Isolation and characterization of putative *Burkholderia* sp. HY1 from mud that able to utilize 2, 2-Dichloropropionate

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ABSTRACT

Halogenated organic compound are extensively used as pesticides, herbicides, and antibiotics in the world and causes pollution. HY1 showed its ability to degrade 2, 2-DCP by observing its growth on 2, 2-DCP liquid minimal media with doubling time. The activity of dehalogenation and growth pattern was directly proportional to chloride ion released using colorimetric assay with maximum chloride ion released. This suggests that a bacterium (HY1) only encode group I dehalogenase and its non-steroselectivity is in agreement with group I haloalkanoic acid such as (2,2DCP, D, L, 2CP). The sequencing result showed that the partial gene sequences were Blastn, while no significant sequence identity was observed. HY1 is gram negative and it showed high ability to break down 2, 2-DCP as energy and carbon sources. The results indicated a broad distribution of dehalogenation gene in different types of microorganisms which can effect on man-made halogenated compounds in the environmental systems.

Keywords: Burkholderia sp., 2, 2-Dichloropropionate, Degradation, dehalogenase gene, 16S rRNA gene

1- INTRODUCTION

Halogenated organic compound are extensively used as pesticides, herbicides, and antibiotics in the world and causes pollution. Environmental contamination from industrial chemicals and herbicide/pesticide from agricultural usage cause a considerable dilemma to the society. 2, 2-Dichloropropionate (abbreviation known 2, 2-DCP) or Dalapon is synthetic halogenated compounds used in herbicides. However, when these compounds contaminate the environment, they can be very hazardous to the ecosystem. Xenobiotic compounds are man-made chemicals that are present in the environment and pollute the environment when present in high concentrations, and xenobiotic as a term can be described as non-natural occurring chemical compound (Rieger et al., 2002).

Now days, some xenobiotic substances are becoming very big problem in the environment system (water, soil) because some of these xenobiotics are resistant to degradation. However, it is believed that microorganisms are capable of degrading all the different complex and resistant xenobiotics found on the earth (Van Pee et al., 2003). Beside this, halogenated compounds are considered the most important group of xenobiotic that can cause pollution. Some of these compounds are very toxic and cause enormous problems to human health and to the environment (Van Pee and Unversucht, 2003). There are many types of soil microorganisms that capable of utilizing such compounds as their sole carbon source for growth while liberation the organically bound chlorine as chloride ions (Hirsch and Alexander, 1960).

The molecular structure of 2, 2-DCP consist of three carbon compound with molecular formula CH$_3$CH(C$_2$) COO (as shown in structure 1).

![Structure 1: Structure of 2, 2-Dichloropropionate](image-url)
There is a carboxylic functional group (-COOH) and two chloride substituent at (Cα) position. The α-chlorination is particularly important within the halogenated propionic series because it results in herbicidal activity.

Biodegradation is one of the main and natural processes that help to remove xenobiotic chemicals such as chloroaliphatic compounds from the environment by microorganisms (Sinha et al., 2009). The microbial degradation of a contaminant typically takes place because microorganisms can benefit from the use of the contaminant as an electron donor and carbon source to support growth. Hydrolytic dehalogenases represent the key position in the degradation of halo aliphatic compounds. These enzymes catalyze the cleavage of carbon-halogen bonds by nucleophilic substitution, replacing the halogen ion by a hydroxyl group derived from water (Schwarze et al., 1996). By using the dehalogenase enzyme chlorinated compounds are being metabolized, chlorine substituents are enzymatically removed to form non-halogenated compounds (Field and Alvarez, 2004). In addition, the lack of biodegradation is often due to the inability of microorganisms to effectively metabolize compounds with chemical structures to which microorganisms have not been exposed during the course of evolution. In other words, recalcitrance generally is the result of a lack of efficient metabolic pathways. Nevertheless, many examples are available of microorganisms that have the ability to metabolize xenobiotic organohalogenics (Sinha et al., 2009). This led to the idea that such microorganisms must have evolved their catabolic pathways during the past few decades, and is thus suited to study the natural assembly of catabolic routes. In general, many types of microorganisms are capable of utilizing halogenated organic compounds as a main carbon source for growth, and the organically bound halogen is liberated as the halide ion. The ability of degradation of these microorganisms refers to possess enzymes which are normally inducible known as dehalogenases that catalyze dehalogenation reactions (Bollag and Alexander, 1970; Hardman and Slater, 1981). Dehalogenases can be classified into three major grouping based on mechanisms of reactions or by substrate specificities. Slater et al., 1997 proposed three basic groups of dehalogenases classification which are: hydrolytic dehalogenase, halo alcohol dehalogenase and co-factor dependent dehalogenases. Many microorganisms produce more than one dehalogenase, which may give a microorganism a survival benefit under variable environmental conditions (Slater et al., 1997). However, why multiple dehalogenases exist in an organism is far from proven (Allison, 1981; Cairns et al., 1996)

