Isolation and molecular characterization of *Bacillus thuringiensis* from different habitats of Nepal and their toxicity to Lepidoptera, potato tuber moth (Phthorimaea *operculella)*

M.Sc. Thesis

Submitted to
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LIST OF ABBREVIATIONS

bp : Base pairs

Bt : *Bacillus thuringiensis*

CBB : Commassie Brilliant Blue

cry : Crystal

DNA : Deoxyribonucleic acid

EDTA : Ethylenediamine tetra acetic acid

EPA : Environmental Protection Agency

IPM : Integrated Pest Management

kb : Kilo base

µg : Microgram

µl : Microlitre

µM : Micromolar

Min : minutes

mM : milimolar

PCR : Polymerase chain reaction

PTM : Potato Tuber Moth

Subsp. : Subspecies

Sp. : Species

TAE : Tris acetate EDTA

TBE : Tris borate EDTA

TE : Tris EDTA

U : Unit

UV : Ultra violet

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ABSTRACT

Potato tuber moth (PTM), *Phythorimae operculella* (Zeller) is a pest of many solanaceous crops including potatoes. It is one of the major constraints to potato production worldwide. Farmers rely extensively on broad spectrum chemical pesticides to check the pests but this has serious side effects on human health, ecosystem and the beneficial insects. Therefore, for sustainable agriculture, the use of biopesticides is very critical. Biopesticides based on *Bacillus thuringiensis* (Bt) have been very popular and successful. Bt is a sporulating, Gram – positive facultative aerobic bacterium. Its principal characteristic is the synthesis of a crystalline inclusion containing the protein known as cry protein during sporulation. These proteins have insecticidal properties. This research was focused on the isolation and molecular characterization of Bt from wide range of habitat of Nepal. A total of 28 different samples were collected from Sauraha/Chitwan, Tulsipur/Dang, lalitpur, Beni/Myagdi, Ghasa/Myagdi, Bhairahawa/Rupandehi and Bandipur/Tanahun. Sodium acetate and quick isolation methods were used to isolate Bt. Gram staining was done to select Gram – positive bacteria. Bt was confirmed by Commassie Brilliant Blue (CBB) staining which stains the crystal into purple blue. A total of nine Bt strains were isolated. Most of them were bipyramidal. The types of cry gene of the isolates were determined by PCR using universal primers specific to cry1 and cry2 genes. Five of the isolated strains were positive for cry1 gene only and three strains were positive for both cry1 and cry2 genes. Cry1 subgrouping reveals the presence of cry1Aa gene in five strains, cry1Ab gene in seven strains and cry1Ac gene in six strains and absence of cry1B, cry1C and cry1D genes in all the isolated and reference strains. The bioassay of isolated strains against PTM showed their effectiveness. The lowest LD_{50} value was 6.67±3.024 µg/ml crude protein for strain d1 and highest LD_{50} value was 36.71±5.68 µg/ml crude protein for reference strain *B. thuringiensis kurstaki*. These isolated strains showed high promise for biopesticides production.

Key words: Bt, biopesticides, crystal proteins, PTM, bioassay.

CHAPTER 1

INTRODUCTION

1.1 Background

In Nepal the use of chemical pesticide is unchecked and rampant as Nepalese farmers are not fully aware of the drawbacks of using excessive chemical pesticides. They consider pesticides as medicine but not the poision that affect wide range of flora and fauna. The most popular pesticides include organochlorine, organophosphorous, arsenic and mercury compounds, phenoxy acid herbicides, atrazine, pyrethroids and dithiocarbamates. (Dich *et al*., 1996). In the context of Nepal, Nepalese farmers were unaware of the of agrochemicals until 1950s. They relied on the use of traditional practices to control the agricultural pests. The use of agrochemicals, such as fertilizer and pesticides started in early 1960s (Manandhar, 2006). The use of chemical pesticides has attained momentum during recent past in order to meet the demand of increasing population. The world population is estimated to increase to 8.5 billion by the year 2020. It has been estimated that up to 15% of crops worldwide are lost because of insect damage alone (James, 2010). It seems quite obvious that the use of chemical pesticides is going to be increased.

The widely held notion that synthetic pesticides as a panacea for the control of agricultural pest is no longer valid as we are seeing the hazardous consequences of overusing it. Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in human and animals. The overdose can be the selective pressure for the evolution of the resistant pest. These insecticides kill many helpful insects such as predators, parasites, etc. (Barkay *et al*., 1989) as these insecticides are generally not specific to particular pests. The control practice so far has increased number of spray per season leading to residue problems in agricultural products. Hence, there is need of eco-friendly biopesticides.

Biopesticides are certain types pesticides derived from natural materials as animals, plants, bacteria and certain animals (EPA). The most commonly used biopesticides are biofungicides (*Trichoderma)*, bioherbicides (*phytopthera*) and bioinsecticides (Bt). Among all, the biocides *Bacillus thuringiensis* is the most important microorganism with entomopathogenic activity against certain insect order (Glazer and Nikaido , 1994). It is a Gram positive spore forming bacteria which produces insecticidal crystals protein. The biopesticide market in 2007 was 2% of the worldwide crop production market of about 600 million US dollars , with 90% of all based on Bt (Sanchis and Bourguet, 2007). Their usage reduces risk of exposure to chemicals, reduce water pollution and causes less harm to human beings, beneficial insects and ecosystem. The first commercial insecticide based on Bt, sporine was produced in France in 1938 to control

flour moth (Ibrahim *et al*., 2010). With advent of molecular biology, the first gene encoding for a Bt crystal protein was cloned by Ernest Schneph and Helen whitely (1981) followed by the launch of insect - resistant transgenic crops commonly known as Bt crops in 1996. The introduction of Bt crops reduced the use of pesticides, also saving on fossil fuels required for spraying (Sanahuja *et al*., 2011). The cumulative reduction in pesticide use for the period 1996-2008 was approximately 356000 tonnes (8.4%) which is equivalent to a 16.1% reduction in the associated net environmental impact as measured by environmental impact quotient (EIQ). This figure is supported by the fact that the cultivation of transgenic plants expressing genetically modified Bt genes has increased considerably in recent years, reaching more than 32 million hectares worldwide in 2006 (James , 2006)

Potato is considered as an important crop for the food security in Nepal. The estimated productivity of potato is 13.641 mt/ha (MOAD , 2012/13). The uncontrolled use of chemical pesticides , climate change, pollution and global warming led to the emergence of very potent pest that damage potato crops. The major potato pests in Nepal are potato tuber moth, red ant, green peach aphid, white grubs, leafminer fly, cut worm, cotton boll worm, semi-looper, epilachna beetle, black blister beetle , flea beetle and wire worm (Giri *et al*., 2014). Among them potato tuber moth (PTM), *Phthorimaea operculella* is one of the most damaging pests of potato in the field and storage and one of the major constraints to potato production worldwide (Rondon *et al*., 2007). The most economically important damage occurs mainly through the larvae's feeding on the tuber. Besides, larvae of *Phthorimaea operculella* also damage the foliage by burrowing through the leaf petioles and creating transparent leaf blisters (Alvarez *et al*., 2005).

The adult phase of PTM is brownish grey moth (wingspan 12 to 16 mm) with tiny dark scattered marks on the forewings. They are active at dusk so they are rarely seen during day. They lay about 50 to 100 or more eggs over about 2 weeks. On potato plants the females usually lay the eggs on the undersides of the leaflets. On tubers in storage they place the eggs near the eyes or near surface scars. The egg is oval, about 0.5mm long and pearly white at first, later becoming yellowish. It hatches in about 5 days in summer and about 14 days in cool weather. The newly emerged larva is about 1mm long and very active, and soon starts to mine. When fully fed it is about 12mm long, with a dark brown head and a body which is greenish if the larva developd in foliage or pinkish grey if it developed in a tuber. Larvae pupate in dead potato leaves, in soil or on stored potato tubers. If these habitats are not available, larvae will seek other protected places for pupation, such as crevices in walls, floors and crates or other locations where the temperature is above freezing. Pupae form a silk cocoon overlaid with soil and debris available nearby. The number of generations each year and the length of the cycle are influenced by temperature. The life cycle (Figure 1.1) can be as short as 2 weeks in summer or as long as 7 months in winter (Alvarez *et al*., 2005).

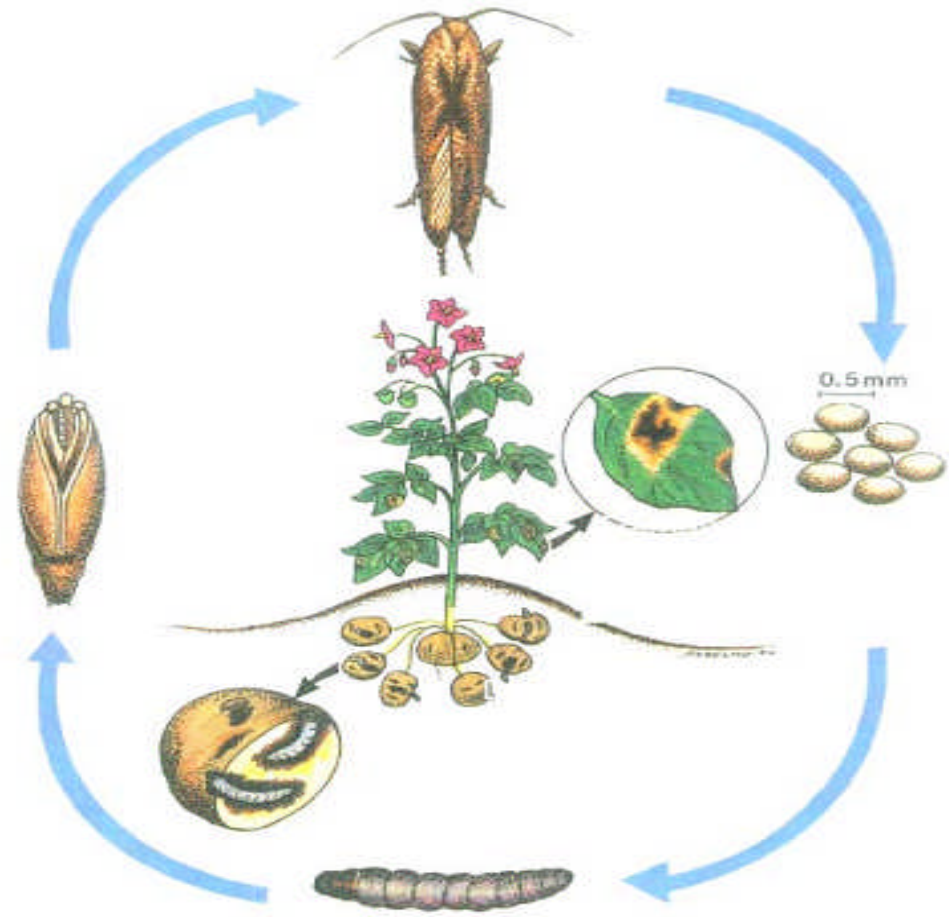


Figure 1.1 life cycle of potato tuber moth (source: Rondon *et al*., 2007)

The economic damage caused by PTM can be significant. Losses of up to 100% have been reported in Nepal during its outbreak in lalitpur (Joshi, 1989). So, effective control of PTM is critical. Furthermore, larval infestation of tubers make potatoes unmarketable. Unfortunately, most of farmers are inclined towards using chemical pesticides which pose serious threat to the farmers, consumers and the environment. So, integrated pest management (IPM) is the novel alternative to the chemical pesticides. The use of biofertilizer is the key pillar of IPM. The application of Bt toxin could be the solution because PTM is lepidopterans and many strains of *Bacillus thuringiensis* harbours cry1, cry2 proteins which show strongest toxicity to Lepidopterans (Crickmore 2000). So, isolation and characterization of cry gene should be done in order assess the effectiveness of toxins produced by novel Bt strains found in diverse habitat of Nepal.

1.2 Hypothesis

Bacillus thuringiensis (Bt) is a gram-positive, rod-shaped, spore-forming bacterium. Bt produce parasporal crystalline inclusion bodies constituted by highly specific insecticidal toxins which are protein by nature. These toxins are mainly active against lepidopteran species and some also show toxicity against dipteran, coleopteran and hemipteran. Potato tuber moth (PTM), *Phthorimaea operculella* is one of the major constraint to potato production and storage. Chemical pesticides are recklessly being used to check PTM. However, it has several drawbacks causing damage to environment as well as human health. Therefore, for sustainable agricultural practice, biopesticides can play crucial role. Bt that harbor cry1 or cry2 gene are toxic to lepidopteran pests. Therefore, This study will identify potent Bt strains that harbor cry1 or cry2 gene that will be effective against PTM. As a result, This study will identify Bt strains that could be developed into biopesticides.

1.2 Objective

1.2.1 General objective

Isolation and characterization of native *Bacillus thuringiensis* collected from different habitat of Nepal.

1.2.2 Specific objectives

- **a.** To isolate *B. thuringiensis* strains from different environments of Nepal.
- **b.** To characterize isolates phenotypically based on parasporal crystal protein.
- **c.** To identify cry gene of the isolates by PCR analysis with universal primers specific to cry1 and cry2 genes.
- **d.** To identify subclass of cry1 gene by PCR analysis with seven pairs of specific primers.
- **e.** To assess the effectiveness of the isolates against potato tuber moth first instar larvae (PTM).

1.3 Rationale and Scope

Potato is an important cash and food crop in Nepal. The annual productivity is around 13.641mt/ha (MOAD, 2012/13) and is the important staple food after rice. The potato tuber moth (PTM), *Phthorimaea operculella (zeller)* is a pest of many solanaceous crops, including potatoes. PTM is one of the major constraints to potato production worldwide (Rondon et al., 2007). In Nepal, the problem is serious as 100% losses have been reported during its outbreak in Lalitpur (Joshi, 1989). To cope with this problem, farmers relied extensively on broad spectrum chemical pesticides. But, the sideffects of chemical pesticides is deleterious to beneficial insects, human health and to the whole ecosystem. So, biopesticides is one of the alternative to chemical pesticides. Biopesticides based on *B. thuringiensis* is most popular. It produces parasporal crystals that have insecticidal activity against insect order (example, Lepidoptera, Diptera, Coloptera, Nematoda etc.). *B. thuringiensis* that harbored cry1 or cry2 gene are toxic to Lepidopteran pests. Isolation of *B. thuringiensis* strains from wide range of habitat in search of potent *B. thuringiensis* strains with cry1 or cry2 gene is crucial step as they can combat PTM.

CHAPTER 2

LITERATURE REVIEW

2.1 *Bacillus thuringiensis*

Bacillus thuringiensis is Gram positive spore forming bacteria grouped into the *Bacillus cereus* group which produces proteinaceous insecticidal crystals during sporulation (Nagamatsu *et al*., 1998 ; Rasko *et al*., 2005). These insecticidal crystals are mainly active against lepidopteran species and some also show toxicity against dipteran and coleopteran (Martin *et al*., 2010). Recent findings have also shown that some toxins are effective against insects order of Hymenoptera, Orthoptera, Hemipetra, Isopetra, Mallophaga, Thisanoptera etc, and some pests such as nematodes and mites (Schinepf *et al*., 1998). The bacteria generally produced two types of insecticidal proteins namely Cry toxins and Cyt toxins. The cry toxin acquired the mnemonic cry because they are found as crystal while Cyt toxin acquired its mnemonic Cyt because of their in vitro cytolytic activity (Crickmore *et al*., 1998).

