MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES

By

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ABSTRACT

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES

Chok Siow Shein

Bacillus thuringiensis is a rod-shaped, spore-forming Gram-positive soil bacterium that forms parasporal crystal proteins during its sporulation phase. These crystal proteins, which comprise of Crystal (Cry) and Cytolytic (Cyt) proteins are able to exhibit toxicity to different insect orders, making them a valuable tool as bioinsecticide. In addition, it was found that the presence of chitinase in *Bacillus thuringiensis* can increase the toxicity to its target organism. On the other hand, certain *Bacillus thuringiensis* strains produce non-insecticidal crystal proteins called parasporin, which was found to exhibit cancer-killing properties, making them a potential candidate for cancer treatment. Thus, the objectives of this study were to characterize the isolated *Bacillus thuringiensis* via morphological identification, electron microscopy, SDS-PAGE analysis and screening of δ-endotoxins and chitinase genes through PCR amplification. All of the bacterial isolates R1, R2, R3, D1C, A2B and C6D were shown to be Gram-positive, endospore-forming bacilli that are able to produce spherical-shaped crystal proteins. Moreover, ovoidal-shaped crystal proteins were observed in R2, R3, D1C and C6D whereas cuboidal-shaped crystal proteins were found in R1 and R2. In SDS-PAGE analysis, all bacterial

isolates exhibited CytA, Cry35, Cry2, Cry3 and Cry11 protein bands. In addition, R1, R3 and A2B isolates were found to exhibit the Cyt2 protein band.

The 94-kDa protein band was observed in R3 and A2B whereas only R1 exhibited Cry34 protein band. In PCR analysis, all bacterial isolates were found to carry *cryI*, *cryII*, *cryII*, *cry10*, *cry11* and *cyt2* genes except R2, which was the only bacterial isolate that did not carry *cryI* genes. The presence of *cryIV* gene was detected in R1 and A2B. All in all, all bacterial isolates were found to carry the Diptera-specific δ-endotoxins genes. No presence of chitinase and parasporin genes can be detected suggesting that all bacterial isolates are non-cytotoxic but insecticidal strains.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Chok Siow Shein

APPROVAL SHEET

The project report entitled **"MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES"** was prepared by CHOK SIOW SHEIN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **CHOK SIOW SHEIN** (ID No: **15ADB06716**) has completed this final year project entitled **"MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIGENOUS *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES"** under the supervision of Ms. ALICIA HO LAI YEE from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Your truly,

(CHOK SIOW SHEIN)

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LIST OF ABBREVIATIONS

A260/A280	Assessment of Nucleic Acid Purity
APN	Aminopeptidase
APS	Ammonium Persulphate
bp	Base pair
Bt	*Bacillus thuringiensis*
Btk	*Bacillus thuringiensis kurstaki*
Bti	*Bacillus thuringiensis israelensis*
CBB	Coomassie Brilliant Blue
Cry protein	Crystal protein
Cyt protein	Cytolytic protein
δ-endotoxin	Delta-endotoxin
g	Gravity
GPI-anchored	Glycophosphatidylinositol-anchored
kDa	Kilo-Dalton
PAGE	Polyacrylamide Gel Electrophoresis
PS	Parasporin
rpm	Revolutions per minute
SEM	Scanning Electron Microscope
SDS	Sodium Dodecyl Sulphate
TEM	Transmission Electron Microscope
TEMED	Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

Every year, insect-borne diseases such as dengue, yellow fever, malaria, filariasis and Japanese encephalitis have contributed significantly to the millions of deaths in more than half of the world's population (El-Kersh, et al., 2016). To date, dengue recorded the highest number of infections in Malaysia since 2015, making it the top of the list of infectious diseases in this country (Hilmi, 2017). In the absence of specific anti-viral treatments and effective vaccines for most of the insect-borne diseases, the effective alleviation of these problems are often crucially relied on insects control programs (Oestergaard, et al., 2007).

Over the years, chemical pesticide is commonly used for the control of insect vectors. Chemical pesticides have demonstrated effectiveness in controlling insect-borne diseases and increasing agricultural productivity. However, the extensive and intensive usage of chemical insecticides have led to several burdens, including increase in human health concerns, such as immune system disorders and cancers, disruption of natural-biological control systems, development of insect resistance and environmental pollution (Schnepf, et al., 1998; Sanchis and Bourguet, 2008; El-Kersh, et al., 2016). Thus, sourcing for alternative methods for vector control is therefore crucial in reducing these problems.

One of the alternatives to substitute chemical compounds for vector control is microbial insecticides. Over the last 6 decades, *Bacillus thuringiensis* has been preferably used as a biological insecticide due to its potency in controlling wide range of agriculture-pests, high host specificity, effectiveness in the control of wide range of agriculture-pests and human disease vectors and it is non-pathogenic to humans (Starnes, Liu and Marrone, 1993). Furthermore, *Bacillus thuringiensis* is able to prevent the development of resistance of pesticides in insects, making them a popular alternative agent of biological control for insect pests (Osman, et al., 2015; El-Kersh, et al., 2016). Hence, screening of new *Bacillus thuringiensis* strains with increased level of insecticidal toxicity with border spectrum of activity have attract the interest of researchers.

Bacillus thuringiensis is a Gram-positive, spore-forming soil bacterium that can be commonly found in soil. The insecticidal activity of this bacterium is mainly due to its ability to produce large amounts of proteins called parasporal crystal proteins during the sporulation phase. These proteins produced by *Bacillus thuringiensis* exhibit insect-killing effects to different insects order such as Lepidopterans, Dipteran, and Coleopterans (Palma, et al., 2014). When a susceptible insect ingests these proteins, they are processed by the alkaline condition in the midgut of the larvae and converted into toxic form (Höfte and Whiteley, 1989). These toxins bind to receptors specifically in the epithelial insect midgut and produce pores, eventually causing the death of the insect.

The parasporal crystal proteins are produced by *Bacillus thuringiensis* alongside the endospore in a sporangium (Sauka, et al., 2010) (**Figure 1.1**). These parasporal crystal proteins are also known as δ-endotoxin. The insecticidal crystal proteins are categorized into two families, Crystal (Cry) and Cytotoxic (Cyt) proteins. Despite the differences in sequence homology, both toxins appear to work synergistically in exerting insect-killing effect. Moreover, it was found that Cry and Cyt proteins interact synergistically in the insect gut to prevent microbial insecticides resistance in insects (Juárez-Pérez, et al., 2002). Recently, it has been reported that the presence of chitinase in *Bacillus thuringiensis* may also increase its insecticidal toxicity by forming perforation at the peritrophic membrane barrier to enhance Cry proteins invasion into epithelial membranes (El-Kersh, et al., 2016; Honda, et al., 2017).

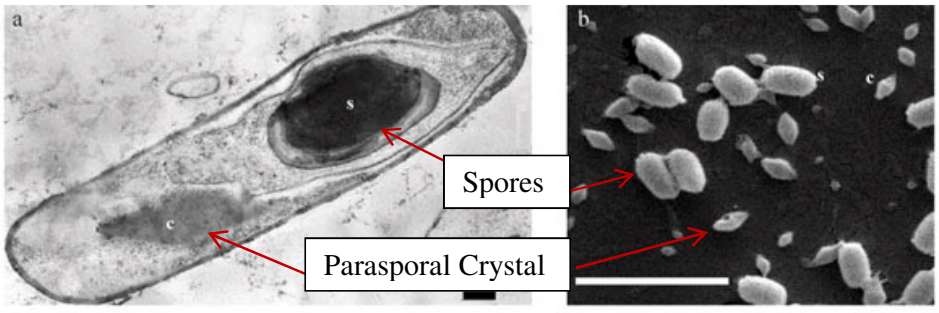

Figure 1.1: Spores and crystals proteins are observed in electron micrographs. (a) Transmission electron microscopy (b) Scanning electron microscopy (Adapted from Sauka, et al., 2010).

Other than Cry and Cyt proteins that give *Bacillus thuringiensis* its insecticidal activity, some *Bacillus thuringiensis* produce non-insecticidal crystal proteins, which were shown to be able to exhibit cytocidal activity against human cancer cells (Mizuki, et al., 1999). The non-insecticidal crystal proteins are called parasporin. It was found that parasporins show strong cytocidal activity against liver cancer cells (HepG2), cervix cancer cells (HeLa), lung cancer cells (A549) and colon cancer cells (CACO-2) (Mizuki, et al., 1999; Ito, et al., 2004; Nagamatsu, et al., 2010). Moreover, parasporins are also able to differentiate between normal and leukemic T cells. Like insecticidal crystal proteins, parasporins are pore-forming protein that increases permeability of plasma membrane of target cells by formation of pore and cause lysis in cancer cells.

Because of the cytotoxic effect on human cancer cells, *Bacillus thuringiensis* has also become a popular research topic in cancer study. The cytocidal potential of parasporins against human cancer cells may lead to their usage as candidates for cancer treatments (Mizuki, et al., 1999). Thus, screening tests for novel anticancer parasporal proteins from *Bacillus thuringiensis* are being carried out (Ohba, Mizuki and Uemori, 2009). Previous study has reported that the natural *Bacillus thuringiensis* populations in Malaysia have shown anticancer activities (Nadarajah, et al., 2008). However, information on the diversity and distribution of parasporin genes among these non-insecticidal *Bacillus thuringiensis* from Malaysia have yet to be discovered.

Therefore, the aims of this research are:

1. To characterize *Bacillus thuringiensis* isolates through morphological identification.

2. To examine the crystals formation in the *Bacillus thuringiensis* isolates using scanning and transmission electron microscopes.

3. To determine the molecular weight of proteins extracted from the *Bacillus thuringiensis* isolates using SDS-PAGE.

4. To investigate the presence of *cry, cyt*, *ps* and *chi* genes in the *Bacillus thuringiensis* isolates through polymerase chain reaction (PCR).

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Bacillus thuringiensis*

Bacillus thuringiensis which was initially named *Bacillus sotto*, was isolated from silkworms in 1901 by a Japanese scientist named Shigetane Ishiwata, who investigated the causative agent of Sotto disease which cause the death of Japan silkworms (Ishiwata, 1901; Cannon, 1995). This bacterium was then rediscovered by a German biologist named Ernst Berliner in 1911, who isolated the bacterium from infected flour moth in Mediterranean. The species name "thuringiensis" was derived from the German state Thuringia, where the moth larvae were found, thus giving the bacterium the name *Bacillus thuringiensis.* Berliner studied the bacterium and found a crystal inclusion body present within the sporulated cells of the bacterium. However, he did not correlate the presence of the inclusion body directly to its pathogenicity (Berliner, 1915; Cannon, 1995). A few years later, Mattes (1927) isolated the *Bacillus thuringiensis* strain discovered by Berliner (1915) and found that this bacterium was able to exhibit toxicity against the corn borer in Europe, but it was not until much later in 1953 when researchers discovered that the insecticidal activity was caused by the inclusion bodies and not the bacterium itself. Hannay (1953) referred the inclusion bodies as parasporal crystal proteins and together with Fitz-James, they demonstrated the insecticidal activity of these pathogenic inclusion bodies

and proposed that they are composed of proteins (Ibrahim, et al., 2010). It was thought that many years before the bacterium was identified, the insecticidal potential of *Bacillus thuringiensis* was already recognized, as some accounts recommended that during ancient Egypt, *Bacillus thuringiensis* spores may have already been in use as an insecticidal agent (Sanahuja, et al., 2011).

2.2 Habitat of *Bacillus thuringiensis*

Bacillus thuringiensis strains can be isolated from many habitats. It is reported that the bacterium seems to be indigenous to many environments. Researchers have suggested that *Bacillus thuringiensis* is an ubiquitous soil microorganism, thus it is normally found in the soil (Dulmage and Aizawa, 1982; Martin and Travers, 1989; Bernhard, et al., 1997). In early work by DeLucca, Simonson and Larson (1981), they observed that 0.5% of more than 46,000 bacterial isolates from various soils in the United States were made up of *Bacillus thuringiensis*. Similarily, Ammouneh, et al., (2011) isolated *Bacillus thuringiensis* from 12.5% of soil samples from Syria which are Lepidoptera-active. In 1989, Martin and Travers compared different *Bacillus thuringiensis* subspecies isolated from 785 soil samples from 30 countries and reported that *Bacillus thuringiensis israelensis* as the most common subspecies. They also claimed that Asia has the most abundant *Bacillus thuringiensis* among the 30 countries they studied and *Bacillus thuringiensis* subspecies *kurstaki* is the most common type in Asia (Martin and Travers, 1989).

Moreover, various subspecies of *Bacillus thuringiensis* that are Lepidoptera, Diptera and Coleoptera-active were also isolated from insects' cadavers. Researchers have isolated *Bacillus thuringiensis* from deceased silk worm, larvae of flour moth, tobacco beetles and also mosquitoes (Berliner, 1915; Kaelin, Morel and Gadani, 1994; Asokan, 2007; Renganathan, et al., 2011). Besides that, various *Bacillus thuringiensis* have been discovered from stored product such as animal feed mills, dried grain residues, dried tobacco residues, stored product dust and mushroom compost (Bernhard, et al., 1997). Kaelin, Morel and Gadani (1994) suggested that the protected and dry stored-environment gave an ecological niche for this bacterium, which was also supported by Meadows, et al. (1992). Interestingly, some researchers proposed that *Bacillus thuringiensis* seems to more commonly isolated in stored products or insects cadavers than in soil (Schnepf, et al., 1998). For instance, Bernhard, et al. (1997) have reported that 45% of *Bacillus thuringiensis* isolates were originated from stored products dust, with only 25% originated from soil. Also, in a study by Meadows, et al. (1992), *Bacillus thuringiensis* was successfully isolated from dried tobacco residues but not in tobacco field soil samples.

Other than that, some researchers have isolated *Bacillus thuringiensis* from conifer trees, phylloplane of deciduous as well as some horticultural crops (Smith and Couche, 1991; Asokan, 2007). Coleopteran-active and Lepidopteran-active *Bacillus thuringiensis* can also be found in leaf surface. In

1991, Smith and Couche (1991) discovered *Bacillus thuringiensis* from surfaces of leaf and suggested that presence of *Bacillus thuringiensis* on phylloplane may be considered as a part of the microflora of many plants. It was later found that *Bacillus thuringiensis* can cause infection of leaf-feeding insect larvae (Smith and Couche, 1991).

2.3 Taxonomy of *Bacillus thuringiensis*

Bacillus thuringiensis have been placed under the *Bacillus cereus* group, which members included *Bacillus cereus, Bacillus weihenstephanensis*, *Bacillus pseudomycoides Bacillus mycoides, Bacillus anthracis* and *Bacillus thuringiensis.* Previous studies have suggested that *Bacillus thuringiensis, Bacillus anthracis* and *Bacillus cereus* should be classified as a single bacterial species (Gordon, Haynes and Pang, 1973; Helgason, et al., 2000). However, sufficient genetic discrimination has been obtained from other studies to differentiate between *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* (Harrell, Andersen and Wilson, 1995; Radnedge, et al., 2003; Chang, et al., 2003).

