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Full Length Research Paper

Isolation and characterization of bacteria from Melaka rubber estate agricultural soil to utilize 2,2-DCP as only carbon source

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Industrial chemicals and pesticides from agricultural activities cause a considerable threat to the environment. 2,2-dichloropropionate (2,2-DCP) is a synthetic halogenated compound used as herbicide. High concentration of 2,2-DCP is toxic if released to the environment and may pollute the soil and ground water source. Using current enrichment technique it was expected to isolate a new bacteria species able to degrade -halocarboxylic acid. Strain Wy1 isolated from soil in Melaka rubber estate was able to utilize 30 mM 2,2-DCP as the sole source of carbon and energy with maximum chloride ion released of 0.27 mmol/L in the liquid growth medium. The biochemical test and 16S rRNA analysis suggested that the bacterial identity was from the genus *Labrys* sp., and therefore it was designated as *Labrys* sp. strain Wy1. The cells doubling time in 30 mM 2,2-DCP liquid minimal medium was 33.44 h. Cell growth was inhibited when grown in liquid minimal medium above 30 mM 2,2-DCP due to the toxicity of 2,2-DCP to the cells. This is the first reported case that the genus *Labrys* sp. is able to degrade 2,2-DCP.

Key words: 2,2-dichloropropionate, dehalogenase, soil bacteria, *Labrys* sp.

INTRODUCTION

Halogenated compounds were used extensively as herbicide and as intermediate chemicals in many industries. Due to the complexity, toxicity, persistence and ubiquitous distribution spreading of these xenobiotic compounds, they have threatened the health and living quality of human and other organisms (Fetzner and Lingens, 1994). Degradation of halogenated compound has been reported since the earlier of 20th century by Penfold (1913).

2,2-dichloropropionate (2,2-DCP) - or Dalapon is an odourless and colourless herbicide used to control and regulate the growth of certain weeds, such as quick grass, Bermuda grass and cattails. It is an effective inhibitor of panothenic acid production (Prasad and Blackman, 1965) and pyruvate utilization in bacteria (Redemann and Meikle, 1955). Degradation of herbicide Dalapon was reported earlier by Magee and Colmer (1959) after observation of bacteria that produce dehalogenase enzyme. Since then, studies on isolation of microbes that potentially produce dehalogenases have been undertaken (Jing and Huyop, 2007; 2008; Schwarze et al., 1997; Weightman et al., 1982; Motosugi et al., 1982; Allison et al., 1982; Hardman and Slater, 1981).

The enzymes which were responsible for the degradation of halogenated compound were dehalogenases, discovered and named by Jensen (1957). Culturing and enrichment of microorganism that can produce dehalogenase in the presence of halogenated compound

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in the environment was the most favorable method. Several dehalogenase producing bacteria were isolated using this technique including were *Methylobacterium* sp. HJ1 (Jing and Huyop*,* 2008), *Pseudomonas putida* PP3 (Senior et al., 1976), *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985), *Pseudomonas* B6P (Mesri et al., 2009) and *Rhizobium* sp. (Berry et al., 1979). Current research also incorporated the methods as described by previous work to increase the probability of isolating reliable bacteria. Therefore, the aim of this study is to isolate and characterize bacteria from Melaka rubber estate agricultural soil which able to utilize 2,2-DCP as only carbon source. Various types of chlorinated pesticides and herbicides were used by the estate owner and we were interested to isolate and characterize the bacteria resistant to chlorinated xenobiotics from the soil.

MATERIALS AND METHODS

Growth media preparation

Routinely, cells were grown in PJC chloride-free minimal media. Stock solution was prepared as 10x concentration of 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 concentrated stock that contained nitriloacetic acid $C_6H_9NO_6$ (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 $CoCl_2.6H_2O$ (10 mg/l) in distilled water (Hareland et al., 1975). Minimal media for growing bacteria contained 10 ml of 10 x 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 at 15 psi). Carbon sources (1 M, 2, 2-dichloropropionate) was sterilised separately and added aseptically to the media to the desired final concentration. In order to prepare solid medium, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilisation. Samples were removed periodically and the growth was determined by measuring turbidity at A_{600nm} and chloride ion liberation at A_{460nm} (Bergman and Sanik, 1957).

