CHARACTERIZATION OF *CRY* GENES AND INSECTICIDAL PROTEINS FROM INDIGENOUS *BACILLUS THURINGIENSIS* TO DEVELOP POTENTIAL BIOPESTICIDE

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ABSTRACT

Indiscriminate use of chemical pesticides, the cause of serious public health problems and environment pollutions as well as the emergence of pest resistance in many developing countries, could be reduced with *Bacillus thuringiensis* (*Bt*) biopesticides for its eco-friendly properties. The work was aimed at controlling vegetable pests by foliar spraying of locally developed potential *Bt* biopesticides as the facts of immunological adverse effects and increasing insect resistance due to the continuous exposure to the *Bt* toxins were reported for transgenic crops. *Bt* isolates were, therefore, isolated from different eco-regions of Bangladesh and characterized based on their phenotype, genetics, proteomics, diversity, toxicity and field efficacy.

Upon biochemical typing of 298 indigenous *Bt* isolates, *Bt indiana* (17%), *kurstaki* (16%), *thuringiensis* (12%) were found to be the most prevalent biotypes in Bangladesh and from the distribution index, an indication of the degree of ubiquity, *Bt thuringiensis* (0.5) and *indiana* (0.5) appeared to be more ubiquitous than all other biotypes followed by eleven (0.43), *kurstaki*, *sotto* and nine (0.4 for each).

Variation in abundance and distribution pattern of 16 different biotypes in Bangladesh was demonstrated in comparison to the other parts of the world. Broad spectrum cytolytic activity was observed to vary with biotypes and 58.36% strains were presumed to insecticidal based on this attribution. The molecular weight based distribution of small plasmids was compared among the biotypes and the maximum diversity was observed among *Bt thuringiensis, kurstaki, indiana* and *israelensis* biotypes.

The screening for insecticidal genes in the native *Bt* strains revealed their presence in varied proportion being *cry1* to be the most prevalent (30.8%) followed by *cry2* (25.5%), *cry3* (22.2%) and *cry9* (7.2%). Prevalence of *cry4*, *cry8* and *cry10* genes were less than 5% and *cry11* gene was not found in any strain. The SDS-PAGE analysis of the partially purified Cry proteins revealed the presence of Cry1- type delta endotoxins including Cry1Aa, Cry1Ab, Cry1Af, Cry1B, Cry1C and Cry1M, Cry2, Cry3, Cry8 and Cry9 proteins which were also supported by the *cry* gene profiles.

Genetic diversity among 177 *Bt* strains were analyzed by RAPD-PCR which revealed 15 genotypes and type 9 and type 11 were found to contain more than 25% of the strains. In

combination of RAPD-PCR and *cry* gene profile analysis, genotype based distribution of *cry* genes was evaluated and Genotype 1, 6, 9, 11 and 12 were found to contain the most of the *cry* genes at different extent while *cry* genes with diversities were most prevalent in genotype 6, 9 and 1. Genetic diversity analyzed thus is highly relevant and significant in discovering novel insecticidal genes in indigenous *Bt* strains and to deal with the problems of emerging resistance towards *Bt* toxins.

Novel toxicity of indigenous *Bt* strains was identified at significant level against the larvae of *Bactrocera cucurbitae* and the efficacy of *Bt* JSc1 was highly comparable with the reference strain *Btk* HD-73 with no statistically significant difference. The complete Open Reading Frame (ORF) of *cry1A-* type gene from *Bt* strain JSc1 was, therefore, obtained by PCR walking technique and was revealed to be identical to *cry1Aa9* and *cry1Aa13* genes. Five conserved block of Cry proteins were identified from the deduced amino acid sequence analysis and the 3-D protein structure constructed by homology modeling revealed its 3-domain protein fold.

A cost effective medium MSeMC-AS was formulated with defatted soybean meal, molasses, marine water, cystine and ammonium sulfate which demonstrated more than 80% increase in yield during scaling up from shake flask to a 3.0 L bioreactor and the growth kinetics of indigenous *Bt* strain JSc1 was also revealed faster than the reference *Btk* HD-73 in this medium. Yield in δ- endotoxin production was enhanced by inhibiting the endogenous protease activity which was demonstrated to be the cause of protein degradation and loss in productivity. From the field trial of *Bt* biopesticide preparation in cabbage, cauliflower and organic tea farming, no significant differences were observed with the active treatments which indicated high feasibility of the prepared *Bt* biopesticide in replacing conventional pesticides with no yield loss.

These results suggest that the *Bt* strains of Bangladesh with their unique and diverse properties as well as potentials as revealed in this study, could potentially be utilized not only in controlling the pests in agriculture but also the vectors, protozoa, nematodes etc of public health concern.

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ABBREVIATIONS

%	Percentage
(v/v)	Volume/ volume e.g. (mL/mL)
(w/v)	Weight/ volume e.g. (g/mL)
°C	degrees Celsius
Bc	*Bacillus cereus*
bp	Base pairs
Bs	*Bacillus anthracis*
BSA	Bovine serum albumine
Bt	*Bacillus thuringiensis*
Btj	*Bacillus thuringiensis* serotype *japonensis*
Btk	*Bacillus thuringiensis* serotype *kurstaki*
Bts	*Bacillus thuringiensis* serotype *sotto*
cfu	Colony forming unit
cm	centimeter
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide
DTT	1,4–dithio–D–threitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
et al	and others
EtBr	Ethidium Bromide
EtOH	Ethanol
Fig.	Figure
g	grams
hrs	Hours
ICP	Insecticidal crystal protein
kb	kilo bases
Kg	kilo gram
l	litre
LB	Luria Bertani
LC_{50}	Lethal concentration of 50% of the population
LC_{99}	Lethal concentration of 99% of the population
M	molar
mg	milligram
min	Minutes
mm	millimeter
mM	millimolar
MW	Molecular weight
n	Number of replicates
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RCBD	Randomized Complete Block Design
rDNA	ribosomal DNA
RH	Relative Humidity

RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	ribosomal RNA
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Seconds
SEM	Scanning electron microscopy
SLPM	Standard liter per minute
Taq	*Thermus aquaticus*
TBE	Tris- borate- EDTA buffer
TE	Tris EDTA buffer
TEMED	Tetramethyl ethylene diamine
δ	Delta
µg	microgram
µl	microlitre
σ	Sigma
(k) Da	(kilo) Dalton
PCM	Phase Contrast Microscope
V	Volt
TCA	Tri- chloro acetic acid
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra violet

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CHAPTER 1

Introduction

1.0 INTRODUCTION

Bangladesh is an agriculture based country where 25% annual loss in vegetable production (Rahman, 2000) is caused by pests which is not affordable. These pests are mainly caterpillar, fruit and shoot borer, stem and pod borer etc. These pests are controlled by indiscriminate use of chemical pesticides and the use has been doubled after every decade according to the report of Department of plant protection, Bangladesh (Meisner and Dasgupta, 2004). Based on a survey by world bank at 2004, major chemical pesticides currently in use in Bangladesh are, organophosphate, carbamate, pyrethroid, 2-dimethylaminopropane-1,3-dithiol, dithiocarbamate etc (Meisner and Dasgupta, 2004). These indicate that pesticides have occupied a strong position in our agriculture by reducing the loss that happens due to the pest infestation.

The problems with these pesticides remain in their non-specificity as affect both beneficial insects as well as noxious pest species and in hazardous as well recalcitrant nature that cause damage to the environment and public health. With their persistence in soil, plant and aquatic bodies, these are being incorporated into the food chain continuously. Bioaccumulation and biomagnifications are the major processes by which pesticides can be shifted from environment (0.04 ppm) to animal and human bodies (25 ppm) through the food chain. It not only affects microbial flora, fauna, fishes and mammalian health but also causes emergence of resistance in the pests. The most alarming and terrible thing is that pesticides are being taken either by eating or drinking or inhaling both consciously and unconsciously by a large population.

As the pesticides are designed to impair the immune, reproductive or nervous systems of insects and killing thereby, an ideal pesticide must be lethal to the targeted pests only, not to the non-target species including human. A wide range of acute and chronic human health problems, unfortunately, have been linked to the indiscriminate uses of chemical pesticides. Acute health effects appear shortly after the exposure to these pesticides such as skin and eye irritations, headaches, dizziness and nausea, weakness, breathing difficulty, mental confusion and disorientation, seizures, coma and even death etc. Chronic health effects normally appear after months or years after the exposure even at minimal level in the environment or from the

ingested food and water contaminated with pesticide residues. These include disorders in nervous, reproductive and immune systems and cancer as well (Aktar *et al.*, 2009). Again, Non-Hodgkin lymphomas, leukemia, lung cancer, aplastic anemia, fetal death, etc were reported to have epidemiological links with chemical pesticides. Even the organophosphates that are used indiscriminately considering safer, affect our central nervous system by inhibiting the acetyl choline esterase.

To get rid of these problems associated with chemical pesticides, Integrated Pest Management (IPM) and Bio-intensive pest management (BIPM) are the recommended and largely practiced methods in the developing countries. Organic farming, rotation of crops, sanitization of fields, protecting natural enemies, replacing chemical insecticides by using biological controls such as biopesticide, transgenic crops etc are employed and emphasized in these practices (Gelernter *et al.*, 1999). Biopesticides from plants, microorganisms, minerals, animals etc have been in use in the developing countries for more than half a century. Biological insecticides are bio-degradable and non toxic to beneficial insects, mammals, humans etc and among the biological pests control agents, *Bacillus thuringiensis* (*Bt*) based insecticide is an excellent example.

Bacillus thuringiensis (*Bt*) is a Gram positive spore former belonging to *Bacillus cereus* group. The key distinguishing feature is its capability to produce insecticidal crystal proteins during sporulation which are highly specific in toxicity against many insect orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera etc. Crystal proteins bind to the epithelial membrane receptors of larval gut being solubilized in the gut environment followed by proteolytic activation. And the sequential oligomerization of the helical domain of the toxin creates lytic pores which cause electrolytes imbalance and starvation and paralyze the larvae leading to death. The high specificity that prevails in this process of toxin-receptor binding based on the structural properties makes the *Bt* toxins lethal only to the specific pests and leaving other biotic elements unharmed. *Bt* has advantages over many synthetic insecticides in that its products have less non-specific activity and are considered more environmentally friendly. This gram-positive, spore-forming bacterium produces one or more insecticidal proteins (Cry proteins) as its main virulence factors. Cry proteins have formed the basis of biological insecticides used to control agricultural, health and forestry pests for more than 70 years (Bravo *et al.*, 2011; Schnepf *et al.*, 1998).

Although many of the components of IPM and BIPM are in practice in Bangladesh but *Bt* based biopesticide is absent to date. Many countries have studied their *Bt* resources nationally and developed *Bt* biopesticides based on the indigenous strains. Recently, transgenic *Bt* brinjal has been introduced by Bangladesh Agriculture Research Institute (BARI) and these *Bt* brinjal varieties are under the field evaluation. Transgenic or genetically modified (GM) crops, the source of nutrition, must pass the GMO test and for *Bt* brinjal, the test performed by Dr. Gallagher was not affirmative (Heinemann *et al.*, 2011). The fact of immunological adverse effects revealed from this test is the cause of great anxieties. Again, this approach for crop protection with genetically engineered plants producing these toxins raised some serious concerns with the reports of increasing insect resistance to *Bt* toxins (Estruch *et. al.*, 1997). This is due to the exposure of the toxins throughout the entire growing season which increases the selection pressure on the insects (McGaughey *et al.*, 1998; Holmes, 1997; Stix, 1998). On the other hand, foliar spraying of *Bt* biopesticide has been established as safer for its specificity, non-integrating nature into the plant system and free of any immunological adverse effects. And, the farmer friendly spraying without extensive protection can be performed targeting the life cycle of the pests, hence, the chances of resistance is low.

In Bangladesh, there was no extensive national study with *Bt* aimed at the production of potential biopesticide, hence, *Bt* biopesticide is not yet in the IPM of Bangladesh agriculture. It has been reported that *Bt* can be present in many different habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, olive tree related-habitats, plant and aquatic environments (Martin and Travers 1989; Meadows *et al.*, 1992; Bel *et al.*, 1997; Ben-Dov *et al.*, 1997; Bravo *et al.*, 1998; Iriarte *et al.*, 2000). In fact, each habitat may contain a novel *B. thuringiensis* strain awaiting discovery which has a toxic effect on a target insect group. So, *Bt* biopesticide from indigenous strains with locally improvised technology and raw materials will be more sustainable and economic. With the concern of developing cost effective biopesticide from potential indigenous *Bt* strains, the work was, therefore, started with the isolation of the strains from different eco-regions of Bangladesh and characterized in terms of their genetics, proteomics, diversity, toxicity and field efficacy.

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CHAPTER 2

Literature Reviews and Objectives

2. LITERATURE REVIEWS

2.1 General Characteristics of *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*), a member of the genus *Bacillus*, is a rod shaped, motile, gram-positive, facultative anaerobe and spore-forming bacterium the size of which varies between 3 and 5 µm. The endospore may remain viable for centuries as it is more resistant than the vegetative cell to heat, drying, disinfection and other destructive agents like other spore-formers. The key distinguishing feature of *Bt* is its ability to form intracellular parasporal crystal shaped δ- endotoxins (Fig 2.1) during sporulation at the stationary phase of its growth cycle (Schnepf *et al.*, 1998). Crystal development does not occur in vegetative stage but begins only after the sporulation phase when growth and nucleic acid synthesis stop. Commonly each sporulating cell contains one crystal, but cell containing two crystals has been seen (Heimpel and Angus, 1963). These δ- endotoxins have been found to be toxic against the larvae of numerous specific insect species in the orders Lepidoptera, Diptera and Coleoptera etc (de Barjac, 1981; Gonzales *et al.*, 1982; Andrews *et al.*, 1987). *Bt* have, therefore, been widely used as bioinsecticide for more than 60 years.

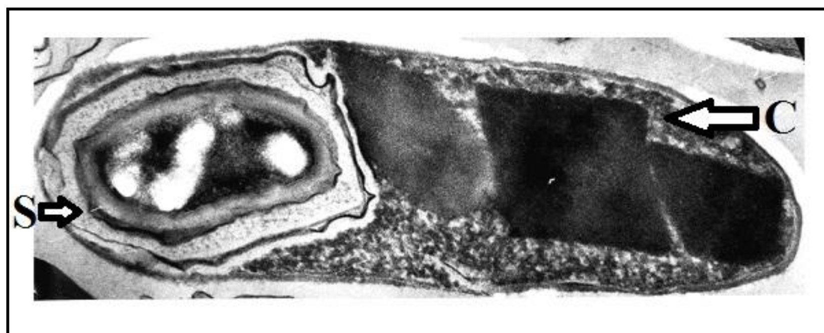

Figure 2.1: Formation of Crystal shaped δ- endotoxin during sporulation in the parasporal body of *Bacillus thuringiensis* (S: Spore, C: Crystal shaped protein).

2.1.1 Life cycle

The life cycle of *Bt* can be divided for convenience into phases and these are Phase I: vegetative growth; Phase II: transition to sporulation; Phase III: sporulation; and Phase IV: spore maturation and cell lysis (Hilbert and Piggot, 2004; Berbert-Molina *et al.*, 2008). The production of the characteristic insecticidal (Cry) proteins deposited in crystals in the mother

cell have been shown to mainly start from the onset of sporulation (Sedlak *et al.*, 2000; Xia *et al.*, 2005; Guidelli-Thuler *et al.*, 2009; Pérez-García *et al.*, 2010).

Life cycle of *Bt* is more complex than that of non-sporulating bacteria. When vegetative cells are growing on rice medium, the cells do not form spores or crystals. However, both sporulation and crystal formation are induced at the end of logarithmic growth when the cells are deprived of carbon and nitrogen sources in the medium. The first appearance of crystal protein is at about two hours after the beginning of stationary phase but deposition into a crystal does not occur until eight hours (Bechtel and Bulla Jr., 1976). Agassie and Lereclus, (1995) reported that the endospore developed in a sporangium consisting of two cellular compartments known as the mother cells and the fore spore. The development process was temporally regulated at the transcriptional level by the successive activation of six sigma factors, which, by binding to RNA polymerase, determine which gene promoters were recognized. These factors were the primary sigma factor of vegetative cells, σA, and five factors that were activated during development and called σH, σF, σE, σG, σK in order of their appearance during sporulation. The σA and σH factors were active before the septum forms, σE and σK in the mother cell and σF and σG in the forespore.

2.1.2 The Genus *Bacillus*: Relation of *Bt* with other *Bacillus* spp.

Bt is very closely related to *Bacillus cereus* and *Bacillus anthracis* (Helgason *et al.*, 2000). The genes encoding δ- endotoxins are very often plasmid mediated and could be transferred to other related species (e.g. *B. cereus, B. anthracis and B. mycoides*) by conjugation and it has been observed that these relatives could express the toxin and produce crystal protein (Hu *et al.*, 2004). *Bt* has also been observed to produce *B. cereus* type enterotoxin, suggesting the conjugative transfer of enterotoxin producing genes from *B.cereus* to *Bt* (Carlson and Kolstø, 1993).

The psychrotolerant *B. weihenstephanensis* has been proposed as a new member of the group (Lechner *et al.*, 1998). Several studies have been dedicated to make comparison between *Bt* and *Bc* on the basis of their characters not related to the production of insecticidal crystal proteins (ICP) which revealed the phenotypic and genotypic discrimination between them to be very difficult (Hansen and Hendriksen, 2001).

2.1.3 History

2.1.3.1 Discovery of *Bt* and its toxicity

Bt was first isolated by the Japanese scientist S. Ishiwata, in 1902, from silkworm larvae (*Bombyx mori*) exhibiting the sotto disease and named that as *Bacillus sotto*. In 1915, Berliner formally characterized the species from a diseased Mediterranean flour moth caterpillar (*Ephestia kuhniella*) in Thuringia province, and linked it to the cause of a disease called Schlaffsucht (Milner, 1994).

In 1956, the main insecticidal activity of *Bt* against Lepidopteran insects due to parasporal crystals was established (Angus, 1956). In 1976, with the discovery of *Bt* subsp. *israelensis* by Margalit and Tahori in Israel, disease causing dipteran insect pests such as mosquitoes and blackflies was successfully controlled (Margalit and Dean, 1985). In 1983, *Bt* subsp. *tenebrionis* has been described effective against the larvae of coleopteran insects (Krieg *et al.*, 1983).

2.1.3.2 Application and research development

Bt first became available as a commercial insecticide, against flour moth, in France in 1938 (Neppl, 2000). The discovery insecticidal activity of *Bt* against Lepidopteran insects, increased the interest of other researchers in crystal structure, biochemistry and mode of action of toxins. In 1950s, *Bt* has been started to be used commercially in US and named as Thuricide. By 1961, *Bt* has been registered as biopesticide to the United States Environmental Protection Agency (USEPA). Dulmage discovered more active *Bt* var. *kurstaki* (HD1), which was commercialized in the USA as Dipel (Glazer and Nikaido, 1995). Up to 1976, *Bt* has been available only for control of Lepidoptera (butterflies and moths), with a highly potent strain *Bt* subsp. *kurstaki* (Dulmage, 1970). In 1980s, biotechnological developments have stimulated researchers to screen for large number of natural *Bt* isolates to find different strains toxic against other insect orders and with these efforts, *Bt* subsp. *aizawai* active against both Lepidoptera and Diptera orders (Glazer and Nikaido, 1995) and *Bt* subsp. *sandiego* active against beetles (Hernstand *et al.*, 1986) has been introduced into markets. *Bt* pesticides have been used in field trials with marked success in the management of pest control programs (Yang & Wang, 1998) and have been applied in a variety of agriculturally important crops such as cotton, corn, potato, soybean and many vegetables (Yang & Wang, 1998). In addition to field trials,

Bt has been sold commercially as a biopesticide for over half a century (Jenkins & Dean, 2001).

At the end of the 1980s, the first report came on the insertion of genes encoding *Bt* toxic proteins into plants. The first transgenic plants expressing *Bt* toxins were tobacco and tomato (Van Frankenhuyzen, 1993). *Bt* field cotton was the first *Bt*-transgenic plant registered by United States EPA (USEPA, 1999). Rapidly developing recombinant DNA technology after 1990 became an important tool to develop genetically manipulated *Bt* pesticides. Today, major *Bt* transgenic crops include corn, cotton, potatoes and rice. They have been commercialized and are in use widely in Canada, Japan, Mexico, Argentina, Australia and United States (Frutos *et al.*, 1999). Both *Bt* in the form of transgenic crop and spray formulations are still being widely used (Lui and Tabashnik, 1997).

2.1.4 Natural habitats of *Bt*

B.thuringiensis is indigenous to many environments including soil **(**Martin and Travers, 1989; Bernard *et al.*, 1997**)**, insect cadavers (Carozzi *et al.*, 1991; Kaelin *et al.*, 1994), stored product dust (Hongyu *et al.*, 2000), leaves of plants (Mizuki *et al.*, 1999), and aquatic environments (Iriarte *et al.*, 2000). Moreover, *Bt* has recently been isolated from marine sediments (Maeda *et al.*, 2000), and also from the soils of Antarctica (Forsty and Logan, 2000). Thus, it is obvious that *Bt* is widespread in nature.

As *Bt* was first isolated from diseased lepidopterans larvae, it has long been thought that the occurance of bacterium is associated with insect breeding environments. Earlier workers have reported that high density populations of *Bt* are often retained in stored-product environments, diseased silkworm larvae, animal feed mill, phylloplanes, insect habitats and insect larvae (Kaur, 2006). However, the normal habitat of the organism is soil and it was found in several reports that *Bt* is also widely distributed in natural soils of various areas of the world (DeLucca *et al.*, 1981; Ohba and Aizawai, 1986; Martin and Travers, 1989; Hastowo *et al.*, 1992).

2.1.4.1 Distribution of *Bt*

Bt is a ubiquitous bacterium that has been isolated from diverse geographical areas and from different sources. It is widely distributed in natural soils of various parts of the world (Martin

and Travers, 1989). It was found in soil in the United States (Saleh *et al.*, 1969), Philippines (Padua *et al.*, 1984), Japan (Ohba and Aizawa, 1986), Indonesia (Hastowo *et al.*, 1992), New Zealand (Chilcott and Wigley, 1993), Taiwan (Chak *et al.*, 1994), Africa (Zelazny *et al.*, 1994), Korea (Lee *et al.*, 1995) and Thailand (Attathom *et al.*, 1994). Martin and Travers, (1989) isolated *Bt* from soil samples in five continents (Africa, Asia, Europe, North and South America) and their associated islands. They found that the frequency of this bacterium is higher in East Asia than in other parts of the world.

It was suggested that soil is the primary habitat of *Bt* in nature. The normal habitat of *Bt* is soil but it also occurrs in other environments. Insectary was found to be a common source of this bacterium. It was isolated from the litters and soil in sericulture farms (Ohba and Aizawa, 1978; Hastowo *et al.*, 1992). Other studies described the habitats of *Bt* as it was isolated from dead and diseased insects (Chanpaisang *et al.*, 1991; Chilcott and Wigley, 1993; Kaelin *et al.*, 1994), insect habitats (Brownbridge and Margalit, 1986; Orduz *et al.*, 1992), grain dust and leaf surface (Smith and Couche, 1991). The diversity of habitat from which subspecies of *Bt* strains have been isolated indicates that the ecology of this bacterium is probably very complex (Itoua-apoyolo *et al.*, 1995).

2.1.4.2 Ecological role and Prevalence

The organism grows naturally as a saprophyte, feeding on dead-organic matter, therefore, the spores of *Bt* persist in soil and vegetative growth occurs when nutrients are available. Because of this, *Bt* can also be found in dead insects. Meadows, (1992) suggested three prevailing hypothetical niches of *Bt* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism. However, the true role of the bacteria is not clear. Although, it produces parasporal crystal inclusions that are toxic to many orders of insects, many *Bt* strains obtained from diverse environments show no insecticidal activity. For example, Maeda *et al.*, (2000) has found that *Bt* strains obtained from marine environments of Japan exhibit no insecticidal activities. The insecticidal activity of *Bt* is rare in nature. For instance, Iriarte *et al.*, (2000) reported that there is no relationship between mosquito breeding sites and pathogenic action level of *Bt* in the surveyed aquatic habitats. However, another study suggests that habitats with a high density of insect mortality were originated by the pathogenic action of this bacterium (Itoua-Apoyolo *et al.*, 1995).

Bt occurs naturally and it can also be added to an ecosystem artificially to achieve insect control. For this reason, the prevalence of *Bt* in nature can be defined as "natural" and "artificial". The habitat is considered as natural when *Bt* can be isolated when there is no previous record of application of the organism for insect control. The artificial habitats of *Bt* are areas sprayed with *Bt* based insecticides (usually a mixture of spores and crystals).

2.2 Classification of *Bt* strains

Bt is composed of an array of serotypes, biotypes, subspecies, strains etc each with its particular properties. The diversity of *Bt* is demonstrated in the almost 71 serotypes and the 92 subspecies described to date based on flagellar (H) antigens. Serotyping does not necessarily indicate the presence of toxicity or toxic genes. Among the recognized subspecies- some of the most commonly used include subspecies *kurstaki*, *israelensis*, *aizawai*, *thuringiensis, sotto, dendrolimus, morrisoni, tenebrionis* etc.

Based on 4 highly relevant and rapid biochemical tests, Martin and Travers, (1989) identified 16 different biochemical types from a total of 8916 isolates from 1115 samples. Biochemical characterization of the strains is important because each serotype has specific physiological characters and one serotype can be subdivided into biotypes based on the different enzymatic reactions. These biotypes also show different toxicity (de Barjac, 1981).

2.3 Virulence factors

2.3.1 The δ (Delta) - endotoxins

The δ-endotoxins are the major component of parasporal crystals which are visible under light and Phase Contrast Microscopes. The crystal is non-toxic until dissolved and activated. *Bt* strains produce two types of δ- endotoxin. They are - the Cry (Crystal) toxins, encoded by different *cry* genes, and the Cyt (Cytolytic) toxins, encoded by *cyt* genes which can augment the Cry toxins, enhancing the effectiveness of insect control.

2.3.1.1 The Cry proteins

Cry toxins are defined as proteins that have significant sequence similarity to existing toxins within the nomenclature or be a *Bt* parasporal inclusion protein that exhibits insecticidal

activity, or some experimentally verifiable toxic effect, to a target organism (Crickmore *et al.*, 1998). Naming of toxins is based solely on amino acid sequence identity and does not take into account their toxicity; thus, toxins that are active against the same order of insectwill not necessarily share any similarity in their names. There are currently around 75 primary subgroups of Cry toxins, i.e., with different primary ranks in the nomenclature (Cry1, Cry2, Cry3, etc.). Their lengths vary from 369 (Cry34) to 1344 amino acids (Cry43) (Adang *et al.*, 2014). In their 1989 review, Höfte and Whiteley identified five conserved sequence blocks in all the Cry toxins (Fig. 2.2). Not all of the toxins contain these blocks and that some only contain a subset of them. Based on experimentally derived structures, and molecular modelling, it is believed that the toxins that contain all, or some, of these conserved blocks are likely to possess the same basic three-domain fold (Pardo-Lo´pez *et al.*, 2013). Figure 2.2 provides a more complete list of currently classified toxins, excluding those that share the same, quaternary rank. Of the 294 toxins in this list, 262 (89%) are predicted to have the three-domain fold and are coloured blue in this figure. It is likely that the majority of Cry toxins produced by *Bt* will be in this three-domain class, although the proportion of these toxins in Fig. 2.2 is likely to be inflated due to the fact that many of them were isolated by PCR techniques based on conserved sequences in existing toxins. Two other classes of Cry toxins have been previously identified: the ETX/MTX-like toxins and the Binary (Bin) like ones. There are 11 toxins in the former group (coloured orange in Fig. 2.2) which show sequence similarity to the Clostridium perfringens epsilon toxin (Bokori- Brown *et al.*, 2011). The mosquitocidal MTX2 toxin from *Lysinibacillus sphaericus* is also related to this class (Berry, 2012).

2.3.1.2 The Cyt Proteins

Beside Cry proteins, some *Bt* strains also synthesize cytolytic proteins. Cyt proteins are parasporal inclusion (crystal) proteins from *Bt* that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein (Crickmore *et al.*, 1998). This class of δ- endotoxins differs in amino acid composition, protein structure and action mechanism from Cry toxins (Thomas and Ellar, 1983; Höfte and Whiteley, 1989). These toxins act synergistically with mosquitocidal Cry toxins (Poncet *et al.*, 1994). The Cyt toxins are only found in Dipteran specific strains, while the Cry toxins are present in many *Bt* strains with wide host range. One Cyt toxin is found in a given *Bt* strain, but two or more subclasses of Cry toxins can exist in a strain.

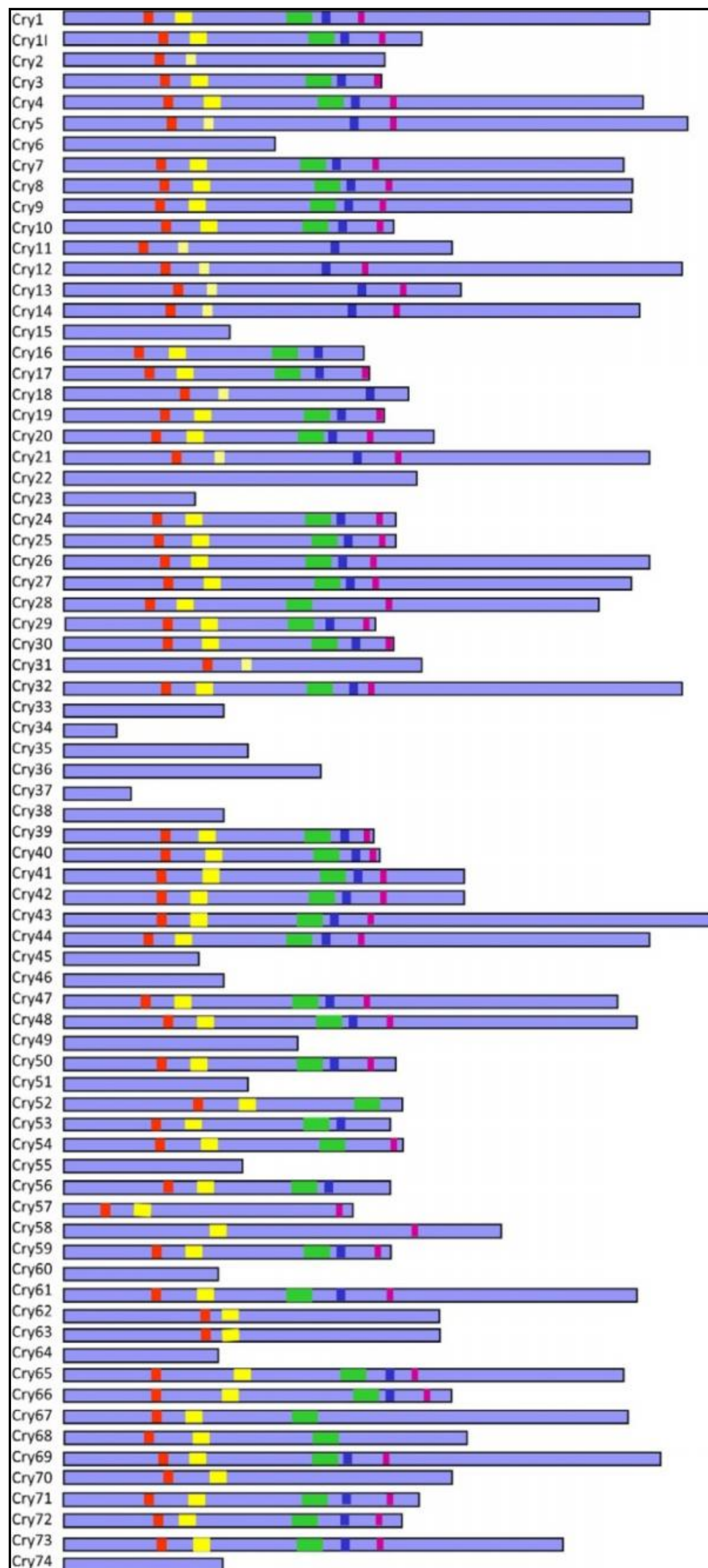

Figure 2.2: Graphical representation of the diversity of *Bt* Cry toxins. The length of each toxin is drawn to scale and the five conserved blocks described in Schnepf *et al.*, (1998) are shown as coloured inserts.

Although both the activated forms of these toxins can lead to pores in lipid bilayers, only the Cyt toxins cause the cytolysis of various eukaryotic cells including erythrocytes (Knowles *et al.*, 1989; Slatin *et al.*, 1990; Gill *et al.*, 1992). Cyt toxins may be used to overcome insecticide resistance and to increase the activity of microbial insecticides (Guerchicoff *et al.*, 2001). Cyt1 and Cyt2 are two cytolytic classes of Cyt toxins that have been identified on the basis of the amino acid identity and are divided into 37 subclasses (Crickmore *et al.*, 2014). Among these subclasses, Cyt1Aa and Cyt2Aa display the highest mosquitocidal activity (Koni and Ellar, 1994). Cyt1A may be used as a practical tool to manage resistance against *B. sphaericus*, which is also a mosquitocidal bacterium. Also other Cyt proteins may increase the insecticidal activity of non-Cyt proteins to other insects (Wirth *et al.*, 2000).

2.3.2 The Vip proteins

In addition to δ-endotoxins, *Bt* produces other toxic protein *viz*., vegetative insecticidal proteins (Vips). One of the interesting features of the Vip proteins is that they do not share sequence homology with the known delta-endotoxins (Estruch *et al.*, 1996) and the gene codes for a 791 amino acid (88.5 kDa) protein. But the mode of action of Vip protein is similar to that of the δ-endotoxins. Destruction of transmembrane potential which might be due to the pore formation may play a vital role in bioactivity. Shotkoshi and Chen, (2003) showed that Vip3A was processed in the larval gut of lepidopteran insects and proteolysis of Vip3A alone was not considered sufficient for insect specificity and further processing was necessary for its bioactivity. And the receptors for finding of Vip3A binding were found to be different from the known Cry1Ac and other receptors. Competition binding assays demonstrated that Vip3A did not inhibit the binding of either Cry1Ac or Cry2Ab2 and vice versa (Lee *et al.*, 2006). Many transgenic crop plants with *vip*3A gene have been produced (maize, rice, cotton, etc.) and tested for protection against the major lepidopteron pests. Vip3A offers an excellent control of bollworm complex, beet armyworm, fall armyworm, and loopers (Shotkoshi and Chen, 2003).

2.3.3 Other virulence Factors of *Bt*

Bt produces various virulance factors other than δ-endotoxins and VIP- toxins. A series of extracellular compounds are ynthesized and contribute to the virulence, such as α-exotoxin, β- exotoxin, phospholipase, protease and chitinase (Levinson, 1990; Lövgren *et al.*, 1990;

Zhang M. *et al.*, 1993; Palvannan and Boopathy, 2005; Hajaij-Ellouze *et al.*, 2006). *Bt* also produces antibiotic compounds having antifungal activity (Stabb *et al.*, 1994). However, the cry toxins are more effective than these extracellular compounds and allow the development of the bacteria in dead or weakened insect larvae. *Bt* strains also produce a protease, which is called inhibitor A. This protein attacks and selectively destroys cecropins and attacins which are antibacterial proteins in insect. As a result of this, the defense response of the insect gets collapsed. The protease activity is specific, because it attacks an open hydrophobic region near the C-terminus of the cecropin and it does not attacks the globular proteins **(**Dalhambar and Steiner, 1984). Also, the spores themselves contribute to pathogenity, often synergizing the activity of the crystal proteins (Johnson and Bishop, 1996).

2.3.3.1 α- exotoxin

There are few reports on α-exotoxin which was identified as lecithinase C or phospholipase C. The enzyme is water soluble, heat labile and toxic to the insects. This thermolabile enzyme is accumulated during the exponential growth and is capable of lysing many types of cell and toxic to mice and the diamondback moth (*Plutella xylostella*) (Dulmage, 1981), *Galleria mellonella* and some sawfly species and others (Rowe, 1987).

2.3.3.2 β- exotoxin

β- exotoxin known as fly factor or thuringiensin, is a thermostable exotoxin secreted by some varieties of *Bt* during the end of exponential growth and beginning of sporulation. It is a low molecular weight, water soluble substance which affects several orders of insect when injected into the body cavity (Heimpel and Angus, 1963). The mode of toxin action of thuringiensin in living system is by inhibition of RNA polymerase enzyme acting competitively with ATP. In human blood cells, it has been shown to increase chromosomal aberration. Because of its mutagenic and teratogenic properties, it is banned in North America and Europe, but is used for insect control in USSR (Rowe, 1987).

2.3.4 Non-insecticidal δ-endotoxins

Another class of δ- endotoxin is mammalian cell recognizing crystal proteins. In natural environments non-insecticidal *Bt* strains are more widely distributed than insecticidal ones

(Ohba and Aizawa, 1986; Mizuki *et al.*, 1999a). This is related to the absence of trypsin recognition sites on the crystal proteins (Gill *et al.*, 1992). Human cancer cell killing activities are associated with parasporal inclusions which do not exhibit insecticidal and haemolytic activity and named as parasporin (Mizuki *et al.*, 1999b).

2.4 Structural Features of Crystal Proteins

The three dimensional structures of the four δ-endotoxins (Cry 1, Cry 2, Cry 3 and Cyt 2A) have been resolved by X-ray crystallography (Li *et al.*, 1991; Grachulski *et al.*, 1995; Li *et al.*, 1996).

The Cry 1, Cry 2, and Cry 3 are remarkably similar, each of them consisting of three domains (Fig 2.3A and Fig 2.4). Domain I is composed of The N-terminal amino acids 1-290 and contains a hydrophobic seven α-helices amphiphatic bundle, with six α-helices surrounding a central helix. This domain contains all of the first conserved blocks and a major portion of the secondary conserved block of amino acid. Computer models showed that helix bundle of this domain could form a pore through the microvillar membrane of midgut epithelial cells.

Domain II extends from amino acid 291-500 and contains three anti-parallel beta-sheets around a hydrophobic core with three-fold symmetry and the conformation is called 'Greek Key'. This domain contains most of the hyper variable region and most of conserved block three and four. The crystal structure of the molecule together with recombinant DNA experiments and binding studies indicate extended loop structures in the beta sheets are the portions of the molecular responsible for initial recognition and binding of the toxin to binding sites on the microvillar membrane.

Domain III is composed of amino acids 501-644 and consists of two anti-parallel beta-sheets within which are found the remainder of conserved block number three along with blocks four and five. This C-terminal domain consists in a 'jelly-roll' formation. This domain is involved in maintaining the structural integrity of the molecule (Lee *et al.*, 1995; Schnepf *et al.*, 1998).

Domain I is involved in membrane insertion and pore formation. Domains II and III are both involved in receptor reorganization and binding. Additionally, a role for domain III in pore function has been found (De Maagd *et al.*, 1996).

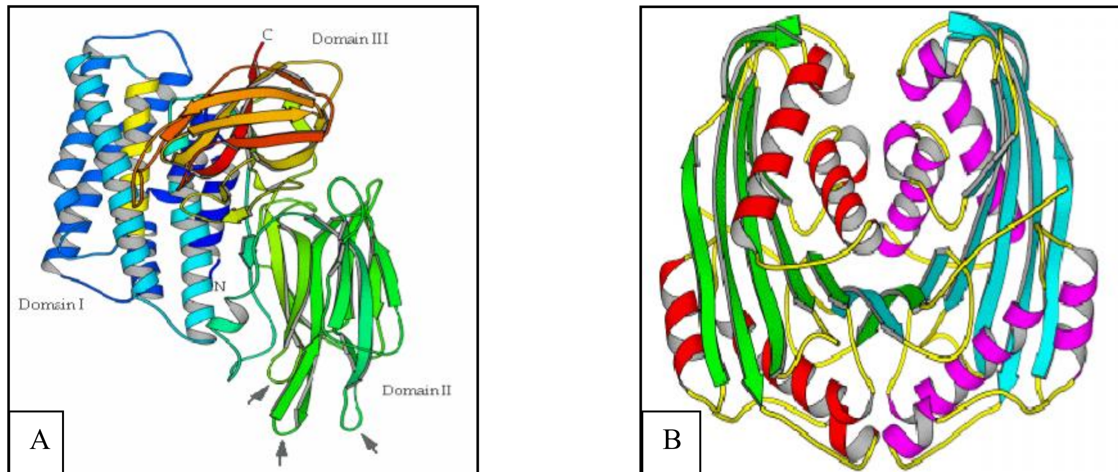

Figure 2.3 Three dimensional structure of δ- endotoxins. **(A)** The structure of Cry3A **(B)** The structure of Cyt2A. (http:// www.bioc.cam.ac.uk/UTOs/Ellar.html)

However, Cyt 2A structure is radically different from the other three structures (Crickmore *et al.*, 1998). It consists of a single domain (Fig 2.3B). The structure of the domain is composed of alpha helix outer layers wrapped around a mixed beta-sheet (Schnepf *et al.*, 1998). The activated cry toxins have two functions: receptor binding and ion channel activity. The activated toxin binds to the specific receptors on the mid-gut epithelia of susceptible insect (Hofman *et al.*, 1988). Binding is a two stage process involving reversible and irreversible binding (Van-Rie *et al.*, 1989). These steps may include toxin binding to the receptor, insertion of the toxin into apical membrane or both. On the other hand, the Cyt toxins have no specific receptor recognition, although, they cause pore formation.

2.4.1 Mode of Action

The crystal proteins of *Bt* show host specificity. For this reason, each type of Cry protein can be toxic to one or more specific insect species. Because of this specific toxicity, they do not affect many beneficial insects, plants and animals including humans. The specificity of these insecticidal crystal proteins (ICPs) derives from their mode of action (Adang, 1991; Gill *et al.*, 1992) as shown in Figure 2.5.

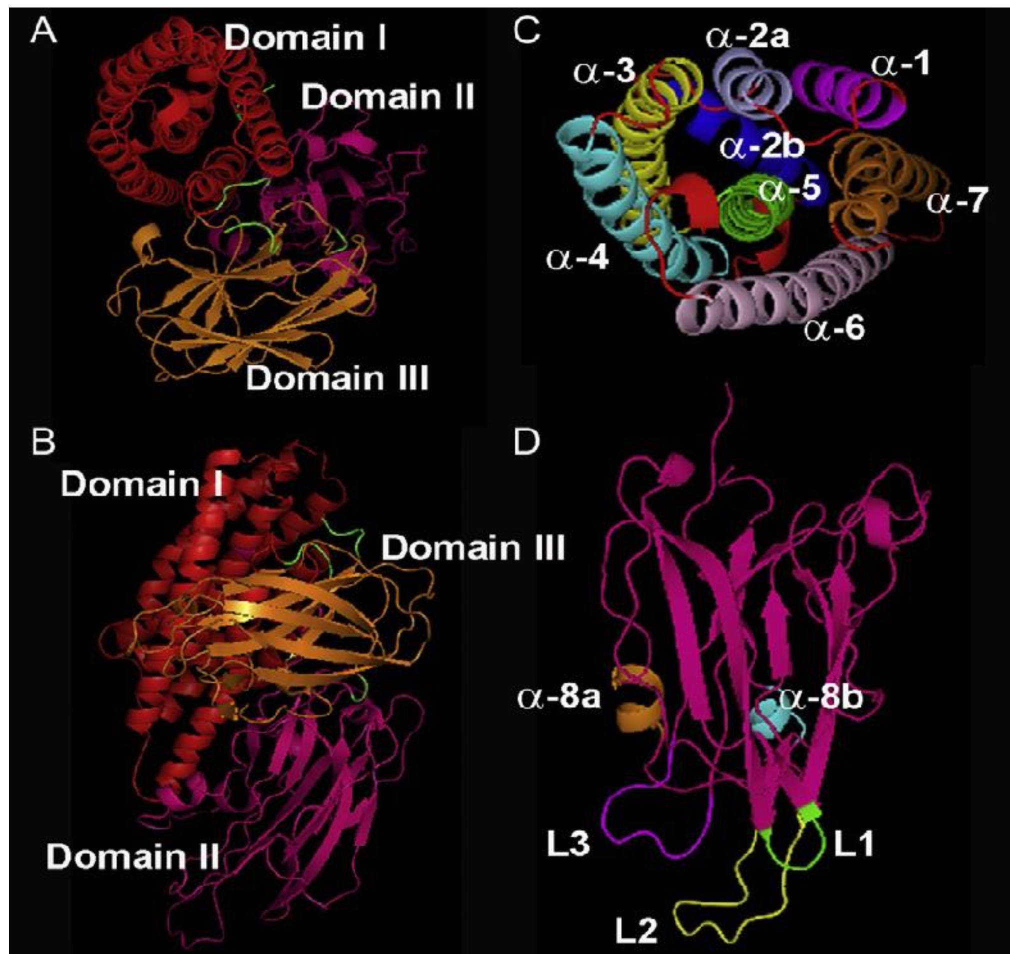

Figure 2.4: Three dimensional structure of Cry1Aa protein and the analysis of its domains.

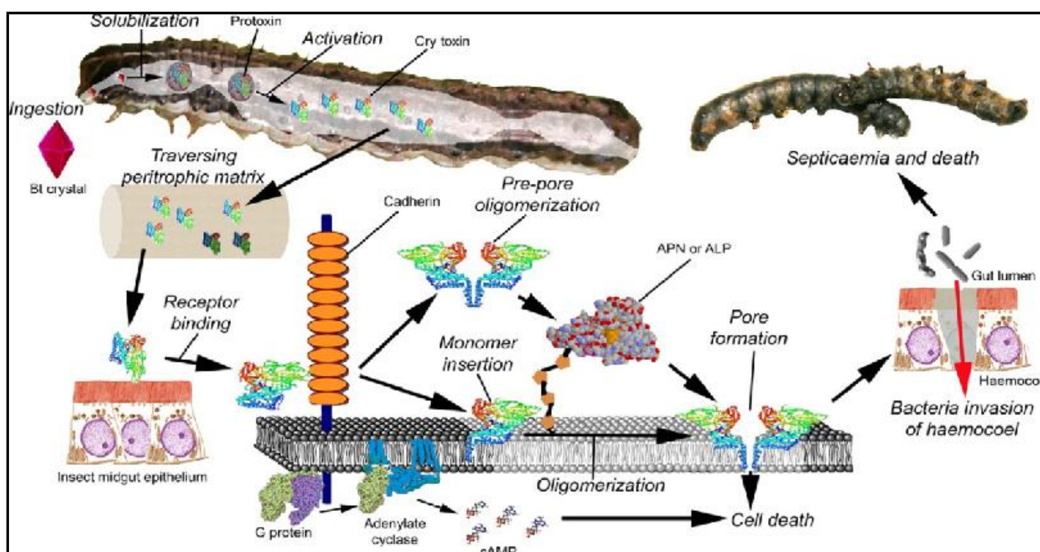

Figure 2.5: The mode of action of Cry proteins in the larval gut.

The parasporal crystals of *Bt* contain the ICPs in the form of protoxins. After ingestion of parasporal crystals by the susceptible insect, the crystals are dissolved in alkaline conditions (pH 10-12) in the insect mid-gut, generating 130 to 135 kDa protein chains called protoxin. These proteins are then processed to the actual toxic fragments of 60- 65 kDa by the gut proteases (Höfte and Whiteley, 1989; Gill *et al.*, 1992).

Finally, these activated toxins bind to specific receptors present in the larval mid-gut epithelia. The activated toxin binding to the specific receptors on the cell membrane creates ion channels or pores. The pore formation causes osmotic shock. As a result of this process, the cell membrane lyses, paralysis occurs and consequently, the insect stops feeding and dies from starvation (Knowles, 1994).

2.5 Genetics of Bacillus thuringiensis

2.5.1 Genome of *Bt*

The genome size of *Bt* strains is about 2.4 to 5.7 Mb (Carlson *et al.*, 1994) and a physical map has been constructed (Carlson and Kolstø, 1993). Comparison with the *B. cereus* chromosomal map suggested that all of these chromosomes have a similar organization in the half near the replication origin while displaying greater variability in the terminal half (Carlson *et al.*, 1996). Most *Bt* isolates have several extra chromosomal elements (plasmids) ranging in size from 2 to >200 kb. Some of these plasmids are circular and some are linear. The parasporal crystal proteins are generally encoded by genes being harboured in large plasmids. Sequence hybridizing studies with *cry* gene probes have been shown that *cry* genes are also found in the bacterial chromosome (Carlson *et al.*, 1994).

2.5.2 The *cry* genes and family

The genes encoding the insecticidal Cry proteins are known as *cry* genes which are found both in the chromosome and large plasmids (Gonzales and Carlton, 1980; Carlson *et al.*, 1994). Many of the Cry protein genes have been cloned, sequenced, submitted in toxin nomenclature and named *cry* genes. To date, over 500 *cry* gene sequences have been organized into 75 holotypes and more than 220 different subgroups on the basis of their nucleotide similarities and range of specificity (Crickmore *et al.*, 2014). For example, the

proteins toxic for lepidopteran insects are encoded by *cry1*, *cry9* and *cry2* genes. The toxins against coleopteran insects are encoded by *cry3*, *cry7*, and *cry8* genes and *cry1Ia1*, which is a subgroup of *cry1* genes. The *cry5*, *cry12*, *cry13* and *cry14* genes express nematocidal toxins, and the *cry2Aa1*, a subgroup of *cry2* and *cry4*, *cry10*, *cry11*, *cry16*, *cry17*, *cry19* as well as *cyt* gene encoded proteins are toxic to dipteran insects (Zeigler, 1999). Each of the *Bt* strains can carry one or more *cry* genes, and therefore, may synthesize one or more crystal proteins. As *cry* genes are very often plasmid mediated, transfer of plasmids among the *Bt* strains is an important reason of diversity in *cry* genes (Thomas *et al.*, 2001).

2.5.3 The transposable elements of *Bt*

The *Bt* strains harbor a large variety of transposable elements, including insertion sequences and transposons and their general characteristics have been extensively reviewed (Mahillon *et al.*, 1994). The *Bt* transposable elements were described with regard to their structural association with the *cry* genes. The first studies on the structural organization of the *cry1A* gene environment showed that genes of this type were flanked by two sets of inverted repeated sequences. Nucleotide sequence analysis revealed that these repetitive elements were insertion sequences that have been designated IS*231* and IS*232*. IS*231* belongs to the IS*4* family of insertion sequences, and IS*232* belongs to the IS*21* family of insertion sequences. Because these elements can transpose, it is likely that they provide mobility for the *cry* genes with which they form typical composite transposons. However, this hypothesis has not been tested experimentally.

In *Bt* subsp. *israelensis*, an IS*231* element (IS*231W*) is adjacent to the *cry11Aa* gene. Although IS*231* elements are frequently associated with *cry* genes, IS*231*-related DNA sequences have also been found in strains of *B. cereus* and *B. mycoides*. In contrast, IS*232* has a much smaller range among the organisms surveyed so far, appearing in only 7 out of 61 *Bt* serovars. The *cry4A* gene of *Bt israelensis* is flanked by two repeated sequences in opposite orientations.

The first transposable element identified in the genus *Bacillus* was isolated from *Bt* following its spontaneous insertion into a conjugative plasmid transferred from *Enterococcus faecalis*.

The genetic and structural characteristics of this transposable element fulfilled the criteria of a Tn element, and it was designated Tn*4430*.

2.5.4 Genetic diversity among the *Bt* strains

The genetic diversity of *Bt* arises from the presence of many different plasmids in each strain, conjugation transfer mechanism, and the transposon-like inverted repeats flanking the endotoxin genes, facilitating a high frequency of DNA rearrangements. Horizontal transfer of protoxin encoding-plasmids may lead to strains producing two different parasporal inclusions. In most species, the major protoxin gene is carried on a low copy number large plasmid (one plasmid per cell). The insertion sequence (IS), observed in transposable elements are especially found in large plasmids and many of these sequences carry protoxin genes. Plasmids that do not include protoxin genes also play a role in the regulation of protoxin synthesis. Plasmids also enhance and provide supplementary growth factors when nutrients are limitted. If protoxin gene is found on a transposable element, it can move into and out of the chromosome. Because of this movement, protoxin sequences may sometimes be present in the chromosome of some subspecies (Aronson *et al.*, 1986). The numbers of both large and small plasmids are between 2 and 11 in one cell (Gonzalez *et al.*, 1981; Lereclus *et al.*, 1993). If plasmids are lost, it will be impossible to distinguish *Bt* from *B. cereus* (Höfte and Whiteley, 1989; Crickmore *et al.*, 1998).

2.6 The *cry* Gene Expression

The expression of *cry* genes occurs during the stationary phase of bacterial growth. The *cry* gene products generally accumulated in the mother cell compartment and form crystal inclusions that form upto 20 to 30 % of the dry weight of sporulated cell (Schnepf *et al.*, 1998). Sporulation and crystal protein synthesis are synchronic processes. In order to coordinate sporulation with the synthesis of the protoxins and their assembly into inclusions, crystal protein synthesis is controlled by a variety of mechanisms occuring at the transcriptional, post-transcriptional and post-translational levels (Agassie and Lereclus, 1995; Baum and Malvar, 1995). The *cry* gene expression can be activated by both sporulation-dependent and sporulation-independent mechanisms. The *cry1Aa* gene is generally expressed during sporulation, as a typical example for sporulation-dependent control. However, *cry3A*

gene is expressed during vegetative growth but it can also be expressed in much lower amounts during sporulation (Schnepf *et al.*, 1998).

The expression level of a gene may also be influenced by its copy number. In fact, the production of *Bt* toxins is not strictly proportional to the *cry* gene copy number. However, it can be decleared that the capacity of *Bt* strains to produce crystal proteins may reach maximum at a certain number of *cry* gene copies in the cell (Agassie and Lereclus, 1995). The stability of mRNA is another important contributor to the high level of toxin production in *Bt*. It has been reported that, the mRNAs half-life encoding the crystal proteins are relatively longer than normal mRNAs (Glathorn and Rapoport, 1973). The determinants of mRNA stability are generally part of untranslated regions of the molecule and classified as 3' and 5' terminal structures according to their location in the mRNA. The fusion of 3' terminal fragment acting as positive retro-regulator, with the 3' end of heterologous genes increases the half-life of their transcripts and consequently their expression levels (Wong and Chang, 1986). Also, the Shine- Dalgarno (SD) sequence present close to 5' end may be a general determinant of mRNA stability in *Bacillus* species (Agassie and Lereclus, 1995). The crystal proteins are generally found in the form of crystalline inclusion in the mother cell compartment. The crystal shape depends on the protoxin composition. This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. The factors, including the secondary structure of the protoxin, the energy of the disulphide bonds and the presence of additional *Bt* specific components affect the structure and the solubility characteristics of *cry* proteins (Schnepf *et al.*, 1998).

2.6.1 Sporulation dependent *cry* gene Expression

Extensive studies of the sporulation of *B. subtilis* have provided detailed information on the complex mechanisms that temporally and spatially control this differentiation process. At the transcriptional level, the development of sporulation is controlled by the successive activation of sigma factors, which bind the core RNA polymerase to direct the transcription from sporulation-specific promoters (Schnepf *et al.*, 1998). These factors are the primary sigma factor of vegetative cells, σA, and five factors called σH, σF, σE, σG, and σK, which appear in that order in a temporally regulated fashion during development. The σA and σH factors are active in the predivisional cell, σE and σK are active in the mother cell, and σF and σG are active in the forespore. The *cry1Aa* gene is a typical example of a sporulation-dependent

cry gene expressed only in the mother cell compartment of *Bt*. Two transcription start sites have been mapped (*BtI* and *BtII*), defining two overlapping, sequentially activated promoters.

2.6.2 Sporulation independent *cry* gene expression

The *cry3Aa* gene, isolated from the coleopteran-active *Bt* var. *tenebrionis*, was found to be expressed during vegetative growth, although at a lesser extent than during the stationary phase (Schnepf *et al.*, 1998). Analysis of *lacZ* transcriptional fusions and primer extension experiments indicates that the *cry3Aa* promoter is weakly but significantly expressed during vegetative growth, is activated from the end of exponential growth until stage II of sporulation (about *T*3), and remains active until stage IV of sporulation (about *T*7). The *cry3Aa* promoter, although located unusually far upstream of the start codon (position 2558), resembles promoters recognized by the primary sigma factor of vegetative cells, σA. A similar promoter was found 542 bp upstream of the start codon of the *cry3Bb* gene. The expression of *cry3Aa* is not dependent on sporulation-specific sigma factors either in *B. subtilis* or in *Bt*. Moreover, *cry3Aa* expression is increased and prolonged in mutant strains unable to initiate sporulation. The results indicate that *cry3Aa* expression is activated by a non-sporulation-dependent mechanism arising during the transition from exponential growth to the stationary phase. The positive effect of mutations preventing the initiation of sporulation suggests that there is an event during sporulation (e.g., the disappearance of σA in the mother cell) that turns off *cry3Aa* expression.

2.7 Isolation and Characterization Methods of *Bt*

2.7.1 Collection of Environmental Samples

Screening samples from different environments may be useful to obtain *Bt* strains with broader host ranges and new toxic properties (Höfte and Whiteley, 1989). The abundance of the bacterium depends mainly on the type of environmental sample. Soil has been shown as the main source of *Bt* novel isolates (DeLucca *et al.*, 1981) as it has been recovered from 70% of soil samples from all over the world (Martin and Travers, 1989). There are some suggestions about the high recovery of *Bt* from soil. First, while collecting sample, the surface is always rejected and the material is taken from at least 5 cm under the surface where UV light damage is not possible and temperature is more stable (Trindade *et al.*, 1996). Second, the soil can act as a reservoir of spores (Akiba, 1986; Martin and Travers, 1989;

Meadows, 1993; Ohba and Aratake, 1994; Lereclus, 1996). *Bt* remain as spore and/or vegetative cell on leaves (Damgaard, 1995) as the appendages on the spore may facilitate the attachment to the surface of plant leaves, leading to the settlement of spores and the colony formation on phylloplane (Mizuki *et al.*, 1999). Hence, leaves can be another source of *Bt*. On the other hand, insect cadavers, stored product dust, etc are also searched very often for *Bt*.

2.7.2 Isolation methods for *Bt*

There are some selective techniques to isolate *Bt* from these environments. Acetate selection method developed by Travers *et al.*, (1987) has been widely used by the researchers for *Bt* isolation (Martin and Travers, 1989; Carozzi *et al.*, 1991; Ben-Dov *et al.*, 1997; Bravo *et al.*, 1998; Hongyu *et al.*, 2000). Sodium acetate at 0.25 M concentration inhibits the germination of spores of *Bt* and some relative species. After a period of bacterial growth, the vegetative cells are eliminated by heat treatment and only sporeformers stay alive. They were then plated on nutrient medium without acetate. After an incubation period, *Bt* colonies can be distinguished from the others by colony morphology and Phase Contrast Microscopic observation. Another method for isolation of *Bt* is based on antibiotic selection. Yoo *et al.*, (1996) used the antibiotics polymyxin B sulfate and penicilin G in isolation to eliminate the cells which have not resistance to these antibiotics. This method however is not used as often as the acetate selection. The efficiency of isolation also depends on the method used. Enrichment techniques are not useful because it has a lower detection limit which is about 103 bacteria per gram of soil. Immunofluorescence-based methods also have a lower detection limit of 105 bacteria per gram of soil in spite of their direct enumeration. The most efficient isolation method so far has been the sodium acetate selection, combined with heat tratment.

Isolation of *Bt* from leaf samples is simpler than soil because the number of acrystalliferous sporeforming bacteria are significantly lower in leaves. A moderate heat treatment at 65°C also eliminates contamination of fungi and non-sporeformers and promotes the germination of *Bt* spores and other sporeformers (Smith and Couche, 1991). Serial dilution method on the other hand can be more effective than this method for the isolation of *Bt* from phylloplanes because most of the viable cells on leaves are found in the vegetative stage and the use of heat treatment can kill these cells (Maduell *et al.*, 2002).

2.7.3 Characterization Methods of *Bt* isolates

The characterization of *Bt* strains has great imprortance as it may help to analyze distribution of *cry* genes and to understand the role of *Bt* in nature. Moreover, it is also important in evaluating toxic potential of the strains against insect orders. The main point in establishing *Bt* strain collections is to have a rapid and accurate characterization method. Upto now, many different methods have been developed to characterize *Bt* strains. The classical methods and phenotypic characterization include crystal morphology, H-serotyping, biochemical reactions, SDS-PAGE, cellular fatty acid analysis and bioassays (de Barjac and Bonnefoi, 1962, 1968). Presence of crystals is a vital clue for identification of *Bt* from other species such as *B. cereus* and *B. anthracis* which do not produce large parasporal inclusions during sporulation (Henderson *et al.*, 1995; Bobrowski *et al.*, 2001). Li and Chen, (1981) identified seven types of crystals showing differences in toxicity to the lepidopteran insects by leaf dip bioassay technique. It is necessary to test each isolate for all target insects, thus it is a long and exhaustive process in screening large number of natural isolates (Ceron *et al.*, 1994). Southern blot analysis to search for known homologous genes (Kornstad and Whiteley, 1986) and analysis of reactivity to different monoclonal antibodies (Höfte *et al.*, 1988) have been used to characterize novel *Bt* isolates. Flagellar (H) antigen serotyping was established for intra specific classification of *Bt* strains (de Barjac and Bonnefoi, 1973). However, they are imprecise predictors of insecticidal activity, expensive and time consuming methods for the identification of novel toxins. On the other hand, DNA fingerprinting, utilization of oligonucleotide probes specific to the *Bt* toxin genes are possible but they are very expensive and time consuming characterization methods for the identification of new strains from large numbers of environmental samples (Bourque *et al.*, 1993). The use of PCR has been a milestone for the analysis of *Bt* strain collections (Carozzi *et al.*, 1991). It is highly sensitive, relatively fast and can be easily used on a routine basis (Ceron *et al.*, 1994). PCR has been used to predict insecticidal activities (Carozzi *et al.*, 1991) to identify *cry* type genes (Bourque *et al.*, 1993; Glaeve *et al.*, 1993; Ceron *et al.*, 1994, 1995) to determine the distribution of the *cry* genes (Chak *et al.*, 1994) and to detect novel *cry* genes (Kalman *et al.*, 1993; Kuo and Chak, 1996). Recently, PCR based different methods have been developed for further characterization of the strains, such as PCR-RFLP which is a two-step strategy where group specific primers are used first, followed by enzymatic digestion of the produced amplicons (Kuo and Chak, 1996); E-PCR based on the use of two sequencial PCR reactions, using a multiplex PCR with specific and universal primers (Juarez-Perez *et al.*, 1997); and

RT-PCR (Shin *et al.*, 1995). Another approach, pulsed field gel electrophoresis of chromosomal DNA digested with an appropriate restriction enzyme recognizing rare sites in the DNA is considered as an accurate typing procedure for closely related bacteria. Thus, it is used for subspecific classification of *Bt* strains and provides more discriminative typing of *Bt* strains than H-serotyping (Rivera and Priest, 2003). Although bioassay remains as an essential tool to determine insecticidal activity exactly, other methods such as serotyping, biotyping, analyzing of plasmid DNA profiles or protein profiles are still necessary for subspecific classification of *Bt* strains. Biochemical characterization of the strains is important because each serotype has specific physiological characters and one serotype can be subdivided into biotypesbased on the different enzymatic reactions. These biotypes also show different toxicity (de Barjac, 1981). PCR analysis of new isolates of *Bt* provides valuable prescreening opportunity that is followed by subsequent insect toxicity assays or other subspecific classifications.

2.7.4 Characterization of *cry* genes by PCR based technique

The identification of toxin genes by PCR can be used to predict the insecticidal activity of a given strain. This method has largely substituted bioassays used in preliminary classification of *Bt* collections because of its rapidity and reliability (Porcar and Juárez-Pérez, 2003). This method also allows the identification of *cyt* gene content of a strain (Bravo *et al.*, 1998). In the premilinary screening, primer pairs designed from highly conserved regions are used to recognize entire *cry* gene subfamilies. Recently, specific primers selected from a variable region are used for amplification (Ben-Dov *et al.*, 1997). The prediction results of PCR must be combined with bioassays in order to decide the potential of isolates as biopesticides (Masson *et al.*, 1998).

Individual *Bt* strains vary in the number and type of toxins they produce. Recent advances in molecular biology have allowed the development of PCR based methods which are rapid and accurate in the identification of *cry* genes present in any isolate and the prediction of its insecticidal activity. PCR amplification of fragments of unexpected size, when gene specific primers are used, may lead to the detection of new *cry* genes (Ceron *et al.*, 1995).

The *cry* genes of *Bt* have been reclassified several times as more individual genes and toxic proteins were identified. Hofte and Whiteley, (1989) introduced the first systematic classification and nomenclature for toxin proteins based on insecticidal activity. The principal

class is designated by Roman letter (I – IV). Thus, *cry*I were classified as active against lepidoptera, *cryII* against lepidoptera and Diptera, *cry*III against Coleoptera, *cryIV* against Diptera, *cryV* against Coleoptera and lepidoptera, while other genes conferring general toxicity were classified as *cyt* genes (Lereclus *et al.*, 1993). Recently, Cyt Parasporin, a *Bt* parasporal protein was shown to be unique in having a strong preferential activity against human cancer cells (Uemori *et al.*, 2007). Subclasses of Cry proteins were later recognized based on their activity within the same group of insect itself; e.g. *cryIC* with high activity against specific Lepidoptera compared with *cryIE* with limited activity (Visser *et al.*, 1990).

However, exceptions to the classification began to arise as more insecticidal genes were identified. Comprehensive re-classification of *cry* genes based on DNA profiles and also in relation to the mode of action of the genes was carried out by Crickmore *et al.*, (1998). Relationships between the *cry* genes are now indicated based on alphanumeric system using Arabic numerals and upper and lower case letters. This replaces the older classification based on the host ranges in which the toxin genes were first observed or tested. Thus the *cry* genes are now recognized using four hierarchical levels based on sequence homology of various proteins in each rank. Proteins with less than 45% sequence homology are considered as a primary rank, while further separation at the secondary and tertiary ranks is based on 78% and 95% homology, respectively. de Maagd *et al.*, (2001) have provided a useful summary of the putative evolutionary relationships between different *cry* genes and listed 31 *cry* genes and their phylogenetic relationships. Further additions to the list of *cry* genes are administered through the database managed by Crickmore *et al.*, (2014). The most significant conclusion from this data is that some *cry* genes, notably *cry*1 and *cry*2 have activity patterns that cross taxonomic boundaries. A particular isolate (HD-2) later identified as *Bt* subsp. *thuringiensis* had a distinct gene (*cry1Ba1*) which showed activity against the house fly (*Musca domestica*, Diptera), cottonwood leaf beetle (*Chrysomela scripta*, Coleoptera) and tobacco hornworm (*Manduca sexta*, lepidoptera) in laboratory assay (Zhong *et al.*, 2000). To identify and to classify *Bt* strains containing *cry* genes toxic to species of lepidoptera, Coleoptera and Diptera, an extended multiplex PCR method was established. The technique enriches current strategies and simplifies the initial large scale screening of *cry* genes by pinpointing isolates that contain specific genes (Ben-Dov *et al.*, 1997), but the process cannot identify the existence of a novel *cry* gene in an isolate whose nucleotide sequence is unknown. A strategy named exclusive PCR or E-PCR would effectively overcome the main

limitation of multiplex PCR. Earlier, a single universal primer was combined with several specific oligonuceotides that recognized the individual genes (Juarez-Perez *et al.*, 1997).

An alternative PCR analysis to screen *cry*7 gene was possible based on the five conserved blocks of amino acids and encoding DNA sequences (Ben Dov *et al.*, 2001). For PCR, thirteen highly homologous primers specific to regions within the genes encoding seven different subgroups of CryI proteins were described. Differentiation among these strains was determined on the basis of electrophoretic patterns of PCR products (Ceron *et al.*, 1994). Balasubramanian *et al.*, (2002) detected a novel gene *cry*32Aa based on the presence of an unusual 42 amino acid long tail at the C terminus and expressed a protein of 139.2 kDa. Beron *et al.*, (2005) designed five degenerate primers for detection of novel *cry* genes from *Bt* strains. An efficient strategy was developed based on a two step PCR approach with these primers in five pair combinations. In the first step, only one of the primer pairs was used, which allowed amplification of DNA fragments encoding protein regions that included consensus domains of protein belonging to different Cry groups. A second PCR is then performed by using first–step amplification products as template and a set of five primer combinations. Cloning and sequencing of the second step amplicons allowed both the identification of known *cry* genes and the detection and characterization of novel *cry* related sequence from *Bt* isolates.

A new family of insecticidal crystal proteins, Cry34Aa1, Cry34 Ab1, Cry34Ac1 of 14 KDa and Cry35 Aa1, Cry35 Ab1 and Cry 35Ac1 of 44 KDa polypeptides were discovered by screening sporulated *Bt* cultures for oral activity against Western corn root worm larvae (Ellis *et al.*, 2002).

Individual studies have employed a variety of strategies to identify new *cry* genes. Bravo *et al.*, (1998) isolated 496 *Bt* from Mexico and the analysis of the strains was based on multiplex PCR with novel general and specific primers that could detect *cry1, cry3, cry5, cry7, cry8, cry9, cry11, cry12, cry 13, cry 14, cry 21* and other *cry* genes. Escudero *et al.*, (2006) reported molecular and insecticidal characterization of a novel *cry* gene encoding a CryII group protein with toxic activity against insects of the Noctuidae, Tortricidae, Plutellidae and Chrysomelidae. PCR analysis detected a DNA sequence with an open reading frame of 2.2 kb which encoded a protein with a molecular mass of 80.9 kDa. This protein was named Cry1Ia7. Song *et al.*, (2003) with the help of a pair of universal primers designed for the conserved region of the gene (1.548 kb) amplified *cry*11 type genes. Analysis of the amplicons digested with *BSP1191* and *Ban1* enzymes revealed four kinds of *cry11* genes.

Sauka *et al.*, (2007) identified new variant of *cry* genes based on the analysis of PCR products. They showed successful amplification of novel *cry1A* genes from 13 *Bt* strains. These bacteria were previously known to harbour at least one *cry1A* gene.

A single *Bt* strain can harbor more than one *cry* genes of a primary rank. The *cry1* primary rank is the best known and contains the highest number of sub ranks. Currently available nucleotide sequence information of many *cry* genes has set a stage for microarray based detection of *cry* gene profile of any isolate. Single micro array hybridization can replace hundreds of individual PCRs. DNA micro arrays are expected to provide platform based technology for quick screening of new *Bt* isolates presenting interesting insecticidal activity (Letowski *et al.,* 2005).

2.7.5 Genotypic Characterization

DNA-based methods used for characterization are specific primed PCR, random amplified polymorphic DNA (RAPD), DNA: DNA colony hybridization (Hansen *et al.*, 1998), rRNA-based probe. Strains with similar plasmid profiles can belong to the same subspecies. Therefore, strains can be classified by comparing the plasmid and crystal patterns (González *et al.*, 1982; Carlton and González, 1985). PCR is a molecular method widely used to characterize the insecticidal bacterium *Bt.* It provides the determination of the presence of a target gene by the amplification of specific DNA fragments.

2.7.6 Toxicity analysis by Bioassay

Classifying isolates based on bioassays against target insects is done with two objectives-firstly, to identify the group of insects against the isolate is effective (lepidopteran active, coleopteran active, *etc.*) and secondly, to find out the toxicity levels. The method of classification through bioassays is tedious and it was considered ambiguous. However bioassays are extremely useful to identify the best isolates against target species of insects and also to study the mechanism of action of the toxic principle.

The criteria for assessment of a strain include larval mortality (Dulmage *et al.*, 1971) and/or decreased feeding. The test larvae could be fed with leaves of plants that have been coated with solutions to be assayed by dipping or spraying (Burges and Thomson, 1971) or force feeding the test larvae with dietary preparation containing *B. thuringiensis.*

2.8 Dominant pest population in Bangladesh agriculture

It has been suggested that an insect becomes an economic pest when it causes a yield loss of 5-10%. In any local pest complex, there are usually few major pests that cause most of the damage and their control is urgently required. The most serious one of the major pests is often designated as the key pest, one or two of which prevail in each agro-ecosystem. More than 200 species out of over 700 insect and mite pest species of different field crops, vegetables, fruit trees, and stored products have been considered major in Bangladesh (Kabir, 1989). Nearly 100 different types of vegetable comprising both local and exotic type are grown in Bangladesh. The dominant pests with the vegetables of Bangladesh are described in the Table 2.1 and few of them are shown in Fig 2.6.

