Full Length Research Paper

Molecular identification and characterization of Dalapon-2,2-dichloropropionate (2,2DCP)-degrading bacteria from a Rubber Estate Agricultural area

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The extensive use of herbicides in agricultural area over the past years resulted in environmental pollution. A total of 4 potential bacterial strains were isolated from the rubber estate using minimal media containing 2, 2-dichloropropionate (2, 2-DCP) as sole source of carbon and energy. The 16S rDNA analysis was carried out for genus identification study. Phylogenetic analysis suggested that strains D1, D6 and D9 were closely related with *Enterobacter cloacae* ATTC13047, whereas, strain Dw was closely related with *Burkholderia* sp. KU-25 with distance values of 0.001 and 0.004 base substitutions per site, respectively. Since strains D1, D6 and D9 belong to the same genus, therefore, D9 and Dw were further analysed. The growth profiles of both D9 and Dw in minimal liquid medium containing 40, 20 and 10 mM 2,2-DCP were studied. Strain Dw growth was approximately 3 times faster than D9. In conclusion, faster growth rate for strain Dw indicates the pragmatic application of the bacterial strains to degrade residual herbicide in the environment.

Key words: Pollutant degradation, herbicide, *Enterobacter*, *Burkholderia*, dehalogenase and halogenated compound.

INTRODUCTION

Halogenated compound were used extensively as herbicide. They are toxic, persistence and poorly degraded in the environment (Alexander, 1999; Rieger et al., 2002). Previous studies showed that halogenated hydrocarbon like insecticides and polychlorinated biphenyl (PCB) were detected as residues in human adipose tissue, serum, and milk. Accumulation of these chemicals may bring harm to the human body (Gyorkos et al., 1985). In previous study, several soil bacteria were isolated based on growth on herbicide Dalapon or 2,2-dichloropropionate (2,2-DCP) (Slater et al., 1979; Macgregor, 1963; Burge, 1969; Berry et al., 1979; Jing et

al., 2008; Huyop et al., 2004).

2,2-DCP has three carbons, with two chloride atoms bind at the α -C position (C_α). 2,2-DCP or Dalapon was often marketed as sodium salt and used to control seasonal weeds and is moderately toxic to human (Figure 1). Extensive use of Dalapon may results in leaching of the herbicide into ground water and cause pollution since it was extremely water soluble (EPA, 1988; Christian and Thompson, 1990).

Dehalogenases are key enzymes in the metabolism of halo-alkanoate compounds. Hill et al. (1999) described systematic approach to amplify two different families of α -halocarboxylic acid (α -HA) dehalogenase genes of group I and group II. Group I dehalogenases were non-stereospecific, whereas group II showing stereospecific, dechlorinating only L- but not D-2-chloropropionic acid. However, only few organism that can degrade β -

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Figure 1. 2,2-dichloropropionic acid with formula molecule of $\text{CH}_3\text{CCl}_2\text{CO}_2\text{H}$.

halocarboxylic acid (β -HA) as reported by Mesri et al. (2009) and Yusn and Huyop (2009). Microorganisms are capable of evolving new enzymes, pathways and regulatory mechanisms for the degradation of almost all xenobiotic compounds due to their short life cycle (Timmis and Pieper, 1999). The only organism so far reported to make all three forms of dehalogenase is a *Rhizobium* sp. (Leigh et al., 1988).

Genomic DNA isolation, PCR amplification of the 16S rDNA genes and direct determination of the PCR-amplified 16S rDNA sequences were carried out by using methods that have been described previously (Hamid et al., 2010a,b). Therefore, current studies aimed on isolation and identification of bacterial species by 16S rDNA gene analysis. The soil sample was taken from rubber estate in Melaka, Malaysia and we were particular interested in isolating new bacteria from this area due to its long time exposure to various kind of halogenated herbicide including Dalapon, Paraquat™ and 2,4-Dichlorophenoxyacetic acid (2,4-D).

