Review of Literature

*Photorhabdus luminescens* is a gram-negative entomopathogenic enterobacterium that exists in a state of mutualistic symbiosis with nematodes of the family *Heterorhabditidae* which is found in the gut of free-living, infective *Heterorhabditis bacteriophora* juveniles (Blackburn et al., 1998: 3036-3041). Maximum growth of these bacteria in nutrient broth occurs at 35-39°C. The natural habitat of *Photorhabdus luminescens* is in the intestinal lumen of the entomo-pathogenic nematodes of *Heterorhabditis bacteriophora* and *Heterorhabditis indica* (Dworkin et al., 2006).

*Photorhabdus* species exist in two forms, primary and secondary variants, which differ in morphological and physiological traits. While the primary variants occur in infective-stage nematodes, secondary variants occur after sustained growth. Primary variants produce extracellular protease, extracellular lipase, intracellular protein crystals CipA and CipB, antibiotics, and exhibit bioluminescence. Secondary variants lack protease, lipase, antibiotic activity, and added to this they have lesser bioluminescence; they further differ in colony morphology, pigmentation, dye adsorption, metabolism, and the ability to support nematode growth and reproduction. It is hypothesized that primary variants correspond to the nematode-associated and insect-infective form, whereas secondary variants are related to late stationary phase cells in infected insects and the re-associative form of the bacteria (Turlin et al., 2006: 2705–2725).

*Photorhabdus* exhibits a complex life cycle, which includes symbiotic stage characterized by colonization of the upper nematode gut, and a pathogenic stage, characterized by release from the nematode into the hemocoel of insect larvae, resulting in rapid insect death caused by bacterial toxins (Joyce et al., 2006: 127–132). This bacterium appears to sense as well as adapt to fresh environment depending on the new hosts, thereby facilitating the production of factors necessary for survival within the host, host-killing and exploitation (Munch et al., 2008: 229-245).

Upon entering an insect host, the nematodes release the bacteria by regurgitation directly into the insect's hemocoel. Once inside the hemocoel, the bacteria replicate rapidly causing lethal sepsis in the host by producing different high molecular weight toxins that kill the insect within 48–72 hours (Han et al., 2001: 239-247). Bioconversion of the
insect cadaver by exoenzymes produced by the bacteria allows the bacteria to multiply and the nematode to reproduce. During this process *P. luminescens* produces antibiotics to prevent invasion of the insect cadaver by other bacterial or fungal competitors. Finally, elimination of competitors allows *P. luminescens* and the nematode to re-associate specifically before leaving the insect cadaver (Forst et al., 1997: 47–72; ffrench-Constant et al., 2003: 433-456).

**Biological insecticidal control**

During the last few decades, erratic application of synthetic insecticides has resulted in problems related to insect resistance and environmental pollution (Roush 1996). To overcome this issue, usage of bio-insecticides as an alternative is being advocated for synthetic insecticides. The important bio-insecticides available are *Bacillus thuringiensis*, insect parasitic nematodes and entomopathogenic bacteria (Hofte&Whiteley 1989: 242-255). Another class of bio-insecticide is naturally occurring soil based entomopathogenic nematodes (EPNs), a symbiotic association between bacteria and nematode, widely used in the horticulture industry of United Kingdom and continental Europe since 1993. Active ingredient of the EPN is the infective juvenile, a developmentally arrested free-living non-feeding form measuring about 500 µ in length and capable of infecting an insect host. The infective juvenile stage of EPN can move through the water films surrounding soil particles and actively seek, locate, and penetrate the insect host. Later on, both the bacteria and the nematodes release toxins and enzymes and cause insect death by inducing acute septicemia. Another important target for EPN is the insect larvae which feed on plant roots. It has been proved that one time application of EPN bio-pesticide provides protection against crop pests for approximately four weeks. Compared with BT toxin, EPNs have several advantages, including the ability to kill a muchbroader range of insects than *Bacillus thuringiensis*. Further, development of EPNs does not depend on specific host nutrients and therefore can be used successfully on various insect species. EPNs are easy to use and apply, either through conventional sprayers or irrigation systems. They actively seek out the insect host, which increases their efficacy. Moreover the infective juveniles can survive for some time without nutrients while they actively search for their host. Their speed in killing insects is comparable to that of
other insecticides; they kill insect hosts within 48 hours (Bedding & Miller, 1981: 211-216; Keith et al., 1989: 554-556).

Entomo-pathogenic nematodes are less likely to kill beneficial insects because these are more active and escape nematode penetration by quickly moving away. EPNs can be grown on artificial media, a characteristic that facilitates their commercial production. When stored at a proper temperature they can stay viable for months (usually three months at a temperature of 60° to 80°F and six months when refrigerated at 37° to 50°F). In insecticidal applications EPNs can be mixed with various fertilizers, insecticides, and herbicides although some compatibility factors must be addressed. The US EPA has pronounced that EPNs are exempt from registration because they occur naturally and require no genetic modification by man. Consequently, EPNs are frequently used in cases where other insecticides are either not effective or are not registered (Chattopadhyay et al., 2004: 33-54).