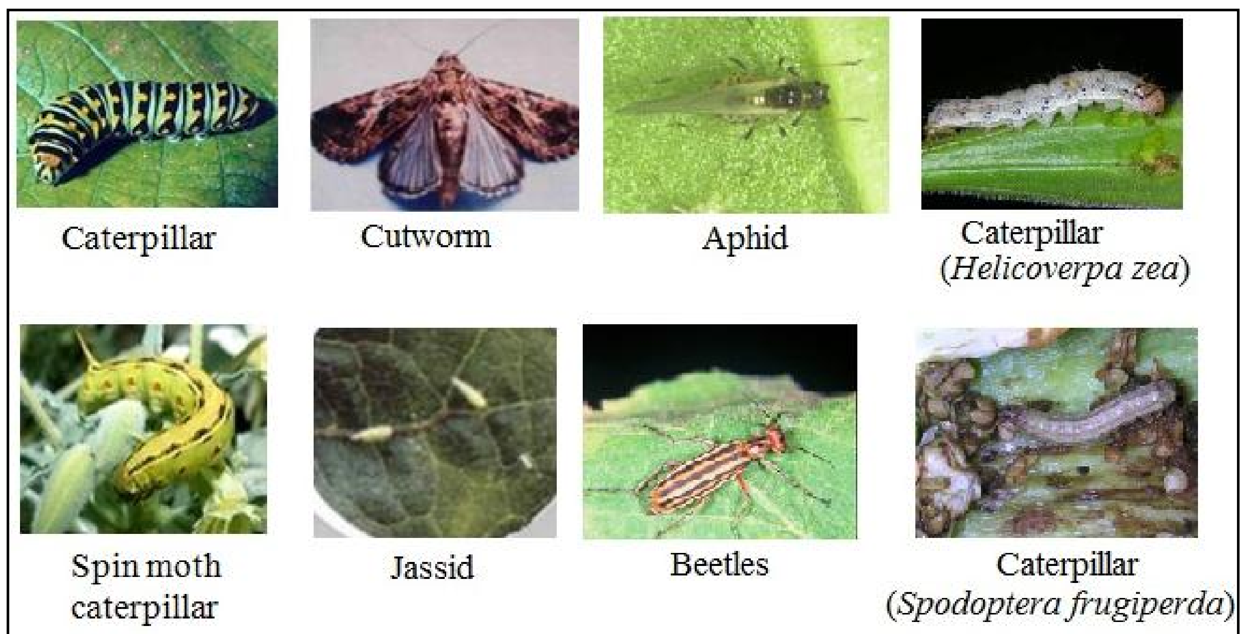

Figure 2.6: Pest in different stages that cause the destruction in agricultural production.

2.8.1 Pest control agents or Pesticides

Early pesticides were the chemical substances. Certain properties made them useful, such as long residual action and effective toxicity to a wide variety of insects. However, the use of them may lead to negative outcomes. The chemical insecticides used today are considered as presumably safer to those used in the past but there are still some concerns. Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in humans and animals (Kegley and Wise, 1998). Also, they can cause accumulation and persistance of toxic residues in soil, water and food; toxicity aganist

beneficial insects and development of pest resistance (Marrone and Macintosh, 1993; Van Frankenhuyzen, 1993; Glazer and Nikaido, 1995). Nevertheless, chemical insecticides have a large market volume, and global sales of them are about $5 billion a year (Glazer and Nikaido, 1995).

Table 2.1: Important vegetables and dominant pests of Bangladesh causing damage

Vegetable Family	Important vegetables	Name of the insect		Family
		Conventional	Scientific	
Crucifera	Cabbage, Cauliflower, Knol-khol, Lettuce, Radish	Caterpillar	*Spodoptera litura*	Lepidoptera: Noctuidae
		Diamondback moth	*Plutella xylostela*	Lepidoptera
		Cabbage butterfly	*Pieris brassica*	Homoptera
Solanaceae	Brinjal	Shoot and fruit borer	*Leucinodes orbonalis*	Lepidoptera: Pyralidae
		Red mite	*Tetranychus* sp.	Acarina: Tetranichidae
		Cut worm	*Agrotis ipsilon*	Lepidoptera
		Leaf Roller	*Eublemma olivacea*	Lepidoptera
		Epilachna beetle	*Epilachna vigintioctopunctata*	Coleoptera: Coccinellidae
			E. dodecastigma	
			E. corupta	
	Tomato	Aphid	*Aphis craccivora*	Homoptera: Aphididae
		Cut worm	*Agrotis ipsilon*	Lepidoptera
		Fruit borer	*Helicoverpa armigera*	Lepidoptera: Noctuidae
	Potato	The potato tuber moth	*Phthorimaea operculella*	Lepidoptera: Gelechiidae
		Aphid	*Myzus persicae*	Hemiptera: Aphididae
Leguminous crops	Country Bean	Flower bud and pod borers	*Maruca testulalis*	Lepidoptera: Pyralidae
			Euchrysops cnejus	Lepidoptera: Lycaenidae
			Heliothis armigera	Lepidoptera: Noctuidae
Cucurbit crops	Gourd, Cucumber, Pumpkin, Squash, melon, watermelon, avocado, papaya, peach, citrus	Melon fruit fly	*Bactrocera (Dacus) cucurbitae*	Diptera: Tephritidae
		Pumpkin beetle	*Raphidopalpa* (*Aulacophora*) *foveicollis*	Coleoptera: Chrysomelidae
			R. abdominalis	
			R. frontalis	
Other crop	Okra	Shoot and fruit borer	*Earias vittella*	Lepidoptera: Noctuidae
		Jassid	*Amrasca devastans*	Homoptera:Jassidae

On the contrary, microbial pesticides are safe for ecosystem. They are non-toxic and non-pathogenic to wildlife and humans. The toxic action of them is often specific to a single group or species of insects, so they do not affect the other insect population in treated areas.

Because they have no hazardous residues to humans or animals, they can also be applied when crop is almost ready for harvest (Neppl, 2000).

2.8.1.1 Classification of pesticide

According to the nature of the pesticides they are grouped into four classes:

i. Plant derivatives e.g. Pyrethrin, Rotenone.

ii. Pure chemicals e.g. Paris green.

iii. Synthetic chemicals :

- Chlorinated hydrocarbon compounds e.g. DDT, Dieldrin, HCH.
- Organophosphate compounds e.g. Diazinon, Malathion, Fenitrothin.
- Carbamates e.g. Propoxur, Bendiocarb.
- Synthetic Pyrinoids e.g. Detamethrin, Permethrin.

iv. Biopesticides

- *Bt* and *Bs*
- Fungi
- Virus

2.8.1.2 Chemical pesticides and its uses in Bangladesh

Chemical pesticides are imported in Bangladesh and some agro-chemical industries formulate and re-pack pesticides. The pesticide market in Bangladesh began in 1956 with 3 tons of pesticides and 500 hand sprayers. In 1995, a total of 9700 MT/KL of pesticides (granular, liquid and power) were applied both in agriculture sector and health programmes in Bangladesh. In the year 1996, Bangladesh used more than 11 thousand metric tons of pesticides and their use has been on the increase as it became more than 16 thousand metric tons in the year 2001 (Meisner and Dasgupta, 2004).

Most of the pesticides applied are insecticides in granular form. Use of herbicides and acaricides is low. Fungicide is also in use. Pesticide consumption in Bangladesh is increasing in relation to acreage of irrigated agriculture. What is most alarming is that pesticide use is very indiscriminate in Bangladesh. There are areas where pesticides are used in excessive

quantities. Such situations make monitoring and assessment of pesticide contamination very difficult.

According to the "Institute of Development Policy analysis", Bangladesh imported about 12,000 metric tons of pesticides in 2001. The use of synthetic organic insecticides developed during the last half of this century to control pests of agricultural importance poses risk to human health including workers in pesticide industries (Pesatori *et al.*, 1994) and threatens serious environmental problems. One conservative estimate suggested that the excessive use of chemical pesticides has already rendered 12 species of reptilians and amphibians as endangered species. The efforts suggesting and developing alternate control strategies for insect pest management have been gaining importance lately.

2.8.1.3 Limitation of chemical pesticides

There are some limitations of chemical pesticides which are as follows -

- Broad spectrum; adverse effects on non-target arthropods
- Outbreak of secondary pests
- Persistent - environmental pollution as the residues are often found in water, food, vegetables, milk and animal hides etc.
- Toxic to mammals and plants: carcinogenic and phytotoxic.
- Pesticide treadmill
- Resistant populations (result of natural selection); cross-resistance; mixed function oxidase.
- Chemical control gets more expensive every year and less effective as resistance develops.
- Rain and the use of water eliminate the effectiveness of chemicals.
- It often kills the natural enemies in agriculture.
- Pose health risk to the workers in pesticide industries and farmers.

2.8.2 Advantages of Biopesticides, an alternative to chemical pesticide

The interests in biopesticides are based on the disadvantages associated with chemical pesticides and certain beneficial features. They are as following:

- Biopesticides do not cause environmental pollution and pose no health risks with the presence of their residues in water, food, vegetables, fiber and fodder as observed for chemical pesticides.
- Biopesticides affect only specific pests whereas chemical pesticides affect from pest to other lives like fish, birds, cattles, mammals etc.
- Biopesticides are often effective in lower doses and decompose quickly and thus result in lower exposures to the atmosphere.
- Biopesticides plays an important role in Integrated Pest Management (IPM) program by unharming the natural enemies as they are specific in toxicity.

2.8.2.1 Types of Biopesticides

There are two types of biopesticides:

i) Predators: There are many invertebrate and vertebrate predators which concentrate their attack during very specific periods of development of vectors, i.e. their eggs, larval stages and adults.

ii) Entomopathogens: Entomopathogens or their products are those biopesticides, most of which act after ingestion by the insects and their mode of action is slow and last for long period in most cases in comparison to chemical pesticides e.g. virus, fungi, protozoa, nematodes and associated Bacteria.

2.8.2.2 Commercial Application of *Bt* preparations as biopesticide

Bt has a successful history of over half a century in controlling the pests and mosquito. The demand of *Bt* based insecticides in agriculture sector declined, in the mid 1970s, because of more effective chemical pesticides. But in the 1980s, *Bt* research was stimulated by progress in biotechnology. First, Schnepf and Whiteley, (1981) cloned a crystal toxin gene from *Bt* subsp. k*urstaki* into *E.coli*, since then much research has been performed to improve target spectra and to find out more infectious strains of *Bt.*

Back in 1995, worldwide sales of *Bt* reached $90 million prompting the motion towards a natural alternative to hazardous synthetic pesticides (Schnepf et al., 1998). In 1998, the number of registered *Bacillus thuringiensis* products in the United States alone had almost exceeded the 200 mark. Although time consuming, it has become well recognized that Cry-based pesticides generally have low costs for development and registration. Astoundingly the cost of *Bt* pesticides is estimated at $1/40^{th}$ that of a comparable novel synthetic chemical pesticide (Becker and Margalit, 1993). The United States is still leading the way with *Bt* pesticide programs already implicated in areas of forestry. These pesticides are based primarily on the strain *Bacillus thuringiensis* HD-1 subsp. *kurstaki* (Dulmage *et al.*, 1970), which produces CrylAa, CrylAb, CrylAc, and Cry2Aa toxins. The huge success that was achieved by these projects was reflected in results throughout the forestry world, encompassing more than one pest species. *Bacillus thuringiensis* subsp. *israelensis* has become one of the most effective and potent biological pesticides in attemps to combat mosquitoes and blackflies, insect pests capable of spreading fatal human diseases. Mosquitocidal activity has been identified through tests conducted with Cry2Aa, CrylAb and Cry1Ca (Haider *et al.*, 1986). Many new uncharacterized isolates containing uncharacterized *cry* genes have also been shown to display mosquitocidal activity (Ragni *et al.*, 1996).

2.8.2.3 Mode of applications

In agricultural use, *Bt* preparations are mostly applied with ground sprayers. Since high volumes of aqueous spray per unit area are needed for adequate coverage of the plant, ground spraying can be impractible in some cases. In recent years, air spraying has been applied from aeroplane (Fig 2.7) have reduced spray volume and made more effective and better controlling of the droplets (Wysokis, 1989). Also the use of air assisted sleeve boom has increased spray penetration, plant coverage and reduces the drift (Navon, 2000). Low persistence of the spore-crystal product on the plant is an important problem in *Bt* applications. When the products of *Bt* were applied to cotton (Fuxa, 1989) and potato (Ferro *et al.*, 1993), persistence was observed as 48 hours. Therefore, timing is the major factor for determining the effectiveness of *Bt* applications. Application early in the season, according to monitoring egg hatching and after sunset instead of in the morning can increase the persistence of *Bt* preparations (Navon, 2000). Laboratory and field assays have showed that younger larvae are more susceptible to *Bt* preparations than older ones (Navon *et al.*, 1990; Ferro and Lyon, 1991). Therefore, larval stage is the suitable period for *Bt* applications.

Figure 2.7: Field application of *Bt* biopesticide in different parts of the world.

2.8.2.4 Spectrum of toxicity

The biological activity of *Bt* strains or their products toward different target organisms has been subject to patent coverage for many years. Many of these patents belong to companies engaged in commercial endeavors, while others remain as a part of the basic research domain. More than 3000 insect species included in 16 orders have been found to be susceptible to different crystal proteins (Lin and Xiong, 2004). Insecticidal crystal proteins are toxic to insects within the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga as well as non-insect organisms such as nematodes, mites, protozoa, and plathelmintes (Feitelson, 1993; Ciche and Ensign, 2003). The toxicity is high against the insects belonging to the first three orders. Lepidopteran and Coleopteran insects are leaf-feeders with chewing mouthparts, whereas Dipterans feed by filtering water. These two different feeding behaviours provide the possible intake of *Bt* spores/ crystals (Borror *et al.*, 1989).

2.8.2.5 Safety of *Bt* Biopesticides

Bt based insecticides have been used commercially to control selected insect pests for approximately 50 years and during this period, no adverse effects on human health or environment were reported (McClintock *et al.*, 1995; EPA, 1998). In the 1990s, the development of natural and recombinant *Bt* products have broadened the insect host range in pest management programs. New formulations based on conventional or genetic engineering methods (encapsulation of the toxins and/or feeding stimulants to increase ingestion), screening of the interactions of *Bt* with insect herbivores and plant allelochemicals or natural enemies of the pests to improve the formulation of biological control strategies, and information and management of insect resistance increased the uses of *Bt* (Navon, 2000). Short persistence and complete biodegradability are other benefits of *Bt* toxins (Bohorova *et al.*, 1997; Copping, 1998). Over synthetic pesticides, the advantages of this organism include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammalians, birds, amphibians and reptiles as well as its relatively low costs of development and registration (Flexner and Belnavis, 1999).

2.8.2.6 Conventional *Bt* Preparations

Most *B.thuringiensis* preparations available on the market contain spores with parasporal inclusion bodies composed of δ-endotoxins. In commercial production, the crystals and spores obtained from fermentation are concentrated and formulated for spray-on application according to conventional agriculture practices (Baum and Malvar, 1995). Although, there are numerous *Bt* strains having insecticidal activity against insect orders (e.g. Lepidoptera, Diptera, Coloptera, Homoptera, Mollaphoga), nematodes and aphids, only a few of them have been commercially developed.

2.8.2.7 Commercial formulations and types

Commercially available *Bt* preparations contain both spore and toxic crystal protein (δ-endotoxin). In the production, spores and crystals obtained from fermentation are mixed with the additives including wetting agents, stickers, sunscreens and synergists (Table 2.2 and 2.3). It is accepted that UV inactivation of the crystal toxin is the major cause for the rapid loss of *Bt* activity. Several approaches such as the use of some chromophores to shield *Bt* preparations against sunlight (Dunkle and Shasha, 1988; Cohen *et al.*, 1991) and enhancing

the melanin-producing mutants of the organism, increase UV resistance and insecticidal activity (Patel *et al.*, 1996). Besides, encapsulations of *Bt* in biopolymers reduce washing of the product from the plant by rain (Ramos *et al.*, 1998).

Table 2.2: Types of *Bacillus thuringiensis* Formulations and their Applications for Insect Pest Control

Formulation	**Application**
Emulsions	Agriculture and forestry
Encapsulations	Agriculture and forestry
Wettable	powders Gardens and agriculture
Granules	Agriculture and forestry
Powders	Forestry
Briquettes	Aquatic systems

In the development of new formulations and optimization of the utilization of biopesticides, knowledge of insect feeding behaviour is a fundamental requirement (Navon, 2000). Some formulations used to stimulate feeding, such as the use of a phagostimulant mixture or a yeast extract in a dustable granular form have been proposed to increase residual toxic activity and to attract to the feed selectively on the *Bt* product than the feed on the plant (McGuire *et al.*, 1990; Navon *et al.*, 1997). These approaches can help to increase the effectiveness of the new *Bt* formulations.

2.8.2.8 Production of *Bt* biopesticides

Although microbial insecticides based on *Bt* and *Bs* are available for use, their high cost makes large-scale application impracticable in developing countries. The use of *B. thuringiensis* as commercial insecticides would remain prohibitively, relatively expensive if the organism is not produced in remarkably large quantities with high titre of insecticidal proteins by large-scale fermentation. More attention has been given to the regulation mechanisms that ensure the efficient production of the insecticidal proteins. Improvement of

bioinsecticides production could be achieved by application of an adequate fermentation technology (Zouari *et al.*, 2002), essentially with use of appropriate media (Zouari and Jaoua, 1999), overcome of metabolic limitations (Zouari *et al.*, 2002), improvement of *Bt* strains through mutagenesis (Ghribi *et al.*, 2004) and adaptation to abiotic stress conditions (Ghribi *et al.*, 2005).

The fermentation of the different isolates of *Bt*, regardless of subspecies, have some general characteristics in common. They all use sugar (usually glucose, molasses, or starch), producing acid during the fermentation. In general, they have similar requirements for proteins or protein hydrolysates, can use NH_4^+ salts, and respond similarly to minerals. However, the individual isolates are unique entities, and a particular medium that may support good growth or toxin production by one isolate may be less satisfactory for another. Different isolates of *Bt* may produce toxins with different spectra of activities.

It was reported that the commercial application of both organisms depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product. The cost of raw materials is one of the principal costs involved in overall *Bt* production. In the conventional *Bt* production process, the cost of raw materials varied between 30 and 40% of the total cost depending on the plant production capacity (Ejiofore, 1991). Therefore, local production of this insecticide in developing countries should depend on the use of production media made of cheap, locally available sources including agro-industrial by-products (Ampofo, 1995). For large scale production of *Bt*, different approaches were investigated to construct media that could support good production of spores and toxins at reasonable costs. Various agricultural and industrial by-products used as raw material in *Bt* and *Bs* production were citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor (El-Bendary, 2006). Recently, other wastes such as sludge and broiler poultry litter were utilized for biopesticides production (Adams *et al.*, 2002; Vidyarthi *et al.*, 2002). In general, two methods of fermentations are used for production of microbial products, submerged fermentation and solid state fermentation.

Thus the wide variety of formulations based on spore-crystal complexes intended for ingestion by target insects, are the result of many years of research. The development of a great variety of matrices for support of the spore-crystal complex enables many

improvements, such as an increase in toxic activity, higher palatability to insects, or longer shelf lives.

Table 2.3: Most Common Commercial *Bacillus thuringiensis*-based Bioinsecticides (Rosas-Garcia, 2009)

Company	Commercial Name	Active Ingredient	Target Pest
Certis	Agree WG	*Bt* v. aizawai	Lepidopterans
Certis	Condor	*Bt* v. kurstaki	Lepidopterans
Certis	CoStar	*Bt* v. kurstaki	Lepidopterans
Certis	Crymax	Genetically engineered strains from *Bt* kurstaki and aizawai	Lepidopterans
Certis	Deliver	*Bt* v. kurstaki	Lepidopterans
Certis	Jackpot WP	*Bt* v. kurstaki	Lepidopterans
Certis	Javelin/Delfin	*Bt* v. kurstaki	Lepidopterans
Certis	Lepinox WDG	*Bt* v. kurstaki	Lepidopterans
Certis	Turix WP/Agree WP	*Bt* v. kurstaki	Lepidopterans
AFA Environment Inc.	Agribac	*Bt* v. kurstaki	More than 30 insect species
Valent Biosciences Corp.	DiPel	*Bt* v. kurstaki	Lepidopterans
Valent Biosciences Corp.	XenTari	*Bt* v. kurstaki	Effective against *Spodoptera* ssp. and *Plutella xylostella*
Valent Biosciences Corp.	Biobit	*Bt* v. kurstaki	Lepidopterans
Valent Biosciences Corp.	Novodor	*Bt* v. tenebrionis	Coleopterans
Valent Biosciences Corp.	VectoBac	*Bt* v. israelensis	Mosquito and fly larvae
Valent Biosciences Corp.	Teknar	*Bt* v. israelensis	Mosquito and black fly larvae
Valent Biosciences Corp.	GnatrolDG	*Bt* v. israelensis	Larval stage of Sciarid mushroom flies
Valent Biosciences Corp.	Foray	*Bt* v. kurstaki	Lepidopterans
Valent Biosciences Corp.	Thuricide	*Bt* v. kurstaki	Lepidopterans and certain leaf-eating worms

2.9 Expression of *cry* genes in transgenic crops

Bt δ-endotoxins are generally safe to vertebrates and beneficial arthropods yet in many cases highly toxic to specific insect pests, thus the genes that encode these δ-endotoxins were among the first to be engineered into plants to confer insecticidal activity (Theunis *et al.*, 1998). Introduction into dicotyledonous plants proved successful in affording resistance to Lepidopteran pests, however the δ-endotoxin genes were expressed at extremely low levels (Fujimoto *et al.*, 1993).

The δ-endotoxin gene can be extensively modified through truncation of the *cry* gene based on the codon region of the transformation vector. This truncation allows for the gene to be highly expressed in transgenic plants and stably inherited for at least two generations (Fujimoto *et al.*, 1993). Monocotyledonous plants have higher G+C content in comparison to dicotyledonous plants, so these modifications also enhance the transformation success of monocotyledonous plants (Fujimoto *et al.*, 1993). Truncation also allows for the transfer of only the sequences required for insecticidal activity. This is achieved by the removal of amino acid sequences of the N-terminus (Lambert *et al.*, 1996). When the ICP enters the insect gut, it is broken down into three subunits. Truncation allows for the expression of only the subunits responsible for the recognition of specific binding sites on the insect gut wall (Jenkins and Dean, 2001). Development of multitoxin systems with combinations that recognize different binding sites would prove useful in implementing deployment strategies to decrease the rate of pest insect adaptation to *Bacillus thuringiensis* toxins (Lee *et al.*, 1997).

Transgenic *Bt* cotton containing *cry1Ac* gene which offers resistance to major bollworms was first commercially released in the world in 1996 and during 2002 in India (Prasad *et al.*, 2009). Besides their long term use as biological insecticides in the form of sprays of spore crystal mixtures, individual *cry* genes have been expressed in transgenic plants to render the crops resistant to insect pests. Since 1996, transgenic maize, cotton and potato expressing *cry* genes have spread to large area, world wide, including India. The first transgenic plants expressing *Bt* toxins were tobacco and tomato (Van Frankenhuyzen, 1993). *Bt* field cotton was the first *Bt* plant pesticide registered by United States EPA (USEPA, 1999). Rapidly developing recombinant DNA technology after 1990 became an important tool to develop genetically manipulated *Bt* pesticides. Today, major *Bt* transgenic crops include corn, cotton, potatoes and rice. They have been commercialized and are in use widely in Canada, Japan, Mexico, Argentina, Australia and United States (Frutos *et al.*, 1999).

2.9.1 Development and Management of Pest Resistance

Insects can develop resistance to nearly every type of insecticides due to genetic variation in large insect population. Besides, there are several other factors increasing the rate of resistance development, which are related to the insect population and insecticide usage. Species with higher reproductive rates, shorter generation times, greater numbers of progeny, and more genetically varied local populations develop a large resistance in the population

more quickly (Pimentel *et al.*, 1996). Also, resistance develops more rapidly against more persistant insecticide due to increasing the time of exposure of susceptible larvae to the toxin (Sanahuja *et al.*, 2011). Similarly, frequent application of non-persistent insecticides can have the same effect (Wood, 1981). Insecticide resistance is a major problem for agriculture, health and economics. The first reported case of resistance to chemical insecticides has occured over 50 years ago. Since then, pesticide resistance has become one of the world's most serious environmental problems because of the concerns on human nutrition due to crop loss, spread of disease by resistant insects, environmental risks in the application of greater amounts of chemicals to the pests which are already gained resistance (Pimental and Burgess, 1985). In 1990s, much evidence on the resistance development of different pests against *Bt* preparations have been reported from Hawaii, Florida, Newyork, Japan, China, the Philippines, Thailand and Malaysia (Iqbal *et al.*, 1996; Lui and Tabashnik, 1997). As a result, insecticide resistance appeared the negative outcome of insecticide usage. In order to overcome resistance problem against *Bt* based pesticides, different management strategies have been developed. Basically, it has been aimed to slow down resistance development as much as possible and to make resistant populations revert to susceptibility (Croft, 1990). Generally, three main approaches are involved in resistance management programs. One approach targets to minimize exposure to toxins and allow for mating between resistant and susceptible insects, thus susceptible traits continue for the next generations. Different strategies based on this approach include tissue-specific and time-specific expression of toxins, mixtures, mosaics, rotations, refuges and occasional release of susceptible males into the field (Wood, 1981). Other approach focuses on combining pest control techniques to provide synergy and improve the efficiency of *Bt* preparations against pests. This includes the strategies of gene stacking, high doses, combination of toxins with completely different modes of action and combination of low toxin dose, other entomopathogenic microbes, plant allochemicals and natural enemies (Murray *et al.*, 1993; Navon, 1993; Trumble and Alvaro-Rodriguez, 1993). Another approach developed for only transgenic *Bt* plants, not spray form, uses trap plants to lure pests away from productive crops (Alstad and Andow, 1995).

2.10 Aims and objectives of the study

The use of *Bt* biopesticide, in controlling the pest in Bangladesh agriculture, can be a useful alternative to chemical pesticides that are causing a massive and silent damage to the environment and public health. The transgenic crops could be a very attractive option but the fact of immunological adverse effects with edible crops and increasing insect resistance due to the uncontrollable exposure of *Bt* toxins leading to selection pressure, made it less preferable. On the other hand, foliar spraying of *Bt* biopesticide has been established as safer for its non-integrating nature into the plant system and hence, free of any immunological adverse effects. Again, the farmer friendly spraying without extensive protection can be performed targeting the life cycle of the pests which reduces the chances of pest resistance. The aim of this study was therefore, to characterize the toxic factors i.e. *cry* genes, insecticidal proteins etc to develop *Bt* biopesticide from potential indigenous *Bt* strains in cost effective manner. So, it is very essential to establish an enriched collection of indigenous *Bt* strains, determining diversity among them and sorting out the potential strains that are active against vegetable pests for their large scale production. Therefore, the main objectives of this study were-

1. Isolation, identification and characterization of *Bt* isolates from different eco-regions of Bangladesh.
2. Analysis of abundance and distribution of *Bt* strains in Bangladesh.
3. Detection and analysis of *cry* genes and insecticidal proteins.
4. Genomic diversity analysis among the indigenous *Bt* strains.
5. Toxicity study of the potential *Bt* strains against vegetable pests by Bioassay both in the lab and in the field.
6. Bioprocess development for large scale and cost effective production of *Bt* biopesticide.

CHAPTER 3

Isolation and characterization of *Bacillus thuringiensis* from Bangladesh: Analysis of abundance and diversity

Isolation and characterization of *Bacillus thuringiensis* from Bangladesh: Analysis of abundance and diversity

3.1 Introduction

Recent developments suggest that biological control with *Bacillus thuringiensis* (*Bt*) based products will become increasingly important. This prompts many researchers to focus on the isolations of native *Bt* strains from different environments which is also very essential to find out novel strains with higher degree and spectrum of toxicity to help coping with the problem of insect resistance (Van Rie *et al.*, 1991). *Bt* has great strain diversity with different toxic potential (Thomas *et al.*, 2001) and many worldwide screening programs have been performed to establish *Bt* strain collections in different countries such as Antarctica (Forsyth and Logan, 2000), China (Hongyu *et al.*, 2000) Colombia (Uribe *et al.*, 2003), Japan (Mizuki *et al.*, 1999), Mexico (Bravo *et al.*, 1998), Philippines (Theunis *et al.*, 1998), Spain (Bel *et al.*, 1997; Iriarte *et al.*, 2000), Taiwan (Chak *et al.*, 1994), United Kingdom (Meadows *et al.*, 1992; Bernhard *et al.*, 1997), United States (Martin and Travers, 1989), and some Asian countries (Ben-Dov *et al.*, 1997). These collections have great importance in analyzing the distribution of toxin producing strains in nature and evaluating their toxic potentials against various insect orders.

Intensive screening programs have identified *Bt* strains from soil, plant surfaces, dead insects, and stored grain samples. The screening for novel isolates has led to the discovery of strains with toxic activity against a broad range of insect orders, including Lepidoptera, Coloeptera, Diptera, Hymenoptera, Homoptera, Molophoga, and Acari (Feitelson *et al.*, 1999). Furthermore, *Bt* strains able to control other insect orders such as Nemalthelmintes, Platyhelmintes, and Sarcomastigophora have been found (Feitelson, 1993). Some *Bt* strains of have also been found to be toxic to nematodes, mites and protozoa (Feitelson, 1993; Feitelson *et al.*, 1999). It is still necessary to search for more toxins, since a significant number of pests remain to be uncontrolled with the available Cry proteins. It is also very important to provide alternatives to overcome the problem of insect resistance, especially, with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (Van-Rie, 1991).

The genetic diversity of *B. thuringiensis* strains shows differences according to the regions where they were isolated. In fact, each habitat may contain novel *B. thuringiensis* which may have some toxic effects on a target spectrum of insects. The characterization of *B. thuringiensis* strain collections may help in the understanding of the role of *B. thuringiensis* in the environment and the distribution of *cry* genes.

In view of these, indigenous *Bt* was isolated from different eco-regions of Bangladesh and their abundance, diversity and distribution were analyzed on the basis of biochemical properties, broad spectrum cytolysis activity, 16S rRNA gene sequence, plasmid profile etc in this chapter.

3.2Materials and methods

3.2.1. Materials

Chemicals and Reagents: Chemicals and Reagents used in this study were listed in Appendix A.

Media: Media were listed in Appendix B.

Buffers and Solutions: Buffers and solutions were presented in Appendix C.

Equipments: Appendix D.

6.2.2 Methods:

3.2.2.1 Bacterial strains

Bacillus thuringiensis kurstaki HD-73, *B. thuringiensis sotto* T84A1*, B. thuringiensis japonensis* Buibui were used as reference strains which were obtained from *Bt* stock collection of Okayama University, Japan (Table 3.2.1).

Table 3.2.1: Reference strains of *B. thuringiensis* used

Strains	BGCS Code	Original Code	Serotype	Genes
B. thuringiensis subsp. *japonensis* Buibui	4AT1	T23 001	23	*cry8ca*
B. thuringiensis subsp. *kurstaki*	4D4	HD-73	3a, 3b, 3c	*cry1Ac*
B. thuringiensis subsp. *sotto*	-	T84A1	4a, 4b	*cry1Aa*

6.2.2.2 Sample collection for the isolation of *Bt*

Samples were collected from different ecosystems such as plane land, river basin, hill tracts, sea beaches etc (Fig 3.2.1) where *Bt* based insecticides were not applied before. Soil (about 10.0 g each) samples were collected from 2- 5 cm below the surface of shadowed and slightly moistened places generally not exposed to sunlight with sanitized spatula into the sterile plastic bags to avoid contamination. Leaves, insects and stored dust products were also collected aseptically. Samples were provided with unique IDs in combination of locations, sample types and orders. They were then kept at room temperature the laboratory.

6.2.2.3 Isolation of *Bacillus thuringiensis*

6.2.2.3.1 Isolation of *Bt* like colonies

Bacillus thuringiensis were isolated from the collected samples and the isolation technique was similar for all types of samples except few variations during the sample processing. For soil specimens, 1.0 g from each sample was added in a 125 ml Erlenmeyer flask containing 20 ml of LB broth (Appendix B) supplemented with 0.25 M Na-acetate (pH 6.8) and incubated in an orbital shaker at 30°C and 200 rpm. For leaf specimens, 1 piece of leaf was inserted into the flask containing LB broth and for insects, their guts were dissected out, crushed and then mixed into the medium. After 4 hours, 0.5 ml of suspension from each sample was transferred into assigned sterile test tubes and heat treated for 10 minutes at 80°C in a water bath. Heat treated suspension was then diluted to 10-folds and inoculated onto T_3-agar medium (Appendix B) (Travers *et al.*, 1987) by spread plate method and incubated at 30°C. In case of appearance of any colony after overnight, incubation period was extended up to 72 hours to allow sporulation.

Figure 3.2.1: Sampling sites for *Bacillus thuringiensis* isolation from Bangladesh as indicated by red ellipses.

6.2.2.3.2 Phase contrast microscopy

After extended incubation in T_3- agar medium, colonies with different morphology from each sample were selected for Phase Contrast Microscopy. Colonies of same morphology were not duplicated only if they were from same sample. A trifle from each colony was then transferred and suspended into a drop of distilled water placed over a microscopic slide by a loop. Colonies containing isolates with glowing spore and juxtaposed dark crystal protein as revealed under Phase Contrast Microscope (Appendix D) were then subcultured and preserved in slants considering them either *Bacillus thuringiensis (Bt)* or *Bacillus sphaericus (Bs)* (Travers *et al.*, 1987). The diameters of the spores were measured using the software ZEN (Zeiss, Germany) and were recorded.

6.2.2.3.3 Differentiation between *Bt* and *Bs* by starch hydrolysis test

According to the Bergey's Manual of Determinative Bacteriology, *Bt* can be distinguished from *Bs* their starch hydrolyzing ability. In this connection, single colony, was inoculated as dot onto the starch hydrolyzing agar medium (Appendix B). After overnight incubation at 37°C, iodine reagent (Appendix C) was added to detect the presence of starch. Iodine reagent complexes with starch to form a blue-black color in the culture medium. Clear halos surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase. Isolates with clear zone around their colonies were considered as starch hydrolyzing, i.e. *Bacillus thuringiensis*.

6.2.2.4 Culture preservation

Bt isolates thus confirmed through starch hydrolysis test, were then preserved both on LB agar slants (Appendix B) for regular use and in 15% Glycerol stock (15% glycerol+ 85% fresh culture in LB broth) for long term storage at -80°C freeze. Subculture was performed when necessary from the glycerol stock and slants on LB agar and single colony was picked every time. The incubation temperature was maintained at or below 30°C for all types of culture conditions so that plasmids are not lost.

6.2.2.5 Scanning Electron Microscopy (SEM)

Bt strains were grown in T_3-agar medium at 30 ^{o}C for 72 hours until sporulation was complete as examined by Phase Contrast Microscope with an oil-immersion lens. The spores and crystals were collected by centrifugation at 4°C at 12,000 g for 10 min (Appendix D), and

the precipitate was washed thrice with ice-cold sterilized double-distilled water. The spore-crystal suspensions were placed on aluminum mount and fixed in after the samples were air-dried overnight. The samples were then coated in an auto fine coater (JEOL JFC-1600). The SEM observation was conducted on an analytical scanning electron microscope (JSM-6490, JEOL, Japan) at a voltage 15 kV following the machine instructions for the devise.

6.2.2.6 Hemolytic test

Bt isolates were checked for their broad-spectrum cytolysins activity by Hemolytic test. The isolates were therefore inoculated (as dots with needle) onto Sheep Blood agar medium (Appendix B) and incubated overnight at 27°C. Isolates forming clear zone of hemolysis around their colonies were considered as hemolytic (Ichikawa *et al.*, 2008).

6.2.2.7 Biotyping of the *Bt* isolates by biochemical tests

Four most relevant biochemical tests i.e. esculin utilization, acid formation from salicin, and sucrose, and lecithinase production were carried out to classify *Bacillus thuringiensis* into 16 biotypes (Table 3.2.2) (Martin and Travers, 1989).

6.2.2.7.1 Esculin hydrolysis test

The *Bt* isolates were inoculated into 5 ml of Esculin Iron broth (Appendix B) in separate test tubes labeled by isolate names and incubated at 37°C for 24- 72 hours. Esculin, a water soluble glycoside, is hydrolyzed by certain bacteria to yield glucose and esculetin. Esculetin reacts with the ferric ions to produce a black colored complex that makes the color of the medium brownish black. Thus, the *Bt* isolates that caused the change in color of the medium from brown to brownish black were considered as esculin utilizer (Qadri *et al.*, 1980).

6.2.2.7.2 Salicin utilization test

The *Bt* isolates were inoculated into 5 ml of Phenol red salicin utilization broth (Appendix B) in separate test tubes labeled by isolate names and incubated overnight at 37°C. Change of color of the medium occurs due to the production of acid during salicin fermentation which indicates the ability of the organism to utilize salicin. Hence, the isolates were considered salicin positive if the broth color was turned into yellow from red (Parry *et al.*, 1983).

Table 3.2.2: Biochemical properties exhibited by different subspecies of *Bacillus thuringiensis*.

Biochemical type (Described subspecies)	Biochemical test results			
	Esculin	Salicin	Lecithinase	Sucrose
1 (*Bt thuringiensis*)	+	+	+	+
2 (*Bt kurstaki*)	+	+	+	-
3 (*Bt indiana*)	+	+	-	+
4 (*Bt galleriae*)	+	+	-	-
5 (*Bt sotto*)	+	-	+	+
6 (*Bt dendrolimus*)	+	-	+	-
7 (*Bt morrisoni*)	+	-	-	+
8 (*Bt darmstadiensis*)	+	-	-	-
9 (undescribed)	-	+	+	+
10 (undescribed)	-	+	+	-
11 (undescribed)	-	+	-	+
12 (*Bt ostriniae*)	-	+	-	-
13 (undescribed)	-	-	+	+
14 (*Bt israelensis*)	-	-	+	-
15 (undescribed)	-	-	-	+
16 (undescribed)	-	-	-	-

"+" sign indicates positive reaction, i.e. utilization of esculin, acid production from salicin and sucrose, and production of lecithinase.

6.2.2.7.3 Lecithin hydrolysis test

The *Bt* isolates were inoculated as a dot in egg-yolk agar medium (Appendix B) with a sterilized needle. Egg yolk contains lecithin and free fats, which are split up by bacterial enzymes lecithinase and lipase. Lecithinase splits lecithin and releases insoluble diglycerides, resulting in the formation of a white opaque zone of precipitation that extends beyond the edge of the colony. A white opaque zone surrounding the colony indicated Lecithinase production and isolates with such ring were considered positive for the test.

6.2.2.7.4 Sucrose utilization test

The *Bt* isolates were inoculated into 5 ml of Phenol red sucrose utilization broth (Appendix B) in separate test tubes labeled properly and incubated overnight at 37°C. Organisms, capable to ferment sucrose, change the color of the medium from red to yellow by acid production and were considered positive.

6.2.2.8 Total DNA preparation

Total DNA of the *Bt* isolates and reference strains was prepared by following the method described by Bravo (Bravo *et al.*, 1998). Single bacterial colony as obtained after overnight incubation at 30°C following streaking on LB agar medium, was transferred into 100 µl sterile de-ionized water in a microfuge tube, vortexed and kept at -70°C for 20 min. It was then incubated in boiling water for 10 min to lyse the cells and briefly centrifuged for 20 s at 12,000×g. The upper aqueous phase transferred into sterile microfuge tubes was used as template and preserved at -20°C for further use. 50-100 ng of DNA from this suspension was used as template in PCR.

6.2.2.9 Plasmid extraction

Plasmid DNA was prepared by following the alkaline lysis method (Crosa and Falkow, 1981). Briefly, the pellet of a 5.0 ml culture grown in LB broth was lysed with 0.85 ml of TE buffer (Appendix C) containing 2 mg/ml of lysozyme (Appendix A), 0.05 ml of 20% SDS solution, and 5 U proteinase-K (Appendix A). After mixing by gentle inversions, the cell suspension was incubated at 37°C for 30 min. 0.03 ml of 3.0 N NaOH was added subsequently to the suspension and mixed gently for 3 min. The suspension was neutralized by addition of 0.06 ml of 2 M Tris-HCl (pH 7.0) and mixed gently. 0.1 ml of 5 M NaCl was then added, and the suspension was mixed by inversions, placed on ice for 15 min, and then centrifuged at 12,000 x g for 15 min at 4°C (Appendix D). The supernatant was transferred into a fresh centrifuge tube, and 2 volume of ice-cold ethanol was added. The microfuge tube was kept at -20 °C for 15 min and then centrifuged at 12,000 x g for 15 min. The supernatant was discarded, and the residue was dried by inverting the tube over a paper towel for a few minutes. The residue was dissolved in 50 µl of TE buffer and kept at -20°C. Plasmid DNA was separated by electrophoresis for 3 hrs and gel images were preserved for analysis as described in section 3.2.2.10.

6.2.2.10 Agarose gel electrophoresis

The DNA samples from the sources like PCR products, plasmids etc were separated in this whole study by Agarose gel electrophoresis (Sambrook *et al.*, 1989). The concentration of agarose (Appendix A) was varied depending of the samples such as 1.5% in 1× TBE buffer (Appendix C) for the analysis of PCR products and 0.75% in 1× TBE buffer for Plasmids. Agarose gel was prepared by dissolving agarose powder completely in 1× TBE buffer by heating and then pouring onto a mold with combs placed when cooled down at temperature 50°C. When the molten agarose solidified and became ready for use, the comb was removed carefully to keep the wells unbroken. The gel was then submerged in the electrophoresis tank with 1× TBE buffer.

The DNA samples were then mixed with 0.2 volume of 6× DNA loading buffer (Appendix C) by micropipette and loaded into the wells of the submerged gel. The electrophoresis was carried out at 60 volt and a DNA size marker was run alongside the samples. The gel was then visualized against UV trans-illumination in a gel documentation system (Alpha imager mini, USA) following staining in Ethidium Bromide (EtBr) (Appendix A) solution (0.5 µg/ml) and de-staining in distilled water.

3.2.2.11 Identification by 16S rRNA gene sequence analysis

16S rRNA gene from indigenous *Bt* isolates was amplified by PCR with universal primers for *Bacillus* spp.: fwd (20F); 5'-GAGTTTGATCCTGGCTCAG-3' (position 9-27), and rev (1500R); 5'-GTTACCTTGTTACGACTT-3' (position 1492-1509) (Soufiane and Cote, 2009). The PCR was performed in a thermal cycler (Appendix D) by 35 cycles (96°C for 50 s, 50°C for 45 s, 72°C for 2 min) with an initial denaturation step at 96°C for 5 min and a final extension step at 72°C for 10 min in 25 µl reaction mixture (forward and reverse primers 0.5 µM each, 50-100 ng of template, 0.5 U of *Taq* DNA polymerase (Appendix A), 200 µM dNTPs, 10 mM Tris, 50 mM KCl and 1.5 mM $MgCl_2$). The PCR product (5 µl) was analysed as described in section 3.2.2.10 and molecular weight was determined.

3.2.2.12 Purification of PCR products

PCR products were purified by using the Wizard® SV Gel and PCR Clean-Up System (Appendix A) and following its protocol if the amplicons were detected by agarose gel electrophoresis. PCR products were purified directly if found to be free of spurious products

whereas bands of desired size were excised from the agarose gel and purified by gel extraction method if mingled with spurious products. Binding of DNA to the minicolumn matrix, washing them well to remove impurities and eluting the DNA finally produced purified PCR products.

3.2.2.13 DNA sequence analysis

The purified PCR products were sequenced with respective primers (both forward and reverse primers if not mentioned otherwise) di-deoxynucleotide method which involves base specific termination of the enzymatic extension of DNA chains by dideoxy analogues (Sanger *et al.*, 1977) at the DNA Sequencing Facility (Centre for Advanced Research in Sciences-CARS, University of Dhaka) using an ABI model 3130 Automated Genetic Analyzer (Hitachi, Japan). The sequences were then corrected if necessary, assembled by DNA baser (version 4) and 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 Genbank database. From the 16S rRNA gene sequences, multiple alignments by ClustalW program and phylogenetic tree analysis based on neighbor-joining method were carried out to compare the *Bt* strains among each other and to determine the genetic distance with the help of MEGA software version 5.22. The sequences were then prepared with appropriate and prescribed annotations, modifiers, and submitted through Sequin and BankIt in the GenBank database (Table 3.3.5).

3.3 Results

6.3.1 Prevalence of *Bacillus thuringiensis*

Total 231 samples (Table 3.3.1) were collected from 26 different districts of Bangladesh (Fig 3.2.1) with the concern of prevailing diversity in the ecosystems that could be divided into 6 different eco-regions. At the end of sample processing, acetate selection, heat treatment and sporulation, 366 isolates were found to produce crystal protein as revealed under Phase Contrast Microscopy (Fig 3.3.1) which could be either *Bacillus thuringiensis* or *B. sphaericus*. Based on the starch hydrolysing capability, 317 isolates (of 366 isolates) were distinguished as *Bacillus thuringiensis* and rest 59 were *Bacillus sphaericus* (Fig 3.3.2A). Thus it was found that 192 samples (83.12%) out of 231 produced at least one *Bt* isolate which were calculated to be 92% for soil samples (171 out of 186), 37.5% for leaf samples (12 out of 32), 55.5% for insect samples (5 out of 9) and 100% for dust samples (4 out of 4).

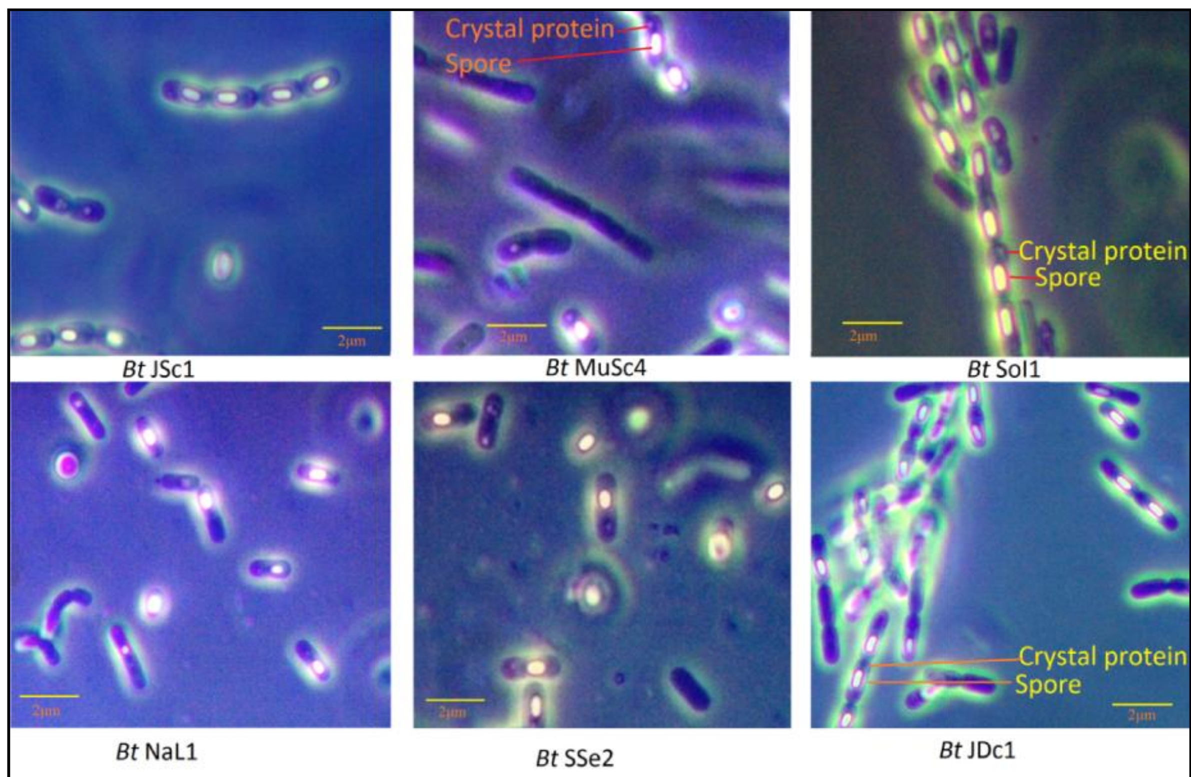

Figure 3.3.1: *Bacillus thuringiensis* like isolates were identified by Phase Contrast microscopy observing the glowing spores and juxtaposed crystal proteins.

Table 3.3.1: Yield of crystal protein producing isolates from different locations of Bangladesh.

Location	Types of sample	Sample number	Samples yielding *Bt* like isolates	Isolates producing crystals proteins
Agargaon, central Dhaka	Soil	2	2	2
	Leaves	1	1	1
Ati, eastern Dhaka	Soil	4	3	3
	Leaves	4	1	1
Chapainawabgonj	Soil	5	5	9
Chittagong	Soil	5	5	11
Chuadanga	Soil	7	3	3
Comilla	Soil	2	2	2
Dakkhinkhan, northern Dhaka	Soil	9	9	44
	Dust	1	1	3
Dinajpur	Soil	3	3	4
FH hall, DU, Central Dhaka	Soil	5	5	17
Inani beach, Cox's Bazar	Sand	1	1	4
Jamalpur	Soil	11	11	10
	Leaves	11	5	6
Jessore	Soil	6	6	11
Jhenidah	Soil	4	4	10
	Dust	3	3	6
Kawla, northern Dhaka	Soil	5	5	7
Keranigonj, Southern Dhaka	Soil	4	4	4
Khulna	Soil	5	5	9
Kustia	Soil	5	5	10
Kuakata, Patuakhali	Soil	4	4	8
Manda, eastern Dhaka	Soil	3	3	7
Meherpur	Soil	5	5	9
Mirpur, central Dhaka	Soil	1	1	1
Munshiganj	Soil	5	4	11
Mymensing	Soil	2	2	4
	Leaves	1	1	1
	Insects	2	2	3
Nandipara, Northern Dhaka	Soil	4	2	2
	Leaves	4	2	2
Narshingdi (1st phase)	Soil	7	5	5
Narsingdi (2nd phase)	Soil	5	5	8
Natore	Soil	5	5	12
Rangamati	Soil	4	4	7
Rangpur	Soil	5	5	10
RH hall, DU, Central Dhaka	Soil	4	4	13
Satkhira	Soil	5	5	8
Savar, western Dhaka	Soil	11	10	10
	Leaves	3	-	-
SD Hall, DU, central Dhaka	Soil	6	5	16
Sherpur	Soil	5	5	12
Sirajgonj	Soil	5	5	9
Sonargaon, Narayanganj	Soil	8	8	8
	Leaves	8	2	2
	Insects	7	3	3
Sylhet	Soil	1	1	2
Tangail	Soil	5	5	13
Uttara,northern Dhaka	Soil	3	1	3
Total		**231**	**192**	**366**

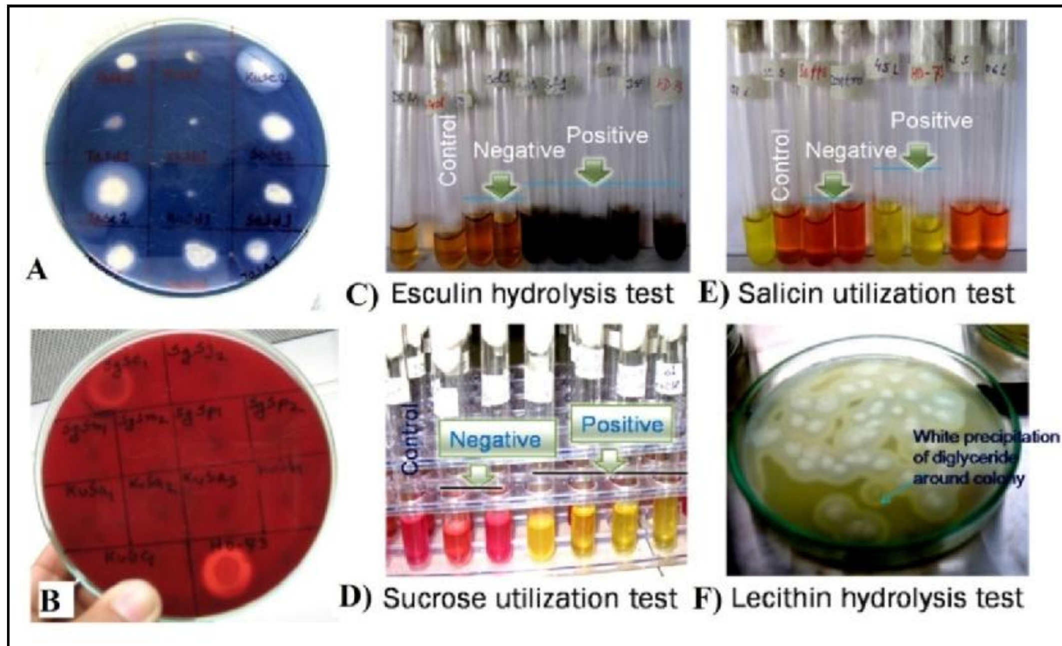

Figure 3.3.2: Biochemical properties. **A)** Starch hydrolysis test to differentiate *Bt* from *Bs*. **B)** Hemolytic test. **C)** Esculine hydrolysis test. **D)** Sucrose utilization test. **E)** Salicin utilization test. **F)** Lecithin hydrolysis test.

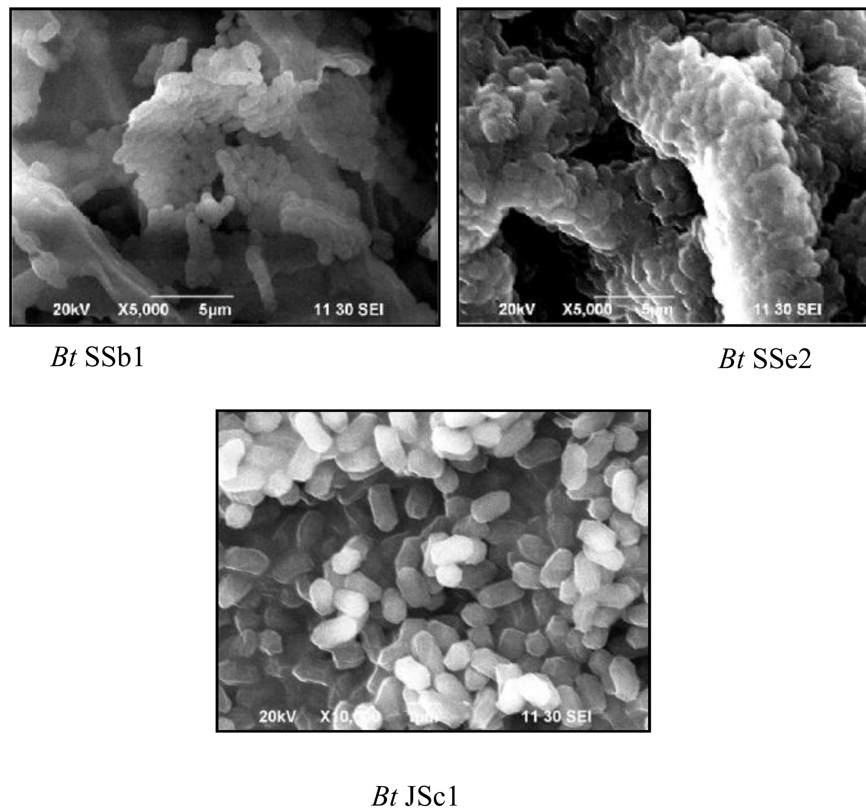

Figure 3.3.3: Scanning electron microscopy (SEM) of indigenous *Bt* isolates.

Table 3.3.2: Biochemical properties of indigenous *Bacillus thuringiensis* isolates.

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
1	AgL1	+	+	-	-	+	-	israelensis
2	AgS1	+	+	+	+	+	+	thuringiensis
3	AgS2	+	+	+	+	+	-	kurstaki
4	AtL1	+	+	+	+	+	+	thuringiensis
5	AtS1	+	+	+	+	+	-	kurstaki
6	AtS2	+	+	-	+	+	-	ten
7	AtS3	+	+	-	+	+	-	ten
8	CgSa2	-						*B. sphaericus*
9	CgSb1	+	+	+	+	+	+	thuringiensis
10	CgSb2	-						*B. sphaericus*
11	CgSc1	+	NH	-	-	+	-	israelensis
12	CgSc2	+	+	-	-	+	+	thirteen
13	CgSd1	+	NH	-	-	-	+	fifteen
14	CgSd2	+	NH	-	-	-	+	fifteen
15	CgSd3	+	NH	+	-	+	-	dendrolimus
16	CgSe1	+	NH	+	+	+	+	thuringiensis
17	CgSe2	+	NH	-	-	-	+	fifteen
18	CgSe3	+	NH	+	+	-	+	indiana
19	ChS1	+	+	-	-	+	-	israelensis
20	ChS2	+	+	-	+	+	-	ten
21	ChS3	+	+	-	+	+	-	ten
22	ChSa1	+	+	+	+	-	+	indiana
23	ChSa2	+	NH	+	-	+	+	*sotto*
24	ChSb2	+	+	+	+	-	+	indiana
25	ChSc1	+	NH	-	-	-	+	fifteen
26	ChSc2	-						*B. sphaericus*
27	ChSd1	+	NH	+	+	-	-	galleriae
28	ChSd2	+	NH	+	+	-	+	*indiana*
29	ChSe1	-						*B. sphaericus*
30	ChSe2	+	NH	-	+	-	+	eleven
31	CiSa1	+	+	+	+	+	-	kurstaki
32	CiSa2	+	+	+	+	+	+	thuringiensis
33	CiSa3	+	+	+	+	+	+	thuringiensis
34	CiSa5	+	+	+	+	+	-	kurstaki
35	CoS1	-						*B. sphaericus*
36	CoS2	+	+	+	+	+	-	kurstaki
37	DD1	+	+	+	+	+	+	thuringiensis
38	DD3	+	+	-	+	-	+	nine
39	DD5	-						*B. sphaericus*
40	DpSa2	+	NH	+	+	-	+	*indiana*
41	DpSb1	+	NH	+	+	-	+	Indiana
42	DpSc1	-						*B. sphaericus*
43	DpSc2	-						*B. sphaericus*
44	DSa1	+	NH	+	+	+	+	thuringiensis
45	DSa2	-						*B. sphaericus*
46	DSa3	+	+	+	+	+	-	kurstaki
47	DSa4	-						*B. sphaericus*
48	DSa5	-						*B. sphaericus*
49	DSa7	+	+	-	+	+	+	Nine
50	DSb1	-						*B. sphaericus*

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
51	DSb2	+	+					
52	DSb3	-						*B. sphaericus*
53	DSb4	+	+	-	-	-	-	sixteen
54	DSb5							
55	DSb6	-						*B. sphaericus*
56	DSb7	+	+	+	-	-	-	darmstadiensis
57	DSc1	-						*B. sphaericus*
58	DSc2	+	+	+	+	+	+	Thuringiensis
59	DSc3	-						*B. sphaericus*
60	DSc5	+	NH					
61	DSd1	-						*B. sphaericus*
62	DSd2	+	NH					
63	DSd3	+	NH					
64	DSe1	+	+	+	+	+	-	kurstaki
65	DSe2	+	+					
66	DSe3	-	+					*B. sphaericus*
67	DSe4	+	+	+	+	+	-	kurstaki
68	DSe5	+	+					
69	DSe6	+	+	+	+	+	-	kurstaki
70	DSe7	-						*B. sphaericus*
71	DSf1	-						*B. sphaericus*
72	DSf2	+	NH					
73	DSf3	-						*B. sphaericus*
74	DSf7	+	+	+	+	+	-	kurstaki
75	DSf8	+	+					
76	DSg1	+	+					
77	DSg2	+	+	+	-	+	-	dendrolimus
78	DSg3	-						*B. sphaericus*
79	DSh1	-						*B. sphaericus*
80	DSh3	+	+					
81	DSh4	+	+	+	-	+	+	sotto
82	DSh5	+	+	+	+	-	-	galleriae
83	DSh7	+	+	+	-	+	+	sotto
84	DSi1	-						*B. sphaericus*
85	DSi2	-						*B. sphaericus*
86	DSi4	+	+					
87	DSi5	+	+	+	-	+	-	dendrolimus
88	FhSa1	+	NH	+	+	-	+	indiana
89	FhSa2	+	NH	-	+	-	-	ostriniae
90	FhSa3	+	NH	+	-	-	-	darmstadiensis
91	FhSb1	+	NH	+	-	+	-	dendrolimus
92	FhSb2	+	+	+	+	-	+	indiana
93	FhSb3	+	NH	-	+	-	+	eleven
94	FhSb4	-						*B. sphaericus*
95	FhSc1	+	NH	-	+	-	+	eleven
96	FhSc2	+	NH	-	-	+	+	thirteen
97	FhSc3	+	NH	-	-	+	+	thirteen
98	FhSc4	-						*B. sphaericus*
99	FhSc5	-						*B. sphaericus*
100	FhSd1	+	NH	+	+	+	-	kurstaki
101	FhSd2	+	NH	+	+	-	+	indiana
102	FhSd3	+	NH	+	+	-	-	galleriae

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
103	FhSd4	+	+	-	-	-	-	sixteen
104	FhSe1	+	NH	+	+	+	-	kurstaki
105	JaL1	+	+	+	+	+	-	kurstaki
106	JaL2	+	+	-	+	+	-	ten
107	JaL3	+	+	+	-	+	+	sotto
108	JaL4	+	+	-	+	+	+	nine
109	JaL5	+	NH					
110	JaL6	+	+	+	+	+	+	thuringiensis
111	JaS1	+	+	-	+	+	+	nine
112	JaS10	+	+	-	+	+	-	ten
113	JaS2	+	+	-	+	+	+	nine
114	JaS3	+	+	+	+	+	+	thuringiensis
115	JaS4	+	+	-	+	-	-	israelensis
116	JaS5	+	+	+	+	+	-	kurstaki
117	JaS6	+	NH					
118	JaS7	+	+	+	+	+	-	kurstaki
119	JaS8	+	+	+	+	+	-	kurstaki
120	JaS9	+	+					
121	JDa1	+	+	+	+	+	-	kurstaki
122	JDa2	+	+	+	+	+	-	kurstaki
123	JDb1	+	+	+	+	+	-	kurstaki
124	JDb2	+	+	+	+	+	-	kurstaki
125	JDc1	+	+	+	+	+	-	kurstaki
126	JDc2	+	+	+	+	+	-	kurstaki
127	JeS1	+	+	-	+	+	+	nine
128	JeSa1	+	NH	+	+	+	+	thuringiensis
129	JeSa2	+	NH	+	-	+	+	sotto
130	JeSb1	+	NH	+	+	-	+	indiana
131	JeSb2	+	NH	-	-	+	-	israelensis
132	JeSc1	+	NH	-	-	+	+	thirteen
133	JeSc2	+	+	+	+	-	+	indiana
134	JeSd1	-						*B. sphaericus*
135	JeSd2	+	NH	+	+	+	+	thuringiensis
136	JeSe1	+	+	-	-	+	+	thirteen
137	JeSe2	+	NH	-	-	+	+	thirteen
138	JSa1	+	+	+	-	+	-	dendrolimus
139	JSa2	-						*B. sphaericus*
140	JSa3	+	+	+	+	+	+	thuringiensis
141	JSb1	+	+	+	+	+	-	kurstaki
142	JSb2	+	+	+	-	+	-	dendrolimus
143	JSc1	+	+	+	+	+	-	kurstaki
144	JSc2	+	+	+	-	+	-	dendrolimus
145	JSc3	+	+	+	+	+	-	kurstaki
146	JSd1	+	+	+	+	+	-	kurstaki
147	JSd2	+	+	+	+	+	-	kurstaki
148	KbSa1	+	NH	+	-	-	+	morrisoni
149	KbSa2	+	NH	+	-	-	-	darmstandiensis
150	KbSb1	+	NH	-	-	+	+	thirteen
151	KbSb2	+	NH	-	-	-	+	fifteen
152	KbSc1	+	NH	+	+	-	+	indiana
153	KbSc2	+	NH	-	-	-	+	fifteen
154	KeS1	+	+	+	+	+	+	thuringiensis

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
155	KeS2	+	+	-	-	+	-	israelensis
156	KeS3	+	+	-	+	+	+	nine
157	KeS4	+	+	-	-	+	-	israelensis
158	KfSa1	+	NH	-	-	+	-	israelensis
159	KfSa2	+	+	-	+	+	+	nine
160	KfSb2	+	+	+	-	-	+	morrisoni
161	KkSa1	+	NH	-	+	-	+	eleven
162	KkSa2	+	NH	-	-	-	+	fifteen
163	KkSb1	+	+	+	-	+	+	sotto
164	KkSb2	+	NH	+	+	+	+	thuringiensis
165	KkSb3	-						*B. sphaericus*
166	KkSc1	+	NH	-	-	+	+	thirteen
167	KkSc2	+	NH	-	+	+	+	nine
168	KkSd1	+	NH	-	-	-	+	fifteen
169	KSa1	+	+	+	+	+	-	kurstaki
170	KSa2	+	+	+	-	+	-	dendrolimus
171	KSb1	+	+	+	+	+	-	kurstaki
172	KSb2	+	+					
173	KSc1	+	+	+	+	+	+	thuringiensis
174	KSc2	-						*B. sphaericus*
175	KSe2	+	+	+	+	+	-	kurstaki
176	KuSa1	-						*B. sphaericus*
177	KuSa2	+	NH	+	-	+	+	Sotto
178	KuSa3	-	NH	-	+	+	+	nine
179	KuSb1	-						*B. sphaericus*
180	KuSc1	-						*B. sphaericus*
181	KuSc2	-						*B. sphaericus*
182	KuSd1	-						*B. sphaericus*
183	KuSd2	+	+	+	-	+	+	sotto
184	KuSe1	+	NH	+	-	+	+	sotto
185	KuSe2	+	NH	-	+	-	+	eleven
186	MaSa1	+	+	+	-	+	-	dendrolimus
187	MaSb1	+	+	+	-	+	+	sotto
188	MaSb2	+	+	-	-	+	+	thirteen
189	MaSb3	+	+	-	-	+	+	thirteen
190	MaSc1	+	+	+	-	+	-	dendrolimus
191	MaSc2	+	+	-	+	-	+	eleven
192	MaSc3	-						*B. sphaericus*
193	MeSa1	+	NH	+	-	-	-	darmstadiensis
194	MeSb1	+	NH	+	+	-	+	thuringiensis
195	MeSb2	+	NH	+	+	-	+	indiana
196	MeSc1	+	NH	+	+	-	+	indiana
197	MeSc2	+	NH	+	+	-	+	indiana
198	MeSd1	+	+	+	+	+	+	thuringiensis
199	MeSd2	+	+	+	+	-	+	indiana
200	MeSe1	+	NH	-	+	-	+	eleven
201	MeSe2	+	NH	+	-	-	+	morrisoni
202	MiSa3	+	+	-	+	-	+	ten
203	MuSa1	+	+	+	-	+	-	dendrolimus
204	MuSa2	+	+	+	-	-	+	morrisoni
205	MuSc1	+	+					
206	MuSc2	+	+	+	+	+	-	kurstaki

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
207	MuSc3	+	+	+	-	+	-	dendrolimus
208	MuSc4	+	+	+	+	+	-	kurstaki
209	MuSd1	+	+	+	+	+	+	thuringiensis
210	MuSd2	+	+	+	+	+	+	thuringiensis
211	MuSd3	+	+	+	-	+	+	sotto
212	MuSe1	-						*B. sphaericus*
213	MuSe4	+	+	-	+	-	+	eleven
214	MyIa1	+	NH	-	+	-	+	eleven
215	MyIa2	-						*B. sphaericus*
216	MyIb1	+	NH	+	+	-	+	indiana
217	MyLa1	+	NH	+	+	-	+	indiana
218	MySa1	+	NH	-	+	-	+	eleven
219	MySa2	+	NH	+	+	-	+	indiana
220	MySb1	+	NH	+	+	-	+	indiana
221	MySb2	+	+	+	+	-	+	indiana
222	NaL1	+	+	+	+	+	-	kurstaki
223	NaL2	+	+	+	+	+	-	kurstaki
224	NaS1	+	+	+	+	+	+	thuringiensis
225	NaS2	+	+	-	+	+	+	nine
226	NaSa1	+	+	-	-	-	-	sixteen
227	NaSa2	+	+	-	+	+	+	nine
228	NaSb1	+	+	-	-	-	-	galleriae
229	NaSb2	+	NH	+	+	-	-	sixteen
230	NaSc1	+	+	-	-	-	-	sixteen
231	NaSc2	+	NH	+	+	-	+	indiana
232	NaSc3	+	+	+	+	-	+	indiana
233	NaSd1	+	+	+	+	-	-	galleriae
234	NaSd2	+	NH	+	+	-	+	indiana
235	NaSd3	+	+	-	+	-	+	eleven
236	NaSe1	+	+	+	+	-	+	indiana
237	NaSe2	+	+	+	+	-	+	indiana
238	NoS1	+	+	+	+	+	+	thuringiensis
239	NoS2	+	+	+	+	+	-	kurstaki
240	NoS3	-						*B. sphaericus*
241	NoS4	+	+	+	+	+	-	kurstaki
242	NoS5	+	+	-	+	+	-	ten
243	NsSa1	+	NH	+	+	-	+	indiana
244	NsSb1	+	NH	+	+	-	+	indiana
245	NsSc1	+	NH	+	+	+	+	thuringiensis
246	NsSc2	+	+	+	-	+	+	sotto
247	NsSd1	+	+	+	-	+	-	dendrolimus
248	NsSd2	+	NH	-	-	-	-	sixteen
249	NsSe1	+	NH	-	-	-	-	sixteen
250	NsSe2	+	NH	-	-	-	+	fifteen
251	RaSa1	+	NH	+	+	-	+	indiana
252	RaSa2	+	+	+	+	-	+	indiana
253	RaSb1	+	NH	+	+	-	+	indiana
254	RaSb2	+	+	+	+	-	-	galleriae
255	RaSc1	+	+	+	-	-	+	morrisoni
256	RaSd1	+	NH	+	+	-	+	indiana
257	RaSd2	+	NH	-	-	-	-	sixteen
258	RhSa1	-						*B. sphaericus*

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
259	RhSa2	+	NH	+	+	+	+	thuringiensis
260	RhSa3	-						*B. sphaericus*
261	RhSb1	+	NH	+	+	-	+	indiana
262	RhSb2	+	+	+	+	-	+	indiana
263	RhSb3	+	NH	+	+	-	+	indiana
264	RhSc1	+	+	-	+	-	+	eleven
265	RhSc2	+	NH	-	-	-	+	fifteen
266	RhSc3	+	NH	+	-	+	+	sotto
267	RhSd1	+	NH	+	+	+	+	thuringiensis
268	RhSd2	+	NH	+	-	-	+	morrisoni
269	RhSd3	+	+	-	+	-	+	eleven
270	RhSd4	+	+	+	-	+	-	dendrolimus
271	RpSa1	+	NH	+	+	+	+	thuringiensis
272	RpSa2	+	NH	+	+	+	-	kurstaki
273	RpSb1	+	NH					thirteen
274	RpSb2	+	+	-	+	-	+	eleven
275	RpSc1	+	+	-	-	+	+	thirteen
276	RpSc2	+	NH	-	+	-	+	eleven
277	RpSd1	-						*B. sphaericus*
278	RpSd2	+	NH	-	+	+	+	nine
279	RpSe1	+	NH	-	-	+	-	israelensis
280	RpSe2	+	+	-	-	-	+	fifteen
281	SaS1	+	+	-	+	-	-	israelensis
282	SaS10	+	+	+	+	+	+	thuringiensis
283	SaS2	+	+	-	+	+	-	ten
284	SaS3	+	+	+	+	+	+	thuringiensis
285	SaS4	+	+	+	+	+	+	thuringiensis
286	SaS5	+	+	-	-	+	-	israelensis
287	SaS6	+	+	+	+	+	-	kurstaki
288	SaS7	+	+	+	+	+	-	kurstaki
289	SaS8	+	+	+	+	+	+	thuringiensis
290	SaS9	+	+					
291	SaSa1	+	+	-	+	-	+	eleven
292	SaSa2	+	NH	+	+	-	-	galleriae
293	SaSb1	+	NH	+	+	-	+	indiana
294	SaSb2	+	NH	-	+	+	+	nine
295	SaSc1	+	NH	+	+	-	+	indiana
296	SaSc2	+	NH	+	+	-	+	indiana
297	SaSd1	+	NH	+	+	-	+	indiana
298	SaSe1	+	NH	+	+	-	+	indiana
299	SgSc1	+	+	-	+	-	+	nine
300	SgSj1	+	NH	-	+	-	+	eleven
301	SgSj2	+	NH	-	-	-	+	fifteen
302	SgSm1	+	NH	-	-	-	+	thirteen
303	SgSm2	+	+	+	-	+	+	sotto
304	SgSn1	+	NH	-	+	-	+	eleven
305	SgSn2	+	NH	+	+	-	+	indiana
306	SgSp1	+	+	-	-	+	+	thirteen
307	SgSp2	+	NH	+	+	-	+	indiana
308	Soi1	+	+	+	+	+	-	kurstaki
309	Soi2	+	+			+		
310	Soi3	+	+	-	+	+	+	nine

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
311	SoL1	+	+	-	-	-	-	sixteen
312	SoL2	+	+	+	+	+	-	kurstaki
313	SoS1	+	+	-	+	+	-	ten
314	SoS2	+	+	-	+	-	-	israelensis
315	SoS3	+	+	-	+	+	-	ten
316	SoS4	+	+	-	+	+	-	ten
317	SoS5	+	+	-	-	+	+	thirteen
318	SoS6	+	+	-	+	+	+	nine
319	SoS7	+	+	+	+	+	-	kurstaki
320	SoS8	+	+	-	+	+	-	ten
321	SpSa1	-						*B. sphaericus*
322	SpSa2	+	+	-	+	-	-	ostriniae
323	SpSb1	+	NH	-	-	+	-	*israelensis*
324	SpSb2	-						*B. sphaericus*
325	SpSb3	+	NH	+	+	-	+	indiana
326	SpSc1	+	NH	-	-	-	-	sixteen
327	SpSc2	-						*B. sphaericus*
328	SpSc3	+	NH	+	+	-	+	indiana
329	SpSd1	+	NH	+	+	-	+	indiana
330	SpSd2	+	NH	+	-	-	+	morrisoni
331	SpSd3	+	NH	-	-	-	+	fifteen
332	Spse2	+	NH	+	-	+	+	sotto
333	SSa1	+	+	+	+	+	+	thuringiensis
334	SSa2	+	+	+	-	+	-	dendrolimus
335	SSa3	+	+	-	+	+	-	ten
336	SSb1	+	+	+	+	+	-	kurstaki
337	SSb2	+	+	+	+	+	-	kurstaki
338	SSc1	+	+	-	-	+	-	israelensis
339	SSc2	+	+	+	+	+	-	kurstaki
340	SSd1	+	+	+	+	+	-	kurstaki
341	SSd2	+	+	+	+	+	+	thuringiensis
342	SSe1	+	NH	-	-	-	+	fifteen
343	SSe2	+	+	+	+	+	-	kurstaki
344	SSe3	+	+	+	-	+	-	dendrolimus
345	SSf1	+	+	+	+	+	+	thuringiensis
346	SSf2	+	+	+	+	+	-	kurstaki
347	SSf3	-						*B. sphaericus*
348	SSf4	+	+	+	+	+	+	thuringiensis
349	SySa1	+	NH	-	-	+	+	thirteen
350	SySa2	+	NH	-	-	-	+	fifteen
351	TaSa1	+	NH	+	+	-	+	indiana
352	TaSa2	+	+	+	+	-	+	indiana
353	TaSa3	+	NH	+	+	-	+	indiana
354	TaSa4	+	+	+	+	-	+	indiana
355	TaSb1	+	+	+	+	-	+	indiana
356	TaSb2	-						*B. sphaericus*
357	TaSb3	+	NH	-	+	-	+	eleven
358	TaSc1	+	NH	-	+	-	+	eleven
359	TaSc2	+	NH	-	-	-	-	sixteen
360	TaSc3	+	NH	+	+	-	+	indiana
361	TaSd1	+	NH	-	+	-	-	ostriniae
362	TaSe1	+	+	+	+	-	+	indiana

Serial no.	Isolate name	Starch hydrolysis	Hemolysis	Es	Sa	Le	Su	Biotype
363	TaSe2	+	NH	-	+	-	-	ostriniae
364	USc1	+	+	+	+	+	+	thuringiensis
365	USc2	+	+	-	+	+	-	ten
366	USc3	+	+	+	+	+	+	thuringiensis
Reference strains used								
367	*Bts* T84A1	+	+	+	-	+	+	Reference
368	*Btk* HD-73	+	+	+	+	+	-	Reference
369	*Btj* Buibui	+	+					Reference

The spore diameter of randomly chosen 23 indigenous *Bacillus thuringiensis* was measured which ranged from 0.6 to 1.02 µm and the average spore diameter was calculated to be 0.76±0.097 µm whereas it was 0.63±0.081 µm for reference *Btk* HD-73 (Fig 1.3.4).

