THE CHARACTERIZATION OF ISOLATED *Bacillus thuringiensis* FROM BLOCKS B, C, I AND ENGINEERING WORKSHOP OF UNIVERSITI TUNKU ABDUL RAHMAN, KAMPAR CAMPUS

By

IRENE CHONG YAN LING

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ABSTRACT

THE CHARACTERIZATION OF ISOLATED *Bacillus thuringiensis* FROM BLOCKS B, C, I AND ENGINEERING WORKSHOP OF UNIVERSITI TUNKU ABDUL RAHMAN, KAMPAR CAMPUS

Irene Chong Yan Ling

Bacillus thuringiensis is a Gram-positive, rod-shaped, endospore forming, facultative anaerobic soil bacterium that has the ability to produce crystal proteins. Their unique ability allows them to produce δ-endotoxins including Crystal (Cry) and Cytolytic (Cyt) proteins which make them valuable tools as biopesticides. The objectives of this study were to characterize the *Bacillus sp.* isolated from Blocks B, C, I and Engineering workshop of Universiti Tunku Abdul Rahman (UTAR), Kampar campus via morphological identification, SDS-PAGE analyses, screening of δ-endotoxin genes through PCR amplification and 16S rDNA gene sequencing. The morphological examination confirmed that all of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 were Gram-positive, rod-shaped, motile and able to produce endospore and crystal protein. The *cryI* gene was carried by all of the bacterial isolates except R3, however only C6D and R2 exhibited the Cry1 protein band on SDS-PAGE. In addition, all bacterial isolates possessed *cryII* gene, with the presence of Cry2 protein band exhibited on SDS-PAGE. Finally, the 16S rDNA gene sequencing analysis showed *Bacillus thuringiensis* as one of the results. In summary, by integrating all of the results, it can be concluded that the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 from UTAR,

Kampar campus showed morphological and molecular characteristics which are similar to *Bacillus thuringiensis*.

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DECLARATION

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

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Irene Chong Yan Ling

APPROVAL SHEET

This project report entitled **"THE CHARACTERIZATION OF ISOLATED *Bacillus thuringiensis* FROM BLOCKS B, C, I AND ENGINEERING WORKSHOP OF UNIVERSITI TUNKU ABDUL RAHMAN, KAMPAR CAMPUS"** was prepared by **IRENE CHONG YAN LING** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by,

____

Ms. Alicia Ho Lai Yee Date: 20th April 2017

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 20th April 2017

PERMISSION SHEET

It is hereby certified that **IRENE CHONG YAN LING** (ID Number: 14ADB06592) has completed this final year project entitled **"THE CHARACTERIZATION OF ISOLATED *Bacillus thuringiensis* FROM BLOCKS B, C, I AND ENGINEERING WORKSHOP OF UNIVERSITI TUNKU ABDUL RAHMAN, KAMPAR CAMPUS"** supervised by Ms. Alicia Ho Lai Yee (Supervisor) from Department of Biomedical Science, Faculty of Science.

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

Yours truly,

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(IRENE CHONG YAN LING)

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LIST OF ABBREVATIONS

g	Gravity
rpm	Revolutions per minute
kDa	Kilo-Dalton
δ-endotoxin	Delta-endotoxins
Cry proteins	Crystal proteins
Cyt proteins	Cytolytic proteins
Bt	*Bacillus thuringiensis*
Btk	*Bacillus thuringiensis kurstaki*
Bti	*Bacillus thuringiensis israelensis*
N-terminus	Amino-terminus
C terminus	Carboxyl-terminus
APN	aminopeptidase
ALP	Alkaline Phosphatase
GPI-anchored	Glycophosphatidylinositol-anchored
SDS	Sodium Dodecyl Sulphate
APS	Ammonium Persulfate
TEMED	Tetramethylethylenediamine
BLASTn	Nucleotide Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
% ID	Percent Identity
bp	Base pair
A260/A280	Assessment of Nucleic Acid Purity
E-value	Expected value

CHAPTER 1

INTRODUCTION

Bacillus thuringiensis is Gram-positive, spore forming bacteria (Bravo, Gill and Soberón, 2007; Tohidi, et al., 2013) that can be naturally found in soil, water, dead insects and grain dust (Valicente and Lana, 2008). It is able to produce δ-endotoxins which specifically kill insects of the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Mallophaga and some invertebrates for instance nematodes (Bravo, Gill and Soberón, 2007; Palma, et al., 2014). The δ-endotoxins produced are toxic to pests and insects, which allows *Bacillus thuringiensis* to be used as a valuable tool and adopted in the Insect Pest Management (IPM) programme.

During the sporulation phase, *Bacillus thuringiensis* produces a sporangium that contains an endospore and insecticidal proteins as shown in Figure 1.1. The parasporal crystal proteins (δ-endotoxins) are comprised of Crystal (Cry) and Cytolytic (Cyt) toxins (Bravo, Gill and Soberón, 2007; Xu, et al., 2014). Once the insects ingest the protoxin, they are solubilized in the alkaline conditions of the insects' midgut. After that, the protoxin is cleaved by the midgut proteases to produce toxin fragments via a proteolytic process. Then, the toxins bind to specific receptors located in the peritrophic membrane to create pores that will cause cell perforation, cell membrane lyse and eventually leading to the insects' death (Bravo, Gill and Soberón, 2007; Schnepf, et al., 1998; Xu, et al., 2014).

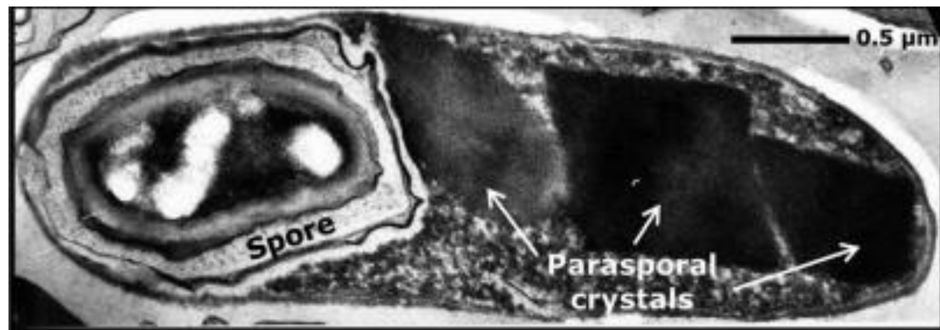

Figure 1.1: The sporulated cell of *Bacillus thuringiensis* subsp. *morrisoni* under transmission electron micrograph (Adapted from Ibrahim, et al., 2010).

The *cryI*, *cryII*, *cryIII* and *cryIV* genes are the four major classes of *cry* genes which encode for Cry toxins that are toxic against Lepidopterans, Lepidopterans and Dipterans, Coleopterans, and Dipterans respectively (Höfte and Whiteley, 1989). In contrast, the Cyt toxins encoded by the *cyt* gene are mostly found in Diptera specific *Bacillus thuringiensis* strains (Bravo, Grill and Soberón, 2007).

Insects are members of the animal kingdom and are characterized in the phylum Anthropoda and in the class Insecta. They are the most successful animals around the world (Australia Museum, 2011) and out of every eight living species, seven are insects (Amateur Entomologists' Society, 1997). Although insects play a major role in the ecosystem, including decomposition of dead plants and animals, as a pollinators, and serve as food for other animals, however, insects can transmit diseases to human and animals, and cause damages to agricultural crops as well as landscape plants. In future years, the agricultural industry will be facing increasing challenges in food production due to worldwide food shortage crisis. Approximately 35% of total losses of

crops occur due to infestations of insects, fungi, bacteria or viruses (Pardo-López, Soberón and Bravo, 2013).

Chemical pesticides were widely used to control insects before the development of biopesticides. Some of the chemical pesticides currently available are extremely toxic to non-target organisms and are harmful to the health of human and animals; causing soil and water pollutions, and increasing the risk of diseases like cancer and autoimmune system disorders. Furthermore, long term exposure to chemical pesticides will cause the insects to develop resistance towards them. Thus, reduction of such dependence on chemicals as the sole control method and sourcing of alternative options for insect control is therefore critical (Devine and Furlong, 2007).

Microbial insecticides have been extensively used as alternatives to substitute chemical pesticides for insect control in main crops. *Bacillus thuringiensis* is a remarkably eco-friendly pest control agent that has the potential to replace the use of chemical pesticides (Jenkins, Roman and Einarsson, 1999). Usage of *Bacillus thuringiensis* is more preferred in order to reduce many problems associated with chemical pesticides, especially environmental pollution and public health problems (Shishir, et al., 2014). Over the past century, biopesticides containing *Bacillus thuringiensis* was used in forestry and agriculture, and for the control of black flies and mosquitoes which are disease vectors. In agriculture, the USDA Cotton Insects Research Laboratory in

Brownsville, Texas isolated a single strain of *Bacillus thuringiensis* known as HD-1 which was eventually included as a product of biological insecticides in 1970 (Jenkins, Roman and Einarsson, 1999; Sanchis and Bourguet, 2008). Another study showed that the commercially available formulations of *Bacillus thuringiensis* were shown to be the most prominent and successful form of biological control used worldwide (Carlton, 1988).

Therefore, the aim of this research were:

1. To characterize the isolated *Bacillus sp.* samples from ex-tin mining soils in Universiti Tunku Abdul Rahman (UTAR), Kampar campus through morphological identification.
2. To determine the δ-endotoxin genes present in the isolated *Bacillus sp.* samples.
3. To identify the isolated *Bacillus sp.* samples through 16S rDNA gene sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Bacillus thuringiensis*

The era of *Bacillus thuringiensis* begun in year 1901, when a Japanese biologist named Shigetane Ishiwata isolated the bacterium from silkworm larvae during his investigation of the cause of sotto disease (sudden-collapse disease). He called the bacterium as Sottokin-Bacillus but this name did not last long (Ishiwata, 1905). After 10 years, Ernst Berliner, a German biologist rediscovered the same bacteria from diseased flour moth larvae and named it *Bacillus thuringiensis* (Berliner, 1911). Berliner's description and name was used instead, as the description by Ishiwata was too brief and inadequate (Milner, 1994). In the year 1915, Berliner reported the presence of inclusion bodies alongside the endospore. The mechanism of the inclusion bodies was not yet discovered then (Berliner, 1915). At nearly the same time, Aoki and Chegãsaki noted that the bacterium was capable of causing disease if the old and sporulated cultures were fed to insects (Aoki and Chegãsaki, 1915). Besides, Mattes also observed the same inclusion bodies in *Bacillus thuringiensis* in 1927 (Mattes, 1927).

In the year 1956, Angus and a team of researchers found that the insecticidal activities against moth insects were due to the presence of parasporal crystals produced by *Bacillus thuringiensis* (Angus, 1953; Hannay, 1953; Hannay and Fitz-James, 1955). With that, researchers begun their interest and started to

work on *Bacillus thuringiensis* crystal's structure, biochemistry and its mechanism. Until the year 1977, only 13 strains of *Bacillus thuringiensis* subspecies were described and their parasporal crystals were only toxic against Lepidopteran larvae. Later, scientists successfully discovered subspecies that were toxic against Dipteran larvae and Coleopteran larvae separately (WHO, 1999). With the advancement of technologies, thousands of *Bacillus thuringiensis* strains were found with their respective toxins against respective species of insects larvae (University of California San Diego, 2016). In more recent decade, studies have also shown that *Bacillus thuringiensis* can produce non-insecticidal proteins besides those which have insecticidal properties (Ibrahim, et al., 2010).

2.2 Taxonomy of *Bacillus thuringiensis* Strains

Bacillus thuringiensis is closely related to *Bacillus cereus* as it is a member of the *Bacillus cereus* group. The members of *Bacillus cereus* group included *Bacillus mycoides, Bacillus cereus* and *Bacillus anthracis* (Helgason, et al., 2000; Jensen, et al., 2003; Roh, et al., 2007). Within this group, phenotypic differentiation is difficult. *Bacillus thuringiensis* and *Bacillus cereus* are motile, while *Bacillus thuringiensis, Bacillus cereus,* and *Bacillus mycoides* are collectively reported as penicillin and hemolytic resistant (Fritze, 2004). According to De Barjac and Bonnefoi (1962), the formation of parasporal inclusion bodies in *Bacillus thuringiensis* is the only phenotypic difference between *Bacillus thuringiensis* and *Bacillus cereus.*

2.2.1 Phenotypic Characterization

The serological analysis of the flagellar (H) antigens were the primary classification of *Bacillus thuringiensis* strain, and has been used ever since (De Barjac and Bonnefoi, 1962). A very motile bacterial culture is required when performing H-serotyping. When producing defined H-antigen serum, the selected reference strains' flagellar suspension from each serotype will be injected into rabbits, and then the bacteria's flagellar suspension is titrated against the serum of defined serotype (Thiery and Frachon, 1997). Nevertheless, this classification by serotype was eventually added with morphological and biochemical identifications (De Barjac, 1981). By the end of 1998, over 69 serotypes and 82 serovars of *Bacillus thuringiensis* were identified and classified according to flagellar H-antigens (Lecadet, et al., 1999).

However, H-serotyping comes with limitations as it might not be enough to represent the molecular characteristics of a strain (Roh, et al., 2007), it is unable to differentiate strains that come with similar H serovar or serotype (Soufiane and Côté, 2009), and it is independent from the characterization of strains in view of the biological activities or the production of parasporal protein (Shisa, et al., 2001). Besides, the long process of H-serotyping made it unsuitable to be used for screening of larger collections of *Bacillus thuringiensis* isolates (Hansen, et al., 1998). Therefore, to anticipate the insecticidal action of the *Bacillus thuringiensis* strains, a combination of serological characterization with toxicity bioassays is necessary as there is no

direct association between the insecticidal activity and serovar (Porcar and Caballero, 2000).

On top of that, presence of crystal proteins is one of the main characterization of *Bacillus thuringiensis*. Phase contrast microscopy has been used to examine the presence of crystal proteins (Ammons, Rampersad and Khan, 2002) and this method is beneficial in the screening of novel environmental isolates (Bravo, et al., 1998). The morphology of the crystal proteins will help to indicate the toxicity of the strain instead of the serotypes (Higuchi, et al., 1998; Mikkola, et al., 1982). As reported by Höfte and Whiteley (1989), insects under the orders of Lepidoptera, Lepidoptera and Diptera, Diptera, and Coleoptera are susceptible to bipyramidal, cuboidal, ovoidal, and rhomboidal inclusions respectively.

Besides, SDS-PAGE can be used to determine the crystal protein composition from *Bacillus thuringiensis* strains. Moreover, to determine the toxicity of *Bacillus thuringiensis* against insects, bioactivity tests can be conducted in order to understand the mechanism of action of toxins and improve commercial formulations (Aronson, Beckman and Dunn, 1986).

2.2.2 Genotypic Characterization

Polymerase chain reaction (PCR) has been used to identify genes that are responsible in encoding *Bacillus thuringiensis* insecticidal toxins by using primers that are homologous to conserved regions of the toxin genes (Carozzi,

et al., 1991; Cleave, Williams and Hedges, 1993). Due to its rapidity and consistency, PCR has replaced the use of bioassays in preliminary classification of *Bacillus thuringiensis* collection (Porcar and Juárez-Pérez, 2003). Thus, the insecticidal activity of a given strain can be determined through the identification of toxin genes including *cry* and *cyt* genes via PCR method. Universal primers can be used to target the highly conserved regions which recognize a group or subfamilies of *cry* genes for preliminary screening purposes but there is also specific primers that target the variable region of an individual *cry* gene (Ben-Dov, et al., 1997; Porcar and Juárez-Pérez, 2003).

