



UNIVERSITI PUTRA MALAYSIA

***BACTERIA-BASED BIOPESTICIDES PRODUCTION USING SHRIMP
POND SLUDGE AS POTENTIAL CHEAP CULTURE MEDIA AND
ENTOMOTOXICITY ACTIVITY AGAINST FRUIT FLIES
(*Bactrocera dorsalis* HENDEL)***

NOORMASSHELA ULUL AZMI

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By

NOORMASSHELA BINTI ULUL AZMI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Science**

April 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science

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April 2015

Chair: Muskhazli Mustafa, PhD

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Biological controls of destructive pest in forest and agriculture sector using bacteria were proven to minimize the problems caused by the usage of chemical pesticides. However, their application is limited due to high production cost through fermentation. It is therefore necessary to develop potential alternatives culture media for a bacteria-based biopesticides production. In this study, shrimp pond sludge and wastewater were used as an alternate culture media for bacteria-based biopesticides production. The alternate media were prepared under three different preparations; without pre-treatment (unhydrolysed), with acid treatment (hydrolysed) and the supernatant obtained after the centrifugation of the hydrolysed sludge/wastewater. Five species of bacteria been tested for the bacteria-based biopesticides production were *Bacillus thuringiensis* ATCC33679, *Bacillus thuringiensis* ATCC10792, *Lysinibacillus sphaericus*, *Klebsiella pneumonia* and *Aeromonas hydrophilla*. The growth rate and sporulation were evaluated throughout the fermentation. *Bacillus thuringiensis* ATCC 10792 cultured in hydrolysed shrimp pond sludge were selected to be used for the bioassay due to the high growth rate ($7.44 \times 10^7 \pm 5.16$ CFU/ml) and sporulation ($5.90 \times 10^7 \pm 7.88$ CFU/ml). Hydrolysed shrimp pond sludge also significantly showed higher growth rate and sporulation compared to unhydrolysed and supernatant. However, variability in the growth rate and sporulation also revealed the preference and requirement of each bacteria to grow and sporulate depends on the species. Further, effectiveness on the usage of alternate culture media was also evaluated on their entomotoxicity level. The bioassay of entomotoxicity test was carried out on the fruit fly larva (*Bactrocera dorsalis* Hendel) and 81.2% mortality was observed. Larval weight ($0.019g \pm 0.001$) and size ($6.97mm \pm 0.97$) observed on the treated larvae showed significant deterioration compared to the control larvae (with $0.027g \pm 0.0003$ weight and $10.08mm \pm 0.22$ in sizes). As well as, the pupation was disrupted in terms of the smaller size of the pupa (30% smaller) and thus, leading to a lower adult fly emergence rate

(65.25%). Furthermore, emerged adult fruit fly showed some physical abnormalities on the morphology (undeveloped, crumpled wings and cranked abdomen) and significantly affecting the survival rate of the flies (100% adult flies died after 4 days emerged from the pupa). Since the *Bt*-based biopesticides have been proven to affect the target insect (*B. dorsalis* Hendel), toxin produced by the bacteria have been identified. Protein separation conducted by SDS-Page showed the existence of a band with approximately 25 kDa molecular weight. The single band was prepared for protein identification using MALDITOF and three highest protein hits were obtained. The three protein hits identified were superoxide dismutase, spore coat protein and probable transaldolase. Among these three proteins identified, spore coat protein possess direct role in virulence towards the target insect. Therefore, it is strongly believed that this protein is the one that exerted the toxicity activities on the target insect with the presence of Cyt protein. On the whole, this study has proven the potential of *Bt* ATCC10792 cultured in shrimp pond sludge are able to produce effective biopesticides.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

**PENGHASILAN RACUN SERANGGA PEROSAK BIOLOGI MENGGUNAKAN
ENAPAN KOLAM UDANG SEBAGAI KULTUR MEDIA MURAH BERPOTENSI
DAN AKTIVITI ENTOMOTOKSIK KE ATAS LALAT BUAH (*Bactrocera*
dorsalis HENDEL)**