2- MATERIALS AND METHODS

2.1 Chemicals

Halogenated compounds of analytical grade were obtained from Sigma-Aldrich, Fluka (Germany) and Fisher Scientific. And the rest of chemicals were of highest purity commercially available.

2.2 Isolation and Cultivation

In this research, the sample of mud was taken from UTM agriculture area in Johor (Malaysia). About five gram of mud was added into 250 ml flask containing 100 ml of minimal media with 5mM of 2, 2-dichloropropionate as the sole carbon and energy source. The minimal media for growing bacteria contained 10ml of 10x concentration basal salt solution [K$_2$HPO$_4$.3 H$_2$O 42.5 g.L$^{-1}$, NaH$_2$PO$_4$.2 (H$_2$O) 10 g.L$^{-1}$, (NH$_4$)$2$SO$_4$ 25 g.L$^{-1}$], and 10ml of 10x concentration metal salt solution [ N(CH$_2$COOH)$_3$.1.0 g.L$^{-1}$, MgSO$_4$.2.0 g.L$^{-1}$, MnSO$_4$.7H$_2$O 0.3 g.L$^{-1}$, ZnSO$_4$.H$_2$O 0.3 g.L$^{-1}$, FeSO$_4$.7H$_2$O 0.12 g.L$^{-1}$, CoCl$_2$.6H$_2$O 0.1 g.L$^{-1}$] per 100 ml of distilled water and 0.001 w/v yeast extract and autoclaved for 20 minutes at 121°C. The neutralized 2, 2-DCP solution was filtered with nylon filter 0.2µm pore size and added to the autoclaved media. The culture was incubated in shaker incubator for 3-7 days at 30°C. In order to prepare solid medium, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilization. Bacterial culture was streaked onto agar plat containing the same ingredients of the minimal media. One
pure colony was obtained after several subcultures by streaking on solid minimal media. The bacterial stain was characterized via morphological / biochemical tests as described in Bergey’s Manual of systematic Bacteriology (Holt et al., 1994). The growth was monitored by measuring the absorbance at $A_{680nm}$.

### 2.3 Dehalogenase Activity (Halide Ion Assay)

Dehalogenase activity was measured by determining the release of chloride ion as described by Bergman and Sanik, (1957) by using spectrophotometer at 460nm. Working solution consists of two different reagents. Reagent A contains 0.25M ferric ammonium sulphate dodecahydrate, Fe (NH4) (SO4)2.12H2O dissolved in 9M nitric acid, HNO3. Reagent B contains mercury thiocyanide, Hg (SCN) 2 dissolved in excess ethyl alcohol. The mixture was vortex vigorously for a few seconds and left a few minutes for sedimentation and clear solution was used to perform the test.

### 2.4 Partial Biochemical Tests

Different types of biochemical tests were carried out including spore staining test, catalase test, urease test, oxidase test, lactose utilization test, indole test, gelatin hydrolysis, motility test and nitrate reduction test to characterize bacteria’s biochemical properties.

