AsPac J. Mol. Biol. Biotechnol. 20115
Vol. 23 (2) : 268-279

Isolation and characterization of a biodegrading 3-chloropropionic acid *Burkholderia cepacia* WH1 isolated from abandoned agricultural land

Wafaa Hassan Muslem[1,3], Fahrul Huyop[3,*], Iffah Izzati Zakaria[4] and Roswanira Abdul Wahab[2,*]

[1]*Department of Biology, College of Science, Al-Mustansiriyah University, Iraq*
[2]*Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia*
[3]*Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia.*
[4]*Iffah Izzati Zakaria, Natural Products and Drug Discovery Center, Malaysian Institute of Pharmaceuticals and Nutraceuticals, National Institutes of Biotechnology Malaysia, Ministry of Science, Technology and Innovation, Block 5-A, Halaman Bukit Gambir, 11700 Pulau Pinang, Malaysia*

Received 4th June 2015 / Accepted 30 June 2015

Abstract. The widespread use of herbicides that may contain the recalcitrant halogenated compound 3-chloropropionic acid poses significant environmental hazards and may prove detrimental to human kind. Therefore, it is important that an environmentally friendly bio-based method to detoxify such substance is developed. Consequently, the research detailed here investigated the isolation and identification of a bacterial strain that could degrade 3-chloropropionic acid (3CP) as its sole carbon source. A dehalogenase producing bacteria capable of utilizing 3CP was successfully isolated from abandoned agricultural land and designated strain WH1. Analysis of 16s rRNA as well as biochemical and morphological tests found that the strain WH1 showed a 96% sequence identity with and characteristics similar to that of *Burkholderia cepacia*. Analysis of phylogeny and BIOLOG confirmed that the strain WH1 was indeed *B. cepacia*. The bacteria grew well at 37°C in media containing 10 mM 3CP but exhibited a rather slow doubling time of 43.62 h, with an optimum chloride ion release of 0.194 µmol Cl^-/mL. Most importantly, analysis by high perfomance chromatography revealed that the *B. cepacia* isolate effectively degraded ~100% of available 10 mM 3CP. This is the first report detailing a *B. cepacia* strain able to competently utilize 3CP as its sole carbon source.

Keywords: 3-chloropropionic acid, Biodegradation, Bioremediation, *Burkholderia* sp., Dehalogenase, Haloalkanoic acid

INTRODUCTION

Halogenated aliphatic compounds are xenobiotic substances produced in large quantities for use in the agricultural and industrial processes (Yan *et al*., 2010; Abdul Hamid *et al*., 2011). These substances symbolize an important class of environmental pollutants due to their widespread use as herbicides, fungicides, insecticides and solvents (Fetzner and Lingens, 1994; Bhatt *et al*., 2007). The utilization of such substances have been linked to several problems such as their persistence and a high tendency to bioaccumulate (Bachas-Daunert *et al*., 2009; Abel *et al*., 2012), their being carcinogenic and genotoxic (Jing and Huyop, 2007; Drivon and Ruppin, 1998) and so potentially harmful to human kind and the natural environment. Discovery of alternative bio-based methods that could naturally detoxify these substances would therefore be timely. Bioremediation may be an economical, safe and environmentally friendly (Janssen e*t al*. 2005, Abdul Hamid *et al*., 2011) means to remove these xenobiotic substances in comparison to the chemical methods *viz*. adsorption, ion exchange, liquid-liquid extraction, and chemical oxidation (Arora and Bae, 2014).

Over the past few decades, dehalogenases have become increasingly popular as biocatalysts for the bioremediation of halogenated environmental pollutants.

* Author for correspondence: Roswanira Abdul Wahab,
Department of Chemistry, Faculty of Science,
Universiti Teknologi Malaysia,
81310 UTM Johor Bahru, Johor, Malaysia,
Telephone: +607 5534148; Fax +607 5566162;
Email: roswanira@kimia.fs.utm.my.
Fahrul Huyop,
Department of Biosience, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia,
81310 UTM Johor Bahru, Malaysia.
Telephone: +607 5534163; Fax +607 5566162;
Email: fzhutm@gmail.com

This may be due to the fact that the enzymes have the natural ability to neutralize the toxic compounds by catalysing the cleavage of carbon-halogen bonds (Thasif *et al.*, 2009). To date, numerous haloalkanoic acid dehalogenases have been isolated from different bacteria and characterised (Hill *et al.*, 1999, Wong and Huyop, 2011). Most pertinently, the haloalkanoic acid dehalogenases have been isolated mainly from soil borne bacteria (Slater *et al.*, 1997); such bacteria may have evolved the ability to metabolize halogenated compounds following continuous exposure. According to literary review, continuous exposure of microorganisms to halogenated compounds is often the driving force for their genetic adaptability to produce dehalogenases that metabolize such compounds (Field and Alvarez, 2004). So far, only few microorganisms have been reported capable of degrading β-halo carboxylic acid (β-HA), including 3-chloropropionic acid (3CP) (Mesri *et al.* 2009; Yusn and Huyop (2009). Among these dehalogenating microorganisms isolated so far, the *Bacillus sp*. CGMCC no 4196 has been by far the most impressive. The bacterium was reportedly capable of degrading halogenated compounds as concentrated as 120 mM and 30 mM of 3−chlorobutyrate (Lin *et al.*, 2011). The study described that the degradation of 3CPA by the bacterium was the highest when the resting cells were utilized as the biocatalysts. Therefore, it was suggested that the *Bacillus sp*. CGMCC no 4196 catalysed the dehalogenation of 3CPA by initially hydrolysing the compound to β-hydroxyl aliphatic acid. This was followed by oxidation of the β-hydroxyl aliphatic acid to β-carbonyl aliphatic acid by the β-hydroxy aliphatic acid dehydroxygenase (Lin *et al.*, 2011).