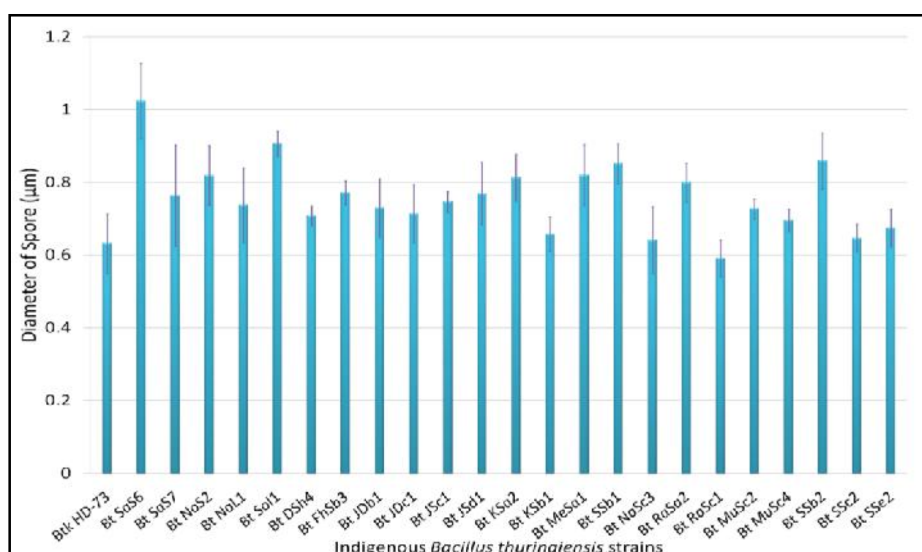

Figure 3.3.4: Variation in spore diameters of randomly chosen 23 indigenous *Bt* isolates.

The average *Bt* index in Bangladesh was calculated to be 0.86 and from the distribution analysis it was observed that almost all parts of Bangladesh are rich in *Bacillus thuringiensis*. *Bt* was most abundant in the Northern and Central part (index 0.90 and 0.89 respectively) and the least abundant was in the sandy beach (index- 0.73) (Table 3.3.3).

The ratio between the *Bt* isolates produced and the fertile samples i.e. yield of isolate per sample was 1.65 (317/192) which varied among different sampling sites (for at least 5 samples) exhibiting Dhaka, central (44/18= 2.44) as most abundant followed by Natore (12/5= 2.4), Tangail (12/5= 2.4) and Jenidah (15/7= 2.14) (Table 3.3.4).

6.3.2 Prevalence and distribution pattern of biotypes

Based on the most significant biochemical tests (Fig 3.3.2C to 3.3.2F), profiles of 298 indigenous *Bt* strains were obtained (Table 3.3.2) and they were classified into 16 biotypes. From this classification, *B. thuringiensis indiana* (17%), *kurstaki* (16%), *thuringiensis* (12%) biotypes were found to be the most prevalent in Bangladesh. *Bt* biotypes *11, 9, sotto, dendrolimus, 13* and *israelensis* were almost equal in their abundances (about or more than 5% of total) whereas biotype *ostriniae* and *darmstadiensis* were the least abundant (Fig 3.3.5).

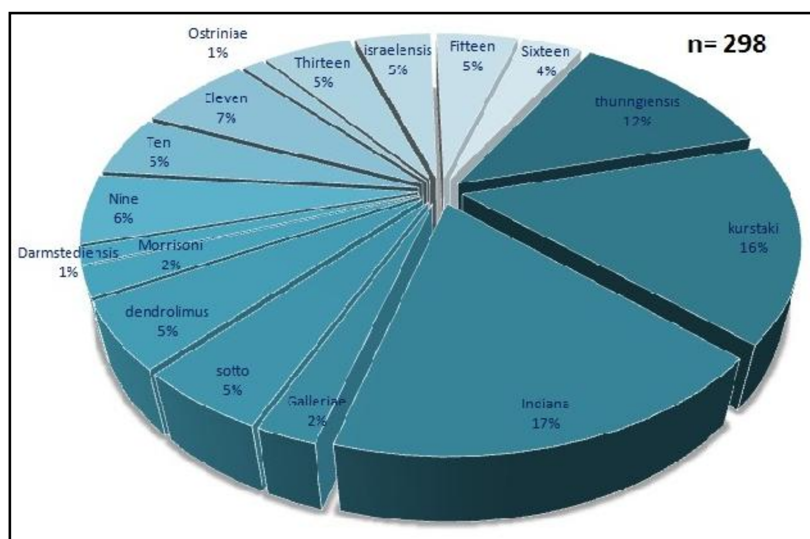

Figure 3.3.5: Abundances of different biotypes among the indigenous *Bt* isolates of Bangladesh.

Table 3.3.3: Distribution of *Bacillus thuringiensis* in different parts of Bangladesh

Eco-regions	Samples	Isolates producing crystal protein	*Bt* isolates	Abundant biotype	Bt index
Central Part	86	148	133	*thu, kur, ind*	0.89
Southern Part	40	66	58	*ind, kur, sotto*	0.87
Northern Part	18	31	28	*Ind, 16,11*	0.90
Hill tracts	22	46	40	*ind, 15*	0.86
River Basin	60	57	47	*10, ind, kur*	0.82
Sandy beach	5	18	11	*thu, kur, 16*	0.73
Total	231	366	317	*ind, kur, thu, 11*	0.86

The *Bt* index was calculated as a number of *Bt* isolates recovered divided by the number of colonies examined. thu= *Bt thuringiensis*, kur= *Bt kurstaki*, ind= *Bt indiana,* 10, 11, 16= other biotypes of *Bt* which do not describe any subspecies yet.

Table 3.3.4: Distribution of different biotypes of *Bt* in Bangladesh

Location (Regions)	Sample number	*Bt* isolate number	Biotypes															
			thu	kur	ind	gal	sot	den	mor	dar	9	10	11	Ost	13	isr	15	16
Chapainawabgonj (N)	5	7	-	-	3	1	1	-	-	-	-	-	1	-	-	-	1	-
Chittagong (H)	5	9	2	-	1	-	-	1	-	-	-	-	-	-	1	1	3	-
Chuadanga (S)	7	3	-	-	-	-	-	-	-	-	-	2	-	-	-	1	-	-
Comilla (H)	2	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cox's Bazar (Sa)	1	4	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dhaka, North (C)	26	42	7	10	-	1	2	3	-	1	3	1	-	-	-	-	-	1
Dhaka, South (C)	4	4	1	-	-	-	-	-	-	-	1	-	-	-	-	2	-	-
Dhaka, East (C)	11	10	1	1	-	-	1	2	-	-	-	2	1	-	2	-	-	-
Dhaka, west (C)	14	10	4	2	-	-	-	-	-	-	-	1	-	-	-	2	-	-
Dhaka, central (C)	19	44	7	9	6	1	1	4	1	1	-	2	4	1	2	2	2	1
Dinajpur (N)	3	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Jamalpur (RB)	22	16	2	4	-	-	1	-	-	-	3	2	-	-	-	1	-	-
Jessore (S)	6	10	2	-	2	-	1	-	-	-	1	-	-	-	3	1	-	-
Jhenaidah (S)	7	15	1	11	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Khulna (S)	5	9	-	-	1	-	-	-	2	1	1	-	-	-	1	1	2	-
Kuakata (Sa)	4	7	1	-	-	-	1	-	-	-	1	-	1	-	1	-	2	-
Kushtia (S)	5	5	-	-	-	-	3	-	-	-	1	-	1	-	-	-	-	-
Meherpur (S)	5	9	2	-	4	-	-	-	1	1	-	-	1	-	-	-	-	-
Munshiganj (RB)	5	10	2	2	-	-	1	2	1	-	-	-	1	-	-	-	-	-
Mymensing (RB)	5	7	-	-	5	-	-	-	-	-	-	-	2	-	-	-	-	-
Natore (N)	5	12	-	-	5	2	-	-	-	-	1	-	1	-	-	-	-	3
Narshingdi (C)	12	12	2	2	2		1	1	-	-	-	1	-	-	-	-	1	2
Rangamati (H)	4	7	-	-	4	1	-	-	1	-	-	-	-	-	-	-	-	1
Rangpur (N)	5	9	1	1	-	-	-	-	-	-	1	-	2	-	2	1	1	-
Sherpur (H)	5	9	-	-	3	-	1	-	1	-	-	-	-	1	-	1	1	1
Shirajgonj (RB)	5	9	-	-	2	-	1	-	-	-	1	-	2	-	2	-	1	-
Shatkhira (S)	5	8	-	-	5	1	-	-	-	-	1	-	1	-	-	-	-	-
Sonargaon (RB)	23	13	-	3	-	-	-	-	-	-	2	4	-	-	1	1	-	1
Sylhet (H)	1	2	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-
Tangail (H)	5	12	-	-	7	-	-	-	-	-	-	-	2	2	-	-	-	1
Total	231	317	37	48	52	7	15	16	7	4	17	15	20	4	16	14	15	11
	Distribution index		0.5	0.4	0.5	0.2	0.4	0.23	0.2	0.13	0.4	0.26	0.43	0.1	0.33	0.36	0.33	0.26

N: northern region, **S**: sourthern region, **C**: central region, **RB**: River Basin, **Sa**: Sandy beach, **H**: Hill tracts.

The distribution pattern of different biotypes in different sampling sites was also analysed which was accomplished by an index i.e. distribution index. The distribution index was calculated by dividing the number of sampling sites with a particular biotype with total number of sampling sites. The distribution index implies the degree of ubiquity for any biotype in this study. Biotypes *thuringiensis* (0.5) and *indiana* (0.5) were found to be more ubiquitous than all other biotypes followed by *eleven* (0.43), *kurstaki*, *sotto* and *nine* (0.4 for each) (Table 3.3.4). Biotypes containing isolates less than 10 were not considered. On the other hand, with more samples, more *Bt* isolates were obtained and more biotypes were also present in north Dhaka and central Dhaka whereas the same consequences were not observed in Jamalpur and Sonargaon (Table 3.3.4).

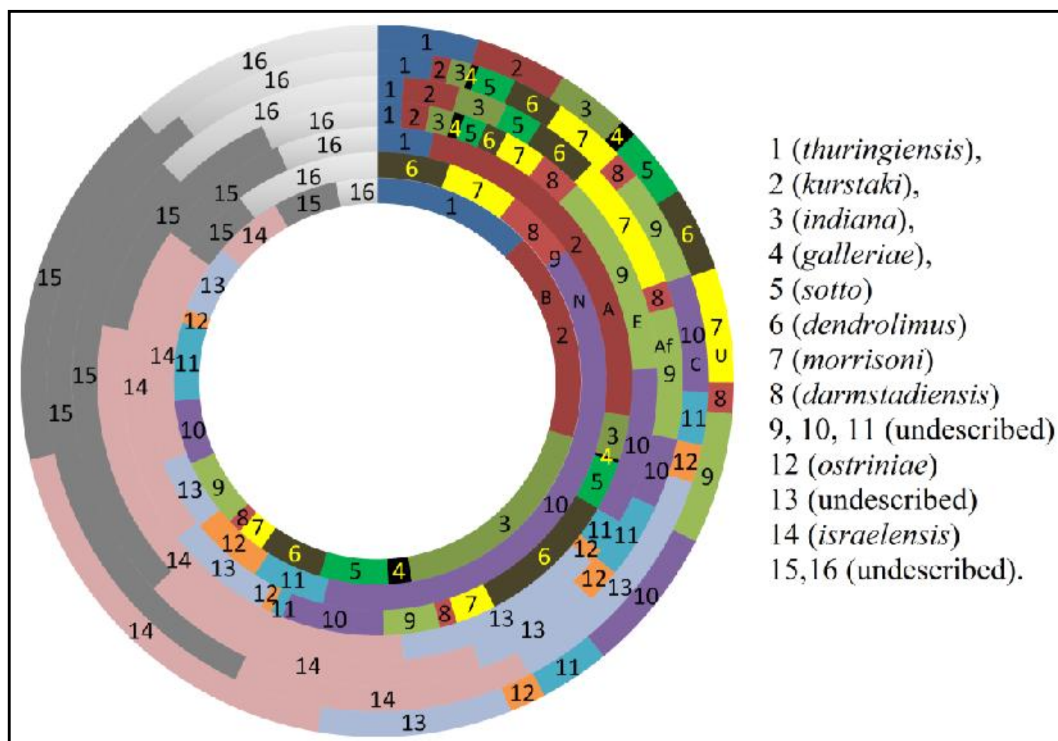

Figure 3.3.6: Comparison of prevalence (%) of different biotypes around the world. Rings from centre to periphery: 1. B- Bangladesh 2. N- New Zealand 3. A- Asia 4. E- Europe 5. Af- Africa 6. C- Central and South America 7. U- United States.

However, the distribution pattern of *Bt* biotypes in Bangladesh seemed to be unique when compared with the patterns of other parts of the world (Fig 3.3.6). *Bt thuringiensis*, *kurstaki* and *indiana* together account for 47% of the total isolates of Bangladesh whereas it is 0%, 30%, 4.4%, 6.9%, 4.6% and 12% for New Zealand, other countries of Asia, Europe, Africa, Central and South America and United States respectively (Martin and Travers, 1989).

Abundance of biotypes *israelensis*, *15* and *16* were found to be the lowest in Bangladesh whereas in other parts of the world abundances are remarkable.

6.3.3 Broad spectrum cytolysin activity:

Three hundred and seventeen *Bt* strains were tested for their broad spectrum cytolysin activity and 58.36% (189) of them were found to have this property hence presumed as insecticidal and rest 41.64% can be explored for anti-cancer cell protein, parasporin. Based on this property, percentages of insecticidal strains in each biotype were also determined (Fig 3.3.2B). The percentages of insecticidal strains among different biotypes were also calculated. The descending order for prevalence of haemolytic strains was like *Bt 10*> *Bt kurstaki*> *Bt dendrolimus*> *Bt thuringiensis*> *Bt 9*> *Bt indiana*> *Bt israelensis* (Fig 3.3.7).

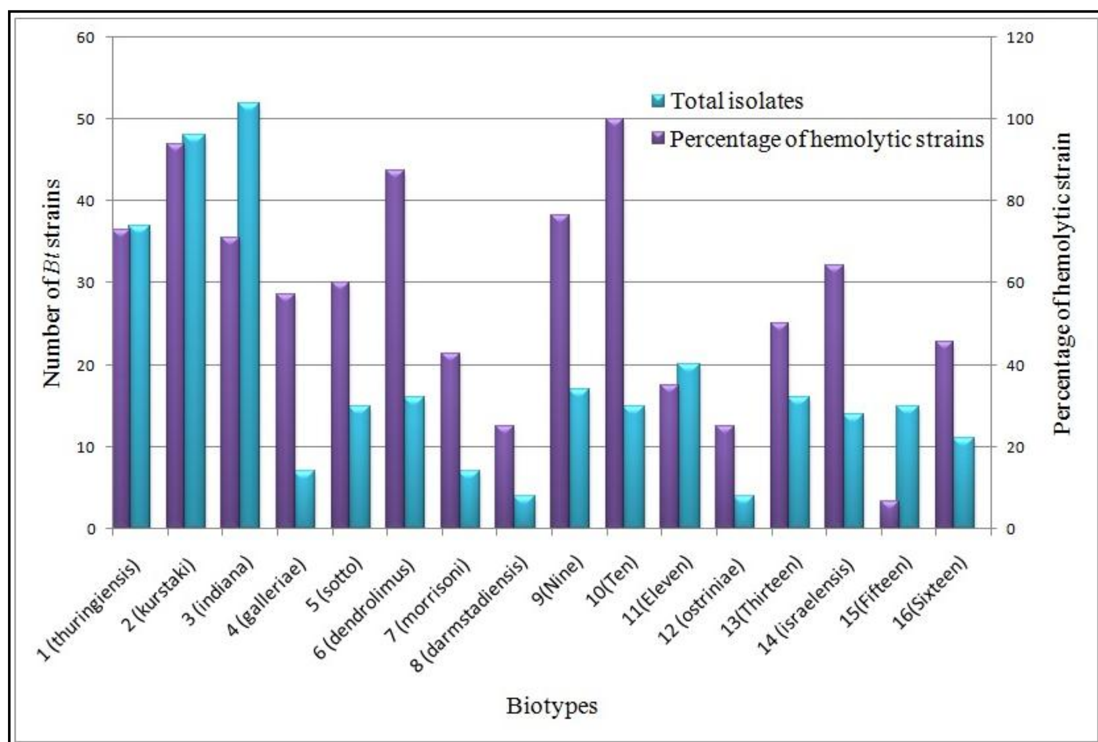

Figure 3.3.7: Percentage of hemolytic *Bt* strains in different biotypes.

6.3.4 16S rRNA gene sequence analysis:

Amplicons of ~1500bp were obtained from PCR amplification of 16S rRNA gene of indigenous *Bacillus thuringiensis* isolates (Fig 3.3.8). The sequences obtained from the amplicons were used for nucleotide blast in NCBI database and the sequences producing significant alignments to them with more than 96% homology were observed to be from different strains of *Bacillus thuringiensis*. GenBank Accession numbers for these strains were (GenBank KF741358- KF74 1360 and GenBank KF812552- KF812557) (Table 3.3.5).

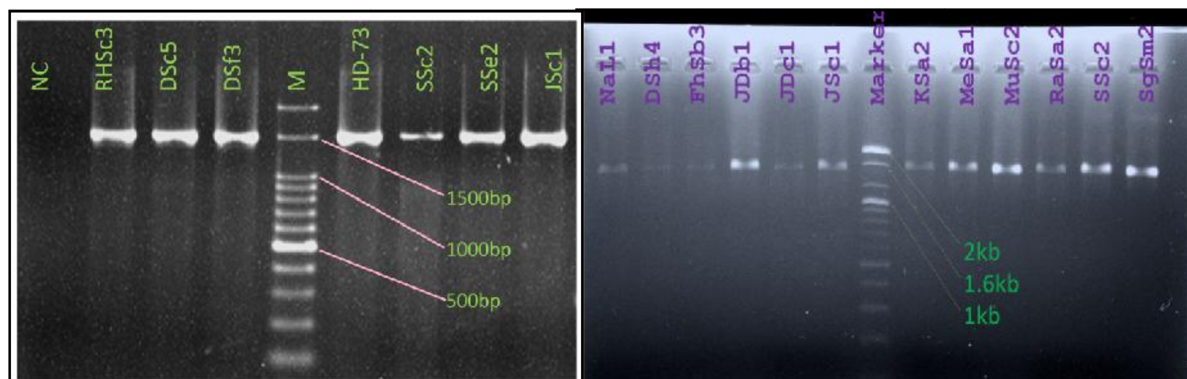

Figure 3.3.8: Amplicons obtained from PCR for 16S rRNA gene analysis (Marker: 100 bp DNA ladder, Bioneer, Korea).

Table 3.3.5: BLASTn results for the sequences of 16S rRNA genes from indigenous *Bt* strains

Strain (accession no.)	Similarity with (Accession no.)	E- value	Homology (%)
CiSa1(GenBank KF812556)	*Bacillus thuringiensis* (HF545006.1)	0.0	98
DSc5 (GenBank KF741360)	*Bacillus thuringiensis* (KC789794.1)	0.0	98
DSf3 (GenBank KF741358)	*Bacillus thuringiensis* (JQ988062.1)	0.0	99
JeSa1(GenBank KF812555)	*Bacillus thuringiensis* (KC789794.1)	0.0	98
JSc1(GenBank KF812553)	*Bacillus thuringiensis* (JQ579628.1)	0.0	97
JSd1(GenBank KF812557)	*Bacillus thuringiensis* (KF017270.1)	0.0	99
Soi1(GenBank KF812554)	*Bacillus thuringiensis* (FJ932761.1)	0.0	99
SSe2 (GenBank KF741359)	*Bacillus thuringiensis* (FJ601906.1)	0.0	96
SSb1(GenBank KF812552)	*Bacillus thuringiensis* (JQ579628.1)	0.0	97

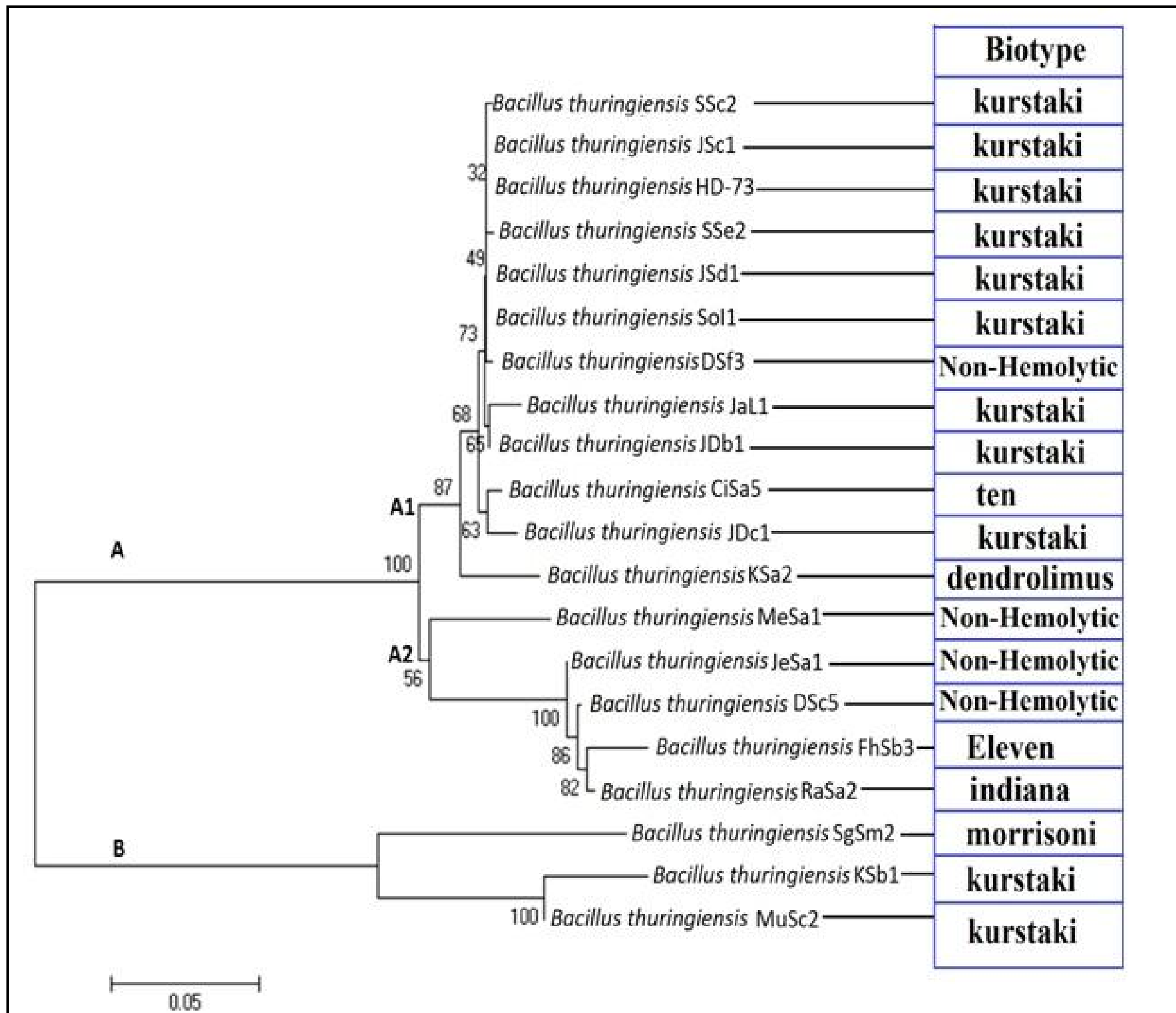

Figure 3.3.9: Neighbor-joining tree showing phylogenetic relationship between indigenous *Bacillus thuringiensis* strains and reference strain *Btk* HD-73 based on the 16S rRNA gene sequence analysis. This is an un-rooted tree reconstructed with 1000 bootstrap replicates based on maximum composite likelihood using tree construction software MEGA version 5.22.

Sequences of amplicons obtained for 16S rRNA gene from the indigenous *Bt* strains were aligned to analyse the phylogenetic relationship between them. Following the bootstrap neighbor joining method, a phylogenetic tree was constructed with 19 indigenous *Bt* strains and reference *Btk* HD-73 (Fig 3.3.9). The tree was observed to contain 2 major distinct phylogenetic groups consisting of clusters A and B. Cluster A, the largest one, contained 16 native *Bt* strains and 1 reference strain whereas cluster B contained rest 3. Nine of the indigenous *Bt* strains from biotype *kurstaki* and reference *Btk* HD-73 remained in the same sub-cluster A1.

6.3.5 Diversity in plasmid profile

Plasmid profiles of *Bt* strains exhibited diversities among themselves. Clearly visible supercoiled plasmids occupied a range of 3 kb to more than 16 kb. 81% of the visible plasmid bands fall into the range of 10-13kb (Fig 3.3.10).

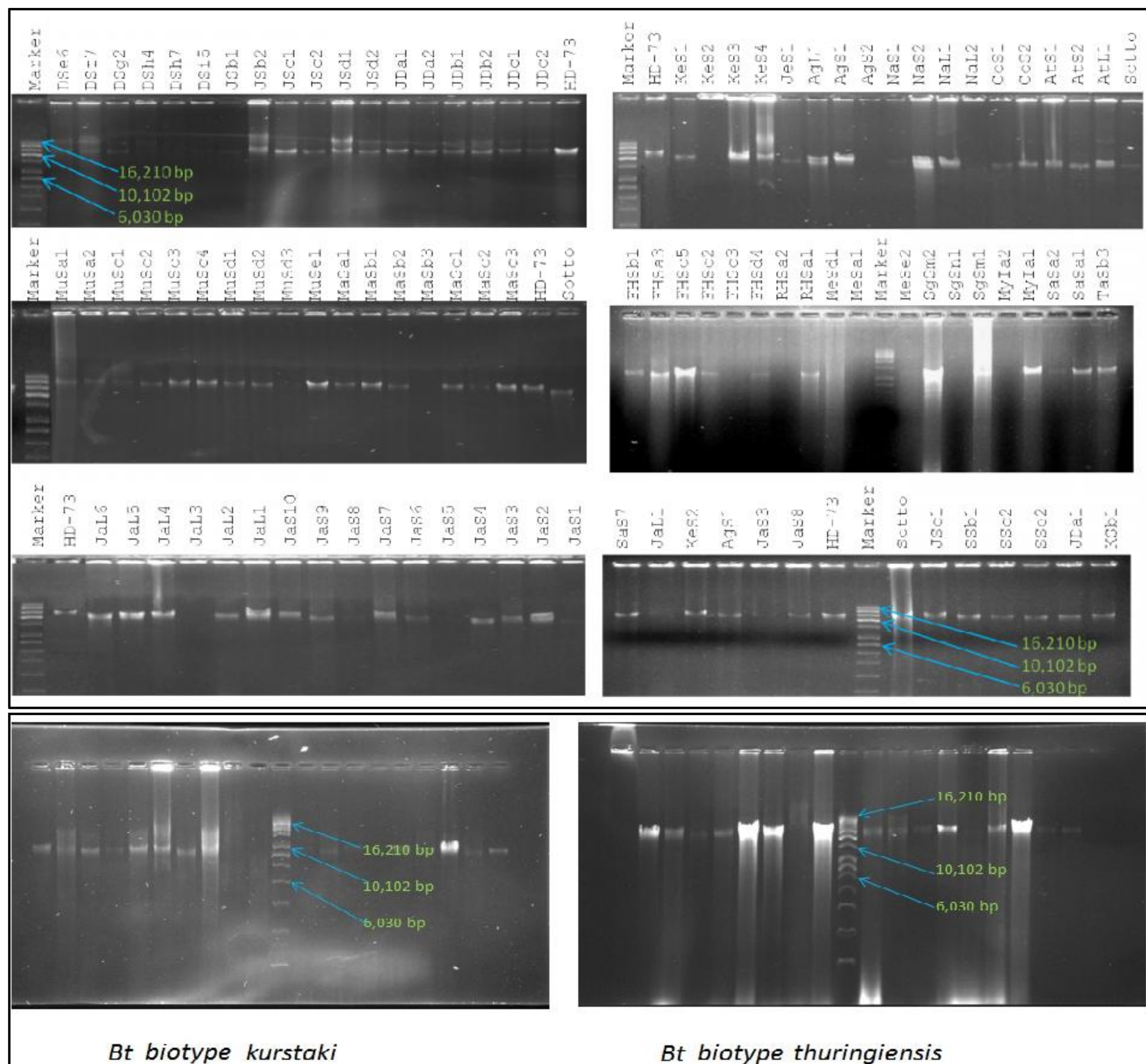

Figure 3.3.10: Plasmid profiles of *Bacillus thuringiensis* strains of Bangladesh. (Names of the strains are labeled over the lanes; Marker: Supercoiled DNA ladder- Appendix A).

As the large plasmids remain in low copy number, their extraction is difficult and not always visible by agarose gel electrophoresis. These large plasmids were visible only in few strains in this study like in KeS4, AgS1, NaL1, AtS2, AtL1, FhSa4 etc (Fig 3.3.10). Hence, the pattern for small plasmids was compared and molecular weight based distribution of plasmids among the biotypes were determined which also renders the degree of diversity among the

strains. Plasmids in *Bt thuringiensis, kurstaki, indiana* and *israelensis* exhibited more diversity as they occupy a wide range of 3 to more than 16 kb (Fig 3.3.11), even it can be seen while plasmids from Bt strains of same biotypes were electrophoresed simultaneously in the same gel (Fig 3.3.10).

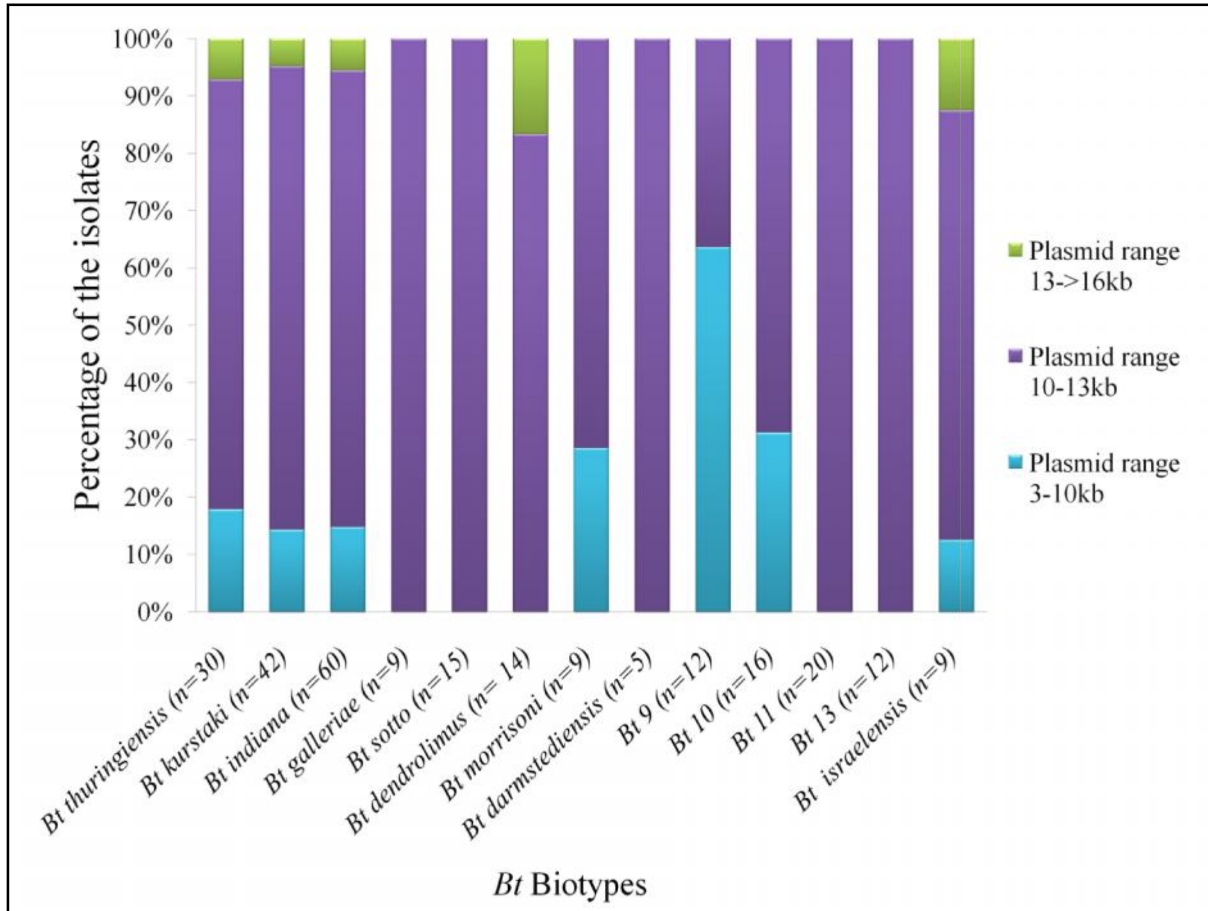

Figure 3.3.11: Distribution of plasmids based on molecular sizes in prevailing *Bt* biotypes of Bangladesh.

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CHAPTER 4

Detection of *cry* genes and δ-endotoxin profile analysis of the indigenous *Bacillus thuringiensis*

Detection of *cry* genes and δ-endotoxin profile analysis of the indigenous *Bacillus thuringiensis*

4.1 Introduction

The insecticidal activity of *Bacillus thuringiensis* (*Bt*) is mainly dependent on its ability to synthesize crystal proteins which upon ingestion by susceptible insects are solubilized and proteolytically activated for specific binding to the receptors in the mid-gut epithelial cell (Höfte and Whiteley, 1989). A δ- endotoxin can be defined as a major protein component of a parasporal crystal showing significant sequence similarity to one or more toxins within the established nomenclature or as a *Bt* parasporal inclusion protein that exhibits insecticidal activity or some experimentally verifiable toxic effect to a target organism (Crickmore *et al.*, 2014). There are two types of δ- endotoxins, the highly specific Cry (from crystal) toxins which act via specific receptors and the non-specific Cyt (cytolytic) toxins, with no known receptors. Both families of toxins are classified exclusively on the basis of their amino acid sequence identity and four ranks have been defined with the boundaries of 45, 78 and 95%.

The existence of diversities in the insecticidal proteins made them effective against a range of important pests and most interesting microbial insecticide alternative to chemical agents. Konecka *et al.*, 2007 reported that proteins of groups Cry1, Cry2, Cry9, and Cry15 were reported to be toxic against the pests of the order Lepidoptera (e.g. *Lymantria dispar*, *Manduca sexta*, *Spodoptera littoralis*, and *Helicoverpa armigera*). Cry proteins belonging to groups 1, 2, 4, 10, 11, 16, 17, 19, 20, 21, 24, 25, 27, 29, 30, 32, 39, and 40 display bioactivity against dipteran insects (e.g. *Anopheles albimanus*, *Aedes aegypti*, *Culex pipens*, *Culex quinquefesciatus*, *Musca domestica*, and *Chrisomonus tepperi*). The toxic activity of Cry1, Cry3, Cry7, Cry8, Cry9, Cry14, Cry23, Cry34, and Cry35 for *Leptinotarsa decemlineata*, *Chrysomela scripta*, *Diabrotica virgifera virgifera*, *Pyrrhalta luteola*, and other coleopteran insects was reported. Cry5 toxin possesses activity against pests of the order Hymenoptera: *Diprion pini* and *Cephalcia abietis*. Some crystal proteins are also toxic for insects of Homoptera, Orthoptera, and Mallophaga (Konecka *et al.*, 2007).

These Cry proteins are encoded by the *cry* genes which are very often located in plasmids and also in chromosomal DNA. Several *cry* genes can be harbored by a single *Bt* strain and up to eight different *cry* genes in a single strain has been reported (Martínez, 2002). In general, the type of *cry* and *cyt* genes present in a strain are correlated to some extent with its insecticidal activity. Thus, the identification of the gene content in a *Bt* strains can be used to predict its insecticidal potential. Several *cry* gene screening projects of *Bt* collection in different regions have been described (Carozzi *et al.*, 1991; Bourque *et al.*, 1993; Kuo and Chak, 1996; Ben-Dov *et al.*, 1997; 1999; Bravo *et al.*, 1998; Kim, 2000). The strategies employed in those screening projects were based on PCR method which has become the most powerful approach to identify the *cry* genes content and to predict the insecticide activity.

The insecticidal potential of a *Bt* strain can more appropriately be ascertained by detection of *cry* genes present followed by analysis of crystal proteins produced by that strain as the presence of a certain gene does not imply its eventual translation. For the identification of Cry proteins various techniques *viz.* immunological characterization (Zouari and Jaoua, 1997), purification and micro-sequencing of the major peptides (Chestukhina *et al.*, 1994), quantitative determination of differential *cry* gene expression at the mRNA level by using reverse transcription PCR (RT-PCR) strategy etc have been used and reported. When the objective is to characterize a large number of strains which might contain more than one δ-endotoxins, use of these techniques becomes infeasible. On the other hand, a conventional technique of molecular weight determination of the crystal proteins by SDS-PAGE analysis can provide more confidences for the presumption with *cry* genes. Thus a binary approach, consisting of characterization of *cry* genes of the *Bt* strains followed by the analysis of the Cry proteins by SDS-PAGE can be very handy to select potential strains.

In view of the above, the aims were to detect *cry1*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9*, *cry10* and *cry11* genes by PCR-based method in the indigenous *Bt* strains in the study of this chapter and to analyze the molecular weight of the Cry proteins. Result from this study will provide useful information in selecting potential *Bt* strains for controlling the targeted pest species with economic importance in Bangladesh.

4.2 Methods

4.2.1 Selection of *cry* genes of interests

From the full list of δ-endotoxins (Crickmore *et al.*, 2014) and the toxin specificity data summary from 'The Canadian Forest Service' (http://cfs.nrcan.gc.ca/projects/119/6), correlations among the *Bt* subspecies, *cry* genes and susceptible insect orders was investigated as shown in Table 4.2.1 and Table 4.2.2. Based on the information, *cry1*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9*, *cry10* and *cry11* genes were targeted for investigation into the indigenous *Bt* isolates.

Table 4.2.1: Insect orders susceptible to the δ-endotoxins

Insect orders	Major specific δ-endotoxin
Lepidoptera	Cry1, Cry2, Cry8, Cry9
Diptera	Cry1, Cry2, Cry4, Cry10, Cry11, cyt1, cyt2
Coleoptera	Cry1, Cry3, Cry8, cty2
Acari	Cry3
Hymenoptera	Cry2, Cry3
Hemiptera	Cry1, Cry2, Cry3

Table 4.2.2: Presence of different δ-endotoxin genes in different *Bacillus thuringiensis* subspecies.

Bt **subspecies**	**Available genes**
Bt thuringiensis	*cry1*
Bt kurstaki	*cry1, cry2, cry3, cry9*
Bt aizawai	*cry1, cry9, cry30, cry39, cry40*
Bt galleriae	*cry1, cry7, cry8, cry9*
Bt sotto	*cry1, cry2, cry14, cry24, cry30*
Bt dendrolimus	*cry1*
Bt morrisoni	*cry1, cry3, cyt1, cry2*
Bt darmstadiensis	*cry5, cyt2*
Bt tenebrionis	*cry3, cyt2*
Bt israelensis	*cry4, cry10, cry11, cry60, cyt1, cyt2*
Bt tolworthi	*cry3, cry9*
Bt japonensis	*cry8, cry9*

4.2.2 Reported oligonucleotide primers

As for the detection of these genes, getting amplicons of expected sizes targeting the conserved regions with the designed primers became an established method. Hence, previously reported literatures were examined for available primers and oligonucleotide primers for *cry1*, *cry1A*, *cry4*, *cry8*, *cry10* and *cry11 genes* were obtained (Table 4.2.3).

Table 4.2.3: Properties of primers used for the dectection of primary groups of *cry* genes by different researchers.

Primer	Sequence	Position	Annealing temperature	Product size	Source
cry1-F	CATGATTCATGCGGCAGATAAAC	2781-2803	54°C	277	(Ben-Dov *et al.*, 1997)
cry1-R	TTGTGACACTTCTGCTTCCCATT	3035-3057			
cry1A-F	CCGGTGCTGGATTTGTGTTA	115-134	54°C	490	(Ben-Dov *et al.*, 1997)
cry1A-R	AATCCCGTATTGTACCAGCG	586-605			
cry4A-F	TCAAAGATCATTTCAAAATTACATG	1706-1730	49°C	459	(Jouzani *et al.*, 2008)
cry4A-R	CGGCTTGATCTATGTCATAATCTGT	2140-2164			
cry8-F	ATGAGTCCAAATAATCTAAATG	1-22	49°C	376	(Bravo *et al.*, 1998)
cry8-R	TTTGATTAATGAGTTCTTCCACTCG	358-376			
cry10-F	TCAATGCTCCATCCAATG	38-55	50°C	348	(Jouzani *et al.*, 2008)
cry10-R	CTTGTATAGGCCTTCCTCCG	366-385			
cry11-F	TTAGAAGATACGCCAGATCAAGC	1522-1544	51°C	311	(Bravo *et al.*, 1998)
cry11-R	CATTTGTACTTGAAGTTGTAATCCC	1814-1832			

4.2.3 Designing of Oligonucleotide Primers

The oligonucleotide primers for the detection of primary groups of *cry* genes such as *cry2*, *cry3*, *cry9* and secondary groups of *cry1* genes such as *cry1Aa, cry1Ac, cry1Ba, cry1Ca* were designed in this study. In this connection, the first step of designing primers to detect a certain gene was to align the available sequences of that particular gene to identify the conserved region. The sequences of the above mentioned *cry* genes were selected from the full list of δ-endotoxin (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/) which are linked to NCBI database and the sequences were aligned using ClustalW program and are depicted in 7 different figures (Fig 4.2.1 to Fig 4.2.7). The conserved regions for different *cry* genes were identified and it was thus ensured that the selected conserved region of each gene remains unique. Primer pairs were then designed targeting the conserved regions by a web-based primer designing program (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

```
gi|309274393|gb|GU073380.1|          ACATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGAA
gi|533206233|gb|KC156702.1|          ACATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGAA
gi|218963750|gb|EU939453.1|          ACATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGAA
gi|220683826|gb|FJ550343.1|          ACATCTTTCTTTTATTAGAGATGTTATTCTAAATGCAGATGAATGGGGAA
gi|142747|gb|M23724.1|BACCRYIB       ACATCTTTCTTTTATTAGAGATGTTATTCTAAATGCAGATGAATGGGGAA
gi|237506878|gb|FJ493542.1|          ACATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGAA
                                     ****************************** *******************

gi|309274393|gb|GU073380.1|          TTTCAGCAGCAACATTACGTACGTATCAAAATCACCTGAGAAATTATACA
gi|533206233|gb|KC156702.1|          TTTCAGCAGCAACATTACGTACGTATCAAAATCACCTGAGAAATTATACA
gi|218963750|gb|EU939453.1|          TTTCAGCAGCAACATTACGTACGTATCAAAATCACCTGAGAAATTATACA
gi|220683826|gb|FJ550343.1|          TTTCAGCAGCAACATTACGTACGTATCGAGATTACTTGAAAAATTATACA
gi|142747|gb|M23724.1|BACCRYIB       TTTCAGCAGCAACATTACGTACGTATCGAGATTACTTGAAAAATTATACA
gi|237506878|gb|FJ493542.1|          TTTCAGCAGCAACATTACGTACGTATCAAAATCACCTGAGAAATTATACA
                                     *************************** * ** ** *** **********

gi|309274393|gb|GU073380.1|          AGAGAGTACTCTAATTATTGTATAACTACGTATCAAACTGCGTTTAGAGG
gi|533206233|gb|KC156702.1|          AGAGAGTACTCTAATTATTGTATAACTACGTATCAAACTGCGTTTAGAGG
gi|218963750|gb|EU939453.1|          AGAGAGTACTCTAATTATTGTATAACTACGTATCAAACTGCGTTTAGAGG
gi|220683826|gb|FJ550343.1|          AGAGATTACTCTAACTATTGTATAAATACGTATCAAAGTGCGTTTAAAGG
gi|142747|gb|M23724.1|BACCRYIB       AGAGATTACTCTAACTATTGTATAAATACGTATCAAAGTGCGTTTAAAGG
gi|237506878|gb|FJ493542.1|          AGAGAGTACTCTAATTATTGTATAACTACGTATCAAACTGCGTTTAGAGG
                                     ***** ******** ********** *********** ******** ***

gi|309274393|gb|GU073380.1|          TTTAAACACCCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
gi|533206233|gb|KC156702.1|          TTTAAACACCCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
gi|218963750|gb|EU939453.1|          TTTAAACACCCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
gi|220683826|gb|FJ550343.1|          TTTAAACACTCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
gi|142747|gb|M23724.1|BACCRYIB       TTTAAACACTCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
gi|237506878|gb|FJ493542.1|          TTTAAACACCCGTTTACACGATATGTTAGAATTTAGAACATATATGTTTT
                                     ********* ****************************************

gi|309274393|gb|GU073380.1|          TAAATGTATTTGAATATGTATCTATCTGGTCGTTGTTTAAATATCAAAGC
gi|533206233|gb|KC156702.1|          TAAATGTATTTGAATATGTATCTATCTGGTCGTTGTTTAAATATCAAAGC
gi|218963750|gb|EU939453.1|          TAAATGTATTTGAATATGTATCTATCTGGTCGTTGTTTAAATATCAAAGC
gi|220683826|gb|FJ550343.1|          TAAATGTATTTGAGTATGTATCTATCTGGTCGTTGTTTAAATATCAAAGC
gi|142747|gb|M23724.1|BACCRYIB       TAAATGTATTTGAGTATGTATCTATCTGGTCGTTGTTTAAATATCAAAGT
gi|237506878|gb|FJ493542.1|          TAAATGTATTTGAATATGTATCTATCTGGTCGTTGTTTAAATATCAAAGC
                                     ************* *********************************** 

gi|309274393|gb|GU073380.1|          CTTCTAGTATCTTCTGGCGCTAATTTATATGCAAGTGGTAGTGGACCACA
gi|533206233|gb|KC156702.1|          CTTCTAGTATCTTCTGGTGCTAATTTATATGCAAGTGGTAGTGGACCACA
gi|218963750|gb|EU939453.1|          CTTCTAGTATCTTCTGGCGCTAATTTATATGCAAGTGGTAGTGGACCACA
gi|220683826|gb|FJ550343.1|          CTTCTAGTATCTTCTGGCGCTAATTTATATGCAAGTGGTAGTGGACCACA
gi|142747|gb|M23724.1|BACCRYIB       CTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGTGGACCACA
gi|237506878|gb|FJ493542.1|          CTTCTAGTATCTTCTGGCGCTAATTTATATGCAAGTGGTAGTGGACCACA
                                     ************** ** ********************************

gi|309274393|gb|GU073380.1|          GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
gi|533206233|gb|KC156702.1|          GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
gi|218963750|gb|EU939453.1|          GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
gi|220683826|gb|FJ550343.1|          GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
gi|142747|gb|M23724.1|BACCRYIB       GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
gi|237506878|gb|FJ493542.1|          GCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTT
                                     **************************************************

gi|309274393|gb|GU073380.1|          TCCAAGTTAATTCAAATTATGTGTTAAATGGCTTTAGTGGCGCTAGACTT
gi|533206233|gb|KC156702.1|          TCCAAGTTAATTCAAATTATGTGTTAAATGGCTTTAGTGGCGCTAGACTT
gi|218963750|gb|EU939453.1|          TCCAAGTTAATTCAAATTATGTGTTAAATGGCTTTAGTGGCGCTAGACTT
gi|220683826|gb|FJ550343.1|          TCCAAGTTAATTCAAATTATGTGTTAAATGGCTTTAGTGGCGCTAGACTT
gi|142747|gb|M23724.1|BACCRYIB       TCCAAGTTAATTCAAATTATGTGTTAAATGGATTTAGTGGTGCTAGGCTT
gi|237506878|gb|FJ493542.1|          TCCAAGTTAATTCAAATTATGTGTTAAATGGCTTTAGTGGCGCTAGACTT
                                     ******************************* ******** ***** ***

gi|309274393|gb|GU073380.1|          ACGCAGACTTTCCCTAATATTGTTGGTTTACCTGGTACTACTACAACTCA
gi|533206233|gb|KC156702.1|          ACGCAGACTTTCCCTAATATTGTTGGTTTACCTGGTACTACTACAACTCA
gi|218963750|gb|EU939453.1|          ACGCAGACTTTCCCTAATATTGTTGGTTTACCTGGTACTACTACAACTCA
gi|220683826|gb|FJ550343.1|          ACGCAGACTTTCCCTAATATTGTTGGTTTACCTGGTACTACTACAACTCA
gi|142747|gb|M23724.1|BACCRYIB       TCTAATACCTTCCCTAATATAGTTGGTTTACCTGGTTCTACTACAACTCA
gi|237506878|gb|FJ493542.1|          ACGCAGACTTTCCCTAATATTGTTGGTTTACCTGGTACTACTACAACTCA
                                      *  * ** *********** *************** *************

gi|309274393|gb|GU073380.1|          CGCATTGCTTGCTGCAAGGGTCAATTACAGTGGAGGAGTTTCGTCTGGTG
gi|533206233|gb|KC156702.1|          CGCATTGCTTGCTGCAAGGGTCAATTACAGTGGAGGAGTTTCGTCTGGTG
gi|218963750|gb|EU939453.1|          CGCATTGCTTGCTGCAAGGGTCAATTACAGTGGAGGAGTTTCGTCTGGTG
gi|220683826|gb|FJ550343.1|          CGCATTGCTTGCTGCAAGGGTCAATTACAGTGGAGGAGTTTCGTCTGGTG
gi|142747|gb|M23724.1|BACCRYIB       CGCATTGCTTGCTGCAAGGGTTAATTACAGTGGAGGAATTTCGTCTGGTG
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gi|237506878|gb|FJ493542.1|        CGCATTGCTTGCTGCAAGGGTCAATTACAGTGGAGGAGTTTCGTCTGGTG
                                   ********************* *************** ************

gi|309274393|gb|GU073380.1|        ATATAGGCGC---TGTGTTTAATCAAAATTTTAGTTGTAGTACATTTCTC
gi|533206233|gb|KC156702.1|        ATATAGGCGC---TGTGTTTAATCAAAATTTTAGTTGTAGTACATTTCTC
gi|218963750|gb|EU939453.1|        ATATAGGCGC---TGTGTTTAATCAAAATTTTAGTTGTAGTACATTTCTC
gi|220683826|gb|FJ550343.1|        ATATAGGCGC---TGTGTTTAATCAAAATTTTAGTTGTAGTACATTTCTC
gi|142747|gb|M23724.1|BACCRYIB     ATATAGGTGCATCTCCGTTTAATCAAAATTTTAATTGTAGCACATTTCTC
gi|237506878|gb|FJ493542.1|        ATATAGGCGC---TGTGTTTAATCAAAATTTTAGTTGTAGTACATTTCTC
                                   ******* **   *  ***************** ****** *********

gi|309274393|gb|GU073380.1|        CCACCTTTGTTAACACCATTTGTTAGAAGTTGGCTAGATTCAGGTTCAGA
gi|533206233|gb|KC156702.1|        CCACCTTTGTTAACACCATTTGTTAGAAGTTGGCTAGATTCAGGTTCAGA
gi|218963750|gb|EU939453.1|        CCACCTTTGTTAACACCATTTGTTAGAAGTTGGCTAGATTCAGGTTCAGA
gi|220683826|gb|FJ550343.1|        CCACCTTTGTTAACACCATTTGTTAGAAGTTGGCTAGATTCAGGTTCAGA
gi|142747|gb|M23724.1|BACCRYIB     CCCCCATTGTTAACGCCATTTGTTAGGAGTTGGCTAGATTCAGGTTCAGA
gi|237506878|gb|FJ493542.1|        CCACCTTTGTTAACACCATTTGTTAGAAGTTGGCTAGATTCAGGTTCAGA
                                   ** ** ******** *********** ***********************

gi|309274393|gb|GU073380.1|        TCGGGGGGGGATTAATACCGTTACCAATTGGCAAACAGAATCCTTTGAGA
gi|533206233|gb|KC156702.1|        TCGGGGGGGGATTAATACCGTTACCAATTGGCAAACAGAATCCTTTGAGA
gi|218963750|gb|EU939453.1|        TCGGGGGGGGATTAATACCGTTACCAATTGGCAAACAGAATCCTTTGAGA
gi|220683826|gb|FJ550343.1|        TCGGGGGGGGATTAATACCGTTACCAATTGGCAAACAGAATCCTTTGAGA
gi|142747|gb|M23724.1|BACCRYIB     TCGGGAGGGCGTTGCCACCGTTACAAATTGGCAAACAGAATCCTTTGAGA
gi|237506878|gb|FJ493542.1|        TCGGGGGGGGATTAATACCGTTACCAATTGGCAAACAGAATCCTTTGAGA
                                   ***** ***  **   ******** *************************

gi|309274393|gb|GU073380.1|        CAACTTTAGGTTTAAGGAGTGGTGCTTTTACAGCTCGAGGTAATTCAAAC
gi|533206233|gb|KC156702.1|        CAACTTTAGGTTTAAGGAGTGGTGCTTTTACAGCTCGAGGTAATTCAAAC
gi|218963750|gb|EU939453.1|        CAACTTTAGGTTTAAGGAGTGGTGCTTTTACAGCTCGAGGTAATTCAAAC
gi|220683826|gb|FJ550343.1|        CAACTTTAGGTTTAAGGAGTGGTGCTTTTACAGCTCGAGGTAATTCAAAC
gi|142747|gb|M23724.1|BACCRYIB     CAACTTTAGGGTTAAGGAGTGGTGCTTTTACAGCTCGCGGTAATTCAAAC
gi|237506878|gb|FJ493542.1|        CAACTTTAGGTTTAAGGAGTGGTGCTTTTACAGCTCGAGGTAATTCAAAC
                                   ********** ************************** ************
```

Figure 4.2.1: Multiple alignments of *cry*2 genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
gi|143083|gb|M30503.1|BACICPCS     TTGGGTAAACTTTAACCGTTATCGCAGAGAGATGACATTAACAGTATTAG
gi|142735|gb|M37207.1|BACCRYCA     TTGGGTAAACTTTAACCGTTATCGCAGAGAGATGACATTAACAGTATTAG
gi|40252|emb|Y00420.1|             TTGGGTAAACTTTAACCGTTATCGCAGAGAGATGACATTAACAGTATTAG
gi|58826237|gb|AY882576.1|         TTGGGTAAACTTTAACCGTTATCGCAGAGAGATGACATTAACAGTATTAG
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     ATTTAATTGCACTATTTCCATTGTATGATGTTCGGCTATACCCAAAAGAA
gi|142735|gb|M37207.1|BACCRYCA     ATTTAATTGCACTATTTCCATTGTATGATGTTCGGCTATACCCAAAAGAA
gi|40252|emb|Y00420.1|             ATTTAATTGCACTATTTCCATTGTATGATGTTCGGCTATACCCAAAAGAA
gi|58826237|gb|AY882576.1|         ATTTAATTGCACTATTTCCATTGTATGATGTTCGGCTATACCCAAAAGAA
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     GTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATTGTCGGAGT
gi|142735|gb|M37207.1|BACCRYCA     GTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATTGTCGGAGT
gi|40252|emb|Y00420.1|             GTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATTGTCGGAGT
gi|58826237|gb|AY882576.1|         GTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATTGTCGGAGT
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     CAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATA
gi|142735|gb|M37207.1|BACCRYCA     CAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATA
gi|40252|emb|Y00420.1|             CAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATA
gi|58826237|gb|AY882576.1|         CAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATA
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     TTCGAAAACCACATCTATTTGACTATCTGCATAGAATTCAATTTCACACG
gi|142735|gb|M37207.1|BACCRYCA     TTCGAAAACCACATCTATTTGACTATCTGCATAGAATTCAATTTCACACG
gi|40252|emb|Y00420.1|             TTCGAAAACCACATCTATTTGACTATCTGCATAGAATTCAATTTCACACG
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gi|58826237|gb|AY882576.1|         TTCGAAAACCACATCTATTTAACTATCTGCGTAGAATTCAATTTCACACG
                                   ********************.*********.*******************

gi|143083|gb|M30503.1|BACICPCS     CGGTTCCAACCAGGATATTATGGAAATGACTCTTTCAATTATTGGTCCGG
gi|142735|gb|M37207.1|BACCRYCA     CGGTTCCAACCAGGATATTATGGAAATGACTCTTTCAATTATTGGTCCGG
gi|40252|emb|Y00420.1|             CGGTTCCAACCAGGATATTATGGAAATGACTCTTTCAATTATTGGTCCGG
gi|58826237|gb|AY882576.1|         CGGTTCCAACCAGGATATTATGGAAATGACTCTTTCAATTATTGGTCCGG
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     TAATTATGTTTCAACTAGACCAAGCATAGGATCAAATGATATAATCACAT
gi|142735|gb|M37207.1|BACCRYCA     TAATTATGTTTCAACTAGACCAAGCATAGGATCAAATGATATAATCACAT
gi|40252|emb|Y00420.1|             TAATTATGTTTCAACTAGACCAAGCATAGGATCAAATGATATAATCACAT
gi|58826237|gb|AY882576.1|         TAATTATGTTTCAACTAGACCAAGCATAGGATCAAATGATATAATCACAT
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     CTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAATTTAGAATTT
gi|142735|gb|M37207.1|BACCRYCA     CTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAATTTAGAATTT
gi|40252|emb|Y00420.1|             CTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAATTTAGAATTT
gi|58826237|gb|AY882576.1|         CTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAATTTAGAATTT
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     AATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTG
gi|142735|gb|M37207.1|BACCRYCA     AATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTG
gi|40252|emb|Y00420.1|             AATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTG
gi|58826237|gb|AY882576.1|         AATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTG
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     GCCGTCCGCTGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATA
gi|142735|gb|M37207.1|BACCRYCA     GCCGTCCGCTGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATA
gi|40252|emb|Y00420.1|             GCCGTCCGCTGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATA
gi|58826237|gb|AY882576.1|         GCCGTCCGCTGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATA
                                   **************************************************

gi|143083|gb|M30503.1|BACICPCS     ATGATCAAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAAT
gi|142735|gb|M37207.1|BACCRYCA     ATGATCAAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAAT
gi|40252|emb|Y00420.1|             ATGATCAAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAAT
gi|58826237|gb|AY882576.1|         ATGATCAAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAAT
                                   **************************************************
```

Figure 4.2.2: Multiple alignments of *cry3* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
gi|3986085|dbj|AB011496.1|      TTTAATGAAATAAATAGCACAGAAGAAAATGGCTGGAAGGCAAGTAACGG
gi|120431603|gb|EF157307.1|     TTTAATGAAATAAATAGCACAGAAGAAAATGGCTGGAAGGCAAGTAACGG
gi|27413809|gb|AF358863.1|      TTTAATGAAATAAATAGCACAGAAGAAAATGGCTGGAAGGCAAGTAACGG
gi|190693067|gb|EU760456.1|     TTTAATGAAATAAATAGCACAGAAGAAAATGGCTGGAAGGCAAGTAACGG
                                **************************************************

gi|3986085|dbj|AB011496.1|      TGTTACTATTAGCGAGGGCGGTCCATTCTTTAAAGGTCGTGCACTTCAGT
gi|120431603|gb|EF157307.1|     TGTTACTATTAGCGAGGGCGGTCCATTCTTTAAAGGTCGTGCACTTCAGT
gi|27413809|gb|AF358863.1|      TGTTACTATTAGCGAGGGCGGTCCATTCTTTAAAGGTCGTGCACTTCAGT
gi|190693067|gb|EU760456.1|     TGTTACTATTAGCGAGGGCGGTCCATTCTTTAAAGGTCGTGCACTTCAGT
                                **************************************************

gi|3986085|dbj|AB011496.1|      TAGCAAGCGCACGTGAAAATTACCCAACATACATCTATCAAAAGGTAGAT
gi|120431603|gb|EF157307.1|     TAGCAAGCGCACGTGAAAATTACCCAACATACATCTATCAAAAGGTAGAT
gi|27413809|gb|AF358863.1|      TAGCAAGCGCACGTGAAAATTACCCAACATACATCTATCAAAAGGTAGAT
gi|190693067|gb|EU760456.1|     TAGCAAGCGCACGTGAAAATTACCCAACATACATCTATCAAAAGGTAGAT
                                **************************************************

gi|3986085|dbj|AB011496.1|      GCATCGACGTTAAAACCTTATACACGATATAAACTAGATGGATTTGTGCA
gi|120431603|gb|EF157307.1|     GCATCGACGTTAAAACCTTATACACGATATAAACTAGATGGATTTGTGCA
gi|27413809|gb|AF358863.1|      GCATCGACGTTAAAACCTTATACACGATATAAACTAGATGGATTTGTGCA
gi|190693067|gb|EU760456.1|     GCATCGACGTTAAAACCTTATACACGATATAAACTAGGTGGATTTGTGCA
                                ************************************* ************
```

```
gi|3986085|dbj|AB011496.1|      AAGTAGTCAAGATTTAGAAATTGACCTCATTCATCATCATAAAGTCCACC
gi|120431603|gb|EF157307.1|     AAGTAGTCAAGATTTAGAAATTGACCTCATTCATCATCATAAAGTCCACC
gi|27413809|gb|AF358863.1|      AAGTAGTCAAGATTTAGAAATTGACCTCATTCATCATCATAAAGTCCACC
gi|190693067|gb|EU760456.1|     AAGTAGTCAAGATTTAGAAATTGACCTCATTCATCATCATAAAGTCCACC
                                **************************************************

gi|3986085|dbj|AB011496.1|      TCGTGAAAAATGTACCAGATAATTTAGTATCTGATACTTATTCTGATGGC
gi|120431603|gb|EF157307.1|     TCGTGAAAAATGTACCAGATAATTTAGTATCTGATACTTATTCTGATGGC
gi|27413809|gb|AF358863.1|      TCGTGAAAAATGTACCAGATAATTTAGTATCTGATACTTATTCTGATGGC
gi|190693067|gb|EU760456.1|     TCGTGAAAAATGTACCAGATAATTTAGTATCTGGTACTTATTCTGATGGC
                                ********************************* ****************

gi|3986085|dbj|AB011496.1|      TCATGTAGTGGAATTAACCGTTGTGAGGAACAACATCAGGTAGATGTGCA
gi|120431603|gb|EF157307.1|     TCATGTAGTGGAATTAACCGTTGTGAGGAACAACATCAGGTAGATGTGCA
gi|27413809|gb|AF358863.1|      TCATGTAGTGGAATTAACCGTTGTGAGGAACAACATCAGGTAGATGTGCA
gi|190693067|gb|EU760456.1|     TCATGTAGTGGAATTAACCGTTGTGAGGAACAACATCAGGTAGATGTGCA
                                **************************************************

gi|3986085|dbj|AB011496.1|      GCTAGATGCGGAGGATCATCCAAAGGATTGTTGTGAAGCGGCTCAAACAC
gi|120431603|gb|EF157307.1|     GCTAGATGCGGAGGATCATCCAAAGGATTGTTGTGAAGCGGCTCAAACAC
gi|27413809|gb|AF358863.1|      GCTAGATGCGGAGGATCATCCAAAGGATTGTTGTGAAGCGGCTCAAACAC
gi|190693067|gb|EU760456.1|     GCTAGATGCGGAGGATCATCCAAAGGATTGTTGTGAAGCGGCTCAAACAC
                                **************************************************

gi|3986085|dbj|AB011496.1|      ATGAGTTTTCTTCCTATATTCATACAGGTGATCTAAATGCAAGTGTAGAT
gi|120431603|gb|EF157307.1|     ATGAGTTTTCTTCCTATATTCATACAGGTGATCTAAATGCAAGTGTAGAT
gi|27413809|gb|AF358863.1|      ATGAGTTTTCTTCCTATATTCATACAGGTGATCTAAATGCAAGTGTAGAT
gi|190693067|gb|EU760456.1|     ATGAGTTTTCTTCCTATATTCATACAGGTGATCTAAATGCAAGTGTAGAT
                                **************************************************

gi|3986085|dbj|AB011496.1|      CAAGGCATTTGGGTTGTATTGCAGGTTCGAACAACAGATGGTTATGCGAC
gi|120431603|gb|EF157307.1|     CAAGGCATTTGGGTTGTATTGCAGGTTCGAACAACAGATGGTTATGCGAC
gi|27413809|gb|AF358863.1|      CAAGGCATTTGGGTTGTATTGCAGGTTCGAACAACAGATGGTTATGCGAC
gi|190693067|gb|EU760456.1|     CAAGGCATTTGGGTTGTATTGCAGGTTCGAACAACAGATGGTTATGCGAC
                                **************************************************

gi|3986085|dbj|AB011496.1|      GTTAGGAAATCTTGAATTGGTAGAGGTTGGTCCATTATCGGGTGAATCTT
gi|120431603|gb|EF157307.1|     GTTAGGAAATCTTGAATTGGTAGAGGTTGGTCCATTATCGGGTGAATCTT
gi|27413809|gb|AF358863.1|      GTTAGGAAATCTTGAATTGGTAGAGGTTGGTCCATTATCGGGTGAATCTT
gi|190693067|gb|EU760456.1|     GTTAGGAAATCTTGAATTGGTAGAGGTTGGTCCATTATCGGGTGAATCTT
                                **************************************************
```

