Malaysian Journal of Microbiology, Vol 10(4) 2014, pp. 249-254

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Identification and characterization of *Raoutella ornithilolytica* and determination of its herbicide 2,2-dichloropropionate (2,2-DCP) degradation potential

Maryam Rajabpour Niknam[2], Fahrul Huyop[2*] and Roswanira Abdul Wahab[1*]

[1]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia,
81310 UTM Johor Bahru, Malaysia.
[2]Department of Biotechnology, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia,
81310 UTM Johor Bahru, Malaysia.
Email: roswanira@kimia.fs.utm.my

Received 13 April 2014; Received in revised form 14 July 2014; Accepted 1 September 2014

ABSTRACT

Aims: The goal of the study is to isolate new bacteria species which are capable to utilizing 2,2-dichlropropionic acid (2-2-DCP) as a sole carbon source from the wastewater sample that was taken from Tioman Island off the coast of Malaysia.
Methodology and results: Genomic DNA from unknown bacterial strain (MR1) was extracted and PCR amplification was carried out using universal primers, Fd1 (5'-AGA GTT TGA TCC TGGCTC AG-3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3') prior to sequencing. The BLASTn and phylogenetic analysis of the 16S rRNA of the MR1 gene found it shares a 95% homology to an aerobic bacillus *Raoutella ornithilolytica*. The bacteria which is usually found in an aquatic and hospital environment was found to possess a distinctive feature of being able to utilize 2,2-DCP as sole carbon source. The bacteria has cell doubling time of 23.11 h and maximum release of chloride ion at 0.257 mmol/L in 20 mM 2,2-DCP. Based on morphological and partial biochemical characteristics, the strain was found to be non-motile, Gram negative bacteria with red colonies that gave a positive catalase reaction.
Conclusion, significance and impact of study: A better understanding of newly isolated microorganisms from the environment which can potentially be used as bioremediation tools in environmental management. This is the first reported case of *Raoutella* sp. that has the unique ability to degrade halogenated compound. Hence, the dehalogenation properties and capabilities of the bacteria deserve to be assessed further.

Keywords: 2,2-dichloropropionic acid, 16S rRNA, phylogenetic analysis, *Raoutella* sp.

INTRODUCTION

2,2-dichloropropionic acid (occasionally abbr. 2,2-DCP) or commercially known as Dalapon is a herbicide and plant growth regulator used to control certain grasses, such as quack grass, Bermuda grass, Johnson grass, as well as cattails and rushes (EPA, 1982). The 2,2-DCP or Dalapon is often marketed as sodium salt and used to control seasonal weeds and is moderately toxic to human (Wong and Huyop, 2012).

These chemical compounds are of non-natural sources and cannot be synthesized biochemically. But there are some microorganisms that have developed massive catabolic potential towards various natural and synthetic compounds which were once considered flawless. In addition, their genetic flexibility and the potential to grow should allow the generation of new traits for coping with xenobiotic compounds (Timmis and Pieper, 1999).

Biodegradation is one of the main and natural processes that help to remove halogenated compound from the environment using microorganisms (Sinha *et al*., 2009). The first reports on biodegradation of chlorinated compounds were first presented by Penfold almost 100 years ago in 1913. And also the controller enzymes, in degradation in 1952 were discovered and named by Jenssen (Kerr *et al*., 2006).

Microorganisms can utilize halogenated aliphatic hydrocarbons as sole source of carbon and energy (Schwarze *et al*., 1997). Dehalogenases are important enzymes in the metabolism of halo-organic compounds (Hill *et al*., 1999). By using this enzyme, chlorinated compounds are metabolized and chlorine substituents are enzymatically removed to form non-halogenated compounds (Field and Alvarez, 2004).

The 2,2-dichloropropionic acid belongs to a class of organic aliphatic herbicide compound, specifically a haloalkanoate molecule with three carbon with both chlorine atoms bound to the alpha position (Cα) of

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

propionate (Figure 1). This compound is solid at room temperature and is very soluble in water-alcohol and ether. The 2,2-dichloropropionic acid also has other trade names on the market, for example Bainex P, Dalacid, Radapon and Unipon (Leasure, 1964; EXOTENT, 1993).