Many Cry proteins have useful pesticidal properties and may be exploited for the control of insect pests in agriculture (Sanchis and Bourguet, 2008). Other proteins produced as parasporal crystals by *B. thuringiensis* strains have no known invertebrate target and have been termed parasporins. Some of this parasporin group of Cry proteins, Toxins such as Cry31A, Cry41A, Cry45A, Cry46A, Cry63A and Cry64A, exhibit strong and specific cytocidal activity against human cancer cells of various origins and have been given the alternative names parasporin-1 (PS1), parasporin-3 (PS3), parasporin-4 (PS4), parasporin-2 (PS2), parasporin-6 (PS6), and parasporin-5 (PS5), respectively (Obha *et al*., 2009;). Additionally*, B. thuringiensis* isolates can also synthesize other insecticidal proteins during the vegetative growth phase; these are subsequently secreted into the culture medium and have been designated as vegetative insecticidal proteins (Vip) (Estruch *et al*., 1996; Warren *et al*., 1998) and the secreted insecticidal protein (Sip) (Donovan *et al*., 2006).

The vegetative cell is rod shaped (2-5 um long and about 1.0 um wide). The spores of the organism are elipsoidal, unswollen and lie in the subterminal position in the cell (Figure 2.1). The best criteria to distinguish *B. thuringiensis* from other *Bacillus* species is the presence of parasporal crystal inclusions which can be easily observed under phase contrast microscope (Bulla *et al*., 1995). Morphology, size and number of crystal inclusions may vary among *B. thuringiensis* strains.

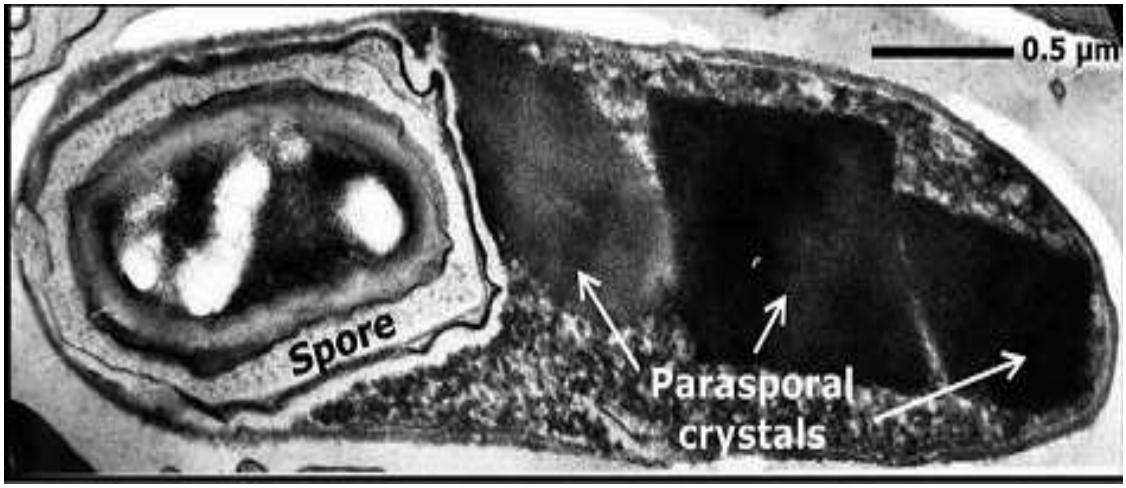


Figure 2.1 Transmission electron micrograph of a sporulated cell *of Bacillus thuringiensis morrisoni* strain. (Source: Ibrahim *et al*., 2010)

2.1.1 Life cycle of *Bacillus thuringiensis*

Bacillus thuringiensis readily proliferates when environmental conditions such as temperature and nutrient availability are conducive while formation of spores have been shown to be induced by signals such as nutrient starvation, cell density and cell cycle progression (Hilbert and Piggot 2004). The life cycle of *B. thuringiensis* can be divided for convenience into phase 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; Molina *et al*., 2008). The production of the characteristic insecticidal (Cry) protein 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). The production of crystal proteins by *B. thuringiensis* during sporulation probably relieves stresss by offsetting water loss during spore formation. Furthermore, the toxin action provides sufficient host nutrients to allow germinatin of dormant bacterial spores (Ibrahim *et al*., 2010).

2.1.2 The *Bacillus cereus* group

This group includes B*. cereus, B. mycoides, B. thuringiensis, B. anthracis, B. pseudomycoides and B. weihenstephanensis* (Chen and Tsen, 2002). *B. anthracis* is the causative agent of anthrax, an acute and often lethal disease in humans and animals. *B. cereus* is an opportunistic human pathogen and may cause food poisoning, eye infections and periodontal disease. The taxonomy within the *B. cereus* group is more controversial and complicated than traditional classification between species and this is linked to the presence or absence of large extra chromosomal

elements (plasmids) within the group. The three species*: B. anthracis*, *B. cereus* and *B. thuringiensis* have been suggested to be variations of the same species, with *B. cereus* as the prototype ancestor (Daffonchio *et al*., 2000). The difference between these species are largely due to genes located on large plasmids (›20kb) that are responsible for the different phenotypic and pathogenic profiles (Rasko *et al*., 2005). Plasmid encoded genes could be transferred to another related species (eg. *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).

2.1.3 Ecology and prevalence

Bacillus thuringiensis was originally associated with certain substrate and affected insects, but with the development of new effective extraction method, a numerous new isolates have been found from wide range of environment (Bernhard *et al*., 1997). Strains have been isolated worldwide from many habitats including soil, insects, stored product dust, deciduous and coniferous leaves (Schinepf *et al*., 1998; Meadow *et al*.,1992; Bel *et al*., 1997; Mizuki *et al*., 1999). Martin and travers (1989) isolated *B. thuringiensis* from 70.4 percent of the 1115 soil of 30 countries that they investigated. They found *B. thuringiensis* in savannah, desert, agricultural and forest soils as well as arctic tundra , urban environmental, beaches and steepes.

2.1.4 History of *Bacillus thuringiensis*

B. thuringiensis was originally discovered by Japanese biologist Ishiwatari in 1901. He isolated it from diseased silkworm (Milner, 1994). It was initially named as *Bacillus sotto* as the cause of "sotto disease" in silkworm. Later, it was named as *Bacillus thuringiensis* by a German scientist Ernst Berliner in 1911. He isolated it from diseased larva of *Ephestia Kuhniella* (flour moth caterpillars) in Thuringia province (Milner, 1994). Berliner studied the bacterium and found inclusion bodies or "Restkorper" alongside the endospore (Ibrahim *et al*., 2010). The isolation of economically important *B. thuringiensis Kurstaki* HD-1 by Dulmage in 1966 was the milestone in the commercial success of biopesticide. It was highly effective against lepidopteran. The discovery of *B. thuringiensis Israelensis* by Margalit and Tahori in Israel was another success. This strain is highly effective for the control of mosquito and other tropical diseases such as malaria and yellow fever.

The first gene coding for a *B. thuringiensis* crystal protein was cloned in 1981 by Ernest Schneph and Helen Whiteley. Since then, biotechnology has evolved rapidly and it did not take long for the first genetically modified plants expressing *B. thuringiensis* insecticidal proteins to be

developed. The industrial-scale production of *B. thuringiensis* is now well controlled and relatively simple and is competitive in terms of cost and this obviously contributes to its success (Sanchis and Bourguet, 2007).

2.2 *Bacillus thuringiensis* insecticidal crystal protein

2.2.1 Nomenclature

Different cry toxins showing well documented toxicity against lepidopterans, coleopterans, hemipterans, dipterans, nematodes, human cancer cells have been established (Figure 2.2). The first systematic attempt to organize the genetic nomenclature relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes. The cryI genes encoded protein toxic to lepidopterans, cryII genes encoded proteins toxic to both lepidopterans and dipterans, cryIII genes encoded proteins toxic to coleopterans and cryIV genes encoded proteins toxic to dipterans alone (Crickmore *et al*., 1998).

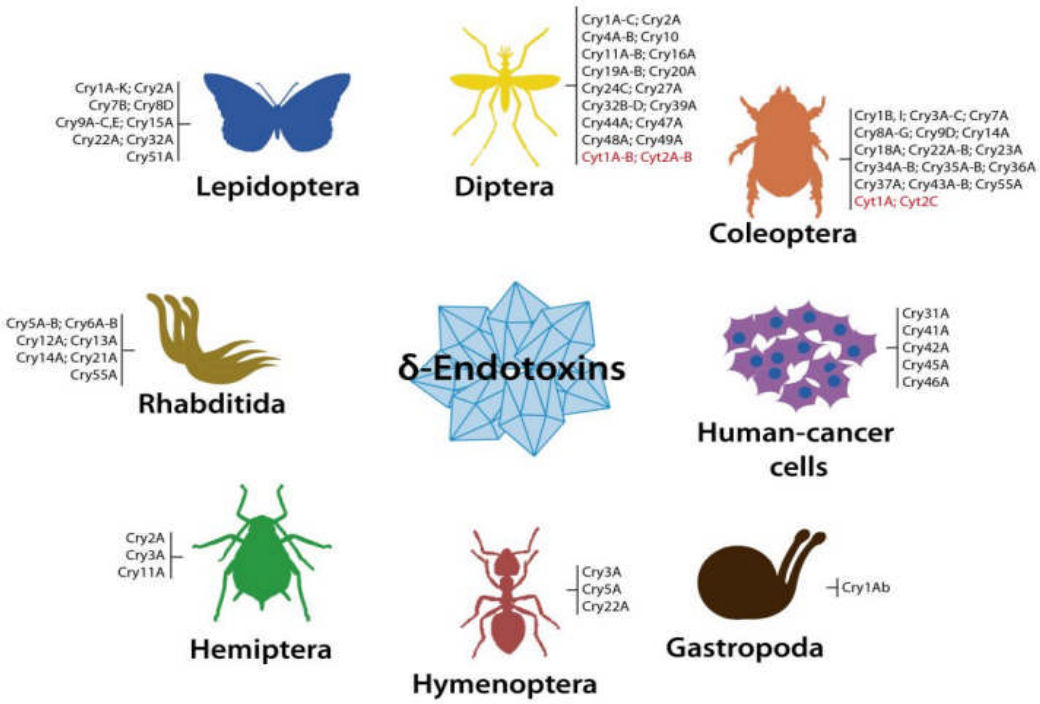


Figure 2.2 summarized view showing the known host spectrum of Bt toxins.

(Source: Palma *et al*., 2014)

There are many problems associated with that sort of classification. The cryIIB gene, for example, received a place in the lepidopteran-dipteran class with cryIIA, even though toxicity

against dipterans could not be demonstrated for the toxin designated cryIIB. Similarly, the protein named cryIC was reported to be toxic to both dipterans and lepidopterans while the protein designated cryIB was reported to be toxic to both lepidopterans and coleopterans (Crickmore *et al*., 1998).

To overcome these problems, the Bt toxin nomenclature committee was created and a novel system of classification was proposed. In this new system, a novel toxin is given a four-rank name depending on its degree of pairwise amino acid identity to previously named toxins. Arabic numbers are used for first and fourth ranks, and uppercase and lowercase letters are assigned for the second and third ranks, respectively. In this way, protein sharing less than 45% pairwaise identity are assigned a different primary rank (an Arabic number, cry1 and cry2); two proteins sharing less than 78% pairwise identity are assigned a secondary rank (a capital letter, example, cry1A and cry1B); protein sharing less than 95% pairwise identity are assigned a different tertiary rank (a lowercase letter, example, cry1Aa and cry1Ab) and finally, to differentiate between proteins sharing more than 95% pairwise identity, a quaternary rank is assigned (an Arabic number, example, cry1Aa1 and cry1Aa2).

2.2.2 Toxin structure

The toxin can be described in terms of their amino acid sequences, protein structure and modes of actions (Crickmore *et al*., 1998). The toxin is composed of three distinct domains (Figure 2.3). Domain I consists of seven α-helices in which central helix α5 is hydrophobic and other six are amphipathic helices which encircled around the central core helix. It has been observed that the isolated Domain I is sufficient for ion channel formation whereas its elimination results in the loss of of binding step (Flores *et al*., 1997). This shows that Domain I plays crucial role in the penetration of the midgut epithelium and pore formation. The α4-α5 helical hairpin is believed to insert into the membrane while other helices are spread out on the surface. It resembles umbrella and this hypothesis is coined as “umbrella hypothesis”(Schwart *et al*., 1997). Mutation in α4 and α5 resulted in the reduction of pore formation capacity (Masson *et al*., 1999). Domain II is made up of three antiparallel β – sheet packed together to form a β – prism with pseudo threefold symmetry (Li *et al*., 1991). It is least conserved domain of all cry protein (Bravo, 1997). Two of sheets are composed of four strands in a Greek key motif and are solvent exposed. The third sheet packs against domain I and is arranged in a Greek- key like motif with three strands and a short alpha – helix (Pigott and Ellar, 2007). Domain III has been shown to contain two antiparallel β- sheets in a jelly roll topology (Boonserm *et al*., 2006). Both sheets are composed of five strands with outer sheet facing the solvent and the inner sheet packing against Domain II. Domain III shows less structural variability than Domain II and the main differences are found in the length, orientation and sequences of the loops (Boonserm *et al*., 2006).

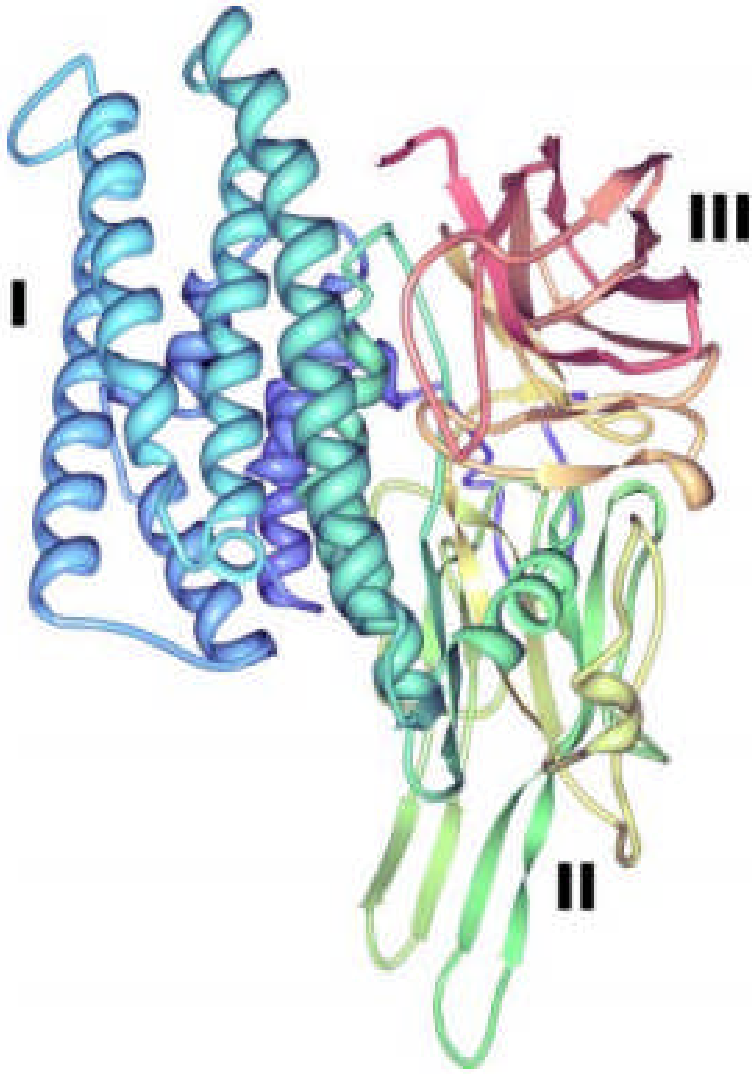


Figure 2.3 Three dimensional structure of cr2Aa toxin (Source: Palm *et al*., 2014)

When the sequences of crystal proteins are aligned, five conserved sequence blocks (Figure 2.4) are common in the majority of them (Maagd *et al*., 2001) Conserved block 1 is in the central helix of Domain I, block 2 is at the domain I-II interface, block 3 is at the boundary between Domain II and III, block 4 is in the central strand of Domain III and block 5 is at the end of Domain III.