Sample preparation

Soil sample (5 g) from rubber plantation estate in state of Melaka was mixed together in minimal medium containing 10 mM of 2,2-DCP and incubated in orbital shaker at 30°C. Bacterial growth was monitored at appropriate time interval. For the isolation of pure colonies, 0.1 mL of aliquot was spread onto solid minimal media contained 10 mM 2,2-DCP. For cells purification, colonies were selected and subcultured onto fresh plates containing similar amount of 2,2-DCP as carbon source. Plates were incubated at 30°C.

Biochemical test

A variety of biochemical tests were carried out including oxidase test, catalase test, gelatine liquefaction test, lactose utilization test, Simmons citrate test, indole test, nitrate reduction test, urease test, triple sugar iron (TSI) test and oxidation fermentation (OF) test to characterize bacteria's biochemical properties. All test media were incubated at temperature ranging from 30 to 35°C separately to observe their optimum bacterial activity.

16S rRNA gene analysis

Genomic DNA from bacterial culture was isolated using Promega® Wizard® Genomic DNA Purification Kit. PCR amplification of the 16S rRNA gene for bacteria identification was carried out using universal primers, FD1 (5'-aga gtttgatcctggctc ag-3') and rP1 (5'-acg gtcataccttgttacgac tt-3') as suggested by Weisburg et al. (1991). Each PCR reaction mixture (total volume 50 µL) contained 1 unit/mL *Taq* polymerase (Promega®), 50 mM Tris-HCl (pH 9.0), 50 mM NaCl, 1.2 mM of $MgCl_2$, 160 µM of each dNTPs, 1µmol of each primer, nuclease free water and 100 ng of DNA template. The PCR protocol consisted of a denaturing process of 94°C for 5 min, followed by 30 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 2 min, incubation at 72°C for 10 min and finally 4°C for infinite time for storage, using GeneAmp® PCR system 9700. Amplicons were purified using QIAGEN® QIAquick PCR purification kit and sequenced by 1st BASE® company (Singapore).

Phylogenetic analysis

The 16S rRNA gene sequences were compared with GenBank database using BLAST (Altschul et al., 1997) by selecting BLASTn method. Multiple sequence alignment of bacteria from different genus were constructed using profile alignment command of CLUSTAL-W from MEGA version 4 (Tamura et al., 2007). Using the same software, phylogenetic trees with bootstrap test (1000 replications) were constructed using Neighbour-Joining method (Saitou and Nei, 1987).

RESULTS

Isolation and characterization of novel bacterium able to utilize 2,2-DCP

After 4 days of incubation the colonies appeared to be white, round shape and their size ranging from 0.5-1.0 mm on 10 mM 2,2-DCP minimal medium plate. Microscopic observation suggested all purified bacterial colonies were of similar type and further analysis by 16S rRNA genes proved they were all clustered in the same genus and initially designated as strain Wy1.

Growth experiment was carried out (Figure 1). Minimal media supplied with 30 mM 2,2-DCP recorded the highest growth with cells' doubling time 33.44 h (Table 1). However, growth was negligible in 10 and 50 mM of 2,2-DCP. Chloride ion assay showed maximum chloride ion released by bacteria grown in 30 mM 2,2-DCP with approximately 0.27 mmol/L (Figure 2). Biochemical tests were conducted as summarised in Table 2 showed Wy1 generally matches with the most related species of *Labrys Neptuniae* sp. Liujia-146.

