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ISOLATION AND IDENTIFICATION OF 3-CHLOROPROPIONIC ACID DEGRADING BACTERIUM FROM MARINE SPONGE

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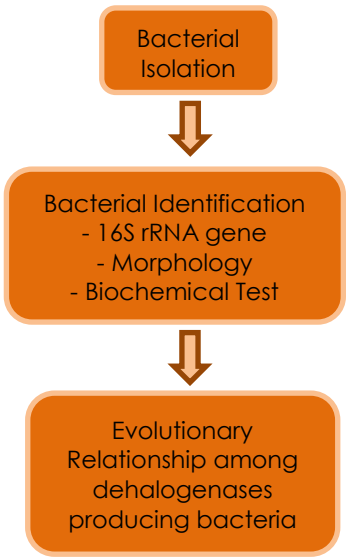
Graphical abstract



Abstract

Synthetic haloalkanoic acids often applied as active components in herbicides are toxic to the environment and harmful to the living organisms. These compounds are widely released to the environment due to their routine use by agricultural activities. In recent years, accumulation of haloalkanoic acids was emerged in marine environment. In this study, a 3CP-degrading bacterium which designated as strain H4 was successfully isolated from marine sponge *Gelliodes* sp. that capable of degrading 3CP as the sole carbon and energy source. The bacteria growth on solid minimal media containing 3CP was the evident for the presence of dehalogenase enzyme. In liquid medium, the doubling time of the cells for strain H4 was 56.82 ± 0.1 h while the maximum chloride ion release was 2.03 ± 0.01 mM. The 16S rDNA sequence of strain H4 was obtained via 16s rRNA gene analysis (1000 bp) and it was closely related to *Bacillus aryabhattai* B8W22 (99% similarity). To the best of knowledge, this report is the first report detailing haloalkanoic acid degrading bacteria from marine sponge in coastal area of Malaysia.

Keywords: Halogenated compound, 3-chloropropionic acid (3CP), haloalkanoic acid, dehalogenation, 16s rRNA gene

1.0 INTRODUCTION

The rapidly growing of chemical industries has resulted in the production of xenobiotic compounds in the environment. Halogenated substances are example of xenobiotics or man-made chemical compounds which are carcinogenic to both of the animals and humans. Halogenated aliphatic compounds are extensively used as solvent, herbicides, insecticides, fungicides and intermediates for chemical synthesis. Herbicide applications help to eliminate unwanted crops or kill certain plants, unfortunately α- and β-chloroacids in the herbicides have brought negative impact to our ecosystem due to its toxicity.

Currently, microbial degradation of halogenated aliphatic compounds is the most preferable method to transform haloalkanoic acids into harmless

product [1-2]. Microorganisms capable of degrading halogenated hydrocarbons as sole of carbon and energy sources are widely distributed and isolated around the world [3-4]. Dehalogenase enzymes produced by these microbes have been proven efficient to degrade a large number of haloalkanoic acid compounds [5-6]. This enzyme could catalyze carbon-halogen bonds and release the halogen from α-carbon of haloalkanoic acid [7].

Previously, many studies were published on bacterial dehalogenation including some were discussing on biodegradation of β-haloalkanoic acids [8-10]. One of the examples, 3-chloropropionic acid is more resistant to degradation compared to other substrates due to the position of halogen in structure molecule. In many years, microorganisms capable of producing α-haloacid dehalogenase have been frequently isolated from soil [11], however only a small number were from marine environment in which none of them could degrade β-haloalkanoic acid [12-13]. Hence, more attention is now being given to marine bacteria, which have become the focus of better resource for new enzymes with novel properties, such as better substrate specificity and catalytic efficiency [13].

Besides, the ocean is a vast reservoir of haloalkanoic acid compounds and also to the pollutant degrading bacteria. The capability of marine sponge to harbor numerous potential microbes has been well discussed and thus makes sponges excellent candidates for the screening of marine bacteria for drug discovery and industrial enzymes [14].

Therefore, in this study, we have isolated and identified a haloalkanoic acid degrading bacterium from marine sponge and this would be crucial to reveal a potential marine microbial for dehalogenation of β-haloalkanoic acid. As a knowledge-basic research, it may stimulate better understanding on the dehalogenation of haloalkanoic acids.

2.0 EXPERIMENTAL

2.1 Sample Preparation

The marine sponge *Gelliodes* sp. was collected from coastal area of Kuantan (gps coordinate: 3.945926, 103.414522) (Figure 1). This area located nearby industrial area of Gebeng and polluted by many wide-range of chlorinated intermediates. A sponge tissue–suspension was prepared by grinding 0.5 g of fresh sponge tissue in 15 ml of sterile natural seawater with a mortar and pestle. The suspension was centrifuged in a speed of 15,000 g per minute. The supernatant was taken for the next analysis.