Oleh

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Kawalan biologi ke atas serangga perosak dalam sektor perhutanan dan pertanian menggunakan bakteria telah terbukti meminimalkan masalah yang berpunca daripada racun perosak kimia. Namun, aplikasinya adalah terhad kerana kos penghasilan melalui fermentasi yang tinggi. Oleh itu, adalah menjadi satu keperluan untuk menghasilkan kultur media alternatif yang berpontensi untuk menghasilkan racun perosak biologi berasaskan bakteria. Dalam kajian ini, enapan kolam udang dan air buangan digunakan sebagai kultur media alternatif untuk penghasilan racun perosak biologi berasaskan bakteria. Media alternatif ini telah disediakan dalam tiga keadaan; tanpa prarawatan (tanpa hidrolisis), rawatan asid (terhidrolisis) dan supernatan yang diperolehi selepas pengemparan enapan/air buangan terhidrolisis. Lima spesies bakteria telah diuji sebagai penghasil racun perosak biologi berasaskan bakteria ialah *Bacillus thuringiensis* ATCC33679, *Bacillus thuringiensis* ATCC10792, *Lysinibacillus sphaericus*, *Klebsiella pneumonia* dan *Aeromonas hydrophilla*. Kadar pertumbuhan dan sporulasi bakteria telah dinilai sepanjang fermentasi. *Bacillus thuringiensis* ATCC 10792 yang dikultur dalam enapan kolam udang terhidrolisis telah dipilih untuk digunakan dalam bioassai berdasarkan sebab kadar pertumbuhan ($7.44 \times 10^7 \pm 5.16$ CFU/ml) dan sporulasinya ($5.90 \times 10^7 \pm 7.88$ CFU/ml) yang tinggi. Enapan kolam udang terhidrolisis juga jelas menunjukkan kadar pertumbuhan dan sporulasi yang lebih tinggi berbanding tanpa hidrolisis dan supernatan. Walau bagaimanapun, variasi dalam kadar pertumbuhan dan sporulasi juga menunjukkan bahawa setiap bakteria mempunyai keutamaan dan keperluan berbeza untuk pertumbuhan dan menghasilkan spora mengikut spesis. Seterusnya, keberkesanan penggunaan kultur media alternatif juga dinilai berdasarkan tahap entomotoksiknya. Bioassai ujian entomotoksik telah dijalankan ke atas larva lalat buah (*Bactrocera dorsalis* Hendel) dan sebanyak 81.2% mortaliti telah diperhatikan. Kemerosotan berat ($0.019g \pm 0.001$) dan saiz ($6.97mm \pm 0.97$) larva yang ketara telah diperhatikan pada larva yang dirawat berbanding larva kawalan (dengan berat $0.027g \pm 0.0003$ dan saiz $10.08mm \pm 0.22$). Di

samping itu, proses pupasi juga telah terganggu dengan saiz pupa yang lebih kecil (30% lebih kecil) dan kadar kemunculan lalat dewasa yang rendah (65.25%). Sementara itu, lalat buah dewasa yang telah terhasil menunjukkan beberapa keabnormalan fizikal pada morfologinya dan ianya telah memberi kesan ke atas kadar kemandirian lalat tersebut (100% lalat mati selepas 4 hari muncul dari pupa). Oleh kerana racun serangga biologi berasaskan *Bt* telah terbukti memberi kesan ke atas serangga sasaran (*B. dorsalis* Hendel), toksin yang dihasilkan oleh bakteria telah dikenalpasti. Pengasingan protein yang dijalankan dengan SDS-Page menunjukkan kewujudan satu jalur dengan anggaran berat molekul sebanyak 25 kDa. Jalur tunggal itu digunakan untuk mengenalpasti protein menggunakan MALDITOF dan tiga jenis protein tertinggi telah diperolehi. Tiga jenis protein yang telah dikenalpasti ialah superoksida dismutase, protein pembalut spora dan probable transaldolase. Antara tiga protein yang telah dikenalpasti ini, protein pembalut spora mempunyai peranan secara langsung didalam virulens terhadap serangga sasaran. Oleh sebab itu, adalah amat diyakini bahawa protein inilah yang telah mendorong aktiviti ketoksikan terhadap serangga sasaran dengan kehadiran protein Cyt. Secara keseluruhannya, kajian ini telah membuktikan potensi *Bt* ATCC10792 yang dikultur di dalam enapan kolam udang berupaya menghasilkan racun perosak biologi efektif.