This present work aimed to isolate and characterize bacteria that could effectively degrade the β-halo carboxylic acid, 3CP. Herein, we report the properties of a new bacteria, designated as WH1, able to utilize 3CP as its sole source of carbon and energy. The bacterium was isolated from agricultural land in the grounds of Universiti Teknologi Malaysia (UTM) which is contaminated with herbicides and pesticides. The identity of the WH1 bacterial isolate was determined using genetic and biochemical methods. Considering the low natural abundance of β–chloroalkanoate-degrading bacteria and the inability of α-chloroalkanoate degrading bacteria to dechlorinate the β–chloro substituted alkanoates, the continuous attempts to isolate β–chlorodegrading bacteria remains crucial. Furthermore, the synergistic interaction between the α-chloro and β–chlorodegrading enzymes may be useful, envisaging their use in bioformulations to effectively treat environments contaminated with both forms of halogenated compounds.

MATERIALS AND METHODS

Chemicals. The various halogenated compounds used in this study were all of analytical grade and purchased from Sigma-Chemical Co. (St. Louis, USA).

Microorganism isolation and identification. The bacterial strain was obtained from agricultural land in UTM that had been extensively exposed to herbicides and pesticides. About 5 g of a soil sample was added into a 250 mL shaker flask containing 100 mL minimal salts medium (pH 7.5) and supplemented with 10 mM 3CP as the sole carbon source. After 10 days of incubation with shaking (200 rpm) at 37°C, an aliquot (0.1 mL) was pour plated onto solidified minimal medium supplemented with 10 mM 3CP. The resulting colonies were selected and screened for 3CP degradation. Among the isolates, a strain that rapidly degraded the β–haloalkanoic acid, 3CP, was chosen for further study.

Growth conditions. The liquid minimal media was prepared as 10× concentrated basal salts which consisted of $K_2HPO_4.3H_2O$ (42.5g/L), $NaH_2PO_4.2H_2O$ (10.0g/L) and $(NH_4)_2SO_4$ (25.0 g/L). The trace metal salts solution was a 10× concentrated preparation that contained nitriloacetic acid (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 the growth of bacteria contained basal salts (10 mL, 10×) and trace metal salts (10 mL, 10×) in 100 mL of distilled water and were autoclaved (121 °C for 15 minutes). The carbon source, 3CP, was neutralised with NaOH (0.2 M) and sterilised by filtration and added to the autoclaved salts medium to form a final concentration of 10 mM.

Determination of bacterial growth. The amount of bacterial growth was determined using the UV-Vis (Perkin Elmer) method, measuring the absorbance of the growth suspension (A_{600nm}) and the release of chloride ions due to degradation of 3CP into the growth medium (A_{460nm}). Determination of the free halide ion during the reaction was carried out using the method of Bergman and Sanik (1957) with some modifications. The chloride ion released was defined as 1 mM chloride, equivalent to 1 µmole Cl-/mL. Next, the cells were harvested by centrifugation (10,000 × g for 10 minutes at 4°C) during the mid-logarithmic phase and the cells were washed with 0.1 M Tris-acetate buffer (pH 7.5) and centrifuged again (10,000 × g for 10 minutes at 4°C). This step was repeated three times before the supernatant was decanted and the cells stored at -20°C until use.

Biochemical tests. In order to ascertain the identity of the isolate, detection of the morphological properties various biochemical tests were carried out: lactose fermentation (MacConkey's), catalase, oxidase, citrate (Simmons), urease (Christensen's), and lactose, motility and gelatine liquefaction.

BIOLOGTM GEN III MicroPlate identification. Biolog is a commercial tool used to identify unknown bacteria, consisting of 94 phenotypic tests that include 71 carbon source utilization assays and 23 chemical sensitivity assays. The test panel provides a "Phenotypic Fingerprint" of the microorganism which can then be used to identify isolates down to the species level. The WH1 isolate was first grown on an agar medium and then suspended in a special "gelling" inoculating fluid (IF) using the manufacturer's recommended cell density. Then, the cell suspension was inoculated into the GEN III MicroPlate at 100 µL per well and the microplate was incubated to allow the phenotypic fingerprint to form. Initially, the wells started out colorless, and following incubation the increased respiration in the wells caused the reduction of the tetrazolium redox dye to form a purple color in the positive colonies. In contrast, the negative wells that corresponded to the negative control (without carbon source) remained colorless. A positive control (supplemented with a carbon source) was also used as a reference for the chemical sensitivity assays. After incubation, the phenotypic fingerprints of the purple wells were compared to the Biolog's extensive species library.

DNA extraction. A DNA purification Kit (Promega Kit) was used to extract the genomic DNA from the WH1 bacterial strain. Following DNA extraction, the concentration of the WH1 bacteria genetic material was approximately 300 ng/µl as measured by Nano-Drop analysis. Next, the extracted DNA was subjected to gel electrophoresis.

