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THESIS

CHARACTERIZATION OF THAI *Bacillus thuringiensis* (Berliner) STRAIN HIGHLY TOXIC TO DIAMONDBACK MOTH, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae)

PRAKAI THAPHAN

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Prakai Thaphan 2011: Characterization of Thai *Bacillus thuringiensis* (Berliner) Strain Highly Toxic to Diamondback Moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae). Doctor of Philosophy (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor: Associate Professor Jariya Chanpaisaeng, Ph.D. 122 pages.

The diamondback moth (*Plutella xylostella* Linnaeus) is the major pest worldwide. One of the alternatives for its control is the utilization of *Bacillus thuringiensis*, an entomopathogenic bacterium characterized by its production of insecticidal crystal proteins. One hundred and twenty one *B. thuringiensis* isolates were tested against *P. xylostella* larvae and selected the high effective isolates in term of high toxicity and UV tolerance. *B. thuringiensis* were shown to be highly effective isolates namely JCPT76, JCPT106, JCPT118 and JCPT121 with the LC_{50} values 8.52×10^3, 1.41×10^4, 7.03×10^3 and 5.36×10^2 spores/ml, respectively. Those isolates were selected for study on UV tolerance. JCPT121 had a higher survival rate at 60 minutes where as JCPT76, JCPT106 and JCPT118 were susceptible. JCPT121 was identified as *B. thuringiensis*. Morphological characteristics of cell, spore and crystal protein by SEM showed that JCPT121 was rod shape, oval spore and produced bipyramidal crystal proteins. The total genomic DNA and plasmid DNA was extracted and *cry* genes were detected by PCR method using 20 pairs of specific primers. PCR products were purified, sequenced and subjected to BLAST search at NCBI. The result revealed that 3 *cry* genes namely *cry*1Ac, *cry*1I and *cry*2A presented in chromosomal DNA while 2 *cry* genes namely c*ry*1I and *cry*2A presented in plasmid DNA. The crystal proteins of JCPT121 were purified by discontinuous sucrose gradient method and analyzed protein profile by SDS-PAGE. The results revealed that JCPT121 composed of approximately 130 kDa and 72 kDa proteins. Screening the toxicity of JCPT121 to insect showed toxicity to *Spodoptera litura, Spodoptera exigua* and *Aedes aegypti* but no toxicity to larvae of *Galleria mellonella*, *Bactrocera dorsalis, Musca domestica, Tenebrio molitor and Tribolium castaneum*.

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Prakai Thaphan

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LIST OF ABBREVIATIONS

cm	centimeter
mm	millimeter
µm	micrometer
g	gram
mg	milligram
h	hour
min	minute
ml	milliliter
µl	microliter
LC_{50}	50% Lethal Concentration
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
bp	base pair
kb	kilobase

CHARACTERIZATION OF THAI *Bacillus thuringiensis* (Berliner) STRAIN HIGHLY TOXIC TO DIAMONDBACK MOTH, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae)

INTRODUCTION

The gram-positive soil bacterium *Bacillus thuringiensis* was originally isolated by Prof. Shigetane Ishiwata, a Japanese biologist, from silkworm and described as "*Bacillus sotto*"(Federici, 2005). *B. thuringiensis* produces crystal protein during sporulation which is toxic to insect upon ingestion. The crystal protein is composed of one or several polypeptides ranging from 24 to 140 kDa molecular mass of protoxin (Hickle and Fitch, 1990). When crystal protein is solubilized in the insect midgut, it is activated by midgut protease. The activated toxin interacts with the larval midgut epithelium causing a disruption in membrane integrity and ultimately leading to insect death (Gill *et al*., 1992).

B. thuringiensis shows genetic diversity with different toxic potential mostly due to plasmid exchange between strains (Thomas *et al*., 2001). Each habitat may contain a novel *B. thuringiensis* strain which is toxic to a target insect group and awaiting discovery. Therefore, *B. thuringiensis* strains have been collected from different environments and characterized to evaluate their toxic potential against various insect orders (Uribe *et al*., 2003). Thailand is located in hot and humid region, an ideal habitat for *B. thuringiensis*. Several isolates of *B. thuringiensis*, discovered from different sources, have been collected from different regions in Thailand. Those isolates may contain different *cry* genes content resulting in production of Cry proteins specific to different insect species.

This study was aimed to characterize highly toxic *B. thuringiensis* against *Plutella xylostella* and tolerant to ultraviolet ray from 121 *B. thuringiensis* isolates collected from national park and wildlife sanctuary in Krabi province (Thaphan, 2003). Results from the study would provide useful information for the selection of suitable strains of *B. thuringiensis* to act as biocontrol agent of *Plutella xylostella*, one economically important insect pest in Thailand.

OBJECTIVES

1. To select the highly effective isolates of *Bacillus thuringiensis* for controlling diamondback moth *(Plutella xylostella*).

2. To characterize the highly effective isolate of *Bacillus thuringiensis* for its biochemical characterization, morphology of spore and crystal protein, *cry* gene sequence, protein profile and insect host range .

LITERATURE REVIEW

Diamondback Moth, *Plutella xylostella* (Linnaeus)

Crufifer is one of the most popular group of vegetables in Southest Asia and many countries throughout the world. It is widely and continuously grown throughout the year. It has many kinds of insect pests in the field. One of the most serious pests of cruciferous crops is the diamondback moth (DBM). The larvae can destroy an entire crop even when intensive control measures are carried out. The annual cost for managing this pest is estimated to be US $ 1 billion throughout the world (Talekar, 1992). Outbreaks of damondback moth in Southeast Asia sometimes cause crop losses of more than 90% (Verkerk and Wright, 1996). The diamondback moth, a native of Europe, can be found throughout the USA, Asia and in all areas of the world where crops are grown. It can also be a problem in greenhouse (Anonymous, 2000).

1. Description

Adult of diamondback moth, grayish-brown moth, has narrow forewings, and conspicuously fringed hind wings with an 18 mm wingspan. When at rest, the light-colored areas that show as anal margins of the forewings in the figure fit together to form diamond-shaped spots, the basis for the common name (Ralph and William, 1987). The minute round egg is pale yellow. Larvae is slightly tapering at both ends. This pale green larva with a black head and scattered black hairs reaches a length of 7 mm when mature. It wriggles rapidly when disturbed, often dropping from the plant and hanging by a silk-like thread. The yellowish pupa is enclosed within a loosely spun, gauze-like cocoon measuring about 7.5 mm in length.

2. General Life Cycle

Diamondback moth adults become active at dusk and continue so in the night. Most adults emerge during the first 8 hr of photophase (Pivnick *et al*., 1990) and mating occurs at dusk of the same day the adults emerge. Female moths start

layings eggs soon after mating. Oviposition period lasts four days, averaging 233.5 eggs per female are laid (Pongpasert, 1985). The majority of eggs are laid before midnight with peak oviposition occurring between 7.00 p.m. – 8.00 p.m. The ratio of eggs laid on the upper and lower leaf surfaces approximates 3:2, and very few eggs are laid on stems and leaf petioles.

Soon after emergence, neonate larvae initiate feeding on foliage. The first-instar mines in the spongy mesophyll tissue, whereas older larvae feed from the lower leaf surface and usually consume all tissue except the wax layer on the upper surface, thus creating a "window" in the leaf. There are four larval instars whose duration depends on temperature (Sarnthoy *et al.,* 1989). The average durations of larval instars were 4, 4, 5, and 5.6 days for the first to fourth instars, respectively. When the fourth-instar has completed its feeding, it constructs an open network cocoon on the leaf surface where it feeds and spends a two-days period of quiescence marking the prepupal stage. The prepupa sheds its larval skin which remains attached to the caudal end of the pupa. The duration of the pupal period varies from 1-15 days depending on temperature (Harcourt, 1957; Pongpasert, 1985). Adults feed on water drops or dew and short-lived. As many as 20 generations per year occuring in the tropics indicates that developmental time from egg to adult is much more rapid in those regions (Liu *et al*. 2002).

3. Host Plants and Damage

The diamondback moth practically feeds only on members of the family Cruciferae, whose members have various edible parts as cabbage, broccoli, cauliflower, collards, kale, brussel sprouts, kohlrabi, turnip, radish, mustard and watercress (Anonymous, 2000). The host range of diamondback moth is limited to crucifers because they contain mustard oils and their glucosides (Gupta and Thorsteinson, 1960; Hillyer and Thorsteinson, 1971). Diamondback moth larvae feed on all plant parts but prefer the undersides of older leaves, cervices between loose leaves and young buds. They eat small holes in leaves and buds or feed superficially

leaving slight perforations instead of holes. Heavy feeding on buds may cause the marketable portion of the plant to fail to develop properly.

In Thailand, diamondback moth is prevalent from February to April when optimum climatic conditions and food plants are more readily available. However, in many areas of the Central plain where crucifers are planted year-round, diamondback moth damage can be observed throughout the year. It is in this area that the insect has been the most serious threat to cruciferous crops for many years. Crucifers grown in the highlands are subjected to lesser degree of damage except during the brief peak outbreak periods of the year. In the lowlands, the diamondback moth problem is much less severe as compared to the other areas, possibly due to rotation with other crops (Rushtapakornchai and Vattanatangum, 1986).

In many countries, synthetic insecticides are used to control diamondback moth. DBM was the first crop insect to be reported resistant to DDT in 1953 in Java, Indonesia (Ankersmit 1953). At present, in many crucifer-producing regions it has shown significant resistance to almost every insecticide applied in the field including new chemistries as spinosyns, avermectins, neonicotinoids, pyrazoles and oxadiazines (Sarfraz and Keddie 2005; Sarfraz *et al*. 2005). Different research groups are testing the potential of various entomopathogens including viruses (e.g. granulovirus), fungi (e.g. *Zoophthora radicans*, *Beauveria bassiana*), bacteria (e.g. *Bacillus thuringiensis*), protozoa (e.g. *Vairimorpha* spp.), and nematodes (e.g. *Steinernema* spp.) as biocontrol agents for diamondback moth. However *Bacillus thuringiensis* is most promising biological control agent and the leading organism used in commercial microbial pesticides (Lambert and Peferoen, 1992; Meadows, 1993).

Bacillus thuringiensis (Berliner)

B. thuringiensis is gram-positive, spore-forming bacterium closely related to several other *Bacillus* species, including *Bacillus cereus* but differs from *B. cereus* by the presence of crystal in mature cell. The mature cell of *B. thuringiensis* is 1.0 to 1.5 microns wide and 4.0 to 5.0 microns long, with peritrichous flagella. The spore and crystal protein produced within the mature cell. The endospore is characteristic feature of *Bacillus* species of 0.5 × 1.0 μm wide and 1.5 μm long (Benson and Smith, 1992). Crystal proteins are of varied shapes as bipyramidal, cuboidal, spherical or irregular crystal protein (Atthathom *et al*., 1995).

1. Exoenzymes and toxins produced by *Bacillus thuringiensis*

1.1 Exoenzymes

On the exponential growth of *Bacillus thuringiensis*, certain *B. thuringiensis* strain are known to produce and secrete exoenzymes, including phosphatidylinositol-specific phospholipase C (PI-PLC), lecithinases, chitinases, and proteases. The PI-PLC is a rare bacterial enzyme and is also produced by *B. cereus*, *Staphylococcus aureus*, and *Clostridium novyi.* The lecithinase C, which is capable of disrupting host membranes, may support in the entry and proliferation of vegetative cells in the host hemocoel. The chitinase and protease have been proposed to disrupt the peritrophic membrane barrier, thus providing access to gut epithelial. (Boucias and Pendland, 1998)

1.2 Toxins

B. thuringiensis produces many kinds of toxin such as δ-endotoxin, exotoxin, haemolysins and enterotoxins (diarrhoeal type) (Glare and O'Callagan, 2002). The hemolysin, referred to as thuringiolysin, is structurally and functionally identical to the cereolysin produced by *B. cereus* (Matsuyama *et al*., 1995). These toxins are capable of lysing vertebrate erythrocytes considered to be important virulence factors in several vertebrate bacterial pathogens. Only two toxins, the

β-exotoxin and δ-endotoxin, are significantly important in agriculture (Dulmage and Aizawa, 1982).

1.2.1 Beta (β) – exotoxin

The beta (β) exotoxin, also called thuringiensin, thermostable toxin or fly toxin, was first detected by MacConnell in autoclave, cell-free broth cultures and detected in several subspecies. This toxin is thermostable (70°C, 15 minutes) with a broad host spectra that kills various lepidopterans, dipterans, hymenopterans, hemipterans, isopterans, orthopterans, nematodes, and mites. It is a low molecular weight and water-soluble toxin which affects insects only when injected into body cavity because it does not pass through the gut wall or is degraded by gut phosphatases (Sebesta *et al*., 1981) At sublethal dose, this toxin affects insect molting and pupation and may result in defection of adult development (Burgerjon *et al*., 1969)

Several laboratories use high-performance liquid chromatography (HPLC) method to detect and quantify β-exotoxin. Two types of β-exotoxin have been found by HPLC (Levinson *et al*., 1990). The type I β-exotoxin detected in *B. thuringiensis* serotypes 1, 9 and 10 contains the disaccharide ribose ether-linked to a phosphorylated glucose residue which is bound to an adenine molecule. This adenine nucleotide analog inhibits mRNA synthesis via blockage of the polymerization reaction catalyzed by DNA-dependent RNA polymerase. Second β-exotoxin, type II, believed to be a uracil nucleotide analog, is isolated from *B. thuringiensis* subsp. *morrisoni* serotype 8ab (Levinson *et al*., 1990). However, very few studies have dealt with type II β-exotoxin and its structure remains unknown.

The production of β-exotoxin I has often been linked to the presence of plasmid of various sizes bearing *cry* gene. Ability to secrete β-exotoxin I and ability to produce crystal protein are transferred to *Bacillus cereus* and *Bacillus* recipient strains by conjugation (Espinasse *et al*., 2002). It has been proposed that β-exotoxin plays a potential regulatory role in *B. thuringiensis* expression. The β-exotoxin or precursor molecule may involve in the regulation of transcription

during sporulation. These molecules are capable of inhibiting both prokaryotic and eukaryotic RNA polymerase. However, the comparative *in vitro* studies demonstrate that the insect RNA polymerase is twice as sensitive as mammalian RNA polymerase which in turn is 300-600 times more sensitive than the prokaryotic RNA polymerase. The inhibitory activity of β-exotoxin insect challenged with β-exotoxin usually stop feeding and die within several days of exposure (Boucias and Pendland, 1998).

In certain cases, β-exotoxin expresses teratogenic effects and disrupts larval and/or pupal molting. Insect adults that survive after β-exotoxin treatment may exhibit reduced longevity and fecundity. β-exotoxin synergizes the activity of the δ-endotoxin against normally resistant insects. This observed potentiation of δ-endotoxin may be caused by the inhibitory effect of β-exotoxin on the regeneration of damaged midgut cells. It should be emphasized that β-exotoxin does not express the specificity of the δ-endotoxin . In vertebrates, β-exotoxin exposure produces lesions in liver, kidney and adrenal glands. Treated chickens lose vigor and produce undersized eggs (Boucias and Pendland, 1998).

As mentioned earlier, β-exotoxin kills a wide spectrum of both pest and non-pest insect hosts (Boucias and Pendland, 1998). Because of vertebrate toxicity, most commercial preparations of *B. thuringiensis* are composed of species or isolates that do not produce β-exotoxin. As a condition for registration for pesticide use on food in the USA, *B. thuringiensis* active ingredient must be tested to show absence of *B. thuringiensis* (McClintock *et al*., 1995)

1.2.2 Delta-endotoxin

The delta-endotoxin of *B. thuringiensis*, which is actually a protoxin, is the major component of the proteinaceous, parasporal crystal formed during sporulation. The delta-endotoxin refers to heat-labile, crystalline toxin, parasporal crystal or just crystal (Heimpel and Angus, 1963). Crystal development does not occur in vegetative rods but happens only when growth and nucleic acid synthesis cease and the cell is committed to sporulation (Heimpel and Angus, 1963).

2. Classification of delta endotoxin (δ-endotoxin) gene

The δ-endotoxin belongs to a large and variable family of insecticidal glycoproteins. The toxins were originally classified by Hofte and Whitely (1989) into 4 classes according to their amino acid sequence homology and insecticidal specificity.

Genes of class *cry* I encode 130-160 kDa protoxins with active toxin embedded in the N-terminal and conserved C-terminal and involve in the packaging of toxin within the crystalline inclusion. CryIA(a), CryIA(b) and CryIA(c) proteins share more than 80% common amino acid sequences. CryIB and CryIC protoxins show 58% and 67% amino acid sequence homology, respectively with CryIA(a) protoxin. CryIA toxin shows activity against Lepidoptera larvae while CryIB is active against coleopteran larvae.

Genes of class *cry* II encode 70-71 kDa protoxins are converted into 65 kDa toxin. The Cry IIA is toxic to both lepidopteran and dipteran larvae, while CryIIB is only toxic to lepidopteran insects. These two toxins are 87% identical and different most between residues 307-822 (Wider and Whitley, 1990).

Genes of class *cry* III, which occur in *B. thuringiensis* subsp. *tenebrionis* and other strains with coleopteran activity, encode Cry IIIA, B, C, D and E toxins (Sekar and Carlton, 1985). The *cry* III genes encode 72 kDa protoxins which are proteolytically activated to 67 kDa and further activated to 55 kDa active toxin in larval gut (Carroll *et al*., 1989).

Genes of class *cry*IV (A, B, C, D) and *cyt* A encode proteins molecular masses in the range of 20-140 kDa. The *B. thuringiensis* subsp. *israelensis* crystals are composed of at least four polypeptides of approximately molecular masses of the followings: 27 kDa (CytA), 58 kDa (CryIVC), 70 kDa (CryIVD, 128 kDa (CryIVB) and 134 kDa (CryIVA).

Two additional classes, *cry*V and *cry*VI genes were added by Fitelson *et al*., (1992). Genes of class *cry* V encode protoxin with 81.2 kDa produced by *B. thuringiensis* subsp. *kurstaki* DSIR 732. These toxins are larvicidal to both coleopteran (*Lepinotarsa decemlinata*) and lepidopteran (*Ostrinia nubilalis*) species, but are more active against lepidopteran larvae (Tailor *et al.*, 1992) and *cry*VI gene encodes protein toxic to nematodes.

Recently, Crickmore *et al*. (2005) has established a new homology-based classification system. Cry proteins are now classified into 19 different classes (see the *Bt*. WWW page at http: //www.susx.ac.uk and users/bafn6/Bt/index.html). The new classification system argues that several ICPs are toxic to more than one other insects, thus the similitude in the amino acid sequences of or known Cry proteins is taken as the only criterion for distributing them into classes and subclasses. Some classes, such as Cry6 and Cry15, do not show any significant homology with the rest of Cry proteins (Figure 1).

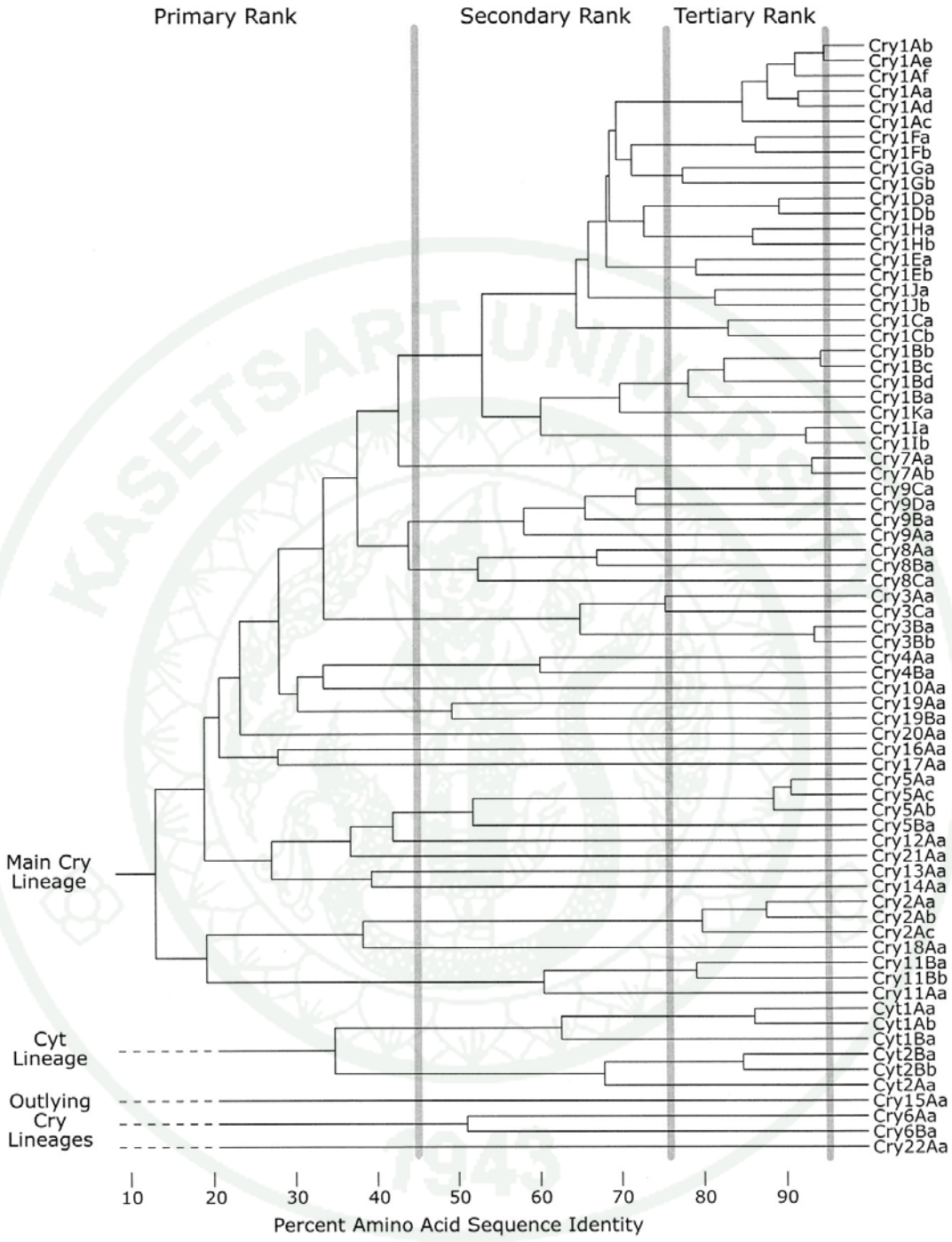

Figure 1 Amino acid homology between *Bacillus thuringiensis* delta-endotoxins.

Source: Crickmore *et al*. (2005)

3. The structure of δ-endotoxin

The first three-dimensional structure of III δ-endotoxin , *B. thuringiensis* var. *tenebrionis,* was determined by X-ray crystallography. All these structures display a high degree of similarity with a three-domain, suggesting a similar mode of action of the Cry three-domain protein family. The N-terminal domain (domain I) is a bundle of seven α-helices in which the central helix-α5 is hydrophobic and is encircled by six other amphipathic helices and this helical domain is responsible for membrane insertion and pore formation. Domain II consists of three antiparallel β-sheets with exposed loop regions which may be responsible for receptor recognition. Domain III is a β-sandwich that may protect the toxin from further degradation during proteolytic processing or moderate toxin bilayer and toxin-toxin interaction (Vontersch *et al*., 1994) (Figure 2).