Figure 1 *Gelliodes* sp. from coastal area of Kuantan

2.2 Growth Experiment

Two stock solutions were prepared in distilled water (Table 1). The bacterial of interest was grown in a minimal media containing 10 mL of 10x basal salts and 10 mL of 10x trace metal salts in 79 mL of distilled water. Then, 1 mL of 3CP stock solution was added to the final volume of 100 mL (10 mM). The sponge suspension was spread on solid minimal media plates which contained 10 mM of 3CP. The plate was then incubated for 4 days at 30°C to allow bacterial growth. The colonies that formed after incubation were isolated and streaked onto agar medium. This process was repeated for few times until a pure culture was obtained. The growth of microorganism in 3CP medium was determined by measuring absorbance at 600 nm every 12 hours by using a UV/VIS a spectrophotometer.

Table 1 Composition of basal salts and trace metalstock solutions

Basal salts solution (10x)	**Amount**
$K_2HPO_4 \cdot 3H2O$	(42.5 g/L)
$NaH_2PO_4 \cdot 2H2O$	(10.0 g/L)
$(NH4)_2SO_4$	(25.0 g/L)
Trace metal salts solution (10x)	**Amount**
$C_6H_9NO_6$	(1.0 g/L),
$MgSO_4$	(2.0 g/L)
$FeSO_4 \cdot 7H2O$	(120.0 mg/L)
$MnSO_4 \cdot 4H2O$	(30.0 mg/L)
$ZnSO_4 \cdot H2O$	(30 mg/L)
$CoCl_2 \cdot 6H20$	(10 mg/L)

2.3 Morphological and Biochemical Analysis

Gram staining was carried out to differentiate between Gram- positive and Gram- negative bacteria while a few of biochemical tests such as catalase test, citrate test, lactose test, triple sugar ion (TSI) test and motility test were carried out to support the identity of bacteria.

2.4 DNA Isolation and 16S rRNA Gene Analysis

Genomic DNA of the isolated bacterium was purified using the instruction provided in Wizard® Genomic DNA Purification Kit. The genomic DNA obtained was

amplified through polymerase chain reaction (PCR) by using universal primer and run by Agarose gel electrophoresis at 100 volts for 60 minutes.

The forward primer (Fd1) has nucleotide sequence of 5' AGAGTTTGATCCTGGCTCAG 3' while the reverse primer (rP1) has nucleotide sequence of 5' ACGGTCATACCTTGTTACGACTT 3' [15]. The program used for amplification of 16S rRNA gene was: initial denaturation 94°C (5 mins), denaturation 94°C (1 min), annealing 55°C (1min) and extension 74°C (4mins). Purification of PCR product was done by QIAquick PCR Purification Kit. Volume of 20 uL purified PCR product was transferred into a sterile tube and sent to 1st Base laboratory, Malaysia for DNA sequencing. Analysis of sequence was done by using the software Bioedit (version 7.2). The 16S rRNA sequences determined in the study were aligned and compared with other sequences of 16S rRNA genes in the Genbank by using Basic Local Alignment Search Tools (BLAST) program. Phylogenetic tree of the identified bacterium was constructed using MEGA 6 software [16].

3.0 RESULTS AND DISCUSSION

3.1 Growth of Bacterial Species on 3-chloropropionic Acid (3CP)

Bacterial strain designated as H4 was originally isolated based on its growth on solid minimal media containing 10mM of 3CP. Several colonies were obviously seen after 48 hours of incubation. They were formed small colonies with circular shape, cream yellow coloured and smooth surface. The colonies indicate the capability of the microbial strains to utilize 3CP as sole source of carbon and energy. In liquid medium, the doubling time of the cells for strain H4 was 56.82 ± 0.1 h while the maximum chloride ion release was 2.03 ± 0.01 mM. Since there was a slow growth, it has suggested that the bacterium took long period of time to adapt in the environment due to the poor uptake system of 3CP compound into bacterial cells.

3.2 Bacteria Identification using 16S rRNA Gene Sequence Analysis

Bacterial strain H4 was subjected to 16S rRNA gene sequence analysis to determine its genus and species. Based on purified DNA of 16S rRNA gene (Figure 2), the expected length of all DNA bands was approximately 1.5 kb when compared to 1 kb DNA ladder.

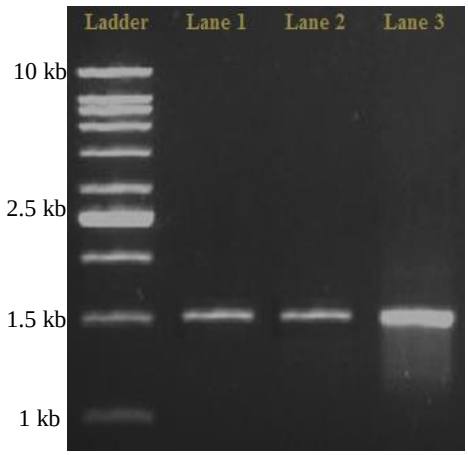


Figure 2 Gel electrophoresis of purified PCR product of amplified 16S rRNA gene fragment on 1.0% (w/v) agarose gel in 1x TAE buffer. Lane 1 and lane 2: H4 strain, lane 3: control *e.coli*

The local similarity results by BLAST suggested that the 16S rRNA gene sequence of bacterial H4 (1000 bp) was closely related to *Bacillus* species in which bacterial H4 possessed highest sequence similarity *with Bacillus aryabhattai* B8W22 (99% similarity). In Figure 3, the bacteria H4 is compared with among dehalogenases producing bacteria and the phylogenetic tree is showing that strain H4 is genetically related with *Bacillus* sp. 3-CPA-15 and confirmed the strain H4 is closely related to *Bacillus* species.

