MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF ISOLATED *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES.

By

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ABSTRACT

MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF ISOLATED *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES

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Microbial pesticides are naturally occurring biological agents that control target pests, yet remain safe to the environment. The most widely used microbial pesticide is from the strain of *Bacillus thuringiensis* which is a Gram-positive, rod-shaped and endospore forming soil bacterium with the ability to produce parasporal crystals comprising of δ-endotoxins that rendered it its insecticidal activity. Certain *Bacillus thuringiensis* strains were also shown to possess non-insecticidal crystal proteins, known as parasporins that exhibit cytotoxicity to human cancer cells. The objectives of this study were to characterise the bacterial isolates via morphological identification, SDS-PAGE, and screening of δ-endotoxins and *chi* genes through PCR amplification. All the isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 were confirmed as *Bacillus thuringiensis*, having characteristics of Gram-positive, rod-shaped, and able to produce endospores and crystal proteins. The *cry1* gene was carried by four of the isolates, however, none of them exhibited Cry1 protein bands on SDS-PAGE nor produced Cry1 crystals which are bipyramidal in shape on the electron micrograph. Among the six isolates expressing *cry2* genes, only two of them produced crystal proteins which are cuboidal in shape, however none of the

isolates exhibited Cry2 protein bands. The four isolates expressing *cry4* genes also did not produce the corresponding protein bands on SDS-PAGE, however, ovoidal-shaped crystals indicative of Cry4 proteins were produced by these four isolates. For *cry10* and *cyt2* genes, their expression in all of the isolates were also not consistent with the production of their respective protein bands in SDS-PAGE. Besides, there were only two isolates which showed both the *cry11* gene expression and Cry11 protein bands. The successful screening of *cry* and *cyt* genes, with the absence of *ps* and *chi* genes in all of the isolates lead to a conclusion that the isolates are all non-cytotoxic, but could be insecticidal strains.

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DECLARATION

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

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Tan Yingying

APPROVAL SHEET

This final year project report entitled "**MORPHOLOGICAL CHARACTERISATION OF ISOLATED *Bacillus thuringiensis* AND SCREENING OF *cry*, *cyt*, *ps* AND *chi* GENES**" was prepared by TAN YINGYING and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman**.**

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PERMISSION SHEET

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I hereby give permission to the University to upload the softcopy of my final year project report 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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(TAN YINGYING)

TABLE OF CONTENTS

		Page
ABSTRACT		**ii**
ACKNOWLEDGEMENTS		**iv**
DECLARATION		**v**
APPROVAL SHEET		**vi**
PERMISSION SHEET		**vii**
TABLE OF CONTENTS		**viii**
LIST OF TABLES		**xi**
LIST OF FIGURES		**xii**
LIST OF ABBREVIATIONS		**xiv**
CHAPTER		
1	INTRODUCTION	1
2	LITERATRE REVIEW	6
	2.1 History of *Bacillus thuringiensis*	6
	2.2 General Characteristics of *Bacillus thuringiensis*	8
	2.3 Life Cycle of *Bacillus thuringiensis*	9
	2.4 Natural Habitat and Distribution of *Bacillus thuringiensis*	10
	2.5 The Genus *Bacillus*: Relation of *Bacillus thuringiensis* with other *Bacillus* spp.	13
	2.6 Classification of *Bacillus thuringiensis* strains	14
	2.6.1 Phenotypic Characterisation	15
	2.6.2 Genotypic Characterisation	17
	2.7 The Parasporal Crystalline Inclusions	19
	2.7.1 The Cry Proteins	19
	2.7.2 Mode of Action of Cry Proteins	21
	2.7.3 The Cyt Proteins	22
	2.8 The Chitinase	24

2.9 Non-insecticidal Parasporin 25
2.10 The Application of *Bacillus thuringiensis* 28

3 MATERIALS AND METHODS 31
3.1 The General Overview of Experimental Design 31
3.2 Chemicals, Media, Reagents and Equipment 32
3.3 Bacterial Samples 32
3.3.1 Maintaining Bacterial Samples 32
3.3.2 Glycerol Stock Preparation 33
3.4 Morphological Identification of Bacterial Isolates 33
3.4.1 Gram Staining 33
3.4.2 Malachite Green Staining 34
3.4.3 Coomassie Brilliant Blue (CBB) Staining 36
3.4.4 Scanning Electron Microscopy (SEM) 36
3.4.5 Transmission Electron Microscopy (TEM) 38
3.5 Protein Identification 40
3.5.1 Protein Extraction 40
3.5.2 Bradford Assay 41
3.5.3 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis 42
3.6 DNA Extraction 45
3.7 Polymerase Chain Reaction (PCR) 46
3.7.1 Touchdown PCR (TD-PCR) 49
3.7.2 Optimisation of Primers 50
3.7.3 PCR Amplification 53
3.8 Agarose Gel Electrophoresis 54

4 RESULTS 55
4.1 Gram Staining 55
4.2 Malachite Green Staining 56
4.3 Coomassie Brilliant Blue (CBB) Staining 58
4.4 Scanning Electron Microscopy (SEM) 60
4.5 Transmission Electron Microscopy (TEM) 63
4.6 Sodium Dodecyl Sulphate – Polyacrylamide Gel 64

Electrophoresis (SDS-PAGE) Analysis

4.7 The δ-endotoxin Genes Gel Electrophoresis 67

5 DISCUSSIONS 73

5.1 Morphological Identification 73

5.2 Scanning and Transmission Electron Microscopy 77

5.3 SDS-PAGE Analysis of the Bacterial Total Proteins 79

5.4 Polymerase Chain Reaction for Screening of *cry*, *cyt* and *chi* Genes 82

5.5 Limitation of Study and Future Works 86

6 CONCLUSIONS 89

REFERENCES 91

APPENDICES 108

LIST OF TABLES

Table		Page
3.1	The components required for making 10% separating gel	42
3.2	The components required for making 4% stacking gel	43
3.3	The components of 6X sample loading buffer	44
3.4	PCR primers used for endotoxin gene amplification	47-48
3.5	Parameters for Touchdown PCR	49
3.6	Standard PCR mixture for a reaction with a final volume of 10 µL	51
3.7	PCR parameters for primer optimization	52
3.8	Parameters for PCR amplification	53
4.1	The summary of the PCR results of *cry1, cry2, cry4, cry10, cry11, cyt2, chi, ps1, ps2* and *ps4* genes of bacterial isolates	72

LIST OF FIGURES

Figure		Page
1.1	A sporulated cell of *Bacillus thuringiensis morrisoni* under transmission electron micrograph	3
3.1	The general overview of experimental design	31
3.2	The general procedure of Gram staining	34
3.3	The general procedure of endospore staining	35
4.1	The Gram staining of the bacterial isolates	56
4.2	The Malachite green staining of the bacterial isolates	57
4.3	The CBB staining of the bacterial isolates	59
4.4	Scanning electron microscopy (SEM) of the bacterial isolates	61-62
4.5	Scanning electron microscopy (SEM) of negative control (BC) *Bacillus cereus*	62
4.6	Transmission electron microscopy (TEM) of the bacterial isolates	63
4.7	The SDS-PAGE of the total protein extracts of bacterial isolates	66
4.8	Screening of the bacterial isolates using Un1 primer (*cry1* gene)	68
4.9	Screening of the bacterial isolates using Cry2gral primer (*cry2* gene)	69
4.10	Screening of the bacterial isolates using Cry4 primer (*cry4* gene)	69

4.11 Screening of the bacterial isolates using Cry10spe primer (*cry10* gene) 70

4.12 Screening of the bacterial isolates using Cry11 primer (*cry11* gene) 70

4.13 Screening of the bacterial isolates using Cyt2 (UN) primer (*cyt2* gene) 71

LIST OF ABBREVIATIONS

A_{260} / A_{280}	Assessment of Nucleic Acid Purity
APS	Ammonium Persulfate
Bt	*Bacillus thuringiensis*
Btk	*Bacillus thuringiensis kurstaki*
Bti	*Bacillus thuringiensis israelensis*
Bc	*Bacillus cereus*
bp	Base pair
Cry proteins	Crystal proteins
Cyt proteins	Cytolytic proteins
δ-endotoxins	Delta-endotoxins
DTT	Dithiothreitol
Eco	*Escherichia coli*
g	Acceleration of gravity
kDa	Kilo-Dalton
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SEM	Scanning electron microscope
TEM	Transmission electron microscope
TEMED	Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

Over the years, application of chemical pesticides have been the monopoly in the control of pests of agricultural and medical importance. Chemical pesticides have consistently demonstrated their worth by controlling insect-borne endemic diseases, protecting plantations, improving crops quality and increasing global agricultural productivity. Most developing countries have increased reliance on these environmentally persistent chemical pesticides, enabling them to obtain much more agricultural products that allow them to become the major crop suppliers in the world, which in turn pay for their much-needed international trade credits (Atreya, 2007; Ecobichon, 2001). Chemical pesticides have undeniably occupied a strong position in agricultural field by reducing the loss of crops due to pest infestation. However, the uncontrolled and continuous use of these chemical pesticides have resulted in environmental contamination or damage, deleterious effects to human health and reduced agricultural sustainability (Recena, et al., 2006; Wilson and Tisdell, 2001).

One of the major problems of chemical pesticides is its non-specificity that causes it to affect noxious pest species as well as the beneficial insects. The unintentional destruction of the beneficial predators of agricultural pests causes the increased virulence of many species of pests (Wilson and Tisdell, 2001). Many cases of human morbidity and mortality caused by chemical pesticides

either environmentally or occupationally have also been reported. One of the main hazardous outcomes of the massive use of pesticides could be the occupational exposure of agricultural workers resulting in health problem, especially in those developing countries (Hurtig, et al., 2003). These externalities from the chemical pesticides have caused the gradual prohibition of its use in agriculture.

Due to the rapidly growing human population, world food production is urgently in demand and the effective way to have increased crop productivity is, undoubtedly, a good pest management system (Abhilash and Singh, 2009; Carvalho, 2006), which should not emphasise the use of chemical pesticides after recognising the seriousness of many pesticide-related problems. Biological controls such as biopesticide is an alternative to chemical pesticide in the effort to halt the massive spread of agrochemical use. Many species of naturally occurring entomopathogens are employed as biological insecticides that are biodegradable and therefore not persistent in the environment. Besides, biological insecticides are also non-toxic to human, mammals and beneficial insects. Among the biological control agents, *Bacillus thuringiensis* is an excellent example and the most widely used microbial pesticide following the discovery of its many new varieties with activity against insect pests in the orders of Lepidoptera, Diptera and Coleoptera (Gupta and Dikshit, 2010; Lacey, et al., 2001).

Bacillus thuringiensis are facultative anaerobic, Gram-positive, spore forming soil bacteria that belonged to *Bacillus cereus* group (Sanahuja, et al., 2011). The proteinaceous toxins located in the parasporal crystals attributed to the insecticidal property of *Bacillus thuringiensis*. The parasporal crystals also known as inclusion bodies that are produced during the sporulation phase (Lacey, et al., 2001) alongside the endospore in a sporangium by *Bacillus thuringiensis* as shown in Figure 1.1. The proteinaceous toxins in the parasporal crystals that are collectively known as δ-endotoxins exhibit highly selective spectrum of activity against insect pests. Microbial insecticides containing these *Bacillus thuringiensis* δ-endotoxins have been employed to replace chemical pesticides for more than 60 years (Romeis, Meissle and Bigler, 2006). Other than Lepidoptera, Diptera and Coleoptera, *Bacillus thuringiensis* also display specific larvicidal activity against insects of orders Hymenoptera, Hemiptera, and some specific invertebrates such as nematodes (Ohba, Mizuki and Uemori, 2009).

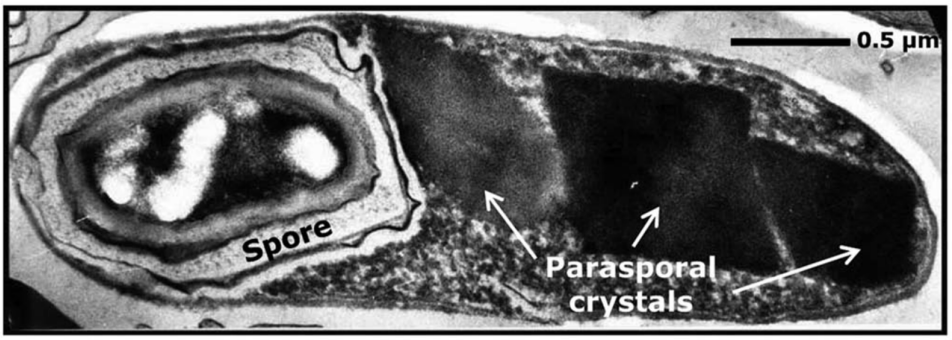

Figure 1.1: A sporulated cell of *Bacillus thuringiensis morrisoni* under transmission electron micrograph (Adapted from Ibrahim, et al., 2010).

Parasporal proteins of *Bacillus thuringiensis* are toxins that are active only inside the insect gut due to its requirement of an alkaline condition and presence of specific protease in insect intestine. Therefore, *Bacillus thuringiensis* toxins do not harm non-target organisms, allowing it safe for use. The parasporal crystals comprise of one or more Cry (crystal) and Cyt (cytolytic) proteins. CryI, CryII, CryIII and CryIV are the four major classes of Cry proteins produced. Their mode of action involves a cascade of events that causes the death of insects that ingest them. Following ingestion and solubilisation in the alkaline environment of insect midgut, cleavage by specific protease produces activated toxin that binds to specific receptor on brush border membrane of the insect gut epithelial cells. The subsequent membrane transport disruption due to lytic pore formation, followed by lysis of cells will ultimately cause insect death (Aronson and Shai, 2001; Lacey, et al., 2001). High specificity of *Bacillus thuringiensis* toxins allows them to exert lethal effect only to specific pests while leaving other organisms unharmed. Highly selective activity of *Bacillus thuringiensis* toxins also enable the survival of natural predators of the target insect pests and other beneficial insects. Besides contributing great potential benefits to agricultural field, *Bacillus thuringiensis* toxins also aid in the control of certain disease-related vectors which fall in the range of insect orders targeted by the toxins.

Other than Cry and Cyt proteins that render *Bacillus thuringiensis* its insecticidal activity, certain strains of this bacterium possess other proteins that exert cytocidal activity rather than insecticidal one. Non-insecticidal strains of *Bacillus thuringiensis* produce parasporal crystal proteins that have shown strong cytocidal activities against different types of human cancer cells. These

non-insecticidal inclusion proteins are known as parasporins. There are currently four families of parasporins: PS1, PS2, PS3 and PS4 with marked differences in their cytotoxicity spectrum and level of activity (Ohba, Mizuki and Uemori, 2009). Similar to Cry toxins, parasporins need to be activated before exhibiting their effect. Human leukaemic T cells (MOLT-4) and human uterus cervic cancer cells (HeLa) have been shown to be killed by the cytocidal activity of activated parasporin (Mizuki, et al., 2000).

Therefore, the aims of this research were:

1. To characterise the *Bacillus thuringiensis* isolates through morphological identification.
2. To examine the crystals formation in the *Bacillus thuringiensis* isolates using scanning and transmission electron microscopes.
3. To determine the molecular weight of proteins extracted from the *Bacillus thuringiensis* isolates using SDS-PAGE.
4. To investigate the presence of *cry*, *cyt*, *ps* and *chi* genes in the *Bacillus thuringiensis* isolates through polymerase chain reaction (PCR) and gel electrophoresis.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Bacillus thuringiensis*

The earliest description of *Bacillus thuringiensis* began in year 1901 by a Japanese bacteriologist, Shigetane Ishiwata who isolated the bacteria from infected silkworms, *Bombyx mori* (Ishiwata, 1905) during his investigation of the cause of the "sotto disease" (Ibrahim, et al., 2010). This bacterium was described by Ishiwata as *Sottokin Bacillus*, which means "sudden death-Bacillus" (Beegle and Yamamoto, 1992; Ishiwata, 1905; Sanchis, 2010). *Sottokin Bacillus* in Japan caused wilt disease in silkworm caterpillars (Milner, 1994). Although Ishiwata had described the pathology of *Sottokin Bacillus* following its ingestion by silkworm larvae and its characteristics (Beegle and Yamamoto, 1992), the description was too brief and incomplete.

