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IDENTIFICATION OF NOVEL BACTERIAL SPECIES CAPABLE OF DEGRADING DALAPON USING 16S RRNA SEQUENCING

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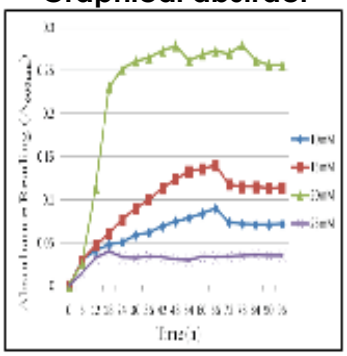
Graphical abstract



Comparison between different growth rates of bacterium JHA1 in different concentrations of 2,2-DCP

Abstract

2,2-dichloropropionic acid (2,2DCP) is used as herbicide in agricultural industry and it is one of the halogenated organic compounds distributed widely in the world causing contamination. In this study, a bacterial strain isolated from contaminated soil where halogenated pesticides applied in Universiti Teknologi Malaysia and it was named "JHA1". Bacterium JHA1 was able to utilize 2,2 dichloropropionate 2,2-DCP or (Dalapon) as a source of carbon and energy. Based on 16S rRNA analysis, the isolate showed 87% identity to Terrabacter terrae strain PPLB. The identity score was lower than 98% so that it was suggested to be new organisms that worth for further investigations if it will be proven that this is novel. Therefore, current isolate was designated as Terrabacter terrae JHA1. The isolate grew in the minimal media containing 10 mM, 15 mM, 20 mM and 25 mM of 2,2- DCP as the sole energy and carbon source and the best growth rate was in 20 mM as the optimum concentration of 2,2-DCP while bacterial growth was inhibited in medium with 30 mM 2,2-DCP.

Keywords: Dalapon, 16S rRNA, identification

Abstrak

Asid 2,2-dikloropropionik (2,2DCP) digunakan sebagai racun herba dalam industri pertanian dan merupakan salah satu daripada sebatian organik terhalogen yang diedarkan secara meluas di dalam dunia lalu menyebabkan pencemaran. Dalam kajian ini, strain bakteria diasingkan daripada tanah yang tercemar di mana racun perosak terhalogen digunakan di Universiti Teknologi Malaysia dan strain bacteria tersebut dinamakan "JHA1". Bakteria JHA1 dapat menggunakan 2,2 dikloropropionat 2,2-DCP atau (Dalapon) sebagai sumber karbon dan tenaga. Berdasarkan analisis 16S rRNA, pencilan tersebut menunjukkan 87% identiti terhadap Terrabacter terrae strain PPLB. Skor identiti adalah lebih rendah daripada 98%, maka pencilan tersebut dicadangkan sebagai organisma baru untuk siasatan lanjut sekiranya dibuktikan bahawa pencilan ini adalah novel. Oleh itu, pencilan ini dinamakan sebagai Terrabacter terrae JHA1. Pencilan ini tumbuh di dalam media minima yang mengandungi 10mM, 15 mM, 20 mM dan 25 mM 2,2- DCP sebagai sumber tenaga dan karbon tunggal dan kadar pertumbuhan yang terbaik adalah di dalam 20 mM sebagai kepekatan optimum 2,2-DCP manakala pertumbuhan bakteria adalah terhalang di dalam medium dengan 30mM 2,2-DCP.

