

ISOLATION, CHARACTERIZATION AND SCREENING OF CYPERMETHRIN-
DEGRADING BACTERIA FROM AGRICULTURAL SOIL

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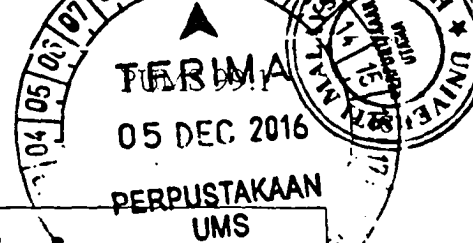
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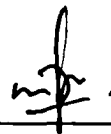
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DECLARATION

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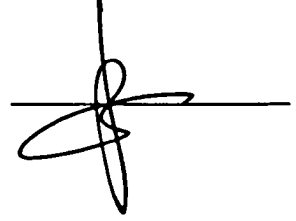
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Foremost, thank to Allah s.w.t. for giving me the patience and strength to finally complete my dissertation. Without His willing, I would not be here, writing this acknowledgement.

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ABSTRACT

Cypermethrin is a common pesticide that have been widely used since 1980's. Its toxicity toward aquatic organisms and humans requires its complete removal from contaminated areas which can done through bioremediation. The aims of this study were to isolate, characterize and screening the potential cypermethrin-degrading bacteria from the agricultural soil. The isolation technique was done through direct plating and about 10 strains were successfully isolated. They were identified as *Sphingomonas paucimobilis*-NADPL-CR1, *Pseudomonas oryzihabitans*-NADPL-CR2, *Staphylococcus aureus*-NADPL-CR3, *Staphylococcus aureus*-NADPL-CR4, *Pasteurella pneumotropica*-NADPL-CR5, *Pseudomonas luteola*-NADPL-CR6, *Pseudomonas aeruginosa*-NADPL-CR7, *Pseudomonas oryzihabitans*-NADPH-CR8, *Haemophilus influenza*-NADPH-CR9 and *Stenotrophomonas maltophilia*-NADPH-CR10. However, only *Staphylococcus aureus* and *Pseudomonas aeruginosa* were able to utilize cypermethrin as their carbon source. The growth of these two strains were tested at different initial cypermethrin concentration and pH in order to determine their optimum condition based on these two variables. *Staphylococcus aureus* had been recorded to grow better and higher in the presence of cypermethrin than *Pseudomonas aeruginosa*.

ABSTRAK

Cypermethrin merupakan racun perosak yang biasa digunakan secara meluas sejak tahun 1980-an lagi. Walaubagaimanapun, toksik yang terkandung di dalam cypermethrin mampu membahayakan hidupan akuatik dan manusia, menyebabkan ia perlu disingkirkan secara menyeluruh dari kawasan tercemar dan cara terbaik adalah melalui kaedah bioremidasi. Tujuan kajian ini adalah untuk pemencilan, pencirian dan penskrinan bakteria yang berpotensi mendegrad cypermethrin dari tanah pertanian. Teknik pemencilan kajian ini dilakukan melalui kaedah langsung dan terdapat 10 jenis bakteria yang telah berjaya dipencilkan. Mereka telah dikenal pasti sebagai *Sphingomonas paucimobilis*-NADPL-CR1, *Pseudomonas oryzihabitans*-NADPL-CR2, *Staphylococcus aureus*-NADPL-CR3, *Staphylococcus aureus*-NADPL-CR4, *Pasteurella pneumotropica*-NADPL-CR5, *Pseudomonas luteola*-NADPL-CR6, *Pseudomonas aeruginosa*-NADPL-CR7, *Pseudomonas oryzihabitans*-NADPH-CR8, *Haemophilus influenza*-NADPH-CR9 and *Stenotrophomonas maltophilia*-NADPH-CR10. Walaubagaimanapun, hanya *Staphylococcus aureus* dan *Pseudomonas aeruginosa* dapat memanfaatkan cypermethrin sebagai sumber karbon mereka. Pertumbuhan kedua-dua strain telah diuji pada kepekatan awal cypermethrin dan pH yang berbeza bagi menentukan keadaan optimum mereka berdasarkan dua pembolehubah tersebut. *Staphylococcus aureus* telah direkodkan sebagai bakteria yang mampu tumbuh dengan lebih baik dan lebih tinggi dalam keadaan dimana terdapat cypermethrin, berbanding dengan *Pseudomonas aeruginosa*.