A decade later, Ernst Berliner, a German scientist, isolated the related strain from the Mediterranean flour moth, *Ephestia kuehniella*, which were collected in a flour mill in the German province of Thuringia (Berliner, 1911; Sanchis, 2010). This insect pathogen was named by Berliner in year 1915 as *Bacillus thuringiensis* and he described it as a Gram-positive, spore forming, rod-shaped bacterium (Berliner, 1915; Milner, 1994). Berliner was credited to naming it

because the description by Ishiwata was not formal and not known to him (Beegle and Yamamoto, 1992).

At the same year the bacterium was rediscovered by Berliner, Aoki and Chigasaki began their studies and works with the bacterium (Sanchis, 2010). They claimed that only the old, sporulated cultures of the bacterium were able to cause disease in insects. Therefore, they reported that the killing activity of the bacteria was due to the toxins in the sporulated cultures (Aoki and Chigasaki, 1916; Beegle and Yamamoto, 1992).

Berliner's culture of *Bacillus thuringiensis* was lost but in year 1927, Mattes was able to reisolate the same organism from the same host (Beegle and Yamamoto, 1992; Mattes, 1927). Mattes' isolate was widely distributed all over the world and used in most of the early commercial products (Beegle and Yamamoto, 1992). Same with Berliner, Mattes had observed, in addition to spores, the inclusion bodies in the *Bacillus thuringiensis*, therefore confirming Berliner's discovery (Ibrahim, et al., 2010). The first commercial insecticide, Sporeine was produced in France in year 1938 (Beegle and Yamamoto, 1992; Milner, 1994).

In year 1953, Christopher Hannay had discovered the inclusion body, as had Berliner and Mattes, during his examination of the sporulating cells (Beegle and Yamamoto, 1992). "Parasporal crystal" was a phrase coined by Christopher Hannay to represent the second body found (Ibrahim, et al., 2010). He speculated that the pathogenicity of *Bacillus thuringiensis* toward the Lepidopterous larvae

was attributed to these inclusion bodies (Beegle and Yamamoto, 1992). Thomas Angus proved Hannay's finding that the toxicity of the bacterium was due to its parasporal crystal. Angus discovered that the crystals exertstheir toxic effect in silkworm only when activated by the gut juice (Beegle and Yamamoto, 1992; Milner, 1994).

In the early 1960s, serological analysis of flagella antigens was applied to classify *Bacillus thuringiensis*. Up until year 1977, there were only 13 subspecies of *Bacillus thuringiensis* that had been described and all the subspecies discovered exert toxic effect against Lepidoptera larvae only (World Health Organisation, 1999). The host range of *Bacillus thuringiensis* became more diverse with the discovery of many other subspecies. Margalit and Tahori discovered the *Bacillus thuringiensis israelensis* in Israel in year 1976 and that subspecies had successfully controlled the Dipteran insects: mosquitoes and blackflies (Margalit and Dean, 1985). Moreover, *Bacillus thuringiensis tenebrionis* that was toxic against Coleopteran insect larvae had been found and described in year 1983 (Krieg, et al., 1983). Until the end of 1998, more than 67 subspecies were identified based on the H flagella antigens (World Health Organisation, 1999).

2.2 General Characteristics of *Bacillus thuringiensis*

As one of the members of the genus *Bacillus*, *Bacillus thuringiensis*, is an ubiquitous Gram positive, facultative anaerobic, rod-shaped, motile and spore

forming soil bacterium. The key defining feature of *Bacillus thuringiensis* that distinguishes it from other *Bacillus cereus* group's members is its ability to produce parasporal crystalline inclusion bodies, which were later known as δ-endotoxins (Sanahuja, et al., 2011; Shishir, et al., 2012). This parasporal crystal was formed during sporulation at the stationary phase of its growth cycle (Schnepf, et al., 1998). The crystal development begins only after sporulation as the crystal-encoding genes are controlled by a dedicated RNA polymerase which is specifically synthesised during spore formation (Sanahuja, et al., 2011). This explained why crystal formation does not occur during vegetative stage. It was later found that the parasporal crystal is derived from highly specific insecticidal toxin. Different types of toxin are produced by different *Bacillus thuringiensis* strains, and each toxin has effect on a narrow taxonomic group of insects. The parasporal crystalline δ-endotoxins are mainly toxic against larvae of insect orders of Lepidoptera, Diptera and Coleoptera (Sanahuja, et al., 2011; Schnepf, et al., 1998). Other insect orders (Hymenoptera, Homoptera, Orthoptera, etc.) that are susceptible to the killing activity of *Bacillus thuringiensis* have been reported as well (Schnepf, et al., 1998). Therefore, *Bacillus thuringiensis* toxin has been widely used as an alternative to chemical pesticides.

2.3 Life Cycle of *Bacillus thuringiensis*

Bacillus thuringiensis has a two-phase life cycle that consists of a vegetative cell division and spore formation (Ibrahim, et al., 2010). The vegetative cell is rod-shaped with the sizes varying between 2 to 5 μm long. *Bacillus thuringiensis*

multiplies by cell division during vegetative cell cycle where a division septum is formed mid-way along the plasma membrane, dividing the vegetative cell into two uniform daughter cells (Ibrahim, et al., 2010; Lambert and Peferoen, 1992).

Asymmetric cell division occurs during sporulation cycle where endospores are formed when nutrients are depleted. The spores are resistant to certain adverse conditions such as heat and drought, allowing *Bacillus thuringiensis* to survive during periods of stress (Lambert and Peferoen, 1992). Crystal proteins are produced by *Bacillus thuringiensis* during sporulation phase and it is a genetically-regulated event (Ibrahim, et al., 2010). The spore and crystal development by *Bacillus thuringiensis* does not start during vegetative cell growth, instead, their formation are initiated only at the end of logarithmic growth of vegetative cells when already deprived of nitrogen and carbon sources. The crystal protein lay alongside the spore and both are released into the environment at the end of sporulation (Lambert and Peferoen, 1992). The crystal production provides additional survival advantage to *Bacillus thuringiensis* by exerting toxic and lethal action to surrounding host insects (Ibrahim, et al., 2010).

2.4 Natural Habitat and Distribution of *Bacillus thuringiensis*

Bacillus thuringiensis is known as a soil microorganism attributed by the fact that its main colonisation was in this environment, as a great deal of its isolation throughout the years was from the soil worldwide. Soil as the main habitat of this bacterium was in agreement with the early works of Bernhard, et al. (1997),

DeLucca II, Simonson and Larson (1981), Martin and Travers (1989), and Ohba and Aizawa (1986). As early as 1981, DeLucca II, Simonson and Larson had reported their findings that 0.50% of a total 46,373 isolates studied was identified as *Bacillus thuringiensis*. In year 1986, Ohba and Aizawa discovered that the *Bacillus thuringiensis* occupied 2.7% (189 out of 6910) of the soils in Japan. Martin and Travers (1989) collected soil samples globally and discovered that 70.40% contained *Bacillus thuringiensis*, with more than 60.0% of the isolates exhibited toxicity against insects of orders Lepidoptera or Diptera. Besides, they also reported that *Bacillus thuringiensis israelensis* was found to be the most common subspecies present in all soil samples.

The isolation of *Bacillus thuringiensis* from soil was also done by researchers in recent years. As reported by Ohba, Wasano and Mizuki (2000), *Bacillus thuringiensis* was recovered from 13.20% of 809 soil samples obtained from Ryukyu Islands, Japan, with 53.0% of the isolates produced ovoid to spherical crystal inclusions. Besides, Assaeedi, Osman and Abulreesh (2011) found the presence of *Bacillus thuringiensis* in 5.0% of the soil samples collected from various areas in Western Saudi Arabia, representing 6.67% of all *Bacillus* species isolated. Similarly, Ammouneh, et al. (2011) recovered *Bacillus thuringiensis* from 12.50% of soil samples from Syria, and 25 of the isolates were found to be Lepidoptera-specific and non Diptera-specific.

Poorer survival and persistence rate was demonstrated by *Bacillus thuringiensis* in phylloplanes as compared to soil environment, because leaf exudates such as

organic acids reduce the leaf surface pH, thus causing bacteria mortality (Devi, et al., 2014; West, et al., 1985). However, the variation of persistence of *Bacillus thuringiensis* on phylloplanes can be due to different varieties of plant species, for example the *Bacillus thuringiensis* strain HD1 cannot survive for long on cotton leaves but is able to grow and persist on the leaves of *Trifolium hybridum* (Bizzarri and Bishop, 2008; Bora, et al., 1994).

In addition to that, Grison, et al. (1976) reported the existence of *Bacillus thuringiensis* spores for few months in shaded pine forests, proving the natural occurrence of *Bacillus thuringiensis* on phylloplane. Based on the study made by Smith and Couche (1991), *Bacillus thuringiensis* were isolated from phylloplanes of conifer and deciduous trees and those isolated strains included the *Bacillus thuringiensis* that are insecticidal to the larvae of the orders Lepidoptera, Diptera and Coleoptera. They also proposed that *Bacillus thuringiensis* might be one of the common microflora on the leaves of many plants.

Furthermore, *Bacillus thuringiensis* were also found in other regions or habitats like rhizosphere, aquatic environment, insect cadavers, etc (Argôlo-Filho and Loguercio, 2013; Raymond, et al., 2010). A population of *Bacillus thuringiensis* was found in rhizosphere in proportion of 260 times higher than in the phylloplanes of the identical plants, which might be due to the availability of richer nutrients in these environments resulting in a more effective colonisation of the bacteria in the rhizosphere (Bisht and Mishra, 2013; Hendriksen and

Hansen, 2002). Ichimatsu, et al. (2000) had reported the recovery of *Bacillus thuringiensis* in relatively high frequency (49.50%) from samples of running and fresh water in Kyushu, Japan. The *Bacillus thuringiensis* isolates represented 4.40% of all *Bacillus* species. Apart from that, isolation of *Bacillus thuringiensis* from insect cadavers was also demonstrated by Ishiwata (1905) and Berliner (1911) from infected silkworms and Mediterranean flour moths repectively.

2.5 The Genus *Bacillus*: Relation of *Bacillus thuringiensis* with other *Bacillus* spp.

Bacillus refers to rod-shaped, aerobic or facultative anaerobic, and highly refractile-endospore-forming bacteria. It encompasses a wide diversity of strains (Gordon, et al., 1973). *Bacillus thuringiensis* is very closely related to *Bacillus cereus* and *Bacillus anthracis* as they share a number of common genotypic and phenotypic characteristics to the extent that they are classified under the same group known as *Bacillus cereus* group (Helgason, et al., 2000; Ibrahim, et al., 2010). There are other members of *Bacillus cereus* group, which are *Bacillus mycoides, Bacillus pseudomycoides* and *Bacillus weihenstephanensis* (Hu, et al., 2004). *Bacillus cereus* is an opportunistic pathogen that causes food poisoning and systemic fatal infections while *Bacillus anthracis* is a causative agent of anthrax characterised by toxaemia and septicaemia (Bhunia, 2008; Helgason, et al., 2000; Ibrahim, et al., 2010). The main unique feature that distinguishes *Bacillus thuringiensis* from the other two *Bacillus* spp. is its formation of proteinaceous crystals during sporulation.

Bacillus thuringiensis can become indistinguishable from *Bacillus cereus* when it loses its ability to form crystal protein due to chemical mutagenesis or plasmid curing (Roh, et al., 2007). The investigation conducted by researchers holding on to the belief that *Bacillus cereus* can be transformed into *Bacillus thuringiensis*-like, had led to the discovery that the parasporal crystal formation of *Bacillus thuringiensis* is conferred by genes located on a plasmid (Berry, et al., 2002; Sanahuja, et al., 2011; Tourasse, et al., 2006). The discovery of the plasmid encoded *cry* genes expressing crystalline inclusion bodies was promptly followed by conjugative transfer of the gene encoding δ-endotoxin from *Bacillus thuringiensis* to other related species (*Bacillus cereus* and *Bacillus anthracis*) and these species have also shown expression of toxin and production of crystal protein during sporulation. The conjugative transfer of *Bacillus cereus* gene to *Bacillus thuringiensis* may also exist, proven by the expression of *Bacillus cereus*-type enterotoxin by some *Bacillus thuringiensis* strains (Hu, et al., 2004; Yuan, et al., 2007).

2.6 Classification of *Bacillus thuringiensis* Strains

Bacillus thuringiensis consists of a wide array of serotypes, biotypes, strains and subspecies each with its particular characteristics. Differences between *Bacillus thuringiensis* strains, especially of their pathogenicity, called for the need to have some reliable modes of classification (De Barjac and Frachon, 1990). The classification and characterisation of *Bacillus thuringiensis* is important especially in helping to evaluate the potential toxicity of different strains against

insect orders, determining the natural role of *Bacillus thuringiensis*, and analysing the distribution of *cry* genes. Of the phenotypic method, flagella (H) serotyping has been widely used and contributed to the establishment of *Bacillus thuringiensis* strains classification. This differentiation of *Bacillus thuringiensis* strains into serological varieties was developed by De Barjac and Bonnefoi (1962), by basing its classification on the bacterial flagella antigen (Lecadet, et al., 1999).

2.6.1 Phenotypic Characterisation

The primary classification of *Bacillus thuringiensis* strains has been accomplished based on serotyping according to their respective flagella (H) antigenic determinants (Al-Momani, et al., 2004; Barjac, 1981; Khyami-Horani, Hajaij and Charles, 2003; Roh, et al., 2007). There are 69 different serotypes and 13 sub-antigenic groups, providing 82 serological varieties among the 3,500 *Bacillus thuringiensis* isolates in the International Entomopathogenic *Bacillus* Centre (IEBC) collection (Lecadet, et al., 1999; Roh, et al., 2007; Xu and Côté, 2008). *Hag* gene of *Bacillus thuringiensis* encodes for flagellin, more specifically through the flagella antigen, which is responsible for inducing the immune reaction required in H serotyping. Each flagellin has its specific amino acid sequence that has correlation to specific H serotypes of *Bacillus thuringiensis* (Ibrahim, et al., 2010; Xu and Côté, 2008). Flagella (H) serotyping is the most commonly used method throughout the world despite its restriction in reflecting only one characteristic of the species (Roh, et al., 2007). However,

the insecticidal activity of *Bacillus thuringiensis* cannot be predicted reliably by using H serotyping (Roh, et al., 2007). Besides, H serotyping is also unable to differentiate *Bacillus thuringiensis* strains from the same serotype or from the same serological variety (Ibrahim, et al., 2010).

Different strains of *Bacillus thuringiensis* have different orders of host insects. This was attributed to the different shapes of parasporal crystals produced by the different *Bacillus thuringiensis* strains. For example, bipyramidal-shaped crystals are toxic against Lepidoptera insects; cuboidal-shaped crystals affect insects of Lepidoptera and Diptera orders; and, rhomboidal-shaped inclusions are toxic to Coleopteran species of insects (Höfte and Whiteley, 1989; Obeidat, Hassawi and Ghabeish, 2004). Therefore, the presence and morphology of crystals can determine the toxicity of the *Bacillus thuringiensis* strains. The sporulating cultures of *Bacillus thuringiensis* strains can be examined under phase contrast microscope before and after cell lysis to identify the presence, and the morphology of crystal proteins produced (Bravo, et al., 1998). Besides that, Coomassie Briliant Blue staining can also be done to confirm the presence of crystals (Lecadet, et al., 1999). Apart from that, SDS-PAGE analysis could be performed to determine the protein profiles of the crystal components from different *Bacillus thuringiensis* strains to aid in their classification.

Furthermore, each serotype possesses different physiological characteristic and can be further subdivided into biotypes according to their specific enzymatic activities, suggesting the importance of biochemical characterisation of *Bacillus*

thuringiensis strains (Barjac, 1981). Moreover, toxicity of each *Bacillus thuringiensis* strain can also be assessed by conducting bioactivity tests that can aid in the understanding of mechanism action of the toxins (Aronson, Beckman and Dunn, 1986).