Moreover, their distinctive pathogenic features also gave a separate species status for these bacteria (Priest, et al., 2004). *Bacillus cereus* is an opportunistic human pathogen that causes eye infection, periodontal diseases and food poisoning. *Bacillus anthracis* is the etiological agent of the lethal disease in

human and animals known as anthrax while *Bacillus thuringiensis* is a bacterium capable of surviving in the midgut and hemocel of insects and produces entomocidal parasporal crystal proteins (Ibrahim, et al., 2010). *Bacillus thuringiensis* and *Bacillus cereus* share many phenotypic characteristics include nutritional requirement, ampicillin resistance and motility. However, *Bacillus thuringiensis* produces parasporal crystal proteins during sporulation phase, making it the only characteristic that can differentiate *Bacillus thuringiensis* from *Bacillus cereus* (Vilas-Boas, Peruca and Arantes, 2007).

2.4 *Bacillus thuringiensis* δ-endotoxins

During sporulation, *Bacillus thuringiensis* produce parasporal crystalline proteins which are toxic against different insect orders. The proteins can be divided into two major types: Crystal (Cry) and Cytolytic (Cyt) proteins, which are also called δ-endotoxins (**Figure 2.1**). Cry proteins are defined as parasporal crystalline proteins produced by *Bacillus thuringiensis* that exhibit toxicity to a target organism. On the other hand, parasporal inclusions that exhibit hemolytic (cytolytic) activity are called Cyt proteins. Both toxins are innocuous to plants, vertebrates and human but highly specific to their target insects (Jisha, Smitha and Benjamin, 2013).

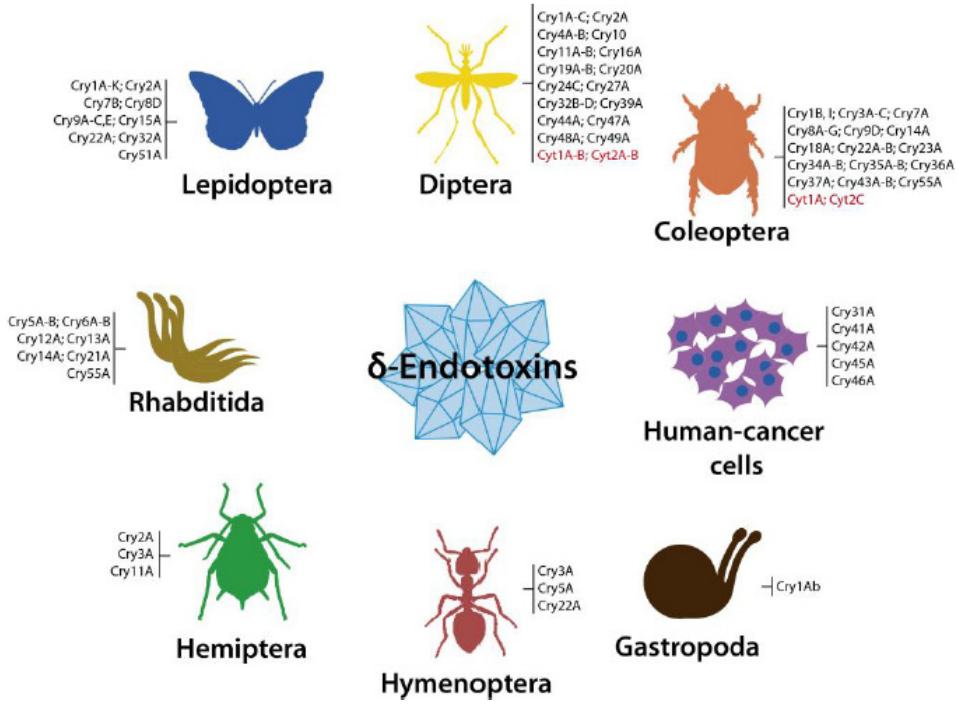

Figure 2.1: Overview of the known host range of *Bacillus thuringiensis* **δ-endotoxins** (Adapted from Palma, et al., 2014).

2.4.1 Crystal (Cry) and Cytolytic (Cyt) Proteins

The first systemic classification and nomenclature for crystal (Cry) proteins of *Bacillus thuringiensis* was based on the insecticidal activity and the number of amino acids proposed by Höfte and Whiteley (1989). Four main classes of Cry proteins were identified: Cry1, Cry2, Cry3 and Cry4, and the genes were designated by Romanized numericals (I-IV): *cryI, cryII, cryIII* and *cryIV*. Generally, *cryI* genes encoded for Cry1 proteins that are toxic against Lepidopteran, *cryII* genes encoded for Cry2 proteins that are toxic against Lepidopteran and Dipterans, *cryIII* genes encoded for Cry3 proteins that are toxic against Coleoptera and *cryIV* genes encoded for Cry4 proteins that are

toxic against Dipterans (Höfte and Whiteley, 1989). Until the year 2012, Crickmore, et al. (1998) have reclassified the Cry proteins based on their DNA profiles and their modes of action. To date, there are 74 classes of Cry proteins (Cry1 to Cry74) that have been identified (*Bacillus thuringiensis* Toxin Nomenclature Committee, 2017) and each individual toxin was reported to show specific toxicity against Lepidopterans, Dipterans, Coleopterans, Rhabditids, Hemipterans, Nematodes, Hymenopteran and Gastropods (Palma, et al., 2014).

Cry1 protein is encoded by the *cryI* genes and has the molecular weight range from 130 kDa to 140 kDa (Cerstiaens, et al., 2001). When Cry1 proteins are ingested by insect larvae, the solubilized inactive protoxin will be cleaved into active toxin with the size range from 60 kDa to 70 kDa. It was reported that *Bacillus thuringiensis morrisoni*, *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis aizawai* carry the *cryI* gene which encodes the Cry1 proteins. The Cry2 protein is 65 kDa in size and enzymatically converted into the size of 60 kDa to 62 kDa when ingested by insect larvae (Liang, et al., 2011). It was found that *Bacillus thuringiensis tolworthi, Bacillus thuringiensis kenyae* and *Bacillus thuringiensis kurstaki* carry the *cryII* genes (Yamamoto, 1983; Moar, et al., 1994). Studies found several subspecies of *Bacillus thuringiensis* that carry *cryIII* gene, which include *Bacillus thuringiensis san diego* and *Bacillus thuringiensis tenebrionis* (Krieg, et al., 1983; Herrnstadt, et al., 1987). The *cryIII* gene encode for Cry3 protein which is 72 kDa in size. Like other crystal proteins, Cry3 proteins are enzymatically converted into the size of 66 kDa

when ingested by insect larvae (McPherson, et al., 1988). Cry4 proteins can be categorized into 4 types: Cry4A (135 kDa), Cry4B (128 kDa), Cry4C (78 kDa) and Cry4D proteins (72 kDa). According to Crickmore, et al. (1998), Cry4C and Cry4D proteins are also referred as Cry10 and Cry11 proteins respectively. It was found that the Cry4A and Cry4B proteins were enzymatically converted into smaller 53 kDa to 78 kDa fragments whereas Cry4C and Cry4D were cleaved into fragments of 58 kDa and 30 kDa respectively, which subsequently exhibit toxic action against target insects (Höfte and Whiteley, 1989). To date, only *Bacillus thuringiensis israelensis* was reported to carry the *cryIV* genes (Sen, et al., 1988; Crickmore, et al., 1998).

Cytolytic (Cyt) proteins are categorized into three different groups: Cyt 1, Cyt2 and Cyt3 (*Bacillus thuringiensis* Toxin Nomenclature Committee, 2017). Unlike the Cry proteins, the genes that encode for the Cyt proteins were designated by numericals (*cyt1*, *cyt2* and *cyt3*). The Cyt proteins are 27 kDa in size and were found to exhibit toxicity against insects in the orders of Diptera, Coleoptera and also Lepidoptera (Höfte and Whiteley, 1989). Interestingly, it was found that the *Bacillus thuringiensis* that produce Cyt proteins, for instance *Bacillus thuringiensis israelensis, Bacillus thuringiensis morrisoni* and *Bacillus thuringiensis medellin* exhibit higher toxicity effect against its target insect through the synergistic interactions with Cry proteins (Pérez, et al., 2005; Palma, et al., 2014).

2.4.2 Mode of Action of Cry and Cyt Proteins

Mode of action of the Cry and Cyt proteins involves several events after their ingestion by insect larvae (Jisha, Smitha and Benjamin, 2013). After the ingestion of crystal proteins, the inactive protoxins are initially solubilized by the alkaline condition in the larvae midgut, then converted into toxic core fragments through the action of protease (Höfte and Whiteley, 1989; Jisha, Smitha and Benjamin, 2013). For Cry proteins, the activated toxins interact with receptors specifically found on the microvilli on the epithelial membranes of the midgut cells such as glycosylphosphatidylinositol (GPI)-anchored aminopeptidase-N (APN), cadherin-like protein (CADR), and glycoconjugate (Rosas-García, 2009; Jisha, Smitha and Benjamin, 2013), which result in the pre-pore oligomeric structure formation. On the other hand, Cyt proteins do not interact with the specific receptors but bind to the membrane lipids directly and cause pores formation (Bravo, Gill and Soberon, 2007; Jisha, Smitha and Benjamin, 2013). Formations of pores cause destruction of cells and eventually cause the death of the larvae. The irreversible formation of toxins and its binding site determine the degree of the toxicity of δ-endotoxins (**Figure 2.2**) (Schünemann, et al., 2012).

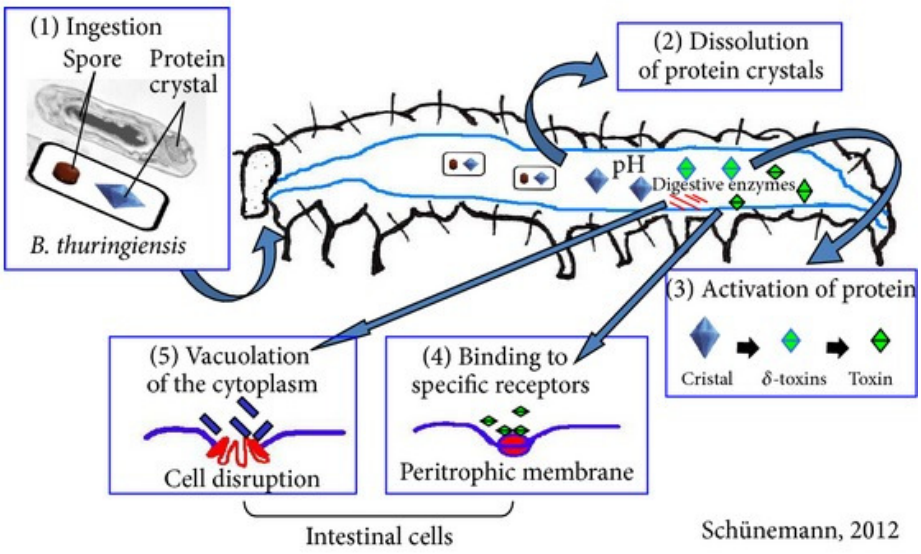

Figure 2.2: Mechanism of crystal proteins of *Bacillus thuringiensis* in larvae of Lepidopteran (Adapted from Schünemann, et al., 2012).

Recently, it has been reported that the presence of Cyt toxins play an important role in preventing resistance to Cry toxins in target insects (Bravo, Gill and Soberon, 2007). It was found that Cyt toxins interact synergistically towards Cry toxins as Cyt toxin functions as receptor molecules, which enhance the binding of Cry toxins to target membranes and cause toxic effect to the insects (Pérez, et al., 2005). Therefore, Cyt proteins may be practical in prevention of resistance in insects and to enhance toxicity of biological insecticides.

2.4.3 Parasporins

For many years, it was believed that the *Bacillus thuringiensis* acquired its insecticidal activity through a host-parasite relationship with the insects. However in natural environments, *Bacillus thuringiensis* isolates with non-insecticidal Cry proteins outnumber the insecticidal ones (Hastowo, Lay and Ohba, 1992; Maeda, et al., 2000; Lee, et al., 2003). This causes researchers to believe that *Bacillus thuringiensis* is not an obligate insect pathogen but an environmental saprophyte (Ohba, Mizuki and Uemori, 2009). As such, researchers hypothesized that non-insecticidal *Bacillus thuringiensis* may have novel biological activities other than insecticidal activity, which leads to the discovery of parasporins, that were found to exhibit toxicity against human cancer cells (Mizuki, et al., 1999; Ohba, Mizuki and Uemori, 2009).

Parasporin was first discovered by Mizuki, et al. (1999), who did a large scale screening of 1744 *Bacillus thuringiensis* isolates from Japan for their cytotoxicity potential against human leukemic T cells (MOLT-4). They discovered 42 strains that were neither insecticidal nor hemolytical, but able to kill leukemic T cells, thus proposing that this is due to parasporal protein inclusions of certain non-insecticidal *Bacillus thuringiensis* strains. The protein was named parasporin and distinguished as Cry category. Some Cry proteins produced by non-insecticidal *Bacillus thuringiensis* such as Cry31, Cry46, Cry41, Cry45, Cry63 and Cry64 exhibit specific and strong cell-killing effect against many human cancer cells (Ohba, Mizuki and Uemori, 2009). Moreover, due to its unexpected remarkable cytotoxicity, the Parasporin Classification and

Nomenclature was established in 2006, and parasporin was defined as parasporal proteins of *Bacillus thuringiensis* that are non-hemolytic but capable of exhibiting toxicity to human cancer cells (Okumura, et al., 2011).

Like Cry protein, parasporin also exhibit cytotoxicity only when digested by proteases such as proteinase K and trypsin (Mizuki, et al., 1999). In 2010, Parasporin Classification and Nomenclature Committee have identified 19 parasporins and they were classified into six families based on amino acid identity: parasporin-1 (PS1), parasporin-2 (PS2), parasporin-3 (PS3), and parasporin-4 (PS4), parasporin-5 (PS5) and parasporin-6 (PS6) (Ohba, Mizuki and Uemori, 2009). In terms of their structure, they can be categorized into two types: three-domain type and pore-forming-toxin (β-PFT) type. PS1, PS3 and PS6 are three-domain type parasporin. Similarly to Cry toxin, they have a three-domain structure. Initially, they are first produced with molecular weight of 80 kDa and are converted to active forms of about 60–70 kDa (Katayama, et al., 2005). On the other hand, PS2, PS4 and PS5 are β-pore-forming-toxin (β-PFT) type. They have significantly similar structure to the aerolysin-type β-PFT, which act as cytolysins via pore-formation (Xu, et al., 2014). They are first produced with molecular weight of 31–37 kDa and are then converted to active forms of about 27–30 kDa (Kitada, et al., 2006, Ekino, et al., 2014).

