DEGRADATION OF 3-CHLOROPROPIONATE (3CP) BY *Rhodococcus* sp. STRAIN HJ1

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ABSTRACT. A soil bacterium isolated from Universiti Teknologi Malaysia (UTM) agricultural field, identified as Rhodococcus sp. was able to degrade and utilize 3-chloropropionate as sole source of carbon and energy. This finding was supported by the ability of this bacterium to grow on 20 mM 3-chloropropionate minimal media batch culture with cells doubling time of 17.1 hr. The bacteria could only grow on β-substituted haloalkanoate and not α-substituted substrate (D,L-2-chloropropionate, 2,2-dichloropropionate, 2,3-dichloropropionate and 2-bromopropionate). The utilization of 3-chloropropionate was observed by the depletion of 20 mM 3-chloropropionate in the growth medium using High Performance Liquid Chromatography (HPLC). The calculated dehalogenase specific activity was 0.013 μmolCl-/ml/min/mg protein in cell free extract. Generally, Rhodococcus sp. was known for its ability in degrading various halogenated compounds as reported in the literature. Our finding was the second reported strain of Rhodococcus sp. able to degrade 3-chloropropionate.

KEYWORDS. 3-chloropropionate, dehalogenase, *Rhodococcus* sp.

INTRODUCTION

Halogenated compounds constitute the most important class of xenobiotic. 3-chloropropionic acid is classified as chlorinated monocarboxylic acid or β-chloro substituted haloalkanoates. This compound can be considered as a possible chemical inclusion in certain pesticides and is carcinogenic. The chemical structure is shown in Figure 1.

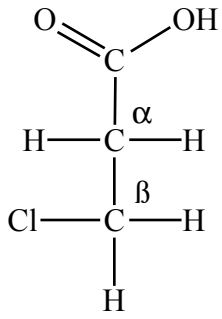


Figure 1. Structure of 3-chloropropionic acid

A wide range of bacterial species has been isolated by enrichment and laboratory culture capable of utilizing halogen-substituted organic acids as their sole sources of carbon and energy (Weightman and Slater 1980; Schwarze *et al*., 1997; Olaniran *et al.,* 2001; Janssen *et al.,* 2005). However, very few studies have been reported regarding degradation of β-chloro substituted haloalkanoates such as 3-chloropropionate. This study is hence considered important since degradation of 3-chloropropionate is poorly understood compared to well studied α-chloro substituted haloalkanoates such as 2,2-dichloropropionate and 2-chloropropionate. In addition, further interest was generated by this subject, when it became apparent that α-chloroalkanoate degrading microorganisms were unable to dechlorinate the β-substituded haloalkanoates, which differ only in chlorine substitution.

In current study, a batch culture method was used for the enrichment and selection of bacterium capable of degrading 3-chloropropionate. The isolated bacterium was then identified using 16S rRNA gene identification technique. The ability of the bacterium in degrading 3-chloropropionate was further confirmed by HPLC analysis and detection of dehalogenase in crude cell free extract.

MATERIALS AND METHODS

Microorganism and growth conditions

A newly isolated bacteria (designated as strain A) was obtained from UTM agricultural soil samples. The bacteria was grown aerobically at 30 °C in minimal medium containing 20 mM 3-chloropropionate as sole source of carbon. Other chemicals used as growth substrate were 20 mM of D,L-2-chloropropionate, 2,2-dichloropropionate, 2,3-dichloropropionate, 3-bromopropionate and 2-bromopropionate, respectively. *Rhodococcus* sp. was inoculated into 20 mM 3-chloropropionate liquid minimal media and incubated at 30°C in a rotary incubator at 180 rpm. Growth was measured at A_{680nm} over 60 hours period. Sample was taken at 12 hours intervals.

16S rRNA gene sequencing

Chromosomal DNA was prepared from late exponential phase culture using Wizard Genomic DNA Purification Kit (Promega, USA). The polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA gene of strain A. 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 forward primer (Fd1), 20 pmol of reverse primer (rP1), 25 µl (2x) PCR master mix (Fermentas) and deionized water. PCR cycle was set as: initial denaturation 94 °C for 5 min, followed by cooling, denaturation 94°C, annealing, 55°C for 1 min; extension, 74°C for 4 min and final extension, 74°C for 10 min. The PCR product was electrophoresed on a 0.8% agarose gel. For sequencing reaction, the PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior sending for sequencing (1st Base Laboratory, Biosyntech, Selangor). 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/2007).