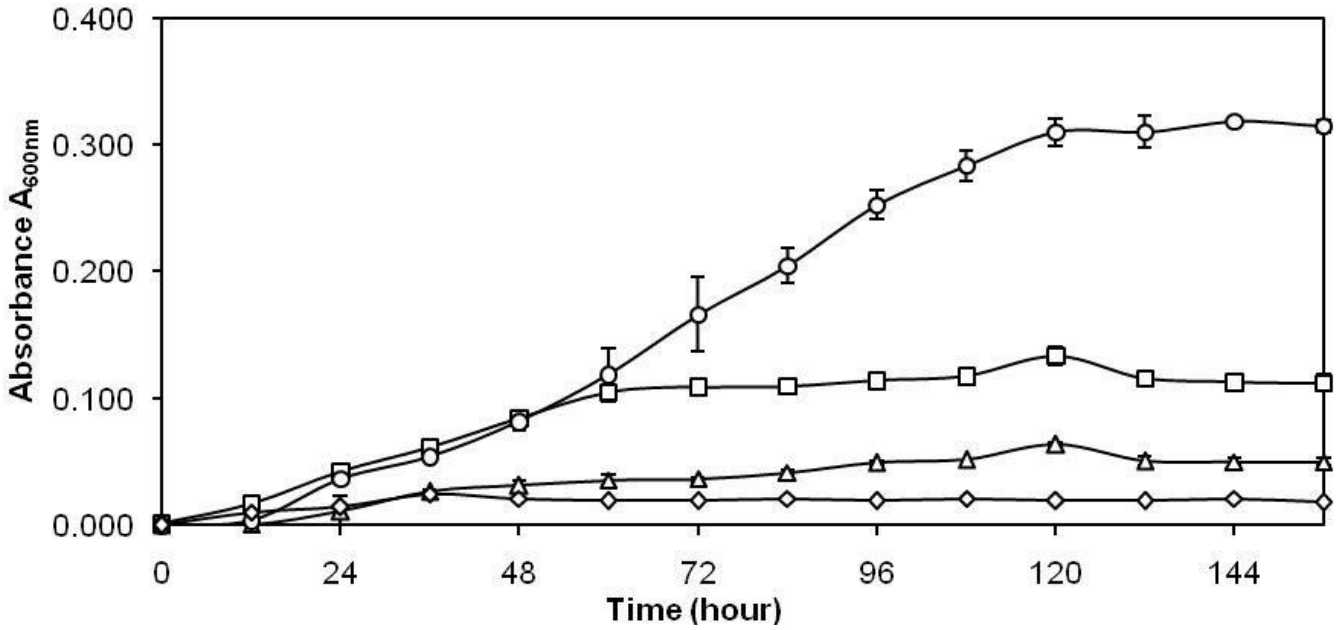

Figure 1. Growth profile of bacterium Wy1 in triplicate minimal media containing four different concentrations of 2,2-DCP: 10 mM (Δ), 20 mM (□), 30 mM (○and 50 mM (◇).

Table 1. Growth experiment of bacterium Wy1.

2,2-DCP concentration(s) (mM)	Maximum absorbance (A_{600nm})	Average cells' doubling time (hour)
10	0.064±0.002	Insignificant
20	0.134±0.007	34.60
30	0.319±0.001	33.44
50	0.025±0.002	Insignificant

16S rRNA gene analysis and biochemical tests and

A total of 1351 bases of 16S rRNA gene were sequenced. BLASTn showed the isolated bacterium matched to the genus *Labrys*, where the first 10 species were highly identical (Table 3). The partial sequence of 16S rRNA gene was submitted to Gene Bank with accession number JF907580. All identical bacteria from BLASTn results were used to construct Neighbour - joining phylogenetic tree with bootstrap test. The tree shows bacteria Wy1 was grouped with *Labrys neptuniae* sp. Liujia-146 with the highest bootstrap value (Figure 3).

DISCUSSION

The main goal of this study is to isolate bacteria which have not been reported to utilize 2,2-DCP as sole carbon and energy source. Generally the bacterial colonies were observed after 4 to 5 days of incubation at 30°C with pH7 in aerobic condition. No colonies were observed in a blank plate contained only minimal media without adding any carbon source. Growth profile showed slow growth of bacterium Wy1 and the maximum absorbance only achieved approximately after 5 days in liquid culture condition (Table 1 and Figure 1). The bacteria grew best in minimal media contain 30 mM 2,2-DCP with the highest absorbance recorded with cells doubling time of 33.44 h. Slow growth of bacteria suggested that the inefficient of the cells uptake system of the growth substrate into the cell (Jing et al., 2010) or weak expression of the dehalogenase gene in the bacteria system. The growth of the isolated bacteria was 3 times slower compared to the previously reported *Rhizobium* sp. in 2,2-DCP liquid medium (Allison et al., 1982). However, detection of chloride ion released into the