Although 16S rDNA gene sequencing has poor classification power for bacteria with similar genera and poor phylogenetic power in species level, it is highly recommended for bacterial classification (Bosshard, et al., 2006; Mignard and Flandrois, 2006). Several studies have reported that *Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis* were considered as a single species (Bavykin, et al., 2004; Helgason, et al., 2000) as the 16S rRNA nucleotide sequence among them exhibited more than 99% similarity (Ash, et al., 1991). As stated by Petti (2007) and Tang, et al. (1998), the identity or similarities of gene sequence among some genera has high score values and cannot be differentiated between species. Thus, the analyses of 16S rDNA and 23S rDNA sequencings have shown that the species in *Bacillus cereus* group have nearly identical and undifferentiated sets of nucleotide sequences (Ibrahim, et al., 2010; Miller, 2008).

Besides, several approaches have been suggested in order to differentiate *Bacillus cereus* group members, including the colony DNA hybridization (Hansen, et al., 1998), amplified fragment length polymorphism (Ticknor, et al., 2001) and PCR-restriction fragment length polymorphism (Chang, et al., 2003). These methods can work together to cluster new strains and aid to identify those strains that cannot be identified by H-serotyping (Hansen, et al., 1998; Joung and Côté, 2001). In addition, random amplified polymorphic DNA (RAPD) (Hansen, et al., 1998) has been used in studies and was shown to be able to differentiate *Bacillus thuringiensis* serovars, identified on the basis of RAPD fingerprints, and even individual strains within the same serotype can be discriminated (Brousseau, et al., 1993; Gaviria-Rivera and Priest, 2003; Pattanayak, et al., 2001).

2.3 Natural Habitats of *Bacillus thuringiensis*

Bacillus thuringiensis are often found naturally in soil. In the year 1981, DeLucca and his team found *Bacillus thuringiensis* denoted in a range of 0.005% - 0.5% of all the isolates of *Bacillus* species in the United States. Ohba and Aizawa (1986) have successfully isolated *Bacillus thuringiensis* from 72% of the 189 soil samples in Japan. Martin and Travers (1989) reported that the *Bacillus thuringiensis* are distributed worldwide. They isolated *Bacillus thuringiensis* from 70.4% of the 1,115 soil samples collected in 30 countries and found *Bacillus thuringiensis* was well represented in agricultural fields, beaches, forests, grasslands, high-altitude mountains, tropical jungles, temperate and tropical caves, scrub wilderness and urban locations. Regionally,

Bacillus thuringiensis kurstaki was most abundant in Asian soils while *Bacillus thuringiensis israelensis* was rich in the United States and Europe (Martin and Travers, 1989).

In another related work, the Coleoptera-active and Lepidoptera-active *Bacillus thuringiensis* serovars are mainly found in soil and phylloplane of coniferous trees, deciduous trees, vegetables, and from other herbs (Damgaard, et al., 1997; Smith and Couche, 1991). Based on Jara, Maduell and Orduz (2006), majority *Bacillus thuringiensis* isolates on phylloplane of maize and bean harbored *cryI* gene which was Lepidotera-specific and some isolates harbored *cryIV* and *cryXI* genes which have protein profiles similar to *Bacillus thuringiensis medellin* and *Bacillus thuringiensis israelensis*. Besides, the Diptera-active *Bacillus thuringiensis*, LLP29 which carried *cyt1* gene was discovered from the phylloplane of *Magnolia denudate* (Zhang, et al., 2010).

Meadows and team (1992) discovered *Bacillus thuringiensis* from stored grain products, and in 1999, the World Health Organization (WHO), reported isolating several *Bacillus thuringiensis* subspecies from insect cadavers of different insect orders. For instance, as reported by Clement (2012), *Bacillus thuringiensis kurstaki* was isolated from *Ephestia kuehniella* (Lepidoptera) whereas *Bacillus thuringiensis israelensis* was found on *Culex pipiens* (Diptera). Besides, *Bacillus thuringiensis tenebrionis* was isolated from *Tenebrio molitor* (Coleoptera) (Clement, 2012). In addition, *Bacillus*

thuringiensis thuringiensis was isolated from the larch fly, *Pristiphora erichsonii* (Hymenoptera) (Morris and Moore, 1983).

Bacillus thuringiensis can be found in aquatic environment. Ichimatsu, et al. (2000) successfully isolated *Bacillus thuringiensis* from still-water (ponds) and running-water (river, streams and ditches) habitats of Kyushu, Japan and this bacteria exhibited larvicidal activity against aquatic Diptera, *Culex pipiens* and *Culex molestus* (mosquito), and *Clogmia albipunctata* (moth-fly). In Spain, isolates of *Bacillus thuringiensis* were discovered in aquatic environment and were active against *Tipula oleracea* (Marsh Crane fly) larvae (Iriarte, et al., 2000).

2.4 *Bacillus thuringiensis* Parasporal Crystalline Proteins

During sporulation time, *Bacillus thuringiensis* produces parasporal crystalline proteins (δ-endotoxin), which included Crystal (Cry) and Cytolytic (Cyt) proteins that are toxic against different range of insect orders (Figure 2.1). The extra chromosomal genes that are primarily found on plasmids were responsible to encode for the production of these δ-endotoxins (Jisha, Smitha and Benjamin, 2013). When the toxins are ingested by insect larvae, the toxins are then solubilized and undergo proteolysis by the midgut proteases to form active toxin. Subsequently, the toxins bind to the specific receptors on the insect's gut membrane, causing its perforation, leading to cell membrane lyses, and eventually causing the death of the larvae (Bravo, Gill and Soberón, 2007; Schünemann, Knaak and Fiuza, 2014).

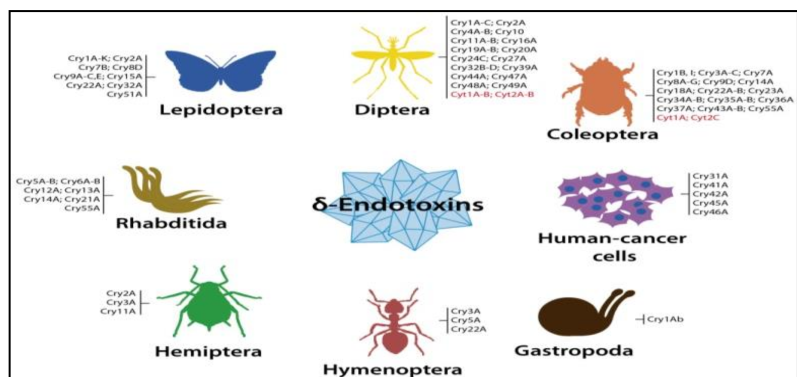

Figure 2.1: Overview of the known host range of *Bacillus thuringiensis* δ-endotoxins (crystal and cytolytic) (Adapted from Palma, et al., 2014).

According to Agaisse and Lereclus (1995), 25% of the dry weight of sporulated *Bacillus thuringiensis* consist of the crystal inclusion products. Based on Schnepf, et al. (1998), the production of crystal proteins by *Bacillus thuringiensis* can be at a maximum of up to 2×10^6 δ-endotoxin molecules. Figure 2.2 shows the stained crystal proteins viewed under phase contrast microscopy at the magnification of 1000X.

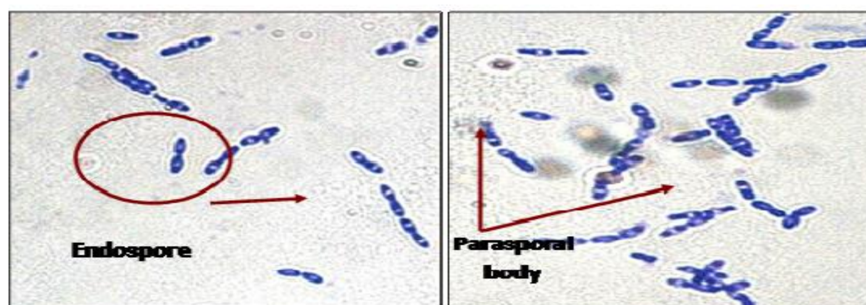

Figure 2.2: The sporulated cells (in circle), formation of endospore and stained parasporal bodies (arrows) under phase contrast microscopy at 1000X magnification (Adapted from Muniady, Rathinam and Subramaniam, 2011).

2.4.1 Crystal (Cry) Protein

In the year 1989, Höfte and Whiteley introduced the first classification and nomenclature for the toxin proteins (Figure 2.3). They classified the Cry proteins into four major classes which were the Cry1, Cry2, Cry3 and Cry4

proteins that are toxic against the insect orders of Lepidopterans, Lepidopterans and Dipterans, Coleopterans, and Dipterans respectively. The classification of the Cry proteins is based on host range specificity and the number of amino acids (Höfte and Whiteley, 1989).

Protein class	Gene type	Host range[a]	No. of amino acids	Predicted molecular mass (kDa)
Cry1	*cryIA(a)*	L	1,176	133.2
	cryIA(b)	L	1,155	131.0
	cryIA(c)	L	1,178	133.3
	cryIB	L	1,207	138.0
	cryIC	L	1,189	134.8
	cryID	L	1,165	132.5
Cry2	*cryIIA*	L/D	633	70.9
	cryIIB	L	633	70.8
Cry3	*cryIIIA*	C	644	73.1
Cry4	*cryIVA*	D	1,180	134.4
	cryIVB	D	1,136	127.8
	cryIVC	D	675	77.8
	cryIVD	D	643	72.4
Cyt1	*cytA*	D/cytol	248	27.4

[a] Specific host ranges: L. Lepidoptera; D. Diptera; C. Coleoptera; cytol., cytolytic and hemolytic.

Figure 2.3: Insecticidal crystal proteins of *Bacillus thuringiensis* (Adapted from Höfte and Whiteley, 1989).

Until the year 2012, 70 classes of Cry proteins (Cry1–Cry70) and 2 classes of Cytolytic proteins (Cyt1–Cyt2) have been classified based on the amino acid sequence similarities (Figure 2.4) (Sarker and Mahbub, 2012). Based on Crickmore, et al. (1998), each Cry group toxins share less than 40% amino acid identity with proteins from other groups. The crystal proteins come in different

shapes for instance Cry1 protein is bipyramidal (Aronson, Beckman and Dunn, 1986), Cry2 protein is cuboidal (Ohba and Aizawa, 1986), Cry3 protein is rhomboidal, flat and square (Herrnstadt, et al., 1987; Höfte and Whiteley, 1989; López-Meza and Ibarra, 1996), and Cry4 protein is ovoidal in shape (Höfte and Whiteley, 1989). Irregular and spherical shape crystal morphologies can also be detected (Onyancha, 2016).

Besides amino acid compositions, Cry proteins can also be classified on the basis of range specificity (Crickmore, et al., 1998). The toxins against Lepidoptera involve Cry1, Cry2 and Cry9 proteins (Crickmore, 2000); toxins against Coleoptera include Cry3, Cry7, Cry8 and Cry9 (De Maagd, et al. 2003; Ellis, et al. 2002); toxins against Diptera such as Cry9, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins (Zeigler, 1999). Other than Lepidopterans, Coleopterans and Dipterans, Cry proteins are also toxic towards the insect order Hymenoptera, and nematodes such as those that are parasitic to human and animals and Rhabditida, as well as mites and protozoa (De Maagd, et al., 2001; Palma, et al., 2014; Schnepf, et al., 1998; Wei, et al., 2003). However, studies have shown that some crystal proteins are toxic against more than one insect order. Cry1B protein is Lepidoptera, Coleoptera and Diptera specific whereas Cyt1A is both Coleoptera and Diptera specific (Palma, et al., 2014).

No	Class	Sub class	No	Class	Sub class	No	Class	Sub class
1	Cry1	241	26	Cry26	1	51	Cry51	2
2	Cry2	68	27	Cry27	1	52	Cry52	2
3	Cry3	19	28	Cry28	2	53	Cry53	2
4	Cry4	14	29	Cry29	1	54	Cry54	3
5	Cry5	12	30	Cry30	11	55	Cry55	2
6	Cry6	4	31	Cry31	10	56	Cry56	2
7	Cry7	21	32	Cry32	7	57	Cry57	1
8	Cry8	38	33	Cry33	1	58	Cry58	1
9	Cry9	30	34	Cry34	11	59	Cry59	1
10	Cry10	4	35	Cry35	11	60	Cry60	6
11	Cry11	7	36	Cry36	1	61	Cry61	3
12	Cry12	1	37	Cry37	1	62	Cry62	1
13	Cry13	1	38	Cry38	1	63	Cry63	1
14	Cry14	1	39	Cry39	1	64	Cry64	1
15	Cry15	1	40	Cry40	4	65	Cry65	2
16	Cry16	1	41	Cry41	4	66	Cry66	2
17	Cry17	1	42	Cry42	1	67	Cry67	2
18	Cry18	3	43	Cry43	4	68	Cry68	1
19	Cry19	2	44	Cry44	1	69	Cry69	2
20	Cry20	3	45	Cry45	1	70	Cry70	3
21	Cry21	3	46	Cry46	8		Cyt1	12
22	Cry22	6	47	Cry47	1		Cyt2	24
23	Cry23	1	48	Cry48	5			
24	Cry24	3	49	Cry49	5			
25	Cry25	1	50	Cry50	3			

Figure 2.4: Classification of Cry and Cyt proteins identified from *Bacillus thuringiensis* in year 2012 (Adapted from Sarker and Mahbub, 2012).

Cry protoxins usually have an additional carboxyl region that is responsible for the formation of crystal proteins but not in their toxicity. When the crystal proteins are subjected to the alkaline conditions in the larval midgut, the carboxyl-terminus and/or the amino-terminus of the protoxins are then proteolytically removed by midgut proteases (De Maagd, et al., 2003; Schnepf, et al., 1998).This is followed by the production of a smaller sized active

protease-resistant toxin that consists of functional domain structures – Domains I, II and III, which are individually responsible in: pore formation and membrane insertion in the insect's midgut epithelium, involved in protein-receptor interactions, and protect the toxin from proteolysis by maintaining the integrity of the toxin molecule structure within the target organism's gut (Deist, et al., 2014).

2.4.1.1 *cryI* Gene

The molecular mass of the Cry1 protein ranges from 130 kDa to 140 kDa (Cerstiaens, et al., 2001). The *cryI* genes encode for Lepidoptera-specific crystal proteins which are bipyramidal in shape (Cannon, 1996). When the Cry1 proteins are ingested by insect larvae, the solubilized inactive protoxin will be cleaved into 60 kDa to 70 kDa fragments to yield the active protease-resistant toxins in the larvae midgut. The examples of *Bacillus thuringiensis* strains that carry the *cryI* gene include, *Bacillus thuringiensis aizawai, Bacillus thuringiensis kurstaki, Bacillus thuringiensis thuringiensis* and *Bacillus thuringiensis morrisoni* (Höfte and Whiteley, 1989).

2.4.1.2 *cryII* Gene

The *cryII* gene encodes for proteins that are active against Lepidopteran and Dipteran species. The molecular mass of Cry2 protoxin encoded by the *cryII* gene is 65 kDa in size and form cuboidal inclusions (Höfte and Whiteley, 1989). This protoxin is enzymatically cleaved by midgut proteases into a molecular mass of 60 kDa to 62 kDa toxin for insecticidal activity (Cherry,

2013). Studies found several subspecies of *Bacillus thuringiensis* that carry *cryII* gene which include, *Bacillus thuringiensis tolworthi, Bacillus thuringiensis kenyae, Bacillus thuringiensis thuringiensis* Berliner and *Bacillus thuringiensis kurstaki* HD-1 (Moar, et al., 1994; Yamamoto, 1983; Yamamoto, Garcia and Dulmage, 1983). In addition, Nicholls, Ahmad and Ellar (1989) suggested that Cry11 and Cry1A proteins may exhibit synergist effect against certain insect species.