2.6.2 Genotypic Characterisation

Polymerase Chain Reaction (PCR) is a useful molecular method that has been widely utilised in classifying and characterising collections of insecticidal *Bacillus thuringiensis* strains. In order to help in characterisation of *Bacillus thuringiensis* strains, PCR can be used to identify *cry* genes types and their distribution, predict their insecticidal toxicities, and detect novel *cry* genes. By amplifying the small DNA fragment, PCR determines the presence of a specific target gene of *Bacillus thuringiensis* samples (Ben-Dov, et al., 2001; Ceron, et al., 1994; Porcar and Juárez-Pérez, 2003). For further characterisation of strains, different PCR-based methods have been developed such as PCR-RFLP, RT-PCR and multiplex PCR.

According to Ben-Dov, et al. (1997), *Bacillus thuringiensis* strains containing *cry* genes that are entomopathogenic can be identified and classified rapidly using an extended multiplex PCR method. Toxicity assays are greatly facilitated by the establishment of this method as it simplifies the initial steps of large-scale screening of *cry* genes. Besides, for the classification of strains and prediction of insecticidal activities, PCR requires only small quantity of DNA yet it allows

rapid, highly sensitive, and simultaneous screening of many *Bacillus thuringiensis* samples (Ben-Dov, et al., 2001).

Apart from that, plasmid profiling and chromosomal DNA profiling are also involved in the genotyping of different strains of *Bacillus thuringiensis*. As speculated by González, Dulmage and Carlton (1981), *Bacillus thuringiensis* strains with the same plasmid profiles are categorised under the same subspecies based on the finding that there is only a single plasmid involved in each strain, and the plasmid can vary in size from one strain to another (González, Brown and Carlton, 1982).

Another approach that allows accurate classification of these closely related bacteria is the pulsed field gel electrophoresis (PFGE) of the bacterial chromosomal DNA. Researchers found correlation between the PFGE types of *Bacillus thuringiensis* strains and *cry* gene composition, making the PFGE method accepted as a classification system which is consistent with toxicity testing, enabling the prediction of insects target range from the strain typing. One of the example reported was the discovery of *cry1A* gene in *Bacillus thuringiensis kurstaki* that was found to be correlated to the toxicity properties of the bacteria against Lepidopteran (Gaviria Rivera and Priest, 2003). An appropriate restriction enzyme is usually involved in this approach to digest the chromosomal DNA after recognising the specific sites on the DNA (Apaydin, et al., 2005; Gaviria Rivera and Priest, 2003; Lövgren, et al., 1998).

2.7 The Parasporal Crystalline Inclusions

The proteinaceous parasporal crystalline inclusions are produced by *Bacillus thuringiensis* upon sporulation. The insecticidal toxins, which are the major components of the parasporal crystal, are often times referred to as δ-endotoxins by Heimpel (1967). The name "δ-endotoxin" was given by Heimpel to represent the parasporal crystalline protein of *Bacillus thuringiensis* because of its fourth order in the discovery of toxic components of the bacterium and its formation inside the cell (Ibrahim, et al., 2010). *Bacillus thuringiensis* strains produce two types of δ-endotoxin, which are Cry (crystal) toxins and Cyt (cytolytic) toxins, with different mechanisms of action. Cry toxins bind to specific receptors on the insect gut epithelia when activated by protease, and form spore oligomeric structures that are insertion competent into the gut membrane (Knowles, 1994; Naimov, et al., 2001; Van Rie, et al., 1990). On the contrary, Cyt proteins insert into the membrane after its direct interaction with the membrane lipid (Sanahuja, et al., 2011). Cyt toxins facilitate the insecticidal activity of *Bacillus thuringiensis* by augmenting Cry toxins' effect. The δ-endotoxin is the virulence factor that can completely distinguish *Bacillus thuringiensis* from its genetic cousins, *Bacillus anthracis* and *Bacillus cereus*.

2.7.1 The Cry Proteins

Cry toxins are the parasporal inclusion proteins of *Bacillus thuringiensis* that exhibit insecticidal activity on host organisms. They have genetic sequences that are significantly similar to existing toxins within the nomenclature (Knowles,

1994; Shishir, 2016). The *cry* gene family is the family of *Bacillus thuringiensis* genes that encode these toxic proteins and the expression occurs only during stationary phase. The synthesis of Cry proteins appeared to be coordinately and tightly regulated by various mechanisms occurred during transcriptional, post-transcriptional and post-translational events. The *cry* genes of *Bacillus thuringiensis* are mostly expressed upon sporulation, while the sporulation process is typically initiated at the transcription level, by the successive activation of sigma factors following their binding to the core RNA polymerase and subsequently the transcription of sporulation-specific gene promoters is directed (Deng, et al., 2014; Ibrahim, et al., 2010; Lin, et al., 1998).

The parasporal crystals of *Bacillus thuringiensis* are originally composed of subunits known as protoxins that are non-toxic in nature. Protoxin, an immediate atoxic precursor, is generated into an insecticidal Cry toxin upon activation (Ibrahim, et al., 2010; Naimov, et al., 2001). The toxic moiety of the Cry protein is located on the N-terminal half of the protein (Hofmann, et al., 1988). The C-terminal portion of Cry toxin is involved in crystal formation and because it is cleaved off inside the insect midgut, it is not considered as part of the mature toxin; whereas the N-terminal portion, as mentioned above, is the toxin itself (Naimov, et al., 2001; Sanahuja, et al., 2011). All the Cry toxins possess the three structural domains: Domain I, Domain II and Domain III, and share a highly similar topology. Domain I functions in the membrane insertion and lytic pores formation in the insects' intestinal epithelium (Bravo, Gill and Soberon, 2007; Sanahuja, et al., 2011; Schnepf, et al., 1998). On the other hand, Domain

II and III are both responsible for receptor binding (Bravo, Gill and Soberon, 2007; Ibrahim, et al., 2010; Sanahuja, et al., 2011).

Cry proteins were classified into four major classes: Cry1, Cry2, Cry3 and Cry4 that are toxic to insects of orders Lepidopteran, Lepidopteran and Dipteran, Coleopteran, and Dipteran respectively (Höfte and Whiteley, 1989). The shape of the Cry toxin produced determines their target host insects. According to Höfte and Whiteley (1989), the criteria used to classify the Cry proteins are their insecticidal spectra and amino acid sequences.

2.7.2 Mode of Action of Cry Proteins

The Cry proteins need to be activated in the insect midgut through the alkaline condition and the presence of specific proteases to initiate their toxicity (Bravo, Gill and Soberon, 2007; Knowles, 1994; Sanahuja, et al., 2011). Therefore, the Cry toxins must first be consumed by the susceptible insect and subsequently solubilised in the alkaline midgut of insect. The differences in the toxicity degree caused by various Cry toxins in insect host can be explained by their differences in the extent of solubilisation (Schnepf, et al., 1998). After that, the protoxins are processed by the specific proteases, that transform the innocuous protoxin into its active form by cleaving off the carboxyl-terminal end (Sanahuja, et al., 2011; Schnepf, et al., 1998).

Activated Cry toxins readily interact with specific receptors located on the brush border of insect midgut (Pardo-Lopez, Soberon and Bravo, 2012; Schnepf, et al., 1998). After receptor binding, Cry toxins are inserted into the apical membrane of the gut epithelial cells of the susceptible insect. This membrane insertion is then followed by induction of ion channels or lytic pores formation in the membrane (De Maagd, Bravo and Crickmore, 2001; Pardo-Lopez, Soberon and Bravo, 2012; Schnepf, et al., 1998). The pores formed cause the disruption to the movement of solutes across the gut epithelial cells and lead to influx of water (Pardo-Lopez, Soberon and Bravo, 2012). This osmotic shock causes lysis of cell membrane and paralysis, eventually leading to death of the organism (De Maagd, Bravo and Crickmore, 2001).

2.7.3 The Cyt proteins

Besides Cry toxin, there is another parasporal inclusion protein known as Cyt toxin that is also synthesised by some *Bacillus thuringiensis* strains. Cyt toxins represent an interesting group of *Bacillus thuringiensis* proteins that can affect the midgut cells of insects and increase insecticidal effect of certain Cry toxins due to their ability to overcome the resistance to Cry toxins in mosquitoes. An example reported by Federici and Bauer (1998) and Pardo-Lopez, Soberon and Bravo (2012) was the Cyt1Aa that was able to suppress resistance of *Chrysomela scripta*, cottonwood leaf beetle towards Cry3Aa protein. Besides, Cyt toxins act synergistically with Cry toxins by acting as Cry-membrane bound receptors (Gómez, et al., 2007; Shishir, 2016; Soberón, López-Díaz and Bravo, 2013).

ELISA, ligand blot and co-immunoprecipitation assays have demonstrated the interactions between Cyt1A and Cry11Aa toxins in which Cyt1A functions as a membrane bound receptor for Cry11Aa to enhance the membrane insertion of Cry11Aa, synergising Cry11Aa toxicity (Soberón, et al., 2007).

Cyt toxins are mostly found in *Bacillus thuringiensis* strains that are active against Dipteran order of insects, whereas Cry toxins are present in many strains with wide host range (Bravo, Gill, and Soberon, 2007). Besides, only one Cyt toxin will be found in a given *Bacillus thuringiensis* strain. Cyt toxins are encoded by two gene families that are highly related, which are Cyt1 and Cyt2 identified on the basis of the amino acid identity. There are 37 subclasses of Cyt toxins, and according to Koni and Ellar (1994), Cyt1Aa and Cyt2Aa exhibit the highest mosquitocidal activity. Cyt1A and Cyt2A consist of a single α-β domain in which two outer layers of alpha helix enfolded around a mixed beta sheet (Schnepf, et al., 1998). The α-helixes possess amphiphilic property while the hydrophobic residues packed against the β-sheet.

According to Li, Koni and Ellar (1996), Cyt toxins are also synthesised as protoxins and in order to be activated, a small portion of the C-terminal and N-terminal ends are removed. Similar to Cry toxins, activation of Cyt toxins require midgut protease in the susceptible insect. Activated Cyt toxins interact with non-saturated membrane lipids and then induce the cation-selective channels formation in the membrane vesicles causing colloid-osmotic lysis of the epithelial cell. Alternatively, the activated Cyt toxins can also exhibit detergent

effect by aggregating non-specifically on the surface of the lipid bilayer that subsequently disassembles the cell membrane and causes cell death (Bravo, Gill and Soberon, 2007; Gómez, et al., 2007; Soberón, López-Díaz and Bravo, 2013).

2.8 The Chitinase

According to Hu, et al. (2009), the expression of chitinase by *Bacillus thuringiensis* is very low and needs to be induced by chitin, leading to its limited field application despite its effectiveness in enhancing the insecticidal activities of *Bacillus thuringiensis* Cry1Ac protein. In 2004, Reyes□Ramírez and colleagues demonstrated the production of chitinase by *Bacillus thuringiensis israelensis* using shrimp water as the only carbon source, with the production reaching maximum level at 120 hours of fermentation. The chitinase enzyme was then concentrated by ultrafiltration and adjusted to pH 5.8 (Reyes□Ramírez, et al., 2004).

The chitinase exhibits it action primarily on the insect larval peritrophic membrane, which is a cylindrical sheet dividing the gut epithelium from the lumen. The peritrophic membrane comprises of a chitin fibril network (Pardo-López, et al., 2009). The mechanical damage and microorganism invasion are restricted by this physiochemical barrier (Kramer and Muthukrishnan, 1997). The solubilised Cry toxins needs to penetrate this membrane in order to interact with their receptors on the apical membrane of the midgut epithelium. Chitinase degrades the chitin to low molecular weight oligosaccharides, resulting in

perforation of peritrophic membrane and increasing the accessibility of insecticidal crystal proteins to their receptors, which enhance the larvicidal effect of *Bacillus thuringiensis* (Hu, et al., 2009; Pardo-López, et al., 2009).

However, despite the potential role of chitinase in enhancing Cry toxin activity, the synergistic effect of *Bacillus thuringiensis* chitinases and the Cry toxins has not been quantitatively demonstrated, which is most possibly be due to the low expression level of chitinase. This is why the molecular cloning of *Bacillus thuringiensis chi* gene has become increasingly important. One useful example could be the successful cloning of one chitinase gene from *Bacillus thuringiensis Pakistani* (Barboza-Corona, et al., 2003).

2.9 Non-insecticidal Parasporin

One considerable issue that arises is the presence or absence of any biological activity of Cry proteins produced by non-insecticidal *Bacillus thuringiensis*. This issue has subsequently lead to the extensive screening of *Bacillus thuringiensis* Cry proteins to investigate their potential for any other biological activity other than insecticidal toxicity, which led to the discovery of parasporins that target human cancer cells of various origins (Mizuki, et al., 1999; Ohba, Mizuki and Uemori, 2009; Palma, et al., 2014). The parasporin proteins were heterogeneous in the spectra of cytotoxicity (Mizuki, et al., 2000).

As reported by Ohba, Mizuki and Uemori (2009), the first attempt on the screening of *Bacillus thuringiensis* strains to discover parasporal inclusion bodies that are cytotoxic to human cancer cells was done by Mizuki, et al. in year 1999. About 1744 *Bacillus thuringiensis* strains were used which consisted of 1700 Japanese isolates from Kyushu University and 44 reference type strains of then-existing *Bacillus thuringiensis* H serovars from the Institute Pasteur, Paris were examined. Among these, 60 strains have their parasporal proteins exhibiting strong haemolytic activity when screened with sheep erythrocytes, which was contributed by the *Bacillus thuringiensis* parasporal cytolysins, Cyt proteins (Mizuki, et al., 1999). The result of 42 positive strains was obtained when the remaining 1684 strains were investigated for *in vitro* cytotoxicity against MOLT-4 cells, which are human leukaemic T cells. Three of the 42 strains were selected to test on MOLT-4, human lung cancer cells (A549) and human uterus cervix cancer cells (HeLa) and their parasporal proteins exhibited strong cytocidal activities. Interestingly, two strains were able to distinguish leukaemic and normal T cells; preferentially killing leukaemic T cells (Mizuki, et al., 1999; Ohba, Mizuki and Uemori, 2009). An anticancer Cry protein was then isolated, and became the new category of *Bacillus thuringiensis* parasporal protein, named parasporin, with discriminate cancer cells killing capacity (Mizuki, et al., 2000).

Parasporins, proteins with molecular weight lower than other Cry toxins are classified into four families: parasporin-1 (PS1), parasporin-2 (PS2), parasporin-3 (PS3) and parasporin-4 (PS4). This type of parasporal proteins affect only altered cells of certain types of cancer and cause no damage to normal cells

(Melo, Soccol and Soccol, 2016; Ohba, Mizuki and Uemori, 2009). Parasporins require activation before exhibiting cytotoxic damage, which can be done enzymatically by treating it with proteinase K and trypsin in an alkaline environment (Melo, Soccol and Soccol, 2016).

As reported by Katayama, et al. (2005), neither the penetration of propidium iodide nor the release of lactate dehydrogenase occurs in PS1Aa1-treated HeLa cells, and the membrane potential in intoxicated cells is not altered. Thus, Katayama, et al. (2005) claimed that PS1Aa1 is not a membrane pore-forming cytotoxic protein. Instead, the overall reduction in the level of DNA synthesis and cellular proteins is the important physiological feature in PS1Aa1-treated HeLa cells. Within 1-3 minutes after treatment with PS1Aa1, HeLa cells undergo early and rapid increase in the concentration of intracellular calcium ions (Ca^{2+}) without any alteration in plasma membrane permeability. PS1Aa1 kills HeLa cells by inducing apoptosis (Ohba, Mizuki and Uemori, 2009).

On the other hand, the second anti-cancer Cry protein, Parasporin-2Aa1 which exhibits extremely high cytocidal activity against HepG2 cells (liver cancer cells) possesses cancer cell-killing action by increasing plasma membrane permeability of cancer cells (Akiba, et al., 2009; Kitada, et al., 2006). PS2Aa1 specifically binds to a putative receptor located in the lipid raft of cancer cells plasma membrane, followed by PS2Aa1 oligomers formation in plasma membranes, leading to pore formation and cell lysis (Akiba, et al., 2009; Ohba, Mizuki and Uemori, 2009). The results of PS2Aa1's killing action are the

leakage of the lactate hydrogenase from the cytoplasm of intoxicated HepG2 cells and the entering of extracellular propidium iodide into the cytoplasm (Katayama, et al., 2005; Ohba, Mizuki and Uemori, 2009). Similar to PS2Aa1, PS3Aa1 also increases plasma membrane permeability of cancer cells and leads to pore formation, as mentioned by Ohba, Mizuki and Uemori (2009).