Jensen (1957) used the perfusion and enrichment technique to isolate five strains of *Pseudomonas* sp. which were able to degrade 2,2-dichloropropionate and other halogenated compound such as dichloroacetate and 2-chloropropionate. Over a decade later, Magee and Colmer (1959) isolated six 2,2-dichloropropionate degrading bacteria which belong to both *Agrobacterium* and *Alcaligenes* having similar properties. Five strains of *Nocardia* and three strains of *Pseudomonas* was isolated by Hirsch and Alexander (1960) following incubation in 2,2-dichloropropionate using the enrichment technique. *Rhizobium* sp. contain more than one dehalogenase that was named DehD, DehL and DehE (Huyop *et al*., 2004). Bioinformatics analysis of the *Rhizobium* dehalogenases indicated the Rhizobial *DehL* and *DehD* were very much different from each other with an 18% sequence identity. The deduced amino acid sequence of *DehE* also showed little identity to *DehD* (14%) and *DehL* (16%) (Huyop *et al*., 2008). The third *Rhizobium* sp. DehE dehalogenase could catalyse the dehalogenation of both isomers of 2-haloacids. *Rhizobium* sp. also produces other dehalogenases such as DehD and DehL which were more substrate specific, unlike the DehE which could act on all of the identified substrate and shows non-sterospecificity.

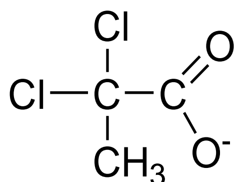

Figure 1: Structure of substrate 2,2-dichloropropionate.

Detoxification process of harmful halogenated compounds by microbial dehalogenases has drawn a considerably great deal of attention as these enzymes were able to cleave the carbon-halogen bonds of the compounds. Studies have shown that microorganisms that have the ability to metabolize a wide range of dichlorinated, monochlorinated, and aliphatic substrates because they generally produce one or more dehalogenases. Some native microorganisms that live in water or soil can readily attack 2,2-dichloropropionic acid and use it as a sole carbon source (Fetzner and Lingens, 1994). In this paper, the properties of a newly isolated bacterium from wastewater in Malaysia that is able to utilize 2,2-dichloropropionate as the sole source of carbon and energy will be discussed.

MATERIALS AND METHODS

Isolation and identification of 2,2-DCP degrading bacteria

The wastewater sample was collected from the Salang station in Tioman Island, located 32 km off the east coast of Peninsular Malaysia in the state of Pahang. The wastewater sample was kept in refrigerator prior to analysis. Fresh samples were placed onto Luria-Bertani (LB) agar plates containing 1.0% tryptone, 0.5% yeast extract and 1.0% sodium chloride (NaCl). The plates were incubated at 30 °C and examined after 16 h. After incubation, several colonies were selected and streaked onto PJC MINIMAL media containing 2,2-DCP as a carbon source and was cultured for 16 h at 30 °C to obtain a pure colony.

Preparation of PJC minimal media

A stock solution for PJC media was prepared as a ten-fold concentration of the trace metal salts containing nitriloacetic acid (1.0 g/L), $ZnSO_4{\cdot}H_2O$ (0.03 g/L), $MnSO_4{\cdot}4H_2O$ (0.03 g/L), $FeSO_4{\cdot}7H_2O$ (0.12 g/L), $MgSO_4$ (2.0 g/L) and $CoCl_2{\cdot}6H_2O$ (0.01 g/L) in distilled water. A ten-fold concentration of Basal salt was prepared according to the method of Hareland *et al.* (1975) containing $(NH_4)_2SO_4$ (25.0 g/L), $K_2HPO_4{\cdot}3H_2O$ (42.5 g/L) and $NaH_2PO_4{\cdot}2H_2O$ (10.0 g/L). The minimal media for growing bacteria containing 10 mL of ten-fold basal salts and 10 mL of ten-fold trace metal salts per 100 mL of distilled water were then autoclaved (121 °C for 15 min at 15 psi). Then 2 mL of 2,2-DCP (1 M) was added into the media as a carbon source by filtration through a nylon filter (0.2 µm pore size) and the media was made up to the desire final concentration. For the preparation of solid medium, bacteriological agar (1.5% w/v) was added prior to sterilization. The bacterial growth was monitored by periodically removing and measuring the turbidity at A_{600}nm.