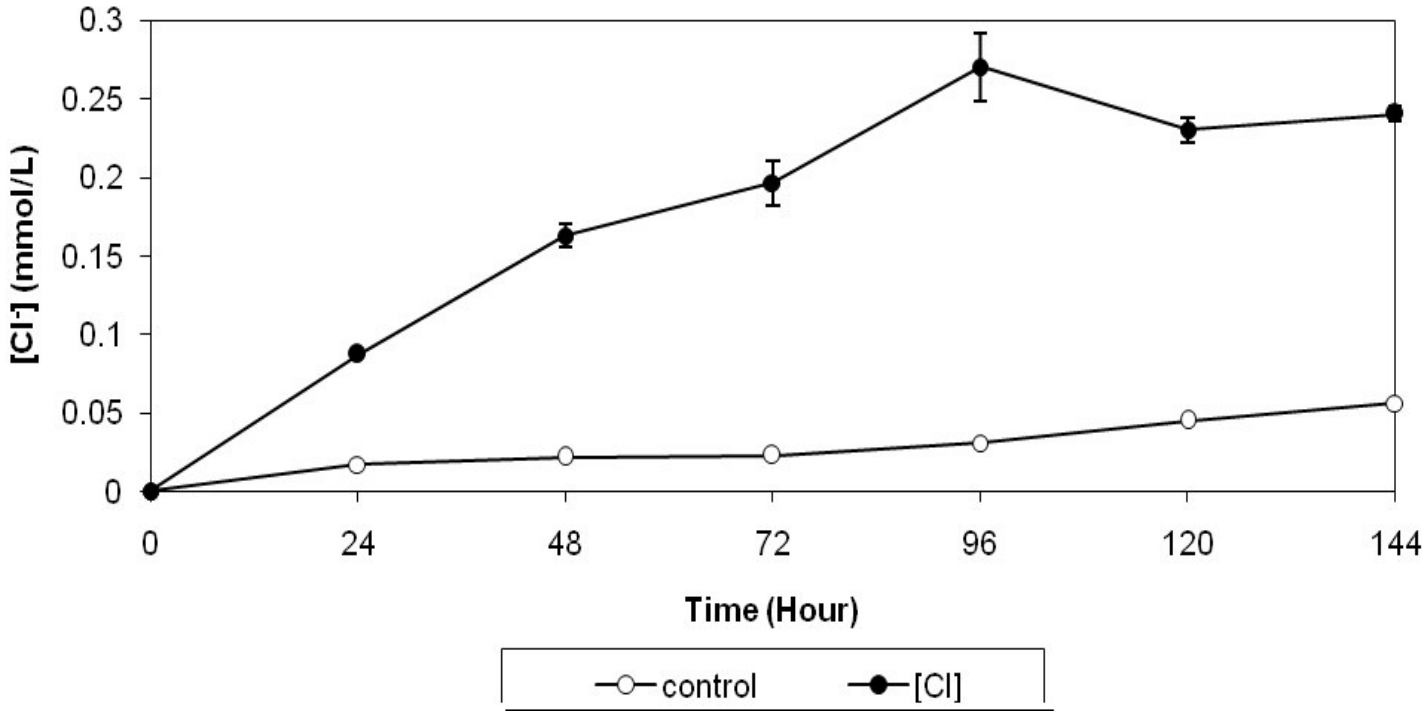

Figure 2. Chloride ion assay of Wy1 cultured in minimal media contain 30 mM 2,2-DCP. Control contains minimal media with 30 mM 2,2-DCP excluding bacteria culture.

Table 2. Comparison of biochemical test with related species.

