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Investigation of growth of *Rhizobium sp.* at low concentrations of halogenated compound

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ABSTRACT

Investigation of growth of *Rhizobium sp.* at low concentrations of halogenated compound

Fahrul Huyop and Ng Hong Jing

Halogenated organic compounds are produced industrially in large quantities and represent an important class of environmental pollutants. Bacteria have evolve several strategies for the enzymes to catalysed dehalogenation and degradation of halogenated compounds. The destruction of halogenated chemicals by microorganisms may be influenced by environmental factors or the structure of the chemical itself. One of the reasons suggested for the lack of degradation of organic compounds by microbes is their low concentration. Such organisms appear to be adapted to low substrate concentrations by having high substrate affinity (low Km value) systems. Our current investigation is to study the possibility of growth of *Rhizobium sp.* at low concentrations of halogenated substrate (2,2-dichloropropionic acid; 2,2DCP). 2,2DCP was chosen because it was widely used as a herbicide (Dalapon). The degradation of low concentrations of 2,2DCP by whole cells of *Rhizobium sp.* has been achieved (cells doubling time: 12 hours). *Rhizobium sp.* was able to grow at 0.2 mM 2,2DCP which was 100x lower than the concentration of the substrate routinely used (20 mM) with cells doubling time of 11 hours. Dehalogenase specific activity for crude extracts from cells grown at 20mM and 0.2 mM 2,2DCP PJC minimal media was calculated and the Km values for both enzymes were 0.1mM respevtively. Apparently, no new dehalogenases are required to allow growth on this low concentration of 2,2DCP as judged by dehalogenase properties which is similar to the growth at low substrate concentration. A new isolated bacterium was identified as *Methylobacterium sp.* by 16S rRNA method was found to be able to degrade 2,2DCP. The bacterium grew in 20mM 2,2DCP PJC minimal medium with a doubling time of 22.80 hours. Degradation of 2,2DCP was detected in growth medium by HPLC technique.

LIST OF ABBREVIATIONS

2,2-DCP	-	2,2-dichloropropionic acid
D,L2CP	-	D,L-2-chloropropionic acid
A	-	Absorbance
BLAST	-	Basic local alignment search tool
DDBJ	-	DNA Data Bank of Japan
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraaceticacid, $(HOOCCH_2)_2N(CH_2)_2N(CH_2COOH)_2$
EMBL	-	European Molecular Biology Laboratory
EtBr	-	Ethidium Bromide
H	-	Hour
kb	-	Kilo base
kDA	-	Kilo Dalton
min	-	Minutes
NCBI	-	National Center for Biotechnology Information
OD	-	Optical Density
PCR	-	Polymerase Chain Reaction
RDP	-	Ribosomal database project
RNA	-	Ribonucleic acid
rDNA	-	Ribosomal DNA
rRNA	-	Ribosomal RNA
TAE	-	Tris-Acetate-EDTA
UV	-	Ultraviolet

UNIVERSITI TEKNOLOGI MALAYSIA

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CHAPTER 1

INTRODUCTION

1.1 Oligotrophy

A less well understood part of the microbial community in soil and in other natural environments are the oligotrophs. Generally these microorganisms can be maintained on media containing less than 15mg/l of organic matter. The study of oligotrophs and slow-growing organisms is an important area in environmental microbiology (Prescot *et al.,* 1996). Oligotrophs are ubiquitous in the environment and have been isolated from soil, rivers, lakes, oceans and tap-water lacking organic substances. There are two types of oligotrophs that have been identified. Those that are able to grow only on a very low concentration of carbon are called obligate oligotrophs and those that are able grow at both low and high concentrations of organic substances are called facultative oligotrophs.

A detailed study was carried out on the enteric bacterium *Enterococcus faecalis*. The natural habitat of this bacterium is in the intestine of humans and animals. When discharged into a natural aquatic system this microorganism needs to adapt for survival in a hostile environment. The ability of *Enterococcus faecalis* to survive in an oligotrophic environment such as in tap water was shown (Hartke *et al.,* 1998).

Many oligotrophic bacteria can be isolated from clinical materials including urine, sputum and vaginal discharges and throat swabs. However, the clinical significance of such oligotrophic bacteria is uncertain (Tada *et al*., 1995).

Slow growth of oligotrophic bacteria was possibly due to the slow process of transport of nutrients into the cell. Dissolved nutrients entering the aquatic environment are assimilated into microorganisms due to permeability and active transport through the cytoplasmic membrane. In some oligotrophic bacteria there is a cellular appendage (prostheca) whose function is to increase the surface area of cell and cytoplasmic membrane for increasing transport of substances into a cell. A biochemical study on prostheca of *Asticcacaulis biprosthecum* revealed that it did not contain DNA, RNA, NADH-oxidase and NADH-dehydrogenase. This highlights the specialised function of prostheca connected with the active transport of nutrients (Jordan *et al.,* 1974). Some oligotrophic bacteria show inhibition of growth in rich nutrient media (Kuznetsoz *et al*., 1979). One reason may be the action of toxic products of metabolism particularly hydrogen peroxide which forms in a number of metabolic reactions. Dubinina (1977) showed that the oligotrophic bacterium *Leptothrix pseudoochraceae* begins to lyse due to accumulation of hydrogen peroxide in a rich medium. The addition of catalase resulted in growth of this organism.

Xenobiotic chemicals that enter the environment are mostly from chlorinated aliphatic acid compounds. Many of these pollutants are present in natural waters only at very low concentrations. However, such concentrations are still sufficient to be a public health and environmental concern (Fauzi *et al.,* 1996). Biodegradation of low substrate concentrations of xenobiotic compounds has been a neglected field of research. The rate of biodegradation at low concentration usually decreases with lower initial substrate concentrations (Boethling and Alexander, 1979). For example the herbicide (2,4-dichlorophenoxy acetic acid) at 2.2 parts/billion was mineralised more slowly in stream water than at higher concentrations (22 ppm). In addition, the carbon of very low concentrations of xenobiotics is often not converted to biomas carbon,

thus other carbon sources may be required for cell growth under these conditions (Subba-Rao *et al.,* 1982).

The bacterial degradation of low concentrations of 1,3-dichloro-2-propanol (1,3-DCP) has been reported. The isolate was identified as *Agrobacterium sp*., which was able to dehalogenate 1,3-DCP with a Km value of 0.1mM (Fauzi *et al*., 1996). However, there is no report so far in the current literature for degradation of low concentration of haloalkanoic aliphatic acids.

1.2 General problem statement

Biodegradation of very low concentrations of xenobiotic compounds has been neglected. It is useful to know about growth of microorganisms in low concentrations of pollutants because of the legal requirements. A government body or legislation may set the concentration of a pollutant down to a level that is not considered harmful. Therefore, if the microorganisms could only remove high concentration of pollutants, they could not be used to meet requirements of the law, since there still will be low concentrations of pollutants in the environment.

The *Rhizobium sp.* had been isolated using 20mM 2,2DCP and it was not known whether the organism could grow at concentrations of the order of one hundreth of this value i.e. could exhibit oligotrophism?. However, should growth be possible at very low substrate concentrations some of the Km values of Rhizobial dehalogenases suggest that the known dehalogenases might function satisfactorily at low substrate concentrations. Alternatively, a different type of dehalogenase with a higher substrate affinity might be needed/present.

1.3 Research objectives

- To determine the ability of *Rhizobium sp*. to degrade 2,2DCP at low concentration.
- To determine the kinetic parameter (i.e. Km values) of *Rhizobium sp.* dehalogenases involved in growth at high and low substrate concentration
- To identify whether the same dehalogenases are involved when growth at low substrate concentration or different dehalogenases are present
- To characterize newly isolate organism that able to degrade 2,2DCP

CHAPTER 2

MATERIAL AND METHODS

2.1 Growth conditions and media

Luria broth (LB) media was described by Miller (1972) and contained yeast extract 10.0 g/l, tryptone 5.0 g/l and NaCl 10.0 g/l.

PJC chloride-free minimal media was prepared as 10x concentrated basal salts containing K_2HPO_4. $3H_2O$ (42.5g/l), NaH_2PO_4. $2H_2O$ (10.0 g/l) and $(NH_4)_2SO_4$ (25.0 g/l). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g/l), $MgSO_4$(2.0 g/l), $FeSO_4$. $7H_2O$ (120.0 mg/l), $MnSO_4$. $4H_2O$ (30.0 mg/l), $ZnSO_4$. H_2O (30 mg/l) and $CoCl_2$ (10.0 mg/l) in distilled water (Hareland *et al.,* 1975).

Minimal media for growing bacteria contained 10ml of 10x basal salts and 10ml of 10x trace metal salts per 100ml of distilled water and were autoclaved (121^{o}C, for 15 minutes). Carbon sources were sterilised separately and added aseptically to the media to the desired final concentration. Liquid minimal cultures were supplemented with yeast extract to a final concentration of 0.05% (w/v) for growth of *Rhizobium*. Liquid cultures were incubated in a Gallenkamp orbital shaker at 200 rpm at the appropriate temperature. The liquid media was solidified by addition of Oxoid bacteriological agar No. 1 (1.5 % w/v) prior to sterilisation.

2.2 Glycerol stocks

Glycerol stocks of organisms were prepared by adding 0.3ml of sterile 50% glycerol to 0.7ml of bacterial culture. The sample was then mixed thoroughly and frozen by standing in dry ice/IMS bath. Stocks were stored at –80^{o}C.

2.3 Measurement of DNA concentration

DNA concentration was estimated by ultraviolet spectrophotometry. An A_{260nm} of 1.0 corresponds to 50μg of double stranded DNA per ml. The DNA purity was estimated from the A_{260nm} / A_{280nm} = ratio. Ratios of less than 1.8 indicate that the preparation is contaminated with protein (Maniatis *et al*., 1982).

2.4 Agarose gel electrophoresis

Restriction enzyme digests were analysed by submarine gel electrophoresis through agarose gels. Generally, 0.8% agarose gel prepared in TAE buffer (40 mM Tris-acetate pH 7.6, 1 mM EDTA + ethidium bromide at 0.5 μg/ml) was used. Samples (between 2 to 5 μl) were mixed with 5μl of gel loading buffer [0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 30% glycerol (w/v)] before loading. Gels were run at a constant 90 volts for 1 to 2 hours (Sambrook *et al.*, 1989).

DNA fragments on the gel were visualised using a UV transilluminator. The sizes were estimated by comparison with a 1 kb ladder (Life Technologies Inc.) standard DNA marker (1μg of DNA marker was used each time). The DNA Ladder could also be used as a means of estimating the amount of DNA present in a sample as the band at 1636 base pairs makes up 10 % (100 ng) of the total DNA present in the marker used.

2.5 Polymerase Chain Reaction (PCR) for Amplification of DNA

PCR reactions were generally carry out in 50µl reaction. The components in the PCR reactions are:

10x buffer with $MgSO_4$(Promega)	5.0µl
dNTPs mix (10mM)	4.0µl
Forward primer (20pmol/µl)	1.0µl
Reverse primer (20pmol/µl)	1.0µl
DNA template	variable(e.g.>0.5µg)
Pfu DNA polymerase (3u/µl)(Promega)	0.3µl
Sterile distilled water to final volume of	50µl

The amplification was carried out using a Techne Progene thermal cycler. The program used was as follows:

Initial denaturation:	95°C	60 sec	1 cycle
Denaturation:	95°C	1.5mins	
Annealing:	between 50 to 55°C	40 sec	25 cycles
Extension:	72°C	1 mins	
Final extension:	72°C	10 mins	1 cycle
Stop:	4°C		

The exact parameters were dependent on a number of factors including estimated length of the final product and predicted annealing temperature of the primers. After completion the reaction mixture was electrophoresed on 0.8% agarose gel.

