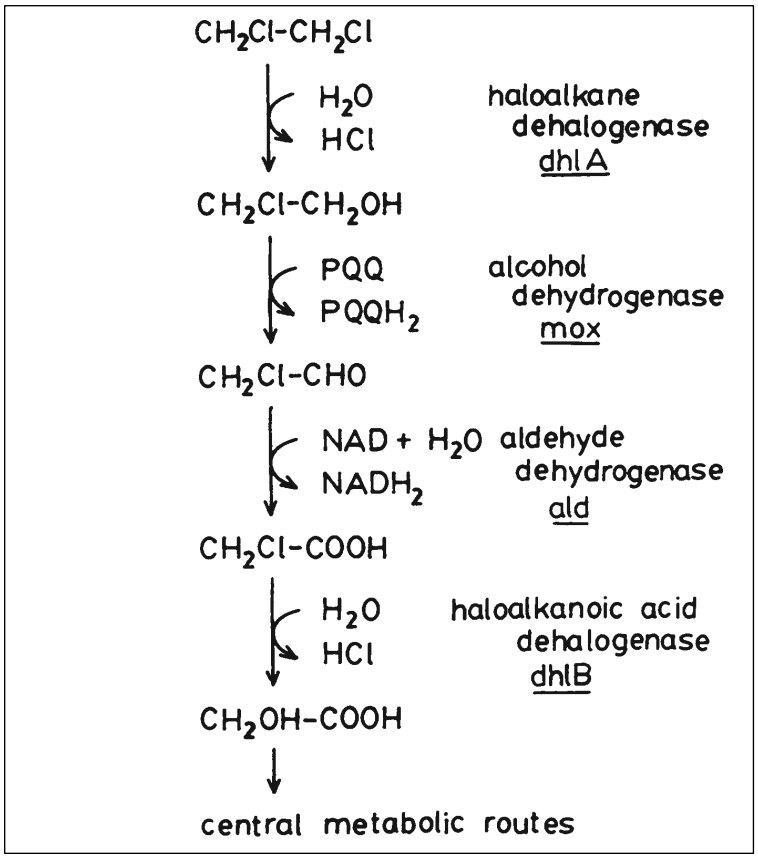

Figure 1.8: Catabolic route for 1,2-dichloroethane of *Xanthobacter autotrophicus* GJ10 and some *Ancylobacter aquaticus* species (Janssen *et al*., 1989)

1.5 ENZYMES INVOLVED IN THE 1,2-DCA DEGRADATION PATHWAY

1.5.1 Haloalkane dehalogenases

Haloalkane dehalogenases are microbial enzymes that can catalyze the net hydrolytic conversion of a chloroalkane or a bromoalkane, yielding a primary alcohol, a proton and a halide (Chovancova *et al*., 2007). The haloalkane dehalogenases belong to the α/β-hydrolase fold family with cleavage of the carbon–halogen bond proceeding via a covalent alkylenzyme intermediate. Haloalkane dehalogenases have a high activity across a broad range of substrates which include chlorinated, brominated and some iodinated primary and secondary haloalkanes (Janssen, 2004).

1.5.1.1 Types of haloalkane dehalogenases

Haloalkane dehalogenase activity has been experimentally confirmed in 12 diverse proteins. Substrate specificities of these dehalogenases are very broad and differ with individual members of the family (Chovancova *et al*., 2007). The crystal structures of three different haloalkane dehalogenases viz., DhlA from *X. autotrophicus* GJ10, LinB from *Sphingobium japonicum* UT26 (formerly *Sphingomonas paucimobilis* UT26) and DhaA from *Rhodococcus* sp. have been determined (Pavlová *et al*., 2007).

Organisms possessing the haloalkane dehalogenase enzyme has been reported (Table 1.5). Statistical analysis of activity by quantitative classification techniques reveals at least three unique specificity classes within this family of enzymes. These are:
(i) Haloalkane dehalogenase of *Xanthobacter flavus, Ancylobacter aquaticus* DH12*, Ancylobacter aquaticus* DH5*, Ancylobacter aquaticus* DH2*, Ancylobacter aquaticus Ancylobacter aquaticus* UV6, *Ancylobacter aquaticus* UV5 (Govender, 2009) *Xanthobacter autotrophicus* GJ10, *Xanthobacter autotrophicus* GJ11, *Ancylobacter aquaticus* AD20, *Ancylobacter aquaticus* AD25 and *Pseudomonas* sp. E4M,

(ii) Haloalkane dehalogenase of *Rhodococcus* sp. HA1, *Rhodococcus* sp. M15-3, *Acinetobacter* sp. GJ70, *Rhodococcus erythropolis* Y2, *Rhodococcus* sp. CP9, *Rhodococcus rhodochrous* NCIMB 13064, *Pseudomonas pavonaceae* 170 and *Mycobacterium* sp. GP1,

(iii) Haloalkane dehalogenase of *Sphingomonas paucimobilis* UT26 (Damborsky and Koca, 1999).

Table 1.5: Bacteria containing haloalkane dehalogenases

Organism	Strain	Gene	Protein	Accession number	
				Gene	Protein
Sphingomonas paucimobilis[a]	UT26	*linB*	LinB	D14594	P51698
Mycobacterium tuberculosis[a]	H37Rv	*Rv2579*	Rv2579	Z77724	Q50642
Mycobacterium tuberculosis[a]	CDC1551	*mt2656*	Rv2579	AE007099	Q50642
Mycobacterium bovis[a]	MU11	*Iso- Rv2579*	Iso- Rv2579	AJ243259	Q9XB14
Mycobacterium smegmatis[a]	MC2_155	*Aal17946*	Aal17946	AY054120	AAL17946
Rhodococcus sp.[a]	M15-3	*dhaA*	DhaA	NA	Q53042
Rhodococcus sp.[a]	HA1	*dhaA*	DhaA	NA	Q53042
Rhodococcus sp.[a]	GJ70	*dhaA*	DhaA	NA	Q53042
Rhodococcus sp.[a]	TB2	*dhaA*	DhaA	NA	Q53042
Rhodococcus erythropolis[a]	Y2	*dhaA*	DhaA	NA	Q53042
Rhodococcus rhodochrous[a]	NCIMB13064	*dhaA*	DhaA	AF060871	Q53042
Pseudomonas pavonaceae[a]	170	*dhaA*	DhaA	AJ250371	Q53042
Mycobacterium sp.[a]	GP1	$dhaA_f$	$DhaA_f$	AJ012627	Q9ZER0
Mesorhizobium loti[a]	MAFF303099	*Mlr5354*	Mlr5354	AP003006	Q98C03
Xylella fastidiosa[a]	9A5C	*Xf1965*	Xf1965	AE004016	Q9PC20
Photobacterium profundum[a]	SS9	*Aal01057*	Aal01057	AF409100	AAL01057
Caulobacter crescentus[a]	CB15	*Cc1175*	Cc1175	AE005795	Q9A919
Mycobacterium tuberculosis[a]	H37Rv	*Rv2296*	Rv2296	Z77163	Q50670
Mycobacterium tuberculosis[a]	CDC1551	*Mt2353*	Rv2296	AE007077	Q50670
Mycobacterium avium[a]	N85	*dhmA*	DhmA	AJ314789	CAC41377
Mycobacterium avium[a]	104	*106*	DhmA	NA	CAC41377
Xanthobacter autotrophicus[a]	GJ10	*dhlA*	DhlA	M26950	P22643
Xanthobacter autotrophicus[a]	GJ11	*dhlA*	DhlA	NA	P22643
Xanthobacter flavus[b]	UE15	*dhlA*	DhlA	NA	

Ancylobacter aquaticus [a]	AD20	*dhlA*	DhlA	NA	P22643
Ancylobacter aquaticus [a]	AD25	*dhlA*	DhlA	NA	P22643
Ancylobacter aquaticus [c]	AD27	*dhlA*	DhlA		
Ancylobacter aquaticus [c]	DH12	*dhlA*	DhlA	FJ573164	
Ancylobacter aquaticus [c]	DH5	*dhlA*	DhlA	FJ573163	
Ancylobacter aquaticus [c]	DH2	*dhlA*	DhlA	FJ573162	
Ancylobacter aquaticus [c]	UV6	*dhlA*	DhlA	FJ573165	
Ancylobacter aquaticus [c]	UV5	*dhlA*	DhlA	FJ573166	

[a] (Jesenka *et al*., 2002), [b] (Song *et al*., 2004), [c] (Govender, 2009)

Figure 1.9 shows the substrates used by the three types of haloalkane dehalogenases. DhlA has greater affinity for shorter chain chlorinated aliphatic compounds such as 1,2-DCA, DCM, and $CHCl_3$, while DhaA prefers the longer chains such as TCA and 1-chlorobutane, while LinB has highest activity on aromatic hydrocarbons e.g. γ-hexachlorocyclohexane. Substrate specificity depends on the size of the substrate molecules as well as to the size of the active site. The active site in LinB is much larger than in other dehalogenases, such as DhlA from *Xanthobacter autotrophicus* GJ10 or DhaA from *Rhodococcus* sp. (Negri *et al*., 2007).

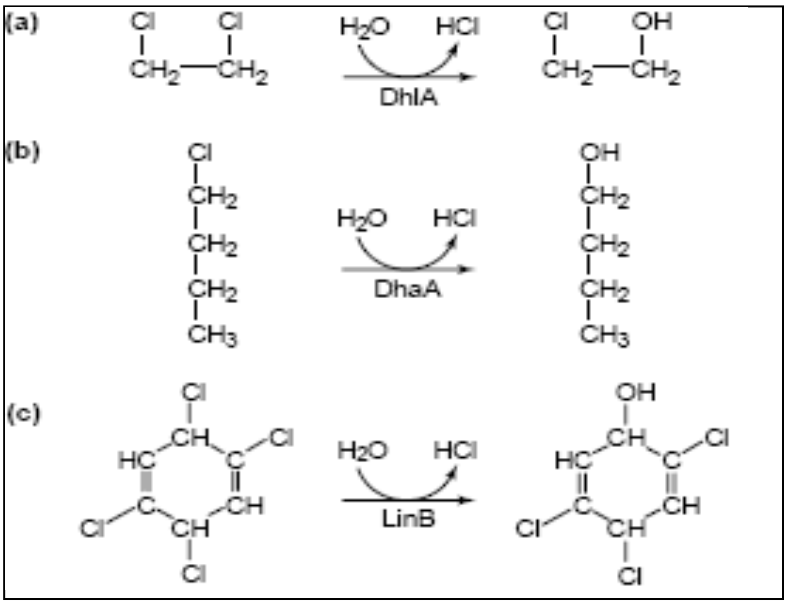

Figure 1.9: Substrates of haloalkane dehalogenases from (a) *Xanthobacter autotrophicus* (DhlA), (b) *Rhodococcus erythropolis* (DhaA), and (c) *Spingomonas paucimobilis* (LinB) (Negri *et al*., 2007)

1.5.1.2 Mechanism and structure of haloalkane dehalogenases

Haloalkane dehalogenases employ a two-step dehalogenation mechanism for the cleavage of carbon-halogen bonds forming an alcohol, a halide and a proton as the reaction products (Fig 1.10). The carbon atom which is attached to the leaving halogen is attacked by a nucleophile in a bimolecular nucleophilic substitution resulting in an alkyl-enzyme intermediate. An activated water molecule then hydrolyzes this intermediate in a nucleophilic addition reaction (Pavlová *et al*., 2007).

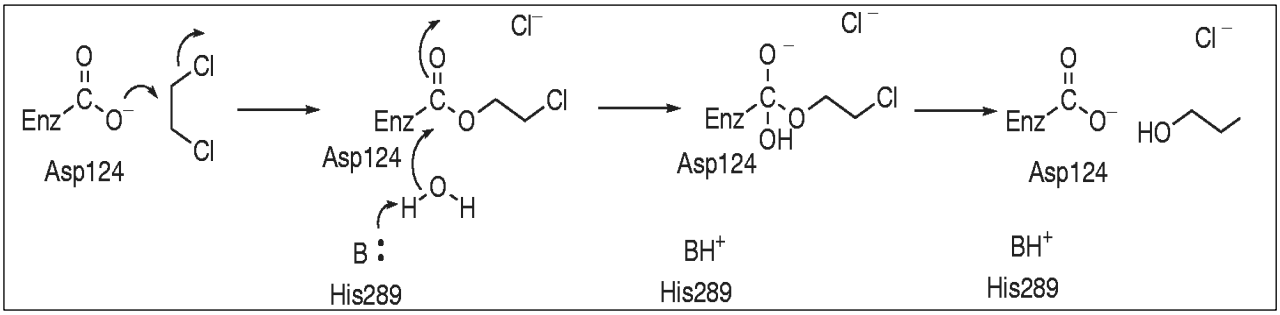

Figure 1.10: Mechanism of haloalkane dehalogenase (Copley, 1996)

Structurally hydrolytic dehalogenases belong to the α/β-hydrolase superfamily. The three-dimensional structures of the three haloalkane dehalogenases reveal two common domains: the α/β-hydrolase core domain (which is conserved in members of the α/β -hydrolase superfamily) and a helical cap domain. The α/β -hydrolase fold is composed of an eight-stranded mostly parallel β-sheet flanked by α-helices and serves as a scaffold for the main catalytic residues. The cap domain composed of a few helices inserted into the catalytic domain usually C-terminally to β strand 6, has been found in the structure of many α/β -hydrolases (not only haloalkane dehalogenases) and is known to influence the substrate specificity of these enzymes. The active site cavity is located between the main domain and the cap domain. A catalytic pentad of residues that is essential for hydrolysis has been identified and it includes aspartic acid (nucleophile), histidine (base), aspartic acid or glutamic acid (catalytic acid), and two halide-stabilizing residues, tryptophan and tryptophan or asparagine (Chovancova *et al*., 2007).

The catalytic pentad and the oxyanion hole are two structural features of haloalkane dehalogenases that are important for their catalytic function. The catalytic pentad is made up of a catalytic triad and two hydrogen bond-donating residues. Catalytic triad is implicated in the covalent bond breakage or formation while the latter provides stabilization to the leaving group (Janssen, 2004). Two of the three residues of the catalytic triad are conserved in DhlA, DhaA and LinB. An aspartic acid localized on a very sharp turn after the strand serves as the nucleophile and histidine localized on the loop after the eighth strand serves as the base (Fig. 1.11). An aspartic acid localized after strand seven in DhlA or a glutamic acid localized after strand six in LinB and DhaA serves as the catalytic acid. One of the two residues involved in the halide binding, i.e. tryptophan located directly next to the nucleophilic aspartate, is invariable. The second halide-stabilizing residue is represented by a tryptophan localized in helix four of the cap domain (in DhlA) or an asparagine positioned on the loop between strand three and the helix one (in DhaA and LinB) (Pavlová *et al*., 2007).

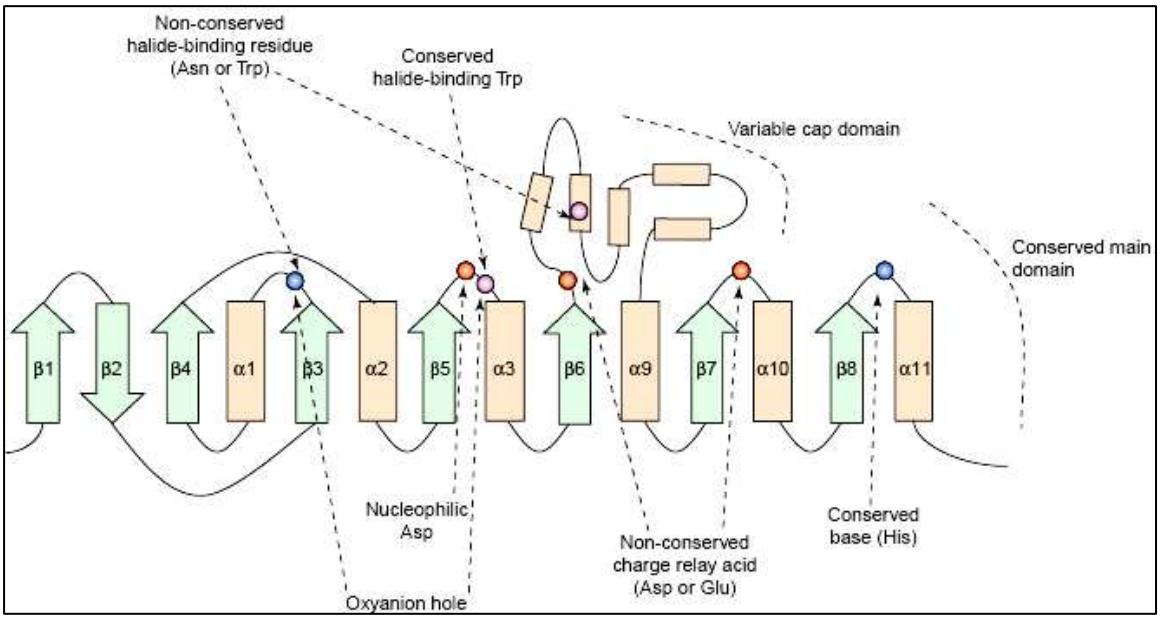

Figure 1.11: General topology of the haloalkane dehalogenase (Janssen, 2004)

1.5.2 Alcohol dehydrogenase

These enzymes are categorized into four types i.e. three alcohol dehydrogenases and one methanol dehydrogenase. Methanol dehydrogenases are located in the periplasmic space of methylotrophic bacteria which catalyze the oxidation of methanol to formaldehyde. The physiological electron acceptor is a soluble cytochrome C_L. The enzyme has a $\alpha_2\beta_2$ tetrametric formation with small β-subunits folding around the surface of the α-subunits. It also contains pyrroloquinoline quinone (PQQ) which is bound within the α-subunit and Ca^{2+} maintaining it in the active site with the required configuration. PQQ is the non-covalently bound prosthetic group of many quinoproteins (Shibata *et al*., 2001).

The other type of alcohol dehydrogenase is a soluble quinoprotein ethanol dehydrogenase derived from *Pseudomonas aeruginosa* that is similar to methanol dehydrogenases. However, these enzymes have a lower affinity to methanol. The remaining two types of alcohol dehydrogenases are soluble and membrane bound quinohaemoprotein alcohol dehydrogenases (Shibata *et al*., 2001). The alcohol dehydrogenase responsible for 2-chloroethanol conversion is the periplasmic quinoprotein alcohol dehydrogenase, also commonly present in methylotrophic bacteria. The tetrameric enzyme is composed of 60-kDa and 10-kDa subunits. The enzyme is induced by methanol or 2-chloroethanol (Janssen *et al*., 1995).

1.5.3 Chloroacetaldehyde (CAA) dehydrogenase

Aldehyde dehydrogenases form a large group of detoxifying enzymes that catalyze the NAD $(P)^+$- dependent oxidations of a broad spectrum of aliphatic and aromatic aldehydes to their corresponding carboxylic acids (Bergeron *et al*., 1998). The 1,2-DCA degrader, *X. autotrophicus* GJ10 show the presence of both a plasmid encoded and chromosomally encoded aldehyde dehydrogenase. The chromosome, however, carries a relatively inactive counterpart (Bergeron *et al*., 1998). Genetic studies have indicated that chromosomally encoded genes are also essential for chloroacetaldehyde metabolism (Janssen *et al*., 1999).

Other acetaldehyde dehydrogenases have been found to play a role in the degradation pathways of other xenobiotic compounds. A variety of enzymes have evolved to metabolize aldehydes to less reactive forms. The most effective pathway for aldehyde metabolism is their oxidation to carboxylic acids by aldehyde dehydrogenases. Regardless of their specificity, these NAD/NADP-dependent enzymes share common structural and functional features that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes (Sripo *et al*., 2002).

1.5.4 Haloacid (HA) dehalogenases

Haloacid dehalogenases catalyze the hydrolysis of halogenated carboxylic acids, such as 2-chloroacetate, an intermediate formed in the 1,2-DCA degradation pathway. Eighteen haloalkanoic acid dehalogenases have been investigated (Hill *et al*., 1999) and these enzymes are generally found to cluster in the phylum *Proteobacteria* (Kerr and Marchesi, 2006).

1.5.4.1 Types of 2-haloacid dehalogenases

2-Haloacid dehalogenases are classified into three groups based on their substrate specificities. L-2-Haloacid dehalogenases catalyze the dehalogenation of L-2-haloalkanoic acids, whereas D-2-haloacid dehalogenase acts on D-2-haloalkanoic acid. DL-2-haloacid dehalogenase acts on both enantiomers with the inversion of the C2 configuration of the substrates (Kurihara *et al*., 2000).

1.5.4.2 Mechanism and structure of the 2-haloacid dehalogenase

2-Haloacid dehalogenases are dimers with two or three domains per subunit. There is a core domain with a Rossmann-fold-like six-stranded parallel β-sheet flanked by five α-helices, a subdomain consisting of a four helix bundle and in some enzymes, a dimerization domain of two antiparallel α-helices (Kurihara *et al*., 2000). The fold in

haloacid dehalogenases is completely different from the α/β-hydrolase fold of the haloalkane dehalogenases. Haloacid dehalogenases use an aspartate based catalytic mechanism that proceeds via a covalent intermediate but there is no histidine to activate a nucleophilic water molecule. In addition, the halide binding site is very different. The activation mechanism of the water molecule is unknown but it has been suggested that another aspartate in the active site fulfills this function (De Jong and Dikstra, 2003).

2-Haloacid dehalogenases catalyze the hydrolysis of halogenated carboxylic acids, such as 2-chloroacetate which is an intermediate in the degradation of 1,2-DCA. They are members of the haloacid dehalogenase (HAD) superfamily (Fig. 1.12). Two reaction mechanisms have been proposed for the L-2-haloacid dehalogenase. Firstly, a carboxylate group of the enzyme attacks the α-carbon atom of the substrate to release the halogen atom. This results in the formation of an ester intermediate which is then hydrolysed to D-2-hydroxyalkanoic acid and regeneration of the active-site carboxylate group. In contrast, the second mechanism involves a water molecule that attacks the substrate to displace the halogen atom (Kurihara *et al*., 2000).

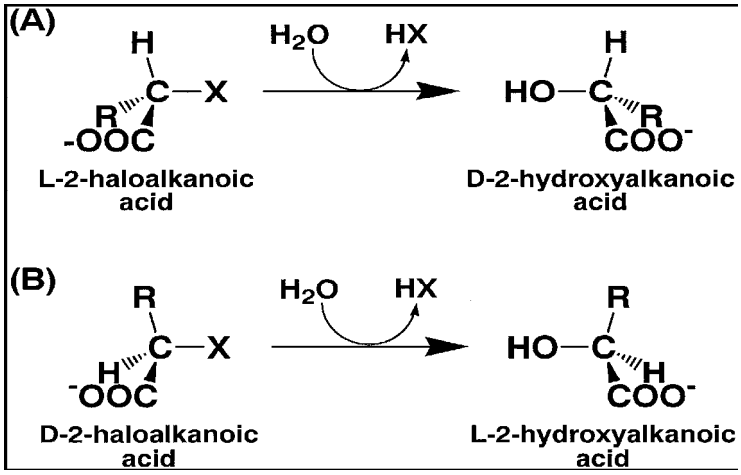

Figure 1.12: Reactions catalyzed by 2-haloacid dehalogenases. (A) L-2-Haloacid dehalogenase (B) D-2 haloacid dehalogenase. R indicates an alkyl group (Kurihara *et al*., 2000)

1.6 BIOREMEDIATION

Bioremediation is an *in situ* clean-up strategy which uses the ability of microorganisms to decrease the concentration and/or toxicity of a considerable number of contaminants. It is a cost-effective, versatile, environment-friendly treatment strategy that has a significant role in the rapidly developing field of environmental renewal (Jain *et al*., 2005).

Bioremediation utilizes the ability of microorganisms to degrade and/or detoxify chemical substances (Jain *et al*., 2005). Strategies involved in bioremediation are bioattenuation, biostimulation and bioaugmentation. Bioattenuation is monitoring the natural progress of degradation over time so as to ensure the decrease of the contaminant with each sampling time. Biostimulation is the intentional stimulation of indigenous microorganisms by electron acceptors/donors, water, or nutrient addition (Widada *et al*., 2002). Bioaugmentation is the addition of contaminant degrading organisms, which have higher activity or greater specificity towards the contaminant (Van den Wijngaard *et al.*, 1993) and can speed-up the degradation process. The recalcitrance of many synthetic chemicals to biodegradation is due to the lack of enzymes at critical steps in catabolic pathways. Therefore the discovery of new catabolic pathways leading to mineralization of these recalcitrant compounds would be valuable and afford a better knowledge of the diversity of catabolic pathways for the degradation of chlorinated compounds as well as bring valuable information for bioremediation processes (Jain *et al*., 2005).

1.6.1 Advantages of bioremediation

Bioremediation is a natural process as it employs the use of living organisms. It is an economical, versatile, environment-friendly and efficient treatment strategy and is gradually making inroads for environmental clean-up treatments (Singh *et al*., 2008). The residues for the treatment are usually harmless products and include carbon dioxide, water, and cell biomass. Bioremediation is also useful for the complete destruction of a wide variety of contaminants with no movement of contaminants from one environmental

medium to another. Another advantage is that it can be carried out on site, without causing too much of interference in normal activities (Vidalli, 2001).