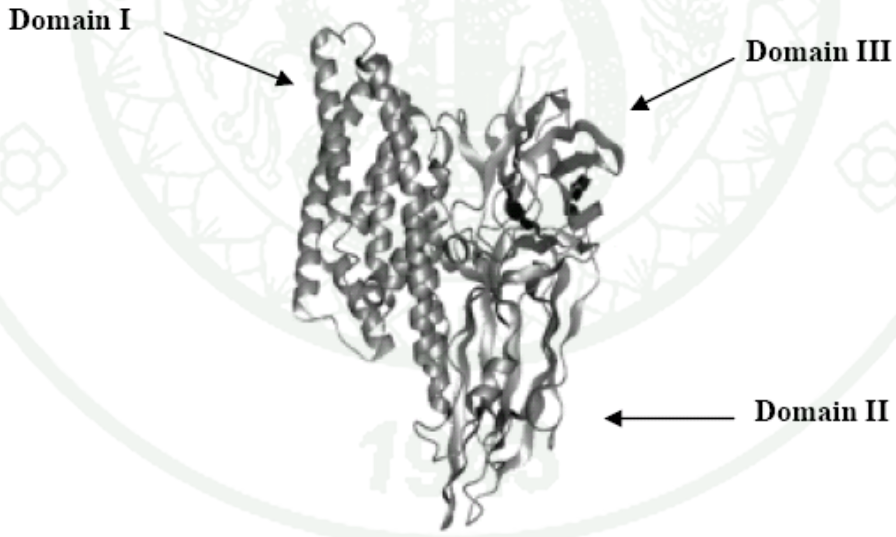

Figure 2 Three-dimensional structure of protein Cry3A, the crystal toxin of *B. thuringiensis*

Source: Li *et al*. (1991)

Domain I shares structural similarities with other PPT like colicin Ia and N and diphtheria toxin supporting the role of this domain in pore formation. Domain II, which is structurally similar to several carbohydrate-binding proteins like vitelline, lectin jacalin and lectin Mpa has been reported. Domain III shares structural similarity with other carbohydrate-binding proteins such as the cellulose-binding domain of 1,4-β-glucanase C, galactose oxidase, sialidase, β-galactosidase (de Maagd *et al*., 2003) (Figure 3).

Cyt proteins have a single α-β domain comprising of two outer layers of α-helix hairpin wrapping around a β-sheet (Li *et al*., 1996). Cyt toxin is structurally related to volvatoxin A2, a PFT cardiotoxin produced by a straw mushroom (*Volvariella volvacea*) (Lin *et al*., 2004).

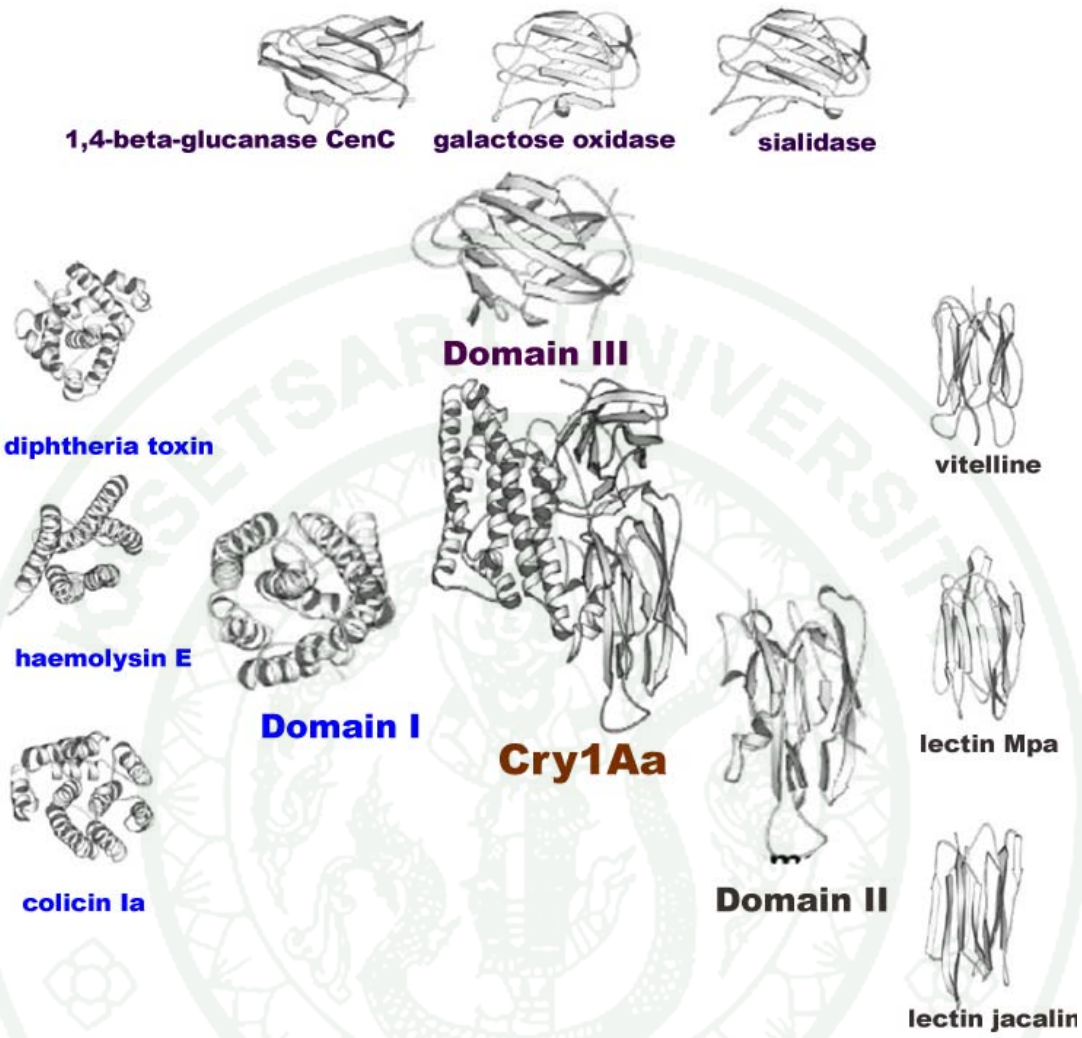

Figure 3 Three-dimensional structure of Cry1Aa protein and comparison of its three domains with similar domains from other proteins .

Source: de Maagd *et al*. (2003**)**

4. Mode of action of three-domain Cry toxins in lepidopteran insects

4.1 Receptor binding in lepidopteran larvae

To date, at least four different protein receptors for Cry1A toxin have been described; a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N(APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Gómez *et al.*, 2007) (Figure 4). In addition, it has been proposed that glycolipids are important Cry-receptor molecules in insects and nematodes (Griffiths *et al.*, 2005).

4.1.1 Cadherin-like receptor (CADR)

Cadherin proteins represent a large family of glycoproteins responsible for intercellular contact. These proteins are transmembrane proteins with a cytoplasmic domain and an extracellular ectodomain with several cadherin repeats (Nagamatsu *et al*., 1999). In the case of cadherin receptor of *Manduca sexta*, it shows that this protein is located in microvilli of midgut cells (Chen *et al*., 2005).

4.1.2 GPI-anchored receptors

The aminopeptidase-N(APN) is a GPI-anchored exopeptidase. APN seems to play an important role in Cry toxin action since a laboratory-selected *Spodoptera exigua* colony resistant to Cry1C does not express APN1, suggesting the lack of APN production being correlated with resistance to Cry1C toxin (Herrero *et al*., 2005). As APN and ALP are both GPI-anchored proteins, these proteins are proposed to be selectivity included in lipid rafts conceived as spatially differentiated liquid-ordered microdomains in cell membranes (Munro, 2003).

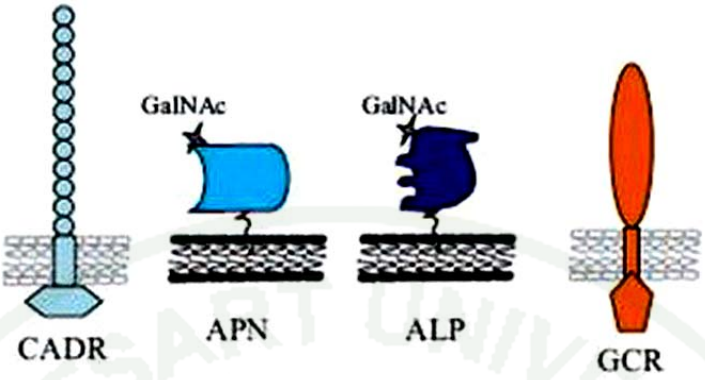

Figure 4 Receptor molecules of Cry1A proteins. CADR, cadherin receptor; APN, aminopeptidase-N, ALP, alkaline phosphatase, GCR, 270kDa glycol-conjugate receptor

Source: Bravo *et al.* (2007)

4.2 Pre-pore formation

Binding of *cry*1Ab toxin to *M. sexta* CADR protein promotes an additional proteolytic cleavage in the N-terminal end of the toxin (helix α-1) facilitating the formation of a pre-pore oligomeric structure important for insertion into the membrane and for toxicity (Gómez *et al.*, 2002). It was reported that oligomeric structure of Cry1Ab and Cry1Ac increased 100-200 folds their binding affinity to the APN receptor, showing apparent dissociation constants of 0.75-1 nM (Pardo-López *et al*., 2006).

4.3 Membrane insertion

The CADR protein receptor is located in soluble membrane in contrast to APN and ALP receptors, which are attached to the membrane by GPI anchors, and are preferentially partitioned into lipid rafts (Zhuang *et al*., 2002) Lepidopteran and dipteran insects have a basic pH (up to pH 11) in their midgut lumen (Gringorten, 1999). Unfolding analyses of pure Cry1Ab structures at different pHs demonstrated

that the molten globe state of pre-pore complex was induced by alkaline pH (Rausell *et al.,* 2004). These analyses also showed that the pre-pore and membrane inserted oligomer, had a more flexible conformation than the monomeric toxin (Rausell *et al.,* 2004). Additionally, in the membrane-inserted pore, only domain I was protected from heat denaturation, suggesting it might be inserted into the membrane in contrast to domain II and III. Finally, the alkaline pH induced a looser conformation of the membrane-inserted domain I important for an active channel formation (Rausell *et al.,* 2004).

A model involving the sequential interaction of toxins to CADR and APN receptor molecules is described as followed. Firstly, the interaction of monomeric toxins with CADR faciliting the formation of pre-pore oligomeric structure gains binding-affinity to APN. Then, the pre-pore toxin binds to APN. As a result, a conformation change occurs and a molten globule state of the toxin is induced. Lastly, the pre-pore oligomeric toxin is inserted into lipid rafts inducing pore formation and cell swelling (Bravo *et al*., 2007) (Figure 5).

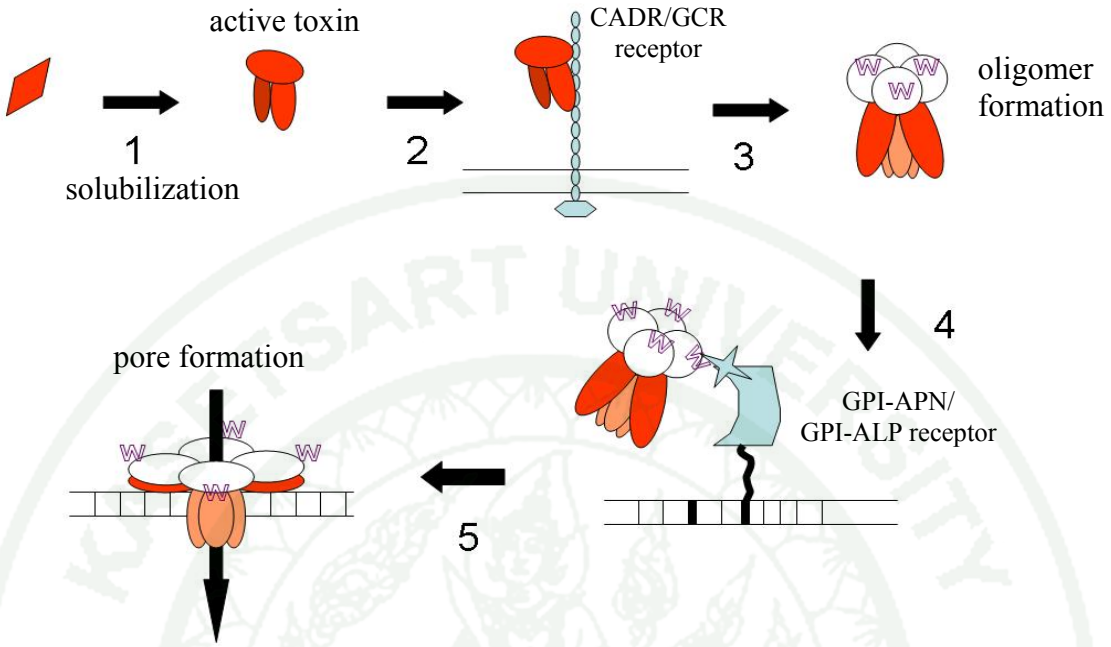

Figure 5 Sequential interaction of Cry toxins with different receptor molecules in lepidopteran larvae. (1) ; (2) binding of monomeric Cry toxin to the first receptor (CADR or GCR), conformational change is induced in the toxin and α-helix 1 is cleaved; (3) oligomer formation; (4) binding of oligomeric toxin to second receptor (GPI-APN or GPI-ALP), a conformational change occurs and a molten globule state of the toxin is induced; (5) insertion of the oligomeric toxin into lipid rafts and pore formation

Source: Bravo *et al*. (2007)

5. Identification of delta-endotoxins by polymerase chain reaction (PCR)

The PCR is composed of three steps among three different temperatures. First, DNA containing the sequences to be amplified is heat denatured (about 95°C) to separate its complementary strands. Then, with a relatively low temperature (about 50°C), the single strand is annealed with two short primer sequences present in large excess. Finally, medium temperature (about 27°C) allows DNA polymerase to synthesize a single strand in a 5′-> 3′ direction of each primer. Then a second cycle of heat denaturation, annealing and primer extension, is carried out. The cycle can be continued without interruption in PCR machines called thermal cycler, simply programmable heating blocks, that accurately and rapidly change the temperature surrounding the reaction mixture. The entire cycles can be repeated up to 30 times and each cycle increases plenty of duplex DNA species bounded by the oligonucleotide primers. In principle, the number of copies of the target sequence is increased exponentially (Vierstraete, 1999) (Figure 6 and 7).

The PCR screening programs have increased knowledge concerning the natural occurrence of single *cry* and *cyt* genes among strains, their combinations within the strains and their occurrence in different geographic locations. Since PCR allows quick and simultaneous screening of many strains, it has been partially used to substitute bioassays in preliminary characterization of *B. thuringiensis* collections. Nowadays, PCR has become a routine screening step for large strain collections, in both public and private research laboratories. However, prediction of insecticidal activity by PCR must always be supported by bioassays, in order to assess the potential of promising isolates as biopesticides (Masson *et al*., 1998).

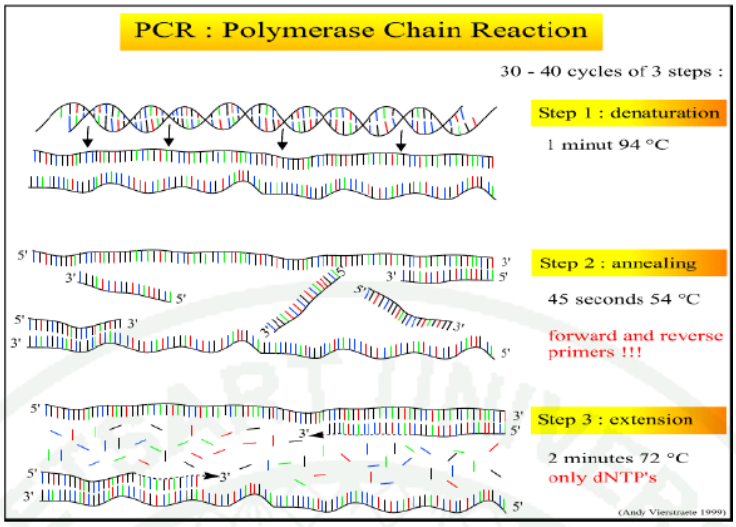

Figure 6 The Polymerase Chain Reaction (PCR). Step1: Denaturation, double helix of DNA separated by heating. Step 2: Annealing, primers anneal to DNA template by decreasing temperature. Step3. Extension, dNTPs incorporated into newly DNA synthesis chains by adjusting temperature to optimum temperature of DNA polymerase.

Source: Vierstraete (1999)

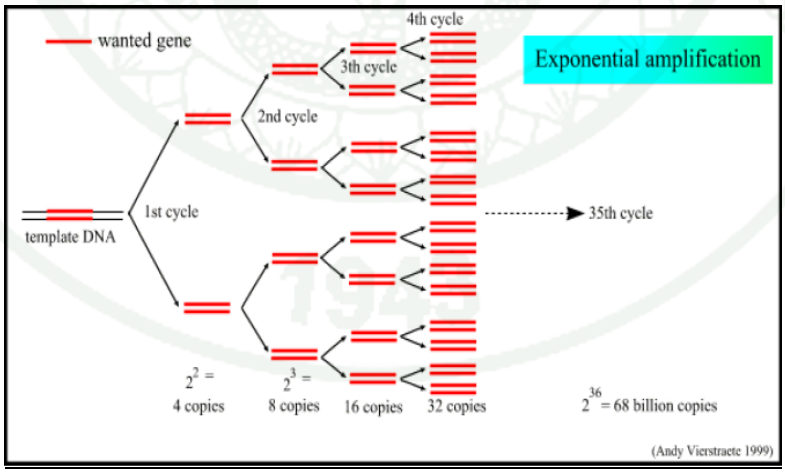

Figure 7 The exponential growth of amplification product

Source: Vierstraete (1999)

The easiest strategy to identify *cry* or *cyt* genes by PCR is the use of a primer pair that specifically recognizes a single *cry* gene. This can be done with a pair of specific primers or by combining a universal primer selected from a conserved block and thus able to anneal to the entire gene family and a specific primer selected from a variable region. However, the high number of *cry* genes known so far makes this gene-by gene strategy inapplicable for large-scaled screening purposes. For practical reasons, primer pairs designed from highly conserved regions and recognizing entire *cry* gene subfamilies are often used in a preliminary screening prior to performing a second PCR with specific primers (Porcar and Juárez-Pérez, 2003).

To date, more than 80 primer pairs specifically recognizing both entire groups (i.e. *cry*1 genes) and individual *cry g*enes have been designed. Primer size varies from 17 to more than 30 nucleotides, and some 'universal' oligonucleotides, directed to the identification of a group or subfamily of genes, are degenerated (Porcar and Juárez-Pérez, 2003).

6. Limitations of insecticidal activity predicted by PCR

In 2003, Porcar and Juárez-Pérez described that the ability of PCR in predicting insecticidal activity depended largely on several factors that could make the prediction erroneous.

6.1 Gene identity

Primer design is a key factor in PCR. The 3′-end of primer is critical in the amplification procedure because a mismatch between the primer and the template occurs at this region. The amplification efficacy can be drastically diminished and may even result in the absence of an amplicon (Kwok, 1994). In consequence, any variation in one or two bases at this region may lead to closely related genes not being detected. In contrast, a substitution at the 5′-end or on the middle of the primer will not significantly affect the amplification and thus different genes can be equally amplified.

6.2 Expression level

The expression level of individual *cry* genes present in any strain can vary greatly. *B. thuringiensis* subsp. *aizawai* strain HD-133 is a good example of such variation. This strain is known to contain six *cry* genes, although only three proteins (Cry1Ab, Cry1C and Cry1D) are expressed in detectable amounts in HD-133 parasporal crystals (Masson *et al.,* 1998). The three remaining genes are either silent due to an insertion within the coding sequence (*cry*1Aa), or expressed at undetectable levels if at all (*cry*2 and *cry*1I).

6.3 Protein interactions

The toxicities of *B. thuringiensis* parasporal crystals depend not only on the activity of their individual components but also on the interactions between such proteins. For example, Cry1A proteins tested against *Lymantria dispar* (Lee *et al.*, 1996) and synergism was found between proteins Cry1Aa and Cry1Ac whereas Cry1Aa and Cry1Ab interacted antagonistically.

6.4 Other virulence factors

It is known that a series of extracellular compounds synthesized by *B. thuringiensis*, such as L-exotoxins, phospholipases, proteases, chitinases and the secreted VIPs (vegetative insecticidal proteins) contribute to virulence. Additionally, spores are also known to synergize with the toxic effect of crystals when tested against some insect species, probably due to the invasion of hemocoel through the midgut, and the subsequent development of septicemia. The highest contribution of the spore to the pathogenicity of *B. thuringiensis* was reported in wax moth (*Galleria mellonella*) larvae.

Ultraviolet radiation

The bactericidal properties of ultraviolet radiation (UV) have been known for almost 150 years. The main bactericide in the environment is the UV component of sunlight. UV radiation can be divided into vacuum ultraviolet (50-200nm), UVC (200-270nm), UVB (270-330nm) and UVA (330-400m). The solar UV radiation that reaches the earth's surface contains wavelengths form 290-380 nm, a considerable amount of which is in the UVA (about 8% of solar output) (Coohill, 1996).

Vacuum ultraviolet has little to relevance to microbial inactivation because it is absorbed by water and oxygen in the air. UVB is responsible for the sun-burn effect and UVA which is responsible for sun tanning (Bolton, 2001). UVC, short-wave radiation, is referred to as the germicidal range because the nucleotides of microorganisms are capable of absorbing the photon energy (600-440 kJ/Einstein) produced at this range, especially 254 nm (Blatchley *et al*., 2001)

1. 254 nm UVC

Many of published papers on the effects of UV on bacteria were conducted at one wavelength, 254 nm. Three reasons for this were: the availability of an inexpensive mercury lamp source (often called a "germicidal" lamp) that emits about 86% (somewhat variable by manufacturer and lamp age) of its energy at 254 nm; this wavelength is near the peak of DNA absorption; bacteria were sensitive enough to this wavelength so that experiments could be conducted over reasonable time periods (Coohill and Sagripanti, 2008).

UVC can be absorbed by the nucleic acids and proteins of microorganisms, preventing the cells from reproducing. There is a wide variety of UV photoproducts produced by 254 nm in bacteria which can affect cell survival. The three most important photoproducts were: the pyramidine (especially thymine) dimer (5, 6 cyclobutane dipyrimidine-pyrimidone (6-4), the major cause of vegetative cell killing by UVC; the pyramidine pyramidine pyrimidone (6-4) series of lesions (called 6-4

photoproducts); and the spore photoproduct (5-thyminyl-5,6-dihydrothymine) (Setlow, 2001).