The primer concentration was calculated using the equation:-

$$\text{Concentration(pmol/µl)} = \frac{100 \times \text{Absorbance}_{260nm} \times \text{dilution factor of the DNA solution}}{\boldsymbol{A}}$$

Where ***A***: (*n*A x 1.54) + (*n*G x 1.17) + (*n*T x 0.92) + (*n*C x 0.75); *n*= is the number of that base in the oligodeoxyribonucleotide

2.6 DNA sequencing and oligodeoxyribonucleotoide synthesis

Sequencing was performed by the 1st Base Laboratory, Malaysia. Initial sequencing of both strands was carried out using an Applied Biosystem PRISM ® 377 automated DNA sequencer by employing forward and reverse PCR primers. Sequences were extended by designing downstream primers based on the available determined sequence.

2.7 Preparation of cell-free extracts

Cell-free extracts were prepared from bacterial cells in mid- to late-exponential phase of growth. Cells from 100ml culture were harvested by centrifugation at 10,000g for 10 minutes at 4°C. The cell pellets were resuspended in 20ml of 0.1M Tris-acetate buffer pH7.6 and centrifuged at 10,000g for 10 minutes at 4°C. The cells were then resuspended in 4ml of 0.1M Tris-acetate buffer pH7.6 and maintained at 0°C for ultrasonication in a Vibra Cells™ ultrasonicator, an ultrasonic disintegrator at a peak amplitude λ=10 microns for 30 seconds. Sonication of *Rhizobium* cell suspensions was generally carried out for 3x30 second periods, with 30 seconds cooling between each sonication. Unbroken

cells and cell wall material were removed by centrifugation at 20,000g for 15 minutes at 4^{o}C.

2.8 Estimation of protein concentration

The protein concentration of crude cell extracts was measured using the biuret method described by Gornall *et al.* (1949). A standard curve of protein concentration was constructed using bovine serum albumin as reference. Cell-free extract (200 µl) was added to the biuret reagent (800 µl) mixed and the colour was allowed to develop for 30 minutes at room temperature before measuring at A_{540nm} against a blank made using 200µl buffer. An A_{540nm} value of 0.1 corresponds to a protein concentration of 2 mg/ml. The concentration of protein in column fractions was estimated by measurement of the A_{280nm} (Warburg and Christian, 1941).

2.9 Assay for dehalogenase activity

Dehalogenase activity was determined as total chloride released at 30^{O}C in an incubation mixture containing:

1-	0.1M Tris-acetate buffer(pH7.6)	4700µl
2-	0.1M halogenated aliphatic acid	50µl
3-	Distilled water and enzyme	
	to a final volume of	5000µl

After 5 minutes equilibration at 30^{O}C, the reaction was initiated by adding cell-free extract. Samples (1.0ml) were removed at appropriate intervals and assayed for halide ions.

2.10 Assay for halide ion

Measurement of free halide released during the dehalogenation reaction was carried out by an adaptation of the method of Bergman and Sanik (1957).

Sample (1ml) was added into 100 µl of 0.25M ammonium ferric sulphate in 9M nitric acid and mixed thoroughly. To this was added 100µl mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The colour was allowed to develop for 10 minutes and measured at A_{460nm} in a Jenway 6300 Series spectrophotometer. Halide concentration was determined by comparison of the absorbance of the test sample against a standard curve of known concentrations of halide.

2.11 Standard curve for chloride and bromide ions

A standard curve for chloride ion was constructed using sodium chloride within the range of 0-0.2µmol. A standard curve for bromide ion (Br^-) using sodium bromide was also constructed within the range of 0 -1.0 µmol Br^-. Standards were known concentrations of sodium chloride and sodium bromide in 100mM Tris-acetate buffer pH 7.6 with colour developed as described in section 2.9. Both standard curves are shown in Figure 2.1 (a) and (b).

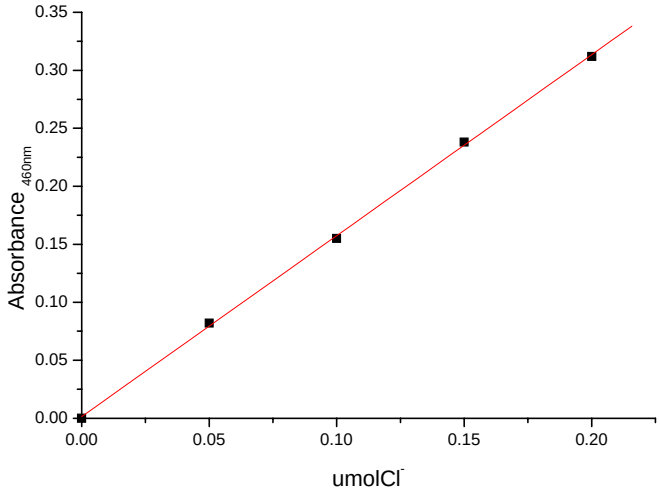


Figure 2.1. (a) Standard curve of Cl^- concentration (0 - 0.2µmolCl^-) against A_{460nm}

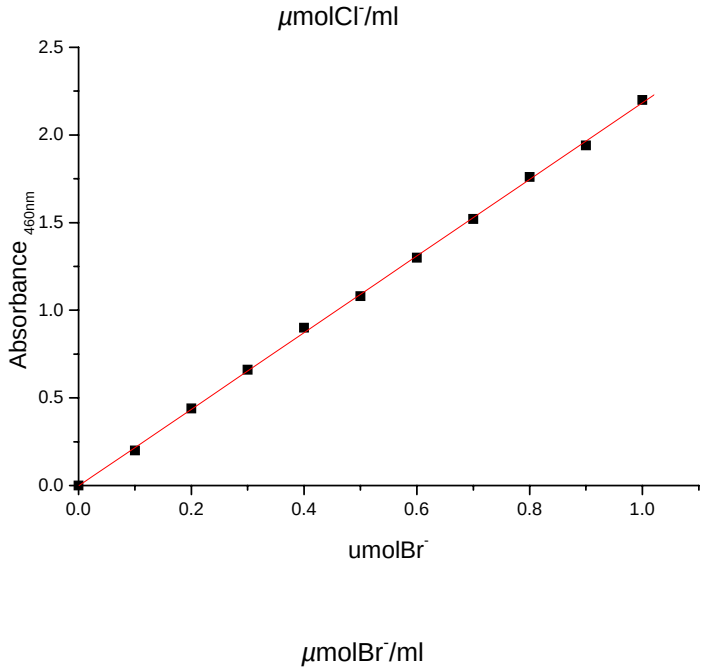


Figure 2.1. (b) Standard curve of Br^- concentration (0 – 1.0µmolBr^-) against A_{460nm}

2.12 HPLC analysis of growth medium

Samples of growth medium were analyzed using HPLC in order to monitor disappearance of 2,2DCP. Samples were filtered through nitrocellulose 0.2 μM filters (Sartorious) to remove bacteria cells and particles which could damage the HPLC equipment.

Samples were separated using an isocratic elution with a mobile phase containing potassium sulphate (20 mM): asetonitile (60:40) in deionized water. Samples were detected with a UV detector equipped with a Supelco C-18 column (250 × 4.6 mm, particle size of 5μm) using a flow rate of 2 ml/min.

2.13 Computer Analysis

International databases were searched using the BLAST programme (Altschul *et al*., 1990) (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences was compared with those in the EMBL database (Guenter *et al.*,, 1998) using FASTA3 at the European Bioinformatics Institute (http://www.ebi.ac.uk) and with those from the Ribosomal Data base Project by using SIMILARITY RANK to identify closely related sequences (Maidak, *et al.,* 1997).

CHAPTER 3

INVESTIGATION OF GROWTH OF *RHIZOBIUM SP.* AT LOW CONCENTRATIONS OF HALOGENATED COMPOUND

3.1 Introduction

In relation to environmental pollution, many investigations have been conducted about the global distribution of persistent organochlorines such as PCBs and DDT. These contaminants are transportable and contaminate all over the world (Iwata, *et al.,* 1993). The destruction of organic chemicals by microorganisms may be influenced by environmental factors or the structure of the chemical itself. One of the reasons suggested for the lack of degradation of organic compounds by microbes is their low concentration (Boethling and Alexander, 1979). Microorganisms that metabolise and grow upon very low concentrations of substrates have been designated as oligotrophs (Poindexter, 1981). Such organisms appear to be adapted to low substrate concentrations by having high substrate affinity (low Km value) systems. In our natural environment many of the polluted substances degraded by microorganisms. Our investigation will focus on *Rhizobium sp.* and its ability to degrade halogenated compound at low substrate concentration.

3.2 Growth of *Rhizobium sp.* on 0.2mM 2,2-dichloropropionate (2,2DCP)

Rhizobium sp. was reported to grow on 2,2DCP, D,L2-CP and D,L2-BP as sole sources of carbon and energy (Berry *et al.,* 1979). However, growth did not occur on 2,2DCP and D,L2-CP at concentrations in excess of 50mM or 20mM respectively, suggesting toxicity of these compounds to the organism (Leigh, 1986). The normal 2,2DCP concentration used to grow *Rhizobium sp.* was

20mM. To investigate the ability of *Rhizobium sp.* to grow on low concentrations of substrate, 0.2mM 2,2DCP, 100x lower than the normal growth concentration, was used.

An initial experiment was carried out to establish whether *Rhizobium sp.* was able to grow on 0.2mM 2,2DCP. The cell inoculum was prepared by growing *Rhizobium sp*. in 20mM lactate minimal medium. Then 15ml of an overnight culture was centrifuged and the cells washed twice with minimal medium before inoculation into 100ml minimal medium supplied with 0.2mM 2,2DCP. Growth was monitored by measuring the amount of chloride ions released at appropriate time intervals. An uninoculated flask treated in the same way was used as a control. This is important to make sure the chloride measured in the growth medium was due to the cells using the 2,2DCP rather than the auto-degradation of the substrate in the growth medium. A typical growth curve is shown in Figure 2.1(a) with a doubling time of approximately 12 hours. From the growth curve the time to harvest cells was set between mid and late logarithmic phase so that at harvesting the cells were still active and producing dehalogenase. For larger scale preparation cells were grown in 8 flasks each with 500ml of medium containing 0.2mM 2,2DCP as a carbon source.

As a control *Rhizobium sp.* grown in 20mM 2,2DCP was also prepared for use in further analysis, as shown in Figure 2.1(b). A doubling time of approximately 11 hours was observed which was more or less the same doubling time for growth at low concentration. Time for harvesting cells was set between mid and late logarithmic phase similar to that for growth at low concentration.