1.6.2 Disadvantages of bioremediation

Regardless of the fact that microorganisms capable of degrading pollutants are usually already present in contaminated soils and aquifers, local environmental conditions could be unfavourable in these areas (Fantroussi and Agathos, 2005). This could make it difficult to extrapolate from bench and pilot-scale studies to full-scale field operations (Vidalli, 2001). Some other concerns of bioremediation are that the products formed after biodegradation may be more persistent or toxic than the parent compounds. Since biological processes are often very specific, bioremediation is limited to those compounds that are biodegradable. Lastly, an obvious disadvantage of bioremediation is that it may take longer than other treatment options (Vidalli, 2001).

Therefore, to gain maximum benefits from this process, molecular biology is being employed to gain a better understanding of the microorganism's natural transformational ability at the genetic level, to accelerate the progress of designer microbes for improved hazardous waste removal (Fantroussi and Agathos, 2005).

1.7 PURPOSE OF THE STUDY

1,2-DCA is a highly recalcitrant and toxic compound, both to humans and the environment. The highly reactive intermediates of 1,2-DCA catabolism, chloroacetaldehyde and halocarboxylic acids, further contribute to its toxicity. This is due to the electrophilic nature of the carbonyl groups found in chloroacetaldehyde and the recalcitrant carbon-chlorine bond in halocarboxylic acids. CAA dehydrogenases play a crucial role in 1,2-DCA degradation as it prevents an accumulation of 2-chloroethanol, which is lethal to the bacterial cell. Also, HA dehalogenase, the last enzyme of the pathway, finally cleaves the recalcitrant carbon-chlorine bond, with the release of

harmless substances. Thus, the presence of these enzymes in 1,2-DCA degrading microorganisms is important for complete degradation to occur. Furthermore, characterizing their degradative ability on 1,2-DCA and other halogenated compounds may enable the possible use of these strains in biological systems for the effective removal of such toxic xenobiotics.

The hydrolytic pathway of 1,2-DCA has extensively been studied in *X. autotrophicus* GJ10, isolated from activated sludge in a wastewater treatment plant. It was one of the first organisms to demonstrate the use a of number halogenated short chain hydrocarbons as carbon sources (Erable *et al.,* 2006). It was found to possess two hydrolytic dehalogenases in its degradation pathway. The haloalkane dehalogenase acts on 1,2-DCA while the haloacid dehalogenase acts on monochloroacetic acid (MCA). Both MCA and 1,2-DCA are regarded as priority pollutants by the Environmental Protection Agency. Thus, there is a need for the removal of such chlorinated compounds from the environment. However, the use of an organism such as *X. autotrophicus* GJ10 is limited for *in situ* bioremediation as it produces extracellular polysaccharides at concentrations higher than 5 mM thus reducing 1,2-DCA uptake into the bacterial cell (Mena-Benetez *et al*., 2008). Since the characteristics of this organism do not make it suitable for certain applications, there is a need to obtain novel 1,2-DCA degraders with different properties which can then be applied in bioremediation strategies for optimal removal of chlorinated compounds. Five *Ancylobacter* strains were previously isolated in our laboratory, based on their ability to utilize 1,2-DCA as a sole carbon source. These isolates were identified by 16S rDNA sequencing and found to be different strains of *Ancylobacter aquaticus*. One of the main objectives of this study was to determine the ability of these strains to utilize 1,2-DCA, as well as other halogenated aliphatic hydrocarbons, as a carbon source. The degradation potential of these organisms will also be compared to the well characterized *X. autotrophicus* GJ10 and two previously characterized *Ancylobacter aquaticus* strains AD25 and AD27. *Ancylobacter aquaticus* AD25 and AD27 were previously isolated from brackish water sediment with 2-chloroethylvinylether supplied as carbon source and was later reported to use 1,2-DCA as sole carbon source. Both strains possess the catabolic pathway elucidated in *X. autotrophicus* GJ10 (Fig. 1.8) as

well as the *dhlA* gene which is identical to the gene of *X. autotrophicus* GJ10. *A. aquaticus* AD25 was also shown to have a higher affinity for 1,2-DCA than *X. autotrophicus* GJ10, as a result of its higher dehalogenase content. Comparing the South African isolates to these well characterized strains might give a better understanding of the degradation potential of these strains and its suitability for bioremediation strategies.

South African *A. aquaticus* strains were also found to follow the catabolic pathway elucidated in *X. autotrophicus* GJ10 (Fig. 1.8). Incidentally, these strains also possess the first enzyme of the pathway, the hydrolytic dehalogenase, encoded by *dhlA* gene. It was therefore of interest to determine if these isolates possess the other important genes involved in the 1,2-DCA degradation pathway and whether homologs of these genes exist. Therefore, the other objective of this study was to investigate the presence of the CAA dehydrogenase and HA dehalogenase encoding genes (latter two enzymes of the pathway) in isolates of *Ancylobacter* using Southern hybridization technique. The control strain used for all experiments was *X. autotrophicus* GJ10 with the *aldB* and *dhlB* genes, encoding the CAA dehydrogenase and haloacetate dehalogenase enzymes respectively, being used as the probes during the hybridization experiments.

1.8 HYPOTHESIS

It is hypothesized that the characterization of 1,2-dichloroethane-degrading *Ancylobacter* strains as well as the genes and enzymes involved in the degradation pathway may provide a better understanding of the potential application of the organisms and/or their enzymes in bioremediation processes.

1.9 OBJECTIVES

1.9.1 To establish the 1,2-dichloroethane degradation potential of *Ancylobacter* strains;

1.9.2 To characterize CAA dehydrogenase and HA dehalogenase-encoding genes in strains of *Ancylobacter* isolates; and

1.9.3 To investigate and compare the specific dehalogenase activities of the *Ancylobacter* strains under various conditions.

1.10 AIMS

1.10.1 To determine the growth rates of the *Ancylobacter* strains in 1,2-dichloroethane and monochloroacetic acid;

1.10.2 To monitor the biodegradation of 1,2-dichloroethane by pure cultures of *Ancylobacter* strains;

1.10.3 To determine the pH and temperature optima of the haloalkane and haloacid dehalogenases in crude extracts of *Ancylobacter* strains;

1.10.4 To determine the substrate range of the dehalogenase enzymes in crude extracts of *Ancylobacter* strains;

1.10.5 To identify the presence of CAA dehydrogenase and HA dehalogenase genes in *Ancylobacter* strains that are capable of growing on 1,2-dichloroethane as a sole carbon source; and

1.10.6 To clone and sequence CAA dehydrogenase and HA dehalogenase genes obtained from these strains.

CHAPTER TWO
MATERIALS AND METHODS

2.1 GROWTH AND MAINTENANCE OF BACTERIAL CULTURES

Xanthobacter autotrophicus GJ10 and seven *Ancylobacter aquaticus* strains, isolated in previous studies (Govender, 2009; Janssen *et al*., 1985; Van den Wijngaard *et al*., 1992), were used in this study (Table 2.1).

Table 2.1: Bacterial strains used in this study

Bacterial strains	Source
X. autotrophicus GJ10	Activated sludge (wastewater treatment plant), Netherlands (Janssen *et al*., 1985)
A. aquaticus AD25	Brackish water sediment (Eems Channel near Delfjizil), Netherlands (Van den Wijngaard *et al*., 1992)
A. aquaticus AD27	Brackish water sediment (Eems Channel near Delfjizil), Netherlands (Van den Wijngaard *et al*., 1992)
Ancylobacter aquaticus DH12	Northern Wastewater Treatment Works, KwaZulu-Natal (Govender, 2009)
Ancylobacter aquaticus DH5	Northern Wastewater Treatment Works, KwaZulu-Natal (Govender, 2009)
Ancylobacter aquaticus DH2	Northern Wastewater Treatment Works, KwaZulu-Natal (Govender, 2009)
Ancylobacter aquaticus UV6	SAPPI Wastewater Treatment, KwaZulu-Natal (Govender, 2009)
Ancylobacter aquaticus UV5	SAPPI Wastewater Treatment, KwaZulu-Natal (Govender, 2009)

Pure strains of these isolates were maintained on Luria-Bertani (LB) agar plates at 30°C. Long term preservation was done by inoculation of cultures into micro-banks, which were stored at -70°C, until use.

2.2 STANDARDIZATION OF BACTERIAL STRAINS FOR INOCULATION INTO GROWTH MEDIA

Pre-cultures of individual isolates (Table 2.1) were inoculated into sterile 100 ml minimal salts media (MSM) (Appendix I), as previously described by Janssen *et al*. (1985), containing 500 µl 1 × trace element solution (Appendix I) and 100 µl 1 × vitamin solution (Appendix I), in 250 ml Wheaton serum bottles. These were incubated at 30^{o}C on a rotary shaker at 150 rpm for three days. Cells were pelleted at 5000 × *g* for 15 min at 4^{o}C in a Beckman Model J2-21 centrifuge, washed twice with 10 mM Tris.SO_4 buffer (pH 7.5) and finally re-suspended in 5 ml of the same buffer. The inocula were standardized, using a spectrophotometer (UltroSpec II, KB), to an optical density of 1.0 at a wavelength of 450 nm, prior to inoculation.

2.3 GROWTH MEASUREMENT STUDIES OF *Ancylobacter aquaticus* ISOLATES ON VARIOUS HALOGENATED COMPOUNDS

The growth patterns of the bacterial isolates in the presence of 1,2-DCA as a sole carbon source was monitored in two different media types. One hundred milliliters of minimal salts media containing either 10 mg/l yeast extract (MMY) or a 1 × vitamin solution (MMV) (Van den Wijngaard *et al*., 1993), was inoculated with a 1% standardized culture of the bacterial isolates. Batch experiments were performed in 250 ml Wheaton serum bottles. 1,2-DCA was added to a final concentration of 5 mM and immediately sealed with Teflon-lined screw caps to prevent evaporation of the volatile compound. Batch experiments with monochloroacetic acid (5 mM) as a sole carbon source were set-up in 250 ml flasks at two pH values of 7 and 9. Batch experiments were also set-up for 1,3-dichloropropene (5 mM), and 1,3-dibromopropane (5 mM) as sole carbon source in 250 ml Wheaton serum bottles. The bottles and flasks were incubated at 30^{o}C with shaking at 150 rpm for 84 h. Two negative controls were used in all experiments. A non-inoculated control was used to determine the abiotic loss of the volatile compounds. The second control contained an organism not known to degrade halogenated compounds i.e.

Escherichia coli DH5αF'. One milliliter samples were removed every 12 h using a sterile syringe and the optical density was measured at 450 nm using a spectrophotometer. Growth rate constants of individual microorganisms were determined based on the growth curve using the equation (Rikvkina *et al*., 2000):

$$k = \frac{\log N_t - \log N_0}{0.301t}$$

2.4 DETERMINATION OF FREE HALIDE RELEASED DURING DEGRADATION OF HALOGENATED COMPOUNDS

The colorimetric method of Bergmann and Sanik (1957) was used to detect and quantify free chlorides in the culture medium during growth of all bacterial isolates investigated (Table 2.1). The method used for the assay was adapted from Olaniran *et al.* (2005). One millilitre samples were removed from Wheaton bottles and centrifuged at 10 000 × *g*. The clear supernatant obtained after centrifugation was treated with 0.2 ml of reagent I (0.03 M ferric ammonium sulphate in 9 M HNO_3) and 0.4 ml of reagent II (saturated mercury thiocyanate in 95% ethanol). This was then incubated for 10 min at room temperature to allow for colour development. The absorbance of the product $Fe(SCN)^{2+}$ was measured spectrophotometrically at 460 nm using a standard UV/VIS spectrophotometer (Ultrospec II, LKB). A standard curve to determine released chloride and bromide concentration was prepared using different dilutions of a 2 mM KCl and 2 mM KBr stock solution, respectively. A 1 ml solution of the different concentrations of KCl and KBr was treated as described for the culture filtrate above. The chloride and bromide ion concentrations were determined from the regression equation obtained from plotting the absorbance readings against the salt concentrations. All assays were performed in triplicate. Standard deviation and T-test analysis was done using Microsoft Excel.

2.5 MEASUREMENT OF DEHALOGENASE ENZYME ACTIVITIES

2.5.1 Preparation of crude extracts

Crude extracts were prepared from 500 ml cultures (refer to Table 2.1) grown to the late exponential phase and harvested by centrifugation at 10 000 × *g* for 10 min. Cells were then re-suspended in 10 mM Tris.SO_4 (pH 7.5) that contained 1 mM β-mercaptoethanol and 1 mM EDTA (TEM buffer) and washed for 10 min in TEM buffer at 10 000 × *g*. Cells were then disrupted by sonication (SONIC RUPTOR 400) under constant cooling (4°C). A crude extract was obtained after unbroken cells and debris had been removed by centrifugation for 30 min at 12 000 × *g*. To protect enzymes against inactivation, extracts were kept at 0 to 4°C. Protein concentration was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as the reference protein.

2.5.2 Dehalogenase enzyme assays

Haloalkane and haloacid dehalogenase activities were measured colorimetrically by monitoring halide release, a modified method of Song et al. (2004). Haloalkane dehalogenase activity was determined by incubating 0.2 ml crude extract at 30°C with 3 ml of 5 mM 1,2-DCA in 10 mM Tris.SO_4 (pH 7.5). After incubation for 60 min at 30°C, 0.2ml of 0.03 M $NH_4Fe(SO_4)_2$ in 6 M HNO_3 was added, followed by 0.4 ml saturated $Hg(SCN)_2$ in ethanol. Chloride liberation was measured spectrophotometrically (Ultrospec II, LKB) at 460 nm. Haloacid dehalogenase activity was determined similarly but with 5 mM monochloroacetic acid in 50 mM glycine.NaOH buffer (pH 9) as the substrate solution. One unit of dehalogenase activity was defined as the amount of enzyme that catalyzed the formation of 1 μM of halide per min. The effect of pH on enzyme activity was determined by incubating the crude extract at different pH conditions using potassium phosphate (pH 6), 10 mM Tris.SO_4 (pH 7-8) and 50 mM glycine.NaOH (pH 9). To determine the substrate range activities of crude enzyme of the

bacterial strains, different substrates were substituted in the haloalkane dehalogenase enzyme activity assay.

2.6 ISOLATION OF TOTAL DNA

Bacterial isolates (Table 2.1) were grown to late exponential phase in 30 ml of LB broth (10 g tryptone, 5 g yeast extract and 5 g sodium chloride per litre). Total genomic DNA was isolated from all bacterial isolates using the CTAB/NaCl method according to Ausubel *et al.* (1989). To obtain a high yield of genomic DNA, the method was scaled up five times and pellet resuspended in a smaller volume of 10 mM Tris.SO_4 buffer (pH 7.5). Genomic DNA was separated by agarose gel electrophoresis. The samples were run on a 1% agarose gel (1 × TAE buffer) at 100 V for 2 h and stained with ethidium bromide (0.25 µg/ml). Gel images were captured using the Chemi-Genius Bio Imaging System (Syngene). Genomic DNA from all South African isolates as well as *X. autotrophicus* GJ10, *A. aquaticus* AD25 and AD27 were quantified using the GeneQuant, RNA/DNA Calculator (Pharmacia).

2.7 RESTRICTION ENZYME DIGESTION

Total genomic DNA isolated from each strain as per Table 2.1 was digested with restriction endonucleases, *Eco*RI; *Pst*I; *Bam*HI and *Hin*dIII, following the manufacturer's recommendations. This was to compare location of genes for all isolates. Reaction mixtures consisted of 1 µg DNA, 1 unit (U) restriction endonuclease, 1 × restriction endonuclease buffer and finally brought up to a volume of 20 µl with sterile double-distilled water. Reactions were incubated at 37°C overnight, followed by gel electrophoresis on a 1% agarose gel prepared using 1 × TAE buffer (as per section 2.6.

2.8 PCR AMPLIFICATION OF ALDEHYDE DEHYDROGENASE (*aldB*) AND HALOACID DEHALOGENASE (*dhlB*) ENCODING GENES PRESENT IN *Xanthobacter autotrophicus* GJ10

PCR primers were designed based on the known sequences of the region flanking the hydrolytic dehalogenase gene (*dhlA*), aldehyde dehydrogenase genes (*aldA* and *aldB*), from *X. autotrophicus* GJ10 by Govender (2009) (Table 2.2). Primers were synthesized by Inqaba Biotech and delivered in a lyophilized form.

Table 2.2: DNA sequences of primer sets

Primer name	Sequence
*dhlB*R	5' CCAATGGCCGCCGCCCATCTC 3´
*dhlB*F	5´ CTGCGCGATGATGCGCAGGCC 3´
*aldB*R	5´ CGGTTAAGCCCCGGGAGGGGG 3´
*aldB*F	5´ GCCCAGCCTTTTCTCGAGCG 3´

Genomic DNA isolated from *X. autotrophicus* GJ10 was used as template DNA in a PCR reaction containing 100 pmol of each primer, 100 µM each of deoxynucleoside triphosphates (dNTPs), 1 × Supertherm *Taq* polymerase buffer and 0.5 U Supertherm *Taq* polymerase (Southern Cross Biotech). The final volume was brought up to 50 µl with sterile double-distilled water. PCR was performed using the PE-900 PCR thermal cycler (Perkin-Elmer). PCR conditions were modified from Govender (2009). Conditions for the *dhlB* primer set were as follows: an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 90 s and a final extension at 72°C for 5 min. PCR conditions for the *aldB* primer sets were the same as for the *dhlB* primers, but, except that the annealing temperature was 62°C. The amplification products were then separated on a 1% agarose gel as described in section 2.6. Amplified PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were again visualized as described in section 2.6.

2.9 SOUTHERN HYBRIDIZATION

Restricted genomic DNA was separated by electrophoresis on a 0.8% agarose gel. A lower percentage gel increases the speed of DNA transfer. Southern Blotting was performed as per Sambrook *et al*., (1989). The gel was incubated with 0.25 mM HCl for 10 min and then treated with denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 2 × 15 min. Denatured DNA fragments were then neutralized with a neutralization buffer for 2 × 15 min. The gel was equilibrated for 20 min in 20 × SSC (3 M NaCl, 0.3 M Sodium Citrate). The treated DNA fragments were subsequently transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech) by overnight capillary transfer. Following the overnight transfer, the membrane was rinsed in 2 × SSC solution and air dried.

Purified PCR products, *dhlB* and *aldB*, obtained from *X. autotrophicus* GJ10 was labeled with digoxigenin-11-dUTP using the nonradioactive DIG High Prime DNA Labeling and Detection Kit (Roche). The labeling reaction was performed according to the manufacturer's instructions. Subsequently, hybridization was carried out at 40-42°C overnight with gentle agitation. This was then followed by the stringency washes. Firstly, the membrane was washed (2 × 5 min) in approximately 30 ml 2 × SSC containing 0.1% SDS at room temperature with constant agitation. Secondly, the membrane was washed (2 × 15 min) in 0.5 × SSC containing 0.1% SDS, (pre-warmed to 65°C), and incubated at 65°C with gentle agitation. Finally, colorimetric detection of the hybridization signals was performed according to the manufacturer's protocol (Roche).

2.10 CLONING AND SEQUENCING OF THE *aldB* AND *dhl*B GENES

2.10.1 Purification of DNA fragments

Total genomic DNA, from organisms producing positive signals, was restricted as per section 2.6 and run on a 1% agarose gel at 50 V for 6 hours. Regions showing a positive

signal on the nylon membrane were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions.

2.10.2 Preparation of electrocompetent *E. coli* cells

Three milliliters of an overnight *E. coli* DH5α'F LB broth culture was used as a pre-culture to inoculate 100 ml LB medium. This was incubated at 37°C, with shaking at 150 rpm, until the cell density reached 0.5 at OD_{600nm}. The culture was then immediately placed on ice to prevent further growth. The cells were harvested by centrifugation at 10 000 × *g* for 10 min. The resultant cell pellet was washed consecutively with 100 ml, 50 ml and 5 ml cold 10% (v/v) glycerol with centrifugation at 10 000 × *g* for 10 min after every wash. Lastly, the pellet was re-suspended in a final volume of 2.5 ml cold 10% glycerol and subsequently transferred as 80 µl aliquots into microfuge tubes. The cells were snap frozen with -70°C absolute ethanol and stored at -70°C until further use.

2.10.3 Isolation and restriction of pBluescript II SK

E. coli DH5α', containing pBluescript II SK, was grown overnight at 37°C in 3 ml LB broth with 100 µg/ml ampicillin. Isolation of the plasmid was performed using the High Pure Plasmid Isolation Kit (Roche) according to the manufacturers' instructions. Briefly, the cells were pelleted and re-suspended in 250 µl lysis buffer with gentle mixing by inverting the tube six times. The lysed solution was then treated with 350 µl cold binding buffer followed by gentle mixing. At this point the solution became cloudy with the formation of a flocculant precipitate. The flocculant was removed by centrifugation at 13 000 × *g* for 10 min. The supernatant was transferred to a high pure filter spin column and centrifuged at full 15 800 × *g* for 1 min. The flowthrough liquid was discarded and the cells were washed with 700 µl wash buffer II. Finally the plasmid DNA was eluted with 50 µl elution buffer. Isolated pBSK was restricted with the corresponding restriction endonucleases, *Eco*RI; *Pst*I; *Bam*HI and *Hin*dIII, for cloning procedures. The reaction mixtures were as follows: 1 U restriction enzyme, 1 U restriction enzyme buffer, 1 U alkaline phosphatase, in a final volume of 20 µl. The reaction was incubated at 37°C for 1 h.

2.10.4 Ligation of inserts to plasmid vector

Linearized pBluescript II SK was purified using the Qiagen PCR Purification Kit (Roche) according to manufacturer's instructions. Ligation of the purified inserts to restricted plasmids took place overnight at room temperature using a 5:1 (insert: plasmid) ratio. Each reaction contained 1 µl plasmid DNA, 5 µl insert, 1 µl T4 DNA ligase and 1 µl T4 ligase buffer which was brought up to a final volume of 10 µl with sterile double-distilled water.

2.10.5 Transformation of *E. coli* DH5α'F

Transformation of electrocompetent cells was performed using the Gene Pulser (BIORAD) using the method by Ausubel *et al.* (1989). The apparatus was set at 25 µF, 2.5 kV and 200 Ω for a 0.2 cm cuvette. Two and a half microlitres of the ligation mixture was added to 80 µl of electrocompetent *E. coli* cells and transferred to a chilled BioRad cuvette. Following electroporation, the cells were immediately suspended in 1 ml of pre-warmed SOC medium (Appendix I), to enhance the survival of transformants. The SOC medium containing transformants were incubated, with shaking, for 1 h at 37°C. To obtain a high number of clones, the cells were then pelleted and spread onto LB agar containing 100 µg/ml ampicillin, 50 µl of 100 mg/ml X-gal and 20 µl of 1M IPTG. The plates were incubated for 16 h at 37°C. Selection of positive clones was based on blue-white selection. The white clones were transferred to a fresh LB agar plate containing ampicillin for further analysis.

2.10.6 Plasmid DNA isolation

Plasmid DNA was isolated using a modified procedure of the alkaline lysis method of Birnboim and Doly (1979). Clones were grown overnight at 37°C in 3 ml LB broth containing 100 µg/ml ampicillin. Bacterial cells were harvested by centrifugation at 15 800 × g for 3 min in a microcentrifuge tube. All traces of the supernatant were removed and the pellet was re-suspended in 100 µl of Solution A (25 mM Tris.Cl; 50 mM glucose; 10 mM EDTA; pH 8) containing 100 µg/ml RNase A. The cell suspension was lysed by addition of 200 µl of freshly prepared Solution B (0.2 M NaOH; 1% SDS)

with immediate inversion of the microcentrifuge tubes and incubated for less than 5 min at room temperature. As soon as the cell suspension turned clear (indicating cell lysis), it was neutralized by the addition of Solution C (3 M sodium acetate, pH 4.8) and incubated on ice for 5 min. Precipitated material was removed by centrifugation at maximum speed (15 800 × *g*) for 15 min. DNA in the supernatant was precipitated by the addition of 2 volumes of cold 100% ethanol and incubation at -70°C for 15 min. The precipitated DNA was centrifuged for 15 min and washed once in 70% ethanol. The DNA was pelleted, air-dried and thereafter dissolved in 20 µl of 10 mM Tris.Cl (pH 8) and stored at -20°C.

