ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF DEHALOGENASE PRODUCING BACTERIA ISOLATED FROM *LABEO ROHITA* AND ITS ENVIRONMENT

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For My Dearest Parents and Family

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ABSTRACT

Microbial dehalogenases are involved in the biodegradation of many types of halogenated compounds. The presence of halogenated compounds in water does not only suppress the immune system of fish but adversely induces serious morbidity and mortality among cultured stocks. In this study, we attempted to screen the gut of pond-reared rohu (*Labeo rohita*) for isolating dehalogenase gene bacteria using molecular technique and tested the degradation ability *in vitro*. The present study shows eight bacterial strains studied were identified as *Enterobacter mori* (MK121001), *Enterobacter cloacae* (MK121003), *Enterobacter cloacae* (MK121004), *Enterobacter cloacae* (MK121010), *Ralstonia solanacearum* (121002), *Acinetobacter baumannii (*MK121007), *Chromobacterium violaceum* (MK121009) and *Pantoea vagans* (121011). Further analysis found three bacterial strains (MK121002, MK121007 and MK121009) were capable of degrading 2,2-dichloropropionic acid (2,2-DCP) as the sole carbon source up to a final substrate concentration of 20 mM. Their mean growth doubling time ranging from 6-23 h with the maximum of chloride ion released of 85%. Another bacterium was isolated from soil samples collected from lake water at Universiti Teknologi Malaysia, Skudai also capable of degrading 2,2-DCP. Phylogenetic analysis indicated that *Serratia marcescens* SE1 strain clearly shared 97% homology to the genus of *Serratia marcescens* according to bioinformatics analysis. *Serratia marcescens* has the ability to degrade 2,2-DCP with cells doubling time of 5 h and maximum chloride ion released of 38 μmolCl^-/mL in the liquid growth medium.

ABSTRAK

Mikrob dehalogenases terlibat dalam biodegradasi di kebanyakkan sebatian halogen. Kehadiran sebatian halogen di dalam air bukan hanya menekankan sistem imun ikan tetapi sebaliknya mendorong morbiditi dan mortaliti yang serius di kalangan stok kultur. Dalam kajian ini, kami cuba menyaring bakteria dari usus ikan rohu kolam pemeliharaan (*Labeo Rohita*) untuk mengasingkan gen dehalogenase bakteria menggunakan teknik molekular dan menguji keupayaan penguraian secara *in vitro*. Kajian menunjukkan lapan jenis bakteria yang dikaji telah dikenal pasti sebagai *Enterobacter mori* (MK121001), *Enterobacter cloacae* (MK121003), *Enterobacter cloacae* (MK121004), *Enterobacter cloacae* (MK121010), *Ralstonia solanacearum* (121002), *Acinetobacter baumannii (*MK121007), *Chromobacterium violaceum* (MK121009) and *Pantoea vagans* (121011). Analisis selanjutnya mendapati tiga jenis bakteria (MK121002, MK121007 dan MK121009) mampu mengurai asid 2,2-dikloropropionik (2,2-DCP) sebagai sumber karbon tunggal sehingga mencapai kepekatan substrat akhir sebanyak 20 mM. Purata masa pergandaan pertumbuhan adalah antara 6-23 jam dengan maksimum ion klorida yang dibebaskan sebanyak 85%. Bakteria seterusnya disaring daripada sampel tanah yang diambil di air tasik di Universiti Teknologi Malaysia, Skudai juga didapati mampu mengurai 2,2-DCP. Analisis filogenetik telah menunjukkan bahawa bakteria SE1 mempunyai 97% homologi dengan spesis *Serratia marcescens* melalui analisis bioinformatik. *Serratia marcescens* mempunyai keupayaan untuk mengurai 2,2-DCP dengan pergandaan sel sepanjang 5 jam dan maksimum ion klorida yang telah dibebaskan adalah sebanyak 38 µmolCl^-/mL dalam medium pertumbuhan cecair.