Chloride ion released (halide ion assay test) in growth medium

Upon degradation of 2,2-DCP as a sole carbon source results in the release of chloride ions as product. The activity of enzyme was determined colorimetrically by measuring the release of chloride ion during dehalogenation reaction (Bergman and Sanik, 1957). The method required preparation of two different solutions. Reagent I is 0.25 M of ferric ammonium sulfate dodecahydrate, $FeNH_4(SO_4)_2$ dissolved in 9 M nitric acid, HNO_3 and reagent II is mercuric thiocyanide, $Hg(SCN)_2$ that mixed in excess ethyl alcohol. The solution was vortexed for a few seconds before 1.5 mL of sample was withdrawn and centrifuged for 5 min at 10,000 rpm. Next, a volume of 1 mL of supernatant is added into a cuvette followed by addition of 0.1 mL of each reagent into the cuvette and was left to stand at room temperature for 10

ISSN (print): 1823-8262, ISSN (online): 2231-7538

min. Then, the mixture was checked for absorbance using a UV spectrophotometer at A_{460}nm.

16S rRNA gene sequencing

Extraction of bacterial DNA was carried out in 20 mM 2,2-DCP minimal media using Promega Wizard® Genomic isolation and purification kit. Concentration of DNA was measured by Nanodrop machine. The polymerase chain reaction (PCR) was carried out by using universal primers, Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3') to amplify the target DNA fragments (Fulton and Cooper, 2005). The PCR cycle for DNA amplification was performed for 30 cycles and was set as initial denaturation 94 °C for 5 min, followed by cooling, denaturation cycle 94 °C for 1 min, annealing cycle 55 °C for 1 min and final extension 72 °C for 10 min. Amplicons were purified using QIAGEN® QIAquick PCR clean up kit and sequenced by 1st Base® (Malaysia).

Phylogenetic analysis

The sequence of 16S rRNA was compared based on the GenBank database using BLAST to define the genetic similarities of the isolated bacteria (Altschul *et al*., 1997). The Phylogenetic tree was constructed and the Neighbor-Joining method was employed using CLUSTAL W from MEGA 5 to show relatedness and distance matrix of the *Raoutella* sp. in the revolutionary pathway (Saitou and Nei, 1987).

RESULTS

Due to the low sensitivity of dehalogenase expression towards the chlorinated compound, slow growth of colony were often observed. Following one month of enrichment cultures, a bacterium with the distinctive ability to utilize 2,2-DCP as sole carbon and energy source was isolated from the wastewater collected on Tioman Island. The basic cellular morphologies of the MR1 bacteria are shown in Figure 2a. The basic cellular morphology of the bacteria revealed it was rod-shaped with smooth-edged red colonies. Gram staining revealed the microorganism to be a Gram negative rod shape bacteria (Figure 2b). Meanwhile, the physiological and biochemical characteristics of the bacterial isolate are described in detail in Table 1.