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LIST OF SYMBOLS

%	percentage
°C	celsius
>	more
<	less
<i>et al.</i>	others
CFI/mL	Colony Forming Unit per millilitre
mL	millilitre
µg/L	microgram per litre
cm ³ / g	centimetre cube per gram
atm-m ³ /mol	atmospheric cubic meter per mol
Pa	Pascal
sp.	species
g	gram
rpm	revolution per minute
H ₂ O ₂	hydrogen peroxide
H ₂ S	hydrogen sulfide

LIST OF ABBREVIATION

API	Analytical Profile Index
OC	Organochlorine
OCP	Organochlorine Pesticide
OP	Organophosphate
OPP	Organophosphate Pesticide
SP	Synthetic pyrethroid
CP	Cypermethrin
DDT	Dichlorodiphenyltrichloroethane
DDD	Dichlorodiphenyldichloroethane
NADPL-CR	Nutrient Agar Direct Plating Lower Agricultural Land- Cream
NADPH-CR	Nutrient Agar Direct Plating High Agricultural Land- Cream
NA	Nutrient Agar
NB	Nutrient Broth
SIM	Sulfide Indole Motility

CHAPTER 1

INTRODUCTION

1.1 Introduction

Rapid increase in world population has increased the demand on food supply which eventually contribute to the risen of agricultural sector in many countries. In this world, approximately one third of the land is covered by agricultural areas (Sachs, 2012; Nijs, 2014) while in Malaysia, around 31% of its land is being arable (Aminuddin *et al.*, 1990). According to Olaniyi *et al.* (2013), the implementation of First to Third Malaysia Plan and the New Economic Policy (1965-1980) have given priority to agricultural development as an important role in economic growth and made agriculture as one of the main land use in Malaysia.

With the increase of agricultural sector, the use of chemical pesticides have also increased in sequence for the control of the pest populations and protection from diseases, in order to enhance the crop production (Yawar *et al.*, 2011; Gahukar, 2012; Abdelhafidh *et al.*, 2015). Maintaining the crops is a challenging part especially when it involves a large area of plantation and yet, pests are the main cause of crop damage (Matthews & Thomas, 2000). According to Murugesan *et al.* (2010) and Verma *et al.* (2014), without pesticides, there will be about 45% possible loss of food production around the world. Plus, the effectiveness of pesticides for better exploitation of plant species in the term of economic interest is well-recognized (Inceer *et al.*, 2009).

Though pesticides are highly beneficial to agricultural sector, the hazardous effects of pesticides to environment are undeniable. Pesticide is a well-known chemical pollutant that latter can contaminate the soil, surface and groundwater.

The environmental contamination occurs when the environment unable to naturally degrade the harmful substances that used by human in the surrounding. This pesticides pollution come from various sources that can be further categorized in either point source or non-point source. Many places have been polluted by pesticides due to their long and widespread of application in agriculture (El-Sheikh & Ashour, 2010). It is more dangerous when the toxicity of pesticides incorporate into the food chain. In some extreme cases, there are pesticides like DDT can even be detected in the human blood who live in the contaminated area (Tang *et al.*, 2014). Besides, pesticides are always considered as a potential mutagenic and carcinogenic agents to non-targeted organism (Inceer *et al.*, 2009). Thus, the concerning part of pesticides application is its effect to the wildlife and human health.

Application of pesticides in Malaysia is very active. As in Sabah, the use of pesticides are important in the plantation of rubber, cocoa and oil palm since 1955 (Conway, 1973). However, the widespread application of these pesticides in agriculture have increased the pollution level in Malaysia. Some hazardous and banned pesticides that belong to organochlorine pesticides (OCPs) have been detected in the environment around Malaysia, and found to cause pollution in the river, sediment and fish (Adbullah, 1995). Since the OCPs are recalcitrant to the environment, Malaysia has prohibited them from being used and have turned to less persistent pesticides like organophosphate (OP) and synthetic pyrethroid (SP) pesticides. However, cypermethrin (CP) which belongs to SP group has also found to contaminate the water body near the applied site (Halimah, 2013), though it is said to be the safest pesticides with less residue compared to OCPs and OPPs (Sukanya & Doss, 2013).