Characterization of the isolates

Cell characteristic was determined under 1000x magnification 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 for example sugar test, gelatin, catalase and citrate were carried out using the standard procedures (Cappuccino and Sherman, 2002).

Determination of 3-chloropropionate concentration

Samples of growth medium were analyzed using HPLC for determination of 3-chloropropionate concentration. Samples were filtered through nitrocellulose 0.2 μM filters (Sartorious, Germany) to remove bacteria cells and particles. 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.

Determination of dehalogenase specific activity

Cells suspended in 0.1 M Tris-acetate buffer pH 7.6 was sonicated on ice using Vibra CellsTM ultrasonicator (Sonics & Materials Inc. USA) operating at 10 % amplitude for 3 minutes. Cell 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 release of halide ion (Bergman and Sanik, 1957). Protein analysis was carried out using Bradford micro assay method with bovine serum albumin (BSA) as the standard.

RESULTS

Identification of 3-chloropropionate degrading bacteria

The 16S rRNA gene from bacterium strain A was successfully amplified by PCR at approximately 1.6 kb in size using Fd1 and rP1 primers (Figure 2.). The partial 16S rRNA gene sequences (1400bp) obtained was compared to the sequence in the database. The 16S rRNA gene sequence was submitted to the gene bank under accession number, AM231909. The sequence alignment showed that strain A was closely matched to the *Rhodococcus* sp. with 100 % sequence identity to other *Rhodococcus* genus (Figure 3.). This bacterium strain A was designated as *Rhodococcus* sp. strain HJ1. This was further confirmed by biochemical characterization.

kb **Lane 1 2 3 4 5**

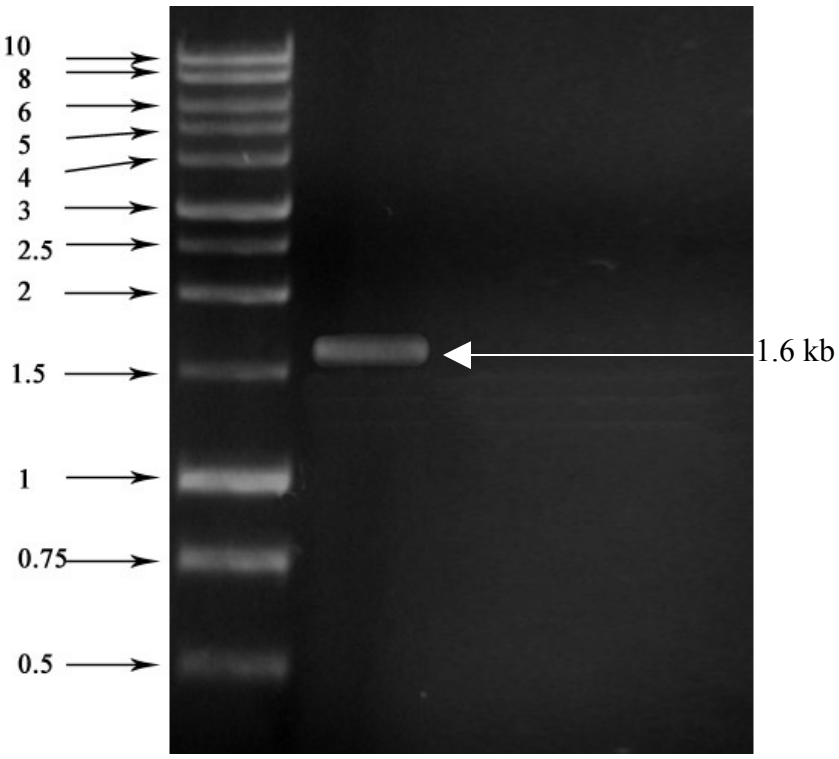


Figure 2. The PCR amplified 16S rRNA gene fragment

Lane 1: Promega 1 kb DNA ladder
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