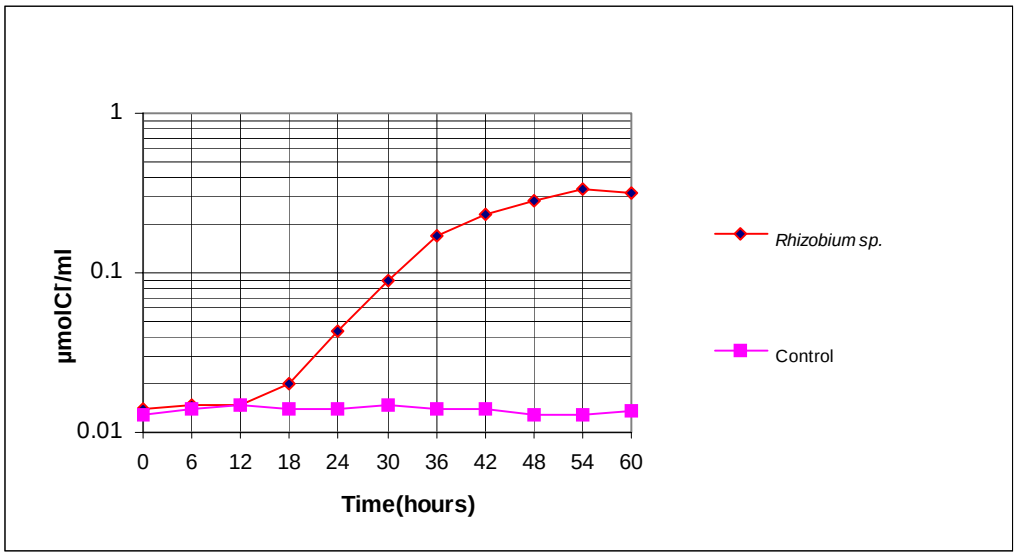


Figure 3.1(a). Growth of *Rhizobium sp*. on 0.2mM 2,2DCP

Rhizobium sp. : inoculated sample with *Rhizobium sp.*

Control : uninoculated sample

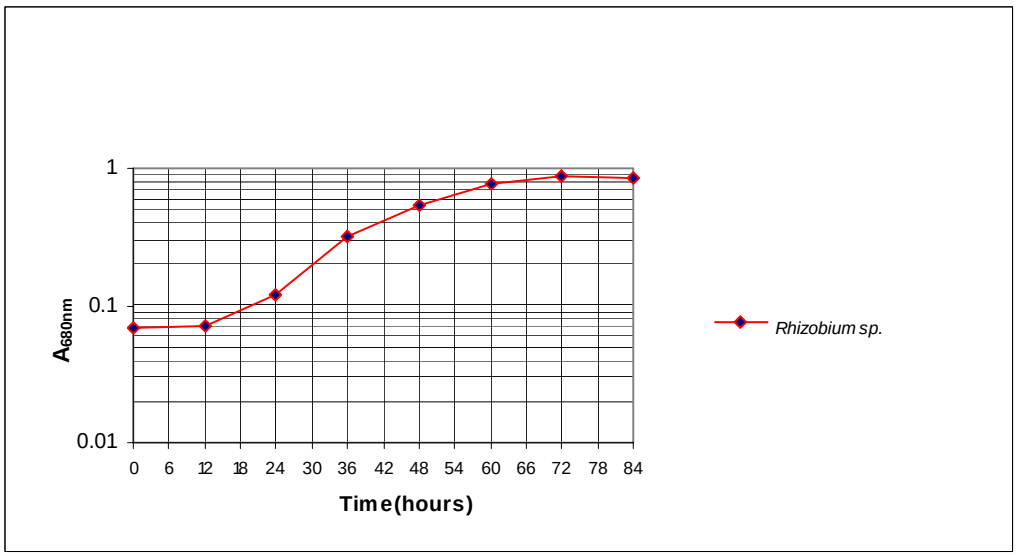


Figure 3.1(b). Growth of *Rhizobium sp*. on 20mM 2,2DCP

3.3 Analysis of cell-free extract from 0.2mM 2,2DCP-grown bacteria by enzyme assay

Table 3.1 shows the dehalogenase specific activity for crude extracts prepared from cells grown on 0.2mM 2,2DCP and on 20mM 2,2DCP. The substrates chosen were based on the specificity of the three dehalogenases the organism is known to produce. DBA is a substrate for DehL and DehE; 2,2DCP a substrate for DehE; MCA a substrate for DehD and DehE; and D,L2-CP a substrate for all three dehalogenases. The results suggest that similar dehalogenase enzymes (DehL, DehE and DehD) are produced under the two growth conditions. The dehalogenase specific activity using DBA as substrate for cells grown in 20mM 2,2DCP is twice that seen for growth at 0.2mM 2,2DCP. Since from the 2,2DCP measurement the amount of DehE in each growth is the same, there must be more DehL in the cells grown on 20mM 2,2DCPP. Such assays of crude extract may not show the presence of any additional dehalogenase(s) in cells grown on low concentration, so the crude extracts were checked by Km values analysis.

Table 3.1. Dehalogenase specific activity for crude extracts from cells grown at low and high concentration

Substrates	**0.2mM 2,2DCP-grown bacteria (μmolCl$^-$/min/mg protein)**	**20mM 2,2DCP-grown bacteria (μmolCl$^-$/min/mg protein)**
Dibromoacetate (DBA)	0.66	1.40
2,2-dichloropropionate (2,2DCP)	0.065	0.055
Monochloroacetate(MCA)	0.130	0.110
D,L-2-chloropropionate (D,L2-CP)	0.22	0.23

3.4 Apparent Km values using crude extract prepared from cells grown on 20mM and 0.2mM 2,2DCP

The apparent Km values for extracts prepared from growth at high and low concentration are shown in Figure 3.2 (a) and (b) using 2,2DCP as substrate. The Km value was 0.18mM in both cases suggested the same dehalogenase might be present in both growth conditions. However, further tests were not carried out such as protein purification and N-terminal amino acid sequencing analysis to confirm types of dehalogeasnes were present.

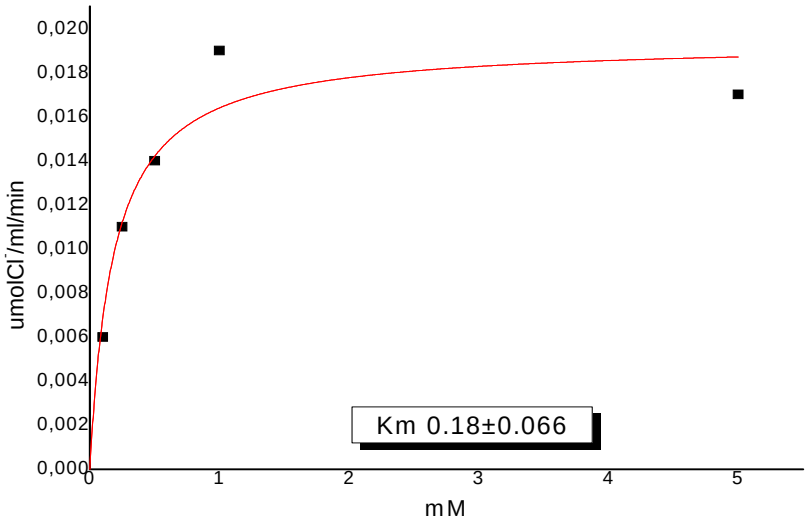


Figure 3.2 (a). Apparent Km using crude extract from *Rhizobium sp*. grown on high (20mM) concentration of 2,2DCP with 2,2DCP as substrate

(The crude extract was prepared in 0.1M Tris-acetate pH7.6 buffer)

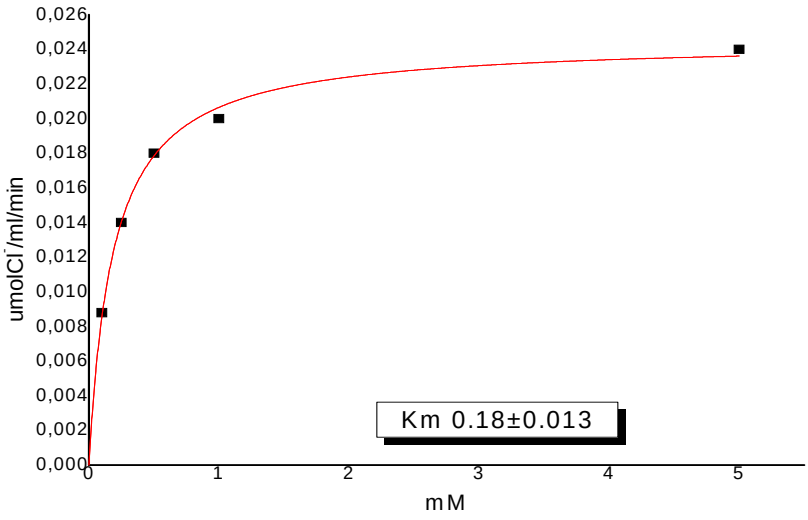


Figure 3.2(b). Apparent Km using crude extract from *Rhizobium sp*. grown on low (0.2mM) concentration of 2,2DCP with 2,2DCP as substrate

(The crude extract was prepared in 0.1M Tris-acetate pH7.6 buffer)

3.5 Discussion

The *Rhizobium sp.* was able to grow on 0.2mM 2,2DCP with a doubling time of approximately 12 hours, similar to the growth rate obtained at 20mM 2,2DCP concentration indicating that the organism was a facultative oligotroph.

Dehalogenases produced during growth on 0.2mM 2,2DCP were analysed by Km analysis to investigate whether growth at low concentration may involve a new dehalogenase enzyme. The Km values were found similar for both growth at high and low substrate concentration.

Maybe further investigation needs to be carried out to determine the N-terminal sequence of each dehalogenase that was present from growth at low 2,2DCP concentration to check its identity.

Rhizobium sp. is the only bacteria reported produced more than one dehalogenases (DehD, DehE and DehL) when grown on 2,2DCP. According to Leigh (1986) when *Rhizobium sp.* grown on 20mM 2,2DCP as sole source of carbon and energy revealed three dehalogenases although DehL and DehD did not play any role in 2,2DCP degradation. In other cases variation in growth condition influenced the nature of dehalogenase production (Hardman and Slater (1981a). According to Allison *et al.,* (1983) different inducers gave different pattern of induction compare to 2,2DCP and it was seen in the current investigation that similar amounts of dehalogenases were observed when growth was at high and low 2,2DCP concentration.

3.6 Conclusion

The present investigation did not show any new dehalogenase from growth at low haloalkanoic acid concentration. The low Km value for 2,2DCP of DehE (0.19mM) as reported earlier (Fahrul *et al.*, 2004) would seem clear DehE enzyme suited to dealing with low concentrations of growth substrate and no other enzyme would appear to be necessary.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF 2,2DCP DEGRADING BACTERIA

4.1 Introduction

Halogenated compounds are extensively used as herbicides, insecticides, fungicides, insulators and lubricants (Chapelle, 1993). Dalapon or 2,2-dichloropropionic acid (2,2DCP) is an example of herbicide and plant growth regulator that used to control specific annual and perennial grasses like Quackgrass, Bermuda grass, Johnson grass as well as rushes. It is selective, meaning that it kills only certain plants, while sparing non-target types of vegetation (Ashton and Crafts, 1973).