MATERIALS AND METHODS

Growth media preparation

The bacteria were grown aerobically in chloride-free minimal media as described earlier (Hareland et al., 1975) containing 2,2-DCP (between 10 to 20 mM) as the sole carbon and energy source. Carbon source-2,2-DCP was prepared as 1 M stock solution. 2,2-DCP (1 M) was filter sterilized using 0.2 μm nylon membrane filter and added to the sterilized minimal media at desired concentration.

Bacteria isolation

Soil sample (5 g) was mixed into minimal growth medium supplied with 5 mM 2,2-DCP. The soil mixture was then incubated at 30°C on a rotary shaker set at 200 rpm over night. The liquid culture from the growth medium was spread onto solid 10 mM 2,2-DCP minimal medium. The potential bacteria were then selected after incubation over 4 to 5 days at 30°C.

Alternatively, customized media was prepared as previously described by Fortin et al. (1998) and Greer et al. (1990). It was used to detect the release of chloride ions if the bacteria were able to utilize halogenated compound. The media contain 1/20 1M of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1/20 1M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg/L yeast extract, 75 mg/L bromothymol blue (pH 7.0) and 2 mM 2,2-DCP. Bromothymol blue changes colour from green to yellow indicating the presence of HCl due to the chloride ion released.

Growth measurement and assay for halide ion

A 1.0 ml of sample was taken from liquid growth medium aseptically at 6 h interval over 30 h growth period. The growth was measured at $A_{680\text{nm}}$. Measurement of free halide released during the dehalogenation reaction was carried out by an adaptation of the method of Bergman and Sanik (1957). Sample (1ml) was added into 100 μl of 0.25 M ammonium ferric sulphate in 9 M nitric acid and mixed thoroughly. To this was added 100 μl mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The colour was allowed to develop for 10 min and measured at $A_{460\text{nm}}$ in a Pye-Unicam SP1750 series spectrophotometer.

DNA isolation and PCR of 16S rDNA

DNA was prepared using Promega® Wizard® Genomic DNA Purification Kit. The rehydrated DNA was stored in -20°C freezer for further use. DNA concentration was estimated using Thermo NanoDrop™ 1000 UV spectrophotometer where absorbance, $A_{260\text{nm}}$ of 1.0 corresponds to 50 μg of double stranded DNA per ml. PCR amplification of 16S rDNA gene using universal primer suggested by Weisburg et al. (1991). PCR was carried out using BIO-RAD™ MyCycler thermal cycler. The primers were fD1 (5' – AGAGTTTGATCCTGGCTCAG – 3') and rP1 (5' – ACGGCTACCTTGTTACGACTT – 3'). The PCR protocol were denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 minute and 72°C for 3 min, and final elongation at 72°C for 5 min. Amplicons were separated (5V/cm) by 1% agarose gel electrophoresis and stained using 6 mg/mL ethidium bromide before visualized under UV light.

Partial dehalogenase gene amplification and analysis

Putative dehalogenase gene was amplified using degenerative primers as suggested by Hill et al. (1999). The primers were dehlI-F (5' - TGGCGVCARMRDCACTBGARTA - 3') and dehlI-R (5' - TCSMADSBRITTBGASGANACRAA - 3'). Each PCR reaction (total volume 50 μl) contains 50 pmol of each primer, 200 mM deoxynucleoside triphosphates, 0.5 U of *Taq* polymerase (Promega®), 50 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl (pH 9.0) and 200 ng of DNA template. PCR was set as initial denaturation at 94°C for 10 min followed by 30 cycles of 94°C for 45 s, 55°C for 2 min, and 75°C for 45 s, with a final elongation step at 75°C for 5 min.

Phylogenetic analysis of 16S rDNA genes

The 16S rDNA sequences were compared with GenBank database using BLASTn method (Altschul et al., 1997). Sequence alignments of bacteria from different genus were constructed using profile

Table 1. General characterization of bacteria D1, D6, D9 and Dw in minimal media supplied with 10 mM 2,2-DCP.