CHAPTER THREE

RESULTS

3.1 DEGRADATION OF HALOGENATED ALIPHATIC HYDROCARBONS

3.1.1 Aerobic utilization of 1,2-DCA

3.1.1.1 Growth pattern of *Ancylobacter* strains in minimal media supplemented with either yeast extract or vitamins

Nine isolates, including recently isolated *Ancylobacter* species and *X. autotrophicus* GJ10 were investigated for their ability to utilize 1,2-DCA as a carbon source, by monitoring their growth patterns over time. Generally, a lag period of 12 h was observed during the growth of the isolates on 1,2-DCA, followed by an increase in growth (Fig. 3.1). Optimum growth was observed in minimal media supplemented with yeast extract after 36 h incubation period for isolate *Ancylobacter aquaticus* DH12 with an optical density of 0.462 at 450 nm (Fig. 3.1). This was followed very closely by *Ancylobacter aquaticus* DH5 with optical density of 0.46, *Ancylobacter aquaticus* UV5 with 0.444 and *A. aquaticus* AD25 with 0.45 after 48 h. Both *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* UV6 reached the end of exponential phase after 60 h with optical density readings of 0.47 and 0.42, respectively (Fig. 3.1). *A. aquaticus* AD27 had the slowest growth and a gradual increase in optical density over the 84 hr incubation period was observed, reaching an optical density of 0.291 at 84 h. This is in contrast to the other isolates which reached the end of the exponential phase after 60 h (Fig. 3.1). No significant increase in turbidity was observed in the un-inoculated control and negative control inoculated with *E. coli* (Fig 3.1).

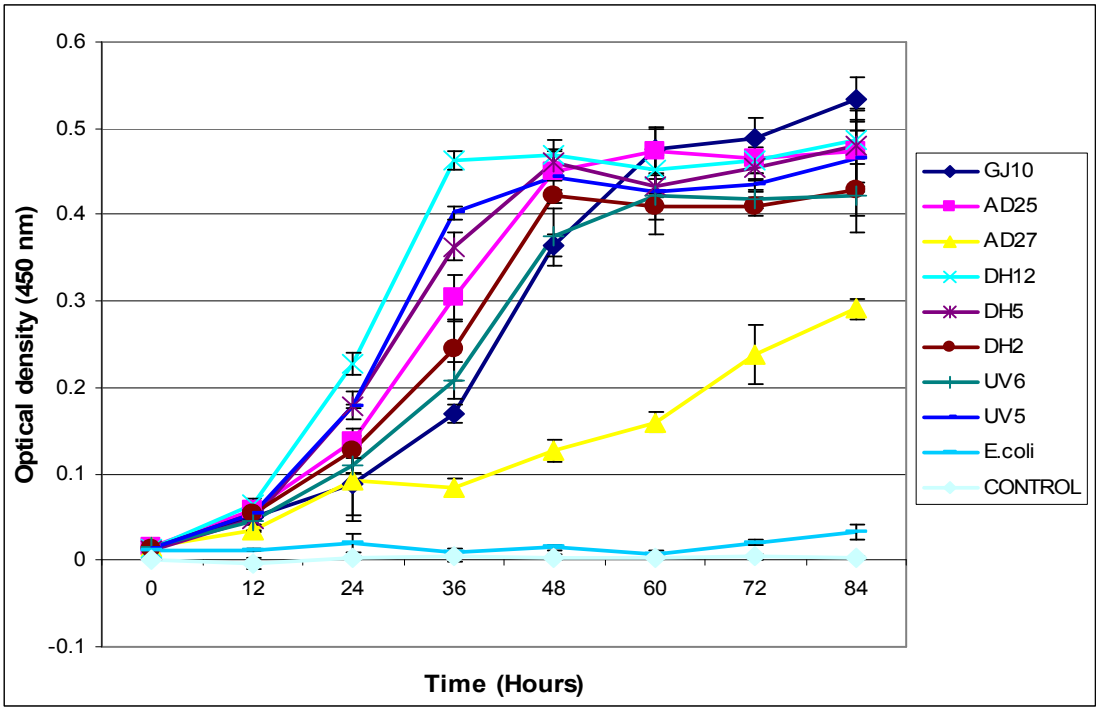

Figure 3.1: Growth pattern of bacterial isolates using 1,2-DCA as sole carbon source supplemented with yeast extract

The growth curves obtained for isolates growing with vitamins as a supplement show a similar trend to those observed for yeast extract. The end of lag phase and beginning of exponential phase was observed after 12 h (Fig. 3.2). After 24 h *Ancylobacter aquaticus* DH12 had the highest optical density value of 0.22, followed by *A. aquaticus* AD25 and *Ancylobacter aquaticus* DH5 with peak optical density values of 0.171 and 0.156, respectively (Fig. 3.2). *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5 had very similar optical density values of 0.12, 0.114 and 0.118 (Fig. 3.2). Within 36 h, *Ancylobacter aquaticus* DH12 and *Ancylobacter aquaticus* DH5 reached the end of the exponential phase with optical density values of 0.502 and 0.484, respectively. While the rest of the isolates reached their end points of exponential growth within 48 h, *A. aquaticus* AD27 showed a very gradual increase in growth over the 84 hr period with an optical density value of only 0.265 at 84 h (Fig. 3.2). This further indicates that *A. aquaticus* AD27 is a slow grow of 1,2-DCA in the MSM with vitamins. No significant increases in turbidity were seen in negative controls.

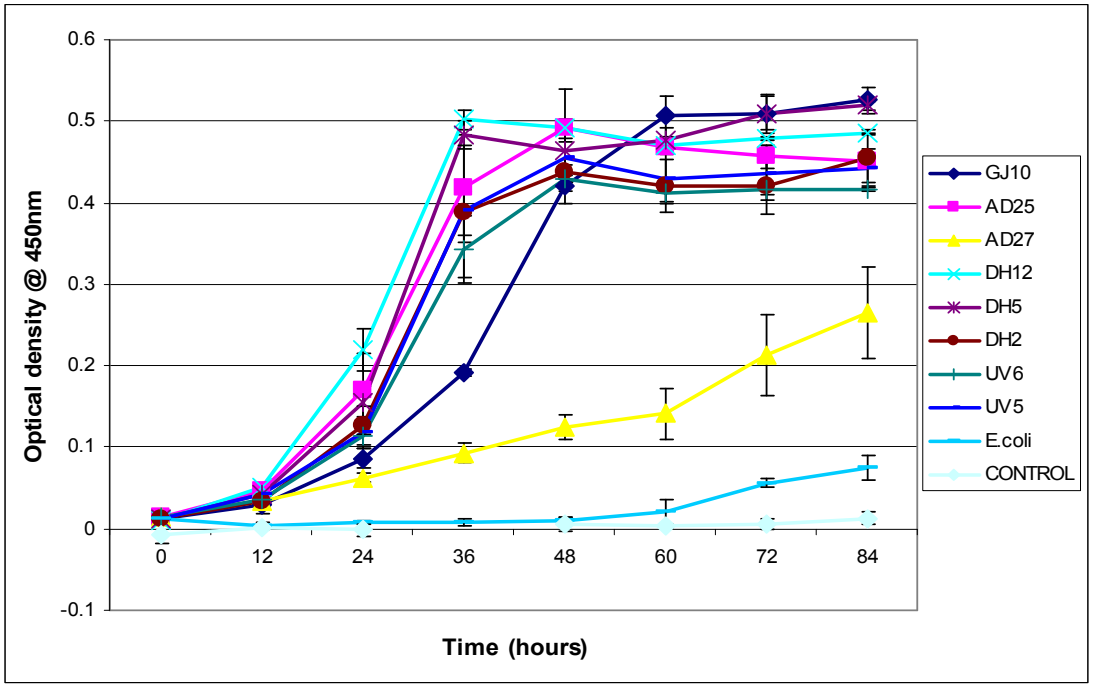

Figure 3.2: Growth pattern of bacterial isolates using 1,2-DCA as sole carbon source supplemented with vitamins

The growth rates of the *Ancylobacter* isolates were determined using two different media types, i.e., minimal media containing yeast extract (MMY) and minimal media containing a vitamin solution (MMV) and indicated in Table 3.1. Results from optical density readings and growth rate constants indicate that all isolates demonstrate better growth on MMV than on MMY. The isolate with the highest growth rate in MMV is *Ancylobacter aquaticus* DH5; however the isolates with the best growth rates in MMY are both *Ancylobacter aquaticus* DH12 and UV5. The slowest growing isolate on both types of media was found to be *A. aquaticus* AD27 with growth rates of $0.04.h^{-1}$ and $0.037.h^{-1}$ in MMV and MMY, respectively. While the other isolates had already reached stationary phase, *A. aquaticus* AD27 was still on a very gradual incline. Most isolates displayed higher optical density values in MMV, thus correlating to the higher growth rate constants achieved for these isolates. Overall, the growth rates obtained for all recently isolated strains were higher than that obtained for previously isolated strains; *X. autotrophicus* GJ10 ($0.085.h^{-1}$ in MMV and $0.068.h^{-1}$ in MMY), *A. aquaticus* AD25 ($0.094.h^{-1}$ in MMV an $0.0812.h^{-1}$ in MMY), and *A. aquaticus* AD27 ($0.04.h^{-1}$ in MMV and $0.037.h^{-1}$ in MMY).

Table 3.1: Growth rates of *Ancylobacter* isolates during growth in 1,2-DCA supplemented either with vitamins or yeast extract

	Growth rate constants (h^{-1})	
	MMV	**MMY**
X. autotrophicus GJ10	0.0846 ± 0.005	0.068 ± 0.02
A. aquaticus AD25	0.094 ± 0.006	0.0812 ± 0.007
A. aquaticus AD27	0.04± 0.011	0.037±0.003
Ancylobacter aquaticus DH12	0.137 ± 0.002	0.118±0.011
Ancylobacter aquaticus DH5	0.147 ± 0.005	0.0906±0.007
Ancylobacter aquaticus DH2	0.102± 0.009	0.082±0.006
Ancylobacter aquaticus UV6	0.0998± 0.004	0.0844±0.004
Ancylobacter aquaticus UV5	0.1± 0.009	0.12±0.009

3.1.1.2 Chloride release during growth of the bacterial isolates in 1,2-DCA in the presence of either yeast extract or vitamins

The free chloride released into the medium over the 84 h incubation period was monitored to determine how well the isolates were able to degrade 1,2-DCA. A clear trend is evident in all isolates whereby a gradual increase in chloride released was observed over the 84-h period. The most effective organism after 24 h is *Ancylobacter aquaticus* DH5 with 0.285 mM chloride released, followed by strains UV5 and DH12 releasing 0.258 and 0.256 mM chloride, respectively. *Ancylobacter aquaticus* DH2 and UV6 released 0.193 and 0.179 mM chloride, respectively (Fig. 3.3). All recently isolated *Ancylobacter* strains released approximately 2-fold higher chloride ions after 24 h than previously isolated strains. Following an 84 h incubation period, the most effective degrader was *Ancylobacter aquaticus* UV6 which released 0.61 mM chloride, which is higher than the 0.58 mM chloride ions released by *X. autotrophicus* GJ10 at the same time (Fig. 3.3). The general trend observed was that most isolates, except *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* UV6 showed more chloride release during the first 48 h than between 48 h-84 h. *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* UV6 both showed a greater release of chlorides after 48 h. This was followed very closely by *Ancylobacter aquaticus* DH5 with value of 0.545 mM. *Ancylobacter*

aquaticus DH12, DH2 and UV5 also produced a higher concentration of free chlorides than the previously characterized isolates *A. aquaticus* AD25 and *A. aquaticus* AD27. Statistical analysis of chloride ion released by the isolates revealed a significant (p-value < 0.05) difference compared to the negative controls used in the study. *E.coli*, after 84 h, showed some release of halides, while the un-inoculated showed no increase at all.

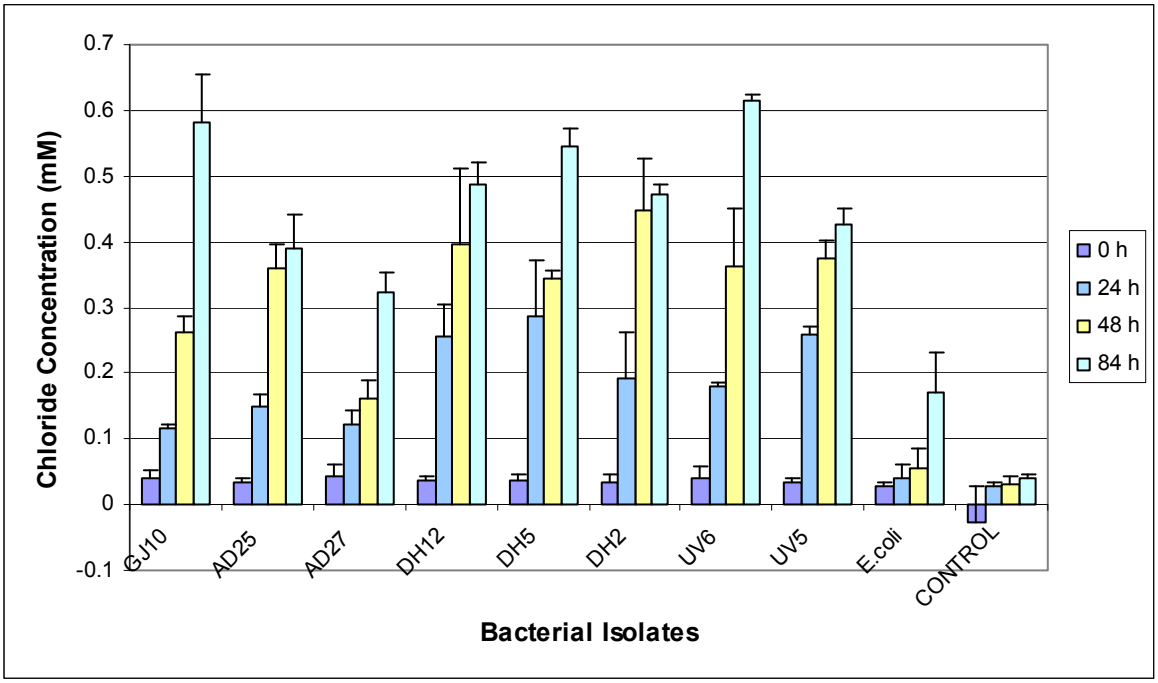

Figure 3.3: Chloride concentration during growth in MSM supplemented with yeast extract in the presence of 1,2-DCA as sole carbon source

The gradual increase of free chloride over the 84 h period is indicative of the ability of the isolates to degrade 1,2-DCA. As shown in Fig. 3.4, the lowest concentration of free chloride was found for isolate *A. aquaticus* AD27 which corresponds to the slow growth of this isolate as shown in Fig. 3.2. After 84 h, the isolate that released the highest amounts of free chloride was *X. autotrophicus* GJ10 with a concentration 0.497 mM. After 24 h, *A. aquaticus* AD25 released the highest amount of free chloride (0.268 mM), followed by strains DH12 and DH5 releasing 0.237 mM and 0.208 mM, respectively (Fig. 3.4). This is in accordance with growth curves for these isolates as they had the highest optical density values after 24 h. After 48 h, the highest concentration of free chloride was released by *Ancylobacter aquaticus* DH2 (0.356 mM). This was followed very closely by strains DH5 and DH12 which released 0.333 and 0.32 mM, chloride

respectively (Fig. 3.4). After 72 h, *Ancylobacter aquaticus* DH2 still had the highest amount of free chloride of 0.469 mM. This was followed by *X. autotrophicus* GJ10 with a free chloride concentration of 0.411 mM, while *A. aquaticus* AD27 still had the lowest chloride concentration of 0.193 mM. Following the 84 h incubation period, *X. autotrophicus* GJ10 was found to release the highest free chloride concentration of 0.497 mM. This was then followed by *Ancylobacter aquaticus* DH2, DH5 and UV5 releasing free chloride concentrations of 0.441, 0.407 and 0.406 mM, respectively. *Ancylobacter aquaticus* DH12, *A. aquaticus* AD25, and *Ancylobacter aquaticus* UV6 lagged behind releasing 0.372, 0.356 and 0.352 mM free chloride, respectively while *A. aquaticus* AD27 released the lowest concentration of 0.333 mM (Fig. 3.4). No significant increase in chloride concentration was observed for both negative controls used.

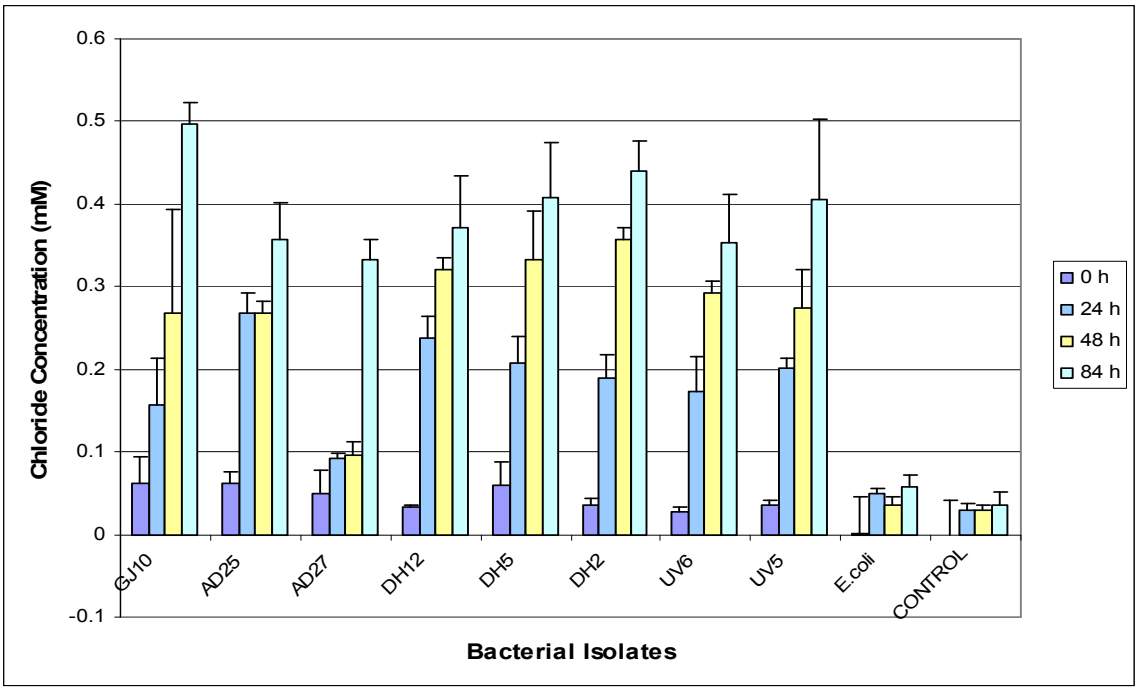

Figure 3.4: Chloride concentration during growth with 1,2-DCA as sole carbon source supplemented with vitamins

3.1.2 Aerobic utilization of MCA by the bacterial isolates monitored in minimal media supplemented with vitamins

3.1.2.1 Growth pattern of bacterial isolates in MCA

Growth of the *Ancylobacter* strains in MCA was monitored at two different pH conditions (pH 7 and pH 9). This was to evaluate whether a neutral or more alkaline pH favoured growth in MCA. At pH 7, all isolates reached the end of their lag phase at 12 h except for *X. autotrophicus* GJ10 and *A. aquaticus* AD27 with extended lag phase up to 24 h (Fig. 3.5). After 24 h, *Ancylobacter aquaticus* DH12 had the highest optical density value of 0.218, followed by *A. aquaticus* AD25 with an O.D of 0.176. *Ancylobacter aquaticus* DH5 and DH2 had very similar values of 0.119 and 0.121, respectively, while *Ancylobacter aquaticus* UV6 and UV5 had O.D values of 0.106 and 0.101. *Ancylobacter aquaticus* DH12 and DH5 reached the end of the exponential phase and beginning of stationery phase after 36 h with O.D values of 0.362 and 0.348, respectively (Fig. 3.5). The remaining isolates reached the end of this phase after 48 h, except for. *X. autotrophicus* GJ10 which took 60 h with a maximum O.D value of 0.367.

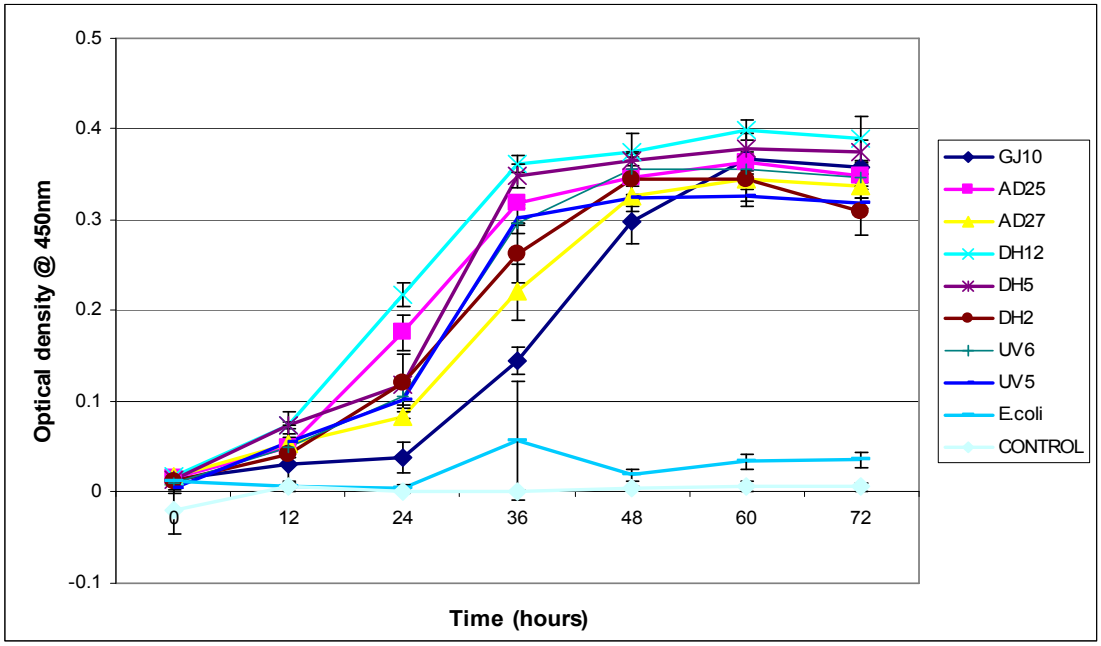

Figure 3.5: Growth pattern of bacterial isolates with MCA as sole carbon source in MSM containing vitamins at pH 7

At pH 9, all isolates demonstrated growth with MCA during the 72 hr incubation period (Fig. 3.6). A similar trend was observed with pH 9 as in pH 7 with *Ancylobacter aquaticus* DH12 having the highest O.D value of 0.144, after 24 h. The slowest grower after 24 h was *X. autotrophicus* GJ10 having an O.D value of 0.06. At 36 h, *A. aquaticus* AD25 had the highest peak O.D value of 0.315, (Fig. 3.6), while *A. aquaticus* AD27 reached the end of exponential phase at 48 h. Other isolates that reached the end of exponential phase at 48 h include *Ancylobacter aquaticus* DH2, UV6, and UV5. *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* DH12 only reached the end of this phase after 60 h. No increase in turbidity seen for un-inoculated control after 72 h, while *E. coli* showed an increase between 48 h and 72 h.

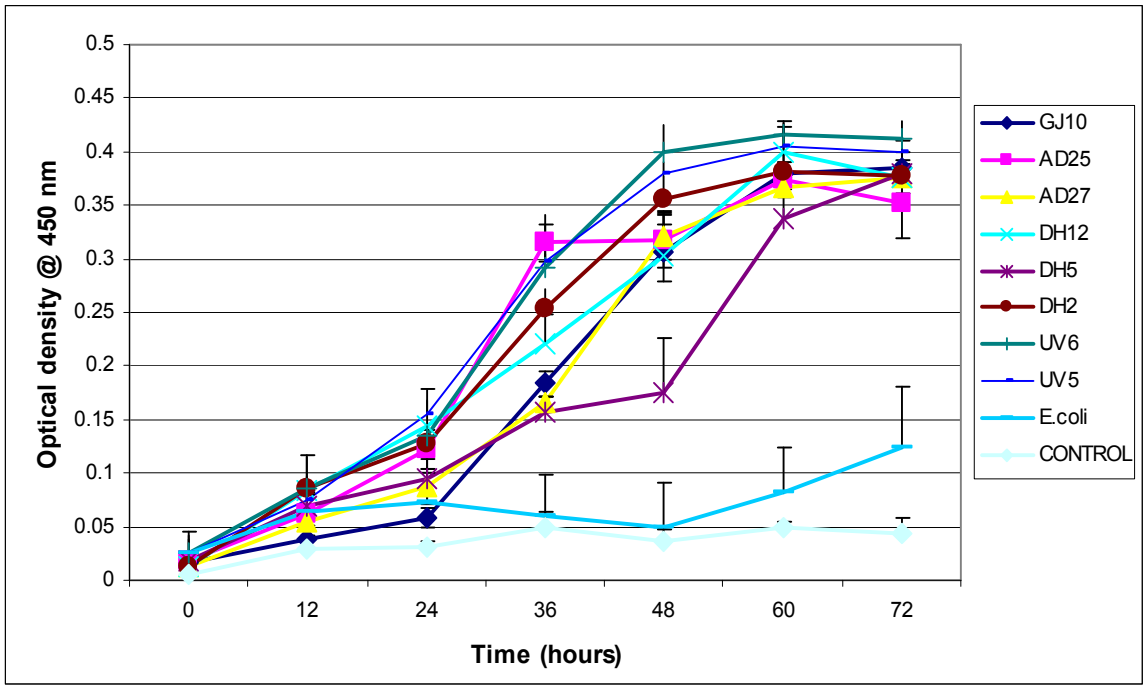

Figure 3.6: Growth pattern of isolates with MCA as sole carbon source in MSM containing vitamins at pH 9

Both *Ancylobacter aquaticus* DH5 and UV5, had the highest growth rate of 0.13.h^{-1}, followed by strains UV6 and AD25 with growth rates of 0.12.h^{-1} and 0.11.h^{-1}, respectively (Table 3.2). The rest of the isolates had growth rates ranging from 0.08-0.096.h^{-1}. *A. aquaticus* AD25 seemed to be unaffected by pH as it had similar growth

rates of 0.112.h^{-1} and 0.11.h^{-1} at both pH 7 and 9, respectively. Comparison of the growth rates of all isolates at pH 7 and pH 9 indicated that a neutral pH of 7 allowed for better growth of all isolates with MCA as the carbon source.

