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BIOLOGICAL CONTROL OF SUBTERRANEAN TERMITE, Coptotermes curvignathus (ISOPTERA: RHINOTERMITIDAE) USING INDIGENOUS BACTERIA FROM ITS GUT AND FORAGING PATHWAY

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By

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Chairman : Associate Professor H'ng Paik San, PhD
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The subterranean termite, *Coptotermes curvignathus*, is one of the most prominent plantation pests that feed exclusively from lignocellulose diets. The lignocellulose digestion of termite is made possible by host-secreted enzymes, specialized gut physiology and microbial gut symbionts. Since termites are very dependant on these microbes to digest the food for survival, termite can be controlled by disturbing the micro-ecology of termite gut by using its own bacteria. Through this study, screening and identification of culturable aerobic bacteria among communities from the gut and foraging pathway of *C. curvignathus* was carried out. Since these bacteria can also lead to pathological states upon reaching favourable conditions, the susceptibility of *C. curvignathus* was evaluated for opportunistic pathogen using culturable aerobic bacteria isolated from termite gut and its foraging pathways. A total of 24 bacteria species have been identified mainly from the family Enterobacteriaceae by using Biolog Gen III. Overall, the bacteria species in the termite gut differ from those of foraging pathway within same location except for *Acinetobacter baumannii* which was the only bacteria species found in both habitats. *Coptotermes curvignathus* was found to be susceptible to both *Microbacterium* sp. and *Serratia marcescens* in varied concentrations by using ingestion and physical contact method under laboratory conditions. *Serratia marcescens* showed the highest mortality of 66.4% and 59.2% at concentrations of 10^9 CFU/mL and 10^6 CFU/mL in ingestion method and was closely followed by *Microbacterium* sp. (65.2%) at concentrations of 10^9 CFU/mL. While on median lethal study, *Pseudomonas aeruginosa* had the effective LC_{50} (10^7 CFU/mL) and LT_{50} at concentration 10^{12} CFU/mL (5 days) under laboratory conditions. *Serratia marcescens* and *P. aeruginosa* was applied onto soil, filter paper and wood. It can be concluded that *S. marcescens* with filter paper application method gave the highest termite mortality (100% in 10^{12} CFU/mL). Furthermore, the ability for bacteria to self-sustain on wood and filter paper is low. *Serratia marcescens* is a chitin degrading bacteria. Nutrient (chitin) was treated on filter paper to allow the growth of *S. marcescens*. From observation, *S.*

marcescens survived longer in chitin-treated filter paper. The termite was forage on chitin-treated filter paper with *S. marcescens*. These results are a valuable tool for the biocontrol of termite. Conclusively, subterranean termite, *C. curvignathus* can be controlled by using indigenous bacteria from its gut and foraging pathway.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAWALAN BIOLOGI TERHADAP ANAI-ANAI, *Coptotermes curvignathus* (ISOPTERA: RHINOTERMITIDAE) DENGAN MENGGUNAKAN BAKTERIA DARI PERUTNYA DAN TAPAK LALUAN

