REVIEW OF LITERATURE
CHAPTER - 2

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The global scenario of crop losses to an estimated 73.8% due to insects and other arthropods, 11.6% from diseases and 9.5% from weeds (Van Rie, 2000) is highly alarming, especially when, hundreds of people die of starvation and malnutrition due to scarcity of food. To minimize the economic loss of agricultural crops, so as to increase the productivity, higher doses of application of pesticides have become increasingly necessary. Ultimately the pesticide residues enter into the food chain resulting in biomagnifications. As a consequence, great impetus is given to find out alternatives to chemical insecticides B. thuringiensis as a class of biopesticides, deserves immediate attention in this regard since they are ubiquitous, easy to multiply, non pathogenic and above all biodegradable.

During sporulation, B. thuringiensis produces crystalline inclusions containing one or more proteins called Pesticidal Crystal Proteins (PCPs) or δ-endotoxins (Rajamohan et al., 1996). The crystals on dissolution in the insect gut liberate protoxins which when proteolytically activated to a toxic fragment, prove to be lethal to the larvae (Schnepf et al., 1998).

Some Bacilli, in addition to PCPs, produce vegetative insecticidal proteins (Vips) during the vegetative growth phase. These are secreted proteins that also damage the insect midgut (Yu et al., 1997).
2.1. Pesticidal crystal proteins: classification and nomenclature

Höfte and Whiteley (1989) proposed a nomenclature system based on sequence homology and insecticidal spectrum of PCPs. However, the classification criteria presented problems since there is no simple relation between sequence and insecticidal spectrum. In order to avoid inconsistencies, a new nomenclature system has recently been introduced, based solely on sequence homology of the full-length PCP proteins (Crickmore et al., 1998). Currently, this system classifies 169 sequences into 28 cry classes and 2 cyt classes, based on a phylogenetic tree. Each toxin name is characterized by four ranks (eg. cry1Ab5) depending on its position in the phylogenetic tree. Although most PCPs are likely to have a common structure and mechanism of action, they are extremely specific. Indeed, even within a particular class of PCPs, e.g. Cry1 proteins, considerable differences in insecticidal spectrum exist. PCPs belonging to the Cry1, Cry4, Cry5, Cry7, Cry8, Cry9, Cry12, Cry14 and Cry21 are pro-toxins of about 120 to 140 kDa, which are proteolytically processed to an active toxic fragment of about 60 kDa. While only few amino acids can be removed from the N-terminus without interfering with biological activity, about half of the protoxin is removed at the C-terminus during activation. PCPs belonging to Cry2, Cry3, Cry10, Cry11, Cry13, Cry16, Cry17, Cry19, Cry19, Cry20 do not contain the typical C-terminal extension and have sometimes been referred to as 'naturally truncated' proteins. Alignment of PCP amino acid sequences showed that sequence variation is not distributed in a random fashion.
2.2. Mode of action of PCPs

When *B. thuringiensis* is ingested by larvae, the first step in the mode of action is that crystal proteins are solubilised from the crystal. The pH in the gut of lepidopteran larvae varies from 9 - 12 and lepidopteran crystal proteins can only be solubilised above pH 9.5 (Knowles and Dow, 1993). Bietlot et al (1993) found other factors such as detergents and reducing potential of the gut to breakup intermolecular bridges leading to solubilisation. Proteases with a trypsin-like activity, either from the insect or from the Bacillus itself, gradually remove some 600 amino acids from the C-terminus of the protein and some 30-50 amino acids from the N-terminus (Jayachandran, 1994; Rajamohan et al., 1996). This generates a protein of some 60kDa, which corresponds to the N-terminal half of the original crystal protein.

This 60-kDa protein is trypsin resistant and is the active core of the crystal protein (Hofte and Whiteley, 1989). Following the processing of the protoxins into the trypsin-resistant core fragment, the δ-endotoxin passes through the pores of the peritrophic membrane and interacts with the gut epithelial cells (Bravo et al., 1997).

