CHAPTER - 6

CONCLUSIONS
Since chemical insecticides are non-specific, non-biodegradable, polluting the environment and also causing emergence of the resistant pests, biological control agents having specificity, biodegradability and non-toxicity to non-target organisms have been sought. *Bacillus thuringiensis* (*Bt*) have been in use as a biopesticide worldwide. *Bt* produces insecticidal crystal proteins (ICPs) during sporulation. Upon ingestion of the ICPs by the susceptible insect larvae, paralysis and death occurs. The gene encoding the ICPs have been successfully transformed into variety of agricultural crops and other microorganisms, for the efficient control of insect pests. But the conventional and safer application of *Bt* lies in its production by fermentation and strain improvement, due to the danger and fear of transgenic crops.

The present study investigates the production of *Bt* in shake flask and bioreactor. Since production of bioinsecticide is basically a biomass production process. By modifying the glucose yeast extract salt (GYS) medium increased biomass and ICPs production was achieved. Uniform growth and short lag phase was obtained at 1% (v/v) inoculum having $10^8$ spores/ml. During the vegetative phase pH falls to acidic side when the glucose is metabolised and raised when starts utilising nitrogen source. pH maintenance (pH 7.0-7.4) is required to avoid the formation of amorphous crystal under acidic condition.

During the present investigation, effect of various carbon and nitrogen sources on the production of *Bt* was studied. In the modified medium higher biomass (4.5 g/l) and crystal protein (0.72 mg/ml) were obtained as compared to the basal medium where biomass (2.50 g/l) and crystal protein (0.25 mg/ml) were obtained.

Since production of *Bt* is a highly aerobic process, the effect of aeration on the production of *Bt* was also investigated. Production of *Bt* was found to be affected by decrease in the $k_{La}$. Higher oxygen transfer rate as well as productivity (0.0135 g/l/h) and yield (168 mg crystal protein / g biomass) were obtained at lower working volume (10% wv) in shake flask. At 40% working volume, productivity (0.0086 g/l/h)
and yield (148 mg crystal protein / g biomass) were obtained. Higher oxygen transfer rate was obtained (k_La 63 / h) during log phase. Increase productivity (0.0153 g/l/h) and yield (167mg crystal protein / g biomass) were obtained when Bt was produced in bioreactor. Higher oxygen transfer rate found at log phase was decreased exponentially during stationary phase, indicated less requirement of oxygen and increased viscosity due to accumulation of biomass. Production of ICPs during fermentation was monitored by immunoassay. The potency of the fermented broth was determined by bioassay. Lower LC_{50} value (38 ± 1.3ng/well) was obtained in case of Helicoverpa armigera indicated susceptibility to the Bt, whereas the less susceptible Spodoptera sp. Showed higher LC_{50} (285 ± 2.6 ng/well).

Effective biopesticide having broader host range and increased larvicidal activity can be develop by using hybrid strains of Bt. The present investigation also dealt with the strain improvement to enhance the larvicidal activity of Bt. Transcipients of Bt var. israelensis and B. sphaericus were obtained by simple conjugation method, in order to exploit this approach with other Bt strain having different host range or specificity. It was found that the transcipients produced the ICPs of both Bt var. israelensis and B. sphaericus. Increased larvicidal activity was seen in case of transcipients when bioassayed against Anopheles mosquito larvae, such transcipients or hybrid strains may be used as a potent mosquito larvicidal agent.

Similarly, an approach of protoplast fusion was attempted, in order to obtained a standardised method for protoplast fusion among Bt strains having different host range. Such methodology can be used as a simple tool for genetic manipulation in these strains, since protoplast fusion may help in transfer of ICPs gene.

Strains of Bacillus thuringiensis produces toxin protein crystals known as insecticidal crystal proteins (ICPs) during sporulation which are deposited outside the exosporium. Two types of ICPs were found in Bt var. kurstaki i.e. bipyramidal and rhomboidal, while amorphous inclusion bodies were seen in Bt var. israelensis. The
ICPs produced by *Bt* are glycoprotein in nature. The carbohydrate present in *Bt* var. *kurstaki* crystals were glucose (4%) and mannose (1%).