According to Ha *et al.* (2014), one of the global concern on the environmental pollution in recent days is the soil contamination in agriculture. Natural soil likely constitute broad metabolic variability with greatest reservoir of microbial diversity (Remenar *et al.*, 2015). The fertility of the soil is important for the crop production. Soil may takes thousands of years to form a good structure that suitable for agriculture but human activities can easily destroy it in a short period of time. The continuous and excessive use of pesticides have been proven to bring damage to the agricultural soils and their ecosystem (Herrero-Hernández *et al.*, 2013). Though some

pesticides like cypermethrin is said to bring less damage to the soil, this kind of pesticide with less adsorption to the soil, can easily contaminate water body through leaching and runoff.

Hence, remediation is needed in order to clean up the pesticide contamination, either in the soil or water body. In fact, the reasons of choosing pesticides in crop protection are made because they are said to be the most economic and provide environmental solution (Leake, 2000). Bioremediation by microorganisms has been recognized as a useful method to degrade many types of persistent pesticides (Geetika *et. al.*, 2013). It is generally considered to be safe, effective and cheap biological approach for pesticide elimination (Zhang *et al.*, 2010). Naturally, the applied pesticide can be degraded by microorganisms inside the soil (Aislabie & Lloyd-Jones, 1995). However, this natural process usually takes time as the process of natural biodegradation is very slow. Hence, the best and most effective way is by finding the suitable microorganisms that able to degrade any particular pesticide and later will able to be applied as bioremediation to pesticide pollution.

1.2 Objectives of Study

This study was carried out in order to determine the cypermethrin-degrading bacteria from the agricultural soil. The specific objectives of this study were:

- (i) To determine the bacterial population from the agricultural soil through direct culture.
- (ii) To isolate and characterize the potential cypermethrin-degrading bacteria.
- (iii) To screen the isolated cypermethrin-degrading bacteria.

1.3 Scope of Study

The scope of this study included the preparation of media and followed by the sampling of an active agricultural site at Kundasang, Sabah. Isolation of the CP-degrading bacteria had been carried out through direct technique. The pure cultures were characterized and screened for the CP-degrading bacteria. The potential CP degraders were further tested at different initial CP-concentration and pH. This biodegradation study on determining the optimal conditions for degrading CP will be performed through the carbon profile by using GC-MS.

1.4 Significance of Study

The findings of this study are important in microbial technology for the treatment of CP pollutant in the soil. Plus, bioremediation is an effective treatment to clean up the pesticide pollution in both aquatic and soil environment. Compare to chemical technology used in the pesticide treatment pollution, bioremediation is more environmental friendly.

The Environmental Relevant Microorganisms (ERM) that locally isolated is more useful to treat the polluted environment in Malaysia. Compare to foreign microorganism, the locally isolated microorganisms are actually having the higher performance and more effective in the local treatment pollution. Plus, CP has been widely used in many oil palm plantations for the control of the rhino beetle population (SGS, 2008; SIRIM, 2014) and the vegetable farms around Malaysia. Hence, the finding can be significantly useful to economic development in Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Pesticides

2.1.1 Definition of Pesticide

The word "pesticide" is said to come from a Latin word known as *pestis* which referred to plague or pest and *caedo* which means kill (Banaszkiewicz, 2010). In general, pesticide is defined as any substance used to protect the crops from the pests by many ways. According to Food and Agriculture Organization (FAO), any substance or mixture of substances that used to kill, repel or control the pest populations including the vector-borne disease, unwanted plants or animals species which can either cause harm or interfere the production, processing, storage, transporting or marketing of food, primary agricultural materials, animal feedstuffs or wood and wood products is known to be pesticide (Koh & Jeyaratnam, 1996). Particularly, it refers to the chemical substances that change the biological processes of the pests (Syed *et al.*, 2013).

2.1.2 Classification of Pesticides

Pesticides can be classified in various ways. Chemically, pesticides can be divided into two major groups which are inorganic and organic. Inorganic and organic pesticides are respectively refer to non-carbon containing substances and the carbon-containing substance that used as pesticides (Joshi, 2006). The inorganic pesticides were found to be used in the ancient era about thousand years ago and contain the elements or natural compounds like arsenic, mercury, sulphur and others (Redcliff, 2002) while most organic pesticides are usually synthetic though they can be produced naturally (Joshi, 2006). The inorganic forms are more toxic compare to the organic pesticides (Briggs *et al.*, 1992). Hence, their functions were then replaced

by the synthetic organic pesticides at which their widespread use started in 1940's, through the finding of successful effects of DDT and other pesticides (Redcliff, 2002).