Test	Wy1	*Labrys neptuniae* sp. Liujia-146	*Labrys methylaminiphilus* sp. nov.[3]
Lactose fermentation (MacConkey's)	+[1]	NR[2]	NR
Catalase	-	-	+
Oxidase	-	-	+
Citrate (Simmons)	-	-	+
Indole	-	-	-
Triple sugar ion (TSI)	-	NR	NR
Urease (Christensen's)	+	+	+
Oxidation fermentation (OF) of glucose	-	-	+
Oxidation fermentation (OF) of lactose	-	NR	-
Motility	-	-	-
Gelatine liquefaction	-	-	-
Nitrate reduction	+	+	+

"+" for positive result, "-" for negative result; NR - Not reported; Miller et al. (2005).

growth medium suggested the utilization of 2,2-DCP as a carbon source.

Figure 3 shows the taxonomic position of Wy1 with other closely related species from different family. Wy1 was clustered together with *Labrys neptuniae* sp. Liujia-146 with high bootstrap value (98), suggesting they were closely related. However, DNA-DNA hybridization test is needed to confirm this finding and define them as same species. According to Chou et al. (2007), *Labrys neptuniae* sp. Liujia-146 was a budding bacteria found in root nodules of *Neptunia oleracea*. This is the first reported case of which *Labrys* sp. could survive solely on 2,2-DCP as carbon source. There has been reported case of *Labrys portucalensis* sp. nov. which capable of degrading fluorobenzene found in contaminated sediment from northern Portugal (Carvalho et al., 2008). *Labrys*