Table 3.2: Growth rate of bacterial strains during growth in MCA as sole carbon source at different pHs.

	Growth rate constants (h^{-1})	
	pH 7	**pH 9**
X. autotrophicus GJ10	0.090 ± 0.019	0.075 ± 0.007
A. aquaticus AD25	0.112 ± 0.015	0.110 ± 0.013
A. aquaticus AD27	0.080 ± 0.006	0.057 ± 0.008
Ancylobacter aquaticus DH12	0.096 ± 0.007	0.080 ± 0.006
Ancylobacter aquaticus DH5	0.130 ± 0.007	0.050 ± 0.011
Ancylobacter aquaticus DH2	0.080 ± 0.013	0.060 ± 0.008
Ancylobacter aquaticus UV6	0.120 ± 0.011	0.0650 ± 0.006
Ancylobacter aquaticus UV5	0.130 ± 0.02	0.0654 ± 0.006

3.1.2.2 Chloride release during growth in MCA

Free chloride concentration in the medium was also monitored at pH's 7 and 9 during the growth period. After 24 h at pH 7, both *Ancylobacter aquaticus* DH12 and DH5 released the highest amounts of free chloride with concentration of 0.186 mM and 0.185 mM, respectively (Fig. 3.7). This is followed by *Ancylobacter aquaticus* UV6 (0.157 mM), *Ancylobacter aquaticus* UV5 (0.147 mM), *A. aquaticus* AD25 (0.143 mM), *A. aquaticus* AD27 (0.126 mM), *Ancylobacter aquaticus* DH2 (0.068 mM) and lastly *X. autotrophicus* GJ10 (0.031 mM) which released the least amount of chloride. Following 36 h of incubation, *Ancylobacter aquaticus* DH12 released the highest amount of free chloride of 0.381 mM. *Ancylobacter aquaticus* UV6 had a chloride concentration of 0.344 mM, followed closely by *Ancylobacter aquaticus* DH5 and *A. aquaticus* AD27 with 0.333 mM and 0.32 mM, respectively. The isolate that released the lowest amount of free chloride after 36 h was still *X. autotrophicus* GJ10 with a concentration of 0.151 mM. Following the end of the 72 hr incubation period all isolates released similar concentrations of free

chloride ranging from 0.256-0.346 mM (Fig. 3.8). Both negative controls did not show any significant increases in chloride concentration.

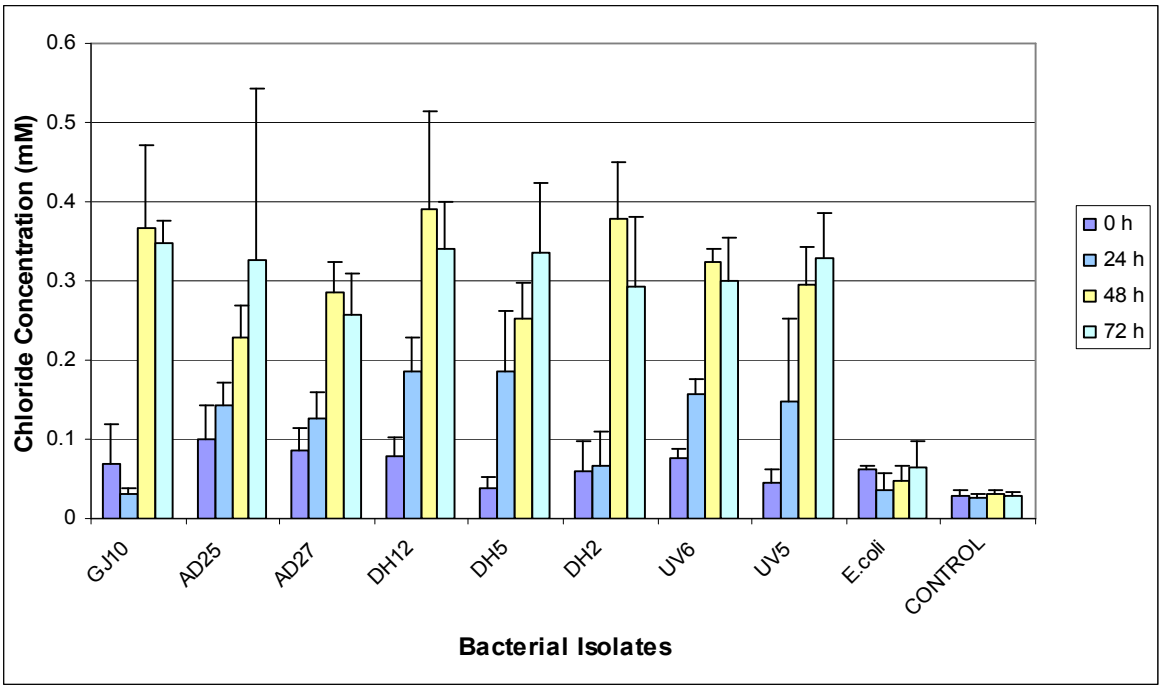

Figure 3.7: Concentration of chloride released during growth on MCA in MSM containing vitamins at pH7

Following the 72 h incubation period at pH 9 (Fig. 3.8), the isolates that released the highest concentration of free chloride were *Ancylobacter aquaticus* DH12 and DH2 with concentrations of 0.407 mM and 0.408 mM, respectively. The isolate that released the least free chloride ions after 72 h was *A. aquaticus* AD25 with 0.224 mM, while the rest of the isolates release free chloride in the range of 0.259-0.386 mM (Fig. 3.8). In all cases, growth was accompanied by halide release which significantly (p-value<0.05) exceeded the values obtained for the un-inoculated controls, which had no significant increases.

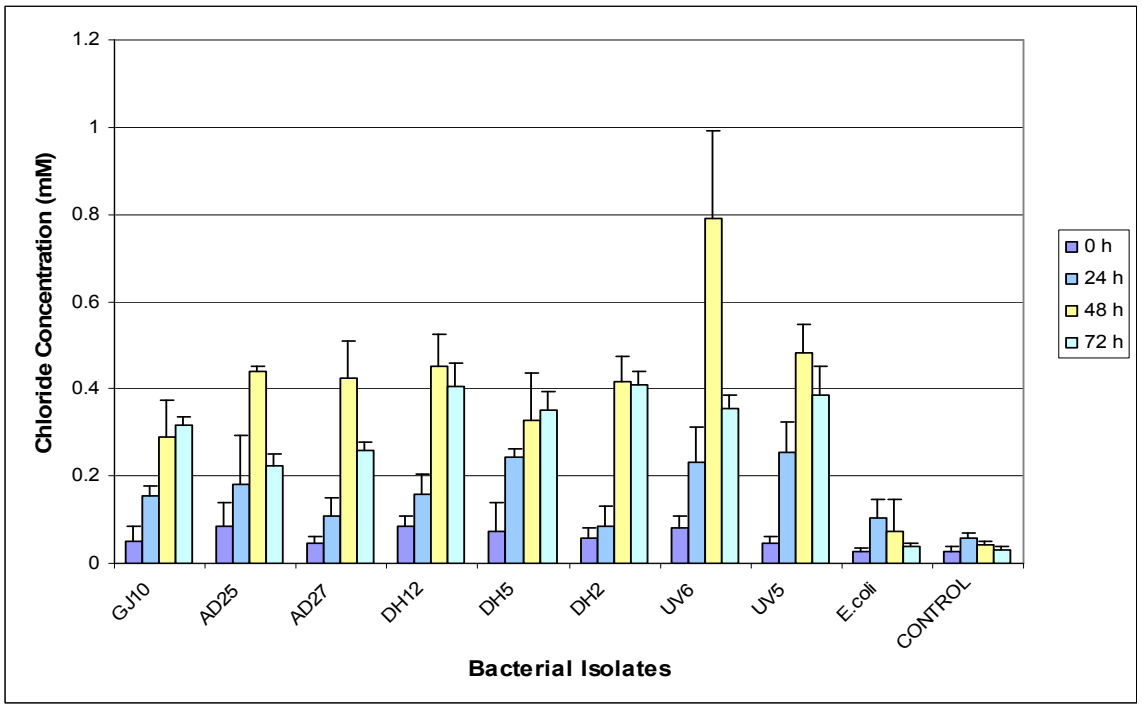

Figure 3.8: Concentration of chloride released during growth on MCA in MSM containing vitamins at pH9

3.1.3 Growth of *Ancylobacter aquaticus* strains with other halogenated aliphatic compounds

Two other aliphatic halogenated compounds, 1,3-dichloropropene (DCP) and 1,3-dibromopropane (DBP), were used as sole carbon sources in batch experiments to determine whether *Ancylobacter* strains can utilize other halogenated compounds. From the results obtained no growth was observed with 1,3-DCP or 1,3-DBP when used as carbon sources, since no increase in turbidity was observed after a 36 h incubation period. This indicates the inability of the organisms to grow in the presence of 1,3-DCP and 1,3-DBP.

3.2 DEHALOGENASE ACTIVITY OF BACTERIAL ISOLATES

3.2.1 Effect of pH and temperature on dehalogenase activity

The effects of pH on haloalkane dehalogenase activity in *Ancylobacter* strains was determined by testing their ability to cleave the carbon-chloride bond in 1,2-DCA under different pH conditions. Crude extracts prepared from cells grown to the late exponential phase in minimal medium with 1,2-DCA as the carbon source was tested for dehalogenase activity at pH 6, 7, 7.5, 8, and 9. Optimum haloalkane dehalogenase activity was observed at pH 8 for all isolates, with no activity detected at pH 6. A gradual increase in dehalogenase activity was observed for all the isolates with increase in pH from 7 to 8 followed by a decrease in activity from pH 8 to pH 9 (Table 3.3). At pH 8, *A. aquaticus* AD25 had the highest haloalkane dehalogenase activity of 110.24 mU/mg protein. *X. autotrophicus* GJ10 (26.66 mU/mg protein) and *A. aquaticus* AD27 (46.77 mU/mg protein) had more than a 4-fold and 2.4-fold lower activity than *A. aquaticus* AD25. All recently isolated strains, except *Ancylobacter aquaticus* DH2 (20.2 mU/mg protein), had higher dehalogenase activity than *X. autotrophicus* GJ10. *Ancylobacter aquaticus* DH12 (64.76 mU/mg protein), *Ancylobacter aquaticus* DH5 (60.47 mU/mg protein), *Ancylobacter aquaticus* UV6 (50.72 mU/mg protein), and *Ancylobacter aquaticus* UV5 (49.46 mU/mg protein) also had slightly higher activity than *A. aquaticus* AD27. *Ancylobacter aquaticus* DH12 had only 1.7-fold lower dehalogenase activity than *A. aquaticus* AD25, while *Ancylobacter* strains DH5; UV6 and UV5 had an approximately 2-fold lower activity, when compared to *A. aquaticus* AD25. *Ancylobacter aquaticus* DH2 showed a lower dehalogenase activity compared to the activity in *X. autotrophicus* GJ10, and an even further 4.8-fold lower dehalogenase activity when compared to the activity demonstrated by *A. aquaticus* AD25 (Table 3.3). *E. coli* showed some activity at all pH conditions.

Table 3.3: Hydrolytic dehalogenase activity (mU/mg protein) of the bacterial isolates using 1,2-DCA as the substrate under different pH conditions

	Specific activity (mU/mg protein)			
	pH 7	**pH 7.5**	**pH 8**	**pH 9**
X. autotrophicus GJ10	18.951±0.014	17.560 ± 0.004	26.657±0.022	21.782±0.021
A. aquaticus AD25	67.445±0.060	101.770 ± 0.067	110.244±0.126	105.072±0.142
A. aquaticus AD27	29.932±0.022	43.276 ± 0.015	46.765±0.150	34.179±0.065
Ancylobacter aquaticus DH12	42.926±0.047	54.860 ± 0.025	64.757±0.049	45.212±0.021
Ancylobacter aquaticus DH5	36.713±0.063	50.870 ± 0.023	60.471±0.161	41.109±0.011
Ancylobacter aquaticus DH2	14.306±0.047	19.534 ± 0.008	20.207±0.005	16.695±0.035
Ancylobacter aquaticus UV6	27.547±0.059	45.878 ± 0.178	50.716±0.058	39.398±0.023
Ancylobacter aquaticus UV5	36.666±0.021	49.082 ± 0.129	49.456±0.065	45.976±0.094
E.coli	12.964±0.047	10.248 ± 0.030	12.371±0.050	13.716±0.031

The effect of pH on the haloacid dehalogenase activity was determined over the same pH range as in haloalkane dehalogenase. As pH increased from 7 to 9, there was a concomitant increase in enzyme activity. The pH that produced maximum haloacid dehalogenase activities in most isolates was pH 9, except for *Ancylobacter aquaticus* DH12 with pH optima at 8 but with 99% activity still retained at pH 9 and isolate *Ancylobacter aquaticus* DH2 with optimum pH at 7.5. *A. aquaticus* AD25 had the highest haloacid dehalogenase activity of 117 mU/mg protein at pH 9, values 1.7-fold higher than that observed for *X. autotrophicus* GJ10. *A. aquaticus* AD27 had an approximately 4-fold and 2-fold lower activity than *A. aquaticus* AD25 and *X. autotrophicus* GJ10, respectively (Table 3.4). *A. aquaticus* AD27 demonstrated very similar activity across the pH range tested. Specific haloacid dehalogenase activity for *Ancylobacter aquaticus* DH12 was a little lower than that observed in *A. aquaticus* AD25 and 1.6 fold lower than *X. autotrophicus* GJ10. *Ancylobacter aquaticus* DH5, UV6 and UV5 had approximately 1.5 to 2- fold lower activities than *A. aquaticus* AD25. Excluding *A. aquaticus* AD27 and *Ancylobacter aquaticus* DH2, the overall specific haloacid dehalogenase activities was approximately 2-fold higher at pH 9 compared to pH 7.5 (Table 3.4). *E. coli* also showed activity at the various pH conditions but was not significant.

Table 3.4: Haloacid dehalogenase activity (mU/mg protein) of the bacterial isolates using MCA as substrate at different pH conditions

	Specific activity (mU/mg protein)			
	pH 7	**pH 7.5**	**pH 8**	**pH 9**
X. autotrophicus GJ10	15.319±0.008	35.986±0.006	51.781±0.009	66.170±0.035
A. aquaticus AD25	59.300± 0.146	56.454±0.066	91.622±0.165	116.9539±0.189
A. aquaticus AD27	30.937±0.006	24.062±0.003	28.407±0.008	27.267±0.0320
Ancylobacter aquaticus DH12	29.135±0.056	51.635±0.015	107.205±0.020	106.056±0.050
Ancylobacter aquaticus DH5	27.722±0.01	37.884±0.010	55.149±0.030	78.427±0.079
Ancylobacter aquaticus DH2	28.59±0.021	36.701±0.243	23.879±0.020	23.06±0.0030
Ancylobacter aquaticus UV6	30.387±0.029	27.619±0.010	32.525±0.007	49.013±0.009
Ancylobacter aquaticus UV5	36.617±0.013	32.417±0.003	36.89±0.010	50.363±0.038
E.coli	13.984±0.011	11.914±0.003	13.003±0.017	10.562±0.0150

The optimum temperature for haloalkane dehalogenase activity observed for all isolates was 30°C (Table 3.5). An increase in temperature from 30°C to 40°C showed an approximately 10% loss in activity for all isolates, indicating that haloalkane dehalogenase enzyme activity is very much stable between 30°C and 40°C. At 50°C, only *A. aquaticus* AD25, *A. aquaticus* AD27 and *Ancylobacter aquaticus* DH12 demonstrated some level of activity.

Table 3.5: Hydrolytic dehalogenase activity (mU/mg protein) of the bacterial isolates using 1,2-DCA as substrate at different temperature conditions

	Specific activity (mU/mg protein)			
	22°C	**30°C**	**40°C**	**50°C**
X. autotrophicus GJ10	5.471±0.004	17.560±0.004	16.245±0.039	ND
A. aquaticus AD25	60.074±0.114	101.771±0.067	90.639±0.029	40.221±0.077
A. aquaticus AD27	15.854±0.046	43.277±0.0160	38.968±0.053	16.019±0.043
Ancylobacter aquaticus DH12	20.433±0.01	54.860±0.025	49.252±0.024	17.314±0.041
Ancylobacter aquaticus DH5	18.626±0.024	50.870±0.023	40.023±0.048	ND
Ancylobacter aquaticus DH2	6.418±0.018	19.534±0.008	17.207±0.081	ND
Ancylobacter aquaticus UV6	19.240±0.051	45.878±0.178	35.503±0.032	ND
Ancylobacter aquaticus UV5	20.846±0.060	49.082±0.13	42.623±0.11	ND
E.coli	3.573±0.058	10.248±0.027	12.889±0.09	ND

* ND – Not detected

3.2.2 Dehalogenase activities of crude extract on various halogenated compounds

The crude extracts obtained from the bacteria were tested on other halogenated compounds to determine the substrate range of the enzyme. From the results obtained, all isolates appear to have higher affinity for 1,3-dichloroproprene (DCP) than 1,2-DCA. A 9.56% increase in specific dehalogenase activity was observed for *A. aquaticus* AD25 in the presence of 1,3-DCP compared to that observed in the presence of 1,2-DCA. Generally, the activity observed with 1,3-DCP is approximately 10% higher for all isolates, than with 1,2-DCA (Table 3.6). Both *X. autotrophicus* GJ10 and *A. aquaticus* AD25 have 2-fold higher activity on 1,3-dibromopropane compared to 1,2-dibromoethane, while the other isolates show similar activity in the presence of these compounds. All isolates had approximately 1.6-fold lower dehalogenase activity with brominated compounds than 1,2-DCA and about 2-fold lower activity when compared to 1,3-DCP. Crude extract from *E.coli* did not show any significant activity. The crude extract of the organisms demonstrated no enzyme activity on other compounds, dichloromethane, dichloroethene and trichloroethene tested.

Table 3.6: Hydrolytic dehalogenase activity (mU/mg protein) of the bacterial isolates using halogenated aliphatic compounds at pH 7.5

	Specific activity (mU/mg protein)			
	1,2-DCA	**1,3-DBP**	**1,3-DCP**	**DBE**
X. autotrophicus GJ10	17.560 ± 0.004	21.757±0.010	56.921±0.017	12.335±0.014
A. aquaticus AD25	101.770 ± 0.067	65.169±0.052	111.501±0.015	29.933±0.009
A. aquaticus AD27	43.276 ± 0.015	26.478±0.012	50.172±0.063	25.225±0.022
Ancylobacter aquaticus DH12	54.860 ± 0.025	32.215±0.036	59.007±0.035	34.053±0.009
Ancylobacter aquaticus DH5	50.870 ± 0.023	29.837±0.029	55.414±0.007	31.092±0.010
Ancylobacter aquaticus DH2	19.534 ± 0.008	10.512±0.035	26.349±0.021	9.471±0.006
Ancylobacter aquaticus UV6	45.878 ± 0.178	32.667±0.028	57.914±0.051	28.963±0.009
Ancylobacter aquaticus UV5	49.082 ± 0.129	33.785±0.021	62.997±0.353	33.022±0.013
E.coli	10.248 ± 0.030	5.977±0.0340	8.759±0.0240	7.29±0.0170

3.3 DETECTION OF HALOACID DEHALOGENASE AND CHLOROACETALDEHYDE DEHYDROGENASE ENCODING GENES IN *Ancylobacter* ISOLATES

3.3.1 PCR amplification of *aldB* and *dhl*B encoding genes from *Xanthobacter autotrophicus* GJ10

Genomic DNA from *X. autotrophicus* GJ10 was used as template for the amplification of the *aldB* and the *dhlB* genes. PCR was successful, producing the correct product sizes of 1550 bp and 800 bp for the *aldB* and *dhlB* gene, respectively (Fig. 3.9). The PCR products were then purified and digoxygenin-labeled, and used in hybridization experiments.

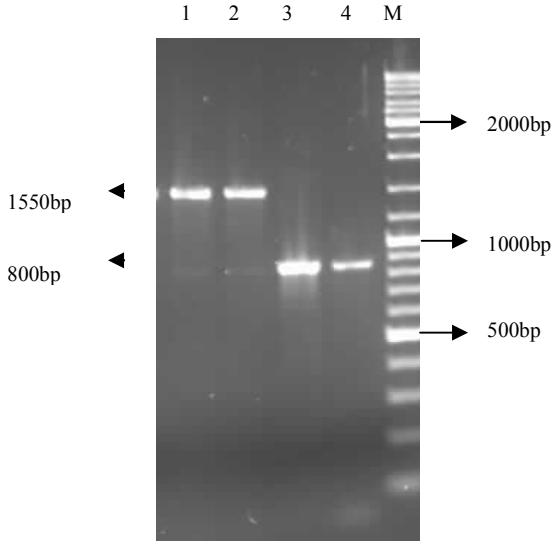

Figure 3.9: PCR amplification of *dhlB* and *aldB* gene probes. Lanes 1 and 2: *aldB* gene; lanes 3 and 4: *dhlB* gene, M: GeneRuler Ladder Mix

3.3.2 Detection of the haloacid dehalogenase (HA) encoding genes in *Ancylobacter* species

Southern blot hybridization analysis with the *dhlB* specific gene probe was used to determine the presence of the gene in all *Ancylobacter* isolates used in this study. Marker

II was DIG-labeled and appears at the end of all blots. Restricted genomic DNA from *X. autotrophicus* GJ10 was used as the positive control for all hybridization experiments. Fig. 3.10A shows *Eco*RI digested total genomic DNA of all isolates and Fig. 3.10B, the corresponding hybridization blot. *Ancylobacter aquaticus* DH12 revealed a single hybridization signal of approximately 5 kb (Fig. 3.10 B, lane 4). This product size is different from that observed in the positive control, which is approximately 8 kb (Fig. 3.10 B, lane 1). This may indicate sequences of homology between the genes in *Ancylobacter aquaticus* DH12 and *X. autotrophicus* GJ10.

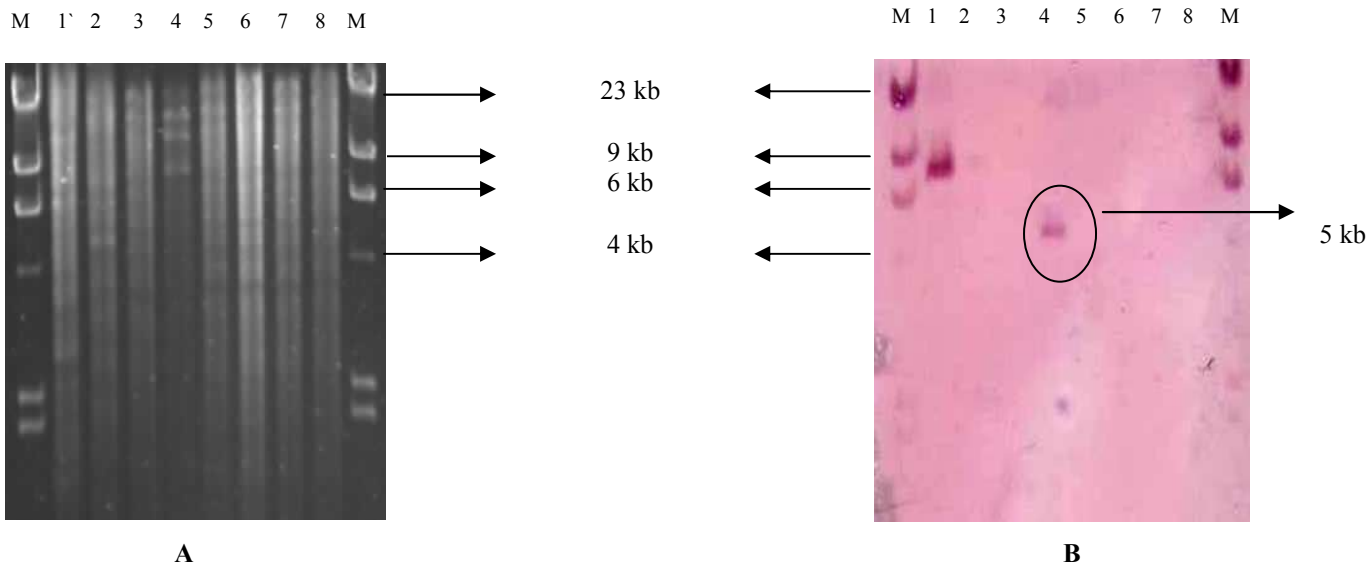

Figure 3.10: A.) *Eco*RI digested total genomic DNA of all isolates B.)Southern hybridization with digoxygenin labeled *dhlB* probe and *Eco*RI digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

Genomic DNA digested with *Bam*HI also produced a single hybridization signal for *Ancylobacter aquaticus* DH12 (Fig. 3.11, lane 4). The size of the positive signal for *Ancylobacter aquaticus* DH12 was approximately 10 kb while a 23 kb signal was obtained for isolate *X. autotrophicus* GJ10 (Fig. 3.11). The presence of a signal for *Ancylobacter aquaticus* DH12 in both restriction digests may suggest that a haloacid dehalogenase gene, with some sequence similarity to *dhlB* gene from

X. autotrophicus GJ10, may indeed be present in this isolate. This assumption can be made based on the detection of a signal obtained for this probe. No other isolate produced a positive signal although all isolates have previously been reported to follow the same degradation pathway as *X. autotrophicus* GJ10. Previous attempts to amplify the *dhlB* gene in these isolates were unsuccessful. This could further indicate that the haloacid dehalogenase present among the rest of the *Ancylobacter* strains may belong to a different subgroup when compared to the *dhlB* in *X. autotrophicus* GJ10. Previously isolated strains *A. aquaticus* AD25 and AD27 also possibly contains a haloacid dehalogenase, however no previous studies, including this study, have confirmed this.