Pyrimidine (thymine and cytosine) bases are ten times more sensitive to UV irradiation than purine (adenine and guanine) bases. UVC light causes damage by causing bonds to form between adjacent thymine in DNA chains. Pyrimidine is more susceptible to UVC, inducing the formation of covalently linked dimers between adjacent bases. If a thymine base absorbs a UV photon, a chemical bond can form between an adjacent thymine base. Dimers inhibit replication of DNA. If enough UV has been absorbed, so as to create enough dimers, DNA cannot replicate and the cells will die. The more radiation, the more formed dimers (Lopez-Malo and Palou, 2005).

In *B. thuringiensis*, the parasporal crystal proteins and spores, which are the primary toxic substances to insects, will be inactivated quickly after exposure to sunlight. The proteins are cross-linked by hydroxyl radicals (· OH) and hydrogen peroxide (H_2O_2) when exposed to UV and ionizing radiation (Wang *et al*., 1999), which leads to a reduced dissolution in insect midgut. Exposure of δ-endotoxin to UV irradiation resulted in the destruction of tryptophan and histidine residues (Pozsgay *et al*., 1987). It was also reported that tryptophan was essential for the interaction of the δ -endotoxin with insect midgut cell receptors and that the destruction of tryptophan residues might result in some changes in the three-dimensional configuration of the toxic protein and consequently the loss of its toxicity (Cohen *et al.,* 1991).

2. Resistance to UV

Resistance to UV irradiation is a factor in the ability of the microorganism to repair damage. The two most common repair mechanisms are photoreactivation and dark repair (Setlow and Setlow, 1996). Photoreactivation requires exposure to light between 310-490nm, to activate DNA photolyase, an enzyme that cleaves the pyrimidine dimers. The amount of photorepair that occurs is dependent on the organism, the amount of UV absorbed, and the nutrient state of the organism (Cohen *et al.,* 1991). Dark repair, which does not require the presence of light, accounts for 99% of UV damage repair. Excision repair, the most common type of dark repair,

involves the following enzyme mediated steps: Repaired endonuclease recognizes the damages DNA and cleaves the DNA strand, exonuclease excises the damaged section, DNA polymerase rebuilds the damaged section, Polynucleotide ligase rejoins the severed strand (Setlow and Setlow, 1996).

MATERIALS AND METHODS

1. *Bacillus thuringiensis* isolates

One hundred twenty one of *B. thuringiensis* isolates were obtained from our collection (Thaphan *et al.*, 2008). The *B. thuringiensis* subsp. *kurstaki* (Bactospien), *B. thuringiensis* subsp. *tenebrionis* (Novador) and *B. thuringiensis* subsp. *israelensis* serotype H14 kindly provided by Assoc. Prof. Jariya Chanpaisaeng was used as the positive control in the experiment.

2. Insect bioassay

2.1 *Bacillus thuringiensis* cultivation

The cultivation method modified from that described by Dubois (1968) was used. One loopful of the bacterial cells of < 24 hours on nutrient agar was cultured in Nutrient broth (Appendix A). The culture was incubated in shaker at 150 rpm at 28°C for 12-18 hours. A 25 ml culture was then used as the inoculum for the cultivation in NB and incubated in an orbital shaker at 190 rpm at 28°C until spores and crystal proteins were obtained. The spores and crystal protein suspensions were kept at 4°C for further studies.

2.2 Diamondback moth rearing

The larvae of *P. xylostella* from Chinese kale fields were collected from Supanburi province. The larvae were maintained in laboratory on Chinese kale leaves. Emerging adult moths were provided with 10% honey solution. Adults were allowed to mate and oviposit on young Chinese kale plants. After hatching, the larvae were fed with fresh Chinese kale leaves. Optimum rearing conditions (27±2°C temperature and 14:10 h light:dark photoperiod) were provided in the laboratory throughout rearing and bioassay periods. The larvae at generation 4 were used in bioassay which reduced resistant from field.

2.3 Bioassay procedure

The toxicities of different *B. thuringiensis* isolates were screened on the second-instar larvae of *Plutella xylostella* as described by Chanpaisaeng *et al*. (1996). In the first screening, the bioassay was performed with highly concentrated spore-crystal suspension. For each isolate, 30 second-instar larvae were used. Mortality was observed after 3 days. Bioassay was replicated with the most active isolates and LC_{50} values were determined for the most active isolates. Three dilutions were used for each isolate and 30 second-instar larvae were used for each dilution. Mortality was observed after 3 days and the LC_{50} was obtained by probit analysis (Finney, 1971) and a highly efficient *B. thuringiensis* isolates for analysis of UV-tolerant was selected.

3. Ultraviolet tolerance

Ultraviolet tolerance of *B. thuringiensis* was observed as described by Chen *et al.,* (2004). A suspension of spore and crystal protein of *B. thuringiensis* approximately 10^9 cell ml^{-1} was mixed and irradiated at a distance of about 12 cm from the UV source at 254 nm. Samples were irradiated at different time intervals. One hundred µl from the irradiated samples was plated them out on NA plates. Cell colonies were measured and survivals were checked after incubation for 24 h at 30 °C. Selection of a high UV tolerant *B. thuringiensis* isolates for biochemical characterization, cell, spore and crystal protein morphology, *cry* gene content, protein profile of crystal protein. Insect host range test was also made.

4. Biochemical characterization

4.1. Gram staining

According to Gram staining procedure, after smearing the colony of *B. thuringiensis* on a drop of water on glass slide, the smear was allowed to dry and heat fixed by moving the slide over flame two or three times. The slide was placed on a slide holder and flooded with crystal violet for 60 sec, then washed for 5 sec with water. After that, the slide was flooded with the iodine solution for a min and rinsed with water for 5 sec. At this point, the specimen was still blue-violet. The ethanol was added dropwise until the blue-violet color was no longer emitted from specimen, then rinsed the slide with the water for 5 sec. The slide was flooded with the safranin O for a min to allow the bacteria to incorporate the safranin O and rinsed with water for 5 sec to remove any excess of dye. The slide was allowed to dry and observed using light microscope. Gram positive cells incorporated little or no counterstain and remained blue-violet in appearance. Gram negative bacteria took on a pink color and were easily distinguished from the Gram positives ones.

4.2 Catalase test

Cultures grown for 24 hours on the maintenance medium were tested for catalase production with 3% (w/v) H_2O_2. One ml of H_2O_2 solution was poured over the surface of an agar culture. Effervescence caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase in the culture under test.

4.3 Motility test

Motility of the strains was tested using motility plates (Appendix A) as described by Guttmann and Ellar (2000). The strains were streak-inoculated onto the middle of the plate from top to bottom and incubated overnight. If a colony was observed to spread out from the inoculation site, the strain was scored as motile; otherwise it was scored as non-motile.

4.4 Biochemical characteristic test using API 50 CHB

The biochemical characteristics tests were performed using the API 50 CHB kit according to the protocol provided by the company

4.4.1 Preparation of the inoculums

B. thuringiensis JCPT121 was cultured on NA plate (Appendix A) and grown at 30°C for 12-16 hr. The *B. thuringiensis* colonies were picked up and prepared a suspension with a turbidity equivalent to 2McFarland in the ampoule of API 50 CHB medium. This ampoule was used to inoculate the API 50 CH strip and homogenized.

4.4.2 Inoculation of the strips

The tubes were filled with the inoculated API 50 CHB medium. The API 50 CH strip was slightly tilted so that bases of the tubes were uppermost which could trap any gas produced. The strip tests were incubated at 37°C under aerobic condition.

4.4.3 Reading the strip

The results of biochemical and carbohydrate fermentation were determined after 24 and 48 hr. A positive test corresponding to an acidification was revealed by the phenol red indicator contained in the medium which changed from red to yellow. But only a change from red to black was observed in Esculin test.

4.5 Crystal and spore staining

Colony of *B. thuringiensis* was smeared on a drop of water on glass slide and allowed to dry and heat fixed by moving the slide over flame. The slide was placed over beaker containing boiling water and then flooded with malachite green for

about 10 min. Removed the slide from the beaker and rinsed for 5 sec with water. The slide was flooded with the safranin for a min and rinsed with water for 5 sec to remove any excess of dye. The slide was allowed to dry and observed using light microscope. Spore and crystal protein took on a green and pink color, respectively.

5. Electron microscopy of cell, spore and crystal protein

To determine the morphology of *B. thuringiensis* JCPT 121, spore and crystal protein inclusions were observed and characterized using scanning electron microscope. The *B. thuringiensis* samples were prepared as follows: the bacterium was cultivated on the surface of NA (Appendix A) for 72 hr. At the time intervals of 12, 24, 48 and 72 hr, a tiny portion of cells was thinly smeared using a small drop of distilled water on acetone cleaned-round coverglass. The coverglass was adhered to a brass specimen holder by silver paste. The sample was allowed to dry at room temperature for 1 hr, vacuum-dried for 2 hr, carbon coated using a vacuum evaporator and then gold-coated for 2 min twice with JEOI, Ion Sputter. The specimen was then observed with JOEL- JES CF-35 scanning electron microscope (SEM) operated at 5 kv.

6. *cry* gene detection by polymerase chain reaction (PCR)

6.1 Genomic DNA extraction

The genomic DNA extraction was modified from that reported by Harwood and Cutting (1990). *B. thuringiensis* was cultivated in nutrient broth. After 18 hr of incubation, the culture was transferred to centrifuge tube and centrifuged at 8,000 rpm for 1 min. The supernatant was carefully decanted and the cell pellet was thoroughly resuspended in 800 µl lysis buffer (Appendix B). A 0.002 g of lysozyme and 60 µl of 20 % sarkosyl were added and incubated at 37°C for 5 min. After incubation, 500 µl of phenol were added and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was carefully removed to a new tube and added 500 µl of phenol: chloroform (29:1). The mixture was mixed thoroughly by inversion of the tube. Phases of DNA in the solution and phenol were separated by centrifugation at 10,000 rpm for 10 min at 4°C. The upper aqueous phase was removed to a new tube and 40 µl of 3 M sodium acetate pH 5.2 and double volumes of cold absolute ethanol were added. The content was mixed by inversion and maintained at -10 °C over night. The precipitated DNA was collected by centrifugation at 12,000 rpm for 10 min at 4°C. and washed with 70% ethanol. The pellet was decanted from ethanol and then air-dried. DNA was finally suspended in 50 µl of ddH_20

6.2 Plasmid DNA extraction

The plasmid DNA extraction was modified from that reported by Birnboim and Doly (1979). *B. thuringiensis* was cultivated in LB medium. After 18 hr of incubation, the culture was transferred into a centrifuge tube and centrifuged at 8,000 rpm for 1 min. The bacterial cells were washed with 1 ml TSE (Appendix B) by centrifugation at 10,000 rpm for 1 min. The supernatant was carefully removed and the cell pellet was thoroughly suspended in 200 µl of solution A (Appendix B) and incubation for 30 min at 37°C. After incubation, 400 µl solution B (Appendix B) was added and the tube was gently vortexed. The suspension became almost clear and slightly viscous. The tube was maintained for 4 min at 0°C and then 300 µl solution C

(Appendix B) were added. The content of the tube was gently mixed by inversion for a few seconds during which a clot of DNA was formed. The tube was maintained at 0°C for 5 min to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate and then centrifuged at 10,000 rpm for 6 min. The supernatant was removed and transferred to a new centrifuge tube. Single volume of phenol: chloroform (1:1) was added and centrifuged at 10,000 rpm for 6 min. The upper phase was carefully removed to a new tube and single volume of chloroform: isoamyl alcohol (24:1) was added. The tube was mixed thoroughly by inversion and centrifuged at 10,000 rpm for 2 min. The upper phase was transferred into a new tube and 500 µl of absolute ethanol was added. The content was mixed by inversion and maintained at -20 °C over night. The precipitated DNA was collected by centrifugation at 12,000 rpm for 10 min at 4°C and washed with 70% ethanol. The pellet was decanted from ethanol and then air-dried. DNA was finally suspended in 50 µl of ddH_20 and stored at 4°C.

6.3 Polymerase Chain Reaction (PCR) amplification and analysis

PCR was carried out in a DNA Thermal Cycler. The 25 µl PCR mixture contained: 1 µl total DNA template, 0.2mM of each deoxynucleotide triphosphate, 0.2 µM concentration of each oligonucleotide primer and 5U Taq DNA polymerase. Oligonucleotide primers of K5Un3, K3Un3, K5Un2 and K3Un2 (Kuo and Chak, 1996), universal primers bound to the specific sequence of various *cry*1-type genes, and the 24 specific primer pairs (Table 1) as described by Juarez-Perez *et al*. (1997) were used for identification of *cry*1 *cry*2 *cry*5 *cry*13A and *cry*14 gene families. The PCR condition was preheated at 95°C for 3 min. PCR amplication was performed for 30 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min. The PCR fragment was analyzed by electrophoresis on 1.2% agarose gel. The PCR product was observed under ultraviolet light and purified by using QIAGEN PCR purification kit (QIAGEN).

Table 1 Specific primer pairs used to the identification of *cry* genes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
5′end of *cry* gene	K5Un3 (CAATGCGTACCTTACAA-TTGTTTAAGTATT)	K3Un3 (CCTCCTGTAAATCCTGG-TCCT)
3′end of *cry* gene	K5Un2 (AGG ACCAGG TTTACA-GGAGG)	K3Un2 (GCTGTGACACGAAGGA-TATAGCCA)
*cry*1Aa	1Aa (TTCCCTTTATTTGGGAATGC)	I(-)(MDATYTCTAKRTCTTGACTA)
*cry*1Ab	1Ab(CGGATGCTCATAGAGGAGAA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ac	1Ac(GGAAACTTTCTTTTTAATGG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ad	1Ad(ACCCGTACTGATCTCAACTA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ae	1Ae(CTCTACTTTTTATAGAAACC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1B	1B(GGCTACCAATACTTCTATTA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1C	1C(ATTTAATTTACGTGGTGTTG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1D	1D(CAGGCCTTGACAATTCAAAT)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1E	1E(TAGGGATAAATGTAGTACAG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1F	1F (GATTTCAGGAAGTGATTCAT)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1G	1G (GCTTCTCTCCAAACAACG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1H	1H (ACTCTTTTCACACCAATAAC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1I	V(+)(ATGAAACTAAAGAATCCAGA)	V(AGGATCCTTGTGTTGAGATA)
*cry*1J	1J (GCGCTTAATAATATTTCACC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1K	1K (TGATATGATATTTCGTAACC)	I(-) (MDATYTCTAKRTCTTGACTA)

Table 1 (Continued)

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
*cry*2A	II(+)(TAAAGAAAGTGGGGAGTCTT)	II(-) (AACTCCATCGTTATTTGTAG)
*cry*5	(TAAGCAAAGCGCGTAACCTC)	(GCTCCCCTCGATGTCAATG)
*cry*12	(CTCCCCCAACATTCCATCC)	(AATTACTTACACGTGCCATACCTG)
*cry*13A	*cry*13A(d)	*cry*13A(r)
	(CTTTGATTATTTAGGTTTAGTTCAA)	(TTGTAGTACAGGCTTGTGATTC)
*cry*14	(ATAATGCGCGACCTACTGTTGT)	(TGCCGTTATCGCCGTTATT)

Remark M = A or C, D = A G or T, Y = T or C, K = G or T, R = A or G

6.4 PCR product purification

The PCR fragments were eluted from the gels and purified by using QIAGEN DNA purification kit (QIAGEN). The procedure of purification was performed according to the instruction manual provided by the company. The DNA fragments were excised from the agarose gel with the clean sharp razor blade. Gel products were bladed with razor on a fluorescent light box into small chunks and transferred to a microcentrifuge tube. The gel slices were weighed in a colorless tube and added 2.5 volumes of 6.6 M sodium iodide to 1 volume and mixed gels. The gel slices were incubated at 50°C until the gel was melted completely. The mixture solution of 1.5 volume of binding buffer was added and placed into a S.N.A.P.^{TM} purification column. The DNA was bound to the column and centrifuged at 5,000 g for 30 sec (repeat 3 times). The flow-through was discarded and column was placed black in the same collection tube. After that, the DNA pellet was added with 0.6 ml washing buffer to S.N.A.P. ^{TM} column and centrifuged at 5,000 g for 30 sec. The column was washed for another 2 times with washing buffer. The DNA pellet was centrifuged at 10,000 g for 1 min and column was placed into a clean microcentrifuge tube. The DNA pellet was eluted with 30 µl TE buffer and the column was centrifuged for 1 min at maximum speed. The product was analyzed by using

electrophoresis in 1.2% (w/v) agarose gel at 100 volt approximately 30 min prior to staining by ethidium bromide and was observed under ultraviolet light.

6.5 *cry* gene sequence

The purified PCR product was sequenced by 1st BASE Laboratories, Malaysia. Comparisons of nucleotide sequences were performed using standard BLAST sequence similarity searching program located at http://www.ncbi.nlm .nih.gov/BLAST/ against previously reported sequences in database.

7. Crystal protein purification

The crystal was purified from the cellular debris by the modified method described by Debro *et al.* (1986). Bacteria were grown at 30°C in NB for 36 to 48 hr. Spores and crystal proteins were recovered by centrifugation at 7000 g for 5 min at 4°C. After centrifugation, sporangial debris was removed from the pellet by washing with ice-cold deionized water. This was repeated up six times until spores and crystals were free from debris when viewed by phase-contrast microscopy. The final pellet was resuspended in deionized water. Spores and crystals were separated using differential ultracentrifugation through a discontinuous sucrose density gradient. A spore/crystal mixture was layered on top of a 10 ml discontinuous sucrose gradient, comprising of 5 ml each of 30% and 60 % (w/v) sucrose. Centrifugation was carried out at 80,000 g for 1 hr at 4°C. Crystals formed a major band at the interface between the 30% and 60 % (w/v) sucrose, while the spores formed a discrete pellet at the bottom of the tube. The crystal band was removed and washed three times in ice-cold deionized water by centrifugation at 10,000 g for 5 min at 4°C to remove the sucrose. The final pellet was resuspended in deionized water and stored frozen at -20°C. Purity of the crystal protein preparation was monitored by phase-contrast microscopy and by the production of bacterial colonies on NA agar plates (Stewart *et al.* 1981) with serial dilutions of crystal preparation. Protein concentration was measured by Bradford protein assay kit.

8. Protein profile of crystal protein (SDS-PAGE)

The molecular weight was estimated using SDS-PAGE (Appendix B) as described by Laemmli (1970) with a prestained SDS-PAGE standard broad range. The gel was run at a constant voltage (100 V) for 1 hr and was stained with 0.1% Coomassie Blue R-250 in 40% methanol, 10% acetic acid and de-stained with 10% acetic acid.

9. Insect host range test

Insecticidal activity of isolate was tested against eight economically important insects pests belonging to 3 Orders, Lepidoptera, Coleoptera and Diptera. The eight insect pests namely *Galleria mellonella* (Lepidoptera: Pyralidae), *Spodoptera litura*, *Spodoptera exigua* (Lepidoptera: Noctuidae), *Tenebrio molitor* and *Tribolium castaneum* (Coleoptera: Tenebrionidae), *Aedes aegypti* (Diptera: Culicidae), *Bactrocera dorsalis* (Diptera: Tephritidae) and *Musca domestica* (Diptera: Muscidae)

9.1 *Spodoptera litura, Spodoptera exigua* and *Galleria mellonella*

The larvae of *S. litura, S. exigua and G. mellonella* and artificial diet were taken from Department of Agriculture. Toxicity was assayed according Chanpaisaeng *et al.* (1996), five of second instar larvae were placed into a cup and were fed with an artificial sterile diet supplemented with one ml of high concentration of spore-crystal suspension.

9.2 *Tenebrio molitor* and *Tribolium castaneum*

Toxicity against *T. molitor* and *T. castaneum* was assayed according to Beron and Selerna (2006). One ml of high concentration of spore-crystal suspension was dropped onto the surface of steriled chicken food in each cup and allowed to dry. Subsequently, 5 sec instar larvae of insects were added into each cup.

9.3 *Aedes aegypti*

According to Beron and Salerno (2006), 1 ml of high concentration of spore-crystal suspension was added to a cup containing 100 ml distilled water. Twenty five second instar larvae of *A. aegypti* were placed in this cup.

9.4 *Bactrocera dorsalis*

Laboratory-reared *B. dorsalis* was supplied as pupae by Department of Agriculture. Toxicity test was performed on 5-day-old larvae 9 (2^{nd} instar). One ml of high concentration of spore-crystal suspension was transferred to a cup containing artificial larval food (Appendix A) (Sutantawong *et al*., 1985).

9.5 *Musca domestica*

For *M. domestica* bioassay according to Zhong *et al.* (2000), 1 ml of high concentration of spore-crystal suspensions was dispersed into sterile dog food and allowed to dry. The surface of the diet was scored to provide crevices for the larvae to begin feeding. Twenty neonate larvae were placed in each cup. Cups were covered with filter paper (Whatman No.3) secured with rubber bands.

The assay was replicated 3 times. Larval mortalities were recorded 24, 48 and 72 h. *B. thuringiensis* subsp. *kurstaki* (Bactospien) *B. thuringiensis* subsp. *israelensis* serotype H14 and *B. thuringiensis* subsp. *tenebrionis* (Novador) was used as the standard strain.

Place and duration

The studies were conducted in the laboratory of the Department of Entomology, Faculty of Agriculture and the laboratory of the Department of Biotechnology, Faculty of Agro – Industry, Kasetsart University, Bangkhen, Bangkok during Feburary 2007 – July 2010.

RESULTS AND DISCUSSION

1. Toxicity of *Bacillus thuringiensis*

1.1 Spore-crystal suspension

All 121 isolates of *B. thuringiensis* were screened for their toxicity against *P. xylostella* larvae. Initially, bioassay was performed by using crude suspension of *B. thuringiensis* containing spores and crystal proteins. Toxicity was expressed as mortality percentage. The collected isolates of *B. thuringiensis* were grouped according to their toxicity to *P. xylostella* as shown in Table 2. The results showed that all isolates of *B. thuringiensis* in this study were toxic to *P. xylostella*. However, their toxicities varied greatly. Two distinct levels of larval mortality were observed in this study: a high mortality of more than 80% and a low mortality of less than 50%. Twenty six *B. thuringiensis* isolates (21.48%) caused 100% mortality. They were isolates No. JCPT3, JCPT4, JCPT7, JCPT14, JCPT15, JCPT16, JCPT17, JCPT18, JCPT45, JCPT46, JCPT65, JCPT71, JCPT74, JCPT75, JCPT76, JCPT77, JCPT78, JCPT79, JCPT82, JCPT84, JCPT88, JCPT89, JCPT106, JCPT118, JCPT120 and JCPT121.

Twenty isolates (16.52%) of *B. thuringiensis* caused 90-99% mortality, including isolates No. JCPT8, JCPT9, JCPT10, JCPT11, JCPT12, JCPT13, JCPT43, JCPT44, JCPT62, JCPT67, JCPT73, JCPT87, JCPT101, JCPT102, JCPT107, JCPT109, JCPT110, JCPT112, JCPT113 and JCPT119. Twenty isolates (16.52%) that caused 80-89% were the isolates JCPT5, JCPT6, JCPT11, JCPT20, JCPT37, JCPT38, JCPT64, JCPT68, JCPT69, JCPT70, JCPT72, JCPT83, JCPT90, JCPT94, JCPT99, JCPT103, JCPT108, JCPT111, JCPT115 and JCPT116.

