

UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF CARBOFURAN -DEGRADING BACTERIA FROM MALAYSIAN SOIL

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PM

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By

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Buat yang tersayang mak serta keluarga yang banyak memberi dorongan dan teristimewa buat suami tercinta yang sentiasa meniupkan semangat dalam diri ini. Jasa kalian tetap dalam ingatan.

-Fazilah



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

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Faculty : Biotechnology and Biomolecular Sciences

Bacteria isolates isolated from soil samples were screened for the ability to degrade Carbofuran. One hundred and fifty isolates isolated from various locations in Selangor, Perak and Johor were screened for the ability to degrade Carbofuran. Five isolates gave positive results with varying degrees of degradation. Based on these results, bacterial Isolate 147 *Pseudomonas* sp. isolated from Bukit Ekspo Universiti Putra Malaysia (UPM) Selangor campus was selected for further studies due to its ability to completely degrade Carbofuran in the minimum amount of time. This bacterium grew on Carbofuran as a sole source of nitrogen in a minimal salts medium. It showed the ability to degrade Carbofuran present in the minimal salts medium containing 100 mg/l Carbofuran as nitrogen source. MTT [3-(4,5-dimethyl-2thiazolyl)-2,5 diphenyl tetrazolium bromide] assay was used to screen bacteria capable of degrading pesticides indicated by the change in colour of the tetrazolium salt from yellow to blue. This bacterium reduced the tetrazolium salt which act as on electron acceptor for the formation of blue formazan. The growth optimization of Isolate 147 was investigated under various conditions. Optimum growth was obtained



at 30°C and pH 8 an additional carbon source such as glucose was needed to provide sufficient energy for the degradation to occur. It also grew abundantly in high concentration of 100mg/l Carbofuran. The degradation of Carbofuran by Isolate 147 was analysed using High Performance Liquid Chromatography (HPLC). This bacterium was able to degrade Carbofuran up to 93.03 % after six days incubation in the minimal salt medium. Enzyme produced by Isolate 147 during Carbofuran degradation was partially purified by 3.6 fold with ion exchange chromatography with an overall yield of 72.6%. It was shown to have a K_m value of 448.5 μ M, optimum activity at 30°C and pH 8.



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

PEMENCILAN DAN PENCIRIAN BAKTERIA YANG BOLEH MENGURAIKAN KARBOFURAN DARIPADA SUMBER TANAH DI MALAYSIA

Oleh

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Mac 2005

Pengerusi : Profesor Dr. Mohd Arif Syed, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Bakteria yang dipencilkan dari sampel tanah telah disaring untuk keupayaan mengurai racun perosak karbofuran. Satu ratus lima puluh kultur bakteria telah dipencilkan dari beberapa lokasi di Selangor, Perak dan Johor dan disaring untuk keupayaan mengurai karbofuran. Lima kultur pencilan telah memberikan keputusan positif yang berbezabeza tahap penguraiannya. Berdasarkan keputusan ini, satu kultur bakteria iaitu *Pseudomonas* sp. telah dikenalpasti (Isolat 147) yang telah di pencilkan dari sampel di Bukit Ekspo, kampus Universiti Putra Malaysia (UPM) Selangor telah dipilih untuk kajian seterusnya kerana keupayaanya mengurai karbofuran dalam masa yang tersingkat. Bakteria ini telah ditumbuhkan di dalam media diperkaya yang mengandungi 100 mg/L karbofuran dan ia telah berupaya menguraikan karbofuran sebagai sumber nitrogen untuk pertumbuhannya. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl tetrazolium bromide]assai telah dijalankan untuk memerhati dan memilih bakteria yang terbaik yang dapat menguraikan karbofuran akan menukarkan warna garam



