Chapter 5

General Discussion

An unidentified entomopathogenic bacterium was first isolated by the Japanese scientist Shigetane Ishiwata, in 1901, from silkworm larvae which exhibits the sotto disease. Ten years later, this bacterium was named as *Bacillus thuringiensis* by E. Berliner in 1911. More than Hundred years later in United States, *B. thuringiensis* genes were incorporated in 69% of cotton and 26% of corn plants as a sprayable biopesticides. *B. thuringiensis* has dominated the market. Later, with development of transgenic technology, its toxins genes were used for transformation of crops to protect against the damage of insect pests. In 1989, the first transformation of cry gene was carried out (Perlak et al., 1990). The insect protecting transgenic cotton was developed and since then *B. thuringiensis* corn and *B. thuringiensis* cotton are planted over about 29 million hectare in 2013. Presently, various cry/vip genes isolated from diverse culture collection of *B. thuringiensis* have been used to develop transgenic plants resistant to insects. In so many previous studies, more than 50,000 *B. thuringiensis* strains have been isolated from different environments (Sadder et al., 2006). The numbers of *B. thuringiensis* bacteria that have been isolated from different types of habitats have steadily risen all over the

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world. The insecticidal activity of *B. thuringiensis* is mainly due to its ability to produce a large amount of proteinaceous δ-endotoxins during the sporulation phase (Burge, 1982).

Use of biological agents against pests have more advantages over chemical insecticides in being highly selective with no side effects self propagating and harmless to human beings, his livestock and other non-target organisms (Dhaliwal and Arora, 1998). The biocontrol potential of certain Cry toxin of *B. thuringiensis* is reported to be 300 times higher than that of synthetic pyrethroids (Feitelson et al., 1992). Thus, there is an increased emphasis on isolation and characterization of novel *B. thuringiensis* strains.

India is considered as one of the centers of biodiversity (Khoshoo, 1994). During the present investigations, an effort was made to identify the native *B. thuringiensis* isolates effective against lepidopteran insects. *B. thuringiensis* were isolated from soil samples predominantly collected from North East regions and some other states of India. This study is a part of the larger effort to sample biodiversity of *B. thuringiensis* from different parts of India and characterize their insecticidal properties and cry gene profiles.

### 5.1 Isolation of *B. thuringiensis* isolates

*B. thuringiensis* was observed in 48.61% of all the soil samples collected from predominantly North East regions and some other states of India. Out of 105 *B. thuringiensis* isolates, 93 *B. thuringiensis* isolates belonged to North East region of India. Four locations (W. Bengal, Mehan in Haryana and Imphal, Churachandpur in Manipur) of sample collection were showed absence of *B. thuringiensis* isolates. The overall recovery of *B. thuringiensis* isolates in this study showed that soils collected from North East regions of India were extraordinarily rich in *B. thuringiensis*. Whereas, Martin and Travers (1989) found that 70% of the soil samples collected from Asia contained *B. thuringiensis*. Bravo et al. (1998) also reported that 81% of the soil samples collected from different habitat of Mexico was contained *B. thuringiensis*. Whereas, some reports showed the lowest percentage of occurrence of *B. thuringiensis* in their sample collections; DeLucca et al. (1981) 0.5%, Meadows et al. (1992) 2.1%, Donovan et al. (1988) 1% and Ejiofor and Johnson (2002) 10%. Arrieta et al. (2004) reported 51.9% of the samples yielded *B. thuringiensis* isolates and our results are also quite similar to their
results. This is probably due to rich microbial load in North East region of India like (Nagaland, Meghalaya, Manipur, Arunachal Pradesh, Mizoram, Sikkim, Assam and Tripura) soils and the method of isolation itself. Kaur and Singh (2000a) also found that soils from different horticultural plantations and forest soils were rich in *B. thuringiensis*.

The isolates showed that colony morphology of *B. thuringiensis* isolates varied and were classified into 4 groups. Colonies of all 105 *B. thuringiensis* isolates were found rough in nature with wavy margin. Out of 216 *B. thuringiensis* isolates, 105 isolates were gram positive. The presence of ‘crystal’ protein is the key factor to differentiate *B. thuringiensis* from other *Bacillus* species (Henderson et al., 1995, Bobrowski et al., 2001). In this study we found that Lepidoptera specific strain AUG-5 produced two types of crystals; bipyramidal as well as spherical. Microscopic observations of the crystals presented diversity of shapes of parasporal crystals. Differences in crystal morphology in *B. thuringiensis* are very common (Wangondu et al., 2003; Arrieta et al., 2004; Armengol et al., 2007). Crystal morphology is not known to be associated with the cry gene profile or insecticidal properties of the isolates. However, the presence of crystals is a distinctive property of *B. thuringiensis*, which distinguishes itself from other bacilli.

### 5.2 Insecticidal toxicity of *B. thuringiensis* isolates

The bioassays showed a wide variation in the toxicity of *B. thuringiensis* against both insects tested. Jarrett and Burges (1982) reported that different *B. thuringiensis* isolates may vary in their insecticidal activity against same species and a given isolate may be very active on one species and inactive against other species. Karim et al. (1999) also reported that native *B. thuringiensis* isolates with same or similar gene content did not showed the same level of insecticidal toxicity. Bioassay conducted against neonates of *H. armigera* showed that most of the *B. thuringiensis* isolates caused 10-54% mortality, whereas AUG-5 caused 95.93% as well as GTG-7 caused 83.33% mortality on the 7th day of treatment. Martin and Travers (1989) evaluated 40.3% of soil isolates active against Lepidoptera. Similar conclusion was reported by Vidyarthi et al. (2002) and Martin et al. (2010) who mentioned that *B. thuringiensis* is mainly active against lepidopterans species. Bioassays conducted against *S. litura* revealed that none of the
isolate highly toxic except AUG-5. AUG-5 caused 73.33% mortality on the 7th day of treatment which was higher than the standard Cry2Ab2 toxin (60%). In case of S. litura most of isolates caused mortality from 10-46%.