Kata kunci: Dalapon, 16S rRNA, identification

1.0 INTRODUCTION

Xenobiotic compounds are defined as foreign materials to a biological system not being found in the nature before they are produced industrially [1]. Herbicides, pesticides, insecticides and antibiotics are halogenated compounds that are considered xenobiotics. 2,2-Dichloropropionic acid (2,2-DCP) known as Dalapon is a halo-aliphatic compound being applied as a herbicide to selectively control certain grasses [2]. Therefore, since Dalapon as a halogenated compound is toxic to the environment, biodegradation of Dalapon through dehalogenation process is of a great interest in bioremediation [3, 4, 5]. Moreover, in order to decrease the negative effects of wide range of pollution caused by the usage of halogenated compounds, not only the use of herbicides should be controlled, but also degrading them biologically should be of significance [6, 7]. This causes the halogenated compounds to be less toxic and more readily biodegradable [8, 9, 10, 11]. Dehalogenase enzyme causes the chlorinated compounds to be metabolised and chlorine substituents to be enzymatically removed and finally non-halogenated compounds to be formed. Thus, isolation of microorganisms capable of producing dehalogenase turned to be an important issue to environmental microbiologists [12]. This study aimed to isolate and characterize a microorganism being capable of growing on 2,2-DCP.

Table 1 Composition of chemicals

2.2DCP concentration	10mM	15mM	20mM	25mM
Distilled water (mL)	79	78.5	78	77.5
Basal salt (mL)	10	10	10	10
Trace metal (mL)	10	10	10	10
"V1", 1M DCP (mL)	1	1.5	2	2.5
Total (mL)	100	100	100	100

2.0 EXPERIMENTAL

2.1 Soil Sample and Growth Media Preparation

Soil sample was from agricultural area located in Universiti Teknologi Malaysia. 15 ml of sterile distilled water mixed with 0.5 g of soil sample and left until the soil particles settled down. On the other hand, the solid and liquid minimal media prepared according to Hareland modification and stored in 4 °C. After that, 0.1 mL of the soil suspension mixture was pipette out and spread on 10 mM of 2,2-DCP plates. The plates was incubated for 3~4 days at 28~31° C to allow bacterial growth. The colonies that formed after incubation was isolated and was streaked onto agar medium by using streak plate method. Streak plate method was repeated until a pure colony was obtained. In addition, the glycerol stock culture of organism was prepared by using aseptic technique and stored at -80 °C [13].

2.2 Bacterial Growth Measurement

The growth curve was performed using spectrophotometer at A_{600} nm to determine the growth of microorganisms in four different 2,2-DCP concentrations of 10 mM, 15 mM, 20 mM and 25 mM in order to find the optimum 2,2 DCP concentration in the media in order to determine the best growth conditions. The pure culture from solid medium was inoculated into 100 mL of the minimal media. After four days of incubation, JHA1 turbidity was measured at A_{600} nm. The measurement of optical density at A_{600} nm was completed every 6 hours interval. Table 1 shows the composition of chemicals in different concentrations of 2,2-DCP in the media as carbon and energy source.

2.3 Gram Staining Procedure

The standard procedure of Gram staining was conducted according to the Tortora procedure [14].

2.4 Biochemical Tests

The chemical tests consist of motility test, nitrate reduction test, oxidase test, catalase test and urease test applied for characterization of isolated microorganism.

2.5 Dehalogenase Enzyme Assay

2,2-DCP degradation results in the production of chloride ions. Over the course of dehalogenation, enzyme activity was measured colorimetrically based on the release of chloride ion [15]. Two different solutions were required to be prepared to follow the steps in the method. Reagent I is 0.25 M of ferric

ammonium sulfate dodecahydrate, $FeNH_4(SO_4)_2$ dissolved in 9 M nitric acid, HNO_3. Reagent II is mercuric thiocyanide, $Hg(SCN)_2$ that mixed in excess ethyl alcohol. The solution was vortexed and then 1.5 mL of sample was centrifuged at 10,000 rpm for 5 min. Afterwards, 1 mL of supernatant and 1 mL of each reagent are added into a cuvette to be remained at room temperature for 10 min before the absorbance is read on a UV spectrophotometer at A_{460} nm.

2.6 Polymerase Chain Reaction

The crude DNA used as template for PCR (Polymerase Chain Reaction) amplification by colony PCR. The genomic DNA was amplified through polymerase chain reaction. A conserved region known as 16S rRNA gene in bacterial DNA used to compare and determine species among many types of prokaryotic microorganisms. In this reaction, universal primer used was as shown in Table 2. Universal PCR primers used to amplify the 16S rRNA gene. The analysis procedures carried out according to Cappuccino *et al.* [16].

