ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF 2,2-DICHLOROPROPIONIC ACID UTILIZING BACTERIA

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ABSTRACT

2,2-dichloropropionic acid (2,2-DCP) is an artificial halogenated compound used as herbicide. A bacterium able to utilize 2,2-DCP as sole carbon source was isolated from soil in Melaka rubber estate. The bacterium was identified as *Labrys* sp. strain Wy1 using bacterium's 16S rRNA partial sequence. The cells doubling time was 34.6 hours in liquid minimal media supplied with 20 mM 2,2-DCP as sole carbon source. Utilization of 2,2-DCP was confirmed by detection of chloride ion released at 0.27 mM. An endophytic bacterium isolated from *Axonopus compressus* which was identified as *Burkholderia cepacia* strain Wy5 was also able to utilize 2,2-DCP as sole carbon source. The bacterium has cells doubling time 2.7 hours and chloride ion released was also detected at 47.28 ± 0.25 mM in minimal media contained 20 mM 2,2-DCP. Cell free extract (CFE) of *Burkholderia cepacia* Wy5 was further characterized due to its higher activity towards 2,2-DCP compared to *Labrys* sp. Wy1. Dehalogenase found in CFE of *Burkholderia cepacia* Wy5 has optimal enzyme specific activity at pH8 (0.83 μmol $[Cl^-]$ min^{-1} mg^{-1}) and 40^oC (0.78 μmol $[Cl^-]$ min^{-1} mg^{-1}). The dehalogenase was also able to react with other α-haloalkanoic acid including monochloroacetic acid, $_{DL}$-2-chloropropionic acid and $_{DL}$-2-bromopropionic acid, but not 3-chloropropionic acid. "Group I" and "Group II" dehalogenase primers were used to amplify dehalogenase gene from both strains Wy1 and Wy5 but only *Burkholderia cepacia* Wy5 showed positive result. The dehalogenase gene fragment amplified was designated "*deh-wy5*" and subsequent analysis showed it belongs to Group I dehalogenase. Customized primers based on $_{D,L}$-*dex* gene were designed to amplify complete sequence of *deh-wy5* due to high similarity between partial sequence of *deh-wy5* and $_{D,L}$-*dex*. Complete sequence of *deh-wy5* was eventually amplified and found to be identical (100%) to $_{D,L}$-*dex*.

ABSTRAK

Asid 2,2-dikloropropionik (2,2-DCP) merupakan bahan buatan berhalogen yang diguna sebagai racun lalang. Sejenis bakteria yang disaring dari sampel tanah ladang getah Melaka didapati mampu menggunakan 2,2-DCP sebagai sumber karbon tunggal. Bakteria tersebut dikenal pasti sebagai *Labrys* sp. strain Wy1 hasil daripada kajian penjujukan 16S rRNA-nya. Bakteria tersebut membiak dalam medium minima yang mengandungi 20 mM 2,2-DCP sebagai sumber karbon tunggal dengan tercatatnya masa gandaan sebanyak 34.6 jam. Pembebasan ion klorida sebanyak 0.27 mM yang dikesan dalam medium minima mengesahkan penggunaan 2,2-DCP oleh bakteria tersebut. Satu lagi bakteria endofit juga disaring dari kandungan daun rumput parit dengan nama saintifiknya *Axonopus compressus*. Bakteria yang dikenal pasti sebagai *Burkholderia cepacia* strain Wy5 juga mampu mengguna 2,2-DCP sebagai sumber karbon tunggal. Bilangan sel bakteria tersebut berganda dalam masa 2.7 jam dan pembebasan ion klorida sebanyak 47.28 ± 0.25 mM dalam medium minima yang mengandungi 20 mM 2,2-DCP juga dapat dikesan. Ekstrak isi sel (CFE) bakteria *Burkholderia cepacia* Wy5 telah diuji secara terperinci memandangkan bakteria tersebut mempunyai kadar penggunaan 2,2-DCP yang lebih tinggi berbanding dengan bakteria *Labrys* sp. Wy1. Dehalogenase yang terdapat dalam ekstrak isi sel bakteria mempunyai aktiviti enzim spesifik optimal yang tercatat pada pH8 (0.83 μmol $[Cl^-]$ min^{-1} mg^{-1}) dan suhu 40^oC (0.78 μmol $[Cl^-]$ min^{-1} mg^{-1}). Dehalogenase tersebut juga dapat bertindak balas dengan asid α-haloalkanoik yang lain termasuk asid monokloroasetik, asid $_{DL}$-2-kloropropionik dan asid $_{DL}$-2-bromopropionik, tetapi tiada tindak balas dikesan dengan asid 3-kloropropionik. Primer dehalogenase "Group I" dan "Group II" telah diguna untuk amplifikasi gen dehalogenase dari kedua-dua strain bakteria Wy1 dan Wy5 tetapi hanya Wy5 memberi hasil positif. Penjujukan separa dehalogenase "Group I" tersebut diberi nama "*deh-wy5*". Disebabkan persamaan yang tinggi antara urutan separa *deh-wy5* dengan gen $_{D,L}$-*dex*, primer yang berasaskan gen $_{D,L}$-*dex* telah direka untuk tujuan amplifikasi gen dehalogenase yang lengkap daripada bakteria strain Wy5. Akhirnya jujukan, lengkap gen dehalogenase *deh-wy5* dapat diamplifikasi dan gen tersebut didapati mempunyai persamaan setinggi 100% berbanding dengan gen $_{D,L}$-*dex*.