However, thirty-seven isolates that caused less than 50% mortality were considered to be ineffective. The 66 isolates causing more than 80% mortalities were selected as effective and used for the secondary screening test. The results suggested that *B. thuringiensis* isolates from Krabi province showed diversity of toxicity

according to Thaphan *et al*., (2008). Thaphan *et al*., (2008) reported that 121 isolates, the same isolate of this study, showed the wide range of toxicity to *Spodoptera litura* and *S. exigua* with mortality ranging from 0 to 100%. The result was also similar to that of Hong yu *et al*. (2000) who reported that *B. thuringiensis* 122 isolates from China showed toxicity against *S. exigua* with mortality ranging from 0 to 100%.

In this study, all *B. thuringiensis* isolates were found to be toxic to *P. xylostella* since the less toxicity caused only 30% mortality. It was important to note that Thai *B. thuringiensis* isolates showed high toxicity to *P. xylostella* than many isolates which showed no toxicity to *P. xylostella* (Gao *et al*., 2008; Maeda *et al*., 2000). Gao *et al.,* (2008) reported that about 40% of 570 *B. thuringiensis* isolated from soil in China showed no toxicity to *P. xylostella*. Maeda *et al*. (2000) reported that the 16 isolates from intertidal brackish sediments in mangroves showed no toxicity against *P. xylostella.*

1.2 Secondary test

For secondary test, 66 isolates of *B. thuringiensis* were assayed against the second-instar larvae of *P. xylostella* to determine LC_{50} values. The toxicities from different isolates of *B. thuringiensis* to *P. xylostella* are presented in Table 3. The results indicated that all isolates were toxic to *P. xylostella* while their toxicity varied greatly. LC_{50} values were in the ranges of 5.36×10^2 - 8.70×10^9 spores/ml. Fifty isolates showed LC_{50} values in the range of 10^6-10^9 spore/ml. These very high values indicated that those *B. thuringiensis* isolates were less toxic to *P. xylostella*.

The toxic isolates were the isolates No. JCPT76, JCPT106, JCPT118 and JCPT121 with the LC_{50} values of 8.52×10^3, 1.41×10^4, 7.03×10^3 and 5.36×10^2 spores/ml, respectively. Based on LC_{50} values, it could be concluded that the isolate JCPT121 was highly effective against *P. xylostella* since these isolates showed LC_{50} value of 6.80×10^2 spores/ml. The isolates No. JCPT106, JCPT118 and JCPT76 were also be considered effective against these species. As a result, 4 isolates were selected for study on UV tolerance.

The LC_{50} values of these strains were lower than those in the previous report using the same methodology. Chanpaisaeng *et al*. (2001) found that the highly effective *B. thuringiensis* named JC150 (*B. thuringiensis* subsp. *galleriae*) and JC590 (*B. thuringiensis* subsp. *kurstaki*) showed LC_{50} at 1.140×10^4 and 4.470×10^3 spores/ml, respectively against *P. xylostella*. The effective strain, *B. thuringiensis* JC590 could significantly control diamondback moth comparing to the control treatment without *B. thuringiensis* spray (Chanpaisaeng *et al*., 2001). Li *et al*. (2002) reported that the toxicities of *B. thuringiensis* K1 to *P. xylostella* were 1.6×10^5 cfu/ml. The result from this study indicated that JCPT121 had high potential as bioinsecticide against *P. xylostella*

The previous report of Thaphan *et al*., (2008) proposed to find the native *B. thuringiensis* isolates that showed high toxicity to 3 species of lepidopteran pest, *Spodoptera litura, Spodoptera exigua* and *P. xylostella*. This report did not mention about *B. thuringiensis* JCPT121 because it showed low toxicity to *S. litura* and *S. exigua*. *B. thuringiensis* showed toxicity against insect at different levels depending on strain due to different *cry* gene content, interactions among different crystal proteins, the expression level of the toxic genes (Cheng *et al*., 1999 and Del Rincón-Castro *et al*., 1999) and the other soluble toxins such as β-exotoxin (Porcar *et al*., 2000). For example, *B. thuringiensis* subsp. *kurstaki* HD-1 efficiently controlled diamondback moth but did not efficiently control *Spodoptera* species while *B. thuringiensis* products such as the *aizawai*-based Xentari (Abbott Laboratories) were introduced for the control of *Spodoptera* species (Porcar *et al*., 2000).

Table 2 Grouping of *B. thuringiensis* based on the toxicity of spore-crystal suspension to second-instar larvae of *Plutella xylostella*

Percentage of larval mortality	Bt isolates (No.)	Total
100	JCPT3, JCPT4, JCPT7, JCPT14, JCPT15, JCPT16, JCPT17, JCPT18, JCPT45, JCPT46, JCPT65, JCPT71, JCPT74, JCPT75, JCPT76, JCPT77, JCPT78, JCPT79, JCPT82, JCPT84, JCPT88, JCPT89, JCPT106, JCPT118, JCPT120, JCPT121	26
90-99	JCPT8, JCPT9, JCPT10, JCPT11, JCPT12, JCPT13, JCPT43, JCPT44, JCPT62, JCPT67, JCPT73, JCPT87, JCPT101, JCPT102, JCPT 107, JCPT109, JCPT110, JCPT112, JCPT113, JCPT119	20
80-89	JCPT5, JCPT6, JCPT11, JCPT20, JCPT37, JCPT38, JCPT64, JCPT68, JCPT69, JCPT70, JCPT72, JCPT83, JCPT90, JCPT94, JCPT99, JCPT103, JCPT108, JCPT111, JCPT115, JCPT116	20
70-79	JCPT 34, JCPT 40, JCPT 85, JCPT 86, JCPT 95, JCPT105	6
60-69	JCPT 23, JCPT 35, JCPT 41, JCPT 42, JCPT47, JCPT48, JCPT 49, JCPT50, JCPT81, JCPT92, JCPT 96, JCPT104	12
50-59	JCPT 2, JCPT 5, JCPT 26, JCPT 28, JCPT 36, JCPT 39, JCPT 91, JCPT 114	8
Below 50	JCPT 1, JCPT 2, JCPT 11, JCPT 21, JCPT 22, JCPT 24, JCPT 25, JCPT 26, JCPT 27, JCPT 28, JCPT 29, JCPT 30, JCPT 31, JCPT 32, JCPT 33, JCPT 36, JCPT 39, JCPT 52, JCPT 53, JCPT 54, JCPT 55, JCPT 56, JCPT 57, JCPT 58, JCPT 59, JCPT 60, JCPT 61, JCPT 63, JCPT 68, JCPT 80, JCPT 91, JCPT 93, JCPT 97, JCPT 98, JCPT 100, JCPT 114, JCPT 117	37

Table 3 Lethal concentration of spore-crystal proteins of *Bacillus thuringiensis* to *Plutella xylostella*

Isolates No.	LC_{50} (spore/ml)	95% fiducial limits		slope
		Lower	Upper	
JCPT3	2.65×10^5	-[1/]	-	0.74 ± 0.19
JCPT4	7.73×10^6	-	-	0.25 ± 0.16
JCPT 5	4.77×10^7	6.66×10^6	1.51×10^8	0.63 ± 0.19
JCPT 6	5.35×10^7	7.64×10^6	1.76×10^8	0.62 ± 0.18
JCPT 7	2.98×10^5	-	-	0.18 ± 0.16
JCPT 8	3.11×10^7	6.76×10^6	7.77×10^7	0.70 ± 0.18
JCPT 9	1.62×10^7	2.54×10^6	4.07×10^7	0.74 ± 0.19
JCPT 10	3.58×10^7	-	-	0.12 ± 0.16
JCPT 11	2.37×10^7	-	-	0.13 ± 0.16
JCPT 12	5.78×10^7	-	-	0.13 ± 0.16
JCPT 13	1.72×10^7	7.10×10^5	3.16×10^7	1.27 ± 0.27
JCPT 14	3.42×10^5	-	-	0.21 ± 0.16
JCPT 15	5.67×10^6	-	-	0.12 ± 0.16
JCPT 16	9.84×10^5	2.75×10^5	6.53×10^7	0.54 ± 0.18
JCPT 17	6.35×10^8	-	-	0.14 ± 0.17
JCPT 18	2.20×10^5	-	-	0.08 ± 0.16
JCPT 20	3.94×10^7	7.55×10^5	1.81×10^8	0.47 ± 0.18
JCPT 35	2.15×10^8	-	-	0.18 ± 0.17
JCPT 37	8.92×10^6	0.002	5.10×10^7	0.36 ± 0.17
JCPT 38	9.36×10^7	-	-	0.14 ± 0.17
JCPT 43	3.35×10^7	7.83×10^5	1.03×10^8	0.91 ± 0.28
JCPT 44	1.33×10^8	3.36×10^7	2.84×10^8	0.19 ± 0.23
JCPT 45	4.21×10^8	1.47×10^8	3.90×10^9	0.60 ± 0.17
JCPT 46	5.00×10^7	3.11×10^5	3.32×10^8	0.38 ± 0.16
JCPT 62	2.66×10^8	-	-	0.29 ± 0.13

Table 3 (Continued)

Isolates No.	LC50 (spore/ml)	95% fiducial limits		slope
		Lower	Upper	
JCPT 64	3.23×10^7	1.36×10^6	1.21×10^8	0.54 ± 0.18
JCPT 65	4.64×10^5	-	-	0.88 ± 0.22
JCPT 66	9.03×10^5	5.93×10^5	2.63×10^7	0.70 ± 0.19
JCPT 67	1.21×10^7	9.10×10^5	3.55×10^7	0.73 ± 0.20
JCPT 68	9.06×10^7	2.55×10^7	2.47×10^8	0.80 ± 0.20
JCPT 70	5.20×10^7	1.16×10^7	1.40×10^7	0.75 ± 0.19
JCPT 71	9.89×10^5	-	-	0.17 ± 0.16
JCPT 72	2.18×10^8	-	-	0.38 ± 0.23
JCPT 73	9.30×10^6	1.37×10^6	2.25×10^7	0.88 ± 0.22
JCPT 74	9.62×10^6	-	-	0.08 ± 0.16
JCPT 75	2.55×10^6	3.81×10^5	3.61×10^8	0.39 ± 0.17
JCPT 76	8.52×10^3	1.65×10^2	2.96×10^4	0.58 ± 0.18
JCPT 77	7.14×10^6	1.79×10^6	1.65×10^9	0.46 ± 0.17
JCPT 78	1.27×10^6	-	-	0.28 ± 0.17
JCPT 79	2.63×10^6	-	-	0.33 ± 0.17
JCPT 82	3.08×10^6	-	-	0.14 ± 0.08
JCPT 83	4.75×10^9	-	-	0.31 ± 0.11
JCPT 84	2.18×10^6	-	-	0.21 ± 0.18
JCPT 87	1.77×10^7	2.85×10^6	4.47×10^7	0.73 ± 0.19
JCPT 88	5.09×10^5	-	-	0.73 ± 0.48
JCPT 89	1.17×10^6	-	-	0.11 ± 0.18
JCPT 90	3.34×10^8	4.61×10^7	9.67×10^8	0.59 ± 0.17
JCPT 94	5.34×10^7	1.37×10^7	1.35×10^8	0.81 ± 0.20
JCPT 99	1.00×10^8	3.33×10^7	2.43×10^8	0.92 ± 0.21
JCPT 101	3.23×10^7	1.62×10^4	1.60×10^8	0.53 ± 0.19

Table 3 (Continued)

Isolates No.	LC50 (spore/ml)	95% fiducial limits		slope
		Lower	Upper	
JCPT 102	1.39×10^{7}	3.62×10^{2}	8.71×10^{7}	0.57 ± 0.21
JCPT 103	2.98×10^{7}	3.79×10^{6}	8.56×10^{7}	0.59 ± 0.17
JCPT 106	1.41×10^{4}	6.90×10^{2}	6.40×10^{5}	0.41 ± 0.18
JCPT 107	7.37×10^{7}	3.21×10^{7}	1.57×10^{8}	0.91 ± 0.18
JCPT 108	1.16×10^{7}	2.34×10^{5}	4.04×10^{7}	0.54 ± 0.18
JCPT 109	1.57×10^{7}	1.59×10^{6}	4.36×10^{7}	0.66 ± 0.18
JCPT 110	4.02×10^{7}	1.88×10^{7}	7.51×10^{7}	1.13 ± 0.21
JCPT 111	8.50×10^{6}	3.04×10^{4}	3.43×10^{7}	0.50 ± 0.17
JCPT 112	1.62×10^{7}	2.54×10^{6}	4.07×10^{7}	0.74 ± 0.19
JCPT 113	2.23×10^{7}	8.21×10^{6}	4.40×10^{7}	1.05 ± 0.22
JCPT 115	9.64×10^{6}	3.93×10^{4}	3.84×10^{5}	0.49 ± 0.17
JCPT 116	8.70×10^{9}	-	-	0.08 ± 0.16
JCPT 118	7.03×10^{3}	57	7.22×10^{4}	0.41 ± 0.18
JCPT 119	1.78×10^{7}	1.12×10^{6}	5.19×10^{7}	0.59 ± 0.17
JCPT 120	1.53×10^{5}	-	-	0.21 ± 0.16
JCPT 121	5.36×10^{2}	0	5.01×10^{3}	0.65 ± 0.29
Bt.*kurstaki*	3.10×10^{2}	0	3.95×10^{3}	0.66 ± 0.33

Remark [1/] fiducial limit could not be generated

2. UV tolerance

B. thuringiensis namely JCPT76 JCPT106 JCPT118 and JCPT121 were all exposed to UV rays of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min. Figure 8 shows the relationship between viability of exposed *B. thuringiensis* and the UV exposure time. Viable counts of the untreated spore-crystal protein suspension were served as the controls.

Viability of all isolates before exposed to UV ray was 10^8 cfu/ml. Figure 8, shows viability of cell and spore to be rapidly decreased about 10^2 cfu/ml after the spore-crystal suspensions exposed to UV ray at 5 min. Viabilities of cell and spore decreased with the increasing exposure times. At 60 min of UV exposed, JCPT121 was observed to have higher viability than JCPT76, JCPT106 and JCPT118 with 55.10 ± 0.16, 4.51 ± 0.16, 4.95 ± 0.04 and 4.45± 0.13 CFU/ml, respectively while the viability *B. thuringiensis* subsp. *kurstaki* (standard) was 4.91± 0.11 cfu/ml (Appendix Figure C1-5).

Some isolates such as JCPT 118, the viability curve was rough which might be caused by the effect of bacterial aggregation as reported by Coohill and Sagripanti (2008). Bacterial aggregation acted as shields to those further downstream from the beam or in the core of a large clump. For example, at 254 nm a bacterium of 1μm in diameter transmitted 61% incident beam if two bacteria shielded a third. Only 37% incident beam would reach the surface of the third bacterium. Furthermore, the error of laboratory techniques might affect the survival curve such as dilution techniques and spread plate techniques.

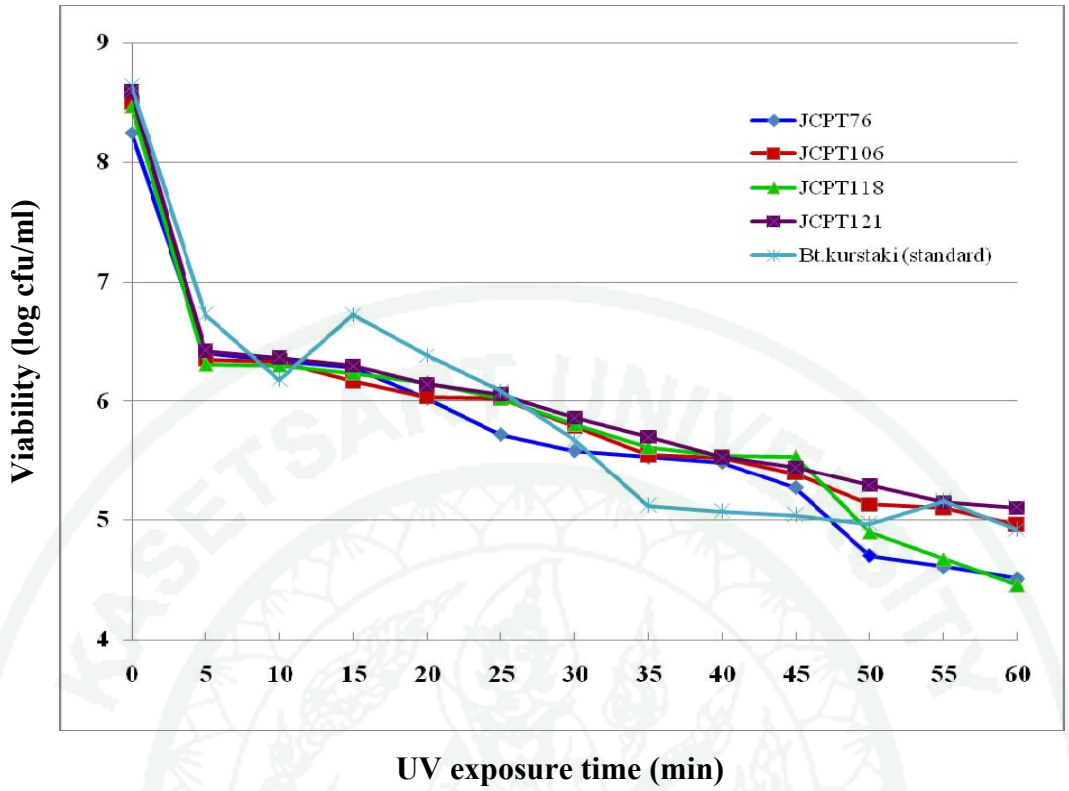

Figure 8 Viability of *Bacillus thuringiensis* exposed to UV ray at interval time.

Viability of all isolates decreased after 5 min of UV exposure. Similarly to Cokmus *et al*., (2000) who reported viability of *Bacillus sphaericus* to be lost after 2.5-4.5 min of UV exposure and larvicidal activity of *Culex quinquefasciatus* larvae was lost when exposed for 12 hours. However viability of JCPT121 was higher than *B. thuringiensis* subsp. *kurstaki* used as the parent strain for study UV tolerant of *B. thuringiensis* -mutant (Saxena *et al.*, 2002). Saxena *et al*. (2002) reported that viability of the parent strain (*B. thuringiensis* subsp. *kurstaki*) lost 80–90% of its colony-forming ability after 2 and 16 min irradiation, respectively, whereas with the same time JCPT121 lost around 30% of its colony-forming ability.

Tolerance to UV ray is the unique characteristic of *Bacillus* for surviving in the environment. The mechanism for protection against UV in some strain is the production of DNA-binding α/β-type small acid-soluble proteins (SASPs) which is a major factor in resistance and long-term survival of spores of *Bacillus* species by protecting DNA spore against damage due to desiccation, heat, toxic chemicals,

enzymes, and UV radiation. (Setlow, 2007; Nicholson *et al*., 2005). Another potential mechanism for protection against UV is the accumulation of absorbing pigments, generally in the spore's outer layers, the coats and outer membrane in particular (Riesenman and Nicholson, 2000; Hullo *et al.,* 2001). There is certain evidence with growing bacteria that pigments in outer layers can protect against UV (Imshenetsky *et al.,* 1979), presumably by absorbing the radiation before it penetrates to the DNA in the spore core or protoplast.

In *Bacillus subtilis* spores, the formation of coat pigment is due to the *cotA* gene product, a copper-dependent laccase generating a probable melanin-like pigment, and melanic pigments shielding microbial cells against UV radiation. Deletion of the *cotA* gene results in albino spores, and these spores are significantly more sensitive to artificial UVB, UVA, and simulated solar radiation than are the spores of its pigmented parent (Hullo *et al.,* 2001). Spores of a *B. thuringiensis* strain that produces a melanin pigment are also significantly more resistant to UVC and 366 nm radiation than are spores of the nonpigmented parent strain (Saxene *et al*., 2002)

According to the primary and secondary screening test, *B. thuringiensis* JCPT121 showed the highest toxicity with LC_{50} 5.36×10^2 cfu/ml to *P. xylostella* and was tolerant to UV ray more than those isolates. For this reason, *B. thuringiensis* JCPT121 was, therefore, chosen as the candidate for study on morphology of cell spore and crystal protein, biochemical characteristics, *cry* genes content, protein profile and insect host range test.

2. Characterization of *Bacillus thuringiensis* JCPT121

2.1 Morphology of cell spore and crystal protein of *B. thuringiensis*

Morphology of cell, spore and crystal proteins of *B. thuringiensis* JCPT121 was studied using scanning electron microscope. The bacterial vegetative cells were rod shape with 0.71 to 0.95 µm wide and 2.26 to 4.04 µm long whereas spore size was 0.75 to 1.00 µm wide and 1.56 to 2.25 µm long. Majority of the *B. thuringiensis* JCPT121 investigated produced bipyramidal shaped crystal of various sizes with 0.5 to 1.37 µm wide and 0.75 to 0.87 µm long (Figure 9).

The size of cell and spore of *B. thuringiensis* JCPT121 was similar to those investigated by many researchers. Attathom *et al*., (1994) reported that the vegetative cells of their *B. thuringiensis* strains were rod shape of 0.8 to 1.5 µm wide and 3.0 to 4.5 µm long and frequently occurred in chains. The oval spores of 0.5 to 0.8 µm wide and 1.0 to 2.0 µm long were found to be produced subterminally with occasional swelling. Isanont (1996) reported that the vegetative cells of *B. thuringiensis* designated as isolates No. 28, 82, 96, 99, 121 and 292 could be measured as 1.02 × 2.90, 0.80 × 2.75, 0.94 × 3.20, 1.26 × 2.90, 0.98 × 2.74 and 0.80 × 2.76 µm, respectively.

B. thuringiensis JCPT121 produced bipyramidal crystals protein. It is well recognized that most *B. thuringiensis* strains produce bipyramidal crystals and within a strain, different size of bipyramidal crystals can be found. Attathom *et al*. (1995) also stated that in general, bipyramidal shaped were typical of *B. thuringiensis* crystal but varied greatly in size among and within subspecies. Isanont *et al.,* (1996) suggested that the bipyramidal shape crystals exhibited great variation in size within and among *B. thuringiensis* isolates, but the cuboidal or spherical shaped crystals exhibited little variation in size.

