

Identification And Characterization Of Unknownbacteria And Assessment Of Its Potential In The Degradation Of Halogenated Compounds

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Abstract: *Aims:*The goal of the study is to identify unknown bacteria isolated from contaminated waste water and assess its potential use in the degradation of halogenated compounds.

Methodology: Bacteria SAI genomic DNA was extracted using genomic DNA Purification Kit and PCR amplification of the 16S rRNA was carried out using universal primers (27F and 1492R).

Results: Biochemical tests, 16S rRNA and phylogenetic analysis suggested SAI belongs to *Acinetobacter* with 98% identity to this genus. SAI growth was not observed in 2, 2-dichloropropionic acid minimal media.

Conclusion: The inability of SAI to grow on 2, 2-dichloropropionic acid minimal media suggesting that it could be toxic to the organism. Hence, there is possibility of it degrading other halogenated compounds since various strains of this species are said to participate in the metabolism of xenobiotic compounds.

Keywords: Bacteria DNA halogenated compound PCR

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I. Introduction

Numerous environmental pollutions results from the release of recalcitrant, artificial organic compounds into the environment. Chemical processing and industrial-scale manufacturing particularly introduce vast amounts of these persistent compounds into the biosphere (Hamid *et al.*, 2013). Halogenated compounds are most common pollutant released into the biosphere owing to their extensive use as insecticides, herbicides and pesticides (Fetzner and Lingens, 1994; Abel *et al.*, 2012). For example, the halogenated compound, 2,2-dichloropropionic acid is a herbicide that can be employed to control some perennial and annual grasses hence, the soil and ground water become contaminated (Jing and Huyop, 2008). It is believed that after the application of these herbicides, reasonable amount persist in the soil and pollute the surface and ground water.

The release of these compounds into the environment resulted to severe pollution and some human health issues because of their persistence, toxicity and bioaccumulation (Olaniran, Pillay and Pillay, 2004; Thasif, Humdan and Huyop, 2009). Studies have shown that intermediate metabolites of halogenated compounds inhibit key reactions. For example, among the effective inhibitors of the kreb cycle is the toxic fluorocitrate synthesised from fluoroacetate, that inhibit aconitase; an enzyme in the cycle (Morrison and Peters, 1954).

Fortunately, a number of bacterial strains that use dehalogenase enzymes to obtain carbon and energy from halogenated compounds have been documented. For instance, the 2,2-dichloropropionic acid degrading bacteria was previously isolated from soil and identified as *Rhizobium* sp. (Berry *et al.*, 1979; Huyop and Nemati, 2010). These bacteria produced all the three forms of haloalkanoic acid dehalogenases and were also able to use 2-chloropropionic acid as sole source of carbon and energy (Cairns, Cornish and Cooper, 1996; Stringfellow *et al.*, 1997; Huyop and Cooper, 2011).

In recent years, there have been an increasing number of researches on dehalogenation of halogenated compounds by various microorganisms. Wong and Huyop, (2012) identified four strains of bacteria from Agricultural area using 2, 2-dichloropropionate as carbon and energy source. Subsequently, Alomar *et al.*, (2014) isolated *Labrys* sp. strain D1 and *Arthrobacter* sp. strains D2 and D3 from herbicides and pesticides contaminated soil which were able to degrade 20 mM monochloroacetic acid with Strain D2 having special ability to further degrade 10 mM 2,2-dichloropropionic acid and some other halogenated compounds. A novel bacteria specie, *Raoutella ornitholytica* was isolated from an Island wastewater sample which showed good growth on 20mM 2,2-dichloropropionic acid (Niknam, Huyop and Wahab, 2014). Researches have also proved that halogenated compounds are deposited in animal organs and waste products (Abel *et al.*, 2012; Ismail,

Wahab and Huyop, 2015). However, most of these organisms reported were isolated from contaminated soil, only few from contaminated water.

Considering the abundant deposition of halogenated compounds in our environments and their deleterious effect to human, isolation and identification of diverse indigenous species of microorganisms from different environments is essential which could be helpful in the bioremediation of the environment. This present work was aimed at identification and characterization of unknown bacteria and assessment of its potential in the degradation of halogenated compounds.