After Mizuki, et al. (1999), several other studies were perofrmed to evaluate the cytocidal activity of *Bacillus thuringiensis* isolates. It was discovered that activated parasporin of certain *Bacillus thuringiensis* exhibit strong cytotoxicity against hepatocyte cancer cells (HepG2), lung cancer cells (A549), uterus cervix cancer cells (HeLa) and colon cancer cells (CACO-2) (**Figure 2.3**) (Mizuki, et al., 1999; Ito, et al., 2004; Nagamatsu et al., 2010). Moreover, it was also reported that parasporins were able to differentiate between normal T cells and leukemic T cells, which may lead to the usage of *Bacillus thuringiensis* parasporin as potential cancer treatment (Mizuki, et al., 1999). In addition, it has been proven in recent studies that the natural *Bacillus thuringiensis* populations in Japan, Canada, Vietnam and Malaysia have parasporin activities (Yasutake, et al., 2007; Nadarajah, et al., 2008; Uemori, et al., 2008).

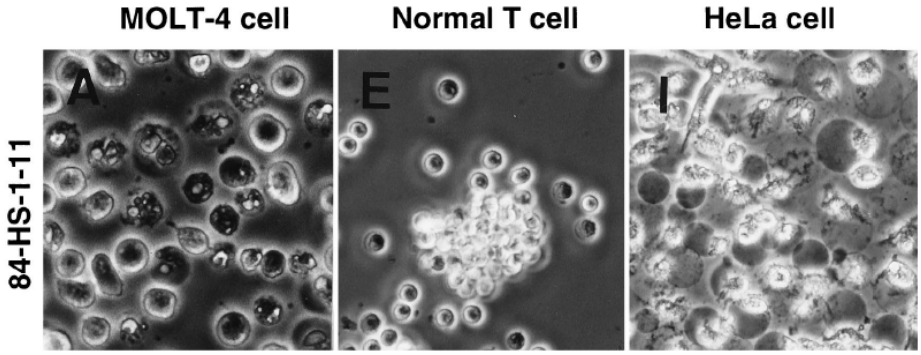

Figure 2.3: Cytocidal activity of parasporal inclusion proteins of *B. thuringiensis* on MOLT-4 cells, normal T cells, and HeLa cells (Adapted from Mizuki, et al., 2000).

2.4.4 Mode of action of Parasporins

Unlike insecticidal Cry proteins, Parasporin-1 (PS1), which corresponds to Cry31, does not cause pore-formation in cancer cells. It has been claimed that PS1 induce apoptosis in HeLa cells by reducing the level of cellular protein and DNA synthesis. It is also reported that PS1 increases the intracellular calcium concentration within 1 to 3 minutes of treatment, which probably activates apoptosis (Katayama, et al., 2007). In contrast, Parasporin-2 (PS2), which corresponds to Cry46, is a pore-forming protein that increases permeability of the plasma membrane of target cells by binding specifically to a putative receptor protein located in the lipid raft of the plasma membrane. This causes formation of huge oligomers in the lipid raft, which then leads to formation of pore and cell lysis (Abe, Shimada and Kitada, 2007; Kitada, et al., 2009). Similarly, Parasporin 3 (PS3) which corresponds to Cry41 also acts as a pore-forming protein as it increases the permeability of target cells (Yamashita, et al., 2005). Parasporin 4 (PS4) which corresponds to Cry45 is cholesterol-independent so it binds to plasma membrane non-specifically (Okumura, et al., 2005). As for Parasporin 5 (PS5) and Parasporin 6 (PS6), there has been no information available for understanding their mechanisms.

2.5 Chitinase

Chitinase is an enzyme that hydrolyzes the β-(1 → 4)-glycosidic linkages in chitin. It is widely distributed in nature and are mainly produced by fungi (Viterbo, et al., 2001), actinomycetes (Akagi, et al., 2006), and also bacteria (Ajit, Verma and Shanmugam, 2006). Through its chitin degradation activity, chitinase was recommended to be used as a fungus control agent. It was suggested by Hayes, et al. (2008) that chitinase can be used as a biocontrol against insects and mosquitoes. This idea was supported by Honda, et al., (2017) who suggested that chitinase is able to form perforations to enhance the Cry proteins invasion into the epithelial cells of the midgut membranes. In the year 2016, it was demonstrated that the presence of chitinase in *Bacillus thuringiensis* increased its insecticidal toxicity as it showed significantly higher larvicidal activity (El-Kersh, et al., 2016).

2.6 Phenotypic Characterization of *Bacillus thuringiensis*

Bacillus thuringiensis is a motile bacterium due to the presence of peritrichous flagella (Ibrahim, et al., 2010). Since 1962, the characterization of *Bacillus thuringiensis* has been achieved by Hemaglutinin (H) serotyping, which is the binding of antibodies to the flagellar antigen (De Barjac and Bonnefoi, 1962). It is reported that specific *Bacillus thuringiensis* H serotypes correlated to specific flagellin amino acid sequences encoded by the *hag* gene (Ibrahim, et al., 2010). By the end of 1998, over 82 serovars and 69 H serotypes of *Bacillus thuringiensis* have been classified (Lecadet, et al., 1999). However, this classification possesses some limitations as it unable to differentiate strains with

similar H serotype and by itself, it is not enough to represent the molecular characteristics of a strain (Roh, et al., 2007). Moreover, this classification is independent of the production of parasporal proteins and biological activities, making the identification of insecticidal action of *Bacillus thuringiensis* difficult as there is no association between serovar and insecticidal activity (Porcar and Caballero, 2000; Soufiane and Côté, 2009).

As such, the investigation on the presence of crystal proteins is also an alternative way to characterize *Bacillus thuringiensis*. The toxicity of a strain against specific groups of insects can be predicted by observing the morphology of the crystals proteins produced (Mikkola, et al., 1982; Higuchi, et al., 1998). There are various shapes of crystal protein according to their Cry protein classes: bipyramidal (Cry1), cuboidal (Cry2), rhomboidal (Cry3), and spherical (Cry4). Refering to Höfte and Whiteley (1989), insects under the order of Lepidoptera, are susceptible to bipyramidal- and cuboidal-shaped crystal proteins, Dipterans are susceptible to bipyramidal, cuboidal and ovoidal-shaped crystal proteins, while Coleopterans are susceptible to bipyramidal and rhomboidal-shaped crystal proteins.

Phase contrast microscopy is commonly used to examine the presence of crystal proteins in *Bacillus thuringiensis* (Ammons, Rampersad and Khan, 2002). In addition to that, the Coomassie Brilliant Blue (CBB) staining can also be used

to examine the presence of crystal proteins in the bacterial isolates (**Figure 2.4**). In fact, it was reported that the CBB staining was preferred over phase contrast microscopy as it allows high throughput evaluation on the presence of crystals in bacterial colonies by increasing the resolution (Rampersad and Ammons, 2005). Other than that, the morphology of crystal proteins can also be analyzed by Scanning Electron Microscopy (**Figure 2.5**) and Transmission Electron Microscopy (**Figure 2.6**) (Sanchis and Bourguet, 2008, Silva, et al., 2010).

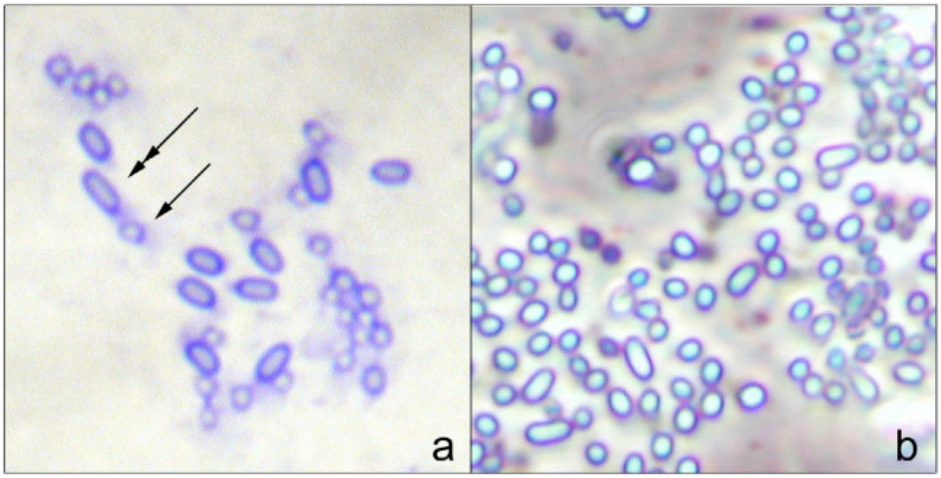

Figure 2.4: Micrographs of *Bacillus thuringiensis* showing spores **(double arrow)** and relatively lightly staining crystals **(single arrow)**. **(a)** Stained **(b)** Phase contrast microscopy (Adapted from Rampersad and Ammons, 2005).

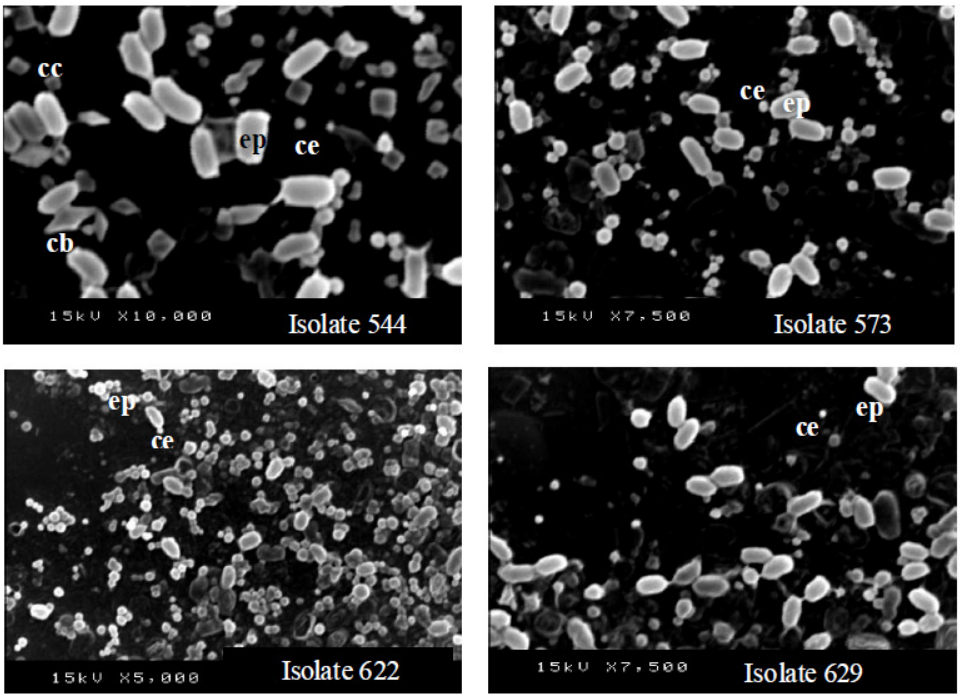

Figure 2.5: SEM of *Bacillus thuringiensis* spores and crystal proteins; ep: spore; ce: spherical crystal; cb: bi-pyramidal crystal; cc: cuboid crystal (Adapted from Silva, et al., 2010).

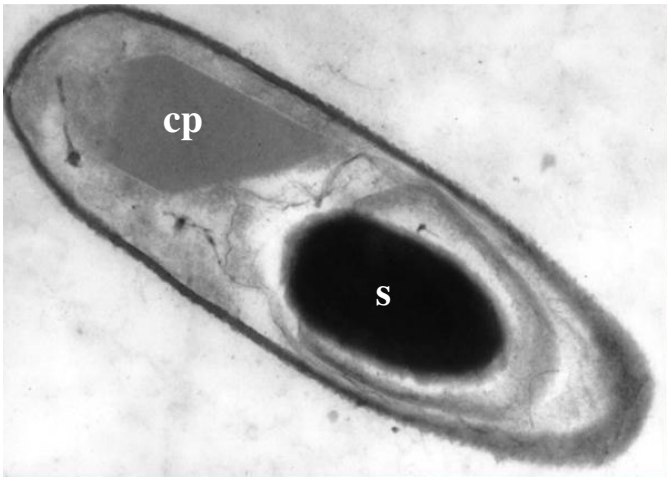

Figure 2.6: Spores (**s**) and crystals proteins (**cp**) are observed in electron micrographs in Transmission Electron Microscope (Adapted from Sanchis and Bourguet, 2008).

2.7 Molecular Characterization of *Bacillus thuringiensis*

It is impossible to predict the toxicity of an unknown *Bacillus thuringiensis* strain by serotyping alone because it does not reflect the specific δ-endotoxin genes that are responsible for the strain's toxicity (De Barjac and Frachon, 1990; Aronson, 1994). Since 1991, Polymerase Chain Reaction (PCR) technology has been used to predict the insecticidal activity of new or known *Bacillus thuringiensis* isolates by using primers that are specific to high homology conserved regions of the crystal protein encoding-genes (Ben-Dov, et al., 1997). PCR is a fast and highly sensitive method to detect the presence or absence of targeted DNA. It requires only small amounts of DNA for analysis and enable quick screening of large *Bacillus thuringiensis* samples simultaneously, making it a better method in characterization than serotyping as the latter requires a long process and is unsuitable to be used for concurrent screenings of large amounts of isolates (Hansen, et al., 1998).

The insecticidal activity of a *Bacillus thuringiensis* strain can be predicted using Polymerase Chain Reaction (PCR) as the result correlates with the strain's toxicity investigated through bioassays (Carozzi, et al., 1991). For instance, PCR primer specific for *cryI* gene is toxic against Lepidopterans, whereas *cryIV* primer that targets the *cryIV* gene is specific against Dipterans. Moreover, Carozzi, et al. (1991) also suggested that PCR can lead to the identification and characterization of novel *cry* genes when unusual PCR products were selected and further analyzed. Over the last decade, PCR has been widely used to identify the δ-endotoxin genes in *Bacillus thuringiensis* isolates (Ben-Dov, et al.; 1997;

Guerchicoff, Ugalde and Rubinstein, 1997). In the research by El-Kersh, et al. (2016), the presence of chitinase (*chi*) gene was confirmed using PCR to study the chitinase-induced synergistic larvicidal effect in *Bacillus thuringiensis*. After the discovery of parasporins, PCR method was established to identify the presence or absence of parasporins in *Bacillus thuringiensis* isolates (Espino-Vázquez, et al., 2012; Brasseur, et al., 2015).

