IDENTIFICATION OF A *Methylobacterium sp.* STRAIN HN2006B BY 16S rRNA GENE ANALYSIS WITH THE ABILITY TO DEGRADE THE HERBICIDE DALAPON

Ng Hong Jing & Fahrul Huyop

Biology Department, Faculty of Science, Universiti Teknologi Malaysia
81310 Skudai Johor, Malaysia

ABSTRACT. *Environmental pollution from industrial chemicals and herbicide/pesticide from agricultural activities cause a considerable problem to society. Consequently, this has led to major research programs, which studied the fate of these chemical pollutants in the natural environment. Microbial dehalogenases are involved in the biodegradation of many important chlorinated compounds. A bacterial strain isolated from UTM agricultural area was identified as Methylobacterium sp. using the 16S rRNA gene. The bacterium was able to utilize the herbicide 2,2-dichloropropionate (DALAPON) as a sole carbon source. The bacterium grew well in minimal media supplemented with 20 mM 2,2-dichloropropionate with a doubling time of 23 hours. Degradation of 2,2-dichloropropionate was detected by HPLC analysis where all 20 mM 2,2-dichloropropionate was utilized from the growth medium. The bacterial cell free extracts were also prepared from the cell grown in 20 mM 2,2-dichloroprionate as a carbon source. The enzyme specific activity was 0.039 µmol Cl^-/min/mg protein towards 2,2-dichloropropionate as a substrate. Methylobacterium sp. is known for its ability to degrade chloromethane or dichloromethane as the sole carbon and energy source as reported in the literature. This is the first reported strain of Methylobacterium sp. that was able to degrade 2,2-dichloropropionate (Dalapon).*

KEYWORDS. Biodegradation, dehalogenase, 2,2-dichloropropionate, Dalapon, *Methylobacterium sp.*

INTRODUCTION

Synthetic chemicals have been widely used and manipulated. The fate of these compounds in environment could threaten the health of humans and mammals. The role of dehalogenases in detoxification or degradation of some of these man-made compounds needs to be investigated. 2,2-dichloropropionic acid (Dalapon) is extensively used as herbicide (Chapelle, 1993). For that reason, microbial dehalogenases that are able degrade Dalapon have been attracting a great deal of attention because of their possible application to bioremediation. In current investigation, samples were taken from UTM agricultural area and bacteria samples were selected for their ability to utilize Dalapon as the carbon source. The outcome from this work will be described further.

MATERIALS AND METHODS

Microorganism and growth conditions. A newly isolated bacteria was obtained from UTM agricultural soil samples. The bacteria was grown aerobically at 30°C in PJC minimal medium containing 20 mM 2,2-dichloropropionate as sole source of carbon. Other chemicals used as growth substrate were 20 mM of D,L-2-chloropropionate, 3-chloropropionate and 3-bromopropionate, respectively. Growth was followed by measurement of the absorbance at A_{680nm} at 12 hours intervals over 100 hours period.

The 16S rRNA gene sequencing. Chromosomal DNA was prepared from overnight culture using Wizard Genomic DNA Purification Kit (Promega). The polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA gene. The universal primers used were Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1(5'-ACG GTC ATA CCT TGT TAC GAC TT-3'). The amplification reactions contained in 50 µl with 300ng template DNA, 20pmol primer (Fd1), 20 pmol of primer (rP1), 25 µl (2x) PCR master mix (Fermentas) containing 0.05u/µl *Taq* DNA Polymerase, 4mM $MgCl_2$ and 0.4mM of each dNTP and deionized water. PCR cycle was set as: initial denaturation 94 °C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 74°C for 4 min and final extension, 74°C for 10 min. The PCR product was electrophoresed on a 0.8% agarose gel and visualized under UV after staining using Ethidium Bromide. For sequencing reaction, the PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior to sending for sequencing (1st Base Laboratory, Biosyntech, Kuala Lumpur) . The sequences were compared to the sequence in the public databases using BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

Characterization of the isolates. The bacteria characteristic was identified using a standard microbiological gram staining technique. Motility test was assessed by the ability of the isolate to migrate from the point of inoculation through semisolid (0.3%) agar tube. Other biochemical characterization of the isolate was carried out using the standard procedures of Cappuccino and Sherman (2002).

HPLC analysis. HPLC was used to detect 2,2-dichloropropionate depletion in the growth medium. It was monitored by high performance liquid chromatography (HPLC) with a UV detector equipped with a Supelco C-18 column (250 × 4.6 mm, 5µm). A mobile phase of potassium sulphate (20mM): asetonitile (60:40) in deionized water was used for separation. Chromatography was carried out at ambient temperature at a flow rate of 2 ml/min at a wavelength of 214.4 nm. Concentration of 2,2-dichloropropionate was calculated from external standards whereas the compounds were identified by their retention times with reference standards.