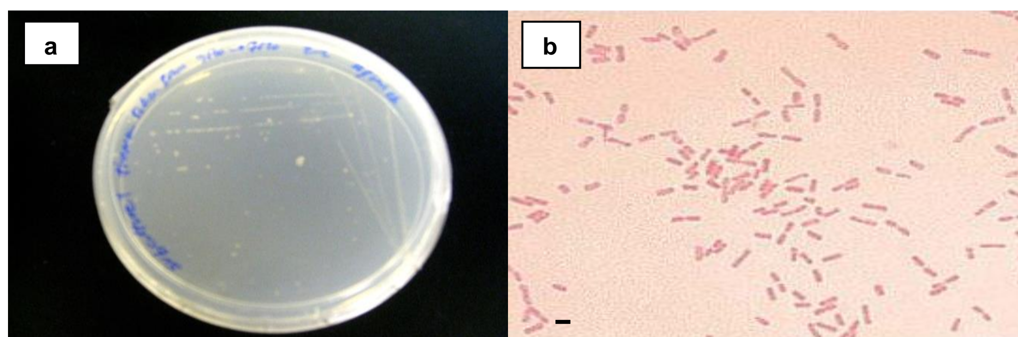

Figure 2: Analysis on the basic cellular morphology of MR1 bacteria. a, Growth on 2,2-DCP after approximately one month with incubation at 30 °C; b, Gram staining procedure showed that the bacteria was rod shape and Gram negative. Bar, 2 µm.

Table 1: Morphological and biochemical characteristics of the isolated bacterium.

Parameters	Properties
Cell shape	Rod shape
Type and color of colony	Single-circular-cream
Size (mm)	1 mm
Gram staining- color	Negative- bright red color
Spore staining	Negative
Margin (outer edge of colony)	Entire
Oxygen requirement	Aerobic
Motility	Non-motile
Catalase	+
Oxidase	-
Indole	+
Urease	+
Gelatin liquefaction	-
Simon citrate	+

+, For positive result; -, For negative result

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Bacterial growth on halogenated compound

Growth of the MR1 bacteria was assessed in various concentrations of 2,2-DCP (10 to 40 mM) which cultures in media containing 10 and 30 mM 2,2-DCP showed development which were insignificant. The exception was the 20 mM media which demonstrated good bacterial growth, while the 40 mM 2,2-DCP was probably toxic as no growth was observed. Following this, the MR1 bacteria was grown in minimal medium supplied with 20 mM 2,2-DCP at 30 °C with agitation at 200 rpm. Growth measurements made at 4 h intervals revealed the cell's doubling time of the bacterium was approximately 23.11 h (Figure 3).

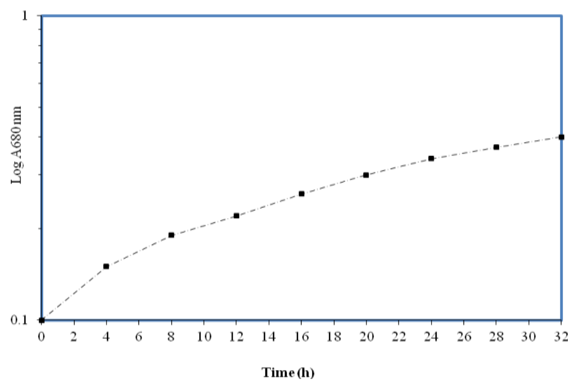

Figure 3: Growth of strain MR1 on 20 mM 2,2-DCP.

Chloride ion released in growth medium

The maximum release of the chloride ion assay for the MR1 bacteria was determined to be 0.257 mmol/L in a 20 mM 2,2-DCP. It can be said that 20 mM of 2,2-DCP was the best concentration to be used as carbon source for its growth. The analysis also confirmed that it was able to degrade 2,2-DCP as a sole carbon source and energy.

Polymerase chain reaction (PCR)

The 1.5 kb of the PCR product of 16S rRNA of the MR1 bacterium was amplified (Figure 4) and subsequent DNA sequencing revealed a complete sequence which consisted of 1500 bp. The BLASTn results showed the bacteria was highly identical to *Raoutella ornithilolytica* (Table 2). From the results, the 2,2-DCP degrading bacterium was designated as *Raoutella ornithilolytica.*

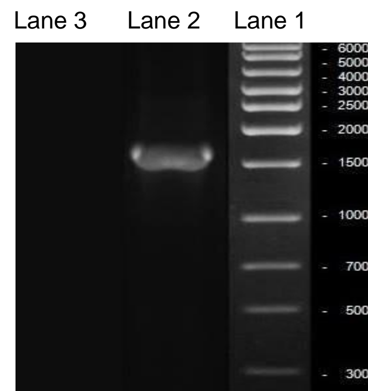

Figure 4: PCR amplification of 16S rRNA gene on an agarose gel (1%). Lane 1, DNA ruler; Lane 2, PCR outcome of amplified 16S rRNA detected under UV light; Lane 3, Control without Fd1 primer.

