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Full Length Research Paper

Characterisation of *Bacillus* strains from volcanic area Gunung Sibayak able to degrade 2,2-dichloropropionic acid

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Halogenated compounds are mainly found in agricultural area. The problem arose because these compounds are toxic to human and are persistent in environment. The aim of the present study was to isolate 2,2-dichloropropionic acid (2,2-DCP) degrading bacteria that can grow at higher temperature rather than at normal 25 to 30°C temperature. A soil bacterium was isolated from the surrounding volcanic area at the feet of Gunung Sibayak, Indonesia using streak plate method. The sample was repeatedly streaked onto 20 mM of 2,2-DCP to get a pure culture. The culture was incubated for 2 days at 30, 40 and 60°C incubator shaker aerobically. The isolated bacterium grew best at 40°C but failed to grow at 60°C. Based on partial 16S rDNA gene sequence, the isolated strain GS1 showed high sequence identity to *Bacillus megaterium* strain 0 to 2, whereas, using biochemical tests suggested that the genus belongs to *Bacillus* sp. as expected. Strain GS1 was a Gram positive, rod in shape and produced spore. The results also suggested that, strain GS1 could degrade 20 mM (2,2-DCP) based on growth experiment. In conclusion, the molecular method identified *Bacillus* genus showing no discrepencies from the biochemical test results. This is the first report of *Bacillus* strains associated with 2,2-DCP degradation using above normal temperature for growth.

Key words: 2,2-Dichloropropionic, *Bacillus* strain GS1, degradation, 16S rDNA and pollutant.

INTRODUCTION

Halogenated compounds constitute the most important class of xenobiotic which mostly make up pollution. Some of these compounds are very toxic and cause enormous problems to human health and to the environment (van Pee and Unversucht, 2003). Several microorganisms reported to utilize 2-halogenated alkanoic acids as a sole carbon source have been isolated (Jing and Huyop, 2008; Ismail et al., 2008; Jing et al., 2008; Thasif et al., 2009; Mesri et al., 2009). The bacterial degradation of 2-haloalkanoic acids is attributed to an enzyme, dehalogenase, which catalyzes the hydrolytic removal of halogens from 2-haloalkanoic acids (Kurihara, 2011). Previous studies also described cloning of dehalogenase in *E. coli* system for dehalogenase enzyme characterization (Cairns et al., 1996; Stringfellow et al., 1997; Yusn and Huyop, 2009; Huyop et al., 2008).

Some halogenated chemicals are easily biodegradable for example 2-chloropropionate that can be enriched from almost any oil sample (Leisinger, 1996). This study initially screened the ability of bacteria in soil sample from volcanic area of Gunung Sibayak to grow at high temperature in the presence of α-haloalkanoate. Research interests are geared towards understanding microbial growth at higher temperature as enzymes from thermophillic organisms often show stability to a number of extrinsic factors such as temperatures and organic solvents. These properties make them useful for industrial biocatalyse (Rye et al., 2009).

In this paper we report the isolation and identification of

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a soil bacterial strain that can grow and degrade 2,2-dichloropropionate (2,2DCP) as sole source of carbon and energy in temperature slightly higher than normal. Based on 16S rDNA gene sequence comparison, the topology of phylogenetic trees was constructed.

MATERIALS AND METHODS

Bacterial cultivation

The sample was taken from soil at volcanic area of Gunung Sibayak, Indonesia. The strain was cultivated aerobically at various temperatures ranging from 30, 40 and 60°C in a 250 ml flask in 100 ml of a minimal medium. The liquid minimal media was prepared as 10x concentrated basal salts containing K_2HPO_4. $3H_2O$ (42.5 g/l), NaH_2PO_4. $2H_2O$ (10.0 g/L) and $(NH_4)_2SO_4$ (25.0 g/L). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (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 growing bacteria contained 10 ml of 10x basal salts and 10 ml of 10 x trace metal salts per 100 ml of distilled water and were autoclaved (121°C, for 15 min). The carbon source (2,2DCP; 3-chloropropionate-3CP) was neutralized with NaOH and filter sterilized separately using Millipore GV filter unit (pore size 0.22 µm) and added to the autoclaved salts medium to an appropriate final concentration. The extent of growth was determined by measuring the absorbance at A_{680nm} and the release of chloride ion.