PCR amplification of the 16S rRNA, gene sequencing and analysis of the 16S rRNA. Using universal primers Fd1 and rP1, as suggested by (Weisburg *et al.*, 1991), the 16S rRNA of the WH1 isolate was amplified and the PCR product was visualized by gel electrophoresis. Chromosomal DNA was prepared from the late exponential phase culture using the Wizard Genomic DNA Purification Kit (Promega, USA). The polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA of strain WH1. The universal primers used were Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ACC TTG TTA CGA CTT'). The total volume of each amplification reaction was 25 µL which consisted of 12 µl PCR master mix (Fermentas Inc. USA), 1 µl template DNA, 1 µl forward primer (Fd1), 1 µl reverse primer (rP1), and 10 µl nuclease free water. 16S rRNAGene amplification was performed for 30 cycles whereby each cycle was set as an initial denaturation phase of 94°C for 5 minutes, followed by denaturation 94°C for 1 minute, annealing 55°C for 1 minute and final extension 72°C for 10 minutes. The PCR product was electrophoresed on an agarose gel (0.8%). For the sequencing reaction, the PCR product was purified using a QIAquick PCR purification kit prior to sequencing (1st Base Laboratory, Selangor). The sequence obtained was compared to other sequences in the public databases using the BLAST search program found in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis of 16S rRNA gene. The 16S rRNA sequence was aligned and compared with the sequences deposited in the Gene Bank database from the National Center for Biotechnology Information (NCBI), using the BLASTn analysis tool. Next, the generated sequences were selected from the BLASTn and added directly into MEGA6 software. The command of CLUSTAL−W was used to align the first 10 sequences obtained from the BLAST analysis. Subsequently, the analysis of phylogeny and alignment of multiple sequences were conducted using CLUSTAL−W and Phylogeny Construction in MEGA6 software (Saitou and Nei, 1987).

HPLC analysis of 3CP degradation. Samples of the bacterial growth media were analysed by high performance liquid chromatography (HPLC) to monitor reduction of the 3CP (10 mM) substrate in the reaction media. Samples were passed through a 0.2 µm nitrocellulose filter (Sartorius) to remove bacterial cells and particulates prior to HPLC analysis. The analysis was performed at a flow rate of 1.0 mL/min using isocratic elution with a mobile phase containing aqueous potassium sulphate (20 mM): acetonitrile (60:40). The fractions of the sample were detected with a UV detector equipped with a ZORBAX Eclipse XDB-C18 (4.6 x 150mm, 5µM particle sizes).

RESULTS

Isolation and Characterization of 3CP Degrading Bacteria. Sampling of the soil obtained from the herbicide-contaminated agricultural land in UTM successfully isolated an indigenous bacterial strain that was designated as strain WH1. The bacterium was found to grow on a minimal media containing 10 mM 3CP as the only carbon source following 6 days of incubation. No colonies were observed on the control plates without 3CP (Figure 1). The basic cellular morphology of the WH1 bacteria showed that the bacteria were generally of circular shape, white (pale brown through sunlight) and formed creamy colored colonies on the 10 mM 3CP minimal agar plates. Gram staining revealed that the WH1 strain was Gram negative, confirmed by formation of post staining pink-red colonies. The morphology and biochemical tests as well as the Biolog™ GEN III Microplate results for the WH1 strain are summarized in Table 1 and Table 2, respectively.

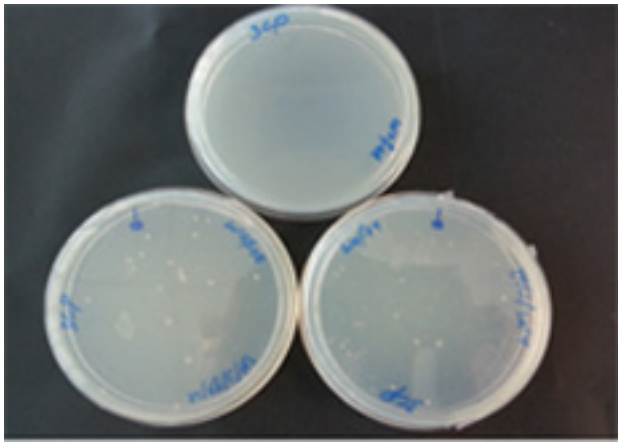

Figure 1. Growth of the dehalogenase producing bacteria WH1 on spread plate minimal media containing 10 mM 3CP following 6 days of incubation at 37°C.

Growth profile and halide ion assay. The WH1 bacteria exhibited a cell doubling time of 43.62 hours in a 10 mM 3CP media with concomitant release of chloride ion in the growth medium (Figure 2). A maximum chloride ion release rate of 0.194 µmol Cl^-/mL was observed for the WH1 strain, and pertinently nearly all of the carbon source (> 99%) in the growth media was consumed after 132 hours of incubation (Figure 3).

Identification of 3-chloropropionate degrading bacteria using 16S rRNA analysis. The 16S rRNA gene of the WH1 isolate was successfully amplified using the universal primers Fd1 and rP1. Gel electrophoresis of the PCR product showed that the amplified gene matched the predicted size of approximately 1500 bp (Figure 4).

Table 1. Morphological and biochemical characteristics of the isolated bacterium WH1 grown on 10 mM of 3CP minimal media.

Parameters	Properties
Size	Small, 0.5 ~ 1 mm
Color	White, pale brown throw light
Shape	Circular, creamy dots
Edge	Entire, sharply defined
Gram	- (Pink/red)
Catalase test	+
Urease test	+
Oxidase test	+
Lactose utilization test (MacConkey Agar)	+
Gelatin hydrolysis test	+
Motility test	+
+: positive result, -: negative result	

Table 2. Extensive biochemical analysis of the bacterial strain WH1 using BIOLOG™ GEN III Microplate.