2.8 Applications of *Bacillus thuringiensis*

Today*, Bacillus thuringiensis* is the most popular biological pest control agent. Most *Bacillus thuringiensis*-based insecticides are formulated mixtures of *Bacillus thuringiensis* spores and δ-endotoxin crystals, which are known to synergize the toxicity of the crystals (Osman, et al., 2015). A number of safety and environment considerations have favored the future development of *Bacillus thuringiensis* as pest control. For instance, *Bacillus thuringiensis* is remarkably easy to use with no irreversible effect to the aquatic ecosystems (Calamari, et al., 1998). Moreover, *Bacillus thuringiensis* bioinsecticides are cheaper to make as compared to chemical pesticides. Not only that, it is not pathogenic to humans, amphibians, birds, mammalians or reptilians. The high specificity on their target insects and invertebrate pests makes *Bacillus thuringiensis* a popular alternative in crops protection (Schnepf, et al., 1998; Sanchis and Bourguet, 2008)

The first commercial insecticide was produced in the year 1938 and was used to control flour moth in France. Also in 1989, *Bacillus thuringiensis* was used in the United States as a pesticide against the gypsy moth (Twardus, 1989). Blackfly and mosquito are vectors of insect-borne diseases such as dengue fever, malaria and onchocercosis. The insecticidal potential towards mosquito and blackfly has led *Bacillus thuringiensis* to become a popular pesticide in insect vector control program (Schnepf, et al., 1998; Sanchis and Bourguet, 2008). In 1977, Goldberg and Margalit reported that *Bacillus thuringiensis israelensis* was proven to be one of the most potent and effective biological pesticides, which is highly toxic to blackfly larvae and mosquito. In 1997, the World Health Organization (WHO) launched the use of *Bacillus thuringiensis* against Dipteran larvae through the Onchocerciasis Control Program (OCP). Since then, *Bacillus thuringiensis* is used in West Africa to control blackfly species that transmit onchocerciasis (Sanchis and Bourguet, 2008). Similarly, Ohba, Mizuki and Uemori (2009) also reported the efficiency of using *Bacillus thuringiensis* to control the population of mosquito vectors that transmit onchocerciasis, lymphatic filariasis, rift valley virus and dengue virus.

Other than biopesticides, the insecticidal potential of *Bacillus thuringiensis* (Bt) is useful to make transgenic plants expressing the insecticidal toxins. Today, Bt cotton and Bt maize are planted largely throughout the world (Sanchis and Bourguet, 2008). In 2015, Osman and team have claimed that more than 90% crops planted in the United States are transgenic crops which produce *Bacillus thuringiensis* δ-endotoxins. Generally, the use of Bt transgenic plants brings many benefits to the agricultural fields. Not only can it significantly reduce the

use of insecticides in agriculture, many improved Bt cotton varieties are confirmed by researchers to be high-yielding (Sanchis and Bourguet, 2008). In addition, the cost of field management is reduced as fewer insecticides are needed due to the continuous production and persistence of insecticidal toxins in transgenic plants (Schnepf, et al., 1998).

However, some studies have reported that microbial insecticides still possess some limitations. For instance, Singh-Ashk (2007) claimed that despite the use of microbial insecticides, there may still be continued damages caused by presence of pests of other species, as one microbial insecticide may only be specific to a particular group of insects. It was reported that the Bt cotton crop in India was damaged by a new insect pest called the mealy bug, which had caused 25% of the Bt cotton to be destroyed (Singh-Ashk, 2007). Therefore, researchers are making more effort in isolating of novel *Bacillus thuringiensis* with better insecticidal spectrum and higher toxicity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of Experimental Design

Figure 3.1 shows the general overview of the experimental design, which is divided into three subsections – morphological identification, molecular identification and protein identification.

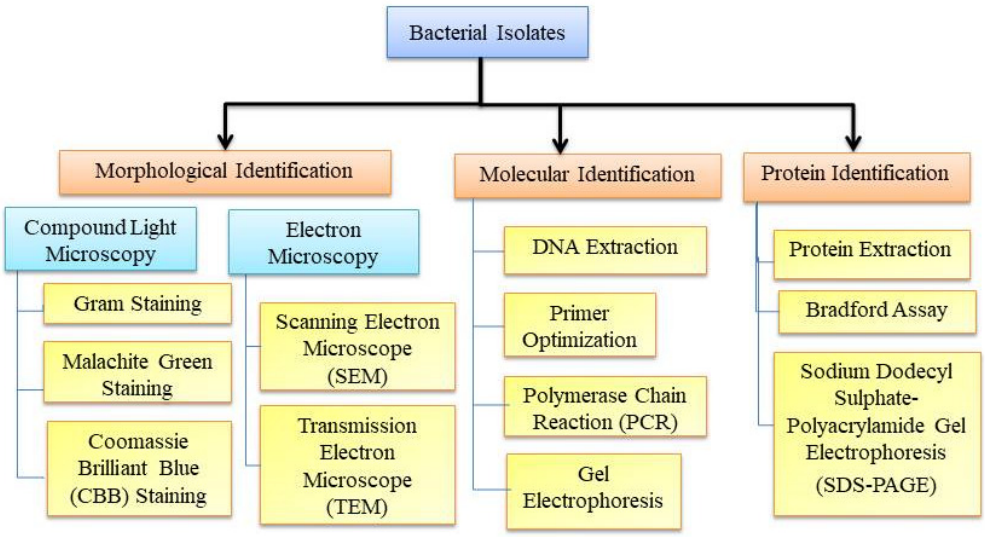

Figure 3.1: Overview of experimental design.

3.2 Chemicals, Media, Reagents and Equipment

The list of chemicals, media, reagents and equipments that were used in this study is tabled in appendices in accordance to their respective manufacturers.

3.3 Bacterial Isolates

Six bacterial samples previously isolated from soil in Kampar were studied in this project, indicated as - R1, R2, R3, D1C, A2B, and C6D. The bacteria sample kept in glycerol stock were taken from -80°C fridge and thawed at room temperature. Then, the bacteria were cultured and maintained in nutrient agar and nutrient broth respectively. The incubation condition was 30ºC and at 200 rpm with constant agitation (for liquid media) overnight.

3.4 Morphological Identification

3.4.1 Gram Staining

Gram staining was performed to verify rod-shaped and Gram-positive bacilli (**Figure 3.2**). A small loop of bacteria sample was smeared onto a drop of 0.85% saline solution on the glass slide. Then, the smear was subjected to air-drying and heat-fixing using a Bunsen burner. Once fixation was completed, the smear was flooded with the crystal violet primary staining for 1 minute, followed by washing with water. After that, the glass slide was stained with gram iodine

mordant for another 1 minute and washed with tap water. Then, the smear was decolorized with 75% ethanol for 10 seconds and washed with water. The slide was left to air dry and viewed under a compound light microscope at the magnification of 1000X with oil immersion. The positive control used was *Bacillus thuringiensis israelensis* and the negative control used was *Escherichia coli*.

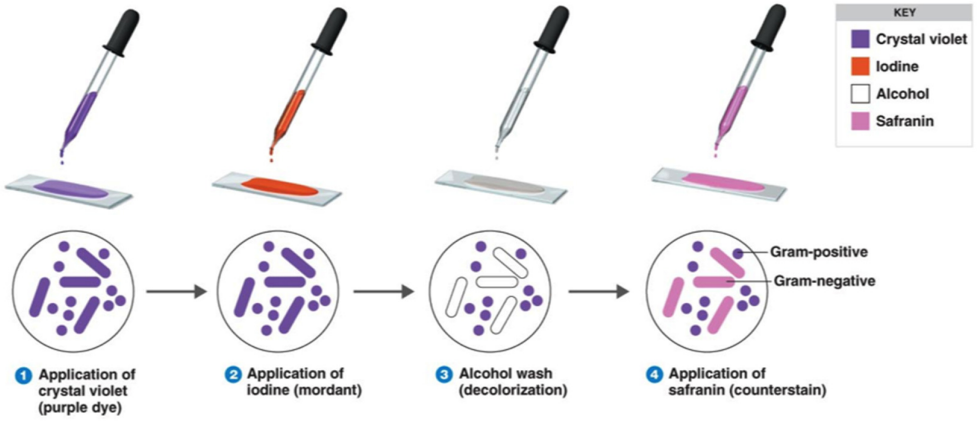

Figure 3.2: Overview of Gram staining (Adapted from Tortora, Funke and Case, 2013).

3.4.2 Malachite Green Staining

Malachite green stain was carried out to confirm the presence of endospores in the bacterial isolates (**Figure 3.3**). After 90 hours of incubation, one loopful of bacteria sample was smeared onto a drop of 0.85% saline solution on the glass slide and subjected to air-drying and heat-fixing. Once fixation was completed, the smear was covered with filter paper soaked with malachite green stain. The

glass slide was then placed on top of a beaker with boiling water and the smear was steamed for 5 minutes. After that, the stained smear was rinsed with tap water and counterstained with safranin for 30 seconds, followed by washing with tap water again. The slide was left to air dry and viewed under a compound light microscope at the magnification of 1,000X with oil immersion. The positive control used was *Bacillus thuringiensis israelensis* and the negative control used was *Escherichia coli*.

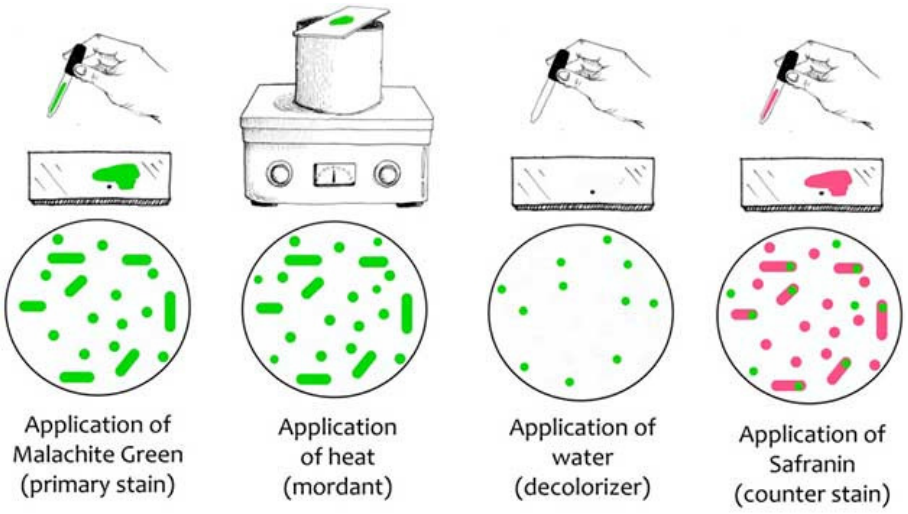

Figure 3.3: Overview of malachite green staining (Adapted from Aryal, 2015).

3.4.3 Coomassie Brilliant Blue (CBB) staining

Coomassie Brilliant Blue (CBB) staining was carried out to confirm the presence of crystal proteins in the isolated bacterial samples. After incubation of 110 hours, one loopful of bacteria sample was smeared onto a drop of 0.85%

saline solution on the glass slide and subjected to air-drying and heat-fixing. Once fixation was completed, the smear was stained with 0.133% CBB for 1 minute. After that, the smear was destained with distilled water for 10 seconds. The slide was then left to air dry and viewed under a compound light microscope at the magnification of 1,000X with oil immersion. The positive control used was *Bacillus thuringiensis israelensis* and the negative control used was *Bacillus cereus*.

3.4.4 Scanning Electron Microscopy (SEM)

The shape of the crystal proteins produced by *Bacillus thuringiensis* isolates were studied using scanning electron microscopy (SEM). A single colony of bacteria was inoculated into a 250 mL conical flask containing 50 mL nutrient broth. The bacteria were incubated at 30ºC for 110 hours until commencement of autolysis phase. The presence of spores and crystal proteins was confirmed by Coomassie Brilliant Blue (CBB) staining prior to SEM processing.

The cells were harvested in a microcentrifuge tube by centrifugation at 6,000 rpm for 1 minute. This step was repeated thrice to increase pellet amount. Supernatant was discarded and the pellets were fixed in 300 µL of 2.5% (v/v) glutaraldehyde in 0.1 phosphate-buffered saline (PBS) at 4ºC overnight. The next day, the glutaraldehyde solution was removed by centrifugation at 1,500 *g* for 10 minutes, followed by washing with 0.1 M PBS. This buffer washing step

was repeated thrice, following by washing of pellets with cold distilled water thrice. After washing, serial dehydration process was performed using different concentrations of ethanol: 25% ethanol for 5 minutes, 75% ethanol for 5 minutes, 95% ethanol for 5 minutes, and 100% ethanol for 5 minutes with three changes. The samples were then further dehydrated by freeze drying for 18 hours.

Prior to viewing using SEM, a small amount of the dehydrated sample was transferred to a carbon tape adhered to a copper stub, followed by coating of the samples with platinum for 1 minute. Then, the copper stub was placed onto a specimen holder and the samples were observed under the SEM at 10,000X to 20,000X magnification.

3.4.5 Transmission Electron Microscopy (TEM)

A single colony from the bacterial isolates R3 and C6D was inoculated into a 250 mL conical flask containing 50 mL nutrient broth respectively. The bacteria were incubated at 30 ºC for 96 hours until the commencement of sporulation phase. The presence of spore and crystal proteins were confirmed by Coomassie Brilliant Blue (CBB) staining prior to TEM processing. The bacteria cells were harvested in a microcentrifuge tube by centrifugation at 1,500 *g* for 5 minutes. Supernatant was discarded and the pellets were fixed in 300 µL of 2.5% (v/v) glutaraldehyde in 0.1 phosphate-buffered saline (PBS) at 4ºC overnight.

After fixation, the samples were centrifuged at 1,500 *g* for 2 minutes. The supernatant was discarded and pellets resuspended in 0.1 M PBS. These steps were repeated twice. After that, the samples were centrifuged again at 1,500 *g* for 2 minutes. The subsequent steps were done in the fume-hood. The supernatant was discarded and the pellet was post-fixed with 1% Osmium tetroxide for 1 hour. After post-fixation, the samples were washed with distilled water twice. After the post-fixation washing steps, the samples were centrifuged to obtain the pellet. Supernatant was discarded and the tube containing the pellet was placed in a water bath set at 45ºC for 15 to 30 minutes. Meanwhile, 3% agar solution was prepared by dissolving agar in boiling distilled water. While it was still molten, the solution was poured into a test tube and the tube was placed in the 45ºC water bath. After calibration of both the molten agar and the pellets to similar temperatures, a drop of the molten agar was transferred to the tube containing the pellets. The pellets were quickly stirred until they broke into small blocks and suspended in the molten agar. Subsequently, the mixture was immediately poured onto a microscope slide and allowed to set for 1 to 2 minutes. The solidified agar containing the pellets was cut into small cubes (1 mm^3) using a sharp blade. These cubes were then subjected to serial dehydration: 50% ethanol for 15 minutes, 75% ethanol for 15 minutes, 95% ethanol for 15 minutes twice, 100% ethanol for 30 minutes twice and 100% acetone for 10 minutes twice.