Large number of polypeptides with widely ranging molecular weight values obtained from the solubilized crystals. Of the various reagents and condition employed, 100 mM Na$_2$CO$_3$, 10 mM EDTA and 1% β-mercaptoethanol was found better solubilizing reagent. The protease enzyme detected in the crude spore crystal complex brought about autolysis of the ICPs during solubilization and under storage condition. The protease occurring in *Bt* are activated under alkaline condition used to solubilize the crystals.

For the characterization of the ICPs such as its nature, amino acid sequence, etc. purification of intact and solubilized ICPs in an essential step. Present study investigated the purification of intact crystal by sucrose density step gradient and the solubilized ICPs by gel filtration chromatography. Two polypeptide bands were obtained (130 and 67 kDa) when the purified protein were resolved on 10% SDS-PAGE.

SDS-PAGE is one of the routine analytical technique used to analyse the protein profile of ICPs of *Bt*. Since there is variation in the SDS-PAGE profile obtained from lab to lab and also there is no uniform method for the analysis of the ICPs, present study attempted to resolve this. Multiple bands were obtained on SDS-PAGE when the crystals were incubated at higher pH (pH > 11) for more than 2 h. A broad smear at the bottom of the gel indicated the extensive degradation of the ICPs.

Since polyclonal anti-*Bt* antibodies reacted with ICPs of other *Bt* having homology, such antibodies can be used to screen new isolates of *Bt* and as an alternative to bioassay. Immunoassay can be used to quantify the active ingredient of *Bt* preparation. The lowest concentration of ICPs detected by dot blot was 78 ng for a *Bt* var. *kurstaki* preparation (LC$_{50}$ value 38 ± 1.3 ng/well). It concludes that biologically
active toxin can be detected by immunological methods such as dot blot as an alternative to bioassays.

By treatment with trypsin and midgut juice of *H. armigera*, an activated toxin was formed having molecular weight ~ 55 kDa. It was also found that the solubilized crystals are toxic to the insect larvae than intact crystals. This might be due to direct interaction of the protoxin on the midgut cells. Since ICPs of *Bt* var. *israelensis* exerts haemolytic / cytolytic activity on *in vitro* method was developed for the assessment of Bti toxin.

*Helicoverpa armigera* is one of the most devasting insect pest causing 40-80% agricultural damage in India. This pest was considered for biological control by *Bacillus thuringiensis* to which it is susceptible. The present study investigated the mode of action and identification of receptor protein from *H. armigera* midgut for the first time.

It seems that first the *Bt* toxin binds to the brush border membrane vesicles followed by disruption of epithelial cells leading to pore formation and leakage of the gut contents. Similar histopathological results were obtained with *A. aegypti* larvae treated with *Bt* var. *israelensis* toxin. *In vitro* binding of *Bt* var *israelensis* toxin to the brush border of *A. aegypti* larval midgut tissue section demonstrated the toxin binding and the presence of receptor in the midgut.

Binding of activated toxin of *Bt* var *kurstaki* to the isolated brush border membrane vesicles of *H. armigera* was demonstrated by dot blot and protein blot. From protein blot analysis, 120 kDa protein was identified as the receptor for cry1A type ICPs of *Bt*.

Aminopeptidase-N was reported as a receptor for *Bt* toxin from various insect species. The presence of aminopeptidase-N activity in the brush border membrane of
*H. armigera* also confirmed the presence of the receptor. Further, the 120 kDa protein was N-terminally sequenced.

During this investigation three aspects were covered (a) production and strain improvement of *Bt*, (b) characterization of insecticidal crystal protein and (c) histopathology and receptor binding studies.

To conclude *Bt* is a potential biological control agent for *H. armigera* and other pests in India and can be exploited to develop a product which is the need of the day.