II. Materials And Methods

Preparation of growth media

The method of Hareland *et al.*, (1975) was used to prepare bacteria growth stock solution. The basal salts 10x concentrated contained 10.0 g/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 42.5 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and 25.0 g/L whereas the trace metal salt 10x concentrated contained 2.0 g/L MgSO_4 , 1.0 g/L $\text{C}_6\text{H}_9\text{NO}_6$, 120.0 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 30.0 mg/L $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 30 mg/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 10 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. The bacteria growth minimal media containing 10 mL of 10x trace metal salts and 10 mL of 10x basal salts per 100 mL distilled water was then autoclaved at 121 °C for 15 min, 15 psi. 2,2-DCP used as carbon source was filter sterilized separately and then added to 100 mL of the growth medium. In order to prepare solid medium, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilization.

Growth Experiment

The bacterial culture isolated from contaminated waste water opposite Universiti Teknologi clinic on nutrient agar plates was inoculated into 100 mL of minimal media containing 10, 20 and 30 mM 2, 2-DCP, separately, as the sole source of carbon and energy. The flasks were incubated at 30°C in a rotary incubator at 150 rpm for 24 hours. Then, 0.1 mL of aliquot from each flask were spread onto their respective solid minimal media and incubated at 30°C for growth observation (Abel *et al.*, 2012).

Partial Biochemical Test

To characterize the bacteria Biochemically, Some Biochemical tests such as catalase test, motility test, MacConkey agar test, oxidase test, nitrate reduction test, citrate test, gelatin liquefaction test, urease test and starch hydrolysis test were carried out.

DNA Extraction and 16S rDNA Gene Amplification

Genomic DNA Extraction Kit (Promega Wizard®) was used to extract the bacterial DNA. After DNA extraction, nano drop ultraviolet Spectrophotometry was used to determine the concentration of the DNA. To amplify the bacterial 16S rDNA gene, polymerase chain reaction was performed using bacterial forward primer 27F (5'-aga gtttgatcmtggc c ag-3') and universal reverse primer 1492R (5'-cgg ttacctgttacgac tt-3') (Jiang *et al.*, 2006). The polymerase chain reaction was performed for 30 cycles with initial denaturation at 94°C (5 min), then cooling, followed by denaturation at 94°C (1 min), annealing at 55°C (1 min) and final extension at 72°C (10 min). Amplicons were purified and sequenced by 1st Base® (Malaysia).

Phylogenetic analysis

The bacteria genetic relationships was identified by aligning and comparing its 16S rRNA sequence with the Gene Bank database sequences from National Center for Biotechnology Information (NCBI) using BLASTn analysis tool (Altschul *et al.*, 1997). Phylogenetic tree was built and the Neighbor-Joining method was used employing CLUSTAL W in MEGA 6 in order to show similarity and distance matrix of the isolated bacteria in the evolutionary route (Saitou and Nei, 1987).

III. Results

SA1 Growth

The bacterial strain SA1 was assessed on solid minimal media containing 10mM, 20mM and 30mM 2, 2-DCP as the sole source of carbon and energy. Bacteria SA1 growth was not observed on the plates which signifying that 2, 2-DCP could be toxic and not a substrate for the bacterial growth. However, the bacterial growth was observed on nutrient agar plate which showed smooth-edged rod-shaped light pink colony. Bacteria SA1 on Nutrient Agar Gram staining and spore staining of the bacteria revealed Gram negative, non-spore forming rod shape bacteria

Physiological and Biochemical property of SA1

The results of physiological and biochemical properties of bacteria SA1 are presented in Table 1. The results revealed that the SA1 shape, size, colony, color observed are rod, 0.9 µm, circular smooth, and light pink, respectively (Table 1). The observed gram and spore staining was negative and bacteria were not motile

under microscope (Table1). Catalase, citrate, urease and MacConkey, all demonstrated positive result. The starch hydrolysis and gelation liquefaction also indicated positive. However, the present of oxidase and nitrate reduction demonstrated negative (Table 1).

Table 1: physiological and biochemical characteristics of bacteria SA1.

Characteristic	Observation
Cell shape	Rod
Size	0.9µm
Colony	Circular smooth
Color	Light pink
Gram staining	Negative
Spore staining	Negative
Motility	Non-motile
Catalase	+
Oxidase	-
Citrate	+
Urease	+
MacConkey	+
Starch hydrolysis	+
Gelation liquefaction	+
Nitrate reduction	-

+, positive result; -, negative result.