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LIST OF SYMBOLS

(v/v)	-	Volume percentage per 100mL volume
(w/v)	-	Mass percentage per 100mL volume
2,4,5-T	-	2,4,5-Trichlorophenoxyacetic acid
2,4-D	-	2,4-Dichlorophenoxyacetic acid
$A_{...nm}$	-	Absorption spectroscopy at …nm light source
BLASTn	-	Basic local alignment search tool – nucleotide
bp	-	Base pairs
CFE	-	Cell free extract
DDT	-	Dichlorodiphenyltrichloroethane
dH_2O	-	Distilled water
HCH	-	Hexachlorocyclohexane
HIA	-	Halide ion assay
kb	-	Kilo bases
MW_r	-	Relative molecular weight
OD	-	Optical density
PCB	-	Polychlorinated biphenyls
PCR	-	Polymerase chain reaction
rpm	-	Revolution per minute
RT	-	Room temperature
V	-	Volts

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Halogenated compounds were used extensively as herbicide and as intermediate chemicals in many industries. Due to their complexity, toxicity, persistence and ubiquitous distribution of these xenobiotic compounds, they have brought threat to the health and living quality of human and other organisms (Fetzner and Lingens, 1994). Physiologists and biochemists have known since the beginning of the 20th century that halogenated compounds will affect metabolic processes as halogenated analogues of intermediary metabolites are toxic (Slater *et al.*, 1995). Degradation of halogenated compound by microorganisms has been reported since the early of 20th century by Penfold (1913). These microorganisms are capable of evolving new enzymes, pathways and regulatory mechanisms for the degradation of almost all xenobiotic compounds due to their short life cycle. The evolution of dehalogenase producing microorganisms using some of these halogenated compounds is scientifically interesting and practically important (Penfold, 1913; Timmis and Pieper, 1999).

2,2-dichloropropionic acid (2,2-DCP) or Dalapon is an odourless and colourless 2-haloalkanoic acid herbicide used to control and regulate the growth of certain weeds, such as quick grass, Bermuda grass and cattails. It effectively inhibits panothenic acid production (Prasad and Blackman, 1965) and pyruvate utilization in bacteria (Redemann and Meikle, 1955). One of the earliest event of degradation of herbicide Dalapon was reported by Magee and Colmer (1959) after observation of bacteria that produce dehalogenase enzyme (Magee and Colmer, 1959). Since then,

studies on isolation of microbes that potentially produce dehalogenases have been undertaken (Berry *et al.*, 1979; Hardman and Slater, 1981; Motosugi *et al.*, 1982; Weightman *et al.*, 1982; Allison *et al.*, 1983; Liu *et al.*, 1994; Schwarze *et al.*, 1997; Nardi-Dei *et al.*, 1999; Huyop *et al.*, 2004; Jing and Huyop, 2007; Huyop *et al.*, 2008). 2,2-DCP is readily removed from the soil by a variety of microorganisms including species of *Pseudomonas, Agrobacterium, Nocardia, Alcaligenes, Arthrobacter* and *Bacillus* (Foy, 1975).

The enzymes responsible for the degradation of halogenated compound were known as dehalogenase, discovered and firstly named by Jensen (1957). Dehalogenases catalyse the hydrolysis of halogen-substituted alkanoic acids yielding either hydroxyalkanoic acids from mono-halogenated acids or oxo-alkanoic acids from di-halogenated compounds products which may be readily metabolized (Hardman and Slater, 1981). Culturing and enrichment of microorganism that can produce dehalogenase in the presence of halogenated compound in the environment was the most favourable method. Jensen (1957) used soil perfusion and enrichment technique to isolate five strains of *Pseudomonas* sp. which able to degrade 2,2-DCP and other α-halogenated substrate such as dichloroacetate and 2-chloropropionate (Jensen, 1957). Several other dehalogenase producing bacteria isolated using this method including *Methylobacterium* sp. HJ1 (Jing *et al.*, 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). Interest in biodegradation of α-substituted halogenated alkanoic acid was increased due to the introduction of Dalapon as herbicide and lead to the isolation of many microorganisms able to grow on 2,2-DCP as sole carbon source (Macgregor, 1963; Burge, 1969; Berry *et al.*, 1979; Kearney and Kellogg, 1985; Jing *et al.*, 2008; Huyop and Nemati, 2010).