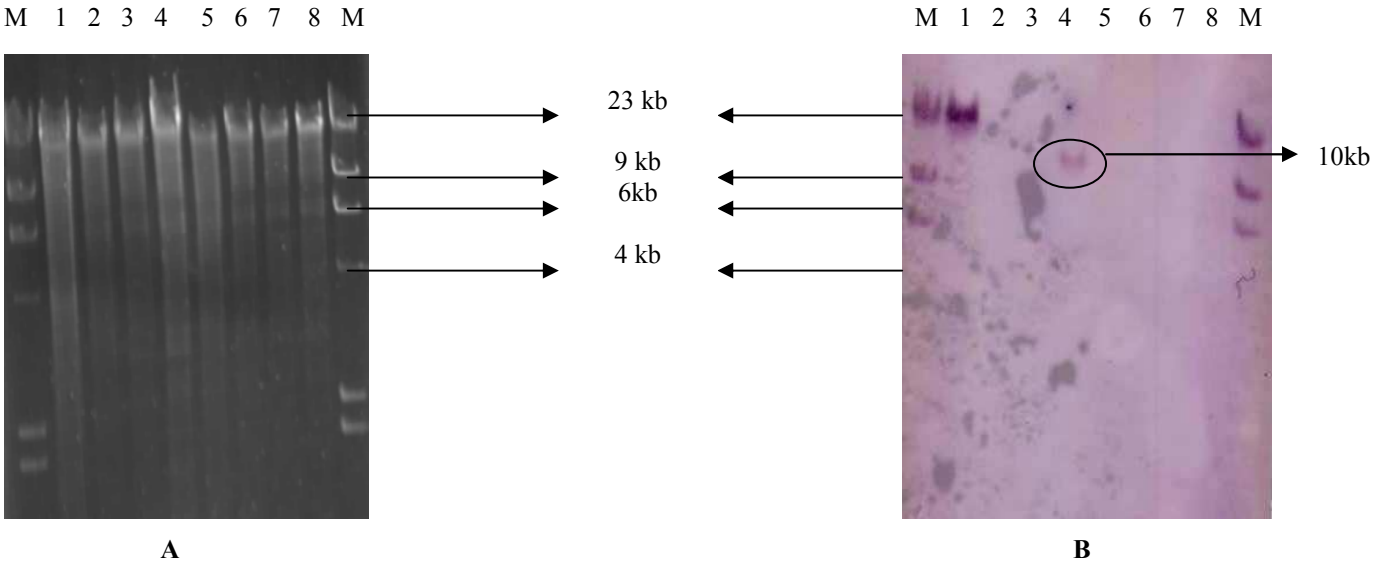

Figure 3.11: A.) *Bam*HI digested total DNA of all isolates. B.) Southern hybridization with digoxygenin labeled *dhlB* probe and *Bam*HI digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

Southern blot analysis of *Hind*III-digested genomic DNA revealed two hybridization signals for *Ancylobacter aquaticus* DH12 (lane 4), while no other isolate produced any signal. *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* DH12, both produced two signals as shown in lanes 1 and 4, respectively. There appeared to be no *Hind*III

restriction site within the *dhlB* gene, therefore two signals was unexpected. The two signals produced could be attributed to partial digestion as indicated in Fig. 3.12.

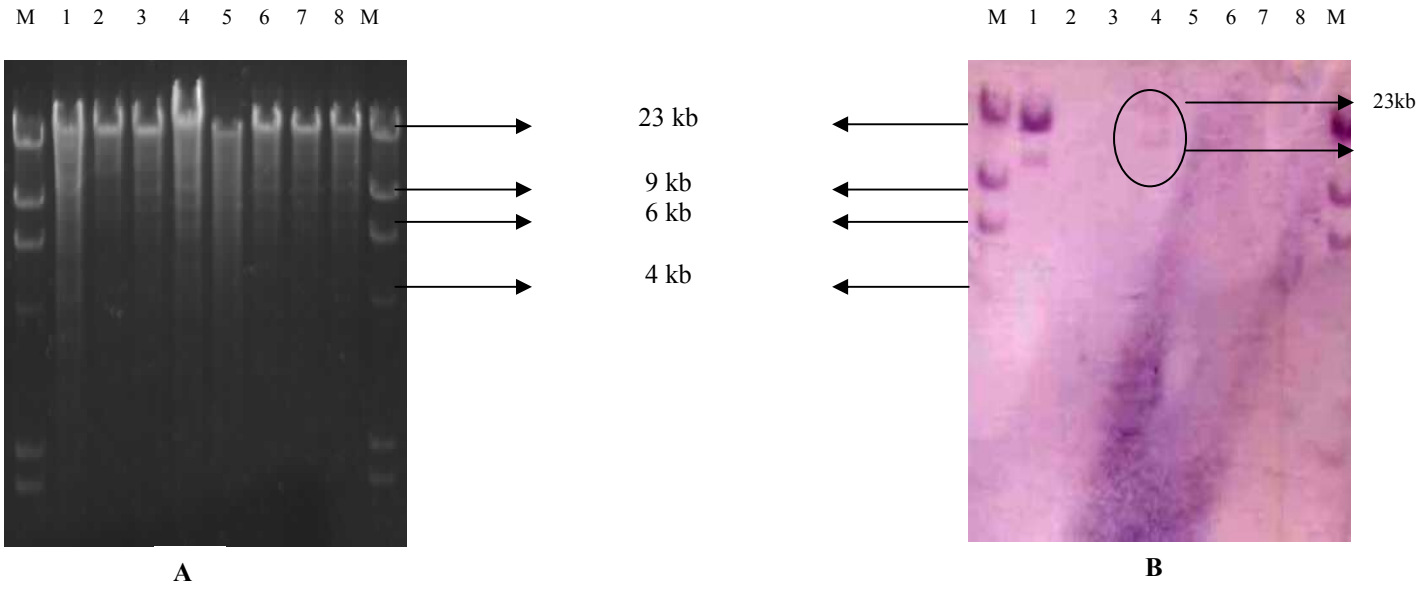

Figure 3.12: A.) *Hind*III digested total DNA of all isolates. B.) Southern hybridization with digoxygenin labeled *dhlB* probe and *Hind*III digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

When *Pst*I-digested genomic DNA was probed against the *dhlB* gene, two hybridization signals, of approximately 9 and 6 kb, were produced for *Ancylobacter aquaticus* DH12 (Fig. 3.13). Results obtained from all blots (Fig 3.10-3.13) further demonstrate that *Ancylobacter aquaticus* DH12 may possibly possess a HA dehalogenase that has sequence similarity to the *dhlB* gene in *X. autotrophicus* GJ10, as the *dhlB* probe was able to hybridize to the different restricted genomic DNA digests. Since the *dhlB* gene in *X. autotrophicus* GJ10 contains a *Pst*I restriction site, two signals should ideally have been observed. However, this was not the case for *X. autotrophicus* GJ10, as multiple signals were produced (Fig 3.13, lane 1), indicating possibly more than one homolog of the gene or incomplete digestion of genomic DNA. An approximately 6 kb signal was

also produced for *Ancylobacter aquaticus* DH5. The presence of a signal for *Ancylobacter aquaticus* DH5 for this blot could be due to different volumes of labeled probe used for the different blots, resulting in the absence of this signal in other blots.

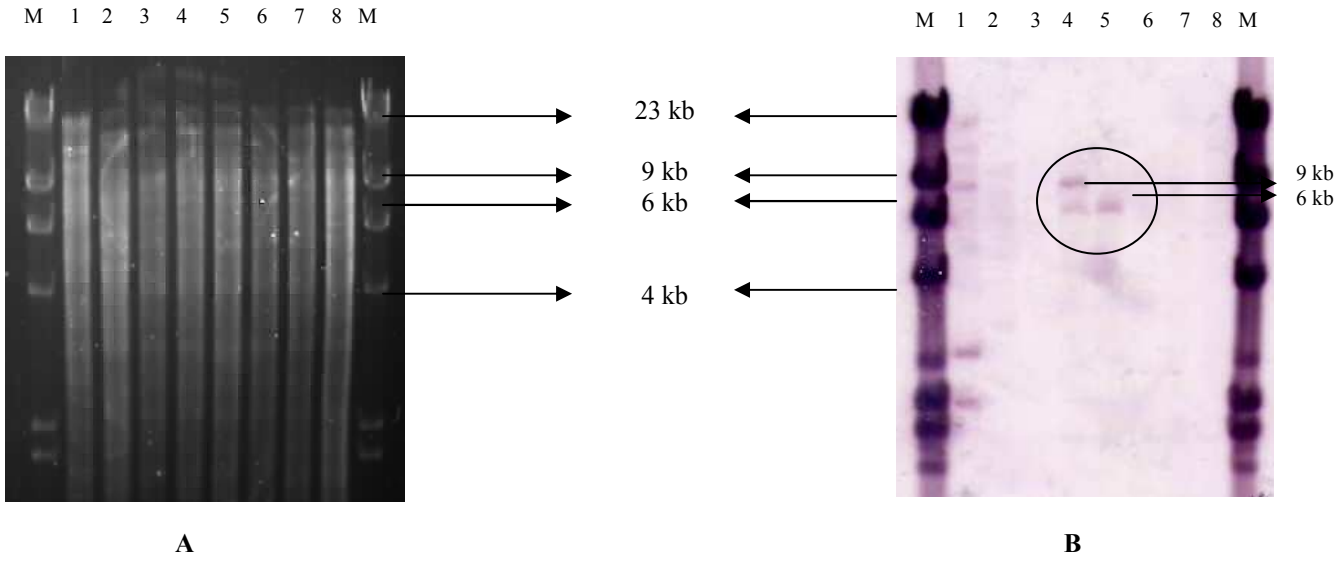

Figure 3.13: A.) *Pst*I digested total DNA of all isolates. B.) Southern hybridization with digoxygenin labeled *dhlB* probe and *Pst*I digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

3.3.3 Detection of CAA dehydrogenase encoding genes in *Ancylobacter* strains

Figure 3.14 shows *Eco*RI digested total genomic DNA of all isolates probed with the *aldB* gene. The positive control, isolate *X. autotrophicus* GJ10 (lane 1), produced two distinct signals, a darker more intense 9 kb signal and a lighter 3 kb signal. *X. autotrophicus* GJ10 is known to possess two aldehyde dehydrogenases, *aldA* and *aldB*, both of which share 84% homology. The two signals could therefore be indicative of the presence of these two genes. However, there appeared to be the presence of a third signal above the 9 kb signal. This could possibly be attributed to partial digestion of the genomic DNA. Digested DNA from the other isolates also produced signals; however of

lighter and varying intensities (Fig. 3.14, lane 2-8). *Ancylobacter aquaticus* DH12 (Lane 4) and *Ancylobacter aquaticus* DH5 (lane 5) appear to have two separate signals. This may indicate that more than one CAA dehydrogenase may be present in these isolates. Due to the lighter intensity of the bands in the other lanes, it is difficult to determine if two signals are also present for the other isolates.

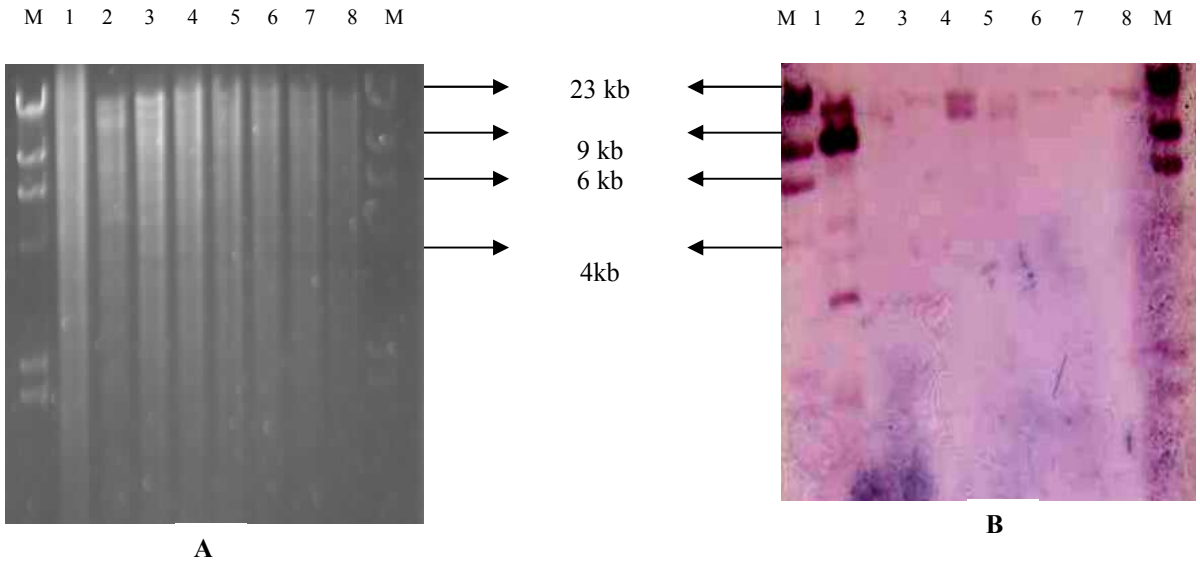

Figure 3.14: A.) *Eco*RI digested total DNA B.) Southern Hybridization with digoxygenin labeled *aldB* probe and *Eco*RI digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

Southern hybridization analysis of *Pst*I digested genomic DNA with *aldB* labeled probe revealed the presence of at least one hybridization signal of the same size for all *Ancylobacter* strains (Fig. 3.15). This indicated that the aldehyde dehydrogenase found in all isolates show similarity to each other. *X. autotrophicus* GJ10 produced a single hybridization band of about 4.5 kb. It was observed that the CAA dehydrogenase-encoding genes in *X. autotrophicus* GJ10 resided on two similar sized *Pst*I fragments, 4.4 kb and 4.5 kb fragments. This could therefore account for the darker more intense band observed in lane 1. The intensity of the bands also varied to an extent, with *Ancylobacter aquaticus* DH12 (lane 4) and *Ancylobacter aquaticus* DH5 (lane 5) having a band of

similar intensity as *X. autotrophicus* GJ10. It is therefore clearly evident from the *Eco*RI-digest and *Pst*I-digests that *Ancylobacter aquaticus* DH12 and *Ancylobacter aquaticus* DH5 possessed more than one homolog of the gene that could be similar to the gene in *X. autotrophicus* GJ10 and this gene may also contain a *Pst*I restriction site. Two signals were observed for *A. aquaticus* AD25 (lane 2), one signal of the same size as the other *Ancylobacter* isolates and an additional larger 5 kb signal, indicating a possible aldehyde dehydrogenase homolog.

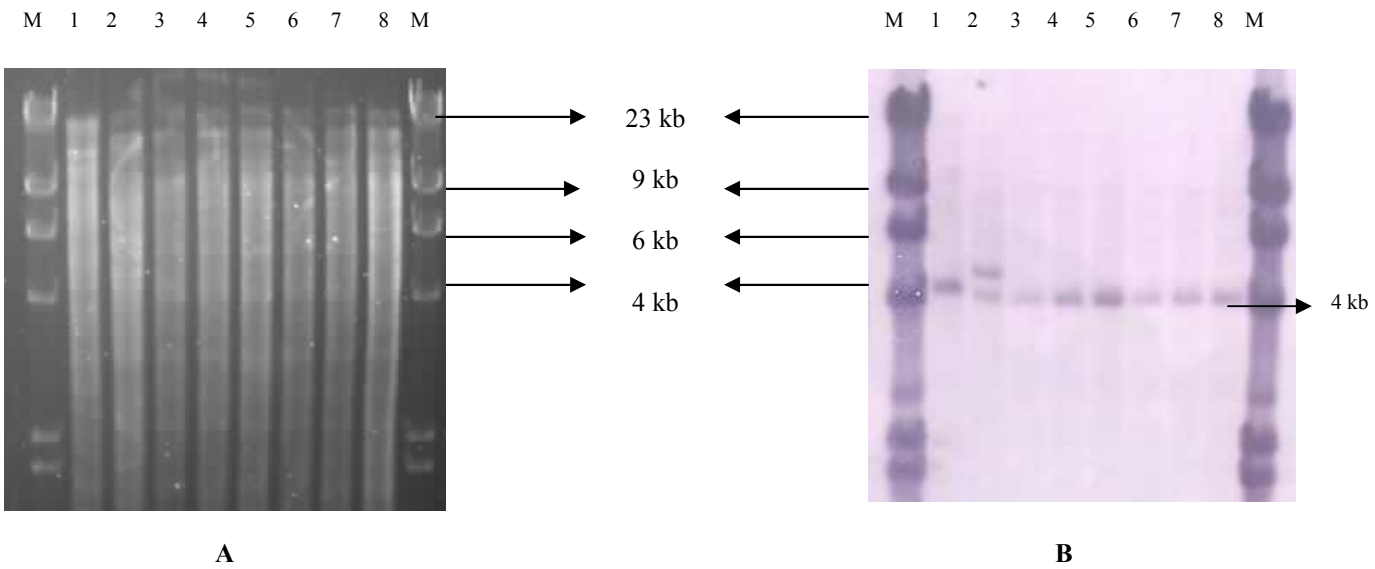

Figure 3.15: A.) *Pst*I digested total DNA B.) Southern hybridization with digoxygenin labeled *aldB* probe and *Pst*I digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

*Hin*dIII-digested genomic DNA probed with *aldB* gene also produced signals for all *Ancylobacter* strains. All signals were approximately 23 kb (Fig. 3.16). Although not very evident, two signals appear to be produced for *X. autotrophicus* GJ10, *A. aquaticus* AD25, *Ancylobacter aquaticus* DH12 and *Ancylobacter aquaticus* DH5. *X. autotrophicus* GJ10 produced two signals at 23 kb, while the other three isolates produced a darker 23kb signal and a second lighter signal of a slightly lower molecular weight (Fig. 3.16). Other strains having a single hybridization signal may suggest that the gene it possesses has sequence similarity to the *aldB* in *X. autotrophicus* GJ10.

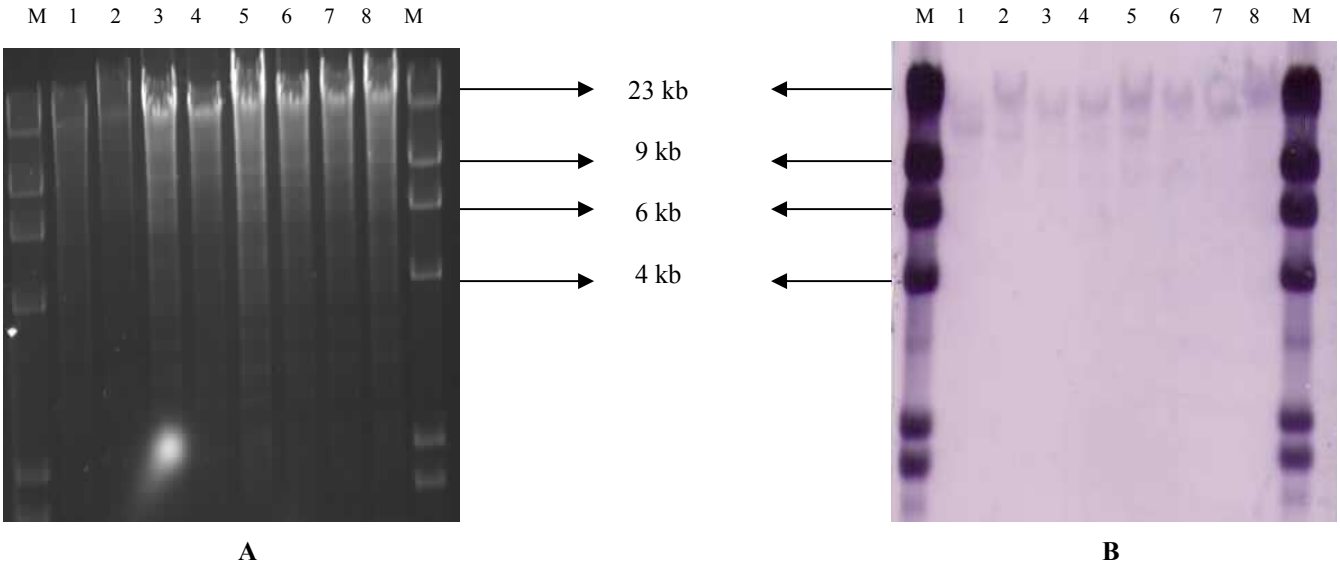

Figure 3.16: A.) *Hind*III digested total DNA B.) Southern hybridization with digoxygenin labeled *aldB* probe and *Hind*III digested total DNA (Lane 1-8: *X. autotrophicus* GJ10, *A. aquaticus* AD25, *A. aquaticus* AD27, *Ancylobacter aquaticus* DH12, *Ancylobacter aquaticus* DH5, *Ancylobacter aquaticus* DH2, *Ancylobacter aquaticus* UV6 and *Ancylobacter aquaticus* UV5)

3.4 CLONING OF THE HA DEHALOGENASE AND CAA DEHYDROGENASE GENES

Positive fragments that were excised from the agarose gel were cloned into pBluescript II SK. This was followed by plasmid isolations, enzyme restrictions, and further hybridizations of the restricted clones. However, after several attempts, cloning of these genes was unsuccessful.

CHAPTER FOUR
DISCUSSION

In the present study, five *Ancylobacter* strains recently isolated in our laboratories were investigated for their ability to utilize and degrade 1,2-DCA and a number of other halogenated hydrocarbons. To establish the effect of organic nutrients on bacterial growth rates, all isolates were cultured in minimal salts media containing either a vitamin solution (MMV) or 10 mg/l yeast extract (MMY). Significant increases in turbidity were observed in both media types tested over the 84 hr incubation period. This therefore confirmed the ability of the five *Ancylobacter* strains to utilize 1,2-DCA as a carbon and energy source for growth. Two species belonging to *Ancylobacter* have previously been reported to be capable of degrading organochloride compounds. These were identified as *A. aquaticus* and *A. dichloromethanicus* which are aerobic facultative methylotrophs. *A. aquaticus* was able to utilize 1,2-DCA, while *A. dichloromethanicus* utilized dichloromethane as a sole carbon source (Van den Wijngaard *et al*., 1992; Xin *et al*., 2006). Members belonging to the genus *Ancylobacter* therefore demonstrate the ability to degrade chlorinated compounds, as was confirmed in this study.