2.4.1.3 *cryIII* Gene

Bacillus thuringiensis san diego (Herrnstadt, et al., 1987), *Bacillus thuringiensis tenebrionis* (Krieg, et al., 1983) and *Bacillus thuringiensis* EG2158 (Donovan, Dankocsik and Gilbert, 1988) are the *Bacillus thuringiensis* strains that express *cryIII* genes. The *cryIII* genes encode for Coleoptera-specific proteins which are 72 kDa in mass and the crystals are rhomboidal in shape. McPherson, et al. (1988) reported the Cry3 proteins can be converted to 66 kDa toxins by removal of the 57 N-terminal amino acid with the help of spore-associated proteases, which is essential for toxin actions.

2.4.1.4 *cryIV* Gene

Bacillus thuringiensis israelensis produces inclusion crystal proteins which are toxic to Dipteran species for instance blackfly and mosquito larvae (Goldberg and Margalit, 1977), but innocuous to Lepidopteran larvae which was reported by Tyrell, et al. (1981). The *cryIV* class of crystal protein gene is made up of a heterogenous group of Diptera-specific crystal protein genes that encode for

the Cry4A (135 kDa), Cry4B (128 kDa), Cry4C (78 kDa) and Cry4D proteins (72 kDa). These proteins will assemble together with the CytA protein with the size of 27 kDa to form an ovoid complex. The Cry4A and Cry4B protoxins were reported to be proteolytically converted into smaller active toxic fragments of 53 kDa to 78 kDa in different studies 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).

2.4.2 Cytolytic (Cyt) Protein

The cytolytic toxin which is another insecticidal protein is specifically encoded by *cyt* genes. The toxin is 27 kDa in size and is Diptera-specific (Höfte and Whiteley, 1989). Moreover, studies have reported that Cyt toxins exhibit toxicity against a broader spectrum of insect orders, including Diptera, Lepidoptera and Coleoptera (Guerchicoff, Delecluse and Rubinstein, 2001) and also nematodes and cancer cells (Van Frankenhuyzen, 2009). Up to the present time, the Bt Toxin Nomenclature Committee has categorized Cyt proteins into three different groups (Cyt1, Cyt2 and Cyt3) (Crickmore, et al., 2017) with their toxicity mostly against mosquitoes and blackflies (Soberón, Lopez-Diaz and Bravo, 2013). *Bacillus thruingeinsis* strains that carry *cyt* gene include, *Bacillus thuringiensis israelensis* and *Bacillus thuringiensis morrisoni* (Palma, et al., 2014). An interesting finding of the Cyt proteins is their capability to suppress insect resistance to certain Cry proteins in some insect species and to exert insecticidal activity with other Cry toxins synergistically (Soberón,

López-Díaz and Bravo, 2013). For instance, the Cyt1Aa toxin is active against *Chrysomela scripta* (cottonwood leaf beetle) and suppresses resistance to the Cry3Aa proteins (Federici and Bauer, 1998).

2.5 The General Mechanism of Action of the Crystal Protein Toxins

When the crystal protein is ingested by insect larvae, the solubilized crystal protein will be digested by protease in the alkaline midgut environment, in which either the N- or C- terminal of the inactive proteins will be removed to become activated protease-resistant three-domain (Domains I, II, III) monomer. Then, the activated toxins interact with specific receptors such as aminopeptidase (APN) and alkaline phosphatase (ALP) on the surface of the epithelial cell, allowing the insertion of toxin into the membrane to create a pore which is permeable to amino acids, sugars and inorganic ions (Carroll, Wolfersberger and Ellar, 1997; Kirouac, et al., 2002). Massive influx of solutes from midgut lumen due to the formation of pores can lead to colloid-osmotic lysis of cell, causing the destruction or damage of cells and eventually lead to the death of the larvae (Figure 2.5).

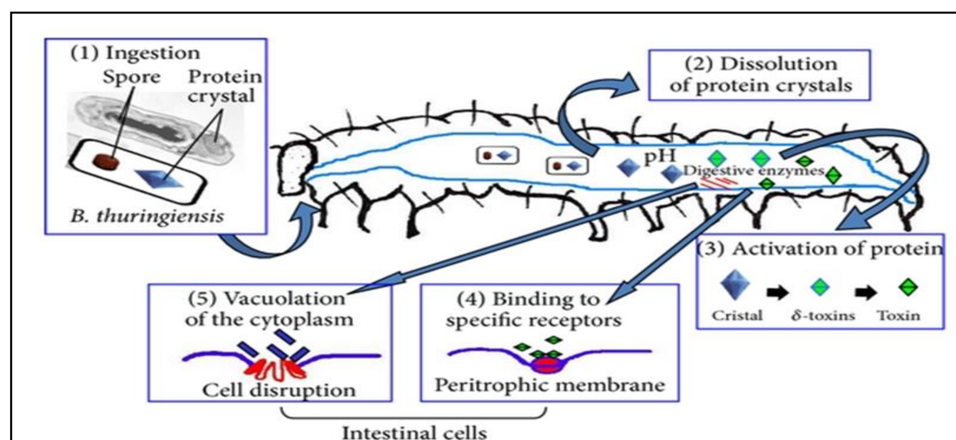

Figure 2.5: The overview mechanism of the *Bacillus thuringiensis* toxin in Lepidoptera (Adapted from Schünemann, et al., 2012).

According to Bravo, et al. (2011) and Xu, et al. (2014), the activated toxins will bind to ALP and Glycophosphatidylinositol-anchored (GPI-anchored) APN receptors with low affinity to induce the concentration and localization of the activated toxins. Besides, when activated toxin bind to the cadherin receptors, proteolytic cleavage at N- terminal end will occur and then pre-pore oligomer will form. These can increase the binding affinity of oligomer to ALP and GPI-anchored APN receptors. The insertion of oligomer into the membrane, causes the formation of pore and eventually leading to cell lysis (Bravo, et al., 2011).

However, there is a difference between Cry and Cyt toxins upon binding to the receptor. As seen in Figure 2.6, Cry toxins will bind to specific receptors of midgut cells, whereas Cyt toxins will bind to the phospholipid bilayer and insert into the peritrophic membrane, leading to the formation of pore (Bravo, Gill and Soberón, 2007; Gill, Cowles and Pietrantonio, 1992; Promdonkoy and Ellar, 2003) or destruction of the membrane by detergent-like interaction (Butko, 2003; Manceva, et al., 2005).

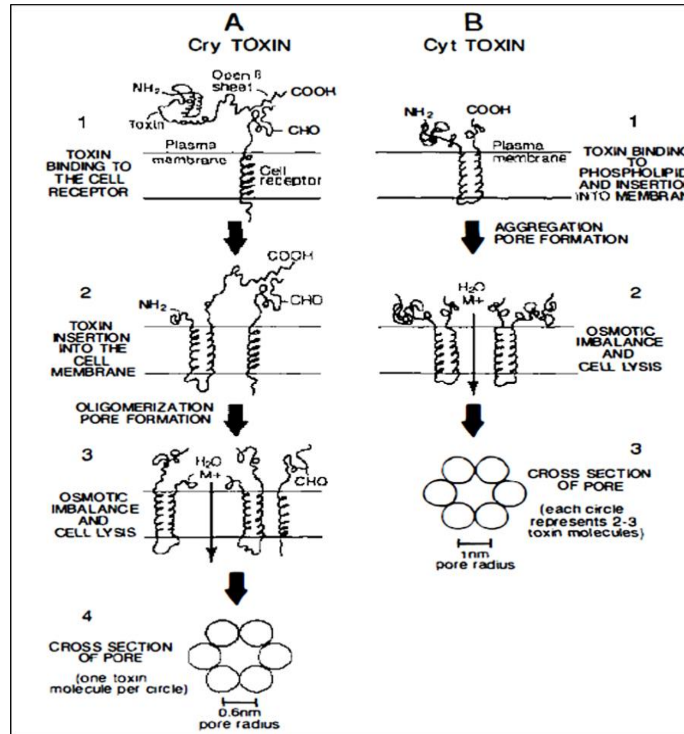

Figure 2.6: Receptor binding of *Bacillus thuringiensis* toxin (Adapted from Gill, Cowles and Pietrantonio, 1992).

2.6 The Application of *Bacillus thuringiensis*

One of the most successful applications of *Bacillus thuringiensis* is their usage in controlling Lepidopteran defoliators in Canada and the United States, due to the ability of *Bacillus thuringiensis kurstaki*-isolate HD1 to produce Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa toxins which are Lepidoptera specific (Bauce, et al., 2004; Van Frankenhuyzen, 2000). Due to the success of *Bacillus thuringiensis kurstaki* in controlling these Lepidopteran defoliators, more and more different strains of *Bacillus thuringiensis* with different Cry proteins that target different insect orders were eventually discovered and used as a biological control agent. Currently, there are over 400 *Bacillus thuringiensis* based biopesticides in the market (Ahmedani, et al., 2008) for insect control in

agriculture (Bravo, et al., 2011). Most of the *Bacillus thuringiensis* biopesticides are spore-crystal formulations that are acquired from different strains of *Bacillus thuringiensis*. These include *Bacillus thuringiensis kurstaki*-isolates HD1 and HD73, *Bacillus thuringiensis israelensis*, *Bacillus thuringiensis tenebrionis, Bacillus thuringiensis san diego* and *Bacillus thuringiensis aizawai*-isolate HD137 which produce different Cry proteins that are toxic against different orders of insects such as Lepidoptera, Coleoptera or Diptera (Schünemann, Knaak and Fiuza, 2014). As a result, the use of chemical insecticides for pests control showed a drastic drop.

In 1977, *Bacillus thuringiensis israelensis*-isolate H14 was discovered and used in vector control due to its high toxicity against mosquito and blackfly larvae, which are the vectors of dengue fever and malaria, and onchocerciasis respectively. *Bacillus thuringiensis israelensis* was used from 1982 to 1997 to control the blackfly species complex (*Simulium damnosum*), which is the transmission vector of *Onchocerca volvulus* that causes river blindness in West Africa (Sanchis and Bourguet, 2008; WHO, 1999). Becker (2000) reported evidence of resistance development in the insect populations toward chemical insecticides. This eventually resulted in the boost on the usage of bioinsecticides, such as the application of *Bacillus thuringiensis* strains for controlling mosquitoes and blackflies due to their high insecticidal activity, lack of resistance development (Bravo, Gill and Soberón, 2007) and showing little or no effect on non-target populations (Calamari, et al., 1998).

Moreover, in the year 2009, over 40 million hectares of *Bacillus thuringiensis* engineered crops were grown globally, which lead to the decrease on the use of chemical pesticides (James, 2009). The important *Bacillus thuringiensis* engineered crops included tobacco, soya, corn, cotton and canola. Besides the reduction on the use of chemical pesticides, bioengineered crops have literally overcome pest related yield losses caused by technical and economical constraints, and they are also environmental friendly (Qaim and Zilberman, 2003; Toenniessen, O'Toole and DeVries, 2003). Studies have shown that in *Bacillus thuringiensis* engineered crops, the Cry proteins were produced continuously, being protected from degradation yet reachable to insects (Bravo, Gill and Soberón, 2007). For instance, the *Bt* engineered corns expressed numerous Cry proteins such as the Cry3Bb, and Cry1A, Cry2Ab and Cry1F that allowed them to be constantly protected from Coleopteran and Lepidopteran pests respectively (Christou, et al., 2006).

In addition to that, it has been found that parasporal proteins of *Bacillus thuringiensis* possess cytotoxic effects on human cancer cells (Ohba, Mizuki and Uemori, 2009; Yamashita, et al., 2005). Numerous studies have been done on different subspecies of *Bacillus thuringiensis* from various countries such as Japan and Canada, which showed that the parasporal proteins produced were able to exert cytotoxic effects especially to human leukemic T cells (Lee, et al., 2000; Lee, et al., 2001) and hepatocyte cancer cells without harming normal T cells and hepatocyte cells when activated (Jung, et al., 2007). Furthermore, non-Cyt inclusion proteins produced by *Bacillus thuringiensis dakota*-isolate

90-F-45-14 expressed high cytotoxic effects toward human leukemic T cells and moderate cytotoxic effects against human cervical cancer cells (Kim, et al., 2000).

Nevertheless, studies have shown that there are some limitations to their effects. The application of *Bacillus thuringiensis* as a biological control agent is threatened by resistance development in insects, moreover, the toxins have poor stability as they can be inactivated by ultraviolet (UV) light, heat and extreme pH (Sanahuja, et al., 2011). Besides, narrow spectrum of activity is also another major threat to the use of *Bacillus thuringiensis* as an insecticide (George and Crickmore, 2012). According to Zhong, et al. (2000), only minority group of the toxins showed activities that spanned two to three insect-orders.

CHAPTER 3

MATERIALS AND METHODS

3.1 The General Overview of Experimental Design

Figure 3.1 shows the general overview of the experimental work flow, which is divided into three different subsections – morphological identification, crude protein extraction and DNA extraction.

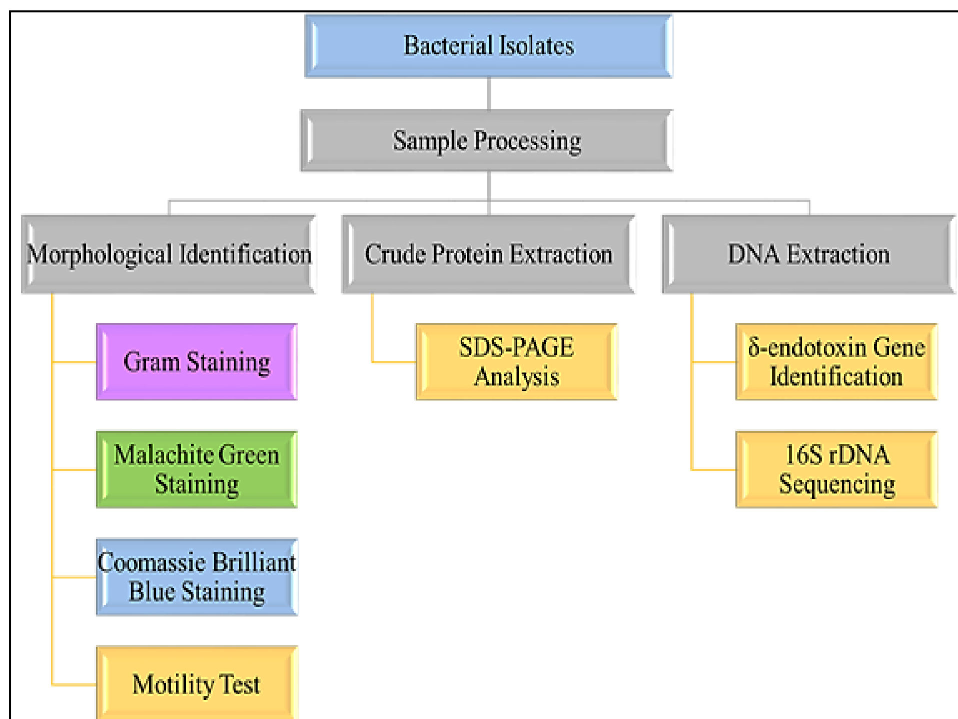

Figure 3.1: The flow chart of the general overview of experimental design.

3.2 Chemicals, Media, Reagents and Equipment

The list of chemicals, media, reagents and equipment that were used in this study are tabled in Appendices A, B and C in accordance to their respective manufacturers.

