UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF CARBOFURAN-DEGRADING BACTERIA FROM MALAYSIA SOIL

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By

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This thesis is dedicated to my mother,who inspired me to do this and to my father,who has taught me to aspire and persevere.

Sukirah

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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Chairperson : Professor Mohd Arif Syed, PhD

Faculty : Biotechnology and Biomolecular Sciences

Carbofuran degrading bacteria were isolated from the soil sample. Isolate 7 was isolated from the soil sample collected from the Marriot Hotel car park in Johor Bahru. The sampling location is known to have no history of carbofuran application or contamination. The Preliminary screening of the MTT (3-[4, 5-dimethylthiazol-2-yl]-,diphenyltetrazoliumbromide) assay screened 148 isolates based on the measurement of growth respiration of the isolated bacteria. Seven isolates as well as Isolate 2, 7, 9, 13, 21, 72 and 138 are shown to have the highest absorbance measured by the assay. These isolates were selected from the blue colour formation of formazan. Selected isolates were subjected to Secondary screening which included the MTT Assay and Direct Plate Count method. Five isolates; Isolate 2, 7, 9, 13 and 21 were chosen from the highest colony count in CFU/ mL and highest absorbance obtained from the MTT assay. The High Performance Liquid Chromatography was used as the detector for the carbofuran degrader which was the Isolate 7. In this study, Isolate 7 gave the reading of 92.98% degradation of 100mg/L carbofuran in the enrichment culture on sixth day of the

incubation period. This followed by Isolate 13 which gave the measurement carbofuran degradation reading of 55.8 %, Isolate 21; 54.09 %, and Isolate 9; 53.6%. The lowest degradation was measured from Isolate 2 with 33.6 % of carbofuran degradation. The control without bacteria accounted only 13 % degraded carbofuran suggesting the carbofuran underwent chemical degradation. The optimum growth conditions were determined for Isolate 7 based on the highest colony count (CFU/mL) of the bacteria on nutrient agar. Glucose as a carbon source and 60 mg/L of carbofuran, in carbofuran enrichment culture were used as optimum parameters for the growth of Isolate 7. The optimum pH of carbofuran enrichment culture was at pH 7 and the optimum temperature was at 25°C, at room temperature. In the designed optimize conditions, the carbofuran degradation of Isolate 7 was two days earlier, which is at 93.05% degradation on the fourth day. Isolate 7 was tentatively identified as *Bacillus* sp. based on the morphological characterization and biochemical test. The carbofuran degrading enzyme for carbofuran showed the K_m value of 0.9571 mM and V_{max} at 0.1142 mmole min^{-1} μg^{-1}. Optimum temperature for the enzyme activity was in the range of 40°C to 45°C and pH was in the range of 8 to 10. The Native PAGE and SDS PAGE showed a lot of protein bands appeared suggesting that the protein was still not purified and several steps must be done to remove the impurities.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk mendapatkan ijazah Master Sains

PEMENCILAN DAN PENCIRIAN BAKTERIA PENGURAI CARBOFURAN DARI TANAH DI MALAYSIA

Oleh

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Ogos 2005

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Bakteria pengurai carbofuran telah dipencilkan dari sampel tanah yang pernah diletakkan pestisid dan tanah yang tidak pernah diletakkan pestisid. Isolat 7 telah dipencilkan dari sampel tanah, tempat letak kereta, Hotel Marriot di Johor Bahru yang diketahui tidak pernah dicemari dan mempunyai sejarah penggunaan carbofuran. Penyaringan peringkat pertama menggunakan assay MTT (3-[4, 5-dimethylthiazol-2-yl]-,diphenyltetrazoliumbromide) telah menyaringkan sejumlah 148 isolat berdasarkan pertumbuhan bakteria. Tujuh isolat termasuk Isolat 2, 7, 9, 13, 21, 72 dan 138 mempunyai absorbans yang tinggi dari keputusan assay MTT. Isolat ini dipilih berdasarkan pembentukan warna biru formazan. Isolat tersebut kemudian diteruskan dengan penyaringan peringkat kedua yang menggunakan kaedah assay MTT dan kaedah pengiraan koloni bakteria. Lima isolat iaitu Isolat 2, 7, 9, 13 dan 21 dipilih kerana mempunyai jumlah koloni yang tinggi berdasarkan CFU/ml dan absorbans yang tinggi dari assay MTT. Keupayaan isolat tersebut untuk menguraikan carbofuran telah dikesan