2.10 The Application of *Bacillus thuringiensis*

According to Bauce, et al. (2004) and James, Miller and Lighthart (1993), *Bacillus thuringiensis kurstaki* was used to control the epidemic of spruce budworm, *Choristoneura fumiferana*, the most prominent Lepidoptera defoliator of coniferous forests in eastern North America. Large scale aerial spraying of chemical pesticides were conducted in Canada and north-eastern United States to protect plants against spruce budworm epidemic, however, the environmental issues and plant resistance to chemical pesticides had led to the discovery and application of *Bacillus thuringiensis kurstaki* as alternative insect control agent. *Bacillus thuringiensis kurstaki*-isolate HD1 has high specificity toward Lepidoptera larvae, and its formulations and application were improved by laboratory research and field trials carried out in Canada and the United States (Bauce, et al., 2004; Van Frankenhuyzen, 1990).

As reported by Becker and Ludwig (1993), *Bacillus thuringiensis israelensis* had been used in controlling the floodwater mosquitoes in the Upper Rhine Valley in Germany for more than ten years. *Bacillus thuringiensis israelensis* – isolate

H-14 was first discovered in year 1977. It is extremely toxic to mosquitoes and black flies larvae, which are vectors of tropical diseases: malaria, onchocerciasis and dengue fever. *Bacillus thuringiensis* commercial products such as Vectobac®, Teknar®, Bactimos® and Skeetal® are utilised to kill mosquitoes and black flies (Sanchis and Bourguet, 2008). In the Onchocerciasis Control Programme (OCP), *Bacillus thuringiensis israelensis*, with high specificity and toxicity against Dipteran larvae, was used extensively in year 1980 in the rivers of West Africa to combat black flies transmitting *Onchocerca volvulus*, a microfilarial parasite that lead to onchocerciasis (Becker, 2000). Progressive amount of *Bacillus thuringiensis israelensis* was applied annually to control black flies larvae epidemic in West Africa due to the increasing resistance developed towards chemical pesticides, subsequently leading to great reduction in the occurrence of the river blindness among people protected by OCP (Becker, 2000; Fillinger, Knols and Becker, 2003).

On the other hand, maize and cotton hybrids of *Bacillus thuringiensis* transgenic crops were planted on 14 million hectares of farm lands in year 1999 (Cannon, 2000). Insecticidal potential of *Bacillus thuringiensis* was fully utilised to produce genetically modified (transgenic) plants that expressed δ-endotoxin genes. In year 1987, transformed tobacco plants that expressed *Bt cry1Ab* gene, whose protein product is resistant to the European corn borer, a pest of maize in the United States and Europe were produced. The transfer of *Bt cry* genes into more plants such as maize was made possible by the invention of electroporation or particle bombardment method of plant transformation (Sanchis and Bourguet, 2008). As reported by Perlak, et al. (1993), Monsanto was the first to develop

and sell *Bt* potatoes in the United States, significantly reducing the use of pesticides and saving costs for the growers. The large scale cultivation of *Bt* maize and *Bt* cotton occurred throughout the world (Marvier, et al., 2007; Sanchis and Bourguet, 2008).

A new crystal protein found in non-insecticidal and non-haemolytic *Bacillus thuringiensis*, known as parasporin protein has the ability to recognize and kill human cancer cells (Ito, et al., 2004; Ohba, Mizuki and Uemori, 2009). These parasporin proteins exhibit cytotoxicity against human leukaemic T cells, MOLT-4. As stated by Kitada, et al. (2006), parasporin-2 gene varies in its cytocidal activities against various human cells due to its target specificity. It was highly toxic against HepG2 cells (human hepatocyte cancer cells) while less toxic to HC cells (normal liver cells). Thus the supplication of non-insecticidal *Bacillus thuringiensis* producing parasporin-2 can aid in the treatment of human cancer cells without harming normal body cells (Akiba, et al., 2009; Kitada, et al., 2006).

Bacillus thuringiensis, however, possesses certain limitations in its application despite its usefulness in insecticidal effect and killing cancer cells. *Bacillus thuringiensis* genetically engineered crops was greatly reduced with the evolving level of pest resistance towards the insecticidal action of *Bacillus thuringiensis* (Lee, et al., 2003). Therefore, attention is focused on discovering new family of δ-endotoxins with different insecticidal action, to ensure the resistance of insects towards the δ-endotoxins' action can be reduced (Sanahuja, et al., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 The General Overview of Experimental Design

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

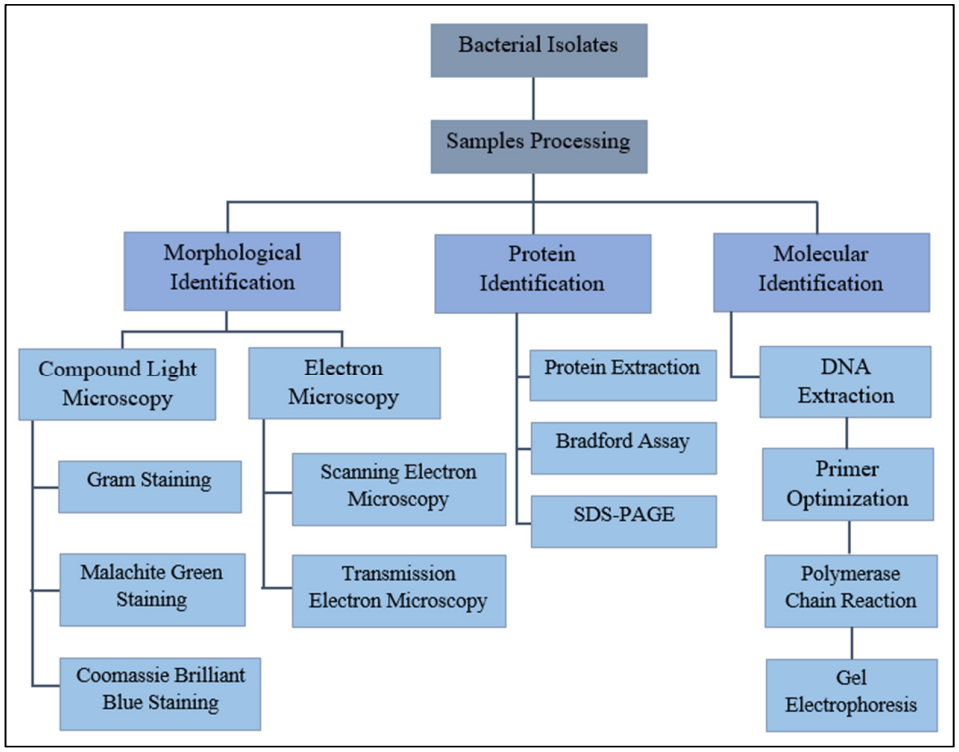

Figure 3.1: The general overview of experimental design.

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 and B in accordance to their respective manufacturers.

3.3 Bacterial Samples

The seven bacterial samples A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 which were previously isolated by seniors were used in this study. Two positive reference strains *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis,* and the negative controls *Escherichia coli* and *Bacillus cereus* were used in the project.

3.3.1 Maintaining Bacterial Samples

The bacterial samples and controls were cultured on nutrient agar/broth. The cultures on nutrient agar were incubated at 30 ºC, whereas the cultures in liquid media were incubated at 30 ºC with constant agitation at 200 rpm. The bacterial cultures were kept in the refrigerator for short-term storage and use.

3.3.2 Glycerol Stock Preparation

Glycerol stock preparation was done for the long term storage of bacterial cultures. The bacterial samples from liquid media cultures were transferred into cryovials to the final glycerol concentrations of 40% and 60% respectively. The glycerol stock was topped up to the final volume after transferring the required volume of liquid media culture into the cryovial as the exact amount of glycerol was almost impossible to be pipetted due to its high viscosity.

3.4 Morphological Identification of Bacterial Isolates

The bacterial samples, positive reference strains and negative controls were streaked on nutrient agar plates and incubated overnight at 30 °C. A white colony with flat to slightly raised elevation and smooth edges were observed on cultures with *Bacillus thuringiensis* positive colonies.

3.4.1 Gram Staining

The isolated bacterial samples were identified as Gram-positive, rod-shaped bacilli by using Gram staining, as shown in Figure 3.2. One drop of 0.85% saline solution was placed on a microscope slide and then a small loopful of bacteria colony was smeared and spread evenly onto the saline solution. The smear on the microscope slide was then subjected to air-drying and heat-fixing using a Bunsen burner. The smear was then flooded with crystal violet dye as primary

staining for 1 minute, followed by washing with running tap water. After that, Gram iodine mordant was used to stain the smear for another 1 minute, and washed with running tap water. The smear was then decolourised with 75% ethanol for 10 seconds, and washed with water again. Lastly, safranin was used as a counterstain to stain the smear for 1 minute, and washed with running tap water. The stained smear was left to air dry. After that, it was observed under a compound light microscope with magnification of 1000X with oil immersion. The procedures were repeated for all the isolates together with positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis* while the negative control was *Escherichia coli.*

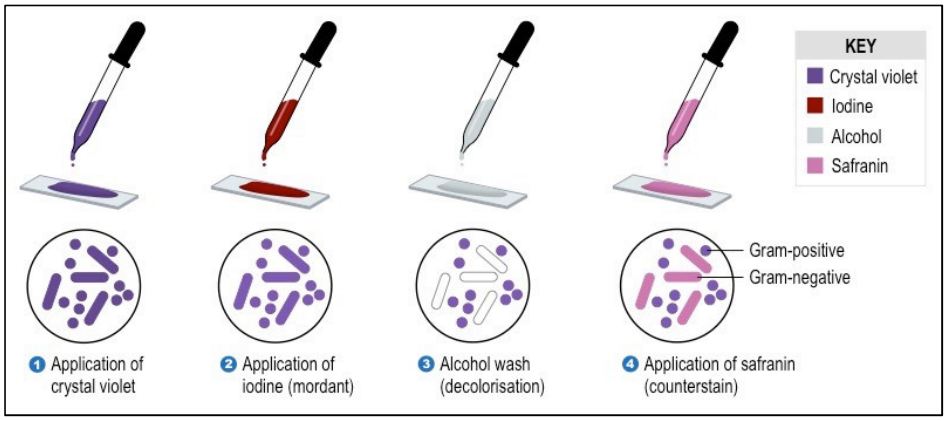

Figure 3.2: The general procedure of Gram staining (Adapted from Tortora, Funke and Case, 2013).

3.4.2 Malachite Green Staining

Malachite green staining was performed to identify the presence of endospores (Figure 3.3). A drop of 0.85% saline solution was placed onto a microscope slide

and a small loopful of bacterial sample cultured for 90 hours was smeared evenly onto it. The smear was then subjected to air-drying and heat-fixing. A filter paper was used to cover the fixed smear and a few drops of malachite green stain was transferred onto the filter paper. After that, the smear was steamed on top of a beaker containing boiling water for 5 minutes. The stained smear was then washed with running tap water and counterstained with safranin for 30 seconds. The smear was again washed with running tap water and left to air dry. A compound light microscope was used to observe the slide with a magnification of 1000X (oil immersion). The procedures were repeated for all the isolates together with positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis* while the negative control was *Escherichia coli.*

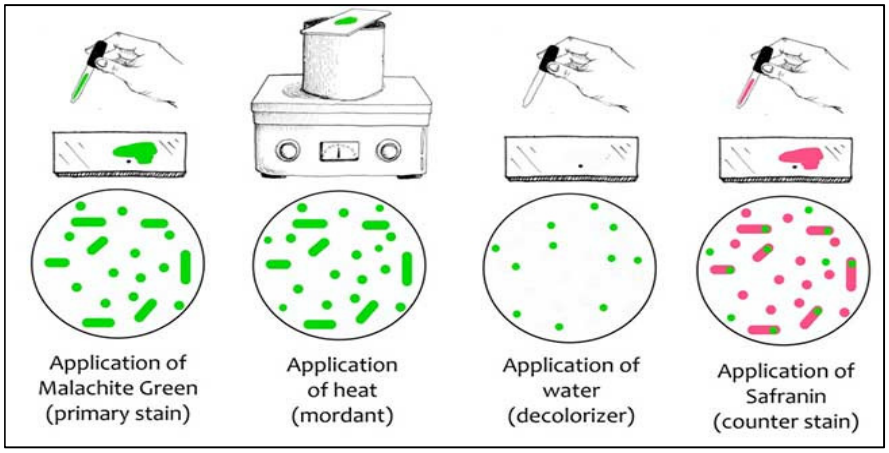

Figure 3.3: The general procedure of endospore staining (Adapted from Aryal, 2015).

3.4.3 Coomassie Brilliant Blue (CBB) Staining

Coomassie Brilliant Blue (CBB) staining was carried out to determine the presence of crystal proteins. After incubation at 30 °C for 110 hours, a loopful of the bacterial sample was smeared onto a drop of 0.85% saline solution on a microscope slide. The smear was then subjected to air-drying and heat-fixing. After that, the smear was stained with 0.133% Coomassie Brilliant Blue (CBB) stain for 1 minute and destained with distilled water for 10 seconds. The smear was then left to air dry before being observed under a compound light microscope with a magnification of 1000X with oil immersion. The procedures were repeated for all the isolates together with positive and negative controls. The positive controls were *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis* while the negative control was *Bacillus cereus.*

3.4.4 Scanning Electron Microscopy (SEM)

A single colony of bacteria was inoculated into a 250 mL conical flask containing 50 mL of sterile nutrient broth. The bacteria were incubated at 30 ºC in an incubator shaker set at 200 rpm for 110 hours until autolysis phase. The presence of spores and crystal proteins was confirmed by Coomassie Briliant Blue (CBB) staining. After that, the bacterial cultures were transferred into a microcentrifuge tube and repetitive centrifugation at 6,000 rpm for 1 minute was conducted to harvest the required amount of pellets. The microcentrifuge tubes were tipped to release the bacterial pellets every time before starting a new cycle of spinning so that the cells will not break during the next cycle of centrifugation.

After the samples collection, the pellets were then fixed in 2.5% glutaraldehyde in Phosphate Buffered Saline (PBS) overnight prior to sample processing.

The next day, the fixed samples which were resuspended in glutaraldehyde were centrifuged at 1,500 *g* for 10 minutes. After that, the supernatant was discarded and the pellets were resuspended in 0.1 M PBS. This phosphate buffer washing step was performed thrice. Subsequently, the samples were washed with distilled water thrice following the same procedures as the buffer washing step. After that, serial dehydration process was conducted as follows:

i. 25% ethanol, 5 minutes
ii. 75% ethanol, 5 minutes
iii. 95% ethanol, 5 minutes
iv. 100% absolute ethanol, 5 minutes (3 changes)

After decanting the supernatant, the samples were kept in a freeze drier overnight. Prior to viewing using the scanning electron microscope (SEM), the dried cells were mounted onto an SEM specimen copper stub with a carbon tape. Lastly, the samples were coated with platinum, followed by placement of the copper stub onto a specimen holder, before being viewed under 10,000X to 20,000X magnification using the SEM.

3.4.5 Transmission Electron Microscopy (TEM)

A single colony of bacteria was inoculated into a 250 mL conical flask containing 50 mL of sterile nutrient broth. The bacteria were incubated at 30 ºC in an incubator shaker set at 200 rpm for 90 hours until sporulation phase. The presence of spores and crystal proteins was confirmed by Coomassie Briliant Blue (CBB) staining. The sporulated cultures were harvested by repeated centrifugation at 6,000 rpm for 1 minute to obtain the required amount of pellets. After discarding the supernatant, the pellets were resuspended in 2.5% glutaraldehyde in 0.1 M PBS for fixation overnight.