Table 2 Universal primers used in this study

Primer
Forward primer "5'-AGA GTT TGA TCC TGG CTC AG-3' "
Reverse primer "5'-ACG GCT ACC TTG TTA CGA CTT-3' "

The PCR products were tested by agarose gel electrophoresis and the gel was observed under UV light. For 16S rRNA genetic material, the band should be observed at approximately ±1.5kbp compared to a DNA marker (ladder). The electrophoresis was started to run by setting the voltage at 90V for 55 minutes [17]. In this project, Promega 1kb DNA Ladder was selected as DNA marker.

2.7 PCR Product Purification

QIA quick Gel Extraction Kit (QIA GEN) was used to extract and purify the targeted DNA bands from agarose gel matrix. The DNA concentration measurement carried out using Nano drop Spectrophotometer and the DNA samples were kept at 20° C.

2.8 DNA Sequencing

The PCR product of 16S rRNA gene from JHA1 as well as the universal primers sent to 1st BASE Laboratories Sdn. Bhd., Malaysia for DNA sequencing.

2.9 DNA Assembly of Full Length 16S rRNA Genes

The full sequence of 16S rRNA genes of bacterial isolates JHA1 was generated from alignment of sequencing data and assembled by using Bioedit version 7.0.5.3 software.

2.10 Homology Search and Construction of Phylogenetic Tree by Using Basic Local Alignment Search Tool for Nucleotide (BLASTn)

The full length sequence of 16S rRNA genes from JHA1 was submitted to BLASTn from NCBI to compare with the nucleotide sequences from Genbank database. Also, JHA1 16S rRNA gene sequences aligned with 16S rRNA gene sequences of organisms including dehalogenating organisms using Clustal W to find conserved regions and comparison purposes.

2.11 Phylogenetic Tree

The Phylogenetic tree was constructed using neighbor-joining method from profile alignment command of CLUSTAL W in MEGA 5 software [18] while 2 bacillus bacteria were used as the out group.

3.0 RESULTS AND DISCUSSION

3.1 Isolation, Bio-chemical Tests and Growth Profile Result

JHA1 was successfully isolated. The size, morphology and gram staining observation were summarized as shown in Table 3.

The growth profiles were constructed by determination of the bacterial growth rate in 10, 15, 20, 25mM of 2, 2-DCP liquid media in temperature-adjustable shaker in 30°C at 200rpm. Different growth rates of the bacterium JHA1 in four different concentrations of 2, 2 DCP are represented in Figure 1. These graphs show that the best 2,2-DCP concentration in minimal media as the sole carbon and energy source is the optimum concentration for the isolates to grow well. *E.coli* was used as the controlling agent in the measurement growth which showed no growth after all in the 2,2-DCP media.

Table 4 summarizes the results of biochemical tests.

Table 3 Morphological and microscopic observations

Properties	Result
Color (Pure colonies)	Red
Margin (outer edge of colony)	Entire
Oxygen requirement	Aerobic
Colony Edge	Raised
Gram	Negative
Microscopic Shape	Circular
Color (Gram-staining)	Pink

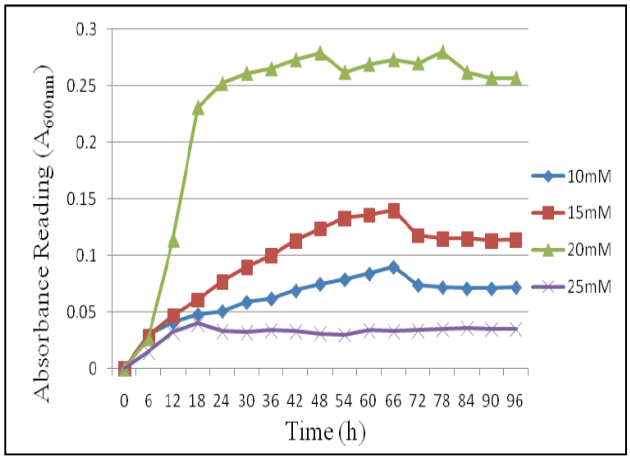


Figure 1 Comparison between different growth rates of bacterium JHA1 in different concentrations of 2,2-DCP