menggunakan alat 'High performance Liquid Chromatography' dan Isolat 7 didapati mengurai carbofuran dengan kadar yang tertinggi, sebanyak 92.98% dari 100mg/L carbofuran di dalam kultur diperkaya carbofuran selama tempoh enam hari. Ini diikuti dengan Isolat 13 yang kadar penguraiannya sejumlah 55.8%, Isolat 21 sebanyak 54.09% dan Isolat 9 sebanyak 53.6% dan akhir sekali Isolat 2 mempunyai kadar yang terendah iaitu sebanyak 33.6%. Sampel kawalan tanpa bakteria mengalami penguraian sebanyak 13% menunjukkan carbofuran mengalami penguraian hasil dari tindakbalas kimia. Keadaan pertumbuhan yang optimum dikenalpasti untuk Isolat 7 berdasarkan jumlah koloni tertinggi pada agar nutrien. Glukosa sebagai sumber karbon dan 100 mg/L carbofuran didalam kultur diperkaya carbofuran adalah parameter yang optimum untuk pertumbuhannya. pH optimum kultur diperkaya carbofuran adalah pH 7 dan suhu optimum adalah 25 °C iaitu pada suhu bilik. Penguraian carbofuran oleh Isolat 7 dengan keadaan pertumbuhan yang optima berlaku lebih cepat selama dua hari, dimana kadar penguraian telah mencapai 93.05 % di dalam tempoh empat hari. Isolat 7 dikenalpasti menyerupai ciri-ciri *Bacillus* sp. kerana berbentuk rod dan mempunyai endospora dan juga menunjukkan ciri-ciri *Bacillus* sp. dari ujian biokimia. Enzim pengurai carbofuran menunjukkan nilai K_m ialah 0.9571 mM dan V_{max} pula 0.1142 mmole min^{-1} μg^{-1} terhadap carbofuran. Suhu optimum untuk aktiviti enzim adalah pada 40°C to 45°C dan pH pada 8 hingga 10. Analisis gel poliakrilimida 'Native' dan gel poliakrilimida SDS (sodium deodosil sulfat) menumjukkan protein masih tidak tulen dan peringkat penulenan yang seterusnya harus dijalankan bagi mendapatkan enzim tulen.

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
CFU	colony forming unit
DDT	dichlorodiphenyltrichloroethane
FAO	Food Agricultural Organisation (of the United Nations)
HPLC	High Performance Liquid Chromatography
Km	Michaelis Menten Constant
MADA	Muda Agricultural Development Authority
MARDI	Malaysian Agricultural Research and Development Institute
mAu*s	mili absorbance unit
MTT	3-[4, 5-dimethylthiazol-2-yl]-,diphenyltetrazoliumbromide
SAM	Sahabat Alam Malaysia
Vmax	maximum initial velocity
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Chemicals have a long history of being used as agricultural pesticides. Around 1000 B.C., an ancient Roman called Homer suggested that sulfur be used on certain plants to control insects while in the 1600s, ants were killed with mixtures of honey and arsenic (Bohmont, 2000). By the late nineteenth century, farmers in America were using Paris Green, a mixture of copper and arsenic to control insect pests in field crops. However, an emergence in pesticide use only began after World War II with the introduction of DDT, BHC, aldrin, dieldrin, endrin and 2, 4–D. These pesticides were inexpensive, effective and popular (Bohmont, 2000).

Unfortunately, the wide use of pesticides gave rise to undesirable side effects by polluting the environment and leaving residues in our food. Pesticides also threaten human health and animal life. This problem needs to be solved to prevent it from becoming worse. Carbofuran is one of the most widely used pesticides in Malaysia for controlling insects. Carbofuran pollutes the environment and according to studies, generates negative effects on animals such as fish and birds. Humans can also be affected because carbofuran has the potential to enter our drinking water via contamination of ground water. Once ingested, carbofuran is known to disrupt transmissions in the central and peripheral cholinergic nervous systems in vertebrates by inhibiting acetylcholinesterase activity (Sharma, 1986).

Bioremediation is the decomposition of non-naturally occurring man-made compounds (xenobiotics) by microorganism in the environment and in recent years,

this technology has generated a great deal of attention. Indigenous microorganisms or isolated microorganisms were used to decompose xenobiotics such as pesticides, petroleum, polyaromatic hydrocarbons (PAH), and heavy metals. Several techniques were invented for the bioremediation of pesticides and three main approaches have been proposed. First, the microorganism can be directly applied to degrade pollutants in a reactor or *in situ*. Secondly, *in situ* spiking of nutrients can be used to stimulate the growth of native microorganism capable of decontamination. Thirdly, cells extract or purified enzyme preparation of microbial origin could be used for decontamination (Chapalmadugu and Chaudry, 1992).

The hyphothesis of this research is that locally isolated bacteria could degrade carbofuran. The objectives of this study are firstly, to isolate and screen carbofuran degrading bacteria from previously treated and untreated Malaysian soil. Secondly is to identify and characterize the best carbofuran degrading bacterium. Thirdly is to partially purify the enzyme(s) involved in carbofuran biodegradation from the chosen bacterium.

CHAPTER 2

LITERATURE REVIEW

2.1 Pesticides

Pesticides are substances that kill or control pests such as insects, fungi, rodents, bacteria, weeds and algae. Pesticides can be categorized according to their primary target organisms such as insecticides, fungicides, rodenticides, bactericides, herbicides, algicides and others. Pesticide compounds can either be organic or inorganic in composition and their molecular structure is varied and complex. In the European Union for example, there are more than 700 different active compounds that were used in 1995 and more formulated products are produced since 1980. Man-made pesticides are synthetic products while biological pesticides are microbial agents such as *Bacillus thuriengiensis, Bacillus sphaericus* and a new strain called *Clostridium bifermentans.* The biological pesticides are used to control mosquito and fly from breeding such as *Simulium* spp.. Fish such as *Gambusia affinis, Poecilia reticulatus*, nematode worms such as *Romanomermis culicivorax* and fungi, *Lagenidium giganteum* are also used against disease vectors (Sulaiman, 1995).

Pesticides play a very important role in the world economy. It has been used in agriculture, forestry, industry, public health and households. The use of pesticides in agriculture has increased crop production by enhancing farm productivity and reducing losses in plantation by killing or controlling pests. This will increase the supply and export of agricultural products and improve the economy of a country