Properties	Results	Properties	Results
D-maltose	–	Tetrazolium blue	–
D-trehalose	–	p-Hydroxy-Phenylacetic Acid	–
D-cellobiose	–	D-Lactic Acid	–
Gentiobiose	–	Methyl Ester	
Sucrose	–	L-Lactic Acid	–
D-turanose	–	Citric Acid	–
Stachyose	–	D-Malic Acid	–
pH 5	–	Nalidixic Acid	–
D-raffinose	–	Lithium Chloride	–
α-D-lactose	–	Potassium Tellurite	–
D-melibiose	–	γ-Amino-Butryric acid	–
β-methyl-D-glucoside	–	α-Hydroxy-Butyric Acid	–
D-salicin	–	β-Hydroxy-D,LButyric Acid	–
N-acetyl-D-mglucosamine	–	α-Keto-Butyric Acid	–
N-acetyl-β-D-mannosamine	–	Acetoacetic Acid	–
N-acetyl-neuraminic acid	–	Propionic Acid	–
1% NaCl	+	Acetic Acid	–
4% NaCl	+	Aztreonam	–
8% NaCl	+	Sodium Butyrate	–
A-D-glucose	–	Pectin	–
D-Mannose	–	D-Gluconic Acid	–
D-Galactose	–	Glucuronamide	–
3-Methyl Glucose	–	Mucic Acid	–
L-Fucose	–	Quinic Acid	–
L-Rhamnose	–	Vancomycin	–
Inosine	–		
Fusidic Acid	–		
D-Serine	–		
D-Sorbitol	–		
D-Mannitol	–		
D-Arabitol	–		
myo-Inositol	–		
D-Serine	–		
Troleandomycin	–		
Rifamycin SV	–		
Minocycline	–		
L-Arginine	–		
Lincomycin	–		
Niaproof 4	–		
+: positive result, -: negative result			

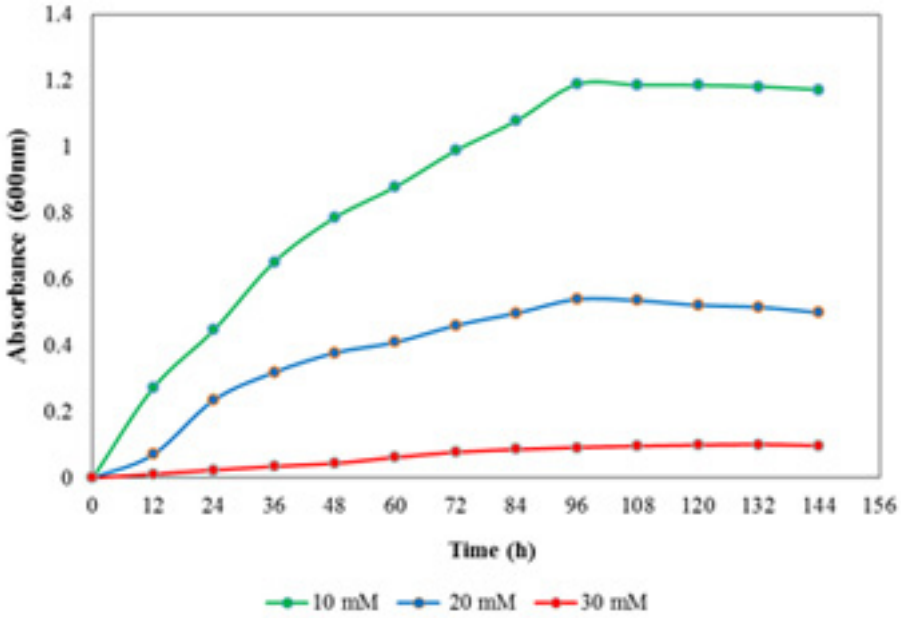

Figure 2. The growth profile for the bacterial strain WH1 in minimal media at concentrations of 10 mM, 20 mM and 30 mM 3CP, respectively.

Sequencing and analysis of 16S rRNA gene. The PCR product of the WH1 isolate was sequenced by 1st BASE ® Malaysia. A total of 1500 bases for the WH1 gene sequenced were aligned with other species using the BLASTn option. The results for the BLASTn search and the significant sequenced alignments suggest that the WH1 strain belongs to the genus *Burkholderia* (Table 3).

Phylogenetic study. Scientific classification of the isolate WH1 was determined as follows: Kingdom – Bacteria; Phylum – Proteobacteria; Class – Betaproteobacteria; Order – *Burkholderiales*; Family – *Burkholderiaceae*; Genus – *Burkholderia*. Bacterial species of the same families of isolates were selected for the phylogenetic analysis. The 16S rRNA sequence of this bacteria species were obtained from NCBI database and

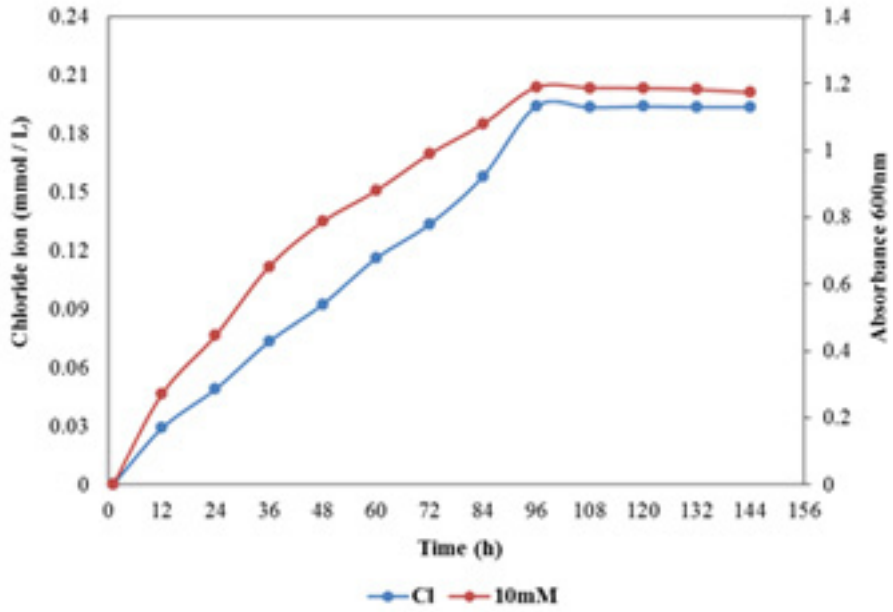

Figure 3. Correlation between the growth of bacteria WH1 and rate of chloride ion release in media supplemented with 10 mM 3CP.