### 2.5 Determination of Bacterial Growth and Halide Ion Released

In order to measure the bacterial growth pattern and dehalogenase activity for the isolated bacteria in 10mM 2, 2-DCP minimal medium, different readings were taken and the turbidity of liquid medium was measured by using spectrophotometer. The readings were recorded every 6 hours intervals by taking 1 ml sample from the growth medium and measured at $A_{680nm}$. The chloride ion in the growth medium was monitored using 1 ml of the same sample for growth monitoring, then aliquot into 100 µl of 0.25 M ammonium ferric sulphate in 9 M nitric acid and then followed by 100 µl of mercuric thiocyanate. After 10 min incubation at room temperature the reading was measured at $A_{460nm}$.

### 2.6 Scanning Electron Microscopy (SEM)

The SEM has allowed researchers to examine a much bigger variety of living things. The Scanning Electron Microscope, or SEM, is a tool for seeing the unseen worlds of micro space via using electrons to create magnified, detailed, black and white, and 3-dimenti onal images. Samples have to be prepared carefully to resist the vacuum inside the microscope.

### 2.7 Measuring of DNA Concentration of Isolated Bacteria

After DNA extraction was done, the process to monitor purified DNA product16S rRNA using Nono-Drop instrument. Blank measurement, calibration and sample measurement are the main steps of using Nano-Drop. The DNA purity was estimated from the $A_{260nm} / A_{280nm}$ = ratio. Ratio less than 1.8 indicates impure DNA.

### 2.8 PCR Amplification for 16S rRNA Gene

The 16S rRNA gene has highly conserved among different species of bacteria includes archaea and it was amplified using colony PCR and sequenced to identify the bacterium isolated. This test was carried out by using universal primers and it is used to amplify the gene 16S rRNA. The sequences of primers are:

**Forward** “5’-AGA GTT TGA TCC TGG CTC AG-3’
**Reverse** “5’-ACG GTC ATA CCT TGT TAC GAC TT -3’

The primers were taken from (Fulton and Cooper, 2005).
25µl of master mix was added into a 100µl micro tube, followed by 5µl of forward primer and 5µl reverse primer. 1µl of rehydrated DNA sample was added into the mixture and excess deionised water was added until 50µl. The PCR thermo cycle used to amplification was Initial Denaturation: 95°C (3 min ) , Denaturation: 95°C (1 min) Annealing: 55°C (1 min ), Elongation: 72°C(4 min) per kb, repeat steps 2-4, 35 cycles Final Elongation: 72°C (10 min) Final Hold: 4°C (∞).

2.9 PCR Amplification for Putative Dehalogenase Gene
In order to detect the dehalogenase gene from the isolated bacteria group I and group II primers were used. The primers were taken from (Fortin et al., 1998). The source of the primers were obtained from *Xanthobacter autotrophicus* dhlB 314, dhlB 637 belonging to group I deh gene. Other primers were obtained from *Moroxella* sp. dehH2 1157, dehH2 1662 belonging to group II deh gene. Table 1 shows the universal primers that used to amplify putative dehalogenase gene.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Primer sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xanthobacter autotrophicus</em></td>
<td>dhlBF314</td>
<td>Janssen et al. ,(1989)</td>
</tr>
<tr>
<td></td>
<td>5’-TCT GGC GGC AGA AGC AGC TGG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dhlBR637</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’- CGC GCT TGG CAT CGA CGC TGA TG-3’</td>
<td></td>
</tr>
<tr>
<td><em>Moroxella sp.</em></td>
<td>dehH2F1157</td>
<td>Kawasaki et al. ,(1981)</td>
</tr>
<tr>
<td></td>
<td>5’-CGG CAC CCT CTA CGA TGT GCA TTC GG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dehH2R1662</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-CAT CCC ATG GAT TCG ACG ATA CAA AGA-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: PCR amplification was performed as described by Hill et al., (1999). The PCR thermo cycle used to amplification was Initial Denaturation: 94°C (2 min ), Denaturation: 94°C (30 sec) Annealing: 55°C (30 sec.), Elongation: 72°C(4 min) per kb, repeat steps 2-4, 35 cycles Final Elongation: 72°C (10 min) Final Hold: 4°C (∞). PCR products were visualized by agarose electro-phoresis

