

SCREEN A DEHALOGENASE GENE USING ISOLATED BACTERIUM FROM  
SEA-SHORE SOIL OF CASPIAN SEA

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I dedicated this dissertation to my beloved parents  
for their endless support and encouragement

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## ABSTRACT

Halogenated organic compounds are widely used in agriculture and industry over the past 100 years. The use of these compounds in the environment led to human health problems and environmental pollution because of their persistence and toxicity. Dalapon or 2,2-dichloropropionic acid is widely used as herbicides and plant growth regulator which cause environmental pollution. In this research, bacterium NR1 was isolated from soil sample taken from Bandar-e Anzali seashore in north of Iran. The result has shown that this bacterium grows in minimal media containing 20mM 2, 2-DCP with doubling time of 0.89 hours, which indicated its ability to degrade 2, 2-DCP. Based on microscopic observation and Gram staining, strain NR1 was identified as rod Gram positive bacterium. Biochemical tests for the bacterium NR1 were positive for oxidase, catalase, gelatin liquefaction, nitrate reduction, TSI, oxidation-fermentation glucose, starch and casein test, while the tests result were negative for lactose utilization, citrate, indole, and urease test. Genomic DNA from bacterium NR1 was extracted and 16S rRNA PCR amplification was carried out using universal primers, Fd1 (5' - AGA GTT TGA TCC TGGCTC AG - 3') and rP1 (5' - ACG GTC ATA CCT TGT TAC GAC TT - 3') before sending for sequencing. NR1 strain 16S rRNA sequences were applied for Basic Local Alignment Search Tool nucleotide (BLASTn) and further analyzed using phylogenetic tree of Neighbour-Joining method (MEGA 5). Phylogenetic analysis indicated that NR1 strain clearly shared 97% homology to the genus of *Bacillus cereus* and therefore designated as *Bacillus cereus* sp. NR1. The PCR analysis of dehalogenase using dh1b\_F\_314/dh1b\_R\_637 primers showed a band with approximate size of >200 bp, suggesting this bacterium carries dehalogenase from class I.

## ABSTRAK

Bahan organik berhalogen digunakan secara meluas dalam bidang pertanian dan industri lebih daripada 100 tahun yang lalu. Penggunaan sebatian ini dalam persekitaran boleh membawa kepada masalah kesihatan manusia dan pencemaran alam sekitar kerana ketoksikan bahan ini. Dalapon atau asid 2,2-dichloropropionic digunakan secara meluas sebagai racun dan pengawal selia pertumbuhan tumbuhan yang menyebabkan pencemaran alam sekitar. Dalam kajian ini, bakteria NR1 telah diasingkan daripada sampel tanah yang diambil dari Bandar-e Anzali pantai di utara Iran. Hasilnya menunjukkan bahawa bakteria ini tumbuh terbaik dalam media yang mengandungi minimum 20mm<sup>2</sup> 2, 2-DCP dengan dua kali ganda masa 0.89 jam, yang menunjukkan keupayaan untuk merendahkan 2, 2-DCP. Berdasarkan pemerhatian mikroskopik dan pewarnaan Gram, ketegangan NR1 telah dikenal pasti sebagai rod bakteria Gram positif. Keputusan ujian biokimia bakteria NR1 positif untuk oxidase, catalase, pencairan gelatin, pengurangan nitrat, TSI, glukosa pengoksidaan-penapaian, kanji dan ujian kasein, manakala hasil ujian itu adalah negatif untuk penggunaan laktosa, sitrat, indole, dan ujian urease. DNA genom daripada bakteria NR1 telah dikeluarkan dan amplifikasi PCR telah dijalankan menggunakan primers sejagat, Fd1 (5' - AGA GTT TGA TCC TGGCTC AG - 3') dan RP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT - 3') sebelum menghantar untuk urutan. 16S urutan rRNA dibandingkan dengan asas Tempatan Penjajaran Search Alat nukleotida (BLASTn) dan seterusnya dianalisis menggunakan pokok filogenetik daripada Jiran-Menyertai kaedah (MEGA 5). Analisis filogenetik menunjukkan bahawa NR1 ketegangan jelas dikongsi 97% homologi kepada genus *Bacillus cereus* dan oleh itu ditetapkan sebagai *Bacillus cereus* sp. NR1. Analisis PCR dehalogenase menggunakan dhlb\_F\_314/dhlB\_R\_637 primer menunjukkan band dengan saiz anggaran > 200 bp, mencadangkan bakteria ini membawa dehalogenase dari kelas I.

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**LIST OF ABBREVIATION**

ABBREVIATION	DESCRIPTION
2,2-DCP	2,2-Dichloropropionate
BLAST	Basic Local Alignments Search Tool
bp	Base Pairs (Nucleotide)
BS	Basal Salt Solution
DNA	Deoxyribonucleic Acid
HIA	Halide Ion Assay
MEGA5	Molecular Evolutionary Genetics Analysis Software version
PCR	Polymerase Chain Reaction
psi	Pound Force per Square Inch
rpm	Round per Minute
RNase / rRNA	Ribonucleic Acidase / Ribosomal Ribonucleic Acid
TM	Trace Metal Solution
UV	Ultra Violet Ray