Dehalogenase Enzyme Specific Activity. Bacterial cells suspended in 0.1 M tris-acetate buffer pH 7.6 were sonicated on ice using Vibra Cells™ ultrasonicator (Sonics & Materials Inc) operating at 10 % amplitude for 3 minutes. Cell free extract was separated from cell debris by centrifugation of the homogenate at 16,000 g at 4 ºC for 20 minutes.

Dehalogenase activity was measured by determining the halide ion released (Bergman and Sanik, 1957). Protein analysis was carried out using Bradford micro assay method with bovine serum albumin (BSA) as the standard.

RESULTS

The 16S rRNA gene sequencing. The 16S rRNA gene from the bacterium was successfully amplified by PCR at approximately 1.6 kb in size using Fd1 and rP1 primer. The partial 16S rRNA gene sequence obtained was deposited in Genbank (Accession no: AM231910) and compared to the sequence in the data base. The sequence alignment showed that it was *Methylobacterium sp.* with 99 % sequence identity (E-value = 0.0). This newly isolate was therefore designated as *Methylobacterium sp.* strain HN2006B. Phylogenetic tree was constructed using Phylip interface to determine the phylogenetic relatedness between *Methylobacterium sp* HN2006B and others *Methylobacterium sp* using *Rhodococcus sp* HN2006A as outgroup (Figure 1).

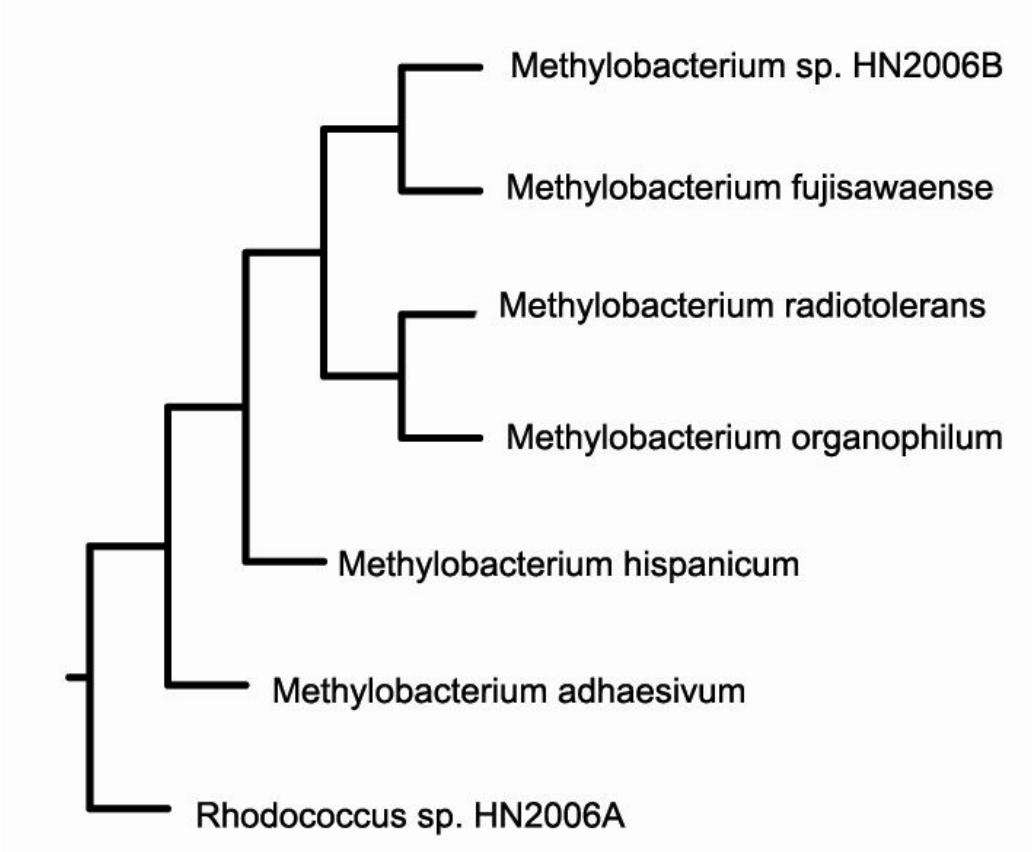

Figure 1. Phylogenetic relatedness between *Methylobacterium sp* HN2006B and others *Methylobacterium sp*.