3.3 Bacterial Samples

Five of the isolated bacterial samples were from Block B, Block C, Block I and Engineering workshop, while the remaining two isolated bacterial samples were from Blocks I and K. Two positive reference strains *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646), and three negative controls *Bacillus subtilis*, *Escherichia coli* and *Acinetobacter baumannii* (ATCC 17978) were used. The bacterial samples and controls were cultured on nutrient agar plates and in nutrient broths, incubated at 30 ℃.

3.4 Morphological Identification of Bacterial Isolates

The seven isolated bacterial samples and two positive reference strains were streaked onto nutrient agar and incubated overnight at 30 ℃. As reported by Renganathan, et al. (2014), after overnight incubation, a white to off-white colony with flat to slightly raised elevation and smooth edges can be observed from *Bacillus sp.* positive colonies (Muniady, Rathinam and Subramaniam, 2011).

3.4.1 Gram Staining

Gram staining was performed to distinguish the isolated bacterial samples as rod-shaped and Gram positive bacilli. The Gram staining procedure (Figure 3.2) was adapted from Bartholomew and Mittwer (1952). A small loopful of bacterial sample was smeared onto a drop of 0.85% saline solution on the microscopic slide, followed by air-drying and heat-fixing. Once fixed, the

smear was flooded with the primary staining, crystal violet dye for 1 minute, then washed with running tap water. The smear was then stained with gram iodine mordant for fixation for another 1 minute, and washed with running tap water again. After that, the smear was decolorized with 75% ethanol for 10 seconds, and then washed with running tap water. Lastly, the smear was counterstained with safranin for 1 minute, and washed with running tap water. The slide was then left to air dry, before being observed under the compound light microscope at the magnification of 1000X (oil immersion). The above procedure was repeated for the rest of the samples together with the positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) while the negative control was *Escherichia coli*.

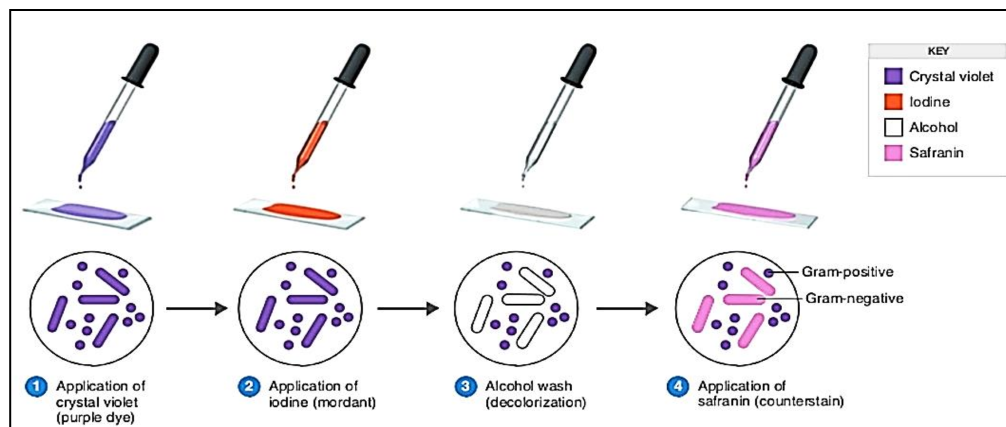

Figure 3.2: The general procedure of Gram staining (Adapted from Tortora, Funke and Case, 2013).

3.4.2 Malachite Green Staining

The malachite green staining which was modified from Bartholomew and Mittwer (1950) was performed to confirm the presence of endospores in the isolated bacterial samples. After 90 hours of incubation, a small loopful of the

bacterial sample was smeared onto a drop of 0.85% saline solution on the microscopic slide, followed by air-drying and heat-fixing. The fixed smear was then covered with filter paper that was soaked with malachite green stain. The slide was placed on top of a beaker containing boiling water and continued steaming for another 5 minutes. The stained smear was then rinsed using running tap water after 5 minutes and counterstained with safranin for 30 seconds. Lastly, the slide was rinsed with running tap water again. The slide was then left to air dry before being observed under the compound light microscope at the magnification of 1000X (oil immersion). The above procedure was repeated for the rest of the samples together with the positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) while the negative control was *Escherichia coli*.

Figure 3.3: The general procedure of endospore staining (Adapted from MicrobeOnline, 2015).

3.4.3 Coomassie Brilliant Blue (CBB) Staining

The Coomassie Brilliant Blue (CBB) staining was used to examine the presence of crystal proteins in the isolated bacterial samples. The CBB staining allows high throughput evaluation on the presence of crystals in bacterial

colonies (Rampersad and Ammons, 2005) by increasing the resolution over phase contrast microscopy. This staining method was modified from Rampersad and Ammons (2005). The bacterial sample that had been incubated at 30 ℃ for 110 hours was smeared onto a drop of 0.85% saline solution on the microscopic slide, followed by air-drying and heat-fixing. The slide was stained with 0.133% Coomassie Brilliant Blue stain for 1 minute followed by destaining with distilled water for 10 seconds. The slide was then left to air dry before being observed under the compound light microscope at the magnification of 1000X (oil immersion). This procedure was repeated for the rest of the samples together with the positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) while the negative control was *Bacillus subtilis*.

3.5 Motility Test

The motility test which was modified from Prask (2005) was used to determine the ability of the isolated bacterial samples to be motile. Two lines were drawn on the agar plate. The bacterial sample was inoculated in between the two lines, and then incubated at 30 ℃ overnight. A motile bacteria will be able to grow out of the two indicated lines. This procedure was repeated for the rest of the samples together with the positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) while the negative control was *Acinetobacter baumannii* (ATCC 17978).

3.6 Crude Protein Extraction

The crude protein extraction method was modified from Luo and Adang (1994). First, the bacterial samples were cultured in 150 ml nutrient broth and incubated for 5 days with constant agitation at 30 ℃ until full autolysis phase. Next, the Coomassie Brilliant Blue (CBB) staining was used to examine the presence of crystal proteins before proceeding to the next step. Once confirmed, the samples were subjected to centrifugation for 15 minutes at 10,000 rpm at 4 ℃. After that, the supernatants were discarded and the pellets were washed and resuspended with saline solution (0.85%), and then re-centrifuged again for 15 minutes at 10,000 rpm at 4 ℃. The supernatants were again discarded and the pellets were washed with saline solution. Subsequently, the pellets were centrifuged for the third time for 15 minutes at 10,000 rpm at 4 ℃. Lastly, the pellets were resuspended in 1 ml of distilled water. The extracted proteins were stored at 4 ℃ until further use.

3.7 Bradford Assay

Bradford assay was used to determine the quantities of protein samples by the measurement of absorbance at 595 nm (Bradford, 1976; Ernst and Zor, 2010). The stock concentration of Bovine Serum Albumin (BSA) was 2.0 mg/ml. A serial dilution was done, and a standard linear graph was generated by using the concentrations of 0.00 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.10 mg/ml, 0.20 mg/ml and 0.40 mg/ml of BSA. The 5X Bradford reagent was diluted to 1X before use and the extracted crude protein samples were diluted to the ratio of 1:50. After that, 10 µl of each BSA concentrations and diluted protein samples

were pipetted into a 96-well plate, then 200 µl of 1X Bradford reagent were added to each well. The absorbance was read at 595 nm wavelength using the microplate reader. Triplicates were performed and the average absorbance values of the standard BSA concentrations obtained were used to plot a standard graph. The concentration of the extracted crude proteins samples were calculated based on the linear equation obtained. The R^2 value of the standard curve plotted was in between 0.95 – 1.0.

3.8 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The protein sizes of the extracted crude protein samples were determined through SDS-PAGE analysis. The procedure of the SDS-PAGE was modified and adapted from Laemmli (1970).

First, two sets of glass plates (Bio-Rad) with the thickness of 0.75 mm were set on the casting frame and fixed on a casting stand. Approximately 2 ml of 70% ethanol were loaded into the glass plates to check for leakages. The ethanol was then removed by using filter paper. Then, 10% separating gel was prepared according to the components and volumes tabulated in Table 3.1.

The 10% separating gel was loaded into the 0.75 mm glass gel plates. After that, 70% ethanol was slowly pipetted on top of the 10% separating gel to remove air bubbles.

Table 3.1: The components required for making 10% separating gel.

Components	Volume (µl)
Distilled water	4000
30% Bis-acrylamide	3330
1.5 M Tris-Hydrochloric acid (pH 8.8)	2500
10% Ammonium Persulfate (APS)	100
10% Sodium Dodecyl Sulfate (SDS)	100
Tetramethylethylenediamine (TEMED)	5

The gel was left to polymerize for 45 minutes followed by the removal of the 70% ethanol using filter paper. After that, the 4% stacking gel was prepared according to the components and volumes tabulated in Table 3.2.

Table 3.2: The components required for making 4% stacking gel.

Components	Volume (µl)
Distilled water	3400
30% Bis-acrylamide	830
0.5 M Tris-Hydrochloric acid (pH 6.8)	630
10% Ammonium Persulfate (APS)	50
10% Sodium Dodecyl Sulfate (SDS)	50
Tetramethylethylenediamine (TEMED)	5

The 4% stacking gel was loaded over the the 10% separating gel. A 10-well comb was carefully inserted into the 4% stacking gel and then the gel was

allowed to solidify. Upon the insertion of the comb, it was crucial to make sure that no bubbles were trapped underneath the teeth. After the stacking gel had solidified, the casting frame together with the glass plates were 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. A 6X sample loading buffer was prepared according to the components and amount listed in Table 3.3.

Table 3.3: The components of 6X sample loading buffer.

Components	Amount
1 M Tris-Hydrochloric acid (pH 6.8)	3750 μl
60% Glycerol	6000 μl
12% Sodium Dodecyl Sulfate (SDS)	1.2 g
0.6 M Dithiothreitol (DTT)	0.93 g
0.06% Bromophenol blue	0.006 g

The 6X sample loading buffer was diluted to 3X. While waiting for the 4% gel to solidify, the extracted crude protein samples were prepared with the loading buffer. First, 10 μl of protein sample were mixed with 5 μl of 3X sample loading buffer. The mixtures were then heated for 10 minutes at 95 ℃ using heat block. After that, the mixtures were spun for 1 minute at maximum speed using table top centrifuge at room temperature. Subsequently, 10 μl of the respective protein samples and 5 μl of 175 kDa Chromatein Prestained Protein Ladder (Vivantis) were loaded into their respective wells. The 1X running

buffer was carefully poured into the chamber to top up the inner chamber and allowed to overflow until the buffer reached the required level in the outer chamber.

The gel tank was connected to a power supply and the gel electrophoresis was run for approximately 1 hour 30 minutes at 80 V. The gel was then stained with Coomassie Brilliant Blue staining for 5 minutes and destained with distilled water overnight. The stained gel was then visualized under UV transilluminator using ChemiDoc™XRS system (Bio-Rad).

3.9 DNA Extraction

The bacterial samples were cultured into 250 ml conical flasks containing 100 ml nutrient broth. The bacteria cultures were incubated overnight with constant agitation at 200 rpm at 30 ℃. Two methods were adopted in the DNA extraction process of the isolated bacterial samples.

3.9.1 Phenol-Chloroform Extraction

The Phenol-chloroform extraction method is the removal of proteins from DNA samples. This method was adapted and modified from Wilson (1997). First, 5 ml of overnight bacterial cultures were transferred into sterile 15 ml-centrifuge tubes and spun for 5 minutes at 6,000 x g/rcf at room temperature, and then the supernatants were discarded. The bacterial pellets were then resuspended in 1.5 ml PBS solution and the suspensions were transferred into sterile 1.5 ml microcentrifuge tubes. After that, the cultures were centrifuged

for 5 minutes at maximum speed of 14,000 rpm, and then the supernatants were discarded. The bacterial pellets were then resuspended in 567 µl Tris-EDTA (TE) buffer by repeated pipetting until the pellets were completely dissolved. Next, lysis buffer was freshly prepared according to the components and amount listed in Table 3.4.

Table 3.4: The components of lysis buffer.

Components	Amount
Lysozyme	60 mg
1 M Tris-Hydrochloric Acid (pH 6.8)	60 µl
0.5 M Ethylenediaminetetraacetic acid (EDTA)	12 µl
10% Triton X-100	300 µl
Sterile distilled water	2628 µl

A volume of 200 µl of lysis buffer was added and allowed to stand for 10 minutes at room temperature. Concurrently, the tubes were inverted every 3 minutes. Next, 30 µl of 10% SDS and 7 µl of 20 mg/ml Proteinase K were added into the tubes, mixed thoroughly by re-suspending the mixture and was incubated for 1 hour at 37 ℃. In addition, 100 µl of 5 M NaCl was added and was mixed thoroughly followed by addition of 80 µl of CTAB/NaCl solution. The solution was mixed thoroughly and again incubated for 20 minutes at 65 ℃ in water bath. The following steps were then carried out in a fume hood. Approximately 780 µl of pre-mixed chloroform/isoamyl alcohol was added and the solutions were mixed well. After that, the mixture were spun for 5 minutes

at 14,000 rpm at room temperature. A white interface was seen after centrifugation which contained denatured proteins. The aqueous and viscous supernatants were aspirated out and transferred into fresh microcentrifuge tubes. An equal volume of phenol/chloroform/isoamyl alcohol was added to the supernatant and was mixed thoroughly. After that, the mixture was spun for 5 minutes at 14,000 rpm at room temperature. The supernatant was then transferred into new microcentrifuge tubes, and 2.5X volume of ice-cooled absolute ethanol was added to precipitate the nucleic acids. The mixture were mixed thoroughly and incubated for at least 30 minutes at -20 ℃. Subsequently, the mixture were spun again for 5 minutes at 14,000 rpm at room temperature.

The supernatants were removed and the DNA pellets were washed with 1 ml of room temperature 70% ethanol and the tubes were gently inverted several times. Again, the pellets were spun for 5 minutes at 14,000 rpm at room temperature and finally the supernatants were removed. The DNA pellets were dried for 10 minutes in 70 ℃ incubator before being dissolved in 50 µl of TE buffer and stored in -20 ℃ for future use. The DNA concentration was determined using nanospectrophotometer. The purity of the DNA (A_{260}/A_{280}) was between 1.8 - 2.0. The extracted DNA for the seven isolated bacterial samples and the two positive reference strains were stored at -20 ℃ for future use.

3.9.2 DNA Extraction Kit

An alternative DNA extraction method using a commercially available DNA extraction kit was also adopted in this research project. A volume of 50 μl of overnight bacterial culture were measured using the spectrophotometer (Bio-Rad) to check for the bacteria concentration ($< 1 \times 10^9$). After that, 1.5 ml of bacteria culture were centrifuged for 1 minute at 13,000 rpm. Then, the DNA was extracted according to the procedure provided by the manufacturer (Real Genomics) with slight modifications. 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. The purity of the DNA should range between 1.8 – 2.0. The extracted DNA for the seven isolated bacterial samples and the two positive reference strains were stored at -20 ℃ for future use.

3.10 The δ-Endotoxin Gene Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis

The PCR amplification was performed to amplify the extracted DNA with different sets of primers which targeted on different Diptera specific genes. The PCR process was carried out in which each of the reactions contained 3 μl of 10 μg DNA template, 0.4 μM of each forward and reverse primers, 5 μl of GoTaq® Green Master Mix 2X (Promega) and 1.2 μl of nuclease free water. The total volume of each reaction was 10 μl. The PCR was performed using thermo cycler (Bio-Rad), with the primers as listed in Table 3.5 and the parameters shown in Table 3.6. In every PCR, a non-template control and two

positive controls which were *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) were used.

Table 3.5: Parameters for PCR amplification.