Polymerase chain reaction (PCR)

The amplified 16S rRNA gene of bacteria SA1 with the length of 1500bp was seen under UV light after carrying out gel electrophoresis for the PCR product (Figure 2) and successive DNA sequencing also showed a complete sequence comprised of 1500bp.

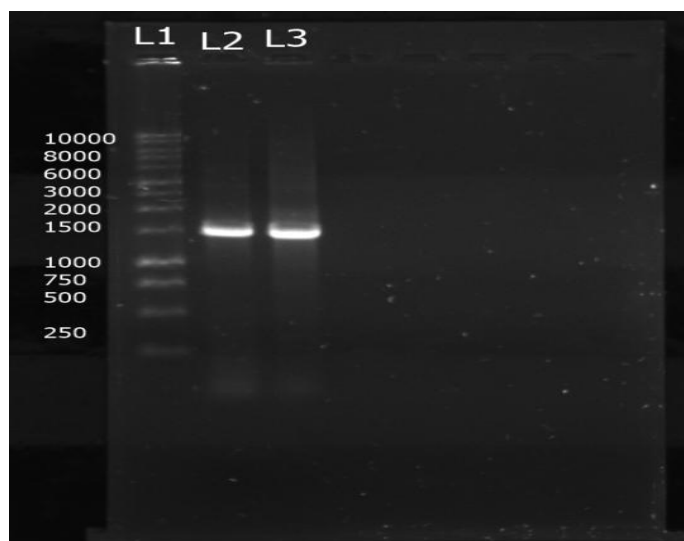


Figure 2: Amplification of 16s rRNA gene on agarose gel. **L:** Lane, **L1:** DNA ladder; **L2:** PCR result of bacteria SA1 and **L3:** Amplified 16S rRNA seen under UV light.

Sequence Analysis of 16S rRNA

Bacteria SA1 16S rRNA gene complete sequence obtained were BLAST in NCBI against the genbank database so as to ascertain its similarity with other species and strains. BLASTn outcome revealed that bacteria SA1 belongs to Acinetobacter with 98% identity to this genus (Table 2). This result was supported by MEGA7 phylogenetic analysis (Fig. 3) and biochemical tests (Table 1).

Phylogenetic tree

The Neighbor-Joining bootstrap test phylogeny tree results of bacteria SA1 are shown in Fig. 3. This was built with MEGA6 using sequences from the BLAST result and about six sequences were selected including bacteria SA1 sequence. According to the tree, bacteria SA1 were found in the midst of Acinetobacter species (Figure 3).

Table 2: Sequence similarity of Bacteria SA1 with other strains from blast result

Accession No.	Description	Max Identity
LC125143.1	<i>Acinetobacter</i> sp. Ue2-1.1	98%
JX867369.1	<i>Acinetobacter</i> sp. Y1 16S	98%
JX221556.1	<i>Acinetobacter</i> sp. YS0810	98%
HM316154.1	Uncultured bacterium clone ncd496f05c1	98%
FJ263930.1	<i>Acinetobacter haemolyticus</i> strain BA56	98%