**Importance of Biopesticides**

Numerous problems are encountered with the applications of synthetic insecticides. Majority of the synthetic insecticides are non-biodegradable, others degrade very slowly and persist in the environment which leads to bio-accumulation as well as biomagnification. For instance, DDT persists in the environment for 15–20 years and accumulates in the tissues, making their concentration increase as they move along the food chain. Insecticides also cause pollution of soil and groundwater and have harmful effects on a wide range of non-target organisms which includes beneficial insects, birds, etc. Over a period of time there is a possibility for new resistant strains of insects to emerge which may require increased dosage of insecticidal application (Chattopadhyay et al., 2004: 33-54). Further, it is noticed that boring, sucking, and root-eating insects have developed resistance towards synthetic insecticides. Because of the harmful effects caused by the application of wide variety of synthetic insecticides, its usage on large scale basis has been banned by the US Environmental Protection Agency (EPA) (Bedding & Miller, 1981: 211–216).

Due to the serious health hazards linked with the synthetic insecticides, biopesticide product such as BT product offers a good alternative for controlling the pest population
affecting agricultural crops. Further, as a result of their selectivity for a specific group of insects, BT-based insecticides are safe to handle, as non-target and non-susceptible organisms are not affected. Unlike many other insecticides, BT-based insecticides do not affect the natural enemies of insects, such as parasitic wasps and lady beetles. All commercial products of BT contain enterotoxins, however these products are harmless if prescribed precautions are taken (Chattopadhayay et al., 2004: 33-54; Poinar, 1986: 95-121).

Bacillusthuringiensis (BT)

*Bacillus thuringiensis* accounts for 90% of the bio insecticide market; it produces insecticidal toxins called delta endotoxins which are proteinaceous and readily biodegradable, and thus have a short half-life inside the insect midgut (Poinar, 1986: 95-121). Bacillus thuringiensis is a common gram-positive aerobic entomopathogenic endospore forming soil bacterium, which produces unique crystalline cytoplasmic inclusion bodies during the process of sporulation (Martin & Travers, 1989: 2437-2442).

*Bacillus thuringiensis* strains contain an array of plasmids with at least one large plasmid on which the toxin genes are present (Reyes-Ramirez and Ibarra, 2008: 125-129; González Jr & Carlton, 1984: 28-38 ; González Jr & Carlton, 1985: 246–252). The insecticidal proteins that are produced by BT are categorized into two families based on amino acid sequence similarity. Namely Cry toxins and (crystal delta endotoxins) and Cyt toxins (cytolysins). Cry toxins have been grouped into four classes: Cry I, CryII, CryIII, and CryIV; and Cyt toxins have been grouped into two classes (Hofte& Whitely, 1989: 242-255).

**Resistance of crop pests to the BT toxins**

There are several studies conducted in the recent past to demonstrate the development of resistance of crop pests towards BT toxin. According to the studies of Hoy (1998: 1787-1795) and Michaud (1997: 4-6), resistance to BT toxin occurs when there is secondary outbreak of genetically variant pests. This is because they possess specialized forms of receptor molecules or are equipped with a mechanism to break down these toxins.
Resistance to BT toxin was first discovered in *Plodia interpunctella* in 1985 and this was followed by other group of insects which include *Ostrinia nubilalis* (European corn borer), *Heliothis virescens* (Tobacco bud worm), *Pectinophora gossypiella* (Pink bollworm moth), *Culex quinquefasciatus* (Mosquito), *Aedes aegypti* (Yellow fever mosquito), *Trichloro plusiani* (Tiger moth), *Leptinotarsa decemlineata* (Colorado potato beetle), *Spodoptera exigua* (Beet armyworm), *Spodoptera littoralis* (Egyptian cotton leaf worm), and *Chryosomela scripta* (Cottonwood leaf beetle) (Wirth et al. 1997: ; Gould et al. 1997: 3519-3523; Liu et al. 1999: 519-522; Frutos et al. 1999: 227-276; Tabashnik, 1994: 7-12).

In order to decrease insecticidal resistance, various methods of resistance management or resistance mitigation are employed (mixtures, mosaics, rotations, refuges and tissue-specific expression of BT toxins). These methods are aimed mainly to delay the resistance of the insects against the toxins and also reverting resistant insects to susceptibility (Tabashnik, 1997: 3488-3490; Caprio, 1998: 1021-1031). Since most of the insects showing resistance to BT toxins even after adapting the resistance management, toxins of *Photorhabdus luminescens* can be used as an alternative to BT toxins as biopesticide.