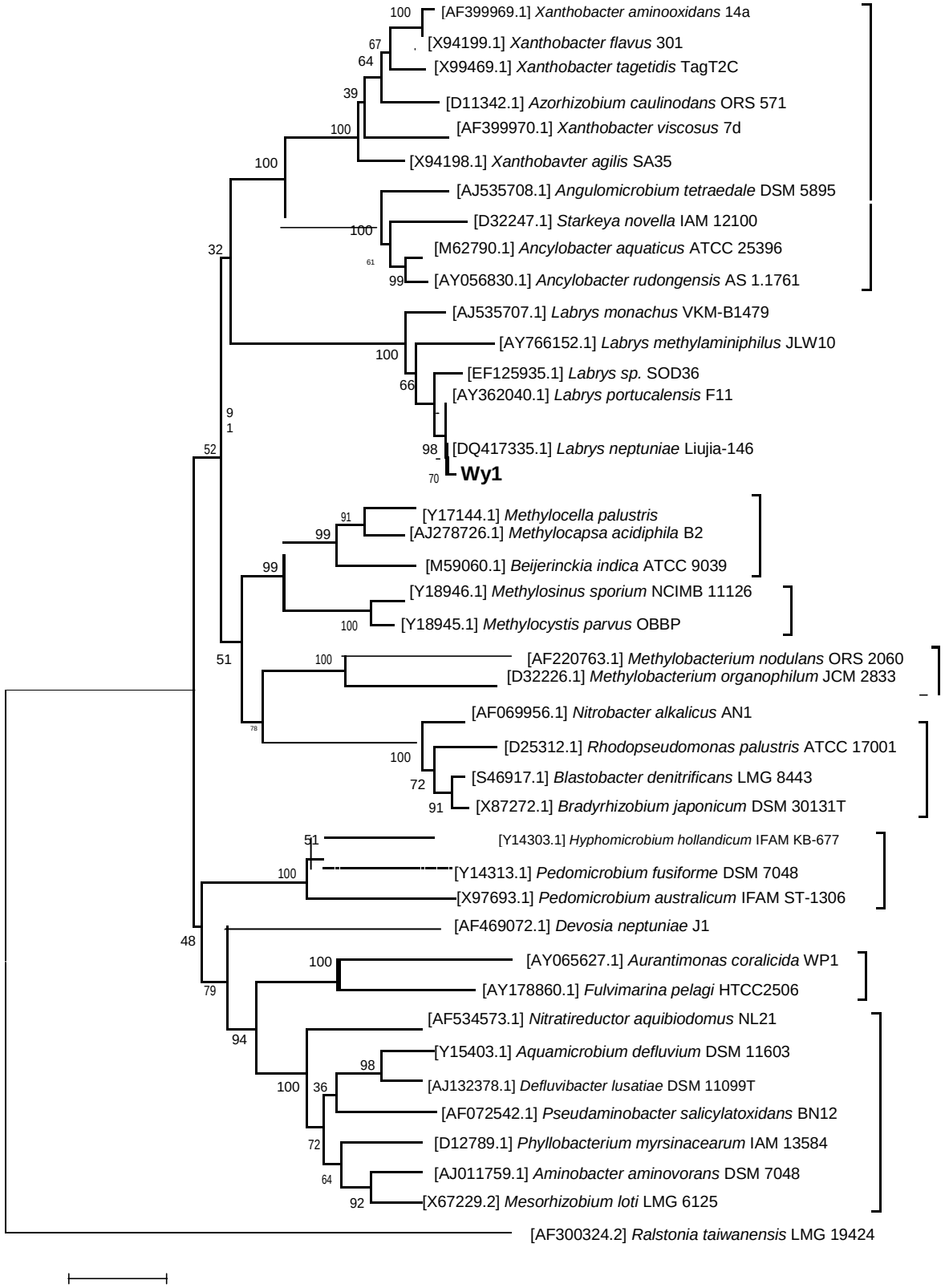

Figure 3. Phylogenetic tree of isolate Wy1 (Bolded) and related bacteria species (Bar: 2% dissimilarity).

Table 3. Species of bacteria identical to Wy1 16S rRNA gene sequence.

Accession number	Description	Maximum score	Query coverage (%)	Maximum identity (%)
DQ417335.1	*Labrys neptuniae* Liujia-146	2392	100	99
AY362040.1	*Labrys portucalensis* F11	2383	100	99
EF125935.1	*Labrys sp.* SOD36	2343	100	98
EF125940.1	Uncultured *Labrys sp.* clone A5	2334	100	98
AB271044.1	*Labrys ginsengisoli*	2331	100	98
EU855784.1	*Alpha proteobacterium* PCNB-21	2287	95	99
AB236172.1	*Labrys methylaminiphilus* G24103	2280	98	98
DQ337554.1	*Labrys methylaminiphilus*	2280	98	98
NR025581.1	*Labrys monachus* VKM B1479	2268	100	97
AB236169.1	*Labrysokinawensis* MAFF 210191	2248	98	97

portucalensis PCNB -21 isolated from polluted soil was also found to be capable of degrading Pentachloronitrobenzene (PCNB) under aerobic and anoxic conditions (Li et al., 2011). Both bacteria were able to use halogenated compound as sole source of carbon. Current study is the first reported case of the respective bacteria from genus *Labrys* which could degrade -haloalkanoic acid.

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REFERENCES

Allison N, Skinner AJ, Cooper RA (1982). The dehalogenases of a 2,2 dichloropropionate-degrading Bacterium. J. Gen. Microbiol., 129: 1283 1293.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res., 25(17): 3389–3402.

Bergman JG, Sanik J (Jr.) (1957). Determination of trace amounts of chlorine in Naphtha. Anal. Chem., 29: 241-243.

Berry EKM, Allison N, Skinner AJ (1979). Degradation of the selective herbicide 2,2-ichloropropionate (Dalapon) by a soil bacterium. J. Gen. Microbiol., 110: 39-45.

Carvalho MF, Marco P, Duqua AP, Pacheco CC, Janssen DB, Castro PML (2008). *Labrys portucalensis*sp. nov. a flurobenzene-degrading bacterium isolated from an industrially contaminated sediment in northern Portugal. Int. J. Sys. Evol. Microbiol., 58(3): 692-698.

Chou YJ, Elliott GN, James EK, Lin KY, Chou JH, Sheu SY, Sheu DS, Sprent JI, Chen WM (2007). *Labrys neptuniae* sp. nov., isolated from root nodules of the aquatic legume *Neptunia oleracea*. Int. J. Syst. Evol. Microbiol., 57:577–581.