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tetrazolium daripada warna kuning ke biru.. Ini kerana bakteria yang dapat menurunkan warna tersebut bertindak sebagai penerima elektron dan membentuk warna biru formazan. Pengoptimakan pertumbuhan bagi Isolat 147 telah dilakukan dengan pelbagai parameter seperti pH, suhu, kepekatan karbofuran dan sumber karbon. Didapati suhu dan pH optima untuk Isolat 147 ialah 30°C dan pH 8. Sumber karbon tambahan seperti glukosa diperlukan untuk membekalkan tenaga yang cukup bagi proses penguraian untuk berlaku. Kepekatan karbofuran yang paling sesuai untuk Isolat 147 ialah 100 mg/L. Analisis kadar penguraian karbofuran dilakukan menggunakan "High Performance Liquid Chromatography" (HPLC). Isolat 147 menunjukkan kadar penguraian karbofuran di dalam media diperkaya iaitu 93.03% selama 6 hari. Enzim yang telah dihasilkan oleh Isolat 147 semasa penguraian karbofuran telah di separa-tulenkan dan didapati hanya 3.6 fold dan sebanyak 72.64% berjaya ditulenkan. Ia mempunyai nilai K_m 448.5 µM dan aktiviti optimum pada 30°C dan pH 8.0.



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LIST OF ABBREVIATIONS

g	gram
HCl	Hydrochloric acid
H ₃ PO ₄	Orthophosphoric acid
H_2O_2	Hydrogen peroxide
K ₂ HPO ₄	dipotasium hydrogen phosphate
KH ₂ PO ₄	Potasium dihydrogen phosphate
KCl	Potasium chloride
Mw	Molecular weight
SDS	Sodium dodecyl sulphate
μg	Microgram
μΙ	Microliter
L	Liter
kD	KiloDalton
CFU	Colony Forming per Unit
rpm	Rotation per minutes



CHAPTER 1

INTRODUCTION

The term "pesticide" covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscacides, nematocides, plant growth regulators and others. Thus far, more than 1000 active substances have been incorporated in the approximately 35,000 preparations, which are known as pesticides. Insecticides represent the greatest proportion of pesticides used in developing countries, whereas herbicide sales have been greater than those of other pesticides in industrialized countries (Balasubramaniam, 1974).

Malaysia is largely an agricultural country and the use of pesticides is prevalent. In 1978, Malaysia has invested about RM 213 million on pesticide and on average the vegetable farmers spends around RM 200 million on pesticide for a half acre of vegetable farms yearly. In 1997, RM 326 million with herbicides still accounting for three quarters of the share at 75.1%. The market of herbicide itself was estimated at RM 245 million in 1997 compared to RM 200 million in 1993 (Source: Malaysian Agricultural Directory and Index, 1999/2000)

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With the significant increase in agrochemical usage in Malaysia, data have shown increases in human poisoning due to exposure to pesticides. The use of pesticides in Malaysia has caused serious concern. Over a ten year period (1979 – 1988), pesticides accounted for 40.3% of total number of 5152 cases of human poisoning in Malaysia (Jeyaratham, 1990)



When applied to crops in a plantation, pesticides can affect unintentional organisms and areas through environmental transport processes. Although not fully supported by facts, pesticide induced chronic toxicity is emerging as a public health concern including cancer, reproductive impairment and irreversible neurotoxicity (Shaw and Chadwick, 1998). There are many factors and processes affecting the environmental fate of pesticides after exposure to a cultivated field. Pesticide when applied to the soil will encounter degradation. Three major degradation processes are involved. They are microbial, chemical and photo degradation. Microbial degradation is the breakdown of pesticides by fungi, bacteria and other microorganisms that use pesticide as a nutrient source.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol N methylcarbamate) is used extensively as a pesticide in Malaysia. It is widely accepted because it acts quickly to kill unwanted pests (Hassall, 1990). When Carbofuran is sprayed on fields, it is meant to come into direct contact with the target organisms and kill. Then it acts very quickly usually within 20 minutes (McEwen, 1979). Carbofuran acts similar to that of organosphoshates by inhibiting the enzyme cholinesterase and causes death of the organisms (Soreq ,1993). It is highly toxic compound if inhaled and slightly toxic if absorbed transdermally (EXTOXNET, 1999). Once Carbofuran have been detected in the environment, they are classified as pollutants and this warrants a remediation step to clean up the environment.