Currently, technological applications of bacterial transformation of halogenated compound can be considered in two major aspects: synthesis of chemical intermediates and degradation of xenobiotic wastes. Dehalogenase can be used as industrial biocatalysts to produce valuable intermediates for chemical synthesis (Huyop and Cooper, 2003). Biotransformation of organic compounds with microbial or enzyme biocatalysts offers new chemical routes for the synthesis of

intermediates and novel products, since these biocatalysts possess chiral specificities and can recognize specific area on a molecule, that are difficult and expensive to achieve by conventional chemistry (Fetzner and Lingens, 1994). For example ICI Biological Products (U.K.) uses *Pseudomonas putida* AJ1/23 to produce $_{L}$-2-monochloropropionate for use in herbicide manufacture from racemic 2-monochloropropionate, which already reached commercial scale (Motosugi *et al.*, 1982). Similarly, the production of optically active 3-halolactate from 2,3-dihalopropionate was also performed with 2-haloalkanoic acid halidohydrolase from *Pseudomonas putida* (Fetzner and Lingens, 1994). In addition, dehalogenating microorganisms were also proved to be useful in a bioremediation process and the application of specialized strains as inocula for the bioremediation of polychlorinated biphenyls (PCP) contaminated soil and groundwater was studied extensively. For example, Hicky *et al.* (1993) used the chlorobenzoate utilizers *Pseudomonas aeruginosa* JB2 and *Pseudomonas putida* Plll and the biphenyl utilizer *Pseudomonas* sp. strain PB133 to mineralize polychlorinated biphenyls in soil (Hickey *et al.*, 1993).

1.2 Rationale of Investigation

Microorganisms with dehalogenating capabilities were proven to be useful in both chemical manufacturing industry and in situ bioremediation of contaminated soil, especially those related to chlorinated xenobiotics. In current investigation, isolating new bacteria with higher reactivity towards chlorinated herbicide compared to other previous research is the main goal. Degradation of chlorinated herbicide, especially 2,2-DCP is chosen due to its more complex structure which resistance to enzymatic attack compared to mono-substituted haloalkane, and also its well-known environmental impact. The source of soil and *Axonopus compressus* (*rumput parit*) used in current research was frequently exposed to various chlorinated herbicide including Dalapon (2,2-DCP) and this could increase the chance of isolate dehalogenating microorganisms. Agricultural soil is a common place to find dehalogenating bacteria, however some endophytes also reported to show resistance to heavy metals and able to degrade organic compounds in the plant, soil or water, and thus also play an important role in pollution control (Germaine *et al.*, 2006), therefore the investigation of whether there is endophyte with dehalogenating capabilities present in *Axonopus compressus*' leaves, especially those possess cryptic dehalogenase, can be a novel approach for isolation of new dehalogenating bacteria. Moreover, these new isolated bacteria could be used in enzymatic production of useful chemicals or as potential bioremediation agent.

The study of 2,2-DCP degradation can also be compared to that of degradation of other chloro-substituted alkanoates, for example, 3-chloropropionic acid, which is an analogue and isomer of 2,2-DCP (Allison *et al.*, 1983). Further interest in this subject was raised when it became apparent that α-chloroalkanoate-degrading microorganisms were unable to utilize β-substituted haloalkanoates, which differed only in chlorine substitution. Only few isolated microorganisms can degrade β-halocarboxylic acid (β-HA) (Mesri *et al.*, 2009; Yusn and Huyop, 2009). Some previous studies have suggested the production of more than one dehalogenase in a few bacterial strains (Goldman *et al.*, 1968; Weightman *et al.*, 1982) and the fungus *Trichoderma viride* (Jensen, 1960). The only microorganism so far reported to produce three forms of dehalogenases which degrade $_{D}$-, $_{L}$- and non-stereospecific isomer of α-haloalkanoate is *Rhizobium* sp. (Leigh *et al.*, 1988).

A limited number of genetic studies which consider the evolutionary mechanisms of dehalogenase have been reported. Isolation of many iso-enzymic forms of dehalogenase from a vast variety of bacterial genera gave rise to the question of their importance in the natural environment and the evolution-relationship of their different forms (Murdiyatmo *et al.*, 1992). The ubiquity of the haloacid halidohydrolases in natural bacterial isolates has led to the suggestion that their importance in being catabolic enzymes cleaving the halo- substituents of halo metabolites as part of degradative pathways for the degradation of more complex halo-organic compounds (Murdiyatmo *et al.*, 1992). The adoption of molecular method might provide an alternative in studying variety of dehalogenases possessed by certain microorganism. Hill *et al.* (1999) described systematic approach to amplify two different families of α-halocarboxylic acid (α-HA) dehalogenase genes of group I and group II based on the knowledge of conserved residues among different dehalogenases. Group I dehalogenases were non-stereospecific, whereas group II showing stereospecificity tendency, dechlorinating only $_{L}$- but not $_{D}$-2-chloropropionic acid. Current investigation adopted the molecular method described by Hill *et al.* (1999) might allowed us to identify cryptic or silent, as well as active dehalogenase genes presence in the bacteria.

1.3 Objectives

I. Isolate, identify and characterize soil and endophytic microorganisms capable of utilizing 2,2-DCP as sole carbon source.

II. Characterization of 2-haloalkanoic acid dehalogenase from cell free extract produced by isolates capable of utilize 2,2-DCP as sole carbon source.

III. Amplification and analysis of 2-haloalkanoic acid dehalogenase gene sequence from isolated microorganisms using designed primers based on conserved gene sequence of known dehalogenases.

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