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I certify that a Thesis Examination Committee has met on 10 April 2015 to conduct the final examination of Noormasshela Binti Ulul Azmi on her thesis entitled "Bacteria-based Biopesticides Production Using Shrimp Pond Sludge As Potential Cheap Culture Media And Entomotoxicity Activity Against Fruit Flies (*Bactrocera Dorsalis* Hendel)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
<i>Bt</i>	<i>Bacillus thuringiensis</i>
CFU/ml	Colony forming unit per milliliter
°C	Degree Celcius
δ	Delta
g	Gram
HCl	Hydrogen chloride
h	Hour
kDa	KiloDalton
LC ₅₀	Lethal concentration 50
μl	Micro liter
mg	Miligram
ml	Milliliter
mmol	Milimole
min	Minute
NCBI	National Center for Biotechnology Information
NA	Nutrient agar
NB	Nutrient broth
%	Percentage
R _f	Retention value
rpm	Round per minute
NaCl	Sodium chloride
spp.	Species (plural)
sp.	Species (singular)
H ₂ SO ₄	Sulphuric acid
V	Volt
v/v	Volume over volume
w/v	Weight over volume



CHAPTER 1

INTRODUCTION

Continuous rise in the number of population throughout the world has generated the demand of producing more foodstuffs for the global needs (Tirado-Montiel *et al.*, 2001). Agriculture and forestry sector has been developed rapidly as a critical resource to satisfy the demand (Glare *et al.*, 2012). Notwithstanding, in our urge to sustain and fulfil the requirement, some of the principal factors in these sectors have been causing damage to the environment. Most significantly, chemicals pesticides have been widely used in field crops for pest control and to increase crop yields.

Application of chemical pesticides to control pest are detrimental for the environment and produce considerable damage to the ecosystem (Wan *et al.*, 2013). Chemical pesticides are primarily difficult to degrade, able to accumulate in the environment and consequently may contaminate water sources and foodstuffs (Alberola *et al.*, 1999). On top of that, some chemical pesticides can act on the non-target organism specifically the human. Van der Werf (1996) stated that due to their inherent toxicity, pesticides may affect other organisms by triggering detrimental side-effects and subsequently affects the whole communities and ecosystem. Thus, the use of chemical pesticides present some serious side effects even though it is known as an effective way to increase the percentage of higher yield.

These days, consumers have given careful consideration to the potential wellbeing effect on the usage of chemical pesticides in the production of food sources and their contamination towards environment (Czaja *et al.*, 2014). Hence, there is an urgent need to discover an alternative approach to avoid the usage of these chemical pesticides. Biological control appears to constitute an alternative strategy for controlling pest, in this manner decreasing the application of chemical pesticides and contributing to the preservation of the nature (Souto *et al.*, 2004).

Fundamentally, biopesticides usually exert less toxic than chemical pesticides. It generally can only affect the target organism and close-related organism, rather than the broad range, conventional chemical pesticides (Gupta and Dikshit, 2010). Biopesticides regularly are powerful in small amounts and decay rapidly. Thus, avoiding the environmental contamination caused by chemical pesticides since the exposures towards environment are reduced.

A biological pesticide is compelling just on the off chance that it has a potential significant effect towards the target pest, market size, successful field performance, low cost production, opinion from the end-user and various technological challenges such as culture condition, formulation and conveyance frameworks (Smitha *et al.*, 2013). Cost for development, time and simplicity of registering and impending rising market on the contrary to

chemical pesticides make biopesticides intriguingly advocates to develop (Brar *et al.*, 2006). Hence, to use biopesticides effectively, proper preparation and planning is important to consistently maintain the performance of the biopesticides.