5.3 cry genes of B. thuringiensis isolates

This study showed the presence of cry1 type genes in the selected lepidopteran specific strains namely, AUG-5, GTG-7, GTG-3S, GTG-4S, GTG-6S, GTG-9 and GTG-70. These B. thuringiensis isolates produced the expected product for cry1 gene, whereas GTG-3S produced an unexpected amplified fragment of 460 bp instead of expected band of 276 bp. This unexpected band of 460 bp could be a new cry gene, as Ceron et al. (1995) and Porcar and Juárez-Pérez (2003) reported that an unexpected amplified fragment might correspond to a new cry gene, using a multiplex PCR with specific primers. Our studies showed the abundance of cry1 type gene in the soil sample. Some similar studies were also reported by some researchers in Mexico (Bravo et al., 1998), Isreal, Kazakhistan and Uzbekistan (Ben-Dov et al., 1996), Tunisia (Jaoua et al., 1996) and some samples of terrestrial and aquatic habitats of Spain (Martinez and Caballero, 2002). Isolates GTG-4, GTG-42 and GTG64 did not produce any product for cry1, cry1A and cry2 genes. The cry1A gene was also detected in B. thuringiensis isolates namely, AUG-5, GTG-7, GTG-3S, GTG-4S, GTG-6S, GTG-9 and GTG-70. However, cry2 gene was found in AUG-5, GTG-7, GTG-4S and GTG-6S with the amplicon of 713, 713, 733 and 733 bp, respectively, as expected which was also seen in standard strain E. coli ECE-126 with PCR product of 713 bp. Only B. thuringiensis isolates GTG-9 and GTG-70 produced 378 bp expected product for cry9 gene along with standard strain E. coli ECE-130.

5.4 Cry protein analysis

The distribution of the cry genes of local B. thuringiensis isolates depended on the geography, type of sample collection site and distribution of insects in that location (Bravo et al., 1998). Chaufaux et al. (1997) reported that the most ubiquitous B. thuringiensis isolates (54%) contained proteins with molecular mass of 130-140 kDa.
Similarly, we found same results in the SDS-PAGE analysis while our research work. *B. thuringiensis* isolates AUG-5, GTG-7, GTG-4S and GTG-6S produced a protein profile similar to that of lepidopteron specific *B. thuringiensis* subsp. *kurstaki* HD-1 with molecular weights of 130, 63-75, 48 and 35 kDa all found highly toxic to Lepidoptera. Isolates GTG-9 and GTG-3S only produced the bands with molecular weights of 63 and 20-25 kDa. HD-73 produced the band of 130 and 63-75 kDa only. In contrast the protein profile of all the *B. thuringiensis* isolates from the different region of India, showed typical Cry1 and Cry2 protein profile having approximately 130kDa, 70-71kDa and 68kDa proteins as described by Höfte and Whiteley (1989).

**5.5 Evaluation of different culture media for the growth of *B. thuringiensis* strain AUG-5**

Eleven different types of media based on agro byproducts were used for the evaluation of *B. thuringiensis* strain AUG-5 in respect of CFU count, spore count and Cry toxin content by using Fermenter. The dry cell mass of AUG-5 obtained by different fermentation media varied. Medium VI produced the highest amount of biomass (5.44 g/L), compared to other media including reference LB medium, whereas the lowest biomass of 1.58±0.17 g/L was produced by LB-1X(BOD). The estimation of Cry toxin (Cry1Ac and Cry2ab) by ELISA produced varied content in all used eleven fermentation media. The Cry1Ac content of spore-crystal complex ranged from 5.6 to 139.3 ng/mg, whereas, Cry2ab content ranged from 0 to 4235 ng/mg of spore-crystal complex. The highest CFU count and spore count were observed in Medium III (84.67±0.88 × 10^8 CFU/mL and 87.33±0.17 × 10^8 spore/mL) and the lowest CFU count and spore count were produced by medium VI (2.77±0.12 × 10^8 CFU/mL and 3.20±0.03 × 10^8 spore/mL). Alves et al. (2010) recovered the highest spore yield of 21.6 × 10^8 spore/mL in the WSM medium which consists of 50% cheese whey and 10% soya bean milk. Efficacy of *B. thuringiensis* spore-crystal complex produced by selected media was evaluated against *H. armigera* and *S. litura*. In case of *H. armigera* Medium II caused the highest mortality (63.3%) after one day of the treatment as compared to other treatments of other media. In case of *S. litura*, reference toxin Cry2Ab2 caused maximal mortality of 60%, while the
spore-crystal complex of Media LB-2X and LB-3X caused about 80% at par, and Media II and III 100% mortality each at 10 µg/g concentration, 7 days after treatment. Further, *S. litura* was less susceptible to the spore-crystal complex than *H. armigera*.

The observation of *cry1*, *cry1A* and *cry2* genes suggests that two isolates AUG-5 and GTG-7 may have a broad spectrum of activity against lepidopteran species. The results of this study are interesting in the sense that they may help developing new strategies for controlling insects of economic importance in India, using very potent *B. thuringiensis* strains that naturally exist in the local environment instead of the current control strategies that are based solely on chemical insecticides.