Oleh

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Anai-anai, *Coptotermes curvignathus* merupakan perosak utama bagi tanaman di ladang. Anai-anai ini mendapat khasiat khususnya daripada lignoselulosa tumbuhan. Penguraian lignoselulosa oleh *C. curvignathus* dilakukan oleh enzim dari perutnya melalui simbion dan mikrob yang terdapat dalam ususnya. Maka kajian ini dijalankan untuk mengenal pasti bakteria aerobik dari usus *C. curvignathus* dan sekitar tapak laluan anai-anai. Apabila bakteria ini dalam keadaan yang sesuai serta CFU yang tinggi, ia dapat memberi kesan patologi terhadap anai-anai. Sejumlah 24 bakteria telah dikenal pasti dengan menggunakan Biolog Gen III dan kebanyakannya adalah daripada keluarga Enterobacteriaceae. Secara keseluruhannya, spesies bakteria dalam usus *C. curvignathus* adalah berbeza daripada spesies bakteria di tapak laluan kecuali *Acinetobacter baumannii* yang merupakan satu-satunya spesies bakteria yang ditemui di kedua-dua habitat. *Serratia marcescens* menunjukkan peratusan kematian tertinggi iaitu 66.4 % dan 59.2 % pada kepekatan 10^9 CFU/mL dan 10^6 CFU /mL dalam keadah makan dan ini diikuti dengan *Microbacterium* sp. (65.2 %) pada kepekatan 10^9 CFU/mL. Selain itu, *Pseudomonas aeruginosa* menunjukkan LC_{50} (10^7 CFU/mL) and LT_{50} yang paling efektif pada kepekatan 10^{12} CFU/mL (5 hari). Peratusan kematian *C. curvignathus* terhadap kayu, tanah, dan kertas penapis yang telah dirawat dengan *S. marcescens* dan *P. aeruginosa* turut dijalankan. Dapat disimpulkan bahawa kertas penapis yang dirawat dengan *S. marcescens* memberi kesan patologi yang paling efektif kepada anai-anai (kematian 100% pada 10^{12} CFU/mL) berbanding dengan kayu dan tanah. Selain itu, keupayaan bakteria untuk mengekalkan jangka hayat atas kayu dan kertas penapis adalah rendah. *Serratia marcescens* merupakan bakteria yang boleh mendegradasikan kitin. Oleh itu, kertas penapis yang telah dirawat dengan kitin dapat memanjangkan pertumbuhan *S. marcescens*. Pemerhatian menujukkan bahawa *C. curvignathus* memakan *S. marcescens* atas kertas penapis yang telah dirawat oleh kitin. Kesimpulannya, anai-anai, *C. curvignathus* dapat dikawal

secara biologi dengan menggunakan bakteria yang ditemui dari usus dan tapak laluan anai-anai.

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I certify that a Thesis Examination Committee has met on 16 November 2016 to conduct the final examination of Wong Wan Zhen on her thesis entitled "Biological Control of Subterranean Termite, *Coptotermes curvignathus* (Isoptera: Rhinotermitidae) using Indigenous Bacteria from its Gut and Foraging Pathway**"** 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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
AWPA	American Wood Protection Association
Bt	Bacillus thuringiensis
$CaCl.2H_2O$	Calcium chloride dehydrate
CCFP	Colloidal chitin treated filter paper
CCFPSS	Colloidal chitin treated filter paper with *S. marcescens*
CDA	Chitin degradation agar
CFU	Colony forming unit
CMC	Carboxymethylcellulose
$CuSO_4.5H_2O$	Copper(II) sulfate pentahydrate
d	Days
DNA	Deoxyribonucleic acid
EBI	Eicosanoid biosynthesis inhibitors
EI	Enzymatic index
G	Relative centrifugal force
h	Hour
H_2BO_3	Dihydrogen borate
HCl	Hydrogen chloride
Ifr	*Isaria fumosorosea*
KCl	Potassium chloride
KOH	Potassium hydroxide
K_2HPO_4	Dipotassium phosphate
LB	Luria bertani
LC_{50}	Lethal concentration 50
LGM	Lembaga Getah Malaysia
LSD	Fisher's Least Significant Difference
LT_{50}	Lethal time 50
$MgSO_4$	Magnesium sulfate
min	Minute
$MnSO_4.H_2O$	Manganese(II) Sulfate Monohydrate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
Na_2MoO_4	Sodium molybdate
NFB	Semisolid malate
OGF	Open grass land and foraging pathway
OGG	Open grass land and termite gut
PCR	Polymerase chain reaction
RPF	Rubber plantation and foraging pathway
RPG	Rubber plantation and termite gut
µL	Microliter
UPM	Universiti Putra Malaysia
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

1.1 General background

In recent decades, there has been a steady increase in the amount of pesticides used. The usage of pesticides in a variety of pest management situations including commercial farming, lawn care/landscaping, forestry, and wood preservation are consequences of pest's habitat destruction by human activity (Resh and Carde, 2009). According to the report from Environmental Protection Agency (EPA), about 5 billion pounds of pesticide's active ingredient were used worldwide and out of that amount, approximately 1 billion pounds are used to control termite (Arthur *et al*., 2011). In the warmer regions of the world, termites are the major wood-destroying structural pests.