2.10 DNA Sequencing and Molecular Analysis
After running the PCR protocol, the PCR products were purified using Promega Wizard® SV Gel and PCR Cleanup system to amplify 16S rRNA gene and putative Dehalogenase gene. After purification, PCR products were sent for sequencing at First BASE Laboratories (Malaysia) Sdn.
3- RESULTS

3.1 Identification of 2, 2-DCP Degrading Bacteria

The isolated bacteria from mud were screened on solid media containing 10mM 2, 2-DCP as a carbon source. After 5 to 7 days incubation at 30 °C, pure colonies were observed on minimal medium agar plates. One potential colony was selected and cultured it in 10mM liquid minimal medium. A bacterium strain HY1 showed its ability to degrade 2, 2-DCP under aerobic conditions in liquid minimal media with doubling time of 42.15 hours (Figure 1). The partial sequencing of 16S rDNA gene suggested that the isolated strain HY1 belongs to genus of *Burkholderia* sp. The morphological and partial biochemical characteristics were summarized in Table 2.

Table 2: Morphological and Biochemical characterization of HY1

<table>
<thead>
<tr>
<th>Properties</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>rod in shape</td>
</tr>
<tr>
<td>Size</td>
<td>Small, 0.5 ~ 1 mm</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Smooth, mucoid and somewhat elevated</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Gram Negative</td>
</tr>
<tr>
<td>Spore staining</td>
<td>-</td>
</tr>
<tr>
<td>Oxygen requirement</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Lactose utilization</td>
<td>-</td>
</tr>
<tr>
<td>Motility test</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate Reduction</td>
<td>-</td>
</tr>
<tr>
<td>Putative Identity</td>
<td><em>Burkholderia</em> sp. 95%</td>
</tr>
</tbody>
</table>

(+) Positive, (-): Negative
Table 3): The top 5 entries in the database that show highest identity to HY1 dehalogenase gene

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>ACCESSION</th>
<th>MAX SCORE</th>
<th>MAX IDENTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menaquinone biosynthesis methyltransferase 1</td>
<td>YP 002541593.1</td>
<td>36.2</td>
<td>28%</td>
</tr>
<tr>
<td>Agrobacterium radiobacter K84 l &gt; qb l ACM29996.1 l Menaquinone biosynthesis methyl transferase protein A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylase involved in Ubiquinone l menaquinone biosynthesis l Rhizobium sp. AP16 l &gt; emb l CCB96325.1 l Methylase involved in Ubiquinone l menaquinone biosynthesis</td>
<td>Zp 10535802.1</td>
<td>35.8</td>
<td>28%</td>
</tr>
<tr>
<td>Serine/threonine kinase l Streptococcus salivarius JIM8777 l &gt; emb l CCB96325.1 l Serine/threonine kinase l Streptococcus saliverius JIM 8777l</td>
<td>YP 006071160.1</td>
<td>35.4</td>
<td>37%</td>
</tr>
<tr>
<td>Hypothetical protein SORBIDRAFT 01q028570 l Sorghum bicolor l &gt; qbl EER91903 l hypotheticl protein SORBIDRAFT 01q028570 l Sorghum bicolor</td>
<td>XP 002464405.1</td>
<td>34.7</td>
<td>52%</td>
</tr>
<tr>
<td>Predicted protein l Naeqleria qruberi l &gt; qb l EFC41182.1 l Predicted protein l Naeqleria qruberi</td>
<td>ZP 10860504.1</td>
<td>33.9</td>
<td>21%</td>
</tr>
</tbody>
</table>

Table 3: The result showed the related genes were identified after BLAST. Referring to the most similarities, methyltransferase has covered 96% of query sequence and only 28% bp is identical. That, the similarity between HY1 dehalogenase genes with other species was low.

Figure 1: Growth curve of bacterium HY1 on 10 mM 2, 2-DCP
3.2 Scanning Electron Microscopy Result
Bacteria HY1 as appeared under SEM, short rods with diameter around 720-980 nm (Figure 3).