Knowles and Dow (1993) postulated that upon pore formation in the columnar cells of the midgut epithelium, there is a rapid influx of ions which leads to depolarization of the apical membrane which in turn causes a rise of intracellular pH, The potassium pump stops and the columnar cells swell and lyse osmotically, leading to disruption of the integrity of the gut epithelium, starvation, and insect death. It appears that the specific action of activated crystal
protein is related to their pore-forming capacity, which in turn is dependent on receptor binding.

2.3. Isolation and Identification of *B. thuringiensis* strains

The toxicity and spectrum is being widened each year with the discovery of novel strains that are active against various insects (Hofte and Whiteley, 1989). Following the early isolation of *B. thuringiensis* from dead insect larvae, these bacteria have been found ubiquitously by using a novel enrichment technique that exploits unique germination properties of the spores (Martin and Travers, 1989) or by simply screening debris, such as soils, leaves, and dead larvae, for spore formers containing parasporal inclusions. One of the most important aspects about establishing a *B. thuringiensis* collection is to have a methodology with which one can rapidly and accurately characterize the strain, the toxin protein, and the gene. This is especially important if the differences among endotoxin genes, carried by a certain strain, are critical for its specificity and toxicity. The bioassay analysis is an exhaustive and time-consuming process because it is necessary to screen all the isolates in all the target insects. Various methodologies viz. (1) Southern blot analysis in search of homologous genes (Kronstad and Whiteley, 1986), (2) Reactivity to different monoclonal antibodies (Hofte and Whiteley, 1989) and (3) Electrophoretic analysis of PCR products using specific primers (Carozzi *et al.*, 1991) have been described to simplify this process. Among the three approaches, PCR analysis is considered to be the
best choice because it is highly sensitive, relatively fast and can be used routinely (Kumar et al., 1996)

As to the wider distribution of *B. thuringiensis* in different environments, various workers have reported isolation from soil, water, saw dust etc (Bravo et al., 1998; Bendov et al., 1997; Helgason et al., 1998; Hongyu et al., 2000)

Chilcott and Wigley (1993) found *B. thuringiensis* in 60 – 100 % of the soil samples that they have collected from New Zealand. Based on the data on the presence of *B. thuringiensis* in 17 soil samples, Hossain et al (1997) were of the opinion that *B. thurngiensis* are ubiquitously distributed in the agricultural soil of Bangladesh. From out of 2362 samples collected from stored products, soil, insect residues, plant material and other unusual materials, 5303 crystal forming isolates were obtained (Bernhard et al., 1997). Similarly Bravo et al (1998) who have isolated *B. thuringiensis* strains in 456 of the 503 Mexican soils suggested that such collection has greatest value, since samples collected were from different climatic regions with a high diversity of insects. Recently, Kaelin and Gadani (2000) reported that 9% of 132 samples of cured tobacco leaves of different types and origins contained *B. thurngiensis*

2.4. Crystal morphology

Controversies about the shapes of crystals were studied by Hameed et al. (1990) where the changes in crystal shapes during various stages of sporulation was observed. Bernhard et al. (1997) in their worldwide collection of *B. thuringiensis* have reported the distribution of crystal morphologies as 45.9%
bypiramidal, 4.4% round, 16.4 irregular and 19.1% pointed. Bravo et al. (1998) reported that isolates producing bipyramidal crystals were more easily distinguished than isolates with rhomboid, oval, or pointed crystal types. While the vast majority of the strains (21 out of 24) produced bipyramidal crystals, 16 isolates showed cuboid or heterogeneous crystals as observed by Kaelin and Gadani (2000)