The samples suspended in glutaraldehyde were centrifuged at 1,500 *g* for 2 minutes. After discarding the supernatant, the pellets were resuspended in 0.1 M phosphate buffer, followed by subsequent centrifugation under the same conditions. This buffer washing step was performed twice. The subsequent steps were done in the fume hood. After discarding the supernatant, the pellets were post-fixed with 1 % Osmium tetroxide for 1 hour. After that, the pellets were washed with distilled water thrice. After post-fixation washing steps, the sample was centrifuged to obtain the pellet. Supernatant was discarded and the microcentrifuge tube containing pellets of fixed cells was placed in a water bath set at 45ºC for 15 minutes. At the same time, 2.00 % agar solution was prepared by dissolving agar in boiling distilled water. The molten agar solution was poured into a test tube which was then transferred to the 45 ºC water bath.

After the temperature of both the agar and the pellets were equilibrated to 45 ºC, a small drop of the agar was transferred to the tube containing pellets. The pellets were stirred until they broke into small pieces and suspended in the molten agar, then the mixture was immediately poured onto a glass microscope slide and allowed to set for 1-2 minutes. The solidified agar containing the cells was cut into 1.0 mm^3 small cubes by using a sharp razor blade. These small cubes were placed in a vial and serial dehydration process was carried out as following: 75% ethanol for 15 minutes, 95% ethanol for 15 minutes twice, 100% ethanol for 30 minutes twice, and finally 100% acetone for 10 minutes twice. After the serial dehydration steps, the cubes were infiltrated overnight in a rotator for three consecutive days with the following mixtures:

i. 1:1 acetone: Spurr's resin mixture (first day)
ii. 100% Spurr's resin mixture (second day)
iii. An exchange of fresh 100% Spurr's resin mixture (third day)

The sample cubes were embedded in 100% Spurr's resin mixture on the fourth day and were cured at 60 ºC for 48 hours.

A razor blade was used to trim the specimen block and to shape the resin into a trapezoid block with sloping sides. The finely-trimmed specimen block was then polished at 0º with a glass knife. Semi thin (1 µm) sectioning was performed with a new glass knife and semi thin sections with interference colours of blue, green, yellow, purple or colourless were collected onto a knife-boat filled with water. The sections were checked under the compound light microscope at 400X magnification after staining with Toluidine Blue to ensure there is at least 80%

of bacterial sample present in every section. After confirming the sections contained the bacterial cells, ultra-thin sectioning (<0.1 µm) was performed and the section with gold or silver interference colours were collected on the dull side of the copper grids. The grids were then incubated in an oven at 60 ºC for 15 minutes to ensure the sections were properly attached onto the grids. The sections were then stained with 2.0% uranyl acetate and lead citrate and allowed to air dry for 30 minutes. After that, the samples were viewed under 10,000X magnification using the transmission electron microscope (TEM).

3.5 Protein Identification

The total proteins of the bacterial samples were examined by performing protein extraction, Bradford assay and lastly, sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) for proteins separation and characterisation.

3.5.1 Protein Extraction

The ProteoSpin™ Detergent Free Total Protein Isolation Kit by Norgen Biotek Corporation was used for the protein extraction step. The cell pellets which were prepared and resuspended in 50 µL of lysis solution were transferred to a lysis tube. Firstly, the lysis tube was capped and vortexed vigorously for 2 minutes, then centrifuged at 14,000 *g* for 2 minutes. A filter column was placed into an

elution tube, and the liquid content in the lysis tube was then transferred into the filter column. The elution tube together with the filter column containing sample was centrifuged at 14,000 *g* for 1 minute to ensure that the entire sample has passed through into the elution tube. The extracted protein was then kept at -20 ºC.

3.5.2 Bradford Assay

The concentrations of the extracted protein samples were measured using the Bradford assay. The 2.0 mg/mL stock concentration of Bovine Serum Albumin (BSA) was diluted to 1.0 mg/mL using distilled water. Following that, a serial dilution was carried out and a standard linear graph was generated by using the BSA concentrations of 0.00 mg/mL, 0.10 mg/mL, 0.20 mg/mL, 0.30 mg/mL, 0.40 mg/mL, 0.50 mg/mL, 0.60 mg/mL and 0.70 mg/mL. The 5X Bradford reagent was diluted to 1X before use. After that, 5 µL of each BSA concentrations and protein samples were pipetted into a 96-well plate, followed by addition of 100 µL of 1X Bradford reagent to each well. The absorbance readings were obtained at 595 nm wavelength using a microplate reader. Triplicates were performed and the standard linear graph was plotted using the average absorbance values of the standard BSA concentrations. Based on the linear equation obtained, the concentrations of the extracted protein samples were calculated. The R^2 value of the standard curve was in between 0.95-1.0.

3.5.3 Sodium Dodecyl Sulphate - Polyarylamide Gel Electrophoresis (SDS PAGE) Analysis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by following the procedures adapted from Laemmli (1970) with modification. 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. In order to check for leakages, 2 mL of 70% ethanol were loaded into the glass plates then subsequently removed by using filter paper. After that, 10% separating gel was prepared according to the components and volumes tabulated in Table 3.1.

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 10% separating gel was loaded into the 0.75 mm glass plates and 70% ethanol was then slowly pipetted on top of the separating gel to remove air bubbles. The gel was left to solidify for 45 minutes followed by the removal of 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 10% separating gel, followed by the careful insertion of a 10-well comb into the 4% stacking gel. The gel was then left to solidify. It was crucial to ensure that no bubbles were trapped underneath the teeth upon the insertion of comb. After the gel has fully solidified, the casting frame with the glass plates were removed from the casting stand and placed into

the gel tank. The 1X running buffer was then poured into the gel tank and the comb was carefully removed from the gel. A 6X sample loading buffer was prepared according to the components and amounts tabulated in Table 3.3.

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

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

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

the gel. The 1X running buffer was poured to top up the inner chamber until it overflowed into the outer chamber and reached the required level in the gel tank.

The gel tank was connected to a power supply and the gel was electrophoresed at 80 V for 90 minutes. After that, the gel was stained with Coomassie Brilliant Blue staining for 10 minutes and destained with distilled water for 1 day. The stained gel was visualised under UV transilluminator using ChemiDoc^{TM}XRS system (Bio-Rad).

3.6 DNA Extraction

Bacterial samples were cultured in 100 mL nutrient broth in 250 mL conical flasks. The bacterial cultures were incubated overnight at 30ºC with a constant agitation at 200 rpm. The DNA of the isolated bacterial samples were extracted using commercial DNA extraction kit (Presto^{TM} Mini gDNA Bacteria Kit) following the manufacturer (Geneaid)'s protocols with slight modification.

A volume of 3 mL of the overnight bacterial culture was transferred and centrifuged at 14,000 rpm for 1 minute in Eppendorf tube. There are five major steps in the protocol of DNA extraction kit, which are sample preparation, lysis, DNA binding, wash and elution. The duration of elution buffer stand in the matrix was extended to about 1 hour instead of 3 minutes, and the elution step was repeated twice. Approximately 100 µL of pre-heated elution buffer was

allowed to stand in the matrix for 30 minutes, the column was then subjected to centrifugation at 14,000 rpm for 1 minute to collect the eluted DNA and elution buffer. The collected elution buffer was pipetted back into the center of matrix, allowed to stand for another 30 minutes and then centrifuged again. After that, the DNA concentration (µg/mL) and purity (A_{260}/A_{280}) were measured using a nanospectrophotometer. The extracted DNA was then kept at -20 °C for future use.

3.7 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed to amplify the extracted DNA with different sets of primers to identify the presence of the target genes. PCR was performed using thermocycler, with the primers as listed in Table 3.4, and each set of primers consists of a forward and a reverse primer. There are ten sets of primers which amplified ten different genes (*cry1, cry2, cry4, cry10, cry11, cyt2, chi, ps1, ps2* and *ps4* genes) that encode for different proteins.

Table 3.4: PCR primers used for endotoxin gene amplification.

Primers	Gene recognised	Forward (f) and Reverse (r) Primer Sequences	Amplicon Size (bp)	References
Un1	*cry1*	(f) 5'-CAT GAT TCA TGC GGC AGA TAA AC-3' (r) 5'-TGT GAC ACT TCT GCT TCC CAT T-3'	277	Halima, et al., 2006
Cry2gral	*cry2*	(f) 5'-GAG TTT AAT CGA CAA GTA GAT AAT TT-3' (r) 5'-GGA AAA GAG AAT ATA AAA ATG GCC AG-3'	500-526	Ibarra, et al., 2003
Cry4	*cry4*	(f) 5'-AAA TTG ATG GTA CTC TTG CCT CTT-3' (r) 5'-TGC GTA ATC CGT AAC TTC TTG TAG-3'	423	Ejiofor and Johnson, 2002
Cry10spe	*cry10*	(f) 5'-TCA ATG CTC CAT CCA ATG-3' (r) 5'-CTT GTA TAG GCC TTC CTC CG-3'	348	Mahalakshmi, et al., 2012
Cry11	*cry11*	(f) 5'-TTT GCA CCA GAT AAT ACT AAG GAC-3' (r) 5'-AAC AAC TGC GAT AAA TAC CAC TCT-3'	485	Ejiofor and Johnson, 2002
cyt2 (UN)	*cyt2*	(f) 5'-ATT ACA AAT TGC AAA TGG TAT TCC-3' (r) 5'-TTT CAA CAT CCA CAG TAA TTT CAA ATG C-3'	355-356	Jouzani, et al., 2008

Table 3.4: PCR primers used for endotoxin gene amplification (continued).

Primers	Gene recognised	Forward (f) and Reverse (r) Primer Sequences	Amplicon Size (bp)	References
chi	*chi*	(f) 5'-ATG GTC ATG AGG TCT C-3' (r) 5'-CTA TTT CGC TAA TGA CG-3'	2027	El-Kersh, et al., 2016
ps1	*ps1*	(f) 5'-TAC AAG CAG GGC GTC CAG-3' (r) 5'-TCT GCT GGA ATT TGC AAT GCT-3'	737	Espino-Vazquez, et al., 2012
ps2	*ps2*	(f) 5'-TGT TGG GAC TGT TCA GTA CG-3' (r) 5'-GTA GTA GAG AAT GAA ACT TCT CCA CC-3'	237	Espino-Vazquez, et al., 2012
ps4	*ps4*	(f) 5'-GAC AGA AAC GTC TCG GAG TT-3' (r) 5'-TAC CAG TGT AAC CAG GAG CA-3'	172	Espino-Vazquez, et al., 2012

3.7.1 Touchdown PCR (TD-PCR)

Touchdown PCR is a method used for increasing sensitivity and specificity in PCR amplification to verify and confirm the annealing temperatures for the primers and to determine the possible samples with positive results for the genes. In this study, touchdown PCR was performed with a constant decreasing temperature of 0.5 ºC for each cycle starting from 58 ºC as shown in Table 3.5. After that, the PCR reaction was continued by using the average annealing temperature of 55 ºC.

Table 3.5: Parameters for Touchdown PCR.

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

3.7.2 Optimisation of Primers

Different primers have different melting temperatures due to their different GC contents and different lengths. The difference in the melting temperature will affect the annealing temperature of the PCR. Primers bind to the DNA template during annealing and enable DNA polymerase to elongate the primer in the extension step. It is crucial to identify the ideal annealing temperature for PCR. Multiple factors such as master-mix concentration, primer concentration, salt concentration and so on can affect the annealing temperature, therefore optimisation of primers was performed rather than theoretically calculate and estimate the annealing temperature.

Optimisation was carried out in which each of the reactions contained 1.0 µL of template DNA, 0.1 µL of each forward and reverse primers, 5 µL of master mix and 3.8 µL of sterile deionised water. The total volume of each reaction was 10 µL as shown in Table 3.5. The DNA template was added in the last step. Nine microlitre of the mixture was first prepared and then transferred to 10 PCR tubes (10 reactions), followed by the loading of the 1.0 µL DNA template into each tube. Using a thermocycler, the gradient temperature was set from 45 °C to 60 °C, and the PCR tubes were arranged in the block. The PCR parameters are shown in Table 3.6. The selected annealing temperature was the temperature which had positive and the brightest product band. The amplified DNA was then assessed and visualised through gel electrophoresis using a gel imager.

Table 3.6: Standard PCR mixture for a reaction with a final volume of 10 µL.

Components	Volume (µL)
PCR master mix	5.0
Forward primer (10 µM)	0.1
Reverse primer (10 µM)	0.1
Template DNA (50-100 µg/µL)	1.0
Sterile deionised water	3.8
Total volume	**10.0**

Table 3.7: PCR parameters for primer optimisation.

Step		Temperature (ºC)	Duration (sec)	Cycle
Initial Denaturation		95	120	1
Denaturation		95	30	35
Annealing	Un1	45 – 60	15	35
	Cry2gral			
	Cry4			
	Cry10spe			
	Cry11			
	Cyt2			
	Ps1			
	Ps2			
	Ps4			
	Chi			
Extension		72	45	35
Final Extension		72	300	1

3.7.3 PCR Amplification

The PCR mixture except DNA template was first prepared in a PCR tube for 7 reactions. After that, 9 µL of the mixture were pipetted into another labelled PCR tube. The 1 µL of DNA templates were then pipetted into their respective PCR tubes. The mixture preparation for a reaction in PCR is 10 µL as final volume. *Bacillus thuringiensis israelensis* was used as positive control, deionised water as non-template control and *Bacillus cereus* as negative control. PCR amplification was performed for all the samples according to the parameters shown in Table 3.8.

Table 3.8: Parameters for PCR amplification.

Step		Temperature (°C)	Duration (sec)	Cycle
Initial Denaturation		95	180* / 120*	1
Denaturation		95	10* / 30*	35
Annealing	Un1	55.5	10	35
	Cry2gral	49.0		
	Cry4	55.0	15	
	Cry10spe	48.40		
	Cry11	55.0		
	Cyt2	45.0		
Extension		72	30* / 45*	35
Final Extension		72	600* / 300*	1

* Duration for primers Un1 and Cry2gral.

* Duration for primers Cry4, Cry10spe, Cry11 and Cyt2.

3.8 Agarose Gel Electrophoresis

The amplified DNA was subjected to agarose gel electrophoresis to assess the result. A 2% agarose gel was prepared using agarose powder and 1X TBE buffer. The prepared 2% agarose gel was placed into the gel tank that was pre-filled with 1X running TBE buffer and the casted agarose gel was made sure that it was fully immersed in the buffer. A volume of 3 µL of 100-bp plus DNA ladder (Thermofisher) together with 1 µL of 6X loading dye containing 20% Novel juice was loaded into the first and the last wells of the agarose gel. Then, 5 µL of amplified product was mixed with 1 µL of 6X loading dye containing 20% Novel juice and loaded into different wells respectively. The gel was electrophoresed at 100 V for 35 minutes, visualised under the UV trans-illuminator using ChemiDoc^{TM}XRS systems (Bio-Rad), then analysed using the Image-Lab^{TM} (Bio-Rad) software.

CHAPTER 4

RESULTS

4.1 Gram Staining

The gram staining of bacterial isolates labelled **A** to **G**, two positive controls labelled **BTK** and **BTI**, and one negative control labelled **ECO** are presented in Figure 4.1. The seven bacterial isolates, same as the two positive controls, appeared rod-shaped and stained blue or violet, assuring that the bacterial isolates were Gram-positive bacilli. Conversely, the negative control labelled **ECO** was Gram-negative indicated by its pink staining and spherical in shape.

Figure 4.1: The Gram staining of the bacterial isolates (**A**) A10C (**B**) D10D (**C**) N6BSS (**D**) 6A3 (**E**) 8A3 (**F**) 8A3S (**G**) 8B3 showed Gram-positive rod-shaped bacilli. (**BTK**) *Bacillus thuringiensis kurstaki* (**BTI**) *Bacillus thuringiensis israelensis* (positive controls). (**ECO**) *Escherichia coli* (negative control). The samples were viewed under compound light microscope at 1000X magnification.

4.2 Malachite Green Staining

The endospore staining of bacterial isolates labelled **A** to **G**, two positive controls labelled **BTK** and **BTI**, and one negative control labelled **ECO** are presented in Figure 4.2. As shown in the figure, vegetative cells were stained red or pink whereas the endospores were stained green. All the bacterial isolates and

positive controls showed the presence of endospore whereas no spores were present in the negative control. The black arrows in Figure 4.2 indicate the presence of spore.