Isolate	BLASTn (% similarity)	Bacterial colony morphology						Size (µm) Diameter/length	[Cl ⁻] µM ¹
		Age (days)	Colour	Size (mm)	Shape	Margin	Surface		
D1	<i>E. cloacae</i> ATCC 13047 (99%)	7	White	~1.0	Circular	Curled	Raised	0.6±0.03/2.4±0.3	30
D6	<i>E. cloacae</i> ATCC 13047 (99%)	7	White	~1.0	Circular	Curled	Raised	0.6±0.01/2.1±0.25	30
D9	<i>E. cloacae</i> ATCC 13047 (99%)	7	White	~1.0	Circular	Curled	Raised	0.7±0.04/2.0±0.22	42
Dw	<i>Burkholderia</i> sp. KU-25 (99%)	5	Pale yellow	~1.0	Circular	Entire	Convex	0.6±0.05/2.3±0.2	100

¹ Chloride ion release in growth substrate contained 1mM of 2,2-DCP.

alignment command of CLUSTAL-W of MEGA version 4 (Tamura et al., 2007). Evolutionary distances were calculated using p-distance (Jukes and Cantor, 1969) and phylogenetic trees with bootstrap test (500 replications) (Felsenstein, 1985) were constructed using Neighbour-Joining method (Saitou and Nei, 1987). All sequences in FASTA format of related genus and species with highest similarity were obtained from National Center for Biotechnology Information (NCBI) GenBank.

RESULTS

Isolation of pure bacteria able to utilize 2,2DCP

Soil sample were obtained from Malacca rubber estate and several bacteria were isolated using minimal media. From 10 bacteria sample, only four isolates were consistently grown on 10 mM 2,2DCP minimal medium. These isolates were designated as strain D1, D6, D9 and Dw.

Analysis of 16S rRNA gene

The bacteria were identified as Gram-negative as summarized in Table 1. The 16S rDNA PCR product was sequenced and analysed with NCBI accession number of JN986806 for D1/D6/D9 and JN986807 for Dw. The phylogenetic analysis

indicates D1, D6 and D9 appeared to be in the same clad with *Enterobacter cloacae* ATCC13047, at 100% bootstrap value (Figure 2). Since D1, D6 and D9 were related, only D9 was further analysed. However, isolate Dw was related to *Burkholderia* with 99% similarity with *Burkholderia* sp. KU-25 (Figure 3).

Halide ion assay and growth experiment

Chloride ion assay was carried out, and maximum concentration of chloride ion [Cl⁻] was shown in Table1. Growth profile of D9 and Dw were summarized in Table 2. However, no growth was detected in 80 mM of 2,2DCP. The doubling time of Dw (3.96 h) was 3 times faster compared with D9 (11.44 h).

Amplification of Group II dehalogenase gene

Group II primers were used to perform PCR on all isolates to detect partial sequence of approximately 400 bp of dehalogenase gene as described by Hill et al. (1999). However, there was no PCR band pattern on agarose gel electrophoresis suggesting D9 and Dw might possess new kind of dehalogenases.

DISCUSSION

The main objective of this study was to isolate new bacteria which could utilize and degrade Dalapon at high rate. There would be higher chance to isolate halogenated compound degrader in frequently polluted agricultural soil (Olaniran et al., 2004; Omori and Alexander, 1978), hence the soil sample was taken from heavily polluted of rubber estate.

Initially, 10 different isolates were obtained through sub-culturing by streak plate technique. Only D1, D6, D9 and Dw were selected for further investigation. Isolate D1, D6, D9 and Dw took shorter times (1~2 days) to form visible colonies, assuming they can efficiently utilized 2,2DCP (Dalapon) as sole carbon source, while other isolates took longer time (> 5 days) to form colonies or never form any colonies after subsequent sub-culturing steps. No colonies were formed from a media without 2,2DCP.

Growth profile for both D9 and Dw strains were established. Based on growth experiment, both bacteria were able to utilize 2,2DCP at faster rate, for example strain Dw. The 16S rDNA analysis suggested that D9 and Dw belongs to *Enterobacter* sp. and *Burkholderia* sp. with 99% nucleic acid sequence identity, respectively.