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LIST OF ABBREVIATIONS

RNA	-	Ribonucleic acid
DNA	-	Deoxyribonucleic acid
rRNA	-	Ribosomal RNA
rDNA	-	Ribosomal DNA
PCR	-	Polymerase Chain Reaction
BLAST	-	Basic Local Alignment Search Tool
PCB	-	Polychlorinated biphenyls
NADH	-	Nicotinamide adenine dinucleotide
EtBr	-	Ethidium Bromide
EDTA	-	Ethylenediaminetetraaceticacid $(HOOCCH_2)_2N(CH_2)_2N(CH_2COOH)_2$
TAE	-	Tris-Acetate-EDTA
UV	-	Ultraviolet
SD	-	Standard Deviation
Sp.	-	Species
F	-	Fluorine
I	-	Iodin
Br	-	Bromine
Cl	-	Clorine
NaCl	-	Sodium Chloride
LB	-	Luria Bertani
HA	-	Haloalkanoic acid
TCA	-	Trichloroacetic acid
MCA	-	Monochloroacetic acid
DCA	-	Dichloroacetic acid

2,4-D	-	2,4-dichlorophenoxyacetic acid
1,2-DCP	-	1,2-dichloropropane
2,2-DCP	-	2,2-dichloropropionic
2,3-DCP	-	2,3-dichloropropionic
1,2-DCE	-	1,2-dichloroethane
4-CBA	-	4-chlorobenzoate
2-MCA	-	2-monochloropropionic acid
1,2-DBE	-	1,2-dibromoethane
2-CBA	-	2-chlorobutyric acid
3-CBA	-	3-chlorobutyric acid
2,2,3-TCBA	-	2,2,3-trichlorobutyric acid
2,2-DCBA	-	2,2-dichlorobutyric acid
2-CPA	-	2-chloropropionic acid
3-CPA	-	3-chloropropionic acid
2,3-CPA	-	2,3-chloropropionic acid
2,2,3-TCPA	-	2,2,3-trichloropropionic acid
1,2-DBE	-	1,2-dibromoethane
4-HBA	-	4-hydroxybutyl acrylate
DDT	-	Dichlorodiphenyltrichloroethane
PAH	-	Polyaromatic hydrocarbons

LIST OF SYMBOLS

A	-	Absorbance
Cl^-	-	Chloride ion
cm	-	Centimeter
g	-	Gram
h	-	Hour
kb	-	Kilo base
M	-	Molar
mg	-	Miligram
mL	-	Mililiter
mM	-	Milimolar
nm	-	Nanometer
pmol	-	Picomolar
psi	-	Per square inch
rpm	-	Renovation per minute
sec	-	Second
V	-	Volts
w/v	-	Mass/volume
α	-	Alpha
µg	-	Microgram
µL	-	Microliter
µmol	-	Micromolar

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CHAPTER 1

INTRODUCTION

1.1 Halogenated Compound in the Environment

Nearly every year millions of tons of halogenated compounds are produced globally as herbicides in agricultural production area. The application of chemically synthesised herbicide in agricultural areas has shown a remarkable success in doubling the yield but at the same time cause adverse environmental issues. Most halogenated compounds are representing an important class of environmental pollutants, partly as a result of their widespread use as biocides, solvents and also due to the improper disposal of wastes, accidental spillage or deliberate release. Accordingly, dehalogenases that catalyse the degradation of these compounds attract a great deal of attention from the viewpoint of environmental technology (Soda *et al.*, 1996).

An abundance of haloorganic compounds are also produced naturally (Fetzner and Lingens, 1994). These substances can be decontaminated using non-biological or microbiological degradation methods which transforms the xenobiotics substances into harmless products. But microbiological methods are favoured because they are economical, safer and environmental friendly. However, naturally occurring halogenated compound are not scarce. This is demonstrated by the relative abundance of halogens as inorganic salts or minerals in soil and freshwater environment.

Recently, environmental contamination of natural water have been a great concern, since most of these herbicide compounds are very persistent, bioaccumulative and their toxicity and carcinogenic properties pose harmful and hazards effects to human and natural environment (Mohn and Tiedje, 1992). As an outcome of this extensive environment input, natural water in rivers and lakes has been contaminated with the trace amounts of herbicides compound. Therefore, lots of studies have been made for microbial degradation of pollutants.