Bacteria Morphology, Staining and Biochemical Characterization. The bacterium was gram negative rod, acid fast with no spore. It formed pink colonies on 2,2 dichloropropionate (Dalapon) PJC minimal media. The overall biochemical characteristics were matched to the genus *Methylobacterium* as indicated in *Bergey's Manual of Systematic Bacteriology* (Holt *et al.,* 1994). This result agreed with 16S rRNA gene analysis.

Bacteria growth in Dalapon. *Methylobacterium sp.* HN2006B was inoculated into 20 mM 2,2-dichloropropionate liquid minimal media and incubated at 30°C in a rotary incubator at 180 rpm. Sample was taken at 12 hours intervals. The growth was achieved with cells doubling time of 23 hours.

Growth of *Methylobacterium sp.* in other halogenated compounds. This experiment was carried out to determine whether carbon-halogen substitution affects bacterial growth. The *Methylobacterium sp.* strain HN2006B was grown on 20 mM 3-chloropropionate, D,L-2-chloropropionate and 3-bromopropionate liquid minimal medium, respectively. The results show that D,L-2-chloropropionate was the only substrate utilized by the isolate as the sole source of carbon and energy with cell doubling time of 26 hours. This experiment suggested that the bacterium produced dehalogenase enzyme that could act on chloride attached to carbon number two only.

Detection of Dalapon using HPLC. *Methylobacterium sp.* was grown in 20 mM 2,2-dichloropropionate liquid minimal medium and the disappearance of 2,2-dichloropropionate was detected when incubation time was increased. From the results obtained, it was concluded that 2,2-dichloropropionate was utilized by *Methylobacterium sp.* strain HN2006B. The peak of 2,2-dichloropropionate in the chromatogram dropped from day 1 to day 5 (Figure 2).

Dehalogenase activity in cell free extracts of 2,2-dichloropropionate – grown bacteria. Cell free extracts was prepared from bacteria grown on 20 mM 2,2-dichloropropionate minimal medium. Dehalogenase specific activity was assayed using 1 mM 2,2-dichloropropionate. By measuring the rate of chloride ion released from the substrate, the average specific activity of dehalogenase on these extracts was found to be 0.039 μmol Cl^-/min/mg protein.

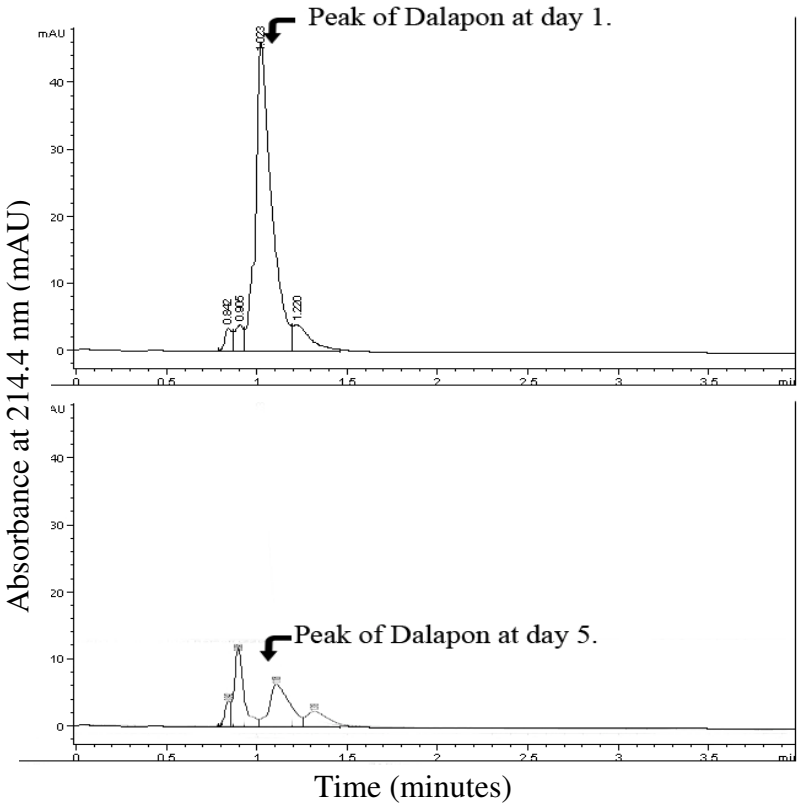

Figure 2. HPLC elution profile of medium from cells utilizing 2,2-dichloropropionate as a source of carbon

DISCUSSION

In current investigation, 16S rRNA of 2,2-dichloropropionate degrading bacteria shared 99% sequence identity to *Methylobacterium sp* with an E-value of 0.0. This result was supported by biochemical analysis. In addition, this bacteria produced pink pigment when grown in 2,2-dichloropropionate (Dalapon) liquid and solid media. Bacteria belongs to the *Methylobacterium sp*. are known to produce pink-pigment (pink slime). For example, a *Methylobacterium sp*. responsible for pink slime produces α-(10→3)-galactan polysaccharide (EPS) (Verhoef *et al.,* 2003). *Methylobacterium sp.* is well known for its ability to degrade chloromethane or dichloromethane as the sole carbon and energy source (McDonald *et al.,* 2002; Studer, 2001; Vannelli *et al.,* 1998 & 1999). However, in current investigation *Methylobacterium sp.* shows its ability to degrade Dalapon. The utilization of 2,2-dichloropropionate (Dalapon) in the liquid medium was coupled with bacterial growth (Figure 3).