Besides, pesticides can also be classified based on their targets or by either mode or time of actions (Arias *et al.*, 2008). For example are the fungicides that target the fungi, herbicides that target the weeds and insecticides that target the insects. Based on the meaning itself, known that pesticides have their own ways of actions as shown in Table 2.1.

Table 2.1 Classification of pesticides by various mode of actions (Arias *et al.*, 2008).

Type of Mode	Actions
Contact	Kills through contact with the pest
Stomach poison	Kills animal pest pests after ingestion
Fumigants	Enters pest as a gas
Non-selective	Toxic to both crop and weed
Selective	Toxic just to weed
Soil Sterilant	Toxic to all vegetation
Post-emergence	Effective when applied after crop or weed emergence
Pre-emergence	Effective when applied after planting and before crop or weed emergence
Pre-plant	Effective when applied before planting
Protectants	Effective when applied before pathogen infects plant
Eradicant	Effective after the pathogen infection
Systematic	Transported through crop or pest following absorption

However, the pesticides can be further classified based on their chemical family and active ingredient through their targets. Table 2.2 shows some examples of this type of classification.

2.1.3 Insecticides

Among all the synthetic pesticides, insecticides can be considered as the oldest pesticide produced in the world (Cioroiu *et al.*, 2013). There are four major classes of synthetic organic insecticides which known as organochlorines (OCs), organophosphates (OPs), carbamates and pyrethroids (Singh, 2007). Thousands of toxic OC, OP, carbamate and pyrethroid pesticides have been synthesized since 1930's due to revolution of agriculture and vector control (Mansingh, 2004).

(i) Organochlorine Pesticides (OCPs)

OCPs are referred to the chlorinated hydrocarbon compound that have been widely used since 1940's for their effectiveness in protecting the crops and combating the vector borne-disease (Betancur-Corredor *et al.*, 2015; Paramasivam *et al.*, 2015; Qu *et al.*, 2015). However, they are found to be ubiquitous in the environment due to their long half-life, which approximately around 4 to 35 years (Qu *et al.*, 2015). Plus, they are particularly characterized by high vapour pressure and strong lipophilicity which eventually toxic to both humans and animals as they can accumulate through the food chain (Tsakiris *et al.*, 2015). OCPs are classified as persistent organic pollutants (POPs) (Pavlikova *et al.*, 2015) which then, their production and use are restricted by the Stockholm Convention (Bosch *et al.*, 2015). However, before this treaty is signed, many countries have already prohibited their use in the early 1970's (Qu *et al.*, 2015; Mekonen *et al.*, 2015).

(ii) Organophosphate Pesticides (OPPs)

According to Ortiz-Hernandez & Sanchez-Salinas (2010), OPs are referred to the very heterogeneous compounds with a phosphoric acid derivative chemical structure. OPs are among the most widely used pesticides (Karimi-Mohajeri & Abdollahi, 2011) that covered the total world market of more than 36% (Baishya & Sarma, 2015). It was first introduced in 1940's through the production of parathion for use of crop protection. However, the popularity of OPPs have only risen after the diminished use of OCPs (Kamrin, 1997) and in fact, they are significantly produced in order to replace the OCPs (Baishya & Sarma, 2015). About more than 100 OP compounds are commercially available as pesticides (Ortiz-Hernandez & Sanchez-

2015). However, the widespread use and accumulation of PSs have increased the public concern with the regard to human health issues (Xiao *et al.*, 2015). According to Khanna *et al.* (2002), PSs have a low acute toxicity to the mammals but known to be potent neurotoxicants to both vertebrates and invertebrates.

2.1.4 Sources of Pesticides in Environment

Pesticides are one of the global anthropogenic pollutants as their occurrence in the environment are due to human activities. In general, they can enter the environment from two sources which known to either be point source or non-point source. Point sources of pollution are usually stationary and the source can be clearly identify (McGuire, 2012) while non-point source pollutions come from diffuse sources and have high variability associated with weather condition (Cestti *et al.*, 2003).

Though most pesticides contaminations come from point sources, around 50 to 70 % of the estimate surface water contaminations are non-point pollutions that caused by the agricultural activities and one of the common pollutants is pesticide (Ritter *et al.*, 2001). Table 2.3 shows the examples of pesticide contamination sources that usually found in the environment.