All bacterial strains, with the exception of *A. aquaticus* AD27, displayed similar growth patterns in both minimal media investigated, demonstrating distinct phases of the growth curve i.e. lag, exponential and stationery. Based on these growth curves, growth rate constants of individual isolates were established and compared in MMV and MMY. Most bacterial isolates demonstrated higher growth rates in minimal media supplemented with vitamins, while *Ancylobacter aquaticus* DH12 had a higher growth rate in the presence of yeast extract. *Ancylobacter aquaticus* UV5 and *A. aquaticus* AD27 displayed similar growth rates in both types of minimal media. *X. autotrophicus* GJ10 and *A. aquaticus* AD25 also grew optimally in vitamins with higher growth rates being observed in the presence of vitamins. These results indicate the requirement of organic nutrients for various microorganisms involved in 1,2-DCA degradation. The overall effect of vitamins on microbial growth was greater than yeast extract. Similarly, Van den Wijngaard *et al*. (1993), investigated the effects of vitamins and yeast extract on the growth of

X. autotrophicus GJ10 in 1,2-DCA. In the presence of yeast extract (10 mg/L) *X. autotrophicus* GJ10 had a growth rate of $0.04.h^{-1}$, while the addition of vitamins resulted in a further increase of $0.09.h^{-1}$. Other previously characterized strains, namely; *A. aquaticus* AD25 and *Pseudomonas* sp. strain DE2 also demonstrated optimal growth when 1,2-DCA was used as a carbon source, in the presence of vitamins (Field and Sierra-Alvarez, 2004). Although vitamins stimulate microbial growth better than yeast extract, the quantity of chlorides released during the 84 h incubation period is overall lower for vitamins. Yeast extract may therefore be a more feasible option in bioremediation strategies for complete removal of chlorinated compounds. Results here do, however, confirm the need for essential co-factors in microorganisms that can utilize chlorinated aliphatics. .

Growth rates obtained for *X. autotrophicus* GJ10 and *A. aquaticus* AD25 in the current study differ from the growth rates reported in previous studies. In the study done by Van den Wijngaard *et al*. (1992), a higher growth rate was observed for *X. autotrophicus* GJ10. This may be due to the growth of the organism in minimal media supplemented with both vitamins and yeast extract, whilst in this study bacterial cells were grown in MMV only. On the contrary, a slower growth rate ($0.04.h^{-1}$) was observed for *A. aquaticus* AD25 as compared to the $0.094.h^{-1}$ obtained in this study. Comparatively, in this study, *A. aquaticus* AD25 was observed to have a higher growth rate of $0.081.h^{-1}$ growing in MMY. The supplemented yeast extract used in the minimal salts media produced a higher growth rate for *A. aquaticus* AD25 than the 30mg yeast extract used in the study by Van der Ploeg *et al*. (1994). Different growth rates obtained in the different studies may be attributed to various factors such as pH, temperature, microbial biomass and the presence of other compounds, in this case the supplement yeast extract, which can affect biodegradation kinetics. Other factors that may significantly influence mineralization include, cell population density, as well as the xenobiotic compound concentration/cell biomass ratio (Gallego *et al*., 2000). These factors should be taken into consideration when designing bioremediation strategies or constructing bioreactors, so as to maximize the benefits of xenobiotic degraders.

During growth in 1,2-DCA, the growth rates observed for the isolates under investigation in this study were similar to other well-known aerobic 1,2-DCA degraders such as *X. autotrophicus* GJ10 ($0.11.h^{-1}$), *Pseudomonas* sp DE2 ($0.08.h^{-1}$), *A. aquaticus* AD20 ($0.08.h^{-1}$) and *Pseudomonas* sp. DCA1 ($0.14.h^{-1}$) (Field and Sierra-Alvarez, 2004), while, the lowest growth rate obtained was *A. aquaticus* AD27. The growth pattern for this organism illustrated only gradual increases in turbidity in both minimal media used with no distinct exponential phase. Accumulation of toxic intermediates may have interfered with the optimum rate at which the enzymes are active thus resulting in slower adaptation of the isolate in 1,2-DCA. It has previously been reported that due to the lack of a regulatory system for dehalogenase synthesis, the haloalkane dehalogenase is constitutively produced (Pries *et al*., 1994). As a result, the reactive intermediates may accumulate and if the enzyme active against these intermediates is not in high content, the organism is unable to cope, leading to bacterial cell death. This may therefore account for the low growth rate observed for *A. aquaticus* AD27 in comparison to the other strains of *Ancylobacter.* These results correlated with a study done by Govender (2009), whereby all five recently isolated *Ancylobacter aquaticus* (DH12, DH2, DH5, UV6 and UV5) demonstrated better growth in the presence of 1,2-DCA than the previously isolated *A. aquaticus* AD27.

The growth pattern of the bacterial isolates obtained in the presence of 1,2-DCA was further supported by the proportional increases of chloride ions released during the incubation period. Higher concentrations of chloride ions were released during the first 24 h for most bacterial isolates, followed thereafter by slower release. A similar trend was observed for *A. aquaticus* AD25 with significant amount of chloride ions being released in the first 24 h of incubation, while most of the chlorides released by *X. autotrophicus* GJ10 were observed after 48 h of incubation. This may be indicative of an initial higher haloalkane dehalogenase activity which may have resulted in producing large quantities of the 2-chloroethanol and chloroacetaldehydes, which are critical intermediates of 1,2-DCA metabolism. High dehalogenase content and an absence of a regulatory system for dehalogenase synthesis in 1,2-DCA degraders has previously been reported (Van den Wijngaard *et al*., 1992). The constitutive production of the haloalkane dehalogenase

result in large amounts of intermediate alcohols being produced, as previously reported by Van den Wijngaard *et al*. (1992). The low content of dehydrogenases may have not been able to deal with the high volumes of 2-chloroethanol and chloroacetaldehydes which are toxic to the bacterial cell. This may have therefore resulted in a slower release of chloride ions after 48 h. *A. aquaticus* AD25 has previously been shown to possess much higher dehalogenase content than *X. autotrophicus* GJ10 (Van den Wijngaard *et al*., 1992). This may explain the higher chloride release observed by *A. aquaticus* AD25 and the other bacterial strains during the first 24 hours of incubation. Statistical analysis revealed a significant ($p<0.05$) difference in the amount of chlorides released by the five recently isolated *Ancylobacter* strains in the presence of 1,2-DCA as compared to the negative controls. However, when the chloride ions released by individual *Ancylobacter* isolates were compared to positive controls *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27, no significant difference was observed ($p>0.05$). Earlier studies have indicated slow aerobic biodegradation, however recent results demonstrate otherwise, with the discovery of a wider range of microorganisms capable of degradation (*X. autotrophicus* GJ10, *A. aquaticus* strains and *Pseudomonas* sp. strain DCA). Other bacteria that have been found to degrade 1,2-DCA under aerobic conditions include *Nitrobacter*, *Nitrosomonas* and *Nitrospira* species. These are oxygenase expressing microorganisms that have the potential to degrade 1,2-DCA through cometabolic processes, with oxygen as the electron acceptor (Kocamemi and Cecen, 2009).

The ability of the *Ancylobacter* strains to degrade other halogenated compounds was further tested. Batch experiments were set up at pH 7 and pH 9 with MCA as a sole carbon source. All bacterial strains were capable of growth in the presence of MCA, with higher growth rates observed at neutral pH of 7. Growth rates obtained for the recently isolated *Ancylobacter* strains were comparable to the previously isolated strains. This study was similar to a study done by of Kerr and Marchesi (2006), also demonstrating better growth and degradation of halocarboxylic acids at a neutral pH. *X. autotrophicus* GJ10 previously demonstrated poor growth in MCA (Van den Wijngaard *et al*., 1992); however a later study done by Torz and Beschkov (2005) suggested otherwise. The latter study correlated with results in this study, whereby growth was evident for *X.*

autotrophicus GJ10 with a growth rate of $0.08.h^{-1}$. Better growth was observed by *X. autotrophicus* GJ10 in both (previous and current) studies when pre-culture of the isolates were initially grown in 1,2-DCA prior to inoculation into fresh media with MCA as carbon source. Higher haloacid dehalogenase content provided by the1,2-DCA may have resulted in an improved growth rate, as previously observed (Torz and Beschkov, 2005).

1,3-Dichloropropene (1,3-DCP), a longer-chained chlorinated compound was also added to batch experiments to determine its use as sole carbon and energy source by the bacterial isolates. No visible change in turbidity was observed after a 36 h incubation period, indicating no growth for all *Ancylobacter* strains under investigation, as well as previously characterized strains. The longer chained structure of 1,3-DCP possibly does not allow the compound to cross the bacterial membrane as easily as a shorter chained compound thus leading to no growth. Alternatively, the presence of the C=C bond may have contributed to the recalcitrance and toxicity of the compound as no visible growth was observed. The other compounds investigated for their use as a carbon source were two brominated hydrocarbons, 1,2-dibromoethane (1,2-DBE) and 1,3-dibromopropane (1,3-DBP). Both compounds failed to induce growth for any of the organisms. Although brominated compounds are less stable than their chlorinated analogs, they also appear to be more toxic, as the carbon-bromine bond is more labile than the carbon-chlorine bond and is thus more reactive (Van der Ploeg *et al*., 1995). No other studies previously reported growth of *Ancylobacter* species or *X. autotrophicus* GJ10 in the presence of brominated compounds. This explains the lack of growth when 1,3-DBP or 1,2-DBE was used as a carbon source.

Specific dehalogenase activities were determined from the crude extracts of the bacterial isolates. Halide production was observed when 1,2-DCA and other halogenated compounds (MCA, 1,3-DCP, 1,3-DBP and 1,2-DBE) were used as substrates in dehalogenase assays. This confirmed the presence of the haloalkane dehalogenase enzyme in the five recently *Ancylobacter* isolated strains. No halides were released when dichloromethane, dichloroethene or trichloroethene were tested as a substrate in the dehalogenase assays. This can be explained, as these compounds are not known to be

degraded aerobically but under anaerobic conditions in the presence of a reductive dehalogenase (Van der Ploeg *et al*., 1995).

The haloalkane dehalogenase is an enzyme with a broad substrate range, capable of hydrolyzing several chlorinated and brominated aliphatic hydrocarbons, as confirmed by all isolates. Although, some of these substrates (1,3-DCP , 1,3-DBP and 1,2-DBE) were efficiently hydrolyzed in the dehalogenase assays, no growth was observed when the same substrates were used as a sole carbon source in batch experiments as indicated earlier in the discussion. It has previously been reported that some of these substrates, which are environmentally important compounds do not support growth of microorganisms (Janssen *et al*., 1989), but the enzymes present can affect hydrolysis by directly cleaving the carbon-halogen bond. These compounds were previously reported to act as substrates for the haloalkane dehalogenase (Janssen *et al*., 1995), and as further confirmed in this study.

Halide production from the individual substrates revealed that enzyme activity for all isolates was highest when 1,3-DCP was used as the substrate. An overall 10% higher activity was observed when compared to 1,2-DCA, for all isolates except *X. autotrophicus* GJ10. This is in contrast to a study done by Janssen *et al*. (1995) whereby higher dehalogenase activity was observed in the presence of 1,2-DCA as compared to 1,3-DCP i.e. 96% activity on 1,3-DCP relative to the 100% activity obtained on 1,2-DCA (Janssen *et al*., 1995). This reduction in activity can possibly be attributed to the subsequent sub-culturing of the bacterial isolates in the absence of 1,2-DCA over several years.

The haloalkane dehalogenase present in *X. autotrophicus* GJ10, *A. aquaticus* AD25 and UV6 had a higher affinity for 1,3-DBP as compared to 1,2-DBE, while the other isolates had similar activity on both compounds. The actual cleavage of the carbon-halogen bond is not rate-limiting in the overall conversion of both 1,2-DCA and 1,2-DBE, however the rate of cleavage of the C-Br bond is faster than the rate of cleavage of the C -Cl bond. This is because bromine is better replaced in bimolecular substitutions than chlorine due

to its weaker activity (Schanstra *et al*., 1996). The differences in activity or affinity of the haloalkane dehalogenases for the various halogenated compounds could be due to the different rates at which these isolates have adapted cleavage mechanisms to survive in the presence of pollutants at contaminated sites.

Specific dehalogenase activities of *A. aquaticus* AD25 and *A. aquaticus* AD27 with 1,2-DCA was higher than that of *X. autotrophicus* GJ10, with a 4-fold and 2.4-fold higher activity, respectively. The specific dehalogenase activities of the recently isolated *Ancylobacter* strains are comparable to the activities of *A. aquaticus* AD27 whilst also higher than that of *X. autotrophicus* GJ10. Results correspond with the findings of Van den Wijngaard *et al*. (1992), whereby, higher dehalogenase activities were reported for *A. aquaticus* AD25 and *A. aquaticus* AD27 as compared to *X. autotrophicus* GJ10. The potential for use of such organisms in bioremediation strategies is immense, as growth rates and enzyme activities of these isolates correlate well with *X. autotrophicus* GJ10 and previously characterized *Ancylobacter* strains.

To determine the pH optima of the dehalogenase activities of the *Ancylobacter* strains, crude extracts of the organisms were used in dehalogenase assays with different pH buffers. Optimum pH obtained for the haloalkane dehalogenase in all *Ancylobacter* strains was pH 8, while the previously isolated strains *A. aquaticus* AD25, *A. aquaticus* AD27 and *X. autotrophicus* GJ10 was further confirmed also at pH 8. It has previously been reported that haloalkane dehalogenase activity in the well-studied *X. autotrophicus* GJ10 cleaved the carbon-chlorine bond optimally at pH 8.2 (Janssen *et al*., 1985), while haloalkane dehalogenase activity in *Mycobacterium avium* N85 was optimum at pH 8 (Jesenska *et al*., 2002). Thus, it was not surprising that the optimum haloalkane dehalogenase activity obtained in this study was the same. Since the haloalkane dehalogenase enzyme present in these *Ancylobacter* strains was found to be identical to the haloalkane dehalogenase possessed by *X. autotrophicus* GJ10 (Govender, 2009), then the characteristics of the enzymes should ideally be the same.

Maximum haloacid dehalogenase activity in *A. aquaticus* AD25, DH5, UV6 and UV5 was observed at pH 9. The L-2-haloacid dehalogenase has been isolated from a few bacterial isolates thus far, which includes *X. autotrophicus* GJ10, *Pseudomonas putida* 109 and *Pseudomonas* sp. strain YL. The pH optimum for maximum haloacid dehalogenase activity in *X. autotrophicus* GJ10 and *Pseudomonas* sp. strain YL was reported to be 9.5. This is similar to the observation made in this study as maximum activity for most of the bacterial isolates was at pH 9. L-2-haloacid dehalogenases from other bacteria also exhibit common properties, having maximum reactivity in the pH range of 9-11 (Kurihara *et al*., 2000). However, the haloacid dehalogenase present in *Ancylobacter aquaticus* DH12 displayed similar activity at both pH 8 and 9. Such activity has been previously reported in a high affinity thermostable L-2-haloacid dehalogenase from *Azotobacter* sp. strain RC26 (Diez *et al*., 1996), indicating that some haloacid dehalogenases do possess a wider pH range. A pH optimum of 9 for most isolates could possibly indicate haloacid dehalogenases fall into the same group as *X. autotrophicus* GJ10. Both *Ancylobacter aquaticus* DH2 and *A. aquaticus* AD27, when grown in minimal salts media with MCA as sole carbon source, had the lowest growth rates among all the isolates used in the study with a corresponding low haloacid dehalogenase activities. *A. aquaticus* AD27, apart from having a low growth rate in MCA, also had low growth rates when 1,2-DCA was used as sole carbon source. This may be due to less efficient dehalogenases or as previously mentioned, due to accumulation of toxic intermediates which resulted in eventual death of bacterial cells. Although *aquaticus* DH2 also had low dehalogenase activity, growth was better as compared to *A. aquaticus* AD27. It has previously been reported that regulation of the 1,2-DCA pathway is at the level of alcohol dehydrogenase and aldehyde dehydrogenase rather than at the haloalkane dehalogenase level. An increase in alcohol dehydrogenase activity during a decreasing growth rate has frequently been observed with different methylotrophs (Van den Wijngaard *et al*., 1992). Thus, although the haloalkane dehalogenase activity is low in *Ancylobacter aquaticus* DH2, enhanced growth rates of this organism may have been due to higher expression levels of the dehydrogenase enzymes compensating for the lower dehalogenase activity, thus enhancing overall growth rate. The mildly alkaline pH optimum observed for haloacid dehalogenase activities observed in these isolates

appeared to be a common feature for L-2-haloacid dehalogenases as reported in previous studies (Asmara *et al*., 1993; Busto *et al*., 1992, Diez *et al.*, 1996; Janssen *et al*., 1985).

The optimum temperature, at which highest haloalkane dehalogenase activity was observed, was 30°C. This temperature was optimum for all strains of *Ancylobacter* isolates and other previously isolated strains as reported by Janssen *et al*. (1985) and Van den Wijngaard *et al*. (1992). Highest activity observed at 30°C may have been due to adaptation of these environmental strains during growth. As temperature varies in the environment, so do the isolates adapt to the varying temperatures. This could account for the stable enzyme activity observed at 40°C, with only a 10% loss in enzyme activity. At a temperature of 50°C, only three strains showed enzyme activity, namely *A. aquaticus* AD25, AD27 and *Ancylobacter aquaticus* DH12. Activity detected at this temperature make the haloalkane dehalogenase in these organisms a thermostable enzyme and can be considered for implementation on an industrial scale.

Overall, low enzyme activities were observed for all dehalogenase assays performed; as compared to specific dehalogenase activities reported in previous studies (Janssen *et al*., 1985; Van den Wijngaard *et al*., 1992). This may have been due to reduced enzyme stability over extended periods of time as well as sub-optimal assay conditions. Lower enzyme activities observed in this study also correlates with a study done by Govender (2009), whereby specific activities were also much lower than that reported in previous studies. This may indicate that the enzymes became less stable over time. Factors that may have contributed to such low enzyme activities included possible protein degradation of crude cell lysates or loss in protein expression due to repeated subculturing on media lacking 1,2-DCA. Loss in activity due to subculturing has been reported in previous studies, for example John *et al*. (2009) reported that several subcultivation steps in the absence of chlorinated ethenes resulted in the reversible loss of the reductive dehalogenase gene expression in the gram-negative anaerobic *S. multivorans*. Other studies have also reported loss of gene expression in the absence of chlorinated aliphatic hydrocarbons as the carbon source (Futagami *et al*., 2006a; Futagami *et al*., 2006b; Krum and Ensign, 2001).

Southern hybridization was performed using the *dhlB* and *aldB* genes from *X. autotrophicus* GJ10 as probes to determine whether genes were located on identical or different restriction fragments. Genomic DNA, extracted from all isolates, was digested with individual restriction enzymes followed by hybridization. Southern blot analysis for the *dhlB* probe indicated signal production for *Ancylobacter aquaticus* DH12 only. Southern blot analysis of the genomic DNA from *Ancylobacter aquaticus* DH12, digested with *Eco*RI and *Bam*HI, revealed signals at 4kb and 10 kb fragments, respectively. This indicates that the *Ancylobacter aquaticus* DH12 genome possibly contains a single copy of the *dhlB* gene without any additional homologous genes. *Pst*I restricted genomic DNA of *Ancylobacter aquaticus* DH12 showed two hybridization signals. Since the *dhlB* gene in *X. autotrophicus* GJ10 possesses a *Pst*I restriction site, two signals were ideally expected. However, multiple signals with two darker and lighter signals being observed. This result concurs with that of Govender (2009), suggesting the possibility of *X. autotrophicus* GJ10 possessing more than one homolog of the *dhlB* gene. Two hybridization signals detected for *Ancylobacter aquaticus* DH12 may indicate either the presence of a *Pst*I restriction site within the encoding gene region or the presence of more than one homolog of the gene. The presence of more than one haloacid dehalogenase in the same organism and with only minor differences in substrate specificities have been demonstrated in other studies (Van der Ploeg *et al*., 1991). Finding more than one copy of the *dhlB* gene in *Ancylobacter aquaticus* DH12 was thus not surprising. It has been suggested that these isoenzymes may have arisen by gene duplication and subsequent divergent evolution (Van der Ploeg *et al*., 1991). Although the *dhlB* probe hybridized to different restriction fragments of *Ancylobacter aquaticus* DH12 and *X. autotrophicus* GJ10, hybridization signals may imply the possible presence of a haloacid dehalogenase gene in *Ancylobacter aquaticus* DH12. In a study done by Song *et al*. (2004), genomic DNA of *X. flavus* UE-15 also hybridized with the *dhlB* probe from *X. autotrophicus* GJ10, but on a different restriction fragment i.e. a 23 kb *Bam*HI fragment, while an approximately 10 kb positive fragment was produced for *Bam*HI restricted genomic DNA of *Ancylobacter aquaticus* DH12. Thus, it is interesting to note that all three organisms,

being 1,2-DCA degraders, appear to possess the *dhlB* gene, however, located on different restriction fragments.

Analysis of blots from southern hybridization experiments revealed the presence of the *aldB* gene in all isolates including previously isolated *Ancylobacter* strains, *A. aquaticus* AD25 and *A. aquaticus* AD27. *Pst*I-digested genomic DNA of all strains produced a single 4 kb hybridization signal in all strains except *A. aquaticus* AD25 which produced the 4 kb signal as well as an additional signal on a larger fragment. This indicated that the *aldB* gene is homologous in all *Ancylobacter* strains as it is observed to be located on the same restriction fragment. These results correlated with Govender (2009), who also demonstrated signals on the same sized fragments for the *aldB* gene probe, in all strains, except *A. aquaticus* AD25 which also produced an additional hybridization signal. This might indicate that *A. aquaticus* AD25 possibly contains more than one copy of the aldehyde dehydrogenase gene. In a study done by Wijngaard *et al*. (1992), *A. aquaticus* AD25 was reported to have a 17-fold higher aldehyde dehydrogenase activity as compared to the activity in *X. autotrophicus* GJ10. Detection of an additional signal for *A. aquaticus* AD25 may therefore indicate the presence of more than one homolog of *aldB* which could be responsible for the higher aldehyde dehydrogenase activity observed.

X. autotrophicus GJ10 was observed to produce a single hybridization signal of approximately 4.5 kb. This is in correlation with a study done by Bergeron *et al*. (1998) where a single 4.5 kb signal was also obtained from *Pst*I digested genomic DNA. The intense dark band observed in this study also corresponds to Bergeron *et al*. (1998) as both the CAA dehydrogenase encoding genes were found to reside on two similar sized *Pst*I fragments of 4.4 and 4.5 kb. The intensity of the signals across all lanes was found to vary. *X. autotrophicus* GJ10, *Ancylobacter aquaticus* DH12 and *Ancylobacter aquaticus* DH5 produced much darker signals as compared to the other isolates. Southern blot analysis of the *Eco*RI digested genomic DNA also revealed that *Ancylobacter aquaticus* DH12 and DH5 produced two similar sized signals, while GJ10 was also observed to have multiple signals. The other isolates produced signals, however of lighter intensities.

This indicates that these *Ancylobacter* strains may contain more than one aldehyde dehydrogenase as previously identified in GJ10 (Bergeron *et al*., 1998). The detection of more than one enzyme in an isolate has been reported in several other bacterial strains, whether these genes are chromosomally or plasmid-borne still remain to be investigated. However, different plasmid and chromosomally encoded aldehyde dehydrogenases have been observed in other organisms such as, *Pseudomonas oleovorans* (Van der Ploeg *et al*., 1994). The detection of haloacid dehalogenases and aldehyde dehydrogenases in these *Ancylobacter* strains could explain their ability to degrade the recalcitrant 1,2-DCA. Several attempts to clone the positive fragments for further characterization were futile as DNA sequencing proved to be unsuccessful on a number of accounts. Re-cloning into a different plasmid vector was also unsuccessful. Therefore, further investigations may still be required to characterize these enzymes on both the molecular and protein levels so as to gain an insight into their possible application for bioremediation of sites contaminated by halogenated organic compounds.

CHAPTER FIVE
CONCLUDING REMARKS

5.1 THE RESEARCH IN PERSPECTIVE

Short-chain halogenated aliphatic hydrocarbons comprise one of the most important groups of chemicals that are produced in large quantities industrially (Song *et al*., 2003). 1,2-Dichloroethane (1,2-DCA), for example, is used for the production of vinyl chloride (chloroethene), a starting compound for the synthesis of polyvinyl chloride (PVC). Other compounds such as tetrachloroethene and trichloroethene are used as degreasing agents, while 1,2-dibromoethane and 1,3-dichloropropene are used as nematocides in agriculture (Van Hylckama Vlieg and Janssen, 2001). The last few decades has seen significant amounts of halogenated aliphatic hydrocarbons being released into the environment due to improper disposal, accidental spillage, or deliberate release, causing numerous cases of environmental pollution (Jain *et al*., 2005).

Various physico-chemical processes, such as land-filling, excavation and incineration, have been developed for treating such pollutants; however, these approaches are frequently prohibitively expensive, non-specific, or have the potential for introducing secondary contamination (Singh *et al*., 2008). Despite the toxicity and recalcitrance of halogenated compounds, microorganisms have evolved various mechanisms to degrade and transform these compounds, utilizing it as carbon and energy source. Bioremediation is thus an attractive alternative method for the clean-up of environmental pollutants. Bioremediation relies on the metabolic capacities of the microorganisms and their ability to adapt to hostile environments in the transformation of xenobiotics to harmless or less toxic compounds (Fantroussi and Agathos, 2005).