Step	**Temperature (℃)**		**Duration (sec)**	**Cycle**
Initial denaturation	95		180	1
Denaturation	95		10	35
Annealing	Un 1	55.5	10	35
	Cry2gral	49.0		
	Cry4Aspe	49.0		
Extension	72		30	35
Final extension	72		600	1

The amplified DNA was assessed using 1.5% agarose gel electrophoresis. The 1.5% gel was placed into a gel tank and immersed in 1X TBE buffer. A volume of 1.5 µl of 100–bp plus DNA ladder (Thermofisher) was loaded into the well of the agarose gel. Then, 5 µl of amplified products were mixed with 1 µl of 6X loading dye and loaded into the different well respectively. The gel was electrophoresed for 35 minutes at 100 V and stained with GelRed for 15 minutes. After that, the gel was visualized under the UV transilluminator using ChemiDoc™XRS system (Bio-Rad).

Table 3.6: The primers used for δ-endotoxin gene amplification.

Primers	Gene recognized	Forward (f) and Reverse (r) Primer Sequences	Amplicon Size (bp)	References
Un1	*cry I*	(f) 5’-CAT GAT TCA TGC GGC AGA TAA AC-3’	277	Halima, et al., 2006;
		(r) 5’-TTG TGA CAC TTC TGC TTC CCA TT-3’		Vidal- Quist, et al., 2009
Cry2gral	*cry II*	(f) 5‘-GAG TTT AAT CGA CAA GTA GAT AAT TT-3’	500 – 526	Ibarra, et al., 2003
		(r) 5‘-GGA AAA GAG AAT ATA AAA ATG GCC AG-3’		
Cry4Aspe	*cry IV*	(f) 5’-TCA AAG ATC ATT TCA AAA TTA CAT-3’	459	Mahalakshmi, et al., 2012
		(r) 5’-CGG CTT GAT CTA TGT CAT AAT CTG T-3’		

3.11 16S rDNA Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis

The PCR amplification for the 16S rDNA sequencing was performed in which the reaction has a final volume of 30 µl, and consisted of 5 µl of 10 µg DNA template, 0.25 µM of each forward and reverse primers, 12.5 µl of DreamTaq Master Mix 2X (Promega) and 12 µl of nuclease free water. The parameters for the PCR reactions were carried out as listed in Table 3.7, using the universal primers U16S_8F [(f) 5'–AGA GTT TGA TCC TGG CTC AG–3'] and U16S_1541R [(r) 5'–AAG GAG GTG ATC CAG CCG CA–3'] (Teo, 2013). The amplified DNA was assessed using 2% agarose gel electrophoresis. The 2% gel was placed into a gel tank and immersed in 1X TBE buffer. A volume of 2.5 µl of 100–bp plus DNA ladder (Thermofisher) was loaded into the last well of the agarose gel. Then, 5 µl of the amplified products were mixed with 1 µl of 6X loading dye and loaded into the different wells respectively. The gel was electrophoresed for 35 minutes at 100 V and stained with GelRed for 15 minutes. After that, the gel was visualized under the UV transilluminator using ChemiDoc™XRS system (Bio-Rad). The expected amplicon size would be at 1500 bp.

3.12 16S rDNA Gene Sequencing and Analysis

The unpurified PCR amplicons were sent to 1st BASE Laboratories Sdn. Bhd. for DNA purification and gene sequencing. The results of the DNA sequences obtained were trimmed using Snapgene. After that, the trimmed sequence were analyzed using Nucleotide Basic Local Alignment Search Tool (BLASTn: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) at the

National Center of Biotechnology Information (NCBI) website. The bacterial isolates were then identified.

Table 3.7: Parameters for PCR amplification in 16S rDNA sequencing.

Step	Temperature (°C)	Duration (sec)	Cycle
Initial denaturation	94	720	1
Denaturation	94	30	30
Annealing	60	30	
Extension	72	90	
Final extension	72	420	1

3.13 Phylogenetic Tree Construction

The sequence obtained was used to construct a phylogenetic tree by using ClustalW2 from EMBL-EBI program (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The phylogenetic relationship and relative identity of the isolated bacterial were compared and deduced based on the constructed tree.

CHAPTER 4

RESULTS

4.1 Colony Morphology of Bacterial Isolates

The colonies have the distinctive *Bacillus*-like characteristic – white to off-white color, circular shape, smooth edge and each colonies showed slight raised elevation with “fried egg” appearance as presented in Figure 4.1.

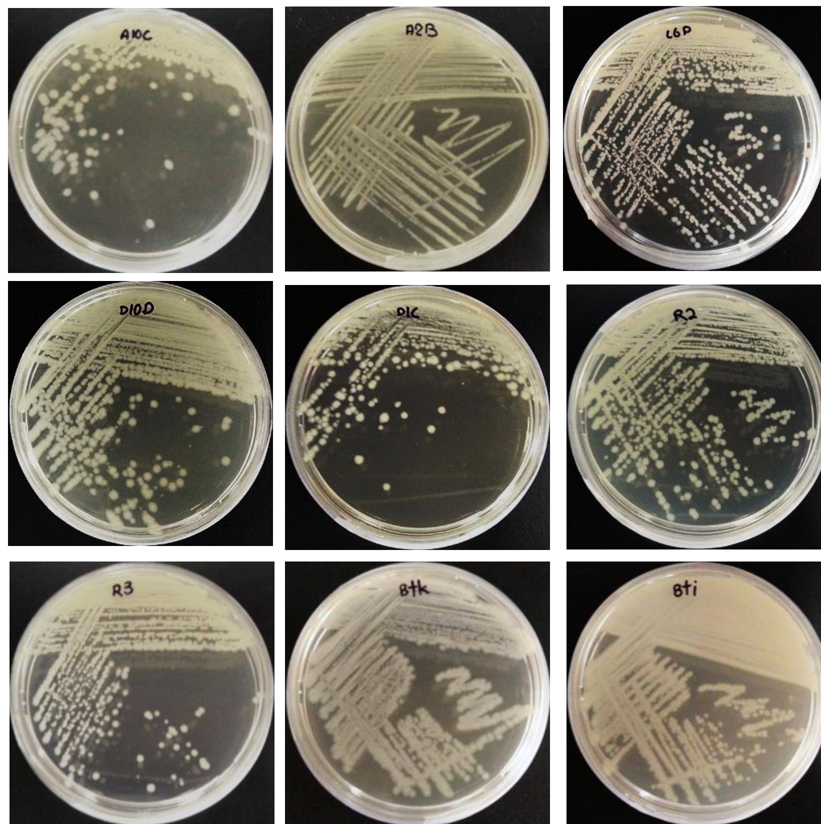

Figure 4.1: The colony morphology of the bacterial isolates (**A**) A10C (**B**) A2B (**C**) C6D (**D**) D10D (**E**) D1C (**F**) R2 (**G**) R3 (**BTK**) *Bacillus thuringiensis kurstaki* ATCC 33679 (positive control) (**BTI**) *Bacillus thuringiensis israelensis* ATCC 35646 (positive control) after overnight incubation at 30 ℃.

4.2 Gram Staining

The Gram staining of bacterial isolates labelled **A** to **G** and two positive controls labelled **BTK** and **BTI** are presented in Figure 4.2. The bacterial isolates appeared as rod-shaped and blue or violet staining assured that the bacterial isolates were Gram-positive. Conversely, the negative control labelled **ECO** appeared as spherical-shaped and pink staining indicated Gram-negative.

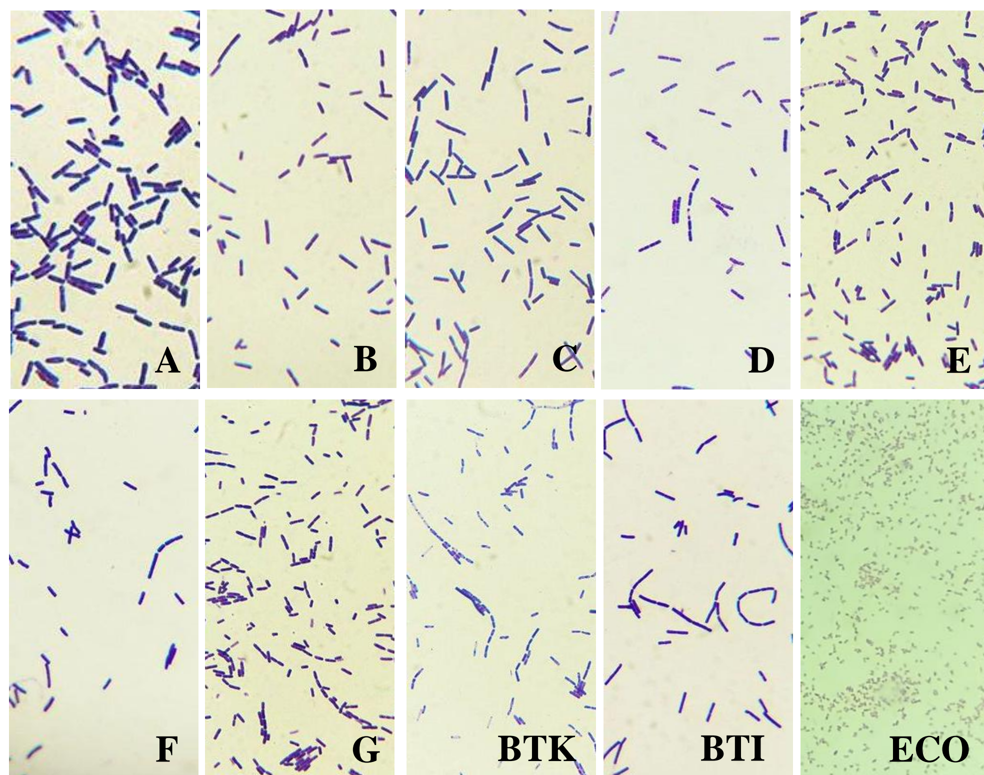

Figure 4.2: The Gram staining of the bacterial isolates (**A**) A10C (**B**) A2B (**C**) C6D (**D**) D10D (**E**) D1C (**F**) R2 (**G**) R3 showed Gram-positive rod-shaped bacilli. (**BTK**) *Bacillus thuringiensis kurstaki* ATCC 33679 (**BTI**) *Bacillus thuringiensis israelensis* ATCC 35646 (positive controls) (**ECO**) *Escherichia coli* (negative control). The samples were viewed under compound light microscope at 1000X magnification.

4.3 Malachite Green Staining

The endospore staining of bacterial isolates labelled as **A** to **G** and two positive controls labelled as **BTK** and **BTI** that are presented in Figure 4.3 showed the presence of endospores, whereas the negative control labelled **ECO** did not show any spore formation. The vegetative cells were stained red whereas the spores were stained green. The black arrows in Figure 4.3 indicate the presence of spores.

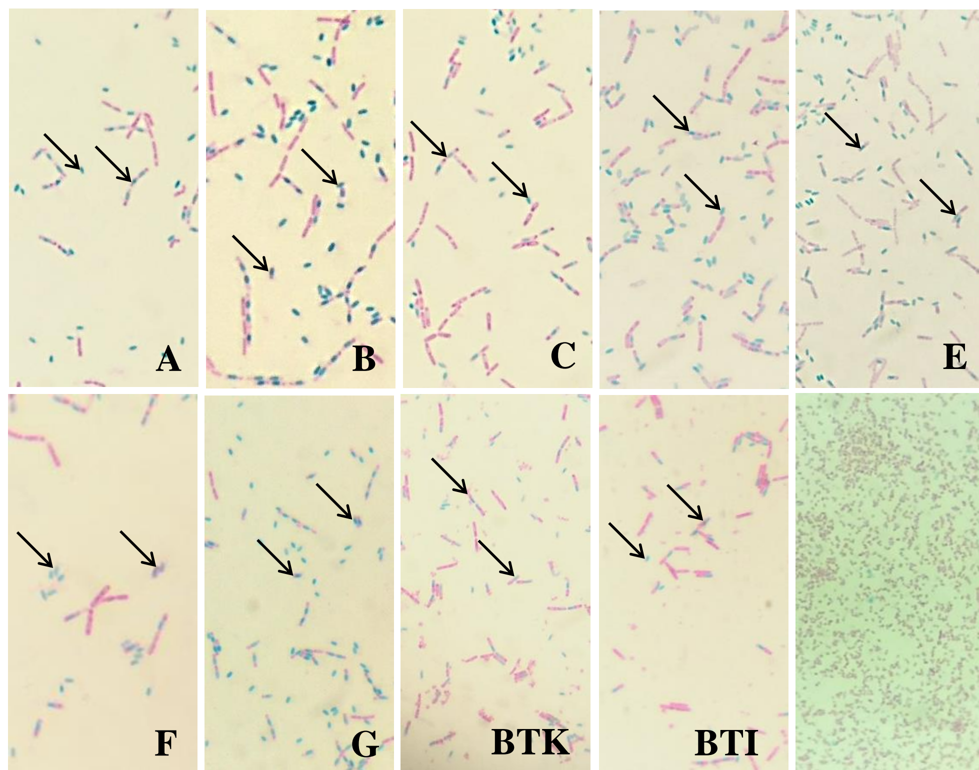

Figure 4.3: The malachite green staining of bacterial isolates (**A**) A10C (**B**) A2B (**C**) C6D (**D**) D10D (**E**) D1C (**F**) R2 (**G**) R3 showed that they are able to produce endospores. (**BTK**) *Bacillus thuringiensis kurstaki* ATCC 33679 (**BTI**) *Bacillus thuringiensis israelensis* ATCC 35646 (positive controls) (**ECO**) *Escherichia coli* (negative control). The black arrows indicate the presence of endospores. The samples were viewed under compound light microscope at 1000X magnification.

4.4 Coomassie Brilliant Blue (CBB) Staining

The CBB staining of bacterial isolates labelled **A** to **G** and two positive controls labelled **BTK** and **BTI** that are presented in Figure 4.4 showed the presence of crystal proteins in comparison to the negative control labelled **BSU** that did not show the presence of crystal protein. The crystal proteins were stained in dark blue color and spherical in shape. The black arrows in Figure 4.4 indicate the presence of crystal proteins.

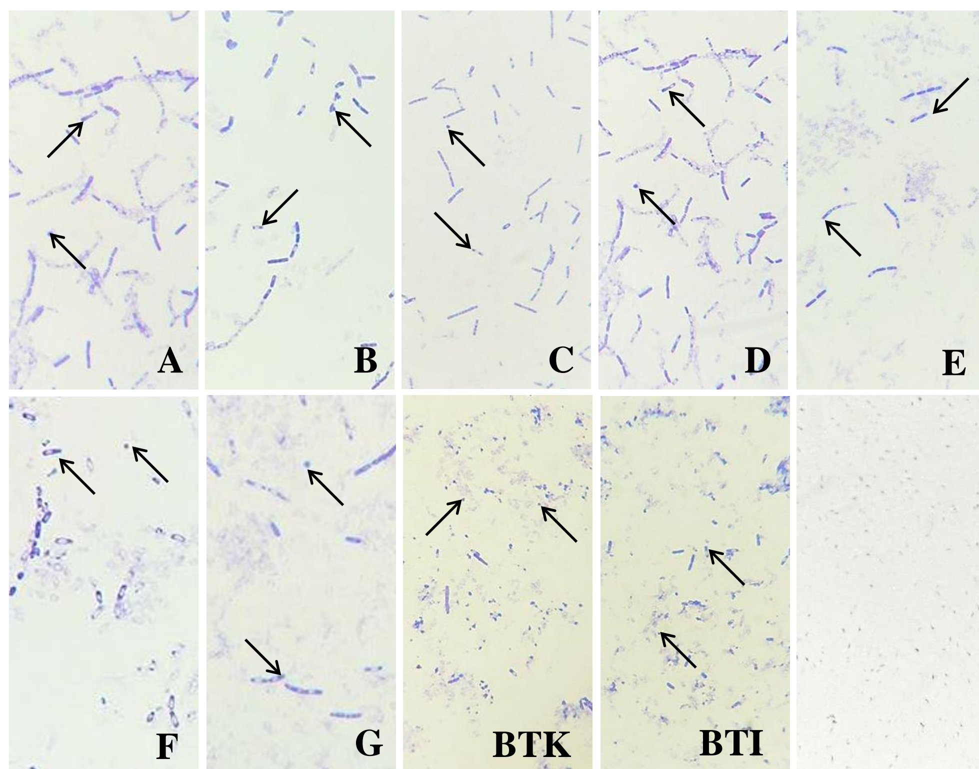

Figure 4.4: The CBB staining of bacterial isolates (**A**) A10C (**B**) A2B (**C**) C6D (**D**) D10D (**E**) D1C (**F**) R2 (**G**) R3 showed that they are able to produce spherical shape crystal proteins. (**BTK**) *Bacillus thuringiensis kurstaki* ATCC 33679 (**BTI**) *Bacillus thuringiensis israelensis* ATCC 35646 (positive controls) (**BSU**) *Bacillus subtilis* (negative control). The black arrows indicate the presence of crystal proteins. The samples were viewed under compound light microscope at 1000X magnification.