Figure 4.2: The Malachite green staining of the bacterial isolates (**A**) A10C (**B**) D10D (**C**) N6BSS (**D**) 6A3 (**E**) 8A3 (**F**) 8A3S (**G**) 8B3 showed that they are able to produce endospores. (**BTK**) *Bacillus thuringiensis kurstaki* (**BTI**) *Bacillus thuringiensis israelensis* (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.

4.3 Coomassie Brilliant Blue Staining

The Coomassie Briliant Blue staining of bacterial isolates labelled **A** to **G** and two positive controls labelled **BTK** and **BTI** are presented in Figure 4.3, showing the presence of crystal proteins which appeared in spherical shape and were stained in dark blue colour. In contrast, the negative control labelled **BC** showed absence of crystal protein. The black arrows in Figure 4.3 indicate the presence of crystal proteins.

Figure 4.3: The CBB staining of the bacterial isolates (**A**) A10C (**B**) D10D (**C**) N6BSS (**D**) 6A3 (**E**) 8A3 (**F**) 8A3S (**G**) 8B3 showed that they are able to produce spherical-shaped crystal proteins. (**BTK**) *Bacillus thuringiensis kurstaki* (**BTI**) *Bacillus thuringiensis israelensis* (positive controls). (**BC**) *Bacillus cereus* (negative control). The black arrows indicate the presence of crystal proteins. The samples were viewed under compound light microscope at 1000X magnification.

4.4 Scanning Electron Microscopy (SEM)

The scanning electron microscopy (SEM) observation of the bacterial isolates labelled **A** to **G** and the positive control labelled **BTI** are presented in Figure 4.4, showing the production of crystal inclusions in varying shapes. Majority of the crystals observed are in spherical shape and some are cuboidal or irregular in shape. The black arrows in Figure 4.4 indicate the presence of crystal inclusions. On the other hand, Figure 4.5 showed the absence of crystal protein production by *Bacillus cereus*, the negative control labelled **BC**.

Among all the seven isolates, none of them showed the presence of bipyramidal and rhomboidal shaped crystals. Crystals in cuboidal shape were found to be present in only two of the isolates, which are A10C and N6BSS. All the bacterial isolates were shown in the scanning electron micrograph to produce spherical shaped crystals. Besides, ovoidal crystals were also produced by bacterial isolates A10C, N6BSS, 8A3 and 8B3, whereas irregular crystals were found in isolates A10C, D10D, N6BSS and 8A3. The positive control *Bacillus thuringinesis israelensis* showed only the presence of spherical, ovoidal, irregular and cuboidal crystals production. The spherical shaped crystals produced by the seven bacterial isolates and the positive control are estimated to be in the sizes ranging from 0.3 µm to 1.0 µm, while ovoidal crystals produced by isolates are within the range of 0.4 µm to 0.7 µm in size. Moreover, cuboidal crystals produced only by isolate A10C are estimated to be in the size of 0.4 µm.

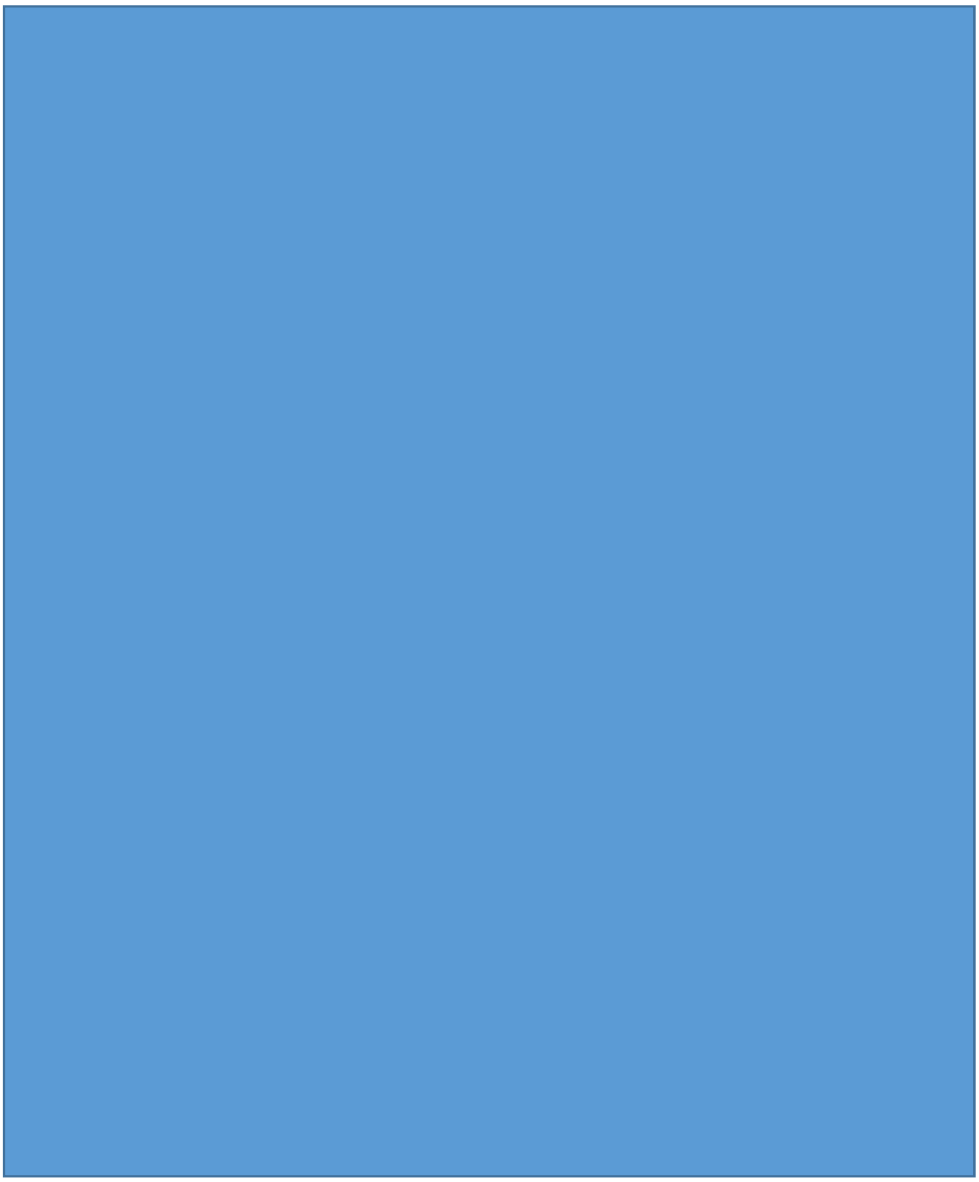

Figure 4.4: Scanning electron microscopy (SEM) of the bacterial isolates labelled (**A**) A10C (**B**) D10D (**C**) N6BSS (**D**) 6A3 (**E**) 8A3 (**F**) 8A3S (**G**) 8B3 and the positive control (**BTI**) *Bacillus thuringiensis israelensis* showed the presence of crystal proteins. The samples were viewed under SEM at 10,000X magnification.

Figure 4.4: Scanning electron microscopy (SEM) of the bacterial isolates labelled (**A**) A10C (**B**) D10D (**C**) N6BSS (**D**) 6A3 (**E**) 8A3 (**F**) 8A3S (**G**) 8B3 and the positive control (**BTI**) *Bacillus thuringiensis israelensis* showed the presence of crystal proteins. The samples were viewed under SEM at 10,000X magnification (continued).

Figure 4.5: Scanning electron microscopy (SEM) of negative control (**BC**) *Bacillus cereus* showed absence of crystal protein. The sample was viewed under SEM at 20,000X magnification.

4.5 Transmission Electron Microscopy (TEM)

Figure 4.6 presents the transmission electron microscopy (TEM) observation of the bacterial isolate labelled **A10C**, showing its ability of spore and crystal formation during sporulation phase. As shown in the figure, a distinct parasporal inclusion body can be observed next to the endospore. Based on the scale of 500 nm : 2 cm, the crystal protein size can be estimated as around 500 nm in diameter.

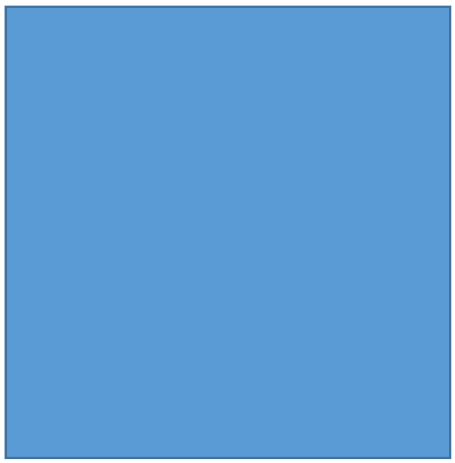

Figure 4.6: Transmission electron microscopy (TEM) of the bacterial isolate labelled A10C. The sample was viewed under TEM at 10,000X magnification. Key: **pb**, parasporal body; **sp**, spore.

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

The presence and molecular weights of total proteins extracted from the bacterial isolates were evaluated and estimated by comparing to the 175 kDa Chromatein Prestained Protein Ladder (Vivantis) as shown in Figure 4.7. The positive and negative controls for this analysis are *Bacillus thuringiensis israelensis* and *Bacillus cereus* respectively. The possible Cry and Cyt proteins found in the total protein extracts were the bands as indicated by arrows of different colours. The orange arrows are indicative of the Cry10 and Cry4C proteins that are 78 kDa in size, and yellow arrows are indicative of the Cry11 and Cry4D proteins that are from 70 kDa to 72 kDa in size. Light green and dark green arrows indicate the Cry2 (65 kDa - 70 kDa) and Cry6A (54 kDa) proteins respectively. The Cry35 proteins at 44 kDa in size are indicated by light blue arrows, while Cyt1 and Cyt2 proteins that are within 27 kDa and 29 kDa in size are indicated by dark blue arrows.

According to Figure 4.7, the Cry6A and Cry35 proteins with band sizes of 54 kDa and 44 kDa respectively are found to be present in all of the bacterial isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 together with the positive control *Bacillus thuringiensis israelensis.* In addition, the Cyt1 and Cyt2 proteins with molecular weight of between 27 kDa to 29 kDa are present in all of the bacterial isolates except 8A3 and 8B3, whereas Cry2 proteins at a molecular weight of between 65 kDa to 70 kDa are present in all of the bacterial isolates except A10C and D10D. Besides, Cry10 and Cry11 proteins are found in only a

few bacterial samples, where Cry10 proteins are present in only A10C and 8A3S, while Cry11 proteins are present in A10C, D10D, 8A3S and 8B3.

Among all of the bacterial isolates, the sample 8A3S possesses most number of extracted proteins, which were in the sizes of 78 kDa, 70 - 72 kDa, 65 - 70 kDa, 54 kDa, 74 kDa and 27 - 29 kDa. In addition to that, only bacterial isolates A10C and 8A3S exhibited a protein band size of around 78 kDa, indicative of either Cry10 or Cry4C protein. The positive reference strain *Bacillus thurngiensis israelensis* on Lane M was shown to exhibit four protein bands indicated as the molecular weights of 70 - 72 kDa, 65 - 70 kDa, 54 kDa and 44 kDa respectively.

Figure 4.7: The SDS-PAGE of the total protein extracts of bacterial isolates. **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8**: *Bacillus thuringiensis kurstaki* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane M**: 175 kDa Chromatein Prestained Protein Ladder.

Legend:

- Cry10, Cry4C proteins at 78 kDa
- Cry11, Cry4D proteins at 70 - 72 kDa
- Cry2 proteins at 65 – 70 kDa
- Cry6A proteins at 54 kDa
- Cry35 protein at 44 kDa
- Cyt1 and Cyt2 proteins at 27 – 29 kDa

4.7 The δ-endotoxin Genes Gel Electrophoresis

The presence of specific genes (*cry, cyt, ps* and *chi* genes) in the isolates were investigated in this study by conducting agarose gel electrophoresis of the PCR products. The PCR analysis utilised 10 pairs of specific primers adapted from several journals (Ejiofor and Johnson, 2002; El-Kersh, et al., 2016; Espino-Vazquez, et al., 2012; Halima, et al., 2006; Ibarra, et al., 2003; Jouzani, et al., 2008; Mahalakshmi, et al., 2012).

Only six out of the ten genes that were examined showed positive results in at least one of the bacterial isolates. The remaining four genes, which are *ps1, ps2, ps4* and *chi* genes were not present in any of the bacterial isolates. According to the PCR results, all bacterial isolates in this study (A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3) possessed at least three specific genes (*cry* and *cyt* genes). Among all, bacterial isolates N6BSS and 8A3 yielded PCR products when amplified with six of the primers, which were Un1, Cry2gral, Cry4, Cry10spe, Cry11 and Cyt2 (UN). Bacterial isolate A10C showed negative result when amplified with Un1 primer whereas 8B3 has one gene, *cry11* gene that was absent. The *cry10* and *cyt2* genes were shown to be present in all bacterial isolates, whereas *cry2* gene was absent only in bacterial isolate 6A3. *Bacillus thuringiensis kurstaki* was used as the positive control for *cry1* and *cry2* genes, while screening of *cry4, cry10, cry11* and *cyt2* genes used *Bacillus thuringiensis israelensis* as positive control. *Bacillus cereus* which was used as the negative control besides the non-template control (NTC) for the screening of the δ-

endotoxin genes showed negative results for all tested genes. The PCR results are presented in Figure 4.7 – 4.12, which are summarised in Table 4.1.

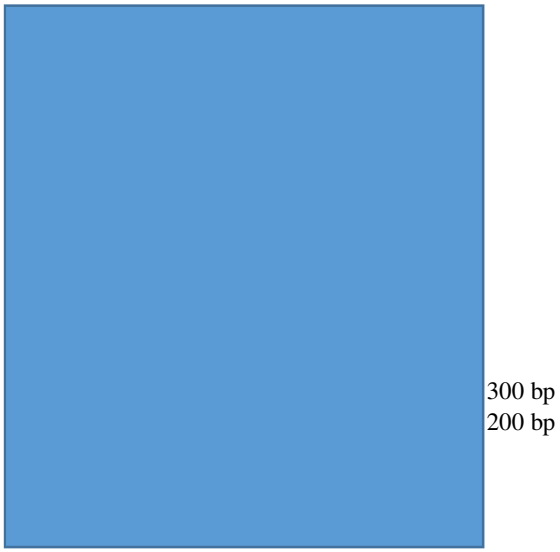

Figure 4.8: Screening of the bacterial isolates using Un1 primer (*cry1* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis kurstaki* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane 10**: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

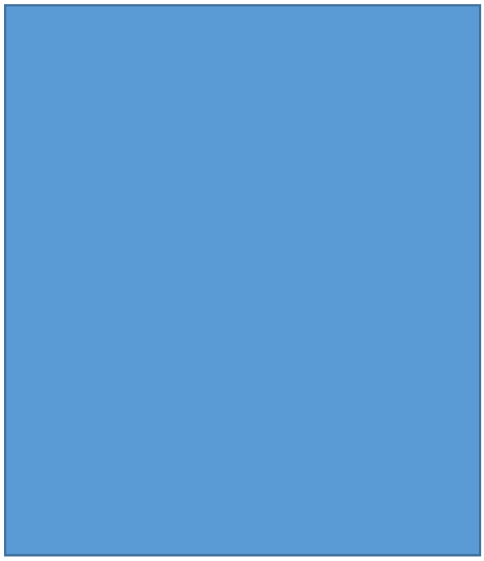

Figure 4.9: Screening of the bacterial isolates using Cry2gral primer (*cry2* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis kurstaki* (positive control); **Lane 9**: *Bacillus cereus* (negative control); Lane 10: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