Figure 4.2.3: Multiple alignments of *cry9* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
cry1Aa_embY09663.1       CTAACACTTACTGTATTAGATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTAT
cry1Ac_gbAY730621.1      TTAACACTAACTGTATTAGATATCGTTGCTCTGTTCCCGAATTATGATAGTAGAAGATAT
cry1Aa_emb               CTAACACTTACTGTATTAGATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTAT
cry1Aa_dbjD00348.1       CTAACACTTACTGTATTAGATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTAT
cry1Ab_gbJN226102.1      TTAACACTAACTGTATTAGATATCGTTTCTCTATTTCCGAACTATGATAGTAGAACGTAT
cry1Ab_gbDQ241675.1      TTAACACTAACTGTATTAGATATCGTTTCTCTATTTCCGAACTATGATAGTAGAACGTAT
cry1Aa_dbjAB026261_1     CTAACACTTACTGTATTAGATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTAT
cry1Ac_gbU87793.1        TTAACACTAACTGTATTAGATATCGTTGCTCTGTTCCCGAATTATGATAGTAGAAGATAT
                          ******* ****************** **** **  * ** ********* ***  ***

cry1Aa_embY09663.1       CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACGAACCCAGTATTAGAAAAT
cry1Ac_gbAY730621.1      CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAAT
cry1Aa_emb               CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACGAACCCAGTATTAGAAAAT
cry1Aa_dbjD00348.1       CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACGAACCCAGTATTAGAAAAT
cry1Ab_gbJN226102.1      CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAAT
cry1Ab_gbDQ241675.1      CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAAT
cry1Aa_dbjAB026261_1     CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACGAACCCAGTATTAGAAAAT
cry1Ac_gbU87793.1        CCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAAT
                         ***************************************** ******************

cry1Aa_embY09663.1       TTTGATGGTAGTTTTCGTGGAATGGCTCAGAGAATAGAACAGAATATTAGGCAACCACAT
```

```
cry1Ac_gbAY730621.1       TTTGATGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAAGAAGTATTAGGAGTCCACAT
cry1Aa_emb                TTTGATGGTAGTTTTCGTGGAATGGCTCAGAGAATAGAACAGAATATTAGGCAACCACAT
cry1Aa_dbjD00348.1        TTTGATGGTAGTTTTCGTGGAATGGCTCAGAGAATAGAACAGAATATTAGGCAACCACAT
cry1Ab_gbJN226102.1       TTTGATGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAGGAAGTATTAGGAGTCCACAT
cry1Ab_gbDQ241675.1       TTTGATGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAGGAAGTATTAGGAGTCCACAT
cry1Aa_dbjAB026261_1      TTTGATGGTAGTTTTCGTGGAATGGCTCAGAGAATAGAACAGAATATTAGGCAACCACAT
cry1Ac_gbU87793.1         TTTGATGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAAGAAGTATTAGGAGTCCACAT
                          ***************** **   ******* * ******   * *******   ******

cry1Aa_embY09663.1        CTTATGGATATCCTTAATAGTATAACCATTTATACTGATGTGCATAGAGGCTTTAATTAT
cry1Ac_gbAY730621.1       TTGATGGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGGGTTATTATTAT
cry1Aa_emb                CTTATGGATATCCTTAATAGTATAACCATTTATACTGATGTGCATAGAGGCTTTAATTAT
cry1Aa_dbjD00348.1        CTTATGGATATCCTTAATAGTATAACCATTTATACTGATGTGCATAGAGGCTTTAATTAT
cry1Ab_gbJN226102.1       TTGATGGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGAGGAGAATATTAT
cry1Ab_gbDQ241675.1       TTGATGGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGAGGAGAATATTAT
cry1Aa_dbjAB026261_1      CTTATGGATATCCTTAATAGGATAACCATTTATACTGATGTGCATAGAGGCTTTAATTAT
cry1Ac_gbU87793.1         TTGATGGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGGGTTATTATTAT
                           * ******** ***** ** ******** ***** ****  ***** **     *****

cry1Aa_embY09663.1        TGGTCAGGGCATCAAATAACAGCTTCTCCTGTAGGGTTTTCAGGACCAGAATTCGCATTC
cry1Ac_gbAY730621.1       TGGTCAGGGCATCAAATAATGGCTTCTCCTGTAGGGTTTTCGGGGCCAGAATTCACTTTT
cry1Aa_emb                TGGTCAGGGCATCAAATAACAGCTTCTCCTGTAGGGTTTTCAGGACCAGAATTCGCATTC
cry1Aa_dbjD00348.1        TGGTCAGGGCATCAAATAACAGCTTCTCCTGTAGGGTTTTCAGGACCAGAATTCGCATTC
cry1Ab_gbJN226102.1       TGGTCAGGGCATCAAATAATGGCTTCTCCTGTAGGGTTTTCGGGGCCAGAATTCACTTTT
cry1Ab_gbDQ241675.1       TGGTCAGGGCATCAAATAATGGCTTCTCCTGTAGGGTTTTCGGGGCCAGAATTCACTTTT
cry1Aa_dbjAB026261_1      TGGTCAGGGCATCAAATAACAGCTTCTCCTGTAGGGTTTTCAGGACCAGAATTCGCATTC
cry1Ac_gbU87793.1         TGGTCAGGGCATCAAATAATGGCTTCTCCTGTCGGTTTTTCGGGGCCAGAATTCACGTTT
                          *******************  *********** ** ***** ** ********* * **

cry1Aa_embY09663.1        CCTTTATTTGGGAATGCGGGGAATGCAGCTCCACC----CGTACTTGTCTCATTAACTGG
cry1Ac_gbAY730621.1       CCGCTATATGGAACTATGGGAAATGCAGCTCCACAACAACGTA-TTGTTGC-TCAACTAG
cry1Aa_emb                CCTTTATTTGGGAATGCGGGGAATGCAGCTCCACC----CGTACTTGTCTCATTAACTGG
cry1Aa_dbjD00348.1        CCTTTATTTGGGAATGCGGGGAATGCAGCTCCACC----CGTACTTGTCTCATTAACTGG
cry1Ab_gbJN226102.1       CCGCTATATGGAACTATGGGAAATGCAGCTCCACAACAACGTA-TTGTTGC-TCAACTAG
cry1Ab_gbDQ241675.1       CCGCTATATGGAACTATGGGAAATGCAGCTCCACAACAACGTA-TTGTTGC-TCAACTAG
cry1Aa_dbjAB026261_1      CCTTTATTTGGGAATGCGGGGAATGCAGCTCCACC----CGTACTTGTCTCATTAACTGG
cry1Ac_gbU87793.1         CCGCTATATGGAACCATGGGAAATGCAGCTCCACAACAACGTA-TTGTTGC-TCAACTAG
                          **  *** *** *    *** *************     **** ****  * * **** *

cry1Aa_embY09663.1        TTTGGGG-ATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATACTTGGTTCAG
cry1Ac_gbAY730621.1       GTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT---TTTAATATAG
cry1Aa_emb                TTTGGGG-ATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATACTTGGTTCAG
cry1Aa_dbjD00348.1        TTTGGGG-ATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATACTTGGTTCAG
cry1Ab_gbJN226102.1       GTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT---TTTAATATAG
cry1Ab_gbDQ241675.1       GTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT---TTTAATATAG
cry1Aa_dbjAB026261_1      TTTGGGG-ATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATACTTGGTTCAG
cry1Ac_gbU87793.1         GTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT---TTTAATATAG
                           *  ***  * * ************ **  **************  *    **  *  **

cry1Aa_embY09663.1        GCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG---CCTCCC
cry1Ac_gbAY730621.1       GGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCT
cry1Aa_emb                GCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG---CCTCCC
cry1Aa_dbjD00348.1        GCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG---CCTCCC
cry1Ab_gbJN226102.1       GGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCT
cry1Ab_gbDQ241675.1       GGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCT
cry1Aa_dbjAB026261_1      GCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG---CCTCCC
cry1Ac_gbU87793.1         GGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCT
                          *   *********  **** * *** ***** ** ** ** *** *** **   *****

cry1Aa_embY09663.1        TAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCACTAGATG
cry1Ac_gbAY730621.1       CAA------ATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGCTGGATG
cry1Aa_emb                TAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCACTAGATG
cry1Aa_dbjD00348.1        TAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCACTAGATG
cry1Ab_gbJN226102.1       CAA------ATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGCTGGATG
cry1Ab_gbDQ241675.1       CAA------ATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGCTGGATG
cry1Aa_dbjAB026261_1      TAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCACTAGATG
cry1Ac_gbU87793.1         CAA------ATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGCTGGATG
                           **      * ***** *** ** **** *** **** ** ** ** ***** ** ****

cry1Aa_embY09663.1        TAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGATTGAGTC
cry1Ac_gbAY730621.1       AAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGATTAAGCC
cry1Aa_emb                TAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGATTGAGTC
cry1Aa_dbjD00348.1        TAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGATTGAGTC
cry1Ab_gbJN226102.1       AAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGATTAAGCC
cry1Ab_gbDQ241675.1       AAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGATTAAGCC
cry1Aa_dbjAB026261_1      TAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGATTGAGTC
cry1Ac_gbU87793.1         AAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGATTAAGCC
```

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                              ************* **** *  ** ****** *    ******** ******** ** *

cry1Aa_embY09663.1            ATGTTACAATGCTGAGC-CAAGC---AGCTGGAGCAGTTTACACCT--TGAGAGCTCCAA
cry1Ac_gbAY730621.1           ATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAGCTCCTA
cry1Aa_emb                    ATGTTACAATGCTGAGC-CAAGC---AGCTGGAGCAGTTTACACCT--TGAGAGCTCCAA
cry1Aa_dbjD00348.1            ATGTTACAATGCTGAGC-CAAGC---AGCTGGAGCAGTTTACACCT--TGAGAGCTCCAA
cry1Ab_gbJN226102.1           ATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAGCTCCTA
cry1Ab_gbDQ241675.1           ATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAGCTCCTA
cry1Aa_dbjAB026261_1          ATGTTACAATGCTGAGC-CAAGC---AGCTGGAGCAGTTTACACCT--TGAGAGCTCCAA
cry1Ac_gbU87793.1             ATGTTTCAATGTITCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAGCTCCTA
                              ***** ***** *  *  ** **   **    ** *** **    *  * ******** *
```

Figure 4.2.4: Multiple alignments of *cry1Aa* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
Cry1Ac_gbGU446674.1           ATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTA
gi|256003037|gb|FJ617446.1|   ATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTA
cry1Ac_gbAY730621.1           ATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTA
cry1Ac_gbEF094884.1           ATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTA
cry1Ac_gbAY225453.1           ATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTA
                              **************************************************

Cry1Ac_gbGU446674.1           ATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGA
gi|256003037|gb|FJ617446.1|   ATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGA
cry1Ac_gbAY730621.1           ATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGA
cry1Ac_gbEF094884.1           ATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGA
cry1Ac_gbAY225453.1           ATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGA
                              **************************************************

Cry1Ac_gbGU446674.1           TTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTTCC
gi|256003037|gb|FJ617446.1|   TTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTTCC
cry1Ac_gbAY730621.1           TTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTTCC
cry1Ac_gbEF094884.1           TTAAATAGTAGTGGAAATAACTTTCAGAATAGAGGGTATATTGAAGTTCC
cry1Ac_gbAY225453.1           TTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTTCC
                              *********************:****************************

Cry1Ac_gbGU446674.1           AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATG
gi|256003037|gb|FJ617446.1|   AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATG
cry1Ac_gbAY730621.1           AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATG
cry1Ac_gbEF094884.1           AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATG
cry1Ac_gbAY225453.1           AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATG
                              **************************************************

Cry1Ac_gbGU446674.1           CTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATT
gi|256003037|gb|FJ617446.1|   CTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATT
cry1Ac_gbAY730621.1           CTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATT
cry1Ac_gbEF094884.1           CTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATT
cry1Ac_gbAY225453.1           CTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATT
                              **************************************************

Cry1Ac_gbGU446674.1           TTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGATAATCTACAATC
gi|256003037|gb|FJ617446.1|   TTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGATAATCTACAATC
cry1Ac_gbAY730621.1           TTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGATAATCTACAATC
cry1Ac_gbEF094884.1           TTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGATAATCTACAATC
cry1Ac_gbAY225453.1           TTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGATAATCTACAATC
                              **************************************************

Cry1Ac_gbGU446674.1           AAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCATTAG
gi|256003037|gb|FJ617446.1|   AAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCATTAG
cry1Ac_gbAY730621.1           AAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCATTAG
cry1Ac_gbEF094884.1           AAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCATTAG
cry1Ac_gbAY225453.1           AAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCATTAG
                              **************************************************

Cry1Ac_gbGU446674.1           GTAATATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATA
gi|256003037|gb|FJ617446.1|   GTAATATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATA
cry1Ac_gbAY730621.1           GTAATATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATA
```

```
cry1Ac_gbEF094884.1          GTAATATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATA
cry1Ac_gbAY225453.1          GTAATATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATA
                             **************************************************
```

Figure 4.2.5: Multiple alignments of *cry1Ac* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
gi|14486713|gb|AF368257.1|     GACGTATTTATATCAAAAAATAGGGGAGTCGGAATTAAAAGCTTATACTC
gi|40264|emb|X06711.1|         GACGTATTTATATCAAAAAATAGGGGAGTCGGAATTAAAAGCTTATACTC
gi|643193386|gb|KJ868173.1|    GACGTATTTATATCAAAAAATAGGGGAGTCGGAATTAAAAGCTTATACTC
gi|13959050|gb|AF363025.1|     GACGTATTTATATCAAAAAATAGGGGAGTCGGAATTAAAAGCTTATACTC
                               **************************************************

gi|14486713|gb|AF368257.1|     GCTACCAATTAAGAGGTTATATTGAAGATAGTCAAGATTTAGAGATATAT
gi|40264|emb|X06711.1|         GCTACCAATTAAGAGGTTATATTGAAGATAGTCAAGATTTAGAGATATAT
gi|643193386|gb|KJ868173.1|    GCTACCAATTAAGAGGTTATATTGAAGATAGTCAAGATTTAGAGATATAT
gi|13959050|gb|AF363025.1|     GCTACCAATTAAGAGGTTATATTGAAGATAGTCAAGATTTAGAGATATAT
                               **************************************************

gi|14486713|gb|AF368257.1|     TTGATTCGTTATAATGCGAAACATGAAACATTGGATGTTCCAGGTACCGA
gi|40264|emb|X06711.1|         TTGATTCGTTATAATGCGAAACATGAAACATTGGATGTTCCAGGTACCGA
gi|643193386|gb|KJ868173.1|    TTGATTCGTTATAATGCGAAACATGAAACATTGGATGTTCCAGGTACCGA
gi|13959050|gb|AF363025.1|     TTGATTCGTTATAATGCGAAACATGAAACATTGGATGTTCCAGGTACCGA
                               **************************************************

gi|14486713|gb|AF368257.1|     GTCCCTATGGCCGCTTTCAGTTGAAAGCCCAATCGGAAGGTGCGGAGAAC
gi|40264|emb|X06711.1|         GTCCCTATGGCCGCTTTCAGTTGAAAGCCCAATCGGAAGGTGCGGAGAAC
gi|643193386|gb|KJ868173.1|    GTCCCTATGGCCGCTTTCAGTTGAAAGCCCAATCGGAAGGTGCGGAGAAC
gi|13959050|gb|AF363025.1|     GTCCCTATGGCCGCTTTCAGTTGAAAGCCCAATCGGAAGGTGCGGAGAAC
                               **************************************************

gi|14486713|gb|AF368257.1|     CGAATCGATGCGCACCACATTTTGAATGGAATCCTGATCTAGATTGTTCC
gi|40264|emb|X06711.1|         CGAATCGATGCGCACCACATTTTGAATGGAATCCTGATCTAGATTGTTCC
gi|643193386|gb|KJ868173.1|    CGAATCGATGCGCACCACATTTTGAATGGAATCCTGATCTAGATTGTTCC
gi|13959050|gb|AF363025.1|     CGAATCGATGCGCACCACATTTTGAATGGAATCCTGATCTAGATTGTTCC
                               **************************************************

gi|14486713|gb|AF368257.1|     TGCAGAGATGGAGAAAAATGTGCGCATCATTCCCATCATTTCTCTTTGGA
gi|40264|emb|X06711.1|         TGCAGAGATGGAGAAAAATGTGCGCATCATTCCCATCATTTCTCTTTGGA
gi|643193386|gb|KJ868173.1|    TGCAGAGATGGAGAAAAATGTGCGCATCATTCCCATCATTTCTCTTTGGA
gi|13959050|gb|AF363025.1|     TGCAGAGATGGAGAAAAATGTGCGCATCATTCCCATCATTTCTCTTTGGA
                               **************************************************

gi|14486713|gb|AF368257.1|     TATTGATGTTGGATGCACAGACTTGCATGAGAATCTAGGCGTGTGGGTGG
gi|40264|emb|X06711.1|         TATTGATGTTGGATGCACAGACTTGCATGAGAATCTAGGCGTGTGGGTGG
gi|643193386|gb|KJ868173.1|    TATTGATGTTGGATGCACAGACTTGCATGAGAATCTAGGCGTGTGGGTGG
gi|13959050|gb|AF363025.1|     TATTGATGTTGGATGCACAGACTTGCATGAGAATCTAGGCGTGTGGGTGG
                               **************************************************

gi|14486713|gb|AF368257.1|     TATTCAAGATTAAGACGCAGGAAGGTCATGCAAGACTAGGGAATCTGGAA
gi|40264|emb|X06711.1|         TATTCAAGATTAAGACGCAGGAAGGTCATGCAAGACTAGGGAATCTGGAA
gi|643193386|gb|KJ868173.1|    TATTCAAGATTAAGACGCAGGAAGGTCATGCAAGACTAGGGAATCTGGAA
gi|13959050|gb|AF363025.1|     TATTCAAGATTAAGACGCAGGAAGGTCATGCAAGACTAGGGAATCTGGAA
                               **************************************************

gi|14486713|gb|AF368257.1|     TTTATTGAAGAGAAACCATTATTAGGAGAAGCACTGTCTCGTGTGAAGAG
gi|40264|emb|X06711.1|         TTTATTGAAGAGAAACCATTATTAGGAGAAGCACTGTCTCGTGTGAAGAG
gi|643193386|gb|KJ868173.1|    TTTATTGAAGAGAAACCATTATTAGGAGAAGCACTGTCTCGTGTGAAGAG
gi|13959050|gb|AF363025.1|     TTTATTGAAGAGAAACCATTATTAGGAGAAGCACTGTCTCGTGTGAAGAG
                               **************************************************

gi|14486713|gb|AF368257.1|     GGCAGAGAAAAAATGGAGAGACAAACGTGAAAAACTACAATTGGAAACAA
gi|40264|emb|X06711.1|         GGCAGAGAAAAAATGGAGAGACAAACGTGAAAAACTACAATTGGAAACAA
gi|643193386|gb|KJ868173.1|    GGCAGAGAAAAAATGGAGAGACAAACGTGAAAAACTACAATTGGAAACAA
gi|13959050|gb|AF363025.1|     GGCAGAGAAAAAATGGAGAGACAAACGTGAAAAACTACAATTGGAAACAA
                               **************************************************

gi|14486713|gb|AF368257.1|     AACGAGTATATACAGAGGCAAAAGAAGCTGTGGATGCTTTATTCGTAGAT
```

```
gi|40264|emb|X06711.1|          AACGAGTATATACAGAGGCAAAAGAAGCTGTGGATGCTTTATTCGTAGAT
gi|643193386|gb|KJ868173.1|     AACGAGTATATACAGAGGCAAAAGAAGCTGTGGATGCTTTATTCGTAGAT
gi|13959050|gb|AF363025.1|      AACGAGTATATACAGAGGCAAAAGAAGCTGTGGATGCTTTATTCGTAGAT
                                **************************************************
```

Figure 4.2.6: Multiple alignments of *cry1Ba* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

```
gi|19880134|gb|AF362020.1|      ATTACAGGACCAGGATTTACAGGAGGGGATATCCTTCGAAGAAATACCTT
gi|37540180|gb|AY015492.1|      ATTACAGGACCAGGATTTACAGGAGGGGATATCCTTCGAAGAAATACCTT
cry1Ca_AY955268.1               ATTACAGGACCAGGATTTACAGGAGGGGATATCCTTCGAAGAAATACCTT
                                **************************************************

gi|19880134|gb|AF362020.1|      TGGTGATTTTGTATCTCTACAAGTCAATATTAATTCACCAATTACCCAAA
gi|37540180|gb|AY015492.1|      TGGTGATTTTGTATCTCTACAAGTCAATATTAATTCACCAATTACCCAAA
cry1Ca_AY955268.1               TGGTGATTTTGTATCTCTACAAGTCAATATTAATTCACCAATTACCCAAA
                                **************************************************

gi|19880134|gb|AF362020.1|      GATACCGTTTAAGATTTCGTTACGCTTCCAGTAGGGATGCACGAGTTATA
gi|37540180|gb|AY015492.1|      GATACCGTTTAAGATTTCGTTACGCTTCCAGTAGGGATGCACGAGTTATA
cry1Ca_AY955268.1               GATACCGTTTAAGATTTCGTTACGCTTCCAGTAGGGATGCACGAGTTATA
                                **************************************************

gi|19880134|gb|AF362020.1|      GTATTAACAGGAGCGGCATCCACAGGAGTGGGAGGCCAAGTTAGTGTAAA
gi|37540180|gb|AY015492.1|      GTATTAACAGGAGCGGCATCCACAGGAGTGGGAGGCCAAGTTAGTGTAAA
cry1Ca_AY955268.1               GTATTAACAGGAGCGGCATCCACAGGAGTGGGAGGCCAAGTTAGTGTAAA
                                **************************************************

gi|19880134|gb|AF362020.1|      TATGCCTCTTCAGAAAACTATGGAAATAGGGGAGAACTTAACATCTAGAA
gi|37540180|gb|AY015492.1|      TATGCCTCTTCAGAAAACTATGGAAATAGGGGAGAACTTAACATCTAGAA
cry1Ca_AY955268.1               TATGCCTCTTCAGAAAACTATGGAAATAGGGGAGAACTTAACATCTAGAA
                                **************************************************

gi|19880134|gb|AF362020.1|      CATTTAGATATACCGATTTTAGTAATCCTTTTTCATTTAGAGCTAATCCA
gi|37540180|gb|AY015492.1|      CATTTAGATATACCGATTTTAGTAATCCTTTTTCATTTAGAGCTAATCCA
cry1Ca_AY955268.1               CATTTAGATATACCGATTTTAGTAATCCTTTTTCATTTAGAGCTAATCCA
                                **************************************************

gi|19880134|gb|AF362020.1|      GATATAATTGGGATAAGTGAACAACCTCTATTTGGTGCAGGTTCTATTAG
gi|37540180|gb|AY015492.1|      GATATAATTGGGATAAGTGAACAACCTCTATTTGGTGCAGGTTCTATTAG
cry1Ca_AY955268.1               GATATAATTGGGATAAGTGAACAACCTCTATTTGGTGCAGGTTCTATTAG
                                **************************************************

gi|19880134|gb|AF362020.1|      TAGCGGTGAACTTTATATAGATAAAATTGAAATTATTCTAGCAGATGCAA
gi|37540180|gb|AY015492.1|      TAGCGGTGAACTTTATATAGATAAAATTGAAATTATTCTAGCAGATGCAA
cry1Ca_AY955268.1               TAGCGGTGAACTTTATATAGATAAAATTGAAATTATTCTAGCAGATGCAA
                                **************************************************

gi|19880134|gb|AF362020.1|      CATTTGAAGCAGAATCTGATTTAGAAAGAGCACAAAAGGCGGTGAATGCC
gi|37540180|gb|AY015492.1|      CATTTGAAGCAGAATCTGATTTAGAAAGAGCACAAAAGGCGGTGAATGCC
cry1Ca_AY955268.1               CATTTGAAGCAGAATCTGATTTAGAAAGAGCACAAAAGGCGGTGAATGCC
                                **************************************************
```

Figure 4.2.7: Multiple alignments of *cry1Ca* genes using ClustalW. The symbol "*" below each column indicates the conserved sequence. The Forward and Reverse primers designed were shown in italic characters marked with grey color indicating the binding site of the primers.

4.2.4 Optimization of PCR conditions:

The PCR conditions i.e. the annealing temperature and Mg^{2+} concentration for the detection of the *cry* genes were optimized. Optimum annealing temperature was determined by performing PCR in a Gradient Thermal Cycler where the annealing temperature was varied from T_m to T_m-5°C keeping all other parameters constant. Temperature at which PCR products of expected sizes were generated, were used as optimum annealing temperatures (Table 4.2.4). Similarly, the optimum concentration of Mg^{2+} was determined by varying in the range of 1.5 mM to 3.0 mM.

Table 4.2.4: Properties of primers designed to dectect *cry* genes.

Primers	Sequence	Position	Annealing temperature	Product size
cry2-F	TGGGGAATTTCAGCAGCAACAT	592-613	55°C	639
cry2-R	AGCTGTAAAAGCACCACTCCTT	1212-1233		
cry3-F	AACCGTTATCGCAGAGAGATG	1359-1379	51°C	525
cry3-R	GTCGTACGTTTGTGTACTTGC	1863-1883		
cry9-F	TGTTACTATTAGCGAGGGCGG	2451-2471	54°C	492
cry9-R	CCCGATAATGGACCAACCTCT	2922-2942		
cry1Aa-F	ATCCAATTCGAACAGTTTCCCA	764-785	55 °C	557
cry1Aa-R	TGCTTGGCTCAGCATTGTAA	1301-1320		
cry1Ac-F	CGGATAGTATTACTCAAATCCCTGC	1403-1427	55 °C	394
cry1Ac-R	ATCACTCCTGCAGTCCCACTA	1776-1796		
cry1Ba-F	TACTCGCTACCAATTAAGAGGTTAT	2376-2400	52 °C	499
cry1Ba-R	GAATAAAGCATCCACAGCTTCT	2853-2874		
cry1Ca-F	CCAGGATTTACAGGAGGGGAT	1539-1559	55 °C	434
cry1Ca-R	ACCGCCTTTTGTGCTCTTTC	1953-1972		

4.2.5 Detection of *cry* genes by PCR

DNA templates prepared from *Bt* strains following the method described in section 3.2.2.8, were mixed with PCR reaction mixture containing 0.2mM dNTPs, 0.5µM of each primer, 1x PCR buffer and 0.5 u of *Taq* DNA polymerase per µl in a reaction volume of 25µl and amplification was performed in a DNA thermal cycler. For all primer sets, PCR was carried out with an initial single denaturation step at 95°C for 2 min and 30 amplification cycles including denaturation at 95°C for 45 s, annealing at temperatures specific for each primer sets (Table 4.2.3 and 4.2.4) for 45 s and extension at 72°C for 60s. Finally an extra extension step was applied at 72°C for 10 min. PCR products (10 µl) were then analyzed by horizontal agarose gel electrophoresis as described in section 3.2.2.10.

4.2.6 Analysis of Cry protein profile

The Cry proteins partially purified from the *Bt* strains were analysed by SDS-PAGE (Öztürk *et al.*, 2009) in a 10% separating gel (Sambrook *et al.*, 1989). Colonies formed on T_3-agar medium upon incubation at 30^oC for 7 days were scrapped off and resuspended in cold sterile de-ionized water. Washing was performed twice with cold sterile de-ionized water to remove exotoxins, once with 1.0 M NaCl containing 5.0 mM EDTA and finally with 5.0 mM EDTA alone. The pellet then resuspended in 1× Laemmli buffer (Appendix C) lacking Bromophenol blue was incubated at boiling temperature for 5 min and the supernatant was collected by centrifugation for 5min at 10,000 rpm (Appendix D). Protein concentration in the supernatant was estimated by Bradford, 1976 method prior to SDS-PAGE analysis to ensure equal amount of proteins loaded in each lane. Upon the completion of electrophoresis, gel was stained in staining solution (0.02% Coomassie Brilliant Blue- G250 in 2% (w/v) phosphoric acid, 5% aluminum sulfate and 10% ethanol) (Kang *et al.*, 2002) for 2 hour. The molecular weight of the proteins was determined with the help of Alphaview SA software.

4.3 Results

4.3.1 Characterization of indigenous *Bt* strains with *cry* gene content

Bt strains belonging to the different biotypes and describing the subspecies of interests, were checked for the presence of the genes as correlated from Table 4.2.1 and Table 4.2.2. Thus, the strains belonging to the biotypes describing *Bt* subspecies *thuringiensis, kurstaki, galleriae, sotto, dendrolimus, morrisoni* were checked for the presence of *cry1* gene, *kurstaki* and *sotto* for *cry2* gene, *kurstaki* and *morrisoni* for *cry3* gene, *israelensis* for *cry4*, *cry10* and *cry11* genes, *galleriae* for *cry8* gene and *kurstaki* for *cry9 gene*. But many subspecies like *aizawai*, *tolworthi*, *tenebrionis*, *japonensis* etc containing *cry1*, *cry3*, *cry8*, *cry9* etc genes could not be traced by biotyping, hence incompatible to the criterion. So, all these genes were also searched in many randomly chosen *Bt* strains which was another criterion. And *cry2*, *cry3* and *cry9* genes were searched in all *cry1* positive *Bt* strains too. Thus the number of indigenous *Bt* strains checked were, 224 for *cry1* gene, 94 for *cry2*, 81 for *cry3*, 69 for *cry9*, 43 for *cry4,* 48 for *cry8,* 42 for *cry10* and 54 for *cry11* genes.

Both expected and spurious bands were observed in agarose gel electrophoresis of the amplicons. Desired amplicons of about 277 bp, 639 bp, 525 bp, 459 bp, 376 bp, 492 bp and 348 bp were obtained for *cry1*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9* and *cry10* genes respectively. Thus the *Bt* strains with showing the presence of desired amplicons were considered as positive for the respective genes. Spurious products are actually generated if mismatch in priming occurs which enhances the chance of getting putative novel *cry* genes as reported in many studies (Jouzani *et al.*, 2008; Bozlağan *et al.*, 2010). Spurious products of different sizes were ignored throughout the study but if any strain was observed with spurious products beside the expected amplicon, it was not ignored rather considered as positive.

4.3.1.1 Analysis of *cry1* gene prevalence

Universal primer sets for *cry1* gene detection (Table 4.2.3) were used for which the expected product size was 277 bp. In 69 *Bt* strains out of 236, amplicons of 277 bp was observed (Fig 4.3.1A), hence considered positive for *cry1* gene. Biotype describing *kurstaki* was found to be most abundant with *cry1* gene as about 78% of the strains were positive for this gene.

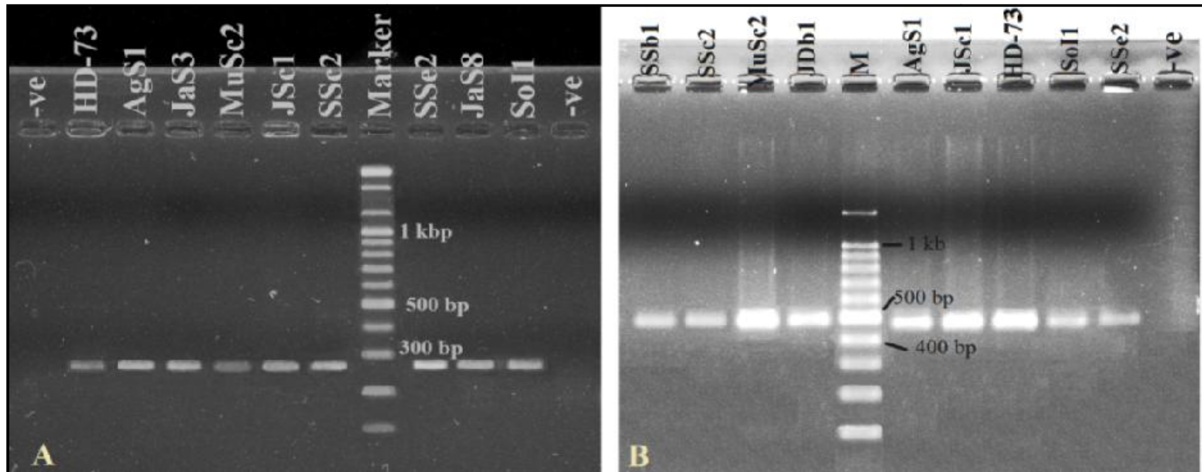

Figure 4.3.1: Presence of *cry* genes was detected by agarose gel electrophoresis of the PCR products yielding amplicons of expected sizes in the indigenous *Bt* strains. A) *cry1* gene with 277 bp amplicons. B) *cry1A* gene with 490 bp amplicons. (Name of *Bt* strains are as per labeled over the lanes; M: 100 bp Marker)

Lepidoptera specific *cry1A*-type gene was searched in *cry1* positive 69 *Bt* strains and 25 of them were found with specific amplicon of 490 bp, hence considered positive (Fig 4.3.1B). This *cry1A*-type gene was found to be the most prevalent (56.25%) in the *Bt* strains of biotype *kurstaki*.

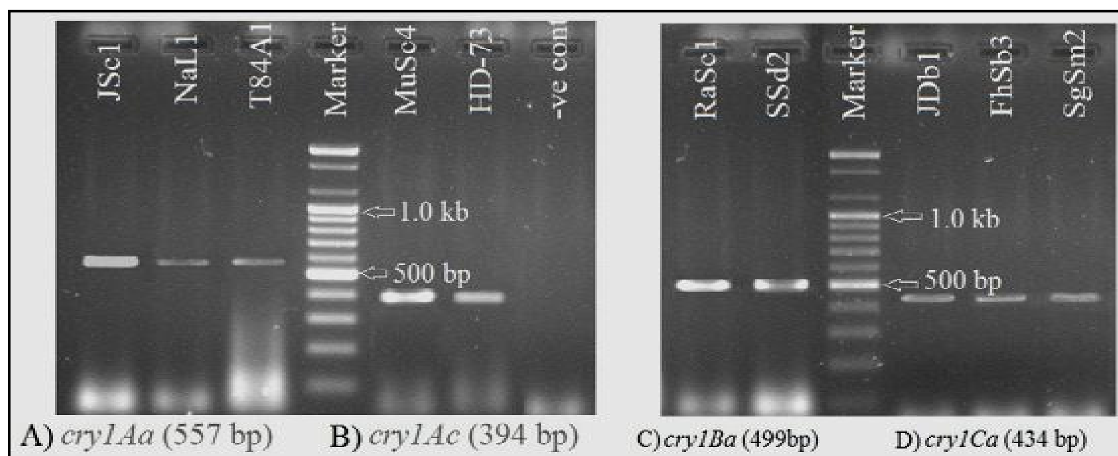

Figure 4.3.2: Detection of *cry1* genes in quaternary ranks as the molecular weight of the amplicons matched the expected size as determined by Agarose gel electrophoresis comparing with the DNA marker. (Name of *Bt* strains are as per labeled over the lanes)

As *cry1Aa* and *cry1Ac* genes are the subgroups of *cry1A*-type genes, 25 out of 69 *Bt* strains were investigated for their presence. But *cry1Ba* and *cry1Ca* were searched in all 69 *Bt*

strains showing positive for *cry1* gene. Out of 25 *cry1A* positive *Bt* strains, fifteen *cry1Aa* (557 bp) and four *cry1Ac* (394 bp) gene positive strains were identified whereas six *cry1Ba* (499 bp) and 15 *cry1Ca* (434 bp) *Bt* strains were identified out of 69 *cry1* positive strains (Fig 4.3.2). *cry1Aa*, *cry1Ac* and *cry1Ca* were prevalent in the *Bt* strains from biotype *kurstaki*.

4.3.1.2 Analysis of *cry2* gene prevalence

The expected product size from the conserved region of *cry2* gene for its detection was 639bp (Table 4.2.4) and twenty four (24) *Bt* strains out of 94, were observed with the presence of amplicons of the desired size as determined by agarose gel electrophoresis (Fig 4.3.3). Thess strains were hence considered positive for *cry2* gene which was 25.5% of the tested strains.

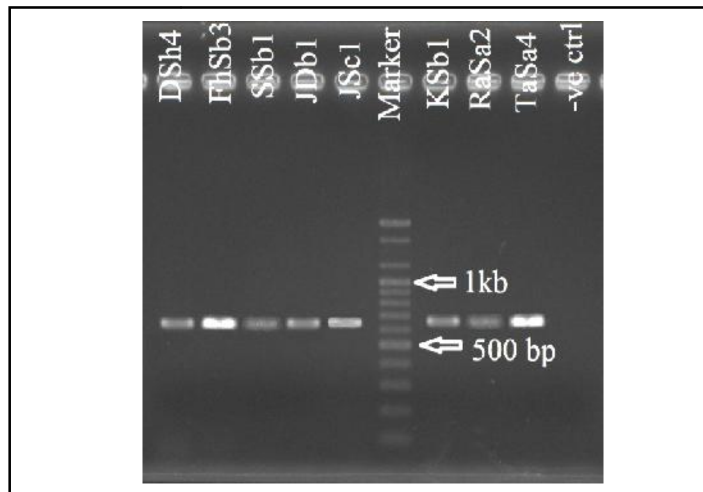

Figure 4.3.3: Amplicon of about 640 bp was revealed upon agarose gel electrophoresis of the PCR products obtained from *cry2* gene identification by PCR.

4.3.1.3 Analysis of *cry3* gene prevalence

For *cry3* gene, the targeted conserved region by the forward and reverse primers was 525 bp. Total DNA from 81 *Bt* strains were used as template in PCR and 18 of them produced amplicons of desired size as nine of them were presented in the agarose gel image (Fig 4.3.4). The prevalence of *cry3* gene was estimated to be 22.2% of the test strains.

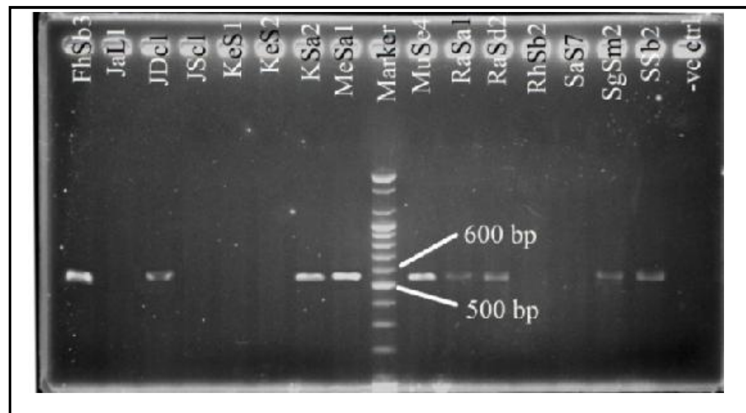

Figure 4.3.4: Agarose gel electrophoresis of the PCR products revealed the presence of *cry3* gene as the amplicons matched the expected target size.

4.3.1.4 Analysis of *cry4A* gene prevalence

The reported primer sets for *cry4A* gene detection (Jouzani *et al.*, 2008) was used for which the amplicon size should be 459 bp (Table 4.2.3). Only 2 *Bt* strains from the biotype describing subspecies *israelensis* out of 43 strains (other biotypes included) were found with the PCR products of desired size (Fig 4.3.5). Even spurious products were not observed in this case.

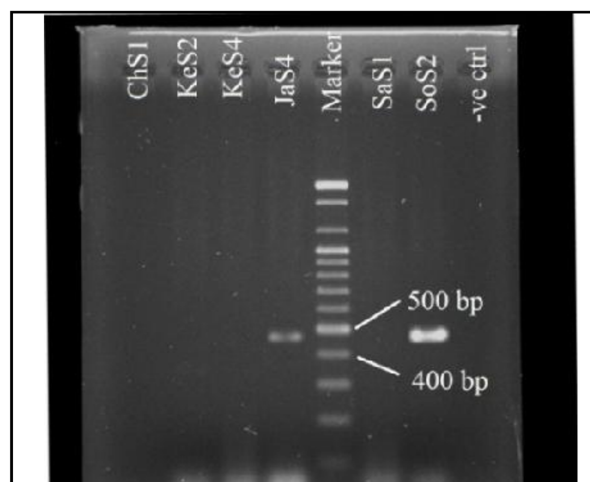

Figure 4.3.5: Detection of *cry4A* gene in the indigenous *Bt* strains by the presence of desired amplicon of 459 bp upon agarose gel electrophoresis of the PCR products.

4.3.1.5 Analysis of *cry8, cry10* and *cry11* gene prevalence

Total 48 *Bt* strains from multiple biotypes were investigated for *cry8* gene with the primers reported by Bravo (Bravo *et al.*, 1998). Amplicon of 376 bp was expected for its detection and 3 strains were observed with the PCR product of desired size as also observed in reference strain *Btj* Buibui (Fig 4.3.6).

Beside the biotype describing subspecies *israelensis*, *Bt* strains from other biotypes were also tested for the presence of *cry10* gene and it was detected only in 1 strain out of 42 (Fig 4.3.6). Expected product size in this gene detection was 348 bp as the primers were chosen from the literature (Jouzani *et al.*, 2008).

In 54 *Bt* strains, *cry11* gene was searched including 12 from biotype describing *israelensis* and rest from randomly chosen biotypes. Primer set was obtained from the previously reported literatures (Bravo *et al.*, 1998) for which the product size was to be 311 bp. But, none of the strains were detected with the expected amplicon of *cry11* gene (not shown).

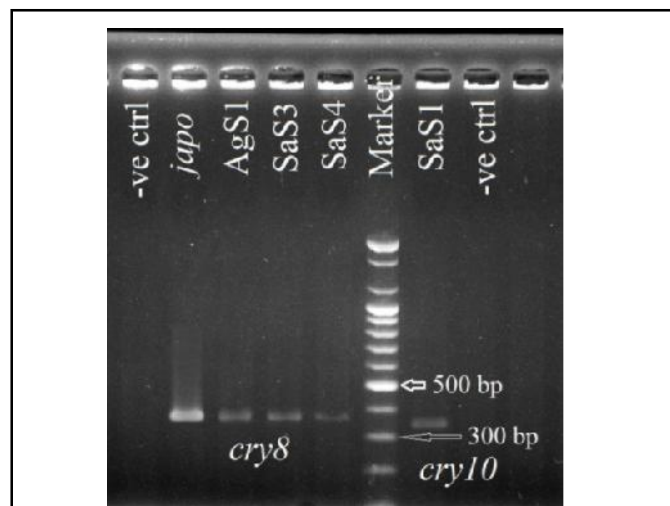

Figure 4.3.6: Detection of amplicons for *cry8* and *cry10* genes by matching them with molecular standards after Agarose gel electrophoresis of the PCR products.

4.3.1.6 Analysis of *cry9* gene prevalence

Out of 69 tested, *cry9* gene was detected in 5 indigenous *Bt* strains. The PCR product of 492 bp was targeted in this case and beside the desired amplicon, spurious products of about 200, 300, 350 and 800 bp was also observed in few strains (Fig 4.3.7). But, only *Bt* strains with specific molecular size were considered positive.

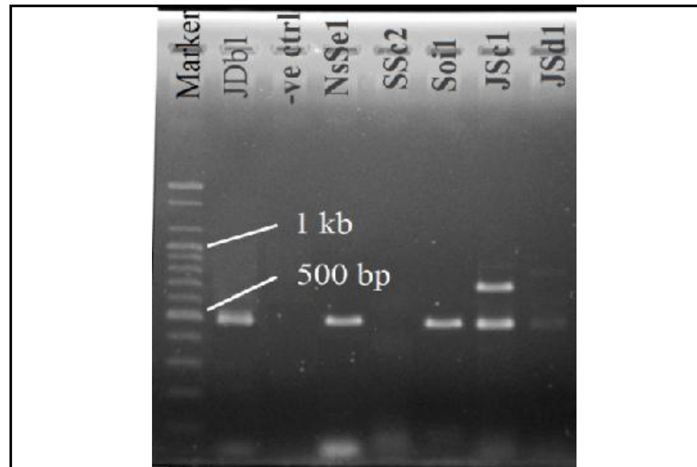

Figure 4.3.7: Agarose gel electrophoresis of the PCR products revealed the presence of *cry9* gene as the amplicon of desired size was observed.

4.3.2 Distribution pattern of *cry* genes

The *cry* genes profile for each of the tested *Bt* strains was obtained by compiling the results of *cry1*, *cry1A*, *cry1Aa*, *cry1Ac*, *cry1Ba*, *cry1Ca*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9*, *cry10* and *cry11* genes detection in those strains (Table 4.3.1). Based on the origins and biotypes of these strains, the distribution patterns were determined and analyzed (Table 4.3.2).

The detection of *cry1* gene was performed in the *Bt* strains of all biotypes and its presence was observed in all the biotypes except *galleriae*, nine, ten, *ostriniae* and thirteen. Maximum occurrence of *cry1* gene was in the biotype describing the subspecies *kurstaki* (78%) followed by sixteen (57%), *indiana* (31%), *thuringiensis* (28%). Lepidoptera specific *cry1A* gene was detected in 36.76% of the strains tested and the distribution was observed in the biotypes *thuringiensis*, *kurstaki*, *sotto*, *morrisoni*, fifteen and sixteen. Again, the occurrences of *cry1Aa* and *cry1Ac* genes were observed only in the biotype *kurstaki* except the strain NsSe2 from biotype fifteen harbouring *cry1Aa* gene. The occurrences for *cry1Aa* and *cry1Ac* were 60% and 16% respectively in the tested strains. Interestingly, *cry1Ac* gene was observed only in the strains harbouring *cry1Aa* gene. And the occurrences of *cry1Ba* and *cry1Ca* were 8.7% and 21.7% respectively, the distribution of which were confined to the biotypes *thuringiensis*, *kurstaki*, *indiana*, *morrisoni* and eleven. In biotype *dendrolimus, cry1Ca* was also observed and its maximum prevalence was in biotype *kurstaki.*

cry2 gene was found to be distributed in 8 biotypes out of 12 tested and the maximum occurrence was observed in *dendrolimus* (66.6%) followed by *kurstaki* (36%) and *indiana* (35%). The sample size for *dendrolimus* was small with only 3 strains whereas for *kurstaki* and *indiana*, it was 33 and 14 respectively. So, the occurrence for *dendrolimus* was not significant enough. Similarly, in the biotype describing *morrisoni*, 2 strains out of 2 were detected with *cry3* genes but *kurstaki* was found with maximum 9 strains to harbour *cry3* gene.

The distribution of dipteran insect specific *cry4A* and *cry10* genes was observed only in the biotype describing subspecies *israelensis*. The occurrences were only 16.6% and 8.3% for *cry4A* and *cry10* genes respectively in this biotype. Similarly, *cry8* gene was observed only in the *Bt* strains from biotype describing the subspecies *thuringiensis* with 20% occurrence. For *cry9* gene, it was occurred in 12.5% of the tested *Bt* strains from the biotype *kurstaki* and in 1 strain tested from the biotype eleven (n=1).

Table 4.3.1: Profiles of *cry* genes for the indigenous *Bt* strains of Bangladesh.

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
1	AgL1	*israelensis*	-								-			-	-
2	AgS1	*thuringiensis*	+	+	-	-	-	-	-	-		+	-		
3	AgS2	*kurstaki*	+	+	-	-	-	-	-	-			-		
4	AtL1	*thuringiensis*	-												
5	AtS1	*kurstaki*	-												
6	AtS2	ten	-								-			-	-
7	AtS3	ten	-												
8	CgSb1	*thuringiensis*	-												
9	CgSc1	*israelensis*	-								-	-		-	-
10	CgSc2	thirteen	-												
11	CgSd1	fifteen	-												
12	CgSd3	*darmstadiensis*	-												
13	CgSe1	*thuringiensis*	-						-						
14	CgSe2	fifteen	-												
15	ChS1	*israelensis*	-								-	-		-	-
16	ChS2	ten	-												
17	ChS3	ten	-								-			-	-
18	ChSa2	*sotto*	-												
19	ChSc1	fifteen	-												
20	ChSd2	*thuringiensis*	+	+								-			
21	CiSa1	*kurstaki*	-												

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
22	CiSa2	*thuringiensis*	-												
23	CiSa3	*thuringiensis*	-												
24	CiSa5	*kurstaki*	+	+			-	-	-	-			-		
25	CoS2	*kurstaki*	+	+			-	-	-	-			-		
26	DpSb1	*indiana*	-												
27	DSa3	*kurstaki*	-												
28	DSc2	*thuringiensis*	-												
29	DSe1	*kurstaki*	+	+			-	-	+	-			-		
30	DSe4	*kurstaki*	-												
31	DSe6	*kurstaki*	-												
32	DSf7	*kurstaki*	+	-			-	-	+	-			-		
33	DSg2	*dendrolimus*	-								-			-	-
34	DSh4	*sotto*	+	+	-	-	-	-	+	-			-		
35	DSh7	*sotto*	-						-	-					
36	FhSa3	*darmstadiensis*	-						-	-					
37	FhSb1	*dendrolimus*	-						-			-			
38	FhSb2	*indiana*	+	+			-	-	+	-			-		
39	FhSb3	eleven	+	+			+	+	+	+			-		
40	FhSc1	eleven	-						-						
41	FhSc2	thirteen									-			-	-
42	FhSc3	thirteen									-			-	-
43	FhSd3	*galleriae*	-												
44	FhSd4	sixteen	-						-		-			-	-
45	FhSe1	*kurstaki*	-						-	-					
46	JaL1	*kurstaki*	+	+			-	-	-	-		-	-		
47	JaL2	ten	-								-			-	-
48	JaL3	*sotto*	+	+			-	-	-	-		-	-		
49	JaL4	nine	-												
50	JaL5		-												
51	JaL6	*thuringiensis*	-												
52	JaS1	Nine	-												
53	JaS10	*ten*	-								-			-	-
54	JaS2	Nine	-												
55	JaS3	*thuringiensis*	+	-			-	-	-	-		-	-		
56	JaS4	*israelensis*	-								+	-		-	-
57	JaS5	*kurstaki*	-						-	-					
58	JaS6		-												
59	JaS7	*kurstaki*	+	-			-	-	-	-			-		
60	JaS8	*kurstaki*	+				-	-	-	-			-		
61	JaS9		-												
62	JDa1	*kurstaki*	+	+	+	-	-	-	-	-		-	-		
63	JDb1	*kurstaki*	+	+	+	+	-	+	+	+		-	+		

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
64	JDc1	*kurstaki*	+	+	+	-	-	+	+	+		-	-		
65	JeS1	Nine	-												
66	JeSa1	*thuringiensis*	-												-
67	JeSa2	*sotto*	-												
68	JeSb2	*Israelensis*	-								-			-	-
69	JeSc1	thirteen									-			-	-
70	JeSe1	thirteen	-												
71	JSa1	*dendrolimus*	-								-	-		-	-
72	JSa3	*thuringiensis*	+	-			-	-	-	-		-	-		-
73	JSb1	*kurstaki*	+	-			-	-	-	-		-	-		
74	JSc1	*kurstaki*	+	+	+	-	-	+	+	-			+		
75	JSc3	*kurstaki*	+	-			-	-	-	-		-	-		
76	JSd1	*kurstaki*	+	+	-	-	-	+	+	-		-	+		
77	JSd2	*kurstaki*	+	+			-	-	-	-			-		
78	KbSa1	*morrisoni*	-						-	-					
79	KbSb1	thirteen	-												
80	KbSb2	fifteen	-												
81	KbSc1	*indiana*	-												
82	KeS1	*thuringiensis*	-						-	-					
83	KeS2	*israelensis*	+	-			-	-	-	-	-		-	-	-
84	KeS3	nine	-												
85	KeS4	*israelensis*	-								-	-		-	-
86	KfSa1	*israelensis*	-								-	-		-	-
87	KfSa2	nine	-												
88	KkSb1	*sotto*	-						-						
89	KkSb2	*thuringiensis*	-												
90	KkSc1	thirteen	-												
91	KkSc2	*thuringiensis*	-												-
92	KkSd1	fifteen	-												
93	KSa1	*kurstaki*	-	-	-	-			-						
94	KSa2	*dendrolimus*	+	-			-	-	+	+	-	-	-	-	-
95	KSb1	*kurstaki*	+	+	+	-	-	-	+	+		-	-		
96	KSc1	*thuringiensis*	-						-						-
97	KSe2	*kurstaki*	+	-			-	-		-			-		
98	KuSa2	*sotto*	-												
99	KuSe1	*sotto*	-												
100	KuSe2	eleven	-								-			-	-
101	MaSb1	*sotto*	-						-		-			-	-
102	MaSb2	thirteen							-		-			-	-
103	MeSa1	*darmstadiensis*	+	-			-	-	+	+		-	-		
104	MeSb1	*thuringiensis*	+				-	-	+	-		-	-		-
105	MeSb2	*indiana*	-							-					

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
106	MeSc1	*indiana*	-												
107	MeSd1	*thuringiensis*	-									-			-
108	MeSd2	*indiana*	-						-	-					
109	MeSe2	*morrisoni*	-								-				
110	MuSa1	*dendrolimus*	+	-			-	+	+	-	-		-	-	-
111	MuSc2	*kurstaki*	+	+	+	+	-	+	-	+	-		-	-	-
112	MuSc4	*kurstaki*	+	+	+	+	-	-	-	+		-	-		
113	MuSd1	*thuringiensis*	-									-			-
114	MuSd3	*sotto*	-												
115	MuSe4	eleven	+				-	-	-	+			-		
116	MyLa1	eleven	-								-			-	-
117	MyLa2	*galleriae*	-									-			-
118	MySa2	*indiana*	-												
119	MySb2	*indiana*	-												
120	NaL1	*kurstaki*	+	+	+	-	+	-	+	-					
121	NaL2	*kurstaki*	-	-											
122	NaS1	*thuringiensis*	-												
123	NaS2	nine	-												
124	NaSa2	eleven	-								-			-	-
125	NaSb2	*galleriae*	-									-			
126	NaSc1	sixteen	+	-			-	-	-	-		-	-		-
127	NaSc2	*indiana*	-												
128	NaSc3	*indiana*	+	-	-	-	+	+	+	-			-		
129	NaSd1	*galleriae*	-												
130	NaSd2	*indiana*	+	-			-	-	-	-			-		
131	NaSd3	eleven	-						-						
132	NaSe1	*indiana*	-												
133	NaSe2	*indiana*	-												
134	NoS1	*thuringiensis*	-									-			
135	NoS2	*kurstaki*	+	+	-	-	-	-	-	-			-		
136	NoS3		-												
137	NoS4	*kurstaki*	+	-			-	-	+	-			-		
138	NoS5	ten	-								-			-	-
139	NsSa1	*indiana*	-						-						
140	NsSb1	*indiana*	-												
141	NsSc1	*thuringiensis*	-												
142	NsSc2	*sotto*	-												
143	NsSd1	*dendrolimus*	-												
144	NsSd2	sixteen	-						-						
145	NsSe1	sixteen	+				-	-	-	-			-		
146	NsSe2	fifteen	+	+	+	-	-	-	-	-			+		
147	RaSa1	*indiana*	+				-	-	-	+			-		

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
148	RaSa2	*indiana*	+				-	-	+	+			-		
149	RaSb1	*indiana*	+				-	-	-	-			-		
150	RaSb2	*galleriae*	-									-			-
151	RaSc1	*morrisoni*	+				+	-	-	+			-		
152	RaSd1	*indiana*	+				-	-	-	-			-		
153	RaSd2	sixteen	+				-	-	+	+			-		
154	RhSa2	*thuringiensis*	+	-			-	-	-	-		-	-		
155	RhSb2	*indiana*	+				-	-	+	-			-		
156	RhSb3	*indiana*	-												
157	RhSc1	eleven	-												
158	RhSc2	fifteen	-												
159	RhSc3	*sotto*	-												
160	RhSd1	*thuringiensis*	-												
161	RhSd3	eleven	-												
162	RhSd4	*dendrolimus*	-												
163	RpSa2	*galleriae*	-									-			
164	RpSb1	thirteen	-												
165	RpSc1	thirteen									-			-	-
166	RpSc2	eleven	-												
167	SaS1	*israelensis*	-								-	-		+	-
168	SaS10	*thuringiensis*	-												
169	SaS2	ten	-									-			
170	SaS3	*thuringiensis*	-						-	-		+			
171	SaS4	*thuringiensis*	+	+	-	-	-	-	-	-		+	-		
172	SaS5	*israelensis*	-								-	-		-	-
173	SaS6	*kurstaki*	+	-			+	-	-	-			-		
174	SaS7	*kurstaki*	+	-			-	-	+	-			-		
175	SaS8	*thuringiensis*	-						-	-					
176	SaSa1	eleven	-						-		-			-	-
177	SaSa2	*galleriae*	-									-			-
178	SaSb1	*indiana*	-												
179	SaSb2	nine	-												
180	SgSc1	nine	-												
181	SgSj2	fifteen	-												
182	SgSm1	thirteen	-								-			-	-
183	SgSm2	*morrisoni*	+	+	-	-	-	+	-	+	-	-	-	-	-
184	SgSn1	eleven	-								-			-	-
185	SgSp1	thirteen	-												
186	SgSp2	*indiana*	-												
187	Soi1	*kurstaki*	+	+	+	-	-	-	-	-		-	+		
188	Soi2		-												
189	Soi3	nine	-												

Serial no.	Bt strains	Biotype	*cry1*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*	*cry2*	*cry3*	*cry4*	*cry8*	*cry9*	*cry10*	*cry11*
190	SoL1		-												
191	SoL2	*kurstaki*	-												
192	SoS1	ten	-												
193	SoS2	*israelensis*	-								+	-		-	-
194	SoS3	ten	-												
195	SoS4	ten	-												
196	SoS5	thirteen	-												
197	SoS6	nine	-												
198	SoS7	*kurstaki*	+	-			-	-	-	-			-		
199	SoS8	ten	-												
200	SpSb3	*indiana*	-												
201	SpSc1	sixteen	+	+	-	-	-	-	-	-	-		-	-	-
202	SpSd1	*indiana*	-												
203	SpSd2	*morrisoni*	-								-	-		-	-
204	SpSd3	fifteen	-												
205	SpSe2	*sotto*	-												
206	SSa1	*thuringiensis*	-												-
207	SSa3	ten	-								-			-	-
208	SSb1	*kurstaki*	+	+	+	-	-	+	+	+			-		
209	SSb2	*kurstaki*	+	+	+	+	-	+		+			-		
210	SSc1	*israelensis*	+	-			-	-	-	-	-		-	-	-
211	SSc2	*kurstaki*	+	+	+	-	-	+	-	+			-		
212	SSd1	*kurstaki*	+	+	-	-	-	-					-		
213	SSd2	*thuringiensis*	+	-			+	+	-	-		-	-		
214	SSe2	*kurstaki*	+	+	+	-	-	+	-	+			-		
215	SSe3	*dendrolimus*	-								-			-	-
216	SSf1	*thuringiensis*	-						-			-			
217	SSf2	*kurstaki*	+	+	+	-	-	+	-	-			-		
218	SSf4	*thuringiensis*	-									-			
219	SySa2	fifteen	-												
220	TaSa2	*indiana*	-						-	-					
221	TaSa4	*indiana*	+	-			-	-	+	-			-		
222	TaSb1	*indiana*	-						-	-					
223	TaSb3	eleven	+	-			-	-	-	-	-		-	-	-
224	TaSc1	eleven	+	-			-	-	-	-			-		
225	TaSc2	sixteen	-						-	-	-			-	-
226	TaSd1	*ostriniae*	-												
227	TaSe1	*indiana*	-						-	-					
228	TaSe2	*ostriniae*	-												
229	USc1	*thuringiensis*	-						-						
230	USc3	*thuringiensis*	+	+	-	-	-	-	-	-		-	-		

The distribution and availability of *cry* genes in different eco-regions of Bangladesh were analyzed based on the origin of each strains and their *cry* genes profiles. Hence, the availability index for each region was calculated by dividing the number of *cry* genes obtained with the number of *Bt* strains tested. The index was maximum for the river basin (0.55) followed by central part (0.51), southern part (0.48) and hill tracts (0.475) (Table 4.2.7).

Table 4.3.2: Distribution of *cry* genes detected in the different biotypes of Bangladesh.

Biotypes	*cry1*	*cry2*	*cry3*	*cry4A*	*cry8*	*cry9*	*cry10*	*cry11*	*cry1A*	*cry1Aa*	*cry1Ac*	*cry1Ba*	*cry1Ca*
thuringiensis	9 (32)	1 (15)	0 (11)	0 (0)	3 (15)	0 (9)	0 (0)	0 (8)	3 (9)	0 (3)	0 (3)	1 (9)	1 (9)
kurstaki	32 (41)	12 (33)	9 (33)	0 (1)	0 (9)	4 (32)	0 (1)	0 (1)	18 (32)	14 (18)	4 (18)	2 (32)	10 (32)
indiana	9 (29)	5 (14)	2 (14)	0 (0)	0 (0)	0 (9)	0 (0)	0 (0)	0 (9)	0 (0)	0 (0)	1 (9)	1 (9)
galleriae	0 (7)	0 (0)	0 (0)	0 (0)	0 (6)	0 (0)	0 (0)	0 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
sotto	2 (13)	1 (5)	0 (3)	0 (1)	0 (1)	0 (2)	0 (1)	0 (1)	1 (2)	0 (1)	0 (1)	0 (2)	0 (2)
dendrolimus	2 (8)	2 (3)	1 (2)	0 (5)	0 (3)	0 (2)	0 (5)	0 (5)	0 (2)	0 (0)	0 (0)	0 (2)	1 (2)
morrisoni	2 (5)	0 (3)	2 (2)	0 (3)	0 (3)	0 (2)	0 (2)	0 (2)	1 (2)	0 (1)	0 (1)	1 (2)	1 (2)
darmstadiensis	1 (3)	1 (2)	1 (2)	0 (0)	0 (1)	0 (1)	0 (0)	0 (0)	0 (1)	0 (0)	0 (0)	0 (1)	0 (1)
nine	0 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ten	0 (13)	0 (0)	0 (0)	0 (6)	0 (1)	0 (0)	0 (6)	0 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
eleven	3 (13)	1 (6)	1 (3)	0 (6)	0 (0)	0 (3)	0 (6)	0 (6)	0 (3)	0 (0)	0 (0)	1 (3)	1 (3)
ostriniae	0 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
thirteen	0 (8)	0 (1)	0 (0)	0 (6)	0 (0)	0 (0)	0 (6)	0 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
israelensis	2 (11)	0 (2)	0 (2)	2 (12)	0 (8)	0 (3)	1 (12)	0 (12)	0 (2)	0 (0)	0 (0)	0 (3)	0 (3)
fifteen	1 (10)	0 (1)	0 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)	0 (1)	0 (1)	0 (1)
sixteen	4 (7)	1 (7)	1 (5)	0 (3)	0 (1)	0 (3)	0 (3)	0 (4)	1 (4)	0 (1)	0 (1)	0 (3)	0 (3)
unbiotyped	2 (11)	0 (2)	1 (2)	0 (0)	0 (0)	0 (2)	0 (0)	0 (0)	0 (2)	0 (0)	0 (0)	0 (2)	0 (2)
Total	69 (224)	24 (94)	18 (81)	2 (43)	3 (48)	5 (69)	1 (42)	0 (54)	25 (68)	15 (25)	4 (25)	6 (69)	15 (69)

Distribution of all the *cry* genes searched in this study but *cry4A* was observed in the central part. The abundances of *cry1*, *cry2*, *cry3*, *cry8* and *cry10* genes in this eco-region were higher than the other regions. In southern part, hill tracts and river basin, *cry1*, *cry2*, *cry3* and *cry9* were present. The abundance of *cry9* in southern part was the maximum and *cry4A* was exceptionally observed in river basin as were *cry8* and *cry10* in the central part.

Table 4.3.3: Distribution of *cry* genes in different eco-regions of Bangladesh.

Regions	Samples	*Bt* strains	*cry* genes obtained	Availability index	*cry1*	*cry2*	*cry3*	*cry4A*	*cry8*	*cry9*	*cry10*
Central Part	86	133	68	0.51	43	13	7	0	3	1	1
Southern Part	40	58	28	0.48	16	6	3	0	0	3	0
Northern Part	18	28	7	0.25	6	1	0	0	0	0	0
Hill tracts	22	40	19	0.475	11	3	4	0	0	1	0
River Basin	60	47	26	0.55	18	1	4	2	0	1	0
Sandy beach	5	11	1	0.09	1	0	0	0	0	0	0

4.3.3 Prevalence of *cry* genes in the indigenous *Bt* strains: a comparative analysis

PCR screening of 230 crystal protein forming *Bt* strains were performed for the *cry* genes and 73 of them were positive for seven different *cry* genes. The prevalence of targeted *cry* genes in the *Bt* strains were calculated and *cry1* gene was found to be the most prevalent (30.8%) followed by *cry2* (25.5%), *cry3* (22.2%) and *cry9* (7.2%) (Fig 4.3.8A). Prevalence of *cry4*, *cry8* and *cry10* genes were less than 5% and no strains with *cry11* gene was found. On the other hand, the prevalence of certain subgroups of *cry1* gene such as *cry1Aa*, *cry1Ac*, *cry1Ba* and *cry1Ca,* searched in this study was also determined (Fig 4.3.8B). Among the subgroups, *cry1Aa* (60%) gene was determined to be the most prevalent followed by *cry1Ca* (21.7%), *cry1Ac* (16%) and *cry1Ba* (8.7%).

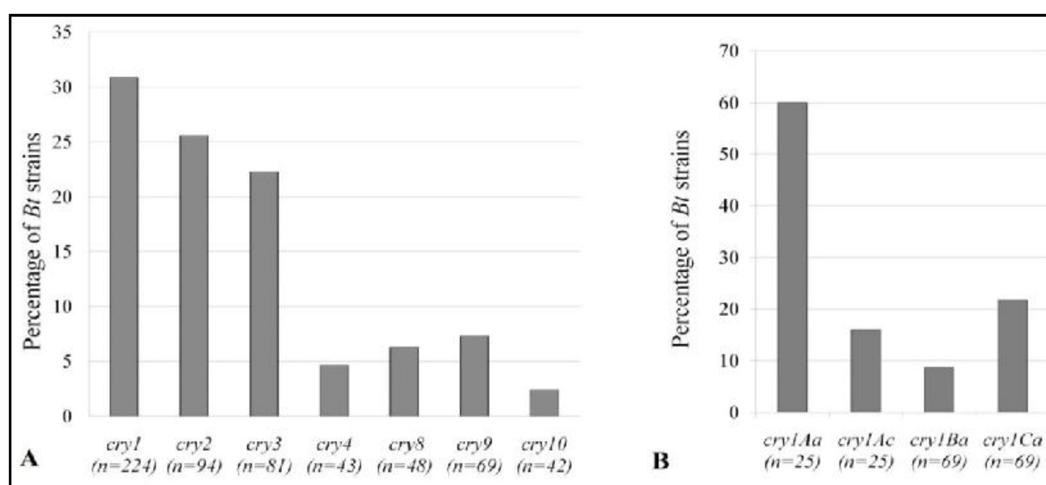

Figure 4.3.8: Prevalence of different *cry* genes in the indigenous *Bt* strains. *cry* genes in A) Primary ranks, B) Tertiary ranks.

So far reported most common *cry* gene in nature belong to *cry1* gene group (Porcar and Juarez-Perez 2003) and similar reports of *cry1* gene to be the most frequent in the collections were made (Ben-Dov *et al.*, 1997; Bravo *et al.*, 1998; Wang *et al.*, 2003). Same pattern was observed in this study as the number of strains containing the *cry1* gene was maximum (69) as compared to the other strains containing the *cry2* (24), *cry3* (18), *cry4* (2), *cry8* (3), *cry9* (5) and *cry10* (1) genes (Fig 4.3.8B). It was also reported that *cry1* genes was the most frequent (49.5%), then *cry3* gene as highly abundant (21.7%) and *cry9* gene less abundant (2.6%) (Bravo *et al.*, 1998). These results showed both similarity and dissimilarity across different geographic regions which might affect the diversity of *cry* gene content of *Bt* strains. The prevalence of *cry2* (25.5%) gene was found to be next abundant in this study followed by *cry3* (22.2%) and *cry9* (7.2%) which is a exception with the reports.

It was found in many studies that concomitant occurrence of *cry1* and *cry2* genes were observed (Ben-Dov *et al.*, 1997; Zhang *et al.*, 2000; Wang *et al.*, 2003). Wang *et al.*, showed that 90.7 % strains among the *cry1* gene positive isolates, also harbored a *cry2* gene (Wang *et al.*, 2003). Similar report was from Ben-Dov that most of the isolates containing *cry1* gene were also positive for *cry2* gene (Ben-Dov *et al.*, 1997). In the present study, it was observed that *cry2* gene was not present in a strain that was devoid of *cry1* gene i.e. 36.23% of the strains positive for *cry1* gene was found to harbor *cry2* gene. Similar stories were found for *cry3* and *cry9* genes as 26% and 7.2% of the *cry1* gene positive strains respectively harbored them.

For the 157 strains detected with no targeted *cry* genes, they may contain other genes as 72 different *cry* gene groups and many subgroups have been defined in the literature to date (Crickmore *et al.*, 2014).

4.3.4 Cry protein profile analysis

The toxicity of any *Bt* strain depends on the expression of the δ-endotoxins though the presence of different *cry* genes are detected. Because the PCR based detection of a certain cry gene alone is not sufficient to expect its eventual translation and cryptic *cry* genes can be arisen by frequent recombination events among the genes present in *Bt* strains. On the other hand, the practice of determination of certain Cry protein by its shape is an obsolete idea and it is also difficult to determine the shape of the δ-endotoxins in maximum cases by Phase Contrast Microscope, hence necessitates Scanning Electron Microscopy (SEM). Detection of expression of a specific Cry protein is mostly performed by antibody based immunoblotting by Western blot analysis. When the objective is to characterize a large number of samples for the identification of potential strains containing more δ-endotoxins, these techniques become infeasible for maintenance. Hence, SDS-PAGE analysis of the Cry protein profiles accompanied by the PCR identification can be a smart shorthand technique for finding potential strains which is supported by the idea that the correlation between Cry proteins and *cry* genes was observed in previous studies. The insecticidal potential of a *Bt* strain can thus more appropriately be ascertained by detection of *cry* genes present followed by analysis of crystal proteins produced by that strain (Kaur, 2006).

In view of this, the δ-endotoxins from 50 indigenous *Bt* strains were purified partially and subject to SDS-PAGE. The analysis was repeated several times and the growth period was also altered for many strains to see the qualitative effects as the expression and the purification process might be the causes of getting differences in the results.

The purified proteins were observed under Phase Contrast Microscope and the shapes of the crystal proteins were presumed by analysis. Crystal proteins of different sizes were observed and the shapes of the proteins as revealed were bipyramidal, rhomboidal, spherical, triangular, cubic, irregular etc (Fig 4.3.9).

To assign the δ-endotoxins in different subgroups, similarity based on the molecular weight was determined. In this connection, the molecular weights of reported Cry and Cyt proteins, one from each quaternary subgroup, was deduced from its amino acid sequence. The reported Cry and Cyt proteins were then placed in a table with their respective molecular weight (Table 4.3.4).

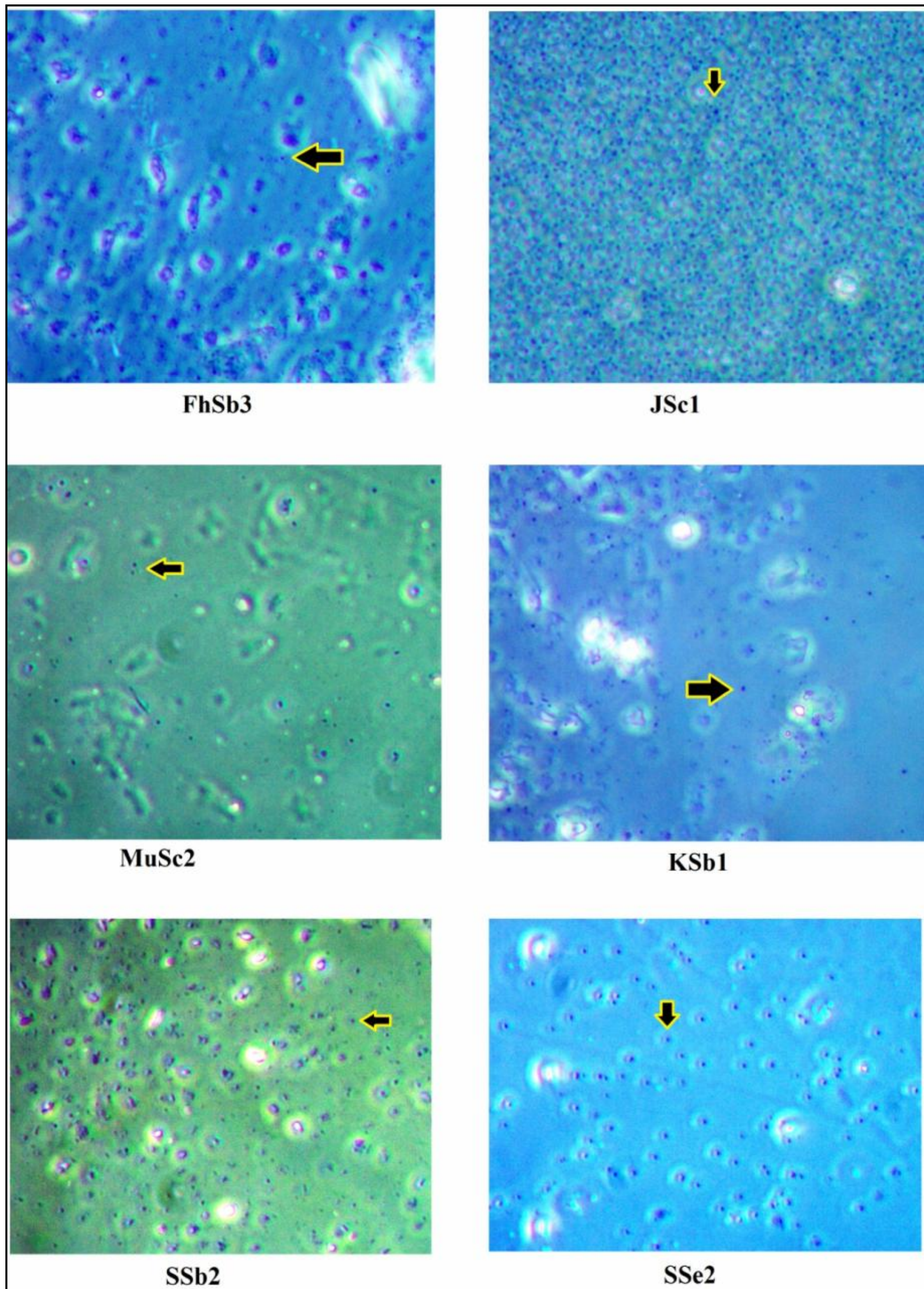

Figure 4.3.9: Presence of crystal proteins in the indigenous *Bt* strains as revealed under Phase Contrast Microscope (Arrows indicate the Crystal proteins).

Table 4.3.4: Molecular weight based arrangement of the δ-endotoxins, reported so far.