4.5 Motility Test

All the bacterial isolates labelled **A** to **G** and two positive controls labelled **BTK** and **BTI** as presented in Figure 4.5 were shown to be motile as the bacteria grew out the lines. Conversely, the negative control labelled (**ABA**) showed that the bacteria did not grow out the lines.

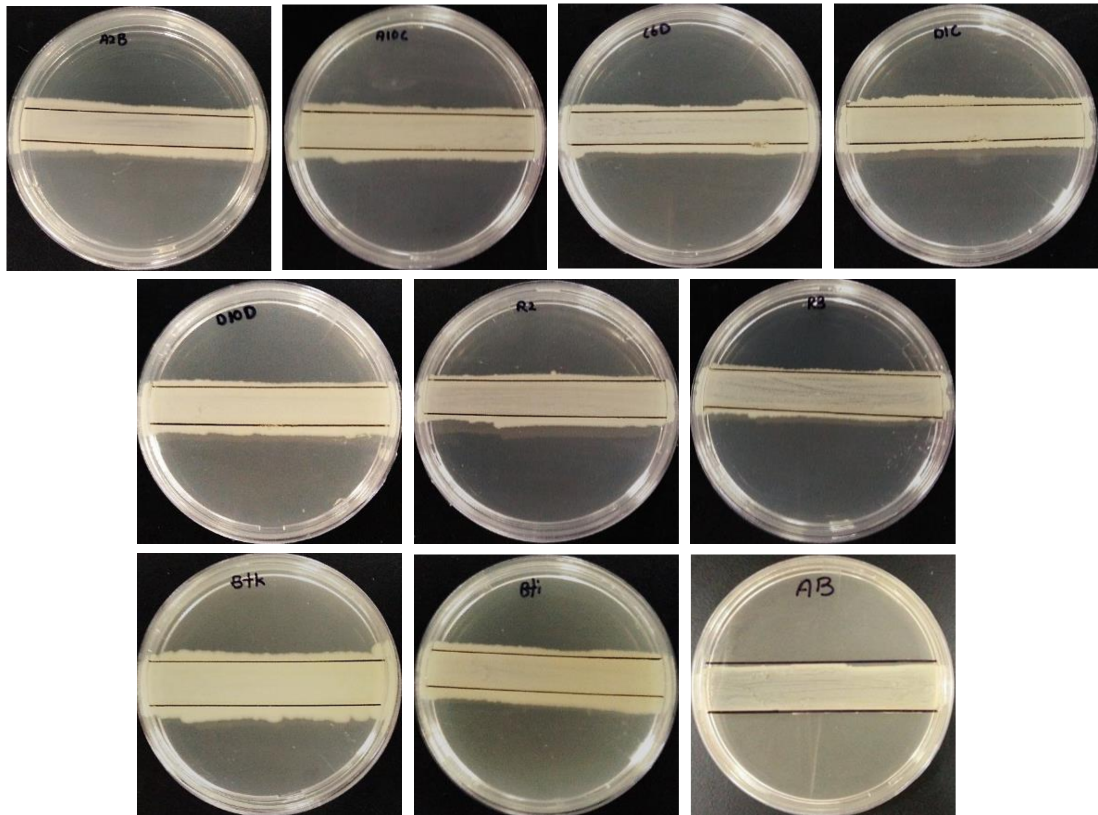

Figure 4.5: The motility test of bacterial isolates (**A**) A2B (**B**) A10C (**C**) C6D (**D**) D1C (**E**) D10D (**F**) R2 (**G**) R3 showed that they were motile. (**BTK**) *Bacillus thuringiensis kurstaki* ATCC 33679 (**BTI**) *Bacillus thuringiensis israelensis* ATCC 35646 (positive controls) (**ABA**) *Acinetobacter baumannii* (negative control).

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

The molecular mass of the extracted crystal proteins from the isolated bacterial samples were estimated by comparing to the 175 kDa Chromatein Prestained Protein Ladder (Vivantis) as shown in Figure 4.6. The red arrows are indicative of Cry1 proteins which are between 130 kDa to 150 kDa in size, the orange arrows are indicative of Cry4B proteins at 127 kDa and the yellow arrows are indicative of Cry2 proteins at 65 kDa to 70 kDa. The green arrows indicate the Cry35 proteins at 44 kDa. The blue arrows are indicative of the Cyt proteins which are between 27 kDa to 29 kDa in size. The indigo arrows indicate the Cry2A and Cry2Aa with the protein sizes of 70 kDa and 65 kDa respectively. The purple arrows indicate the sizes of 78 kDa and 72 kDa which are the Cry4C and Cry4D proteins respectively.

The isolated bacterial samples showed crystal proteins with molecular weights ranging from 27 kDa to 134 kDa. All of the bacterial isolates on Lanes 1, 2, 3, 4, 5, 8 and 9 respectively showed vivid protein bands with the molecular sizes at approximately 65 kDa to 70 kDa, 44 kDa and 27 kDa. In addition, the bacterial isolates A2B, D10D, D1C and R3 showed a protein band size of around 127 kDa, whereas bacterial isolates C6D and R2 showed protein bands at around 130 kDa to 150 kDa in size. Besides, the bacterial isolates C6D, D10D, D1C, R2 and R3 showed protein bands with the size of 44 kDa. Lane 6 indicate the positive control *Bacillus thuringiensis kurstaki* ATCC33679, which exhibited 127 kDa, 70 kDa and 65 kDa protein sizes. On the other hand, the positive control *Bacillus thuringiensis israelensis* ATCC 35646 was shown

to produce 4 bands which are indicated as the sizes of 127 kDa, 78 kDa, 72 kDa and 27 kDa crystal proteins respectively. The negative control *Bacillus subtilis* did not exhibit any protein bands that could be correlated with the Cry and Cyt proteins.

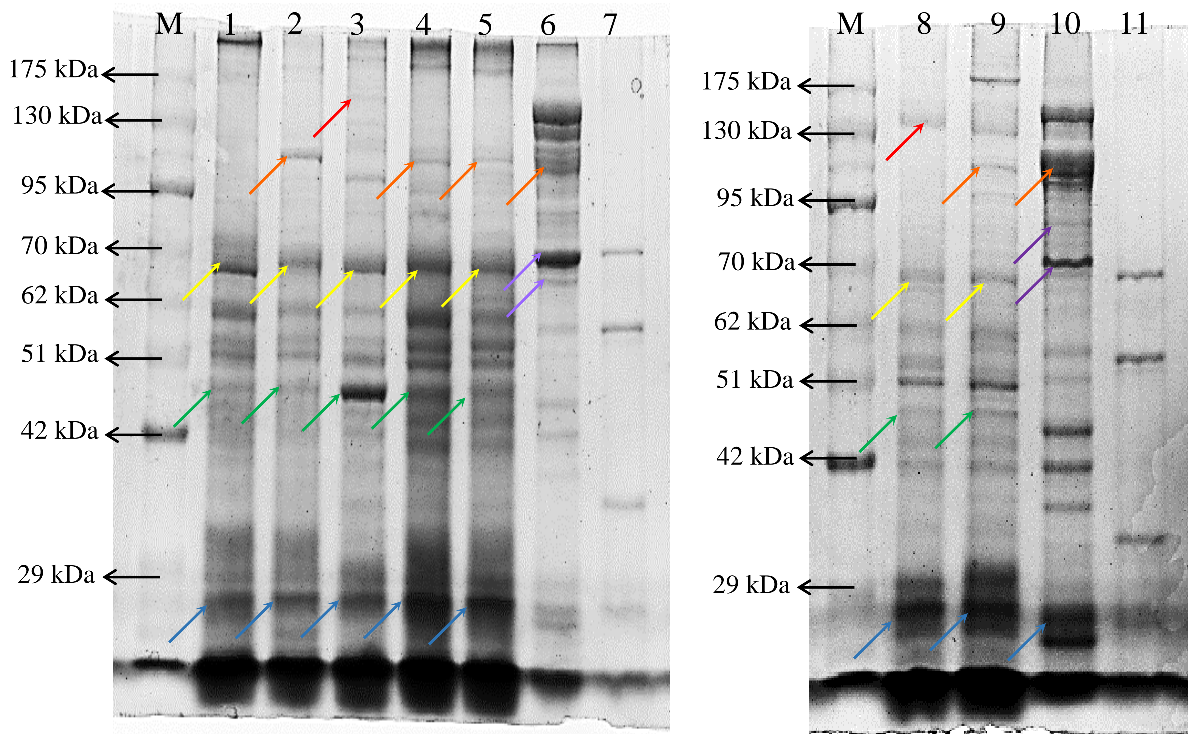

Figure 4.6: The SDS-PAGE of the total protein extracts of bacterial isolates. Lane M: 175 kDa Chromatein Prestained Protein Ladder; Lane 1: A10C; Lane 2: A2B; Lane 3: C6D; Lane 4: D10D; Lane 5: D1C; Lane 6: *Bacillus thuringiensis kurstaki* ATCC 33679 (positive control); Lane 7: *Bacillus subtilis* (negative control); Lane 8: R2; Lane 9: R3; Lane 10: *Bacillus thuringiensis israelensis* ATCC 35646 (positive control); Lane 11: *Bacillus subtilis* (negative control).

Legend:

- (red arrow) Cry1 proteins at 130 -150 kDa
- (orange arrow) Cry4B proteins at 127 kDa
- (yellow arrow) Cry2 proteins at 65 – 70 kDa
- (green arrow) Cry35 protein at 44 kDa
- (blue arrow) Cyt proteins at 27 – 29 kDa
- (light purple arrow) Cry2A, Cry2IAa proteins at 70 kDa and 65 kDa
- (dark purple arrow) Cry4C and Cry4D proteins at 78 kDa and72 kDa

4.7 The δ-endotoxin Gene Gel Electrophoresis

In this study, the distribution of *cryI*, *cryII* and *cryIV* genes in the isolated bacterial samples from Universiti Tunku Abdul Rahman (UTAR), Kampar campus was evaluated.

The characterization was based on PCR analysis using specific primers which were adapted from several journals (Halima, et al., 2006; Ibarra, et al., 2003; Mahalakshmi, et al., 2012; Vidal-Quist, et al., 2009). The PCR results showed that each bacterial isolates contained at least one *cry* gene either *cryI* gene at 277 bp or *cryII* gene at 500 - 526 bp or both are present (Figures 4.7, 4.8 and 4.9). The bacterial isolates A10C, A2B, C6D, D10D, D1C and R2 showed the presence of *cryI* and *cryII* genes whereas R3 showed only *cryII* gene. However, none of the bacterial isolates possessed the *cryIV* gene. The results are summarized in Table 4.1.

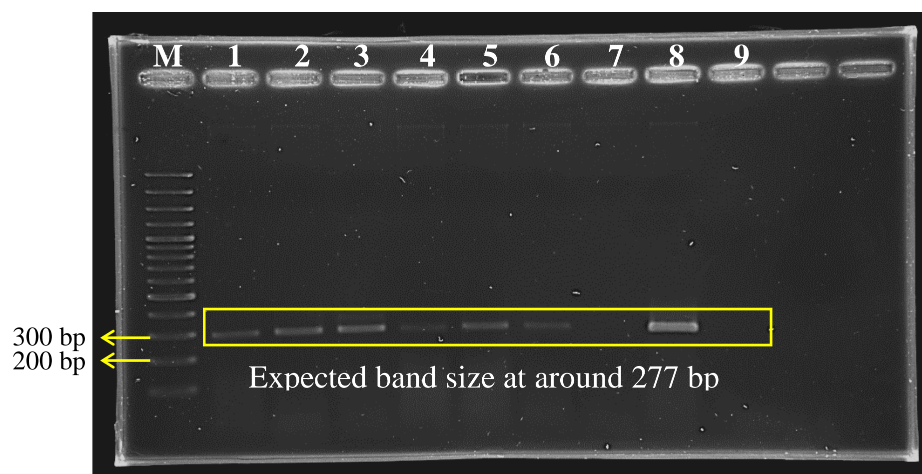

Figure 4.7: Screening of the bacterial isolates using Un1 primer (*cryI* gene). Lane M: 100-bp plus DNA Ladder (Thermofisher). Lane 1: A10C; Lane 2: A2B; Lane 3: C6D; Lane 4: D10D; Lane 5: D1C; Lane 6: R2; Lane 7: R3; Lane 8: *Bacillus thuringiensis kurstaki* ATCC 33679 (positive control); Lane 9: Non-template control.

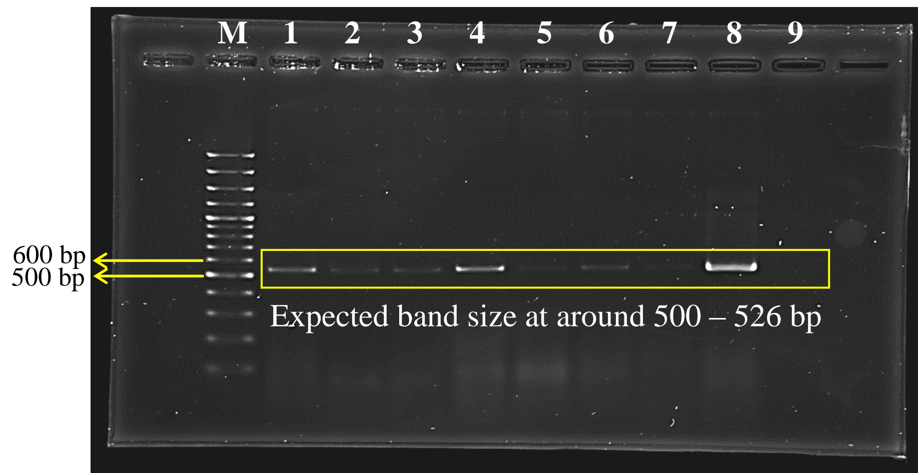

Figure 4.8: Screening of the bacterial isolates using Cry2gral primer (*cryII* gene). Lane M: 100-bp plus DNA Ladder (Thermofisher). Lane 1: A10C; Lane 2: A2B; Lane 3: C6D; Lane 4: D10D; Lane 5: D1C; Lane 6: R2; Lane 7: R3; Lane 8: *Bacillus thuringiensis kurstaki* ATCC 33679 (positive control); Lane 9: Non-template control.