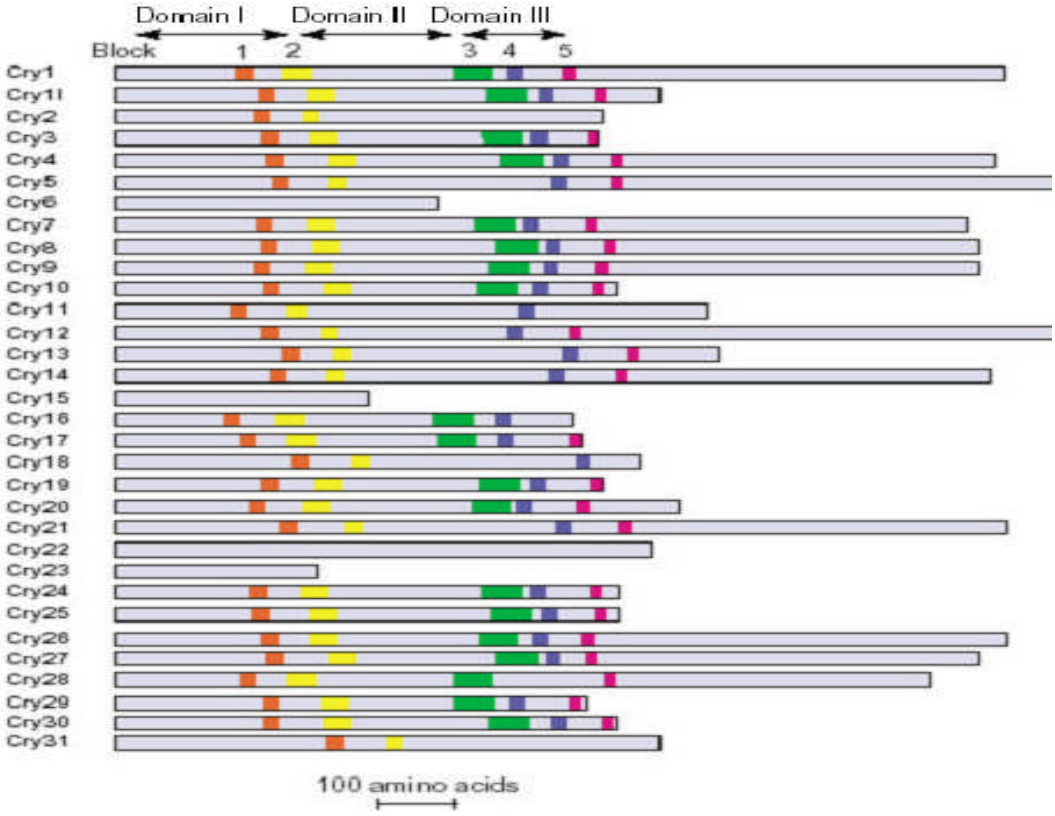


Figure 2.4 Relative lengths of cry protoxins and position of the five conserved blocks (Source: Maagd *et al.*, 2001)

2.2.3 Mode of action of the toxin

At first, endotoxin has to be ingested. Generally larva ingest a mixture of spores and endotoxins feeding on decaying soil matter or plant storage material. The crucial part of the process is the solubilization of protein by protease that takes place under alkaline condition of the insect midgut (Hofmann *et al.*, 1988). The degree of toxicity among cry protein is correlated with the degree of solubilization of protein. The reduction in solubility is speculated to be one of the potential mechanism for insect resistance (Jisha *et al.*, 2013). There are two mechanism of the action of cry toxin. One is the formation of ionic pores and other is the mg^{+2} dependent signal cascade pathway.

In ionic pore formation model, (Figure 2.5) the toxin whose molecular weight is around 135kDa is cleaved by the digestive protease of the host to generate mature toxin of about 65kDa. This toxin is the amino terminal part of the protoxin. The carboxy – terminal part of molecule don't attribute toxicity. Nevertheless, it plays an important role in the formation of disulfide bridges linking endotoxin in the crystal. The high pH and reducing condition prevailing in the guts of

most susceptible insects is paramount for the disruption of disulfide bridges. The activated toxin interact with specific high affinity receptors in the midgut brush border membrane. This reversible binding result in conformational change which facilitates a second cleavage that removes the N- terminal α -1, by a membrane protease. The removable of α-1 result in the formation of oligomers. The oligomerised activated toxin that is bound to membrane receptors then inserts the central hydrophobic helix α-4 and α-5 into the apical membrane of midgut cells causing osmotic shock, bursting of the midgut cells and finally ending in the insect death (Schnepf *et al*., 1998). Furthermore, the spore may germinate in the gut of insect leading to propagation (Yang and Wang, 1998). The requirement for the alkaline condition, specific protease and specific receptors explains why Bt is harmless to mammals (which have acidic gut and lack the corresponding receptors) and why each toxin has a narrow host range (Sanahuja *et al*., 2011).

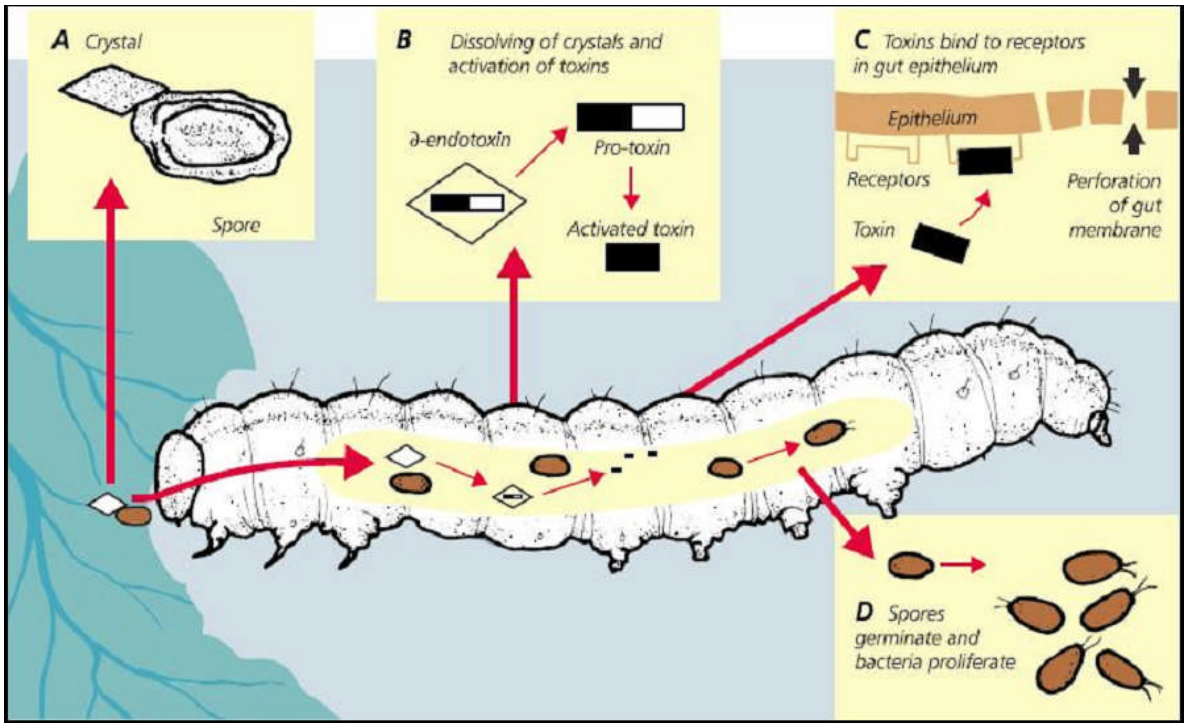


Figure 2.5 Mode of action. A, Toxin crystal and spore are ingested. B, Activation of toxin. C, Binding of toxin to receptor and perforation of the gut epithelium. D, Germination and proliferation of bacteria. From WHO Environmental Health Criteria, No. 217.

In mg^{+2} dependent signal cascade pathway, (Figure 2.6) activation is triggered by the interaction of the monomeric 3-domain with the primary receptor, the cadherin protein (Zhang *et al*., 2005; Zhang *et al*., 2006). This triggers a pathway involving stimulation of the stimulatory G protein α-subunit and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (cAMP) levels and activation of protein kinase A (PKA). Activation of the AC/PKA signaling pathway initiates a series of cytological events that includes membrane blebbing, appearance of nuclear ghosts and cell swelling followed by cell lysis (Tsuda *et al*., 2003).

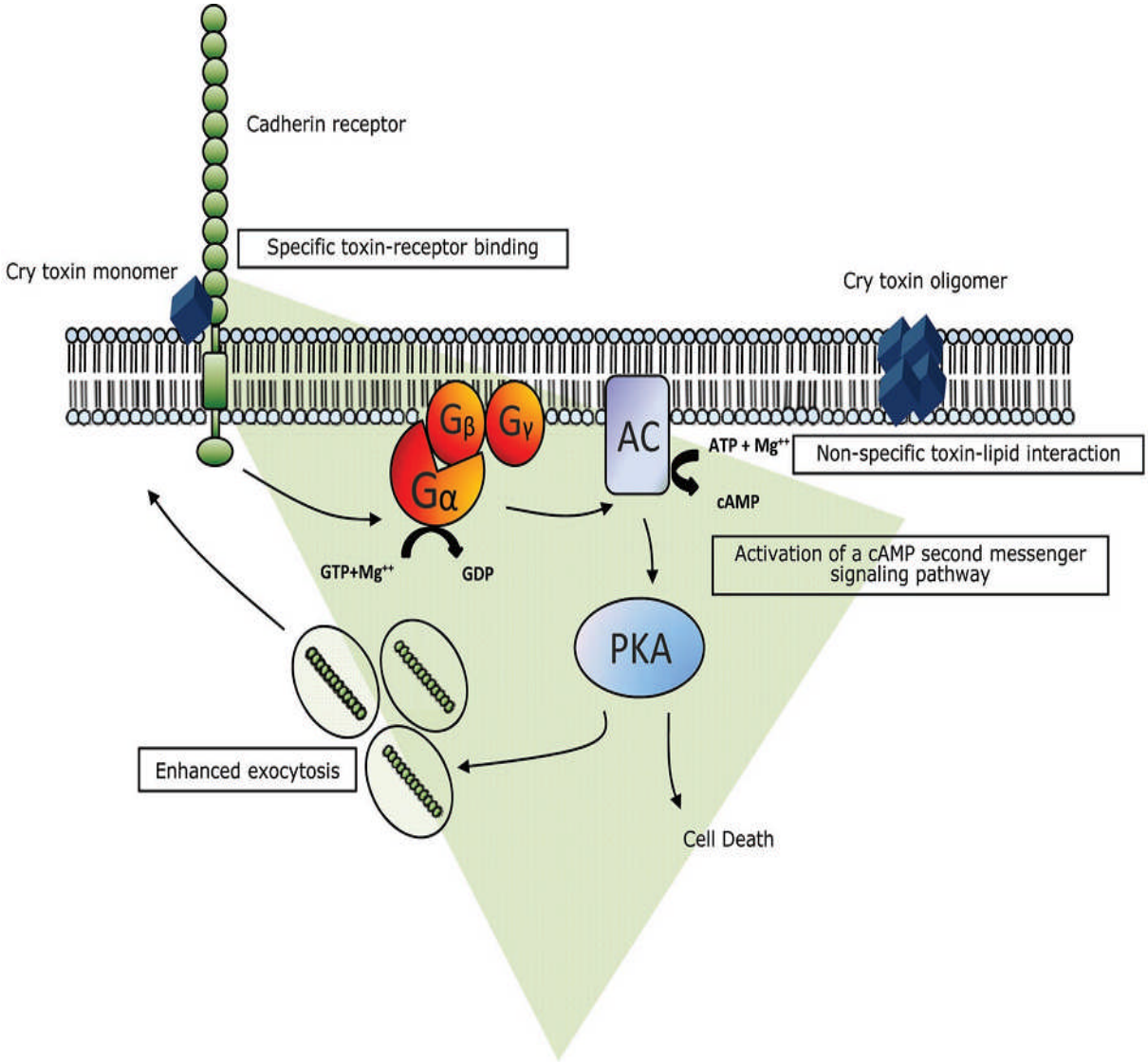


Figure 2.6 Proposed mechanism for cry toxin action by signal transduction pathway. (Source: Ibrahim *et al*., 2010).

Genetic features *of Bacillus thuringiensis*

2.3.1 The *B. thuringiensis* Genome

B. thringiensis strains have a genome size of 2.4 to 5.7 million bp (Carlson and Kolsto, 1993). Physical maps have been constructed for two *B. thuringiensis* strains. Comparison with *B. cereus* chromosomal maps suggests 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 *B. thuringiensis* isolates have extra chromosomal elements, some of them are circular and other linear (Carlson, 1994). It has been recognized that the proteins comprising the parasporal crystals are generally encoded by large plasmid (Schnepf *et al*., 1998).

2.3.2 The transposable elements of *B. thuringiensis*

B. thuringiensis species harbor a large variety of transposable elements, including insertion sequences and transposons. The first studies on the structural organization of the cry1A gene environment showed that genes of this type were flanked by the two sets of inverted repeated sequences. Nucleotide sequence analysis revealed that these repetitive elements were insertion sequences that have been designated IS231 and IS232 (Lereclus *et al*., 1992).

Regarding the role of the transposable elements in *B. thuringiensis*, it is postulated that they are involved in the amplification of cry genes. A second possible role is one of the mediating the transfer of plasmids by a conduction process involving the formation of cointegrate structure between self conjugative plasmids and chromosomal DNA or nonconjugative plasmids (Green *et al*., 1989). Thus, a major adaptive function for these transposable elements may be the horizontal dissemination of the genetic materials, including cry genes within the *B. cereus – B. thuringiensis* species (Schnepf *et al*., 1998).

2.3.3 The cry gene expression

Cry gene is expressed during the stationary phase and produce crystal inclusion that can account for about 20%-30% of the dry weight of the sporulated cells. The cry protein synthesis appear to be controlled by a variety of mechanism occurring at transcriptional, posttranscriptional and post translational level (Schnepf *et al*., 1998)

At the transcriptional level, the development of sporulation is controlled by the successive activation of sigma factors, which bind the core of RNA polymerase to direct the transcription from sporulation-specific promoters (Piggot and Hilbert, 2004). These factors are the primary sigma factors of vegetative cells, σ^A and five factors called σ^H, σ^F, σ^E, σ^G and σ^K, which appears in that order in a temporally regulated fashion during development. The σ^A and σ^H factors are

active in the predivisional cell, σ^E and σ^K are active in mother cell and σ^F and σ^G are active in the forespore (Moran, 1993).

The cry1A gene is a typical example of a sporulated dependent cry gene expressed only in the mother cells compartment of *B. thuringiensis*. Two transcriptional start sites have been mapped (Bt I and Bt II), defining two overlapping sequentially activated promoters. The cry3Aa, on the other hand, is sporulation independent cry gene expression. It was found to be expressed during vegetative growth (Malvan *et al*., 1994). The expression of cry3Aa is not dependent on sporulation specific sigma factors. Moreover, cry3Aa expression is increased and prolonged in mutant strains unable to initiate sporulation. The results indicate that cry3Aa expression activated by a non-sporulation-dependent mechanism during the transition from exponential growth to the stationary phase (Salamitou *et al*., 1996).