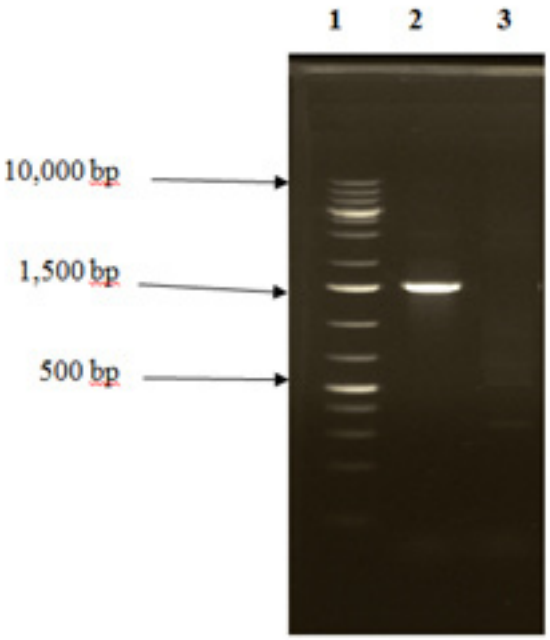

Figure 4. Gel electrophoresis of PCR product from the 16S rRNA gene of the bacterial strain WH1. The PCR amplification of 16S rRNA gene on an agarose gel (1%). Lane 1: DNA Ladder (1 kb), Lane 2: Amplification of 16S rRNA gene from bacterium WH1, Lane 3: Negative control (dH_2O+ Fd1 + rP1 + PCR mix).

alignment were made using CLUSTAL W provided by MEGA6. A Neighbour-Joining phylogeny tree with boot strap value was constructed (Figure 5). The results inferred that the bacterium WH1 may be designated as *Burkholderia cepacia.*

HPLC analysis of 3CP concentration in the bacterial growth medium. Samples of the growth medium were analysed using HPLC at daily intervals to monitor the presence of 3CP. The study revealed that the substrate 3CP was almost fully utilized by the *Burkholderia cepacia*, consistent with the disappearance of a peak that corresponded to 3CP after 96 hours. In contrast, the concentration of 3CP in the negative control minimal medium (without bacteria) remained constant, inferred from the relatively similar area of the peak at 0 and at 96 hours, hence conclusively indicating the non-utilization of 3CP. The HPLC results for the isolated *Burkholderia* sp. are summarized in Table 4.

Table 3. The BLASTn results according to NCBI database.

Microorganisms	Accession Number	Maximum Score	Maximum Identity
Burkholderia cepacia strain 2EJ5 16S rRNA gene partial sequence	GQ383907.1	2097	96%
Burkholderia cepacia GG4 chromosome l 16S rRNA gene partial sequence	CP003774.1	2091	96%
Burkholderia cepacia 16S rRNA gene partial sequence	AB681697.1	2091	96%
Burkholderia cepacia gene for 16S rRNA gene partial sequence	AB626115.1	2091	96%
Burkholderia cepacia 16S rRNA gene complete sequence	FN908409.1	2091	96%

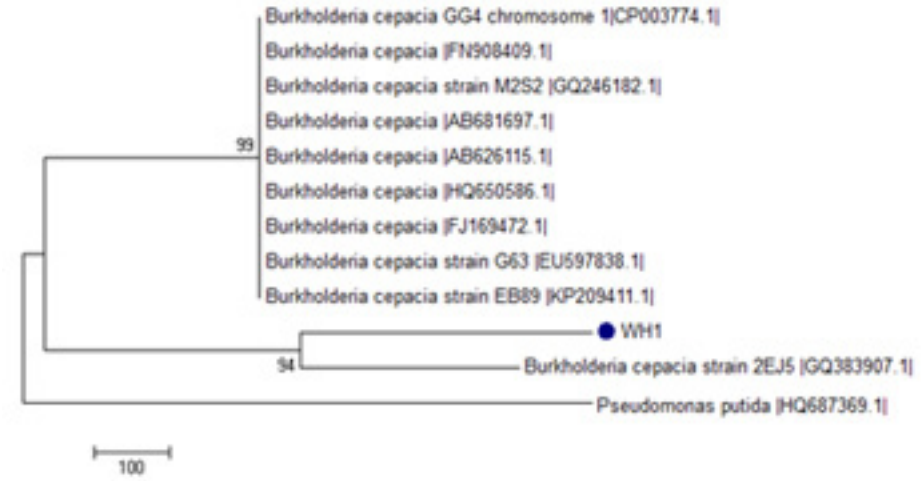

Figure 5. Neighbour-Joining phylogeny tree of the bacterial isolate WH1. The accession number of each species of bacteria is also provided.

Table 4: Summary of HPLC growth medium analysis for the isolated bacteria strain WH1 using Quantitative HPLC analysis concentration formula.