Table 2.3 Sources of pesticides

Sources of Pesticides	Examples	References
Point sources	Wastewater generated from the pesticide factories.	Faisal <i>et al.</i> , 2012;
	Vegetable washing facilities. Come from the farm due to spillage, leakage or any washing activities.	Ortiz-Hernandez & Sanchez-Salinax, 2010
Non-point sources	Movement of pesticides in the stream or sea after broad applications.	Neumann <i>et al.</i> , 2002;
	Rainfall-induced surface runoff.	Probst <i>et al.</i> , 2005;
	Input by drift, direct spraying or from the atmosphere in precipitation.	Waxman, 1998

2.1.5 Fate of Pesticides in the Environment

Generally, when pesticides are applied to the crops, less than 1 % of the pesticides reach the targeted pests while the other 99 % have lost to surrounding (Pimentel & Burgess, 2012). According to Llorent-Martínez *et al.* (2011), more than 98% of the insecticides and 95% of the herbicides sprayed do not reach the targeted species.

Fate of pesticides in the environment are characterized by some complex processes that take place in different environmental compartment like soil, plant, air, surface and groundwater (Queyrel *et al.*, 2016). The fate of pesticides is affected by the biotic and abiotic factors (Aislabie & Lloyd-Jones, 1995) that can be classified into a few major processes like the adsorption, transfer and degradation (Srivastava *et al.*, 2010). Transfer includes processes that move the pesticides away from the target site like volatilization, spray drift, runoff, leaching, absorption and crop removal.

Figure 2.1 shows some of the processes during the pesticides application which will eventually decide their fate in the environment.

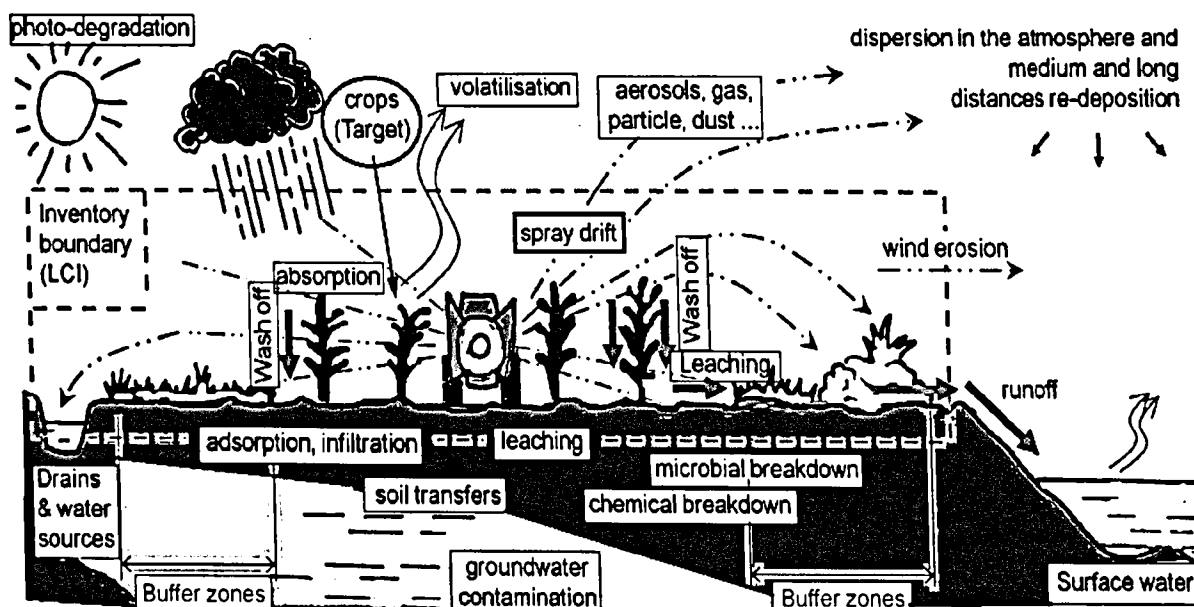


Figure 2.1 Examples of mechanisms from pesticides application to emissions in air, soil and water (Zelm *et al.*, 2014)

(i) Soils

Pesticides can enter the soil through either spray drift or direct application (Arias-Estevéz, *et al.*, 2008) and can be bounded to the soils due to the adsorption phenomenon. Adsorption is the binding of pesticides to the soil particles through the

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