After the dehydration process, the cubes were infiltrated with acetone: Spurr's resin mixture (1:1) overnight, followed by a new change of 100% Spurr's resin mixture and infiltrated for another 5 hours in a rotator. After that, the cubes were embedded in 100% Spurr's resin mixture and cured at 60 ºC for 48 hours.

Subsequently, the resin blocks were roughly trimmed with sharp blade, then finely trimmed with a a glass knife using an ultramicrotome. Semi thin (1 µm) sectioning were performed with a new glass knife and the thin sections were collected on a knife-boat filled with water. The sections were stained with Toluidine Blue and were checked under a compound light microscope under 400X magnification to confirm that the sections contained at least 80 % of the bacteria samples. After that, ultra-thin sectionings (less than 0.1 µm) were performed and the sections were collected on the dull side of a copper grid. The grid was allowed to dry in 60 ºC oven for 15 minutes to allow proper attachment of the sections onto the grid. The sections were then stained with 2.0% lead citrate and uranyl acetate and allowed to dry for about 30 minutes. After that, the samples were viewed at 6,300X magnification using a Transmission Electron Microscope.

3.5 Molecular Identification

3.5.1 DNA Extraction

The bacterial samples were cultured into 250 mL conical flasks containing 100 mL nutrient broth. The bacterial cultures were incubated overnight at 30ºC with constant agitation at 200 rpm. An extraction kit (Presto™ Mini gDNA Bacteria Kit) was used for DNA extraction following the manufacture's protocols. A volume of 3 mL of overnight bacteria culture was transferred and centrifuged at 14,000 *g* for 1 minute in a 1.5 mL microcentrifuge tube. Then, the DNA was extracted according to the procedure provided by the manufacturer (Geneaid) with slight modification. The duration of elution buffer stand in matrix was extended to about 1 hour and the elution step was repeated twice. After that, the DNA concentration and purity (A_{260} / A_{280}) was measured using nanospectrophotometer and was stored at -20ºC for future use.

3.5.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) amplifications using different set of primers were performed to identify the presence of the target genes. Ten sets of primers were used to detect the presence of the possible δ-endotoxin, parasporin and chitinase genes in the bacteria samples (**Table 3.1**). The standard PCR mixture for a reaction was prepared with components as shown in **Table 3.2** with a final volume of 10 µL.

Table 3.1: PCR primers used for gene amplification.

Primers	Gene Recognized	Forward and Reverse Primer Sequences	Product size (bp)	Reference
Un1	*cryI*	(f) 5'- CATGATTCATGCGGCAGA TAA AC – 3'	277	Halima, et al. (2006)
		(r) 5'- TTGTGACACTTCTGCTTCCCA TT – 3'		
Cry2gral	*cryII*	(f) 5'- GAGTTTAATCGACAAGTAGATAATTT – 3'	500-526	Ibarra, et al. (2003)
		(r) 5'- GGAAAAGAGAATATAAAAATGGCCAG – 3'		
Cry4	*cryIV*	(f) 5'- AAATTGATGGTACTCTTGCCTCTT – 3'	423	Ejiofor and Johnson (2002)
		(r) 5'- TGCGTAATCCGTAACTTCTTGTAG – 3'		
Cry10spe	*cry10*	(f) 5'- TCAATGCTCCATCCAATG – 3'	348	Mahalakshmi, et al. (2012)
		(r) 5'- CTTGTATAGGCCTTCCTCCG – 3'		
Cry11	*cry11*	(f) 5'- TTTGCACCAGATAATACTAAGGAC	485	Ejiofor and Johnson (2002)
		(r) 5'- AACAACTGCGATAAATACCACTCT – 3'		

Table 3.1: PCR primers used for gene amplification (Continued).

Primers	**Gene Recognized**	**Forward and Reverse Primer Sequences**	**Product size (bp)**	**Reference**
cyt2 (UN)	*cyt2*	(f) 5'- ATTACAAATTGCAAATGGTATTCC – 3'	355-356	El-Kersh, et al. (2016)
		(r) 5'- TTTCAACATCCACAGTAATTTCAAATGC – 3'		
chi	*chi*	(f) 5'- ATGGTCATGAGGTCTC – 3'	2027	El-Kersh, et al. (2016)
		(r) 5'- CTATTTCGCTAATGACG – 3'		
ps1	*ps1*	(f) 5'- TACAAGCAGGGCGTCCAG – 3'	737	Espino-Vazquez, et al. (2012)
		(r) 5'- TCTGCTGGAATTTGCAATGCT – 3'		
ps2	*ps2*	(f) 5'- TGTTGGGACTGTTCAGTACG – 3'	237	Espino-Vazquez, et al. (2012)
		(r) 5'- GTAGTAGAGAATGAAACTTCTCCACC – 3'		
ps4	*ps4*	(f) 5'- GACAGAAACGTCTCGGAGTT	172	Espino-Vazquez, et al. (2012)
		(r) 5'- TACCAGTGTAACCAGGAGCA		

Table 3.2: Standard PCR mixture for a reaction.

Components	Volume (µL)
PCR master mix	5.0
Forward primer (10 µM)	0.1
Reverse primer (10 µM)	0.1
Template DNA (50-100 µg/µL)	1.0
Sterile deionized water	3.8
Total volume	**10**

3.5.3 Optimization of Annealing Temperature for DNA Amplification

The primers listed in **Table 3.1** were optimized prior to PCR amplification. Ten tubes containing the reaction mixture as listed in **Table 3.2** were prepared. Using a thermocycler, the gradient temperature was set from 45 ºC to 60 ºC, and the PCR tubes were arranged in the block. The optimization parameters are shown in **Table 3.3**. The selected annealing temperature was the temperature which had positive and the brightest product band. The amplified DNA was then assessed and visualized using gel electrophoresis.

Table 3.3: PCR setting for optimization of annealing temperature.

Step	Temperature (ºC)	Duration (sec)	Cycle
Initial denaturation	95	120	1
Denaturation	95	30	35
Annealing Un1 Cry2gral Cry4 Cry10spe Cry11 Cyt2 (UN)	45-60	15	35
Extension	72	300	35
Final extension	72	300	1

3.5.4 PCR Amplification

The PCR for the amplification of *cryI*, *cryII*, *cryIV*, *cry10*, *cry 11* and *cyt2* genes were performed using thermocycler (Eppendorf), with the parameters shown in **Table 3.4**. In every PCR, *Bacillus cereus* was used as negative control and deionized water was used as a non-template control. For amplification of *cryIV*, *cry10*, *cry 11* and *cyt2* genes. *Bacillus thuringiensis israelensis* was used as the positive control whereas *Bacillus thuringiensis kurstaki* was used as the positive control for amplification for *cryI* and *cryII* genes.

Table 3.4: PCR setting for PCR amplification.

Step	Temperature (ºC)		Duration (sec)	Cycle
Initial	95		120	1
denaturation	95			
Denaturation			30	35
Annealing	Un1	55.5	15	35
	Cry2gral	49.0		
	Cry4	55.0		
	Cry10spe	48.4		
	Cry 11	55.0		
	Cyt2 (UN)	45.0		
Extension	72		45	35
Final extension	72		300	1

3.5.5 Touchdown PCR

Touchdown PCR is a method that can be used to increase the specificity of the reaction without actually determining the annealing temperature. In this study, touchdown PCR was performed using the primers for the *chi*, *ps1*, *ps2* and *ps4* genes to run preliminary screening for the presence of the respective genes on the bacterial isolates as there were no positive reference strains. The parameter for touchdown-PCR is shown in **Table 3.5**.

Table 3.5: PCR setting for Touchdown PCR amplification.

Step		Temperature (ºC)	Duration (sec)	Cycle
Initial denaturation		95	120	1
Denaturation		95	30	22
Annealing	chi	58 (Δ -0.5)	15	22
	ps1			
	ps2			
	ps4			
Extension		72	45	22
Denaturation		95	30	20
Annealing	chi	55	15	20
	ps1			
	ps2			
	ps4			
Extension		72	45	20
Final Extension		72	300	1

3.5.6 Agarose Gel Electrophoresis

After PCR amplifications, the amplified DNA was assessed using agarose gel electrophoresis. A 2% agarose gel and 1X TBE buffer were prepared. For gel electrophoresis of *chi* genes, 1.5% agarose gel with 1X TBE buffer were prepared. The gel was placed into a gel tank and immersed in 1X TBE buffer. A volume of 3 µL of 100-bp Plus DNA ladder (Thermofisher), and 5 µL of the

respective amplified products that were mixed with 1 µL of 6X loading dye containing 20% Novel juice, were each loaded into their respective wells. For *chi* gene gel electrophoresis, 1-kb Plus DNA ladder was used. The gels were electrophoresed for 25 minutes at 100 V and visualized under UV trans-illuminator using the ChemiDoc™ XRS systems (Bio-Rad).

3.6 Protein Identification

3.6.1 Protein Extraction

The bacterial samples were cultured into 250 mL conical flasks containing 100 mL nutrient broth. The bacterial cultures were incubated overnight at 30 ºC with constant agitation at 200 rpm. Total protein extraction was done using a protein extraction kit (ProteoSpin Total Protein Isolation Kit) following the manufacturer's protocols.

A volume of 3 mL of overnight bacteria culture was transferred into a microcentrifuge tube and was centrifuged at 6,000 rpm for 1 minute to harvest the pellet. Supernatant was discarded and 50 µL of lysis solution was added into the tube. The resuspended pellet was then transferred to lysis tube. Then, the proteins were extracted according to the procedure provided by the manufacturer with slight modification. The liquid content was allowed to stand in the filter column for about 1 hour and the protein elution step protein was repeated twice.

3.6.2 Bradford Assay

Bradford Assay was carried out to determine the concentration of the extracted proteins. A standard linear graph was generated using eight concentrations (0.0 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL and 0.7 mg/mL) of Bovine Serum Albumin (BSA). The 5X Bradford reagent was diluted into 1X before use. After that, 5 µL of each BSA concentrations and extracted protein samples were pipetted into a 96-well plate, then 100 µL of 1X Bradford reagent were added to each well. The absorbance was read at 595 nm wavelength using the microplate reader. The assay was performed thrice to obtain the average absorbance values of the standard BSA concentrations and used to plot a standard graph with the r^2 value of 0.95-1.00. The concentration of the extracted protein samples were calculated based on the linear equation obtained.

3.6.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The total protein profile of six bacterial isolates were determined through SDS-PAGE analysis. First, two sets of glass plates with the thickness of 0.75 mm were set on the casting frame and fixed on a casting stand. Then, 2 mL of 70% ethanol were loaded into the glass plates to check for leakages. The ethanol was then removed by using filter paper. After that, 10% separating gel was prepared with components and volume as shown in **Table 3.6** and was loaded into the glass plates.

Table 3.6: Preparation of 10% separating gel

Components	Volume (µL)
Distilled water	4000
30% Bis-acrylamide	3330
1.5M Tris-hydrochloric acid (pH 8.8)	2500
10% Ammonium persulphate (APS)	100
10% Sodium dodecyl sulphate (SDS)	100
Tetramethylethylenediamine (TEMED)	5

Subsequently, 70% ethanol was pipetted gently on top of the 10% separating gel to remove air bubbles and the gel was left to polymerize for 30 minutes. After the gel had solidified, the 70% ethanol was gently removed by filter paper and 4% stacking gel was prepared with components and volume as shown in **Table 3.7**. Then, the 4% stacking gel was loaded over the 10% separating gel and a 10-well comb was carefully inserted into the 4% stacking gel. The gel was then allowed to polymerize for 30 minutes. After the gel had solidified, the glass plate was removed from the casting stand and placed into the gel tank. Then 1X of running buffer was poured into the gel tank and the comb was carefully detached from the 4% stacking gel.

Table 3.7: Preparation of 4% stacking gel

Components	Volume (µL)
Distilled water	3400
30% Bis-acrylamide	830
0.5M Tris-hydrochloric acid (pH 6.8)	630
10% Ammonium persulphate (APS)	50
10% Sodium dodecyl sulphate (SDS)	50
Tetramethylethylenediamine (TEMED)	5

A 6X sample loading buffer was prepared with components and volume as shown in **Table 3.8** and was diluted to 3X. A volume of 10 µL of the extracted protein samples were mixed with 5 µL of 3X sample loading buffer. The mixtures were then heated at 95ºC for 10 minutes using heat block. The mixtures were spun at maximum speed for 1 minute at room temperature using table top centrifuge.

Table 3.8: Preparation of 6X sample loading buffer

Components	Amount
1.0 M Tris-hydrochloric acid (pH 6.8)	3750 µL
60% Glycerol	6000 µL
12% Sodium dodecyl sulphate	1.2 g
0.6 M Dithiothreitol (DTT)	0.93 g
0.06% Bromophenol blue	0.006 g

A volume of 20 µL of the respective protein samples and 5 µL of 175 kDa Chromatein Prestained Protein Ladder (Vivantis) were loaded into their respective wells. The gel tank was connected to a power supply and the gel was electrophoresed at 80 V for 90 minutes. The gel was then stained with Coomassie Brilliant Blue staining for 5 minutes and destained with distilled water overnight. Then, the stained gel was visualized under UV trans-illuminator using the ChemiDoc™ XRS systems (Bio-Rad).

CHAPTER 4

RESULTS

4.1 Gram Staining

The Gram staining of bacterial isolates labeled **A** to **F**, and positive control labeled **BTI** are presented in **Figure 4.1** showed that the bacterial isolates and positive control appeared as purple and rod-shaped, indicating that they are Gram-positive bacilli while the negative control labeled **ECO** appeared as pink and rod-shaped in shape indicating Gram-negative bacilli.

Figure 4.1: Gram staining of the bacterial isolates viewed under compound light microscope at 1000X magnification. **(A)** R1 **(B)** R2 **(C)** R3 **(D)** D1C **(E)** A2B **(F)** C6D showed Gram- positive rod-shaped bacilli. (**BTI**) *Bacillus thuringiensis israelensis* (positive control). (**ECO**) *Escherichia coli* (negative control).

4.2 Malachite Green Staining

The Malachite Green staining of bacterial isolates labeled **A** to **F**, and the positive control labeled **BTI** are presented in **Figure 4.2**. All bacterial isolates and the positive control showed the presence of endospore while the negative control labeled **ECO** did not show any formation of spore. The vegetative cells were stained pink whereas the spores were stained green. The black arrows in **Figure 4.2** indicate the presence of endospore.