Thus, a huge amount of repair costs have been spent to suppress the wood feeding termite. In terms of economic loss, around USD 40 billion has been spent annually on repairing the destroyed wood of building, forest and other commercial products responsible by termites (Rust and Su, 2012). Synthetic pesticide such as chlorpyrifos, bifenthrin, imidacloprid, endosulfan, and lindane remains as the primary methods used to prevent and suppress termite attack on wooden materials and plantation (Su, 1994). However, the excessive use of chemicals on controlling termites is a serious environmental concern. This situation was showed by bans on the use of many organochlorines by United Nations Environment Program (UNEP) and Food and Agriculture Organization (FAO) as there are harmful to human health (FAO, 2016; Monica *et al*., 2009).

As a consequence of these developments, the focus in termite management has shifted alternatively to biological methods. Biological control (biocontrol) can be defined as the control or reduction of damage caused by termites due to declining in the number and destructive activity of termites using the entomopathogenic microorganisms or with the product of a natural biological process. Biocontrol constitutes a more environmentally acceptable alternative to chemical control. When successfully implemented, it can yield permanent, cost-effective management of pest populations with minimal environmental disturbance (Culliney and Grace, 2000). The earliest biocontrol used on termites was reported by Beal and Kais in 1962, which identified *Aspergillus flavus* link as a fungal pathogen of *Reticulitermes* sp. (Chouvenc *et al*., 2011).

The entomopathogenic microorganisms that has an antagonistic effect on killing termites belong to different genera, i.e., *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Enterobacter*, *Micrococcus*, *Neisseria*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Kanchana *et al*., 2007; Yuvraj, 2007; Osbrink *et al*., 2001). Numerous studies showed the ability of different entomopathogenic microorganisms to cause the death of termites (Chouvenc, *et al*., 2011; Grace, 2003). The use of nematode and fungus as biocontrol agents against termite became a common trend. Entomopathogenic fungus such as *Metarhizium anisopliaes* that act as a parasite to the termites has shown promise in suppressing termite growth (Milner and Staples 1996; Gunner, 1994). On the other hand, entomopathogenic nematodes such as Steinernematid and Heterorhabditid have received great attention in the 1990s as biopesticides. Nematodes have the advantage of being easy to apply, compatible with many pesticides, and finding their hosts either actively or passively (Changlu *et al*., 2002; Smart, 1995). Field studies have proven that *Heterorhabditis* sp. is able to remove termite colony in plantation and wooden structure (Lenz, 2005).

1.2 Statement of problem

The formidable defenses systems of termites continue to evolve in order to fend off attempts of infection by entomopathogenic microorganisms particularly nematodes and fungus. Unsatisfactory results of entomopathogenic nematodes and fungi as pest control agents are reported by Shapiro-Ilan et al. (2002 and 2012). Entomopathogenic nematodes and fungi although exhibit different attacking system on termites, both have similar infection which is via penetration essentially on the host cuticle (Shapiro-Ilan *et al*., 2006). Termite's cuticle itself is a highly heterogeneous structure that can vary greatly in composition even during the various life-stages. Usually, mechanisms that are involved in resistance to fungi are defense mechanisms. Chouvenc *et al*. (2009) demonstrated the importance of cellular encapsulation in termites as a defense mechanism against the propagation of the fungal hyphae into the host hemocoel. The cellular encapsulation was found to be omnipresent in cases of cuticle penetration by the fungus and the sclerotized nodule resulting from the encapsulation was efficient in blocking the fungus from invading the hemocoel. The other common problems with the use of nematodes are their bioavailability and efficacy (Gaugler, 1988). Gaugler (1988) noted the striking differences in efficacy that exist between Steinernematid and Heterorhabditid nematodes and that various species of the respective families have differing effects depending on the host infected. Epsky and Capinera (1988) also reported an ability of the subterranean termite *Reticulitermes tibialis* to detect and avoid *Steinernema feltiae*.