2.5. SDS-PAGE analysis of PCPs

Analysis of B. thuringiensis crystals using SDS-PAGE has revealed that most lepidopteran-active crystals contain 130- and/or 65 kDa proteins whereas mosquito active crystals contain 130-, 65-, and 28- kDa proteins (Hofte and Whiteley, 1989). Chilcott and Wigley (1993) also found that isolates active against diperans contained proteins with molecular weights of 130, 68, 28 KDa. All B. thuringiensis isolates characterised by Chak et al., (1994) showed three typical protein profiles around 130-140 kDa. By carrying out insect gut binding assays, Rajamohan and Dean (1996) have also shown that proteins in the molecular weight range of 65- kDa do bind to insect guts. Numerous strains synthesize crystals made up of proteins with sizes differing from the already known toxins (Chaufaux, et al., 1997)

Ogiwara et al., (1991) suggested that the appearance of a doublet is a common occurrence of gut juice-activated toxins which may reflect minor N-terminal or C-terminal processing. Frankenhuyzen et al. (1993) found that processing of the protoxin in 1% silkworm gut juice resulted in a predominant
toxin band that migrated as a doublet in 56 to 59 kDa range. The importance of all these studies suggests that a clear understanding is essential for trypsin treated PCPs for knowing its insecticidal activity.

As suggested by Lambert et al. (1992) it is likely that 65- kDa proteins are breakdown products of 130- kDa high molecular weight proteins. Zhang et al., (2000) found that the proteinase inhibitors had no effect on the in vivo digestion of protein, and the trypic activity has suggested that the mode of action of proteinase inhibitors is to cause hyper production of trypsin, which results in the large depletion of sulphur-containing amino acids required for the synthesis of proteolytic enzymes and causes antinutritional effects.

2.6. Larval mortality

Bioassays are routinely used to detect the potency of commercial and experimental B. thungiensis preparations. They are tested in vitro by allowing reared larvae or in vivo by exposing to field conditions (Dulmage et al., 1970).

Martin and Travers (1989) found 40.3% of their isolates to be active against lepidoptera and 22.7% against mosquitoes. Chilcott and Wigley (1993) observed that the percentage of strains obtained from the soil with toxicity against the lepidopteran larvae alone ranged from 37 to 88%, 43 to 100% against the dipteran, 0-6% against the coleopteran, 45 to 77% both the lepidopteran and dipteran and 20-60% with no activity against lepidopteran, dipteran or coleopteran. Bernhard et al. (1997) tested single-dose assays of 5303 B. thuringiensis isolates against Agrotis ipsilon, 5136 against H. virescens, 3077
against *Peris brassicae*, and 3028 against *S. littoralis*. The 2789 isolates which had been tested against all four lepidopteran species were grouped according to their spectra of insecticidal activity using the principle that strains with 25% or less corrected mortality were rated inactive whereas strains giving more than 25% mortality were rated active. The most active strain from Mexican collection active against *Trichoplusia ni* larvae was reported (Bravo et al., 1998) to harbour *cry1Aa1, cry1Ab1, cry1Ac1* and *cry1D1* genes.

The biopesticides viz., Centari, Delfin and Dipel 8C were found to cause cent percent mortality within 48h after treatment (Shenhmar and Brar, 1997). Vaidya (1997) recorded 80% larval mortality after 48h of exposure to *B. thuringiensis* formulation Centari 3G (*B. thuringiensis* var. *aizawai*) at 0.1% concentration

Strains containing *cry1C* and *cry1D* genes were found to pose highest threat to *S. frugiperda* larvae (Ceron et al., 1994). Similarly Frankenhuuyzen et al. (1993), reported the toxicity of *cry1C* against *Spodoptera* spp., *cry1D* against *S. littoralis* and *cry1E* against *S. exigua*. Bai et al., (1993) observed minimal sensitivity of *cry1Ab, cry1B, cry1D, cry1Aa* and *cry1Ac* to *S. exempta*