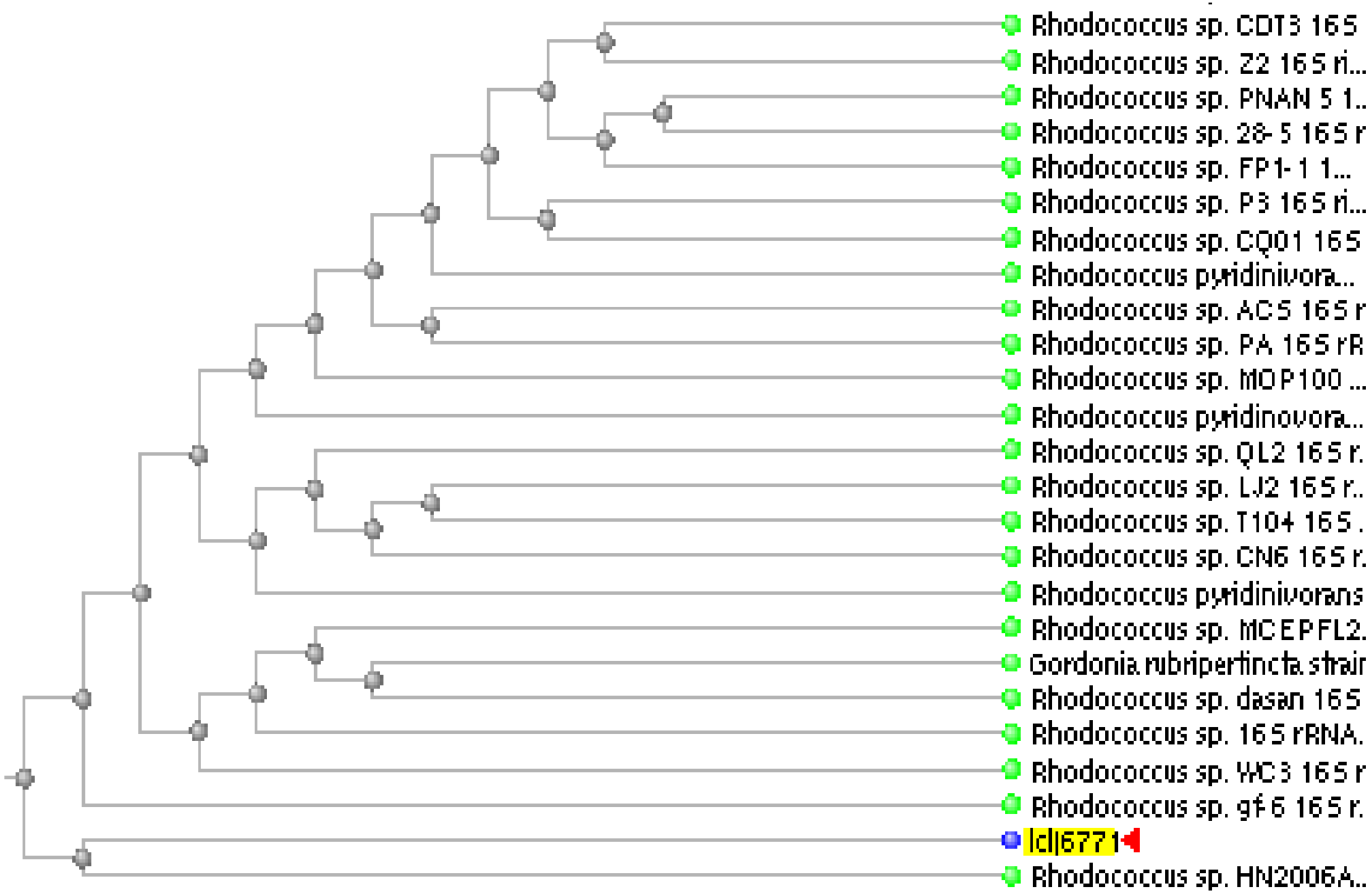


Figure 3. Dendogram showing the genetic relationships of many of the major groups of environmental isolated organisms based on the 1400bp 16S rRNA gene sequence. The *Rhodococcus* sp. were downloaded from the GeneBank, and the sequence for *Unknown lcl6771* was generated in our laboratory under accession number AM231909.