1,2-DCA has been given much attention as a model compound as it can be biologically degraded by microorganisms under several geochemical conditions. 1,2-DCA is subject to both aerobic and anaerobic biodegradation. Aerobic biodegradation has been most

extensively studied as these conditions result in the complete mineralization of 1,2-DCA to harmless substances namely; CO_2, H_2O and Cl^- (Janssen *et al*., 1985). A number of pure cultures capable of metabolizing 1,2-DCA aerobically has been isolated (Hage & Hartmans, 1999; Janssen *et al*., 1985; Stucki *et al*., 1983; Van den Wijngaard *et al*., 1992). The present study investigated the aerobic biodegradation of 1,2-DCA by five recently isolated South African *Ancylobacter* strains. This involved establishing the effect of vitamins and yeast extract on bacterial growth rates as well as determining the specific dehalogenase activities of the individual isolates (*Ancylobacter aquaticus* DH12, DH5, DH2, UV6 and UV5) under various conditions. The presence of 1,2-DCA degrading encoding genes, CAA dehydrogenase and HA dehalogenase, was also investigated.

To determine the bacterial growth rates and the effect of organic nutrients on bacterial growth, all isolates, including previously characterized strains (*Ancylobacter aquaticus* AD27, *Ancylobacter aquaticus* AD25 and *Xanthobacter autotrophicus* GJ10), were grown in batch culture containing either a vitamin solution or yeast extract (10 mg/l), and 5 mM 1,2-DCA. The ability of the five isolates to aerobically utilize 1,2-DCA as a sole carbon and energy source was confirmed. Furthermore, some of these growth rates were higher than that obtained for the previously characterized *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27. Although *X. autotrophicus* GJ10 is a well known 1,2-DCA degrader, the rate at which it can degrade 1,2-DCA is limited by the rate at which CAA can be metabolized. Thus, the use of the South African isolates in bioremediation strategies may be promising in future applications and result in improving degradation rates of 1,2-DCA at contaminated sites.

The ability of some microorganism to degrade 1,2-DCA is dependent on the addition of an organic nutrient. Some microorganisms may require vitamins or yeast extract whilst others may not require either. *X. autotrophicus* GJ10 (Janssen *et al*., 1985), *X. flavus* UE15 (Song *et al*., 2004) and *A. aquaticus* strains AD25 and AD27 (van den Wijngaard *et al*., 1992) all have a requirement for various vitamins and co-factors. The current study confirmed the requirement for vitamins and yeast extract, with higher growth rate constants being obtained for previously characterized strains, *X. autotrophicus* GJ10 and

A. aquaticus AD25, growing in minimal media supplemented with vitamins. The addition of vitamins to the batch cultures containing the South African isolates also had an overall greater impact on microbial growth than yeast extract. However, yeast extract resulted in the release of a higher concentration of chlorides over the 84 h incubation period than vitamins. The highest growth rate obtained in minimal salts media supplemented with vitamins was *Ancylobacter aquaticus* DH5 while *Ancylobacter aquaticus* DH12 had the highest growth rate in minimal media supplemented with yeast extract. This indicates that organisms of the same species may require different preferences for organic nutrients when involved in 1,2-DCA degradation. Therefore, prior to applying organisms in bioremediation strategies, it would be important to determine if the organism requires an organic nutrient and if so, what is the feasibility and effectiveness of the nutrient in stimulating microbial growth.

To further investigate the ability of the five South African isolates to utilize 1,2-DCA, specific haloalkane and haloacid dehalogenase activities were determined under various conditions. Optimum pH for the haloalkane and haloacid dehalogenase was observed at pH 8 and pH 9, for most isolates, respectively. The optimum temperature for haloalkane dehalogenase activity was observed at 30°C, while activity was still stable at 40°C with only 10% loss in activity. The stability of enzyme activity under various physical conditions makes these organisms useful in the bioremediation of sites contaminated with chlorinated compounds, as they would be able to withstand and adapt to various environmental conditions.

Previously characterized *A. aquaticus* AD25 demonstrated the highest dehalogenase activity while the five recently isolated *Ancylobacter* strains had higher activities than the previously characterized *A. aquaticus* AD27 and *X. autotrophicus* GJ10. The South African isolate with the highest dehalogenase activity, for both the haloalkane and haloacid dehalogenase was *Ancylobacter aquaticus* DH12. This was followed very closely by *Ancylobacter aquaticus* DH5. Haloalkane dehalogenase present in all the isolates display a wide substrate range with compounds such as 1,3-dichloropropene, 1,2-dibromopropane and 1,3-dibromopropane being used as substrates in the dehalogenase

assays. These properties therefore make the isolates useful in bioremediation of sites contaminated with a wide variety of halogenated hydrocarbons.

Dehalogenation is the critical step in the biodegradation of chlorinated aliphatic hydrocarbons as these enzymes cleave the carbon-chlorine bond. There are at least two distinct groups of hydrolytic dehalogenases based on their substrate range, namely; haloalkane dehalogenases and haloacid dehalogenases. The haloalkane dehalogenase is the first enzyme of the 1,2-DCA degradation pathway and is encoded for by the *dhl*A gene. This gene has thus far been identified in a few bacterial strains (Janssen *et al*., 1985;; Song *et al*., 2004; Van den Wijngaard *et al*., 1992). The recently isolated South African strains investigated in this study also possessed the first enzyme of the pathway, the hydrolytic dehalogenase, encoded for by *dhl*A gene (Govender, 2009). This therefore may have contributed to the high growth rates obtained when 1,2-DCA was used as a carbon source.

Although, the ability to grow with 1,2-DCA is attributed to the presence of two hydrolytic dehalogenases, observations have demonstrated the special role plasmid encoded aldehyde dehydrogenases play in the degradation pathway of *Xanthobacter autotrophicus* GJ10. In the absence of the plasmid, the organism is unable to grow in the presence of 2-chloroethanol, an intermediate of the pathway (Van der Ploeg *et al*., 1994). The ability of *X. autotrophicus* GJ10 to overcome the toxicity of these intermediates is attributed to the production of three different aldehyde dehydrogenases with the high expression of a plasmid encoded chloroacetaldehyde dehydrogenase (Van der Ploeg *et al*., 1994).

It was therefore of interest to determine whether South African isolates possessed the other important genes, namely; chloroacetaldehyde dehydrogenase and haloacid dehalogenase encoding genes, or their homologs. The presence of the genes was confirmed by using the Southern hybridization technique. The control strain used was *Xanthobacter autotrophicus* GJ10, while the *aldB* and *dhlB* genes were used as the probes during hybridization experiments. Positive hybridization signals were obtained for

restricted genomic DNA of all recently and previously isolated strains of *Ancylobacter* when *aldB* was used as the probe. More than one hybridization signal was observed for the positive control *X. autotrophicus* GJ10 as well as *Ancylobacter aquaticus* DH12 and DH5. The ability of isolates to utilize chlorinated aliphatic hydrocarbons may be attributed to the presence of the chloroacetaldehyde dehydrogenase. The presence of multiple signals for GJ10 was expected as it was shown previously to contain three aldehyde dehydrogenases. The presence of more than one aldehyde dehydrogenase for *Ancylobacter aquaticus* DH12 and DH5 may have accounted for the highest growth rates obtained for these organisms as indicated earlier. Hybridization experiments performed to determine the presence of the haloacid dehalogenase gene indicated positive signals for *Ancylobacter aquaticus* DH5 and DH12. The detection of the HA dehalogenase genes in *Ancylobacter aquaticus* DH12 and DH5 may explain the degradative ability of these isolates with 1,2-DCA as the sole carbon source.

It is promising that 9 different aerobic 1,2-DCA degrading bacteria isolated at different locations globally (*X. autotrophicus* GJ10-Netherlands; *X. flavus* UE-15-Korea, *A. aquaticus* AD25, AD27-Netherlands; *Ancylobacter aquaticus* DH12, DH5, DH2, UV6, UV5-South Africa) carry similar, if not identical genetic information for 1,2-DCA degradation. These microorganisms may lead to the natural attenuation of 1,2-DCA residues in the environment or might be used as a basis for achieving targeted bioremediation of 1,2-DCA isomers and other halogenated compounds.

5.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE STUDY

Bioremediation is an effective strategy in the removal of chlorinated aliphatic hydrocarbons, as it involves microorganisms that have evolved various mechanisms, over many decades, to degrade these toxic xenobiotics. The *Ancylobacter aquaticus* characterized in this study demonstrated this ability, since 1,2-DCA was effectively utilized and degraded. Such specialized bacterial cultures with good potential to utilize halogenated aliphatics may thus be applied in several biological technologies. One such

example is the use of biological waste gas purification. The elimination efficiency of this technology depends not only on the process technology used but also on the degradation properties of the bacterial strains involved, thus indicating the suitability of the *Ancylobacter aquaticus* for application in such technologies.

Several challenges and limitations may be encountered in bioremediation strategies involving the use of the *Ancylobacter* strains. Challenges may include the impact of the indigenous microbes on the individual isolates, the survival of the isolates at sites contaminated with a mixture of xenobiotics, and the possible high cost associated with additional nutrients required by the isolates. Thus, it is imperative that the South African isolates are characterized further, such as determining chlorinated compound concentrations that are toxic to the isolates, substances or chemicals present at contaminated sites that inhibit degradation and the effect of individual isolates versus bacterial consortia.

The characterization of xenobiotic degraders at a molecular level may gain insight on the genes encoding the degradative enzymes. Therefore in this study, Southern hybridization technique was used to determine the presence of degradative genes in the *Ancylobacter aquaticus* Results obtained indicated the presence of the CAA dehydrogenase and HA dehalogenase genes in two *Ancylobacter* strains; however, attempts to determine DNA sequences of these genes were unsuccessful. Future work should determine the DNA sequences of these genes present in *Ancylobacter aquaticus* DH12 and DH5 as well as compare these sequences to previously characterized genes. Molecular microbiology techniques provide a remarkable arsenal of molecular tools and may also be used in monitoring, detecting and quantifying the microbes. Thus, in order to maximize the potential benefits of microbes in combating pollution problems, it is vital that we have a fundamental understanding of their degradative potential under various conditions, its biochemical systems and its molecular biology, prior to implementing bioremediation strategies. Further lab based degradation experiments should be performed in order to evaluate the feasibility of applying 1,2-DCA degraders or their enzymes to on-site field bioremediation strategies.

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APPENDIX I

1. Composition of buffers and media

1.a Minimal Salts Media (per litre): (as per Section 2.2)

5.37 g of Na_2HPO_4 $12H_20$,

1.36 g of KH_2PO_4,

0.5 g of $(NH_4)_2SO_4$,

0.2 g of $MgSO_4$ '$7H_20$,

5 ml of a salts solution

1.b 1 x Trace element solution (per litre): (as per Section 2.2)

- 530 mg $CaCl_2$
- 200 mg $FeSO_4.7H_2O$
- 10 mg $ZnSO_4.7H_2O$
- 10 mg H_3BO_3
- 10 mg $CoCl_2$
- 10 mg $MnSO_4.5H_2O$
- 10 mg $NaMoO_4.2H_2O$
- 2 mg $NiCl_2.6H_2O$

1.c 1 x Vitamin solution (per litre): (as per Section 2.2)

- 0.012 g biotin
- 1 g choline chloride
- 1 g calcium (D)-pantothenate
- 2 g *i*-inositol
- 1 g nicotinic acid
- 1 g pyridoxine chloride
- 1 g thiamine chloride
- 0.2 g *p*-aminobenzoic acid

- 0. 1 g cyanocobalamin)

1.d 50 x TAE buffer (per litre) (as per Section 2.6)

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA, pH 8.5

1.e SOC medium (per litre) (as per Section 2.10.5)

- 20 g bacto-tryptone
- 5 g bacto-yeast extract
- 20 mM glucose
- 0.5 g NaCl
- 2.5 mM KCl
- 1 mM $MgCl_2$

2. Standard Curves of Absorbance vs Concentration

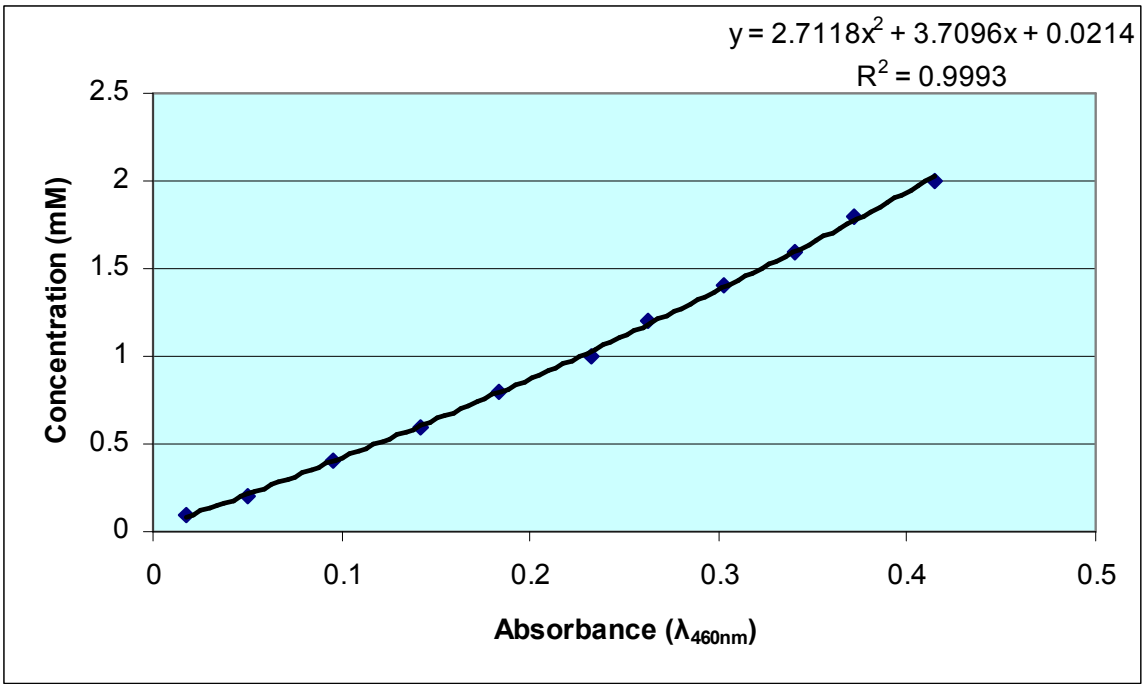

Figure 2.a: Standard curve for estimating the concentration of free chlorides released during cleavage of the carbon-chlorine bond

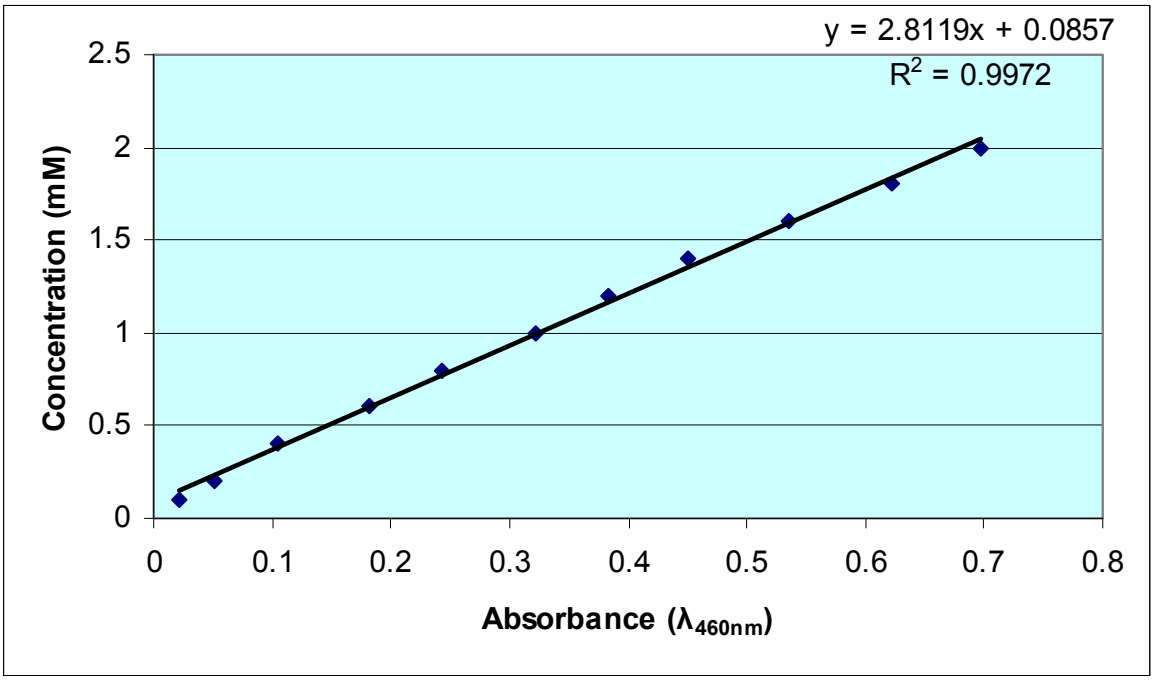

Figure 2.b: Standard curve for estimating the concentration of free bromides released during cleavage of the carbon-bromine bond.

3. Biodegradation of Halogenated Aliphatic Hydrocarbons

The data represented in Tables 3.i – 3.xxxv was used to generate the relevant Figures shown in parenthesis.

Table 3.i: Average triplicate optical density values for different bacterial isolates during the growth measurement studies with 1,2-DCA as sole carbon source in MMY (Fig 3.1)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.013333	0.049667	0.087333	0.170000	0.364667	0.476667	0.488000	0.533333
AD25	0.015000	0.059333	0.138000	0.304000	0.449667	0.473333	0.464333	0.474667
AD27	0.015667	0.034333	0.092000	0.084333	0.126667	0.159333	0.237667	0.291333
DH12	0.014000	0.065333	0.227667	0.462667	0.469000	0.452333	0.463667	0.487333
DH5	0.012333	0.048000	0.179333	0.363000	0.460333	0.433000	0.453667	0.479333
DH2	0.012667	0.054000	0.127333	0.243667	0.422000	0.408667	0.409667	0.429000
UV6	0.015667	0.045667	0.109333	0.208667	0.375000	0.422667	0.417000	0.423000
UV5	0.012667	0.055000	0.178333	0.402000	0.444333	0.426333	0.435000	0.465667
E.coli	0.011667	0.011667	0.019000	0.010000	0.014667	0.008000	0.020333	0.033000
CONTROL	0.000333	-0.00300	0.002000	0.004000	0.003667	0.002333	0.004000	0.002667

Table 3.ii: Average triplicate standard deviation values of optical density for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMY (Fig 3.1)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.001528	0.008622	0.040723	0.011358	0.012097	0.024826	0.025159	0.025968
AD25	0.001000	0.003786	0.005000	0.026851	0.025697	0.025146	0.025384	0.047816
AD27	0.002082	0.000577	0.039345	0.010970	0.012097	0.011930	0.034078	0.012097
DH12	0.001732	0.006028	0.012097	0.010408	0.017000	0.024214	0.014844	0.022723
DH5	0.001155	0.006245	0.016042	0.015716	0.013013	0.013000	0.005508	0.041789
DH2	0.001528	0.008185	0.025403	0.03581	0.007211	0.015044	0.010408	0.030447
UV6	0.002082	0.000577	0.009074	0.022008	0.033061	0.045938	0.010583	0.042755
UV5	0.000577	0.006928	0.002517	0.007937	0.004041	0.014468	0.005568	0.032470
E.coli	0.000577	0.001155	0.010583	0.004359	0.002517	0.003606	0.002887	0.008888
CONTROL	0.000577	0.006083	0.002000	0.005292	0.003786	0.001528	0.003000	0.002082

Table 3.iii: Average triplicate optical density values for different bacterial isolates during the growth measurement studies with 1,2-DCA as sole carbon source in MMV (Fig 3.2)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.012667	0.030333	0.086667	0.191000	0.421333	0.506667	0.510333	0.526333
AD25	0.015000	0.047000	0.171000	0.417667	0.492667	0.467667	0.457000	0.451333
AD27	0.015000	0.033333	0.062667	0.093000	0.124000	0.141000	0.213000	0.265333
DH12	0.011000	0.051333	0.220000	0.502333	0.491000	0.470000	0.479000	0.486333
DH5	0.012667	0.042000	0.156000	0.483667	0.463333	0.477333	0.508333	0.519500
DH2	0.011667	0.034333	0.126667	0.388000	0.437667	0.420667	0.421000	0.455333
UV6	0.013667	0.035667	0.114000	0.343000	0.429667	0.411333	0.415333	0.416667
UV5	0.012000	0.041667	0.118000	0.389667	0.454333	0.429333	0.436333	0.443333
E.coli	0.013000	0.004667	0.008667	0.008000	0.011000	0.020333	0.056333	0.075333
CONTROL	-0.00633	0.001000	-0.00067	0.002344	0.005667	0.003000	0.006667	0.013333

Table 3.iv: Average triplicate standard deviation values of optical density for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMV (Fig 3.2)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.001528	0.005859	0.012423	0.004359	0.007234	0.02444	0.021079	0.016010
AD25	0.001732	0.006245	0.044034	0.065858	0.046318	0.005508	0.014177	0.005033
AD27	0.002646	0.014503	0.005033	0.011533	0.015133	0.031177	0.050239	0.056359
DH12	0.001000	0.001528	0.026287	0.011930	0.023000	0.003464	0.002646	0.004041
DH5	0.004726	0.001732	0.017349	0.017786	0.038423	0.015373	0.023671	0.004950
DH2	0.000577	0.005686	0.024007	0.026907	0.037899	0.033292	0.035157	0.030925
UV6	0.002082	0.003512	0.016093	0.040927	0.004726	0.010116	0.004509	0.002517
UV5	0.001000	0.008083	0.002000	0.080277	0.024685	0.030238	0.033531	0.023714
E.coli	0.001000	0.004041	0.002309	0.003606	0.004583	0.016503	0.006028	0.015308
CONTROL	0.011846	0.001000	0.008622	0.002000	0.008963	0.002000	0.006658	0.007234

Table 3.v: Average triplicate chloride concentration values for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMY (Fig 3.3)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.038793	0.033800	0.114563	0.197829	0.263223	0.442077	0.352707	0.579910
AD25	0.035043	0.085317	0.148107	0.285803	0.357846	0.294555	0.362164	0.390113
AD27	0.042547	0.121209	0.122311	0.139025	0.161148	0.255164	0.291726	0.323434
DH12	0.036289	0.082830	0.256692	0.280857	0.395443	0.334183	0.418170	0.487642
DH5	0.036294	0.084006	0.285618	0.316793	0.343834	0.367530	0.373528	0.545217
DH2	0.033814	0.027594	0.193042	0.367826	0.448147	0.457989	0.468768	0.471499
UV6	0.038816	0.076536	0.179367	0.205855	0.363554	0.388452	0.444390	0.614821
UV5	0.032559	0.086540	0.257800	0.231023	0.373123	0.388827	0.366110	0.426965
E.coli	0.028832	0.030075	0.041306	0.089064	0.055120	0.032566	0.090343	0.171928
CONTROL	-0.02716	0.012839	0.026361	0.030089	0.030089	0.012839	0.030075	0.041342

Table 3.vi: Average triplicate standard deviation values of the chloride concentrations for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMY (Fig 3.3)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.013112	0.005696	0.008868	0.020530	0.022247	0.113569	0.207802	0.073901
AD25	0.005695	0.023788	0.018357	0.093860	0.037605	0.042666	0.053018	0.051322
AD27	0.017309	0.044574	0.022200	0.013414	0.026547	0.026836	0.054829	0.029023
DH12	0.007463	0.030443	0.048623	0.030079	0.117457	0.019601	0.096176	0.032946
DH5	0.009881	0.014407	0.084610	0.071995	0.011001	0.040483	0.069202	0.027908
DH2	0.011997	0.005678	0.068383	0.060080	0.079418	0.130162	0.087647	0.015493
UV6	0.018812	0.034086	0.006011	0.035730	0.086545	0.052677	0.180567	0.009719
UV5	0.006449	0.01438	0.014582	0.023510	0.029276	0.061477	0.062539	0.022994
E.coli	0.003720	0.005688	0.018478	0.010082	0.029748	0.009867	0.013382	0.060122
CONTROL	0.055364	0.024548	0.008592	0.011980	0.011980	0.024548	0.024960	0.005688

Table 3.vii: Independent T-Test comparing all bacterial isolates for the release of chloride ions in MMY (Fig 3.3)

	Isolate									
Isolate	GJ10	AD25	AD27	DH12	DH5	DH2	UV6	UV5	*E.coli*	CONTROL
GJ10		0.665053	0.288259	0.383297	0.633538	0.142688	0.533617	0.719593	0.00201	0.018334
AD25	0.639018		0.154156	0.156386	0.651128	0.105538	0.956971	0.631873	0.068526	0.007065
AD27	0.288259	0.154156		0.022826	0.072473	0.023043	0.028767	0.024554	0.079743	0.003006
DH12	0.383297	0.458502	0.022826		0.272273	0.247209	0.161937	0.190424	0.041227	0.001723
DH5	0.633538	0.651128	0.072473	0.272273		0.041004	0.433725	0.898034	0.008929	0.002851
DH2	0.142688	0.105538	0.023043	0.247209	0.041004		0.026901	0.04548	0.015969	0.000757
UV6	0.533617	0.956971	0.028767	0.161937	0.433725	0.026901		0.322198	0.032573	0.000112
UV5	0.719593	0.631873	0.024554	0.190424	0.898034	0.04548	0.322198		0.016427	0.001576
E.coli	0.00201	0.068526	0.079743	0.041227	0.008929	0.015969	0.032573	0.016427		0.060212
CONTROL	0.018334	0.007065	0.003006	0.001723	0.002851	0.000757	0.000112	0.001576	0.060212	