	Incubation time (hours)				
	0	**24**	**48**	**72**	**96**
WH1					
Concentration of 3CP (mM)	10	1.267	1.67	1.611	0.14
Area of peak (mAU*s)	8635.33887	8635.33887	1449.7583	1391.8446	74.66859
Total degradation (%)	98.6				
Control	1.267	1.255	1.283	1.257	1.268
Area of peak (mAU*s)	8635.33887	8634.1226	8634.391	8633.8412	8633.389
Concentration of 3-CP (mM)	10	10	10	10	10
Total degradation (%)	0				

Note: Calculation Using Quantitative HPLC analysis concentration formula, Concentration unknown= $\frac{\text{Area unknown}}{\text{Area know}}$ Known concentration

DISCUSSION

The Gram negative WH1 bacterial strain was determined to be a *Burkholderia* sp. following biochemical and morphological tests. Apart from being Gram-negative, *Burkholderia* sp. is also a motile, rod-shaped and obligatory aerobic bacterium (Zuleta *et al.*, 2014). The findings of this study are consistent with previous reports of the *Burkholderia* sp. as one of many bacteria prevailing in the natural environment such as in soils, rhizospheres, water, plants, fungi, animals, hospital environments and infected humans (Coenye and Vandamme, 2003; Chiarini, *et al.*, 2006 and Vial *et al.*, 2011). *Burkholderia* has been described to be able to survive for prolonged periods in moist environments. A previous study reported that the *Burkholderia* sp. is able to adhere on plastic surfaces (including those of medical devices) and produce several enzymes such as elastase and gelatinase (Mahenthiralingam

et al., 2005), so a relatively low virulence *Burkholderia* sp. was expected.

The significantly low rate of utilization of 3CP by the *Burkholderia* sp. (43.62 hours) reflects the low sensitivity of the dehalogenase expression in the bacteria towards the substance (Weightman and Slater, 1987). Review of literature revealed the cell doubling time of the *Burkholderia* sp. to be comparatively sluggish compared to other previously reported β-halogenated degrading bacteria. A study by Schlömann, *et al*., (1990) reported that *Pseudomonas cepacia* was able to grow in media supplemented with 2 mM 4-fluorobenzoate (Schlömann, *et al*., 1990) and exhibited a cell doubling time of 26−28 hours. Similarly, *Pseudomonas* sp. B6P clocked an impressive cell doubling time of approximately 7−8 hours in a 20 mM 3CP minimal media (Mesri *et al*., 2009), one of the fastest cell doubling times ever reported for a *Pseudomonas* sp. that produces β-dehalogenase. Jing *et al*. (2008) reported a rather short cell doubling time for the *Rhodococcus* sp. HJ1 at 10−11 hours grown in a 20 mM 3CP minimal media (Jing *et al*., 2008). On the contrary, comparison to other α-halogenated degrading microorganisms, *Raoutella ornithilolytica* afforded a cell doubling time of 23.11 hours in 20 mM 2,2−DCP (Niknam *et al*. 2014). Interestingly, the *Arthrobacter* sp. S1 that could utilize a myriad of α-halo compounds such as α-halocarboxylic acid (αHA), 2,2−dichloropropionic acid (2,2-DCP) and D,L−2-chloropropionic acid (D,L−2-CP) as well as β-halocarboxylix acid (βHA) and 3−chloropropionic acid (3CP) as sole carbon source, exhibited cell doubling times of 5 ± 0.2 hours, 7 ± 0.1 hours and 10 ± 0.1 hours, respectively (Bagherbaigi *et al*., 2013). The variety of observed microbial growth speeds in media supplemented with 3CP may correlate with the well-known toxicity of this halogenated compound (Jing and Huyop, 2007). The sluggish uptake of the compound as the sole carbon source and energy for growth observable in the *Burkholderia* sp. in this study may be due to the bacteria being more sensitive towards the β-haloacid 3CP (Niknam *et al*. 2014).

According to Towner and Cockayne (1993), the molecular approach is a common way to find a new genus/species. The 16S rRNA gene sequencing is particularly useful as the 16S rRNA gene is present in all bacteria and is a universal target for bacterial identification that is highly accurate, reliable and reproducible (Kolbert and Persing, 1999; Drancourt *et al*., 2004). The identification of variations in the nucleotide sequence described would allow for the most precise identification and typing of microorganisms. In this investigation, 16S rRNA gene sequence of the 3CP degrading bacterial strain showed 96% identity to *Burkholderia* sp. However, analysis of the 16S rRNA gene sequence alone is normally not sufficient to provide conclusive differentiation of strains within a species. Therefore, such deficiency was overcome by the use of BIOLOG®, a molecular tool that complements other species confirmation methods, as a single method of identification can be potentially misleading (Abel *et al*., 2012). The results from the Biolog analysis conclusively indicated that the *Burkholderia* sp. was indeed *Burkholderia cepacia*.

The ability of the *Burkholderia cepacia* isolate to degrade 3CP was confirmed by HPLC analysis of the bacterial growth media supplemented with the substance. It is widely accepted and also proven in the literature (Lin *et al*., 2011) that the product that results from the degradation of 3CP is 3-hydroxypropionic acid (3HP). However, it is impossible to determine the concentrations of 3HP in the growth culture as the liberated 3HP quickly utilized as the growth substrate in the bacterial culture. The consistent decline in the concentration of 3CP in the growth media with the increase in the incubation time strongly suggested that *Burkholderia cepacia* was capable of degrading and utilizing the substance as a carbon and energy source. Hence, it is suggested that the ability of *Burkholderia cepacia* to utilize 3CP in this study may be associated with the existence of a cluster of enzymatic reaction or reactions that favoured the dehalogenation of 3CP (Selvamani *et al*., 2015). The findings in this study also suggest that the β-dehalogenase in *Burkholderia cepacia* may have an important role in the degradation of β−chlorinated xenobiotics in the environment.