Generally, 2,2DCP is not readily bind or adsorb to soil particles. Even in muck soil, as little as 20% of applied 2,2DCP may adsorb. Since it is not adsorb to soil particles, 2,2DCP had a high degree of mobility in all soil types and leaching does occur. However, 2,2DCP movement in soil may prevented by rapid breakdown of the herbicide into naturally-occurring compound by soil microorganisms. Biodegradation is the main route of 2,2DCP disappearance from soil.

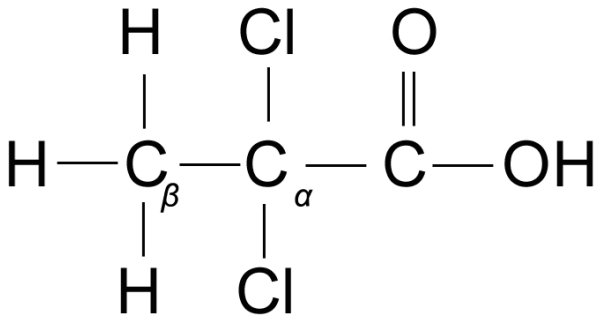


Figure 4.1. The molecular structure of 2,2-dichloropropionic acid (Dalapon)-(2,2DCP)

Some soil microorganisms utilized halogenated aliphatic compound as sole sources of carbon and energy and liberated halogen atom in form of halogen ion (Cl^- or Br^-). This reaction was called dehalogenation and was catalyzed by the dehalogenase enzyme (Jensen, 1960). 2,2-dichloropropionic acid was readily removed from the soil by variety of microorganisms for example: *Pseudomonas, Agrobacterium, Nocardia, Alcaligens, Arthrobacter, Bacillus sp.* (Jensen, 1957a; Foy, 1975) and *Rhizobium sp*. (Leigh *et al.,* 1986).

Since microbial dehalogenation of 2,2DCP was an important step in 2,2DCP detoxification (Schwarze *et al.,* 1997) and thus, it was interesting to isolate a microorganism that was capable to degrade the halogenated aliphatic compounds in the environment. In current study, a microorganism capable in degrading 2,2DCP was isolated and characterized. It was identified based on 16S rRNA gene. It was hoped that such studies would give a greater understanding of the dehalogenase enzyme system and 2,2DCP-degrading microorganisms.

4.2 Isolation of 2,2DCP degrading bacteria

A mixed culture from UTM plantation was streaked onto 20 mM 2,2DCP PJC minimal medium. Three morphologically different colonies were observed. Colonies formed were repeatedly streaked on the same type of medium. Only one of them grew well in 2,2DCP PJC liquid medium minimal medium and picked for further analysis. This bacterium was designated as Bacterium B.

4.3 Identification of bacterium B by 16S gene sequencing

4.3.1 DNA analysis of Bacterium B

Genomic DNA of bacterium B was prepared using Wizard genomic DNA kit (promega). Figure 4.2 showed the genomic DNA prepared from bacterium B. PCR reaction was carried out using the prepared genomic DNA as a template for 16S rRNA gene amplification. The fragment amplified was approximately 1.6 kb. The PCR condition generated a single fragment, with appropriate controls.

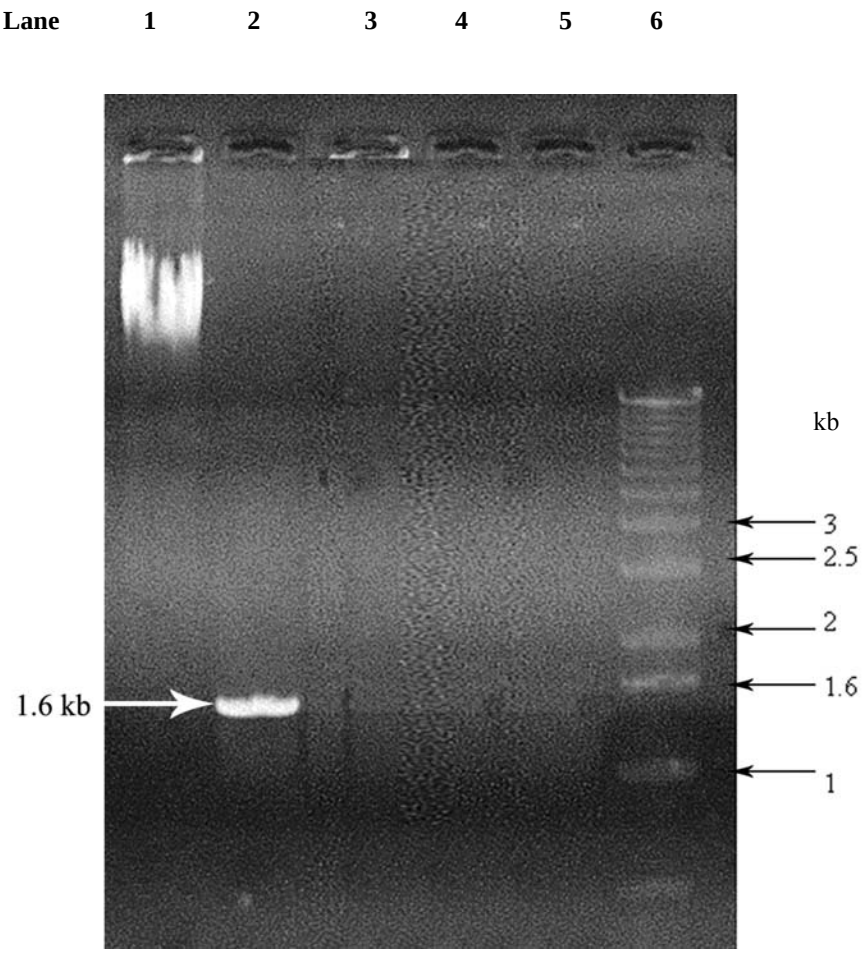


Figure 4.2. Agarose gel electrophoresis of undigested genomic DNA and 16S rRNA gene fragment

Lane 1: Genomic DNA (0.25 µg/µl) prepared from bacterium B

Lane 2: The amplified 16S rRNA DNA fragment

Lane 3: Negative control without DNA template

Lane 4: Negative control without FD1 primer

Lane 5: Negative control without rP1 primer

Lane 6: 1 KB DNA ladder (Invitrogen)

4.3.2 Sequencing of the PCR Product

The 16S rRNA gene fragment was sequenced using FD1 and rP1 primers. Figure 4.3 showed the partial nucleotide sequence of 16S rRNA gene from the isolated bacterium. The sequence comprises of 1307 nucleotides lacking the very proximal 5' and terminal 3' regions corresponding to the universal primers used. This sequence was submitted to the GenBank with accession number of **AM231910 (Appendix A).**

The 16S rRNA gene sequence was compared to the sequences in the GenBank database. The result revealed that the 2,2DCP degrading bacteria matched 99% identity with *Methylobacterium sp.* (Figure 4.4). From the results obtained, it was clear that the bacterium B belongs to *Methylobacterium sp.* It shared 99 % identity to the series of bacteria listed in the database (Table 4.1).

```
1    tgagtaacgc gtgtgaacgt gccttccggt tcggaataac cctgggaaac tagggctaat
61   accggatacg cccttatggg gaaaggttta ctgccggaag atcggcccgc gtctgattag
121  ctagttggtg gggtaacggc ctaccaaggc gacgatcagt agctggtctg agaggatgat
181  cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag tggggaatat
241  tggacaatgg gcgcaagcct gatccagcca tgccgcgtga gtgatgaagg ccttagggtt
301  gtaaagctct tttatccggg acgataatga cggtaccgga ggaataagcc ccggctaact
361  tcgtgccagc agccgcggta atacgaaggg ggctagcgtt gctcggaatc actgggcgta
421  aagggcgcgt aggcggcgtt ttaagtcggg ggtgaaagcc tgtggctcaa ccacagaatg
481  gccttcgata ctgggacgct tgagtatggt agaggttggt ggaactgcga gtgtagaggt
541  gaaattcgta gatattcgca agaacaccgg tggcgaaggc ggccaactgg accattactg
601  acgctgaggc gcgaaagcgt ggggagcaaa caggattaga taccctggta gtccacgccg
661  taaacgatga atgccagctg ttggggtgct tgcaccgcag tagcgcagct aacgctttga
721  gcattccgcc tggggagtac ggtcgcaaga ttaaaactca aaggaattga cgggggcccg
781  cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg cagaacctta ccatcctttg
841  acatggcgtg ttacccagag agatttgggg tccacttcgg tggcgcgcac acaggtgctg
901  catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc
961  cacgtcctta gttgccatca ttcagttggg cactctaggg agactgccgg tgataagccg
1021 cgaggaaggt gtggatgacg tcaagtcctc atggccctta cgggatgggc tacacacgtg
1081 ctacaatggc ggtgacagtg ggacgcgaag gagcgatctg gagcaaatcc ccaaaagccg
1141 tctcagttcg gattgcactc tgcaactcga gtgcatgaag gcggaatcgc tagtaatcgt
1201 ggatcagcat gccacggtga atacgttccc gggccttgta cacaccgccc gtcacaccat
1261 gggagttggt cttacccgac ggcgctgcgc caaccgcaag gaggcag
```

Figure 4.3. Bacterium B 16S rRNA partial sequence lacking the very proximal 5' and terminal 3' regions (Accession number AM 231910)

```
Bacterium B          1    TGAGTAACNCGTGTGAACGTGCCTTCCGGTTCGGAATAACCCTGGGAAACTAGGGCTAAT  60
                          ||||||||| |||| ||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium    69    TGAGTAACGCGTGGGAACGTGCCTTCCGGTTCGGAATAACCCTGGGAAACTAGGGCTAAT  128

Bacterium B         61    ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGGAAGATCGGCCCGCGTCTGATTAG  120
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   129    ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGGAAGATCGGCCCGCGTCTGATTAG  188

Bacterium B        121    CTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGAT  180
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   189    CTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGAT  248

Bacterium B        181    CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT  240
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   249    CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT  308

Bacterium B        241    TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTT  300
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   309    TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTT  368

Bacterium B        301    GTAAAGCTCTTTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT  360
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   369    GTAAAGCTCTTTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT  428

Bacterium B        361    TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTA  420
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   429    CCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTA  488

Bacterium B        421    AAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAATG  480
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   489    AAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAATG  548

Bacterium B        481    GCCTTCGATACTGGGACGCTTGAGTATGGTAGAGGTTGGTGGAACTGCGAGTGTAGAGGT  540
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   549    GCCTTCGATACTGGGACGCTTGAGTATGGTAGAGGTTGGTGGAACTGCGAGTGTAGAGGT  608

Bacterium B        541    GAAATTCGTAGATATTCGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGACCATTACTG  600
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   609    GAAATTCGTAGATATTCGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGACCATTACTG  668

Bacterium B        601    ACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG  660
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   669    ACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG  728

Bacterium B        661    TAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCGCAGCTAACGCTTTGA  720
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   729    TAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCGCAGCTAACGCTTTGA  788

Bacterium B        721    GCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCG  780
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   789    GCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCG  848

Bacterium B        781    CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCATCCTTTG  840
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   849    CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCATCCTTTG  908

Bacterium B        841    ACATGGCGTGTTACCCAGAGAGATTTGGGGTCCACTTCGGTGGCGCGCACACAGGTGCTG  900
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   909    ACATGGCGTGTTACCCAGAGAGATTTGGGGTCCACTTCGGTGGCGCGCACACAGGTGCTG  968

Bacterium B        901    CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  960
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   969    CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  1028

Bacterium B        961    CACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCGGTGATAAGCCG  1020
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium  1029    CACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCGGTGATAAGCCG  1088

Bacterium B        1021   CGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTTACGGGATGGGCTACACACGTG  1080
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium   1089   CGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTTACGGGATGGGCTACACACGTG  1148
```

```
Bacterium B       1081   CTACAATGGCGGTGACAGTGGGACGCGAAGGAGCGATCTGGAGCAAATCCCCAAAAGCCG  1140
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium  1149   CTACAATGGCGGTGACAGTGGGACGCGAAGGAGCGATCTGGAGCAAATCCCCAAAAGCCG  1208

Bacterium B       1141   TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGT  1200
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium  1209   TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGT  1268


Bacterium B       1201   GGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT  1260
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium  1269   GGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT  1328

Bacterium B       1261   GGGAGTTGGTCTTACCCGACGGCGCTGCGCCAACCGCAAGGAGGCAG  1307
                         |||||||||||||||||||||||||||||||||||||||||||||||
Methylobacterium  1329   GGGAGTTGGTCTTACCCGACGGCGCTGCGCCAACCGCAAGGAGGCAG  1375
```