Table 4 List of biochemical tests result

No.	Test	Result
1.	Motility	Negative
2.	Oxidase	Positive
3.	Catalase	Positive
4.	Urease	Positive
5.	Nitrate Reduction	Positive

3.2 Chloride Ion Released in Growth Medium

The maximum release of the chloride ion for the JHA1 bacteria was 0.282 mmol/L in a 20 mM 2,2-DCP. It can be concluded that 20 mM of 2,2-DCP was the optimum concentration for carbon source.

3.3 Polymerase Chain Reaction Result

The PCR products were tested using gel electrophoresis in order to assure that the purification was carried out successfully. In this project, Promega 1kb DNA Ladder was selected as DNA marker. Figure 2 shows the amplified 16S rRNA gene with the length of 1500bp.

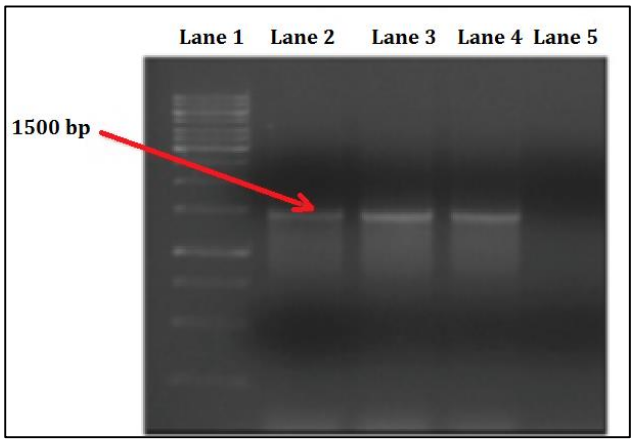


Figure 2 PCR product under UV-light-Lane 1: 1kb DNA ladder, Lane 2, 3 and 4: *Terrabacter terrae* JHA1 and Lane 5: Control without primer

The concentration of PCR products was adjusted to be 40 ng/µl or higher before sending for sequencing.

3.4 DNA Sequencing Result

Bioedit version 7.0.5.3 software was used to view the sequencing results generated by 1st BASE Laboratories Sdn. Bhd., and the full sequence of 16S rRNA was aligned and assembled. The size of partial sequence obtained was 988 nucleotides shown in Figure 3.

3.5 Homology Search by Using Basic Local Alignment Search Tool (BLAST)

The full sequence of JHA1 was then BLASTn in National Center of Biotechnology Information (NCBI). Table 5 shows the top ten BLAST search result of strain JHA1 which showed 87% similarity to Intrasporangium calvum DSM 43043 accession no. CP002343.1. But, in order to perform a better classification, phylogenetic tree was constructed.

Bosshard in 2003 used ≥99% similarity to define a species and ≥95% to ≥99% to define a genus [19] and Fox in 1992 proposed that there be a difference of at least 5 to 15 bp in the whole 16S rRNA gene sequence to define a species [20]. Also, Turenne in 2001 designated the reportable range for a species as <0.8 to 2.0% [21]. Moreover, they suggested that a sequence could obviously be called unique representing an organism whose sequence has not yet been deposited and thus might be a novel species, if there were at least 20 to 38 bp differences in sequence.

Since the highest identity found for the isolate was less than 98%, it was not considered as a known organism. The organism is worth for further investigations to ascertain the name of genus and species if the investigations prove the novelty. Also, multiple sequence alignment performed between 16S rRNA sequence of bacterium JHA1 and *Terrabacter terrae* strain PPLB using ClustalW. Phylogenetic tree showed evolutionary relationship of isolates JHA1 on the basis of 16S rRNA.