MW (kD)	Name	MW (kD)	Name	MW (kD)	Name	MW (kD)	Name
13.2206	Cry34Aa1	70.9936	Cry2Ae1	79.0346	Cry18Aa1	129.1965	Cry7Ca1
13.6017	Cry34Ab1	71.0375	Cry57Aa1	79.1125	Cry22Ab1	129.3226	Cry7Gc1
13.6307	Cry34Ac1	71.1728	Cry16Aa1	79.2495	Cry22Aa2	129.392	Cry7Aa1
14.1806	Cry37Aa1	71.3584	Cry2Ak1	79.5565	Cry54Ba1	129.3994	Cry9Ba1
14.8393	Cry34Ba1	71.6513	Cry17Aa1	79.5565	Cry54Ab1	129.4287	Cry47Aa
26.1938	Cyt2Ca1	71.9786	Cry1Na1	79.7334	Cry52Ba1	129.7265	Cry9Aa1
27.3411	Cyt1Aa1	72.3488	Cry11Aa1	80.941	Cry1Ig1	129.7315	Cry7Da1
27.5072	Cyt1Ab1	72.3848	Cry2Ba1	81.025	Cry1Ie1	129.7761	Cry9Ca1
29.2134	Cry23Aa1	73.0269	Cry3Ca1	81.0495	Cry31Aa1	129.7786	Cry7Ab1
29.2354	Cyt2Aa1	73.3064	Cry56Aa1	81.2104	Cry1Ic1	129.8272	Cry9Ee1
29.7159	Cyt2Bc1	73.5379	Cry40Da1	81.2643	Cry1Ia1	129.881	Cry48Ab
29.8431	Cyt2Ba1	73.8667	Cry40Ca1	81.2954	Cry1Ib1	129.8961	Cry9Ea1
29.906	Cyt1Ba1	74.0358	Cry3Aa1	81.3441	Cry11Ba1	130.2228	Cry9Ed1
30.0594	Cyt2Bb1	74.3125	Cry30Ga1	81.4032	Cry1Id1	130.2231	Cry7Ea1
30.0798	Cry45Aa	74.3853	Cry3Bb1	81.7784	Cry31Ab1	130.2438	Cry9Ec1
33.0166	Cry46Ab	74.4361	Cry29Aa1	84.4077	Cry11Bb1	130.4264	Cry8Ca1
33.7985	Cry64Aa1	74.4605	Cry59Aa1	84.5639	Cry63Aa1	130.4534	Cry8Bb1
33.8552	Cry60Aa1	74.7429	Cry19Aa1	84.7826	Cry31Ac2	130.4534	Cry8Ca2
33.8869	Cry33Aa1	75.1593	Cry3Ba1	84.7826	Cry31Ad1	130.5335	Cry8Ja1
33.982	Cry51Aa1	75.3117	Cry40Aa1	86.1393	Cry20Aa1	130.5706	Cry7Ba1
34.2777	Cry38Aa1	75.4326	Cry40Ba1	86.7471	Cry31Ac1	130.6236	Cry1Ab1
34.9571	Cry60Ba1	75.5127	Cry39Aa1	88.1429	Cry13Aa1	130.7059	Cry4Cc1
37.4476	Cry46Aa	75.643	Cry25Aa1	88.5366	Cry20Ba1	130.7062	Cry4Cb1
37.5469	Cry15Aa1	75.7885	Cry53Aa1	88.5982	Cry41Ba1	130.7883	Cry61Aa1
40.3208	Cry55Aa1	75.8163	Cry53Ab1	88.5982	Cry66Aa2	130.8682	Cry9Eb1
40.3208	Cry55Aa2	75.8486	Cry18Ba1	90.5818	Cry70Ba1	130.9695	Cry1Db1
43.8109	Cry35Ab1	75.9068	Cry24Ba1	91.3008	Cry42Aa1	131.0098	Cry8Aa1
43.812	Cry35Ba1	75.959	Cry24Aa1	91.7279	Cry70Bb1	131.0626	Cry1Hb1
43.819	Cry35Ac1	76.2917	Cry50Ba1	93.4772	Cry58Aa1	131.0632	Cry7Gd1
44.0679	Cry6Ba1	76.3227	Cry54Aa1	93.6938	Cry41Aa1	131.1591	Cry8Ad1
44.33	Cry35Aa1	76.7436	Cry62Aa1	93.8418	Cry41Ab1	131.21	Cry9Fa1
53.2756	Cry49Aa	77.0061	Cry50Aa1	94.4346	Cry27Aa1	131.2393	Cry8Ga1
53.517	Cry49Ab1	77.1283	Cry30Fa1	95.3746	Cry67Aa2	131.2852	Cry26Aa1
54.0754	Cry6Aa1	77.4018	Cry30Db1	98.4718	Cry41Ba2	131.3582	Cry7Ia1
57.9648	Cyt1Da1	77.4382	Cry30Ca1	101.6854	Cry5Ad1	131.361	Cry9Bb1
58.6096	Cry36Aa1	77.4385	Cry30Ba1	101.6997	Cry8Ka1	131.4407	Cry5Da1
60.3317	Cyt1Ca1	77.5904	Cry1Nb1	103.1809	Cry1Af1	131.4407	Cry5Da2
69.5249	Cry22Ba1	77.6219	Cry30Ea1	117.8359	Cry65Aa1	131.5461	Cry14Ab1
69.613	Cry22Bb1	77.7605	Cry10Aa1	125.7135	Cry28Aa1	131.5706	Cry8Ea1
69.7294	Cry2Ac1	77.8444	Cry30Aa1	127.3138	Cry7Fa1	131.6485	Cry9Dc1
70.5143	Cry2Af1	78.0236	Cry44Aa	127.3718	Cry7Fa2	131.6945	Cry14Aa1
70.55	Cry2Ai1	78.0496	Cry30Da1	127.6836	Cry7Gb1	131.7993	Cry7Ha1
70.7412	Cry2Ab1	78.0724	Cry24Ca1	127.7642	Cry4Ba1	131.9722	Cry8Db1
70.7521	Cry2Ad1	78.259	Cry18Ca1	127.898	Cry7Ga2	131.9994	Cry1Gc1
70.8053	Cry2Ag1	78.3046	Cry1Ma1	127.9756	Cry7Kb1	132.0044	Cry1Ga1
70.8524	Cry2Aa1	78.491	Cry19Ba1	128.0609	Cry8Da1	132.1267	Cry9Db1
70.9805	Cry2Ah1	78.5531	Cry52Aa1	128.4384	Cry7Ka1	132.2287	Cry9Da1

MW (kD)	Name	MW (kD)	Name	MW (kD)	Name		
132.4815	Cry1Da1	136.4912	Cry8La1	144.1473	Cry32Ia1		
132.7209	Cry8Na1	136.5075	Cry7Ja1	144.1742	Cry43Ca1		
132.7359	Cry8Pa1	137.0973	Cry7Cb1	144.2895	Cry32Da1		
132.7606	Cry1Ja1	137.1209	Cry8Ra1	144.9797	Cry32Ja1		
132.7632	Cry8Qa1	137.1448	Cry69Aa1	144.99	Cry21Ba1		
132.7632	Cry8Qa2	137.2118	Cry8Bc1	145.0297	Cry21Da1		
132.8683	Cry1Cb2	137.3792	Cry1Ka1	145.5684	Cry32Mb1		
132.9057	Cry1Gb1	137.9125	Cry8Ib1	146.0252	Cry32Ma1		
132.9808	Cry1Ha1	138.2118	Cry8Ac1	146.1721	Cry43Cb1		
132.9825	Cry1Dc1	138.3192	Cry9Ga1	146.3969	Cry21Ca1		
133.0292	Cry1Jd1	138.8574	Cry4Ca1	146.5975	Cry32Oa1		
133.0826	Cry8Fa1	139.085	Cry1Be1	147.3734	Cry32Qa1		
133.108	Cry7Fb2	139.0853	Cry32Pa1	148.7543	Cry5Ca1		
133.1331	Cry1Aa1	139.1967	Cry32Aa1	148.7543	Cry5Ca2		
133.2531	Cry1Ea1	139.2807	Cry32Ua1	149.7462	Cry43Ba1		
133.2783	Cry7La1	139.308	Cry1Bf1	151.1974	Cry43Aa1		
133.2783	Cry8Ab1	139.3794	Cry8Ta1	152.3139	Cry32Fa1		
133.2915	Cry1La1	139.5571	Cry32Ab1	152.4404	Cry5Aa1		
133.3117	Cry1Jc1	139.6476	Cry1Ba1	153.3864	Cry32Ta1		
133.3308	Cry1Ac1	139.6554	Cry1Bd1	161.0408	Cry32Ra1		
133.3509	Cry1Fb1	139.7706	Cry1Bb1	165.2932	Cry32Ha1		
133.3772	Cry1Ad1	139.7758	Cry5Ba1	170.4662	Cry32Ka1		
133.3935	Cry1Ag1	139.854	Cry32Eb1				
133.4205	Cry1La2	140.4524	Cry1Bc1				
133.4292	Cry1Ai1	140.4744	Cry1Bg1				
133.5438	Cry8Ba1	141.1392	Cry1Bi1				
133.5537	Cry1Jb1	141.5338	Cry32Ea1				
133.6026	Cry1Eb1	141.5469	Cry32Hb1				
133.6222	Cry1Fa1	141.7342	Cry32Cb1				
133.7382	Cry1Ae1	141.9838	Cry5Ab1				
133.8448	Cry8Ma1	142.0652	Cry1Bh1				
134.0318	Cry1Ah1	142.2664	Cry12Aa1				
134.0835	Cry7Bb1	142.5548	Cry32Ba1				
134.5206	Cry4Aa1	142.5548	Cry32Ca1				
134.8367	Cry1Ca1	142.5859	Cry32Ga1				
134.938	Cry8Ha1	142.6137	Cry43Cc1				
135.4147	Cry8Ia1	143.0916	Cry32Na1				
135.5521	Cry48Aa	143.3942	Cry32Sa1				
136.0319	Cry19Ca1	143.5775	Cry32La1				

Molecular weight of the δ-endotoxins, prominent bands revealed in polyacrylamide gels after SDS-PAGE analysis (Fig 4.3.10), were determined comparing the Rf values with the standard protein marker by Alphaview SA software version 3.4.0 (USA). Based on the molecular weights obtained thus for the Cry proteins from indigenous *Bt*, presence of the respective proteins was presumed. On the other hand, no δ-endotoxins was assigned and labeled as did not match (DNM) if not found in the catalogue. It has been observed that the

molecular weights of several Cry proteins are very similar and differ in decimal. Again, determination of the molecular weight by SDS-PAGE is never 100% accurate. So, the Cry proteins were presumed as any of those with close molecular weights in that ranges and the easiest and simplest way of confirmation could be the PCR detection. Thus, the Cry protein profiles for the tested strains were deduced (Table 4.3.5) and further matched with their respective *cry* gene profiles (Table 4.3.1).

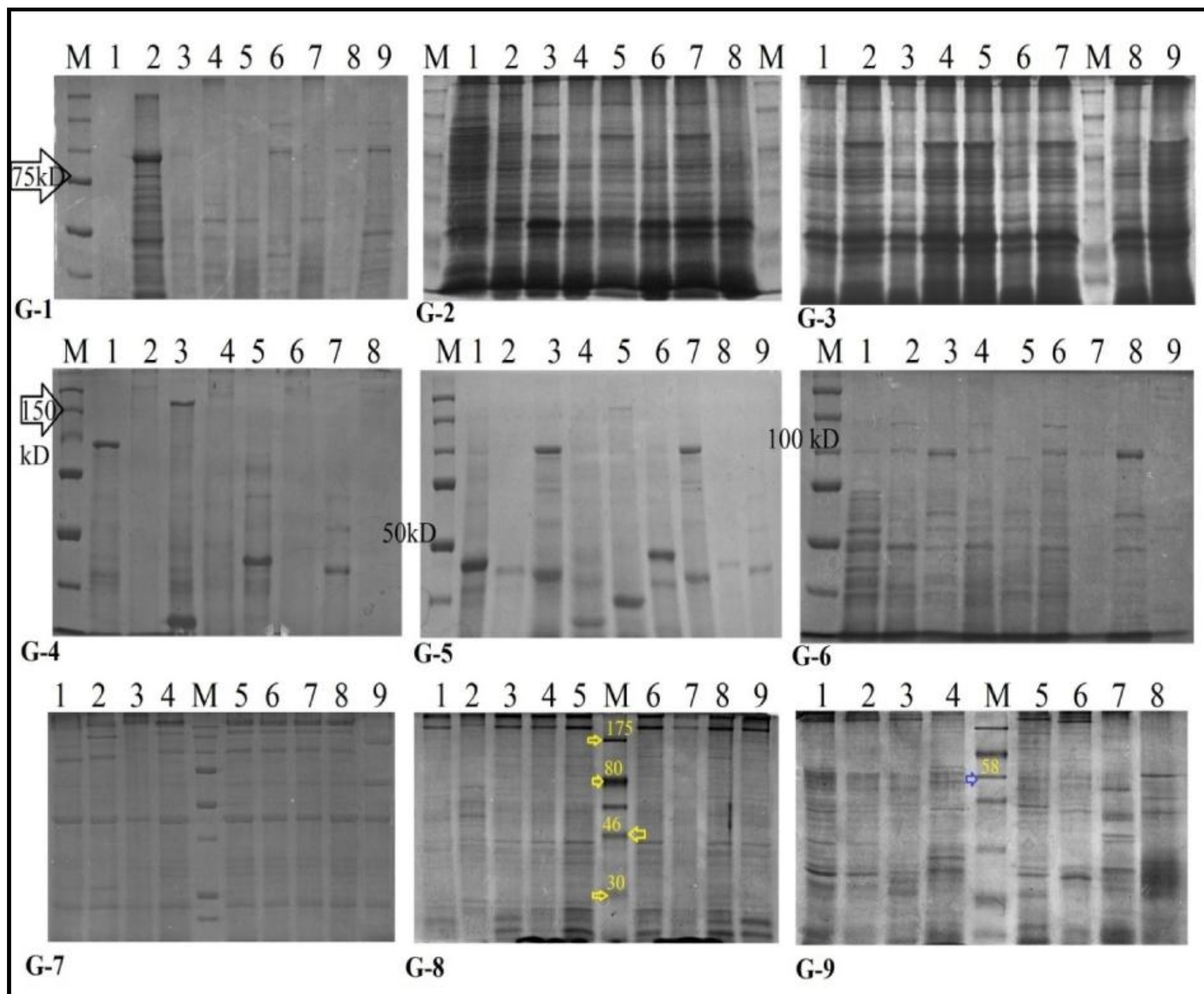

Figure 4.3.10: SDS-PAGE (10%) analysis of partially purified δ- endotoxins from the indigenous *Bt* strains. The names of the strains were mentioned in the Table 2.3.5 according to the gel number. M: (Gel 1, 4-7: Precision plus protein standards, All blue; Gel 2, 3, 8 and 9: Pre-stained protein Marker)

Table 4.3.5: Presumptive classification of δ- endotoxins based on their molecular weight. (r- reference strains, ns: not searched for *cry* genes, Underlined Names of Cry proteins in bold characters indicate their confirmation by PCR)

Gel	Lane	*Bt* strains (number of *cry* genes)	Bands observed	Presumed Cry proteins
1	1	SoS3 (0)		
	2	NoS1 (0)	94, 79, 48	27A, 18/22/52/54, (DNM)
	3	NoS4 (2)	96	67A
	4	NoS5 (0)	62, 56	(DNM), Cyt1D
	5	AgL1 (0)	57	Cyt1D
	6	JaL3 (1)	103, 43	**1A**f
	7	JaL5 (0)	57	Cyt1D
	8	JaL6 (0)	103	1Af
	9	SoL1 (0)	143, 96, 50	32L/N/S, (DNM), (DNM)
2	1	FhSb3 (5)	160, 130, 75, 65, 57, 26	32R, **1A**, **3**B, (**D1**), Cyt1, Cyt2
	3	SSc2 (4)	130-35, 57, 54, 40, 26	**1A**(a-c)/**1C**, Cyt1D, 6A, 55A, Cyt2
	6	RaSa2 (3)	130-35, 40, 26	1A(a-c)/1C, 55A, Cyt2
	7	JDc1 (4)	130-35, 57, 54, 40, 26	**1A**(a-c)/**1C**, Cyt1D, 6A, 55A, Cyt2
3	2	KSa2 (3)	103, 69, 60, 54, 53, 26	1Af, **2**A/22B, Cyt1C, 6A, 49A, Cyt2
	4	SSe2 (3)	103, 54, 53, 26	**1A**f, 6A, 49A, Cyt2
	5	JDb1 (6)	103, 54, 53, 26	**1A**f, 6A, 49A, Cyt2
	8	SSb2 (4)	103, 73, 54, 53, 26	**1A**f, **3**C, 6A, 49A, Cyt2
4	2	SaS4 (2)	130	**1A**b/7/**8**/9
	3	JaS2 (0)	179, 65, 26	(DNM), (D1), Cyt2
	5	HD-73 (r- 1)	65	(D1)
	6	T84A1 (r- 1)	65	(D1)
5	1	NaS1 (0)		
	2	SaS9 (ns)		
	3	SaS10 (0)	101	5A/ 8K
	4	CoS1 (ns)	48, 40, 27	(DNM), 55A, Cyt1A
	5	AtS2 (0)	34	38A/ 60B
	6	SoS4 (0)	48, 40, 27	(DNM), 55A, Cyt1A
	7	SoS7 (1)	103	1Af
6	1	AgS1 (2)		
	2	AtS3 (0)	50	
	3	SoS1 (0)	98	41B
	4	SoS3 (0)		
	5	SoS5 (0)	95	67A
	6	AgL1 (0)	57	Cyt1D
	7	JaL3 (1)	103	**1A**f
	8	SoL1 (0)	98, 65, 49	41B, (D1), (DNM)
	9	HD-73 (r- 1)	133, 57	1Ac, Cyt1D
7	1	AgS1 (2)	130, 90, 65	**1A**b/1D/4/7/**8**/9/61, 70B, (D1)
	2	JaS3 (1)	149, 130, 90, 27	43B, 1Ab, 70B, Cyt1A
	3	JaS8 (1)	103	**1A**f
	4	JSa1 (0)	100	(DNM)
	5	JSc1 (4)	132, 103, 90, 65	**1C**, **1A**f, (**D1**)
	6	JSc2 (ns)	130, 103, 65	1Ab/1D/4/7/8/9/61, 1Af, (D1)
	7	SSc2 (4)	130-5, 103, 65	**1A**(a-c) / **1A**f, (D1)
	8	SSe2 (3)	130-5, 103, 65	**1A**(a-c) /**1A**f, (D1)
	9	HD-73 (r- 1)	134, 65	1Ac, (D1), 6B/35A

	1	Sol1 (2)	130-5, 65	**1A**(a-c) /**9**A, (D1)
	2	JDb1 (6)	74, 65, 54, 29	**3**A/19/29/30/59, (D1), 6A, Cyt1/2
	3	JDc1 (4)	134, 27	**1A**, Cyt1
	4	JSc1 (4)	138	**9**G,
8	5	KSb1 (3)	134, 54, 27	**1A**(a-c)/1C, 6A, Cyt1
	6	MuSc4 (3)	134, 27	**1A**(a-c)/1C, Cyt1
	7	SSe2 (3)	134	**1A**(a-c)/1C, (D1), Cyt1, Cyt2
	8	SSc2 (4)	134, 65, 27, 26	**1A**(a-c)/7/8, (D1), Cyt2
	9	SSb1 (4)	134, 65, 26	**1A**(a-c)/7/8, (**D1**), Cyt2
	1	FhSb3 (5)	32, 27	46A, Cyt1
	2	JSd1 (4)	27	Cyt1
	3	RaSc2 (ns)	27	Cyt1
9	4	NaSc3 (4)	65, 60, 54, 29, 27	(D1), Cyt1C, 6A, Cyt1/2, Cyt1
	5	KSa2 (3)	27	Cyt1
	6	SaS7 (2)	27	Cyt1
	7	MeSa1 (3)	54, 40, 34.9, 27	6A, 55A, 60B, Cyt1
		DSh4 (2)		
		RaSc1 (3)	65, 29	(D1), Cyt1/2
		MuSc2 (4)	60, 54	Cyt1C, 6A
		RaSc1 (3)	65, 60, 54, 29, 27	(D1), Cyt1C, 6A, Cyt1/2, Cyt1
Not in figure		DSh4 (2)	103, 40, 29, 26	**1A**f, 55A, Cyt1/2, Cyt2
		CoS2 (2)	103, 74, 65, 35	**1A**f, 3A/3B/19/29/30/59, (D1), 60B
		JaS8 (1)	37, 30	
		NoS4 (2)	78, 72, 53, 29	**1**M, **2**B, 49A, Cyt1/2
		NaL1 (3)	140, 72, 65, 29	**1B**, **2**B, (**D1**), Cyt1/2

It was observed from this analysis that Cry1- type delta endotoxins were expressed in 26 indigenous *Bt* strains out of 50 tested. Cry1A- type proteins including Cry1Aa, 1Ab and 1Af were expressed in 24 of them, differentiation among which was done based on their molecular weight. Among them, 18 strains i.e. AgS1, CoS2, DSh4, FhSb3, JaL3, JaS8, JDb1, JDc1, JSc1, KSb1, MuSc4, SaS4, SoI1, SoS7, SSb1, SSb2, SSc2 and SSe2 were confirmed from their *cry* gene profiles. Cry1B- type protein was observed in the strain NaL1 which was also confirmed from its *cry* gene profile.

Cry1C- type proteins were observed in the *Bt* strains JDc1, JSc1, KSb1, MuSc4, RaSa2, SSc2 and SSe2 which was confirmed for the strains JDc1, JSc1, SSc2 and SSe2 based on their *cry* gene contents. Cry1M was also observed only in the *Bt* strain NoS4.

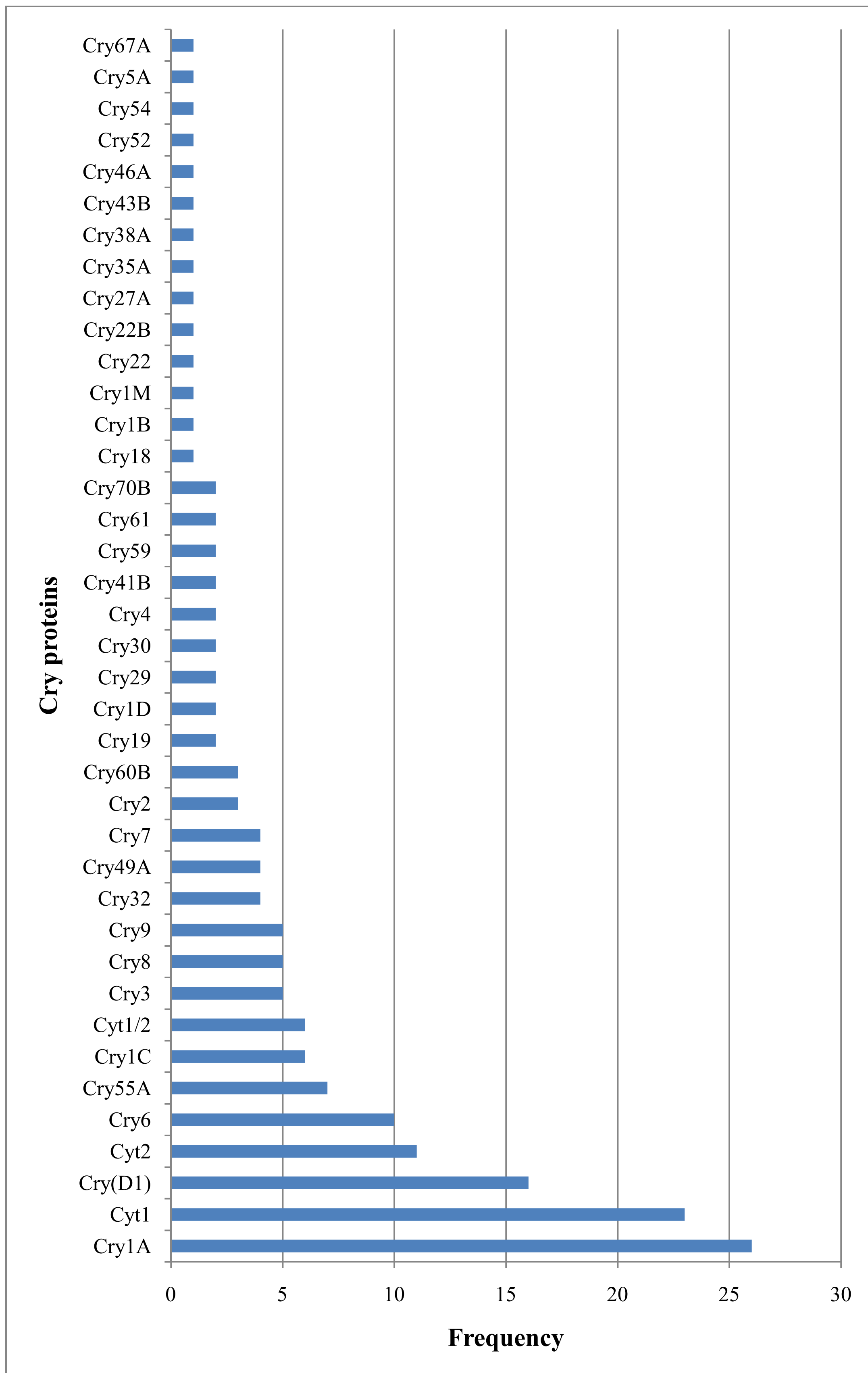

Figure 4.3.11: Frequency of the presumed different δ-endotoxins in 50 indigenous *Bt* strains.

Cry2- type proteins were observed in 3 strains, i.e. NaL1, KSa2 and NoS4 and these were also supported by the PCR results. Cry3- type proteins were confirmed in the strains CoS2, FhSb3, JDb1, SSb2 except CoS2 both by PCR detection of gene and presence of δ-endotoxin bands in SDS-PAGE analysis. Presence of Cry8- type proteins was confirmed in the strains AgS1 and SaS4 by both SDS-PAGE and PCR analysis. Cry9- type proteins were observed in 4 strains and 2 of them i.e. SoI1 and JSc1 were confirmed from their *cry* gene profile.

The denotation of D1 has been used for the 65- kDa protein which might be Cry2A- type protein or the degraded product of Cry1Aa-type protein (Arango *et al.*, 2002; Armengol *et al.*, 2007; Seifinejad *et al.*, 2008; Bukhari and Shakoori, 2010) and this has been shown as underlined bold character if confirmed by PCR for *cry2* gene. For other *cry1*, *cry2*, *cry3* and *cry9* gene positive strains, bands for Cry2- type δ-endotoxins were not observed which may be due to the little or no expression. The observation of protein bands with 57, 29, 27 and 26 kDa suggests the presence of Cyt proteins which may exert toxicity against Dipteran insect orders.

The prevalence of the δ-endotoxins of different molecular weights was analyzed among the 50 indigenous *Bt* strains studied in this purpose. Cry1A-type protein was found to be most prevalent followed by Cyt1-type δ-endotoxin, a 65- kDa protein which might be Cry2A- type protein or the degraded product of Cry1Aa-type protein, , Cyt2, Cry6, Cry55A, Cry1C etc (Fig 4.3.11).

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CHAPTER 5

Genetic diversity analysis of indigenous *Bacillus thuringiensis* strains by RAPD-PCR

Genetic diversity analysis of indigenous *Bacillus thuringiensis* strains by RAPD-PCR

5.1 Introduction

The key to the toxicity of *Bacillus thuringiensis* against the insect larvae is the specific molecular interactions of the insecticidal proteins with the membrane receptors followed by pore formation in the insect mid-gut epithelium. The degree and spectrum of toxicity of *Bt* insecticidal proteins against different insect species are variable. There are currently around 75 primary subgroups of Cry toxins, 3 for Cyt toxins and 4 for Vip toxins (Adang *et al.*, 2014) and more than 300 different members so far reported are present in these subgroups (Crickmore *et al.*, 2014). The remarkable diversity is because of a high degree of genetic plasticity or variations that occurs among the *Bt* strains due to many intrinsic factors like the presence of many different plasmids in each strain and their conjugal transfer, recombination between chromosomal DNA and plasmids, involvement of transposon-like inverted repeats flanking the endotoxin genes in high frequency causing DNA rearrangements etc and some extrinsic factors like mutation, nutritional influences etc (Kaur *et al.*, 2006). Genetic diversity among the *Bt* strains is, therefore, a blessing in view of its entomopathogenic phenomenon which also facilitates the chances of getting more and more *Bt* strains with novel toxicities and target spectrum.

Few additional virulence factors that enhance the toxicity of certain strains are also variable among the strains. The processing rate of Cry protoxin is also important for insect susceptibility which was evidenced with enhanced activity in engineered Cry toxins with increased activation rates compared to wild-type toxins (Walters *et al.*, 2010). These virulence factors are also subject to the variation due to the genetic diversities beside the insecticidal proteins.

Development of insect resistance against any insecticide is a common occurrence with no exception for *Bt* toxins. The facts behind the resistance and cross-resistance of insect pests to *Bt* toxins includes- i) reduction of binding of toxins to receptors in the mid-gut of insects, ii) reduced solubilisation of protoxin, iii) alteration of proteolytic processing of protoxins and iv) toxin degradation and or precipitation by proteases etc (Bruce *et al.*, 2007). To overcome the

resistance problems and to control a significant number of other pests, efforts should be continuing to discover more *Bt* strains with genetic variations, expressing novel and diverse toxins with improved activity besides protein engineering with the existing pool.

Hence, the genetic diversity analysis among the *Bt* strains is highly significant for maximum utilization of the resources and several different techniques were reported in this occasion, like M13 fingerprinting (Miteva *et al.*, 1991), arbitrary primer PCR (Brousseau *et al.*, 1993), PCR using conserved primers for 16S to 23S ribosomal intergenic spacer sequences (Bourque *et al.*, 1995), DNA hybridization using variable region of 16S rDNA (te Giffel *et al.*, 1997), AFLP fingerprinting, RAPD-PCR (Welsh and McClelland, 1990) etc. Ribotyping, either by PCR or DNA hybridization failed to detect the diversity among *Bt* strains which might be because of the use of one single gene or operon and the evolutionarily conserved nature of rDNA. However, when the whole genome was used for identification of *Bt* strains by M13 DNA fingerprinting and arbitrarily primed PCR considerable diversity among *Bt* serovars representing different serotypes had been detected. On the other hand, the detection of diversity among the *Bt* strains by RAPD-PCR technique is faster, less labor-intensive and more reliable in comparison to other molecular typing methods (Bostock *et al.,* 1993; Sikora *et al.,* 1997).

Considering the high relevance of these issues, the study of this chapter was designed to determine the genomic diversity among the 177 indigenous *Bt* strains, previously isolated and characterized from Bangladesh (as described in chapter 3) and to reveal the distribution pattern of *cry* genes in terms of their diversity.

5.2 Methods

5.2.1 Bacterial strains and growth conditions

Genomic diversity analysis was performed with 177 indigenous *Bt* strains along with the reference *Bt kurstaki* HD-73, *Bt sotto* T84A1 and *Bt japonensis* Buibui. LB agar and broth were used for culture maintenance, propagation and subculture throughout the study.

5.2.2 Total DNA preparation

Total DNA was prepared from the indigenous *Bt* isolates streaked on LB agar medium. (Bravo *et al.*, 1998) After 12 hours of incubation at 30°C, a single colony transferred into 100 µl of sterile de-ionized water in a microfuge tube was vortexed and kept at -70°C for 30 min. It was then incubated in boiling water for 10 min to lyse the cells and briefly centrifuged for 20 s at 12,000×g. The upper aqueous phase transferred into sterile microfuge tubes was used as template and preserved at -30°C for further use. 50-100 ng of DNA from this suspension was used as template in PCR-RAPD analysis.

5.2.3 RAPD-PCR analysis

RAPD-PCR was performed using the primers consisting of 10 bases with minimum 60% G+C content and devoid of any internal repeat (Kumar *et al.*, 2010). PCR was carried out within a reaction volume of 25 μl [1× PCR Master mix (Appendix A), 2.0 µM of primer (OPA 03: 5'- AGCTCAGCCA -3'), 50-100ng of template DNA] in a thermal cycler (Appendix D) by 35 cycles (95 ºC for 1 min, 40 ºC for 1 min, 72ºC for 1 min) with an initial denaturation step at 95º C for 4 min and a final extension step at 72ºC for 15 min (Kumar *et al.*, 2010). PCR products (15µl) were then analyzed in 1.5% (w/v) agarose gel by horizontal electrophoresis at 60V for 1h in 1× TBE (89 mM Tris (pH 7.6), 89 mM boric acid, 2 mM EDTA) buffer and gel images were captured after visualization against UV trans-illumination in a gel documentation system (Appendix D) following staining in (EtBr) solution and de-staining in distilled water. Molecular weight of the DNA bands in those gels was then determined by using Alphaview SA software (version 3.4.0.0).

5.2.4 Data analysis and dendrogram construction

Binary matrix was prepared for each strain from the gel images based on the presence or absence as scored 1 or 0 respectively for 16 individual bands (100, 150, 200, 225, 275, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000 and 1100 bp). Based on the binary matrices, similarity and distance matrices were calculated following dice coefficient method. These data were used in cluster analysis by UPGMA method to construct the dendrograms (http://insilico.ehu.es/dice_upgma/). For maximum accuracy of comparison, all isolates were processed with the same batch of PCR master mix.

5.2.5 Genotyping and estimation of diversity index

Throughout the whole study, threshold level was chosen at 0.2 in the scale bar. Each cluster was considered a separate genotype if distances among the strains in that cluster were less than 0.2 in scale bar. Thus the genotypes were identified among the tested strains as a whole and also in terms of their biotypes and location. Again, the ratio between the number of clusters and isolates for a set of strains was considered as their diversity index. Based on this criterion, the diversity index for each of the biotypes and locations were estimated and compared.

5.2.7 Determination of distribution of *cry* genes in the genotypes

Detection of *cry* genes belonging to the primary subgroups such as *cry1*, *cry2, cry3, cry4*, *cry8*, *cry9* and *cry10* as well as to the secondary and tertiary subgroups of *cry1* primary subgroup such as *cry1Aa*, *cry1Ac*, *cry1Ba*, *cry1Ca* was performed with 230 *Bt* strains (Table 4.3.1). Combining the results for all these genes, *cry* gene profile was obtained for each strain. Again, each strain for its RAPD profile belongs to a certain genotype. Thus, the number of *cry* genes in each genotype was determined and their distribution was determined.

6.3 Results

5.3.2 RAPD profile based genotyping

A total of 177 *Bt* strains was employed for RAPD-PCR amplification with decamer OPA 03 that produced random amplified polymorphic DNA bands among the strains. These bands were then compared with the DNA standard (100 bp DNA ladder, Bioneer) and RAPD profiles of the strains were found to be comprised of 0- 11 bands at varied numbers and varied combinations (Fig 5.3.1). By scrutinizing the molecular weight of the bands for different strains, binary matrices could be obtained for each strains based on 16 different molecular weights (100, 150, 200, 225, 275, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000 and 1100 bp).

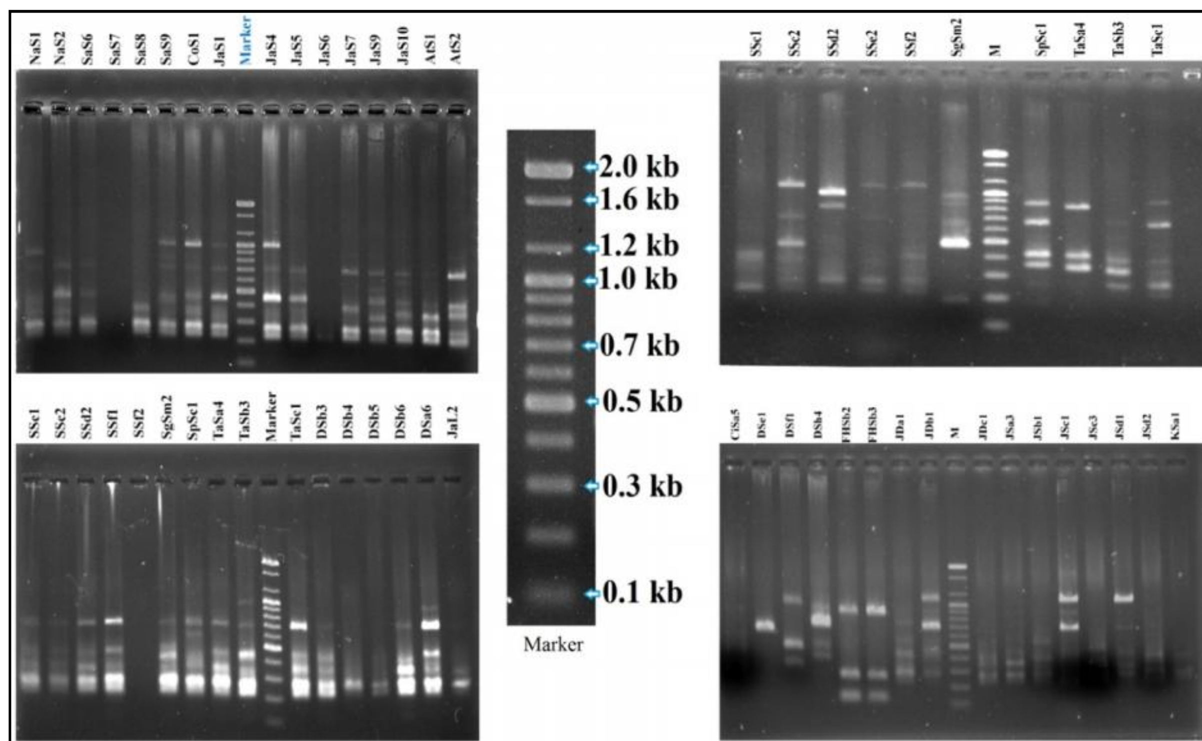


Figure 5.3.1: Representative gel images showing random amplified polymorphic DNA (RAPD) produced from *Bt* strains with the primer OPA 03. Binary matrix for each strain was prepared from these gels based on the presence or absence of bands. (Marker: 100 bp DNA ladder, Bioneer).

While obtaining binary matrices, 15 strains were found to produce no amplicon. Polymorphism based on these 16 individual bands was calculated and 100% polymorphism was not observed in any strain. Maximum 68.8% polymorphism was seen in 0.4% of the strains tested whereas 25% polymorphism (16.1% of test strains) was most prevalent followed by 31.3% (15.3% of test strains) and 18.8% (13.1% of test strains) (Table 5.3.1).

Table 5.3.1: Prevalence of polymorphism diversity in the indigenous *Bt* strains (n=177).

Polymorphism (%)	6.3	12.5	18.8	25	31.3	37.5	43.8	50	56.3	62.5	68.8	75	81.3	87.5	93.8	100
Prevalence (%)	2.5	10.6	13.1	16.1	15.3	12.3	12.7	5.5	3.8	1.3	0.4	0	0	0	0	0

Dendrogram was constructed by UPGMA clustering method with the RAPD profile of all isolates (Fig 5.3.2). The binary matrix of each strain is the numerical RAPD profile which was used to prepare the distance matrix and similarity matrix among the strains by dice coefficient comparison method. From the dendrogram analysis, the *Bt* strains were found to be divided into two major clusters, cluster A and cluster B (Fig 5.3.2). Cluster B was found to be smaller and A was larger further divided into smaller sub-clusters A1 and A2. Sub-cluster A2 was large enough and found to be further branched into small clusters with significant number of strains denoted as A2a and A2b.

Again as binary matrix was prepared for each strain based on the 16 polymorphic DNA bands, (16^2 =) 256 numbers of different banding patterns are possible. So, the genetic varieties in this study could be more than the number of isolates. Thus, quantitative comparison of genetic diversities among different sets of strains e.g. biotypes or locations became possible upon standardization. The height of the clades in scale bar, an indication of distance among the strains could be standardized in this case. The height of the start point of branching was found at 0.45 in the scale bar. A middle height at 0.2 was therefore considered as the threshold level to distinguish the clusters as separate genotype throughout the whole study. Thus heights of 15 clades (clusters) were found to be more than 0.2 and these were considered as separate genotypes (Fig 5.3.2). Among them, genotype 2, 4 and 10 were simplicifolious (single leafed), genotype 14 was bifolious (two leaved), genotype 8 and 15 were trifolious (three leaved) and rest others were polyfolious (more than three leaved). Genotype 9 and 11 were the largest, each containing more than 25% of the strains (Fig 5.3.3).

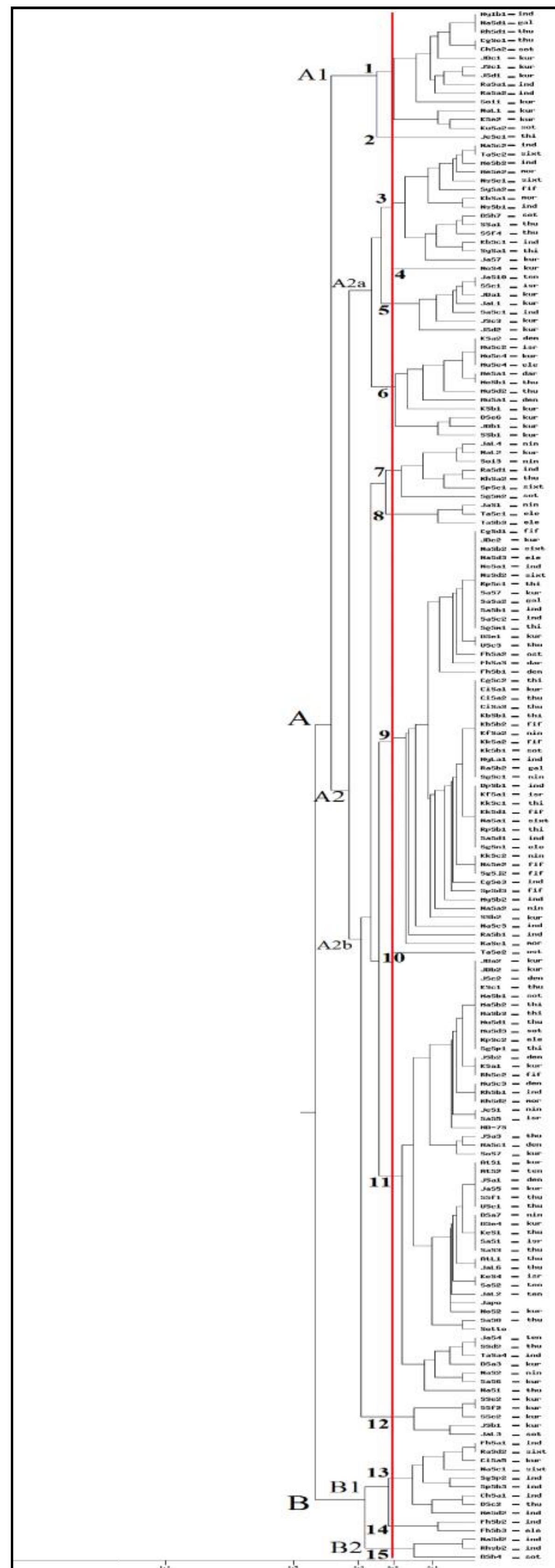


Figure 5.3.2: Dendrogram exhibiting the genetic distance among the selected strains of *Bacillus thuringiensis* based on their RAPD-PCR patterns in the range from 100 bp to 1.1 kb as compared using the Dice coefficient and the UPGMA clustering algorithm.

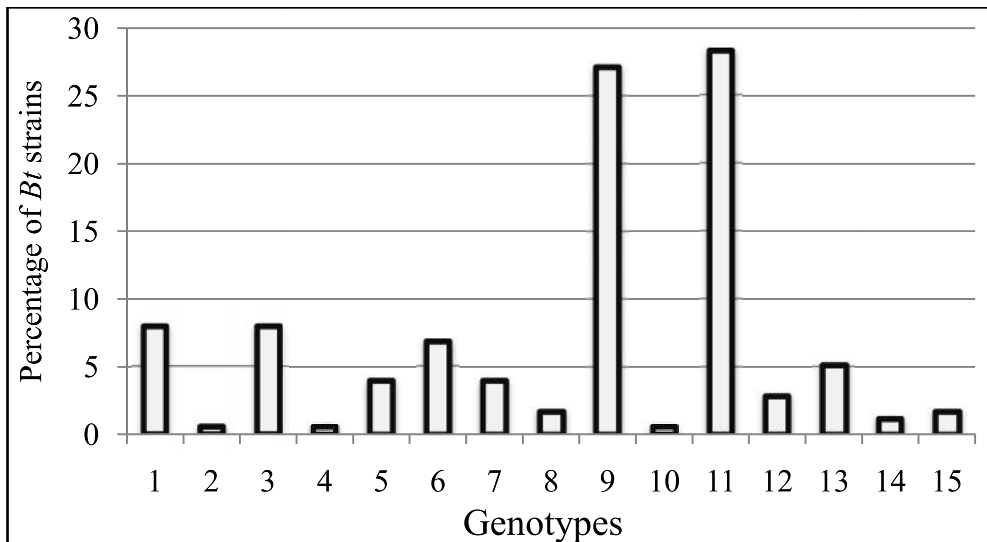


Figure 5.3.3: Prevalence of different genotypes among the indigenous *Bt* strains of Bangladesh.

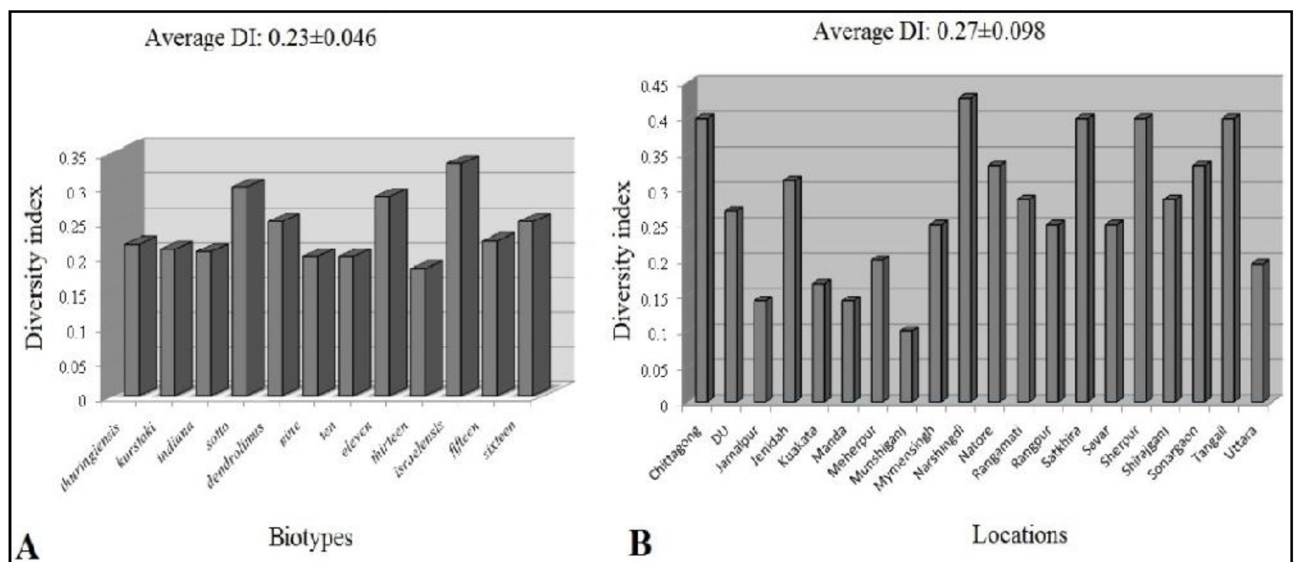


Figure 5.3.4: Comparison of diversity indices (DI) as calculated based on the ratio of number of clusters beyond the threshold level and number of strains for A) the selected biotypes B) the selected locations.

5.3.3 Comparison of diversity between biotypes and locations

Based on this threshold height or distance, the diversity indices (DI) were calculated, as the ratio between the number of clusters and the number of strains, within biotypes and locations. In case of biotypes, genetic diversity was maximum in *Bt israelensis* followed by *sotto*, and eleven and minimum was in biotype 13, ten and nine (Fig 5.3.4A). In case of the locations,

maximum diversity was observed among the strains of Narshingdi and the minimum was for Munshiganj (Fig 5.3.4B). The average diversity index for locations was 0.27±0.098 which was higher than that for biotypes (0.23±0.046).

5.3.4 Pattern of distribution for *cry* genes

As the genotypes and *cry* gene profiles of the strains were thus retrieved, it was analyzed whether the distribution of *cry* genes is random or genotype oriented. So, the distribution of different *cry* genes in different genotypes was analyzed and from the graphical presentation (Fig 5.3.5) it was found that *cry* genes were present in all genotypes except genotype 10. The abundances of the *cry* genes were maximums in genotypes 1, 6, 9, 11 and 12. Though genotype 9 and 11 were found to be the largest containing more than 25% of the strains, only genotype 9 of them was significant with different *cry* genes besides genotypes 1 and 6 as compared to the number of strains. Comparing the ratio between the number of *cry* genes and strains in the genotypes, genotype 6 (2.167) was found to be most significant followed by genotype 1 (1.285), genotype 9 (0.29), genotype 11 (0.18) and genotype 3 (0.14). On the other hand, 6 types of *cry* genes were present in genotypes 1, 6, 9 and 11.

5.3.5 Comparison between different similarity parameters

Another comparison was performed with 20 *Bt* strains (indigenous- 19, reference- 1) in terms of their 16S rRNA gene sequence based phylogeny, Biotype, RAPD based genotype and number of available *cry* genes (Fig 5.3.6). Phylogenetically close 12 strains as in sub-cluster A1 were observed to have similar biochemical properties since from the same biotype *kurstaki* except strain DSf3 (non-hemolytic), strain CiSa5 (biotype ten) and KSa2 (*dendrolimus*). Again in sub-cluster A2, 3 strains out of 5 were non- hemolytic and 2 out of 3 strains in cluster B were from biotype *kurstaki*. Though the biochemical properties of most of them conformed to the phylogenetic relatedness, their RAPD genotypes were variable e.g. five genotypes (1, 5, 6, 11 and 12) were visible among the strains of sub-cluster A1 which were from the biotype *kurstaki*. This genetic diversity might be caused due to the presence of many different plasmids in each strain, conjugation transfer mechanism and the transposon-like inverted repeats flanking the endotoxin genes and facilitating a high frequency of DNA rearrangements in variable regions.

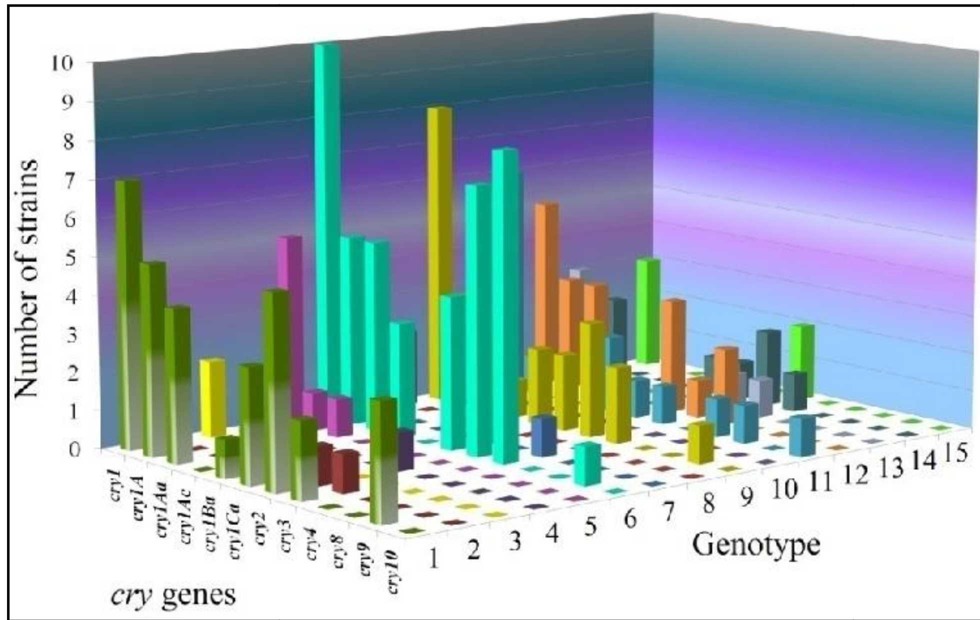
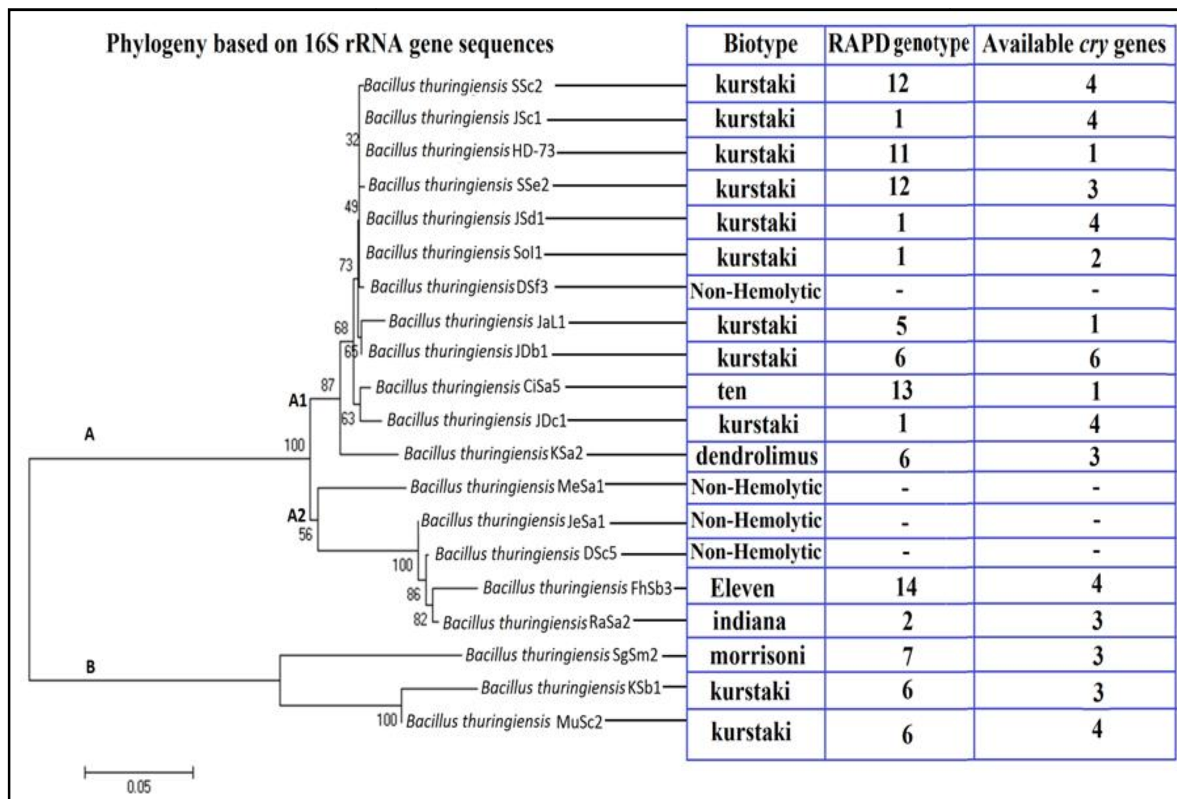


Figure 5.3.5: Distribution of different *cry* genes in the different genotypes established for the *Bt* strains by RAPD-PCR method. Prevalence of different genes in each genotype is indicated with same colored column i.e. color varied with genotypes not genes.



Strain	Biotype	RAPD genotype	Available *cry* genes
Bacillus thuringiensis SSc2	kurstaki	12	4
Bacillus thuringiensis JSc1	kurstaki	1	4
Bacillus thuringiensis HD-73	kurstaki	11	1
Bacillus thuringiensis SSe2	kurstaki	12	3
Bacillus thuringiensis JSd1	kurstaki	1	4
Bacillus thuringiensis Sol1	kurstaki	1	2
Bacillus thuringiensis DSf3	Non-Hemolytic	-	-
Bacillus thuringiensis JaL1	kurstaki	5	1
Bacillus thuringiensis JDb1	kurstaki	6	6
Bacillus thuringiensis CiSa5	ten	13	1
Bacillus thuringiensis JDc1	kurstaki	1	4
Bacillus thuringiensis KSa2	dendrolimus	6	3
Bacillus thuringiensis MeSa1	Non-Hemolytic	-	-
Bacillus thuringiensis JeSa1	Non-Hemolytic	-	-
Bacillus thuringiensis DSc5	Non-Hemolytic	-	-
Bacillus thuringiensis FhSb3	Eleven	14	4
Bacillus thuringiensis RaSa2	indiana	2	3
Bacillus thuringiensis SgSm2	morrisoni	7	3
Bacillus thuringiensis KSb1	kurstaki	6	3
Bacillus thuringiensis MuSc2	kurstaki	6	4

Figure 5.3.6: Comparison of relatedness between the *Bt* strains determined by 16S rRNA gene sequence, biochemical properties, RAPD genotyping and availability of *cry* genes.

On the other hand, correlation persisted for the highly conserved phenotypes like biochemical properties and genotypes such as 16S rRNA etc. The number of available *cry* genes among these strains was also variable. It can, therefore, be said that the report of conformity between phylogenetic and phenotypic i.e. biotype or serotype (biotype in this case) relatedness was also evidenced in this study though RAPD- genotyping and *cry* gene profile did not follow the pattern.

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CHAPTER 6

In-vivo toxicity study of potential indigenous *Bacillus thuringiensis* strains against *Bactrocera cucurbitae*

In-vivo toxicity study of potential indigenous *Bacillus thuringiensis* strains against *Bactrocera cucurbitae*

6.1 INTRODUCTION

Bacillus thuringiensis (*Bt*) biopesticides, for their highly specific mode of actions, are the key components of Integrated Pest Management (IPM) strategies aimed at preserving natural enemies of pests and managing insect resistance (Kumar *et al.*, 2008). *Bt* biopesticides are eco-friendly as free of recalcitrant residues which upon bioaccumulation and biomagnifications might become carcinogenic, mutagenic, teratogenic or allergenic etc (Zahm *et al.*, 1997). Hence, *Bt* biopesticides have served as valuable alternatives of synthetic chemical pesticides in agriculture, forestry and mosquito control for last many decades (Mohan and Gujar, 2000).

Bangladesh, a country of subtropical climate, produces different vegetables covering an area of ca. 498,073 acres. But the yield per unit area is low as 25% annual yield losses occurs in vegetables due to the pests alone (Rahman, 2000). More than 200 major species of insects and mites of different field crops, fruit trees, and stored products have been recorded from Bangladesh (Rahman, 2000). The melon fruit fly, *B. cucurbitae* (Diptera: Tephritidae), is one of the widely distributed and detrimental vegetable pests damaging about 81 host plants (Hollingsworth and Allwood, 2000) mainly from cucurbitaceous crops (Dhillon *et al.*, 2005) and it causes significant losses in different cucurbits (includes cucumber, melon, watermelon, squash, pumpkin, gourds etc) of Bangladesh too. Female melon fly is capable of destroying large numbers of fruits in its lifespan of 10 months to a year as one may deposit up to 1,000 eggs in soft tender fruit tissues by piercing them with the ovipositor (Mohan and Gujar, 2000). Maggots feed inside the fruits, flowers and stems and young larvae cause necrotic regions, often introducing various pathogens and hasten fruit decomposition (Dhillon *et al.*, 2005).

A lot of control measures were reported against melon fruit fly such as bagging of fruit, field sanitation, monitoring and control with parapheromone lures/cue-lure traps, host plant resistance, chemical control, wide area management, male-sterile technique, transgene based

embryo-specific lethality system, quarantine and also biological control (Dhillon *et al.*, 2005). In Bangladesh, with no exception, chemical insecticides are the major and mostly administered control measure for melon fruit fly. Continuous and indiscriminate use of these insecticides for their long residual action and toxicity to a wide spectrum of insects has polluted the environment largely by bioaccumulation, bio-magnification etc and led to the emergence of resistance in agricultural pests and vectors of human diseases simultaneously (Marrone and MacIntosh, 1993). So, eco-friendly pest management or organic farming is an undeniable necessity in the agriculture (Frankenhuyzen 1993; Margalit *et al.*, 1995; Salehi *et al.*, 2005) and *Bt* biopesticide, therefore, should be the most appropriate choice.

Although mortality of few species from genus *Bactrocera* were reported to be caused by the δ- endotoxins of *Bt*, no such development was found against *B. cucurbitae* (Ansari *et al.*, 2012). Worldwide continuous screening programs for new strains with different combinations of crystal proteins as well as the discovery of new toxins have broadened the activity spectrum of *Bt* toxins. The study of this chapter was therefore designed combining in vitro and in vivo molecular techniques with a view to finding potential *Bt* strains, toxic against *B. cucurbitae* (melon fruit fly).

6.2 METHODS

6.2.1 Insect rearing

Larvae were maintained on locally developed semi liquid artificial diet. Adult melon flies were stocked in a stainless steel framed cage (120×120×90 cm) covered with stainless iron net. The culture was usually supplied with a laboratory diet (yeast extract: casein: sugar- 1: 1: 2) and water soaked cotton. In general, 2000-2500 adult fruit flies were maintained in a stock cage. Temperature and relative humidity (RH) of the rearing room were maintained at 28±2°C and 70-80% respectively. To collect huge numbers of eggs the matured flies in the cage were provided with a piece of sweet gourd for ovi-position. The piece of sweet gourd was removed after 2 hours from the adult cage and placed in a plastic bowl with sawdust for further larval development.

6.2.2 Preparation of spore-crystal mixture

Spore-crystal mixture for bioassay was prepared from the twenty nine *cry1* gene positive isolates and three reference strains by inoculating them in 100ml of T_3-liquid medium (Obeidat *et al.*, 2004) and incubating for 5 days at 30°C with continuous shaking at 250 rpm. Cultures were centrifuged at 5000 rpm for 15 min to separate the culture from medium. Pellets (spores and crystal protein mixture) were washed twice with 20 ml of cold sterile distilled water and centrifuged at 5000 rpm for 5 min. The pellets were re-suspended in 20 mL of sterile distilled water and incubated for another 2 days at 30°C with continuous shaking at 250 rpm and used for bioassay (Fig 6.2.1).

6.2.3 Estimation of spore count

The spore counting was performed by taking 1.0 ml of spore-crystal suspension and treating with heat at 80°C for 10 min. The heat treated suspension was then serially diluted and plated on the LB agar medium by spread plate technique. The plates were then incubated at 37°C for 24 hrs. The spores survived the heat but all other vegetative cells were killed in this process. So, the colonies that were germinated from the spores after incubation were counted and multiplied by the dilution factor to estimate the actual number.

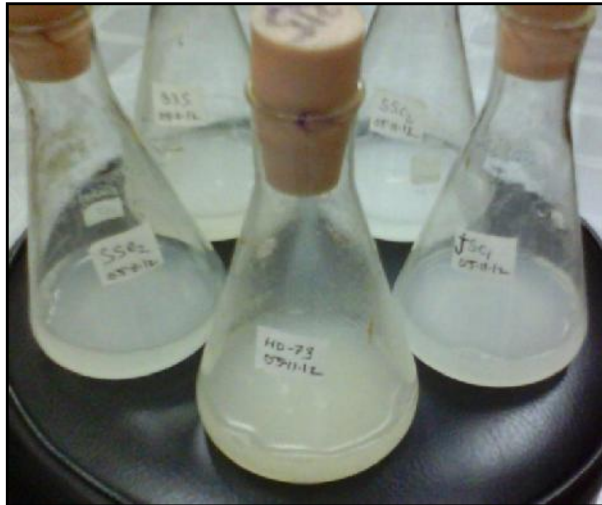


Fig 6.2.1: Spore-crystal protein mixture prepared for bioassay.

6.2.4 Bioassay

The toxicity of the *Bt* strains was analyzed in vivo against the 3rd instar larvae of melon fruit fly, *B. cucurbitae* by bioassay. The mortality of the larvae, fed on the processed diet in which spore-crystal mixture of different *Bt* strains was mixed, was recorded and their efficacy was statistically analyzed. In this regard, larval diet was prepared by boiling sweet gourd to avoid fungal contamination and mashing them into paste (Fig 6.2.2A & B). Then, 1.0 ml of spore-crystal suspension of the *Bt* strains were mixed with 10 g of the sweet gourd paste (Fig 6.2.2C) and 20 larvae were placed in each petridish and the plates were incubated in an incubator maintaining temperature at 28±2°C and relative humidity (RH) at 70±10%, with a photoperiod of 16:8 (L: D). The mortality of the larvae, fed on this processed diet, was scored up to 7 days for each *Bt* strain in parallel a control diet supplemented with sterile distilled water to correct the test mortality using Abbot's formula (Daffonchio *et al.*, 1998).

6.2.5 Data collection and statistical analysis

Bioassay was consisted of two steps including screening for potential strains in the initial step and determination of lethal concentration values in the final step for the strains causing more than 50% mortality.

Figure 6.2.2: A) *Bt* spore-crystal was mixed with previously boiled 10 g of sweet gourd mash at room temperature measured in each plate. **B)** Larvae were transferred into the plates to feed the diet being mixed with spore-crystal protein of *Bt*. **C)** Diet supplemented with *Bt* preparation for *Bactrocera cucurbitae* larvae to be fed in the bioassay.

In the initial step, undiluted spore-crystal suspension was mixed with the larval diet standardizing the volume (keeping approximately equal spore) for each strain and numbers of dead larvae were recorded. It was performed in triplicate and the average percentage of mortality was determined for each strains. *Bt* strains causing more than 40% mortality were tested again in the same manner and strains exerting death to more than 50% of the larvae were selected for the next step.

In the final step, the spore-crystal suspension of *Bt* strains, with average percentage of mortality more than 50%, was serially diluted in sterile distilled water up to 2^{-1}, 2^{-2} and 2^{-3} folds and mixed with the diet. Number of dead larvae in different concentrations of different treatments (spore-crystal mixture of *Bt* strains) were recorded for further analysis to determine LC_{50} and LC_{99} values and It was also performed in triplicate. As the concentration of spores in the suspension was the basis for estimation of Lethal Concentration values, logarithmic scale was used for simplification. From the data of dead larvae for different treatments, concentrations causing death of 50% and 99% of larvae were determined by Probit Analysis- Finney Method [Lognormal Distribution] for the treatments using Statplus 2009 software for Windows. All bioassays were repeated 3 times, and means were analyzed using one-way analysis of variance (ANOVA) and compared as least significant differences (LSD). The acceptance level of statistical significance was $\alpha=0.05$.

6.2.6 Identification of putative factors of toxicity

To identify the factors behind the variation in toxicity, the conserved regions of *cry1* gene from the potential strains were purified, sequenced as described in section 3.2.2.12 and 3.2.2.13 and the sequences were aligned by ClustalW using BioEdit software (version 7.2.5). On the other hand, the *cry* gene profiles of the strains were compared with the data from section 4.3.2 and Cry protein profiles of respective strains were also compared with the data from section 4.3.4. Thus, reference *Btk* HD-73, *Bts* T84A1 and indigenous *Bt* strains JSc1, SSc2 and SSe2 which exhibited significant toxicity against *B. cucurbitae* were analyzed simultaneously.

6.3 RESULTS

6.3.1 Bioassay

The melon fruit fly, *B. cucurbitae* belong to the insect order Diptera: Tephritidae. Usually, Dipteran insects are susceptible to the Cry2, Cry4, Cry10 and Cry11 δ-endotoxins. It was also reported that Cry1Ab and Cry1Ac proteins exert toxicity against few insect from the order Diptera (The Canadian Forest Service: http://cfs.nrcan.gc.ca/projects/119/6). Again there are many reports of concomitant occurrence of *cry1* and *cry2* genes (Ben-Dov *et al.*, 1997; Zhang *et al.*, 2000; Wang *et al.*, 2003). So, the *Bt* strains that were detected with *cry1* gene, were selected for bioassay in this study. Thus the bioassay was performed with 40 indigenous *Bt* strains, 2 reference strains and a control.

As the *Bt* preparations collected from the culture medium were resuspended in sterile distilled water and the incubation period was extended for another 2 days in the same condition, complete sporulation was attained and the crystal proteins were completely dissociated or released from the spore as revealed under Phase Contrast Microscope (Fig 6.3.1). It was observed from the Phase Contrast Microscopy that Cry proteins of different types were present in different *Bt* strains. So, the estimation of Cry protein concentration would not necessarily represent the concentration of the functional ones i.e. Cry proteins exerting the toxicity. With this view in mind, spore concentration, as a universal parameter, was employed to compare the efficiency among different strains in Bioassay (Fig 6.3.2).

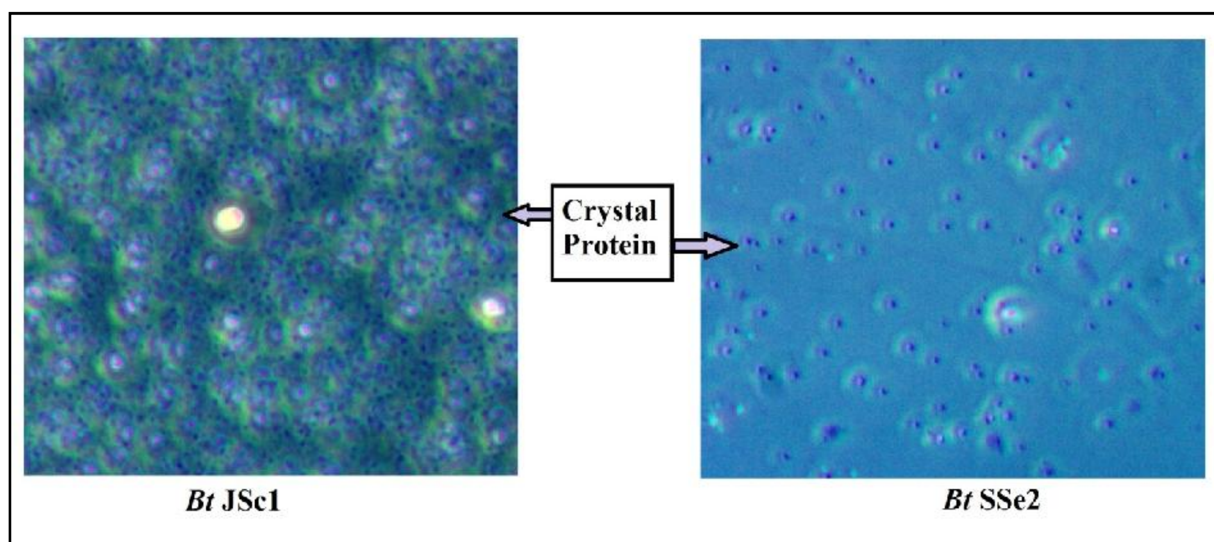


Figure 6.3.1: Presence of crystal proteins in the *Bt* preparations that was mixed in the processed diet for bioassay.

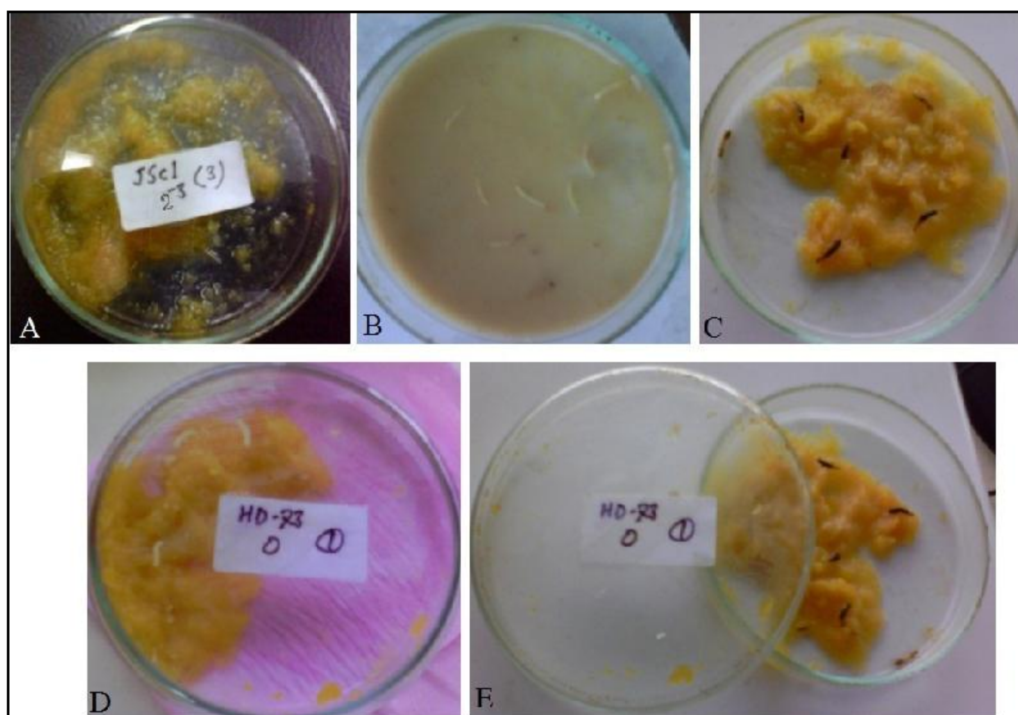


Figure 6.3.2: Observation at different stages of Bioassay. **A)** Larvae were transferred to *Bt* preparation mixed diet. **B)** After 3 days, larvae stopped movement if not touched. **C)** After 7 days larvae turned into black- indication of death. **D)** Bioassay with *Btk* HD-73- Day 1, **E)** Dead larvae- at day 7.