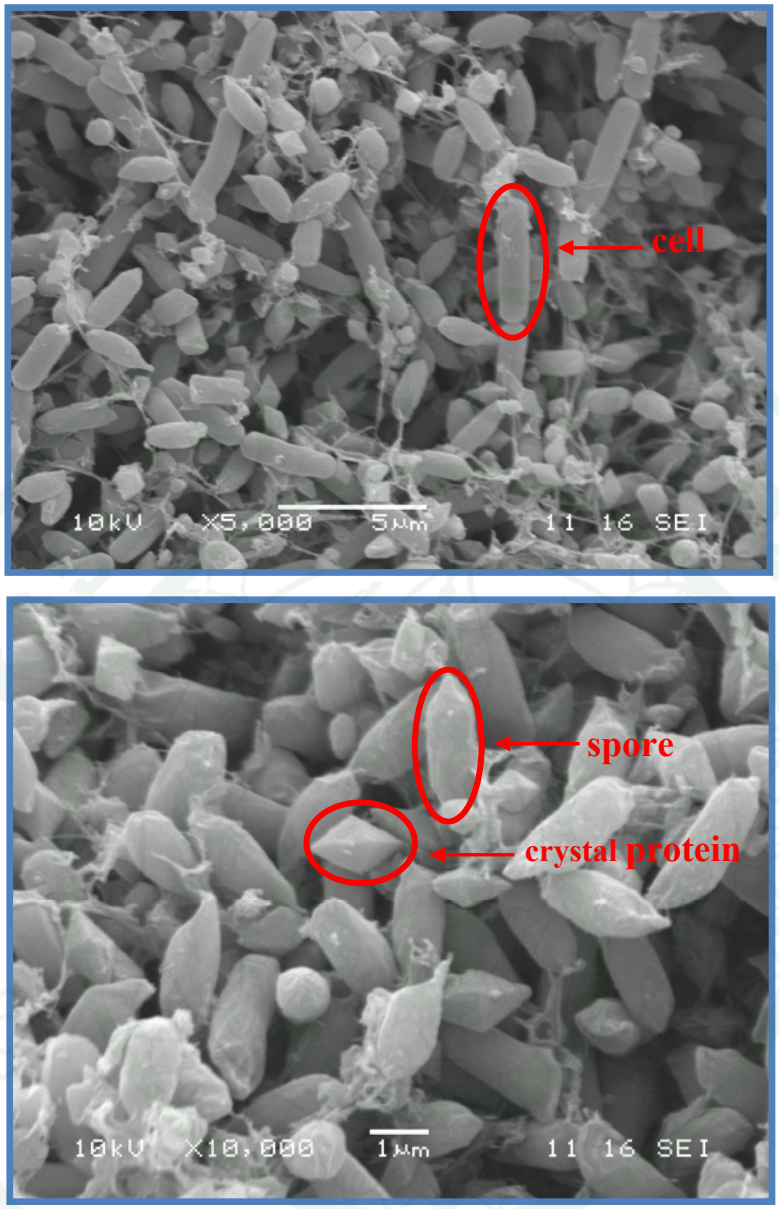

Figure 9 Cell, spore and crystal proteins of *Bacillus thuringiensis* JCPT121 by scanning electron microscope.

2. 2 Biochemical characterization test

As shown in Table 4. *B. thuringiensis* JCPT121 was determined to be rod, Gram-positive, catalase positive and motile. Based on comparison of their characteristics with Bergey's manual, *B. thuringiensis* JCPT121 could be identified as genus *Bacillus.* For species determination, the method using biochemical technique was applied in identifying the type of *B. thuringiensis* JCPT121. These isolates were subjected to carbohydrate fermentation pattern analysis by API 50 CHB test strip. Carbohydrate fermentation patterns indicated that *B. thuringiensis* JCPT121 was able to ferment Glycerol, D-Arabinose, D-Ribose, D-Galactose, D-Glucose, D-Fructose, N-Acetylglucosamine, Amygdalin, Arbutin, Esculin ferric citrate, Salicin, D-Celiobiose, D-Maltose, D-Saccharose (Sucrose), D-Treharose, and Glycogen (Table 5). By using API database correlation version 4.0, this isolate which exhibited a rate of 99.7% identities, was identified as belonging to the species of *B. cereus*. The method was unable to differentiate *B. thuringiensis* from *B. cereus*. In this case, the additional test, observation for the crystal proteins, was then performed and found the isolate to produce crystal protein. According to morphology, physiological, carbohydrate fermentation tests, this could be identified as *B. thuringiensis*

Table 5 shows the API profile of *B. thuringiensis* JCPT121 to be similar to that of Logan and Berkeley (1984). They identified of *Bacillus* strains using the API system and suggested that API system was unable to separate *B. thuringiensis* and *B. cereus*. The ability to ferment carbohydrate was different among *B. thuringiensis* isolates as indicated by Hernandez *et al.* (1998). They reported that *B. thuringiensis* subsp. *konkukian* (serotype H34) was utilized only trehalose, maltose and glucose. Swiecicka *et al*. (2002) stated that *B. thuringiensis* strains isolated from intestines of small, free-living mammals (Rodentia and Insectivora) from Poland were positive tested for galactosidase, D-glucose, D-fructose, ribose, maltose, N-acetyloglucosamine, trehalose, starch, glycogen fermentation.

Table 4 Characteristics of *Bacillus thuringiensis* JCPT121

Test	Result
Morphology	rod
Gram's stain	+
Catalase	+
Motility	+
Spore	+
Crystal proteins	+

Table 5 Carbohydrate fermentation patterns of *Bacillus thuringiensis* JCPT121

Test	*Bacillus thuringiensis* JCPT121	*Bacillus thuringiensis*	*Bacillus cereus*
1. Glycerol	+	+	+
2. Erythriol	-	-	-
3. D-Arabinose	+	-	-
4. L-Arabinose	-	-	-
5. D-Ribose	+	+	+
6. D-Xylose	-	-	-
7. L-Xylose	-	-	-
8. D-Adonitol	-	-	-
9. Methyl-βD-Xylopyranoside	-	-	-
10. D-Galactose	+	-	+/-
11. D-Glucose	+	+	+
12. D-Fructose	+	+	-
13. D-Mananose	-	-	-
14. L-Sorbose	-	-	-
15. L-Rhamnose	-	-	-

Table 5 (Continued)

Test	*Bacillus thuringiensis* JCPT121	*Bacillus thuringiensis*	*Bacillus cereus*
16. Dulcitol	-	-	-
17. Inositol	-	-	+/-
18. D-Manitol	-	-	-
19. D-Sorbitol	-	-	-
20. Methyl-αD-Mannopyranoside	-	-	-
21. Methyl-αD-Glucopyranoside	-	+/-	+/-
22. N-Acetylglucosamine	+	+	+
23. Amygdalin	+	+/-	+/-
24. Arbutin	+	+	+
25. Esculin ferric citrate	+	+	+
26. Salicin	+	+	+
27. D-Celiobiose	+	+	+
28. D-Maltose	+	+	+
29. D-Lactose (bovine origin)	-	-	+/-
30. D-Melibiose	-	-	-
31. D-Saccharose (Sucrose)	+	+/-	+/-
32. D-Treharose	+	+	+
33. Inulin	-	-	-
34. D-Melezitose	-	+/-	+/-
35. D-Raffinose	-	+/-	-
36. Amidon (starch)	-	+	+
37. Glycogen	+	+	+
38. Xylitol	-	-	-
39. Gentiobiose	-	+/-	+/-
40. D-Turanose	-	+/-	+/-

Table 5 (Continued)

Test	*Bacillus thuringiensis* JCPT121	*Bacillus thuringiensis*	*Bacillus cereus*
41. D-Lyxose	-	-	-
42. D-Tagatose	-	-	-
43. D-Fucose	-	-	-
44. L-Fucose	-	+/-	-
45. D-Arabitol	-	-	-
46. L-Arabitol	-	-	-
47. Potassium gluconate	-	+/-	-
48. Potassium 2-ketogluconate	-	-	-
49. Potassium 5-ketogluconate	-	-	-

Remark +, acid production (positive)
-, no acid produced

3. *cry* gene detection by polymerase chain reaction (PCR)

3.1 Identification by universal primers

The detection of *cry*1, *cry*2, and *cry*9 genes in *B. thuringiensis* JCPT 121 isolate selected for this study was accomplished using primer pairs specific to each *cry* gene as described in Table 1. Two pairs of universal primers designed by Kuo and Chak (1996) were used to detect *cry* genes by the size of their PCR products. The chromosomal DNA and plasmid DNA of *B. thuringiensis* JCPT 121 isolates were served as template in reactions (Figure 10). The result showed that chromosomal DNA and plasmid DNA of *B. thuringiensis* JCPT 121 isolates reacted positively to K5Un2 and K3Un2 by producing a fragment 1600 bp, and to K3Un3 and K3Un3 by producing a fragment of 1400 bp (Figure 11).

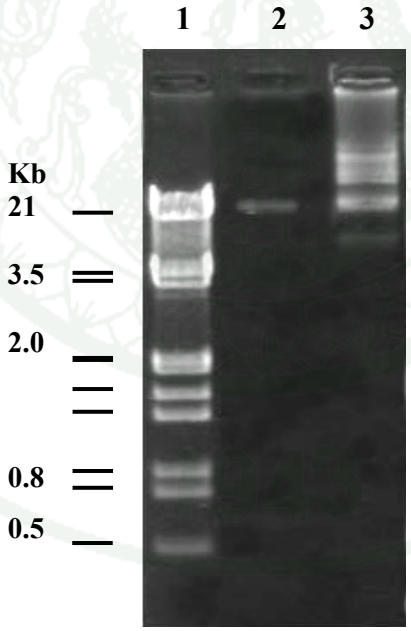

Figure 10 Agarose gel (0.8%) electrophoresis of chromosomal DNA and plasmid DNA of *B. thuringiensis* JCPT121. Lane 1, molecular weight markers (λ DNA cleaved by *Hind* III and *Eco*RI) with sizes (in kilobases) as indicated on the left. Lane 2, chromosomal DNA. Lane 3, plasmid DNA.

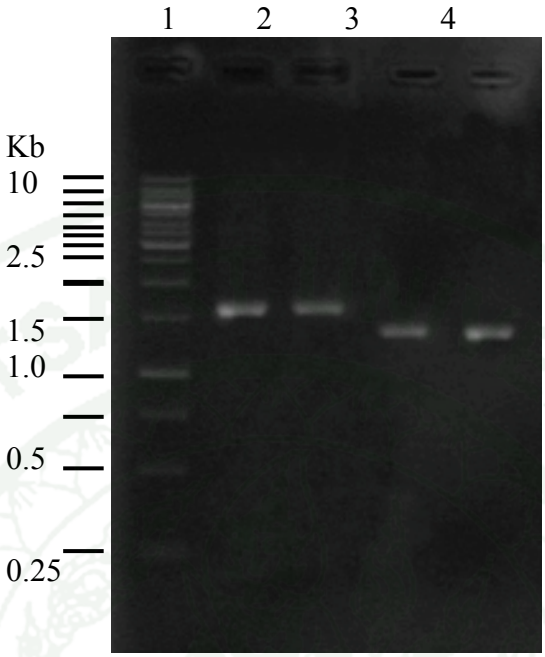

Figure 11 Agarose gel (1%) electrophoresis of PCR products amplified from *B. thuringiensis* JCPT121 with universal primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2, plasmid DNA of *B. thuringiensis* JCPT121 with K5Un2 and K3Un2 (1600 bp). Lane 3, chromosomal DNA of *B. thuringiensis* JCPT121 with K5Un2 and K3Un2 (1600 bp). Lane 4, plasmid DNA of *B. thuringiensis* JCPT121 with K5UN3 and K3UN3 (1400 bp). Lane 5, chromosomal DNA of *B. thuringiensis* JCPT121 with K5Un3 and K3Un3 (1400 bp).

3.2 Identification by specific primers

The specific primers designed by Juarez-Perez and Ferrandis (1997) were used to identify 20 genes (Table 1). The results showed that on chromosomal DNA, PCR products of *cry*1Ac, *cry*1I, *cry*2A and *cry*14 gene were detected (Figure

12-14) whereas PCR products of *cry*1I and *cry*2A gene were found on plasmid DNA (Figure 15-16).

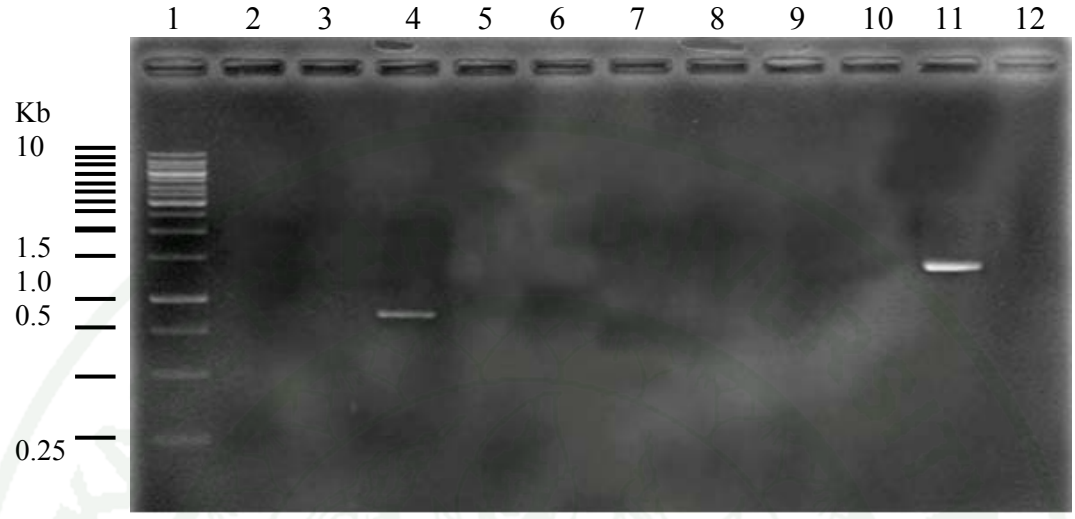

Figure 12 Agarose gel (1%) electrophoresis of PCR products amplified from chromosomal DNA of *B. thuringiensis* JCPT121 with specific primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2 to 12 , PCR products obtained from chromosomal DNA. Lane 2, primers for *cry*1Aa gene. Lane 3, primers for *cry*1Ab gene. Lane 4, primers for *cry*1Ac gene. Lane 5, primers for *cry*1Ad gene. Lane 6, primers for *cry*1Ae gene. Lane 7, primers for *cry*1B gene. Lane 8, primers for *cry*1C gene. Lane 9, primers for *cry*1D gene. Lane 10, primers for *cry*1E gene. Lane 11, positive control. Lane 12, water control.

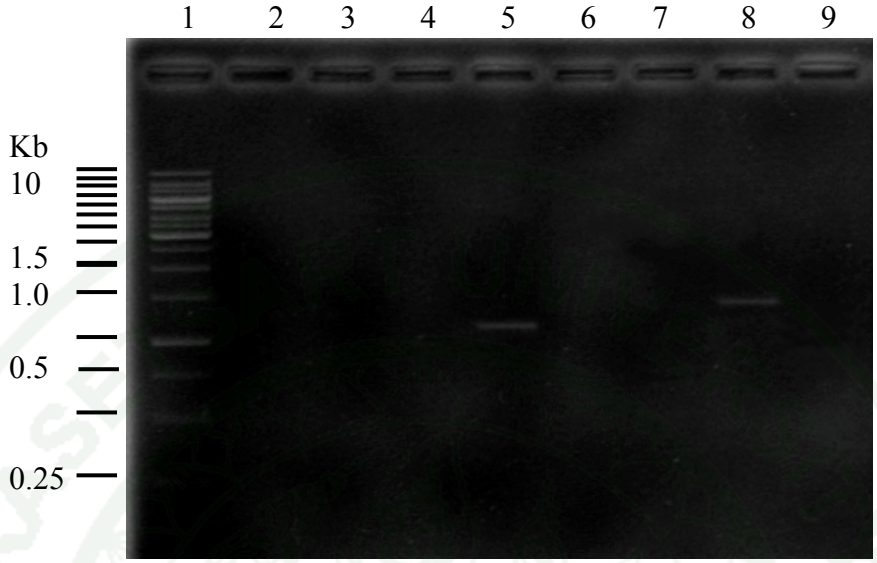

Figure 13 Agarose gel (1%) electrophoresis of PCR products amplified from chromosomal DNA *B. thuringiensis* JCPT121 with specific primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2 to 9, PCR products obtained from chromosomal DNA. Lane 2, primers for *cry*1F gene. Lane 3, primers for *cry*1G gene. Lane 4, primers for *cry*1H gene. Lane 5, primers for *cry*1I gene. Lane 6, primers for *cry*1J gene. Lane 7, primers for *cry*1K gene. Lane 8, positive control. Lane 9, water control.

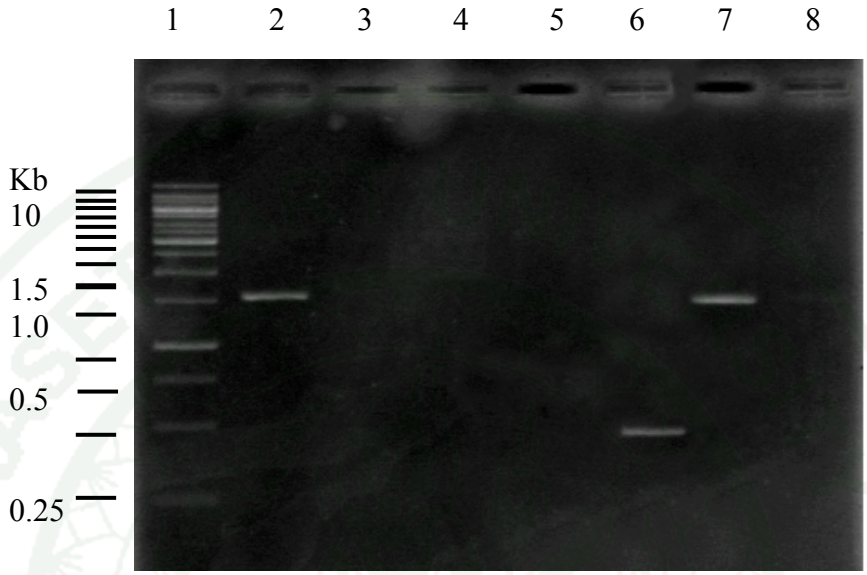

Figure 14 Agarose gel (1%) electrophoresis of PCR products amplified from chromosomal DNA *B. thuringiensis* JCPT121 with specific primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2 to 7, PCR products obtained from chromosomal DNA. Lane 2, primers for *cry*2A gene. Lane 3, primers for *cry*5 gene. Lane 4, primers for *cry*12 gene. Lane 5, primers for *cry*13A gene. Lane 6, primers for *cry*14 gene. Lane 7, positive control. Lane 8, water control.

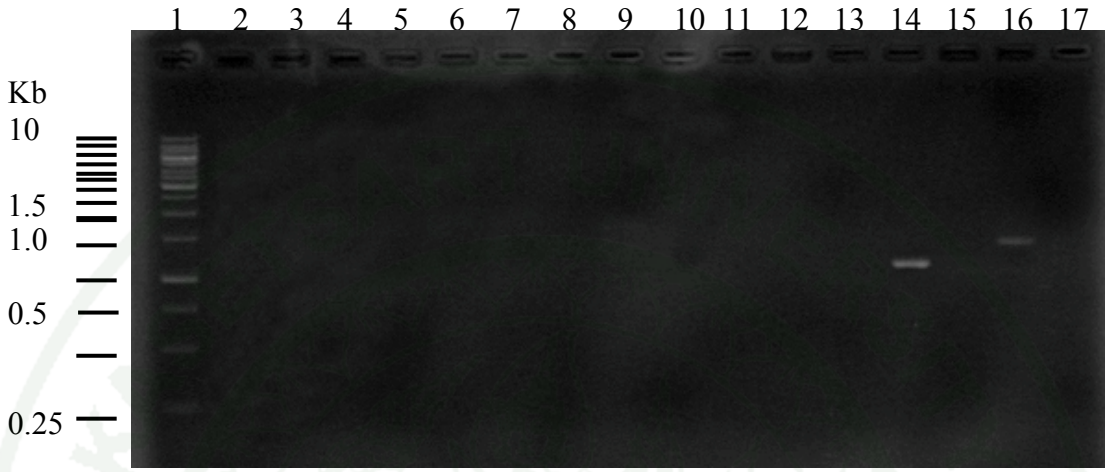

Figure 15 Agarose gel (1%) electrophoresis of PCR products amplified from plasmid DNA *B. thuringiensis* JCPT121 with specific primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2 to PCR products obtained from plasmid DNA. Lane 2, primers for *cry*1Aa gene. Lane 3, primers for *cry*1Ab gene. Lane 4, Lane 5, primers for *cry*1Ad gene. Lane 6, primers for *cry*1Ae gene. Lane 7, primers for *cry*1B gene. Lane 8, primers for *cry*1C gene. Lane 9, primers for *cry*1D gene. Lane 10, primers for *cry*1E gene. Lane 11, primers for *cry*1F gene. Lane 12, primers for *cry*1G gene. Lane 13, primers for *cry*1H gene. Lane 14, primers for *cry*1I gene. Lane 15, primers for *cry*1J gene. Lane 16, positive control. Lane 17, water control.

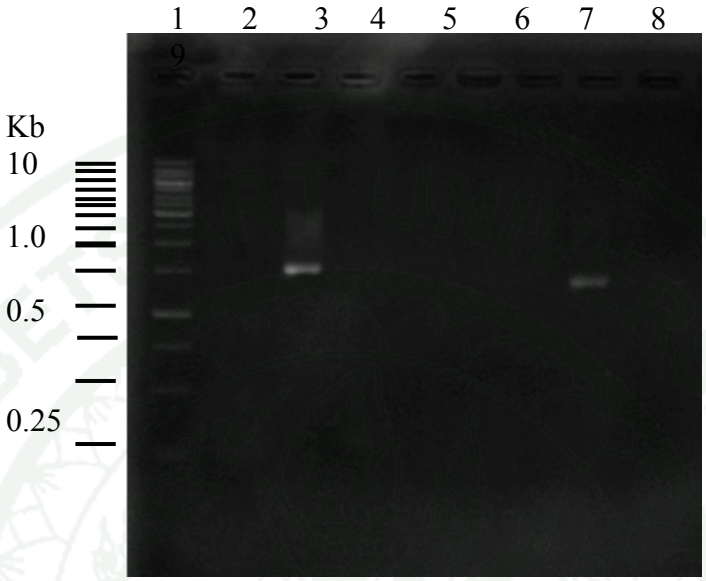

Figure 16 Agarose gel (1%) electrophoresis of PCR products amplified from plasmid DNA *B. thuringiensis* JCPT121 with specific primers. Lane 1, molecular weight markers (1 kb) with sizes (in kilobases) as indicated on the left. Lane 2 to 9, PCR products obtained from chromosomal DNA. Lane 2, primers for *cry*1K gene. Lane 3, primers for *cry*2A gene. Lane 4, primers for *cry* 5gene. Lane 5, primers for *cry*12 gene. Lane 6, primers for *cry*13A gene. Lane 7, primers for *cry*14 gene. Lane 8, positive control. Lane 9, water control.

The PCR products of four *cry* genes were sequenced in order to confirm the correction of PCR amplification. The sequences obtained from 1st Base laboratories showed that in chromosomal DNA, PCR product of *cry*1Ac, *cry*1I *cry*2A and *cry*14 were found to have 767, 1,070, 1,059 and 595 bp, respectively. In plasmid DNA, PCR product of *cry*1I and *cry*2A consisted of 1,071, and 1,035 bp, respectively (Appendix Figure D1-D6).