Most of Southeast Asian countries like Malaysia, Thailand, Indonesia and Vietnam have banned the use of herbicide compounds since 1990s but the residues are still detected in water, soil or sediments at the significant levels (Ibrahim *et al.*, 2002). A variety of halogenated compounds such as haloacids, which are produced by chemical industries in vast quantities are degraded through dehalogenation by microbial dehalogenases that involve carbon-halogen cleavage (Copley, 1998). A critical step in degradation of organohalides is the cleavage of the carbon-halogen bond (Haggblom *et al.*, 2000). Naturally occurring carbon-halogen covalent bonds are found widely throughout the environment in animals and plant. The role of many of these compounds is suggested to inhibit the growth of competing species for example production of antibiotics. However, it is the release of man-made compounds that has raised awareness of environmental issues relating to halogenated compounds.

Dehalogenation is the critical step in the degradation of chlorinated aliphatics because the reaction occurs as the first step in the degradative pathway. A variety of microbial enzymes which catalyze carbon-halogen bond cleavage have been described (Fetzner and Lingens, 1994; Janssen *et al*., 1994; Janssen *et al*., 2001; Slater *et al*., 1997; Leisinger, 1996). Hydrolytic dehalogenases represent the key position in the degradation of haloaliphatic compounds. The mechanism involve enzymes catalyse the cleavage of carbon halogen bonds by nucleophilic substitution, replacing the halogen ion by a hydroxyl group derived from water. Dehalogenation is also used for degradation of chlorinated aliphatic acids for example degradation of α-chloro substituted haloalkanoates, 2,2-dichloropropionate (2,2-DCP) and monochloroacetate (MCA) (Kerr and Marchesi, 2006; Sui-Yi *et al*., 2007).

Microorganisms capable of utilizing halogenated aliphatic hydrocarbons as sole sources of carbon and energy are widely distributed and a large number of dehalogenase producing bacteria were previously isolated including *Methylobacterium* sp. HJ1 (Jing and Huyop, 2008), *Pseudomonas putida* PP3 (Senior *et al*., 1976), *Anthrobacter autotrophicus* GJ10 (Janssen *et al*., 1985), *Pseudomonas* B6P (Mesri *et al*., 2009) and *Rhizobium* sp. (Berry *et al*., 1979). From these bacterial sources, a number of enzymes involved in the degradation of halogenated compounds have been purified and characterized (Tsang and Sam, 1999; Magnuson *et al*., 2000; Van Der Ploeg *et al*., 1991).

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*., 1983).

1.2 Problem Statement

The pollution of rivers and streams with chemical contaminants has become one of the most critical environmental problems of the century. As a result of the pollutants transport from industrial areas into the environment and their chemical persistence, many freshwater systems are faced with spatially or temporally alarming high level of xenobiotics chemical (Brack *et al*., 2002; Diez *et al*., 2002). Some of these chemicals are biodegradable and quickly decay into harmless forms, while others are non-biodegradable and remain dangerous for a long time. Now, there is a growing concern worldwide over the indiscriminate use of such chemicals, resulting in environmental pollution and toxicity risk to aquatic organisms (Khan, 1996).

Fish are able to take up and retain chemicals dissolved in water via active or passive processes. They can be used to detect and document pollutants released into their environment. The gut microbiota of marine and freshwater fish has been widely investigated during the last two decades (Cahill, 1990; Ringo *et al*., 1995; Hansen and Olafsen, 1999). So far, there is no information or study have reported on isolation of bacteria from the gut of *Labeo rohita* fish that able to degrade 2,2-DCP as sole carbon source. In addition, *Labeo rohita* fish was used as cheap source of protein in Myanmar. Current study will focus in this area. Nowadays, public concern about the possible hazardous effects of halogenated compound on human and their environment has been neglected. Therefore, it is very crucial to understand the role of potential microorganism in biodegradation process.

1.3 Research Objectives

Dehalogenase producing microorganisms have been frequently isolated from soil and marine environment but none from other animals. So far, there is no study that has been reported on the association of pollutant degrading bacteria in the gut of *Labeo rohita* fish. In current study, the justification of isolating bacteria that can degrade 2,2-DCP from soil and *Labeo rohita* freshwater fish is because to observe the variation of dehalogenase gene from two different sources. 2,2-DCP was used as a model of investigation as it was available in the environment due to widely use of herbicide. Therefore, the primary objectives of this study were to (i) identify and characterize novel bacteria strains that capable of degrading several selected halogenated compound as carbon and energy source from the gut of *Labeo rohita* freshwater fish and also from soil, (ii) to characterize the ability of the bacteria to degrade 2,2-DCP.

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