Table 3.viii: Average triplicate chloride concentration values for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMV (Fig 3.4)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.061416	0.03256	0.156242	0.26876	0.267277	0.297162	0.411167	0.497267
AD25	0.062557	0.037526	0.268637	0.232318	0.268690	0.298763	0.312356	0.356354
AD27	0.050105	0.046270	0.091595	0.119695	0.095441	0.192566	0.192911	0.332824
DH12	0.033796	0.122774	0.237720	0.271297	0.320379	0.348000	0.378002	0.372025
DH5	0.058877	0.042511	0.208473	0.311001	0.333117	0.353624	0.335806	0.407215
DH2	0.036287	0.025114	0.188655	0.294383	0.356330	0.465470	0.469307	0.440938
UV6	0.027594	0.047637	0.173034	0.287798	0.291657	0.283513	0.310739	0.352517
UV5	0.035043	0.038811	0.201758	0.243100	0.274222	0.278048	0.348273	0.406279
E.coli	0.000745	0.019063	0.050004	0.041291	0.036294	0.042549	0.047637	0.058791
CONTROL	-0.00173	0.023875	0.030078	-0.00044	0.030075	0.025114	0.026355	0.036316

Table 3.ix: Average triplicate standard deviation values of chloride concentration for different bacterial isolates during growth with 1,2-DCA as sole carbon source in MMV (Fig 3.4)

	Hours							
Isolate	0	12	24	36	48	60	72	84
GJ10	0.032839	0.007452	0.0566	0.119648	0.126972	0.136556	0.023012	0.026371
AD25	0.013577	0.002156	0.024744	0.020853	0.014064	0.033122	0.053806	0.046026
AD27	0.028700	0.012026	0.005819	0.010179	0.017202	0.022764	0.058949	0.024304
DH12	0.002153	0.065607	0.026343	0.010381	0.014258	0.014429	0.086497	0.061215
DH5	0.029490	0.004321	0.031893	0.051935	0.058231	0.030065	0.047457	0.067872
DH2	0.006467	0.003715	0.029159	0.116341	0.014466	0.058178	0.014758	0.036595
UV6	0.005678	0.032783	0.043049	0.113046	0.014353	0.018787	0.047748	0.060288
UV5	0.005695	0.017679	0.011894	0.030187	0.046682	0.053256	0.004068	0.097566
E.coli	0.045463	0.031896	0.005728	0.015098	0.009863	0.017704	0.032783	0.013557
CONTROL	0.043320	0.002143	0.007743	0.106253	0.005688	0.003715	0.005680	0.016292

Table 3.x: Independent T-Test comparing all bacterial isolates for the release of chlorides in MMV (Fig 3.4)

	Isolate									
Isolate	GJ10	AD25	AD27	DH12	DH5	DH2	UV6	UV5	*E.coli*	CONTROL
GJ10		0.72017	0.163521	0.11063	0.180189	0.029845	0.348821	0.117836	0.004762	0.007619
AD25	0.72017		0.623824	0.434748	0.122391	0.013281	0.04448	0.338249	0.017274	0.011722
AD27	0.163521	0.623824		0.367	0.154523	0.087543	0.68848	0.365841	0.001013	0.000364
DH12	0.11063	0.434748	0.367		0.651523	0.317697	0.806757	0.460844	0.008825	0.011552
DH5	0.180189	0.122391	0.154523	0.651523		0.549297	0.272702	0.993044	0.012832	0.007544
DH2	0.029845	0.013281	0.087543	0.317697	0.549297		0.06152	0.626978	0.005671	0.005018
UV6	0.348821	0.04448	0.68848	0.806757	0.272702	0.06152		0.586784	0.01952	0.013584
UV5	0.117836	0.338249	0.365841	0.460844	0.993044	0.626978	0.586784		0.024561	0.027639
E.coli	0.004762	0.017274	0.001013	0.008825	0.012832	0.005671	0.01952	0.024561		0.095072
CONTROL	0.007619	0.011722	0.000364	0.011552	0.007544	0.005018	0.013584	0.027639	0.095072	

Table 3.xi: Average triplicate optical density values for different bacterial isolates during the growth measurement studies with MCA as sole carbon source in MMV at pH 7 (Fig 3.5)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.014333	0.031667	0.038333	0.145000	0.297333	0.367333	0.357333
AD25	0.016333	0.049333	0.176333	0.318000	0.346333	0.364333	0.349000
AD27	0.018333	0.053333	0.084000	0.221000	0.325667	0.344667	0.337667
DH12	0.017000	0.073333	0.218333	0.362000	0.375667	0.399000	0.389333
DH5	0.014000	0.073667	0.119000	0.348333	0.364667	0.378000	0.374000
DH2	0.012333	0.042333	0.120667	0.263333	0.345000	0.345000	0.308667
UV6	0.010667	0.048667	0.106000	0.295333	0.355333	0.355333	0.347000
UV5	0.004000	0.056000	0.101667	0.301667	0.325000	0.327000	0.319000
E.coli	0.013000	0.007333	0.005000	0.056333	0.019000	0.033667	0.036333
CONTROL	-0.02000	0.006333	0.001000	0.001333	0.005333	0.006333	0.005667

Table 3.xii: Average triplicate optical density values for different bacterial isolates during the growth measurement studies with MCA as sole carbon source in MMV at pH 9 (Fig 3.6)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.016000	0.038667	0.058667	0.183667	0.305667	0.379333	0.385333
AD25	0.019000	0.062000	0.122667	0.315000	0.318000	0.374333	0.351333
AD27	0.013333	0.054667	0.087333	0.165667	0.320667	0.366667	0.376667
DH12	0.012000	0.083333	0.144333	0.22000	0.303000	0.399000	0.376000
DH5	0.018000	0.070000	0.095000	0.156667	0.175000	0.338000	0.379667
DH2	0.012333	0.085333	0.127667	0.253667	0.355000	0.382000	0.378000
UV6	0.026000	0.086333	0.135000	0.291667	0.400333	0.415333	0.413000
UV5	0.026000	0.074333	0.155000	0.297667	0.380333	0.405000	0.399000
E.coli	0.025667	0.064667	0.072333	0.060667	0.05000	0.081667	0.124000
CONTROL	0.005667	0.030000	0.031667	0.049000	0.037333	0.049667	0.043667

Table 3.xiii: Average triplicate standard deviation values of optical density for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 9 (Fig 3.6)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.005196	0.010017	0.009074	0.012423	0.027301	0.01115	0.014012
AD25	0.002646	0.019698	0.018037	0.017349	0.026889	0.008505	0.031390
AD27	0.005508	0.004041	0.015948	0.006506	0.022030	0.008505	0.015308
DH12	0.010536	0.033171	0.034646	0.028213	0.038432	0.028931	0.011790
DH5	0.007000	0.013748	0.019000	0.014640	0.051507	0.084658	0.030370
DH2	0.009609	0.029704	0.012342	0.019140	0.022605	0.015524	0.025942
UV6	0.016371	0.010017	0.006000	0.012220	0.025325	0.011372	0.016703
UV5	0.012288	0.007767	0.012530	0.043247	0.011060	0.016462	0.018520
E.coli	0.020648	0.015503	0.027934	0.038371	0.040447	0.042158	0.056347
CONTROL	0.008145	0.008718	0.004933	0.015395	0.010066	0.005508	0.014189

Table 3.xiv: Average standard deviation values of optical density for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 9 (Fig 3.6)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.004933	0.022143	0.016010	0.014799	0.024007	0.003055	0.007095
AD25	0.003786	0.011372	0.019553	0.005292	0.019140	0.009292	0.008718
AD27	0.004509	0.011930	0.008660	0.031241	0.017243	0.006351	0.012858
DH12	0.001000	0.004163	0.012897	0.009539	0.019218	0.011269	0.024214
DH5	0.005000	0.015275	0.004583	0.012741	0.004933	0.017321	0.013229
DH2	0.001155	0.009609	0.031533	0.032347	0.029513	0.029513	0.025813
UV6	0.001528	0.004041	0.009644	0.001528	0.017098	0.017098	0.010149
UV5	0.004583	0.017578	0.020502	0.016073	0.002000	0.007211	0.005568
E.coli	0.009539	0.002517	0.003606	0.06521	0.006000	0.007638	0.008505
CONTROL	0.026458	0.005033	0.001000	0.000577	0.001528	0.005033	0.004163

Table 3.xv: Average triplicate chloride concentration values for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 7 (Fig 3.7)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.067897	0.030073	0.031319	0.151102	0.367166	0.340398	0.346681
AD25	0.100727	0.055071	0.142978	0.194064	0.228475	0.187432	0.326523
AD27	0.086609	0.082805	0.126239	0.325046	0.286339	0.310881	0.256745
DH12	0.078993	0.10568	0.186124	0.380914	0.391351	0.320633	0.340046
DH5	0.037552	0.056276	0.185301	0.333117	0.252611	0.263458	0.336321
DH2	0.058948	0.058935	0.067784	0.231276	0.379131	0.280875	0.293800
UV6	0.075147	0.129037	0.157199	0.344496	0.324523	0.350219	0.300136
UV5	0.046281	0.112144	0.146845	0.299897	0.295966	0.341408	0.327596
E.coli	0.062536	0.030089	0.036332	0.037606	0.047545	0.033803	0.065173
CONTROL	0.028837	-0.01401	0.026355	0.030089	0.030075	0.032577	0.027594

Table 3.xvi: Average triplicate standard deviation values of the chloride concentrations for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 7 (Fig 3.7)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.051560	0.004296	0.007757	0.060451	0.104112	0.082702	0.029223
AD25	0.043286	0.022551	0.027948	0.045643	0.040219	0.040381	0.216254
AD27	0.027436	0.026984	0.032728	0.071843	0.036706	0.033712	0.053462
DH12	0.024326	0.025464	0.041328	0.093177	0.122420	0.124950	0.059620
DH5	0.014113	0.011480	0.077410	0.058231	0.044388	0.051369	0.087641
DH2	0.037639	0.036427	0.041569	0.052734	0.070261	0.032676	0.086613
UV6	0.012189	0.054356	0.018473	0.080536	0.016713	0.090132	0.054121
UV5	0.015149	0.035121	0.105890	0.027233	0.047340	0.058122	0.057708
E.coli	0.003769	0.011980	0.019781	0.024924	0.018738	0.007755	0.031497
CONTROL	0.007441	0.038429	0.005680	0.011980	0.005688	0.013447	0.005678

Table 3.xvii: Independent T-Test comparing all bacterial isolates for the release of chloride ions in MMV containing 5mM MCA at pH 7 (Fig 3.7)

	Isolate									
Isolate	GJ10	AD25	AD27	DH12	DH5	DH2	UV6	UV5	E.coli	CONTROL
GJ10		0.888281	0.197946	0.856664	0.886275	0.459694	0.426962	0.66183	0.012639	0.002255
AD25	0.888281		0.656517	0.939546	0.95731	0.718754	0.837981	0.991709	0.19976	0.134468
AD27	0.197946	0.656517		0.228536	0.100539	0.576316	0.17996	0.297356	0.009314	0.020691
DH12	0.856664	0.939546	0.228536		0.946946	0.638825	0.579955	0.869095	0.012199	0.013232
DH5	0.886275	0.95731	0.100539	0.946946		0.660593	0.533482	0.920146	0.014113	0.029072
DH2	0.459694	0.718754	0.576316	0.638825	0.660593		0.872883	0.26445	0.063639	0.03138
UV6	0.426962	0.837981	0.17996	0.579955	0.533482	0.872883		0.507238	0.017453	0.01353
UV5	0.66183	0.991709	0.297356	0.869095	0.920146	0.26445	0.507238		0.032324	0.010347
E.coli	0.012639	0.19976	0.009314	0.012199	0.014113	0.063639	0.017453	0.032324		0.221896
CONTROL	0.002255	0.134468	0.020691	0.013232	0.029072	0.03138	0.01353	0.010347	0.221896	

Table 3.xviii: Average triplicate chloride concentration values for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 9 (Fig 3.8)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.050145	0.079043	0.153324	0.240608	0.289679	0.312437	0.317649
AD25	0.083123	0.152756	0.180690	0.283485	0.439274	0.279817	0.224374
AD27	0.047524	0.130047	0.109640	0.283708	0.425856	0.399988	0.259155
DH12	0.086573	0.157206	0.157402	0.346913	0.451341	0.230170	0.407012
DH5	0.074410	0.091617	0.243038	0.269102	0.328489	0.346604	0.350943
DH2	0.056339	0.089122	0.085531	0.236450	0.418398	0.352446	0.408250
UV6	0.082794	0.123688	0.232992	0.604598	0.791230	0.370116	0.355021
UV5	0.047524	0.104467	0.254283	0.362613	0.484244	0.239414	0.386064
E.coli	0.028835	0.077792	0.105796	0.089400	0.074486	0.057580	0.038782
CONTROL	0.028843	0.043793	0.058783	0.088031	0.041271	0.036284	0.031319

Table 3.xix: Average triplicate standard deviation values of the chloride concentrations for different bacterial isolates during growth with MCA as sole carbon source in MMV at pH 9 (Fig 3.8)

	Hours						
Isolate	0	12	24	36	48	60	72
GJ10	0.033745	0.031500	0.023510	0.051071	0.084641	0.053484	0.016661
AD25	0.057223	0.082301	0.112180	0.116514	0.011374	0.061295	0.026534
AD27	0.013526	0.025139	0.041116	0.046482	0.084622	0.054099	0.016849
DH12	0.021593	0.020003	0.045440	0.055201	0.074300	0.069905	0.051585
DH5	0.066141	0.014448	0.018972	0.069749	0.107629	0.010461	0.042834
DH2	0.025104	0.023822	0.047586	0.036079	0.057740	0.128863	0.030101
UV6	0.025357	0.035412	0.078036	0.053809	0.199036	0.111629	0.031279
UV5	0.013499	0.034128	0.070273	0.084691	0.062713	0.062267	0.064557
E.coli	0.006449	0.033161	0.039998	0.052597	0.070562	0.022955	0.009396
CONTROL	0.009853	0.017131	0.011292	0.044783	0.008642	0.003731	0.007757

Table 3.xx: Independent T-Test comparing all bacterial isolates for the release of chloride ions in MMV containing 5mM MCA at pH 9 (Fig 3.8)

Isolate	Isolate									
	GJ10	AD25	AD27	DH12	DH5	DH2	UV6	UV5	E.coli	CONTROL
GJ10		0.061805	0.090529	0.111249	0.316895	0.061506	0.308961	0.228677	0.000932	0.001765
AD25	0.061805		0.098715	0.017714	0.036492	0.002985	0.005411	0.067454	0.007761	0.004065
AD27	0.090529	0.098715		0.048508	0.098268	0.016287	0.009334	0.061216	0.004256	0.002269
DH12	0.111249	0.017714	0.048508		0.02704	0.941033	0.268571	0.781978	0.004809	0.00456
DH5	0.316895	0.036492	0.098268	0.02704		0.063462	0.915848	0.628174	0.003962	0.00429
DH2	0.061506	0.002985	0.016287	0.941033	0.063462		0.113872	0.711737	0.001672	0.001177
UV6	0.308961	0.005411	0.009334	0.268571	0.915848	0.113872		0.488823	0.004632	0.002868
UV5	0.228677	0.067454	0.061216	0.781978	0.628174	0.711737	0.488823		0.014601	0.013204
E.coli	0.000932	0.007761	0.004256	0.004809	0.003962	0.001672	0.004632	0.014601		0.224954
CONTROL	0.001765	0.004065	0.002269	0.00456	0.00429	0.001177	0.002868	0.013204	0.224954	

Table 3.xxi: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at pH 7

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
X. autotrophicus GJ10	0.091187	1.728165	18.9518±0.013929
A. aquaticus AD25	0.406546	27.41947	67.4450±0.060079
A. aquaticus AD27	0.486154	14.55168	29.93227±0.022137
Ancylobacter strain DH12	0.662889	28.45534	42.92622±0.047438
Ancylobacter strain DH5	0.523038	19.20241	36.71324±0.062837
Ancylobacter strain DH2	0.480006	6.866741	14.30553±0.047066
Ancylobacter strain UV6	0.438512	12.0795	27.54658±0.058683
Ancylobacter strain UV5	0.552545	20.25984	36.66643±0.021031
E.coli	0.642911	8.334696	12.96400±0.046581

Table 3.xxii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at pH 7.5

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min	Specific activity (mU/mg protein)
GJ10	0.091187	1.601288	17.56041 ± 0.003861
AD25	0.406546	41.3745	101.7709 ± 0.067075
AD27	0.486154	21.0391	43.27666 ± 0.01575
DH12	0.662889	36.36619	54.86012 ± 0.025487
DH5	0.523038	26.60696	50.87008 ± 0.023229
DH2	0.480006	9.376355	19.53382 ± 0.007745
UV6	0.438512	20.11801	45.87792 ± 0.177997
UV5	0.552545	27.12014	49.08225 ± 0.128812
E.coli	0.642911	6.588791	10.24838 ± 0.030465

Table 3.xxiii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at pH 8

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min	Specific activity (mU/mg protein)
GJ10	0.091187	2.430767	26.65683±0.022445
AD25	0.406546	44.81921	110.244±0.126076
AD27	0.486154	22.73525	46.76556±0.150997
DH12	0.662889	42.92663	64.75685±0.049088
DH5	0.523038	31.62902	60.47179±0.161516
DH2	0.480006	9.699713	20.20747±0.004501
UV6	0.438512	22.2399	50.71678±0.057545
UV5	0.552545	27.32667	49.45603±0.065255
E.coli	0.642911	7.953445	12.37100±0.050287

Table 3.xxiv: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at pH 9

Isolate	Final protein concentration (mg/ml)	Reaction rate (µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.986289	21.78249±0.021452
AD25	0.406546	42.71667	105.0723±0.142224
AD27	0.486154	16.61633	34.17917±0.065145
DH12	0.662889	29.9705	45.21192±0.021833
DH5	0.523038	21.50177	41.10942±0.010972
DH2	0.480006	8.013734	16.69506±0.035432
UV6	0.438512	17.27636	39.39771±0.022957
UV5	0.552545	25.4038	45.97599±0.094088
E.coli	0.642911	8.818435	13.71643±0.031040

Table 3.xxv: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using MCA as substrate at pH 7

Isolate	Final protein concentration (mg/ml)	Reaction rate (µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.396916	15.31918±0.008062
AD25	0.406546	24.10824	59.30022± 0.145943
AD27	0.486154	15.04037	30.93748±0.005642
DH12	0.662889	19.3134	29.13517±0.055835
DH5	0.523038	14.49971	27.72212±0.009697
DH2	0.480006	13.72327	28.58977±0.021633
UV6	0.438512	13.32523	30.38741±0.029171
UV5	0.552545	20.23279	36.61747±0.013138
E.coli	0.642911	8.990179	13.98356±0.011800

Table 3.xxvi: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using MCA as substrate at pH 7.5

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	3.281473	35.98604±0.00611
AD25	0.406546	22.95143	56.45475±0.066418
AD27	0.486154	11.69788	24.06212±0.002676
DH12	0.662889	34.22878	51.63574±0.015131
DH5	0.523038	19.81485	37.88418±0.010772
DH2	0.480006	17.61667	36.70091±0.243317
UV6	0.438512	12.11115	27.61876±0.009711
UV5	0.552545	17.91208	32.41742±0.002917
E.coli	0.642911	7.65960	11.91394±0.002507

Table 3.xxvii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using MCA as substrate at pH 8

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min	Specific activity (mU/mg protein)
GJ10	0.091187	4.72178	51.78106±0.008556
AD25	0.406546	37.24865	91.62232±0.164698
AD27	0.486154	13.81012	28.4069±0.008271
DH12	0.662889	71.06553	107.2057±0.019512
DH5	0.523038	28.84504	55.14908±0.030182
DH2	0.480006	11.46225	23.87938±0.020146
UV6	0.438512	14.26281	32.52549±0.007344
UV5	0.552545	20.38332	36.88989±0.010413
E.coli	0.642911	8.35987	13.00316±0.016645

Table 3.xxviii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using MCA as substrate at pH 9

Isolate	Final protein concentration (mg/ml)	Reaction rate (µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	6.033901	66.17034±0.035646
AD25	0.406546	47.54709	116.9539±0.189173
AD27	0.486154	13.25615	27.2674±0.032343
DH12	0.662889	70.30313	106.0556±0.049768
DH5	0.523038	41.02049	78.42742±0.078609
DH2	0.480006	11.06887	23.05985±0.00266
UV6	0.438512	21.49293	49.01335±0.009164
UV5	0.552545	27.82813	50.36358±0.037706
E.coli	0.642911	6.790649	10.56235±0.015501

Table 3.xxix: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at 30°C

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	0.498952	5.471723±0.003764
AD25	0.406546	24.42292	60.07424±0.113961
AD27	0.486154	7.707748	15.85455±0.045598
DH12	0.662889	13.54521	20.43359±0.009914
DH5	0.523038	9.742241	18.62627±0.024750
DH2	0.480006	3.081094	6.418861±0.018224
UV6	0.438512	8.436807	19.23964±0.051711
UV5	0.552545	11.51808	20.84552±0.060141
E.coli	0.642911	2.297352	3.573361±0.058020

Table 3.xxx: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at 30°C

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.601288	17.56041±0.003861
AD25	0.406546	41.37450	101.7709±0.067075
AD27	0.486154	21.03910	43.27666±0.015750
DH12	0.662889	36.36619	54.86012±0.025487
DH5	0.523038	26.60696	50.87008±0.023229
DH2	0.480006	9.376355	19.53382±0.007745
UV6	0.438512	20.11801	45.87792±0.177997
UV5	0.552545	27.12014	49.08225±0.128812
E.coli	0.642911	6.588791	10.24838±0.027452

Table 3.xxxi: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at 40°C

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.481311	16.24468±0.039332
AD25	0.406546	36.84881	90.6388±0.028251
AD27	0.486154	18.94449	38.96813±0.053255
DH12	0.662889	32.64861	49.25198±0.023976
DH5	0.523038	20.93365	40.02322±0.047782
DH2	0.480006	8.259598	17.20727±0.080538
UV6	0.438512	15.56832	35.50264±0.031509
UV5	0.552545	23.55086	42.62254±0.109548
E.coli	0.642911	8.28679	12.88949±0.090291

Table 3.xxxii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,2-DCA as substrate at 50°C

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	ND	ND
AD25	0.406546	16.35185	40.22144±0.076921
AD27	0.486154	7.787927	16.01948±0.043414
DH12	0.662889	11.4775	17.31435±0.040563
DH5	0.523038	ND	ND
DH2	0.480006	ND	ND
UV6	0.438512	ND	ND
UV5	0.552545	ND	ND
E.coli	0.642911	ND	ND

Table 3.xxxiii: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,3-DBP as substrate

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.983952	21.75686±0.009875
AD25	0.406546	26.49435	65.16943±0.051874
AD27	0.486154	12.87225	26.47775±0.011707
DH12	0.662889	21.35482	32.21475±0.035826
DH5	0.523038	15.60605	29.83733±0.029222
DH2	0.480006	5.045798	10.51194±0.035158
UV6	0.438512	14.32507	32.66747±0.028166
UV5	0.552545	18.66789	33.7853±0.021105
E.coli	0.642911	3.84293	5.977394±0.033743

Table 3.xxxiv: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using 1,3-DCP as substrate

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	5.190484	56.92107±0.016822
AD25	0.406546	45.33043	111.5015±0.015205
AD27	0.486154	24.39158	50.17259±0.062969
DH12	0.662889	39.11509	59.00697±0.034596
DH5	0.523038	28.98366	55.4141±0.006607
DH2	0.480006	12.64783	26.3493±0.021217
UV6	0.438512	25.39584	57.91371±0.050623
UV5	0.552545	34.80866	62.997±0.353866
E.coli	0.642911	5.631483	8.759357±0.023863

Table 3.xxxv: Hydrolytic dehalogenase activity (mU/mg protein) of all isolates using DBE as substrate

Isolate	Final protein concentration (mg/ml)	Reaction rate µM/min)	Specific activity (mU/mg protein)
GJ10	0.091187	1.12476	12.3346±0.01443
AD25	0.406546	12.16928	29.93337±0.009039
AD27	0.486154	12.26301	25.22456±0.022554
DH12	0.662889	22.57331	34.0529±0.009039
DH5	0.523038	16.26216	31.09175±0.009875
DH2	0.480006	4.545905	9.470512±0.005853
UV6	0.438512	12.70042	28.96254±0.00859
UV5	0.552545	18.24611	33.02195±0.012886
E.coli	0.642911	4.6865	7.289505±0.017181