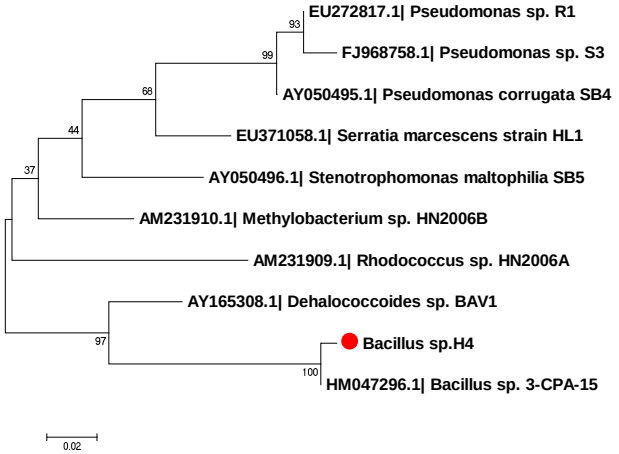


Figure 3 Evolutionary relationships of taxa of *Bacillus* sp. H4 among dehalogenase producing bacteria

In previous study, *Bacillus* sp. CGMCC no. 4196 was isolated from mud in Hangzhou, China [17]. This strain exhibited the ability to utilise high concentration of 3CP as sole carbon source for growth. Moreover, the metabolic pathway of β-chlorinated aliphatic acid was elucidated. While in this study, *Bacillus* sp. H4 has been confirmed to dechlorinate 3CP and 3-hydroxypropionic acid (3HP)

was predicted to be the product of degradation as similarly discussed for the strain CGMCC no. 4196. Both strains may have shared similar characteristic especially in dehalogenation potential since they are closely related according to the genetic distance.

Chiba and his group (2008) had successfully found a *Bacillus* strain that produce haloacid dehalogenase in marine environment [18]. However, the study only emphasize on degradation of monobromoacetic acid, monochloroacetic acid and D,L-2chloropropionic acid. While Huang and his colleagues (2011) had successfully isolated 2-haloacid dehalogenase-producing bacterium, designated as DEH99, from the marine sponge *Hymeniacidon perlevis* [13]. This is the first bacteria isolated from marine sponge that can degrade L-isomers of 2-chloropropionic acid, 2-bromopropionic acid and iodoacetic acid. However, study on bacteria in exhibiting the capability to degrade β-haloalkanoic acid from marine sponge has not documented yet. Therefore, this finding has revealed a new knowledge in environmental microbiology for bioremediation.

3.2 Morphological and Biochemical Characteristic

In support to the 16s rRNA gene analysis, morphological and biochemical analyses were carried out. The isolated bacteria grew well at 30°C with pH 6.8 under aerobic conditions and produce chloride ions as a result of hydrolytic dechlorination of 3CP. In morphological observations using light microscopy (1000x magnification), all cells are rod-shaped, and gram-staining revealed that the strain is gram positive (Figure 4). The positive results were obtained from biochemical test for TSI and motility test, and negative for catalase, citrate and lactose tests (Table 2). These showing that this bacterium is mobile by means of flagella and could ferment the three sugars in TSI test. However, the strain does not have ability to utilize citrate as sole of carbon source, produce exoenzyme casein and express enzyme catalase to detoxify hydrogen peroxide. According to the Bergey's manual, most of all results from the tests are corresponding to the *Bacillus* sp.

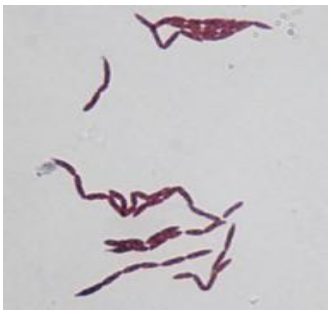


Figure 4 Gram stain of *Bacillus* sp. H4

Table 2 Biochemical test for *Bacillus* sp. H4

Catalase Test	Citrate Test	Lactose Test	TSI Test	Motility Test
-	-	-	+	+

4.0 CONCLUSION

In conclusion, we have found *Bacillus* sp. H4 from marine environment that possesses hydrolytic dehalogenation activity towards β-haloalkanoic acid using a medium containing 3-chloropropionic acid. This will help us to identify haloacid dehalogenases of marine microorganism and investigate halogenation/ dehalogenation processes in the halogen cycle of marine ecology.

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