Table 2: The BLASTn results according to NCBI database.

Accession No.	Description	Max Identity
KC456520.1	*Raoutella ornithilolytica* strain ALK014 16S rRNA gene	95%
JF418157.1	*Raoutella ornithilolytica* strain C8 16S rRNA gene	95%
AB364958.1	*Raoutella ornithilolytica* gene for 16S rRNA	95%

Analysis and sequencing of 16S rRNA

Identification of the PCR product done in "ab1" format Sequence Scanner revealed a complete sequence of the MR1 bacteria. The complete sequence of the 16S rRNA of bacterial strain MR1 gene was then compared to the sequences in the Genbank database NCBI (Table 2). Results of the BLASTn of the MR1 bacterial strain showed 95% homology to the three different strains of *Raoutella ornithilolytica* bacteria.

Phylogenetic tree

In this study, ten dehalogenase producing bacteria were selected from BLASTn analysis for construction of phylogenetic tree using MEGA 5 version 5.2.1. The Neighbor-Joining phylogeny tree of isolated bacteria (strain MR1) was constructed (Figure 5).

DISCUSSION

In this experiment a bacteria strain called the MR1 was isolated from Tioman Island and have the distinctive ability to utilize and degrade 2,2-DCP as a sole carbon source and energy. The 16S rRNA analysis showed that it was of a genus *Raoutella,* a reportedly aerobic bacillus usually found in aquatic environment which produced the dehalogenase enzyme. Cell growth analysis afforded the *Raoutella* to have cell doubling time (23 h) longer than that of *Serratia* sp. (5 h) and *Rhizobium* sp. (12 h) previously reported by Allison *et al.* (1983). Based on the findings, it can be said that the *Raoutella* sp. showed the

ISSN (print): 1823-8262, ISSN (online): 2231-7538

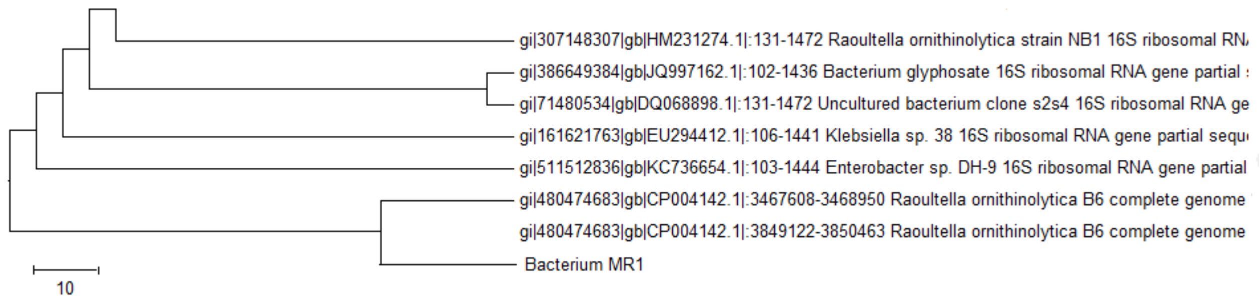

Figure 5: Phylogenetic tree showing evolutionary relationship of isolates MR1 on the basis of 16S rRNA. Bacterium MR1 (*Raoutella ornithinolytica*).

lowest rate of utilizing 2,2-DCP due to low expression of the dehalogenase enzyme and also a poor bacterial uptake system. Furthermore, the 2,2-DCP is a toxic halogenated compound could have also led to sluggish uptake of the 2,2-DCP as sole carbon source and energy for growth of the *Raoutella* sp.