AGATATTTCAGGAGGACACATATGGGGCAGTCGACGGTGCGGTTG
GGTACCGCTTCAGGTGTGACAACTCCTGGGATGTCGACGGTGCGG
TTGGGTACCGCTTCAGGTGTGACAACTCCTGGGTACGTCCATTCCG
TAAACGATGGGCTCTAGCCGTTATAAGGGCCCATTCCATGGGTTC
CGAGCCGCAGATAACGCATTAAGCCCCCAGAGCGCCTGGGGAGT
ACGGCCACAAGGCTAATACTCAAAAGAATTGACGGGGGCCCCCA
CAAGCGGCGGATCCTGCTGATTAATTCGGTGCAACGGGAAGAACC
TTACCAAGGCTTGACATACGCGGGATTCCCTCAGAGATGGGTGCG
TCTTCGGACTGGTGTACAGGCGGGTCATGGTTGTCGTCAGCTAGT
GTCGTGAGATATTGGGTTAAGTCCCGCAAGGAGCGCAACCCTCGT
TCTACGTTGCCAGCCCTCGATGGTGGGGACTCATAAGAGACTCCC
GGTAACAGGTCATCTGGGAGGAAGGTGGGGATGATGACATATCA
TCATGCCAAACCTTATGTCTTGGGCTTCACGCATGCTACAATGGCG
GGTACAAAGGGGTGAGAAACCGTAAGGTGGAGCAAATCCCAAAA
AACCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCACGAA
GTGGGAGTCGCTAGTAATCGCAGATCAGAAACGGTGCGGTGAAT
AAGTTCCGGGGCTTTGTACACACCCCCCCGCAAGTCAAGAAATCG
GTAACGGGACCCGAACCCGGTGGCCCAACCCTTGTGGAGGGAGC
CGTCCGCAATATTTTGGTGTCCCCCTTTTTTTACNCTNCCCCTTGCA
AATAAACTCTACCCATACTAAAGCGTCCCGTATCGAAGGCGCATT
CNGTGGGTTGTAGTCANNNNNCNTTCCACCCCCNAACCTAAAAAC
GCCGACTTACTGCNCCCCTGTTACNCC

Figure 3 The 16S rRNA gene sequence obtained from strain JHA1

3.6 Phylogenetic Study

16S rRNA gene sequence of top ten Blast search result together with the 16S rRNA gene sequence of bacterium JHA1 were chosen to perform alignment by MEGA5 software in order to construct the Neighbour-Joining phylogeny of JHA1. Moreover, 2 bacillus bacteria were used as the out groups in the constructed phylogenetic tree. The details were shown in Figure 4.

Bacterium JHA1 was clustered within a clad consisting *Terrabacter terrae* strain PPLB according to the Figure 4. So, it was suggested that bacterium JHA1 is closely related to *Terrabacter* sp.

4.0 CONCLUSION

In this study, bacterial strain JHA1 was isolated from the soil sample taken from UTM agricultural area which grew well in 20mM of 2,2- dichloropropionate as the optimum concentration of 2,2-DCP at 30°C while bacterial growth was inhibited in 30mM as the toxic concentration of 2,2 DCP.

Result of 16S rRNA analysis and Blast search showed that bacteria strain JHA1 had 87% similarity to *Terrabacter terrae* strain PPLB. The result of the BLAST search was supported by MEGA5 phylogenetic analysis. Since, the highest identity was lower than 98%, the isolate worth further investigations to ascertain the name of genus and species if the investigations prove the novelty.

Further study such as study of enzyme dehalogenase can be carried out in terms of isolation and expression of the gene responsible for the production of dehalogenase may provide useful information about the enzyme responsible.