Figure 3: Scanning Electron Microscopy Result
Bacteria HY1 as appeared under SEM, short rods with diameter around 720-980 nm

3.3 Amplification of 16S rRNA Gene
Amplification reaction through Polymerase Chain Reaction (PCR) was carried out using extracted bacterial DNA. Amplification of the PCR product were amplified by using universal primer Fp1 and Rp1 and monitored with electrophoresis gel. Figure 4 showing the bonds of PCR outcome and the amplified band 1.5 Kb compared with the 1 Kb DNA ladder.
Figure 4: Amplification of 16S rRNA Gene

![Figure 4: Amplification of 16S rRNA Gene](image)

Lane 1: DNA Ladder (1 kb) from Promega
Lane 2: Amplification of 16S rRNA gene for bacterium HY1
Lane 3: Negative control of Rp1
Lane 4: Negative control of Fd1

Figure 4: The result of gel electrophoresis for PCR product (16S rRNA gene) for bacterium HY1 detected under UV light

3.4 Amplification of Putative Dehalogenase Gene

In current investigation, dehalogenase gene of bacterium HY1 was isolated in order to determine the isolated gene belongs to group I or group II according to Hill et al., (1999) classification system. According to this classification, dehalogenase genes can be classified into two families of bacterial α-halocarboxylic acid (α-HA) dehalogenase genes, called group I and group II deh genes. The two families are evolutionarily unrelated and together represent almost all of α-HA deh genes described to date. Group I include dehalogenase genes in bacteria that able to degrade haloalkoinic acid such as 2, 2-DCP, D, L and 2-chloropropionic acid. Group II include dehalogenase genes in bacteria that able to degrade haloacetic acid such as Monochloropropionic acid, Dichloropropionic acid and Trichloropropionic acid. The PCR reaction was carried out as previously described. Then, the PCR product was observed using gel electrophoresis. The result of Gene Flash photo showed clear band (approximately 398 bp) of dehalogenase gene that related to group I. On the other hand, there is no band was detected for dehalogenase gene that related to group II.

3.5 Sequencing and Molecular analysis of 16s rDNA

The PCR product was purified by using Wazard Genomic DNA Purification Kit (Promega) to make sure the genomic DNA of the bacterium HY1 was purified well. The purification was done successfully. After that, the PCR product had been sent for sequencing to 1st Base ® Company. The result had been received through e-mail with ab1 format and the file was viewed using Chromatogram Viewer and region of sequence with good signal quality. Using BioEdit software, the result was viewed and the alignment between the forward and reverse sequences was done in order to obtain partial length of 16S rDNA
sequence. The sequencing result obtained from First Base Company was blasted using NCBI nucleic acid data base. The first 10 sequences with maximum identity 95% to 96% to the bacterium HY1 were obtained.

Bacterium HY1 was identified using the 16S rRNA gene sequence analysis. The PCR amplification of 16S rRNA gene was sequenced 1442 bp and deposited in NCBI GenBank under accession number (000000). The highest sequence identity was to *Burkholderia* sp. showing 95% identity.

### 3.6 Phylogenetic Tree Analysis

In order to investigate the evolution relationship of bacterial strain HY1 among related species. Phylogenetic tree was established using BLAST-Webpage and MEGA5 Software. Ten different species from BLAST analysis related to the bacterium HY1 were chosen together with sequence of HY1 to perform alignment using Neighbor-joining method and MEGA5 software. The result of multiple alignments for phylogenetic tree showed that HY1 strain was located among *Burkholderia* sp. Figure 6 showed bacterium HY1 was clustered with a bootstrap value of 1000 and scale bar 0.5 substitutions per site with a clad consisting *Burkholderia* sp. this suggested that bacteria HY1 was closely related to the respective *Burkholderia* sp.

### 3.7 Pairwise-Distance Analysis

The result of Pairwise-Distance estimates the evolutionary divergence between sequences. The number of base substitutions per site from between sequences is shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the p-distance method in MEGA5. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). From the depicted phylogram, there are eleven operational taxonomic units (OTUs) which include HY1 strain and ten closest bacteria presented on the phylogram. This untreated phylogram was depicted with five internal nodes (hypothetical taxonomic units) represents the ancestor of the OTUs. According to the data, HY1 was mostly related to the *Burkholderia* sp.