Colonies of bacterium was observed as having a rough surface with a smooth margin, round and raised elevation. It also formed milky-orange colonies on nutrient agar and 3-chloropropionate-containing medium. Microscopic analysis showed that it was a Gram-positive rod and arranged in chains. The cells were acid-fast and no spores were demonstrated by malachite green staining. The bacterium also showed positive results in utilizing lactose, gelatin liquefaction, catalase and citrate tests. However, the isolate do not produce oxidase and are non-motile. The overall biochemical characteristics were matched to the genus *Rhodococcus* sp. as indicated in *Bergey's Manual of Systematic Bacteriology* (Holt *et al.*, 1994).

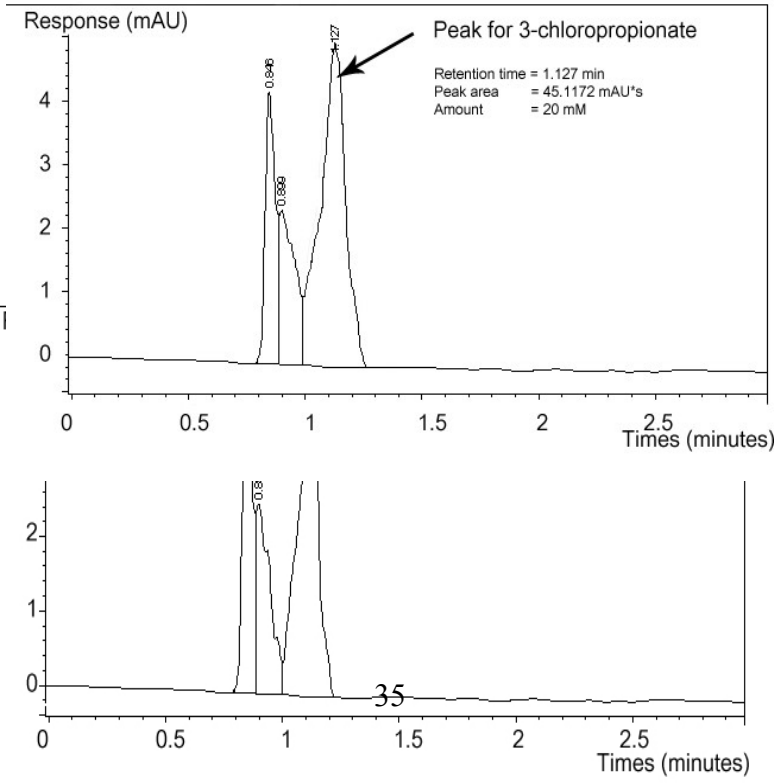
Growth of *Rhodococcus* sp.

The growth was achieved with cells doubling time of 17.1 hours on 3-chloropropionate as the carbon source. Growth of *Rhodococcus* sp. in other halogenated compounds was carried out to determine whether carbon-halogen substitution affect bacterial growth. The *Rhodococcus* sp. was grown on 20 mM D,L-2-chloropropionate, 2,2-dichloropropionate, 2,3-dichloropropionate, 3-bromopropionate and 2-bromopropionate liquid minimal medium, respectively. The results show that 3-bromopropionate was the only substrate utilized by the isolate as the sole source of carbon and energy with cells doubling time of 22.46 hours. This experiment suggested that the bacterial produced dehalogenase enzyme that could act on β-chloro substituted haloalkanoates only.

The amount of 3-chloropropionate in growth medium can be measured using HPLC. *Rhodococcus* sp. that grown in 20 mM 3-chloropropionate liquid minimal medium and the disappearance of 3-chloropropionate was detected when incubation time was increased. The peak of 3-chloropropionate in the chromatogram dropped from day 1 to day 2 (Figure 4.). It was concluded that the 3-chloropropionate was fully utilized by *Rhodococcus* sp. Dehalogenase specific activity can be measured using the rate of chloride ion released from the substrate and the average specific activity of dehalogenase on these extracts was found to be 0.013 μmol Cl^-/min/mg protein.