The liberation of chloride ions via the microbe-assisted dehalogenation reaction was monitored by a chloride ion assay (Bergman and Sanik, 1975). The released chloride ions reduced the mercury (III) cyanide to produce mercury (II) chloride, releasing cyanide ions that subsequently interacted with ferric ion (Fe^{3+}) to give the colored ferric thiocyanide complex (Bergman and Sanik, 1957). Based on the slow colorimetric development, it can be said that the *Burkholderia cepacia* exhibited a rather slow rate of 3CP utilization, attributable to a poor uptake system and/or low expression of the dehalogenase enzyme (Jing *et al*., 2010). No growth was observed in the media supplemented with 30 mM 3CP. The outcome suggested that the 3CP was probably toxic to the bacteria at higher concentrations and the presence of excess 3CP could not be tolerated, hence no bacterial growth was observed in this medium. The study therefore proposed that the reaction for the dehalogenation of 3CP by *Burkholderia cepacia* is by hydrolytic dehalogenation similar to that of the Group II dehalogenase enzyme. This mechanism begins with nucleophilic substitution whereby the hydroxide ion of water attacks the electrophilic carbon atom on the chloride group. This results in the transfer of electron pairs from the electron-rich hydroxide to the electron-poor carbon atom. Since carbon can accommodate only eight electrons in its valence shell, the carbon-chlorine bond subsequently breaks as the carbon-oxygen bond begins to form (van Pée and Unversucht, 2003). The chloride ion is then liberated with the pair of electrons that was once bonded to the carbon atom.

According to literary review, organisms capable of utilizing a halogenated organic compound as their sole carbon and energy source generally have some common characteristics. However, there are some basic steps that must first be satisfied before any classification can be

made. First, the organism must either possess or able to synthesize a dehalogenase enzyme capable of removing the substituent halogen(s) in response to the presence of such compound in the environment. Secondly, the dehalogenation product should be non-toxic and easily converted to an intermediate compound in the organism's central metabolic pathway. Thirdly, the halogenated compound should be able to enter the cell either passively or by active transport in order to reach the site of dehalogenase activity. Finally, the halogenated compound should be non-toxic to the organism at normal intracellular concentrations.

CONCLUSION

Discovery of a dehalogenase-producing *Burkholderia cepacia* makes such bacteria very unique since there are no reports to date of such a species able to degrade 3CP as a sole carbon source. Therefore, *Burkholderia cepacia* is undoubtedly an uncommon source of 3CP dehalogenase enzyme in nature and the dehalogenation properties and capabilities of the bacteria deserve to be assessed and further optimized. The β-halogenated degrading class of dehalogenases is highly valuable in the bioremediation of environments contaminated with the recalcitrant β-halogenated xenobiotic compounds. In view of the current preferred use of chemicals for weed management in the agricultural sector, an increase in utilization and contamination of ecosystems with halogenated compounds are therefore expected. The problem is further exacerbated with the ever growing human population, resulting in the expansion of agricultural land area used for planting crops to meet the increasing food demand. Therefore, the feasibility of utilising α- and β-dehalogenases to complement the current treatments in detoxifying halogenated compounds in the environment merits special consideration. Such enzymes may be useful in the bioremediation of environments contaminated with these classes of organohalogens.

REFERENCES

Arora, P. K. and Bae, H. (2014). Bacterial degradation of chlorophenols and their derivatives. *Microb. Cell Fac*. 13: 1-17.

Abdul Hamid, T. H. T., Hamid, A. A. A., Zulkifly, A. H., S. Hamdan,S., Ariffin, S. H. Z. and Huyop F. (2011). Purification and properties of a new dehalogenase enzyme from *Pseudomonas* sp. B6P grow in 3-chloropropionate(3CP). *Afr. J. Biotechnol*. 10:610-614.

Abel, E., Pakingking, R. V., Pagador, G., Wint, M. T. and Huyop, F. (2012). Characteristics of dehalogenase from bacteria isolated from the gut of pond-reared rohu (Labeo rohita) juveniles in Myanmar. *Adv. Biosci. Biotechnol* 3: 353-361.

Bagherbaigi, S., Gicana, R. G., Lamis, R. J., Nemati, M. and Huyop, F. (2013). Characterisation of Arthrobacter sp. S1 that can degrade α and β-haloalkanoic acids isolated from contaminated soil. *Annals Microbiol*. 63:1363–1369.

Bhatt, P., Kumar, M. S., Mudliar, S., and Chakrabarti, T. (2007). Biodegradation of chlorinated compounds - A review. Crit. Rev. Environ. Sci. Technol. 37: 165-198.

Bachas-Daunert, P. G., Sellers, Z. P. and Wei, Y. (2009). Detection of halogenated organic comopounds using immobilized thermophilic dehalogenase. *Anal. Bioanal. Chem.* 395: 173-1178.

Bergman, J. G. and Sanik, J. (1957). Determination of Trace Amounts of Chlorine in Naptha. *Anal. Chem.* 29: 241-243.

Chiarini, L., Bevivino A., Dalmastri C., Tabacchioni, S. and Visca. P. (2006). *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol*. 14: 277–286.

Coenye, T. and Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ. Microbiol.* 5: 719–729.

Drancourt, M., Berger, P. and Raoult, D. (2004). Systematic 16S rRNA gene sequencing of atypical clinical isolates identified 27 new bacterial species associated with humans. *J. Clinical Microbiol*. 42: 2197-2202.

Drivon, G. and Ruppin, C. (1998). Process For The Preparation of 3-chloro-propionic Acid.U. S. Patent Documents.

Fetzner, S. and Lingens, F. (1994). Bacterial Dehalogenases: Biochemistry, Genetics and Biotechnological Applications. *Microbiol. Mol. Biol. Rev.* 58: 641- 685.

Field, J. A. and Alvarez, S. R. (2004). Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds. *Rev. Environ. Sci. Biotechnol.* 3: 185-254.