Figure 4.2: Malachite green staining of the bacterial isolates viewed under compound light microscope at 1000X magnification. **(A)** R1 **(B)** R2 **(C)** R3 **(D)** D1C **(E)** A2B **(F)** C6D showed that they are able to produce endospore. (**BTI**) *Bacillus thuringiensis israelensis* (positive control) (**ECO**) *Escherichia coli* (negative control). The black arrows indicates the presence of endospores.

4.3 Coomassie Brilliant Blue (CBB) Staining

The Coomassie Brilliant Blue (CBB) staining of bacterial isolates labeled **A** to **F**, and the positive control labeled **BTI** are presented in **Figure 4.3**. All bacterial isolates and the positive control showed the presence of crystal proteins while the negative control labeled **BC** did not show any formation of crystal proteins. The crystal proteins are stained in dark blue color. The black arrows indicate the presence of crystal proteins.

Figure 4.3: CBB staining of the bacterial isolates viewed under compound light microscope at 1000X magnification. **(A)** R1 **(B)** R2 **(C)** R3 **(D)** D1C **(E)** A2B **(F)** C6D showed that they are able to produce crystal proteins. (**BTI**) *Bacillus thuringiensis israelensis* (positive control) showed that they are able to produce crystal proteins. (**BC**) *Bacillus cereus* (negative control). The black arrows indicate the presence of crystal proteins.

4.4 Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopy (SEM) images of bacteria isolates and positive control *Bacillus thuringiensis israelensis* are presented in **Figures 4.4** to **4.10**. The SEM results showed that all of the bacterial isolates and the positive control were able to produce spherical-shaped crystal proteins. The negative control *Bacillus cereus* (**Figure 4.11**) did not show any formation of crystal proteins. The red arrows indicate the presence of crystal proteins whereas the yellow arrows indicate the presence of endospores.

The presence of crystal proteins with various shapes and their sizes were noted. **R1**, **R2** and **C6D** showed the formation of spherical-shaped crystal proteins with the size of 0.3 µm whereas **R3**, **D1C** and **A2B** showed the formation of spherical-shaped crystal proteins with the sizes range from 0.3 to 0.6 µm. Moreover, **R2, R3** and **D1C** showed the formation of ovoidal-shaped crystal proteins approximately 1.3 µm in size whereas cuboidal-shaped crystal proteins with the sizes range from 0.3 to 0.4 µm were present in isolates **R1** and **R2**. No bacterial isolates showed the presence of bipyramidal or rhomboidal-shaped crystal proteins.

Figure 4.4: Scanning electron microscopy of bacteria isolate **R1** at 20,000X magnification showed presence of endospores and formation of cuboidal and spherical-shaped crystal proteins.

Figure 4.5: Scanning electron microscopy of bacterial isolate **R2** at 20,000X magnification showed presence of endospores and formation of spherical, cuboidal and ovoidal-shaped crystal proteins.

Figure 4.6: Scanning electron microscopy of bacterial isolate **R3** at 10,000X magnification showed presence of endospores and formation of spherical and ovoidal-shaped crystal proteins.

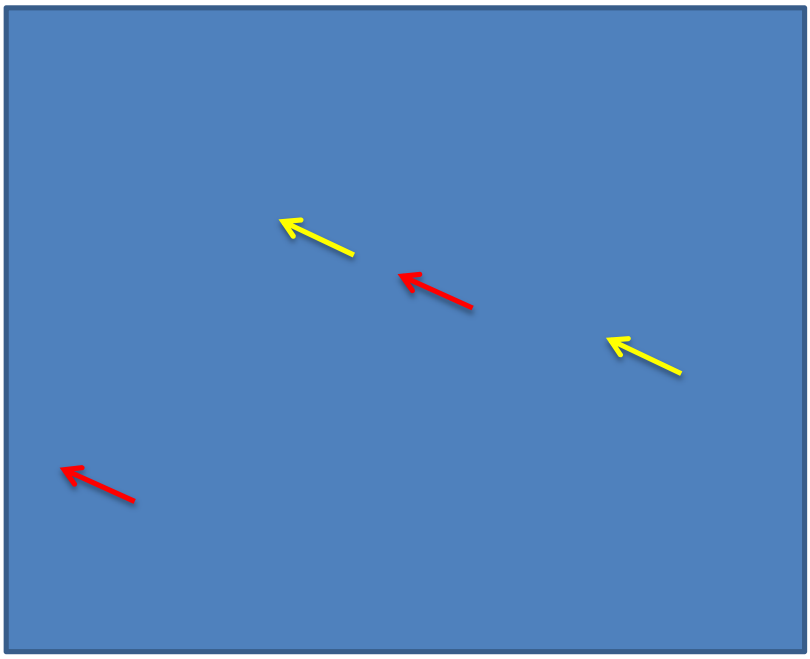

Figure 4.7: Scanning electron microscopy of bacterial isolate **D1C** at 10,000X magnification showed presence of endospores and formation of spherical and ovoidal-shaped crystal proteins.

Figure 4.8: Scanning electron microscopy of bacterial isolate **A2B** at 10,000X magnification showed presence of endospores and formation of spherical-shaped crystal proteins.

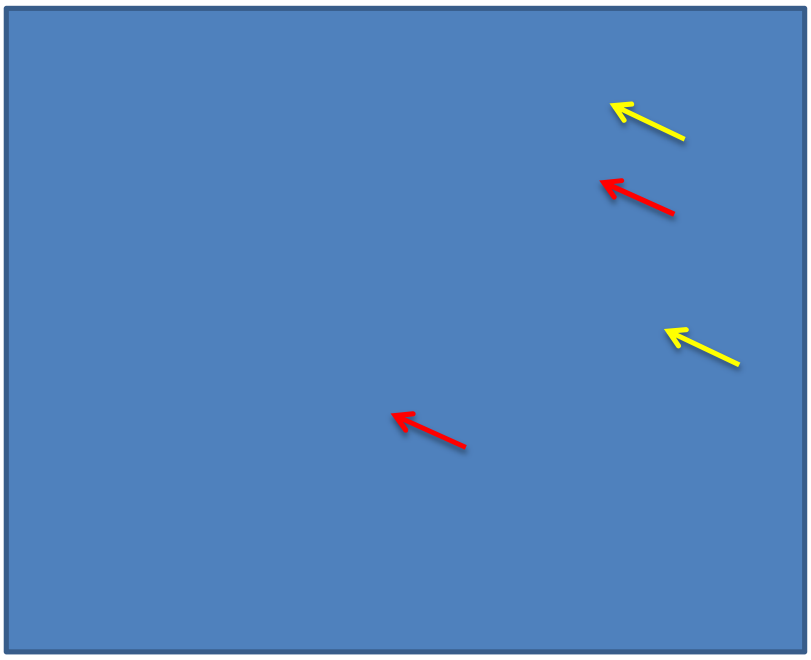

Figure 4.9: Scanning electron microscopy of bacterial isolate **C6D** at 20,000X magnification showed presence of endospores and formation of spherical-shaped crystal proteins.

Figure 4.10: Scanning electron microscopy of *Bacillus thuringiensis israelensis* (positive control) at 10,000X magnification showed presence of endospores and formation of bipyramidal and spherical-shaped crystal proteins.

Figure 4.11: Scanning electron microscopy of *Bacillus cereus* (negative control) at 20,000X magnification showed presence of endospores but no crystal protein formation.

4.5 Transmission Electron Microscopy (TEM)

The Transmission electron microscopy (TEM) images of two bacterial isolates **R3** and **C6D** are presented in **Figures 4.12** and **4.13** showed the formation of endospores and presence of ovoidal-shaped crystal proteins next to the endospore, with the size of approximately 1.3 µm. The yellow arrows indicate the presence of endospores whereas the red arrows indicate the presence of crystal proteins. **Table 4.1** shows the summary of the isolates with the respective crystal protein shapes.

Figure 4.12: Transmission electron microscopy of bacterial isolate **R3** at 6,300X magnification showed the presence of endospore and ovoidal-shaped crystal protein.

Figure 4.13: Transmission electron microscopy of bacterial isolate **C6D** at 6,300X magnification showed the presence of endospore and ovoidal (**ov**) and spherical (**sp**) shaped crystal proteins.

Table 4.1: Summary of the shapes of the crystal proteins produced by bacterial isolates.

Shape	**R1**	**R2**	**R3**	**D1C**	**A2B**	**C6D**	**BTI**	**BC**
Spherical	+	+	+	+	+	+	+	-
Ovoidal	-	+	+	+	-	+	-	-
Cuboidal	+	+	-	-	-	-	-	-
Bipyramidal	-	-	-	-	-	-	+	-
Rhomboidal	-	-	-	-	-	-	-	-

*(+) show the presence of respective crystal protein shape and (-) show the absence of respective crystal protein shape. BTI: *Bacillus thuringiensis isrelensis*. BC: *Bacillus cereus.*

4.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The total protein profile of each bacterial isolates, and the positive and negative controls are as shown in **Figure 4.14**. All of the bacterial isoates exhibited protein bands within 14 kDa to 95 kDa. The molecular weight of CytA, Cry2, Cry35 and Cry3 or Cry11 proteins are 25 kDa, 44 kDa, 65 kDa and 70 kDa respectively. In addition to that, the presence of protein band with the molecular weight of 29 kDa was observed in **R1**, **R3** and **A2B**. Bacterial isolates **R3** and **A2B** exhibited protein band of around 94 kDa in size wheareas only **R1** showed protein band of approximately 14 kDa in size, which corespond to the size of Cry34 protein. The positive control, *Bacillus thuringiensis israelensis* exhibited the protein bands approximately at molecular weights of approximately 25 kDa, 29 kDa, 44 kDa and 70 kDa, which correlated to the sizes of CytA, Cyt2, Cry35 and Cry 3 or Cry11 proteins respectively. The presence of protein bands with the molecular weight of 29 kDa, 44 kDa and 70 kDa were observed in the negative control *Bacillus cereus*, which are similar to the molecular weights of Cyt2, Cry35, and Cry3 or Cry11 proteins. Other than that, no presence of protein bands that correlated to crystal proteins were observed in *Bacillus cereus*.

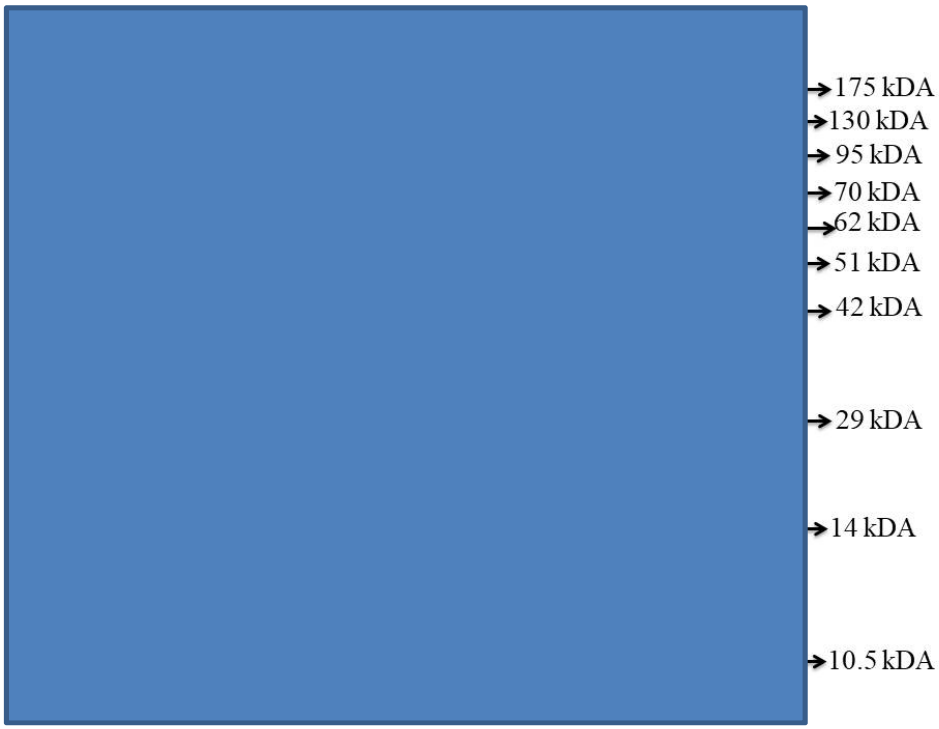

Figure 4.14: The SDS-PAGE of the total protein extracts of bacterial isolates. Lane 1: R1; Lane 2: R2; Lane 3: R3; Lane 4: D1C: Lane 5: A2B; Lane 6: C6D; Lane 7: *Bacillus thuringiensis israelensis* (positive control); Lane 8: *Bacillus cereus* (negative control); Lane M: the 175 kDa Chromatein Prestained Protein Ladder.

Legend:

Crystal proteins at 94 kDa
Cry 3 or Cry 11 proteins at 70 kDa
Cry 2 proteins at 65 kDa
Cry 35 proteins at 44 kDa
Cyt2 proteins at 29 kDa
CytA proteins at 25 kDa
Cry34 proteins at 14 kDa

4.7 The δ-endotoxin Genes Gel Electrophoresis

The distribution of δ-endotoxin genes *cryI, cryII, cryIV, cry10, cry11, cyt2,* parasporin genes *ps1, ps2 and ps4* and chitinase gene *chi* in the bacterial isolates were evaluated. The PCR results showed that all bacterial isolates contained *cryII, cry10, cry11* and *cyt2* genes (**Figure 4.15** to **4.20**). The presence of *cryI* genes can be observed in all of the bacteria isolates except **R2** while *cryIV* gene can be observed only in **R1** and **A2B**. *Bacillus thuringiensis kurstaki* was used as positive control for screening of *cryI* and *cryII* genes, wheareas *Bacillus thuringiensis israelensis* was used as reference strain for detection of *cryIV*, *cry10*, *cry11* and *cyt2* genes. However, none of the bacterial isolates and the positive controls contained parasporin and chitinase genes. All of the tested genes were not observed in the negative control *Bacillus cereus*. The results are summarized in **Table 4.2**.