2.3.4 Regulation of cry gene expression

The stability of mRNA is an important contributor to the high level of toxin production in Bt. The half life of cry mRNA, about 10 minutes, is at least fivefold greater than the half-life of an average bacterial mRNA (Glatron *et al*., 1972). The putative transcriptional terminator of cry1Aa gene (a stem-loop structure) acts as a positive retroregulator. The fusion of a DNA fragment carrying the terminator with 3'end of heterologous genes increases the half-life of their transcripts two- threefolds, which in turn increases the expression of their gene products. It has been demonstrated that the processive activities of 3'-5' exoribonucleases are impeded by 3' stem loop structures (Wang and Chang, 1989). It is likely that the cry1Aa transcriptional terminator increases the cry mRNA stability by protecting it from exonuclease degradation from the 3' end (Schnepf *et al*., 1998).

Agaisse and Lereclus (1996) identified a perfect Shine-Delgarno (SD) sequence (GAAAGGAGG) in Bt required for the stabilization of cry3A mRNA . It is quite possible that the stability of mRNA resulted from interaction of the 3'-end of 16s rRNA of the 30S ribosomal subunit and Shine-Delgarno. The binding of 30S ribosomal subunit to this sequence probably protects the mRNA from 5'-3' ribonuclease activity, resulting in the stable transcripts.

The cry proteins generally forms crystalline inclusions in the mother cell compartment. Depending on their protoxin composition, the crystals have various forms: bipyramidal, cuboidal, flat rectangular, irregular, spherical and rhomboidal. This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. However, the crystals have to be solubilized rapidly and efficiently in the gut of insect larvae to become biologically active. The structure and the solubility characteristics of a crystal presumably depend on such factors as the secondary structure of protoxin, the energy of the disulfide bonds and the presence of additional Bt specific components (Schnepf *et al*., 1998). The

presence of cysteine rich region in the C-terminal half of the protoxin also may contribute to a stable crystal structure through formation of disulfide bond (Bietlot *et al*., 1996). Those toxin that lacks the cysteine-rich C-terminal region, example, truncated cry toxin, may overcome that problem by forming intermolecular salt bridges and hydrophobic interactions that can stabilize crystal structure (Baum and Malvar, 1995).

The organization and clustering of cry genes in operons have been demonstrated in numerous *B. thuringiensis* strains (Widner). Specifically, cry1Ac, cry1F, cry2Ac are constituents of operon (Sedlak *et al*., 2000). It has been suggested that the presence of operon facilitates differential gene expression rendering parasporal crystals containing different cry toxin, each of which contains a specific individual toxin. A consequence of this scenario could be the synergism among the various cry toxins. Similarly, the arrangement of cry genes in operons enhance genetic recombination that might generate new toxin or combination (Sedlak *et al*., 2000)

2.4 *Bacillus thuringiensis* resistance

All insecticides create selection pressure on target population (Tabashnik, 1994; Gould, 1998). Human have been extensively using pesticides for a long period of time. This leads to the emergence of the potent pesticide resistance pests. For example, certain population of aphids, *Aphis gossypii* have become resistant to almost all major classes of insecticides known (Bourguet and Sanchis, 2007). Thus, all insecticides have a high probability of becoming inffective. The same scenario may be true to *B. thuringiensis* toxin. The first evidence of this process was observed in 1985, when resistant mealmoths (*Plodia interpunctella*) were found in grain stores that had been sprayed with *B*. *thuringiensis* spores (Bourguet and Sanchis, 2007).

There have been many laboratory-selected and field-selected resistance strains have been reported. Examples of laboratory-selected insect resistance to individual cry toxins include the Indian-meal moth (*Plodia interpunctella*), the almond moth (*Cadra cautella*), the Colorado potato bettle (*Leptinotarsa decemlineata*), the cottonwood leaf beetle (*C. scripta*), the cabbage looper, the coootn leaf-worm (*Spodeptera littoralis*) (Schnepf *et al*., 1998). Similarly, the first case of field-selected resistance to Bt was reported from Hawaii where populations of diamondback moth (*P. xylostella*) showed different level of susceptibility to a formulated Bt product (Dipel)(Schnepf *et al*., 1998). Resistance to Btk products and resulting failure in diamondback moth control has resulted in the extensive use of *B. thuringiensis* subspecies *aizawai*-based insecticides in certain locations (Jisha *et al*., 2013).

2.4.1 Mechanism of *B. thuringiensis* resistance

Different mechanism (Figure 2.7) have been proposed for the mode of resistance of insect to Bt pesticides such as reduction of binding of toxins to receptors in the midgut of insects, reduced solubilization of protoxin, alteration of proteolytic processing of protoxin and toxin degradation or precipitation by protease (Bruce *et al*., 2007)

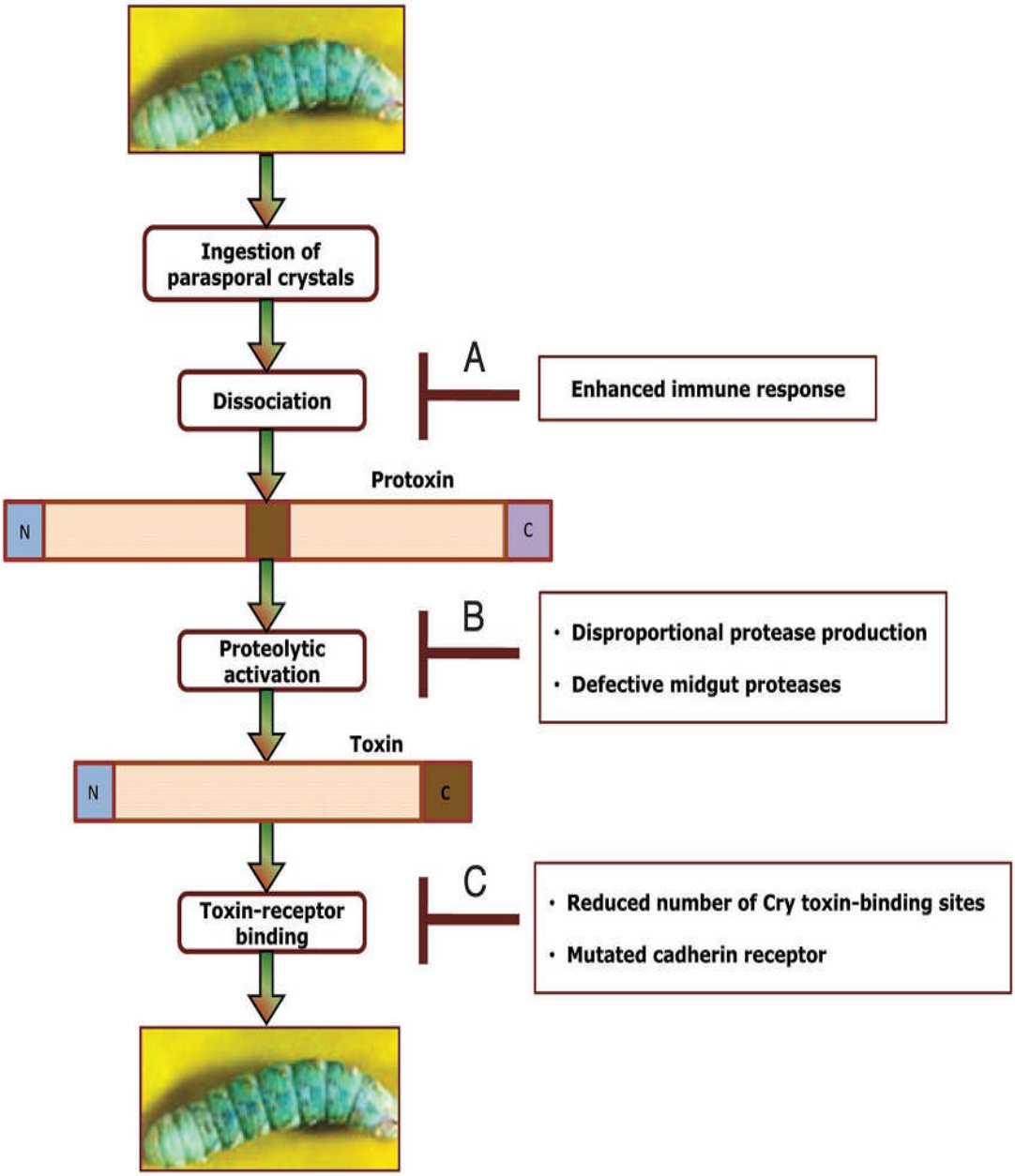


Figure 2.7 Possible mechanisms of insect resistance to *B. thuringiensis*. (Source: Ibrahim *et al*., 2010).

When in continual touch with *B. thuringiensis*, insects exhibit physiological changes and enhanced immune response. A heightened immune response primarily involves changes in the activity of the mucuosal surface, causing increased secretion of proteases and pro-coagulants. For example, a 75-KDa pro-coagulant protein from the gut juice of the spruce budworm which exhibits elastase-like activity has been shown to bring about precipitation of the protoxin of *B. thuringiensis sotto* (Milner *et al*., 1998). Precipitation leads to sequestering of the toxin and limiting its accessibility to its target receptor (Ibrahim *et al*., 2010).

Midgut proteases are involved in the solubilization and activation of *B. thuringiensis* protoxins. After ingestion of cry protein, the protease in the midgut of lepidopteran species can activate cry protoxin by cleaving highly basic residues like Arginine and lysine. Reduction in protease activity results in reduced activity of *B. thuringiensis* protoxin to active toxin. For example, a *B. thuringiensis* resistance in a strain of *P. interpunctella* was found to be associated with a significant reduction in midgut protease (zhu *et al*., 2000). In contrast, up regulation of midgut protease in some cases, has found to be associated with *B. thuringiensis* resistance. For example , enhanced protease activities could increase degradation of cry1C toxin in *Spodoptera littoralis* (Shao *et al*., 1998). Furthermore, the aminopeptidase is associated in the pathogenesis of *B. thuringiensis* toxins as receptors of the toxins (Bravo *et al*., 2004). A cry1Ac resistance strains of *S. exigua* was related to the lack of the mRNA transcript encoding amionopeptidase (Herrero *et al*., 2005).

Toxin binding to toxin binding sites is critical. The reduction in toxin binding site or mutation has been implicated in resistance (Gahan *et al*., 2001). Cadherins are believed to be a primary binding receptor to cry protein in Lepidoptera, dipteral and Coleoptra (Bravo *et al*., 2011). The binding of cry protoxin to cadherins induce a conformational change in cry toxin that facilitates the formation of pre-pore toxin oligomer and finally lead to osmotic imbalance of the insect gut (Gomez *et al*, 2002).

2.4.2 *Bacillus thuringiensis* resistance management

The objective of resistance management is to keep the frequency of resistance gene low for insect control. Different strategies have been proposed. Some of them are use of multiple toxins (stacking or pyramiding), crop rotation, high or ultrahigh dosages and high-dose refuge strategies.

The high dose-refuge strategy is one of the most reliable and popular strategy. It involves growing plants of Bt crops producing large amount of toxin along-side non-Bt crops (referred to as refuge zone), in which the larvae of target insects are not exposed to the toxin (Alstad and andow, 1995). Resistance is the consequence of genetic mutation. The wild type susceptible allele can be denoted by ‘S’ and the mutant type resistance allele can be donoted by ‘R’. If the

Bt plant produce sufficiently large amounts of toxin, it will kill all SS homozygotes and RS heterozygotes. Only few RR homozygotes will emerge. Then RR individuals are likely to mate with SS individuals from the refuge zones. The offspring of these crosses will consists mostly of susceptible RS heterozygotes. As a result, the frequency of the resistance alleles will decrease (Bourget and Sanchis, 2007). However, the success of this strategy is based on several requirements. First, wild susceptible insects have to migrate into the Bt field and mate in a random way with the resistant individuals. Asynchronal larval development in refuge and treated plots can undermine the effectiveness of this strategy (Liu *et al*., 1997).

Another robust method is the use of gene stacking. This approach involves the expression of two or more *B. thuringiensis* toxins with different spectrum of activities. In theory, resistance against one cry protein could arise through a single point mutation in the gene encoding its receptor. The chances of two mutations arising simultaneously in its receptors, two independent activity toxins would be much lower (Sanahuja *et al*., 2010). Additionally, Bt cry toxin could be combined with other insecticidal proteins. The multiple attack strategy assumes that within a population, if insects homozygous for one resistance gene are rare, then insects homozygous for multiple resistance genes are extremely rare. A critical condition for the success of this strategy is that each of the insecticides on its own should have high mortality for susceptible homozygotes (Schnepf *et al*., 1998).

2.5 Characterization of *Bacillus thuringiensis*

2.5.1 Classical methods

The classical method for characterization include crystal morphology, flagellar serotyping, biochemical reaction and bioassays.

The presence of crystal protein facilitates the isolation of *B. thuringiensis* from other species such as *B. cereus* and *B. anthracis* which donot produce large parasporal inclusions during sporulatin. Shishir *et al*., 2012 studied crystal morphology of 57 isolates from different habitats of Bangladesh. Five different types of parasporal crystal protein (spherical, bipyramidal, irregular pointed, cuboidal and irregular shaped) were observed among the isolates. However, the production of the parasporal crystal, the defining quality of *B*. *thuringiensis* is too narrow a criterion for taxonomic purposes (Schnepf *et al*., 1998).

Biochemical tests include different tests such as starch hydrolysis, urease production, mannose and salicin fermentation, esculin utilization, lecithinase production etc. Martin and Travers (1989) performed fourteen biochemical tests to identify isolates. The most relevant

biochemical tests were found to be esculin utilization, acid formation from salicin and sucrose and lecithinase production. They divided the *B. thuringiensis* isolates into sixteen biochemical types.

Classification of *B. thuringiensis* strains has been accomplished by H-serotyping, the immunological reaction to the bacterial flagellar antigen. The 'hag' gene encodes flagellin , which is responsible for eliciting the immunological reaction in H-serotyping. Specific flagellin amino acid sequences have been correlated to specific Bt H-serotypes and at least 69 H-serotypes and 82 serological varieties (serovars) of Bt have been characterized (Lacadet *et al*., 1999). H-serotyping, however, is limited in its capability to distinguish strains from the same H-serotype or from the same serovar (Cote and Soufiane, 2009).

The bioassay are extremely useful to identify the best isolates against target species of insects but it is somewhat tedious and sometimes it is ambiguous (Ben-Dov *et al*., 1999).

2.5.2 Molecular characterization of *Bacillus thuringiensis*

Identification of novel *B. thuringiensis* isolates by bioassay is somewhat tedious and redundant. So, molecular techniques, in particular, polymerase chain reaction (PCR) is the best alternative. It is quick and allows simultaneous screening of many *B. thuringiensis* samples to classify them and to predict their insecticidal activities (Ben-Dov *et al*., 1999).

The discovery of novel cry gene is vital because the existing cry gene will pose less threat to the pest as they developed resistance. So, characterization and discovery of novel cry gene is one of the main step in the search of more potent cry toxin. Different procedures have been brought into practice such as PCR, PCR hybridization, PCR-RFLP (restriction fragment length polymorphism) (Ye *et al*., 2012). The efficacy of PCR for cry gene identification relies on the alternation of conserved and variable nucleotide regions. By designing oligonucleotides to be used as primers either from conserved blocks or from variable regions, it is possible to recognize either entire gene subfamilies or specific individual genes (Ye *et al*., 2012).