Figure 4.4. Sequence comparison for bacterium B 16S ribosomal RNA gene (percent identity: 99 %)

Table 4.1. Top 5 entries in the database that showed highest identity to bacterium B

Bacteria	**Sequence Identity**
Methylobacterium sp. F05	99%
Methylobacterium sp. F15	99%
Methylobacterium fujisawaense	99%
Methylobacterium sp. F73	99%
Methylobacterium sp. A1	99%

4.4 Bacteria morphology, staining and biochemical characterization

The identification of bacterium B using 16S rRNA gene sequence was supported by the bacterial staining and biochemical analysis. Bacterium B colony was observed as a rough surface, a smooth margin and a raised elevation. It formed pink colonies on nutrient agar and 2,2-dichloropropionate minimal medium (Table 4.2). It was a gram-negative rod bacterium. The cells were acid-fast and no spores were demonstrated by malachite green staining. Bacterium B also demonstrated its ability in utilizing lactose, gelatin liquefaction, producing catalase, oxidase, motile and grew on citrate (Table 4.3). The biochemical test results were compared to *Bergey's Manual of Systematic Bacteriology* (Holt *et al.*, 1994) and were agreeing well with the finding of 16S rRNA analysis of bacterium B as *Methylobacterium sp.*

Table 4.2. Colony characteristic on 2,2DCP PJC minimal medium

Characteristics	**Observation**
Size of colony	Small
Pigmentation	pink
Form (Shape of the colony)	Smooth Circular
Margin (Outer edge of colony)	Entire (Sharply defined, even)
Elevation	Raised (Slightly elevated)

Table 4.3. Morphological and biochemical characteristics of *Methylobacterium sp.*

Biochemical test and staining	Result
Gram Stain	Gram negative
Acid Fast	Positive (Blue)
Spore Stain	No Spore
Grow Behavior	Aerobic
Oxidase	Positive
Catalase	Positive
Citrate	Positive
Gelatin Liquefaction	Positive
Lactose Utilization	Positive
Motility	Positive

4.5 Growth of *Methylobacterium sp.* in 2,2DCP PJC minimal medium

Methylobacterium sp. was inoculated into 100 ml PJC minimal liquid medium containing 5 mM, 10 mM, 20 mM and 40 mM 2,2DCP as sole source of carbon, respectively. The flask was incubated at 30°C in a rotary incubator with 180 rpm. The maximum growth was achieved in 20mM 2,2DCP PJC minimal medium. Figure 4.5 showed the growth patterns of bacteria in different 2,2DCP concentration. *Methylobacterium sp.* grew readily on 5 to 20 mM 2,2DCP. However, no growth was observed when growth at 40 mM suggested that the substrate was toxic to the cell at higher concentration. The cell doubling time was calculated for each growth curve as summarized in Table 4.4.

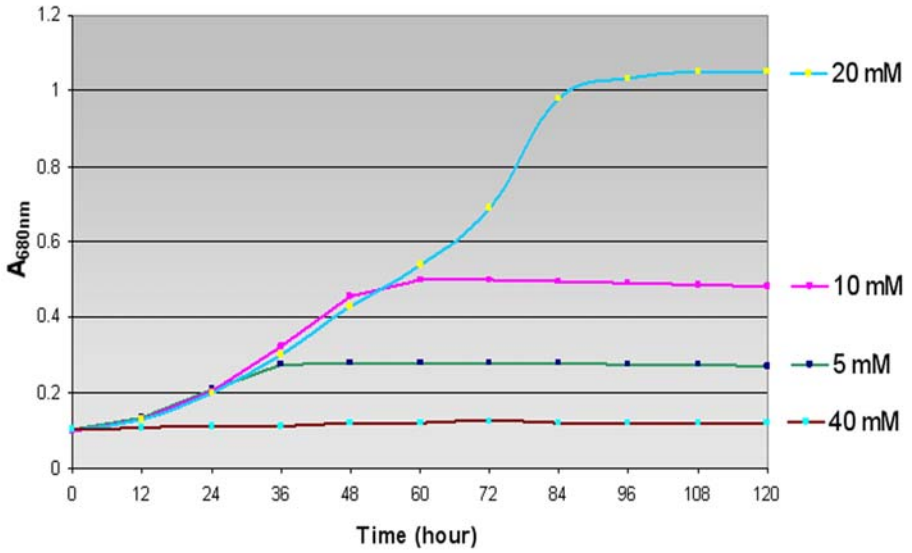


Figure 4.5. Growth curves on 5, 10, 20 and 40 mM 2,2DCP for *Methylobacterium sp.*
(Values are a mean of triplicate determinations).

Table 4.4. Growth properties of bacteria in different 2,2DCP concentration

Substrate Concentration	**Highest A680nm**	**Doubling time**
5 mM	0.279	24.27
10 mM	0.499	19.67
20 mM	1.052	22.80
40 mM	0.122	NG

*NG: no growth

4.6 Growth of *Methylobacterium sp.* in other halogenated compounds

Table 4.5 showed growth at various types of halogenated compound. Both of these compounds failed to support growth at 40mM in liquid PJC minimal media. The compounds were toxic to the cell at higher concentration.

Figure 4.6 showed the growth curves of *Methylobacterium sp.* when grown on 2,2-dichloropropionate and D,L-2-chloropropionate, with doubling time of 22 and 26 hours, respectively. This experiment suggested that *Methylobacterium sp.* could only act on chloride attached to carbon number 2.

Table 4.5. Growth properties of *Methylobacterium sp.* in different substrates

Substrate	**A $_{680nm}$**	**Growth doubling times**
3-chloropropionate	NG	NG
D,L-2-chloropropionate	1.091	26.40 hours
2,2-dichloropropionate	1.052	22.80 hours
2,3-dichloropropionate	NG	NG
3-bromopropionate	NG	NG

NG : No Growth

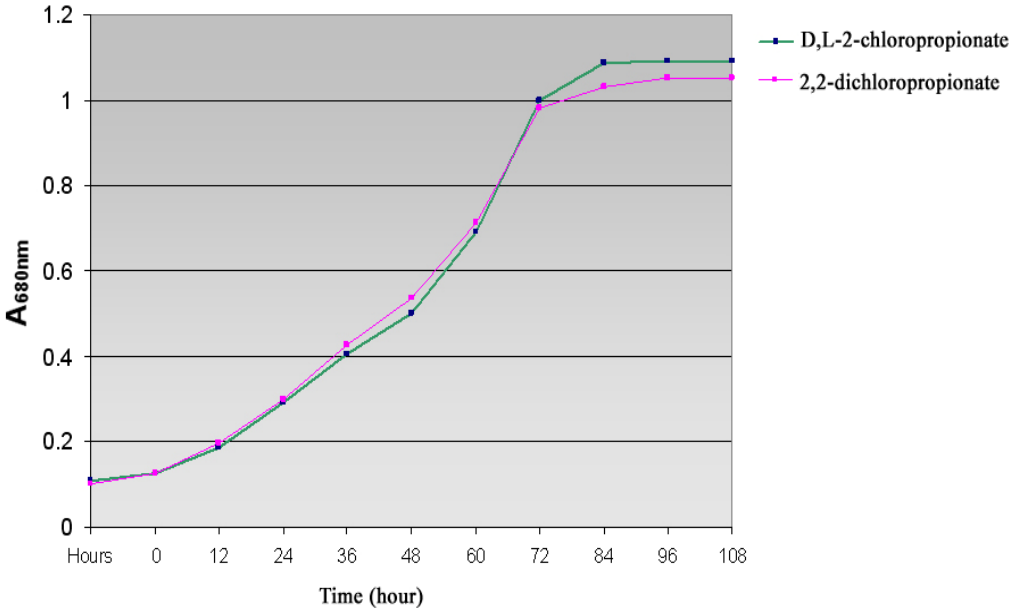


Figure 4.6. Grow curve of *Methylobacterium sp.* when grown on different types halogenated substrate. (Values are a mean of triplicate determinations).

4.7 HPLC Analysis of Growth Medium

4.7.1 2,2DCP Calibration Curve

A standard curve was constructed from a series of known concentration of 2,2-dichloropropionate (2,2DCP) within the range of 0 to 20 mM. Table 4.6 showed the data used to construct the calibration curve for 2,2-dichloropropionate whereas Figure 4.7 showed the calibration curve constructed.