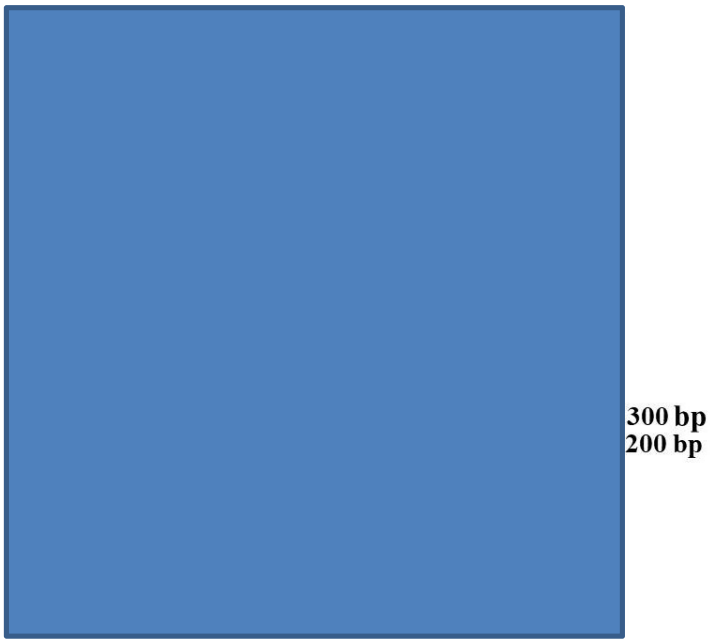

Figure 4.15: Screening of the bacterial isolates using Un1 primer (*cry1* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis kurstaki* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

Figure 4.16: Screening of the bacterial isolates using Cry2gral primer (*cry2* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis kurstaki* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

Figure 4.17: Screening of the bacterial isolates using Cry4 primer (*cry4* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis israelensis* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

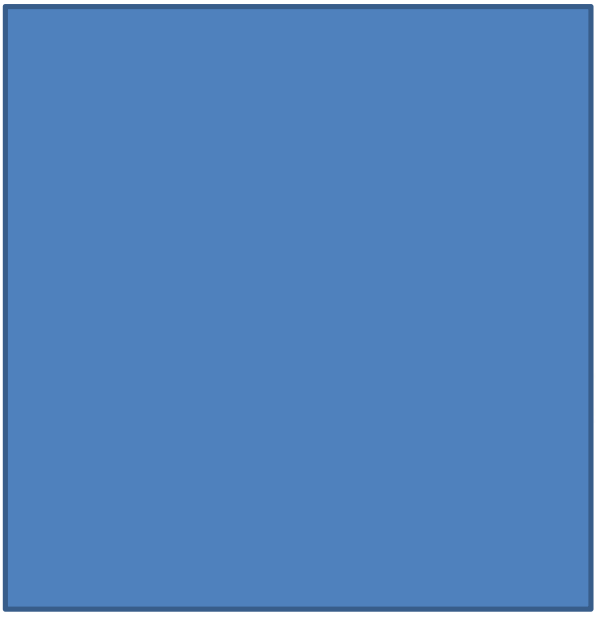

Figure 4.18: Screening of the bacterial isolates using Cry10spe primer (*cry10* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis israelensis* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

Figure 4.19: Screening of the bacterial isolates using Cry11 primer (*cry11* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis israelensis* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

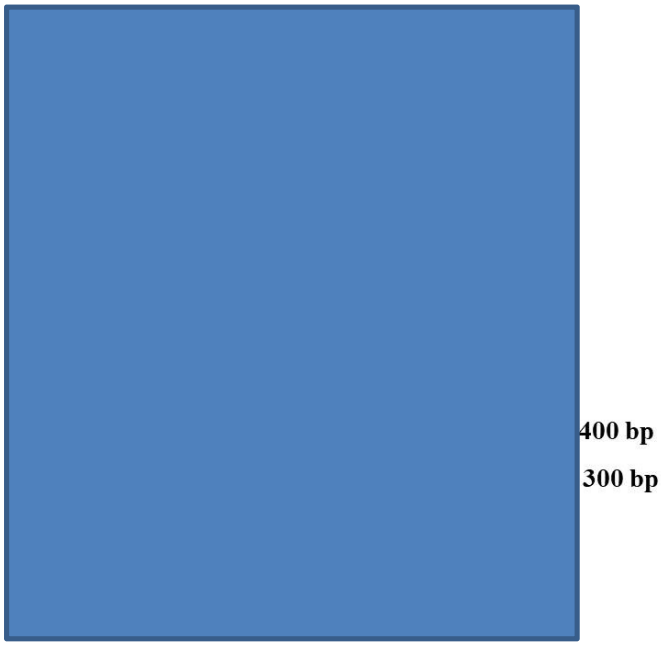

Figure 4.20: Screening of the bacterial isolates using Cyt2 (UN) primer (*cyt2* gene). **Lane 1**: R1; **Lane 2**: R2; **Lane 3**: R3; **Lane 4**: D1C: **Lane 5**: A2B; **Lane 6**: C6D; **Lane 7**: *Bacillus thuringiensis israelensis* (positive control); **Lane 8**: *Bacillus cereus* (negative control); **Lane 9**: Non-template control; **Lane M**: 100 bp DNA ladder (Thermofisher).

Table 4.2: Summary of PCR results

Genes	R1	R2	R3	D1C	A2B	C6D	BTI	BTK	BC
cry1	+	-	+	+	+	+		+	-
cry2	+	+	+	+	+	+		+	-
cry4	+	-	-	-	+	-	+		-
cry10	+	+	+	+	+	+	+		-
cry11	+	+	+	+	+	+	+		-
cyt2	+	+	+	+	+	+	+		-
chi	-	-	-	-	-	-	-	-	-
ps1	-	-	-	-	-	-	-	-	-
ps2	-	-	-	-	-	-	-	-	-
ps4	-	-	-	-	-	-	-	-	-

*(+) show the presence of respective gene and (-) show the absence of respective gene. BTI: *Bacillus thuringiensis isrelensis*. BTK: *Bacillus thuringiensis kurstaki.* BC: *Bacillus cereus.*

CHAPTER 5

DISCUSSION

5.1 Morphological Identification

All of the bacterial isolates R1, R2, R3, D1C, A2B and C6D were shown to be Gram-positive and rod-shaped, which was similar to the positive control *Bacillus thuringiensis israelensis.* These results are in agreement to previous researches conducted on *Bacillus thuringiensis* (Quesada-Moraga, et al., 2004; Muniady, Rathinam and Subramaniam, 2011), who reported that *Bacillus thuringiensis* are Gram-positive bacilli. During the process of Gram-staining, the pore size of the thick peptidoglycan layer of Gram-positive *Bacillus thuringiensis* shrink when alcohol is added, resulting in the retention of the crystal violet-iodine complexes even after washing, thus being shown as purple color. As for Gram-negative bacteria, the presence of alcohol dissolves the lipopolysaccharide layer of the cell membrane due to the thin peptidoglycan layer. As such, the crystal violet-iodine complexes are not able to be retained in Gram-negative bacteria after washing, leading to the appearance of red or pink color due to the uptake of safranin dye (Tortora, Funke and Case, 2004).

After 90 hours of incubation, all of the bacterial isolates R1, R2, R3, D1C, A2B and C6D were shown to be able to produce endospores, which was similar to

positive control *Bacillus thuringiensis israelensis.* These results correlate with the findings of Jisha, Smitha and Benjamin (2013), who reported that *Bacillus* spp. are capable of producing endospores to allow the bacteria to survive in extreme environmental conditions such as lack of nutrients. Spores are resistant to UV radiation, heat and chemicals because they are comprised of a tough proteinaceous covering called keratin. When the environment becomes more favorable, the endospore can reactivate itself to the vegetative state. Due to the tough coating, endospores resist to staining, thus the heating process was required to soften the coat of the endospores and enable the uptake of the malachite green dye. As the dye is insoluble in water, the washing step will decolorize the vegetative cells but not the endospores. The addition of safranin was used as counter stain to stain the decolorized vegetative cells. The vegetative cells would be stained pink while the endospores internally are stained green (Tortora, Funke and Case, 2004).

In addition to that, at 110 hours of incubation, all of the bacterial isolates R1, R2, R3, D1C, A2B and C6D and positive control *Bacillus thuringiensis israelensis* were shown to able to produce crystal proteins, which corresponds to the findings of Höfte and Whiteley (1989), Muniady, Rathinam and Subramaniam (2011) and Jisha, Smitha and Benjamin (2013). *Bacillus thuringiensis* is a closely related member within the *Bacillus cereus* group as they share many similar phenotype and genotype features, making it difficult for them to be distinguished from each other. Nevertheless, according to Höfte and Whiteley (1989), *Bacillus thuringiensis* is the only *Bacillus* sp. among the two that is able to produce crystal proteins, which makes it the only phenotypic

characteristic that can allow them to be distinguished from the other members of the *Bacillus cereus* group. Instead of phase contrast microscopy, Coomassie Brilliant Blue (CBB) staining was used to examine the presence of crystal proteins as CBB staining has improved resolution over phase contrast microscopy, allowing even very small parasporal bodies to be visualized (Rampersad and Ammons, 2005).

5.2 Crystal Proteins of *Bacillus thuringiensis*

The distinct shapes of the crystal proteins produced by the *Bacillus thuringiensis* can be used to identify their crystal protein classes and also the range of toxicity that they may exhibit against different insect orders (Höfte and Whiteley, 1989). There are various shapes of crystal proteins noted so far: bipyramidal, cuboidal, spherical, rectangular, ovoidal and also irregular-shaped. Although CBB staining can be used to verify the presence of crystal proteins, their definitive shapes and sizes are not entirely distinguishable due to the limitations of magnification of the compound light microscope. Therefore, two types of electron microscopy techniques were utilized, which were the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM), to visualize the shapes and sizes of the crystal proteins produced by the bacterial isolates.

Based on the electron micrographs generated, all of the bacterial isolates R1, R2, R3, D1C, A2B and C6D and positive control *Bacillus thuringiensis*

israelensis produced spherical-shaped crystal proteins with the sizes ranging from 0.3 to 0.6 µm, which are similar to the findings from previous studies, who noted all of their *Bacillus thuringiensis* strains showed formation of spherical-shaped crystal proteins with similar sizes (Saadoun, et al., 200; Silva, et al., 2010 and Monnerat, et al., 2005). The observation of spherical-shaped crystal proteins in positive control *Bacillus thuringiensis israelensis* also corresponds to the result of Saadoun, et al. (2001) who reported that *Bacillus thuringiensis israelensis* are able to produce spherical inclusions. Crystal proteins that come with spherical-shaped are the Cry4 proteins, which are found to have toxic effect against insect order Dipterans. In 2005, Monnerat, et al. claimed that their mosquito-killing *Bacillus thuringiensis* strains produce spherical-shaped crystal proteins, suggesting that the production of spherical-shaped crystal proteins associate with the strains' toxicity against mosquitoes. Not only that, Silva, et al. (2010) noted that the spherical formations could alternatively be correlated to the toxicity of *Bacillus thuringiensis* against Coleopterans. In present study, all of the bacterial isolates were shown to exhibit ability to produce spherical-shaped crystal proteins, suggesting that all bacterial isolates have potential in exhibiting toxicity against insect orders of Diptera and Coleoptera.

In addition, according to Höfte and Whiteley (1989), Cry4 proteins may also exists as ovoidal-shaped, which were observed in the bacterial isolates R2, R3 and D1C in SEM images and R3 and C6D in TEM images with the size of 1.3 µm. This observation further implicates the possibility that all of the bacterial isolates are able to exhibit toxicity against mosquitoes.

On the other hand, presence of cuboidal-shaped crystal proteins with the sizes ranging from 0.3 to 0.4 µm were observed in two of the bacterial isolates namely R1 and R2, which are similar to the findings of Silva, et al. (2010), who noted all of their *Bacillus thuringiensis* strains showed formation of cuboidal-shaped crystal proteins with similar sizes. According to previous studies, cuboidal crystal that represent the Cry2 protein was found in *Bacillus thuringiensis* strains that exhibit toxicity to insects from the orders of Lepidoptera and Diptera, thus suggesting that bacterial isolates R1 and R2 may have potential to exhibit toxicity to Lepidopteran and Dipteran order of insects (Höfte and Whiteley, 1989).

Besides that, bipyramidal-shaped crystal proteins that represent the Cry1 proteins that are toxic to Lepidopterans were not found in any of the bacterial isolates, but were exhibited by the positive control *Bacillus thuringiensis israelensis*, which correlate to the findings of Höfte and Whiteley (1989), who reported that *Bacillus thuringiensis israelensis* able to produce bipyramidal inclusions. The absence of bipyramidal-shaped crystal proteins in all bacterial isolates implicates that the bacterial isolates may not exhibit toxicity to Lepidopterans. All in all, the number and diversity of the crystal shape in this study are in agreement to a previous study done by Cinar, et al. (2008), who noted that spherical-shaped crystal proteins are the most abundant crystal shapes produced by *Bacillus thuringiensis* isolates, followed by cuboidal and lastly bipyramidal-shaped.

5.3 SDS-PAGE Analysis

SDS-PAGE is a method used to separate proteins based on their molecular sizes. Based on the result, the positive control *Bacillus thuringiensis israelensis* showed the presence of protein bands at sizes of 25 kDa, 29 kDa, 44 kDa and 70 kDa, representing CytA, Cyt2, Cry35 and Cry3 or Cry11 proteins respectively, which are corresponding to the findings in the research of Höfte and Whiteley (1989) research.

All of the bacterial isolates R1, R2, R3, D1C, A2B and C6D showed similar results in their protein profiles when compared to reference strain. The presence of protein bands with the sizes of 25 kDa, 44 kDa, 65 kDa and 70 kDa in all lanes indicate the presence of CytA, Cry35, Cry2, and Cry3 or Cry11 proteins in all of the bacterial isolates. These results are similar to the findings of Höfte and Whiteley (1989), Arrieta, Hernández and Espinoza (2004) and Monnerat, et al. (2005), who reported the presence of Cry2, Cry3, Cry11 and Cry35 proteins with the sizes mentioned above in their *Bacillus thuringiensis* strains. Generally, the CytA, Cry35, Cry2, Cry3 and Cry11 proteins were specific against the insect orders of Lepidoptera and Diptera, thus it can be suggested that all of the bacterial isolates may exhibit toxicity against these two insect orders (Crickmore, et al., 1998). Moreover, three of the bacterial isolates R1, R3 and A2B exhibited the protein bands with the size of 29 kDa, which

represent the Cyt2 proteins that are specific to Dipteran. Thus, it can be suggested that R1, R3 and A2B may exhibit insecticidal property against insects from the order of Diptera.

Interestingly, the presence of distinct protein bands with approximately 94 kDa were observed in two of the bacterial isolates R3 and A2B. A previous study by Restrepo, et al. (1997) reported that *Bacillus thuringiensis* subsp. *medellin*, which showed toxicity to larvae of mosquito, produced parasporal crystalline inclusions with sizes of 94 kDa, thus suggesting that R3 and A2B may exhibit toxic effect against insect order Dipetera. Moreover, a clear protein band with the size of 14 kDa was observed in R1, which was also reported in a study by Ellis, et al. (2002), who noted the presence of parasporal inclusion bodies with molecular mass of 14 kDa. These were represented as the Cry34 proteins, which were found in *Bacillus thuringiensis* isolates that show insecticidal activity against western corn rootworm (WCR) larvae. Thus, it can be anticipated that R1 may also exhibit toxic effect against WCR larvae.