For the mining of novel cry genes, multiplex PCR can be used. Bravo *et al* (1998) used general and specific primers that could detect the cry1, cry3, cry5, cry7, cry8, cry9, cry11, cry12, cry13, cry14 and cry21 genes. Six pairs of general primers were used in this method. Strains for unique PCR product profiles were obtained with general primers were further characterized by additional PCRs with specific primers. 49.5 percent of strains contained cry1 genes along with 33 percent different cry1-type profiles. Around 14 percent of strains did not give any PCR product. So, they came to conclusion that so many strains may harbor potentially novel cry genes. Crickmore *et al*. 2002 classified over 200 cry genes into 47 classes and subclasses based on amino acid sequences similarity. Recently, the classification of cry genes has been changed

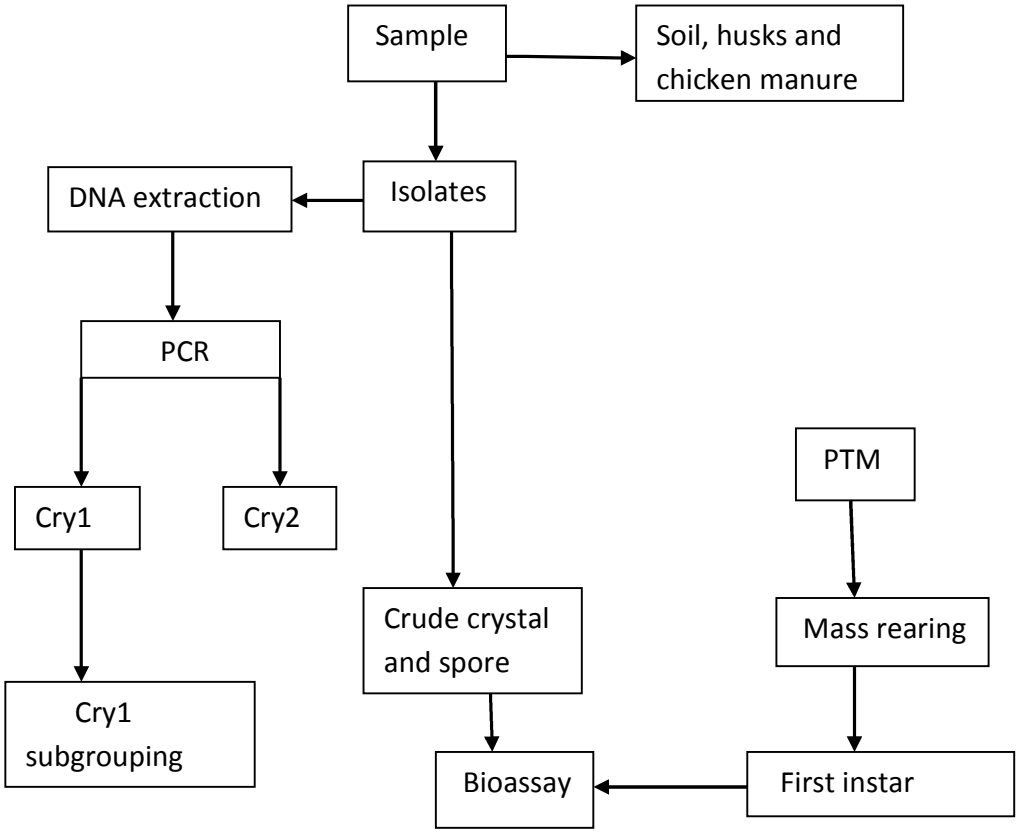
into 73 classes and many subclasses. The recent classification of cry genes and its list presented in table 2.1

Table 2.1 Number of classes and subclasses of cry genes

Sl. No.	**Class**	**Sub class**	**Sl. No.**	**Class**	**Sub class**	**Sl. No.**	**Class**	**Sub class**
1	cry1	269	26	cry26	1	51	cry51	2
2	cry2	77	27	cry27	1	52	cry52	2
3	cry3	19	28	cry28	2	53	cry53	2
4	cry4	17	29	cry29	2	54	cry54	5
5	cry5	13	30	cry30	13	55	cry55	3
6	cry6	4	31	cry31	11	56	cry56	4
7	cry7	37	32	cry32	28	57	cry57	2
8	cry8	58	33	cry33	1	58	cry58	1
9	cry9	36	34	cry34	11	59	cry59	2
10	cry10	5	35	cry35	11	60	cry60	6
11	cry11	8	36	cry36	1	61	cry61	3
12	cry12	1	37	cry37	1	62	cry62	1
13	cry13	1	38	cry38	1	63	cry63	1
14	cry14	2	39	cry39	1	64	cry64	1
15	cry15	1	40	cry40	4	65	cry65	2
16	cry16	1	41	cry41	4	66	cry66	2
17	cry17	1	42	cry42	1	67	cry67	2
18	cry18	3	43	cry43	7	68	cry68	1
19	cry19	3	44	cry44	1	69	cry69	3
20	cry20	4	45	cry45	1	70	cry70	3
21	cry21	10	46	cry46	3	71	cry71	1
22	cry22	7	47	cry47	1	72	cry72	1
23	cry23	1	48	cry48	5	73	cry73	1
24	cry24	3	49	cry49	5			
25	cry25	1	50	cry50	3			

(Source: http://www/lifesci.sussex.ac.uk/home/neil_crickmore/Bt/toxins2.html)

METHODOLOGY



Plan of the work

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

The materials, different media and reagents used in this thesis work are given in Appendices 1, 2 and 3, respectively.

3.2 Methods

3.2.1 Sample collection

A total of 28 different samples were collected from different geographical regions of Nepal comprising terai, hilly and Himalayan regions. The collected samples are shown in table 3.1. Approximately, 5 gm of soil samples were taken from at least 5cm below the ground level by scrapping off the surface with a sterile spatula. They were placed in zip locked plastic bags aseptically, transported to the laboratory and stored at 4°C until processed.

Table 3.1 Locations , types and number of samples

S.I No.	Location	Type of sample	No. of Samples
1	Ghasa, Myagdi	soil	4
2	Beni, Myagdi	soil	2
3	Lalitpur	chicken manure husks	3 3
4	Sauraha, Chitwan	soil	5
5	Tulsipur, Dang	soil	5
6	Bandipur, Tanahun	soil	4
7	Bhairahawa, Rupendehi	soil	2

Fig. 3.1 Location of collected samples

3.2.2 Isolation

For the isolation of *B. thuringiensis* from soil, acetate selection method (Travers *et al*., 1987) was used whereas quick isolation method (Muniady *et al*., 2011) was used to isolate *B. thuringienisis* from chicken manure and husks. In acetate selection method, 5 gram of sample was suspended in 10 ml of nutrient broth containing 0.25M sodium acetate (pH 6.8). The suspension was vortexed vigorously and incubated overnight for microbial growth at 37°C in a shaking water bath. Heat treatment was applied for 5 minutes at 80°C to eliminate vegetative and non-sporeforming cells. After that, they were plated on nutrient agar plates and incubated overnight at 37°C. Finally, suspected *B. thuringiensis* colonies which were white, spreadout and seem to be fried egg on plate (Travers *et al*., 1987) were labeled and subcultured to get pure culture

In quick isolation method, 5 gram of each (chicken manure and husk samples) were weighed and suspended in 100ml of sterile distilled water in 250ml conical flasks. This procedure was done in order to homogenize. Then, 1ml of aliquots were given heat-shock treatment at 80°C shock treatment, the suspensions were vortexed and five-fold serial dilution was done. Then, 20ul of each aliquot of serial dilutions were plated on nutrient agar using spread plate technique and incubated at 30°C for 24 hours. The plates were examined for suspected *B. thuringiensis* colonies.

3.2.3 Gram Staining

Gram staining was done in order to select the rod-shaped, Gram positive ones. First, the samples were smeared onto a slide with a drop of distilled water, followed by air drying and heat fixing. Then, Gram staining procedure were carried out with primary staining with crystal violet, followed by fixation with iodine, decolouration with acetone and lastly counter staining with safranin. The slides were left to air dry and observed under light microscope. Blue coloured cells were Gram positive whereas pink coloured cells were Gram negative.

3.2.4 *Bacillus thuringiensis* Reference Strains

Three different *B. thuringiensis* strains present at NAST were used as reference strains. They were *B*. *thuringiensis Kurstaki, B. subtilis* and *B.thuringiensis Israeliensis*.

3.2.5 Commassie Brilliant Blue (CBB) staining

The CBB stain allows high throughput evaluation of bacterial colonies for the presence of crystals (Rampersad and Ammons, 2005). The isolates that were selected earlier using the Gram staining techniques were first inoculated into sterile 100ml conical flasks containing 50ml T3 media. The samples were then incubated in a orbital shaker for 110 hours at 30°C with 100rpm agitation. A loop of sample was transferred onto clean glass slide followed by air-drying and heat fixing. The slides were stained using 0.133% Commassie Blue stain in 50% acetic acid (Rampersad *et al*., 2002) for about 1 minute and then rinsed off using the prepared destaining solution. The slides were dried and visualized under light microscope for crystals.

3.2.6 DNA Isolation

DNA isolation was performed by the method of Ceron *et al* (1993). *B. thuringiensis* strains were grown for 12 hours on a nutrient agar plates. A loop of cells from a single colony was transferred to 0.1ml of water and the mixture was boiled for 10 minutes to lyse the cells. The resulting cell lysate was briefly spun (10sec, 10,000rpm). Then, concentration of DNA was measured using biophotometer (Eppendorf, Germany).

3.2.7 Oligonucleotide Primers For Polymerase Chain Reaction (PCR).

In this study, 2 pairs of universal primers reported by Bendov *et al*. (1997,1999), for cry1 and cry2 genes were used. Their sequences and the expected sizes of their PCR products are shown in table 3.2.

Table 3.2 Universal Primers of cry1 and cry2 genes

Universal primers	Expected PCR Product Size
For cry 1 genes Un1,D_1 5'-CATGATTCATGCGGCAGATAAAC-3' R_1 5'-TTGTGACACTTCTGCTTCCCATT-3,	274-277 bp
For cry 2 genes Un2, D_2 5'-GTTAATTCTTAATGCAGATGAATGGG-3' R_2 5'-CGGATAAAATAATCTGGGAAATAGT-3'	689-701 bp

Similarly, 7 pairs of specific primers reported by Ceron *et al*. (1993) for sub-grouping cry1 genes were used. The expected sizes of their PCR products, gene recognized and sequence of primers are shown in table 3.3.

Table 3.3 cry1 specific Primers

S.I No.	Primer pair	Genes recognized	Size of PCR products	Sequence
1	CJ1-CJ2	cry1Aa	249 bp	5' TTATACTTGGTTCAGGCCC 3' 3' TTGGACCTCTCAAGGTGTAA 3'
2	CJ3-CJ2	cry1Ad	171bp	5' CAGCCGATTTACCTTCTA 3' 3' TTGGAGCTCTCAAGGTGTAA 3'
3	CJ4-CJ5	cry1Ab	216 bp	5' AACAACTATCTGTTCTTGAC 3' 3' CTCTTATTATACTTACACTAC 3'
4	CJ6-CJ7	cry1Ac	180 bp	5' GTTAGATTAAATAGTAGTGG 3' 3' TGTAGCTGGTACTGTATTG 3'
5	CJ8-CJ9	cry1B	367 bp	5' CTTCATCACGATGGAGTAA 3' 3' CATAATTTGGTCGTTCTGTT 3'
6	CJ10-CJ11	cry1C	130 bp	5' AAAGATCTGGAACACCTTT 3' 5' CAAACTCTAAATCCTTTCA 3'
7	CJ12-CJ13	cry 1D	290 bp	5' CTGCAGCAAGCTATCCAA 3' 5' ATTTGAATTGTCAAGGCCTG 3'

3.2.8 cry Gene Identification by Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 25 µL reaction volumes containing 12.5 µl of 2x master mix (Promega Co.), 50 ng of template DNA, 0.5 pM each of forward and reverse primers, 3.0 mM of $Mgcl_2$ and 3.5 µL of sterile double distilled water to maintain the volume. Amplification were carried out in PCR machine (Bioer XP thermal cycler, China) with the program set for 25 cycles of denaturation temperature at 92 °C for 1 min, annealing at 53°C for 1 min, extension at 72 °C for 1 min with an extra step of extension at 72 °C for 10 minutes. After amplification, PCR products were electrophoresed at 1.5% agarose gel. Gels were visualized in a gel documentation system (Syngene, UK).

3.3 Bioassay Of Target Insects

3.3.1 Mass rearing and maintenance of PTM population

PTM adults were collected from well established mass rearing laboratory of Entomology Division, NARC, Khumaltar, Lalitpur. Mass rearing of PTM was done as described by Dangi and Giri (2010). Adults (50 pairs) were placed on the egg laying boxes covered with mesh cloth and black muslin cloth. Adults were fed with a diluted honey solution:water (1:10). The diluted honey drops were kept on the edges of plastic box over the black cotton cloth for feeding the PTM. Adults female moth laid eggs in black muslin cloth. The eggs are collected after 24 hours and replaced the black muslin cloth with new one. The eggs were allowed to hatch in hatchling box at 25°C. Larvae emerged 4 to 5 days after incubation. The one day neonate larvae were used for the experiment.

3.3.2 Spores and crystal mixture preparation

The spore and crystal mixture prepared according to Shishir *et al*. (2010) with slight modification. Isolates of *B. thuringiensis* with parasporal bodies were cultured in 100ml of T3 - liquid media and incubated for 5 days at 30°C with continuous shaking at 100rpm. Liquid culture were centrifuged at 5000rpm for 15minutes. Pellets (spores and parasporal protein crystal) were washed with 20ml sterile distilled water and centrifuged at 5000rpm for 5 minutes. Washing procedure was repeated twice. The supernant were discarded and the pellet were dried in a desiccator at 37°C and used it as spore crystal mixture.

3.3.3 Treatment procedure

At first the potato tubers were washed thoroughly with distilled water. The potato were cut into three pieces and placed on the clean tissue for few minutes. Different concentration of spore/crystal mixture were prepared; 20ug/ml, 40ug/ml, 60ug/ml, 80ug/ml and 100ug/ml. Each 50 gram potato were dipped into particular concentration of solution for about 20seconds. Thereafter, treated tubers were air dried for few minutes and placed into bioassay containers. Containers were labeled properly. 25 neonates larvae were inoculated on treated potato per container using a fine paint brush. Containers were closed with a lid (mesh window for ventilation) and incubated at 25°C. The experiment is replicated thrice. Mortality was recorded after 7 days. During evaluation, the developed larvae from neonates were considered as survivors.

3.3.4 Calculation of LD_{50}

LD_{50} represents the concentration at which 50% of the population responds (die). LD_{50} was calculated using probit analysis. Probit analysis is a type of regression used to analyze bionomial response variables. It transforms the sigmoid dose-response curve to a straight line that can be analyzed by regression either through least squares or maximum likelihood.
To calculate LD_{50}, first of all, the mortality in each concentration were converted to mortality percentage. The mortality percentage was corrected using Schneider-Orelli's (1947) formula:

$$\text{Corrected} = \frac{\%\ \text{of responded} - \%\ \text{of responded in control}}{100\% - \text{responded}\%\ \text{in control}} \times 100$$

The corresponding probits of each corrected mortalities were recorded by looking up the Finney's table (Finney, 1952). The graph of log concentration Vs probits was constructed using excel (2007). The graph displayed equation of the line. The value X was calculated from the equation by placing probit (Y=5). The inverse of log value (X) was calculated which was the LD_{50}.

3.3.5 Stastistical analysis

All the experiments were performed in triplicates and the data represent the mean $\pm$ standard deviation from three independent assays. The LD_{50} values were calculated using Microsoft Excel 2007 software. The graphPad Prism version 5 was used to construct charts and for stastistical analysis. An unpaired t- test (two- tailed) was applied to compare the LD_{50} between isolated strains and reference strain(4D1). P value < 0.001 was taken as extremely significant, 0.001 to 0.01 as very significant, 0.01 to 0.05 as significant and >0.05 as non significant.