Table 4.6. Data used to construct the 2,2DCP calibration curve

No	Peak Name	Level	Mean Area	Amount
1	2,2DCP	1	56.236	5 mM
2	2,2DCP	2	130.323	10 mM
3	2,2DCP	3	274.321	20 mM

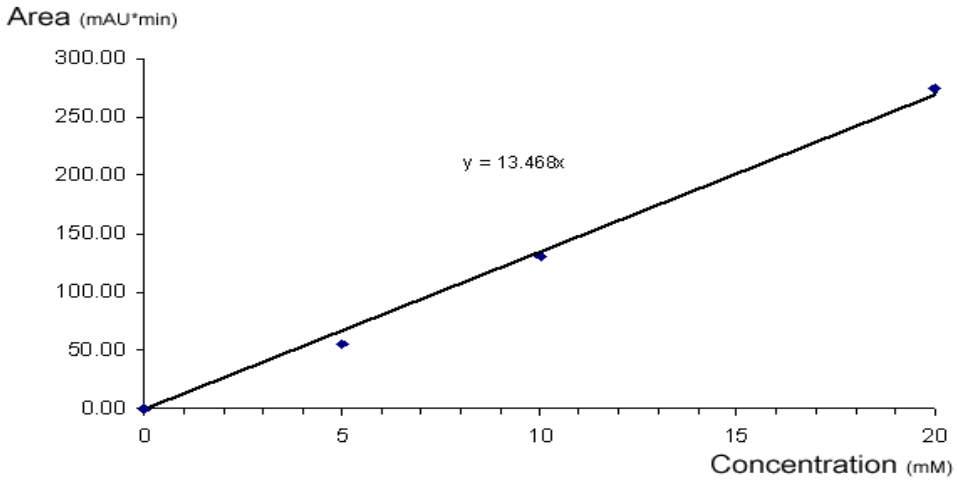


Figure 4.7. Calibration curve for 2,2DCP

4.7.2 Detection of 2,2DCP in growth medium

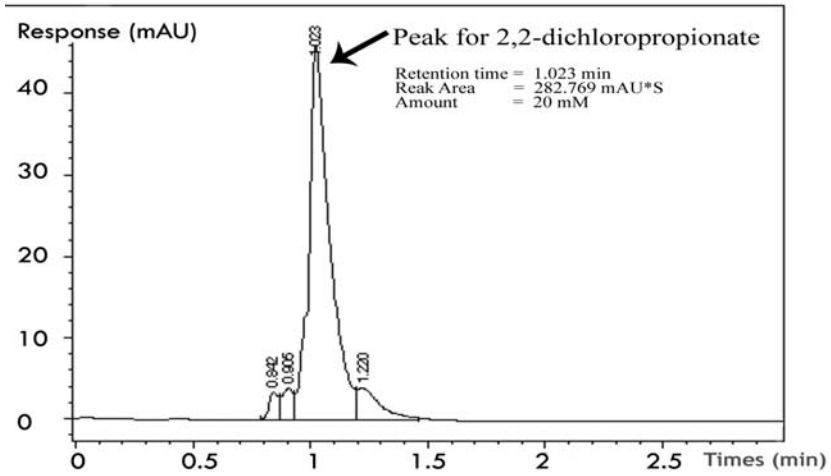
Methylobacterium sp. was grown in 20 mM 2,2DCP PJC minimal medium. Growth medium was then analyzed using HPLC at daily intervals to monitor the presence of 2,2DCP. The chromatogram showed the area of the peak corresponding to 2,2DCP decreased from 20 mM at day 0 to 0 mM at day 4. The results were summarized in Table 4.7.

Table 4.7. Comparison between the 2,2DCP consumed and turbidity of *Methylobacterium* growth medium. (Values are a mean of triplicate determinations).

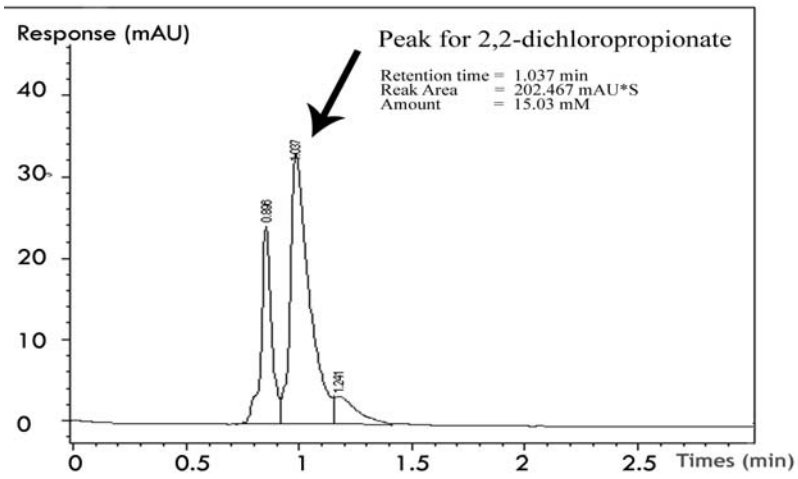
Days	**Bacteria Growth ($A680_{nm}$)**	**Amount of 2,2DCP left**
0	0.102	100 %
1	0.199	86 %
2	0.428	72 %
3	0.712	35%
4	1.302	0%

From the results obtained, it was concluded that 2,2DCP was fully utilized and depleted by *Methylobacterium sp*. The traces of 2,2DCP was not observed in the growth medium after 4 days of incubation period. Nevertheless, depletion of 2,2DCP was coupled with bacteria growth suggested that 2,2DCP was carbon and energy source for *Methylobacterium sp*. Figure 4.8 showed the peak of 2,2DCP in the chromatogram dropped from day 0 to day 4.

(i)



(ii)



(iii)

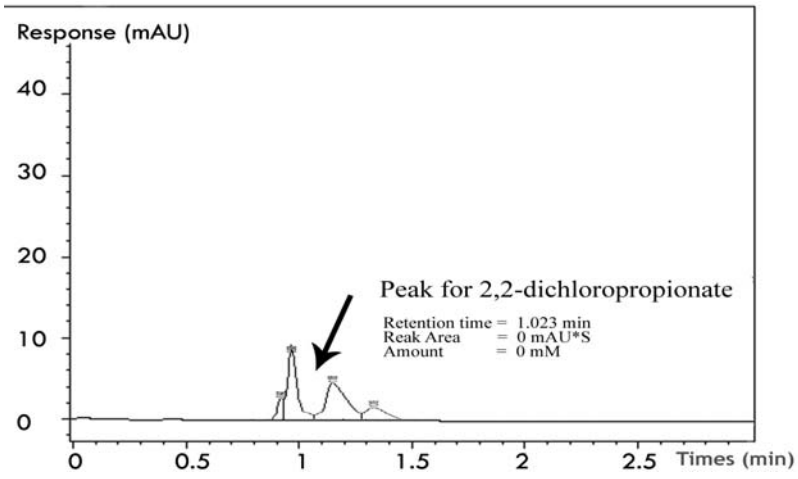


Figure 4.8. HPLC elution profile of medium from cells utilizing 2,2DCP as source of carbon. Medium was analysed at i) day 0, ii) day 2 and (iii) day 4

4.7.3 Dehalogenase activity in cell free extracts of 2,2DCP grown bacteria

Cell free extracts were prepared from bacteria grown on 2,2DCP as sole carbon source and were assayed for dehalogenase activity using 1 mM 2,2DCP as substrate. By measuring the rate of chloride ion released from the substrate (Figure 4.9), the average specific activity of dehalogenase on these extracts was found to be 0.039 μmol Cl^-/min/mg protein. Details as in **Appendix B.**

4.8 Discussion

In current investigation, 16S rRNA of 2,2DCP degrading bacteria shared 99% to *Methylobacterium sp*. Since any identification close to 99% is well above acceptance level, it was possible to confirm that the 2,2DCP degrading bacteria belongs to *Methylobacterium sp*. In addition, this was supported by staining properties and biochemical analysis.

The 2,2DCP degrading bacteria belongs to genus *Methylobacterium* was supported by its novel ability to produce pink pigment. Certain *Methylobacterium* species are known to be pink-pigmented bacteria that produce so called pink slime. For example, a *Methylobacterium sp*. which is known to be responsible for so called pink slime produced a (10→3)-galactan polysaccharide (EPS) was described by Verhoef *et al.* (2003).

Methylobacterium is a facultative *Methylobacteriumlotroph*, meaning it has the ability to grow by reducing carbon compounds with one or more carbon atoms but no carbon-carbon bonds (Madigan *et al.,* 2003). It grows on methylobacteriumlamine, methanol, and C_2, C_3, and C_4 compounds, including the methanol emitted by the stomata of plants. They are non-motile rod-shaped and are obligate aerobic; they are also called PPFMs - pink-pigmented facultative *Methylobacteriumlotrophs*.

Methane is the end product of anaerobic degradation of organic matter; therefore, methane-oxidizing bacteria can be found using this methane in such habitats as in wetland rice fields (Eller and Frenzel, 2001). This bacterium can be found mostly in soils, on leaves and in other parts of plants (Lidstrom and Christoserdova, 2002; Aken *et al.,* 2004), or even dust, air, freshwater and aquatic sediments (Gallego *et al.,* 2005). One species, *Methylobacterium podarium*, is thought to be part of the natural human foot microflora. *Methylobacterium* have even been

found living inside the human mouth. Samples have been isolated from the tongue, supra- and subgingival plaques that have shown growth of *Methylobacterium*, especially *M. thiocyanatum*.

Methylobacterium was well known for their ability in degrading chloromethane or dichloromethane as the sole carbon and energy source (Vannelli *et al.,* 1998, 1999; Studer, 2001; McDonald *et al.,* 2002). However, no *Methylobacterium* that able to degrade 2,2DCP was reported so far.

There are four basic criteria which must be fulfilled in order for a given halogenated compound to be utilized by an organism as sole source of carbon and energy. Firstly, the organism must either possess or synthesize dehalogenase in response to the halogenated compound which is capable of removing the substituent halogen(s) from the compound. Secondly, the dehalogenation product should be non-toxic and easily converted to an intermediate of the organism's central metabolic pathway. Thirdly, the halogenated compound should be able to enter cell either passively or by active transport in order to reach the site of dehalogenase activity, and finally, the halogenated compound should be non-toxic to the organism at normal intracellular concentrations.

When *Methylobacterium sp*. grew on 2,2DCP and D,L-2-chloropropionate (D,L2CP) as sole source of carbon and energy, the above criteria were satisfied. Of these compounds, the isolate grew more rapidly on 2,2DCP compared to D,L2CP. Both halogenated compound failed to support growth at concentration exceeding 40mM; presumably the intracellular concentration of halo-aliphatic acid had reached a toxic level.

Senior *et al*.,(1976) reported that *Pseudomonas putida* PP3 utilized 0.5 g carbon L^{-1} of 2,2DCP as sole carbon source with specific growth rates 8.33 hours in continuous culture. However, mean generation time for *P. putida* PP3 growing on

2,2DCP closed culture system was not mentioned. Allison (1981), reported that growth of *Rhizobium sp*. on 2,2DCP closed culture system with the doubling time of 12 hours. In current study, the doubling time of *Methylobacterium* in the same substrate was around 20 hours, or 2 times slower compared to *Rhizobium sp*. The difference maybe due to different affinity towards 2,2DCP between the two organisms.

Allison (1981) described that differential plots of culture extinction at 680 nm against medium chloride ion concentration were linear during exponential growth of *Rhizobium sp.* in 2,2DCP PJC minimal medium, indicating that the rate of 2,2DCP dehalogenation was proportional to the growth rate of the *Rhizobium sp.* Since 2,2DCP was the sole carbon source in the experiment, the results also indicated that the dehalogenation product was being utilized by the organism for metabolism and growth.

In order to further confirm the relationship between the 2,2DCP and bacterial growth, HPLC was employed in current study. The degradation and utilization of 2,2-dichloropropionate was initiated from the beginning of the growth. Table 4.7 clearly illustrates that when the bacteria number increased in the growth medium, 2,2DCP concentration dropped gradually. HPLC results suggested that growth of the bacterium in the medium was proportional to the utilization of 2,2DCP in the PJC growth medium.

Dehalogenase activity was detected in cell-free extracts of *Methylobacterium sp*. grown on 2,2DCP by measuring the rate of halide ion released from the substrate. Specific activity was expressed as μmol Cl^-/min/mg protein. The specific activity of dehalogenase present in *Methylobacterium* was 0.039 μmol Cl^-/min/mg protein.The average specific activity of dehalogenase in cell free extract of

Rhizobium sp. described by Allison (1981) was found to be 0.243 μmol Cl^- /min/mg protein. In current study, specific activities of dehalogenase present in *Methylobacterium* crude cell free extract was 82% lower compared to *Rhizobium sp*. Since *Methylobacterium sp.* was a slow grower (40% slower as described earlier) compared to *Rhizobium sp*, it was not surprised that specific activity of dehalogease from *Methylobacterium sp*. had much lower enzyme specific activity.