Frankenhuuyzen et al. (1993) also suggested that *cry1Aa, cry1Ab, cry1Ac, cry1B and cry1C* were toxic to *P. xylostella* Of the *cry1Aa, cry1Ab, cry1Ac, cry1C, cry1D* and *cry1Aa* genes present in *B. thuringiensis* active against *P. xylostella*, *cry1Ab* was significantly toxic (Monnerat et al., 1999)

The LT$_{50}$ values of *H. armigera* were less than 24h for *B. thuringiensis* at a PCP concentration of 10µg/g of synthetic diet (Ingle et al., 1997). Natarajan and
Srinivasan, (1999) recorded 100%, 85% and 20% mortality in first, second and third instar larvae of *H. armigera* respectively at a PCP concentration of 20μg/ml.

2.7. Field evaluation of Indigenous *B. thuringiensis*:

Employing Principle Component Analysis (PCA) Leong et al., (1980) reported that pathogenicity is the combined effect of multiple environmental factors like sunlight, leaf temperature and vapour pressure deficit. Vimaladevi et al. (1996) found that *B. thurinigiensis* var. *kurstaki* was as effective as Monocrotophos (0.05%), a chemical insecticide, in causing feeding cessation and mortality of *A. janata* in castor when compared to *S. litura*. Kalra et al. (1997) in their studies on field evaluation of *B. thuringiensis* for the control of *Sylepta* sp. on *Sterculia urens* suggested that up to 72 hours after application, there was a consistent decrease in larval population. Navon (2000) based on his studies has indicated that insecticidal power of *B. thuringiensis* is a) instar dependent, b) solar sensitive, c) dependent on microbial load and d) presence of allelochemicals on the phylloplane.

*S. litura* is an important polyphagous pest of cultivated crops primarily in the tropical and subtropical regions (Amonkar et al., 1985) Insecticides often are used preventively to suppress *Spodoptera* populations from reaching the economic threshold level (Zaz, 1990).

Rao et al. (1997) showed that when *B. thuringiensis* var. *kurstaki* products were combined with *B. thuringiensis* there was a significant increase in larval mortality of *A. janata*. Although *B. thuringiensis* products containing cry1C protein
have demonstrated high levels of efficacy against *Spodotera* sp., Van Rie *et al.* (2000) have shown that there is the potential for lepidopteran larvae to develop resistance to this protein.

2.8. PCR Analysis of *cry* genes

PCR was suggested as a rapid method for detection and differentiation of *B. thunngiensis* strains, which allows better follow-up in commercial formulation and in the field. Besides, Bravo *et al.* (1997) exploited PCR to predict insecticidal activities to identify *cry* type genes and determine their distribution, and to detect new genes. Further, they identified 22 *cry*-type profiles from 126 field-collected *B. thunngiensis* strains. The preliminary screening by universal primers was preferred as it saves effort by sorting the strains for the specific screening, which then produces a PCR product with a unique size for each *cry* gene. Jung *et al.* (1997) characterized *B. thunngiensis* mutant and natural isolates using PCR analysis. Hongyu *et al.* (2000) reported that the *B. thunngiensis* isolates depended on geography and type of warehouses.

Since new strains can be isolated more rapidly than they can be assayed on insects, the PCR was preferred for identification of strains with the greatest likelihood of containing novel genes by Carozzi *et al.* (1991). Kuo and Chak (1996), based on the partial nucleotide sequence of the PCR products confirmed that their predicted *cry7* type gene was novel. Similarly Bravo *et al.* (1997) sequenced PCR products of 423 bp long DNA using UN7 and UN8 primers from
**B. thuringiensis var. indiana** and **B. thuringiensis var. tochigienesis** which showed about 80 – 90% homology to all the three cry8 genes.