*Photorhabdus luminescens* genome

The complete genome sequence of *Photorhabdus luminescens* strain TT01 is 5,688,987 base pairs (bp) long and contains 4,839 predicted protein-coding genes, which encodes a large number of adhesins, toxins, hemolysins, proteases and lipases, and contains a wide array of antibiotic synthesizing genes. These proteins are likely to play a role in the elimination of competitors, host colonization, invasion and bioconversion of the insect cadaver, making *P. luminescens* a promising model for the study of symbiosis and host-pathogen interactions (Duchaud et al., 2003: 1307-1313).

Waterfield et al., 2001(185-191) identified four toxin-complex loci (*tca*, *tcb*, *tcc* and *tcd*) from *P. luminescens* strain W14. Among these complexes, *tca* locus encodes for a protein complex which has high oral toxicity against the target insect *Manduca sexta*(Bowen et al., 1998; Marokhazzi et al., 2003). Strain TT01 contains the *tcc* and *tcd* loci, an incomplete *tca* locus and five newly identified *tc* loci (Duchaud et al., 2003: 1307-1313).
*Photorhabdus luminescens* secretes many enzymes that contribute to insect death and result in bioconversion of the insect cadaver (Bowen et al., 2000: 69-74). *Photorhabdus luminescens* strain TT01 encodes ten triacylglycerol lipase, phospholipase A and D like proteins. Both contain the lipase-specific consensus sequence in their amino-terminal moiety. *Yersinia enterocolitica* phospholipase A contributes to pathogenesis in a mouse model (Schmiel et al., 1998: 3941-3951), suggesting a role in virulence for the TT01 homolog (Duchaud et al., 2003: 1307-1313).

The gene *plu2096* encodes a protein similar to the *Pseudomonas aeruginosa* lectin PA-1, which recognizes specific carbohydrates exposed on the host cells and functions as an adhesin and a cytotoxin that plays a role in the initiation of infection (Avichezer et al., 1992: 23023-23027). The putative *plu1561* gene product is similar to a Ca2+-dependent adhesion molecule of the cadherin family in *Dictyostelium discoideum* (Wong et al., 1996: 16399-16408). Another *Photorhabdus luminescens* gene, *plu2433*, may encode an adhesion required for insect colonization, because *Plu2433* is similar to the recently discovered *Erwinia carotovora* virulence factor (Evf) allowing this bacterium to colonize the gut epithelium of *Drosophila melanogaster* (Basset et al., 2003: 205-209).

**Toxicity of insecticidal toxin from *Photorhabdus luminescens***

*Photorhabdus* is a highly virulent pathogen of a wide range of insect larvae (Clarke et al., 2008: 2159-2167). Even though toxins of *Photorhabdus luminescens* have both injectable and oral activities against a wide range of insects, the normal mode of delivery of this toxin is directly into the insect hemocoel (Bowen & Ensign, 1998: 3029-3035). Purified Tca was shown to disrupt the insect midgut epithelium in a manner similar to the δ-endotoxins from ‘Bt’. Effect of Tca on the midgut are similar to other gut-active toxins such as the δ-endotoxins (Blackburn et al., 1998: 3036-3041).

Toxin complex a (Tca), a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*, has been found to be orally toxic to both the, *Leptinotarsa decemlineata* (Colorado potato beetle) and *Bemisia tabaci* (the sweet potato whitefly) . The 48 hour LC50 for Tca against neonate *L. decemlineata* was found to be 2.7 ppm, and the growth of 2nd instar *L. decemlineata* exposed to Tca for 72 hours was almost entirely inhibited at concentrations above 0.5
ppm indicating that Tca is lethal at much lower concentrations and is capable of rapid killing of insects. Tca would ultimately prove fatal to young *L. decemlineata* larvae at concentrations below 1 part per million and there was a very little growth occurred at concentrations above 0.4 to 0.5 ppm (Blackburn et al., 2005: 1-11). The effect of Tca on the midgut epithelium of *L. decemlineata* larvae was similar to that described for larvae of *Manduca sexta* (Blackburn et al., 1998: 3036-3041).

Entry of Tca into insect midgut may be either oral or ingestion, but the symptoms are very similar to the symptoms caused by *Bacillus thuringiensis* (Endo & Nishiitsutsuji-Uwo, 1980: 14-21). Ingestion of Tca leads to apical swelling and blebbing of large cytoplasmic vesicles by the columnar cells, leading to the eventual extrusion of cell nuclei in vesicles into the gut lumen. Globlet cells are apparently affected in the same fashion but the proximal cause is not determined (Blackburn et al., 1998: 3036-3041). Waterfield et al., (2001: 185-191) proved that the combination of three genes, tcdA, tcdB, and tccC, is essential for oral toxicity to *M. sexta* when expression in *E. coli* is used.

The toxicity of the proteins encoded by plu4093 and plu4437 loci was verified experimentally by Duchaud et al., (2003). Two Escherichia coli clones, containing the recombinant BAC1A02 (plu4093–plu4092) and BAC8C11 (plu4437–plu4436), were shown to be toxic toward insects. BAC1A02 exhibited oral toxicity toward three mosquito species, *Aedes aegypti*, *Culex pipiens* and *Anopheles gambiae*, as well as toward the lepidopteran *Plutella xylostella*. BAC8C11 showed insecticidal activity toward *Plutella xylostella* (Duchaud et al., 2003: 1307-1313).