Molecular analysis

The chromosomal DNA of bacteria was prepared using Genomic Wizard Kit ® and sent for sequencing to 1st Base Laboratory, Malaysia using initial primers as described by Fulton and Cooper (2005). The PCR conditions were based on method of Hamid et al., 2010(a) and (b). The full 16S rRNA gene sequencing was subjected to MEGA4 software (Tamura et al., 2007) and was also analyzed using BLASTn option (http://www.ncbi.nlm.nih.gov/BLAST/). The results were graphically displayed online on the Distribution of Blast Hits on the Query Sequence.

Morphology and biochemical analysis

The morphology and biochemical tests were carried out in order to determine the characteristics of the isolated bacterium. Gram staining, spore staining and mobility test are included in the morphology experiment, while the biochemical tests of the bacteria involves various techniques such as oxidase, catalase, gelatin liquefaction, lactose utilization, citrate, nitrate reduction, indole, oxidation fermentation and urease tests.

Scanning electron microscope

The bacterial culture from LB plate were collected and fixed with 4% glutaraldehyde for 12 to 24 h at 4°C and washed with 0.1 M sodium cacodylate buffer for 3 changes at 10 min each. Then, they were post-fixed with 1% osmium tetroxide for 2 h in the same buffer. By applying a serial concentration of alcohol (20 to 80%), the fixed cells were being dehydrated for every 10 to 20 min. Then, the specimens were put into critical dryer for 30 min. To examine the cells, they were coated with gold using a sputter coater and observed under a scanning electron microscope (XL 30 ESEM, Philips- UPM, Serdang, Malaysia).

Reconstruction of phylogram using Mega4 software

Phylograms of unknown bacteria were reconstructed using Mega4 Software (Tamura et al., 2007). Using an integrated web browser, known 16S rDNA gene sequences of related dehalogenase-producing bacteria obtained from NCBI were pasted into alignment explorer, ClusterW. All sequences were aligned and the output was used to reconstruct a phylogram. Neighbor-Joining method was used to infer the evolutionary history of the unknown bacteria (Saitou and Nei, 1987). The evolutionary history of the taxa was analysed from the bootstrap consensus of 500 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985), with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree is then drawn to scale where, P-distance method is used to compute the evolutionary distance and is in the units of the number of base substitutions per site (Tamura et al., 2004). Complete deletion option was used to eliminate all positions containing gaps and missing data.

RESULTS

Bacteria isolation of halogenated compund

Several morphologically different colonies were observed on solid 20 mM 2,2DCP minimal medium but not on 10 mM 3-chloropropionate (3CP) after overnight growth at 30°C and 40°C. However, no growth was detected at 60°C. Colonies formed were repeatedly streaked on the same type of medium in order to obtain a pure colony. One of the colonies (GS1-strain) grew well in liquid minimal medium supplied with 20 mM 2,2DCP as sole source of carbon in shake flask culture with a doubling time 8 h at 40°C.

Characterization of the isolates

Morphology screening and biochemical analysis

Pleminary experiment suggested that the identity of the isolated bacteria belongs to *Bacillus* sp. (Table 1). The biochemical tests were also carried out to further identify the identity of the isolated bacterium. It showed negative results with oxidase, lactose utilization and indole test, whereas the isolate showed positive results with catalase, gelatine liquefaction, citrate utilization, nitrate reduction and urease test. Latter, it was identified as oxidative organism based on oxidation fermentation test. The bacterium was then designated as *Bacillus* sp. strain GS1.

Scanning electron microscopy (SEM) analysis

The cellular morphology was examined with an electron scanning microscope as shown in Figure 1. The isolate

Table 1. Some basic physiological morphology properties of strain GS1.