Besides identification of the cry gene content of a strain, altered versions of a gene of known subclass can be detected if the PCR product was either significantly smaller or significantly larger than the predicted size (Masson et al., 1998). However, to claim a gene as novel, Theunis et al., (1998) suggested that it requires isolation of full-length clones of the genes, production of purified proteins in an expression system, and insect bioassays. Park et al. (2000) have made various combinations of enhancer elements and evaluated them to determine their capacities to enhance the synthesis and crystallization of truncated cry1C (cry1C-t) molecules. They also showed that inclusions of cry1C-t can be produced by combining mRNA stabilising sequences and helper protein genes in constructs containing truncated cry1C-t.

### 2.9. Immuno-blotting of expressed PCPs

Immuno-blotting experiments by Ceron et al. (1995) revealed the detection of cry1E and cry3a toxins in natural isolates using specific monoclonal antibodies. ELISA and Western blot analysis performed with cry1A, cry2A and cry3 specific polyclonal antisera were used to investigate the immunological relatedness of **B. thuringiensis** strains to predict their insecticidal activities (Theunis et al., 1998). To directly determine which genes were expressed and crystallized in the parasporal inclusions, Masson et al. (1998) employed HPLC and dot blot analysis of total mRNA.
2.10. Mass multiplication of *B. thuringiensis*

Yang and Wang (2000) reviewed the status of current *B. thuringiensis* fermentation research and development and suggested the following three areas of future study. 1) process study and genetically engineered multiple-toxin *B. thuringiensis* producers, 2) process optimisation that specifically improves toxin protein yield (in lieu of spore count) and 3) identification of factors that specifically influence the sporulation and toxin-expression phase

Although microbial insecticides based on *B. thuringiensis/B. sphaericus* have been in use in biocontrol programmes, their high cost makes large-scale application impracticable in developing countries. Therefore, Ampofo (1995) was of the opinion that local production of biopesticides in developing countries would be successful if only cheap and readily available raw materials are used in their production. In the design of a fermentation medium, he suggested that consideration must be given to its requirements for carbon, nitrogen and minerals

Hameed *et al.* (1990) employed several media ingredients locally available in Egypt for their ability to support δ-endotoxin production by their native *B. thuringiensis* strains. Soybeans, black eyed beans, common peas and lentils supported good production of toxin whereas peanuts, fodder yeast, cheese, whey and corn steep liquor gave only low amounts of toxin

A sizable increase in biomass and spore productivities was achieved by either one fed batch or continuous culture, however, the specific insecticidal
activity obtained was lower compared to batch culture (Rossa et al., 1992).

Knowledge of growth parameters and their interrelationships should help to achieve optimum results when either laboratory or plant production of *B. thuringiensis* is intended. A short exponential phase was found where the maximum cell density was reached at 7-h followed by spore formation from 18-h after inoculation of *B. thuringiensis* under modified air lift bioreactor. Total crude endotoxins also continued to increase upon the onset of cell growth and sporulation. (Liu and Tzeng, 2000)

Invariably, for all raw materials employed for scale-up of *B. thuringiensis / B. sphaericus* basal salts were employed by Faloci et al., (1990) and Rossa et al. (1992). Differences in toxicity was observed based on levels of trace elements in various raw materials used in the preparation of the media (Ampofo, 1995)

Ejiofor and Okafor (1988) recorded growth of between $5.5 \times 10^7$ and $7.0 \times 10^{10}$ cfu/ml after 40 h of growth. However, for maximum sporulation, 72 h fermentation period was required (Ampofo, 1995).

Formulations of *B. thuringiensis* using dried bovine blood and extracts of leguminous seeds have been reported in Nigena (Ejiofor and Okafor, 1986, Ejiofor and Okafor, 1988).

Statistical procedures provide an alternative methodology to optimise a particular process by considering mutual interactions among the variables and gives an estimate of the combined effect of these variables on final result. In this
context, Murthy et al. (2000) reported that response surface methodology (RSM) is one such statistical technique, based on the fundamental principles of statistics, randomisation, replication and duplication, which simplifies the optimisation by studying the mutual interactions among the variables over a range of values in a statistically valid manner.