Figure 2. Neighbour joining tree of strains D1, D6 and D9 based on 16S rDNA gene sequences. The sequence of *Pantoea agglomerans* AB299-HQ706104.1 was used as the out group. Scale Bar 0.005 indicates sequence divergence.

Figure 3. Neighbour joining tree of strain Dw based on 16S rDNA gene sequences. The sequence of *B. brasiliensis* M130-AJ238360.1 was used as the out group. Scale Bar 0.005 indicates sequence divergence.

Both strains could not withstand growth at 80 mM 2,2DCP possibly due to the toxic effect of 2,2DCP. Slow growth was observed for D9 strain compared to Dw possibly due to inefficient substrate uptake system gene in D9 compared to Dw. Yu et al. (2007) suggested the uptake of halogenated compound into the bacterial cell was due to haloacid permease. Previous study

identified haloalkanoic permease uptake system gene in the dehalogenase producing bacteria was essential for substrate uptake as proposed by Jing et al. (2010).

In previous investigation there was report on *Burkholderia cepacia* MBA4 which could degrade monobromoacetate(MBA), monochloroacetate(MCA), D,L-2-chloropropionate(D,L2CP), and D, L-2-bromopropionate

Table 2. Growth profile of bacteria D9 and Dw in triplicates on 10, 20, 40 and 80 mM 2,2-DCP minimal medium.

Isolate	BLAST (% similarity)	2,2-DCP concentration							
		10 mM		20 mM		40 mM		80 mM	
		M.A. ¹	D.T. ²	M.A.	D.T.	M.A.	D.T.	M.A.	D.T.
D9	<i>Enterobacter cloacae</i> ATCC 13047 (99%)	0.48±0.001	11.59	0.15±0.001	12.24	0.28±0.005	11.44	NG ³	NG ³
Dw	<i>Burkholderia</i> sp. KU-25 (99%)	0.21±0.003	7.48	0.34±0.002	06.16	0.66±0.001	6.96	NG ³	NG ³

¹ Maximum absorbance at A_{600nm}. ² Doubling time (hours). ³ NG: No growth.

Figure 4. Conserve region (shaded) of group II α -haloacid dehalogenase (α HA). The primers were designed based on these conserved residues.

(D,L2BP) (Tsang and Sam, 1999). The dehalogenase enzyme produced by *B. cepacia* MBA4 is a dimeric with 45 kDa protein designated as *hdlIva* (Murdiyato et al., 1992).

This study is the first reported case that *Enterobacter* sp. could degrade halogenated compound like 2,2DCP. *E. cloacae* is a rod-shaped, Gram-negative bacterium from the *Enterobacteriaceae* family (Nishijima, 1999). This bacterium is always

associated with urinary and respiratory tract infections (Barnes et al., 2003). In addition, other study reported that some *Enterobacter* sp. involved in degradation of complex halogenated compound like monochlorobiphenyls and dichlorobiphenyls as sole sources of carbon and energy (Adebusoye et al., 2007).

Several attempts were made to amplify Group II dehalogenase gene from D9 and Dw. But it was

unsuccessful possibly due to the dehalogenase(s) of the isolate is evolutionary unrelated to Group II halohydrolase. Alternatively, dehalogenase in D9 and Dw themselves might belongs to new kind of dehalogenases. The degenerate primers used were based on the conserve region of several amino acids as guideline for primer design as demonstrated by Nardi-Dei et al. (1997) (Figure 4). The small area of conserve region might or might

not necessary shared by other novel dehalogenases. Some dehalogenase gene such as *dehH1* from *Moraxella* sp. strain B (Kawasaki et al., 1992) and *dehII* from *Rhizobium* sp. NHG3 (Higgins et al., 2005) could not be amplified using Group I or II dehalogenase primers during PCR reaction.

In conclusion, the isolation of enteric bacteria from soil with a special ability to utilize 2,2DCP as sole source of carbon and energy suggests dehalogenase genes are well distributed across microbial genera. High growth rate of bacterium Dw indicates the pragmatic application of the bacterial strains to degrade residual Dalapon or 2,2DCP in the environment. The discovery of new microbes with new kind of dehalogenases is very important for future enzyme study.

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