**LIST OF SYMBPLS**

SYMBOL	DESCRIPTION
$^{\circ}\text{C}$	Degree Centigrade Celsius
$[\text{Cl}^-]$	Concentration of Chloride Ion
$1000X$	1000 Times Magnification
$A_{600\text{nm}} / A_{460\text{nm}}$	Absorbance at 600 Nanometre / 460 Nanometre
$g / \text{mg} / \mu\text{g}$	Gram / Milligram / Microgram
$L / \text{mL} / \mu\text{L}$	Litre / Millilitre / Microlitre
$M / \text{mM}$	Molar / Millimolar
$\text{mm} / \mu\text{m}$	Millimetre / Micrometer
$\text{mmol/L} / \text{mg/L}$	Millimole per Litre / Milligram per Litre
$V$	Voltage
$vs$	Versus
$w/v$	Weight per Volume Percentage

## CHAPTER 1

### INTRODUCTION

#### 1.1 Background

Sustainable biology aims at an improved efficiency of usage natural resources to keep biological balance, and to satisfy human demands for chemical products. Therefore, microorganisms have been used to produce different enzymes and also synthetic drugs for a long time and new techniques are always been developed to increase its proficiency (Slater *et al.*, 1997).

Although a wide variety of halogenated compound that occur within the environment are of natural origin, the common use of these compounds in agriculture and industry over the past century has produced numerous xenobiotic pollutants into the environment (Chapelle, 1993). The existence of these compounds in the environment leads to human health problems and environmental pollution because of their persistence and toxicity (Jing and Huyop, 2007). Halogenated compounds are widely used as solvents, pesticides, insecticides, herbicides, fungicides, lubricants, hydraulic and intermediate for chemical synthesis process (Fetzner and Lingens, 1994). Dalapon or 2,2-dichloropropionic acid is an example of xenobiotic compounds utilized as herbicides and plant growth regulator used to control certain grasses, such as Johnson grass, quack grass, Bermuda grass, as well as cattails and rushes (EPA, 1988). This compound is selective; therefore it kills only certain plants, while sparing non-target types of vegetation. The main food crop use of

dalapon is on sugar beets and sugarcane. It is also used on various fruits, potatoes, carrots, asparagus, alfalfa and flax (Rieger *et al.*, 2002).

Biodegradation is one of the major natural processes that help to remove halogenated compound from the environment via microorganisms (Sinha, *et al.*, 2009). Microorganisms can utilize halogenated aliphatic hydrocarbons as sole sources of carbon and energy (Schwarze *et al.*, 1997). Hydrolytic dehalogenases show the important position within the degradation of haloaliphatic compounds (Hill, *et al.*, 1999). The mechanism of these enzymes is to catalyze the cleavage of carbon-halogen bonds by nucleophilic substitution, replacing the halogen ion by a hydroxyl group derived from water (Field and Alvarez, 2004; Schwarze, *et al.*, 1997).

Dalapon had very little impact and is unlikely to harm the soil microflora or soil fertility at normal concentrations of 2.6 and 26 ppm, while there is a considerable effect on the microflora and its activities at abnormal concentrations of 266 and 2,660 ppm (Greaves *et al.*, 1981). Thus, we are able to assume that the microorganism has an opportunity to acquire carbon from synthetic compound and adapt to this environment. A lot of studies have been done on microorganism which is capable of degrading and utilizing 2, 2-DCP by dehalogenase enzyme (Kearney *et al.*, 1965; Jing *et al.*, 2008), therefore this research is also focused on the isolation and identification of soil microorganism capable of degrading 2,2-dichloropropionic acid as sole sources of carbon and energy.

## **1.2 Problem Statement**

Recently, the environmental pollution is very important as people are more demand for a “greener”, or sustainable life-style. Excessive usage of haloaliphatic hydrocarbons in industry and agriculture cause considerable environmental pollution as well as human health problems due to improper disposal of wastes, accidental spillage, or deliberate release. Therefore, the use of bioremediation technology is

preferred and lots of studies have been done for microbial degradation of those pollutant chemicals (Song, *et al.*, 2003).

### **1.3 Objectives**

1. To isolate bacteria that can degrade 2, 2-dichloropropionate from seashore soil.
2. To identify and characterize the isolated bacteria based on its microscopic observation and biochemical tests.
3. To determine 16S rRNA gene sequence of the isolated bacteria for species identification and phylogeny study.

### **1.4 Research Scope**

The source of sample was obtained from seashore soil of Caspian Sea in Bandar-e Anzali, north of Iran. The microorganism was isolated by providing a medium containing 20mM 2,2-dichloropropionate as the only carbon source. Streak plate method and microscopic examination was used for isolating pure colony. The pure colony of bacterium was tested for its growth curve by using spectrophotometer. Production of chlorine by degradation of 2,2-DCP was identified by halide ion assay. Different biochemical tests were done on the isolated bacterium such as catalase test, citrate test, gelatin liquefaction test, casein hydrolysis test, starch test, triple sugar iron test, oxidation-fermentation glucose test, urease test, mac conkey test, indole test, nitrate reduction test and motility test. 16S rRNA genomic analysis was done to find out the sequence of 16S rRNA gene of bacterial DNA in order to compare with genomic database for the microorganism's identity. The evolutionary relationship distance of the organism to the nearest known organisms was found out by constructing phylogeny tree. Dehalogenase primers were used to screen the existence of dehalogenase gene.

## **1.5 Significance**

The result of the research allow us to prove the existence of specific microorganism which can grow in a polluted environment and posses natural properties which can degrade the pollutants and use them as sole sources of carbon and energy. Moreover this research will demonstrate the efficiency of protocol based on previous research. There might be possible that a new strain of bacteria which is previously unknown for its response to 2,2-dichloropropionate will be isolated.



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