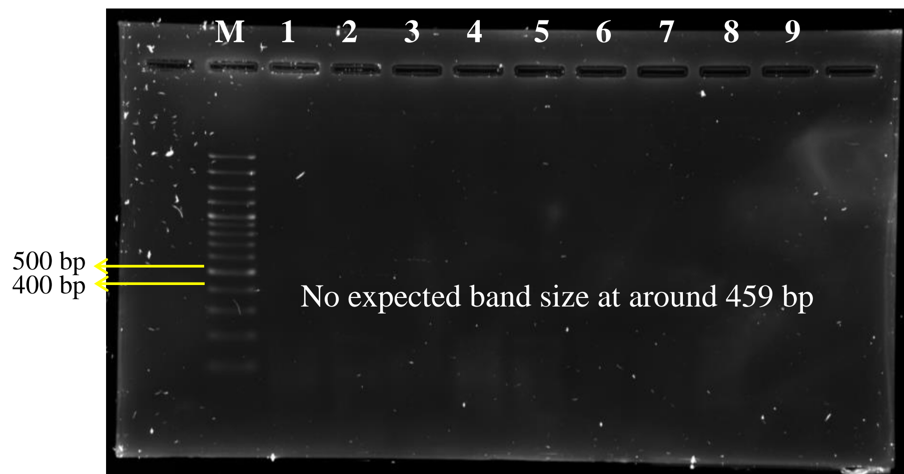

Figure 4.9: Screening of the bacterial isolates using Cry4Aspe primer (*cryIV* gene). Lane M: 100-bp plus DNA Ladder (Thermofisher). Lane 1: A10C; Lane 2: A2B; Lane 3: C6D; Lane 4: D10D; Lane 5: D1C; Lane 6: R2; Lane 7: R3; Lane 8: *Bacillus thuringiensis israelensis* ATCC 35646 (positive control); Lane 9: Non-template control.

Table 4.1: The summary of the PCR results of *cryI*, *cryII* and *cryIV genes* of bacterial isolates.

Genes	A10C	A2B	C6D	D10D	D1C	R2	R3	*Btk*	*Bti*
cryI	+	+	+	+	+	+	-	+	
cryII	+	+	+	+	+	+	+	+	
cryIV	-	-	-	-	-	-	-		-

*"+" show the presence of respective gene and "-" show the absence of respective gene

4.8 16S rDNA Gene Sequencing

For the final molecular evaluation, the 16S rDNA gene sequencing of the bacterial isolates were examined using universal primers U16S_8F and U16S_1514R. The expected band size for all the bacterial isolates was 1,500 bp as displayed in Figure 4.10.

Due to resource constraints, only the 16S rDNA-amplified products of A10C, A2B, C6D, D10D and D1C were selected based on the δ-endotoxin gene results and sent to 1st Base for purification and gene sequencing. Subsequently, the nucleotide blast results of A10C, A2B, C6D, D10D and D1C with the highest percentage and lowest E-value are summarized in Tables 4.2.

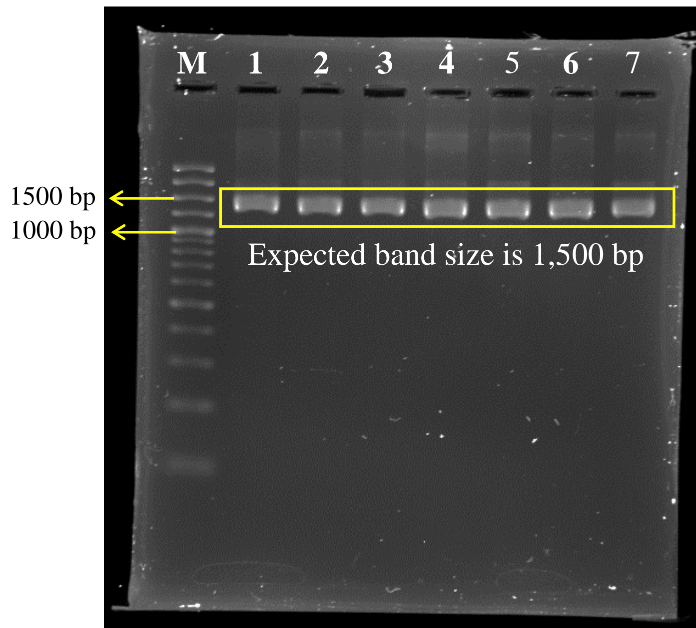

Figure 4.10: 16S rDNA-amplified PCR products on 2% agarose gel. Lane M: 100-bp plus DNA Ladder (Thermofisher). Lane 1: A10C; Lane 2: A2B; Lane 3: C6D; Lane 4: D10D; Lane 5: D1C; Lane 6: R2; Lane 7: R3

Based on the BLASTn results, the five bacterial isolates are *Bacillus spp*. and the possible species are *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus anthracis.* However, from the results of the 16S rDNA gene sequencing, the bacterial isolates cannot be specifically differentiated up to species level as the scores, query coverages, expected values and the identity among the isolates are identical. Thus, the phylogenetic tree could not be constructed using the sequencing results.

Table 4.2: BLASTn analysis of the 16S rDNA sequences of bacterial isolates.

Isolates	BLASTn Identity	Score (bits)	Query Coverage (%)	E-Value	Identity (%)	Accession
A10C	*Bacillus thuringiensis* strain NG 16S ribosomal RNA gene, partial sequence	1804	100	0.0	100	KY657578.1
	Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, partial sequence					KY628813.1
	Bacillus anthracis strain Sterne 34F2 genome					CP019726.1
	Bacillus thuringiensis strain NBBT7 16S ribosomal RNA gene, partial sequence					KY611801.1
A2B	*Bacillus thuringiensis serovar finitimus* YBT-020, complete genome	1969	100	0.0	100	CP002508.1
	Bacillus cereus AH187, complete genome					CP001177.1
	Bacillus thuringiensis strain DMB 16S ribosomal RNA gene, partial sequence	1965			99	DQ513324.1
	Bacillus thuringiensis strain NG 16S ribosomal RNA gene, partial sequence	1963				KY657578.1
C6D	*Bacillus thuringiensis* strain NG 16S ribosomal RNA gene, partial sequence	1358	100	0.0	100	KY657578.1
	Bacillus cereus strain TWV503 16S ribosomal RNA gene, partial sequence					KY630563.1
	Bacillus cereus strain TWV103 16S ribosomal RNA gene, partial sequence					KY630562.1
	Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, partial sequence					KY628813.1

Table 4.2: BLASTn analysis of the 16S rDNA sequences of bacterial isolates (Continued).

Isolates	BLASTn Identity	Score (bits)	Query Coverage (%)	E-Value	Identity (%)	Accession
D10D	*Bacillus thuringiensis* strain NG 16S ribosomal RNA gene, partial sequence	1890	100	0.0	100	KY657578.1
	Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, partial sequence					KY628813.1
	Bacillus anthracis strain Sterne 34F2 genome					CP019726.1
	Bacillus thuringiensis strain NBBT7 16S ribosomal RNA gene, partial sequence					KY611801.1
D1C	*Bacillus thuringiensis* strain NG 16S ribosomal RNA gene, partial sequence	1846	100	0.0	100	KY657578.1
	Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, partial sequence					KY628813.1
	Bacillus anthracis strain Sterne 34F2 genome					CP019726.1
	Bacillus thuringiensis strain NBBT7 16S ribosomal RNA gene, partial sequence					KY611801.1

CHAPTER 5

DISCUSSION

5.1 Morphological Identification

The *Bacillus thuringiensis* is closely related to *Bacillus cereus*, *Bacillus mycoides* and *Bacillus anthracis* which are collectively known as the *Bacillus cereus* group. Phenotypic identification among the *Bacillus cereus* group members is often difficult. Several morphological identification has been done to distinguish *Bacillus thuringiensis* from the other *Bacillus cereus* group members.

The bacterial isolates were cultured on media at 30 ℃ which is the optimum growth temperature. This is because the bacteria were isolated from soil and 30 ℃ is similar to soil temperature (Bahig, et al., 2008; Renganathan, et al., 2014).

The colony morphologies of bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 appeared to be white to off white in color, circular shaped, and have smooth edges with slight elevation that resembles fried egg appearance, which were similar to the two positive controls *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646). The results are consistent with *Bacillus thuringiensis* colony morphology in the findings of Chai, et al. (2016), El-kersh, et al. (2012), Mahalakshmi, et al. (2012), and Travers, Martin and Reichelderfer (1987).

However, the *Bacillus thuringiensis* cannot be differentiated from *Bacillus cereus* based on colony morphology alone. This is because the colony morphologies between *Bacillus thuringiensis* and *Bacillus cereus* are rather indistinguishable; as they both show the typical circular or irregular colonies in white or grey; granular, milky or matt aspect (De Respinis, et al., 2006). Nevertheless, these characteristics help to distinguish the former pair from the two other members *Bacillus mycoides* and *Bacillus anthracis* as the colonies of *Bacillus mycoides* are characterized by having fungal-like growth on agar plates in which they are filaments projecting outward and curving to the left or to the right (Stratford, Woodley and Park, 2013). Moreover, the colony characteristics of *Bacillus anthracis* on nutrient or blood agar show matt appearance, fairly flat, markedly tacky, white or grey-white, non-hemolytic on blood agar, and often having curly tailing at the edges (Turnbull, 1999).

All of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 were shown to be Gram positive and rod-shaped which were similar to the two positive strains, *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646). The results obtained were corresponding to previous *Bacillus thuringiensis* related studies (Muniady, Rathinam and Subramaniam, 2011; Quesada-Moraga, et al., 2004; Zakeel, Dissanayake and Weerasinghe, 2010). Due to the thick peptidoglycan layer of the Gram-positive bacteria, upon addition with alcohol, the pore size of the layer will shrink, resulting in the retention of the crystal violet-iodine complexes within the Gram-positive bacteria even after washing. Alcohol

dissolves the lipopolysaccharide layer of the cell membrane of Gram-negative bacteria and exposes the thin peptidoglycan layer of the bacteria which is unable to retain the crystal violet-iodine complexes after washing. Thus, Gram positive bacteria will remain purple colour while the Gram negative will take up the safranin dye and appear red or pink (Tortora, Funke and Case, 2013).

After 90 hours of incubation, all of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 together with the two positive strains *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) were shown to have the ability to produce spores which is in agreement with Jisha, Smitha, and Benjamin (2013), Kassogué, et al. (2015), and Shakoori, et al. (2011). Jisha, Smitha, and Benjamin (2013) reported that the *Bacillus* spp. are known to have the ability to produce endospores. The formation of endospores allow the bacteria to survive harsh environmental conditions such as nutrient depletion until favorable growth conditions are restored (Paredes-Sabja, Setlow and Sarker, 2011). The endospore coat is a multilayered shell, thus the heating process in endospore staining enables the spore's coats to soften and be able to uptake the dye (Cappuccino and Sherman, 2014). The malachite green dye is water-soluble and does not adhere well to the cell, thus, the decolorizing agent can easily decolorize the vegetative cells but not decolorize the endospore. After decolorization, the cells become colorless, leaving the endospores stained green (MicrobeOnline, 2015; Tortora, Funke and Case, 2013).

In addition, all the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 together with the two positive strains *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) were shown to have the ability to produce crystal proteins which correlates with the findings of Kassogué, et al. (2015), Muniady, Rathinam and Subramaniam (2011), Rampersad and Ammons (2005), and Shakoori, et al. (2011). Generally, crystal morphology can be categorized into five groups, which include bipyramidal, rectangular, spherical, ovoid and irregular shaped (Maeda, et al., 2001; Shishir, et al., 2012). In the present study, the crystal proteins produced from all of the bacterial isolates including the two positive strains *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) were in spherical shape. The observation on *Bacillus thuringiensis israelensis* (ATCC 35646) corresponds to the result of Saadoun, et al. (2001), who reported that *Bacillus thuringiensis israelensis* was able to produce spherical inclusions. Although *Bacillus thuringiensis kurstaki* is commonly reported to produce bypiramidal and cuboidal crystals, the present finding is in agreement with the findings of Silva, et al. (2004), who found spherical crystal contents in two *Bacillus thuringiensis kurstaki* strains, S701 and S764 besides having their typical bipyramidal and cuboidal crystals. As mentioned earlier, the *Bacillus thuringiensis* cannot be distinguished from *Bacillus cereus* through colony morphology identification. According to Martin and Travers (1989), of all the *Bacillus* species, *Bacillus thuringiensis* is the only species that can produce crystal proteins which made it the only phenotypic characteristic that allows them to be distinguishable from the *Bacillus cereus*. In this study, Coomassie

Brilliant Blue (CBB) staining was used to examine the presence of crystal proteins instead of phase contrast microscopy. As reported by Rampersad and Ammons (2005), the presence of crystal proteins can be recognized by using CBB staining as it allows high throughput evaluation of the bacterial colonies for the presence of crystals by increasing the resolution over phase contrast microscopy. Moreover, CBB staining allowed very small crystal proteins to be readily seen with stained specimens as compared to phase contrast microscopy (Rampersad and Ammons, 2005).

Motility test helps to further discriminate *Bacillus thuringiensis* from *Bacillus anthracis* and *Bacillus mycoides*. The bacterial isolates together with the two positive controls *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646) exhibited the ability to be motile which is in agreement to Salvetti, et al. (2009). Among the *Bacillus cereus* group members, *Bacillus cereus* and *Bacillus thuringiensis* are motile whereas *Bacillus anthracis* and *Bacillus mycoides* are non-motile. *Bacillus thuringiensis* are motile through presence of peritrichous flagellum (Bouillaut, et al., 2005; Logan, 2005; Maheswaran, et al., 2010). Salvetti, et al. (2009) reported that the *Bacillus cereus* and *Bacillus thuringiensis* use peritrichous flagella as motility attachment and the motility of *Bacillus thuringiensis* follows a swarming pattern which allows the bacteria to proliferate over solid surfaces. The *Bacillus thuringiensis* has elongated and hyperflagellated swarm cells that promotes propagation of cells on solid media (Ghelardi, et al., 2002).

5.2 The Characteristic of Crystal Proteins of the Bacterial Isolates

5.2.1 The SDS-PAGE Analysis of Bacterial Total Protein

The SDS-PAGE is a technique used to separate protein compositions based on size, charge and shapes. With reference to the positive control *Bacillus thuringiensis kurstaki* ATCC 33679, it was shown to produce three protein bands of approximately at 127 kDa, 70 kDa and 65 kDa in size respectively which is similar to the study conducted by Fakruddin, et al. (2012), whereby the *Bacillus thuringiensis kurstaki* ATCC 33679 was shown to possess 140 kDa, 130 kDa, 75 kDa, 70 kDa, and 60 kDa proteins. On the other hand, the *Bacillus thuringiensis israelensis* ATCC 35646 showed different protein profiling. In the present study, *Bacillus thuringiensis israelensis* ATCC 35646 exhibited four band sizes at around 127 kDa, 78 kDa, 72 kDa and 27 kDa representing the Cry4B, Cry4C, Cry4D and Cyt proteins respectively which is in agreement to Höfte and Whiteley (1989).