The screening for potential *Bt* strains were performed in the 1st phase of the bioassay and in this initial study, 17 indigenous strains out of 40 were found to cause more than or equal to 30% mortality of the larvae tested (Fig 6.3.3). The assay was performed in triplicate for each strain and the assay for all strains was not performed simultaneously due to the unavailability of the larvae and work volume. But it was a continuous process and the results of the bioassays were compiled and compared based on the control and reference strains.

Among the two reference strains used, *Btk* HD-73 was found to cause significant toxicity. Some larvae were found to die in control i.e. without any treatment which was used to correct the test mortalities. Then, six indigenous *Bt* strains JaS8, JSa1, JSc1, JSc2, SSc2 and SSe2 causing more than 40% mortality (Fig 6.3.3) were retested together beside reference *Btk* HD-73 and *Bts* T84A1 (Fig 6.3.4 A & B).

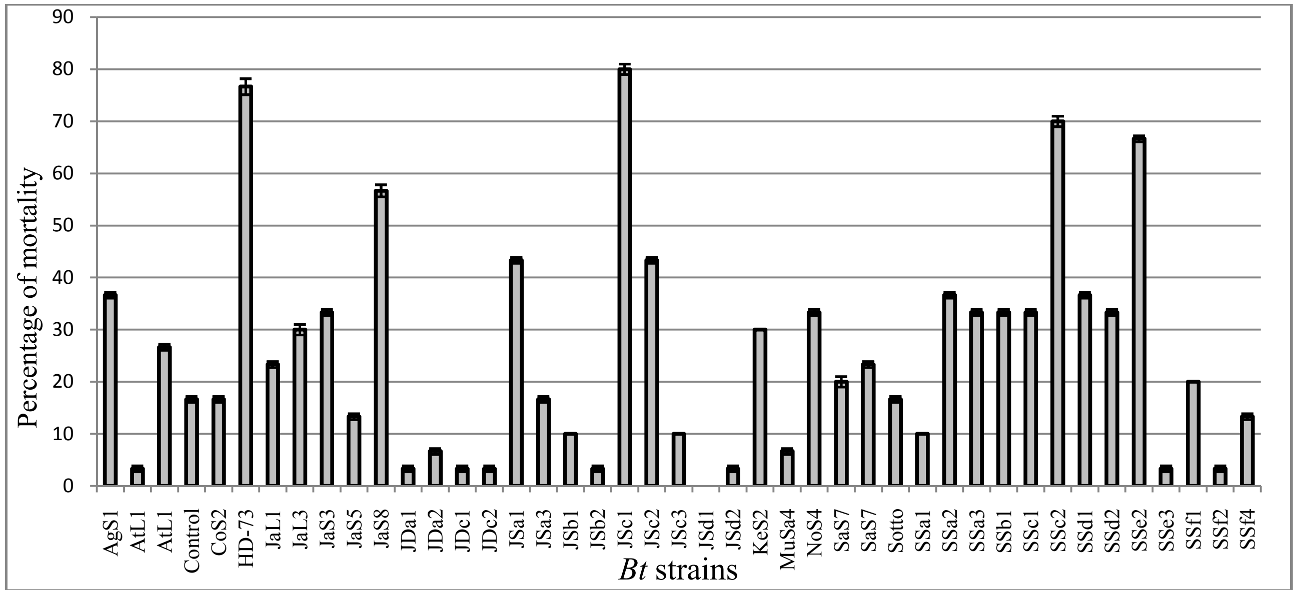


Figure 6.3.3: Rate of mortality caused by the spore-crystal protein mixture of different *Bt* strains.

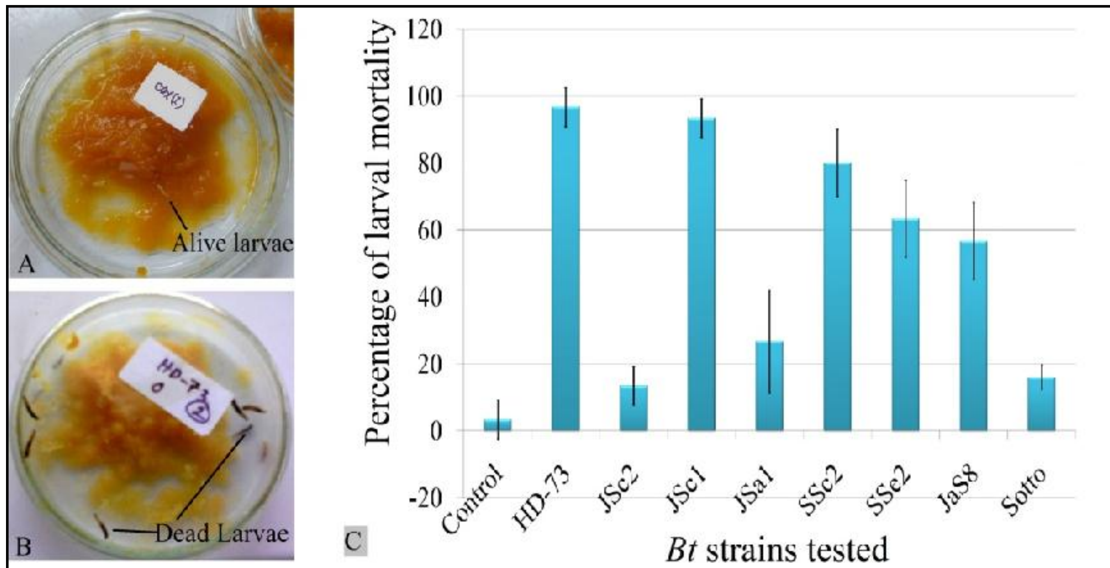


Figure 6.3.4: Bioassay performed with 3rd instar larvae of *B. cucurbitae*. Fate of the larvae that were fed with the diet supplemented with A) Sterile distilled water, B) Spore- Cry protein mixture of *Bt*. C) Efficiency of the indigenous and reference *Bt* strains in causing death to the test larvae.

These *Bt* strains were again found to exhibit significant toxicity against the 3rd instar larvae of *B. cucurbitae* (Fig 6.3.4C). Mortalities were recorded this time as JSc1-93%, SSc2- 80%, SSe2- 63% and JaS8- 56% among the indigenous *Bt* strains while reference strain, *Btk* HD-73 exhibited 96% mortality and *Bts* T84A1 only 16% (Fig 6.3.4C).

6.3.2 Determination of lethal concentrations (LC)

Then, the bioassay was repeated with the *Bt* strains causing more than 50% mortality for the determination of LC_{50} and LC_{99} values. In this connection, original spore-crystal mixture was diluted for each strain up to 2^{-1}, 2^{-2} and 2^{-3} dilutions. The spore concentration for each strain was calculated in logarithmic scale and the mortality for each concentration were recorded (Table 6.3.1).

Table 6.3.1: Mortality of *B. cucurbitae* larvae at different spore concentrations by *Bt* strains

Log_{10} [spore count/ml]	Average mortality caused by the *Bt* strains (n)					Test larvae (n)
	HD-73	JSc1	SSc2	SSe2	JaS8	
7.778	20	19.33	16	12.66	11.33	20
7.477	13.33	14.66	6.66	11.33	5.33	20
7.176	10.66	12.66	4	4.66	2.66	20
6.875	8	11.33	2	1.33	1.8	20

This was performed in triplicate and the average value of the mortality of larvae was considered for probit analysis. Thus for each spore concentration, Probit Analysis - Finney Method [Lognormal Distribution] was performed with the α-value 0.05 (for confidence interval) using the software Statplus 2009.

Table 6.3.2: LC_{50} and LC_{99} values estimated for indigenous and reference *Bt* strains.

Strains	LC_{50}	LC_{99}	X_2	Df	*p*-level
***Bt* SSc2**	7.54	8.55	1.589	2	0.451
***Bt* SSe2**	7.56	8.67	1.019	2	0.600
***Bt* JSc1**	6.89	8.60	0.512	2	0.774
***Bt* JaS8**	7.75	8.87	0.303	2	0.859
***Btk* HD-73**	7.10	8.25	1.033	2	0.596

LC_{50} and LC_{99}: log (spore concentration ml^{-1}). **X_2:** Chi-square; **Df**: Degree of freedom

The LC_{50} and LC_{99} values varied from 6.89 to 7.75 and from 8.25 to 8.87 respectively (Table 5.3.2). The lowest LC_{50} value was observed for the indigenous *Bt* strain JSc1 (LC_{50}- 6.89) indicated the highest potency in causing death of 50% of the larvae whereas for reference *Btk* strain HD-73, LC_{50} was 7.12 i.e. approximately 0.3 log higher spore concentration was required in this case. On the other hand, *Bt* strain SSc2 (LC_{50}- 7.54) and SSe2 (LC_{50}- 7.56) demonstrated comparable LC_{50} values and JaS8 exhibited the maximum spore requirements

(LC_{50}- 7.75). For LC_{99} values i.e. causing 99% larval mortality, reference *Btk* strain HD-73 was found to be the most potentials while *Bt* strain JSc1 (LC_{99}- 8.60) was very close to that (LC_{99}- 8.25).

6.3.3 Analysis of Variance (One-Way)

Table 6.3.3: Statistical values of mortality of *B. cucurbitae* caused by different *Bt* strains. Calculation was excerpted from the ANOVA (One- Way)

SUMMARY:

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
HD-73	4	52	13	755.555
JaS8	4	21.133	5.283	167.239
JSc1	4	58	14.5	877.777
SSc2	4	28.666	7.166	320.444
SSe2	4	30	7.5	312.444
Total	20		9.489	33.276

ANOVA:

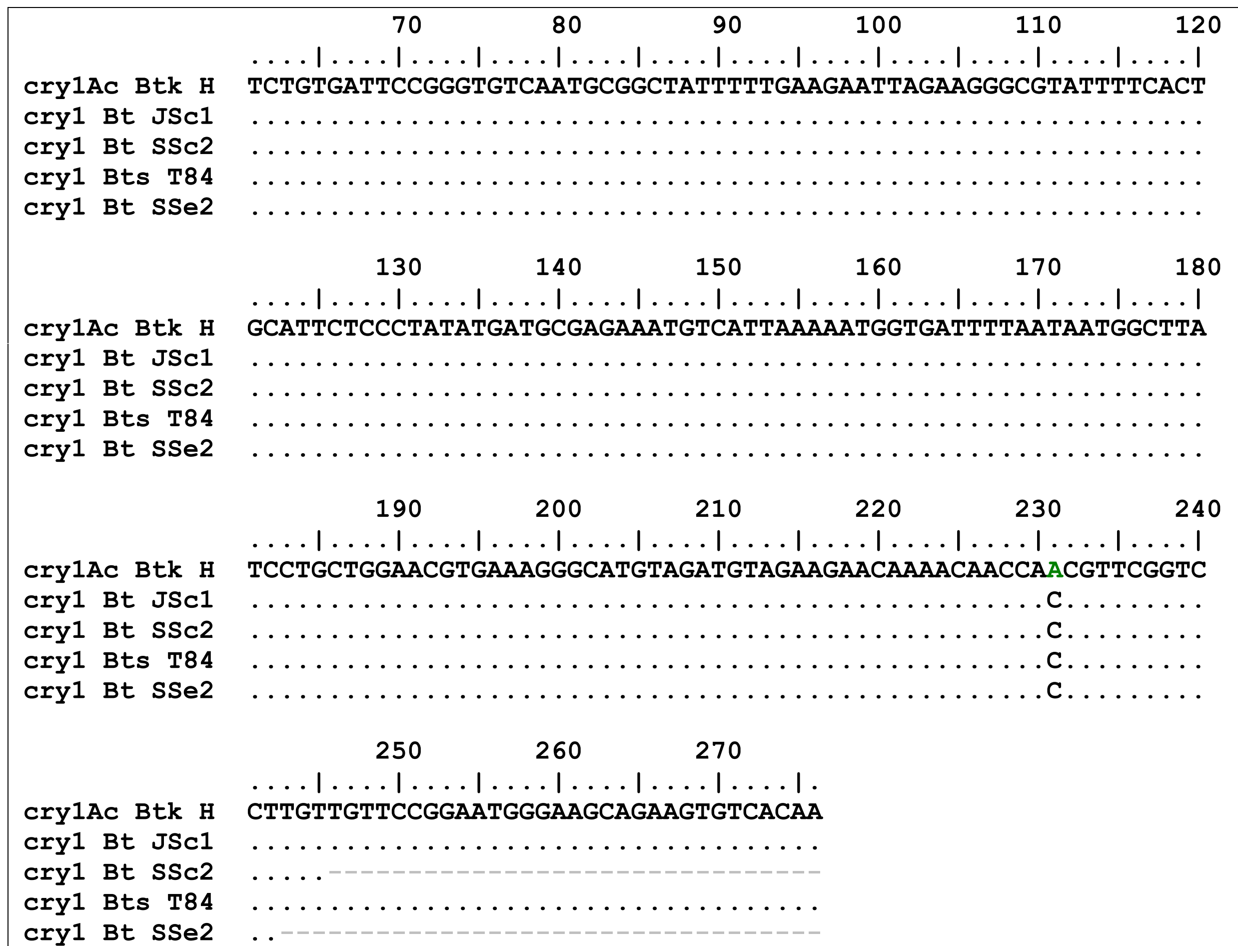
Source of Variation	*d.f.*	*SS*	*MS*	*F*	*p-level*	*F crit*	*Omega Sqr.*
Between Groups	4	257.896	64.474	2.583	0.07969	3.0555	0.2405
Within Groups	15	374.363	24.957				
Total	19	632.260					

Statistical data obtained from the ANOVA (One- Way) produces an F value indicating that at α=0.05 the larval mortality does not significantly deviate from the average mortality throughout the bioassay replicates (F= 2.583; df=19 ; P=0.07969).

6.3.4 Causes of toxicity and its variation based on the gene and protein profile

It was observed from the alignment of sequences of *cry1* conserved region, obtained from the indigenous *Bt* strain JSc1, SSc2, SSe2 and two reference strains *Btk* HD-73 and *Bts* T84A1 that the indigenous strains were more similar to of *Bts* T84A1. Mismatches were observed at 24 and 231 base positions with *Btk* HD-73 (Fig 6.3.5).

```
                       10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
cry1 Btk HD73  ATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAGCTTATCTGCCTGAGCTG
cry1 Bt JSc1  .......................C....................................
cry1 Bt SSc2  .......................C....................................
cry1 Bts T84  .......................C....................................
cry1 Bt SSe2  .......................C....................................
```



```
                     70        80        90       100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
cry1Ac Btk H    TCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAGAAGGGCGTATTTTCACT
cry1 Bt JSc1    ............................................................
cry1 Bt SSc2    ............................................................
cry1 Bts T84    ............................................................
cry1 Bt SSe2    ............................................................

                    130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|
cry1Ac Btk H    GCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTGATTTTAATAATGGCTTA
cry1 Bt JSc1    ............................................................
cry1 Bt SSc2    ............................................................
cry1 Bts T84    ............................................................
cry1 Bt SSe2    ............................................................

                    190       200       210       220       230       240
                ....|....|....|....|....|....|....|....|....|....|....|....|
cry1Ac Btk H    TCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAAACAACCAACGTTCGGTC
cry1 Bt JSc1    ..................................................C.........
cry1 Bt SSc2    ..................................................C.........
cry1 Bts T84    ..................................................C.........
cry1 Bt SSe2    ..................................................C.........

                    250       260       270
                ....|....|....|....|....|....|....|.
cry1Ac Btk H    CTTGTTGTTCCGGAATGGGAAGCAGAAGTGTCACAA
cry1 Bt JSc1    ....................................
cry1 Bt SSc2    .....-------------------------------
cry1 Bts T84    ....................................
cry1 Bt SSe2    ..----------------------------------
```

Figure 6.3.5: Alignment of the sequences of *cry1* genes (conserved regions) of indigenous and reference *Bt* strains. Mismatches at 24 and 231 base positions with *Btk* HD-73.

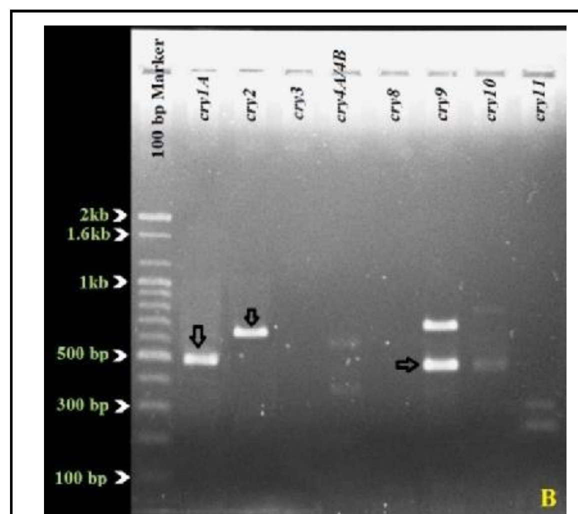


Figure 6.3.6: Product size of causative *cry* genes in *Bt* JSc1 rendering toxicity against melon fruit fly. Expected amplicons of approximately 490, 639 and 492 bp were observed in case of detecting *cry1A*, cry2 and *cry9* genes as indicated with arrows whereas spurious amplicons of

about 564 and 365 for *cry4A/4B*, 667 for *cry9*, 480, 700 bp for *cry10* and 200, 270 for *cry11* genes were observed.

Upon agarose gel electrophoresis of the PCR products, the presence of *cry1*, *cry2, cry3* and *cry9* genes in the indigenous *Bt* strains was revealed whereas reference *Btk* HD-73 (*cry1Ac*) and *Bts* T84A1 (*cry1Aa*) were positive for only *cry1* gene. *Bt* JSc1 was found to harbour *cry1*, *cry2* and *cry9* genes (Fig 6.3.6) whereas *Bt* SSc2 and SSe2 harbour *cry1* and *cry3* genes (Table 6.3.4). The correlation between the *cry* genes profile and LC_{50} values of these *Bt* strains was observed in this study (Table 6.3.4). Thus, the presence of *cry2* and *cry9* genes has a positive effect on the toxicity as the LC_{50} value for *Bt* JSc1 was found to be the lowest among all the *cry1* gene harbouring *Bt* strains. The effect of *cry3* gene was also observed since the toxicity was found to be more intense in strains SSc2 and SSe2 than JaS8 that lacked the genes. The toxicity of *Bt* strains exhibited against *B. cucurbitae* can therefore be concluded as the synergistic effects of *cry1*, *cry2*, *cry3* and *cry9* gene products.

Table 6.3.4: Comparison between the *cry* gene profiles of *Bt* strains tested in bioassay

Strains	*cry1*	*cry2*	*cry3*	*cry4A/4B*	*cry8*	*cry9*	*cry10*	*cry11*	LC_{50}
Bt SSc2	✓	✕	✓	✕	✕	✕	✕	✕	7.54
Bt SSe2	✓	✕	✓	✕	✕	✕	✕	✕	7.56
Bt JSc1	✓	✓	✕	✕	✕	✓	✕	✕	6.89
Bt JaS8	✓	✕	✕	✕	✕	✕	✕	✕	7.75
Btk HD-73	✓	✕	✕	✕	✕	✕	✕	✕	7.10
Bts T84A1	✓	✕	✕	✕	✕	✕	✕	✕	-

6.3.5 Cry protein profile of the potential *Bt* strains

SDS-PAGE analysis (Fig 6.3.7) of the partially purified Cry proteins revealed that diversified Cry proteins are synthesized by the indigenous *Bt* strains at different expression level. Based on the molecular weight of the proteins (Table 6.3.5), Cry1, Cry2, Cry3 and Cry9 proteins can be presumed to be expressed. From the analysis, common 23, 45, 50 kDa protein bands were observed for all *Bt* strains which were not considered as Cry proteins. Another 67 kDa common band was observed in all *Bt* strains except strain JaS8 which might be the degraded product of Cry1 protein (Armengol *et al.*, 2007). An unusual but prominent band at 103 kDa

was common in all indigenous strains. A faint band in the range of 130-140 kDa was observed for *Bt* JSc1 which can be both Cry1 and Cry9 protein whose expression level seems to be low. The molecular weight of Cry1Ac protein of reference *Btk* HD-73 was determined 133 kDa.

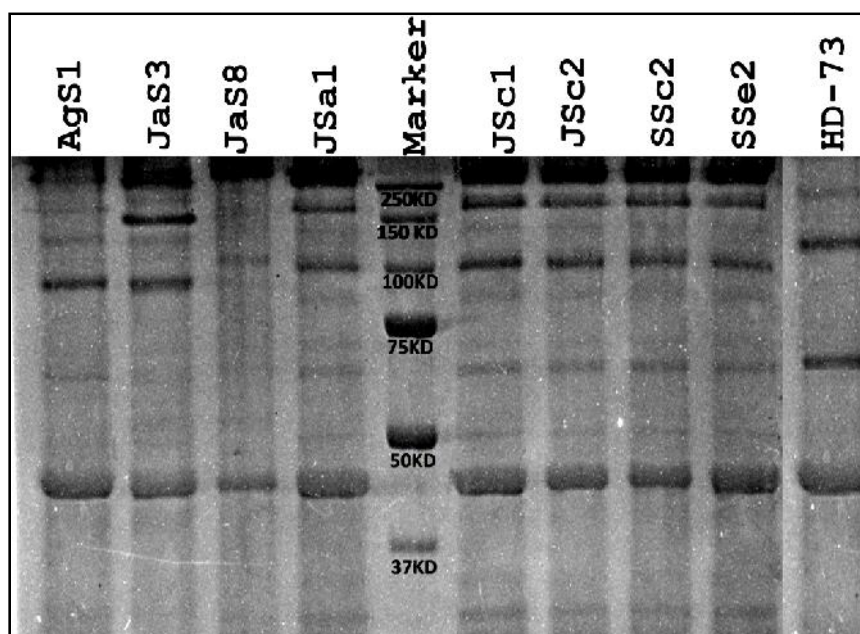


Figure 6.3.7: SDS-PAGE analysis of partially purified Cry proteins of *Bt* strains. Lanes have been labeled with the names of the strains. (HD-73: Reference strain *Btk* HD-73) (**M**: Precision plus protein standards, All blue, Appendix A).

Table 6.3.5: Determination of MW of the partially purified Cry proteins

Strain	MW of the visible protein bands (kDa)	Presumptive Cry proteins
Bt SSc2	66, 73, 103, 134	Cry1, Cry3
Bt SSe2	66, 73, 103, 134	Cry1, Cry3
Bt JSc1	66, 73, 103, 134	Cry1, Cry2, Cry9
Bt JaS8	56, 103	Cry1
Btk HD-73	66, 134	Cry1Ac

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CHAPTER 7

Analysis of *cry1Aa*-type gene Open Reading Frame (ORF) from indigenous *Bt* strain JSc1 retrieved by PCR walking

Analysis of *cry1Aa*-type gene Open Reading Frame (ORF) from indigenous *B. thuringiensis* JSc1 retrieved by PCR walking

7.1 Introduction

The novel insecticidal genes of *Bacillus thuringiensis* were reported to be identified by different approaches (Kaur, 2002). A cumbersome and time-consuming traditional process in order to identify a novel *cry* gene was insect bioassay followed by cloning which might result already known *cry* genes although found to be highly toxic against a target insect (Theunis *et al.*, 1998). Southern hybridization, another approach, used for the identification of homologous *cry* genes (Kronstad and Whiteley, 1986) as the sequence homology among different *cry* gene families were 45, 70, 95% for primary, secondary and tertiary ranks and specific probes were designed in this connection. However, it became cumbersome as well as slow with the increase of newly discovered *cry* genes. Since homologous probes for the entire known *cry* genes would have to be used for detection, a mixture of various *cry* gene sequences has been used as probe for detection of diverse cry genes in a single step to improve efficiency of this method (Beard *et al.*, 2001).

PCR-based methods have been developed for the identification of *Bt* isolates with novel gene profiles (Porcar and Juarez-Perez, 2003) which facilitated large scale, first-tier screening by its high sensitivity and rapidness. *Bt* strains harboring novel and also the less frequently observed *cry* genes have been identified by PCR using specially designed primers targeting the highly conserved regions (Bravo *et al.*, 1998; Porcar and Juarez- Perez, 2003; Beard *et al.*, 2001; Tounsi *et al.*, 2003; Wang *et al.*, 2003).

An elegant method based on the combination of PCR and restriction fragment length polymorphism (RFLP) strategies was designed to detect novel *cry* genes (Kuo and Chak, 1996). However, when more than four *cry* genes are present in a strain, the restriction profile may become difficult to analyze. Also, a high degree of similarity among the *cry* genes from secondary or tertiary subgroups may make it difficult to detect differences among these genes. A second PCR, using alternative primers, which recognize other regions of the genes, followed by restriction analysis and a long electrophoresis run in order to achieve a better resolution of restriction fragments can overcome this problem. Unexpected restriction

products may denote the presence of a new type of gene. A novel *cry1A*-type gene was detected in a *Bt* isolate from China by using a combination of PCR and restriction analysis of the amplified products (Wang *et al.*, 2003). A novel *cry1Ie1* gene was also identified from a *Bt* isolate by the combined PCR-RFLP technique (Song *et al.*, 2003).

Another PCR strategy known as exclusive PCR (E-PCR), has been devised for detection of novel genes, in which the amplification of already known *cry1* genes is followed by a second conditional amplification using three primers, that will occur only if a new putative *cry1* gene(s) is present in the strains (Juarez-Perez *et al.*, 1997). This method uses several specific primers designed to recognize only one type of *cry* gene and 'universal' primers designed to detect *cry* gene families in a multiplex PCR. A single universal primer is combined with many specific oligonucleotide primers that recognize individual *cry* genes. The universal primers are designed to have degeneracy of sequence in order to increase the probability of amplification of sequences having low homology within the gene family.

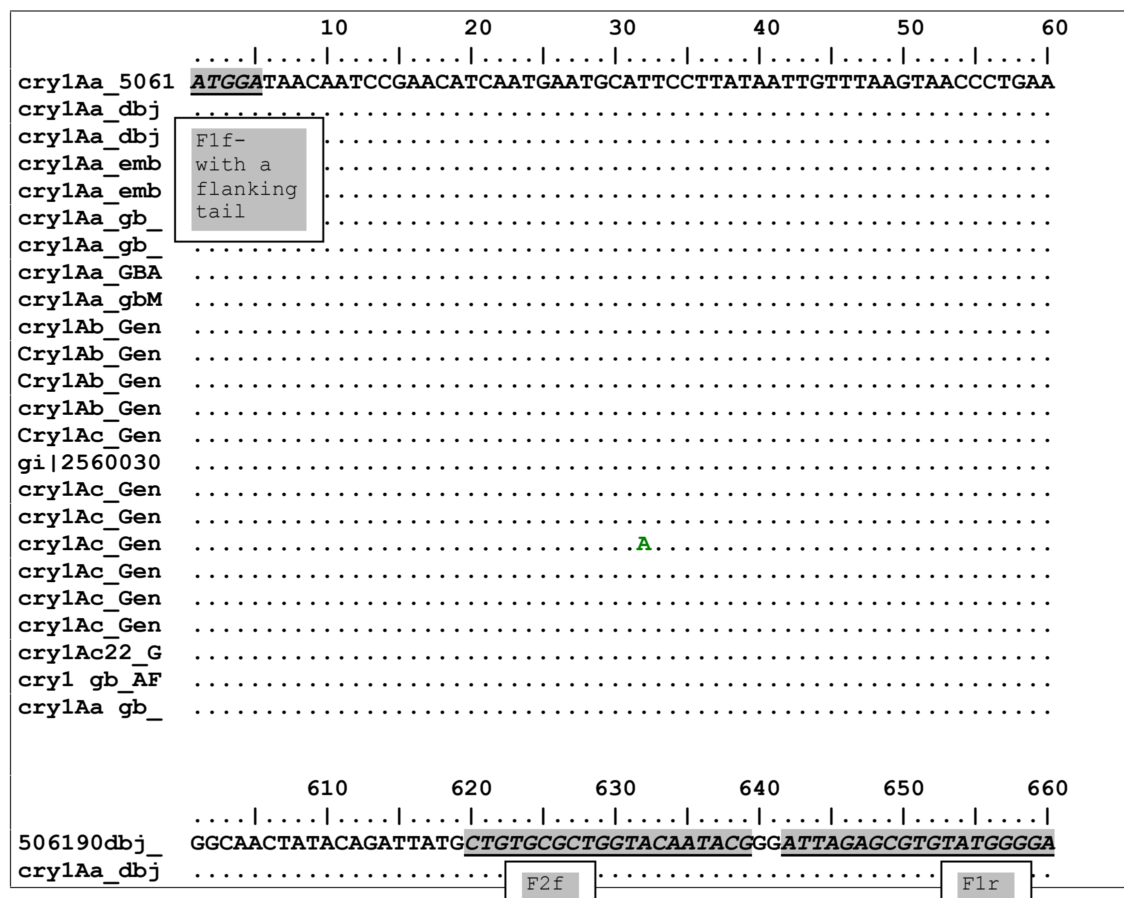
A 'PCR walking' strategy was devised for the identification of variants of specific *cry* genes, by employing a series of primers designed to anneal throughout the length of *cry* gene sequence in a single multiplex PCR reaction (Kalman *et al.*, 1993). A modification of the expected PCR profile would indicate the presence of a new *cry* gene. This method was used for detection of the *cry1Cb1* gene. However, this method has only limited application as it is restricted to closely related genes within the same group and relatively large number of primers is required to analyze each group of genes. A novel *cry1Ab18* gene was cloned from a *Bt* isolate by a PCR-based strategy using a set of three highly specific oligonucleotide primers designed to amplify full-length open reading frame (ORF) of all the known *cry1Aa*, *cry1Ab* and *cry1Ac* genes in our laboratory (Stobdan *et al.*, 2004).

Indigenous *Bt* strain JSc1 harboring *cry1A*-type gene as identified by PCR detection and sequence analysis was found to exert significant toxicity against the larvae of *Bactrocera cucurbitae*. To retrieve the sequence of the open reading frame of the *cry1A*-type gene, PCR walking strategy was employed in this study and six sets of primers were designed for six overlapping fragments chosen from the alignment of the available *cry1Aa*, *cry1Ab* and *cry1Ac* genes.

7.2 Methods

7.2.1 Oligonucleotide Primer Designing

The oligonucleotide primers to amplify the overlapping fragments of *cry1Aa*-type gene were designed in this study. The first step, in this regard, was to align the available sequences of *cry1Aa*-type genes to identify the conserved regions based on what the overlapping fragments could be chosen. The sequences of the *cry1Aa*, *cry1Ab* and *cry1Ac* genes were obtained from the full list of δ-endotoxin (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/) which are linked to NCBI database and the sequences were aligned using ClustalW program (Fig 7.2.1). The conserved regions of the sequences were identified from the alignment and overlapping fragments were chosen in such a manner that the primer binding sites remain in the conserved regions and amplification targets were limited to 500- 800 bp. Again the forward primer of the fragment-1 and reverse primer of fragment-6 were chosen aligning respectively the available 5' and 3' flanking regions from *cry1Aa*, *cry1Ab* and *cry1Ac* genes (Fig 7.2.2 and Fig 7.2.3 respectively) so that the complete ORF could be retrieved. Primer pairs were then designed targeting the chosen regions by a web-based primer designing program (http://www.ncbi.nlm.nih.gov/tools/primer-blast).



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cry1Aa_dbj   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_GBA   ............................................................
cry1Aa_gbM   ............................................................
cry1Ab_Gen   ...............C.......A....................................
Cry1Ab_Gen   ............................................................
Cry1Ab_Gen   ...............C.......A....................................
cry1Ab_Gen   ...............C.......A....................................
Cry1Ac_Gen   .......................A.......................A............
gi|2560030   .......................A.......................A............
cry1Ac_Gen   .......................A.......................A............
cry1Ac_Gen   .......................A.......................A............
cry1Ac_Gen   ............................................................
cry1Ac_Gen   ............................................................
cry1Ac_Gen   .......................A.......................A............
cry1Ac_Gen   .......................A.......................A............
cry1Ac22_G   .......................A.......................A............
cry1 gb_AF   ............................................................
cry1Aa gb_   ............................................................

                      670       680       690       700       710       720
             ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_   CCGGATTCTAGAGATTGGGTAAGGTATAATCAATTTAGAAGAGAGCTAACACTTACTGTA
cry1Aa_dbj   ............................................................
cry1Aa_dbj   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_GBA   ............................................................
cry1Aa_gbM   ............................................................
cry1Ab_Gen   ..................A....A....................AT.......A......
Cry1Ab_Gen   ............................................................
Cry1Ab_Gen   ..................A....A....................AT.......A......
cry1Ab_Gen   .......T..........A....A....................AT.......A......
Cry1Ac_Gen   ............................................AT.......A......
gi|2560030   ............................................AT.......A......
cry1Ac_Gen   ............................................AT.......A......
cry1Ac_Gen   ............................................AT.......A......
cry1Ac_Gen   .....................................................A......
cry1Ac_Gen   ............................................................
cry1Ac_Gen   ............................................AT.......A......
cry1Ac_Gen   ............................................AT.......A......
cry1Ac22_G   .....................................C......AT.......A......
cry1 gb_AF   ............................................................
cry1Aa gb_   ............................................................

                     1210      1220      1230      1240      1250      1260
             ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_   TCCACTATATATAGACAAAGGGGTACAGTCGATTCACTAGATGTAATACCGCCACAGGAT
cry1Aa_dbj   ............................................................
cry1Aa_dbj   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_GBA   ............................................................
cry1Aa_gbM   ............................................................
cry1Ab_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
Cry1Ab_Gen   ............................................................
Cry1Ab_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1Ab_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
Cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
gi|2560030   ...G..G....C...A....C..A..G..A.....G..G....A......A......A..
```

F3f

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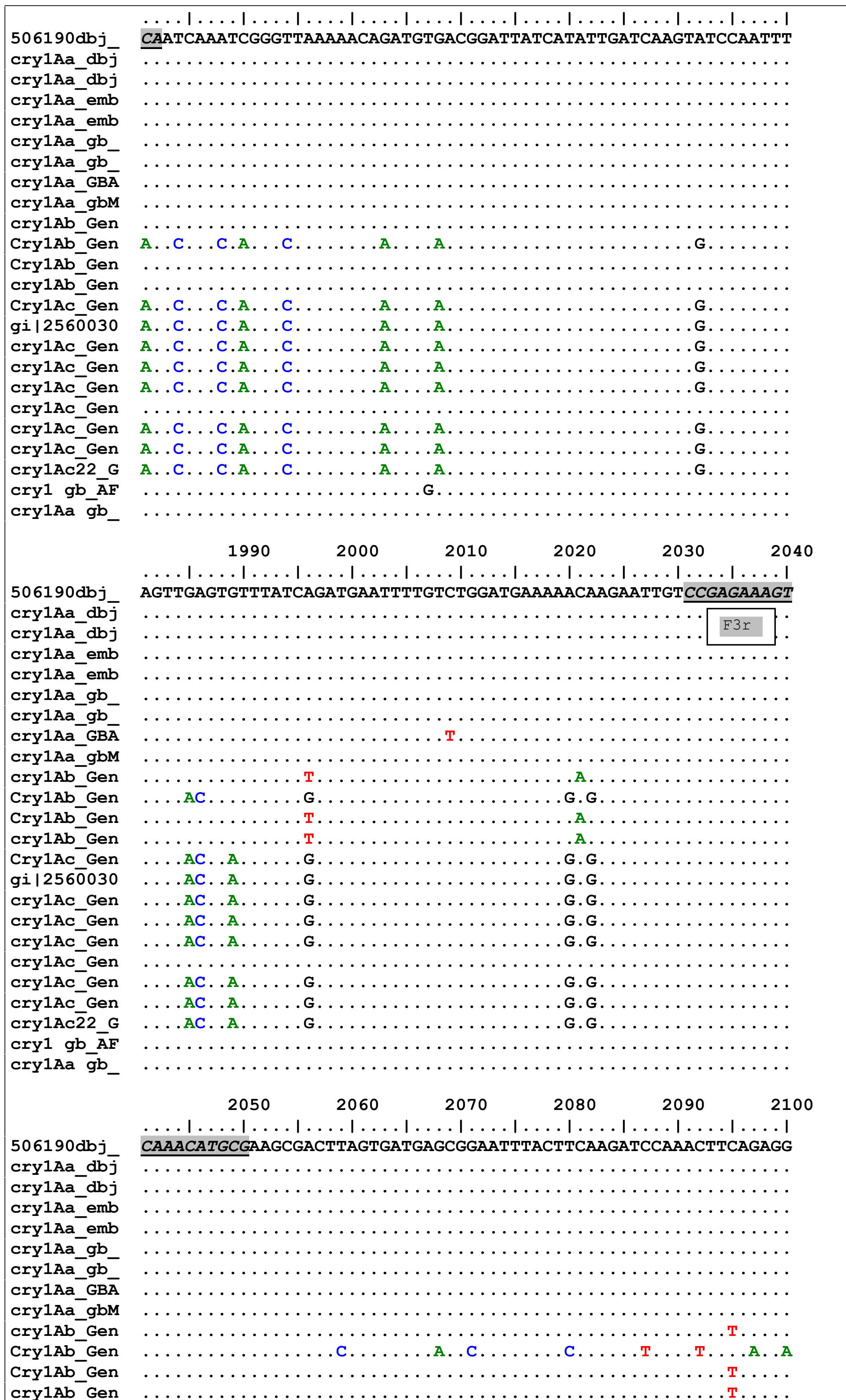
cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A......A......A..
cry1Ac_Gen   ............................................................
cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1Ac_Gen   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1Ac22_G   ...G..G....C...A....C..A..G..A.....G..G....A.............A..
cry1 gb_AF   ............................................................
cry1Aa gb_   ............................................................

                       1330      1340      1350      1360      1370      1380
             ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_   CAAG----CAGCTGGAGCAGTTTACACCT--TGAGAGCTCCAACGTTTTCTTGGCAGCAT
cry1Aa_dbj   ....----.......................--...........................
cry1Aa_dbj   ....----.......................--...........................
                                 F2r
cry1Aa_emb   ....----.......................--...........................
cry1Aa_emb   ....----.......................--...........................
cry1Aa_gb_   ....----.......................--...........................
cry1Aa_gb_   ....----.......................--...........................
cry1Aa_GBA   ....----.......................--...........................
cry1Aa_gbM   ....----.......................--...........................
cry1Ab_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
Cry1Ab_Gen   ....CAGCTG.----.........--.ACCT.............................
Cry1Ab_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ab_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
Cry1Ac_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
gi|2560030   TC..GCTCT..TA---.T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac_Gen   TC..GCTCT..TA---.T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac_Gen   ....----.......................--...........................
cry1Ac_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac_Gen   TC..GCTTT..TAAT..T...G..AGTA.AA.A........T.T...C......ATA...
cry1Ac22_G   TC..GCTTT..TAAT..T...G..AGTA.AA..........T.T...C......ATA...
cry1 gb_AF   ....----.......................--......T..CT....CT.C...TAG..
cry1Aa gb_   ....----.......................--...........................

                       1870      1880      1890      1900      1910      1920
             ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_   TGAGGCAGAATATGATTTAGAAAGAGCACAAAAGGCGGTGAATGAGCTGTTTACTTCTTC
cry1Aa_dbj   ............................................................
cry1Aa_dbj   ............................................................
                                                           F4f
cry1Aa_emb   ............................................................
cry1Aa_emb   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_gb_   ............................................................
cry1Aa_GBA   ............................................................
cry1Aa_gbM   ............................................................
cry1Ab_Gen   ............................................................
Cry1Ab_Gen   C.....T......A..C.G........G..G.............C.........G...A.
Cry1Ab_Gen   ............................................................
cry1Ab_Gen   ............................................................
Cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
gi|2560030   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac_Gen   ............................................................
cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac_Gen   C.....T......A..C.G........G..G.............C.........G...A.
cry1Ac22_G   C.....T......A..C.G........G..G.............C.........G...A.
cry1 gb_AF   .................................C.GC......C................
cry1Aa gb_   ............................................................

                       1930      1940      1950      1960      1970      1980
```



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Cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
gi|2560030    ..................C........A..C........C......T....T....A..A
cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
cry1Ac_Gen    ..................C........A..C........C......T....T....A..A
cry1Ac22_G    ..................C........A..C........C......T....T....A..A
cry1 gb_AF    ............................................................
cry1Aa gb_    ............................................................

                       2530      2540      2550      2560      2570      2580
              ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_    CATTGATGTAGGATGTACAGACTTAAATGAGGACCTAGGTGTATGGGTGATCTTTAAGAT
cry1Aa_dbj    .........T................[ F5f ].T................A..C.....
cry1Aa_dbj    ..........................       ..........................
cry1Aa_emb    ............................................................
cry1Aa_emb    ............................................................
cry1Aa_gb_    ............................................................
cry1Aa_gb_    ............................................................
cry1Aa_GBA    ............................................................
cry1Aa_gbM    ............................................................
cry1Ab_Gen    .........T........................T................A..C.....
Cry1Ab_Gen    .................................TT....C...........A..C.....
Cry1Ab_Gen    .........T........................T................A..C.....
cry1Ab_Gen    .........T........................T................A..C.....
Cry1Ac_Gen    ............................................................
gi|2560030    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ..................................T.........................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac22_G    ............................................................
cry1 gb_AF    ............................................................
cry1Aa gb_    ............................................................

                       2590      2600      2610      2620      2630      2640
              ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_    TAAGACGCAAGATGGGCACGCAAGACTAGGGAATCTAGAGTTTCTCGAAGAGAAACCATT
cry1Aa_dbj    ...[ F4r ].....C..T...........A........A....................
cry1Aa_dbj    ...       ..................................................
cry1Aa_emb    ............................................................
cry1Aa_emb    ............................................................
cry1Aa_gb_    ......................................................G.....
cry1Aa_gb_    ............................................................
cry1Aa_GBA    ............................................................
cry1Aa_gbM    ............................................................
cry1Ab_Gen    ...............C..T...........A........A....................
Cry1Ab_Gen    ......A........C..T..G.A.A....A........A..............G.TT..
Cry1Ab_Gen    ...............C..T...........A........A....................
cry1Ab_Gen    ...............C..T...........A........A....................
Cry1Ac_Gen    ............................................................
gi|2560030    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac_Gen    ............................................................
cry1Ac22_G    ............................................................
cry1 gb_AF    ......CG....................................................
cry1Aa gb_    ............................................................
```

```
                     3070      3080      3090      3100      3110      3120
                ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_      GGAATGGGAAGCAGAAGTGTCACAAGAAGTTCGTGTCTGTCCGGGTCGTGGCTATATCCT
cry1Aa_dbj      ............................................................
cry1Aa_dbj      ............................................................
cry1Aa_emb      ............................................................
cry1Aa_emb      ............................................................
cry1Aa_gb_      ............................................................
cry1Aa_gb_      ............................................................
cry1Aa_GBA      ............................................................
cry1Aa_gbM      ............................................................
cry1Ab_Gen      ............................................................
Cry1Ab_Gen      A....................C....................A.................
Cry1Ab_Gen      ............................................................
cry1Ab_Gen      ............................................................
Cry1Ac_Gen      ............................................................
gi|2560030      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac22_G      ............................................................
cry1 gb_AF      ............................................................
cry1Aa gb_      ............................................................

                     3130      3140      3150      3160      3170      3180
                ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_      TCGTGTCACAGCGTACAAGGAGGGATATGGAGAAGGTTGCGTAACCATTCATGAGATCGA
cry1Aa_dbj      ............................................................
cry1Aa_dbj      ............................................................
cry1Aa_emb      ............................................................
cry1Aa_emb      ............................................................
cry1Aa_gb_      ............................................................
cry1Aa_gb_      ............................................................
cry1Aa_GBA      ............................................................
cry1Aa_gbM      ............................................................
cry1Ab_Gen      ............................................................
Cry1Ab_Gen      ......T...........A..............G..C........G..C...........
Cry1Ab_Gen      ............................................................
cry1Ab_Gen      ............................................................
Cry1Ac_Gen      ............................................................
gi|2560030      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac_Gen      ............................................................
cry1Ac22_G      ............................................................
cry1 gb_AF      ............................................................
cry1Aa gb_      ............................................................

                     3550      3560      3570      3580      3590      3600
                ....|....|....|....|....|....|....|....|....|....|....|....|
506190dbj_      GGACAGC-GTGGAATTACTCCTTATGGAGGAATAGTCTCATGCAAACTCAGGTTTAAATA
cry1Aa_dbj      .......-...........T..............A.A.ATGCTTT.AAAT.TAAGGTG.G
cry1Aa_dbj      .......-...........T...................................-----
cry1Aa_emb      .......-...........................-------------------------
cry1Aa_emb      .......-....................................................
cry1Aa_gb_      .......-...........................-------------------------
cry1Aa_gb_      .......-...C.......................-------------------------
cry1Aa_GBA      .......-...........T...............-------------------------
cry1Aa_gbM      .......-....................................................
cry1Ab_Gen      .......-...........T..............A-------------------------
Cry1Ab_Gen      ...T...-...........................-------------------------
Cry1Ab_Gen      .......-...........T...........-----------------------------
```

F6f F5r F6r

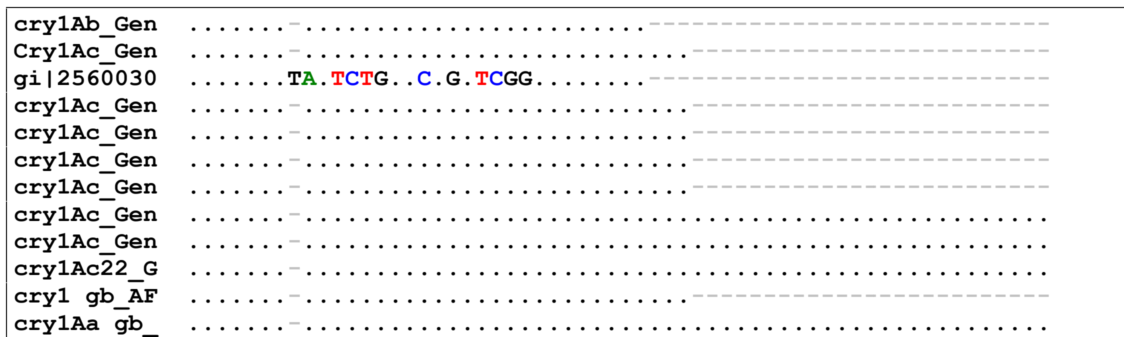

Figure 7.2.1: Alignment of available sequences of *cry1Aa*, *cry1Ab* and *cry1Ac* genes with the help of BioEdit program (Hall, 1999). Primers were selected from the conserved sequences and indicated by underlined marked characters (F1f-Fragment 1 forward primer, F1r-Fragment 1 reverse up to Fragment 6). Dots mean identical amino acids, blue means similar nucleotide.

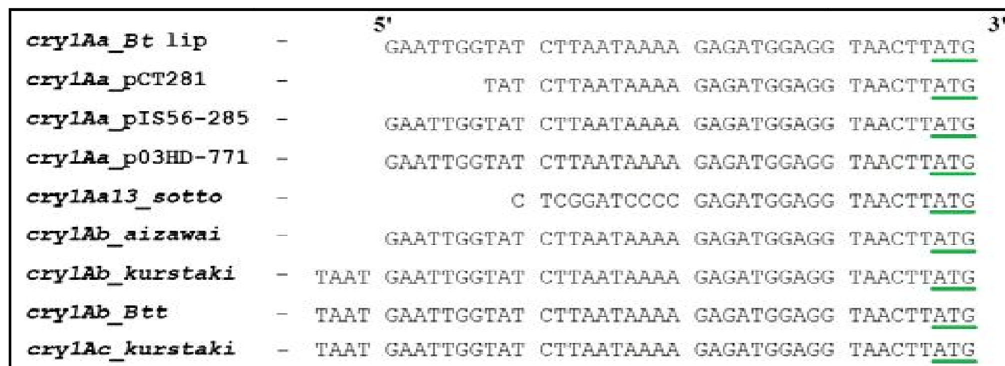

Figure 7.2.2: Alignment of the 5'-flanking regions of available *cry1Aa*, *cry1Ab* and *cry1Ac* genes to choose priming site upstream of the start codon.

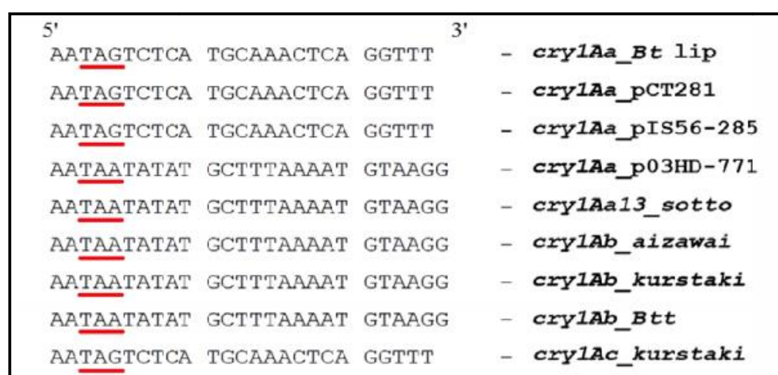

Figure 7.2.3: Alignment of the 3'-flanking regions of available *cry1Aa*, *cry1Ab* and *cry1Ac* genes to choose priming site downstream of the stop codon.

7.2.2 PCR amplification of overlapping fragments and sequencing

DNA template from the *Bt* JSc1 was added into six PCR reaction mixtures i.e. per µl containing 0.2mM dNTPs, 0.5µM of each primer (both forward and reverse primers for six

different fragments), 1× PCR buffer and 0.5 u of *Taq* DNA polymerase (Appendix A) in 25µl reaction volume and amplification was performed in a DNA thermal cycler. For all primer sets, PCR was carried out with an initial single denaturation step at 95°C for 2 min and 30 amplification cycles including denaturation at 95°C for 45 s, annealing at temperatures specific for each primer sets (Table 7.2.1) for 45 s and extension at 72°C for 60 s. Finally an extra extension step was applied at 72°C for 10 min. PCR products (5 µl) were then analyzed by horizontal agarose gel electrophoresis as described in section 3.2.2.10.

The PCR products were purified directly as described in section 3.2.2.12 and their sequences were obtained with both forward and reverse primers as described in section 3.2.2.13.

Table 7.2.1: Properties of the primers designed to amplify the overlapping fragments of *cry1Aa*- type gene.

Primer		Sequence (5'-3')	Length	Annealing temperature	Product length (bp)
Fragment 1	Fwd	AGAGATGGAGGTAACTTATGGA	22	53 ^{o}C	678
	Rev	GTCCCCATACACGCTCTAAT	20		
Fragment 2	Fwd	CTGTGCGCTGGTACAATACG	20	55 ^{o}C	727
	Rev	GCTCTCAAGGTGTAAACTGCT	21		
Fragment 3	Fwd	AGATGTAATACCGCCACAGGA	21	54 ^{o}C	787
	Rev	CGCATGTTTGACTTTCTCGG	20		
Fragment 4	Fwd	GTGAATGAGCTGTTTACTTCTTCCA	25	55 ^{o}C	700
	Rev	GCCCATCTTGCGTCTTAATCT	21		
Fragment 5	Fwd	ATGAGGACCTAGGTGTATGGGT	22	55 ^{o}C	588
	Rev	ACGCTGTGACACGAAGGATA	20		
Fragment 6	Fwd	GGAAGCAGAAGTGTCACAAG	20	53 ^{o}C	504
	Rev	GCATGAGACTATTCCTCCATAAG	23		

7.2.3 Sequence assembling and analysis

The sequences of the amplified fragments were then assembled with the help of the software, DNA Baser (version 4). As the fragments were overlapped during assembly, the errors in nucleotide bases that occurred in first few bases due to the weak signals could be corrected where necessary by matching with the overlapping sequences of other fragments. The complete Open Reading Frame was then determined detecting the start and stop codons and the amino acid sequence was deduced by the software MEGA version 5.22. The protein sequence was then aligned and compared with Cry1Aa, Cry1Ab and Cry1Ac proteins by

ClustalW and its phylogenetic relation was inferred by neighbor-joining method. The complete sequence was then deposited in GenBank database as described in section 3.2.2.13.

7.2.4 Building 3-D Model for Cry1Aa-type Protein

Three dimensional model of Cry1Aa protein was built with ProMod Version 3.70 from a fully automated server, SWISS-MODEL (http://swissmodel.expasy.org/). Homology modeling typically comprises the following steps: (i) template identification, (ii) template selection, (iii) model building and (iv) model quality estimation (Sali and Blundell, 1993; Schwede *et al.*, 2008). The deduced amino acid sequences were used in this process to build the models based on the target-template alignment. Coordinates which are conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized by using a force field. When the loop modeling with ProMod (Guex *et al.*, 1997) does not give satisfactory results, an alternative model was built with MODELLER (Sali and Blundell, 1993). The global and per-residue model quality has been assessed using the QMEAN scoring function (Benkert *et al.*, 2011)

7.3 Results

7.3.1 Analysis of PCR product

Upon electrophoresis of the PCR products in agarose gel slab submerged in 1×TBE buffer, sharp and bright bands were observed for all targeted fragments (Fig 7.3.1). The sizes of the PCR products were revealed to be approximately 680 bp for fragment 1; 730 bp for fragment 2; 790 bp for fragment 3; 700 bp for fragment 4; 600 bp for fragment 5 and 500 bp for fragment 6 which were very close to the expected sizes.

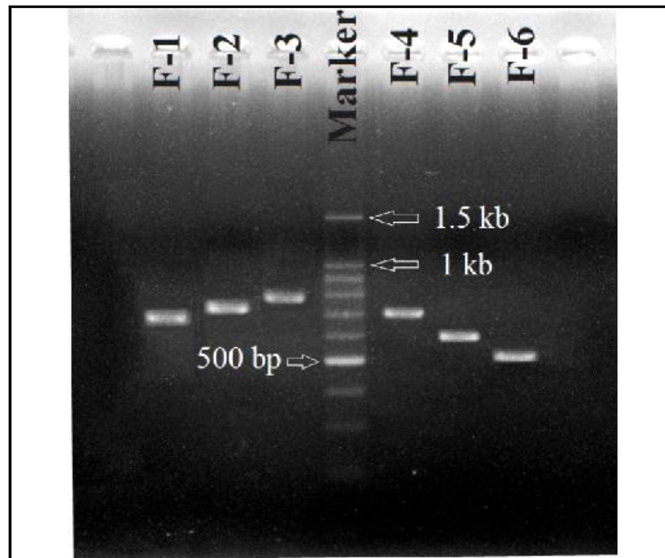

Figure 7.3.1: Observation of amplicons by agarose gel electrophoresis obtained from overlapping fragments targeting PCR.

Then, the PCR products were purified and sequenced. Sequencing was performed with both forward and reverse primers to ensure the accuracy of the sequence results. Thus, the sequences of six fragments were double in number i.e. six 5'- 3' sequences (Fig 7.3.2) and six 3'- 5' sequences (Fig 7.3.3). The sequences were scrutinized very carefully if any misread nucleotide was present and corrected where necessary. The sequences of the overlapping fragments were then assembled with the help of bioinformatics software, DNA Baser (version 4). Fragments were assembled automatically by the software recognizing the overlaps (Fig 7.3.4). Thus the full length sequence of the gene was obtained and comparisons of nucleotide sequences were performed using standard BLAST sequence similarity searching program located at http://www.ncbi.nlm.nih.gov/BLAST/. The blast result indicated that the maximum identity of the nucleotide sequence was observed with *cry1Aa*-type genes.

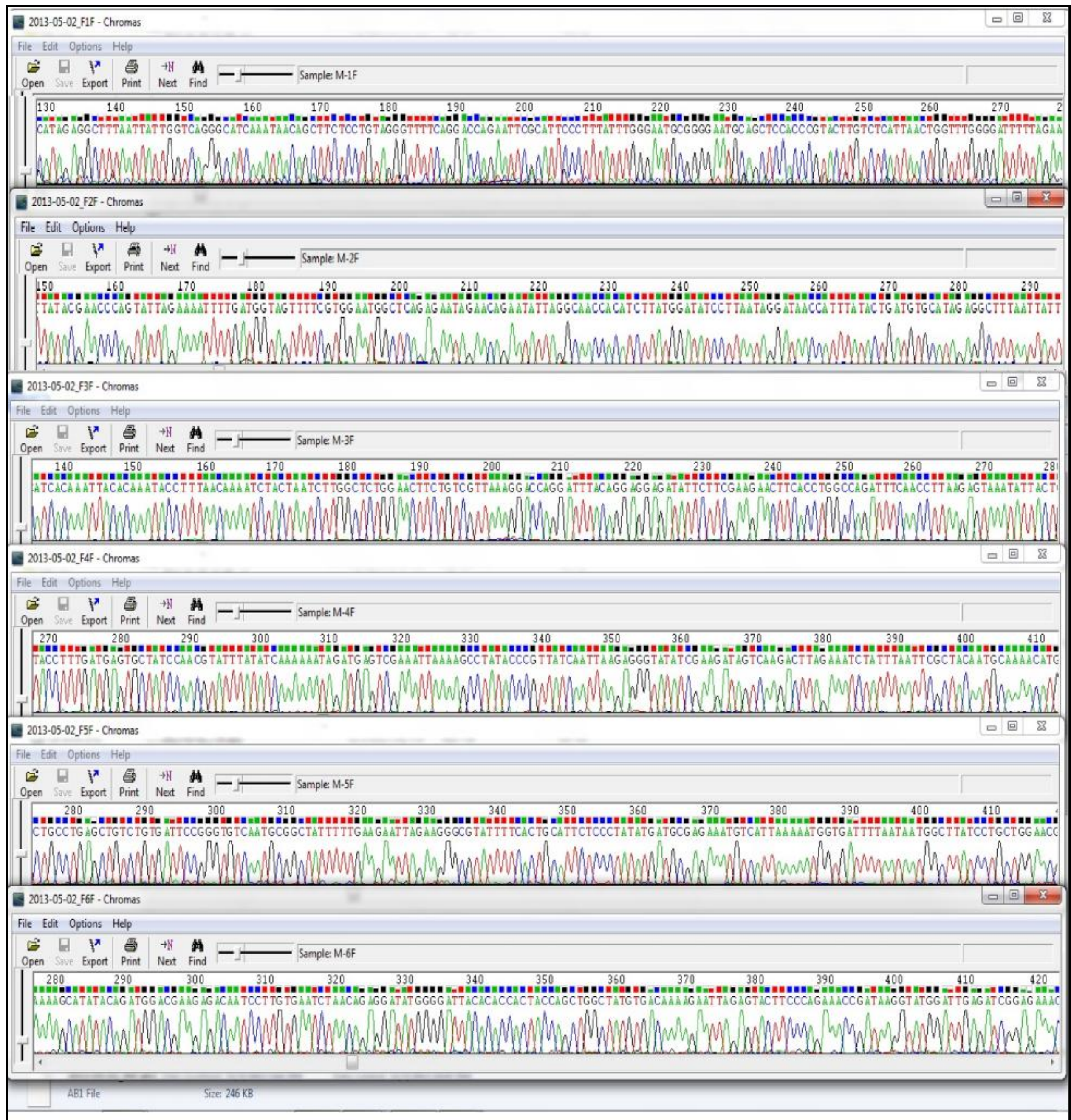

Figure 7.3.2: Representative chromatogram of the sequences for six overlapping fragments obtained with forward primer.

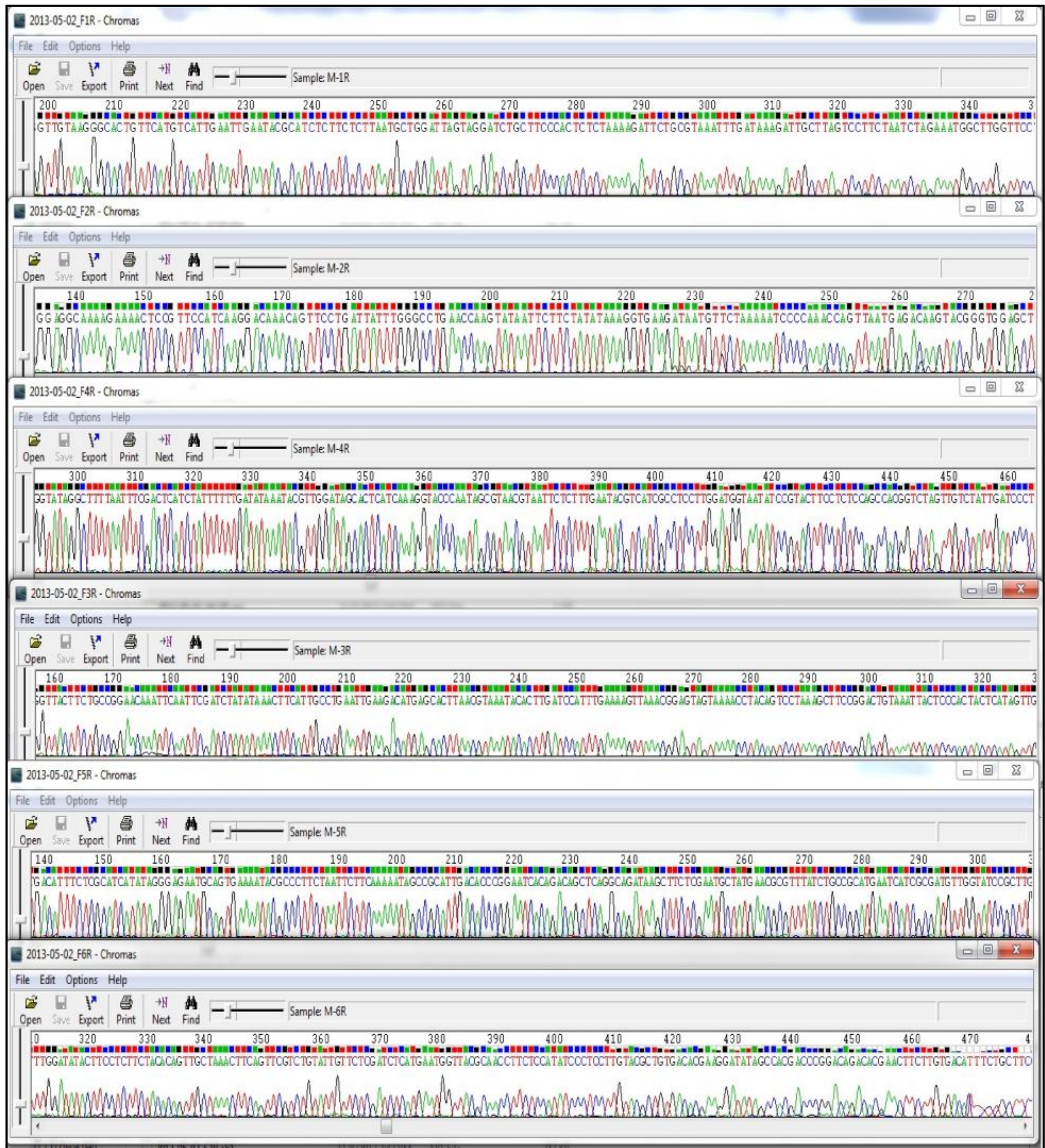

Figure 7.3.3: Representative chromatogram of the sequences for six overlapping fragments obtained with reverse primer.

Figure 7.3.4: Representative chromatogram of the assembled sequences of six overlapping fragments (Small common fragments are from initially sequenced conserved regions).

7.3.2 Complete Open Reading Frame (ORF) of *cry1Aa*-type gene

The start codon and stop codon were then identified by aligning the sequence with other *cry1Aa*- type genes and the complete ORF was determined (Fig 7.3.5). It was then deposited in the NCBI GenBank Database and an accession number (KM924540.1) was provided for this sequence.

```
>seq [organism=Bacillus thuringiensis] [strain=JSc1] complete sequence of cry1Aa- type gene
```

```
ATGGATAACAATCCGAACATCAATGAATGCATTCCTTATAATTGTTTAAGTAACCCTGAAGTAGAAGTATTAGGTGGAGA
AAGAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTTTTGAGTGAATTTGTTCCCGGTG
CTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGAATTTTTGGTCCCTCTCAATGGGACGCATTTCTTGTACAAATT
GAACAGTTAATTAACCAAAGAATAGAAGAATTCGCTAGGAACCAAGCCATTTCTAGATTAGAAGGACTAAGCAATCTTTA
TCAAATTTACGCAGAATCTTTTAGAGAGTGGGAAGCAGATCCTACTAATCCAGCATTAAGAGAAGAGATGCGTATTCAAT
TCAATGACATGAACAGTGCCCTTACAACCGCTATTCCTCTTTTTGCAGTTCAAAATTATCAAGTTCCTCTTTTATCAGTA
TATGTTCAAGCTGCAAATTTACATTTATCAGTTTTGAGAGATGTTTCAGTGTTTGGACAAAGGTGGGGATTTGATGCCGC
GACTATCAATAGTCGTTATAATGATTTAACTAGGCTTATTGGCAACTATACAGATTATGCTGTGCGCTGGTACAATACGG
GATTAGAGCGTGTATGGGGACCGGATTCTAGAGATTGGGTAAGGTATAATCAATTTAGAAGAGAGCTAACACTTACTGTA
TTAGATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTATCCAATTCGAACAGTTTCCCAATTAACAAGAGAAAT
TTATACGAACCCAGTATTAGAAAATTTTGATGGTAGTTTTCGTGGAATGGCTCAGAGAATAGAACAGAATATTAGGCAAC
CACATCTTATGGATATCCTTAATAGGATAACCATTTATACTGATGTGCATAGAGGCTTTAATTATTGGTCAGGGCATCAA
ATAACAGCTTCTCCTGTAGGGTTTTCAGGACCAGAATTCGCATTCCCTTTATTTGGGAATGCGGGGAATGCAGCTCCACC
CGTACTTGTCTCATTAACTGGTTTGGGGATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATACTTGGTTCAG
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GCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTGCCTCCCTAACGACCAACTTGCCTTCCACT
ATATATAGACAAAGGGGTACAGTCGATTCACTAGATGTAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATT
TAGCCATCGATTGAGTCATGTTACAATGCTGAGCCAAGCAGCTGGAGCAGTTTACACCTTGAGAGCTCCAACGTTTTCTT
GGCAGCATCGCAGTGCTGAATTTAATAATATAATTCCTTCATCACAAATTACACAAATACCTTTAACAAAATCTACTAAT
CTTGGCTCTGGAACTTCTGTCGTTAAAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAACTTCACCTGGCCAGAT
TTCAACCTTAAGAGTAAATATTACTGCACCATTATCACAAAGATATCGGGTAAGAATTCGCTACGCTTCTACTACAAATT
TACAATTCCATACATCAATTGACGGAAGACCTATTAATCAGGGTAATTTTTCAGCAACTATGAGTAGTGGGAGTAATTTA
CAGTCCGGAAGCTTTAGGACTGTAGGTTTTACTACTCCGTTTAACTTTTCAAATGGATCAAGTGTATTTACGTTAAGTGC
TCATGTCTTCAATTCAGGCAATGAAGTTTATATAGATCGAATTGAATTTGTTCCGGCAGAAGTAACCTTTGAGGCAGAAT
ATGATTTAGAAAGAGCACAAAAGGCGGTGAATGAGCTGTTTACTTCTTCCAATCAAATCGGGTTAAAAACAGATGTGACG
GATTATCATATTGATCAAGTATCCAATTTAGTTGAGTGTTTATCAGATGAATTTTGTCTGGATGAAAAACAAGAATTGTC
CGAGAAAGTCAAACATGCGAAGCGACTTAGTGATGAGCGGAATTTACTTCAAGATCCAAACTTCAGAGGGATCAATAGAC
AACTAGACCGTGGCTGGAGAGGAAGTACGGATATTACCATCCAAGGAGGCGATGACGTATTCAAAGAGAATTACGTTACG
CTATTGGGTACCTTTGATGAGTGCTATCCAACGTATTTATATCAAAAAATAGATGAGTCGAAATTAAAAGCCTATACCCG
TTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAGAAATCTATTTAATTCGCTACAATGCAAAACATGAAACAG
TAAATGTGCCAGGTACGGGTTCCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGC
GCGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAATGTGCCCATCATTCCCATCATTT
CTCCTTGGACATTGATGTTGGATGTACAGACTTAAATGAGGACTTATGTGTATGGGTGATATTCAAGATTAAGACGCAAG
ATGGCCATGCAAGACTAGGAAATCTAGAATTTCTCGAAGAGAAACCATTAGTAGGAGAAGCACTAGCTCGTGTGAAAAGA
GCGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATTGTTTATAAAGAGGCAAAAGAATCTGT
AGATGCTTTATTTGTAAACTCTCAATATGATAGATTACAAGCGGATACCAACATCGCGATGATTCATGCGGCAGATAAAC
GCGTTCATAGCATTCGAGAAGCTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTA
GAAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTGATTTTAATAATGGCTTATC
CTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAAACAACCACCGTTCGGTCCTTGTTGTTCCGGAATGGGAAG
CAGAAGTGTCACAAGAAGTTCGTGTCTGTCCGGGTCGTGGCTATATCCTTCGTGTCACAGCGTACAAGGAGGGATATGGA
GAAGGTTGCGTAACCATTCATGAGATCGAGAACAATACAGACGAACTGAAGTTTAGCAACTGTGTAGAAGAGGAAGTATA
TCCAAACAACACGGTAACGTGTAATGATTATACTGCGACTCAAGAAGAATATGAGGGTACGTACACTTCTCGTAATCGAG
GATATGACGGAGCCTATGAAAGCAATTCTTCTGTACCAGCTGATTATGCATCAGCCTATGAAGAAAAAGCATATACAGAT
GGACGAAGAGACAATCCTTGTGAATCTAACAGAGGATATGGGGATTACACACCACTACCAGCTGGCTATGTGACAAAAGA
ATTAGAGTACTTCCCAGAAACCGATAAGGTATGGATTGAGATCGGAGAAACGGAAGGAACATTCATCGTGGACAGCGTGG
AATTACTTCTTATGGAGGAATAG
```

Figure 7.3.5: Nucleotide sequence of the *cry1Aa*- type gene from indigenous *Bacillus thuringiensis* strain JSc1 of Bangladesh (ATG: Start codon, TAG: Stop codon).

7.3.3 Analysis of the deduced amino acid sequence

The amino acid sequence of the protein expressed by the *cry1Aa*- type gene was deduced from the 3543 bp long open reading frame (ORF) which encodes a polypeptide of 1180 amino acids (AJG01595.1), with no N-terminal signal peptide (Fig 7.3.6) as predicted (Petersen *et al.*, 2011) from a web based program (http://www.cbs.dtu.dk/services/ProP/).

A ClustalW comparison (Thompson *et al.*, 1994) of the deduced amino acid sequence of the presumed Cry1A- type protein with other known Cry protein sequences like Cry1Aa, Cry1Ab and Cry1Ac (Fig 7.3.7) using the program BioEdit (version 7.2.5) helped to identify the characteristic five conserved blocks (Schnepf *et al.*, 1998) of 3- domain Cry proteins. The structure of the protein was thus presumed to possess the same basic three-domain fold as it is believed that the toxins containing all, or some, of these conserved blocks are likely to have this structure (Pardo-Lo´pez *et al.*, 2013).

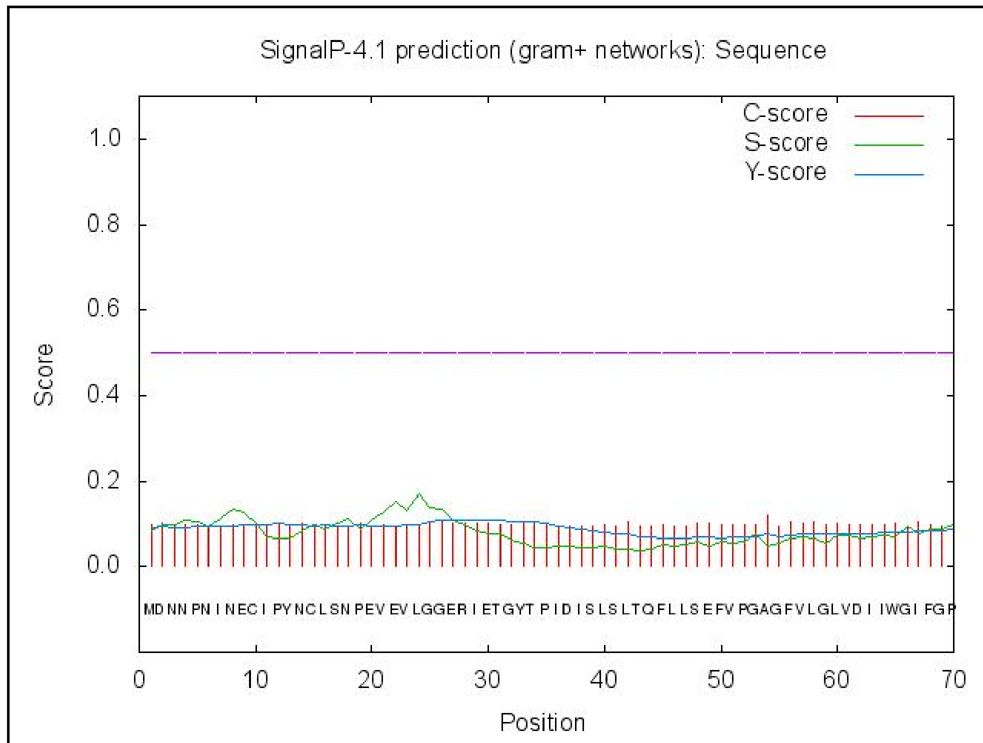

Figure 7.3.6: The graphical output from SignalP shows the three different scores, *C*, *S* and *Y*, for each position in the sequence to determine the signal peptide.

From this analysis, no amino acid sequence differences were observed for the Cry1Aa-type protein in its open reading frame with Cry1Aa9 and Cry1Aa13 protein whereas huge differences were observed with Cry1Ab and Cry1Ac proteins. The molecular weight (M_r) of this full-length polypeptide was predicted to be ca. 133 kDa from the web based program, Swiss-Model (http://swissmodel.expasy.org/).