One of the alternative methods is to use microorganisms to degrade or to transform nitrogenous compounds through aerobic or anaerobic processes. Insecticides bioremediation via enzymatic action is the focus of extensive study after the



isolation of a bacterial species (Dane *et al.*, 1993). The microbial degradation of a pesticide molecule has a great effect on the environmental fate and efficacy of the compound. So the load of environmental contamination caused by commercial application of pesticides can be reduced.

The main objectives of this research are:

- 1. To isolate bacteria from soil sample.
- 2. To identify the most active Carbofuran biodegrading bacteria in soil.
- 3. To partially purify the enzymes responsible for degrading Carbofuran.



CHAPTER 2

LITERATURE REVIEW

2.1 Pesticides Contamination in the Worldwide Environment

Pesticides are substances used for the destruction or control of pest. They include insecticides, herbicides, fungicides, rodenticides, molluscides and others. Pesticides may be of the broad-spectrum type that kills a wide range of organisms or the selective type, which destroys only one organism or a few specific organisms. In the early part of this century the increase in world population resulted in a greater demand for food. It has led to a substantial increase in the production and use for agrochemical such as pesticides and fertilizers (Hassall,1990)

Pesticides are known to contribute significantly in reducing losses and thereby increasing food production and quality. Past trend indicated, that the most of the pesticides used in the developing countries have frequently been employed in the control of vectors of human disease and on industrial export crops. Approximately 1000 pesticide formulations are used throughout the world today. The annual worldwide agriculture use of pesticides has been estimated to be in the order of 5 million tons, of which about 70% is used for agriculture and the remainder by public health agencies and government agencies for vector control and for domestic purposes (Shaw and Chadwick, 1998).



In the developing countries, particularly in North America, Western Europe and Japan, pesticides have come to play an extremely important role in the maintenance of high agriculture productivity. Despite the uses of pesticides, about 35% of the crops are lost from pests, diseases and weeds. Worldwide FAO estimates that an average of 38% of the cotton crop is saved from destruction by pests through the effective use of pesticides. However, pesticides have been known to affect a number of non-target organisms. It has been estimated that only 0.1% of the pesticides applied to crops reach the target pests and more than 99% of the applied pesticides affect non-target organisms (Shaw and Chadwick, 1998).

A good example is the decline in padi field fish populations due to increased pesticide usage, causing severe economic hardship and nutritional deficiencies among the poorer padi farmers. In the rice growing areas of central Thailand, farmers used the food irrigation method where when they drain the water from the rice paddies into the rivers and canals, carrying pesticides residues to nearby agricultural farms. In December 1982, this practice resulted in the complete destruction of the aquaculture industry in central Thailand. Over US\$10 million worth of fish were killed and about 5 million kilograms of fish were lost due to pesticides poisoning. This affected people throughout the country because fish is a major source of cheap protein.

Pesticides induced chronic toxicity is emerging as a public health concern including cancers, reproductive impairment and irreversible neurotoxicity. According to the World Health Organization (WHO), over half a million people are poisoned each year by pesticides and five thousand of the victims die (USEPA, 1987).



A study by Copplestone (1985) states that about 60-70 percent of all cases of unintentional acute poisoning cases due to occupational exposure. Those in developing countries such as Malaysia are especially at high risk due to inadequate education, training and safety systems.

2.2 Toxicology of Pesticides in General

Pesticides (whether insecticides, herbicides or fungicides) by their nature and purpose are poisons. Even if their amount is minimal in comparison to that of silt, their impact on the environment may be considerable. Since 1962, the used of pesticides in the Unites States has increased more than two fold. It now endangers groundwater quality in most of the States (Zakrzewski,2002)

Recently, concern about the effects of pesticides on human health and on the ecosystem began to move beyond cancer. It appears that some chlorinated hydrocarbon pesticides exert a multitude of toxic effects. These pesticides are neurotoxic, mutagenic and teratogenic. They exert their toxic effects on the reproductive system and suppressed the immune system. Davis *et al.*,. (1993) has been shown that these compounds act by mimicking or inhibiting estrogen receptors. It not only affect women's health but also believed to be responsible for a decrease in sperm count and a rise in testicular cancer in human as well as abnormal sexual development in some wildlife species.