The size of PCR product obtained in accordance with Juarez-Perez *et al*. (1997) who reported that PCR product expected of IAc and I(-) primer was 844 bp. Masson *et al*. (1998) reported that PCR product of *cry*1I obtained from V(+) and V(-) primer was 1,137 bp and PCR product of *cry*2A obtained from II(+) and II(-) primer was 1,556 bp. Ejiofor *et al*. (2002) reported that PCR product expected of *cry*14 from *cry*14F and *cry*14R primer was 456 bp.

Comparisons of nucleotide sequences of four *cry* genes - *cry*1Ac, *cry*1I *cry*2A and cry14, were performed against GenBank database program retrieved from http://www.ncbi.nlm.nih.gov/BLAST/. The result showed that the sequences of the chromosomal DNA *cry*1Ac had high homology to *cry*1Ac gene (HM061081.1) with 99% identity. Sequences of *cry*1I gene had high homology to *cry*1Ia genes (EU887515.1), 99% identities for chromosomal DNA and 99% identities for plasmid DNA. Sequences of *cry*2A gene had high homology to *cry*2Ab genes (AF441855.1), 94% identities for chromosomal DNA and 92% identities for plasmid DNA. Sequences of the chromosomal DNA *cry*14 obtained had high homology to Tryptophan 2,3-dioxygenase gene (ZP04174944.1) with 98% identity. The results obtained by using Blast-N program showed that sequences of three *cry* genes as *cry*1Ac, *cry*1I and *cry*2A expressed high homology to those *cry* genes (Table 6, Table 7 and Appendix Table D1-D6). Sequencing of *cry*14 had highly homology to Tryptophan 2,3-dioxygenase gene (ZP04174944.1) (Appendix Table D4) which suggested that the sequences of *cry*14 gene was not *cry*14 of *B. thuringiensis*. The result was similar to that of Poojitkanon *et al*, (2008) who detected *cry*14 on chromosomal DNA of *B. thuringiensis* JC590 and found that the sequences of *cry*14 PCR products were not *cry* gene groups in database.

Sequence comparison on two *cry* gene as *cry*1I and *cry*2A gene located on both chromosomal DNA and plasmid DNA were aligned using EMBOSS in order to compare these genes between chromosomal DNA and plasmid DNA. The sequences of *cry*1I and *cry*2A had high homology with 98 % and 94 % sequences identity, respectively. (Table 8, Appendix Figure D7-D8)

Table 6 Sequences identity of PCR products on chromosomal DNA using Blast-N program

PCR product of gene	Identity (%)	Score	E-value	Reference gene	Accession Number
*cry*1Ac	99	1406	0	*cry*1Ac *Bacillus thuringiensis* strain S3299-1	GU446674.1
*cry*1I	99	1958	0	*cry*1Ia *Bacillus thuringiensis* isolate Bt11 (cry1Ia)	EU887515.1
*cry*2A	94	1600	0	*cry*2Ab *Bacillus thuringiensis* ly30	AF441855.1
*cry*14	98	1115	0	Tryptophan 2,3-dioxygenase *Bacillus cereus* AH1273	ZP04174944.1

Table 7 Sequences identity of PCR products on plasmid DNA using Blast-N program

PCR product of gene	Identity (%)	Score	E-value	Reference gene	Accession Number
*cry*1I	99	1962	0	*cry*1Ia *Bacillus thuringiensis* isolate Bt11 (cry1Ia)	EU887515.1
*cry*2A	92	1515	0	*cry*2Ab *Bacillus thuringiensis* ly30	AF441855.1

Table 8 Sizes of PCR products of *cry* genes and sequences alignment of *cry* genes between chromosomal DNA and plasmid DNA using EMBOSS Pairwise Alignment Algorithms.

Gene	Sizes (bp) on chromosomal DNA	Sizes (bp) on plasmid DNA	Identity (%)	Gap (%)	Score
*cry*1I	1070	1071	98.2	1.6	5254
*cry*2A	1059	1035	91.1	4.5	4619

B. thuringiensis JCPT121 expressed high effienciency against diamondback moth larvae when compared with 120 *B. thuringiensis* isolates. PCR analyses with specific primers effective to insect in Order Lepidoptera found chromosomal DNA to carry 3 genes; *cry*1Ac, *cry*1I and *cry*2A, and plasmid DNA carried 2 genes; *cry*1I and *cry*2A. Tabashnik *et. al.* (1994) also reported that these genes in *B. thuringiensis* JCPT121 (*cry*1Ac, *cry*1I and *cry*2A) had high toxicity to diamondback moth. The same as van Frankenhuyzen (2009) who reported that *cry*1Ac showed toxicity against 50 species of lepidopteran insects, for example *Helicoverpa armigera*, *H. zea*, *S. exigua*, *S. litura*, S. *littoralis, Trichoplusia ni, Ostrinia nubilalis and P. xylostella*. Tailor *et al*. (1992) also indicated that *cry*1Ia was active against *O. nubilalis, Leptinotarsa decemlineata*, *Diabrotica* sp and *P. xylostella* while *cry*2Ab was toxic to 11 lepidopteran insects especially *P. xylostella*. Appearance of the high toxicity *cry* genes as *cry*1Ac, *cry*1I and *cry*2A and the presence of *cry*1I and *cry*2A gene in chromosomal DNA that resulted in higher stability of these genes could be the best explanation for high efficiency of *B. thuringiensis* JCPT121 against diamondback moth and other pests.

Sixteen of *cry* gene as *cry*1Aa, *cry*1Ab, *cry*1Ad, *cry*1Ae, *cry*1B, *cry*1C, *cry*1D, *cry*1E, *cry*1F, *cry*1G, *cry*1H, *cry*1J, *cry*1K, *cry*5, *cry*12 and *cry*13A were not observed in this study as. The results agreed with to Poojitkanont *et al.* (2008) who did not find 12 *cry* genes namely *cry*1Aa, *cry*1Ad, *cry*1Ae, *cry*1B, *cry*1F, *cry*1G, *cry*1H, *cry*1J, *cry*1K, *cry*5, *cry*12 and *cry*13A on *B. thuringiensis* JC590. Thammasittirong and Attathom (2008) reported that three *cry* genes - *cry*1F, *cry*1H and *cry*1K, were not detected from 162 *B. thuringiensis* recovered from different geographical regions in

Thailand. It was suggested that these *cry* genes could involve in the toxicity toward insect species. It could be predicted that *cry* genes rarely detected in the effective isolates, might not be important in toxicity. The absence of *cry* genes such as *cry*1F, *cry*1G, *cry*1H, *cry*1J and *cry*1K, then indicated that they might not involve in the toxicity toward lepidopteran insects.

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4. Crystal protein purification

B. thuringiensis JCPT121 crystal proteins was purified by centrifugation on a discontinuous sucrose gradient. Both light microscopy (Figure 17) indicated that residual spore was contaminated in purified crystal protein around 10^5 spores/ml. Comparison of primary and final suspensions showed that this purification removed around 10^5spores/ml and recovering at 80% of initial amount of proteins concentrated (Table 8). Thomas and Ellar (1983) purified crystal protein of *B. thuringiensis* subsp. *kurstaki* using sucrose gradient technique and it was found that residual spore contamination in the purified crystal was determined as ≤0-2% by viable colony counts on CCY agar. Similarly, this study also observed residual spore as 0.002 %. In the study, crystal protein purification using discontinuous sucrose gradient gave better results in terms of quality and quantity of the crystal protein obtained. It was a practical, easy and inexpensive method.

Table 9 Quantitative description of the primary and final crystal suspensions from the crystal purification process.

Parameter	Suspension	
	Primary	Final
volume (ml)	10	5
Viable count (cfu/ml)	5.1×10^{12}	1.2×10^{7}
Spore count (spores)	3.2×10^{10}	6.7×10^{5}
% initial spores	100	0.002
OD 595	0.797	0.698
Protein concentrated (mg/ml)	1.432	1.246

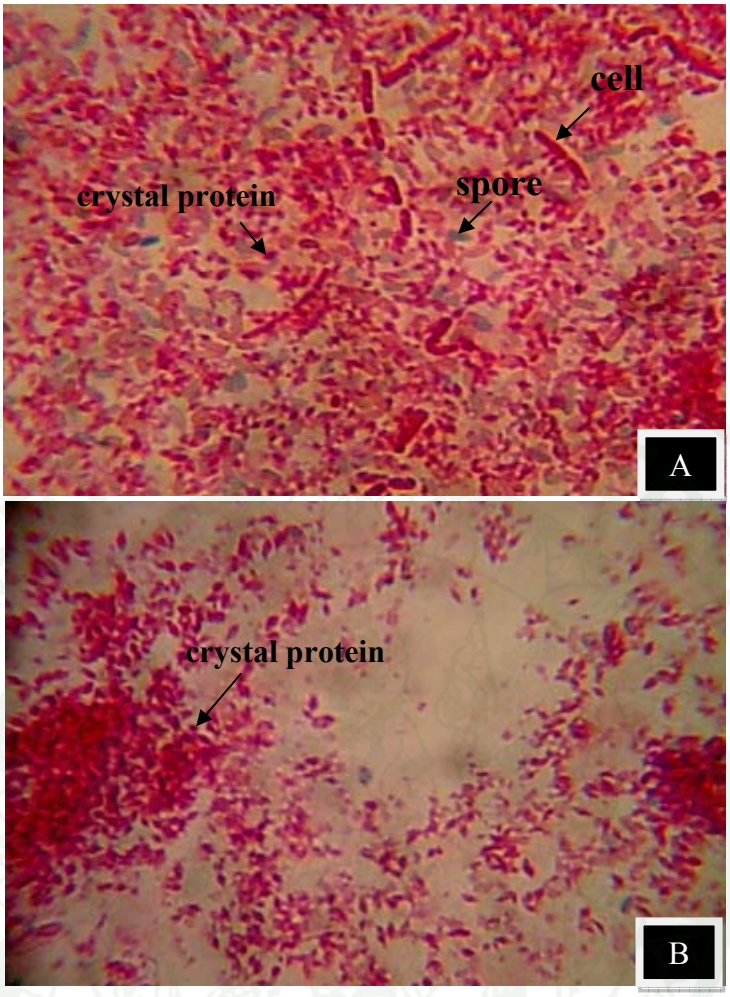

Figure 17 Crystal proteins of *Bacillus thuringiensis* JCPT121 before (A) and after (B) purification by sucrose gradient

5. Protein profile

SDS-PAGE analysis of crystal protein of *B. thuringiensis* JCPT121 and *B. thuringiensis* JC82, the standard strain, revealed that *B. thuringiensis* JCPT121 was composed of approximately 130 kDa and 72 kDa protein(Figure 18). However, *B. thuringiensis* JC82 was composed of a single protein of approximately 130 kDa. The protein profile of JC82 obtained in this study agreed with Isanont (1996) who stated that JC82 serotype 3a:3b:3d and centari (the commercial strain) was composed of 130 kDa protein. The protein profile obtained was also similar to the previous report of Kao *et al*. (1996) who demonstrated that seven *B. thuringiensis* isolates were active against *P. xylostella* containing mostly ~135 and 70 kDa.

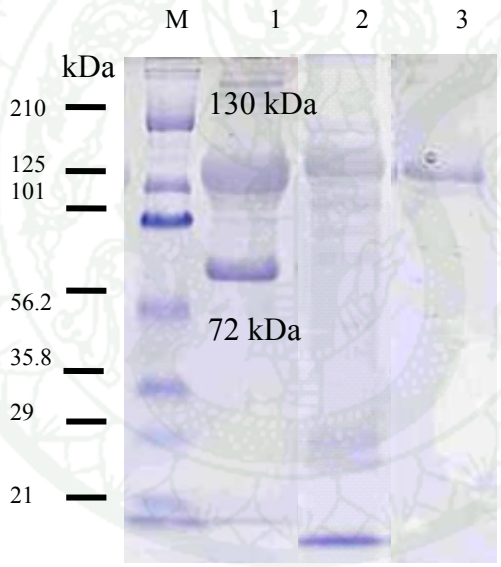

Figure 18 SDS-PAGE analysis of protein profiles of crystal proteins . M: molecular (After purification); Lane 2: Cell, spore and crystal protein of *B. thuringiensis* JCPT 121 (Before purification); Lane 3 crystal protein of *B. thuringiensis* JC82

Hofte and Whiteley (1989) indicated that various *cry* genes encoded different sizes and shapes of crystal protein. For example, *cry*I encoded 130-140 kDa bipyramidal crystal, *cry*II encoded 62-75 kDa cuboidal crystal protein, *cry*III encoded 65-75 kDa rhomboidal crystal and *cry*IV and cyt encoded 130-140 kDa and 27 kDa ovoid crystal. As Hofte and Whiteley (1989) confirmed *B. thuringiensis* JCPT121 contained *cry*I and *cry*II gene since their crystal proteins showed molecular weights of approximately 130 and 72 kDa. However, crystal proteins with equal molecular weight could be different in shape of crystal protein, *B. thuringiensis* JCPT121 containing *cry*IIA but did not produce cuboidal crystal protein. Only bipyramidal crystal protein was observed.

Protein profile according to *cry* gene detection found *cry*1Ac and *cry*2 on *B. thuringiensis* JCPT121. *cry*1I and *cry*14 genes were also detected but the 85 kDa and 20 kDa belonging to Cry1I and Cry14 protein, respectively were not found. The *cry*1I genes are usually located approximately 500 bp downstream of other cry1 genes, but *cry*1I genes sometimes may not be expressed due to the lack of an upstream promoter-like sequence (Kostichka *et al.* 1996). In fact, *cry*1I genes are usually either silent or expressed in vegetative phase and secreted into the growth suspension (Kostichka *et al.*, 1996; Song *et al*., 2003; Tounsi *et al*., 2003)

The protein profiles of 130 and 70 kDa were most frequently detected on *B. thuringiensis* strains isolated from many regions. For example, Beron and Salerno (2006) isolated *B. thuringiensis* in Argentina and found 75% of the strains to exhibit two polypeptides with molecular masses of 130 and 70 kDa. A few strains showed only 130-to 140-kDa polypeptides. Arrieta and Espinoza (2006) characterized 146 *B. thuringiensis* from Costa Rican natural ecosystem and noticed that strains with bipyramidal crystals to frequently present two protein, one of high molecular weight of 120 or 140 kDa and another of 65 or 60 kDa.

6. Insect host range test

Based on percentage mortality of the tested insects, it could be concluded that the *B. thuringiensis* JCPT121 was highly effective against *S. litura, S. exigua* and *A. aegypti* with 93.33, 100 and 96.66 % mortality, respectively. However, these isolates were considered to have no activity against *G. mellonella*, *B. dorsalis, M. domestica, T. molitor and T. castaneum* (Table 9).

The *cry*1Ac and *cry*2A-type genes exhibited a wide spectrum of toxicity to insect pests as Diptera and Lepidopteraand (Schnepf *et al*., 1998; van Frankenhuyzen, 2009). Hence, *B. thuringiensis* JCPT121 expressed toxicity against *P. xylostella* and *A. aegypti*.

Table 10 Insecticidal activity of *Bacillus thuringiensis* JCPT121 against 8 economically important insect pests.

Insect species	Mortality (%)			
	*Bt.*JCPT121	*Bt. kurstaki*	*Bt. israelensis*	*Bt. tenebrionis*
Spodoptera litura	93.33	100	0	0
Spodoptera exigua	100	100	0	0
Galleria mellonella	0	100	0	0
Bactrocera dorsalis	0	0	0	0
Musca domestica	3.33	0	0	0
Aedes aegypti	96.66	0	100	0
Tenebrio molitor	0	0	0	0
Tribolium castaneum	5	0	0	0

Some insect tested such as *G. mellonella*, *B. dorsalis, M. domestica, T. molitor and T. castaneum* were not susceptible to *B. thuringiensis* JCPT 121 due to the lack of *cry* gene coding toxin for pathogenic activity. van Frankenhuyzen (2009) demonstrated that *G. mellonella was* susceptible to Cry9Aa, *T. molitor and T. castaneum* were susceptible to Cry3A and *M. domestica* was susceptible to Cry1B. However, the Cry toxin was toxic to *B. dorsalis* was still unclear.

B. thuringiensis subsp. *tenebrionis* produces endotoxin , which is specific for coleopteran pest, including insects from the Chrysomelidae, Curculionidae and Scarabaeidae families. However, for two insect species tested, the crystal-spore suspension of *B. thuringiensis* subsp. *tenebrionis* did not show any insecticidal activity. The results were in agreement with those of Abdel-Razek (2002) who studied the efficacy of *B. thuringiensis* subsp. *tenebrionis* (commercial and *B. thuringiensis* subsp. *Morrisoni* for the control of lesser grain borer (*Rhyzopertha dominica*) Grain weevil (*Sitophilus granaries) and T. confusum. B. thuringiensis* subsp. *morrisoni* gave the highest mortality to the adults of *R. dominica* and *T. confusum* while *B. thuringiensis* subsp. *tenebrionis* did not give any actual mortality to all insects tested.

P. xylostella control caused the most serious problem for farmer. The availability of biological agent to against this insect can solve many of the toxicological environmental problems caused by insecticides. The study strongly recommends Thai *B. thuringiensis* JCPT121 which is toxic for *P. xylostella* larval and resistant to UV ray. Interestingly, this isolates not only kill *P. xylostella* but also *A. aegypti* whereas other *B. thuringiensis* especially the commercial strain affects only one. The isolates could be excellent substitutes for insecticides or *B. thuringiensis* commercial strain. However, it should be tested in the greenhouse and farmer fields to evaluate the potential toxicity.

CONCLUSION

One hundred and twenty one *B. thuringiensis* isolates were tested against *P. xylostella* larvae and the highly effective isolates in terms of toxicity and UV tolerance were selected. *B. thuringiensis* shown to be highly effective isolates were JCPT121, JCPT106, JCPT118 and JCPT76 with the LC_{50} values of 6.80 $\times 10^2$, 2.16 $\times 10^4$, 3.17 $\times 10^4$ and 6.80 $\times 10^2$ cfu/ml, respectively. Those isolates were selected for study on UV tolerance. After exposed to UV ray at 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mins, the viability of cells and spores in all isolates decreased with the increasing exposure time. JCPT121 had a higher survival rate at 60 mins of UV exposure whereas JCPT76, JCPT106 and JCPT118 were susceptible. Therefore, *B. thuringiensis* JCPT121 was selected for study on morphology of cell spore and crystal protein, biochemical characteristics, *cry* genes content, protein profile and insect host range test.

Morphological characteristics of cell spore and crystal protein using scanning electron microscope demonstrated the bacterial vegetative cells to be rod shape with 0.71 to 0.95 µm wide and 2.26 to 4.04 µm long whereas spore size of 0.75 to 1.00 µm wide and 1.56 to 2.25 µm long. Majority of the *B. thuringiensis* JCPT121 investigated produced bipyramidal shaped crystal of various sizes of 0.5 to 1.37 µm wide and 0.75 to 0.87 µm long.

Biochemical tests with API 50 CHB kits, expressed *B. thuringiensis* JCPT121 to be able to ferment Glycerol, D-Arabinose, D-Ribose, D-Galactose, D-Glucose, D-Fructose, N-Acetylglucosamine, Amygdalin, Arbutin, Esculin ferric citrate, Salicin, D-Celiobiose, D-Maltose, D-Saccharose (Sucrose), D-Treharose, and Glycogen. According to morphological, physiological, carbohydrate fermentation tests, this could be identified as *B. thuringiensis*.

The total genomic DNA and plasmid DNA was extracted and *cry* genes were detected by PCR method using 20 pairs of specific primers. PCR products were purified, sequenced and subjected to BLAST search at NCBI. There *cry* genes namely *cry*1Ac, *cry*1I and *cry*2A were observe to be present in chromosomal DNA while *cry* genes namely c*ry*1I and *cry*2A in plasmid DNA.

The crystal proteins of *B. thuringiensis* JCPT121 were purified by discontinuous sucrose gradient method and analyzed protein profile by SDS-PAGE and JCPT121 was found to be composed of approximately 130 kDa and 72 kDa proteins. Screening the toxicity of JCPT121 to insect showed toxicity to *S. litura*, *S. exigua* and *A. aegypti* but no toxicity to larvae of *G. mellonella*, *B. dorsalis*, *M. domestica*, *T. molitor* and *T. castaneum*.

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APPENDICES

Appendix A

Media and artificial diet

1. Nutrient Broth (NB) and Nutrient Agar (NA)

Yeast extract	3.0 g/l
Peptone	5.0 g/l

Combine ingredients and sterilize by autoclaving for 15 mins at 121 °C. . For NA, agar (15g/l) was added to the liquid media before autoclaving.

2. Luria-Bertani medium (LB medium)

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
NaCl	5.0 g/l

The pH was adjusted to 7.0 with 5N NaOH (~0.2 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sterilization was achieved by autoclaving for 15 mins at 121°C. For agar plate, agar (15g/l) was added to the liquid media before autoclaving

3. Motility medium

Peptone	10.0 g/l
NaCl	5.0 g/l
Agar	3.0 g/l

Combine ingredients and boil to dissolve the agar. Sterilization was achieved by autoclaving of 15 min at 121°C.

4. Fruitfly larval artificial diet

Larval diet based on wheat-yeast formula was used for laboratory rearing of the fruit flies (Sutantawong *et al*., 1985). The formulation was as follows:

Methyl-p-hydroxybenzoate	1	g
Sodium benzoate	1	g
Sugar	120	g
Dried yeast	36	g
Wheat bran	260	g
HCl (con.)	2	ml
Distilled water	580	ml

The prepared diet was put in the shallow plastic tray (23 × 32 × 5 cm). Each tray was filled up to a depth of 2 cm (1,000 g of diet). The eggs were put on top of 2 tissue paper strips (5.5 × 11 cm) placed across the surface of larval diet. Eggs were smeared on the larval diet using a fine camel's hair-brush. The diet tray was covered by the addition of second inverted tray to maintain high humidity necessary for larval hatching. Subsequently, the diet was held in rearing room until the larvae were fully grown.

Appendix B

Reagents and solutions

1. Reagent for chromosomal DNA and plasmid DNA extraction

1.1. Lysis buffer

50mM EDTA	10 ml
0.1 M NaCl	10 ml
H_2O	80 ml

1.2. Solution A

20% (w/v) sucrose	50 ml
10 mM Tris HCl (pH 8.0)	10 ml
10 mM EDTA (pH 8.0)	10 ml
50 mM NaCl	5 ml
H_2O	25 ml

Solution A can be prepared in batches of approximately 100 ml, autoclaved for 15 mins at 15 lb/in2 and stored at 4°C.

1.3. Solution B

0.2 M NaOH

1% SDS

The solution was freshly prepared from stock solution of 2 M NaOH and 10% SDS.

1.4. Solution C

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

1.5. TSE buffer

10 mM Tris.Cl (pH 8.0)	10 ml

30 mM NaCl	3 ml
10 mM EDTA	10 ml

2. Reagent for agarose electrophoresis

1. Tris-acetate (TAE)

1x TAE buffer was used as an electrophoresis buffer throughout the study. The working solution of 1x TAE buffer was prepared from stock solution of 10x TAE buffer, as follows:

Tris-base	4.84 g
Glacial acetic acid	1.15 ml
0.5 M EDTA.2Na (pH 8.0)	2.00 ml

The volume of the solution was adjusted to 1 litter with distilled water and sterilized by autoclaving for 15 mins at 121°C.