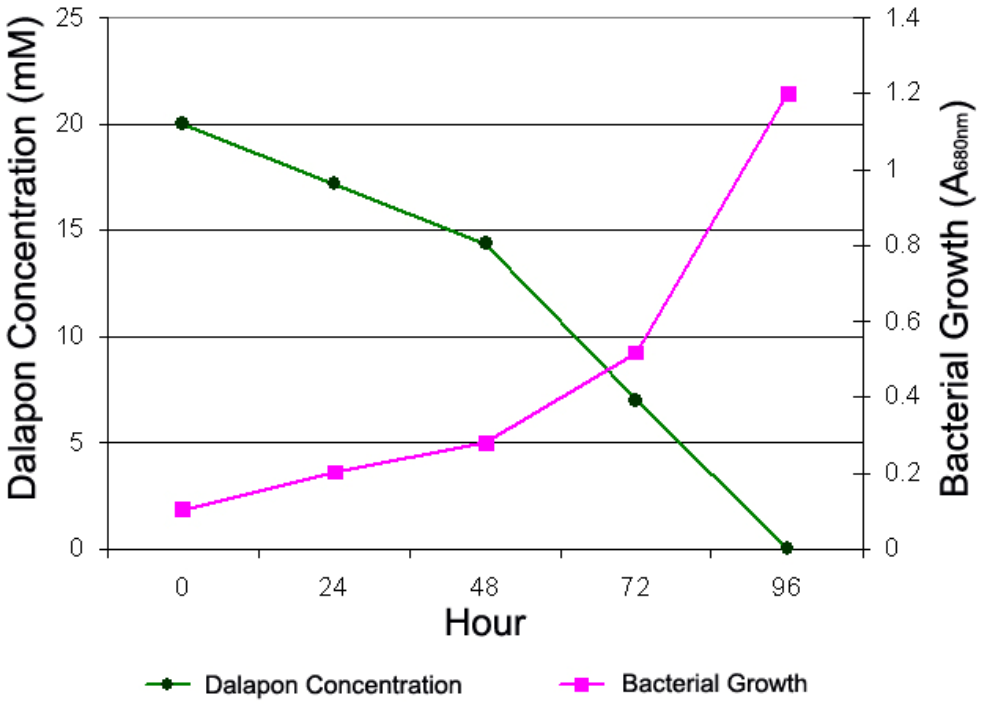

Figure 3. Correlation between growth of *Methylobacterium sp.* HN2006B and depletion of 2,2-dichloropropionate (Dalapon) in growth medium. (Values are a mean of triplicate determinations).

The ability of *Rhizobium sp*. to grow on haloalkanoic acids was previously studied by Berry *et al.,* (1976) and Allison (1981). The doubling times during growth on 2,2-dichloropionic acid had been reported as 12.4 hours (Berry *et al.,* 1976) and 11.8 hours (Allison, 1981), respectively. In current investigation, *Methylobacterium sp.* strain HN2006B grew on the same substrate when present as sole carbon source both in liquid and solid media. The observed doubling time during growth on 2,2-dichloropropionate was 23 hours, thus two times slower than result reported earlier. Little information is available regarding the physiology of newly isolated organism and subtle environmental changes may account for the observed variation in growth rates.

CONCLUSIONS

A bacterial isolate from soil was found to have a good potential in degrading 2,2-dichloropropionate, which is among the most active compounds used in herbicides. The organism was identified as *Methylobacterium sp.* strain HN2006B. This is the first

reported strain from *Methylobacterium sp*. that was able to degrade 2,2-dichloropropionate so far. The bacteria produced dehalogenase specific for carbon-halogen no. 2 where it could not grow in other compounds which differed in halogen substitution at carbon-halogen no. 3 for example 3-chloropropionic acid and 3-bromopropionic acid. The ability of *Methylobacterium* in degrading Dalapon was confirmed by HPLC analysis. Production of dehalogenase enzyme by *Methylobacterium sp*. strain HN2006B was confirmed by enzyme assay analysis.

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