Nevertheless, in a recent study by Schwarze *et al*. (1997), two *Agrobacterium tumefaciencs* species, *A. tumefaciens* RS4 and *A. tumefaciens* RS5 were found to grow on 2,2DCP with a doubling time of 8.5 and 4.7 hours, respectively. Their dehalogenase specific activity in crude extract were 0.247 μmol Cl^-/min/mg protein and 0.647 μmol Cl^-/min/mg protein, respectively. This indicated that the growth rate was correlated with dehalogenase specific activity. Slower growth rate in *Methylobacterium sp.* in current study was caused by lower dehalogenase activity.

4.9 Conclusion

A bacterial species isolated from soil that able to degrade 2,2DCP was identified as *Methylobacterium sp.* based on 16S rRNA gene analysis. This was the first reported strain that able to grow on 2,2DCP since *Methylobacterium* was facultative Methylobacteriumlotroph for example some strains were able to degrade chloromethane and dichloromethane but no *Methylobacterium* was reported to degrade 2,2DCP so far. The ability of *Methylobacterium sp.* in degrading 2,2DCP was confirmed by HPLC analysis. Other than 2,2DCP the bacteria also grew well in D,L2CP. However, it could not grow in other compound which differed in halogen substitution position such as 3-chloropropionate (3CP) and 3-bromopropionate (3BP). This suggested that the bacteria dehalogenase system could only act on β-halocarboxylic acids. Finally, production of dehalogenase enzyme by *Methylobacterium* was confirmed by enzyme assay. Crude cell free extract of *Methylobacterium* showed week activity

toward 2,2DCP with specific activity of 0.039 μmol Cl^-/min/mg protein. This agreed by the slow growth in 20 mM 2,2DCP PJC minimal medium.

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APPENDIX: A

```
ID   AM231910   standard; genomic DNA; PRO; 1307 BP.
AC   AM231910;
SV   AM231910.1
DT   24-FEB-2006 (Rel. 86, Created)
DT   24-FEB-2006 (Rel. 86, Last updated, Version 1)
DE   Methylobacterium sp. HN2006B partial 16S rRNA gene, strain HN2006B
KW   16S ribosomal RNA; 16S rRNA gene.
OS   Methylobacterium sp. HN2006B
OC   Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
OC   Methylobacteriaceae; Methylobacterium.
RN   [1]
RP   1-1307
RA   Ng H.;
RT   ;
RL   Submitted (22-FEB-2006) to the EMBL/GenBank/DDBJ databases.
RL   Huyop F.Z., Biology Department, Faculty of Science, Universiti Teknologi
RL   Malaysia, Universiti Teknologi Malaysia, Skudai, Johor, 81310, MALAYSIA.
RN   [2]
RA   Ng H.;
RT   "Degradation of herbicide by soil microorganism and cloning of a haloacid
RT   permease gene";
RL   Unpublished.
FH   Key             Location/Qualifiers
FH
FT   source          1..1307
FT                   /country="Malaysia"
FT                   /db_xref="taxon:373519"
FT                   /mol_type="genomic DNA"
FT                   /organism="Methylobacterium sp. HN2006B"
FT                   /strain="HN2006B"
FT                   /isolation_source="agriculture soil"
FT   rRNA            <1..>1307
FT                   /gene="16S rRNA"
FT                   /product="16S ribosomal RNA"
XX
SQ   Sequence 1307 BP; 307 A; 317 C; 422 G; 261 T; 0 other;
     tgagtaacgc gtgtgaacgt gccttccggt tcggaataac cctgggaaac tagggctaat        60
     accggatacg cccttatggg gaaaggttta ctgccggaag atcggcccgc gtctgattag       120
     ctagttggtg gggtaacggc ctaccaaggc gacgatcagt agctggtctg agaggatgat       180
     cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag tggggaatat       240
     tggacaatgg gcgcaagcct gatccagcca tgccgcgtga gtgatgaagg ccttagggtt       300
     gtaaagctct tttatccggg acgataatga cggtaccgga ggaataagcc ccggctaact       360
     tcgtgccagc agccgcggta atacgaaggg ggctagcgtt gctcggaatc actgggcgta       420
     aagggcgcgt aggcggcgtt ttaagtcggg ggtgaaagcc tgtggctcaa ccacagaatg       480
     gccttcgata ctgggacgct tgagtatggt agaggttggt ggaactgcga gtgtagaggt       540
     gaaattcgta gatattcgca agaacaccgg tggcgaaggc ggccaactgg accattactg       600
     acgctgaggc gcgaaagcgt ggggagcaaa caggattaga taccctggta gtccacgccg       660
     taaacgatga atgccagctg ttggggtgct tgcaccgcag tagcgcagct aacgctttga       720
     gcattccgcc tggggagtac ggtcgcaaga ttaaaactca aaggaattga cgggggcccg       780
     cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg cagaacctta ccatcctttg       840
     acatggcgtg ttacccagag agatttgggg tccacttcgg tggcgcgcac acaggtgctg       900
     catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc       960
     cacgtcctta gttgccatca ttcagttggg cactctaggg agactgccgg tgataagccg      1020
     cgaggaaggt gtggatgacg tcaagtcctc atggcccttа cgggatgggc tacacacgtg      1080
     ctacaatggc ggtgacagtg ggacgcgaag gagcgatctg gagcaaatcc ccaaaagccg      1140
     tctcagttcg gattgcactc tgcaactcga gtgcatgaag gcggaatcgc tagtaatcgt      1200
     ggatcagcat gccacggtga atacgttccc gggccttgta cacaccgccc gtcacaccat      1260
     gggagttggt cttacccgac ggcgctgcgc caaccgcaag gaggcag                    1307
     //
```

Appendix: B

Specific activity calculation

Raw data of enzyme assay when using cell free extract from *Methylobacterium.*

Minutes	A_{460}	Corrected A_{460}	Cl^- (mM)
0	0.198	0	0
5	0.242	0.044	0.046
10	0.297	0.099	0.101
15	0.351	0.153	0.155
20	0.394	0.196	0.199

Since 400 µl of cell free extract used in 10ml incubation mixture during enzyme assay, the dehalogenase specific activity can be calculated as below:

[*y* µmol Cl^- /ml] / *z* minute = *X* µmol Cl^- /ml /min

[*X* x 1000 /*a*] / *b* mg /ml protein in a sample = specific activity

Where *a*: Amount of cell free extract: 400/10 = 40 µl/ml

b: Measurement of protein concentration at Abs_{595} :

(0.145-0.0122) / 0.021 = 6.45 mg/ml

Therefore, dehalogenase specific activity towards 2,2-dichlororpopionate was 0.039 µmol Cl^-/min/mg protein.

Benefits Report Guidelines

A. Purpose

The purpose of the Benefits Report is to allow the IRPA Panels and their supporting experts to assess the benefits derived from IRPA-funded research projects.

B. Information Required

The Project Leader is required to provide information on the results of the research project, specifically in the following areas:

- Direct outputs of the project;
- Organisational outcomes of the project; and
- Sectoral/national impacts of the project.

C. Responsibility

The Benefits Report should be completed by the Project Leader of the IRPA-funded project.

D. Timing

The Benefits Report is to be completed within three months of notification by the IRPA Secretariat. Only IRPA-funded projects identified by MPKSN are subject to this review. Generally, the Secretariat will notify Project Leaders of selected projects within 18 months of project completion.

E. Submissin Procedure

One copy of this report is to be mailed to :

IRPA Secretariat
Ministry of Science, Technology and the Environment
14th, Floor, Wisma Sime Darby
Jalan Raja Laut
55662 Kuala Lumpur

Benefit Report

1. Description of the Project

A. Project identification

1. Project number : 09-02-06-0124 EA001

2. **Project title : Investigation of growth of Rhizobium sp. at low concentrations of halogenated compound**

3. Project leader : Dr. Fahrul Zaman Huyop

B. Type of research

Indicate the type of research of the project (Please see definitions in the Guidelines for completing the Application Form)

☐ Scientific research (fundamental research)

☒ Technology development (applied research)

☐ Product/process development (design and engineering)

☐ Social/policy research

C. Objectives of the project

1. Socio-economic objectives

Which socio-economic objectives are adressed by the project? (Please indentify the sector, SEO Category and SEO Group under which the project falls. Refer to the Malaysian R&D Classification System brochure for the SEO Group code)

Sector :	Science & Engineering
SEO Category :	Natural Sciences, Technologies and Engineering
SEO Group and Code :	S50106 Applied Sciences Technologies

2. Fields of research

Which are the two main FOR Categories, FOR Groups, and FOR Areas of your project? (Please refer to the Malaysia R&D Classification System brochure for the FOR Group Code)

a. Primary field of research

FOR Category :	Biological Sciences
FOR Group and Code :	F10808- Biotechnology
FOR Area :	Microbial Biotechnology

b. Secondary field of research

FOR Category :	Biological Sciences
FOR Group and Code :	F10803 Microbiology
FOR Area :	Other Microbiology

D. Project duration

What was the duration of the project ?

_____36_____ Months

E. Project manpower

How many man-months did the project involve?

_____66_____Man-months

F. Project costs

What were the total project expenses of the project?

RM__145,215-65_____

G. Project funding

Which were the funding sources for the project?

Funding sources	Total Allocation (RM)
_*IRPA*_____	___171,200-00_____
_____	_____
_____	_____
_____	_____

ll. Direct Outputs of the Project

A. Technical contribution of the project

1. **What was the achieved direct output of the project :**

For scientific (fundamental) research projects?