All of the bacterial isolates had minimal difference in their protein profiles when compared to the reference strains. All of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 possess 27 kDa to 29 kDa, 44 kDa and 65 kDa to 70 kDa proteins which suggested the presence of Cyt, Cry35 and Cry2 proteins which is in agreement with Arrieta, Hernández and Espinoza, (2004), Fakruddin, et al., (2012), and Höfte and Whiteley, (1989) as they also reported the Cry2, Cry35 and Cyt proteins have the corresponding protein band sizes as mentioned above. Only the protein sizes of 27 kDa to 29 kDa that were present correlated to the reference strain, *Bacillus thuringiensis israelensis* (ATCC

35646). The bacterial isolates A2B, D10D, D1C and R2 possess Cry4B protein with the band size of around 127 kDa (Höfte and Whiteley, 1989) which correlated with the reference strains, *Bacillus thuringiensis kurstaki* (ATCC 33679) and *Bacillus thuringiensis israelensis* (ATCC 35646). The bacterial isolates C6D and R2 were shown to possess Cry1 protein with the size of 130 kDa to 150 kDa which is in agreement to Höfte and Whiteley (1989). Nevertheless, the bands for Cry1 proteins were non-conclusive as the bands may also represent the Cry9 protein which has the size of 130 kDa to 140 kDa, or the Cry7 and Cry8 proteins in which their sizes are 130 kDa as reported by Arrieta, Hernández and Espinoza, (2004). The bacterial isolates C6D, D10D, D1C, R2 and R3 possess a band size of around 44 kDa which suggested the presence of Cry35 proteins (Fakruddin, et al., 2012). In addition, the protein profiling of all bacteria isolates through SDS-PAGE indicate the absence of Cry3 protein which is 75 kDa in size. In short, it can be assumed that the bacterial isolates may contain *cryI*, *cryII*, *cryIV*, *cryVII*, *cryVIII*, *cryIX*, *cry35* and *cyt* genes which produce Cry1, Cry2, Cry4, Cry7, Cry8, Cry9, Cry35 and Cyt proteins. The presence of Cry proteins were further determined by performing the polymerase chain reaction (PCR) to screen for the δ-endotoxin genes that can produce these crystal proteins.

Different proteins have different toxicity against the various insect orders. In the protein profiling, the dominant protein group was sized between 65 kDa to 70 kDa and 27 kDa to 29 kDa which supported the hypothesis that the *cryII* and *cyt* gene was present. All of the bacterial isolates possess Cry2 and Cyt

proteins, so it can be suggested that all of the bacterial isolates may exhibit toxicity against Lepidopterans and Dipterans (Crickmore, 2000; Ellis, et al., 2002; Höfte and Whiteley, 1989; Wang, et al., 2003). Nevertheless, only bacterial isolates C6D, D10D, D1C, R2 and R3 possess Cry35 proteins that may exhibit toxicity against Coleopterans (Ellis, et al., 2002). Therefore, SDS-PAGE can be used to further characterize the *Bacillus thuringiensis* from the other *Bacillus cereus* group members through protein profiling. In addition, it can also be applied to predict the toxicity profile of the *Bacillus thuringiensis* against insect orders.

5.2.2 PCR for Screening of δ-endotoxin Genes

The extracted DNA using the DNA extraction kit were used instead of the phenol-chloroform method although the latter gave high yield of DNA. This is because the phenol-chloroform method yielded low A_{260}/A_{230} nm ratio which indicated the presence of phenol contamination, loss of DNA pellet and the presence of PCR inhibitors such as phenol (Psifidi, et al., 2015).

The Un1 primer which was adapted from Halima, et al. (2006) and Vidal-Quist, et al. (2009) was used to identify the *cryI* gene. Six out of the seven bacterial isolates which were A10C, A2B, C6D, D10D, D1C and R2 were shown to possess the *cryI* gene which is in agreement to the findings of the above researchers as they reported similar *cryI* genes in their isolated *Bacillus thuringiensis* which recorded a band size of around 277 bp. The Cry2gral primer which was adapted from Ibarra, et al. (2003) was used to detect the

presence of *cryII* gene. In the present study, all of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 possessed the *cryII* gene at 500 bp to 526 bp. The Cry4Aspe primer which was adapted from Mahalakshmi, et al. (2012) was used to identify the *cryIV* gene. However, in the present study, none of the bacterial isolates possessed *cryIV* gene at the band size of 459 bp. This may be due to the primer used targets a specific *cryIVA* gene which may not be present in any of the bacterial isolates. Among the seven bacterial isolates, six of them A10C, A2B, C6D, D10D, D1C and R2 carry both *cryI* and *cryII* genes, only R3 possessed *cryII* gene, whereas none of the bacterial isolates possess the *cryIV* gene.

In the present study, *cryI* and *cryII* were the genes found in the bacteria isolated from the ex-tin mining soils in UTAR, Kampar Campus. In Eastern Asia, it has been reported that, *cryI* and *cryII* genes are the most abundant of all *cry* genes (Martin and Travers, 1989; Wang, et al., 2003; Zhang, Yu and Deng, 2000). However, the present findings did not correlate to the findings in Brazil whereby Nunes-Pinto and Fiúza (2003) reported the *cryIX* genes to be more abundant than *cryI* and *cryII* genes. In addition, in a previous finding in Mexico it was also shown that the most abundant *cry* genes in a majority of bacterial isolates were *cryIX* genes (Rosas-García, et al., 2008). Therefore, it is obvious that the distribution of the *cry* genes is highly depend on the geographical areas.

By comparing the results of the protein profiling through SDS-PAGE and PCR amplification of the δ-endotoxin genes, bacterial isolates A2B, D10D, D1C and R3 showed the presence of Cry4 protein bands but did not possess the *cryIV* gene. Besides, all bacterial isolates except R3 possessed *cryI* gene, however only bacterial isolates C6D and R2 exhibited the Cry1 protein band on SDS-PAGE. This indicates that the presence of genes did not yield the corresponding proteins. According to Armengol, et al. (2007), in most of their bacterial isolates, there is minimal to no correlation between protein profiling and *cry* gene patterns. These differences might be due to the detected genes could possibly be coding for proteins with low level of expressions or are inactive (Armengol, et al., 2007). Porcar and Juárez-Pérez (2003) reported their bacterial isolates were known to have six Cry proteins but, only three proteins were expressed. The non-expressed protein might have been silenced due to insertion within the gene coding region.

Moreover, the low level of proteins can be due to differences in sporulation time of the strains, as the time required for completion of sporulation varies from strain to strain and depends on growth medium conditions. Some sample may have yet to reach autolysis phase (Armengol, et al., 2007). Protein bands would not be detectable by Coomassie Brilliant Blue (CBB) staining when the protein concentration is insufficient or low. More sensitive staining techniques such as silver staining can be used to visualize the bands. According to Iriarte, et al. (2000), they reported that a strain of *Bacillus thuringiensis* which has a 66 kDa protein, was not visibly shown on SDS-PAGE gel staining with

Coomassie Brilliant Blue 250, however it was detectable after silver staining. In addition, it is also possible that the detected genes may code for similar, non-active, low expressed proteins, and these crystal proteins are controlled by weak promoters (Armengol, et al., 2007). Another plausible reason for the differences between protein profiles of the same *cry* gene could be due to some environmental factors that can turn on or turn off the expressions of some *cry* genes (Agaisse and Lereclus, 1995).

5.3 The 16S rDNA Sequence Analysis

The 16S rDNA sequencing was used in the classification of taxonomic affinities in a wide range of taxa (Baker, Smith and Cowan, 2003). The U16S_8F and U16S_1541R primers which were adapted from Teo (2013) were used to identify the gene sequence of the bacterial isolates at the size of 1,500 bp. According to Wang and Qian (2009), bacterial universal primers targeted on the conservative regions to generate amplicons of variant regions which is useful in taxonomic classification. Chen, et al. (1989) and Relman (1999) reported that the bacterial universal primers are often chosen as the conserved regions at the beginning and the end of ~ 1,500 bp where the sequences of variable regions are covered. It has been reported that the amplicons of variant regions in 16S rDNA, which are the V3 and V6 regions provide sufficient phylogenetic information for the bacteria being tested (Huse, et al., 2008). However, based on the result obtained, the primers used could only allow for confirmation of the bacteria up to genus level, *Bacillus* sp. The species of the bacterial isolates remained unknown as the BLASTn analysis showed that the

bacterial isolates could be *Bacillus thuringiensis*, *Bacillus cereus* or *Bacillus mycoides*.

Ash, et al. (1991) reported that the 16S rDNA gene sequencing of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* showed at least 99% similarity in their sequences. The number of nucleotide bases which were different among these species group was only around four to nine nucleotides. Thus, it becomes difficult to distinguish these species genotypically through 16S rDNA gene sequencing as the coverage rate of the 16S rDNA is more than 90%, which means the primer can anneal to conserved regions in the majority of the bacterial 16S rDNA (Wang and Qian, 2009). Besides, Bavykin, et al. (2004) and Helgason, et al. (2000) reported that the *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* were considered as a single species due to the high similarity in their nucleotide sequence. As such, the screening of δ-endotoxin genes and examination of the presence of crystal proteins become the main criteria to distinguish the *Bacillus thuringiensis* from other non-*Bacillus thuringiensis* species.

5.4 Limitation of Study and Future Works

The shapes of the crystal proteins can help to identify the classes of the crystal proteins and their toxicity against different insect orders. In the present study, the definitive shapes and the sizes of the crystal proteins produced by the bacterial isolates were not able to be distinguished as a result of limited magnification of the compound light microscope. The Scanning Electron

Microscope (SEM) and Transmission Electron Microscope (TEM) can be used in future studies in order to further examine the crystal proteins produced by these isolated bacterial samples.

The use of silver staining to detect the presence of crystal proteins in SDS-PAGE can be performed as the silver can detect proteins in concentrations as low as 2 ng, whereas Coomassie Brilliant Blue (CBB) staining cannot detect the protein with concentration lower than 10 ng. The SDS-PAGE analysis can help to determine the molecular weights of the proteins but cannot assure the presence of proteins. In order to determine the presence of the proteins of interest, Western blotting can be performed. Western blotting uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. Besides, genes coding for specific crystal proteins can be cloned into a vector, transformed into host cells, allowing the host cell to replicate and eventually obtaining larger amounts of the targeted crystal proteins.

The screening of δ-endotoxin genes was time consuming as it required optimization and usage of various primers. However, this procedure should be continued and further improvised so that it not only further distinguishes the *Bacillus thuringiensis*, but also evaluates the toxicity of the toxins.

In addition, apart from Diptera specific δ-endotoxin genes, screening of other δ-endotoxin genes that target Lepidoptera, Coleoptera, Hymenoptera,

Hemiptera, Mallophaga and some invertebrates such as nematodes can also be performed. For instance, the *cryI* gene that encodes for Lepidoptera specific protein and *cryIII* gene that encodes for Coleoptera specific protein. Larvicidal test can also be performed to determine the larvicidal properties of the bacterial isolates against different insect orders. Apart from this, PCR can also be performed to confirm that the δ-endotoxin genes are only present among *Bacillus thuringiensis* and none in non-*Bacillus thuringiensis* strains.

The identification of *Bacillus thuringiensis* through 16S rDNA gene sequencing does not show differential results. The analyses of the 16S rDNA gene sequencing can only differentiate the bacterial isolates to genus level. The similar scores, query coverages, expected values and the identity among the isolates resulted in the failure of the phylogenetic tree construction. Thus, other genotyping characterization such as pulsed-field gel electrophoresis, multiplex PCR and other alternative methods together with the morphology identification can be done in future to distinguish *Bacillus thuringiensis* isolates. On the other hand, VITEK® 2 can also be performed as it has been known to be able to separately identify *Bacillus thuringiensis* from *Bacillus cereus* through their biochemical profilings. The VITEK® 2 is an automated system used to identify unknown bacteria using a colorimetric reagent card (ID card) and interpret the result automatically.

CHAPTER 6

CONCLUSION

Although the 16S rDNA gene sequencing analyses could not give a definitive result and showed the possibility of the bacterial isolates to be either *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus mycoides*, however it can be concluded that the bacterial isolates were *Bacillus thuringiensis* through morphological identification and screening of δ-endotoxin genes. This is because only *Bacillus thuringiensis* possess the ability to produce crystal proteins. All of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 were shown to be Gram positive, rod-shaped, able to produce endospores and crystal proteins, and are motile.

In the SDS-PAGE analyses, all bacterial isolates were shown to exhibit 27 kDa Cyt proteins and 65-70 kDa Cry2 proteins. Bacterial isolates A2B, D10D, D1C and R2 possessed Cry4 protein with the band size of around 125 kDa, whereas C6D and R2 are the only bacterial isolates that were shown to possess Cry1 protein at the size of 130 kDa to 150 kDa. Based on the screening of δ-endotoxin genes through PCR amplification, all bacteria isolates exhibited *cryI* and *cryII* genes at 277 bp and 500 bp to 526 bp respectively except for R3 bacterial isolates which was the only bacterial isolate that carried the *cryII* gene at 500 bp to 526 bp.

In conclusion, all of the bacterial isolates A10C, A2B, C6D, D10D, D1C, R2 and R3 were *Bacillus thuringiensis* spp. Additional screening of other δ-endotoxin genes through PCR and observation of crystal proteins produced through electron microscopy could be necessary to further evaluate the subspecies.

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APPENDICES

Appendix A

List of instruments and apparatus used with the respective manufacturers

Instruments/Apparatus	Manufacturers
Autoclaved Machine	Hirayama, Japan
Centrifuge Tube (15 ml & 50 ml)	Axygen Scientific, USA
Gel Electrophoresis Set	Major Science, Taiwan
High Speed Microcentrifuge Machine	Thermo Fisher Scientific, USA
Table-top Microcentrifuge Machine	Profuge
Microwave Oven	Sharp, Japan
Mini-Protean Tetra System	Bio-Rad, US
Nano-photospectrometer	IMPLEN,
Petri Dishes	Labmart
pH Meter	Sartorius
Vortex	Gemmy Industrial Corp., Taiwan
Shaking Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
SmartSpec Plus Spectrophotometer	Bio-rad, US
Cuvette	Bio-rad, US
UV Transilluminator	Bio-rad, US
Heat block	BioShake IQ
Weighing Balance	Sartorius
Hot Plate Stirrer	Harmony
Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Fume hood	ESCO Micro (M) Sdn. Bhd., Malaysia
Compound Light Microscope	Leica, Japan

Appendix B

List of chemicals, media and reagents used with the respective manufacturers

Chemicals/Media/Reagents	Manufacturers
Nutrient Agar	Merck, Germany
Nutrient Broths	Merck, Germany
Absolute Ethanol	Qrec, Malaysia
Safranin O	Bendosen
Gram’s Iodine	Lab Stain
Crystal Violet	Lab Stain
Sodium Chloride	RCI Labscan
Malachite Green	R&M Marketing, Essex
Coomassie Brilliant Blue (R-250)	BioBasic, Canada
Methanol	Merck, Germany
Glacial Acetic Acid	Qrec, Malaysia
Glycerol	Qrec, Malaysia
100-bp plus DNA Ladder	Thermofisher
6X DNA Loading Dye	Thermofisher
30% Bis-Acrylamide	Bio basic
Agarose Powder	1st Base
Chromatin Prestained Protein Ladder	Vivantis
Sodium Dodecyl Sulphate (SDS)	Chem Solution
Tetramethylethylenediamine (TEMED)	Alfa Aeser
Ammonium Persulfate (APS)	Sigma Aldrich
DTT	Thermofisher
Tris-HCl	Chem Solution
Advance DNA Stain	Midori Green

Appendix C

Preparation of Media

Preparation of Nutrient Agar

A total amount of 6.4 g of nutrient agar powder were dissolved in 800 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 0.85% Saline Solution

A total weight of 8.5 g of Sodium Chloride salt was dissolved in 1000 ml of distilled water in media bottle, autoclaved and stored for prior to usage.

Preparation of Nutrient Broths

A total amount of 16 g of nutrient broths powder were dissolved in 800 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.