Furthermore, efficiency of entomopathogenic nematodes and fungi locating, infecting and killing an insect are all profoundly affected by the host (Gouge *et al*., 1999). Entomopathogenic nematodes and fungi differ in their abilities to survive different environmental conditions. The ability to penetrate soil, method of host attack, and ability to handle environmental extremes are the key factors for satisfactory biological control results. Entomopathogenic nematodes are living organisms, and both biotic and abiotic factors can be detrimental during applications. The nematode and fungi efficacy only can be enhanced by specific matching of the most appropriate environmental factors, e.g., temperature, humidity, and sunlight, which are likely to affect adhesion, germination, growth (and penetration) on insect cuticles and their components (Shapiro-Ilan *et al*., 2010). Thus, another entomopathogenic microorganism should be introduced which co-exist together with termites under same environment condition as biological control for termites.

Beside the urgent need to search for new entomopathogenic microorganism, the suitable application method of the new bio-pesticides should be carried out as well. Most of the research on termite biological control has followed the concepts of classical biological control (Lacey *et al*., 2001; Ferron, 1978). Due to the cryptic habitat and social organization of termites, biological control in termites had to be modified from strategies used in agricultural crops. Inundative biological control refers to the release of overwhelming numbers of a mass-produced biological control agent in the expectation of achieving a rapid reduction of a pest population without necessarily achieving continuing impact. This method was used for termite species commonly with a central nest structure, one-piece nesting type, or intermediate nesting type (Abe, 1987). Unfortunately, the inundative method is not realistic for termite species with a diffuse nest structure (extended nesting type), such as subterranean termites, because only a small fraction of the colony is accessible. Occurrence of an epizootic in subterranean termite species relies upon transmission of the pathogenic agent among all individuals in the colony, which is difficult due to avoidance of the treated areas by healthy individuals (Shapiro-Ilan *et al*., 2012).

1.3 Justification

The selective pressures on the pathogen and the target host have led to a co-evolutionary arms race that is increasingly being recognized as having led to some startling outcomes especially bacteria as bio-pesticides. The emerging of entomopathogenic bacteria as an alternative biopesticide in controlling pest, particularly in the field of agriculture and forestry has fascinated scientists. This is because they can be easily mass produced and at a lower cost and co-exist together with termites (Roy *et al*., 2009). Termites depend upon the microbes in their gut or digestive tract to digest the complex sugars in wood into simpler

molecules as food and bacteria is one of the three microbes groups exist in termite guts.

Most of the antagonistic bacterial for termite research focused on *Bacillus thuringiensis* (Bt). The potential of bacteria to reduce termite populations in laboratory scale has proven favorable (Roy *et al*., 2009). However, field trials showed microbes have narrow environmental tolerances, limited mobility and thus are unable to self-sustain. This mainly related to the usage of exotic microbes on controlling termites (Lacey *et al*., 2001). Besides, the termite's immune response, social behavior and group living also contribute significantly to disease resistance (Rosengaus *et al*., 2011). These inconsistent performances are major constraint to their widespread use as biocontrol agents in commercial agriculture. Usually, biocontrol agents from areas of soil, water and also disease plant are being searched to control exotic species. However, it's not easy to introduce an exogenous bacteria to a new locale where they do not occur naturally. This is due to their survivability and self-sustainability (bioavailability) on field as mentioned earlier. To overcome the constraints mentioned, the search for pathogenic bacteria from the bacteria flora that originate from within termite and their surrounding might (call indigenous bacteria) increase the bacterial tolerance to environmental factor. An increased tolerance could lead to improved efficiency in reducing termite pest.