CHAPTER 4

RESULT

4.1 Isolation of *Bacillus thuringiensis*

A total of 28 samples were studied. Samples were collected from geographical regions of Nepal comprising lowland terai to the Himalayan regions. *B. thuringiensis* was isolated from soil samples using acetate selection method (figure 4.2) (Travers *et al*., 1987) and quick isolation method (Muniady *et al*., 2011) from manure and husk samples. A total of 140 *B. thuringiensis* like white colonies ,spreadout and seemed fried eggs on plate (Travers *et al*., 1987) were selected as the possible *B. thuringiensis*. Gram staining was done in order to select Gram positive bacteria. Gram positive bacteria were subcultured to obtain pure culture (figure 4.3). Then, each of the Gram positive was inoculated into sporulating media (T3) and incubated at 30°C for 5 days with constant shaking (100rpm). Finally, Comassie brilliant blue staining (Rampersad *et al*., 2002) was done to observe the presence and shape of crystals in order to confirm *B*. *thuringiensis.*

A total of 9 *B. thuringiensis* strains were isolated. Out of 140 colonies examined, the number of *B. thuringiensis* isolates were expressed as index of the number of colonies examined (Table 4.1). Bt index represents the ratio of *B. thuringiensis* isolates to the number of colonies examined. It measures the success in isolating *B. thuringiensis*.

Table 4.1 Bt index showing no. of samples and colonies examined and no. of Bt. Isolates obtained.

Location/ district	Number of sample	Number of colony examined	Number of Bt. isolates	Bt index
Ghasa/ Myagdi	4	20	2	0.10
Local chicken manure/ lalitpur	3	15	2	0.13
Local rice husks/ lalitpur	3	15	0	0.00
Sauraha/ Chitwan	5	25	1	0.04
Tulsipur/ Dang	5	25	2	0.08
Bandipur/ Tanahun	4	20	2	0.10
Beni/ Myagdi	2	10	0	0.00
Bhairahawa/ Rupandehi	2	10	0	0.00
Total	28	140	9	

4.2 Morphology of crystal proteins of *Bacillus thuringiensis* Isolates

Four different crystal morphology were observed. Out of 9 Bt isolates, 6 produced bipyramidal (figure 4.4) (B) crystals, 1 produced round (R) crystals (figure 4.5), 1 produced bipyramidal and oval (B&O) crystals and 1 produced bipyramidal and irregular (B&I) crystals. The reference strain *B. thuringiensis kurstaki* produced bipyramidal crystals, strain *B. thuringiensis israelensis* produced round crystals while *B. subtilis* didn't produced any crystals.

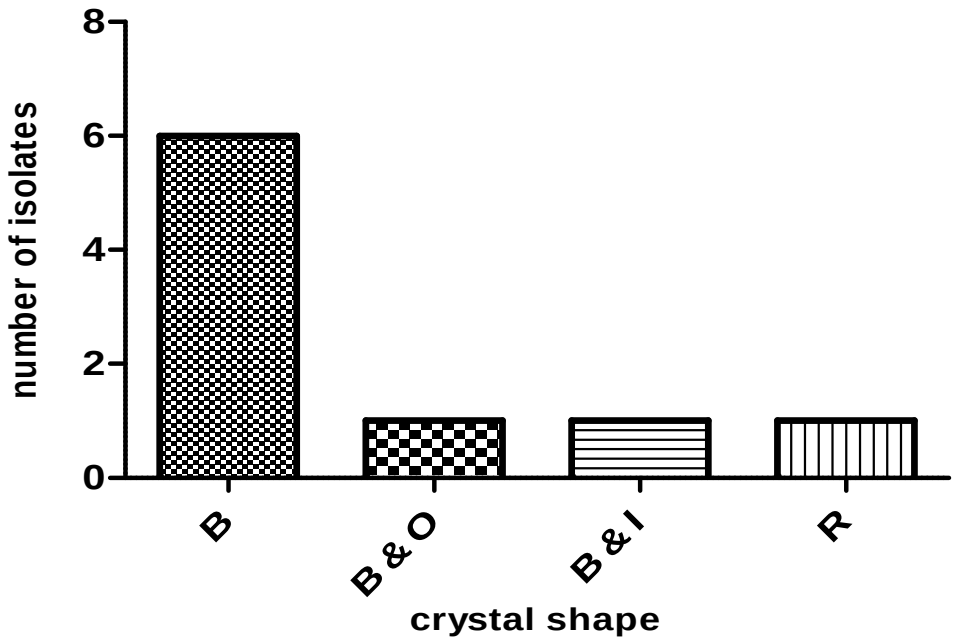


Figure 4.1 Morphology of crystal protein of *B. thuringiensis* isolates

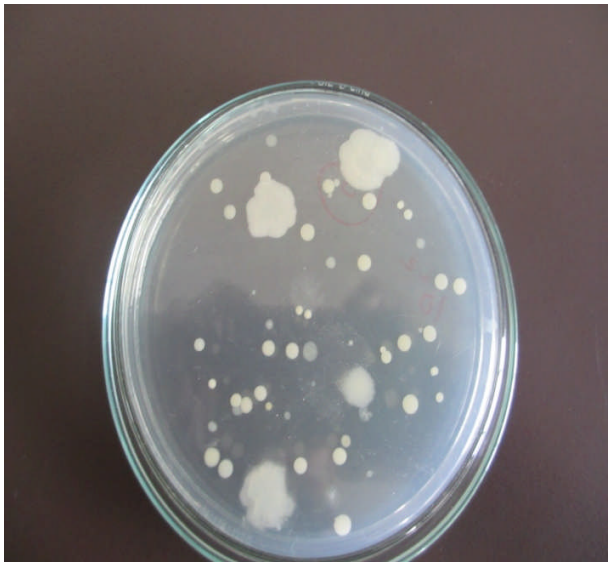


Figure 4.2 colonies after sodium acetate selection

Figure 4.3 pure culture of isolate

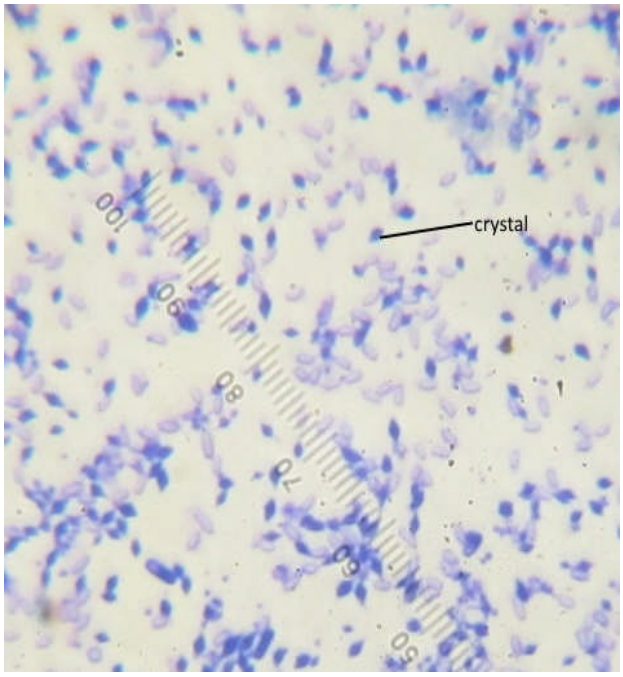


Figure 4.4 bipyramidal crystals

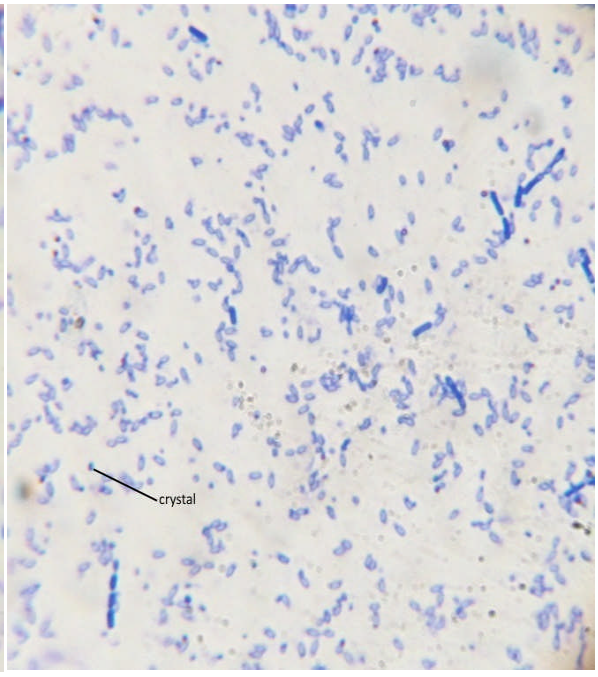


Figure 4.5 round crystals

4.3 Cry1 and cry2 gene analysis of *Bacillus thuringiensis* isolates

B. thuringiensis strains can harbor one or more cry genes. The types of cry gene of the isolates were determined by PCR using universal primers for cry1 and cry2 genes. The universal primers (Table 3.2) for cry1 gene produced PCR products of expected size at around 274 bp (figure 4.6.A) whereas for cry2 gene PCR product is around 690 bp (4.6.B) . Out of nine isolates, five were positive only for cry1 gene whereas three were positive for both cry1 and cry2 genes and strain b1 was negative for both cry1 and cry2 genes. The reference strain *B. thuringiensis kurstaki* was positive for both cry1 and cry2 genes. The other two reference strains *B. thuringiensis israelensis* and B. *subtilis* were negative for both cry1 and cry2 genes.

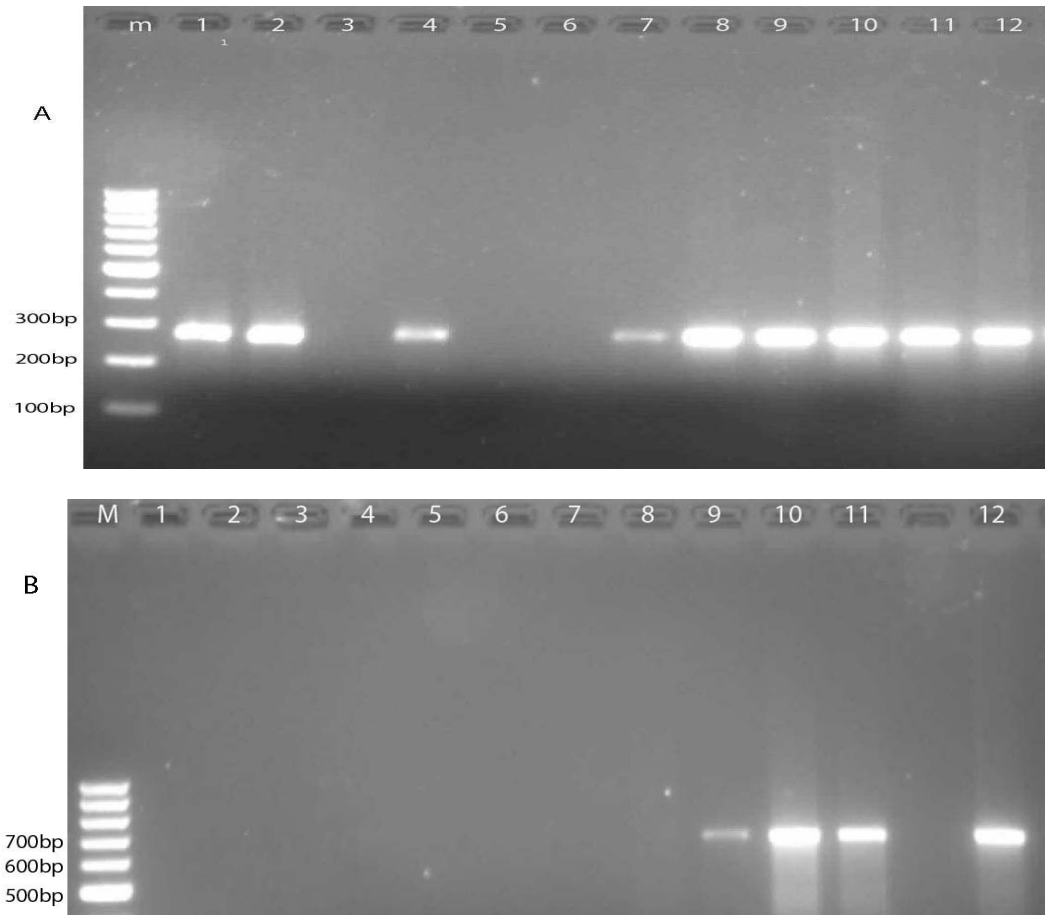


Figure 4.6 PCR products of isolates (A) cry 1 and (B) cry2 genes. **Lanes : M.** DNA ladder 100 bp ; **1.** c1 ; **2.** c2 ; **3.** b1 ; **4.** g1 ; **5.** *B. subtilis* ; **6.** *B. thuringiensis subsp. Israelensis* ; **7.** ch1 ; **8.** d1 ; **9.** d2 ; **10.** g2 ; **11.** *B. thuringiensis subsp. Kurstaki* ; **12.** b2.

4.4 cry1 subgroups genes of *Bacillus thuringiensis*

The seven sets of primers (Table 3.3) are able to identify cry1A to cry1D genes subgroups, including subdivisions into cry1A genes (Figure 4.7). Out of nine isolated strains, seven strains reacted with at least one pair of cry1 set of specific primers. Table 4.2 summarizes the cry1 gene contents of the strains. Five isolated strains had cry1Aa gene. Seven isolated strains had cry1Ab gene and six isolated strains had cry1Ac gene. All the strains lacked cry1B or cry1C or cry1D gene. The isolated strains b1 and c1 as well as reference strains *B. subtilis* and *B. thuringiensis israelensis* did not react with any of cry1 set of specific primers. The reference strain *B. thuringiensis kurstaki* had cry1Aa, cry1Ab and cry1Ac genes. Strains possessing specific genes are presented in Table 4.3.