Waterfield et al., (2005: 47-52) confirmed that, PirA and PirB from two different *Photorhabdus* strains have insecticidal activity against caterpillars of the moth *Galleriamellonella* but show no oral activity against a second moth species *Manducasexta* when injected together (Waterfield et al. 2005: 47-52). PirAB shows larvicidal activity against both *Aedes aegypti* and *Aedes albopictus* larvae but did not affect the *Mesocyclops thermo cyclopoides* predator. PirAB expressed the strongest toxicity compared to PirA, PirB, or the mixture of PirA plus PirB (Ahantarig et al., 2009: 4627-4629).
Daborn et al., (2002: 10742-10747) showed that a single large Photorhabdus gene, makes caterpillars floppy (mcf), is sufficient to allow *Escherichia coli* to persist within and also kill an insect. The predicted high molecular weight Mcf toxin has little similarity to other known protein sequences but carries a BH3 domain and triggers apoptosis in both insect hemocytes and the midgut epithelium.

Yang et al., (2006: 2254-2261) reported that that recombinant *Escherichia coli* expressing PVC-containing cosmids from *Photorhabdus* has injectable insecticidal activity against larvae of the wax moth. Injection of *Photorhabdus* PVC products destroys insect hemocytes, which undergo dramatic actin cytoskeleton condensation (Yang et al., 2006: 2254-2261).

**Insecticidal Toxins of *Photorhabdus luminescens***

More toxin genes were predicted in the *Photorhabdus luminescens* genome than in any other bacterial genome sequenced yet (Duchaud et al., 2003: 1307-1313). A large number of these toxins may be involved in the killing of a wide variety of insects. Some may act synergistically and result in the quick death of the host. In addition, some may kill insects by interfering with their development (Daborn et al., 2002: 10742-10747).

Toxins produced by *Photorhabdus luminescens* are classified into four major groups and these are: (i) Toxin complexes (Tcs); (ii) *Photorhabdus* insect related (Pir) proteins; (iii) Makes caterpillars floppy (Mcf) toxins and (iv) *Photorhabdus* Virulence Cassettes (PVC) (ffrench-Constant et al., 2007: 436-451; Yang et al., 2006: 2254-2261).

**Toxin complexes (Tc’s)**

Toxin complexes (Tc) are high molecular weight, multi-subunit, insecticidal toxins produced both by Gram-negative and Gram-positive bacteria (waterfield et al., 2001a: 185-191). Using several chromatographic techniques, Bowen et al., (1998: 3029-3035) were successful in separating four different toxin complexes which were coined as Tca, Tcb, Tcc and Tcd. Although native gel electrophoresis showed that each of these toxin complexes can migrate as a single band on a non-denaturing gel, when run on a denaturing SDS-PAGE gel, each complex gets fragmented into relatively small sized polypeptides. The tc genes are encoded alongside other genes with putative virulence
functions (Yang et al., 2006; Waterfield et al., 2001a: 185-191). Tc-like genes have been identified in both other insect-associated bacteria such as *Serratia entomophila* and non-insect-associated bacteria like some *Pseudomonas* species (Waterfield et al., 2001b: 5017-5024).

The three complexes show significant similarity to one another; therefore three basic types of genetic elements have been identified. They are

1. *tcdA*-like element, equivalent to the combination of *tcaA* and *tcaB*,
2. *tcdB*-like element, equivalent to the *tcaC*
3. *the* *tccC*-like element.

*tcdA*-like elements are responsible for establishing primary toxicity, while the *tcdB/tccC*-like elements are potentially toxic to insects (Pinheiro & Ellar, 2007: 2372–2380).

According to the classification of insecticidal toxin complexes proposed by Ffrench Constant et al., (2007: 436-451), each mature complex is made up of three components namely: A, B and C. And also each gene name is associated with its anticipated complex component, A, B or C. For example, in case of Tcd toxin present in *Photorhabdus luminescens*, subunit A is TcdA, subunit B is TcdB and subunit C is TccC (Fig 1).
Fig 1: Schematic diagram of ABC classification of tc genes of *Photorhabdus luminescens* W14 (Ffrench-Constant et al., 2007)

“Makes Caterpillars Floppy” Toxins

Another toxin referred to as “makes caterpillars floppy” toxins 1 (Mcf1) and 2 (Mcf2) acts upon injection (Ffrench-Constant et al., 2007: 436-451; Yang et al., 2006: 2254-2261; Daborn et al., 2002: 10742-10747) to insect gut and they are encoded by PAI II (Waterfield et al., 2002: 541-545; Waterfield et al., 2004: 240-250), along with other
hemagglutinin-like proteins. Mcf1 has been shown to promote apoptosis in the midgut, producing a characteristic “floppy” phenotype in the infected insect, as well as in mammalian cells (Dowling et al., 2004: 345-353); it mimics BH3 domain proteins that are found in mitochondria and have proapototic actions (Yang et al., 2006: 2254-2261) as in its N-terminal domain, this protein has a Bcl2-homology 3-like domain (BH3 domain). Its central domain is of hydrophobic character with high similarity of the translocation domain of the Clostridium difficile toxin B, while the C-terminal domain of Mcf1 resembles the repeats-in toxin (RTX) like toxins of another bacterium (Actinobacillus pleuropneumoniae) (Dowling et al., 2004: 345-353).