Fetzner S, Lingens F (1994). Bacterial Dehalogenases: Biochemistry, Genetics, and Biotechnological Applications. Microbiol. Rev., 58(4): 641–685.

Hardman DJ, Slater JH (1981). Dehalogenases in soil bacteria. J. Gen. Microbiol., 123:117- 128.

Hareland WA, Crawford RL, Chapman PJ, Dagley S (1975). Metabolic Function and Properties of 4-Hydroxyphenyl-Acetic Acid 1 Hydroxylase from *Pseudomonas acidovorans.* J. Bacteriol., 121:272 285.

Janssen DB, Scheper A, Dijkhuizen L, Witholt B (1985). Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ1O. App. Environ. Microbiol., 49(3): 673-677.

Jensen HL (1957).Decomposition of chlorosubstituted aliphatic acids by soil bacteria. Can. J. Microbiol., 3:151-164.

Jing NH, Huyop F (2007). Dehalogenation of chlorinated aliphatic acid by *Rhodoccus*sp. As. Pac. J. Mol. Biol. Biotechnol., 15(3):147-151.

Jing NH, Huyop F (2008). Enzymatic dehalogenation of 2,2 dichloropropionic acid by locally isolated *Methylobacterium* sp. HJ1. J. Biol. Sci., 8(1): 233-235.

Jing NH, Wahab RAB, Hamdan S, Huyop F (2010). Cloning and DNA sequence analysis of the haloalkanoic permease uptake gene from *Rhizobium* sp. RC1. Biotechnol., 9(3): 319-325

Li R, Zheng JW, Ni B, Chen K, Yang XJ, Li SP, Jiang JD (2011). Biodegradation of pentachloronitrobenzene by *Labrys portucalensis* pcnb-21 isolated from polluted soil. Pedosphere, 21(1): 31-36.

Magee LA, Colmer AR (1959). Decomposition of 2,2 dichloropropionicacid by soil bacteria. Can. J. Microbiol., 5: 255-260.

Mesri S, Wahab RAB, Huyop F (2009). Degradation of 3 Chloropropionic acid (3CP) by *Pseudomonas* sp. B6P isolated from a rice paddy field. Ann. Microbiol., 59(3): 447-451

Miller JA, Kalyuzhnaya MG, Noyes E, Lara JC, Lidstrom ME, Chistoserdova L (2005). *Labrysmethylaminiphilus* sp. nov., a novelfacultativelymethylotrophic bacterium from a freshwater lake sediment. Int. J. Sys. Evol. Microb., 55:1247-1253.

Motosugi K, Esaki N, Soda K (1982). Purification and properties of a new enzyme, DL-2-haloacid dehalogenase from *Pseudomonas* sp. J. Bacteriol., 150(2): 522-527

Penfold WJ (1913). The inhibitory selective action on bacteria of bodies related to: a contribution monochloroacetic acid to the theory of cell intoxication. J. Hyg., 13: 34-48

Prasad R, Blackman GE (1965). Studies in the physiological action of 2,2-Dichloropropionic acid III. Factors affecting the level of accumulation and mode of action. J. Exp. Bot., 16: 545-568

Redemann CT, Meikle RW (1955). The inhibition of several enzyme systems by 2,2-dichloropropionate. Arch. Biochem. Biophy., 59(1): 106 112.

Schwarze R, Brokamp A, Schmidt FRJ (1997) Isolation and characterisation of dehalogenases from 2,2-dichloropropionic acid degrading soil bacterium. Curr. Microbiol., 34: 103-109.

Saitou N, Nei M (1987). The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. Mol. Bio. Evol., 4: 406-425.

Senior E, Bull AT, Slater JH (1976). Enzyme evolution in a microbial

community growing on the herbicide Dalapon. Nature, 263(5577): 476–479.
Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Bio. Evol., 24: 1596-1599.
Weightman AJ, Weightman AL, Slater JH (1982). Stereospecificity of 2 Monochloropropionate dehalogenation by the two dehalogenases of *Pseudomonas putida* PP3: evidence for two different dehalogenation mechanisms. J. Gen. Microbiol., 128: 1755-1762.
Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173(2): 697 703.