### 3.8 Sequencing and Analysis of Putative Dehalogenase Gene

The purified PCR product was sent to First Base Company to do DNA sequencing. The outcome of sequencing was viewed by Sequence Scanner software. From the sequencing result, only the PCR product that contained dehalogenase gene (group I) was gave positive result of sequencing. This proved the result of agarose electrophoresis in Figure 5. The partial sequence of forward primer of dehalogenase gene (dhlB-314) was received from First Base Company and the length of this sequence is 404 bp. Open Reading Frame (ORF) is a graphical analysis tool can be used to find all open reading frames for the target sequence. ORF was used to examine the forward sequence of dehalogenase gene. After BLAST the sequence in ORF Finder, three frames were detected. The longest frame begins at position 107 bp until 403 bp. Total lengths is 297 bp and coded for 98 amino acids. The nucleotide sequence result and its converted amino acid sequence of best frame predicted by ORF Finder as shown in Figure 7. From the result above, ORF Finder encodes 99 amino acids that started from first ATG codon at 120th nucleotide which encoded for Methionine. Furthermore, the length of deduced sequence was very short. Thus, the stop codon did not detect for this sequence. Moreover, in order to determine the most related protein identity for dehalogenase gene that isolated from bacterium HY1. The sequence was examined in Genebank BlastX analysis tool. The result showed the related genes were identified after BLAST. Referring to the most similarities, methyltransferase has covered 96% of query sequence and only 28% bp is identical. That, the similarity between HY1 dehalogenase gene with other species was low.
**Figure 5:** Amplification of Putative Dehalogenase Gene

Lane 1: DNA Ladder (1 kb) from Promega
Lane 2: Dehalogenase gene for bacterium HY1 related to Group I
Lane 3: Negative control (Deh- gene Group I)
Lane 4: Negative control (Deh-gene Group II)
Lane 5: Dehalogenase gene for bacterium HY1 related to Group II

**Figure 5:** PCR product for Deh-gene (gel electrophoresis) for bacterium HY1 detected under UV light

**Figure 6:** Phylogenic Tree Analysis

**Figure 6:** The Neighbour-Joining phylogeny tree of HY1 using MEGA5 software. The scale bar represents 0.5 substitutions per site.
Figure 7: Sequencing and Analysis of Putative Dehalogenase Gene

```
M K A I A I L D A L R
ATG AAG GCG ATC GCG ATC TTG GAC GCG TTG AGG
R I P T A C K H F V W
AGG ATC CCG ACA GCT TGC AAA CAT TTT GTA TGG
V L T D P K L L A V S
GTT TTA ACT GAT CCA AAA CTC CTT GCT GTC TCA
L N W I Q P V L K S D
CTG AAT TGG ATT CAA CCC GTC CTC AAG AGC GAT
L F L D Y A T G L V L
CTT TTT CTT GAC TAC GCC ACC GGG CTC GTG CTT
K S D G E D E T R K V
AAG TCT GAC GGG GAA GAT GAA ACC AGG AAA GTT
S S R F R F A D G L T
TGC TCT GGC TTC AGA TTT GCA GAT GGT TTG ACG
L L A L C L E P I I R
CTG CTC GCA CTC TGT CTG GAA CCA ATC ATT CGA
P A S S A G R
CCT GCT TCT TCT GCC GGA AGA
```