- ☐ Algorithm
- ☐ Structure
- ☐ Data
- ☐ Other, please specify : _____

For technology development (applied research) projects :

- ☒ Method/technique
- ☐ Demonstrator/prototype
- ☐ Other, please specify : _____

For product/process development (design and engineering) projects:

- ☐ Product/component
- ☐ Process
- ☐ Software
- ☐ Other, please specify : _____

2. How would you characterise the quality of this output?

- ☒ Significant breakthrough
- ☐ Major improvement
- ☐ Minor improvement

B. Contribution of the project to knowledge

1. How has the output of the project been documented?

- ☐ Detailed project report
- ☐ Product/process specification documents
- ☑ Other, please specify : _MSc. Thesis

2. Did the project create an intellectual property stock? 'No'

- ☐ Patent obtained
- ☐ Patent pending
- ☐ Patent application will be filed
- ☐ Copyright

3. What publications are available?

- ☐ Articles (s) in scientific publications — How Many: _____
- ☑ Papers(s) delivered at conferences/seminars — How Many: _one_____
- ☐ Book
- ☑ Other, please specify : ___M.Sc. thesis _____

4. How significant are citations of the results?

- ☐ Citations in national publications — How Many: _____
- ☐ Citations in international publications — How Many: _____
- ☑ None yet
- ☐ Not known

lll. Organisational Outcomes of the Project

A. Contribution of the project to expertise development

1. How did the project contribute to expertise?

- ☐ PhD degrees — How Many: _____
- ☒ MSc degrees — How Many: _____one_____
- ☐ Research staff with new specialty — How Many: _____
- ☐ Other, please specify: _____

2. How significant is this expertise?

- ☒ One of the key areas of priority for Malaysia
- ☐ An important area, but not a priority one

B. Economic contribution of the project?

1. How has the economic contribution of the project materialised?

- ☐ Sales of manufactured product/equipment
- ☐ Royalties from licensing
- ☐ Cost savings
- ☐ Time savings
- ☒ Other, please specify : Fundamental & Academic achievement

2. How important is this economic contribution ? Not Available

- ☐ High economic contribution — Value: RM_____
- ☐ Medium economic contribution — Value: RM_____
- ☐ Low economic contribution — Value: RM_____

3. When has this economic contribution materialised?

- ☐ Already materialised
- ☐ Within months of project completion
- ☐ Within three years of project completion
- ☐ Expected in three years or more
- ☒ Unknown

C Infrastructural contribution of the project

1. What infrastructural contribution has the project had?

- ☐ New equipment Value: RM _____
- ☐ New/improved facility Investment : RM _____
- ☐ New information networks
- ☒ Other, please specify: Preparation of enzymes/ Enzyme technology equipments

2. How significant is this infrastructural contribution for the organisation?

- ☐ Not significant/does not leverage other projects
- ☒ Moderately significant
- ☐ Very significant/significantly leverages other projects

D. Contribution of the project to the organisation's reputation

1. How has the project contributed to increasing the reputation of the organisation

- ☐ Recognition as a Centre of Excellence
- ☐ National award
- ☐ International award
- ☐ Demand for advisory services
- ☐ Invitations to give speeches on conferences
- ☐ Visits from other organisations
- ☒ Other, please specify: for post graduates and internal research for further study

2. How important is the project's contribution to the organisation's reputation ?

☐ Not significant

☒ Moderately significant

☐ Very significant

1V. National Impacts of the Project

A. Contribution of the project to organisational linkages

1. Which kinds of linkages did the project create?

- [ ] Domestic industry linkages
- [ ] International industry linkages
- [x] Linkages with domestic research institutions, universities
- [ ] Linkages with international research institutions, universities

2. What is the nature of the linkages?

- [ ] Staff exchanges
- [ ] Inter-organisational project team
- [ ] Research contract with a commercial client
- [x] Informal consultation
- [x] Other, please specify: attending short courses and instituitional visit

B. Social-economic contribution of the project

1. Who are the direct customer/beneficiaries of the project output?

Customers/beneficiaries:	Number:
Department of environmental studies	n/a
_____	_____
_____	_____

2. How has/will the socio-economic contribution of the project materialised ?

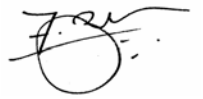
- [ ] Improvements in health
- [ ] Improvements in safety
- [x] Improvements in the environment
- [ ] Improvements in energy consumption/supply
- [ ] Improvements in international relations
- [x] Other, please specify: knowledge in pollutant degradation; at low pollutant degradation

3. How important is this socio-economic contribution?

- [x] High social contribution
- [ ] Medium social contribution
- [ ] Low social contribution

4. When has/will this social contribution materialised?

- [ ] Already materialised
- [ ] Within three years of project completion
- [ ] Expected in three years or more
- [x] Unknown

Date: 21.11.2006 **Signature:** 

End of Project Report Guidelines

A. Purpose

The purpose of the End of Project is to allow the IRPA Panels and their supporting group of experts to assess the results of research projects and the technology transfer actions to be taken.

B. Information Required

The following Information is required in the End of Project Report :

- Project summary for the Annual MPKSN Report;
- Extent of achievement of the original project objectives;
- Technology transfer and commercialisation approach;
- Benefits of the project, particularly project outputs and organisational outcomes; and
- Assessment of the project team, research approach, project schedule and project costs.

C. Responsibility

The End of Project Report should be completed by the Project Leader of the IRPA-funded project.

D. Timing

The End of Project Report should be submitted within three months of the completion of the research project.

E. Submission Procedure

One copy of the End of Project is to be mailed to :

IRPA Secretariat
Ministry of Science, Technology and the Environment
14th Floor, Wisma Sime Darby
Jalan Raja Laut
55662 Kuala Lumpur

End of Project Report

A. Project number : 09-02-06-0124 EA001

Project title : Investigation of growth of Rhizobium sp. at low concentrations of halogenated compound

Project leader: Dr. Fahrul Zaman Huyop

Tel: **07-5534556** **Fax: 07-556 6162**

B. Summary for the MPKSN Report (for publication in the Annual MPKSN Report, please summarise the project objectives, significant results achieved, research approach and team strucure)

Halogenated organic compounds are produced industrially in large quantities and represent an important class of environmental pollutants. Bacteria have evolved several strategies for the enzymes to catalysed dehalogenation and degradation of halogenated compounds. The destruction of halogenated chemicals by microorganisms may be influenced by environmental factors or the structure of the chemical itself. One of the reasons suggested for the lack of degradation of organic compounds by microbes is their low concentration. Microorganisms that metabolise and grow upon very low concentrations of substrates have been designated as oligotrophs. Such organisms appear to be adapted to low substrate concentrations by having high substrate affinity (low Km value) systems. Our current investigation is to study the possibility of growth of *Rhizobium sp.* at low concentrations of halogenated substrate (2,2-dichloropropionic acid; 22DCP). 22DCP was chosen because it was widely used as a herbicide (Dalapon).

The degradation of low concentrations of 22DCP by whole cells of *Rhizobium sp*. has been achieved (cells doubling time of 12 hours). *Rhizobium sp*. was able to grow at 0.2 mM 22DCP which was 100x lower than the concentration of the substrate routinely used (20 mM; cells doubling time 11 hours). Dehalogenase specific acitivity for crude extracts from cells grown at 0.2 mM 22DCP showed the specific activity for 22DCP (0.065 μmolCl^-/min/mg protein) and also had the affinity for 22DCP (Km 0.18 $\pm$ 0.01 mM). Similar study was carried out using cell free extract prepared from cells grown at 20 mM 22DCP to see whether new dehalogenases were involved. Apparently, no new dehalogenases are required to allow growth on this low concentration of 22DCP as judged by dehalogenase properties which is similar to the growth at low substrate concentration.

C. Objectives achievement

- **Original project objectives** (Please state the specific project objectives as described in Section ll of the Application Form)

1. To determine the ability of *Rhizobium sp*. to degrade 2,2DCP at low concentration.
2. To determine the kinetic parameter (i.e. Km values) of *Rhizobium sp.* dehalogenases involved in growth at high and low substrate concentration
3. To identify whether the same dehalogenases are involved when growth at low substrate concentration or different dehalogenases present

- **Objectives Achieved** (Please state the extent to which the project objectives were achieved)

All of the objectives stated were achieved

- **Objectives not achieved** (Please identify the objectives that were not achieved and give reasons)

D. Technology Transfer/Commercialisation Approach (Please describe the approach planned to transfer/commercialise the results of the project)

It is important at this stage biodegradation of very low concentrations of xenobiotic compounds has been neglected. It is useful to know about growth of microorganisms in low concentrations of pollutants because of the legal requirements. A government body or legislation may set the concentration of a pollutant down to a level that is not considered harmful. Therefore, if the microorganisms could only remove high concentration of pollutants, they could not be used to meet requirements of the law, since there still will be low concentrations of pollutants in the environment.

In nature the concentration of nutrients (including some pollutants that can be used as a source of carbon) in a given habitat varies (due to weather etc.). Some of the pollutants degrader may include oligotrophic bacteria that can survive or utilise substrates at low nutrient flux may be present in nature. Experimental study of such bacteria need to be carried out and their properties need to be clarified.

E. Benefits of the Project (Please identify the actual benefits arising from the project as defined in Section lll of the Application Form. For examples of outputs, organisational outcomes and sectoral/national impacts, please refer to Section lll of the Guidelines for the Application of R&D Funding under IRPA)

- **Outputs of the project and potential beneficiaries** (Please describe as specifically as possible the outputs achieved and provide an assessment of their significance to users)

In government and non-government organisation: to tackle pollutant at a very low concentration in the environment.

(i.e. MARDI, Kepala Batas, Pulau Pinang: herbicide widely use to kill weeds in Padi area)
Some of the herbicide may be toxic and released of the toxic compound into the environment may cause environmental pollution. Some of these herbicides are biodegradable and some are not.

This study only demonstrated that some of the microbes has some potential of degrading pollutants when the compounds are present at low concentrations.

- **Organisational Outcomes** (Please describe as specifically as possible the organisational benefits arising from the project and provide an assessment of their significance)

1. Establishment of General Microbiology laboratory
2. Establishment of protein preparation and characterization techniques

- **National Impacts** (If known at this point in time, please describes specifically as possible the potential sectoral/national benefits arising from the project and provide an assessment of their significance)

n/a

F. Assessment of project structure

- **Project Team** (Please provide an assessment of how the project team performed and highlight any significant departures from plan in either structure or actual man-days utilised)

Group meeting was carried out every six months to discuss students/research associates progress. Several short courses were attended at Institute Bioscience in UPM to master skills in : 1. DNA sequencing (Workshop) 2. Protein Preparation & Purifications (Techniques)

- **Collaborations** (Please describe the nature of collaborations with other research organisations and/or industry)

1. Regular meeting
2. Short Course attended (Institute Biosains UPM)

G. Assessment of Research Approach (Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficulty encountered)

1. To establish growth of *Rhizobium sp*. under low 2,2DCP concentrantion (0.20mM) using shake flask culture. (to see whether cells is able to grow at 100^{th} times lower than the normal growth condition).
2. Scale up process: grow cells up to at least in 8L culture (using shake flask technique)
3. Preparation of cell-free extract using standard extraction technique
4. Non-denaturing polyacrylamide gel analysis: to obtain the protein profiles from growth at low substrate concentration compared to high substrate concentration
5. Kinetic analysis will be carried out to see the Km values between the enzymes prepared from low and high concentration of growth substrate.
   (Lap top computer equip with latest version computer software to measure kinetic analysis ie. Microcal Origin Ver. 6.5 or Enzfitter from SIGMA)

H. Assessment of the Project Schedule (Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)

The project completed was completed in October 2006 after 6/7 months extension from **31st March 2006**. There is no changing in the plan and research activities. However, some of the research activities may take longer times to accomplished and time period extension is necessary.

I. Assessment of Project Costs (Please comment on the appropriateness of the original budget and highlight any major departure from the planned budget)

Overall budget is sufficient within 3 years allocation. The latest statement of account indicated that only 85% from the total budget was fully utilized.

J. Additional Project Funding Obtained (In case of involvement of other funding sources, please indicate the source and total funding provided)

n/a

K. Other Remarks (Please include any other comment which you feel is relevant for the evaluation of this project)

The investigation is important to project the properties of bacterial enzymes and possibly to asses the very basic concept of oligotrophy by examining the enzyme properties of the isolated bacterial species. The primary purpose to imply that oligotrophy does exist. And, it is suitable for exploitation of habitats low in pollutants as sole of carbon source.

Date : 22nd November 2006 **Signature :**