(i)

(ii)



(iii)

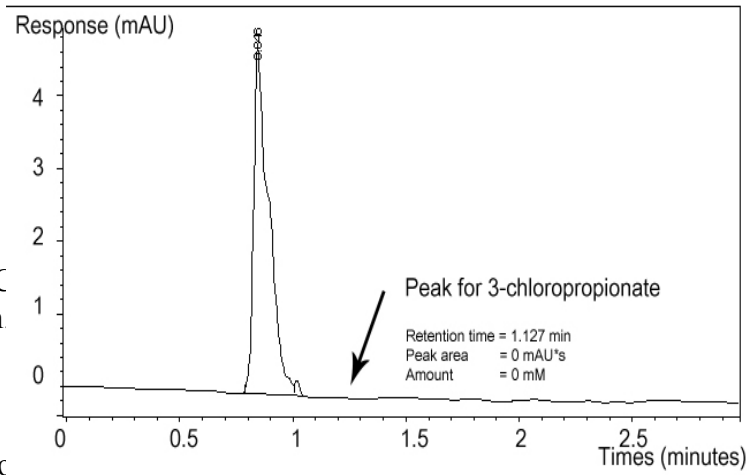


Figure 4. HPLC opropionate as source of carbon 2

DISCUSSION

The 3-chloropr e than 99.6% similarities to *Rhodococcus* sp. Therefore, we can possibly confirmed that the 3-chloropropionate degrading bacteria isolated belongs to *Rhodococcus sp*. This result was supported by biochemical analysis in its ability in utilizing lactose, gelatin liquefaction, producing catalase and grew on citrate. These naturally occurring organisms are of considerable environmental and biotechnological importance due to their broad metabolic diversity and array of unique enzymatic capabilities. For example, *Rhodococcus* sp. are of interest to desulphurization of fossil fuels (Whyte *et al.,* 1998), the industrial production of acrylamide and degradation of hydrophobic and halogenated pollutants (Maeda *et al.,* 1995; Seto *et al.*, 1995; Fournier *et al.*, 2002; Haroune *et al.*, 2002). In addition, *Rhodococcus* sp. is well known to degrade herbicides such as thiocarbamate (de Schrijver *et al.*, 1997) and *S*-Ethyl dipropylthiocarbamate (Shao and Behki, 1995). This was the second reported strain of *Rhodococcus* to show its ability in degrading 3-chloropropionate. The first reported *Rhodococcus* sp. in degrading 3-chloropropionate was described by Hughes (1988).

Growth experiment showed that *Rhodococcus* sp. was able to grow in minimal media supplied with 20 mM 3-chloropropionate, however, growth above 20 mM 3-chloropropionate was inhibited. This suggested that the higher concentration of 3-chloropropionate was toxic to the bacteria.

HPLC analysis showed that the utilization of 3-chloropropionate was initiated from the beginning of the growth. An accurate and precise detection and quantification of 3-chloropropionate was described earlier by Hymer and Cheever (2004) using gas chromatography (GC). Our investigation was the first to develop a simple and effective method to measure 3-chloropropionate depletion using HPLC. Other methods to detect dehalogenase activity are coulometric titration (Slater *et al*,. 1985) and microplate fluorimetric assay to determine the biodegradation of 2-chloropropionate (Marchesi, 2003).

The specificity of *Rhodococcus* sp. dehalogenase was shown when the microorganism was not able to utilize other than 3-chloropropionate and 3-bromopropionate. This investigation provides further evidence for the position of the halogen substituent in governing the susceptibility of chlorinated aliphatic acids to microbial attack. In current study, specific activities of dehalogenase present in *Rhodococcus* sp. crude cell free extract was only 0.013 μmol Cl^-/min/mg protein. Lower dehalogenase activity from *Rhodococcus* sp. agreed by the slow growth in 20 mM 3-chloropropionate minimal medium.

CONCLUSIONS

The results presented here offer information about the biodegradation of a β-chloro substituted alkanoate, 3-chloropropionate, which was among the most active compounds used in pesticides. The ability of *Rhodococcus* sp. in degrading 3-chloropropionate was confirmed with HPLC analysis. The presence of dehalogenase enzyme in *Rhodococcus* sp. was detected by the enzyme assay using crude cell free extract. This bacterium seems likely to play an important role in the degradation of chlorinated xenobiotics in the environment.

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