In this study, the indigenous bacteria from *C. curvignathus* guts and foraging pathway are screened and identified. This species of termite is among the most important lignocelluloses digesting insects. They depend upon the microbes in their gut or digestive tract to digest the complex sugars in wood into simpler molecules that they can use for food. Termite gut contains a lot of beneficial microbes which can digest cellulose such as *Bacillus*, *Paenibaccillus*, *Streptomyces*, *Actinobacteria* group, and *Klebsiella* (Konig *et al*., 2002). Since termites are very dependant on these microbes to digest the food to survive, in this case, termite can be controlled by disturbing the micro-ecology of termite gut by using its own bacteria. One of the methods is to increase the colony forming unit of the bacteria inside the guts itself. This will increase the horizontal transmission among termite and will enhance colony mortality. However, increased concentration might decrease the time to death of infected termite. These resulting infected termites do not have the time to return to the central nest. Thus, the effective method to apply the entomopathogenic bacteria isolated from the termite guts and foraging pathway are studied. It was important to know whether indigenous pathogenic bacteria would infect the termite either through oral or through physical contact to the infected area. If termite can be infected through physical contact with the bacteria, pathogenic bacteria can be applied onto the soil.

Therefore, in this study, the biocontrol of termite (*Coptotermes curvignathus*) was carried out using the bacteria located of inside the termite guts and foraging pathway to reduce the environmental condition effects. The isolated bacteria were screened for its pathogenic effect towards termite. Those identified entomopathogenic bacteria were applied into the termite colony using two different methods; oral and physical contact under laboratory conditions.

1.4 Objectives

The overall aim of this study was to determine the potential of indigenous bacteria isolated from termite guts and its foraging pathway as biocontrol agent against subterranean termites *Coptotermes curvignathus*.

The specific objectives of this study were:

1. To identify of the culturable aerobic bacteria isolated from the gut of *C. curvignathus* and its surrounding feeding area (foraging pathway) by using the phenotypic fingerprint with Biolog's extensive species library.

2. To evaluate the susceptibility of *C. curvignathus* to opportunistic pathogen using the identified bacteria isolated from termite gut and its foraging pathways by using ingestion method and physical contact method.

3. To determine the Lethal effects (LC_{50} and LT_{50}) of entomopathogenic bacteria identified on the *C. curvignathus.*

4. To determine the efficacy of entomopathogen bacteria applied on three different growth media against *C. curvignathus*

5. To develop biocontrol method via bait system using *S. marcescens* applied on filter paper as bait against *C. curvignathus*.

1.5 Scope of study

Chapter 1 gives a general background and justification of the study. Chapter 2 illustrates a comprehensive literature on the termite biology, host specificity of entomopathogenic bacteria to termites, strategies for the use of

entomopathogenic bacteria against termites, behavior and abiotic factors which enhance or limit the potential for epizootic spread, together with the combination use of microbial and microbes with synthetic pesticide.

Chapter 3 introduces the bacteria occurrence from the gut of *C. curvignathus* and its surrounding feeding area (foraging pathway) by isolation of culturable aerobic bacteria. A general morphological and biochemical (cellulose and nitrogen) characteristics of isolated strain from termites guts and foraging pathway was determined. Chapter 4 presents the susceptibility of *C. curvignathus* to opportunistic pathogen using bacteria isolated from chapter 3. Bacterial suspensions were prepared in varied concentrations and diagnosed with two application method. A biocontrol score was developed to determine the ability of bacteria with lowest possible concentration to induce high mortality for termite. Then, the lethal effect (LC_{50} and LT_{50}) of five bacteria which occupied the highest biocontrol score were determined.

Two bacteria with strongest lethal effect identified in chapter 4 were used in chapter 5. The bacteria were treated on filter paper, soil and wood. The survival condition of *C. curvignathus* toward the bacteria treated filter paper, soil and wood were summarized in chapter 5. Chapter 6 presents the evaluation on survival rate and biochemical characteristic of *Serratia marcescens.* Besides, we improved the growth media (filter paper) with chitin. Then, we observed on the interaction of biocontrol agent, *S. marcescens* with *C. curvignathus.* Finally, chapter 7 concludes the study and provides recommendations for further work.

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