Mcf2 N-terminus shows similarity to a type III secreted protein of a plant pathogen (Pseudomonas syringae) but it lacks the BH3-like domain (Waterfield et al., 2003: 265-270). mcf2 locus is located next to a type I secretion system operon. Both Mcf1 and Mcf2 have C-terminal domains that support their export via a type I secretion system (Wilkinson et al., 2009:302-323). These toxins, when expressed recombinantly in Escherichia coli, allow the bacterium to survive inside the insect and promote its death and they have been possibly evolved to provide the bacterium with toxicity against different kinds of insects. A different site of action within the insect is also possible for the existence of two different proteins with the same properties (Waterfield et al., 2003: 265-270).

Photorhabdus Virulence Cassettes (PVC)

The “Photorhabdus virulence cassettes” genes show sequence similarities to known toxins such as the Mcf of P. luminescens or the toxin A of C. (ffrench-Constant et al., 2007: 436-451). Each of these cassettes encodes for 15-20 proteins, about 30nm wide, that resemble R-type pyocins, a type of bacteriocin. The protein products of the PVCs have no direct antibacterial activity, but do destruct insect hemocytes. These proteins also show similarities to phage tail and base plate assembly proteins, fimbrial usher and proteins from other pathogenic bacteria (Yang et al., 2006: 2254-2261). Their loci can be found clustered between a type IV conjugation pilus and the mukB locus, a locus involved in plasmid stability. Furthermore, their effector proteins are located always downstream of the PVCs and are flanked by transposon sequences, indicative of a
possible mechanism of insertion in the PVC or even their movement among different PVCs (Yang et al., 2006: 2254-2261).

**Photorhabdus Insect Related (Pir) toxins**

Photorhabdus insect related (Pir) are binary proteins encoded by *pirAB* gene located at 2 different loci namely *pirA* (*plu4093-4092*) and *pirB* (*plu4437-4436*) of *P. luminescens* TT01 genome (ffrench-Constant et al., 2007: 436-451; Yang et al., 2006: 2254-2261). These proteins show similarities to the δ-endotoxins of *Bacillus thuringiensis*, thus making them a possible substitute for Bt (*Bacillus thuringiensis* toxin) recombinant crops (ffrench-Constant et al., 2007: 436-451). PirA shows little similarity to known proteins, but its partner PirB shows high homology with the N-terminal region of the pore-forming domain of the Cry2A insecticidal toxin, suggestive of the existence of a similar motif in these binary proteins. PirB also has similarities with a developmentally regulated protein from the beetle *Leptinotarsa decemlineata* which appears to have juvenile hormone esterase (JHE) activity (Waterfield et al., 2005: 47-52.; Wilkinson et al., 2009: 302-323). However, till date, there are no experimental data available in the Protein Data Bank related to the tertiary structure of Pir toxins. Nevertheless, there are several studies that have been conducted to understand the 3D structure of different types of cry toxins from *Bacillus thuringiensis*.

**General features of Cry toxins from Bacillus thuringiensis**

Cry toxins are globular molecules containing three structural domains connected by single linkers. One of the important features of the members of this family is the presence of protoxins with two different lengths. One large group of protoxins is approximately twice as long as the majority of the toxins. The C-terminal extension found in the long protoxins is dispensable for toxicity and is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd et al., 2001: 193-199).

Several experimental investigations have been conducted in the recent past to reveal the structural co-ordinates of Cry toxins using X-ray crystallographic techniques and the same has been deposited into the protein data bank, with each of these having a unique
Classification of the Cry proteins is based on amino acid sequence identity. The name given to any particular toxin depends on its occupancy at a particular node position in the phylogenetic tree. A new toxin that joins the tree to the left of the leftmost boundary will be assigned a new primary rank (numerical number such as 1, 2, 3 ……n). Toxin that enters the tree between the left and central boundaries will be assigned a new secondary rank (an uppercase letter like A, B, C etc.). It will have the same primary rank as the other toxins within that cluster. A toxin that enters the tree between the central and right boundaries will be assigned a new tertiary rank (a lowercase letter like a, b, c, etc.). Finally, a toxin that joins the tree to the right of the rightmost boundary will be assigned a new quaternary rank (another Arabic number). Toxins with identical sequences but isolated independently will receive separate quaternary ranks (Cickmore et al., 1998: 807-813). This classification scheme in fact is adopted for the different types of cry toxins whose structural coordinates are deposited in Protein Data Bank. To quote an example, a δ-endo toxin isolated and purified from *Bacillus thuringiensis* has been designated as “Cry2Aa”. This methodology of classification is also utilized to correlate with the taxonomic order of susceptible insect species of agricultural significance