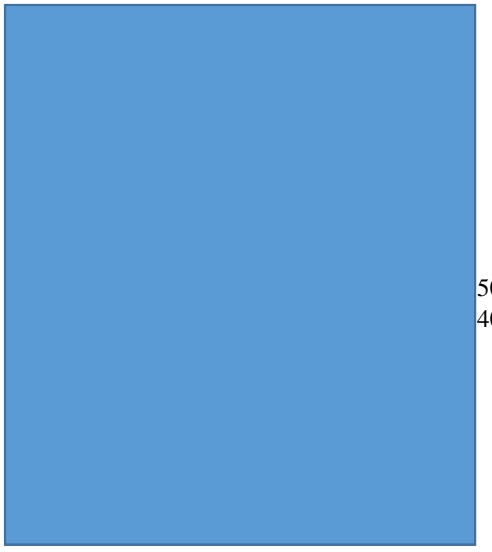

Figure 4.10: Screening of the bacterial isolates using Cry4 primer (*cry4* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis israelensis* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane 10**: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

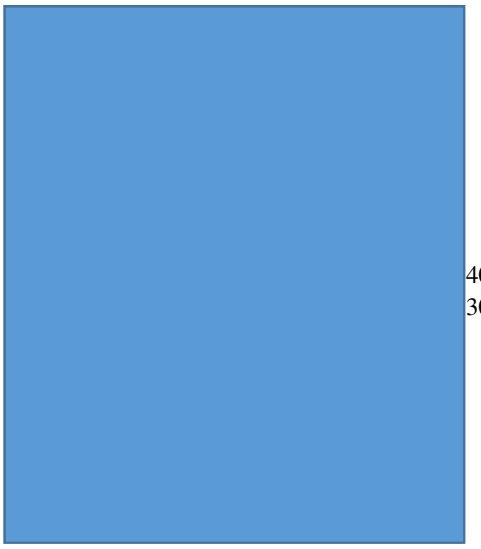

Figure 4.11: Screening of the bacterial isolates using Cry10spe primer (*cry10* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis israelensis* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane 10**: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

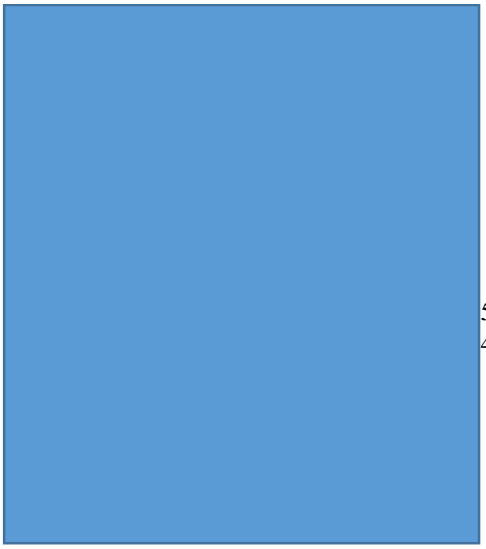

Figure 4.12: Screening of the bacterial isolates using Cry11 primer (*cry11* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis israelensis* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane 10**: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

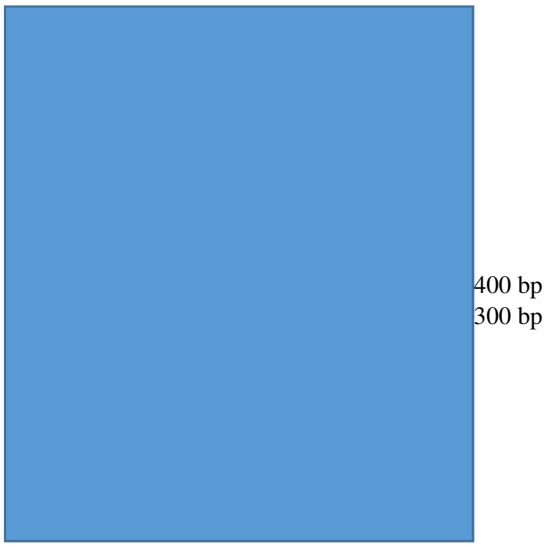

Figure 4.13: Screening of the bacterial isolates using Cyt2 (UN) primer (*cyt2* gene). **Lane 1**: A10C; **Lane 2**: D10D; **Lane 3**: N6BSS; **Lane 4**: 6A3; **Lane 5**: 8A3; **Lane 6**: 8A3S; **Lane 7**: 8B3; **Lane 8***: Bacillus thuringiensis israelensis* (positive control); **Lane 9**: *Bacillus cereus* (negative control); **Lane 10**: Non-template control; **Lane M**: 100-bp plus DNA ladder (Thermofisher).

Table 4.1: The summary of the PCR results of *cry1, cry2, cry4, cry10, cry11, cyt2, chi, ps1, ps2* and *ps4* genes of bacterial isolates.

Genes	A10C	D10D	N6BSS	6A3	8A3	8A3S	8B3	Btk	Bti	Bc
cry1	-	-	+	+	+	-	+	+		-
cry2	+	+	+	-	+	+	+	+		-
cry4	+	-	+	-	+	-	+		+	-
cry10	+	+	+	+	+	+	+		+	-
cry11	+	-	+	-	+	+	-		+	-
cyt2	+	+	+	+	+	+	+		+	-
chi	-	-	-	-	-	-	-	-	-	-
ps1	-	-	-	-	-	-	-	-	-	-
ps2	-	-	-	-	-	-	-	-	-	-
ps4	-	-	-	-	-	-	-	-	-	-

* "+" shows the presence of respective gene and "-" shows the absence of the respective genes.

CHAPTER 5

DISCUSSION

5.1 Morphological Identification

Bacterial isolates in this study were confirmed as *Bacillus thuringiensis*; a member belonging to the *Bacillus cereus* group that comprises of six members: *Bacillus cereus, Bacillus anthracis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis* and *Bacillus weihenstephanensis*. Confirmation of the identity of the bacterial isolates as members of *Bacillus cereus* group were verified through several morphological examinations conducted in the study. The characteristics of Gram-positive, rod-shape and spore-forming are features that are shared among members of the *Bacillus cereus* group; whereas formation of parasporal crystal inclusions was the determining criterion of these *Bacillus thuringiensis* isolates to distinguish them from the other members of the *Bacillus cereus* group.

Gram staining of the bacterial isolates showed that they are Gram-positive bacilli, confirming the basic morphological characteristics of *Bacillus thuringiensis*. Similar to the two positive control strains of *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis*, all seven bacterial isolates namely A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 were stained purple indicating their Gram-positive properties and appeared in rod shape indicating that they are

bacilli. In contrast, the negative control, *Escherichia coli* was noted as Gram-negative bacteria due to the pink colour appearance after the Gram staining process. These results correspond to previous studies related to *Bacillus thuringiensis* (Beveridge and Davies, 1983; Muniady, Rathinam and Subramaniam, 2011; Quesada-Moraga, et al., 2004). Cell wall of Gram-positive bacterium consists of large network of peptidoglycan to which the teichoic acids are attached. Shrinkage of the pore size along this thick peptidoglycan layer upon addition of alcohol results in the retention of the crystal violet-iodine complex within the Gram-positive bacterium, which was not washed out in the ethanol decolourisation step. This eventually resulted in the dark violet or purple stain of the Gram-positive bacterium. Conversely, the thin peptidoglycan layer of Gram-negative bacterium is not strong or thick enough to retain the crystal violet-iodine complex during the decolourisation step, resulting in red or pink counter stain of Gram-negative bacterium (Beveridge, 2001; Beveridge and Davies, 1983).

Apart from that, malachite green staining was performed on the bacterial isolates after 90 hours of incubation when they have reached their sporulation phase. Together with the two positive strains *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis*, all of the bacterial isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 were shown to be capable of forming endospore during their sporulation phase. The results obtained can be supported by findings of Jisha, Smitha and Benjamin (2013), Shakoori, et al. (2011), and Smitha, et al. (2013). As reported by Bukhari and Shakoori (2010), and Muniady, Rathinam and Subramaniam (2011), endospores are stained green while

vegetative cells are stained in pink colour by safranin, the counter stain. According to Figure 4.2, all of the bacterial isolates produced endospores that are stained in green colour and majority of the endospores were matured and have been released from the bacterium. There were also some endospores still remaining within the vegetative cells and visible in the green stain. Sporulation starts at the onset of stationary phase during nutrient depletion or other harsh condition experienced by the bacterial cells. Endospores possess extraordinary resistance properties enabling them to survive in harsh environment and these properties attribute to their spore coat. Due to the resistance of spore coat – a multilayer shell, the malachite green dyes are forced into the endospores via heating. Because of its resistance to the staining process, the endospore is equally resistant to destaining or decolourisation step. Therefore, the malachite green dye will be retained inside the endospore. On the other hand, the high water solubility of the dye which resulted in its poor adherence to the cell wall and the disruption of vegetative cell by heating process, caused the malachite green dye to be easily removed from the vegetative cells, leaving them counter-stained pink by safranin (Acharya, 2015; McKenney, Driks and Eichenberger, 2013).

In addition to that, Coomassie Brilliant Blue (CBB) staining was conducted after 110 hours of incubation to detect the presence of crystal proteins in the bacterial isolates. In correlation with the findings of Apaydin, et al. (2005), Çetinkaya (2002), El-Kersh, et al. (2016), and Rampersad and Ammons (2005), all of the bacterial isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3, together with the two positive strains *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis israelensis* were noted for their ability to produce crystal proteins, as shown in

Figure 4.3. There are several morphologies of the parasporal crystalline inclusions that can be produced by *Bacillus thuringiensis* which include bipyramidal, cuboidal, spherical, ovoidal, rectangular, irregular and etc (Apaydin, et al, 2005; Çetinkaya, 2002). However, the shape of the crystal proteins produced by the isolates cannot be determined solely from the Coomassie Brilliant Blue staining. This is due to the fact that CBB staining can only detect the presence of crystals in the bacterial isolates by staining the crystal proteins in dark blue colour but without providing sufficient information on their shapes due to the limited magnifications. Therefore, scanning and transmission electron microscopes were performed to confirm the shape of the crystals after detecting their presence in the isolates through Coomassie Brilliant Blue staining.

The crystal formation is the only phenotypic feature that makes *Bacillus thuringiensis* distinguishable from other members of the *Bacillus cereus* group. In this study, Coomassie Brilliant Blue staining was used rather than phase contrast microscopy for the detection of crystal proteins. This is due to the higher resolution of Coomassie Brilliant Blue staining over the phase contrast microscopy which allowed for high throughput evaluation of the bacterial isolates for the presence of crystal proteins. Moreover, Coomassie Brilliant Blue staining is sensitive enough to detect smaller sized crystals or of low abundance (Rampersad and Ammons, 2005).

5.2 Scanning and Transmission Electron Microscopy

There are a number of studies conducted regarding the evaluation of the crystals formation of *Bacillus thuringiensis* via the scanning and transmission electron microscopies. In correlation with the previous findings (Herrnstadt, et al., 1986; Mahillon and Delcour, 1984; Shao, Liu, and Yu, 2001) and with the CBB morphological findings of the present study, all of the bacterial isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 along with the positive control *Bacillus thuringiensis israelensis* were shown to have crystal proteins production as determined by SEM. Despite the high proportion of bipyramidal crystals produced in most varieties of *Bacillus thuringiensis* which have specific toxicity to Lepidopteran, most of the crystal proteins produced by the bacterial isolates and positive strain *Bacillus thuringiensis israelensis* were spherical in shape, moreover some ovoidal, cuboidal and irregular crystals were also noted in certain bacterial isolates. The ability of *Bacillus thuringiensis israelensis* to produce spherical crystals corresponds to the findings reported by Ibarra and Federici (1986), and Saadoun, et al. (2001).

Spherical shaped crystals found in all the bacterial isolates suggest that these isolates might be able to exhibit toxicity against insect of order Diptera. Besides, the bacterial isolates A10C, N6BSS, 8A3 and 8B3 are also assumed to be Dipteran-specific indicated by the ovoidal crystal observed in these isolates via SEM analysis. Moreover, cuboidal crystals indicative of Cry2 proteins possessing toxicity to Lepidopteran and Dipteran insects were found in isolates A10C and N6BSS, leading to an assumption that these two isolates might be able

to kill insects of orders of Lepidoptera and Diptera. There was no crystal protein observed in the electron micrograph of *Bacillus cereus* that served as negative control.

Referring to Figure 4.6, the micrograph from the transmission electron microscopy observation indicated the presence of an inclusion body alongside the endospore which was present at the middle of the sporulated cell. However, there may be a possibility that a second inclusion body may be present as well beside the major inclusion body. The observation of two inclusion bodies that closely lie against one another is quite common, as indicated by Hernández-Soto, et al. (2009), who reported the presence of two inclusion bodies may be due to different crystallisation of each protein encoded by different open reading frame (ORF). In the present study, the inclusion body was present in an amorphous semi-spherical shape and had diffused border, instead of a defined shape. The transmission electron microscopy observation of the isolate A10C is consistent and in correlation to the results of CBB staining and SEM analysis. The irregular shaped crystals were observed in both scanning and transmission electron micrographs of isolate A10C. During autolysis phase, the crystal inclusion produced will be released from the sporangium. Based on the transmission electron microscopy image, it can presumed that the endospore was produced concurrently with the formation of crystal inclusions by the bacterial isolate A10C after at least 48 hours of incubation.

5.3 SDS-PAGE Analysis of the Bacterial Total Proteins

SDS-PAGE is a technique used to separate charged molecules by their molecular masses or weights by using an electric field. The protein profiles of various bacterial isolates in the study were developed by using SDS-PAGE with *Bacillus thuringiensis israelensis* and *Bacillus cereus* as the positive and negative reference strains respectively. As shown in Figure 4.7, *Bacillus thuringiensis israelensis* had protein bands at molecular sizes of 70 kDa to 72 kDa, 65 kDa to 70 kDa, 54 kDa and 44 kDa, which represent Cry11 or Cry4D, Cry2, Cry6A and Cry35 proteins respectively, in agreement with the works of Höfte and Whiteley (1989) and Pereira, et al. (2013).

All of the bacterial isolates except 8B3 had minimal differences in their protein profiles in comparison to that of the positive reference strain. Bacterial isolate 8B3 was shown to possess Cry11/Cry4D, Cry2, Cry6A and Cry35 proteins with the band size of around 70 - 72 kDa, 65 - 70 kDa, 54 kDa and 44 kDa (Fakruddin, et al., 2012; Shakoori, 2009; Shishir, 2016), almost identical to the crystal proteins present in the positive strain *Bacillus thuringiensis israelensis*. All of the bacterial isolates A10C, D10D, N6BSS, 6A3, 8A3, 8A3S and 8B3 together with positive strain contained the 54 kDa and 44 kDa proteins, which suggested the presence of Cry6A and Cry35 proteins. The findings were in agreement with the studies of Arrieta, Hernández and Espinoza (2004), Fakruddin, et al. (2012) and Shishir (2016), who reported the protein bands of Cry6A and Cry35 were corresponding to the band sizes as mentioned above. Bacterial isolates A10C and 8A3S were shown to possess Cry10 protein at the molecular size of 78 kDa

which was corresponding to the finding of Pereira, et al. (2013). However, the bands for Cry10 proteins were not conclusive as the Cry4C proteins may also be represented by the bands. As reported by Höfte and Whiteley (1989), Cry4C proteins have molecular size of around 77.8 kDa. In addition, protein bands which appeared in Lanes 1 and 8 at molecular sizes of 70 kDa to 72 kDa indicate the respective bacterial isolates as having the Cry11 proteins, corresponding to findings of Shakoori (2009) and Yudina, et al. (2003). Nevertheless, the bands may also represent the Cry4D proteins as reported by Höfte and Whiteley (1989), that have molecular size of 72.4 kDa.

Furthermore, protein bands at molecular size of 65 kDa to 70 kDa are suggestive of the presence of Cry2 proteins in the bacterial isolates A10C, D10D, N6BSS, 8A3, 8A3S and 8B3, which agreed to findings of Fakruddin, et al. (2012). Besides, Cyt1 and Cyt2 proteins are present in the bacterial isolates A10C, D10D, N6BSS, 6A3 and 8A3S, indicated by the protein bands in Lane 1, 2, 3, 4 and 6 at molecular sizes of 27 kDa to 29 kDa which also correspond to the findings of Fakruddin, et al. (2012) and Pereira, et al. (2013). Apart from that, the protein profiling of all the bacterial isolates via SDS-PAGE analysis showed the absence of Cry1 protein of which the molecular size is 130 kDa to 150 kDa. In short, the presumption can be deduced from the SDS-PAGE analysis that the bacterial isolates may contain *cry2, cry4, cry6, cry10, cry11, cry35, cyt1* and *cyt2* genes which encode for Cry2, Cry4C, Cry4D, Cry6A, Cry10, Cry11, Cyt1 and Cyt2 proteins respectively. Polymerase Chain Reaction (PCR) was then carried out for further determination of the presence of these crystal proteins by screening for the δ-endotoxin genes encoding these proteins.