```
                        10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ      MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
cry1Aa_dbj      ............................................................
Cry1Aa13_G      ............................................................
cry1Ab_Gen      ............................................................
cry1Ac_Gen      ............................................................

                        70        80        90       100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ      VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
cry1Aa_dbj      ............................................................
Cry1Aa13_G      ............................................................
cry1Ab_Gen      ............................................................
cry1Ac_Gen      ............................................................

                       130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|
```

```
Cry1Aa-typ   PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
cry1Aa_dbj   ............................................................
Cry1Aa13_G   ............................................................
cry1Ab_Gen   ............................................................
cry1Ac_Gen   ............................................................

                    190       200       210       220       230       240
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV
cry1Aa_dbj   ............................................................
Cry1Aa13_G   ............................................................
cry1Ab_Gen   .........................H....................I.............
cry1Ac_Gen   ............................................................

                    250       260       270       280       290       300
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   LDIVALFSNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL
cry1Aa_dbj   ............................................................
Cry1Aa13_G   ............................................................
cry1Ab_Gen   ....S..P.....T............................S..G..KYESTFDGYTQY
cry1Ac_Gen   .......P..................................S..G..KYESTFDGYTQY

                    310       320       330       340       350       360
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   NRITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPP-VLVSLTGLGIFR
cry1Aa_dbj   ...............................................-............
Cry1Aa13_G   ...............................................-............
cry1Ab_Gen   .HLYGCSRRILLVRASN----NGFSCRVF.ARIH.SAIW.Y.KCSSTTTYCCS..Q.VY.
cry1Ac_Gen   .HLYGCSGLLLLVRASN----NGFSCR.F.ARIHVSAIW.H.KCSSTTTYCCS..Q.VY.

                    370       380       390       400       410       420
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   TLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYRQRGTVDSLDVIPPQDN
cry1Aa_dbj   ............................................................
Cry1Aa13_G   ............................................................
cry1Ab_Gen   ....T....P-FNI.I...Q.S.......AYG--.SS...AV..KS.......E....N.
cry1Ac_Gen   ....T....P-FNI.I...Q.S.......AYG--.SS...AV..KS.......E....N.

                430       440       450       460       470       480
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   SVPPRAGFSHRLSHVTMLSQAAG--AVYTLRAPTFSWQHRSAEFNNIIPSSQITQIPLTK
cry1Aa_dbj   .......................--...................................
Cry1Aa13_G   .......................--...................................
cry1Ab_Gen   N....Q.........S.FRSGFSNSS.SII...M...I............TNY.NTFNKI
cry1Ac_Gen   N....Q.........S.FRSGFSNSS.SII...M...I..........A.DYYSNPCSE.

                    490       500       510       520       530       540
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   STNLG--SGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQ
cry1Aa_dbj   .....--.....................................................
Cry1Aa13_G   .....--.....................................................
cry1Ab_Gen   YSW.WNFCRRTRIYRRRYSSKNFTWPD.TLRVN-Y--T.ITKI.GKNSL.FYHKF.I---
cry1Ac_Gen   LSFWFCNFR-TRIYWW.LSIK-WKHSERVYSSNS.P-IDIYQI.SSCT.CFCNPDSP---

                    550       560       570       580       590       600
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   FHTSIDGRPINQGNFSATMSSGSN--LQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSG
cry1Aa_dbj   ........................--..................................
Cry1Aa13_G   ........................--..................................
cry1Ab_Gen   --PY.NRK...........WEFTVRK.DCRFYYS.L.KWIKCIYVKC.CLQFRQSLYR.N
cry1Ac_Gen   --VNWGNSS.FSNTVP..AT.L.T--IKFWL.KCQC.YIF-R-YSRCKFWDCR-SDNRQI
```

```
                       610       620       630       640       650       660
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     NEVYIDRIEFVPAEVTFEAE-----YDLERAQKAVNELFTSSNQIG---LKTDVTDYHID
cry1Aa_dbj     ....................-----.....................---...........
Cry1Aa13_G     ....................-----.....................---...........
cry1Ab_Gen     ICSGRSNLGRIFRKS.KGG.-AVYFFQSN.VKNRCDG.SY..I.FS-VFI-ILSGKKR.V
cry1Ac_Gen     IYSSYCNTRGISGKSAEGG.CAVYV.KPT..KNKC.G.SY..V.FSYVFIGILSGKAR.V

                       670       680       690       700       710       720
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     QVSNLVECLSDEFCLDEKQELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDI
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     RE.QTC.AT-A..TSRS.LR-----DQTT.PWL..KYG---YYHPRR.RIQ.EL.YAIGY
cry1Ac_Gen     RE.QTC.ATQTQ.TPRF.FQ-----R..T.TWVG.KYR---DYHPRRGRIR-KL.HTIRY

                       730       740       750       760       770       780
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     TIQGGDDVFKENYVTLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLI
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     LVLS--N..ISKNRVEIKSLYPLPIKRV.RRSRLRN.FNSLQC.T.NSKCARYGFLMAAF
cry1Ac_Gen     LVLS--NI.VSKNRIKIKSLYPLSIKRV.RRSRLRN.FNSLQCKT-NSKCARYGFLMAAF

                       790       800       810       820       830       840
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     RYNAKHETVNVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSH
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     SPKSNRK--------------------------M.PSFPSFLLGHCWMY.--L.GLRCMG
cry1Ac_Gen     SPKSNRKVWRAESMRATPME.LRLFV.---WRKV.PSFASFLLRHCRMY.--L.GPRCMG

                       850       860       870       880       890       900
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     HFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKW
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     DIQD.ARWP.KTRKSRISRRETISRRSTSSCEKS.--.KM.RQTKI.MGNKYCL.GKRIC
cry1Ac_Gen     DL-D.ARWARKTRESRVSRRETISRRSASSCEKS.--.KM.RQTKI.MGNKYRL.GKRIC

                       910       920       930       940       950       960
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     RDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYLPE
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     .CFIC..SI-ITSG.QHRDD.CGRTRSH.R--S.S.AVCDSGCQCG---YFR..R..F--
cry1Ac_Gen     .CFIC..SISITSG.EYCHD.CGRTCSH.R--S.S.AVCDSGCQCG---YFR..R..F--

                       970       980       990       1000      1010      1020
               ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ     LSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNHRS
cry1Aa_dbj     ............................................................
Cry1Aa13_G     ............................................................
cry1Ab_Gen     HCIL.-----.C.KCHKWFWLILLERERACRCRRTKQPPFGP.CSGM.SRS.T-----..
cry1Ac_Gen     HCIL.-----.C.KCHKWFWLILLERERACRCRRTKQPTFGP.CSGM.SRS.T-----..

                       1030      1040      1050      1060      1070      1080
```

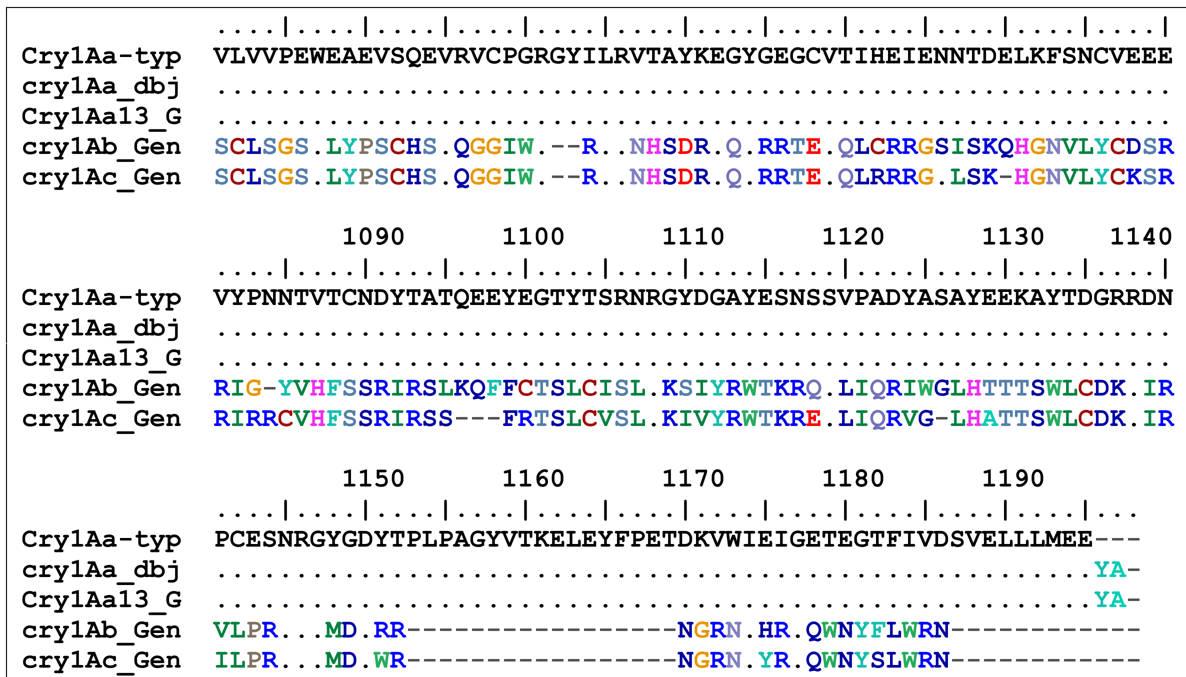

Figure 7.3.7: Alignment of the Cry1Aa- type protein with other Cry1A proteins of tertiary rank by BioEdit sequence alignment editor using ClustalW method showing the conserved blocks of amino acids. (YQVPL…RWG: Block 1; DWVRY…TNPV: Block 2; FSWQH…DIL: Block 3; QRYRV…AST: Block 4; VYID…FVP: Block 5) "." Dots mean identical amino acids, blue means similar aa.

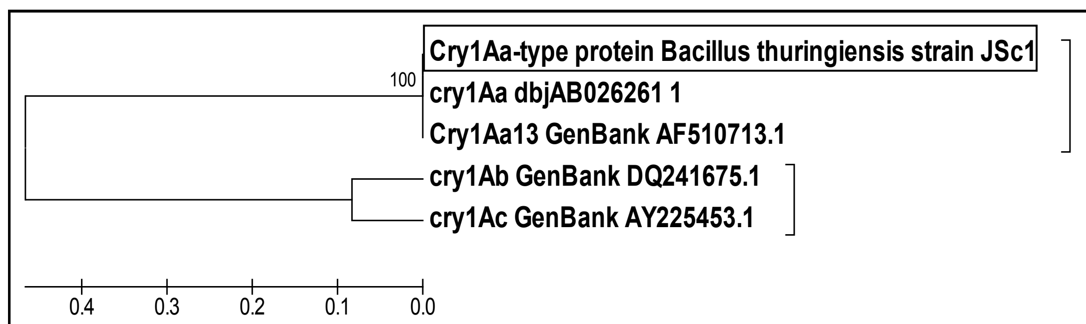

Figure 7.3.8: Evolutionary relationships of Cry1Aa-type protein with other closely related Cry proteins as determined by bootstrap test and inferred using the Neighbor-Joining method.

On the other hand, from BLAST analysis of the protein being studied revealed close homology to the Cry1Aa class of proteins than Cry1Ab or Cry1Ac classes and showed 100% similarity to Cry1Aa9 and Cry1Aa13 (Fig 7.3.8).

7.3.4 Protein structure simulation

After the amino acid sequence analysis, PDB file for the deduced amino acid sequence was generated by homology modeling from the web-based automated server, SWISS-MODEL (http://swissmodel.expasy.org/). Template search with Blast and HHBlits against the SWISS-MODEL template library produced 26 templates for BLAST against the primary amino acid sequence and for the HHblits, 82 templates were found. Alignment of the target sequence with the templates of maximum homology (Cry1Aa: 1ciy.1.A and Cry1Ac: 4ary.1.A) revealed that 99.66% sequence identity was present with Cry1Aa protein (Table 7.3.1) whereas 75.39% was present with Cry1Ac.

Table 7.3.1: Properties of the 13 templates out of 108 matched against the target obtained from SWISS-MODEL template library

Template	Seq Identity	Oligo-state	Found by	Method	Resolution	Seq Similarity	Range	Coverage	Description
1ciy.1.A	99.66	monomer	BLAST	X-ray	2.25Å	0.6	33 - 609	0.5	CRYIA(A)
1ciy.1.A	97.79	monomer	HHblits	X-ray	2.25Å	0.6	33 - 609	0.5	CRYIA(A)
4ary.1.A	75.39	monomer	BLAST	X-ray	2.95Å	0.52	31 - 606	0.49	CRY1AC
4arx.1.A	75.75	monomer	BLAST	X-ray	2.35Å	0.52		0.48	CRY1AC
4ary.1.A	73.16	monomer	HHblits	X-ray	2.95Å	0.51		0.48	CRY1AC
4arx.1.A	73.24	monomer	HHblits	X-ray	2.35Å	0.51		0.48	CRY1AC
3eb7.1.A	39.5	monomer	BLAST	X-ray	2.30Å	0.4		0.47	Cry8Ea1
3eb7.1.A	37.41	monomer	HHblits	X-ray	2.30Å	0.39		0.47	Cry8Ea1
1dlc.1.A	35	monomer	HHblits	X-ray	2.50Å	0.38		0.47	CRYIIIA
4qx0.1.A	35	monomer	HHblits	X-ray	2.80Å	0.38		0.47	cry3Aa
1ji6.1.A	33.21	monomer	HHblits	X-ray	2.40Å	0.38		0.47	CRY3BB
1ji6.1.A	36.1	monomer	BLAST	X-ray	2.40Å	0.39		0.46	CRY3BB
4qx0.1.A	38.27	monomer	BLAST	X-ray	2.80Å	0.4		0.45	cry3Aa

```
              10        20        30        40        50        60
     ....|....|....|....|....|....|....|....|....|....|....|....|
```

```
Cry1Aa-typ    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
1ciy.1.A      ----------------------------................................
1ciy.1.A      ............................................................
4ary.1.A      ............................................................

                      70        80        90       100       110       120
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
1ciy.1.A      ............................................................
1ciy.1.A      ............................................................
4ary.1.A      ............................................................

                     130       140       150       160       170       180
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
1ciy.1.A      ...........................L................................
1ciy.1.A      ...........................L................................
4ary.1.A      ............................................................

                     190       200       210       220       230       240
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV
1ciy.1.A      ............................................................
1ciy.1.A      ............................................................
4ary.1.A      ............................................................

                     250       260       270       280       290       300
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    LDIVALFSNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL
1ciy.1.A      ............................................................
1ciy.1.A      ............................................................
4ary.1.A      .......P..................................S..G..RS..S.......

                     310       320       330       340       350       360
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    NRITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPP-VLVSLTGLGIFR
1ciy.1.A      .S.............................................-............
1ciy.1.A      .S.............................................-............
4ary.1.A      .S......A...YY.......M...........T...Y.TM.....QQRI.AQL.Q.VY.

                     370       380       390       400       410       420
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    TLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYRQRGTVDSLDVIPPQDN
1ciy.1.A      ............................................................
1ciy.1.A      ............................................................
4ary.1.A      ....T....-PFNI.I...Q.S.......AYGT-SS....AV..KS.......E....N.

                     430       440       450       460       470       480
              ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ    SVPPRAGFSHRLSHVTMLSQAAG--AVYTLRAPTFSWQHRSAEFNNIIPSSQITQIPLTK
1ciy.1.A      .......................--...................................
1ciy.1.A      .......................--...................................
4ary.1.A      N....Q.........S.FRSGFSNSS.SII...M...I..........A.DS.....AV.

                     490       500       510       520       530       540
              ....|....|....|....|....|....|....|....|....|....|....|....|
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Cry1Aa-typ   STNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPL-----SQRYRVRIRYASTT
1ciy.1.A     .........................................-----..............
1ciy.1.A     .........................................-----..............
4ary.1.A     GNF.FN.-..IS........LV.LN.S.NNIQN.GY.EV.IHFPST.T.....V....V.

                     550       560       570       580       590       600
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   NLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNS
1ciy.1.A     ............................................................
1ciy.1.A     ............................................................
4ary.1.A     PIHLNVNWGNSS.FSNTVP..AT.LD....SD.GYFESANA.TS.L.N---IVGVRN.SG

                     610       620       630       640       650       660
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   GNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVE
1ciy.1.A     ..........................----------------------------------
1ciy.1.A     ..........................----------------------------------
4ary.1.A     TAG.I...F..I.VTA.-------------------------------------------

                     670       680       690       700       710       720
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   CLSDEFCLDEKQELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDD
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     730       740       750       760       770       780
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   VFKENYVTLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHE
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     790       800       810       820       830       840
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   TVNVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDID
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     850       860       870       880       890       900
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   VGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKL
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     910       920       930       940       950       960
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   EWETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYLPELSVIPGV
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------
                     970       980       990      1000      1010      1020
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   NAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNHRSVLVVPEW
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------
                    1030      1040      1050      1060      1070      1080
             ....|....|....|....|....|....|....|....|....|....|....|....|
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```
Cry1Aa-typ   EAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEVYPNNTV
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     1090      1100      1110      1120      1130      1140
             ....|....|....|....|....|....|....|....|....|....|....|....|
Cry1Aa-typ   TCNDYTATQEEYEGTYTSRNRGYDGAYESNSSVPADYASAYEEKAYTDGRRDNPCESNRG
1ciy.1.A     ------------------------------------------------------------
1ciy.1.A     ------------------------------------------------------------
4ary.1.A     ------------------------------------------------------------