Raoutella ornithilolytica is a Gram negative, rod-shape, non motile and aerobic bacteria. Members of the *Raoultella* genus can grow at 10 °C in soil, water, and in mammalian system (Bergey, 1984) and are commonly found in an aquatic environment, fish and insect (Solak *et al*., 2011). Although previously there have been many virulence factors expressed by *R. ornithilolytica* (Nakajo *et al*., 2004; Ngugi *et al*., 2007) namely, reports detailing their resistance towards antibiotics such as penicillin, ampicillin, gentamicin, chloramphenicol, rifampin, cephalothin, cephotaxime, streptomycin, amoxicillin (Al-Hulu *et al*., 2009). It is described to be an opportunistic pathogen found in human clinical specimens, mainly from the respiratory tract and blood. It has been associated with diverse infections, such as enteric fever-like syndrome, pancreatitis, and mastitis in cattle (Morais *et al*., 2009). However, this is the first reported case of *R. ornithilolytica* with the unique ability to degrade 2,2-DCP as a carbon source.

The 16S rRNA gene is used basically for identifying bacteria. The specific gene is highly conserved between different varieties of bacteria and also archaea. DNA of the bacteria was extracted before PCR reaction was performed (Ventura and Zink, 2002). The Phylogenetic tree is constructed using neighbor-joining method from profile alignment command of CLUSTAL W from MEGA 5 (Saitou and Nei, 1987). An important point in all bacteria species is 16S rRNA which is a universal target by the highest accuracy for identification of bacteria.

CONCLUSION

This paper provides an identification of a bacteria strain that was taken from wastewater in Tioman Island. Based on biochemical analysis, the MR1 bacteria produces a dehalogenase enzyme that could degrade 2,2-DCP as a sole carbon source and energy. Evaluation of the morphology, 16S rRNA sequence and evoluationary reflationary relationships inferred from the constructed phylogenetic tree, the MR1 bacteria exhibited close similarity to *R. ornithilolytica*. Discovery of a dehalogenase producing *R. ornithilolytica* makes this bacteria very unique as there is yet any reports on such species able to degrade 2,2-DCP as a sole carbon source. The bacteria is certainly an uncommon source of 2,2-DCP dehalogenase enzyme in nature and therefore, its dehalogenation properties and capabilities deserves to be assessed and further improved.

ACKNOWLEDGEMENT

Author would like to thank Universiti Teknologi Malaysia and Research University Grant (GUP 08J13) for partially supporting and financing this project.

REFERENCES

Al-Hulu, S. M., Al-Charrakh, A. H. and Al-Saadi, M. A. (2009). Isolation and characterization of *Raoultella ornithinolytica* from clinical specimens in Hilla city. *Iraqi Medical Journal of Babylon* **7, 42-47.**

Allison, N., Skinner, A. J. and Cooper, R. A. (1983). The dehalogenases of a 2,2-dichloropropionate-degrading bacterium. *Journal of General Microbiology* **129, 1283-1293.**

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acid Research* **25, 3389-3402.**

Bergey, D. H. (1984). Bergey's Manual of Systematic Bacteriology. Lippincott, W., Wilkins. New York. **pp. 78-80.**

Bergman, J. G. and Sanik Jr., J. (1957). Determination of trace amounts of chlorine in naphtha. *Analytical Chemistry* **29, 241-243.**

EPA: Environmental Protection Agency, USA. (1988). Health Advisory Summary: Dalapon. *Office of Drinking Water,* Washington DC. **pp. 6-43.**

EXTOXNET: Pesticide Information Profile-Dalapon: A Pesticide Information Project of Cooperative Extension (1993). Offices of Cornell University, Michigan State University, Oregon State University

ISSN (print): 1823-8262, ISSN (online): 2231-7538

and University of California at Davis, accessed 24th March 2014.