The crystal proteins of *Bacillus thuringiensis* have various insect host range, including Lepidopteran, Dipteran, Coleopteran, etc. Referring to the protein profiling, Cry2 proteins that were found to be present in N6BSS, 6A3, 8A3, 8A3S and 8B3 make these bacterial isolates presumably toxic to insect order of Lepidopteran. Crystal proteins belonging to Cry4, Cry10 and Cry11 have the ability to exhibit specific toxicity to insects of Dipteran. In addition, Cyt proteins are also toxic to Dipteran insects. Furthermore, Cry35 proteins are specifically toxic to insects of Coleopteran order (Crickmore, 2000; Ellis, et al., 2002; Fakruddin, et al., 2012). Based on these specificity host range of different crystal proteins, assumption can be made that bacterial isolates A10C, D10D, 8A3S and 8B3 possessing protein bands at 70 kDa to 72kDa may exhibit insecticidal activity against Dipterans, and there are two (A10C and 8A3S) among these four bacterial isolates that also exhibit the Cry10 protein bands, strengthening the assumption regarding toxicity of these two isolates against Dipterans. All of the bacterial isolates contain Cry35 proteins, which suggested that all of them may exhibit toxicity against Coleopterans. Moreover, Cry6A proteins which are toxic to Rhabditida (nematodes) were found in all the bacterial isolates. SDS-PAGE analysis can be fully utilised in characterising the *Bacillus thuringiensis* isolates and at the same time, in predicting the toxicity profile of the crystal proteins of the *Bacillus thuringiensis* isolates against various insect orders. However, SDS-PAGE analysis cannot be used to distinguish *Bacillus thuringiensis* from *Bacillus cereus* due to their high similarity in their protein profiles.

5.4 Polymerase Chain Reaction for Screening of *cry, cyt* and *chi* Genes

There are ten different pairs of primers that were used in the evaluation of the distribution of δ-endotoxin and *chi* genes in the seven bacterial isolates. The Un1 primer, adapted from Halima, et al. (2006) and Vidal-Quist, Castañera and González-Cabrera (2009), was used in the screening for *cry1* gene in the bacterial isolates. The bacterial isolates N6BSS, 6A3, 8A3 and 8B3 were shown to harbour the *cry1* gene, represented by the 277 bp amplification fragment which is in agreement to the findings of researchers mentioned above.

The Cry2gral primer which was adapted from Ibarra, et al. (2003) was used to detect the presence of *cry2* gene in the bacterial isolates. The present study showed that all of the bacterial isolates except 6A3 had yielded the expected PCR product at 500 - 526 bp when amplified with the Cry2gral primer, corresponding to the study of Ibarra, et al. (2003). In addition, the Cry4 primer used to identify the *cry4* gene in the bacterial isolates was adapted from Ejiofor and Johnson (2002) who reported the presence of *cry4* genes which was represented by amplification fragment at 423 bp. There are four bacterial isolates A10C, N6BSS, 8A3 and 8B3 in the present study that showed the formation of the corresponding 423 bp amplification fragment, indicating the presence of *cry4* gene in these isolates, in correlation to studies of above researchers and Ferrandis, et al. (1999).

The *cry10* gene was targeted using Cry10spe primer adapted from Ibarra, et al. (2003), Mahalakshmi, et al. (2012) and Vidal-Quist, Castañera and González-

Cabrera (2009). All of the bacterial isolates A10C, D10D, N6BS, 6A3, 8A3, 8A3S and 8B3 were shown to harbour *cry10* gene by producing PCR product when amplified with Cry10spe primers, which correlated to the above studies reporting the presence of similar *cry10* gene in their bacterial isolates. Besides, the Cry11 primers adapted from Ejiofor and Johnson (2002) was used to detect the presence of *cry11* genes in the bacterial isolates. Referring to Figure 4.12, the bacterial isolates designated A10C, D10D, 8A3 and 8A3S yielded the amplification fragment at around 485 bp, indicating the harbouring of *cry11* genes in these four out of the seven bacterial isolates.

Cyt2 (UN) primer used to screen for the presence of *cyt2* gene was adapted from Jouzani, et al. (2008), who reported the PCR product size amplified with Cyt2 (UN) primer is around 355 - 356 bp. Similar to *cry10* gene, *cyt2* gene was found to be present in all of the bacterial isolates A10C, D10D, N6BS, 6A3, 8A3, 8A3S and 8B3, representing by the amplification fragment at the corresponding 355 - 356 bp.

Chi primers used to identify *chi* gene was adapted from El-Kersh, et al., 2016. Besides, the Ps1, Ps2 and Ps4 primers adapted from Espino-Vázquez, et al. (2012) were also used for the screening of *ps1, ps2* and *ps4* genes respectively. However, in the present study, none of the bacterial isolates showed any bands at the corresponding amplicon sizes of any of these four genes. An assumption can be made from the absence of *ps* genes is that all of the bacterial isolates studied are not cytotoxic strains of *Bacillus thuringiensis*, instead they are all insecticidal.

By comparing the successful amplification of δ-endotoxin genes using PCR to the results of protein profiling through SDS-PAGE and the electron micrograph of SEM and TEM, most of the bacterial isolates showed minimal or no correlation of their gene expression to the respective protein band production and crystal shapes observed. Bacterial isolates N6BSS, 6A3, 8A3 and 8B3 possess *cry1* genes but did not exhibit Cry1 protein bands at 130 kDa to 150 kDa. Also, based on the electron micrograph, none of the bacterial isolates had shown the production of bipyramidal crystal, which indicates the Cry1 protein. Absence of Cry2 protein bands in the bacterial isolates also did not correspond to the presence of *cry2* genes. Bacterial isolates A10C and D10D, which were shown to harbour the *cry2* gene in PCR amplification did not yield the Cry2 protein band in SDS-PAGE, while bacterial isolate 6A3 showing protein band at 65 kDa to 70 kDa was not having the *cry2* gene shown by the PCR gene profile. Despite the presence of *cry2* genes in six out of the seven isolates, only two of them, which are A10C and N6BSS, have shown the corresponding crystal shape (cuboidal) in SEM analysis.

Although the *cry4* gene was expressed by isolates A10C, N6BSS, 8A3 and 8B3, SDS-PAGE protein profile showed that these isolates did not yield the Cry4 protein band. In the contrary, the four bacterial isolates expressing *cry4* gene were shown to produce the ovoidal shaped crystals indicative of Cry4 protein. Besides, Cry10 protein bands were exhibited only by A10C and 8A3S despite the presence of *cry10* gene in all of the bacterial isolates. Moreover, the expressions of the *cry11* gene and the Cry11 protein by the bacterial isolates D10D, N6BSS, 8A3 and 8B3 were also not consistent. All the bacterial isolates

showed positive results when amplified with Cyt2 (UN) primers, however, there were two bacterial isolates, 8A3 and 8B3, which showed no Cyt2 protein band in SDS-PAGE.

Overall, it can be said that the presence of genes in particular bacterial isolates did not necessarily yield the corresponding proteins. The findings of Armengol, et al. (2007) had emphasised the inconsistency of the SDS-PAGE protein profile and the *cry* gene profile. Most of their isolates had no correlation found between the presence of *cry* genes to the Cry proteins. According to Armengol, et al. (2007), the bacterial isolates showing PCR amplification but did not yield protein band at respective size could be due to the PCR amplified genes encoded for proteins that have relatively low expression to the extent that they cannot be detected through SDS-PAGE. Besides, Porcar and Juárez-Pérez (2003) had reported the non-expressed proteins could also be due to truncation or silencing. In the contrary, presence of crystal proteins in isolates that did not show any respective PCR amplification product was also explained by Armengol, et al. (2007) that the proteins might be coded by genes that are undetectable by specific primers used in the study.

As mentioned above, low level of protein expression may have caused the absence of the protein band in SDS-PAGE. This statement can be further explained by few reasons, including the insensitivity of Coomassie Brilliant Blue (CBB) staining that is commonly used for detection of protein bands in SDS-PAGE gel. This causes the proteins that are expressed in relatively low or

insufficient level could not be detected, which is in agreement to the finding of Iriarte, et al. (2000) who reported that protein bands with concentration lower than 10 ng cannot be detected by CBB staining. Next, certain isolates might have insufficient protein concentrations at the time of SDS-PAGE analysis, as different strains or isolates have different sporulation time, and some might not have reached the autolysis phase to release higher amounts of crystal proteins for detection (Armengol, et al., 2007).

5.5 Limitation of Study and Future Works

One of the limitations displayed in this study was in the detection of protein bands in SDS-PAGE where Coomassie Brilliant Blue (CBB) staining was used. CBB staining cannot detect proteins with concentration lower than 10 ng, resulting in the false negative result in that the proteins might have actually been expressed out but at insufficient level, as such it could be mistakenly analysed as being absent. To improve the sensitivity of staining procedure to detect the protein bands, silver staining can be used as it can detect proteins in concentrations as low as 2 ng. SDS-PAGE can be used to determine the molecular weight of the proteins and predict the types of proteins present in a particular bacterial sample based on comparison to molecular weight marker. However, it cannot ascertain the presence of a particular protein. To identify the presence of a protein of interest in a bacterial sample that contains various proteins, Western blotting can be performed. Western blotting is a technique that uses specifically labelled antibodies to identify proteins of interest that have been

separated by gel electrophoresis based on their molecular weight. Unlike SDS-PAGE, there is only one band that should be visible in the Western blot since the specific antibody targets only the protein of interest. The amount of the target proteins present in the bacterial sample is represented by the intensity or thickness of the band.

Based on the gene profiling of PCR amplification, there were no *ps* genes found in the bacterial isolates. To further confirm the result, cytotoxicity assay with human leukaemia T cells (MOLT-4) and other human cancer cells can be performed on the bacterial isolates. The procedure of the assay involves the observation of cytopathic effect (CPE) on the cancer cell lines using phase contrast microscopy. If negative result is obtained, this indicates that the bacterial isolates are non-cytocidal but are insecticidal strains, confirming the results of the present findings.

The screening of δ-endotoxin genes using PCR amplification is time-consuming and tedious as it required optimisation procedures and usage of various pairs of primers. However, it should be continued as it not only helps to characterise the *Bacillus thuringiensis* isolates according to the δ-endotoxins, but also helps in the evaluation of the toxicity spectra against different insect orders. In the present study, the bacterial isolates have been screened for the presence of Lepidopteran specific δ-endotoxin genes: *cry1* and *cry2* genes; and Dipteran specific δ-endotoxin genes: *cry4, cry10, cry11* and *cyt2*. In future works, screening of Coleopteran specific δ-endotoxin genes such as *cry35* gene can be considered.

Moreover, electrospray ionisation - Fourier transform ion cyclotron resonance (ESI - FTICR) mass spectrometry can be performed to precisely determine the molecular weight of proteins separated on the SDS-PAGE gel. As mentioned before, there are several protein bands present within a molecular weight range in SDS-PAGE gel but cannot be differentiated accurately as different crystal proteins may have almost similar and overlapping molecular sizes. For example, the Cry10 protein and Cry4C protein that have almost similar molecular sizes. Moreover, PCR products can also be further analysed using ESI - FTICR as the mass differences of a single base substitution can be accurately measured.

CHAPTER 6

CONCLUSION

The morphological identification of the bacterial isolates using staining procedures confirmed the isolates as one of the members of *Bacillus cereus* group having characteristics of Gram-positive, rod-shaped and endospore formation. The ability of crystal protein formation identified via CBB staining, SEM and TEM concludes that the bacterial isolates are *Bacillus thuringiensis* as crystal formation is its distinguishable feature. Moreover, the observation of crystal morphology via electron microscopy allowed the presumption regarding the toxicity spectra of the bacterial isolates to be made. As spherical crystals were predominantly present among all the seven bacterial isolates, it suggests that their toxicity property is mainly against Dipteran insects. Ovoidal-shaped crystals indicative of Cry4 proteins that exhibit toxic effect against insects of Dipteran order were also observed in bacterial isolates A10C, N6BSS, 8AS and 8B3. Besides, there are two isolates A10C and N6BSS that produced the cuboidal crystals representing the Cry2 proteins, suggesting their toxicity against Lepidopteran and Dipteran insects.

In SDS-PAGE analysis, all bacterial isolates were shown to yield 54 kDa Cry6A proteins and 44 kDa Cry35 proteins. Cry2 and Cyt proteins were also shown to be present in many of the bacterial isolates, where Cry2 protein bands were exhibited by N6BSS, 6A3, 8A3, 8A3S and 8B3, while bacterial isolates A10C,

D10D, N6BSS, 6A3 and 8A3S exhibited Cyt protein bands at 27 kDa to 29 kDa. In contrast, Cry10 protein bands were exhibited by relatively less isolates, which are A10C and 8A3S only. The bacterial isolates A10C, D10D, 8A3S and 8B3 exhibited protein band size of 70 kDa to 72 kDa correlating to Cry11 protein.

Based on the PCR gene profile, *cry10* and *cyt* genes were shown to be expressed by all of the isolates at band size of around 348 bp and 355 – 356 bp respectively. All of the isolates except 6A3 showed the expression of *cry2* gene, indicated by the amplification fragment at 500 – 526 bp. Moreover, there are four out of the seven isolates yielded the amplification fragment of *cry1* gene. The *cry4* and *cry11* genes were also expressed by four bacterial isolates at 423 bp and 485 bp respectively. The *ps1*, *ps2*, *ps4* and *chi* genes were not expressed by any of the bacterial isolates.

There are isolates that expressed the δ-endotoxin genes but did not yield the corresponding protein band sizes nor the respective crystal shapes. It can be presumed that the δ-endotoxin genotype, protein profile and the crystal shapes are not in correlation to each other, and the presence of genes did not necessarily yield the corresponding proteins. Moreover, the absence of *ps* and *chi* genes in all the seven isolates suggests that the isolates are all insecticidal strains lacking cytotoxic properties.

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APPENDICES

Appendix A

List of instruments and apparatus used with respective manufacturers

Instrument/Apparatus	Manufacturers
Autoclaved Machine	Hirayama, Japan
Centrifuge Tube (15 ml & 50 ml)	Axygen Scientific, USA
Compound Light Microscope	Leica, Japan
Freeze Dryer	Scanvac COOLSAVE™, Denmark
Gel Electrophoresis Set	Major Science, Taiwan
Heat Block	BioShake IQ
Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Microwave Oven	Sharp, Japan
Mini-Protean Tetra System	Bio-Rad, US
Nano-photospectrometer	IMPLEN
Petri Dishes	Labmart
pH Meter	Sartorius
Shaking Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Scanning Electron Microscope	JEOL (JSM-670IF), USA
Table-top Microcentrifuge Machine	Profuge
Transmission Electron Microscope	CM12, Philips
Vortex	Gemmy Industrial Corp., Taiwan
UV Transilluminator	Bio-rad, US

Appendix B

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

Chemicals/Media/Reagents	Manufacturers
Nutrient Agar	Merck, Germany
Nutrient Broth	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
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
PCR Master Mix	1st Base
Novel-Juice	GeneDireX
DNA Extraction Kit	Geneaid, Taiwan

Appendix C

Preparation of Media

Preparation of Nutrient Agar

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

Preparation of Nutrient Broth

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

Preparation of 6X Sample Buffer for SDS-PAGE

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

Preparation of Loading-dye-Novel-juice Mixture

A total amount of 100 mL of novel juice was added with 400 mL of 6X loading dye to an Eppendorf tube. The solution was then mixed thoroughly and kept in dark under -20ºC.

Preparation of 1.5 % Agarose Gel

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

Preparation of 2.0 % Agarose Gel

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