Table 5 Top ten BLAST search result of JHA1

Sequences producing significance alignments:						
Accession	Description	Max Score	Total Score	Query coverage	E value	Max ident
JF342354.1	Uncultured bacterium clone A6 16S rRNA gene, partial sequence	824	824	71%	0.0	88%
CP002343.1	Intrasporangium Calvum DSM 4303, complete genome	785	1571	71%	0.0	87%
FJ423552.1	Terrabacter sp. ON10 16 S rRNA gene, partial sequence	785	785	71%	0.0	87%
AM690744.1	Terrabacter lapilli partial 16S rRNA gene, type strain LR-26T	785	785	71%	0.0	87%
DQ125903.1	Uncultured bacterium clone AKAU4164 16S ribosomal RNA gene	785	785	71%	0.0	87%
AY944176.1	Terrabacter terrae 16S rRNA gene, partial sequence	785	785	71%	0.0	87%
AJ566282.1	Intrasporangium calvum 16S rRNA gene, type strain DSM 4304	785	785	71%	0.0	87%
AF005023.1	Terrabacter tumescens 16S rRNA gene, partial sequence	785	785	71%	0.0	87%
X83812.1	T. tumescens 16S rRNA gene	785	785	71%	0.0	87%
FJ006904.1	Terrabacter sp. WPCB144 16S rRNA gene, partial sequence	784	784	71%	0.0	87%

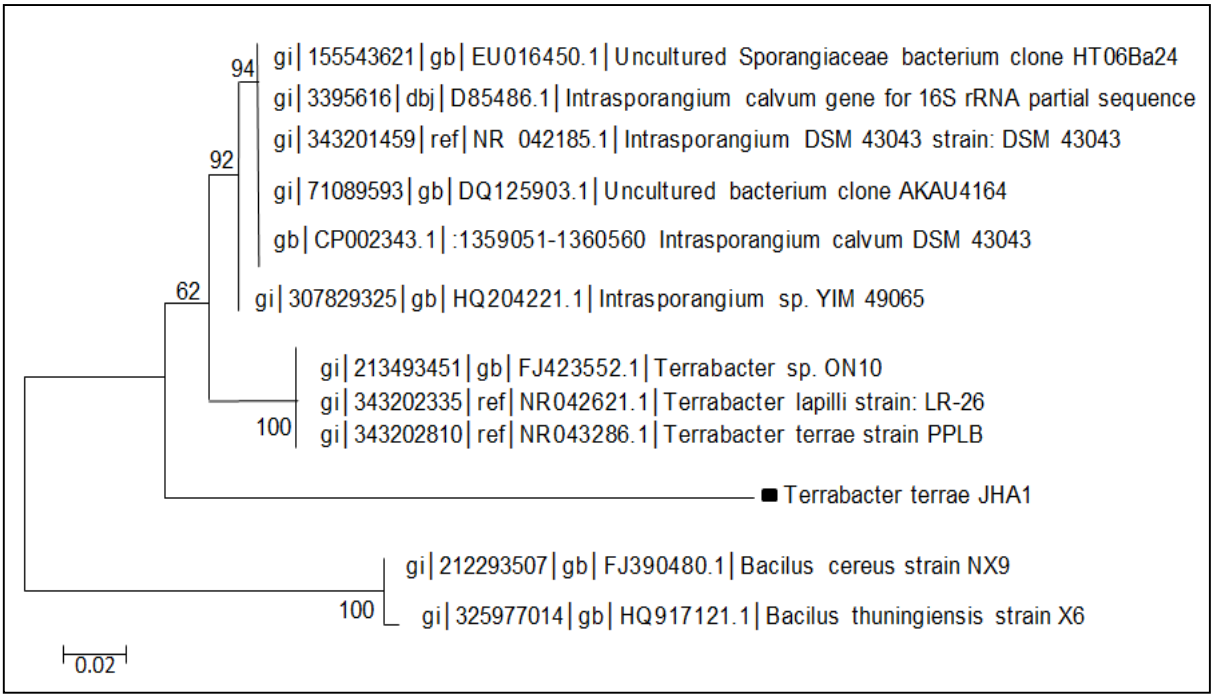


Figure 4 MEGA5 Neighbour-Joining phylogenetic tree: JHA1

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