- “Cry1: Lepidoptera”
- ” Cry2 : Lepidoptera”
- “Cry3 : Coleoptera”
- “Cry2 and Cry4: Diptera” (Morse et al., 2001)

All the Cry toxins made up of 3 domains display a high degree of structural similarity suggesting a similar mode of action. The N-terminal region of domain I is a bundle of seven α-helices in which the central helix-α5 is hydrophobic and is encircled by six other amphipathic helices; and this helical domain is responsible for membrane insertion and pore-formation. While domain II consists of three anti-parallel β-sheets with exposed loop regions, domain III is rich in β- sheets, present in the sandwich form. Exposed regions in domain II and domain III are involved in receptor binding (Bravo et al., 2005: 363-382; Boonserm et al., 2006: 3391-3401).
Domain I is found to share structural similarities with other Pore Forming Toxin (PTF) like colicin Ia and N and diphtheria toxin, indicting the role of this domain in pore-formation. In case of domain II, structural similarities with several carbohydrate-binding proteins like vitelline, lectin jacalin, and lectin Mpa have been reported de Maagd et al., (2003: 409-433). Domain III shares structural similarity with other carbohydrate-binding proteins such as the cellulose binding domain of 1, 4-β-glucanase C, galactose oxidase, sialidase, β-glucoronidase, the carbohydrate-binding domain of xylanase and β-galactosidase (de Maagd et al., 2003: 409-433). These similarities suggest that Domain III of Cry toxins may bind to carbohydrate moieties and thus carbohydrate moiety could have an important role in the mode of action of Cry toxin (Griffitts et al., 2005: 922-925). This observation is based on the fact that in the nematode Caenorhabditis elegans, mutations in the bre genes responsible for the synthesis of specific glycolipid lead to the development of resistance towards Cry5 toxin, suggesting glycolipid are important receptor molecules for Cry5 (Griffitts et al., 2005: 922-925).

The studies conducted by Audtho et al., (1999: 4601-4605) on the crystal structure of Cry2Aa from Bacillus thuringiensis subsp kurstaki revealed that this toxin is made up of 633 amino acid residues, referred to as protoxin. This contains the N-terminal peptide (49 amino acid residues) that gets cleaved upon activation, resulting in the formation of 3 domain mature toxin (Audtho et al., 1999: 4601-4605). Overall topology of Cry2Aa toxin being similar to the activated toxins Cry3Aa (Li et al. 1991: 815-821) and Cry1Aa (Grochulski et al., 1995: 447-464), suggests that removal of the activation peptide helps in exposing the interior region of toxin rather than altering its conformation. However, Cry3Aa and Cry1Aa toxins show little sequence identity to Cry2Aa.

Potential receptors for insecticidal toxins

The four putative Cry toxin receptors have already been identified. These receptors include aminopeptidase-N (APN), alkaline phosphatase (ALP), cadherins and glycoproteins (Pardo-López et al., 2006: 10329-10332; Muñoz-Garay et al., 2009: 2229-2232). Alkaline phosphate may act as a Cry1Ac receptor in Helicoverpa armigera (Ning et al., 2010: 666-671), Manduca sexta (McNall & Adang, 2004: 999-1002) and Heliothis virescens (Jurat&Adang, 2004: 3127-3129) and as a Cry1Aa receptor in Aedes aegypti.
In *Helicoverpa armigera*, ALP is a 68-kDa GPI-anchored membrane glycoprotein. Binding of Cry1Ac to ALP was demonstrated by ligand blot analysis of BBMV and was dependent on the existence of GalNAc residues in *H. armigera* ALP. It has been proposed as a crucial step during toxin processing (Ning et al., 2010: 666-671). Another potential receptor is Aminopeptidase N, which belongs to the zinc-binding metalloprotease family of proteins. The C-terminal stalk of the APN binding site has been found to be rich in N-acetylgalactosamine (GalNAc), and this region acts as the binding site of the Cry1Ac toxin (Knight et al., 1994: 429–436; Knight et al., 2004: 101–112).

Voltage-gated sodium channel is one of the neurotoxic receptor. The selective conductance of sodium ions across the plasma membrane by the voltage-gated sodium channel underlies the propagation of action potentials in neuronal cells of both vertebrates and invertebrates. The critical role of the sodium channel in the functioning of the nervous system has made it the target of a diverse array of toxins during evolution (Wang&Wang, 2003: 151–159). During an action potential the sodium channel undergoes transitions between closed-resting, activated and inactivated functional states, and toxins binding to specific sites on the channel either alter the equilibrium between these functional states or block the channel pore (Catterall, 1980: 15–43). There are at least ten separate binding sites for ligands on the sodium channel, including those for local anaesthetics and anti-convulsants (Wang&Wang, 2003: 151–159).