**Figure 7:** Nucleotide and deduced amino acid sequence of HY1 (atg is start codon)

### 4- DISCUSSION

Halogenated organic compound to be utilized by an organism as sole source of carbon and energy, there are some basic steps must be satisfied. 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. In this research, aseptic methods were greatly emphasized during each of every experiment carried out to eliminate all probable contamination to the media as well as equipments used. All the required apparatus and media were autoclaved in 121°C, 15 psi for 20 minutes. The sample of mud was taken from UTM agricultural area to test whether this area has been exposing to halogenated compounds and also to isolate indigenous bacteria that have capability to break down the chloroaliphatic compounds.
Minimal media supplemented was prepared according to Hareland et al., (1975) with 10mM of 2, 2-dichloropropionate as a sole source of carbon. After incubation period at 30°C for 3 days, colonies were purified using streak plate method several times (Tortora et al., 2007). Then, pure culture of microorganism was inoculated into the liquid media, and then incubated for 24 hours at 200rpm with shaking at 30°C (Jing and Huyop, 2007). To verify the relationship between 2, 2-DCP and bacterial growth, measuring of growth rate and chloride ion released were carried out. However, bacteria HY1 was grown in minimal media supported with 10mM 2, 2-DCP and the doubling time was 42.15 hours and maximum growth rate was 0.547 at A₆₈₀ nm. The bacteria utilize 2, 2-dichloropropionate as their carbon source by eliminating chlorine atom from compound and produce pyruvate (Slater et al., (1997). The liberation of chloride ion via dehalogenase was monitored by chloride ion assay (Bergman and Sanik, 1975). Chloride ion released will react with mercury (III) cyanide to produce mercury (II) chloride releasing cyanide ions. These cyanide ions will react with ferric ion (Fe³⁺) to produce highly coloured ferric thiocyanate complex (Bergman and Sanik, 1957). For the maximum liberation rate of chloride ion was 0.612×10⁻³ mole/L also after 42 hours at A₄₆₀ nm.

Moreover, morphological and biochemical tests were carried out to determine the identity of HY1. The result of gram staining showed that HY1 is Gram negative bacteria as observed under light microscope. A few biochemical tests were performed such as catalase test, oxidase test, nitrate reduction test, motility test and so on. Each result was recorded. The results of identification were supported by biochemical and morphological tests indicated that the isolated bacterium related to genes Burkholderia sp. Furthermore, using molecular approach is a common way in finding a new genus/species. Based on the experimental result that obtained from 16S rRNA gene sequence of 2, 2-dichloropropionate degrading bacteria shared 95% to Burkholderia sp. and the result of dehalogenase gene amplification showed that dehalogenase gene related to group I and non sterospecific according to Hill et al., 1999 classification system.

In this experiment two universal primers were used and each primer has it owns specificity. For instance, primer from Xanthobacter autotrophicus was used to obtain dehalogenase gene from bacteria that specific to degrade haloalkinic acid such as 2,2-DCP, 2CP. The primer from Moroxella sp. was used to obtain dehalogenase gene from bacteria that specific to degrade haloacetic acid such as MCA, DCA and TCA. In this research, and in order to identify dehalogenase enzyme for the isolated bacterium HY1, the isolated dehalogenase gene was sent for DNA sequencing. The partial gene sequence was Blasted using BLASp but there is no significant sequence identity was observed because the short of this sequence. It suggests may be it belongs to other group of dehalogenase.

Conclusion
Several types of halogenated organic compounds have been found and widely spread in agriculture areas as herbicides. 2, 2-dichloropropionate was one of these compounds which were studied well during the last decade. However, the presence of these compounds in soil, water made some microorganisms to produce certain enzymes that able to utilize the compound as energy and carbon sources. The isolated bacterium HY1 was obtained from mud sample that was taken from UTM agriculture area and it was effectively grown on minimal media containing 2, 2- dichloropropionate (2, 2-DCP) as sole carbon source. A bacterium HY1 grew well in the liquid broth medium containing 10 mM of 2, 2-DCP and it showed high ability to break down 2, 2-DCP in to free chloride ion and pyruvate molecule in the halide ion assay. The bacterium grew well in 10mM 2, 2-DCP at 30°C and the optimum doubling time was 43.12 hours and the optimum chloride ion released was 0.612mmol/L. As a result, the capability of bacterium HY1 to grow on the halogenated compound gave evidence that the isolated bacteria able to produce dehalogenase
enzyme. The biochemical and morphological tests of bacterium HY1 showed that the bacterium has high similarity to genes *Burkholderia* sp. The result of 16S rRNA gene sequencing gave 95% identity of isolated bacterium HY1 to *Burkholderia* sp. Furthermore, the result of MEGA5 phylogenetic analysis also supported that strain HY1 belongs to *Burkholderia* sp.

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6- REFERENCES