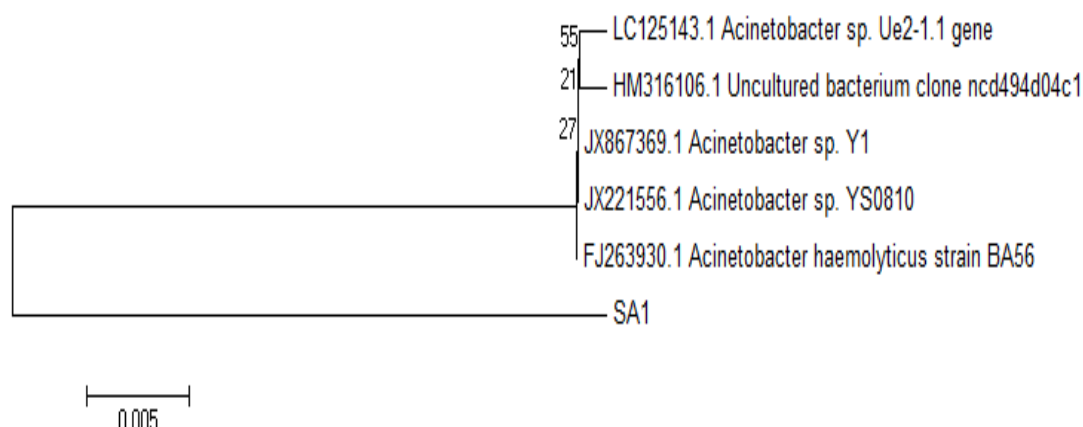


Figure 3: Bacteria SA1 MEGA6 Neighbour-Joining phylogeny tree

The biochemical test results of the isolated bacteria were compared to the biochemical property of *Acinetobacter* strain in earlier research work of Abel *et al.*, (2012)(Table 3).

Table 3: Comparison of SA1 Staining and biochemical tests results to Abel, et al. (2012).

Test	SAI	A.baumannii
Motility	-	-
Catalase	+	+
Oxidase	-	-
Citrate	+	+
Nitrate reduction	-	+

+: positive result; -: negative result.

IV. Discussion

The 16S rRNA gene analysis is a suitable parameter for bacterial classification due to the fact that 16S rRNA gene is universal among bacteria and is conserved except that it has adequate variation to make a distinction between taxa (Gutell *et al.*, 1985). From the growth experiment result, the bacteria growth was not observed on 2,2-DCP minimal media, it may be suggested that the dehalogenase gene in the bacteria could be silenced as reported by Abel *et al.*, (2012) or 10mM, 20mM and 30mM 2,2-DCP may be too toxic to the organism. According to Yu *et al.*, (2007) some bacteria can grow on halogenated compounds due to their capability of producing haloacidpermease that enhance the halogenated compound uptake into their cells. However, some organisms are said to produce dehalogenases that are substrate specific, for example Huyop *et al.*, (2008) purified and characterized DehL enzyme from *Rhizobium* sp., which was found to act on Monochloroacetic acid, and Dichloroacetic acid but not on 2,2 Dichloropropionic acid and Trichloroacetic acid.

The 16S rDNA gene analysis verified the organism's identity (Table 2) and it revealed that the bacteria belong to the genus *Acinetobacter*. Bacteria SA1 gram staining, motility and oxidase tests indicated negative results as well as catalase and citrate test showed positive results, with exception of nitrate reduction test. These findings are in line with the results of Abel, *et al.* (2012) that isolated *Acinetobacter baumannii* from the gut of pond-reared rohu (*Labeorohita*) in Myan-mar was capable of utilizing 2,2-DCP as carbon and energy source. Though no growth of bacteria SA1 was observed on 2,2-DCP minimal media as demonstrated in *Acinetobacter baumannii* by Abel, *et al.* (2012), but they still possess similar characteristics.

Acinetobacter species are widespread in nature, and usually found in soil and water. Their capability to live on moist and dry surfaces and also to withstand exposure to numerous universal disinfectants permits some of them to live in a hospital environment (Doughari *et al.*, 2011). Generally, *Acinetobacter species* are said to be Gram negative bacilli, non-spore forming, non-motile, non-fermentative and strictly aerobic ((Doughari *et al.*, 2011). All strains of *Acinetobacter species* are found to grow between 20° to 30°C but most strains have 33° to 35°C as optimal temperature. They are oxidase negative, catalase positive mostly nitrate negative (Constantiniu *et al.*, 2004) and partially ferment lactose when cultured on MacConkey agar, though some strains may show variation. Various strains of these species participate in metabolism of xenobiotic compounds (Briganti *et al.*, 1997; Abel *et al.*, 2012).

V. Conclusion

This research focused on identification and characterization of unknown bacteria isolated from contaminated waste water and assessment of its potential in the degradation of halogenated compounds. From the growth experiment result, Bacteria SA1 growth was not observed on 2,2-DCP minimal media. 16s rRNA analysis result and BLAST search showed that bacteria SA1 is 98% similar to *Acinetobacter specie* which was supported by MEGA6 phylogenetic analysis and biochemical tests. Though bacteria SA1 growth was not observed on 2,2-dichloropropionic acid minimal media, there is possibility of it degrading other halogenated compounds since various strains of this species are said to participate in the metabolism of xenobiotic compounds. Therefore its ability to utilize other halogenated compounds needs to be assessed.

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