**Computational studies on insecticidal toxins**

Extensive studies have been conducted to understand the structure and function of toxin proteins from various organisms such as *Bacillus thuringiensis* (Kashyap et al., 2010: 280-285), Scorpion and Snake venom (Doley et al., 2010: 31-41) using *in silico* approach.

**Studies on phylogenetic relationship**

Phylogenetic relationships among δ-endotoxins from *Bacillus thuringiensis* and their different functional domains were studied by Bravo (1997: 2793-2801). In order to estimate the phylogenetic relationships of δ-endotoxins and each of their functional domains, the genetic distances among the Cry sequences were calculated with the
PROTDIST program of PHYLIP 3.5 (Felsenstein, 1993) package. FITCH program was later used to estimate phylogenies from the distance matrix data under the additive tree model. Phylogenetic analyses were also done by the parsimony method using the PROT-PARS program. Output obtained at the end of the analysis shows that some of the different protoxin classes (Cry1, Cry3, and Cry7) formed independent clusters. For instance, all Cry1 protoxins formed one single clade. In contrast to this, all the nematode-specific protoxins such as Cry5, Cry12, and Cry13 got clustered together with Cry14 toxin, indicating their close relationship during evolutionary process. Other protoxins such as Cry8 and Cry9 were also arranged in the same branch where Cry2 and the Cry11 protoxins are located, suggesting a common origin for these protoxins (Bravo, 1997: 2793-2801).

Phylogenetic study was also carried out for individual domains of Cry toxins from *Bacillus thuringiensis* and the tree was constructed using PRODIST and CONSENSE program of Phylip 3.5 (Felsenstein, 1993). The tree that was generated for Domain 1 consisted of 3 main groups, wherein the first group consist of lepidopteran-specific, second groups consist of Coleopteran – and Lepidopteran-Coleopteran specific toxins and third group had a mixture of different toxins (nematode, dipteran and Cry2 lepidopteran-specific toxins). Tree constructed for domain II suggests that this domain is probably derived from different evolutionary roots, since some sequences showed infinite distances and could not be grouped in the same tree. Phylogenetic tree for third domain showed infinite distances of Cry2 and Cry11 toxins from the rest of the Cry family, suggesting a very different origin (Bravo, 1997: 2793-2801).

**Molecular modeling and docking studies**

The prediction of 3D structures of proteins from its primary sequence using comparative protein modelling techniques offers an alternative for studying structure – function relationship in toxins. In the recent past, several studies have been conducted to predict the 3D structures of toxins from wide variety of organisms. For instance, comparison of homology modeled structures with experimentally solved 3D structures of venom-toxins has enabled identification of putative functional residues involved in binding and catalytic site, which were subsequently experimentally validated (Church and Hodgson
Molecular simulations of toxin–receptor complexes have been used for determination of critical interacting residues on the surface of toxins (Ellis et al., 2001: 5942–5953; Cui et al., 2001: 1659–1669).

Hsueh-Fen Juan and his co-workers (1999: 500–510) were successful in constructing and comparing the 3D structures of 3 classes of venom toxins (short and long chains of cobra toxin and angusticeps- type toxin [denoted as FS2]) using homology modelling approach. The results of their study strongly suggest that these 3 classes of toxins shared similar structural features (i.e., a three-loop tertiary structure with eight or ten critically important cysteines situated at the base to form anhydrophobic core and three flexible external loops extending outwards), however, they displayed distinct differences in the overall peptide flexibility and interior microenvironment suggesting some intrinsic differences in the surface hydrophobicity of the peptide segments present on the surface loops. Moreno-Murciano et al., (2002) have used similar kind of technique for predicting the 3D structure of ‘Obtustatin’- a novel disintegrin (cysteine-rich peptide) from the venom of Vipera lebetina obtusa. Even though its primary sequence displays many of the strongly conserved features of known disintegrins, the integrin binding loop of ‘Obtustatin’ contains a novel KTS motif instead of the classical RGD motif.

Tanje et al., (2012: 61-76) were successful in predicting the 3D structures of pure Cry1Ac toxin of Bacillus thuringiensis and aminopeptidase N (APN) receptor from Manduca sexta based on comparative protein modeling approach. Further, in the same study that was carried out by Tanje et al., (2012: 61-76), binding efficacy of Cry toxin towards the APN receptor was evaluated by replacing domain III of Cry1Ac with crystal structure of garlic-lectin (1KJ1). The results of docking studies suggest that chimeric protein “DIDII-garlic lectin” had better binding affinity towards the APN receptor compared to the native Cry1AC protein.