However, SDS-PAGE analysis cannot distinguish proteins that come with similar molecular sizes. For instance, Cry3 and Cry11 share the molecular sizes of approximately 70 kDa, thus making it hard to verify the presence by merely observing the protein bands. In addition to that, all bacterial isolates that exhibited the Cry35, Cry3 and Cry11 protein bands are non-conclusive as the protein bands with the similar sizes of around 44 and 70 kDa were also observed in negative control *Bacillus cereus*. This can be due to the presence of a protein

of approximately 42 kDa called the 41.9-kDa protein, which are found to be produced by *Bacillus thuringiensis* and also *Bacillus cereus* (Palma, et al., 2014). Also, there were appearance of protein bands at the size of 70 kDa in *Bacillus cereus*, which represent Cry3 and Cry11 proteins, thus making the presence of Cry3 and Cry11 in bacterial isolates non-conclusive. This also may be due to the presence of protein with the size closely to 70 kDa such as chitinase, who has the size of 74 kDa. SDS-PAGE analysis may be able to determine the protein sizes, but does not ensure the presence of the crystal proteins. Therefore, the ability of the bacterial isolates to produce the crystal protein classes were further examined by performing polymerase chain reaction (PCR) to screen for the δ-endotoxin genes that can produce these crystal proteins.

5.4 PCR Analysis of δ-endotoxin Genes

Polymerase Chain Reaction (PCR) was carried out to screen for the presence of δ-endotoxin genes in the bacterial isolates. The δ-endotoxin genes were detected using their respective primers adapted from previous studies. Based on the results, all bacterial isolates R1, R2, R3, D1C, A2B and C6D were found to carry *cryII, cry10,* cry11 and cyt2 genes at their respective band sizes (Ejiofor and Johnson, 2002; Ibarra, et al., 2003; Mahalakshmi et al., 2012; El-Kersh, et al., 2016). Out of the six isolates, five of them which are R1, R3, D1C, A2B and C6D possess the *cryI* genes at 277 bp, which corresponds to the findings by Halima, et al. (2006). The presence of *cryIV* genes can be observed in only two bacterial isolates R1 and A2B at the band size of 423 bp, which correlate to

findings of Ejiofor and Johnson (2002), making it the least abundant *cry* genes present among the six *cry* genes tested.

The distribution of the *cry* and *cyt* genes in the bacterial isolates were similar with Lone, Malik and Padaria (2017), who reported that the *cryI* genes were the most abundant genes, followed by *cyt2*, *cry 11* and lastly *cryIV* genes. However, these results are not in agreement with Rosas García, et al. (2008), who claimed that the *cryIV* genes are more abundantly found than *cryI* and *cryII* genes. Not only that, none of the bacterial isolate contained any of the parasporin and chitinase genes, which was in contrast with the findings of Espino-Vazquez, et al. (2012) and El-Kersh, et al. (2016). This could be due to the strains of the *Bacillus thuringiensis* were isolated from different geographical areas, resulting in different distribution of δ-endotoxin genes among the bacteria isolates. This theory is supported by Djenane, et al. (2017), who explained that the contrast in the distribution of the δ-endotoxin genes with previous studies could be a consequence of the adaptation of *Bacillus thuringiensis* to the biotope.

The results obtained through electron microscopy, SDS-PAGE and PCR were evaluated as the shapes of the crystal proteins formation could be associated to the type of Cry protein and consequently to its *cry* gene (Höfte, et al., 1988). The spherical-shaped Cry4 proteins were shown to be present in all bacteria isolates, and this result corresponds to the findings in PCR analysis, which showed that all bacteria isolates carry either the *cryIV* genes or *cry10* and *cry11*

genes, which are also referred as *cryIVC* and *cryIVD* genes respectively (Crickmore, et al., 1998). Similarly, *cry11* gene was observed in all bacteria isolates in PCR analysis, and this correlate with the results of the protein profile in SDS-PAGE, which showed the presence of Cry11 protein bands in all of the bacterial isolates.

However, some of the shapes, proteins and gene profiles of bacterial isolates did not show any correlation. The appearance of Cry2 protein bands in SDS-PAGE in all bacterial isolates corresponds to the result in PCR analysis, which showed the presence of *cryII* genes in all bacteria isolates. However, cuboidal-shaped crystal proteins, which represent the Cry2 crystal protein, were only observed in R1 and R2. Also, only three of the bacterial isolates R1, R3 and A2B showed the presence of Cyt2 protein band, although result of the PCR analysis showed all of them to be carrying the *cyt2* gene. The bacterial isolates R1 and A2B which were found to possess the *cryIV* gene did not show any bands that corresponds to Cry4 proteins. In addition to that, although bacterial isolates R1, R3, D1C, A2B and C6D were found to carry *cryI* genes, none of these bacterial isolates showed appearance of Cry1 protein bands in SDS-PAGE nor the formation of bipyramidal-shaped crystal protein, which represent the Cry1 protein. Not only that, all bacterial isolates showed the presence of *cry10* genes, but none of the bacterial isolates showed the Cry10 protein bands. Moreover, all of the bacteria isolates exhibited presence of the Cry3 protein band, but in electron microscopy, no rhomboidal-shaped crystal proteins can be observed.

The differences in the shapes, proteins and genes profile in *Bacillus thuringiensis* isolates were also reported in the studies of Porcar and Juarez-Perez (2003) and Silva, et al. (2010) studies, thus it is suggested that the presence of genes does not necessarily yield the corresponding proteins. In 2008, Cinar, et al. mentioned that there is no correlation between the insecticidal crystal shapes of the isolates and *cry* genotypes. In 1995, Agaisse and Lereclus also concluded that the expression of δ-endotoxin genes can be turned on or off due to environmental factor, which explained the difference in the shapes and protein profiles of the bacteria isolates in this study, as the electron microscopy and SDS-PAGE analysis were not carried out at the same time period. This theory was supported by Armengol, et al. (2007), who claimed that the sporulation of each bacteria isolates depends on growth medium condition and varies from strain to strain, and some isolates might not have reached the autolysis phase to release higher amounts of crystal proteins for detection. In addition, Armengol, et al. (2007) also supported the idea that there is minimal to no correlation between the protein profiling and the δ-endotoxin genes as it is possible that the detected δ-endotoxin genes are inactive or the proteins are expressed in low amount, which then cannot be detected by using Coomassie Brilliant Blue (CBB) staining.

5.3 Limitation of Study and Future Work

In present study, SDS-PAGE was performed using extracted total proteins, which may lead to the presence of protein bands that are not related to the crystal proteins. Thus, additional purification step can be carried out to obtain a pure crystal proteins suspension, so that the protein bands obtained can represent the presence of the actual crystal proteins present. Similary, instead of whole bacteria suspension, pure crystal proteins suspension should be used for the study of the shapes of crystal proteins using electron microscopy, as whole bacteria suspension contains lysed bacteria and cell debris, which makes the identification of the shapes of crystal proteins difficult.

Nevertheless, the analysis of the crystal proteins using SDS-PAGE possess some limitations as this method helps to determine the protein sizes, but does not ensure the presence of proteins. Also, SDS-PAGE cannot distinguish proteins that come with similar molecular size. Thus instead of SDS-PAGE, Western blotting can be performed, whereby specific antibodies that binds to specific crystal proteins are used to confrim the presence of the respective crystal proteins. Not only that, Western blot can also confirm the expression of δ-endotoxin genes. The analysis of the protein profiles in *Bacillus thuringiensis* can be difficult as the expression of crystal proteins varies from strains to strains, and the environment factor may turn off the δ-endotoxin gene expression, resulting in low amount of crystal proteins. Therefore, silver staining could be used instead of Coomassie Brilliant Blue (CBB) staining to detect any low concentration of crystal proteins. In order to yield higher amount of crystal proteins, cloning can be carried out by inserting the genes coding for specific

crystal proteins into a host cell, and eventually larger amount of crystal proteins can be obtained when the host cell replicates.

In future works, screening of other δ-endotoxins that encodes for proteins that target different organisms such as Lepidoptera, Coleoptera, Hymenoptera, Gastropoda or Hemiptera can be carried out to fully assess the toxicity potential of the bacterial isolates. Larvicidal activity of different species of insect larvae can also be done to confirm the toxicity of bacterial isolates. Although parasporin genes were not detected in this study, screening of other δ-endotoxins genes that show cytotoxic activity against cancer cells or cytotoxic assay can also be carried out to verify the cytotoxic effect of bacterial isolates against cancer cells.

CHAPTER 6

CONCLUSION

All of the bacterial isolates R1, R2, R3, D1C, A2B and C6D were shown to be Gram-positive, rod-shaped *Bacillus thuringiensis* that produce endospores and crystal proteins. In electron microscopy, all bacterial isolates were able to produce spherical-shaped crystal proteins, suggesting that all bacterial isolates have potential to exhibit insecticidal property against insect orders from the order of Diptera. Ovoidal-shaped crystal proteins which also show toxic effect to Diptera can be observed in R2, R3, D1C and C6D. On the other hand, cuboidal-shaped crystal proteins were found in R1 and R2, which are known to be able to exhibit toxic effect against Lepidopteran and Dipteran, suggesting that R1 and R2 have potential in exhibiting toxic effect against these two insect orders.

In SDS-PAGE analysis, all bacterial isolates R1, R2, R3, D1C, A2B and C6D were shown to exhibit 25 kDa, 44 kDa, 65 kDa, and 70 kDa protein bands, indicating the presence of CytA, Cry35, Cry2, Cry3 and Cry11 proteins respectively, thus suggesting that all of the bacterial isolates have potential exhibit toxicity against Lepitopterans and Dipterans. In addition, bacterial isolates R1, R3 and A2B isolates were found to exhibit the protein size of Cyt2 protein, whereas R3 and A2B showed presence of 94 kDa proteins that can cause toxic effect to Diptera. Not only that, R1 was the only bacterial isolate

that exhibits Cry34 proteins which was found to be able to kill rootworm (WCR) larvae.

In PCR analysis, all bacterial isolates were found to carry *cryI*, *cryII*, *cryII, cry10, cry11* and *cyt2* genes except for R2 which was the only bacterial isolate that did not carry *cryI* genes. The presence of *cryIV* gene was detected in two bacterial isolates R1 and A2B. The differences in the shapes, protein and gene profile in *Bacillus thuringiensis* isolates suggested that the shapes of the crystal proteins and *cry* genotypes are not correlated, and the presence of genes does not necessarily yield the corresponding proteins. As a conclusion, all bacterial isolates were shown to carry Diptera-specific δ-endotoxins genes. No presence of chitinase and parasporin genes can be detected, suggesting that the bacterial isolates may not possess cytotoxic activity. However, cytotoxic assay or screening of other genes that can kill cancer cells can be carried out to further confirm the absence of parasporin genes in bacterial isolates.

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APPENDICES

Appendix A

List of instruments and apparatus used with respective manufacturers

Instrument/Apparatus	Manufacturers
Autoclaved Machine	Hirayama, Japan
Centrifuge Tube (15 ml & 50 ml)	Axygen Scientific, USA
Compound Light Microscope	Leica, Japan
Freeze dryer	Scanvac COOLSAFE™, Denmark
Gel Electrophoresis Set	Major Science, Taiwan
Heat block	BioShake IQ
Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Microwave Oven	Sharp, Japan
Mini-Protean Tetra System	Bio-Rad, US
Nano-photospectrometer	IMPLEN
Petri Dishes	Labmart
pH Meter	Sartorius, Gemmy Industrial Corp.
Shaking Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Scanninge Electron Microscope	JEOL (JSM-6701F),USA
Table-top Microcentrifuge Machine	Profuge
Transmission Electron Microscope	CM12, Philips
Vortex	VELP® Scientific, Europe
UV Transilluminator	Bio-rad, US

APPENDICES

Appendix B

List of chemicals, media and reagents used with respective manufacturers

Chemicals/Media/Reagents	Manufacturers
Agarose Powder	1st Base
Ammonium Persulfate (APS)	Sigma Aldrich
30% Bis-Acrylamide	Bio basic
Coomassie Brilliant Blue (R-250)	BioBasic, Canada
Chromatin Prestained Protein Ladder	Vivantis
Crystal Violet	Lab Stain
Dithiothreitol (DTT)	Thermofisher
DNA Extraction Kit	Geneaid, Taiwan
100-bp Plus DNA Ladder	Thermofisher
1kb Plus DNA Ladder	Thermofisher
6X DNA Loading Dye	Thermofisher
Gram's Iodine	Lab Stain
Glycerol	Qrec, Malaysia
25% Glutaraldehyde	Sigma-Aldrich
Malachite Green	R&M Marketing, Essex
PCR Master Mix	Promega
Novel-juice	GeneDireX
Nutrient Agar	Merck, Germany
Nutrient Broths	Merck, Germany
Total Protein Isolation Kit	ProteoSpin, Norgen
Safranin O	Bendosen
Sodium Dodecyl Sulphate (SDS)	Chem Solution
Tetramethylethylenediamine (TEMED)	Alfa Aeser
Tris-HCl	Chem Solution

APPENDICES

Appendix C

Preparation of Media

Preparation of Nutrient Agar

A total amount of 8 g of nutrient agar powder were dissolved in 1000 mL of distilled water in media bottle and autoclaved. Then, the media was poured into sterile agar plate and allowed to solidify at room temperature.

Preparation of Nutrient Broths

A total amount of 20 g of nutrient broths powder were dissolved in 1000 mL of distilled water in media bottle and autoclaved. Then, the media was stored in media bottle for prior to usage.

Preparation of 6X Sample Buffer for SDS-PAGE

A volume of 3.75 ml of 1 M Tris-HCl with the pH of 6.8 was dissolved in 6 ml of glycerol. Then, 1.2 g of SDS powder, 0.93 g of DTT powder and 0.006 g of bromophenol blue powder were added to the mixture and mixed well. Then, the mixture was topped up with water to 10 ml and stored at -20□ for prior to usage

Preparation of Loading-dye-Novel-juice

A total amount of 100 mL of novel juice was added with 400 mL of 6X loading dye to an Eppendorf tube. The mixture was mixed thoroughly and kept in the dark condition under -20°C.

Preparation of 1.5 % Agarose Gel

A total amount of 0.45 g of agarose powder was mixed with 30 mL of 1X TBE buffer. The mixture was heated in the microwave to dissolve the powder, then poured onto a gel cast and waited for 15 minutes for the gel to solidify.

Preparation of 2.0% Agarose Gel

A total amount of 0.3 g of agarose powder was mixed with 15 mL of 1X TBE buffer. The mixture was heated in the microwave to dissolve the powder, then poured onto a gel cast and waited for 15 minutes for the gel to solidify.