Table 4.2 amplification of cry1 genes by specific primers

S.N	Primer pair	c1	c2	b1	g1	B.subtilis	Bt subsp. israelensis	Ch1	d1	d2	g2	Bt subsp kurstaki	b2
1	CJ1-CJ2	-	-	-	+	-	-	+	+	+	+	+	-
2	CJ3-CJ2	-	-	-	-	-	-	-	-	-	-	-	-
3	CJ4-CJ5	-	-	-	+	-	-	+	+	+	+	+	+
4	CJ6-CJ7	-	-	-	+	-	-	+	+	+	+	+	+
5	CJ8-CJ9	-	-	-	-	-	-	-	-	-	-	-	-
6	CJ10-CJ11	-	-	-	-	-	-	-	-	-	-	-	-
7	CJ12-CJ13	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.3 Identification of cry1 subgroup genes present in different *B. thuringiensis* strains

strains	**Genes identified by PCR analysis**
c1	cry1Ab
c2	none
b1	none
g1	cry1Aa, cry1Ab, cry1Ac
B. subtilis	none
B. thuringiensis israelensis	none
ch1	cry1Aa, cry1Ab, cry1Ac
d1	cry1Aa, cry1Ab, cry1Ac
d2	cry1Aa ,cry1Ab, cry1Ac
g2	cry1Aa, cry1Ab, cry1Ac
B. thuringiensis kurstaki	cry1Aa, cry1Ab, cry1Ac
b2	cry1Ab, cry1Ac

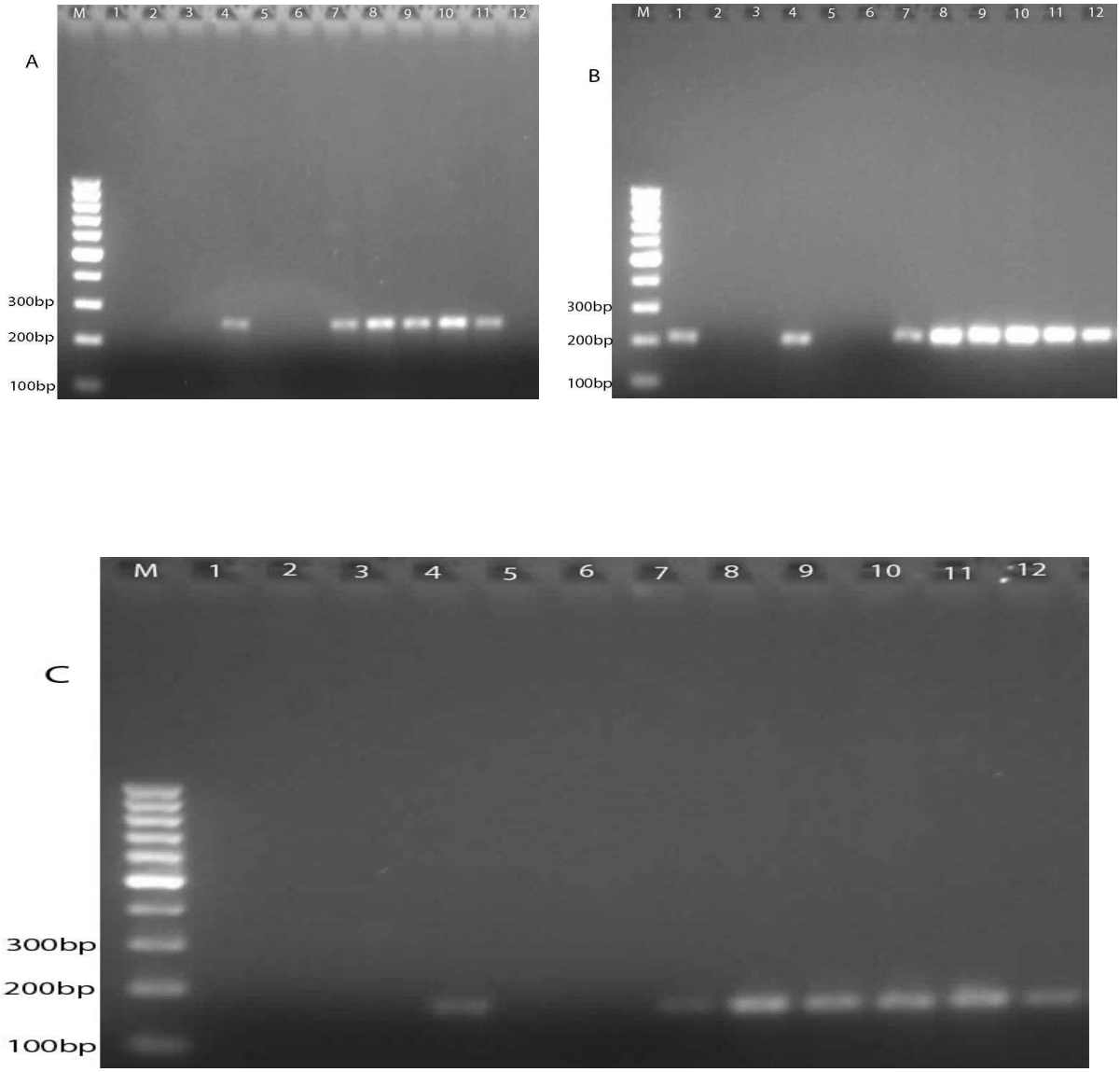


Figure 4.7 Amplification of cry1 gene by specific primers (A) CJ1-CJ2, (B) CJ4-CJ5 and (C) CJ6-CJ7. **Lanes : M.** DNA ladder 100 bp ; **1.** c1 ; **2.** c2 ; **3.** b1 ; **4.** g1 ; **5.** *B. subtilis* ; **6.** *B. thuringiensis subsp. Israelensis* ; **7.** ch1 ; **8.** d1 ; **9.** d2 ; **10** g2 ; **11.** *B. thuringiensis subsp. Kurstaki* ; **12.** b2.

4.5 Determination of LD_{50} value

Out of nine *B. thuringiensis* isolates, five strains were used for bioassay (figure 4.9, 4.10 and 4.11) along with two reference strains *B. thuringiensis kurstaki* and *B. thuringiensis israelensis*. The isolated strain b1 and reference strain *B. thuringiensis israelensis* had no effect on the potato tuber moth (PTM) first instar larvae at all. The strain d1 was found more effective against PTM first instar larvae with LD_{50} value 6.67±3.028 µg/ml crude protein. The LD_{50} values of different isolates are given in table 4.4

Table 4.4 LD_{50} values of *B. thuringiensis* strains

B.thuringiensis strains	LD_{50} value (µg/ml crude protein)
d1	6.67± 1.749
g2	15.73± 1.89
g1	19.09± 0.81
C1	25.09± 4.36
B. thuringiensis kurstaki	36.71± 5.68

Results are expressed as mean± SD of three independent experiments.

4.5.1 Stastical comparision of LD50 values

The LD_{50} value of Bt isolates versus reference strain *B. thuringiensis kurstaki* was analyzed with unpaired t-test (two tailed) to determine if a significant difference occurred between them (figure 4.8). All the results showed significant differences.

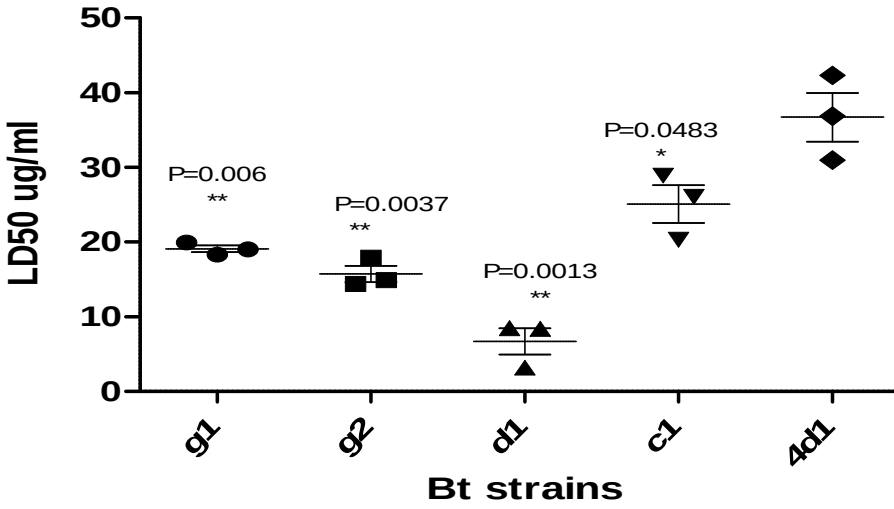


Figure 4.8 Comparasion of LD_{50} of *B. thuringiensis* isolates vs reference strain *B. thuringiensis kurstaki* (4d1)

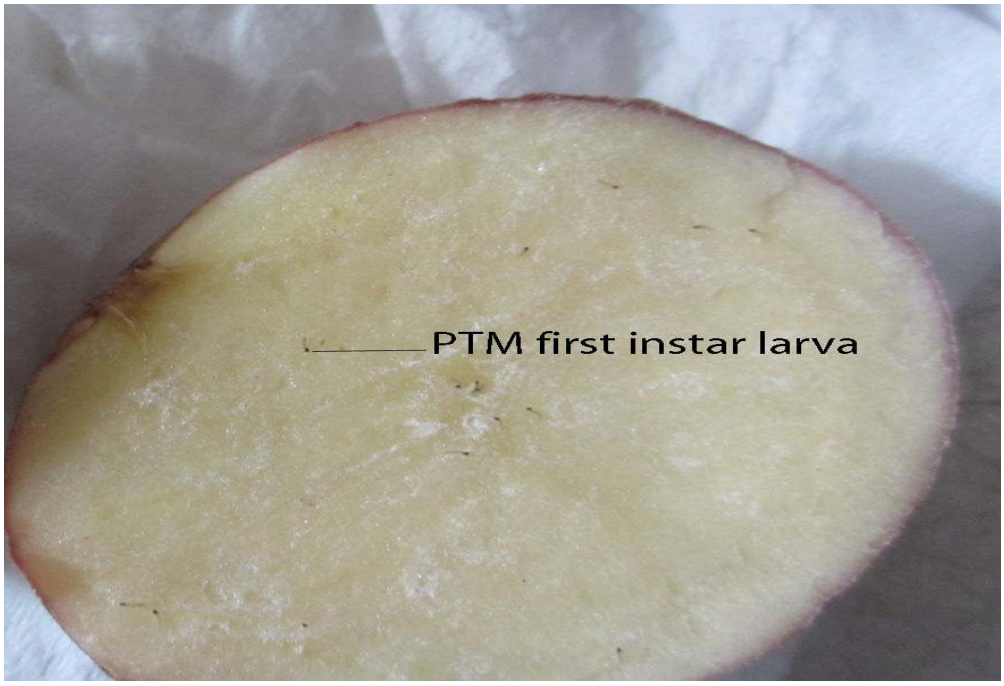


Figure 4.9 First instar larva of potato tuber moth (PTM)

Figure 4.10 Experimental set up for bioassay against PTM

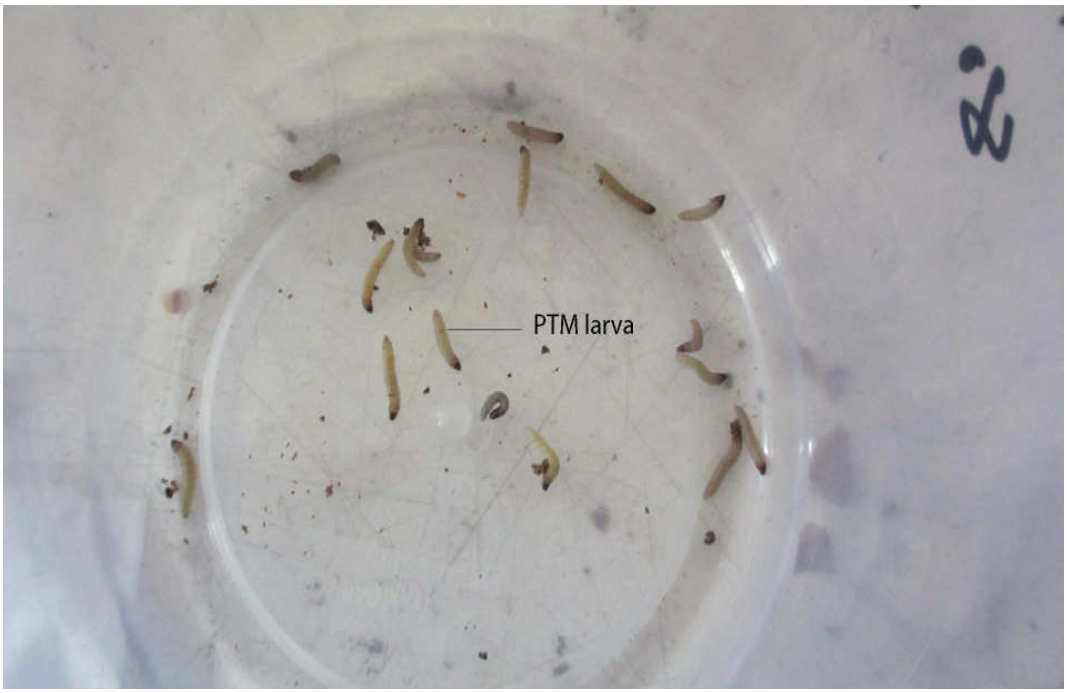


Figure 4.11 Fully developed larva from first instar larva of PTM

Chapter 5

DISCUSSION

Rapid population growth is a major concern for developing countries like Nepal. The population of Nepal is nearly 30 million and the rate of population growth is around 1.6 percent. The Nepalese population is expected to grow for long time. To feed the growing population from limited arable land, productivity per unit area is necessary. However, due to climate change, the insect pests are also affected and new biotypes are evolved. Therefore, higher doses of chemical pesticides are being applied for their control. In this regard, it is not surprising in the surge of pesticides. However, the chemical pesticides have several drawbacks. It adversely affects the ecosystem including accumulation of toxic residue in the nature, leading health problems in mammals and development of insect resistance (Fadel and Sabour, 2002). Therefore, it is not prudent to depend on chemical pesticides totally. The sound management of agricultural pest is to fully incorporate integrated pest management in the agricultural practices. One of the key pillar of integrated pest management is the use of biopesticides. The biopesticides based on *B. thuringiensis* is widely used. The bacteria is a Gram-positive that can exhibit a wide range of toxicity to different insects orders such as Diptera, Lepidoptera and Coleoptera (Martin *et al*., 2010). The isolation of potent strains from wide range of environment is the critical step. Although, Nepal is a small country, it has diverse habitat making it a potential source of many potent *B. thuringiensis* strains.

In this study, acetate selection method proposed by (Travers *et al*., 1987) was used to isolate *B. thuringiensis* from the soil samples. Using this method, seven strains of *B. thuringiensis* were isolated. Quick isolation method as proposed by (Muniady *et al*., 2011) was used to isolate *B. thuringiensis* strains from chicken manure and husks samples. Using this method two strains of *B. thuringiensis* were isolated. Different methods of isolation have been used but acetate selection method have been widely used by many scientists so far. In acetate selection method, the *B. thuringiensis* strains were usually inhibited by 0.25M sodium acetate concentration. The inability of most *B. thuringiensis* strains to germinate in the presence of a acetate buffer allows the use of this trait to screen for this organisms in the soil samples. When the soil sample is subjected to growth media buffered with 0.25M sodium acetate, the unwanted organisms will grow. The unwanted organisms are eliminated when the media is subjected to heat treatment. Quick method, on the other hand, just rely on the heat treatment. Without heat treatment, the ratio of unwanted bacteria (non sporeforming) to wanted bacteria (spore forming) would have been too high (Travers *et al*., 1987).

In this study, one hundred forty colonies were selected as potential *B. thuringiensis* colonies which seemed like fried eggs on plate (Travers *et al*., 1987). Initially Gram staining was done in

order to eliminate Gram negative bacteria. The Gram positive bacteria were subcultured to get the pure colonies. The colonies were cultured in T3 media for sporulation. Although, heat treatment eliminates many undesirable bacteria, some close relatives of *B. thuringiensis* namely *B. anthracis* and *B. cereus* still persist. Based on their similar morphology and genetic makeup it has been proposed that the three species should be considered a single species (Helgason *et al*., 2000). This highlights the importance of media T3 which favours the growth of *B. thuringiensis* suppressing other *Bacillus* species. The *B. thuringiensis* strain was confirmed by Comassie brilliant blue stain (Rampersad and Ammons, 2005). It stained the crystals into purple blue. The resolution of crystals by comassie brilliant blue stain is higher than that of amido black stain (Rampersad and Ammons, 2005).

In this study, twenty two soil samples were collected from different geographical regions of Nepal comprising terai to Himalayan region. The prevalence of *B. thuringiensis* was expressed as Bt index. It is the ratio of *B. thuringiensis* to number of colonies observed. It is an important measure of success in isolating *B. thuringiensis*. The highest Bt index for soil was 0.10 representing from Ghasa, Myagdi. The Bt index of Beni, Myagdi and Bhairahawa, Rupendehi were zero. The Bt index for chicken manure from Kathmandu was 0.13. It showed that Bt index varied from place to place. Besides, the number of samples, location of samples, method of isolation and method of collection of samples greatly affect the Bt index. Therefore, Bt index is not always consistent. For example, Hongyu *et al*., (2000) reported that *B. thuringiensis* was more abundant in husks than in soil. On the contrary, in this study, the Bt index was nil for husk samples.