Properties	Details
Colony morphology	Rough surface and circular
Pigmentation	Creamy-yellowish
Gram staining	Positive
Physical morphology	Rod-shape
Width	0.7µm ± 0.1
Length	2.0 µm ± 0.1
Motility	-
Spore staining	+

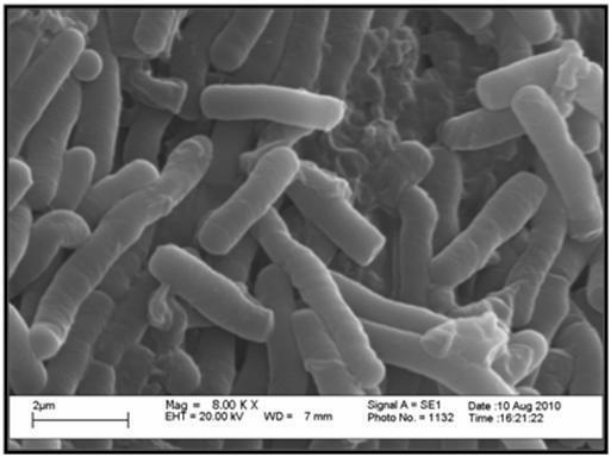

Figure 1. Scanning electron micrograph of strain GS1 (8,000× magnification).

GS1 was rod in shape as expected.

Analysis of 16S rDNA gene sequence for genus identification

Genomic DNA was prepared using Wizard Genomic DNA kit (Promega). The PCR amplification using appropriate primers revealed a single fragment of approximately 1.5 kb for each strain. The PCR products were purified using QIAquick PCR purification kit (Qiagen) for DNA sequencing. In order to get some idea concerning genera and species type, the nucleotide sequencing data were than analyzed using BLASTn online analysis tool to identify the closest phylogenetic relatives. The gene sequences were compared to the sequences in the GenBank database - NCBI (U.S National Centre for Biotechnology Information). GS1 showed the highest sequence similarity (99% sequence identity with e-value=0) to the genus *Bacillus megaterium* strain 0 to 2 (Accession number: GQ870260.1) as expected (Figure 2).

Evolutionary relationship of *Bacillus* sp. Strain GS1

Phylogenetic tree was established using BLAST-Webpage (NCBI). According to Table 2, strain GS1 was located among *Bacillus* sp. Further analysis was carried out by taking ten different related species of *Bacillus* sp. as operational taxonomic units (OTUs) in order to investigate the evolutionary relationship of *Bacillus* sp. strain GS1 among related species (Tamura et al., 2004). The multiple alignments were then constructed using ClustalW in MEGA4. Figure 3 strongly suggest that *Bacillus* sp. GS1 was not located within the large different homologous sites. However, it was closely related to the *B. megaterium* strain 0 to 2 with genetic distance 0.01 base substitutions per site.

DISCUSSION

The present study has shown that GS1 grows specifically only on α-haloalkanoate but not on β-haloalkanoate as sole source of carbon and energy. The isolate grew rapidly on 2,2DCP (8 h) compared to *Rhizobium* sp. (11.8 h) as reported earlier by Allison (1981). Four basic criteria must be fulfilled in order for a given halogenated compound to be utilized by an organism as sole of carbon and energy. First, the organism must synthesize dehalogenase in response to the halogenated compound, which enables it to remove the substituent halogen(s) from the compound. Secondly, the dehalogenation product should be non-toxic and easily converted to an intermediate of the organism's central metabolic pathways. Thirdly, the halogenated compound should be able to enter the cell either passive or by active transport in order to reach the site of dehalogenase activity, and finally the halogenated compound should be non-toxic to the organism at normal intracellular concentrations. The inability of GS1 to grow on 3CP is possibly due to the four reasons given.

Mesophilic bacteria are organisms that grow best in temperatures ranging from 20°C to about 40°C. GS1 failed to grow at higher temperature (60°C). The optimal growth temperature of this organism is 40°C. Since the current isolate was able to grow at 40°C, possibly it belongs to this group. The optimal growth temperature for *Sulfolobus tokodaii* was 75 to 80°C (Rye et al., 2009). Therefore, dehalogenase from this organism has maximum activities at elevated temperatures up to 60°C.