                     1150      1160      1170      1180
             ....|....|....|....|....|....|....|....|....|...
Cry1Aa-typ   YGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
1ciy.1.A     ------------------------------------------------
1ciy.1.A     ------------------------------------------------
4ary.1.A     ------------------------------------------------
```

Figure 7.3.9: Alignment of the protein sequence (1180 aa) of *B. thuringiensis* strains JSc1 with the highly similar templates used for protein modeling. Dots mean identical amino acids, blue means similar aa.

The target protein differed from the Cry1Aa protein (PDB 1ciy.1.A) only by Arg302 instead of Ser302 in matured form (Fig 7.3.9).

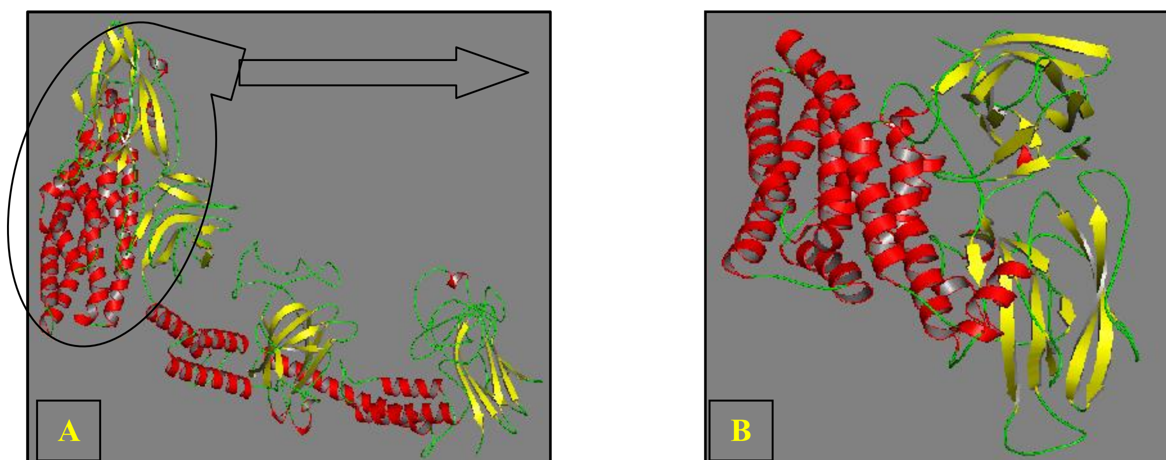

Figure 7.3.10: 3- D modeling of Cry1Aa-type protein from *Bacillus thuringiensis* strains JSc1. A) Overall view of the protein in its protoxin form as the helix, sheet and chain are indicated by red, yellow and green colors respectively. The marked portion as indicated with a oval shaped ring gets cleaved upon proteolytic activation and the matured protein is generated. B) 3-domain Cry1Aa in its active form i.e. cleaved protein.

It was found that the matured 3-domain Cry1Aa protein which is activated from protoxin upon solubilization and proteolytic cleavage, is consisted of the amino acids from Thr33 to Arg609. Thus two structures were built with the PDB file, one for protoxin and another for

matured form, the active toxin (Fig 7.3.10). An active toxin of 576 residues is generated upon proteolytic digestion the molecular mass of which was estimated to be 64419 Da.

CHAPTER 8

Production of *B. thuringiensis* biopesticide preparation in cost effective manner and its field application

Production of *Bacillus thuringiensis* biopesticide in cost effective manner and its field application

8.1 INTRODUCTION

Bacillus thuringiensis (Bt), for its insecticidal δ-endotoxin (Cry protein) has been the most widely used biopesticide in agriculture (Crickmore *et al.*, 1998). The high cost of *Bt* products is due to the production being located mostly in developed countries incurring higher production costs and cost for the shipment to the operational sites as well. The expense for raw materials is one of the principal costs involved in overall *Bt* production. In conventional *Bt* production process, the expense of raw materials varied between 30% and 40% of the total costs depending on the plant production capacity (Ejiofore, 1991). Therefore, production of *Bt* biopesticide based on locally available cheap raw materials including agro-industrial by products in developing countries like Bangladesh will reduce the cost significantly (Hasan *et al.*, 2011).

Bt biopesticides are usually composed of spores and crystals protein mixtures, harvested from the production media, readily produced by aerated liquid fermentation. They are easily harvested and have a long shelf life when formulated properly (Ghribi *et al.*, 2007). Optimizing different culture conditions and regulating some critical factors, it is possible to obtain higher yield in terms of cell mass, Cry protein concentration and toxicity to develop efficient *Bt* formulations (Dulmage *et al.*, 1990 a). Critical factors e.g. sugars have significant impact on cell growth but when used at high concentrations, they can cause adverse effects on sporulation due to the acids produced by *Bt* δ-endotoxin from carbohydrates (Dulmage *et al.*, 1990 a) and moreover, the balance of the ratio between carbon and nitrogen, itself is directly important for the crystal protein production (Farrera *et al.*, 1998).

Other important components for the production of crystal proteins are the trace minerals (Rose, 1979). Again, amino acids are important in the formation of spores and crystal proteins (Sachidanandham *et al.*, 1997). Moreover, it was reported that decreasing the proteolytic activity in the fermentation medium increased the accumulation of δ-endotoxin in the insecticidal crystal proteins (Ennouri *et al.*, 2013).

The study in this chapter was, therefore, carried out with a view to develop a cost effective medium for large scale production of *Bt* biopesticide with locally available cheap raw materials such as defatted soybean extract and molasses as nitrogen and carbon source respectively and sea water as the substituent of basal salts. The present study also reports the efficacy of *Bt* biopesticide preparation from indigenous strains JSc1 in cabbage, cauliflower and organic tea farming.

8.2 Methods

8.2.1 Bacterial strain and culture conditions

Reference strain *B. thuringiensis kurstaki (Btk)* HD-73 and the indigenous *Bt* strain JSc1 were used in this study. *Bt* JSc1 was isolated from Jhenidah destrict of Bangladesh (As shown in Chapter 3). LB agar was used for culture maintenance, subculture and spore count of the *Bt* strains. Incubation temperature was maintained at 30°C for all types of cultures and the liquid cultures were incubated in an orbital shaker at 180 rpm.

8.2.2 Inoculum preparation

Bt strains were streaked on LB- agar plate from the slant and was incubated overnight at 30°C. An isolated colony was picked from the LB- agar plate aseptically with a loop and was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of LB broth and incubated overnight at 30°C and 180 rpm. The cell density of the culture medium was measured after overnight incubation at OD_{600nm} using uninoculated LB broth as blank. The overnight culture was then used as inoculum for all relevant fermentation experiments and each time, inoculum was added into the medium in such a manner that the culture medium starts with an OD_{600nm} = 0.1 if not otherwise stated.

8.2.3 Sampling and sample analysis

Samples were collected aseptically from the culture broth during the fermentation processes at every 24 hours intervals i.e. 3 sample at 24, 48, 72 hours in a Bio-safety cabinet. Samples were collected in sterile microfuge tubes as aliquots for microscopic analysis, determination of spore count and crystal protein concentration.

8.2.3.1 Microscopic studies

The stage of the growth in the fermentation process was monitored by Phase Contrast Microscopy. A single drop from the collected samples was placed on a clean slide and was then covered with a cover slip. Excess liquid from edge of the cover slip was blotted by a tissue paper. Then the specimen was observed carefully under a Phase Contract Microscope (Appendix D) to detect the sporulation stage.

8.2.3.2 Determination of spore count

The spore counts were determined from 1.0 ml of culture medium being sampled at different time intervals during fermentation. The culture medium was heated at 80°C for 10 min to kill

the vegetative cells. Then it was serially diluted and highest two dilutions (0.1 ml) were inoculated on LB agar by spread plate technique. After overnight incubation at 37°C, the colonies were counted and multiplied by the dilution factor to estimate their actual number which was performed in triplicate.

8.2.3.3 Estimation of crystal protein concentration

The purification of crystal protein was performed as described in section 4.2.6 with few modifications. Finally the purified crystal protein was resuspended in 1.0 ml of 0.1 N NaOH and the concentration in the supernatant was estimated by Bradford method (Bradford, 1976).

8.2.4 Optimization of cystine concentration

As cystine plays an important role in enhancing the sporulation and endotoxin synthesis (Vora and Shethna, 1999), optimum cystine concentration affecting growth and sporulation was determined in this study. Hence, 10% cystine stock was prepared by suspending 6.0 g of cystine in 60.0 ml of phosphate buffer [pH 6.8] (Appendix C) and the growth media were supplemented with cystine in the range from 200 to 600 mg/l in separate conical flasks. The media were then inoculated with *Bt* strain inoculum and fermentation was carried out in an orbital shaker (Appendix D). The spore count and the crystal protein concentration from the collected samples were then determined.

8.2.5 Selection of efficient and cheap substrates (N and C source)

To select cheap raw materials, defatted soybean meal and defatted mustard seed meal were tested in different formulations like whole, or extract, and or in the presence of cystine and molasses. To prepare either 10% soybean meal (**Sm**) medium or 10% mustard seed meal (**Mm**) medium, defatted soybean or defatted mustard seed meal were finely ground and 10g of each was mixed in separate 250 ml conical flask with 90 ml of basal salt solutions (Appendix C) (Vora and Shethna, 1999).

On the other hand, 10 g of finely ground soybean meal or mustard seed meal were separately boiled in distilled water with a final volume of 100 ml and was then soaked for overnight. The liquid fraction was then extracted and the final volume was adjusted to 100 ml to prepare soybean extract (**Se**) medium or Mustard extract (**Me**) medium.

The pH in all cases was maintained at 7.2 ± 0.2 before autoclaving at 121°C and 15 psi for 15 min. The formulated media were then allowed to cool down to room temperature and then inoculated with the inoculums prepared as described in section 8.2.2. The efficiency of defatted soybean meal and defatted mustard seed meal was then compared based on the spore count and crystal protein concentration obtained from the samples collected during the fermentation process following the methods in sections 8.2.3 to 8.2.5. The efficiency was also compared by supplementing 0.5% of molasses and 300 mg/l of cystine in the above formulated media.

Table 8.2.1: Media formulations made in combination of different C& N sources.

Formulations	**Ingredients**
Se	Soybean extract
Me	Mustard extract
SmC	Soybean meal + Cysteine
SmM	Soybean meal + Molasses
MmMC	Mustard meal+ Cysteine+ Molasses
MmC	Mustard meal + Cysteine

8.2.6 Substitution of trace elements with sea water

It was shown that diluted sea water can be used as an important source of minerals (Ghribi *et al.*, 2007). So, basal salts were substituted with 20% sea water (v/v) in the tested medium and the efficiency was compared in parallel by spore count and crystal protein concentration.

8.2.7 Control of crystal protein degradation from protease

It was suspected that some proteolytic enzyme might be responsible for the degradation of crystal protein which was confirmed by checking protease activity and protein concentration as a function of time. PMSF (0.1 mM) and ammonium sulfate (AS) [4% (w/v)] were supplemented into the medium to inhibit the protease synthesis by *Bt* strains (Pinghui *et al.,* 1969) and the effect was determined by protease assay and estimation of protein concentration. SDS-PAGE analysis was performed with the partially purified crystal proteins in a 10% separating gel recovered from AS supplemented and non-supplemented medium to see its influence on inhibiting protease activity (Sambrook *et al.*, 1989).

Protease assay

Protease activity was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. In this enzyme assay, 400 µl of the culture supernatant and 400 µl of 1% azo-casein solution (Appendix C) was taken in microfuge tube. The mixture was then incubated in a water bath at 37°C for 60 minutes. The reaction was stopped by adding 135 µl of 35% TCA and kept on ice for 15 minutes. The solution was then centrifuged at 13000 rpm for 10 minutes and 750 µl of the supernatant was collected in which equal volume of freshly prepared 1.0 N NaOH was added by gentle mixing. Absorbance (OD_{440nm}) of the solution was then measured using the solution from a parallel reaction as blank where TCA was added before the enzyme. Enzyme activity was then estimated from the absorbance [OD_{440nm} = 1.0 is equivalent to 100 U Enzyme activity] (Kreger and Lockwood, 1981).

8.2.8 Cost effective medium formulation for *Bt* biopesticide production

A cost effective medium, with 0.5% (w/v) molasses as carbon source, 10% (w/v) soya bean extract as nitrogen source, 300 mg/l cystine supplement as growth promoter, 20% (v/v) sea water as substituent of trace elements and 4% (w/v) ammonium sulphate as protease inhibitor, was formulated for *Bt* biopesticide production. The pH of the medium was adjusted to 6.8 before autoclaving at 121ºC for 20 min. It was also compared with LB broth and Glucose-Peptone medium (GPM) [10% (w/v) glucose and 5% (w/v) peptone] (Vora and Shethna, 1999) based on spore count and crystal protein concentration. For biopesticide production, 100 ml of formulated culture medium in 500 ml Erlenmeyer flasks were inoculated with *Bt* HD-73 and *Bt* JSc1 inoculum and incubated for 72 hrs at 30ºC and 180 rpm. Sample was collected at 24 hrs interval for spore count, estimation of crystal protein concentration and proteolytic enzyme assay.

8.2.9 Bioinsecticide production in 3.0 L bioreactor

Production experiments were carried out at 30ºC in a 3.0 L fully controlled bioreactor (Appendix D) containing 2.0 L of finally optimized medium. 30% dissolved oxygen level in the medium was automatically controlled by the aeration, set at 1.0 SLPM and agitation at

250 rpm. Dissolved oxygen was continuously monitored by an oxygen sensor without controlling the pH.

8.2.10 Field trial

Bt preparation containing both Cry proteins and spores, produced on the formulated medium by indigenous *Bt* strain JSc1 in a 3.0 L bioreactor, was applied in the field. The efficacy was analyzed both qualitatively and quantitatively against the caterpillars in comparison to a positive control, chemical pesticide currently in use and a negative control, water treatment usually. *Bt* preparations were usually applied at a concentration of 0.3 mg/ml of alkali soluble proteins i.e. crystal protein.

To obtain statistically valid data, randomized complete block design (RCBD) was followed as the experimental design (Fig 8.2.1). In this design, treatments are both replicated and blocked, which means that plots are arranged into blocks and then treatments are assigned to plots within a block in a random manner. This design is most effective if the patterns of non-uniformity (changing soil types, drainage patterns, fertility gradients, direction of insect migration into the field, etc) in a field are identified. If the potential sources of variations are not identifiable, this design can still be used keeping the blocks as square as possible which usually keeps the plots within a block most uniform.

A	B	A	C
B	C	C	A
C	A	B	B
Block 1	**Block2**	**Block3**	**Block4**

Figure 8.2.1: Model of Randomized Complete Block Design.

8.2.10.1 Application of *Bt* preparation in cabbage and cauliflower farming

The efficacy of *Bt* JSc1 spore-crystal protein preparation was tested against the pests of Cabbage and Cauliflower in Narshingdi. *Bt* preparation was applied besides control (Water) and chemical pesticides (lambda cyhalothrin, emamectin benzoate) to control the pests with cabbage and cauliflower such as *Helicoverpa armigera, Spodoptera litura, Plutella xylostella, Trichoplusia ni* etc which belong to the Lepidoptera insect order.

Treatments were both replicated and blocked and they were applied along 4 blocks, each containing 3 replicates. Experiment was performed with 360 plants, 30 plants in each replicate and 90 plants in each block. Survival data of plants from pest infestation and damage from each replicate was collected and statistically analyzed.

8.2.10.2 Application *Bt* preparation in organic tea farming

Bt preparation was applied in organic tea farming to protect the leaves from caterpillars in the Kazi & Kazi organic tea estate (Panchagarh) beside the conventional treatments, garlic formulation and neem formulation, upto 10 weeks. Each treatment was applied in every week and the numbers of plants survived from pest infestation were recorded for individual treatments. The data were then analysed statistically and the efficacy was determined.

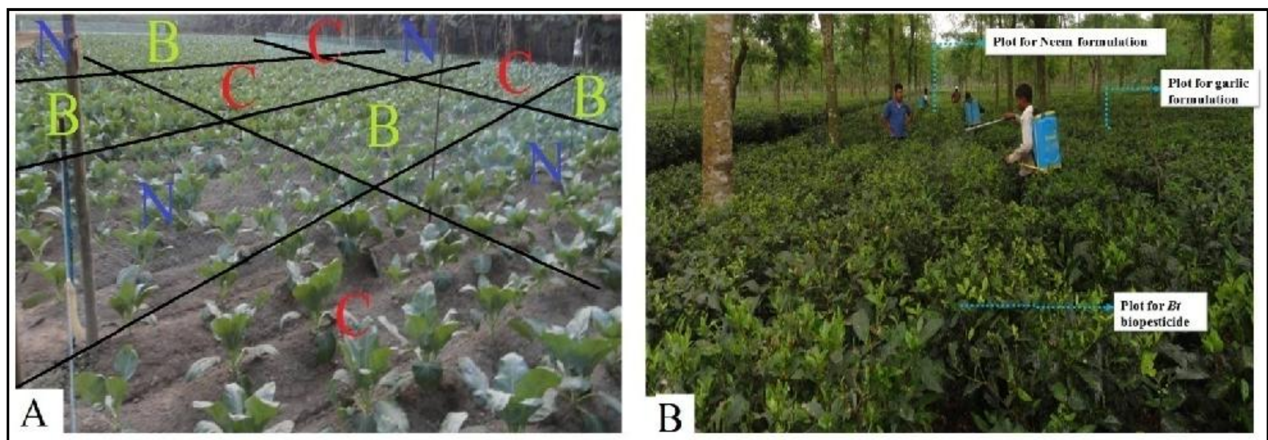

Figure 8.2.2: Application of different treatments **A)** In cabbage farming following Randomized Complete Block Design (RCBD). C: chemical pesticide; N: no treatment, B: Biopesticide. **B)** In organic tea farming.

8.3 Results

8.3.1 Enhanced sporulation and δ-endotoxin synthesis by cystine

Cystine plays an important role in enhancing both sporulation and endotoxin production (Vora and Shethna, 1999). In view of this, cystine at various concentrations starting from 100- 700 mg/l was supplemented in the liquid growth medium to determine its optimum concentration for maximum sporulation and δ-endotoxin synthesis by reference *Btk* strain HD-73. The experiment was performed with a control (without cystine) in parallel and it was observed that cystine supports enhanced sporulation and endotoxin yield. The maximum sporulation (11.31 Log cfu/ml) and δ- endotoxin yield (0.215 mg/ml) were obtained at 300 mg/l cystine (Fig 8.3.1). On the contrary, with the increase of cystine concentrations such as at 500 and 600 mg/l, both sporulation and δ- endotoxin production was decreased.

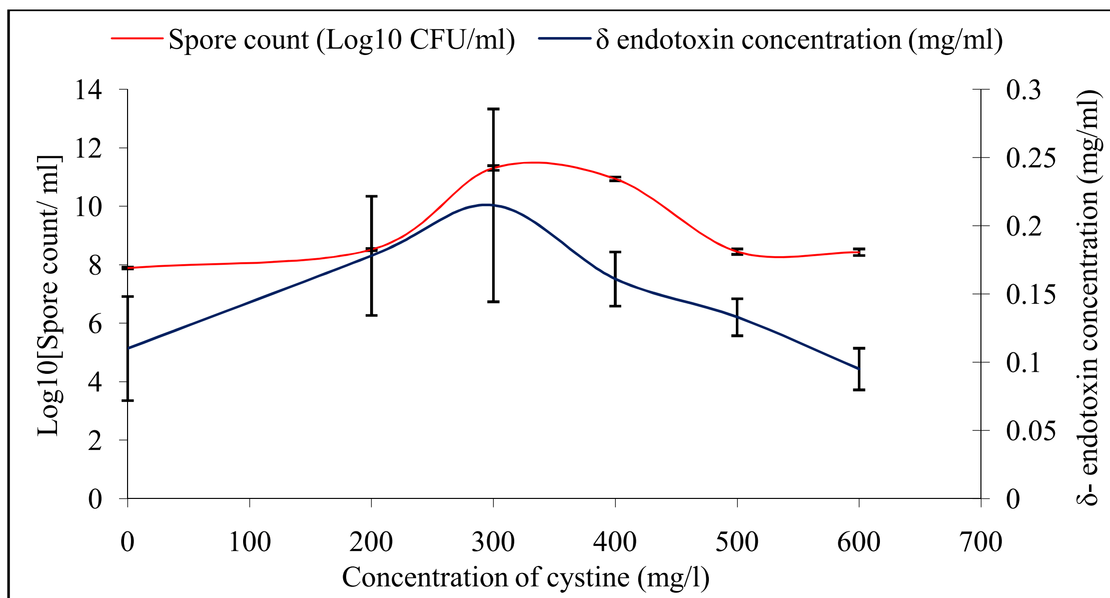

Figure 8.3.1: Determination of optimum concentration of cystine (300 mg/l) on sporulation (11.31 Log10 CFU/ml) and δ-endotoxin synthesis (0.215 mg/ml) of *Btk* HD-73.

8.3.2 Feasibility of molasses and soybean extract as efficient C & N source

Among the available cheap raw materials, defatted mustard seed meal and defatted soybean meal were compared by formulating them in different way as mentioned in methodology. The spore count determined for Soybean extract medium (Se), Mustard extract medium (Me),

Soybean meal cystine medium (SmC), Soybean meal molasses medium (SmM), Mustard meal cystine medium (MmC) and Mustard meal molasses cystine medium (MmMC) were respectively 10.34, 10.24, 9.5, 9.38, 9.32 and 8.2 log (Fig 8.3.2A) and the δ- endotoxin concentrations were respectively 0.326, 0.201, 1.66, 1.92, 1.26, 1.27 mg/ml (Fig 8.3.2B). It was observed from the comparison that soybean meal supported better sporulation and δ-endotoxin production than mustard seed meal as observed in Se medium (10.34 log & 0.326 mg/ml) and Me medium (10.24 log, 0.201 mg/ml). On the other hand, δ-endotoxin production was better in the meal form than the extract form for both of the substrates whereas the spore count was lower in this case. It may be due to the inseparable spores attached with the meal while resuspended in this purpose. Positive impacts in sporulation and δ-endotoxin production were observed when supplemented with 0.5% (w/v) molasses and optimum cystine for both of the substrates and soybean meal was superior. Thus defatted soybean meal or extract was found to be better than defatted mustard seed meal in all combinations.

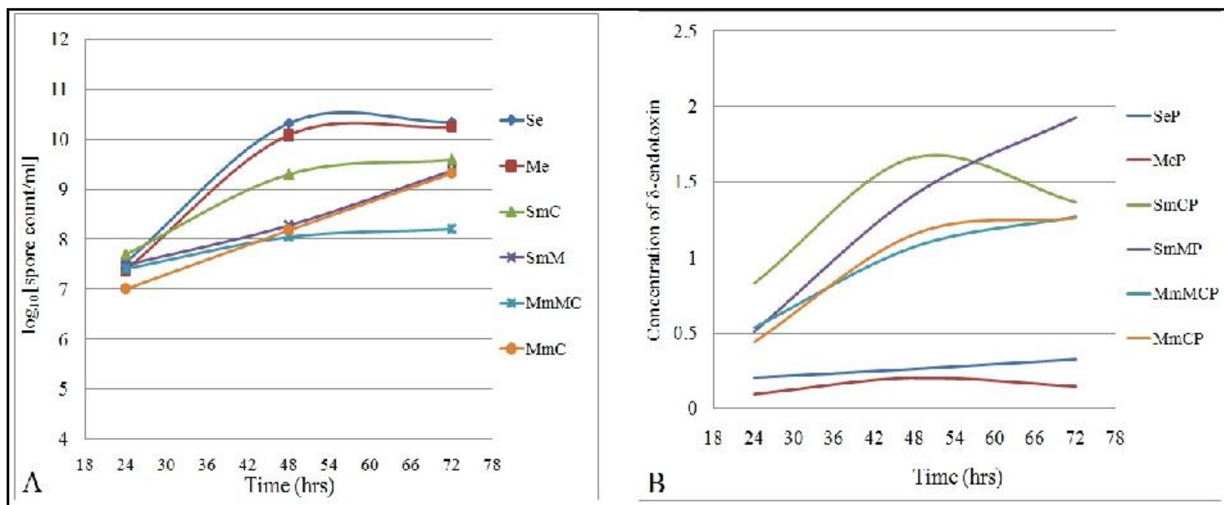

Figure 8.3.2: Comparison between cheap and available substrates by different formulations based on **A)** Spore count **B)** δ- endotoxin concentration (Se: defatted soybean extract, Me: defatted mustard extract, SmC: soybean meal cystine, SmM: soybean meal molasses, MmC: mustard meal cystine, MmMC: mustard meal molasses cystine, addition of P in figure B indicates for protein from those media).

8.3.3 Efficacy of marine water as Basal salts substituent

The efficacy of marine water (20%) in sporulation and δ-endotoxin synthesis was comparable with that of the basal salts. With both ingredients, maximum sporulation and δ-endotoxin

synthesis were obtained at 72 hours and 24 hours respectively (Fig 8.3.3). The maximum yield with sea water was 90.88% spores and 74.29% δ-endotoxin concentration of Basal salt did. This made it feasible to be used as one of the component in cheap medium formulation for Bt production in large scale level.

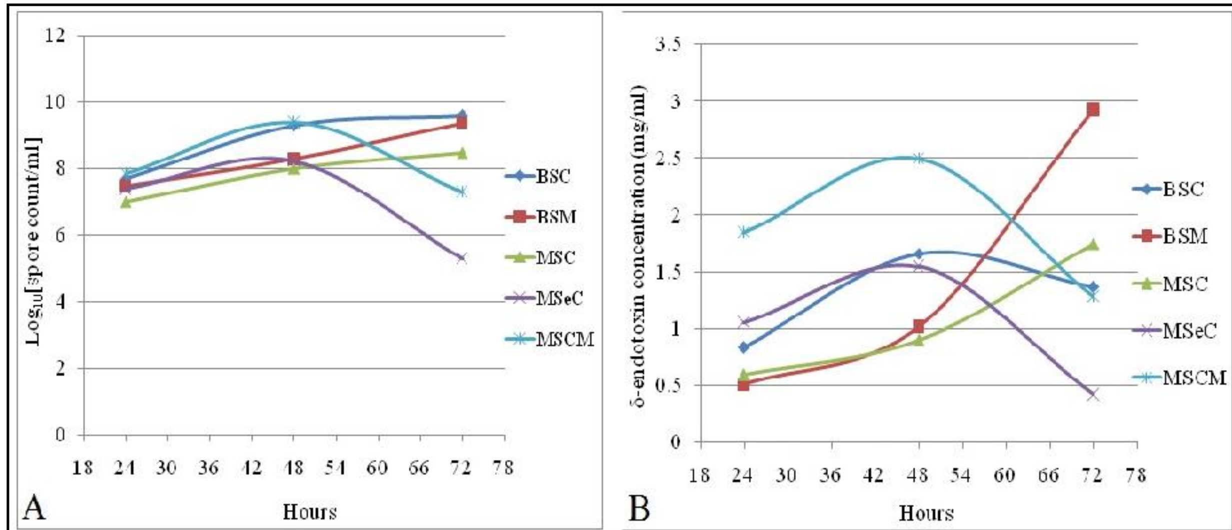

Figure 8.3.3: Comparison between basal salts and marine water with different medium formulations based on **A)** Spore count and **B)** δ- endotoxin synthesis (BSC: Basal salt-soybean-cystine, BSM: Basal salt-soybean-molasses, MSC: Marine water-soybean meal-cystine, MSeC: Marine water- soybean extract- cystine, MSCM: Marine water-soybean meal-cystine-molasses).

8.3.4 Prevention of proteolytic degradation of δ-endotoxin

Besides increasing the yield of δ- endotoxin, its protection from endogenous protease degradation is also important. It was observed that sharp decrease in δ- endotoxin concentration was occurred simultaneously with the rise of endogenous protease activity as determined by protease assay after 24 hours (Fig 8.3.4). When the protease activity reached its maximum i.e. 105.9 U/ml gradually, the δ-endotoxin concentration was also reduced to its minimum (0.075 mg/ml) from the peak (0.312 mg/ml). Protease inhibitor PMSF was added into the culture medium at 0.1 mM and 0.3 mM concentration to examine the proteolytic degradation of δ-endotoxin which inhibited the protease activity partially (Data not shown), an indication of proteolytic degradation of δ-endotoxin.

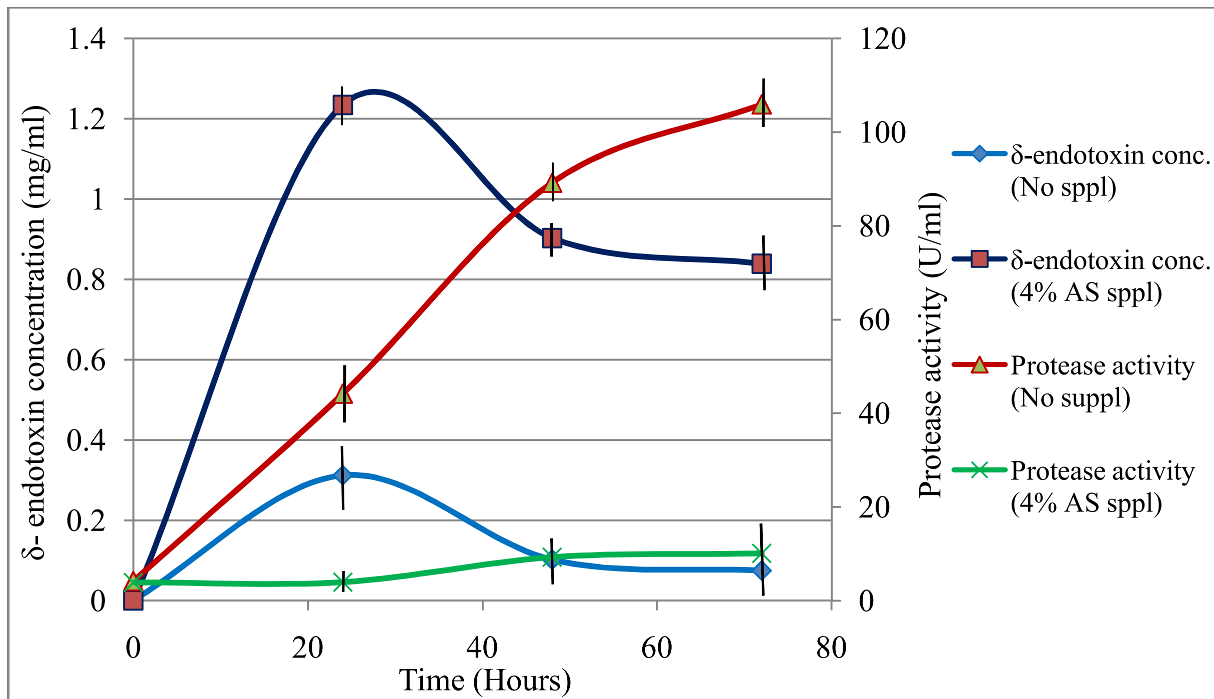

Figure 8.3.4: Effect of endogenous protease on δ-endotoxin degradation and its control by Ammonium sulphate supplement (Spl/suppl: Supplement, AS: Ammonium sulfate).

Ammonium sulfate (4%) was used as an alternative to PMSF and the protease activity was monitored up to 72 hours at 24 hours interval. Ammonium sulfate (4%) resulted in maximum endotoxin yield (1.2 mg/ml) as well as restricted the protease activity within 10.1 U/ml (Fig 8.3.4). This result corresponded to a 295% increase in δ- endotoxin productivity (51.43×10^{-3} g/L/h) in presence of 4% ammonium sulfate.

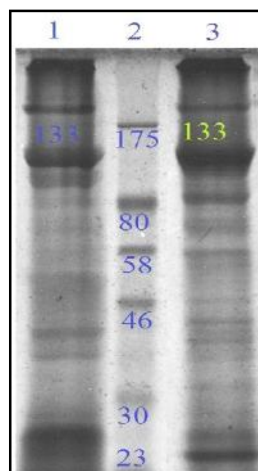

Figure 8.3.5: The yield of Cry1Ac protein by *Btk* HD-73 in MSeMC medium was analyzed by SDS-PAGE with the partially purified δ-endotoxin. **Lane 1**: Medium without $(NH_4)_2SO_4$; **Lane 2**: Marker (ColorPlus Prestained protein marker, Broad range, NEB); **Lane 3**: 4% $(NH_4)_2SO_4$ supplemented medium. Yield intensity was observed to be doubled in the presence of $(NH_4)_2SO_4$.

The effect was also visualized by SDS-PAGE analysis of the partially purified δ-endotoxin from Ammonium sulfate supplemented medium and non- supplemented medium (Figure 8.3.5). *Btk* HD-73 was used for fermentation as it is known for a single Cry protein i.e. Cry1Ac protein of 133 kD. Equal amount of protein was loaded in both lanes so that the difference can be understood and the concentraion of the partially purified Cry1Ac protein was observed to be higher in the presence of ammonium sulfate than in its absence.

8.3.5 Potentiality of optimized medium on sporulation and δ- endotoxin yield

Thus, selection of efficient C & N sources with molasses & soybean meal based on their feasibility study and regulation of a critical factor i.e. addition of cystine helped to formulate an economic media (SMC) which produced 8.47 log spores and 2.248 mg/ ml δ- endotoxin which are 1.5 fold and 17 fold higher respectively than that of commercial LB medium. But scaling up with this medium in a bioreactor might create some problems due to the insoluble particles of the soybean and soybean extract was therefore preferred over soybean meal. The yield of δ-endotoxin (0.42 mg/ ml) reduced in this formulation (SeMC) though the spore count (8.596) was higher. And the replacement of basal salts or trace element with marine water in MSeMC formulation reduced both yields (7.8 log & 0.311 mg/ ml) a little. Prevention of proteolytic degradation by PMSF improved the δ- endotoxin concentration (0.43 mg/ ml) which was improved dramatically with 4% ammonium sulphate (1.42 mg/ ml). Thus MSeMC-AS formulation based on locally available cheap raw materials was optimized (Fig 8.3.6) for shake flask culture which can be scaled up into bioreactor successfully.

8.3.6 Comparison between indigenous and reference strains in their productivity

Finally, the optimized formulation containing soybean extract, molasses, sea water, cystine and 4% ammonium sulfate was used for the production of *Btk* HD-73 and the indigenous *Bt* JSc1 under controlled conditions in a 3.0 L bioreactor with an working volume of 2.0 L. Maximum δ-endotoxin yields were 2.1 mg/ ml and 2.63 mg/ ml by *Btk* HD-73 and *Bt* JSc1 respectively at 24 hours (Figure 8.3.7). Protease activity was negligible for both strains in the finally formulated medium. The indigenous *Bt* JSc1 resulted in 25% higher δ-endotoxin yield than the reference *Btk* HD-73.

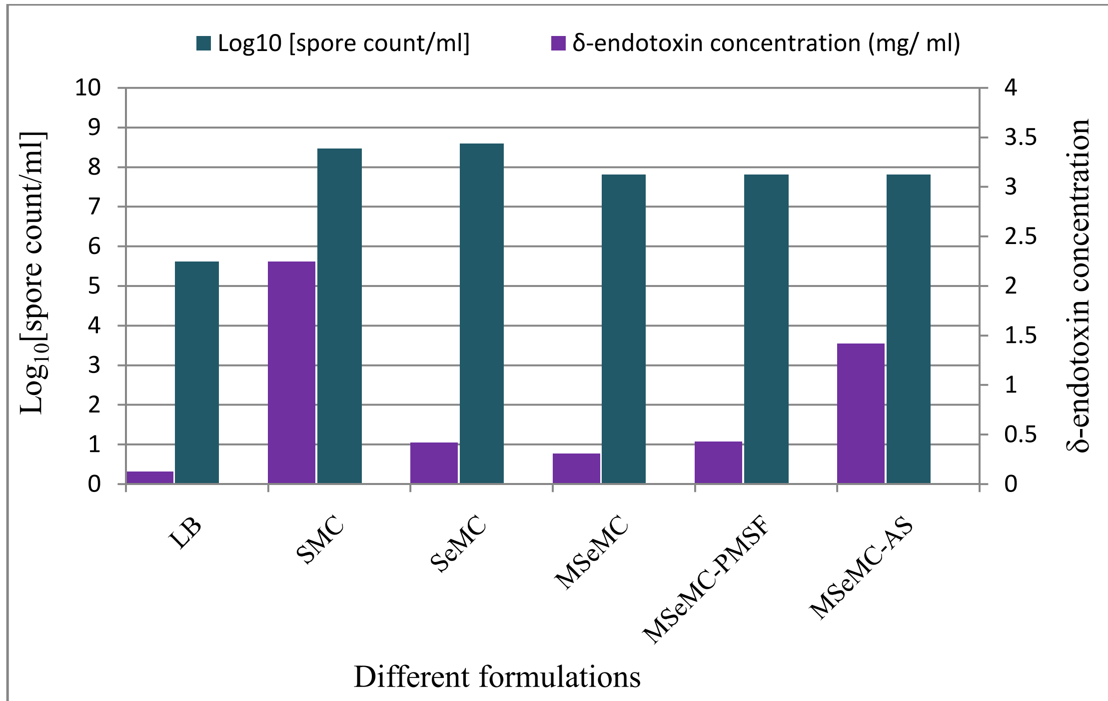

Figure 8.3.6: Optimization of an economic medium formulation by regulating critical components.

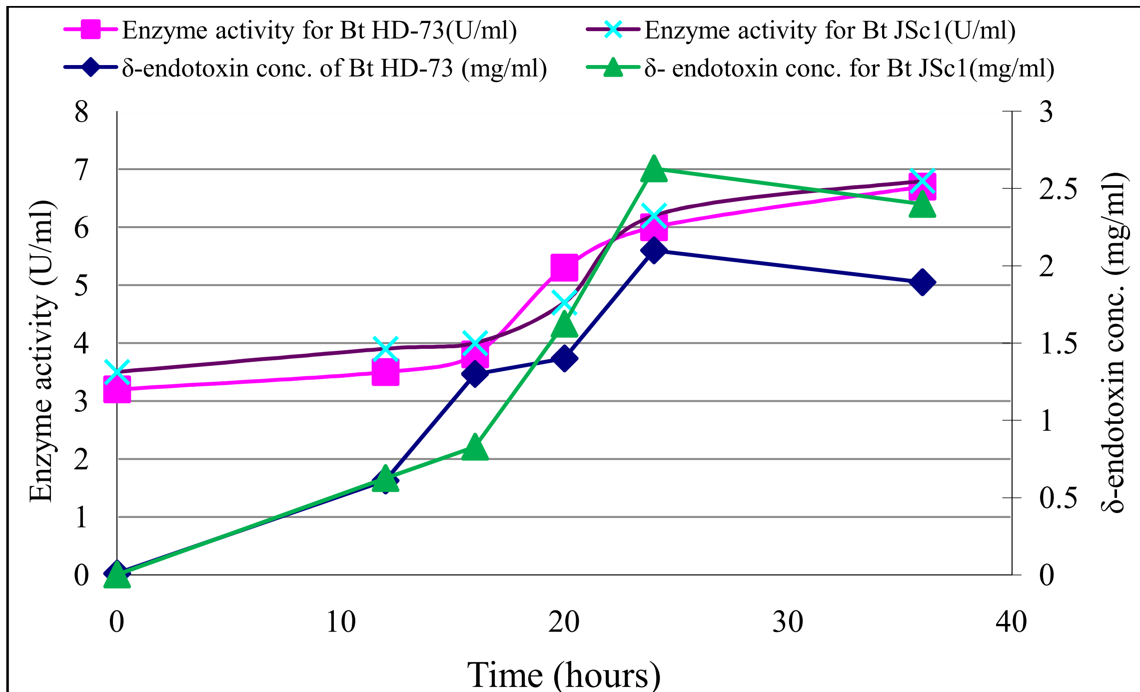

Figure 8.3.7: Production kinetics of *Bt* JSc1 and *Btk* HD-73 in MSeMC-AS medium in a 3.0 L bioreactor.

8.3.7 Efficacy of *Bt* preparation in cabbage and cauliflower farming

Bt biopesticide was applied in the cabbage and cauliflower farming following Randomized Complete Block Design (RCBD) as shown in the figure 8.2.1. Numbers of healthy crops from each block were recorded and each of them was weighed. As for the qualitative results, both *Bt* biopesticide and chemical pesticide protected the cabbage and cauliflower from pest infestation equally and untreated plants were found completely damaged by the pests (Fig 8.3.8).

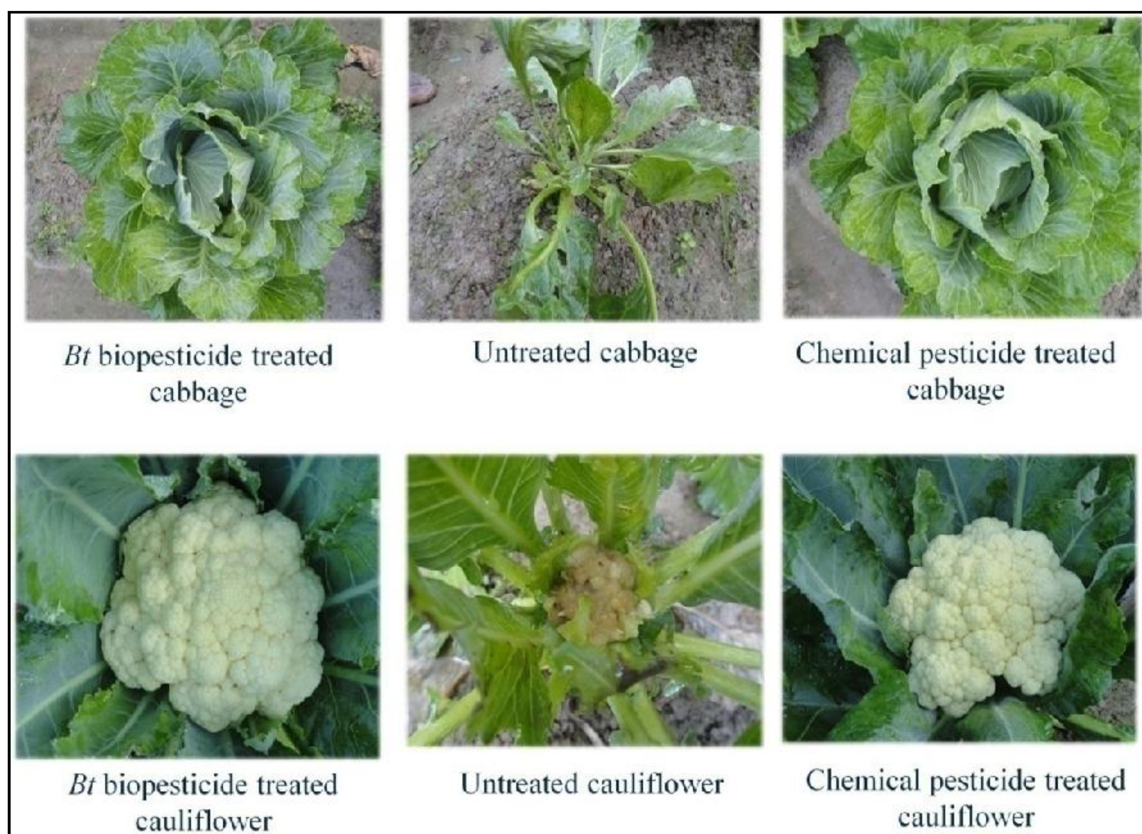

Figure 8.3.8: Impact on pest infestation and growth of cabbage and cauliflower by different treatments. Untreated plants were found damaged and *Bt* biopesticide produced comparable results with chemical pesticides.

The quantitative analysis of the treatments was performed based on the percentage of the survived and healthy cabbage and cauliflower plants and their production yield. Results were found to be comparable of *Bt* biopesticide and chemical pesticide treatments i.e. about 90% whereas only about 25% plants survived without any treatment (Fig 8.3.9). The average weight of cabbage was slightly higher for *Bt* biopesticide (1.885 kg) than that of chemical pesticides (1.855 kg) and it was opposite for cauliflower (Bt- 1.36 kg, Chemical- 1.56 kg).

And the average weight for untreated plants was almost 38% less in both cases (cabbage- 1.165 kg; cauliflower- 0.918 kg) (Fig 8.3.9).

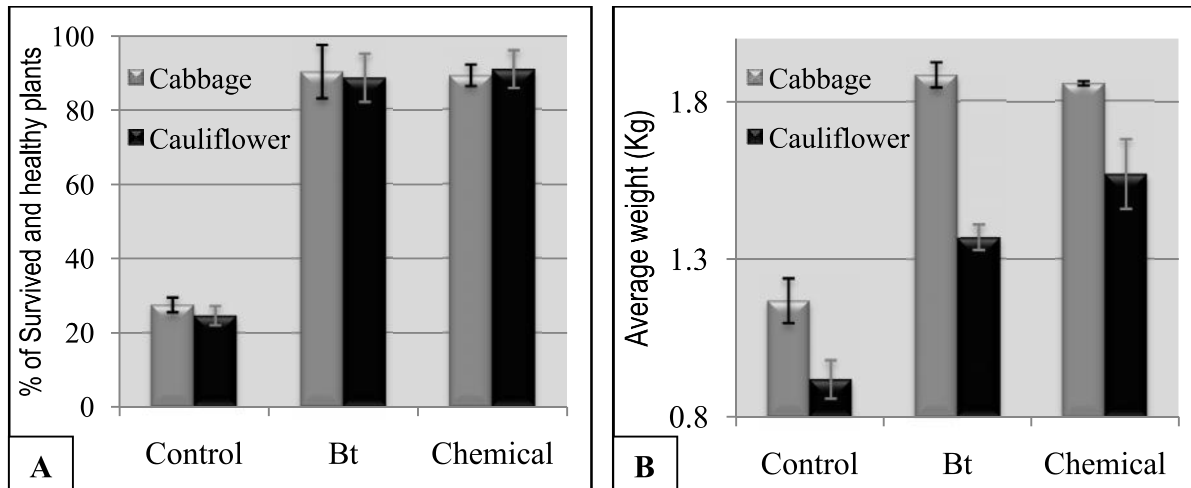

Figure 8.3.9: Comparison between the *Bt* preparation and chemical pesticides in terms of**- A)** Survival rate against pest infestation, **B)** Productivity of cabbage and cauliflower.

8.3.8 Efficacy of *Bt* preparation in organic tea farming

Efficacy of *Bt* biopesticide was found to be parallel to the other treatments i.e. garlic formulation and neem formulation that are currently in use in organic tea garden up to 10 weeks.

Neem formulation treatment was recorded with highest number of survived plants (more than 75%) at 4th week but it dropped as the treatment was stopped after 4th week and was resumed at 6th week. On the other hand, *Bt* biopesticide and garlic formulation were recorded with relatively lower survival percentage but both of them retained their impacts over time even though there was an interruption. Both *Bt* biopesticide and garlic formulation protected maximum numbers of plants after 10th week of their application but neem formulation could not regain its initial maximum protection efficiency. The differences between these three treatments were further analyzed by ANOVA test.

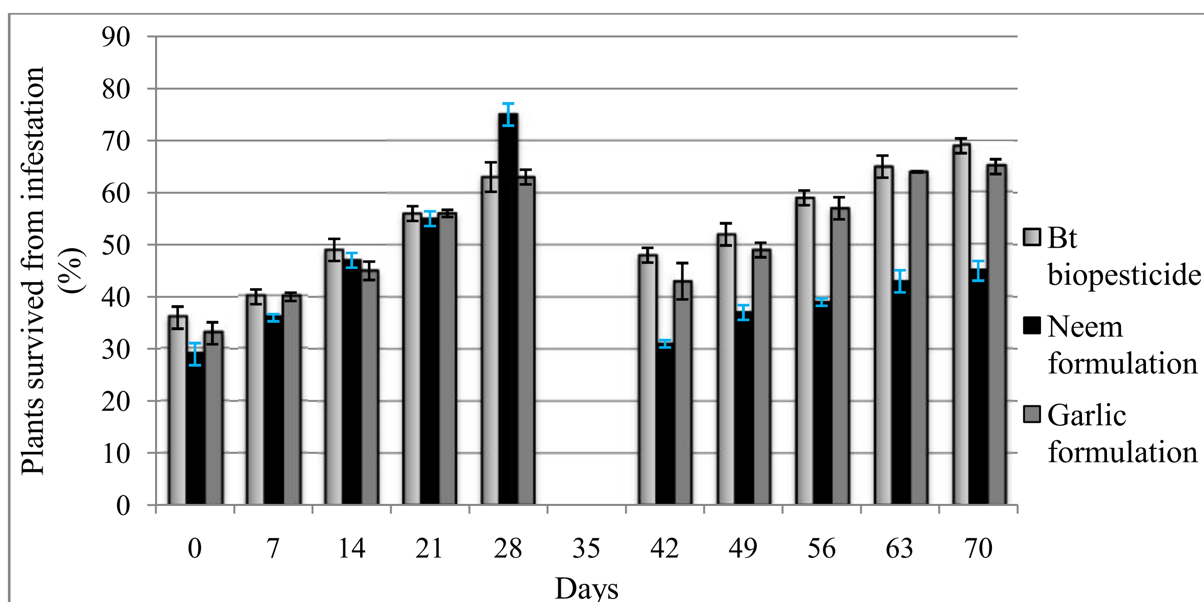

Figure 8.3.10: Comparison of potentialities of *Bt* biopesticide, Neem formulation and Garlic formulation in protecting the tea plants from pest infestation.

8.3.9 Analysis of Variance (One-Way)

8.3.9.1 Cabbage and cauliflower farming

Table 8.3.1: Statistical data obtained from the ANOVA (Two- Way) test performed on the average survival of cabbage and cauliflower plants from the pest infestation.

ANOVA for Randomized Complete Block Design (RCBD)

Source of Variation	Cabbage					Cauliflower				
	d.f.	SS	MS	F	P-value	d.f.	SS	MS	F	P-value
Treatment	2	6021	3010.6	175.658	0.00000473	2	6795	3398	661.151	0.0000000922
Block	3	89	29.6	1.724	0.261	3	103	34	6.676	0.0244
Residual	6	103	17.1			6	31	5		
Total	11	6213				11	6929			

In Cabbage farming, statistical data obtained from the ANOVA (Two- Way) produces an F value indicating that at α=0.05, statistically significant differences among the treatments

prevailed (Bt biopesticide, Chemical pesticide and Control) (F= 175.658; df=11; P-value= 0.00000473).

Also for Cauliflower production, statistical data obtained from the ANOVA (Two- Way) produces an F value indicating that at α=0.05, there were statistically significant differences among the treatments (*Bt* biopesticide, Chemical pesticide and Control) (F= 661.151; df=11; P-value= 0.0000000922) .

As significant differences among the treatments were determined statistically (Table 8.3.1), pair wise difference analyses among the multiple treatments were calculated by Fisher Least Significant Difference method.

Table 8.3.2: Fisher Least Significant Difference (LSD) Method for Multiple Comparison Test

Group vs Group (Contrast)	Difference (taking the absolute value)		Least Significant Difference	
	Cabbage	Cauliflower	Cabbage	Cauliflower
Biopesticide vs Chemical pesticide	1.5	3.75	7.154864	3.868907
Biopesticide vs Control	48.25	48.5	7.154864	3.868907
Chemical pesticide vs Control	46.75	52.25	7.154864	3.868907

From the analysis, the data provides much evidences to conclude that, at α=0.05 level of significance, *Bt* biopesticide and Chemical pesticide do not differ significantly whereas the pairs *Bt* biopesticide and control as well as Chemical pesticide and Control differ significantly in Cabbage farming. It means that the efficacy of *Bt* biopesticide and Chemical pesticide in controlling the pests with cabbage exists highly in comparison to no treatment. But there was no significant difference among these two efficient treatments (Table 8.3.2). This suggests that the substitution of chemical pesticide currently in use in cabbage farming with our *Bt* biopesticide preparation will be feasible in terms of controlling pests.

Again, in Cauliflower farming, similar results were observed i.e. *Bt* biopesticide and Chemical pesticide do not differ significantly at α=0.05 level of significance whereas the pairs *Bt* biopesticide and control; and Chemical pesticide and Control differ significantly at α=0.05 level of significance. This clearly indicates that the substitution of chemical pesticide with our *Bt* biopesticide preparation is possible.

8.3.9.2 Organic tea farming

Table 8.3.3: Statistical data obtained from the ANOVA (One- Way) test performed on the average survival of tea plants from the pest infestation

SUMMARY (Organic Tea farming):

Treatments	*Sample size*	*Sum*	*Mean*	*Variance*
Bt biopesticide	11	592	53.8181	32902
Garlic formulation	11	568	51.6363	30448
Neem formulation	11	487	44.2727	23221
Total	33		49.9090	136.5852

ANOVA (Organic Tea farming):

Source of Variation	*d.f.*	*SS*	*MS*	*F*	*p-level*	*F crit*	*Omega Sqr.*
Between Groups	2	550.3636	275.1818	2.1609	0.1328	3.31583	0.06573
Within Groups	30	3820.3636	127.3454				
Total	32	4370.7272					

Statistical data obtained from the ANOVA (One- Way) produced an F value indicating that at α= 0.05, there was no statistically significant difference between the mean survival of tea plants for all the treatments throughout the field trial replicates (F= 2.1609; df= 32; P= 0.1328). This inferred that these three treatments, *Bt* biopesticide formulation, Garlic formulation and Neem formulation to be of equal effects in organic tea farming (Table 8.3.3). Hence, *Bt* biopesticide preparation can be used in this field in parallel.

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CHAPTER 9

Discussion and Conclusions

9.0 DISCUSSION AND CONCLUSIONS

9.1 DISCUSSION

The indiscriminate use of agricultural pesticides is causing serious health problems and environmental pollutions in many developing countries including Bangladesh. These pesticides are extremely hazardous and recalcitrant in nature which upon mobilization through irrigation, farming and flooding results in bioaccumulation and biomagnifications and thus exist in the food chain. They not only affect soil health and microbial flora, water bodies and aquatic lives i.e. fauna and fishes but also affect animal and human health (Jackson, 1991; Ramaswamy, 1992). Emergence of resistance in the pests is another major problem associated with chemical pesticides. This necessitates "Integrated pest management (IPM)" which accommodates all possible eco-friendly pest control methods by making greater use of economical, sustainable and environmentally safe alternatives such as the use of biopesticides. Research on biopesticides development necessitates the isolation of naturally occurring potential *Bacillus thuringiensis* strains and the determination of their specific target pests. No comprehensive study has been done in Bangladesh aiming at its large scale production and application for controlling the pests particularly with vegetables.

The study was, therefore, performed to isolate and identify *Bt* strains from different areas of Bangladesh effective against different pests affecting particularly vegetables. This was followed by the characterization of toxin genes and proteins, selection of the most effective *Bt* isolates by bioassay, their large scale production and application in the field.

Continuous search for *Bacillus thuringiensis* (*Bt*) strains expressing toxins with novel and improved activity keeps tremendous importance for replacing the prevailing chemical pesticides as well as to fight the pest resistance. The research was, therefore, carried out with a view to develop a *Bt* collection source characterized with their genetic and toxic properties leading to biopesticide production.

Prevalence of *Bacillus thuringiensis* in Bangladesh

Sample was collected from different parts of Bangladesh in a manner so that almost the entire eco-regions of the country could be represented and the isolation *of Bt* with diversities from different eco-systems as well as the analysis of abundance and distribution could be accomplished. So, 26 different districts of Bangladesh that covered plane lands, river basin,

hilly regions, sandy beaches and agricultural lands were included in this study (Fig 3.2.1) and samples comprising soil, leaf, insect cadaver, dust etc were the sources for *Bt* isolation as described in many literatures (Öztürk *et al.*, 2009).

Two hundred and thirty one samples were processed with acetate selection and heat treatment so that the spores other than *Bacillus thuringiensis* are germinated and heat-killed. Then the left alone *Bt* spores were allowed to germinate in T_3- agar medium for overnight which was extended up to 72 hours for complete sporulation as protein crystals are synthesized during this time. Phase contrast microscopy was performed to identify the isolates synthesizing crystal proteins (Fig 3.3.1) and 366 isolates were obtained with this property. Acetate selection method was preferred over ampicillin-polymyxin B selection method (DeLucca *et al.*, 1981) as it was proved to be more efficient in isolating even those *Bt* strains that are susceptible to the antibiotic treatment and do not survive (Travers *et al.*, 1987).

It was discussed that *Bacillus sphaericus* responded similarly in this isolation process but was distinguished by Phase contrast microscopy as lacking parasporal crystal proteins (Travers *et al.*, 1987). Again some strains of *B. sphaericus* synthesize a parasporal inclusion or crystal which contains proteins toxic for larvae of a variety of mosquito species (Baumann *et al.*, 1991). So, this stalemate process needed some additional confirmation for confident differentiation of *Bt* from *Bs*. In this regard, after obtaining the parasporal crystal proteins producing *Bt* and *Bs* like isolates, the starch hydrolysis test was employed according to the Bergey's Manual of Determinative Bacteriology to differentiate among these two species and isolates with starch hydrolyzing ability were identified as *Bacillus thuringiensis*. Upon starch hydrolysis test, 317 out of 366 parasporal crystal protein synthesizing isolates were confirmed as *Bacillus thuringiensis*. Thus it was observed that 83.12% (n=192) of the samples were *Bt* isolate producing and the distributions were calculated to be 92% (n=171) for soil samples, 37.5% (n=12) for leaf samples, 55.5% (n=5) for insect samples and 100% (n=4) for dust samples (Table 3.3.2).

The spore diameter of native *Bt* strains determined in this study ranged from 0.6 to 1.02 μm and the average spore diameter was calculated 0.76±0.097 μm in this study and native *Bt* spores were found 20% bigger than the reference *Btk* HD-73 (0.63±0.081 μm). Deviation in spore diameters up to ±0.097 for local *Bt* strains is a clear indication of high diversities among the strains of Bangladesh.

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Bt index, most often used as an indication of how easily the *B. thuringiensis* was isolated, was 0.86 in this study and it was variable across different ecosystems of Bangladesh. Northern and central parts of the country were observed with higher index than the rest other parts and *Bt* was less available in the sandy beaches, yet it was 0.73 (Table 3.3.3).

Bt is known for a lot of important and diverse functions like bacteriocin, chitinase, Vip toxins, cytocidal proteins and δ endotoxin production which are not common for all. To sort out the strains with specific functions, typing is very important for which Serotyping and biotyping are two established methods for rapid classification into subspecies with a presumptive function. Biotyping was preferred over serotyping, in this study, as it is less cumbersome, and a rapid process and neither serotyping nor biotyping reveal the expression of insecticidal δ- endotoxins. The biochemical typing was performed based on the esculin hydrolysis, salicin utilization, lecithinase production, and sucrose utilization ability, which were reported to be the most variable among *Bt* isolates and these tests enabled devising a simplified system for categorizing into 16 biochemical types (Martin and Travers, 1989). Ten out of 16 biotypes were described for known subspecies and named on them though few biotypes were found to represent more than one subspecies but named with the prevalent one (Martin and Travers, 1989). Categorizing the *Bt* strains, characterization and further analysis in this study were performed based on the biotyping.

Again, *Bt* has been reported to produce parasporin, another type of parasporal crystal protein which has anti-cancer cell activity. It was shown that parasporal proteins from non-hemolytic *Bt* strains are mainly non-insecticidal but may have anti-cancer cell activity (Mizuki *et al.*, 1999). So, hemolytic activity or broad spectrum cytolysin activity for presumptive identification of insecticidal isolates and biochemical typing were performed with all the *Bt* isolates obtained. The biochemical tests thus revealed the available biotypes describing *Bt indiana*, *kurstki* and *thuringiensis* as the most prevalent in Bangladesh.

Distribution of *Bt*

The distribution pattern of different biotypes in different sampling sites was also analysed which was accomplished by an index i.e. distribution index. The distribution index was calculated by dividing the number of sampling sites with a particular biotype with total number of sampling sites. The distribution index implies the degree of ubiquity for any biotype in this study. Biotypes *thuringiensis* (0.5) and *indiana* (0.5) were found to be more ubiquitous than all other biotypes followed by *eleven* (0.43), *kurstaki*, *sotto* and *nine* (0.4 for

each) (Table 3.3.4). Biotypes containing isolates less than 10 were not considered. On the other hand, with more samples, more *Bt* isolates were obtained and more biotypes were also present in north Dhaka and central Dhaka whereas the same consequences were not observed in Jamalpur and Sonargaon (Table 3.3.4).

The abundance of *Bt* biotypes around the world (Martin and Travers, 1989) was compared with that of Bangladesh. The abundance pattern in Bangladesh was slightly similar with that of Asian countries but high variations were discernible with the abundance pattern of other regions (Fig 3.3.6). Biotypes 14 (*israelensis*), 15 and 16 account for 30-58% of the *Bt* strains different parts of the world whereas it was 13.8% for Bangladesh. On the other hand, biotype 1 (*thuringiensis*), 2 (*kurstaki*) and 3 (*indiana*) together account for 47.2% *Bt* strains in Bangladesh while only 4.4- 12% was observed in the other parts of the world other than Asia. The percentage was 30% for Asian countries which is much closer to Bangladesh. Martin and Travers showed that biotypes not yet described represented 51.9% of the total isolates worldwide (Martin and Travers, 1989). For Bangladesh it was calculated 30.5% in this study.

The insecticidal *Bt* strains as presumed by their broad spectrum cytolysin activity were found to be 58.36% (n=189) and rest 41.64% can be explored for anti-cancer cell protein, parasporin. Based on this criterion, percentage of insecticidal strains in each biotype was also calculated (Fig 3.3.2B) which revealed the prevalence to be *Bt 10*> *Bt kurstaki*> *Bt dendrolimus*> *Bt thuringiensis*> *Bt 9*> *Bt indiana*> *Bt israelensis* (Fig 3.3.7). On the other hand, biotype *darmstadiensis* and *ostriniae* were found with no hemolytic strains and only 7% of the strains were hemolytic in biotype 15. Less than 40% hemolytic strains were present in biotypes *galleriae, morrisoni, 11, 13, 16* and more than 60% hemolytic strains were present in rest other biotypes (Fig 3.3.7). 100% strains from *Bt* 10, 93% from *kurstaki* and 85% from *dendrolimus* were hemolytic.

Diversity analysis based on 16S rRNA and plasmids

The 16S rRNA gene sequence analysis was performed with randomly selected 19 strains. Sequences obtained following PCR with universal primer set for *Bacillus* were analyzed and they were identified as *Bacillus thuringiensis* by blastn program. The sequences of 9 strains have been submitted into the NCBI database and accession numbers were provided against them (Table 3.3.5). This technique has been used as a molecular identification tool for *Bt* and the claims of its ability to discriminate *Bt* in different H-serotypes also was reported (Joung and Cote, 2002; Soufiane and Cote, 2009; Poornima *et al.*, 2010). In the present study, a

dendrogram was constructed based on neighbour-joining method by aligning the sequences which is indicative of the phylogenetic relationship among the strains. Two main clusters (A and B) were generated from the dendrogram analysis (Fig 3.3.9) with two sub-clusters in cluster A. Sub-cluster A1 is the largest with 12 strains in which *Bt* strains mainly from biotype *kurstaki* were found and in cluster A2, non-hemolytic *Bt* strains were prevalent.

Bt strains harbor a varied number of extra-chromosomal elements i.e. plasmids with different molecular mass ranging in size from 2 to 200 kb in general. Some of these plasmids are circular and some are linear, and most of the *cry* genes are located in these large plasmids (Carlson *et al.*, 1996). Plasmid exchange between strains as well as recombination between *cry* genes from different backgrounds occur in *Bt* strains (Feitelson *et al.*, 1999). Usually mega plasmids are present in low copy numbers and small or cryptic plasmids are present in high copy numbers in *B. thuringiensis*. So it is likely to observe more small plasmids than mega plasmids in agarose gel electrophoresis. Again depending on the variation in techniques, successful extraction of small plasmids may vary also. Plasmid was therefore extracted with a view to compare the pattern among indigenous *Bt* strains. In our study, large plasmids were observed in many strains but may be due to their presence in low copy numbers (Lereclus *et al.*, 1993), the bands were faint (Fig 3.3.10).

Within *B. thuringiensis* species, a large variation of plasmid patterns has been found, reflecting higher strain diversity. The pattern for small plasmids was therefore compared and molecular weight based distribution of plasmids among the biotypes was determined which in other sense renders the diversity as well. Plasmids from *Bt thuringiensis*, *kurstaki*, *indiana* and *israelensis* biotypes occupied a wide range of 3kb to more than 16 kb thus indicating the presence of more diversity among the strains (Fig 3.3.11).

Prevalence of *cry* genes

The toxicity of any *Bt* strain is dependent on the expression of the δ-endotoxins or Cry toxins and there are currently around 75 primary subgroups of Cry toxins- i.e. with different primary ranks in the nomenclature (Cry1, Cry2, Cry3, etc.) (Crickmore *et al.*, 2014). These toxic crystal proteins are encoded by the *cry* genes and one *Bt* strain may harbor more than one *cry* genes. Hence, with a view to classifying potential strains from our collection of *Bt*, *cry* genes toxic against agriculturally important pests i.e. *cry1*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9, cry10* and *cry11* genes were searched in this study.

In this detection process, few sets of rules were followed as to which *Bt* strains to be checked for the presence of which *cry* genes. Gene contents of different *Bt* subspecies was an important guideline in this process. *Bt* strains belonging to different biotypes describing these subspecies were checked for the presence of corresponding genes as correlated from Table 4.2.1 and Table 4.2.2. Thus the strains belonging to the biotypes describing *Bt* subspecies *thuringiensis, kurstaki, galleriae, sotto, dendrolimus, morrisoni* were examined for the presence of *cry1* gene, *kurstaki* and *sotto* for *cry2* gene, *kurstaki* and *morrisoni* for *cry3* gene, *israelensis* for *cry4*, *cry10* and *cry11* genes, *galleriae* for *cry8* gene and *kurstaki* for *cry9 gene*. But many subspecies like *aizawai*, *tolworthi*, *tenebrionis*, *japonensis* etc containing *cry1*, *cry3*, *cry8*, *cry9* etc genes could not be traced by biotyping, hence incompatible to the criterion. So, all these genes were also searched in many randomly chosen *Bt* strains which was another criterion. And *cry2*, *cry3* and *cry9* genes were searched in all *cry1* positive *Bt* strains too. Thus the number of *Bt* strains checked were, 224 for *cry1* gene, 94 for *cry2*, 81 for *cry3*, 69 for *cry9*, 43 for *cry4,* 48 for *cry8,* 42 for *cry10* and 54 for *cry11* genes and *cry1A*, *cry1Aa*, *cry1Ac*, *cry1Ba*, *cry1Ca* genes were also searched in *cry1* positive *Bt* strains.

From the screening of the above mentioned *cry* genes, 73 strains out of 230 were positive for at least one of the *cry* genes searched for. The prevalence of these *cry* genes were calculated and *cry1* gene was found to be the most prevalent (30.8%) followed by *cry2* (25.5%), *cry3* (22.2%) and *cry9* (7.2%) (Fig 4.3.8A). Prevalence of *cry4*, *cry8* and *cry10* genes were less than 5% and *cry11* gene was not found in any strain. The prevalence of tertiary subgroups of *cry1* gene such as *cry1Aa*, *cry1Ac*, *cry1Ba* and *cry1Ca,* searched in this study was also determined (Fig 2.3.8B). Among the subgroups, *cry1Aa* (60%) gene was determined to be the most prevalent followed by *cry1Ca* (21.7%)*,* *cry1Ac* (16%) and *cry1Ba* (8.7%). Again, strains harboring maximum number of *cry* genes were also determined. *Bt* strain JDb1 was found to harbor maximum 6 different *cry* genes (*cry1Aa*, *cry1Ac*, *cry1Ca*, *cry2*, *cry3* and *cry9*) and strain FhSb3 was positive for 5 *cry* genes (*cry1A*, *cry1Ba*, *cry1Ca*, *cry2* and *cry3*). Eight *Bt* strains were found positive for 4 different *cry* genes, 12 strains for 3 *cry* genes, 18 strains for 2 *cry* genes and 33 strains were found to harbor only 1 *cry* gene.

Comparison between *cry* gene frequencies and distributions

The reports on *cry* genes distribution are quite variable worldwide. Certain *cry* genes have been recognized as abundant with *Bt* strains everywhere in the world, such as *cry1*-type genes. As for the frequency, certain *cry* genes are more prevalent in some areas than the others, such as *cry1A*, *cry1C*, *cry1D* and *cry2* were more commonly found in Asia (Chak *et*

al., 1994; Ben-Dov *et al.*, 1997; Kim, 2000; Zhang *et al.*, 2000) than those from Latin America (Bravo *et al.,* 1998; (Gislayne *et al.*, 2004). So far reported most common *cry* gene in nature belong to *cry1* gene group (Porcar and Juarez-Perez, 2003) and similar reports of *cry1* gene to be the most frequent in the collections were made (Ben-Dov *et al.*, 1997; Bravo *et al.*, 1998; Wang *et al.*, 2003). Same pattern was observed in this study as the number of strains containing the *cry1* gene was maximum (69) as compared to the other strains containing the *cry2* (24), *cry3* (18), *cry4* (2), *cry8* (3), *cry9* (5) and *cry10* (1) genes (Fig 4.3.8B). It was also reported that *cry1* genes was the most frequent (49.5%), then *cry3* gene as highly abundant (21.7%) and *cry9* gene less abundant (2.6%) (Bravo *et al.*, 1998). These results showed both similarity and dissimilarity across different geographic regions which might affect the diversity of *cry* gene content of *Bt* strains. The prevalence of *cry2* (25.5%) gene was found to be next abundant in this study followed by *cry3* (22.2%) and *cry9* (7.2%) which is an exception revealed in the present study.

Frequency and distribution of *cry* genes might be influenced by biological, geographical and ecological factors as Wang *et al.*, (2003) found more isolates harbored *cry1C*, *cry1D* and *cry1B* gene and less strains contained a *cry*1E gene in China than the results described by Zhang *et al.*, (2000). It was found in many studies that concomitant occurrence of *cry1* and *cry2* genes were observed (Ben-Dov *et al.*, 1997; Zhang *et al.*, 2000; Wang *et al.*, 2003). Wang *et al.* showed that 90.7 % strains among the *cry1* gene positive isolates, also harbored a *cry2* gene (Wang *et al.*, 2003). Similar report was from Ben-Dov *et al.* that most of the isolates containing *cry1* gene were also positive for *cry2* gene (Ben-Dov *et al.*, 1997). In the present study, it was observed that *cry2* gene was not present in a strain that lacked *cry1* gene i.e. 36.23% of the strains positive for *cry1* gene was found to harbor *cry2* gene. Similar stories were found for *cry3* and *cry9* genes as 26% and 7.2% of the *cry1* gene positive strains respectively harbored them.

The significance of 157 *Bt* strains detected with no *cry* genes searched in this study is that they may contain other genes out of 75 different *cry* gene groups and subgroups as have been defined in the literature to date (Crickmore *et al.*, 2014).

Analysis of novel *cry* gene sequences, being reported in a quick process, requires reliable and rapid methods for assessing the insecticidal potential of these genes. Presence of many cryptic *cry* genes, arising out of frequent recombination events among the genes, was also reported in many *Bt* strains (Kaur, 2006). Therefore, the presence of a PCR band alone is not sufficient and its translation needs additional confirmatory tests. The determination of certain

Cry proteins by their shapes is an obsolete practice and it is also difficult to determine the shape of the δ-endotoxins in maximum cases by mere Phase Contrast Microscope, hence necessitates Scanning Electron Microscopy (SEM). Yet, the shape of a crystal protein does not necessarily imply the degree and spectrum of its toxicity.

Analysis of Cry proteins

The insecticidal potential of a *Bt* strain can more appropriately be ascertained by detection of *cry* genes present followed by analysis of crystal proteins produced by that strain. Various techniques have been adopted for analysis of Cry proteins. Immunological characterization (Zouari and Jaoua, 1997), proteomics-type approach involving purification and micro-sequencing of the major peptides (Chestukhina *et al.*, 1994) etc have been used for the identification of Cry proteins. PCR amplification is also useful for quantitative determination of differential *cry* gene expression at the mRNA level by using reverse transcription PCR (RT-PCR) strategy. However, use of monoclonal antibodies for the detection of Cry proteins has got limitations due to the cross-reactivity among different Cry proteins. Again, this technique becomes infeasible when characterization of a large number of strains to identify potential ones which might contain more than one δ-endotoxins. Hence, a binary approach consisting of characterization of *Bt* strains in terms of their *cry* genes content followed by the analysis of the Cry proteins present can be the most feasible and economic process in *Bt* biopesticide research as potential strains with putative toxicity are actually obtained in any of the above mentioned techniques which need to be confirmed further by Bioassay.

In this study, SDS-PAGE analysis of the Cry proteins and determination of their molecular weights were performed initially. Then, the molecular weights of the Cry proteins, one from each tertiary subgroup, reported so far in the full list of δ-endotoxins, were deduced from their amino acid sequences with the help of bioinformatics. A table was prepared with the Cry proteins and their corresponding molecular weights (Table 4.3.4) which was used to identify the unknown proteins from their molecular weight. The identified putative Cry proteins of certain strain were then confirmed by its corresponding *cry* genes profile. It was observed from the molecular weight table (Table 4.3.4) that the reported δ-endotoxins ranged from 13-170 kDa.

The *Bt* strains, used for the Cry protein analysis, were chosen in a manner so that the strains positive for single and multiple *cry* genes and detected with no *cry* genes are covered in the study. The purified proteins were observed under Phase Contrast Microscope (PCM) and the

shapes of the crystal proteins were presumed to be of different sizes and the shapes of the proteins as revealed were bipyramidal, rhomboidal, spherical, triangular, cubic, irregular etc (Fig 4.3.9). But the shapes were not recorded for the strains because it was very difficult to conclude about the shapes by PCM only which might be ambiguous.

The δ-endotoxins from 50 indigenous *Bt* strains were purified partially and subjected to SDS-PAGE. The molecular weight of the Cry proteins obtained from the indigenous *Bt* strains were in the range from 26 kDa to 179 kDa. Protein bands with similar molecular weight of the tabulated Cry proteins (Table 4.3.4) were considered as Cry proteins only. It was observed that the molecular weights of several Cry proteins are very similar and differ in decimal (Table 4.3.4). Again, the determined molecular weight by SDS-PAGE is never 100% accurate. So, the Cry proteins were presumed as any of those with close molecular weights in that ranges. The Cry protein profiles for the tested strains were deduced (Table 4.3.5) and further matched with their respective *cry* gene profiles (Table 4.3.1).

It was observed from this analysis that 16 *Bt* strains, detected with no *cry* genes (searched in this study) were not found to produce any Cry protein bands of respective molecular weights except JaL6 and JaS2 producing bands of 103 and 65 kDa which are similar to the products of *cry1Af* and degraded *cry1A*(*a-c*) or *cry2* genes respectively. Cry protein was also purified from 4 *Bt* strains that were not searched for any *cry* genes and among them SaS9, CoS2 and RaSc2 did not exhibited any Cry protein bands whereas JaSc2 showed the presence of 130, 103 and 65 kDa bands which might be Cry1Ab/1D/4/7/8/9/61, Cry1Af and degraded Cry1A(a-c) or Cry2 proteins respectively.

Cry1- type δ endotoxins including Cry1Aa, Cry1Ab, Cry1Af, Cry1B, Cry1C and Cry1M were observed in 26 indigenous *Bt* strains out of 50 tested. Occurrence of Cry1C- type protein was observed only in the strains expressing Cry1A- type proteins whereas Cry1B and Cry1M were not accompanied by any other Cry1- type proteins. From the *cry* gene profile analysis of these strains, 18 strains were confirmed for Cry1A- type proteins, 1 strain for Cry1B- type protein, 4 strains for Cry1C- type protein and 1 strain for Cry1M- type protein was confirmed.

Cry2- type proteins of about 70 kDa were observed in 3 strains, i.e. NaL1, KSa2 and NoS4 and the results were also supported by the PCR detection of *cry2* genes. On the other hand, the molecular weight of the Cry2 proteins were also demonstrated to be about 65- 66 kDa in few reports (Arango *et al.*, 2002; Armengol *et al.*, 2007; Seifinejad *et al.*, 2008; Bukhari and

Shakoori, 2010) though the deduced molecular weight (M_r) from their amino acid sequences were found to be around 70 kDa. Hence, the 65- kDa protein, the denotation D1 was used for which, might be Cry2A- type protein or the degraded product of Cry1Aa-type protein and this has been shown as underlined bold character for 4 strains that were confirmed by PCR for *cry2* gene.

Presence of 72-75 kDa protein bands in SDS-PAGE analysis was presumed as Cry3- type proteins in the strains CoS2, FhSb3, JDb1 and SSb2 which were confirmed in those strains except CoS2 from their *cry* gene profiles. Protein band of 130 kDa revealed in SDS-PAGE analysis might be the translated product of Cry1Ab/ Cry1D/ Cry4/ Cry7/ Cry8/ Cry9/ Cry61 Cry8- type proteins which was observed in the strains AgS1 and SaS4. These strains were found to be positive for *cry8* gene and hence the protein band was confirmed as Cry8- type protein. Similarly, 130 kDa band presumed as Cry9- type protein was confirmed in the *Bt* strain SoI1 by PCR detection of *cry9* gene. In the strain JSc1, Cry9- type protein was presumed for a 138 kDa band and it was also confirmed from the *cry* gene profile. The observation of protein bands with 57, 29, 27 and 26 kDa suggests the presence of Cyt proteins which may exert toxicity against Dipteran insect order.

The reason for not getting the bands for *cry1*, *cry2*, *cry3* and *cry9* genes in the strains detected as positive may be the little or no expression of these genes. *Bt* strains can exhibit different protein profile regardless of the type of *cry* genes, which they carry. In other words, the same protein profile might not be exhibited by two different strains which carry same *cry* genes. This may be due to some environmental factors which can turn on and off the expression of some *cry* genes (Agassie and Lereclus, 1995). There may also be post transcriptional and post-translational regulation of *cry* genes for which the protein profiles might differ. Again the purification process may also influence the recovery of different Cry proteins.

The prevalence study of the δ-endotoxins of different molecular weights among the 50 indigenous *Bt* strains revealed the predominance of Cry1A-type protein as it was to be according to the most of the reports (Chak *et al.*, 1994; Ben-Dov *et al.*, 1997; Kim, 2000; Zhang *et al.*, 2000). It was followed by Cyt1-type δ-endotoxins which will make the strains toxic to the Dipteran insect order (Fig 4.3.11). Thus the analysis of *cry* genes and δ-endotoxins profile of the indigenous *Bt* strains helped to find out the potential *Bt* strains against agriculturally important insect orders.

Genetic diversity analysis by RAPD-PCR

The specific typing of *B. thuringiensis* enables tracking of strains dispersed in the environment and assist in the discovery of new strains. The existing serotyping scheme, while having provided an invaluable basis for *Bt* classification for a long time, provides no information about the genetic relatedness of strains within groups and between groups and does not necessarily indicates the degree and spectrum of toxicity (Rivera and Priest, 2003). Contrarily, Random amplification of polymorphic DNA (RAPD) is a modified method of polymerase chain reaction (PCR) in which a single arbitrary primer recognizes differences in the prevalence and positions of annealing sites in the genome producing a spectrum of amplicons that are considered to reflect the genomic composition of the strain and may vary along the strains (Williams *et al.*, 1990; Welsh and McClelland, 1990). The advantage of this method is that no prior knowledge of the genome under research is necessary and this is a faster, less labor-intensive in comparison to other molecular typing methods (Bostock *et al.*, 1993; Sikora *et al.*, 1997). From several studies it was found that the RAPD analysis could effectively distinguish between the *Bt* strains (Kumar *et al.*, 2010). Hence, the analysis of genetic diversity among the indigenous *Bt* strains was done by RAPD-PCR method in this study.

The decamer OPA 03 was used as the common primer for all the strains as it was reported to produce 100% polymorphism (Kumar *et al.*, 2010) and also observed to be efficient in few initial screenings in this study. Binary matrices, i.e. the RAPD profiles obtained for the strains after the PCR and agarose gel electrophoresis image analysis, were used to construct the dendrogram by UPGMA clustering method. As the binary matrices were prepared based on the 16 polymorphic DNA bands, (16^2 =) 256 numbers of different banding patterns are possible. So, the genetic varieties in this study could be more than the number of isolates. When the qualitative analysis of branching pattern for the strains was performed from the dendrogram, no such quantitative information was produced to compare the different sets of strains. Hence, to perform quantitative comparison of genetic diversities among different sets of strains such as biotypes or locations, a standard parameter i.e. the heights of the clades were chosen as they indicate the distance among the strains. The height of the maximum distance of branching was found at 0.45 in the scale bar (Fig 5.3.2). A middle height at 0.2 was therefore considered as the threshold level to distinguish the clusters as separate genotype throughout the whole study. Thus heights of 15 clades (clusters) were found to be more than 0.2 and these were considered as separate genotypes (Fig 5.3.2). Among them,

genotype 2, 4 and 10 were simplicifolious (single leafed), genotype 14 was bifolious (two leaved), genotype 8 and 15 were trifolious (three leaved) and rest others were polyfolious (more than three leaved). Genotype 9 and 11 were the largest, each containing more than 25% of the strains (Fig 5.3.3).

The average diversity index (DI), the ratio between the number of clusters and the number of strains, for locations (0.27±0.098) was higher than that for biotypes (0.23±0.046). This indicates that the genetic diversity among the strains of a certain location might not be resulted from the influence of abiotic factors only such as UV, salinity, trace elements, pH, organic maters etc rather a phenotypical pattern was maintained as the DI among the strains with similar biochemical properties was found to be lower across the different locations.

The distribution of different *cry* genes in different genotypes revealed that *cry* genes were present in all genotypes except genotype 10. Though genotype 9 and 11 were the largest sets of strains (more than 25% of the strains for each), genotype 9 was significant with different *cry* genes besides genotypes 1 and 6. The ratio between the number of *cry* genes and strains was maximum for genotype 6 (2.167) followed by genotype 1 (1.285), genotype 9 (0.29), genotype 11 (0.18) and genotype 3 (0.14). On the other hand, maximum 6 types of *cry* genes were present in genotypes 1, 6, 9 and 11. Thus, it was clear from this analysis that though the *cry* genes were observed in varied frequencies in most of the genotypes, they were most abundant in terms of number and type in genotype 6, 1 and 9. Again, the presence of same *cry* gene in different genotypes increased the chances that the degree and spectrum of toxicity might be variable i.e. genes except *cry4*, *cry8* and *cry10* were found to be present in multiple genotypes.

Correlation among Phylogeny, Biotype, RAPD genotype and available *cry* genes

The comparison among 16S rRNA gene sequence based phylogeny, Biotype, RAPD based genotype and number of available *cry* genes (Fig 5.3.6) revealed that phylogenetically close strains were observed to have similar biochemical properties. Though the biochemical properties of most of them conformed to the phylogenetic relatedness, their RAPD genotypes were variable. This genetic diversity might be due to the presence of many different plasmids in each strain and high frequency of DNA rearrangements in variable regions by conjugation transfer mechanism and the transposon-like inverted repeats flanking the endotoxin genes. Plasmid DNA exchange in nature is well documented in *B. thuringiensis* strains and has been implicated as the source of the remarkable diversity of *cry* genes (Carlson and Kolstø, 1993). Other comparisons of soil isolates of *B. cereus* and *B. thuringiensis* strains, selected with no

regard for insect toxicity, have demonstrated extensive chromosomal DNA exchange with no apparent clonal population structure (Hu *et al.*, 2004).

On the other hand, correlation persisted for the highly conserved phenotypes like biochemical properties and genotypes such as 16S rRNA etc as these are regulated by the in house conserved genes. The number of available *cry* genes among these strains was also variable. It can, therefore, be said that the report of conformity between phylogenetic and phenotypic i.e. biotype or serotype (biotype in this case) relatedness was also evidenced in this study though RAPD- genotyping and *cry* gene profile did not follow the pattern.

Novel toxicity of *Bt* against *Bactrocera cucurbitae*

Discovery of novel potential *Bt* strains is necessary to solve the problems of resistance as reported in many pests against many *Bt* biopesticide formulations and transgenic *Bt* crops. The melon fruit fly, *B. cucurbitae* (Diptera: Tephritidae), is one of the widely distributed and detrimental vegetable pests damaging about 81 host plants (Hollingsworth and Allwood, 2000) mainly from cucurbitaceous crops (Dhillon *et al.*, 2005) and it causes significant losses in different cucurbits (includes cucumber, melon, watermelon, squash, pumpkin, gourds etc) of Bangladesh too. *Bt* toxin was reported to cause mortality (more than 65-80%) to olive fruit fly, *Bactrocera oleae* (Ansari *et al.*, 2012) but there was no report of *Bt* toxicity against melon fly, *B. cucurbitae* nor it is listed in the toxin specificity data summary. So the particular interest of this study was to isolate and identify potential *Bt* strains with novel toxicity against the melon fruit fly (*B. cucurbitae*), an important vegetable pest of Bangladesh.

Bt strains used in this study were mainly hemolytic and were selected based on the presence of *cry1* gene. Though the Dipteran insect orders have been found to be susceptible to *Bt* subsp. *israelensis* mostly for Cry4, Cry10 and Cry11 proteins, Cry1Ab and Cry1Ac proteins were also found to exert toxicity against them (The Canadian Forest Service: http://cfs.nrcan.gc.ca/projects/119/6). In this connection, toxicity of *Bt* strains harbouring *cry1A*-type gene was tested against the 3rd instar larvae of *B. cucurbitae*.

The larvae were fed on sweet gourd paste in which *Bt* spore-Cry protein suspension was mixed. The presence of Cry proteins in the suspension was confirmed by Phase Contrast Microscopy (Fig 6.3.1). The larvae raised on that artificial diet were observed up to 7 days and the unaffected larvae were observed to be grown up into the pupae and finally matured into flies. On the other hand, the effect of Cry toxins over the larvae was evidenced as their

movement and feeding was gradually stopped and finally they turned into black and died (Fig 6.3.2).

The experiment was repeated for thrice and each time it was done in triplicate to evaluate the results statistically. After a series of experiments, four indigenous *Bt* strains and one reference strain was found to cause more than 50% mortality. Hence, the lethal concentrations for these strains were determined. The logarithmic value of spore count/ml was the basis for LC_{50} and LC_{99} determination instead of protein because actual amount of the active protein should be confirmed for the strains expressing more than one protein. From this analysis, LC_{50} value of *Bt* JSc1 and LC_{99} value of *Bt* SSc2 were found to be highly comparable to those of *Btk* HD-73 (Table 6.3.2). The result of the bioassay performed in the experiment revealed that the indigenous *Bt* strain JSc1 and reference *Btk* HD-73 are highly toxic to the melon fly larvae (Fig 6.3.4).

As 16S rRNA gene sequence analysis was performed previously (Fig 1.3.9), the genetic distance among the strains found toxic in this chapter was searched. Interestingly, all the *Bt* strains that were found to be highly toxic against the larvae of melon fruit fly were observed in the same sub-cluster A1 (Fig 1.3.9). On the other hand, the alignment of sequences of *cry1* gene from the potential strains obtained from bioassay revealed that the sequences are more similar to *cry1Aa* of *Bts* T84A1 rather than *Btk* HD-73. Hence, the variation in toxicity is very likely. But the toxicity of *Bts* T84A1 was very low compared to the indigenous strains and reference *Btk* HD-73 (Fig 6.3.4). So, some other gene products i.e. Cry proteins might be involved in the toxicity against *B. cucurbitae*. To get an insight into this, the *cry* gene profiles of the potential strains were checked for the genes *cry2*, *cry3*, *cry4*, *cry8*, *cry9*, *cry10* and *cry11* as most of the lepidopteran and dipteran vegetable pests were reported to be susceptible to the proteins encoded by these genes. Interestingly, the indigenous strains were detected with few of the genes searched in this regard. Besides *cry1* gene, *Bt* JSc1 was positive for *cry2* and *cry9* genes (Fig 6.3.6) and *Bt* SSc2 as well as SSe2 were positive for *cry3* genes (Table 4.3.1) in chapter 4. The PCR products of expected sizes only were the basis to presume the presence of a gene and non-specific products were disregarded. From this analysis, the toxicity was found to be correlated with the *cry* gene profiles of the *Bt* strains (Table 6.3.4). The lowest LC_{50} value was obtained for *Bt* JSc1 which was detected with *cry1*, *cry2* and *cry9* genes whereas it is slightly higher for reference *Btk* HD-73 that harbours only *cry1Ac* gene. The LC_{50} values of *Bt* SSc2 and SSe2 are higher than that of *Bt* JSc1 and *Btk* HD-73 as they lack *cry2* and *cry9* genes as well as *cry1* gene in them are not similar to

cry1Ac. But the presence of *cry3* gene enhanced their toxicity more than *Bt* JaS8 harbouring only *cry1* gene.

The Cry protein profile of the indigenous *Bt* strains revealed the presence of Cry1, Cry2, Cry3 and Cry9 proteins (Table 6.3.5). Thus the Cry protein profile of *Bt* JSc1 with Cry1, Cry2 and Cry9, strain JaS8 with Cry1 and strain SSc2 as well as SSe2 with Cry1 and Cry3 comply with the *cry* gene profiles. It can, therefore, be concluded that the synergistic effects of Cry proteins encoded by *cry1*, *cry2*, *cry3* and *cry9* are the causes of novel toxicity of the indigenous *Bt* strains against *B. cucurbitae* and Cry1Ac protein is the toxic agent in *Btk* HD-73.

Analysis of *cry1Aa*-type gene from *Bt* JSc1 retrieved by PCR walking

Since, the toxicity of indigenous *Bt* JSc1 against *B. cucurbitae* is a novel report, it was decided to sequence the *cry1A*-type gene from the organism and to analyze its amino acid sequence. As the length of the *cry1Aa*- type gene is about 3.5 kb, it was decided to target for six overlapping fragments of the gene so that the amplicons remain in the range of 500- 800 bp to obtain good quality sequences. Again, to retrieve the complete sequence of the open reading frame, forward primer of the 1st fragment and the reverse primer of the 6th fragment were chosen from the flanking regions, upstream and downstream of the start and stop codons respectively (Figure 7.2.2 & 7.2.3). The landing sites for other primers were chosen in such a manner that they remain in the conserved regions of *cry1Aa*, *cry1Ab* and *cry1Ac* genes mostly. Thus the expected amplicon sizes of the fragments were within 500- 800 bp (Table 7.2.1). After amplification, purification and sequencing of the products, the complete ORF was obtained by assembling the overlapping fragments and the corresponding amino acid sequence was deduced and analyzed.

Whether the protein contained any signal peptide or not, was predicted by a web based program (Fig 7.3.6). To accomplish it, the neural networks in SignalP produce three output scores for each position in the input sequence: i) *C-score* (raw cleavage site score) which is trained to distinguish signal peptide cleavage sites from everything else and to be high at the position immediately after the cleavage site (the first residue in the mature protein), ii) *S-score* (signal peptide score) which are trained to distinguish positions within signal peptides from positions in the mature part of the proteins and from proteins without signal peptides, and iii) *Y-score* (combined cleavage site score) i.e. a combination (geometric average) of the C-score and the slope of the S-score, resulting in a better cleavage site prediction than the raw

C-score alone. This is due to the fact that multiple high-peaking C-scores can be found in one sequence, where only one is the true cleavage site. The Y-score distinguishes between C-score peaks by choosing the one where the slope of the S-score is steep. The maximal values of these three scores were estimated (max. C score at position 54 aa= 0.114; max. Y score at 28 aa= 0.111; max. S score at 24 aa= 0.173; mean S: 1-27 score 0.112; D: 1-27 score 0.112; D-cutoff= 0.450; presence of SP= 'NO'). In the result, mean S indicates the average S-score of the possible signal peptide (from position 1 to the position immediately before the maximal Y-score) and *D-score* (discrimination score) indicates a weighted average of the mean S and the maximal Y scores. As the D-score did not surpass the D-cutoff, presence of no signal peptide was not detected in this polypeptide sequence and for non-secretory proteins all the scores represented in the SignalP output should ideally be very low i.e. close to the negative target value of 0.1 as it was for the analyzed sequence.

Three domain structure of the protein (Pardo-Lo´pez *et al.*, 2013) was confirmed by aligning the amino acid sequences with Cry1Aa, Cry1Ab and Cry1Ac protein sequence and detecting the five conserved blocks. The evolutionary history was inferred using the Neighbor-Joining method where the optimal tree with the sum of branch length= 1.01529245 is shown (Fig 7.3.8). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 5 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1053 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.

Protein structure simulation by homology modeling

From the homology modeling results, a total of 103 templates were found to match the target sequence at different similarity index (Table 7.3.1). For each identified template, the template's quality has been predicted from features of the target-template alignment. The templates with the highest quality have then been selected for model building. This list was filtered by heuristic down from which 3 models were built based on the maximum sequence coverage, similarity and identity.

For each identified template, the template's quality has been predicted from features of the target-template alignment. The templates with the highest quality have then been selected for model building. Models were built based on the target-template alignment using Promod-II. Coordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodeled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is regularized by using a force field. Usually, an alternative model is built with MODELLER (Sali *et al.*, 1993), if the loop modeling with ProMod-II (Guex *et al.*, 1997) does not give satisfactory results. The global and per-residue model quality was assessed using the QMEAN scoring function (Benkert *et al.*, 2011). For improved performance, weights of the individual QMEAN terms have been trained specifically for SWISS-MODEL. Ligands present in the template structure are transferred by homology to the model when the following criteria are met: (a) The ligands are annotated as biologically relevant in the template library, (b) the ligand is in contact with the model, (c) the ligand is not clashing with the protein, (d) the residues in contact with the ligand are conserved between the target and the template. If any of these four criteria is not satisfied, a certain ligand will not be included in the model. The model summary includes information on why and which ligand has not been included.

The signal sequence (Fig 7.3.6) though not obtained (Petersen *et al.*, 2011) from the scoring, the exclusion of initial 33 amino acids gave the idea that this might work as an analogue of the signal peptide. This might include a basic N-terminal segment followed by a stretch of uncharged residues.

Cost effective productions of *Bt* biopesticide

The reasons behind the absence of *Bt* biopesticide in the Integrated Pest Management of Bangladesh are- i) lack of awareness among the importers and farmers, ii) fear of inefficiency, acceptability and cost, iii) narrow spectrum of activity and iv) no local production etc. All these problems can be solved with the availability that could be achieved by industrial production of *Bt* biopesticide which will ultimately facilitate its application. In this regard, higher yield in spore and δ-endotoxin production with low cost medium is one of the prerequisites to keep the product within farmers' buying capacity. Hence, the present study was performed to formulate a low cost medium regulating different cultural components for higher yield and to evaluate their efficacy to control the pests with cabbage, cauliflower and tea plants.

The first and foremost task in this process development was selecting cheap carbon and nitrogen sources available for the growth and propagation of *Bacillus thuringiensis*. Among the raw materials as described in section 6.1, defatted soybean meal, defatted mustard seed meal and molasses are certain inexpensive and available agro-industrial raw materials in Bangladesh. Defatted mustard seed meal was reported to be a suitable substrate for *Bt* biopesticide production (Hasan *et al.*, 2011) and high yield was demonstrated with defatted soybean and groundnut seed meal extracts when supplemented with cystine (Vora and Shethna, 1999). Hence, defatted soybean meal and mustard seed meal were compared as N-source and molasses was tested as carbon source in presence and absence of cystine and different formulations like extract, or whole, and or presence of cystine and molasses were used in the present study (Table 8.2.1).

Initially, the optimum concentration of cystine was determined using reference *Btk* strain HD-73 in commercial medium as lower concentration of cystine or cysteine (Rajalakshmi and Shethna, 1980) promotes growth, sporulation and crystal formation in *B. thuringiensis,* while at higher concentrations, only the vegetative growth is observed (Rajalakshmi and Shethna, 1980). This increase could be explained as the fact that cystine might have interfered with some of the macromolecular changes during sporulation and parasporal crystal formation (Rajalakshmi and Shethna, 1980). The influence of cystine was therefore checked at different concentrations and at a concentration of 300 mg/l, it was found to support optimum sporulation and δ-endotoxin synthesis.

As N- source, defatted soybean was found to be more preferable to defatted mustard seed meal. It was observed from the comparison between these two at their best performance that soybean produced 0.1 log higher spore count and 62.2% higher δ- endotoxin yield than the defatted mustard seed meal (Fig 8.3.2). On the other hand, when these substrates were evaluated in the form of whole meal or extract, whole meal formulation was found to produce better δ- endotoxin than did the formulation with extract for either of the substrates. But the spore count did not respond in the similar fashion which may be due to the inseparable condition of the spores, adhered with the solid particles of the meal while resuspended for enumeration purpose (Fig 8.3.2). To avoid this problem which also interfered in the bioreactor cultivation during scaling up, soybean extract was, therefore, preferred over soybean meal. On the other hand, the efficiency of molasses (0.5% w/v) as C- source was proved as positive impacts both in sporulation and δ-endotoxin production (Fig 8.3.2).

Since, the target of the work was to reduce the production cost of the biopesticide, the key cost incurring ingredients were tried to be tuned up beside carbon and nitrogen sources such as minerals or basal salts. Marine water is a good source of some trace elements or minerals and there are some reports of using marine water instead of basal salts to reduce the cost (Ghribi *et al.*, 2007). They reported that the use of sea water increased the final growth (CFU count) as compared to that obtained when culturing *Bt* cells in starch-based medium with the required basal salts, added individually and inferred that NaCl present in the marine water, in fact, stimulate the growth of *Bt* cells. Diluted marine water (20%) was therefore used and compared as alternative of the basal salts in this study and the performance was found to be very close to that of basal salts i.e. yield for sea water was 90.88% spore count and 74.29% δ-endotoxin concentration of basal salts output (Fig 8.3.3). So another cost incurring ingredient i.e. minerals could successfully be replaced with sea water without much difference on yield. It was reported that 20% sea water of Mediterranean sea improved the yield in δ-endotoxin concentraion and spore count by 2% and 4% respectively (Ghribi *et al.*, 2007) whereas slightly lower yield was observed in this study. It indicates that the yield might be variable based on the source of sea water too which needs further study.

Few problems like the reduction in δ-endotoxin concentration were observed when the N-source was changed to extract form of defatted soybean meal from its whole meal. On the other hand, after a certain time period, the δ- endotoxin concentration was found to decrease drastically due to the endogenous proteolytic enzyme which was confirmed by checking the protease activity of the culture supernatant and protein concentration kinetically with time (Fig 8.3.4). As *Bacillus spp*. are renowned for producing different types of proteases it is not unusual for *Bt*. It was further confirmed by using PMSF to inhibit the protease activity which improved δ- endotoxin concentration partially and it might be due to the fact that the *Bt* strain secreted not only serine protease but also some other classes of proteases as it could not be inhibited by this serine protease inhibitor (PMSF) (Fig 8.3.4). The use of PMSF became a contradiction with the objective of the work as cost would increase. So, the alternative of PMSF was searched and it was found that ammonium sulfate could be a good choice for protease inhibition (Pinghui *et al.*, 1969). To reduce the costs in inhibiting the protease activity, PMSF was replaced with ammonium sulfate and it produced much better yield in δ-endotoxin concentration as protease synthesis was inhibited to a great extent (from 100 to 10.1 U/ml) and also resulted in higher δ- endotoxin yield (from 0.312 to 1.23 mg/ml). The effect of ammonium sulfate was also visible by SDS-PAGE analysis as 133 kD Cry protein in

Btk HD-73 was doubled in amount in the presence of ammonium sulfate in SeMC medium (Fig 8.3.5).

Thus in combination of stepwise selection, exclusion and supplementation, an economic medium, (MSeMC-AS) was formulated with 10% defatted soybean extract, 0.5% molasses, 20% marine water, 300 mg/l cystine and 4% ammonium sulfate and was found feasible and efficient for the production of both optimum spores and δ-endotoxin in shake flask (Fig 8.3.6). The formulation was then employed in bioprocess development in a 3.0 L bioreactor under controlled conditions of cascade agitation and aeration which revealed more than 80% increase in yield during scaling up. The reference *Btk* strain HD-73 and indigenous *Bt* strain JSc1 were used in this study and the indigenous strain demonstrated faster growth kinetics than that of reference strain, determined on the basis of sporulation and δ- endotoxin concentration. The optimum fermentation period was found to be 24 hours for both reference and indigenous strains to recover δ-endotoxin at its maximum yield which also reduced the power consumption as well as cost.

In reviewing the whole process, it could be observed that molasses is cheap, available throughout the year and easy to store; the defatted soybean meal is also a low cost, readily available item and easy to handle; marine water can also serve as a good source of minerals which can be obtained at nominal cost. Thus the cost for the production of *Bt* biopesticide might receive a 20-fold reduction and this low cost medium will facilitate large scale industrial production of *Bt* biopesticide in Bangladesh which will facilitate its application in the agriculture.

Field Trial and statistical analysis

The *Bt* preparation was then applied in the cabbage and cauliflower farming following Randomized Complete Block Design (RCBD) and in organic tea farming (Fig 8.2.2). In RCBD design, treatments (*Bt* biopesticide, chemical pesticide and negative control) were blocked and replicated, which means that plots were arranged into blocks and then treatments were assigned to plots within a block in a random manner. This design is most effective if the patterns of non-uniformity (changing soil types, drainage patterns, fertility gradients, direction of insect migration into the field, etc) in a field are identified. If the extraneous variability associated with field is not removed prior to testing for a treatment effect, it will show up in the MSE term, making it more difficult to detect treatment effects via an F-test. In other words, the denominator in the F-test will be larger than needed because it contains

variability associated with field. If the potential sources of variation are not identifiable, this design can still be used keeping the blocks as square as possible which usually keeps the plots within a block most uniform. The goal of RCBD is to maximize the differences among the blocks while minimizing the differences within the block.

As cabbage and cauliflower damaging pests such as *Helicoverpa armigera*, *Spodoptera litura*, *Plutella xylostella, Trichoplusia ni* belong to Lepidopteran insect order, they were challenged by spore-crystal preparation of *Bt* JSc1 in parallel to chemical pesticide and control treatments. *Bt* biopesticide preparation was found to exert almost similar impact on cabbage and cauliflower growth as did the chemical pesticide. At the final stage of the farming, it was observed that the untreated plants were completely damaged due to the pest infestation whereas chemical pesticide or *Bt* biopesticide treated plants were protected and healthy cabbage and cauliflower were grown. The damage to the untreated plants is the indication of pest infestation level which might vary with seasons and locations. For cabbage, more protection was provided by *Bt* biopesticide than the chemical pesticide but for cauliflower, it was opposite with slight difference. The efficacy of *Bt* biopesticide was compared with chemical pesticide statistically to see if there is any significant difference among the pesticides used and to carry out the analysis, number of healthy crops from each block was recorded.

The percentage of survived and healthy cabbage and cauliflower plants were found to be very close for *Bt* biopesticide and chemical pesticide treatment i.e. about 90% whereas only about 25% plants survived without any treatment (Fig 8.3.9A). The average weight of cabbage was slightly higher for *Bt* biopesticide treated plants than that of chemical pesticides and it was opposite for cauliflower. And the average weight for negative control was almost half in both cases (Fig 8.3.9A).

When the statistical analysis for the efficacy of *Bt* biopesticide was performed in comparison to the chemical pesticide by ANOVA (Two- Way), it produced the F values indicating that at $\alpha=0.05$, significant differences among the treatments prevailed both in cabbage and cauliflower farming (Table 8.3.1). As significant differences among the treatments were determined, pair wise difference analysis among the multiple treatments was calculated by Fisher Least Significant Difference method.

From the analysis, the data provided much evidences to conclude that, at $\alpha=0.05$ level of significance, *Bt* biopesticide and Chemical pesticide do not differ significantly whereas the

pairs *Bt* biopesticide and control as well as Chemical pesticide and Control differ significantly in Cabbage farming. It means that the efficacy of *Bt* biopesticide and Chemical pesticide in controlling the pests with cabbage exists highly in comparison to the absence of any treatment. This suggests that the substitution of chemical pesticide currently in use in cabbage farming with the *Bt* biopesticide preparation will be feasible for controlling pests i.e. the yield.

On the other hand, similar results were observed in Cauliflower farming i.e. *Bt* biopesticide and Chemical pesticide did not differ significantly at α=0.05 confidence level whereas the pairs *Bt* biopesticide and control and Chemical pesticide and Control differed significantly at α=0.05 level of significance. This clearly indicates that the substitution of chemical pesticide with the *Bt* biopesticide preparation is possible.

Bt biopesticide was applied in the tea garden along with the garlic formulation and Neem formulation currently in use up to 10 weeks. Treatment was stopped after 4th week and was resumed at 6th week. Survival of the plants from pest infestation was recorded in this time frame. Neem formulation was most effective up to fourth week but the protection was dropped drastically which might be due to its short residual activity. On the other hand, *Bt* biopesticide and garlic formulation retained their impacts better than that of Neem formulation up to 2 weeks. Both *Bt* biopesticide and garlic formulation protected maximum numbers of plants at 10th week but Neem formulation could not regain its initial maximum protection efficiency. May be the larvae those were exposed to the Neem formulation survived as a result of interruption in pesticide administration and short residual activity. And resistance might be evolved with them and transferred to the offspring.

Statistical data obtained from the One- Way ANOVA produced an F value indicating that at α= 0.05, there is no statistically significant difference among the mean survival of tea plants for all the treatments throughout the field trial replicates (F= 2.1609; df= 32; P= 0.1328). As no significant differences were evidenced, pair wise difference analysis for these three treatments was not necessary, hence it was not performed. From this analysis, it could be concluded that these three treatments are of equal effects in organic tea farming. But for longer residual activity, *Bt* biopesticide preparation was found to be more feasible in organic tea farming.

9.2 CONCLUSION

The aim of this research was to isolate indigenous *Bacillus thuringiensis* (*Bt*) strains from different eco-regions of Bangladesh and to characterize them based on their genetics, proteomics, distribution and diversity, toxicity and field efficacy etc. A comprehensive study was, therefore, carried out and the following outcomes were obtained which will definitely facilitate the application of *Bt* biopesticide in Bangladesh agriculture.

- A total of 317 *B. thuringiensis* isolates were obtained in this process and the abundance of *Bt* in Bangladesh was highly remarkable as the average *Bt* index was observed to be 0.86.

- Upon biochemical typing, biotypes describing subspecies *Bt indiana* (17%)*, kurstaki* (16%) and *thuringiensis* (12%) were estimated to be the most prevalent as well as ubiquitous in Bangladesh.

- The distribution pattern of *Bt* biotypes in Bangladesh appeared to be unique when compared with the other parts of the world.

- Identification of *Bt* strains and their biotyping into different classes were found to conform with representative 16S rRNA gene sequence analysis results.

- The molecular weight based distribution of small plasmids and its diversity among the biotypes revealed that high genetic diversities prevail among the strains which were also revealed in RAPD-PCR analysis. This result suggests that the degree and spectrum of the toxicity of indigenous *Bt* strains could be diverse to be used as efficient weapons to fight the resistance problems with pests.

- The insecticidal potentials of indigenous *Bt* strains were presumed to be against the larvae of Lepidoptera, Diptera, Coleoptera, etc insect orders since *cry1* (30.8%), *cry2* (25.5%), *cry3* (22.2%) and *cry9* (7.2%) genes were determined to be the most prevalent with the corresponding translated products i.e. Cry proteins.

- The novel toxicity of indigenous *Bt* strains was identified against the larvae of melon fruit fly (*Bactrocera cucurbitae*) and with lowest LC_{50} value, potentiality of *Bt* strain JSc1 was

highly comparable with the reference *Btk* strain HD-73. This discovery could be the basis for developing efficient and eco-friendly biopesticide as well as transgenic control measure against melon fly.

- The amino acid sequence of Cry1Aa type protein, gene sequence of which was retrieved by PCR walking, revealed its five conserved block to be classified as a 3 domain δ-endotoxin. The 3-D protein structure was constructed for the corresponding protein by homology modeling and its mode of toxicity was thus understood to be similar to other 3 domain proteins.

- A cost effective medium, MSeMC-AS was formulated with cheap raw materials which is anticipated to receive a 20-fold reduction in production cost for *Bt* biopesticide in Bangladesh. More than 80% increase in yield in a 3.0 L bioreactor was obtained by this medium. Again, the growth kinetics of indigenous *Bt* strain JSc1 was faster than that of the reference *Btk* HD-73. Hence, the industrial production of *Bt* biopesticide in this low cost medium with indigenous organism of higher growth kinetics will make it economic and promote its application thereby in the Bangladesh agriculture.

- The outcomes of the field trial in cabbage, cauliflower and organic tea farming suggested the replacement of conventional pesticides with the *Bt* biopesticide preparation to be highly feasible unaffecting the yield.

So, the indigenous *Bt* strains characterized in the present study, demand further exploration to determine the degree and spectrum of their toxicity against agriculturally important pests and other important properties.

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CHAPTER 10

REFERENCES

10. REFERENCES

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications:

1. ***Shishir, A.***, Roy, A., Islam, N., Rahman, A., Khan, S. and Hoq, M. M. (2014). Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their *cry* gene profile. *Frontiers in Environmental Science, 2*, *20*.
2. ***Shishir, A.***, Akter, A., Bodiuzzaman, M., Aktar, N., Rahman, M., Shakil, M., Ilias, M., Khan, S. N., & Hoq, M. (2012). Molecular characterization of indigenous *Bacillus thuringiensis kurstaki* isolates from Bangladesh and toxicity of *Btk* HD-73 against melon fruit fly, *Bactrocera cucurbitae. In proceedings of 1st AFSA Conferences on Food Safety and Food Security* held in Osaka, Japan.
3. ***Shishir, A.***, Akter, A., Hassan, M. H., Kibria, G., Ilias, M., Khan, S. N., & Hoq, M. (2012). Characterization of locally isolated *Bacillus thuringiensis* for the Development of Eco friendly Biopesticides in Bangladesh. *JBiopest, 5*(Suppl), 216-222.

In press:

1. Hoq, M., Mamun, A. A., ***Shishir, A. M.,*** Khan, M. M., Akand, M. N. R., & Khan, S. N. (2015). Bioprocess development for eco-friendly microbial products and its impacts on bio-industry establishment in Bangladesh. *In proceedings of CARES 1st International conference on Biotechnology* held in Dhaka, Bangladesh from May 25-26, 2013.

Accepted:

1. ***Shishir, A. M.,*** Akter, A., Bodiuzzaman, M., Alam, M. M., Hossain, M. A., Khan, M. S. A., Ilias, M., Khan, S. N., & Hoq, M. Novel toxicity of *Bacillus thuringiensis kurstaki* HD-73 and indigenous *Bt* strains from Bangladesh against melon fruit fly, *Bactrocera cucurbitae* (Dip., Tephritidae).
In Biocontrol science and will be published in ***June, 2015***.

Presentations in Conferences:

1. Md. Mozammel Hoq, ***Md. Asaduzzaman Shishir***, Anamika Bhowmik, Md. Nahinur Rahman, Shakila Nargis Khan. **A holistic approach in *Bacillus thuringiensis* biopesticides production for the food security, public health and environment.** *2nd AFSA Conferences on Food Safety and Food Security* held in Dong Nai, Vietnam from August 15-17, 2014.

2. Md. Mozammel Hoq, Mohammad Ilias, Shakila N. Khan, ***Md. Asaduzzaman Shishir***, Arpita Roy, Namista Islam, Md. Nahinur Rahman and Arafat Al Mamun**. Development of *Bacillus thuringiensis* biopesticide for eco-friendly pest management for food security.** *Presented at the "Asian Congress on Biotechnology"*, December 15-19, 2013, IIT, New Delhi, India.

3. Md. Nahinur Rahman Akand, ***Md. Asaduzzaman Shishir***, Arafat A. Mamun, Shakila N. Khan, **Md. Mozammel Hoq**. **Potentiality of *Bacillus thuringiensis* JSc1 for controlling pests with cauliflower and cabbage in the field.** Presented at the "International Conference of Biotechnology" by Cares, May 25-26, 2013, Hotel Westin, Dhaka.

4. ***Md. Asaduzzaman Shishir***, Md. Bodiuzzaman, M Nahinur Rahman, Arafat Al Mamun, Alamgir Rahman, Mohammad Ilias, Shakila N. Khan, Md. Mozammel Hoq*. **Introduction of efficient indigenous *Bt* biopesticide in integrated pest management (ipm) to control vegetable pests in Bangladesh.** *CARES 1st International conference on Biotechnology* held in Dhaka, Bangladesh from May 25-26, 2013.

5. Md. Mozammel Hoq, ***Md. Asaduzzaman Shishir***, Shakila N. Khan, Arpita Roy, Namista Islam. **Abundance and diversity of *Bacillus thuringiensis* for controlling pest in Bangladesh agriculture.** *113th General Meeting of American Society for Microbiology* held in Denver, Colorado from May18-21, 2013.

6. ***Asaduzzaman Shishir***, Asma Akter, Bodiuzzaman, Nasima Aktar, Mushfiqur Rahman, Md. Shakil, Mohammad Ilias, Shakila Nargis Khan and Mozammel Hoq. **Molecular characterization of indigenous *Bacillus thuringiensis kurstaki* isolates from Bangladesh and toxicity of *Btk* HD-73 against melon fruit fly, *Bactrocera cucurbitae.*** *1st AFSA Conferences on Food Safety and Food Security* held in Osaka, Japan from September 15-17, 2012.

7. ***Asaduzzaman Shishir***, Shakila Nargis Khan and Md. Mozammel Hoq. **Characterization of *Bacillus thuringiensis* from different habitats of Bangladesh.** *Presented at the Meeting on Biopesticdes Nomenclature* organized by Neil Christopher, University of Sussex, UK, 18 – 19th March, 2012.

8. ***Md. Asaduzzaman***, Kaniz Fatema, Mohammad Ilias, Shakila Nargis Khan, **Md. Mozammel Hoq**. **Isolation and characterization of indigenous *Bacillus thuringiensis* from Bangladesh for Developing Eco-friendly Biopesticides.** *26th Annual Conference, Bangladesh Society of Microbiologists, 21 Jannuary, 2012,* Chittagong University, Chittagong

9. ***Md. Asaduzzaman***, Asma Akther, Mohammad Ilias, Shakila Nargis Khan, Md. Mozammel Hoq. **Characterization of Locally Isolated *Bacillus thuringiensis* for the Development of Eco-friendly Biopesticides in Bangladesh.** *Presented at the 3rd Biopesticide International Conference (BIOCON-2011),* November 28 – 30, 2011, Tamil Nadu, India.

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APPENDICES

APPENDIX - A

CHEMICALS AND REAGENTS

All chemicals were of analytical grade and were purchased from a variety of suppliers.

Name of chemicals	Source
Acrylamide	Carl Roth, Germany
Agar	Merck, Germany
Agarose	Promega, USA; Carl Roth, Germany
Aluminium sulfate	Merck, India
Ammonium persulphate	Wako, USA
Ammonium sulfate	BDH, England
Azo-casein	Sigma, USA
Bis-acrylamide	Carl Roth, Germany
Boric acid	Merck, India
Bromophenol Blue	Wako, USA
Bovine serum albumin (BSA)	Sigma, USA
Calcium chloride ($CaCl_2$)	Merck, Germany
Coomassie Brilliant Blue G250	Thermo Scientific, USA
Di potassium hydrogen orthophosphate	Merck, Germany
Di sodium hydrogen orthophosphate	Merck, Germany
Dithiothreitol (DTT)	American Bioanalytical, USA
dNTPs	TaKaRa, Japan
EDTA	BDH, England
Ethanol (EtOH)	Merck, Germany
Ethidium bromide (EtBr)	Sigma, USA
Ferric ammonium citrate	Sigma, USA
Glacial acetic acid	Merck, Germany
Glycerol	Sigma, USA
Glycine	Wako, USA
Hydrochloric acid (HCl)	Merck, Germany
Immersion oil	Merck, Germany
Iodine	Sigma, USA
Isoamyl alcohol	Merck, Germany
Isopropanol	Merck, Germany
Potassium chloride	Merck, Germany
Potassium dihydrogen orthophosphate	Merck, Germany
Lysozyme	Wako, USA
Methanol	Merck, Germany
Magnesium chloride	Merck, Germany
Manganese chloride	Merck, Germany
Molasses	Local market
Na_2CO_3	Sigma, USA
Na_2HPO_4	Merck, Germany
Sodium chloride	Merck, Germany

Sodium dihydrogen orthophosphate	Merck, Germany
PCR product purification kit	Wizard® SV Gel and PCR Clean-Up System, Promega, USA
PCR Master mix	Promega; New England Biolab, USA
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
Phenol red	Sigma, USA
Phenyl methyl sulphonyl flouride	Sigma, USA
Proteinase- K	Nacalai tesque, Japan
Protein marker	Precision plus protein standards (All blue), Bio-Rad, USA; Pre-stained protein Marker, NEB, England
Ribonuclease A	Nacalai tesque, Japan
Salicin	Sigma, USA
Sodium acetate	Merck, Germany
Sodium dodecyl sulphate (SDS)	Wako, USA
Sodium hydroxide (NaOH)	Merck, Germany
Soluble starch	Merck 1.01252
Sucrose	Merck, Germany
Supercoiled DNA Ladder	Invitrogen, USA
Taq DNA polymerase	TaKaRa, Japan; NEB, USA; Thermo, USA
Tri-chloro acetic acid	BDH, England
Tris base	Invitrogen, USA
Tryptone	BD, USA
Tryptose	BD, USA
Yeast Extract	1st Base, Singapore
1 kb DNA Ladder	TaKaRa, Japan
100 bp DNA Ladder	TaKaRa, Japan; Bioneer, Korea

APPENDIX - B

MEDIA FOR GROWTH AND BIOCHEMICAL TESTS

LB broth and agar media

Ingredients	***Per Liter***
NaCl	10.0 g
Tryptone	10.0 g
Yeast extract	5.0 g
Final pH	7.1±0.1
Agar (for LB agar)	15.0 g

T_3 broth and agar media

Ingredients	***Per Liter***
Tryptone	3.0 g
Tryptose	2.0 g
Yeast extract	1.5 g
$MnCl_2$	0.005 g
Phosphate buffer (pH: 6.8)	0.05 M
Agar (for T_3 agar)	15 g

Starch hydrolysis agar

Ingredients	***Per Liter***
Nutrient broth	13.0 g
Soluble Starch	4.0 g
Final pH	7.1±0.1
Agar	15.0 g

Sheep blood agar

Ingredient	***Per Liter***
Nutrient broth	13.0 g
Sheep blood	50.0 ml
Agar	15.0 g

Sheep blood was added after autoclaving the rest of ingredients and when the temperature came down to 50°C.

Esculin iron broth

Ingredient	***Per Liter***
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
pH	7.1± 0.2

Salicin utilization broth

Ingredients	***Per Liter***
Tryptone	10.0 g
NaCl	5.0 g
Salicin solution	50 ml
Phenol red solution	2 ml

Salicin solution was added aseptically at RT after autoclaving the rest and when the temperature came down to 50°C.

Sucrose utilization broth

Ingredients	***Per Liter***
Tryptone	10.0 g
NaCl	5.0 g
Sucrose	5.0 g
Phenol red solution	2 ml

Egg Yolk Nutrient Agar for Lecithin hydrolysis test

Ingredient	***Per Liter***
Nutrient broth	13.0 g
Egg yolk emulsion	100 ml
Agar	18.0 g

Ingredients except egg yolk emulsion were dissolved in 900 ml of distilled water and the solution was sterilized by autoclaving at 121°C for 15 min. After cooling medium to 50°C, sterile egg yolk emulsion was added aseptically to the medium.

In case of all the media mentioned above, the ingredients were dissolved in distilled water by stirring with gentle heating and the media were sterilized by autoclaving at 121°C for 15 min if not stated otherwise.

APPENDIX - C

BUFFERS AND SOLUTIONS

Agarose Gel loading dye (6×, 200ml)

Bromophenol Blue (0.5 g), Xylene Cyanol (0.5 g) and Glycerol (30 ml) were mixed well and the volume was adjusted to 200 ml with deionized water.

Azo-casein solution (1.0%)

Azo-casein solution was prepared by dissolving 1.0 g of azo-casein in 100 ml 0.05 M Tris-HCL buffer (pH: 8.5). The solution was preserved at $4^{o}C$.

Basal salt solution

Per liter: $MgSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.1 g; $FeSO_4.7H_2O$, 0.02 g; $ZnSO_4.7H_2O$, 0.02 g; $CaCl_2$, 0.01 g; KH_2PO_4, 1.0 g.

Bradford Reagent

100 mg of Coomassie blue G250 in 50 mL of 95% ethanol and the solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman No. 1 filter paper and stored in an amber bottle at RT. The reagent was also filtered before use as the dye might precipitate from the solution.

EDTA(0.5 M)

186.1 g of $Na_2EDTA.\ 2H_2O$ (disodium ethylene diamine tetra-acetic acid) and 20g of NaOH pellets were added to 800 mL distilled water and dissolved by stirring on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at RT.

Egg Yolk Emulsion

Fresh eggs were swabbed with disinfectant and then submerged in 50% alcohol solution for about 45 minutes. The yolks from the whites of the eggs were separated aseptically by sterile pipette into a sterile Duran bottle up to 100 ml and 0.85 g of NaCl dissolved in 100 ml water was sterilized at 121°C for 15 min. Equal volume of egg yolk and sterile saline solution were mixed vigorously in a sterile Duran bottle aseptically. Solution is stirred until the egg yolk is completely dispersed and kept at $4^{o}C$ for further use.

Ethidium bromide solution (staining solution)

Thirty (30) µl of Ethidium bromide (EtBr) was dissolved in 150 ml 1×TBE buffer and kept in the dark place.

Gram's Iodine Solution

1.0 g of Iodine and 2.0 g of Potassium iodide were ground in a mortar and dissolved by adding water slowly up to 300 ml and the prepared solution was mixed well by stirring.

1× Laemmli buffer

Ingredients were mixed in a way so that the final concentration becomes 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT and 0.1% bromophenol blue.

NaCl (3 M)

175.3 g of NaCl was dissolved in distilled water to a final volume of 1L. The solution was autoclaved and stored at RT.

NaOH (10 M)

40 g of NaOH pellet was dissolved in distilled water to final volume of 100 mL. The solution was stored in an airtight bottle at RT.

Normal saline

Normal saline was prepared by dissolving 0.85g NaCl in 100ml of distilled water and sterilized by autoclaving.

Phenol Red Solution

0.1 g phenol red was dissolved in 28.2 ml 0.01M NaOH and 221.8 ml water to prepare a 250 ml solution of Phenol red.

Phosphate buffer

Na_2HPO_4 was dissolved in distilled water to make a 0.05 M solution and the pH was adjusted to appropriate value with 0.05 M NaH_2PO_4.

Salicin (100 mg/ml)

1.0 g Salicin was added into 10 ml of deionized water and heated gently to dissolve completely. Then it was sterilized through filtration by a 0.45µ Whatman syringe filter.

Sodium acetate

40.81 g of Na_2 (CH_3COOH).H20 was dissolved in 80 mL of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted 100 mL with distilled water and the solution was sterilized by autoclaving. It was stored at 4˚C.

SDS (10%)

10g of SDS (Sodium dodecyl sulfate) (Siga) was added to 80 mL of distilled water and dissolved by stirring on a magnetic stirrer slowly to avoid foaming. The final volume was adjusted to 100 mL with distilled water and stored at RT.

Tris-HCl (1.0 M)

121.1 g tris-base was dissolved in 800 mL of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

TBE buffer (10×)

108.8 g of Tris base and 55 g boric acid were mixed and dissolved in 800 ml of deionized water and 40 ml of 0.5 M EDTA (pH 8.0) was added into the solution. The volume was adjusted to 1000 ml with deionized water.

TE buffer (pH 8.0)

10 mM tris-Cl (pH 8.0), 1 mM EDTA was prepared by diluting concentrated stocks of 1M tris-Cl (pH 8.0) and 0.5 M EDTA. The buffer was stored at 4˚C.

Reagents for SDS-PAGE

1. **30% acrylamide-bisacrylamide solution:**

Acrylamide	: 29.0 g
Bisacrylamide	: 1.0 g
Distilled water	: 100 ml

2. **10% ammonium persulphate (APS)**

APS	: 1.0 g
Distilled water	: 10 ml

Stored at 4°C

3. **0.1% BPB (Bromophenol blue solution) or tracking dye**

Bromophenol blue	: 0.1 g
Distilled water	: 100 ml

4. **Staining solution**

Coomassie brilliant blue G-250	: 0.20 g
Phosphoric acid (85%)	: 20 ml
Aluminium sulfate	: 50 g
Absolute Ethanol	: 100 ml

Deionized water was added to adjust the volume up to 1000 ml.

5. **Sample loading buffer**

0.5 M tris-Cl (Upper gel buffer)	: 10 ml
10% SDS	: 10 ml
1.0M DTT	: 5 ml
Glycerol	: 10 ml
Distilled water	: 14 ml

6. **Electrophoresis buffer (pH 8.3)**

Tris-base	: 3.0 g
Glycine	: 14.4 g
10% SDS	: 10 ml
Distilled water	: 1000 ml

7. Upper gel buffer (pH 6.8)

Tris-base	: 18.17 g
SDS	: 0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	: Up to 11 ml

8. Lower gel buffer (pH 8.8)

Tris-base	:18.17 g
SDS	:0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	:Up to 11 ml

APPENDIX- D

LIST OF EQUIPMENTS

Equipment	**Model/ Company**
Autoclave	: Hirayama model HL-42, AE, Japan
Bioreactor, 3.0 L	: New Brunswick, USA
Biosafety cabinate	: ESCO BSC ClassII, Singapore
Centrifugation	: Biofuge Primo (Heraeus)
	: Eppendorf, Centrifuge, 5415D, USA
Electronic fine balance	: KERN, ABS, Germany.
Electrophoresis buffer tank (Hori.)	: Bio-Rad, USA
Electrophoresis buffer tank (Verti.)	: Bio-Rad, USA
Freezer (-30°C)	: SIEMENS, Germany
Gel Documentation	: Alphaimager mini, USA
Glassware sterilizer	: Binder, USA
High speed refrigerated centrifuge	: TOMY, MX-305, Japan
Incubator	: Red line by Binder, USA
Magnetic stirrer	: CIMAREC, Thermo scientific, USA
Micropipettes	: Eppendorf research, USA
Orbital shaker	: New Brunswick, USA and N-BIOTEK, Korea
pH meter	: INOLAB WTW series, Germany
Phase Contrast Microscope	: Primo Star, Carl Zeiss, Germany
Power supply	: Powerpac Basic, Bio-Rad, USA
Refrigerator (4°C)	: Royal Freshtech, Sharp
Sonicator	: Omni Ruptor-4000, USA
Spectrophotometer	: Genesys 5, Thermo scientific, USA
	: Nanodrop 2000, Thermo scientific, USA
Thermo stated shaking water bath	: N-BIOTEK Korea; GFL, 1083, Germany
Vortex mixer	: VM-2000, DIGISYSTEM
PCR thermal cycler	: MJ Mini, Bio-Rad, USA