The three dimensional structure of all the three domains of Cry1Ab17 (Kashyap et al., 2010: 280-285), Cry1Ab21 (Kashyap et al., 2011a: 142-147) and Cry1Ab22 (Kashyap et al., 2011b: 202-206) from Bacillus thuringiensis was generated using MODLLER software by Kashyap et al., (2011b: 202-206). Upon closer examination of 3D models of
Cry toxins it was found that overall topology of all three domain are same except domain I. Domain I shows differences in their structure. CryAb17 and CryAb21 were made up of both α-helices and β-sheets while CryAb22 made up of only helix.

The 3D models were subjected to series of tests for evaluating its consistency and reliability. Backbone confirmation was evaluated by the inspection of the Psi/Phi Ramachandran Plot using the RAMPAGE web server. The Ramachandran plot showed that 92.5% of CryAb21 and 93.5% of CryAb17 and CryAb22 of residues have Φ and Ψ angles in the core of favored regions. The energy criterion was evaluated with ProSA (Wiederstein & Sippl, 2007: W407–W410) web server. The Z-score of evaluated model was -8.92, -9.39 for CryAb17 and CryAb21 respectively. The superimposed backbone traces for these toxins displayed very low RMSD (Kashyap et al., 2011a: 142-147).

Shan et al., (2011: 427-432) predicted the structure of Cry1Ac Toxin-binding Alkaline Phosphatase Receptor from Helicoverpa armigera with the help of swiss model in the expasy server. The obtained model was validated by ERRAT and ProSA. In this case ERRAT score was 77.378. The backbone conformation and non-bonded interactions of the homology model are all within the normal range. Therefore, all the evaluations indicate that the homology model structure is quite reasonable.

Homology modeling was employed for the structure prediction using Cry2Aa as template protein, a high-resolution X-ray crystallography structure. The model predicted for the B. thuringiensis LDC-9 Cry10Aa protein reveals a partial N-terminal domain only due to its partial sequence of 104 amino acids. B. thuringiensis Cry10Aa model contains three domains such as domain I, a bundle of eight alpha helices with the central relatively hydrophobic helix surrounded by amphipathic helices while domain II and III contain mostly beta-sheets. Significant structural differences within domain II in this model among all Cry protein structures indicates that it is involved in recognition and binding to cell surfaces. Comparison of B. thuringiensis israelensis predicted structure with available experimentally determined Cry structures reveals identical folds. The distribution of electrostatic potential on the surface of the molecules in the model is non-uniform and identifies one side of the alpha-helical domain as negatively charged.
indicating orientation of toxic molecules toward the cell membrane during the initial binding with a cell surface receptor (Mahalakshmi & Shenbagarathai, 2010: 363-378).

A homology model of the housefly voltage-gated sodium channel was developed by O’reilly et al., (2006: 255-263) to predict the location of binding sites for the insecticides fenvalerate, a synthetic pyrethroid, and DDT an early generation organochlorine. S4 to S5 regions and P-loop helices from the X-ray crystal structure (2A79) of the Shaker rat-brainKv1.2 channel and the S6 helices from the crystal structure (1ORQ) ofKvAP (Aeropyrum pernix Kv channel) provided the structural template for a homology model of the housefly voltage-gated sodium channel in an activated state. Then the model was generated using the Biopolymer module of SYBYL [Version 7.0, Tripos Inc., St. Louis, MO, U.S.A.]. The final model was subjected to 500 rounds of conjugate-gradient minimization in SYBYL using the Tripos force-field (O'Reilly et al., 2006: 255-263).

Homology models of the three Anopheles minimus P450 enzymes were constructed using the multiple template alignment method. Amino acid sequences of CYP6AA3 (GenBank: AAN05727.1), CYP6P7 (GenBank: AAR88141.1), and CYP6P8 (GenBank: AAR88142.1) were aligned against protein structures deposited in Protein Data Bank using PSI-BLAST. Comparative modeling of CYP6AA3, CYP6P7, and CYP6P8 was performed using a restrained-based approach implemented in MODELLER9v6. Refinement of models was performed using Amber10 package to reduce steric clashes among residues. The AMBER ff03 all atom force field was applied. The proteins were solvated in TIP3P water molecules with 12 Å cutoff. Dock 4.0 program was employed to dock ligands into active-site cavity of target models using Lamarckian genetic algorithm, consisting of 200 runs and 270000 generations, with the maximum number of energy evaluation set to 2.5 × 106. The predicted enzyme model structures were compared and used for molecular docking with insecticides and compared with results of in vitro enzymatic assays. The three model structures comprise common P450 folds but differences in geometry of their active-site cavities and substrate access channels are prominent. The CYP6AA3 model has a large active site allowing it to accommodate multiple conformations of pyrethroids. The predicted CYP6P7 active site is more constrained and less accessible to binding of pyrethroids (Lertkiatmongkol et al., 2011: 321).
Based on these studies, it is possible to conclude that information about the 3D structures of the various insecticidal toxins from the *Photorhabdus luminescens* and its interaction with receptors is not yet available. Therefore the present study attempts to predict the structure and function of insecticidal toxin from *Photorhabdus luminescens* and its interaction with its respective receptors using computational tools.