A number of biopesticides (virus, bacteria, fungi, plant extracts and pheromones) have been commonly used to control different species of pest in forestry and agricultural sector (Copping and Menn, 2000). *Bacillus pumilus*, *B. thuringiensis*, *B. subtilis*, *Streptomyces* spp., and *Pseudomonas* spp. are among the bacteria that successfully used as biopesticides (Raudales and Gardener, 2008). Despite the increased interest in biologicals for biopesticides in recent years, Vu *et al.* (2010) revealed that biopesticides have not yet be able to replace chemical pesticides in pesticide market.

Obviously, there are some reasons contribute to the weak penetration of biopesticides in the market. For instance, toxicity exerted by biopesticides is slow action and remain only for a short time (Lachhab *et al.*, 2001). Lack of studies on their mode of action also affects the marketing strategies. Other than that, economically there are various issues in production, high cost and short expiry duration.

In commercial bacteria-based biopesticides production, 30-40% from the total cost is the cost for the raw materials depending on the capacity of the production (El-Bendary, 2006; Chang *et al.*, 2007). Subsequently, local biopesticides production in evolving countries should rely upon the usage of raw materials that are cheap, easily accessible such as by-products from agriculture or industrial sector (Fadel and Sabour, 2002). El-Bendary (2006) also reported that for a large scale production of biopesticides, distinctive methodologies ought to be examined to develop alternate media that are able to sustain sporulation and production of toxin in sensible costs.

A number of reported studies utilized alternative media for the bacteria based biopesticides production as the substrate were successfully replaced with wastewater sludge and agro-industrial wastes (rice straw, wheat bran, corn steep liquor, maize starch, etc.) (Tirado-Montiel *et al.*, 2001; Lachhab *et al.*, 2001; Vidyarthi *et al.*, 2002; Brar *et al.*, 2004; Yezza *et al.*, 2004, 2005; Zhuang *et al.*, 2010). In fact, application of wastewater as an alternative raw material in generating value-added products is a lot better than the conventional management of sludge disposal such as landfilling and incineration (Zhuang *et al.*, 2011). Wastewater provides a good source of nutrients (nitrogen, carbon, phosphorus, etc) for the microbial biological processes that validated its potential to be used as alternate fermentation media for the production of biopesticides, specifically bacteria-based biopesticides (Vidyarthi *et al.*, 2002; Yezza *et al.*, 2004, 2005, 2006; Brar *et al.*, 2009).

On the other hand, shrimp pond sludge is other option for the high-cost production problems of bacteria-based biopesticides. Periodic discharge of shrimp pond sludge to maintain the water quality in the pond is one of critical issues feared as the discharged effluent bring along high concentrations of suspended solids and nutrients, predominantly nitrogen (N) that can disrupt the ecosystem (Jackson *et al.*, 2003). The release of high loads of nutrients and

suspended solids can possibly have unfavorable consequences on the receiving waters causing algal blooming and anoxic conditions (Islam *et al.*, 2004).

Hence, using sludge or wastewater as a raw material for the production of biopesticide offers a practical clean-up alternative for sludge or wastewater disposal, and provides an economical production as alternative media for commercialization. The potential of shrimp pond sludge and wastewater as alternative media for biopesticides production needed to be explored and developed in our quest to find the solution of the high-cost production on the raw materials.

This study aims to evaluate the effectiveness of the bacteria-based biopesticides production in alternative culture media against the target insect. This study was divided into three experiments, each with its own objective. Five strains of bacteria were selected as potential bacteria-based biopesticides. Evaluation on growth and sporulation of each bacteria in the alternative culture media (shrimp pond sludge and wastewater) was conducted to proceed with the bacteria-based biopesticides production on the target pest, the larva of fruit-flies (*Bactrocera dorsalis* Hendel). Once the entomotoxicity level of the bacteria-based biopesticides have been determined and justified, identification of the bioactive compound produce by the bacteria-based biopesticides that was conducted.

Therefore, our objectives are:

1. to evaluate the growth performance and sporulation of bacteria in different alternative culture media.
2. to determine the entomotoxicity activity of the selected bacteria-based biopesticides and alternative culture media against the target insect, the larva of fruit-flies (*Bactrocera dorsalis* Hendel).
3. to identify the bioactive compound in the bacteria-based biopesticides produced.

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