2. Gel loading buffer

Bromophenol blue	0.025 g
Glycerol	3.0 ml

3. Ethidium bromide solution (10 mg/ml)

The ethidium bromide solution was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water. The solution was stored in light-tight container at room temperature.

3. Reagents for SDS-PAGE

1. Acrylamide/Bis (30%T, 2.67%C)

Add 14.6 g of acrylamide and 0.4 g of N'N'- bismethylene – acrylamide to 50 ml of distilled water and keep at 4 °C.

2. 1.5M Tris-HCl pH 8.8

The 1.5 M Tris-HCl was prepared by dissolving 18.15 g of Tris base (MW 121.1) in 50 ml of distilled water. The pH was adjusted to 8.8 by adding concentrated HCl. The volume of the solution was adjusted to 100 ml with distilled water and kept at 4 °C.

3. 0.5M Tris-HCl pH 6.8

The 0.5 M Tris-HCl was prepared by dissolving 6g of Tris base (MW 121.1) in 50 ml of distilled water. The pH was adjusted to 6.8 by adding concentrated HCl. The volume of the solution was adjusted to 100 ml with distilled water and kept at 4 °C.

4. 10% Sodium dodecyl sulphate (SDS)

The stock solution of 10% SDS was prepared by dissolving 10 g of sodium dodecyl sulphate in 100 ml of sterilized distilled water. Sterilization is not required for the preparation of this stock solution.

5. 5X Running buffer, pH 8.3

Add 6 g of Tris base (MW 121.1), 43.3 g of Glycine and 3 g of SDS in 600 ml of distilled water and keep at 4 °C.

4. SDS-PAGE

Molecular weight of the purified crystal protein was determined by SDS–PAGE according to the method of Laemmli (1970). The preparation was conducted as follows:

1. Separating gel 10 ml

A separating gel solution containing 1.5 M Tris pH 8.8 5.0 ml, Deionized water 3.52 ml, 10% SDS 0.2 ml, 30% acrylamide solution 4.9 ml, 10% ammonium persulfate 0.05 ml and N, N, N', N'-tetramethylethylene diamide (TEMED) 0.02 ml were mixed immediately and poured into the gel apparatus to about comb foot. The surface of the gel was then layered with water-saturated n-butanol and allowed to polymerize. The top layer was drawn and the gel surface was washed with distilled water once.

2. Stacking gel 10 ml

Stacking gel solution containing 0.5 M Tris pH 6.8 5.0 ml, Deionized water 12.2 ml, 10% SDS 0.2 ml, 30% acrylamide solution 2.66 ml, 10% ammonium persulfate 0.20 ml and N, N, N', N'- tetramethylethylene diamide (TEMED) 0.02 ml were mixed immediately before bring poured into the separating gel. Comb was inserted immediately and allowed for polymerization.

3. Sample preparation

Crystal protein at the concentration of 1 mg/ml was mixed with and equal volume of SDS-PAGE sample buffer containing 0.5M Tris-HCl (pH 6.8) 2.5 ml, glycerol 2.0 ml, 10% (w/v) sodium dodecylsulfate (SDS) 4.0 ml, b-mercaptoethanol 1.0 ml and bromophenol blue 1.0 ml. After boiling for 10 mins. 10 µl of the sample was applied to 10% SDS-polyacrylamide gel. Protein standard used for determination molecular masses were prestained SDS-PAGE broad range.

4. Electrophoresis procedure

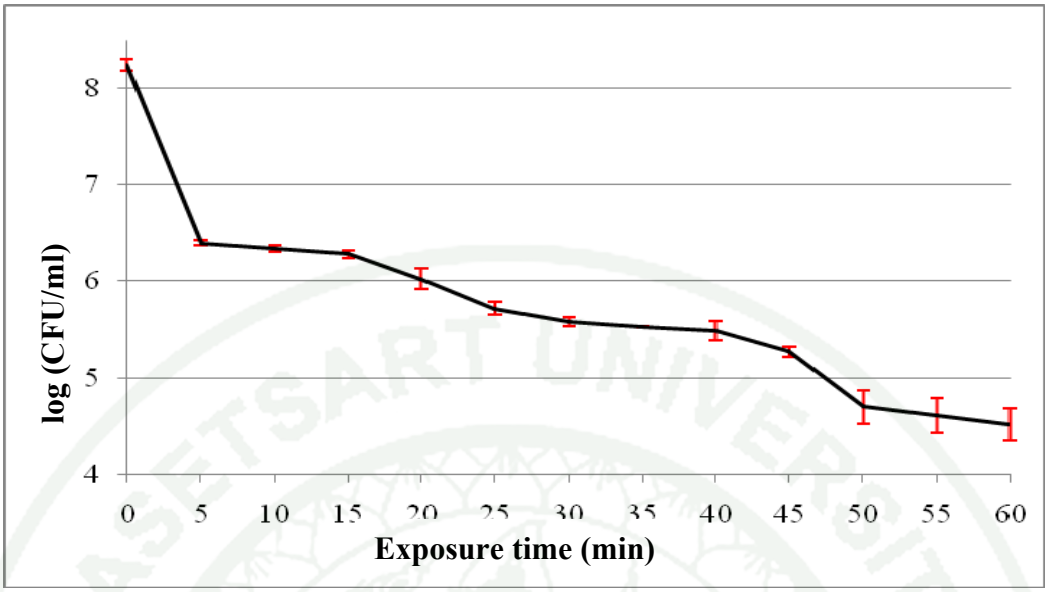
Electrophoresis was performed in 5X Running buffer at a constant 90 voltage for 1 hour.

5. Coomassie Brillaint Blue Staining

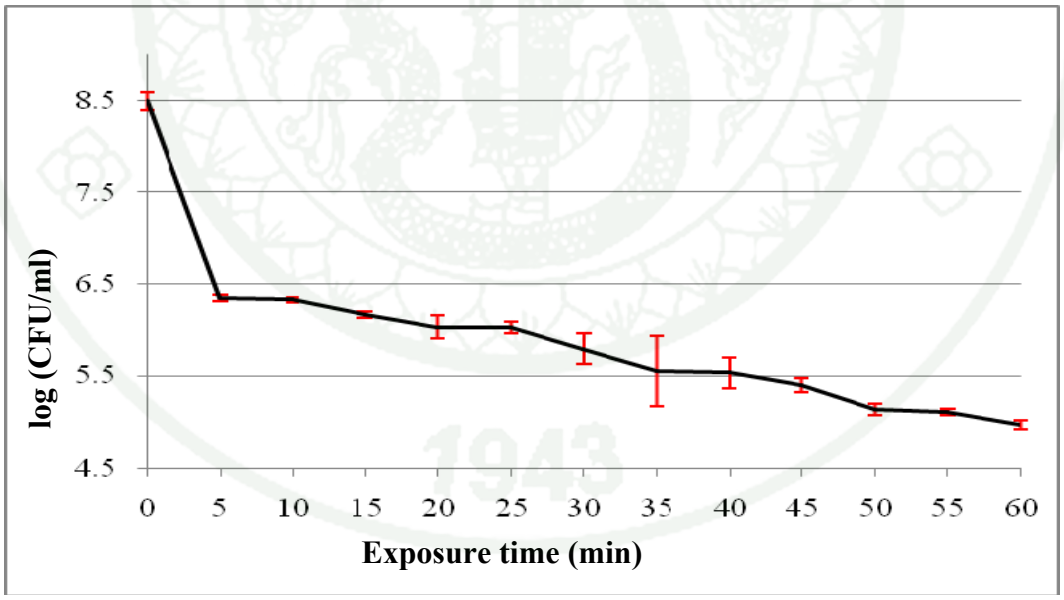
The gel was stained with coomaassie brilliant blue R-250 in 45% methanol and 1% acetic acid. As soon as the bands became visible, the gel was destained with 45% methanol and 1% acetic acid and kept in destaining solution at 4°C.

Appendix C

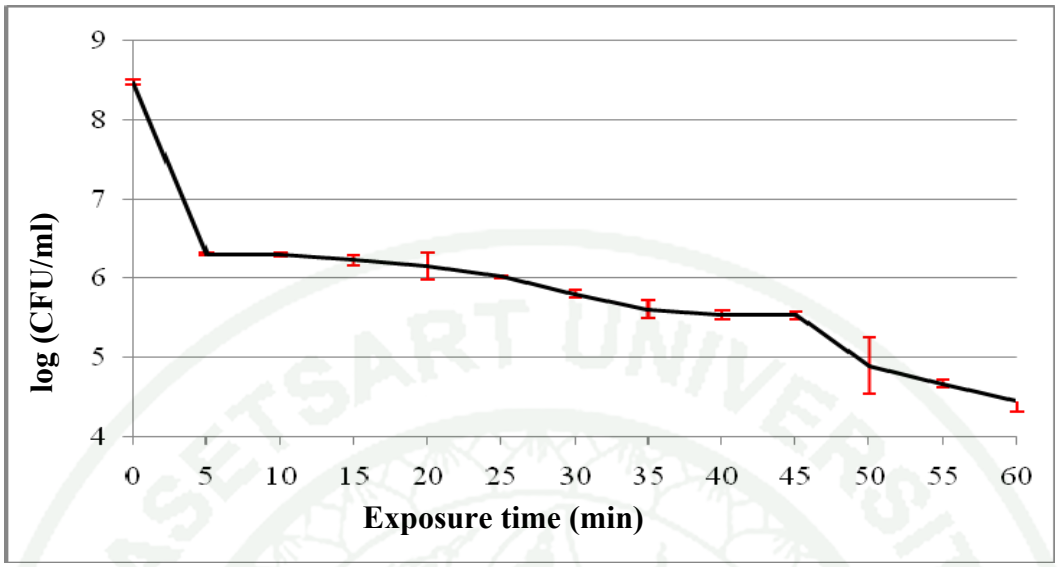
Data of growth



Appendix Figure C1 Semi logarithm plot of *Bacillus thuringiensis* JCPT76 growth curve after exposed to UV ray.

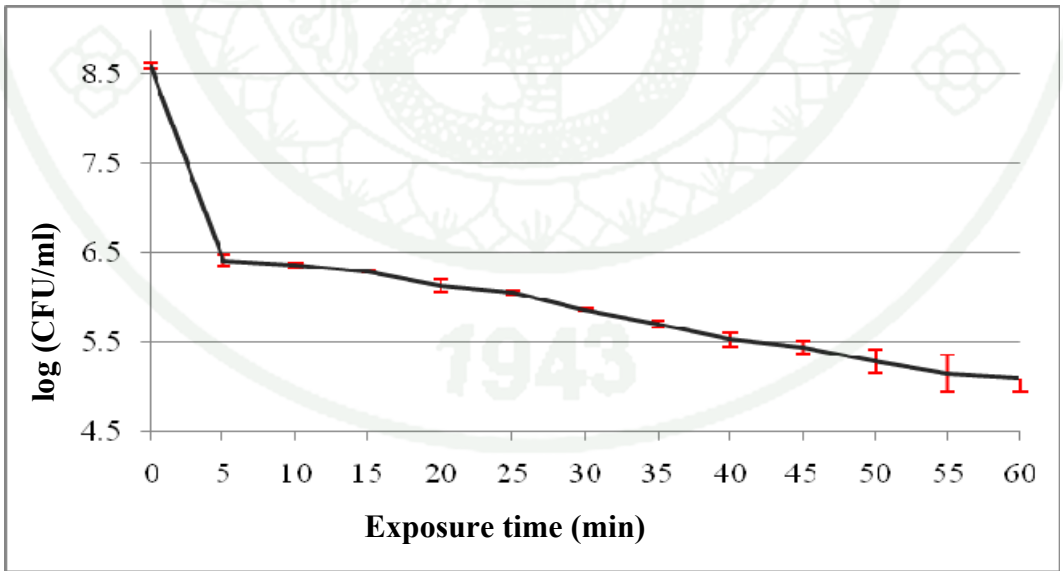


Appendix Figure C2 Semi logarithm plot of *Bacillus thuringiensis* JCPT106 growth curve after exposed to UV ray.

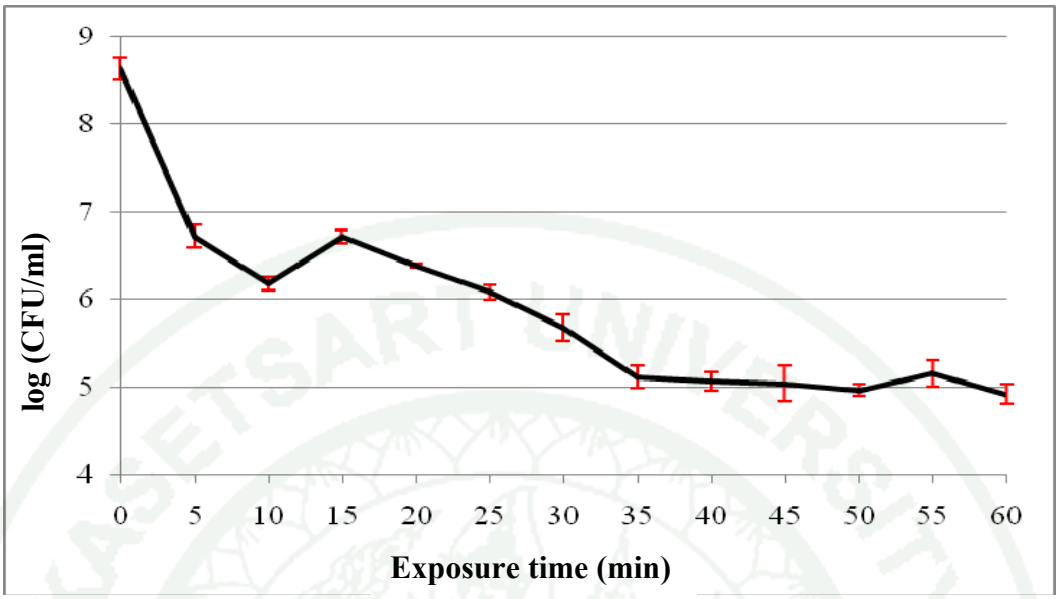


Appendix Figure C3 Semi logarithm plot of *Bacillus thuringiensis* JCPT118 growth curve after exposed to UV ray.

.



Appendix Figure C4 Semi logarithm plot of *Bacillus thuringiensis* JCPT121 growth curve after exposed to UV ray.



Appendix Figure C5 Semi logarithm plot of *Bacillus thuringiensis* subsp. *kurstaki* growth curve after exposed to UV ray.

Appendix D

Nucleotide sequence and analysis

```
  1 TGGTGGGGAN TTAGTTAGGA TTAAATAGTA GTGGAAATAA CATTCAGAAT
 51 TTGAAGTTCC AATTCACTTC CCATCGACAT CTACCAGATA TCGAGTTCGT
101 GTACGGTATG CTTCTGTAAC CCCGATTCAC CTCAACGTTA ATTGGGGTAA
151 TTCATCCATT TTTTCCAATA CAGTACCAGC TACAGCTACG TCATTAGATA
201 ATCTACAATC AAGTGATTTT GGTTATTTTG AAAGTGCCAA TGCTTTTACA
251 TCTTCATTAG GTAATATAGT AGGTGTTAGA AATTTTAGTG GGACTGCAGG
301 AGTGATAATA GACAGATTTG AATTTATTCC AGTTACTGCA ACACTCGAGG
351 CTGAATATAA TCTGGAAAGA GCGCAGAAGG CGGTGAATGC GCTGTTTACG
401 TCTACAAACC AACTAGGGCT AAAAACAAAT GTAACGGATT ATCATATTGA
451 TCAAGTGTCC AATTTAGTTA CGTATTTATC GGATGAATTT TGTCTGGATG
501 AAAAGCGAGA ATTGTCCGAG AAAGTCAAAC ATGCGAAGCG ACTCAGTGAT
551 GAACGCAATT TACTCCAAGA TTCAAATTTC AAAGACATTA ATAGGCAACC
601 AGAACGTGGG TGGGGCGGAA GTACAGGGAT TACCATCCAA GGAGGGGATG
651 ACGTATTTAA AGAAAATTAC GTCACACTAT CAGGTACCTT TGATGAGTGC
701 TATCCAACAT ATTTGTATCA AAAAATCGAT GAATCAAAAT TAAAAGCCTT
751 TACCCGT
```

Appendix Figure D1 Nucleotide sequence of 758 bp *cry*1Ac-PCR product amplified chromosomal DNA from *Bacillus thuringiensis* JCPT121

```
   1 GNAATGCGAA GTAGTAAATC TCTACGGATT CACTAAAAAA TGAAACAGAT
  51 ATAGAATTAC AAAACATTAA TCATGAAGAT TGTTTGAAAA TGTCTGAGTA
 101 TGAAAATGTA GAGCCGTTTG TTAGTGCATC AACAATTCAA ACAGGTATTG
 151 GTATTGCGGG TAAAATACTT GGTACCCTAG GCGTTCCTTT TGCAGGACAA
 201 GTAGCTAGTC TTTATAGTTT TATCTTAGGT GAGCTATGGC CTAAGGGGAA
 251 AAATCAATGG GAAATCTTTA TGGAACATGT AGAAGAGATT ATTAATCAAA
 301 AAATATCAAC TTATGCAAGA AATAAAGCAC TTACAGACTT GAAAGGATTA
 351 GGAGATGCCT TAGCTGTCTA CCATGATTCG CTTGAAAGTT GGGTTGGAAA
 401 TCGTAATAAC ACAAGGGCTA GGAGTGTTGT CAAGAGCCAA TATATCGCAT
 451 TAGAATTGAT GTTCGTTCAG AAACTACCTT CTTTTGCAGT GTCTGGAGAG
 501 GAGGTACCAT TATTACCGAT ATATGCCCAA GCTGCAAATT TACATTTGTT
 551 GCTATTAAGA GATGCATCTA TTTTTGGAAA AGAGTGGGGA TTATCATCTT
 601 CAGAAATTTC AACATTTTAT AACCGTCAAG TCGAACGAGC AGGAGATTAT
 651 TCCGACCATT GTGTGAAATG GTATAGCACA GGTCTAAATA ACTTGAGGGG
 701 TACAAATGCC GAAAGTTGGG TACGATATAA TCAATTCCGT AGAGACATGA
 751 CTTTAATGGT ACTAGATTTA GTGGCACTAT TTCCAAGCTA TGATACACAA
 801 ATGTATCCAA TTAAAACTAC AGCCCAACTT ACAAGAGAAG TATATACAGA
 851 CGCAATTGGG ACAGTACATC CGCATCCAAG TTTTACAAGT ACGACTTGGT
 901 ATAATAATAA TGCACCTTCG TTCTCTGCCA TAGAGGCTGC TGTTGTTCGA
 951 AACCCGCATC TACTCGATTT TCTAGAACAA GTTACAATTT ACAGCTTATT
1001 AAGTCGATGG AGTAACACTC AGTATATGAA TATGTGGGGA GGACATAAAC
1051 TAGAATTCCG AACAATAGGA
```

Appendix Figure D2 Nucleotide sequence of 1070 bp *cry*1I-PCR product amplified chromosomal DNA from *Bacillus thuringiensis* JCPT121

```
   1 CNNNNGNTAT TNTAGGTAAC TCGAATATTT GGAATTTCCT ATTGAAGATA
  51 ACTCTTAAAT AAAGATTGTA ACTATTTCCA TTCCCTCTAA GCGTATAACG
 101 AGCTGTCGTG TTGTTTTGTT CAAATCTTAA AGAATCACCT TGATTTCCAA
 151 ATTTTTCAGA AATAAATGTT CGAGTTTGAT TATTCACTTG AGTGGCATGT
 201 ATTGGCGAAA TAGTAAATCC TGTATAATCA TTTGGCGCTA AATGAATCAT
 251 AGAACCATTT TCATTAACGG CATAGATATT ATTTTTTCTG TTATGCACAG
 301 ATACCATATA AGCTCGTGCT CCACCAGGTG TTCCTGAAGG ACTTTCTATA
 351 TTTCTTATTT CATTATAGTG TAACGGTCTT GTTAAATCTT CATTTCTAAC
 401 AACTAAAGGA ACCCCAGAAA TATTACGAAT AAAATAATCT GGGAAATAGT
 451 TTGAATTACC GCGGGCTGAA AAAGCACCAC TCCTTAAACT TAAAGTTGTC
 501 TGAAAGGATT CTGTCTGCCA ATTTGTAANG GTGGCAACGC CCTCCCGATC
 551 TGAACCTGAA TCTAGCCAAC TTCTAACAAA TGGTGTTGAC AATGGAGGGA
 601 GAAATGTGCT ACAATTAAAA TTTTGATTGA ACTTAGACGC CCCTATATCA
 651 CCAGATGAAA CTCCTCCACT GTAATTAACC CTTGCACTAT GCAATGCGTG
 701 AGTTGTAGTA CAACCCGGTA AACCAACTAT ATTAGGGAAG GTAATAGAAA
 751 GCCTAGCACC ACTAAATCCA TTTAATATAT AATTCGAATT AACTTGGAAA
 801 AGAGAATATA AAAATGGCCA GTCTTGTGCT GTAAATGATT GTGTCTGCTG
 851 TGGTCCACTA CCACTTGCAT ATAAATTAGC AGCCAGAAGA TACCATAAGA
 901 CTTTGATATT TAAACAATGA CCAGATAGAT ACATACTCAA ATACATTTAA
 951 AAAACATATA TGTTCTAAAT TCTAACATAT CGTGTAAACG AGTGTTTAAA
1001 CCCTTTAAAC GCATTTTGAT ACGTATTTAT ACAATAATTA AAAATAATCC
1051 CTTGGAAAA
```

Appendix Figure D3 Nucleotide sequence of 1070 bp *cry*2A-PCR product amplified chromosomal DNA from *Bacillus thuringiensis* JCPT121

```
  1 TGCGTATATC ACGGTTTAAC ACATCTTTAT GAATAGGGAA TCCTTCTTTT
 51 ACTAATGCTT GAATCGCGAC ATCATATAGA CTTGGTGCAT GTAGCGCTTT
101 ATGAAGTCGT GCATGTAATT CTGGATCCTT TTCATAAATT TTTAATGCAT
151 GTGGCGTTTT ATAGCCAAGT GCATACTCAA TCATACGATA TTGATACGAT
201 TGAAAACCTG AAGCTTGACC GAGTGAATCA CGAAACTCAA TATATTCTGA
251 TGGTGTTAAT GTCGCAAGAA TATCCCAAGA TTGAATAATT TGAGACTGAA
301 TTTTTGATAC ACGTGCTAAC ATTTTAAAAG CTGGTTGTAA TTTATCTTGT
351 TTAATAGATT CAATCGCCGC ATTTAGCTCA TGTAAAATGA GCTTCATCCA
401 AAGCTCACTT GCTTGGTGAA TAACGATAAA TAACATTTCA TCATGATGGT
451 CTGATAATCT TTTTTGAGAA GATAGTAAAC TATCTAATTG TAAATATTCC
501 CCATACGTCA TATTCTCTTT AAAATCCGTA TGAATCCCTT TTTCCATAAT
551 TACTTTTTCA TTTTCTTTCA TATTAGCCAA ACGCCCCTTT TATAT
```