Hareland, W. A., Crawford, R. L., Chapman, P. J. and Dagley, S. (1975). Metabolic Function and Properties of a 4- Hydroxyphenylacetic Acid 1 Hydroxylase from *Pseudomonas acidovorans. J. Bacteriol.* 21: 272-285.

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

Janssen D. B., Dinkla, I.J.T., Poelarends, G.J. and Terpstra, P. (2005). Bacterial Degradation of Xenobiotic Compounds: Evolution and Distribution of Novel Enzyme Activities. *Environ. Microbiol.* 7: 1868-1882.

Jing N. H., Wahab R. A., Taha A. M., Rashid N. A. A., Huyop AM (2008). A further characterization of 3-chloropropionic acid dehalogenase from *Rhodococcu*s sp. HJ1. Res. *J. Microbiol.* 3: 482-488.

Jing, N. H., and Huyop, F. (2007). Dehalogenation Of Chlorinated Aliphatic Acid by *Rhodococcus* sp. *Asia Pac. J. Mol. Biol. Biotechnol.* 15: 147-151.

Jing, N. H., Wahab R. A., Hamdan, S. and Huyop, F. (2010). Cloning and DNA sequence analysis of the haloalkanoicpermease uptake gene from *Rhizobium* sp. RC1. *Biotechnol*, 9: 319-325.

Kolbert, C. P. and Persing, D. H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr. Opin. *Microbiol.* 2: 299-305.

Lin, C-J., Yang, L-R., Xu, G. and Wu, J.P. 2011. Biodegradation and metabolic pathway of β-chlorinated aliphatic acid in *Bacillus* sp. CGMCC no.4196. *Applied Microbial and Cell Physiology*, 90: 689-696.

Mahenthiralingam, E., Urban, T. and Goldberg J (2005). The multifarious, multireplicon *Burkholderia cepacia* complex. *Nature Rev. Microbiol.* 3: 144-56.

Mesri, S., Wahab, R. A. and Huyop, F. Z. (2009). Degradation of 3- Chloropropionic Acid (3CP) by *Pseudomonas* Sp. B6p Isolated from a Rice Paddy Field. *Annals Microbio*l. 59: 446.

Niknam, M. R. M., Huyop, F. and Abdul Wahab, R. (2014). Identification and characterization of *Raoutella ornithilolytica* and determination of its herbicide 2,2-dichloropropionate (2,2-DCP) degradation potential. *Malaysian J. Microbiol.* 10: 249-254.

Saitou, N., and Nei, M. (1987). The Neighbor -Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evo.*4: 406-425.

Selvamani S., Wahab R. A. and Huyop, F. Z. 2015. A novel putative non-ligninolytic dehalogenase activity for 3-chloropropionic acid (3CP) utilization by *Trichoderma asperellum* strain SD1. M*alaysian J. Microbiol*. in press.

Schlömann, M., Schmidt, E. and Knackmuss, H. J. (1990). Different types of dienelactone hydrolase in 4-fluorobenzoate-utilizing bacteria. *J. Bacteriol.* 172: 5112.

Slater, J. H., Bull, A. T. and Hardman, D. J. (1997). Microbial dehalogenation of halogenated alkanoic acids. *Adv. Microbial Physiol.* 38: 133-176.

Thasif, S., Salehhuddin, H. and Huyop, F. (2009). Degradation of D,L-2chloropropionic acid by bacterial dehalogenases that shows stereospecificity and its partial enzymatic characteristics. *Biotechnol.* 8: 264-269.

Towner, K. J. and Cockayne, A. (1993). Analysis of nucleic acid profiles. In: Molecular Methods for Microbial Identification and Typingpp. 29-63. Towner K. J. and A. Cockayne (eds.) Chapman & Hall, London, UK.

Van Pée, K. H. and Unversucht, S. (2003).Biological Dehalogenation and Halogenation Reactions. *Chemosphere* 52: 299-312.

Vial, L., Annelise, C, Groleau, M. C., and Déziel, E. (2011). The various lifestyles of the *Burkholderia cepacia* complex species: a tribute to adaptation. *Environ. Microbiol* 13: 1-1.

Weightman, A.J. and Slater, J.H. (1987). The Problem of Xenobiotics and Recalcitrance.In: Lynch, J.M., and Hobbie, J.E. eds, *Microorganism in Action: Concepts, Application in Microbial Ecology. Blackwell Scientific Publication.* 322-347.

Weisburg, W., Barns, S. M., Pelletier D. A. and Lane, D. J. (1991).16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol* 173: 697-703.

Wong, W. Y. and Huyop, F. (2011). Characterization of a Labrys sp. strain Wy1 able to utilize 2,2-dichloropropionate (2,2-DCP) as sole source of carbon. Afr. J. Microbiol. Res. 5: 3282-3288.

Yan, S., Subramaniam, S. B., Tyagi, R. D. , Surampalli, R. Y. and Zhang, T. C. (2010). Emerging contaminants of environmental concern: Source, transport, fate, and treatment. *Pract Periodical Haz Toxic Radioactive Waste Manage* 14: 2-20.

Yusn, T. Y., Huyop, F. (2009). Degradation of 3 -Chloropropionic Acid by E. coli JM109 Expressing Dehalogenase (deh) Gene Used as Selection Marker. *J. Biotechnol.* 8: 385-388.

Zuleta, L., Cunha, C. D., De Carvalho, F. O., Ciapina, L., Souza, R., Mercante, F. B., De Faria, S., Baldani, J., Straliotto, R., Hungria, M. and De Vasconcelos, A. (2014). The complete genome of *Burkholderia* phenoliruptrix strain BR3459a, a symbiont of Mimosa flocculosa: Highlighting the coexistence of symbiotic and pathogenic genes. BMC Genomics, 15: 535.