Bacteria taxonomy using 16S rDNA is a common method in the characterization and identification of microorganism, such as the taxonomy of actinomycetes (Colquhoun et al., 1998), taxonomy of extremophiles (Takami et al., 1997; Sorokin et al., 2000) and taxonomy of hydrocarbon degrading bacteria (Wang et al., 1995). BLASTn analysis in current investigation revealed strain GS1 shared at least 99% identity to the sequence of *Bacillus* sp. This was supported by the biochemical and morphological analysis of the genus. This study also

Figure 2. The 16S rDNA partial sequence comparison. GS1 current study; Sbjct: *Bacillus megaterium* strain O-2 (Total sequence identity: 99% identity).

Table 2. *Bacillus* species and its accession number obtained from NCBI database.

Organisms	Accession No.
B. megaterium strain 0-2	GQ870260.1
Bacillus sp. 210_64	GQ199766.1
Bacillus sp. M9H	DQ323079.1
Bacillus sp. 210_20	GQ199722.1
Bacillus sp. 210_18	GQ199720.1
B. megaterium strain NY-3	EU918562.1
Bacillus sp. 210_16	GQ199718.1
Bacillus sp. NyZ43	HQ231222.1
Bacillus sp. 210_55	GQ199757.1
Bacillus sp. 210_33	GQ199735.1

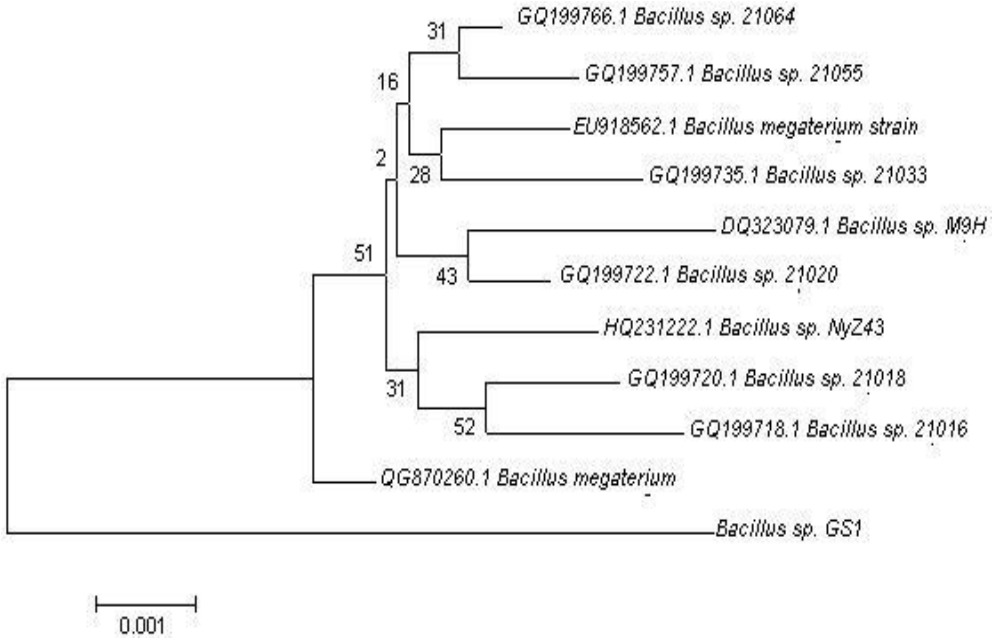

Figure 3. Phylogenetic relationships between *Bacillus* sp. GS1 and other bacteria in the same genera. The scale bar represents 0.001 substitutions per site. Bootstrap values are shown at the nodes (based on 500 resembling).

reported that this strain had a potential to degrade halogenated compound by producing dehalogenase enzyme and so far, this is the first reported *Bacillus* strains associated with 2, 2-DCP degradation above normal temperature for growth. Other reported strains from the same genus are dichloromethane degrading gene isolated from *Bacillus curculans* WZ-12 (Wu et al., 2009) and degradation of low concentration of monochloroacetic acid by *Bacillus* sp. TW1 (Zulkifly et al., 2010).

In conclusion there are many types of dehalogenases, often involved to degrade complex halogenated compounds. Dehalogenases have important applications in biodegradation, apart from having potential in degradation of toxic halogenated compounds, dehalogenase is also important in industrial applications in the production of chiral haloalkanoic acids and hydroxyalkanoic acids. Therefore, this study is significantly important in understanding the distribution of α-haloalkanic degrading bacteria that might be very useful in bioremediation and industrial applications.

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