Appendix Figure D4 Nucleotide sequence of 595 bp *cry*14-PCR product amplified chromosomal DNA from *Bacillus thuringiensis* JCPT121

```
   1 TTTCTAGCAT GCGAAAGTAG AAAAATCTCT ACGGATTCAC TAAAAAATGA
  51 AACAGATATA GAATTACAAA ACATTAATCA TGAAGATTGT TTGAAAATGT
 101 CTGAGTATGA AAATGTAGAG CCGTTTGTTA GTGCATCAAC AATTCAAACA
 151 GGTATTGGTA TTGCGGGTAA AATACTTGGT ACCCTAGGCG TTCCTTTTGC
 201 AGGACAAGTA GCTAGTCTTT ATAGTTTTAT CTTAGGTGAG CTATGGCCTA
 251 AGGGGAAAAA TCAATGGGAA ATCTTTATGG AACATGTAGA AGAGATTATT
 301 AATCAAAAAA TATCAACTTA TGCAAGAAAT AAAGCACTTA CAGACTTGAA
 351 AGGATTAGGA GATGCCTTAG CTGTCTACCA TGATTCGCTT GAAAGTTGGG
 401 TTGGAAATCG TAATAACACA AGGGCTAGGA GTGTTGTCAA GAGCCAATAT
 451 ATCGCATTAG AATTGATGTT CGTTCAGAAA CTACCTTCTT TTGCAGTGTC
 501 TGGAGAGGAG GTACCATTAT TACCGATATA TGCCCAAGCT GCAAATTTAC
 551 ATTTGTTGCT ATTAAGAGAT GCATCTATTT TTGGAAAAGA GTGGGGATTA
 601 TCATCTTCAG AAATTTCAAC ATTTTATAAC CGTCAAGTCG AACGAGCAGG
 651 AGATTATTCC GACCATTGTG TGAAATGGTA TAGCACAGGT CTAAATAACT
 701 TGAGGGGTAC AAATGCCGAA AGTTGGGTAC GATATAATCA ATTCCGTAGA
 751 GACATGACTT TAATGGTACT AGATTTAGTG GCACTATTTC CAAGCTATGA
 801 TACACAAATG TATCCAATTA AAACTACAGC CCAACTTACA AGAGAAGTAT
 851 ATACAGACGC AATTGGGACA GTACATCCGC ATCCAAGTTT TACAAGTACG
 901 ACTTGGTATA ATAATAATGC ACCTTCGTTC TCTGCCATAG AGGCTGCTGT
 951 TGTTCGAAAC CCGCATCTAC TCGATTTTCT AGAACAAGTT ACAATTTACA
1001 GCTTATTAAG TCGATGGAGT AACACTCAGT ATATGAATAT GTGGGGGAGG
1051 ACATAAACTA GAATTCCGAA C
```

Appendix Figure D5 Nucleotide sequence of 1071 bp *cry*1I-PCR product amplified plasmid DNA from *Bacillus thuringiensis* JCPT121

```
   1 TTATAGTAAC TCGAATAGTG GAATTTCCTA TTGAAGATAC TCTTAAATAA
  51 AGATTGTAAC TATTTCCATT CCCTCTAAGC GTATAACGAG CTGTCGTGTT
 101 GTTTTGTTCA AATCTTAAAG AATCACCTTG ATTTCCAAAT TTTTCAGAAA
 151 TAAATGTTCG AGTTTGATTA TTCACTTGAG TGGCATGTAT TGGCGAAATA
 201 GTAAATCCTG TATAATCTTT TGGCGCTAAA TGAATCATAG AACCATTTTC
 251 ATGAACGGCA TGGATATTAT TTTTTCTGTT ATGCACAGAT ACCATATAAG
 301 CTCGTGCTCC ACCAGGTGTT CCTGAAGGAC TTTCTATATT TCTTATTTGN
 351 TTATAGTGTA ACGGTCTTGT TAAATCTTCA TTTCTAACAA CTAAAGGAAC
 401 CCCAGAAATA TTACGAATAA AATAATCTGG GAAATAGTTT GAATTACCGC
 451 GGGCTGAAAA AGCACCACTC CTTAACCTTA AAGTTGTCTG AAAGGATTCT
 501 GTTTGCCAAT TTGTAACGGT GGCAACGCCC TCTCGATCTG AACCTGAATC
 551 CAGCCAACTT CTAACAAATG GTGTTGACAA TGGAGGGAGA ANTGTGCTGC
 601 AATTAAAATT GTGATTGANA TGAGACGCCC CTATATCACC AGATGAAACT
 651 CCTCCGCTGT AATTAACCCT GGCACTATTC AATGCGTGAG TTGTAGTACN
 701 ACCCGGTAAA CCAACCTATA TTAGGGAAGG TATTAGAAAG CCTAGCACCA
 751 CTAAATACCA TTTAACACAT AATTCGAATT AACTTGGAAA AGAGAATATA
 801 AAAATGGCCA GTCTTTGTGC TGTAAATGAT TGNGTCTGCT GTGGTCCACT
 851 ACCACTTNGC ATATAAATTA GCNGCCGAGA AGATACTNNT AAGACTTCTG
 901 ATATTTAAAC NANNGACCAG ATNGNNACAT ACTTCAAATA CATTTAAAAA
 951 CATATATGTN CNAAATTTCT AACATATCGT GTAAACGAGN GTTTAACCCT
1001 TNTAAACGCN NNTTTGATAC GTANTTTATA CAATA
```

Appendix Figure D6 Nucleotide sequence of 1035 bp *cry*2A-PCR product amplified plasmid DNA from *Bacillus thuringiensis* JCPT121

Alignment of nucleotide sequence

```
1I_c      -----GNAATGCGAA-GTAGTAAA-TCTCTACGGATTCACTAAAAAATGAAACAGATATA 53
1I_p      TTTCTAGCATGCGAAAGTAGAAAAATCTCTACGGATTCACTAAAAAATGAAACAGATATA 60
                 ******* **** *** **********************************

1I_c      GAATTACAAAACATTAATCATGAAGATTGTTTGAAAATGTCTGAGTATGAAAATGTAGAG 113
1I_p      GAATTACAAAACATTAATCATGAAGATTGTTTGAAAATGTCTGAGTATGAAAATGTAGAG 120
          ************************************************************

1I_c      CCGTTTGTTAGTGCATCAACAATTCAAACAGGTATTGGTATTGCGGGTAAAATACTTGGT 173
1I_p      CCGTTTGTTAGTGCATCAACAATTCAAACAGGTATTGGTATTGCGGGTAAAATACTTGGT 180
          ************************************************************

1I_c      ACCCTAGGCGTTCCTTTTGCAGGACAAGTAGCTAGTCTTTATAGTTTTATCTTAGGTGAG 233
1I_p      ACCCTAGGCGTTCCTTTTGCAGGACAAGTAGCTAGTCTTTATAGTTTTATCTTAGGTGAG 240
          ************************************************************

1I_c      CTATGGCCTAAGGGGAAAAATCAATGGGAAATCTTTATGGAACATGTAGAAGAGATTATT 293
1I_p      CTATGGCCTAAGGGGAAAAATCAATGGGAAATCTTTATGGAACATGTAGAAGAGATTATT 300
          ************************************************************

1I_c      AATCAAAAAATATCAACTTATGCAAGAAATAAAGCACTTACAGACTTGAAAGGATTAGGA 353
1I_p      AATCAAAAAATATCAACTTATGCAAGAAATAAAGCACTTACAGACTTGAAAGGATTAGGA 360
          ************************************************************

1I_c      GATGCCTTAGCTGTCTACCATGATTCGCTTGAAAGTTGGGTTGGAAATCGTAATAACACA 413
1I_p      GATGCCTTAGCTGTCTACCATGATTCGCTTGAAAGTTGGGTTGGAAATCGTAATAACACA 420
          ************************************************************

1I_c      AGGGCTAGGAGTGTTGTCAAGAGCCAATATATCGCATTAGAATTGATGTTCGTTCAGAAA 473
1I_p      AGGGCTAGGAGTGTTGTCAAGAGCCAATATATCGCATTAGAATTGATGTTCGTTCAGAAA 480
          ************************************************************
```

Appendix Figure D7 The nucleotide sequence alignment of *cry*1I gene from chromosomal DNA (1I_c) and *cry*1I gene from plasmid DNA (1I_c) using EMBOSS Pairwise Alignment Algorithms.

Note * single, fully conserved residue,
- no consensus

```
1I_c      CTACCTTCTTTTGCAGTGTCTGGAGAGGAGGTACCATTATTACCGATATATGCCCAAGCT 533
1I_p      CTACCTTCTTTTGCAGTGTCTGGAGAGGAGGTACCATTATTACCGATATATGCCCAAGCT 540
          ************************************************************

1I_c      GCAAATTTACATTTGTTGCTATTAAGAGATGCATCTATTTTTGGAAAAGAGTGGGGATTA 593
1I_p      GCAAATTTACATTTGTTGCTATTAAGAGATGCATCTATTTTTGGAAAAGAGTGGGGATTA 600
          ************************************************************

1I_c      TCATCTTCAGAAATTTCAACATTTTATAACCGTCAAGTCGAACGAGCAGGAGATTATTCC 653
1I_p      TCATCTTCAGAAATTTCAACATTTTATAACCGTCAAGTCGAACGAGCAGGAGATTATTCC 660
          ************************************************************

1I_c      GACCATTGTGTGAAATGGTATAGCACAGGTCTAAATAACTTGAGGGGTACAAATGCCGAA 713
1I_p      GACCATTGTGTGAAATGGTATAGCACAGGTCTAAATAACTTGAGGGGTACAAATGCCGAA 720
          ************************************************************

1I_c      AGTTGGGTACGATATAATCAATTCCGTAGAGACATGACTTTAATGGTACTAGATTTAGTG 773
1I_p      AGTTGGGTACGATATAATCAATTCCGTAGAGACATGACTTTAATGGTACTAGATTTAGTG 780
          ************************************************************

1I_c      GCACTATTTCCAAGCTATGATACACAAATGTATCCAATTAAAACTACAGCCCAACTTACA 833
1I_p      GCACTATTTCCAAGCTATGATACACAAATGTATCCAATTAAAACTACAGCCCAACTTACA 840
          ************************************************************

1I_c      AGAGAAGTATATACAGACGCAATTGGGACAGTACATCCGCATCCAAGTTTTACAAGTACG 893
1I_p      AGAGAAGTATATACAGACGCAATTGGGACAGTACATCCGCATCCAAGTTTTACAAGTACG 900
          ************************************************************

1I_c      ACTTGGTATAATAATAATGCACCTTCGTTCTCTGCCATAGAGGCTGCTGTTGTTCGAAAC 953
1I_p      ACTTGGTATAATAATAATGCACCTTCGTTCTCTGCCATAGAGGCTGCTGTTGTTCGAAAC 960
          ************************************************************

1I_c      CCGCATCTACTCGATTTTCTAGAACAAGTTACAATTTACAGCTTATTAAGTCGATGGAGT 1013
1I_p      CCGCATCTACTCGATTTTCTAGAACAAGTTACAATTTACAGCTTATTAAGTCGATGGAGT 1020
          ************************************************************

1I_c      AACACTCAGTATATGAATATGTGGGG-AGGACATAAACTAGAATTCCGAACAATAGGA 1070
1I_p      AACACTCAGTATATGAATATGTGGGGGAGGACATAAACTAGAATTCCGAAC------- 1071
          ************************* ************************
```

Appendix Figure D7 (Continued)

Note * single, fully conserved residue,

- no consensus

```
(2A_c)      CNNNNGNTATTNTAGGTAACTCGAATATTTGGAATTTCCTATTGAAGATAACTCTTAAAT 60
(2A_p)      ---------TTATAG-TAACTCGAATAGT-GGAATTTCCTATTGAAGATA-CTCTTAAAT 48
                     ** *** *********** * ******************* *********

(2A_c)      AAAGATTGTAACTATTTCCATTCCCTCTAAGCGTATAACGAGCTGTCGTGTTGTTTTGTT 120
(2A_p)      AAAGATTGTAACTATTTCCATTCCCTCTAAGCGTATAACGAGCTGTCGTGTTGTTTTGTT 108
            ************************************************************

(2A_c)      CAAATCTTAAAGAATCACCTTGATTTCCAAATTTTTCAGAAATAAATGTTCGAGTTTGAT 180
(2A_p)      CAAATCTTAAAGAATCACCTTGATTTCCAAATTTTTCAGAAATAAATGTTCGAGTTTGAT 168
            ************************************************************

(2A_c)      TATTCACTTGAGTGGCATGTATTGGCGAAATAGTAAATCCTGTATAATCATTTGGCGCTA 240
(2A_p)      TATTCACTTGAGTGGCATGTATTGGCGAAATAGTAAATCCTGTATAATCTTTTGGCGCTA 228
            ********************************************** **********

(2A_c)      AATGAATCATAGAACCATTTTCATTAACGGCATAGATATTATTTTTTCTGTTATGCACAG 300
(2A_p)      AATGAATCATAGAACCATTTTCATGAACGGCATGGATATTATTTTTTCTGTTATGCACAG 288
            ********************** ******** *************************

(2A_c)      ATACCATATAAGCTCGTGCTCCACCAGGTGTTCCTGAAGGACTTTCTATATTTCTTATTT 360
(2A_p)      ATACCATATAAGCTCGTGCTCCACCAGGTGTTCCTGAAGGACTTTCTATATTTCTTATTT 348
            ************************************************************

(2A_c)      CATTATAGTGTAACGGTCTTGTTAAATCTTCATTTCTAACAACTAAAGGAACCCCAGAAA 420
(2A_p)      GNTTATAGTGTAACGGTCTTGTTAAATCTTCATTTCTAACAACTAAAGGAACCCCAGAAA 408
              ********************************************************

(2A_c)      TATTACGAATAAAATAATCTGGGAAATAGTTTGAATTACCGCGGGCTGAAAAAGCACCAC 480
(2A_p)      TATTACGAATAAAATAATCTGGGAAATAGTTTGAATTACCGCGGGCTGAAAAAGCACCAC 468
            ************************************************************

(2A_c)      TCCTTAAACTTAAAGTTGTCTGAAAGGATTCTGTCTGCCAATTTGTAANGGTGGCAACGC 540
(2A_p)      TCCTTAACCTTAAAGTTGTCTGAAAGGATTCTGTTTGCCAATTTGTAACGGTGGCAACGC 528
            ******* ************************* ************* ***********
```

Appendix Figure D8 The nucleotide sequence alignment of *cry* 2A gene from chromosomal DNA (2A_c) and *cry* 2A gene from plasmid DNA (2A_p) using EMBOSS Pairwise Alignment Algorithms.

Note * single, fully conserved residue,

- no consensus

```
(2A_c)      CCTCCCGATCTGAACCTGAATCTAGCCAACTTCTAACAAATGGTGTTGACAATGGAGGGA 600
(2A_p)      CCTCTCGATCTGAACCTGAATCCAGCCAACTTCTAACAAATGGTGTTGACAATGGAGGGA 588
            **** ***************** ************************************

(2A_c)      GAAATGTGCTACAATTAAAATTTTGATTGAACTTAGACGCCCCTATATCACCAGATGAAA 660
(2A_p)      GAANTGTGCTGCAATTAAAATTGTGATTGANATGAGACGCCCCTATATCACCAGATGAAA 648
            *** ****** ********** *******  * *************************

(2A_c)      CTCCTCCACTGTAATTAACCCTTGCACTATGCAATGCGTGAGTTGTAGTACAACCCGGTA 720
(2A_p)      CTCCTCCGCTGTAATTAACCCTGGCACTATTCAATGCGTGAGTTGTAGTACNACCCGGTA 708
            ******* ************* ******* ****************** ********

(2A_c)      AACCAAC-TATATTAGGGAAGGTAATAGAAAGCCTAGCACCACTAAAT-CCATTTAATAT 778
(2A_p)      AACCAACCTATATTAGGGAAGGTATTAGAAAGCCTAGCACCACTAAATACCATTTAACAC 768
            ******* ********** ********** ********* ******** *

(2A_c)      ATAATTCGAATTAACTTGGAAAAGAGAATATAAAAATGGCCAGTCTT-GTGCTGTAAATG 837
(2A_p)      ATAATTCGAATTAACTTGGAAAAGAGAATATAAAAATGGCCAGTCTTTGTGCTGTAAATG 828
            ********************************************** ************

(2A_c)     ATTGTGTCTGCTGTGGTCCACTACCACTTG-CATATAAATTAGCAGCC-AGAAGATAC-C 894
(2A_p)     ATTGNGTCTGCTGTGGTCCACTACCACTTNGCATATAAATTAGCNGCCGAGAAGATACTN 888
           **** ************************  ************* *** *********

(2A_c)     ATAAGACTT-TGATATTTAAACAATG-ACCAGATAGATACATACT-CAAATACATTTAAA 951
(2A_p)     NTAAGACTTCTGATATTTAAACNANNGACCAGATNGNNACATACTTCAAATACATTTAAA 948
           ******** ************ *   ******* *  ******* **************

(2A_c)     AAACATATATGTTCTAAATT-CTAACATATCGTGTAAACGAGTGTTTAAACCCTTTAAAC 1010
(2A_p)     AA-CATATATGTNCNAAATTTCTAACATATCGTGTAAACGAGNGTTTAACCCTTNTAAAC 1007
           ** ********* * ***** ******************** ****** ** * *****

(2A_c)     GCATTTT-GATACGTATTT-ATACAATAATTAAAAATAATCCCTTGGAAAA 1059
(2A_p)     GCNNNTTTGATACGTANTTTATACAATA---------------------- 1035
           **   ** ******** ** ********
```

Appendix Figure D8 (Continued)

Note * single, fully conserved residue,

- no consensus

Appendix Table D1 Sequence comparison of *cry*1Ac genes on chromosomal DNA with other *cry*1AC genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position (+/+)
HM061081.1	*cry*1Ac	ZQ-89	99	1479-2244
GU294785.1	*cry*IAc	HD07	99	1858-2623
EU282379.1	*cry*1Ac	*kurstaki* strain W015	99	1561-2326
EU583385.1	*cry*1Ac	DOR3	99	48-813
AY925090.2	*cry*1Ac	*kenyae* strain HD-549	99	1479-2244

Appendix Table D2 Sequence comparison of *cry*1Ia genes on chromosomal DNA with other *cry*1I genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position
EU887515.1	*cry*1Ia	Bt11	99	43-1113
DQ535488.1	*cry*1Ia	-	99	43-1113
AY262167.1	*cry*1I	-	99	43-1113
AF517127.1	*cry*1Ia	-	99	126-1196
AJ315121.1	*cry*1Ia	*kurstaki*	99	354-1424

Appendix Table D3 Sequence comparison of *cry*2A genes on chromosomal DNA with other *cry*2 genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position (+/-)
AF441855.1	*cry*2Ab	ly30	94	1679-647
AF164666.1	*cry*2Ab	-	94	1909-877
X55416.1	*cry*IIB	*kurstaki* HD-1	94	2552-1520
M23724.1	*cry*2B	-	94	1909-877
X55416.1	*cry*IIB	*kurstaki* HD-1	94	1520-2552

Appendix Table D4 Sequence comparison of *cry*14 genes on chromosomal DNA with other genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position (+/+)
ZP04174944.1	Tryptophan 2,3-dioxygenase	*Bacillus cereus* AH1273	98	589-5
ZP04169190.1	Tryptophan 2,3-dioxygenase	*Bacillus mycoides* DSM 2048	98	589-5
ZP04197792.1	Tryptophan 2,3-dioxygenase	*Bacillus cereus* AH603	98	589-5
ZP04295160.1	Tryptophan 2,3-dioxygenase	*Bacillus cereus* AH621	97	589-5
ZP00743510.1	Tryptophan 2,3-dioxygenase	*Bacillus thuringiensis* serovar *israelensis*	98	580-5

Appendix Table D5 Sequence comparison of *cry*1I genes on plasmid DNA with other *cry*1I genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position (+/+)
EU887515.1	*cry*1Ia	Bt11	99	35-1106
DQ535488.1	*cry*1Ia	-	99	35-1106
AY262167.1	*cry*1I	-	99	35-1106
AF517127.1	*cry*1Ia	-	99	118-1189
AJ315121.1	*cry*1Ia	*kurstaki*	99	346-1417

Appendix Table D6 Sequence comparison of *cry*2A genes on plasmid DNA with other *cry*1I genes in database at NCBI with Blast-N program

Genbank Accession No	Gene	*B. thuringiensis* subsp./strain	Identities (%)	Position (+/-)
AF441855.1	*cry*2Ab	ly30	92	664-1684
AF164666.1	plasmid *cry*2Ab	-	92	894-1914
X55416.1	*cry*IIB	HD-1 plasmid	92	1537- 2557
M23724.1	*cry*2B	-	92	664-1684
DQ361266.1	*cry*2Ab	-	92	664-1684

Appendix E

Data of insect bioassay

Appendix Table E1 The insecticidal activity of spore-crystal suspension (10^9 spores-crystals mL^{-1}) on lepidopteran, dipteran, coleopteran at 24 hours after ingestion.

Insect species	mortality			Total	Mortality[1/]
	Rep.1	Rep.2	Rep.3		
Spodoptera litura	10	6	8	24	80
Spodoptera exigua	8	9	9	26	86.67
Galleria mellonella	0	0	0	0	0
Bactrocera dorsalis	0	0	0	0	0
Musca domestica	0	0	0	0	0
Aedes aegypti	3	2	5	10	16.66
Tenebrio molitor	0	0	0	0	0
Tribolium castaneum	0	0	0	0	0

Remark [1/] Corrected mortality using Abbott's formula

Appendix Table E2 The insecticidal activity of spore-crystal suspension (10^9 spores-crystals mL^{-1}) on lepidopteran, dipteran, coleopteran at 48 hours after ingestion.

Insect species	mortality			Total	Mortality[1/]
	Rep.1	Rep.2	Rep.3		
Spodoptera litura	10	10	8	28	93.33
Spodoptera exigua	10	10	9	29	96.67
Galleria mellonella	0	0	0	0	0
Bactrocera dorsalis	0	0	0	0	0
Musca domestica	0	0	0	0	0
Aedes aegypti	17	13	10	40	66.66
Tenebrio molitor	0	0	0	0	0
Tribolium castaneum	1	0	0	1	0.01

Remark [1/] Corrected mortality using Abbott's formula

Appendix Table E3 The insecticidal activity of spore-crystal suspension (10^9 spores-crystals mL^{-1}) on lepidopteran, dipteran, coleopteran at 72 hours after ingestion.

Insect species	mortality			Total	Mortality[1/]
	Rep.1	Rep.2	Rep.3		
Spodoptera litura	10	10	8	28	93.33
Spodoptera exigua	10	10	10	30	100
Galleria mellonella	0	0	0	0	0
Bactrocera dorsalis	0	0	0	0	0
Musca domestica	0	0	2	2	3.33
Aedes aegypti	20	18	20	58	96.66
Tenebrio molitor	0	0	0	0	0
Tribolium castaneum	3	0	0	3	5

Remark [1/] Corrected mortality using Abbott's formula

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