Crystal morphology can provides valuable insights on the target insects. For example, bipyramidal crystals are mostly effective against Lepidopteran while round crystals are generally mosquitocidal. The round crystals producing *B. thuringiensis israelensis* is widely used in control of mosquito in many parts of world. In this study, bipyramidal crystals were more abundant. These crystals producing strains are more likely to be effective against wide range of Lepidopteran pests. One strain also produces round crystals. Therefore, it could be effective against mosquito larvae. Two of the strains had two types of crystals, one with bipyramidal and oval and other with bipyramidal and irregular. Generally, bipyramidal, oval, round, cuboidal, square, rhomboidal, irregular shaped were reported. Obeidat *et al*., (2004) had reported cuboidal, bipyramidal and spherical shaped crystals.

The cry gene encodes toxic crystal proteins. *B. thuringiensis* can harbor one or more cry genes. So far, seventy three types of cry gene have been discovered (www/lifesci.sussex.ac.uk/home/neil_crickmore/Bt/toxins2). The cry1 and cry2 genes are more abundant cry genes. The cry1 gene is further classified into two hundred sixty nine subclass and cry2 into seventy seven subclass according to recent nomenclature of *B. thuringiensis* strains. In

this study, five strains of *B. thuringiensis* harbored only cry1 gene. Three strains harbored both cry1 and cry2 genes. The reference strain *B. thuringiensis kurstaki* harbored both cry1 and cry2 genes. The other reference strains *B. subtilis* and *B. thuringiensis israelensis* did not contain either cry1 or cry2 genes. From this study, it showed that cry1 gene is most prevalent in the nature. Most studies have reported that cry1 and cry2 genes were more prevalent than any other cry genes (Wang *et al*., 2003). Wang *et al*., (2003) had found that 90.7% strains harbored both cry1 and cry2 genes. In this study, 33.33% harbored both cry1 and cry2 genes. Besides cry1, other cry genes such as cry8 and cry9 secrete crystals that are also effective against Lepidoptera (Bravo *et al*., 1998). The cry2 gene secrete crystal that are effective against both Dipterian and Lepidopteran (Bravo *et al*., 1998). Similarly, cry4 (Carozzi *et al*., 1991), cry10, cry16, cry17 (Mazier *et al*., 1997) , cry24 (Ibarra *et al*., 2003) secrete crystals that are effective against Diptera. Further, subgrouping of cry1 showed the prevalence of cry1Aa, cry1Ab, cry1Ac genes but absence of cry1B, cry1C and cry1D. One of the isolated strain c2 harbored cry1 gene but it was not amplified by any one of the seven pairs of specific primers of cry1 gene. This showed that the strain c2 harbored other type of cry1 subclass. That subclass of cry1 gene could be new or may be similar to any one of the 269 subclass of cry1 gene. That surmise could be verified only after sequencing that gene.

Altogether, seven strains were used for bioassay against PTM (potato tuber moth). Five of the strains were isolated and the remaining two were reference strains. The isolated strain b1 and reference strain *B. thuringiensis israelensis* did not have any effect on PTM first instar larvae. Their ineffectiveness against PTM is more likely due to shape of crystals. In this case their crystal were round. Hence, there is strong correlation between shape of crystals and their toxicity. The LD_{50} value of *B. thuringiensis kurstaki* was 36.71±5.6 µg/ml crude protein. The LD_{50} value of d1 strain was 6.67±3.028 µg/ml crude protein which was lowest in this study. It can be said that d1 strain is comparatively more potent than any other tested strains. The LD_{50} value of BTK (commercial strain formulation Biolep, strain Z-52) was around 290µg/ml (Pandey *et al*., 2003). The LD_{50} values of tested strains were far lower than BTK strain. It showed that the isolated strains from this present study are far better than the commercial strain. Therefore, it can be generalized that instead of relying on commercial products, it is more wise to rely on locally isolated strains.

The LD_{50} values of isolated strains versus reference strain *B. thuringiensis kurstaki* was analysed with unpaired two tailed t- tests (Graphpad prism, version 5.0). Data from study revealed significant difference between isolated strains and reference strain showing that isolated strains were better at killing PTM first instar larvae than the reference strain. The result is quite encouraging as the strains could be used as the substitute for chemical pesticides. If these isolated strains are developed into biopesticides, it would substantially reduce the economic loss of farmers due to PTM.

Potato (*Solanum tuberosum, Solanaceae*) is an important food and cash crop of Nepal. The potato tuber moth (PTM*), Phthorimaea, Gelechiidae*) is one of the major pests attacking potato. The larvae mine into potato leaves and tuber. It was reported from Kathmandu, Kavrepalanchowk, Nala, Panchkhal, Nuwakot, Dhadhing, Makawanpur and Bara districts of Nepal (Joshi, 1989). At present, Nepalese farmers are totally dependent on chemical pesticides which is on long term is not sustainable. Nepalese farmers still consider pesticides as medicine to kill pests but not poision that affect the other living organisms. The use of highly hazardous chemicals such as Phorate (thimet) is still been used against soil insects without considering their side effects (Dahal, 1995). The use of hazardous and obsolete pesticides are widespread because of farmers illiteracy and lack of strict government regulations. In addition, the weak economic condition of farmers is the important contributing factor for status quo. Hence, it is more prudent to opt for biopesticides. The use of safer and biodegradable biopesticides have a number of advantages. One of these advantage results from their high level of selectivity. They are often used in organic agriculture which is becoming increasingly popular with consumers. Similarly, many studies have also highlighted the benefits of exploiting Bt based pesticides for protection of crops and forests. Progress in molecular genetics has also made it possible to use Bt cry genes for the construction of plants resistant to insects. In India, after introduction of Bt cotton it not only became self sufficient but also became second largest producer and exporter of cotton by doubling its production within five years. Nepal must take some urgent steps in the feasibility and research on biopesticides because the development of alternative biopesticides is critical for sustainable agriculture.

Summary

Lepidopteran pests cause severe damage to crop production in many areas of the world. Potato tuber moth (PTM), *Phthorimae operculella* (Zeller) is one of the devastating lepidopteron that attack many solanaceous crops, including potato. The control of PTM is critical in Nepal as potato is the second staple food after rice. The pest is difficult to control and many farmers have relied extensively on the use of insecticides for PTM control. However, this practice have several drawbacks causing severe human health hazards as well as environmental pollution. Therefore alternative approaches to pest control are needed. One of the ecofriendly method is the use of biopesticides, especially based on *B. thuringiensis* (Bt) is very promising. Bt produce endotoxin crystal (cry) protein that are known to have toxicity against variety of insects pests. This study aimed at screening potent Bt strains from wide range of habitat of Nepal.

Sodium acetate selection method was used to screen potential Bt strains from soil samples whereas Quick isolation method was used for chicken manure and husks samples. A total of 28 samples from different parts of Nepal were screened for potential Bt strains. 140 Bt like (white, spreadout) colonies were studied. Gram staining was initially done to eliminate Gram – negative bacteria. Comassie brilliant blue (CBB) staining was done to confirm Bt which stains crystal into purple blue. The morphology of crystals were also studied. Most of the isolates had bipyramidal crystals. *B. thuringiensis* strains can harbor one or more cry genes. The cry gene types of the isolates were determined by PCR using universal primers for cry1 and cry2 genes. Out of nine isolates, five were positive only for cry1 gene whereas three were positive for both cry1 and cry2 genes. The seven sets of specific primers are able to identify cry1A to cry1D genes subgroups, including subdivisions into cry1A genes. Five strains had cry1Aa gene. Seven strains had cry1Ab gene and six strains had cry1Ac gene. All the strains lacked cry1B or cry1C or cry1D genes. Bioassay were performed against PTM. Five strains were used for bioassay. The isolated strain b1 had no effect on PTM but other strains c1, d1, g1 and g2 were effective against PTM. The lowest LD_{50} value was 6.67±3.028 µg/ml crude protein for strain d1. Finally, The LD_{50} value of Bt isolates versus reference strain *B. thuringiensis kurstaki* was analyzed with unpaired t-test (two tailed) to determine if a significant difference occurred between them. All the results showed significant differences. Therefore, the isolated strains have potential to be developed into biopesticides.

Conclusion

Potato tuber moth (*Phthorimaea operculella*) is the most destructive pest of potato in Nepal. It is also a serious pest of tomatoes and tobacco. Since, the economy of most Nepalese depend largely on agriculture, their control is indispensable. The best alternative is the use of biopesticides as they are very selective toward their target as well as biodegradable. The biopesticides based on Bt is quite popular and effective. *B. thuringiensis* is found in diverse environment and the insecticidal protein found in diverse environment are quite different attributing their toxic potential against wide range of economically important pests. Hence, Nepal is a very good place to isolate different strains of *B. thuringiensis* as Nepal has diverse geography and climate.

In this study, out of nine isolates, five of them were tested against potato tuber moth (PTM). Four of them were effective against PTM and comparatively better than the reference strain *B. thuringiensis kurstaki*. The discovery of highly toxic isolates reveals the usefulness of screening for novel *B. thuringiensis* strains. The further application of these strains in biological control programmes requires optimization condition of the microorganism using low cost substrates. In addition, the toxic cry gene of these strains could be used to contruct transgenic plants resistant to insects.

Recommendations

The study of *Bacillus thuringiensis* is very promising as it is one of the major source of biopesticides. With the emergency of many resistant pest, the study of *Bacillus thuringiensis* seem even more promising. This study aimed at isolating potent strains of *Bacillus thuringiensis*. Many further works can be done. Some of them are

- Extensive screening of novel Bt strains from wide range of Nepal.
- Identification of many cry genes by PCR.
- Bioassay against many economically important pests.
- Mass production of crystal protein using low cost substrates.
- Toxin producing gene, cry gene may also be cloned from plasmid into E. coli with a suitable vector to produce target proteins rapidly and economically.

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APPENDICES

APPENDIX 1: MATERIALS

A. EQUIPMENTS

Autoclave

Centrifuge

Distillation Unit

DNA Thermal Cycler

Electric balance

Electric Heater

Elecrophoretic tank

Glasswares

Hot air oven

Hot water bath

Incubator

Laminar Flow

Microcentrifuge

Micropipettes

Microscope

Microwave Oven

pH meter

Refrigerator

Spectrophotometer

UV illuminator

Vortex

Water bath shaker

Micropipettes tips

Ependorff tubes

B. MEDIA

Culture media

Agar powder

Beef extract

Nutrient agar

Nutrient broth

Peptone

Tryptone

Yeast extract

C. Chemicals/reagents

Staining reagents

Acetic acid

Acetone

Alcohol

Comassie brilliant blue

Bromophenol Blue

Calcium chloride

Crystal violet

Disodium hydrogen phosphate

Glycerol

Iodine

Malachite green

Mono sodium hydrogen phosphate

Safranine

D. Others:

Sodium Acetate

Hydrochloric acid

Methanol

Sodium hydroxide pellets

Ferric Citrate

APPENDIX 2: NAME AND COMPOSITION OF MEDIA

CULTURE MEDIA

1. **Lauria Bertani Broth**

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Final pH (at 25°C)	6.8 ±0.2

2. **Lauria Bertani Agar**

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00

Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	6.8 ±0.2

3. **Nutrient Broth**

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Final pH (at 25°C)	7.4 ±0.2

4**. Nutrient Agar**

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	7.4 ±0.2

5. **T3 Agar Medium Used for Sporulation**

Ingredients	(Grams/Litre)

Tryptone	3
Tryptose	2
Yeast extract	1.5
Mangane chloride	0.005
Agar	15
Sodium phosphate	0.05 M

APPENDIX 3. COMPOSTION OF TEST AND STAINING REAGENTS

1. **Crystal Violet**

Solution A:

Crystal violet	2 gm
95% ethanol	20 ml

Solution B:

Ammonium oxalate	0.8 gm
Distilled water	30.0 ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water.

Then solution A and B were mixed.

2. **95% Ethyl Alcohol**

5 ml of distilled water was added to 95 ml of absolute alcohol to make 95% ethyl alcohol solution.

3. **Gram's Iodine**

Iodine crystal	1.0 gm
Potassium iodine	2.0 gm

Distilled water 300 ml

Iodine and potassium iodine were dissolved in distilled water.

4. **Coomassie brilliant blue (0.25%)**

50% ethanol is mixed with 7% acetic acid in water.

5. **Safranin**

Safranin (2.5% solution in 95% ethyl alcohol) 10 ml

Distilled water 100 ml

APPENDIX 4. BUFFERS AND STOCK SOLUTIONS

1. **50 X TAE**

242 g Tris base is dissolved in deionized water, 57.1 ml glacial acetic acid and 100ml. 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionized water.

2. **1 X TAE.**

20 ml of 50X TAE buffer is taken and the volume is adjusted to 1000 ml with deionized water to obtain 1000 ml 1X TAE buffer.

3. **5 X TBE**

54 g Tris Base and 27.5 g boric acid are weighed and dissolved in nearly 800 ml of deionized water. And 20 ml 0.5 M EDTA pH 8.0 is added. The volume is brought to 1000 ml with deionized water.

4. **1X TBE**

200 ml 5X TBE is taken and the volume is brought to 1000 ml with deionized water.

5. **1X TE (pH 8.0**)

10 mM Tris (pH 8.0), 1mM EDTA

6. **Tris-HCl (1 M, pH 8.0)**

121.1 g Tris base is dissolved in 800 ml of deionized water. pH is adjusted to 8.0 with concentrated HCl. Volume is adjusted to 1000 ml with deionized water. The solution issterilized by autoclaving.

7. **EDTA (0.5 M, pH 7.5, 8.0 and 9.5)**

186.1 g of EDTA is dissolved in 800 ml of deionized water and pH is adjusted to desired value with 10 N NaOH. Volume is brought to 1000 ml with deionized water. The solution is sterilized by autoclaving.

8. **Sodium Acetate (3M, pH 5.2)**

408.1 g sodium acetate (3 H2O) is dissolved in 800 ml deionized water and pH is adjusted to 5.2 by glacial acetic acid. Volume is brought to 1000 ml. The solution is sterilized by autoclaving.

9. **Ammonium Acetate (10M)**

770 g of ammonium acetate is dissolved in 800 ml of distilled water. Volume is adjusted to 1000ml. The solution is sterilized by filtration.

10. **Ethidium Bromide (10 mg/ml)**

1 g of ethidium bromide is dissolved in 100 ml of deionized water by strring for several hours. The solution is stored in a dark bottle at room temperature.

11. **6X Gel Loading Buffer (20 ml)**

2 ml of 10x TBE, 6 ml of glycerol and 12 ml deionized water are mixed. Bromophenol blue is added with toothpick until obtaining sufficient color of the solution.

APPENDIX 5. Isolation of *Bacillus thuringiensis* from different places and their sources

Table I (Soil sample from Ghasa, Myagdi, g)

Sample	No. of isolates	Codes of isolates
g1	1	g1
g2	1	g2
g3	0	
g4	0	

Table II (Chicken manure from Lalitpur, c)

Sample	No. of isolates	Codes of isolates
c1	1	c1
c2	1	c2
c3	0	

Table III (Soil sample from Sauraha, Chitwan, ch)

Sample	No. of isolates	Codes of isolates
ch1	1	ch1
ch2	0	
ch3	0	
ch4	0	
ch5	0	

Table IV (Soil sample from Tulsipur, Dang, d)

Sample	No. of isolates	Codes of isolates
d1	1	d1
d2	1	d2
d3	0	
d4	0	
d5	0	

Table V (Soil sample from Bandipur, Tanahun, b)

Sample	No. of isolates	Codes of isolates
b1	1	b1
b2	1	b2
b3	0	
b4	0	