Fetzner, S. and Lingens, F. (1994). Bacterial dehalogenases: Biochemistry, genetics, and biotechnological applications. *American Society for Microbiology-Microbiology Review* **58, 641-685.**

Field, J. A. and Alvarez, S. R. (2004). Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds. *Reviews in Environmental Science and Biotechnology* **3, 185-254.**

Fulton, C. K. and Cooper, R. A. (2005). Catabolism of sulfamate by *Mycobacterium* sp. CF1. *Environmental Microbiology* **7, 378-381.**

Hareland, W. A., Crawford, R. L., Chapman, P. J. and Dagley, S. (1975). Metabolic function and properties of 4-hydroxyphenyl-acetic acid 1-hydroxylase from *Pseudomonas acidovorans*. *Journal of Bacteriology* **121, 272-285.**

Hill, K. E., Marchesi, J. R. and Weightman, A. J. (1999). Investigation of two evolutionarily unrelated halocarboxylic acid dehalogenase gene families. *Journal of Bacteriology* **181, 2535-2547.**

Hirsch, P. and Alexander, M. (1960). Microbial decomposition of halogenated propionic and acetic acids. *Canadian Journal of Microbiology* **6, 241-249.**

Huyop, F. Z., Tan, Y. Y., Ismail, M., Wahab, R. A. B. and Cooper, R. A. (2004). Overexpression and characterisation of non-stereospecific haloacid dehalogenase E (DehE) of *Rhizobium* sp. *Asia Pacific Journal of Molecular Biology and Biotechnology* **12, 15-20.**

Huyop, F. Z., Ng, H. J. and Cooper, R. A. (2008). Overexpression, purification and analysis of dehalogenase D of *Rhizobium* sp. *Canadian Journal of Pure and Applied Sciences* **2, 389-392.**

Jensen, H. L. 1957. Decomposition of chloro-substituted aliphatic acids by soil bacteria. *Canadian Journal of Microbiology* **3, 151-164.**

Kerr, L. M. and Marchesi, J. R. (2006). Isolation of novel bacteria able to degrade alpha-halocarboxylic acids by enrichment from environmental samples. *Chemosphere* **64, 848-855.**

Leasure, J. K. (1964). Metabolism of herbicides: The halogenated aliphatic acids. *Journal of Agricultural and Food Chemistry* **12, 40-43.**

Magee, L. A. and Colmer, A. R. (1959). Decomposition of 2,2-dichloropropionic acid by soil bacteria. *Canadian Journal of Microbiology* **5, 255-260.**

Morais, P., Daporta, M. T., Bao, A. F. and Campello, M. G. (2009). Enteric fever-like syndrome caused by *Raoultella ornithinolytica* (*Klebsiella ornithinolytica*). *Journal of Clinical Microbiology* **47, 868-869.**

Nakajo, K., Nakazawa, F., Iwaku, M. and Hoshino, E. (2004). Alkali-resistant bacteria in root canal systems. *Oral Microbiology and Immunology* **19, 390-394.**

Ngugi, K., Tsanuo, K. and Iddi Boga, H. (2007). Benzoic acid-degrading bacteria from the intestinal tract of *Macrotermes michaelseni* Sjostedt. *Journal of Basic Microbiology* **47, 87-92.**

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4, 406-425.**

Schwarze, R., Brokamp, A. and Schmidt, F. R. J. (1997). Isolation and characterization of dehalogenases from 2,2-dichloropropionate-degrading soil bacteria. *Current Microbiology* **34, 103-109.**

Sinha, S., Chattopadhyay, P., Pan, L., Chatterjee, S., Chanda, P., Bandyopadhayay, D., Das, K. and Sen, S. (2009). Microbial transformation of xenobiotics for environmental bioremediation. *African Journal of Biotechnology* **8, 6016-602.**

Solak, Y., Gul, E. E., Atalay, H., Genc, N. and Tonbul, H. Z. (2011). A rare human infection of *Raoultella ornithinolytica* in a diabetic foot lesion. *Annals of Saudi Medicine* **31, 93-94.**

Timmis, K. N. and Pieper, D. H. (1999). Bacteria designed for bioremediation. *Trends in Biotechnology* **17, 201-204.**

Ventura, M. and Zink, R. (2002). Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. *FEMS Microbiology Letters* **217, 141-154.**

Wong, W. Y. and Huyop, F. Z. (2012). Molecular identification and characterization of dalapon-2,2-dichloropropionate (2,2DCP)-degrading bacteria from a rubber estate agricultural area. *African Journal of Microbiology Research* **6, 1520-1526.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538