2.1 Introduction

Lepidopteran pests are major limitation to high production of food crops (Karthikeyan, 2012). *Helicoverpa armigera* is a major lepidopteran pest found in different countries of Asia, Africa and America and losses $5 billion annually in crop yield and for pest management (Gujar et al., 2007). *Bacillus thuringiensis* is a Gram positive sporulating bacteria belongs to bacillaceae family. It produces different insecticidal proteins like Cry toxin, VIP toxin, β-exotoxin and chitinase etc. Bt and its subspecies are effective against worldwide pests belonging to Lepidoptera, Coleoptera, Dipteral, Hymenoptera and Homoptera. Advantages of Bt bioinsecticides includes target specific killing, lack of toxicity to humans and ease of commercial production (Bravo et al., 2011; Deist et al.,2014). Transgenic *Bt* crops have reduced significantly use of insecticides and increased yield of crops and profits to farmers (Shi., 2013). These benefits prompted farmers to plant *Bt* crops from 1 million hectors in 1996 to 76 million till 2013 (Shi et al.,2013). However, Bt toxin has limitations such as ineffective to target more than one type of insects due to narrow spectrum toxicity, ineffective to sucking pests such as Homoptera, Hemiptera and Thysanoptera (Catarino et al., 2015; Patel et al., 2013). Though *Helicoverpa armigera*, *Plutella xylostella* and *Helicoverpa zea* belonged to lepidoptera, they shown variation in susceptibility to Cry toxins (Gujar et al., 2007; Kumar and Gujar, 2005, Siegfried et al., 2000).

The most widely studied strategies to improve the toxicity of Cry toxins include mutation in specific region of toxin by site directed mutagenesis (Liu, 2006; Rajamohan, 1996), enhancement in pore formation activity by domain swapping between Cry toxins (Caramori 1991; Naimov,2001), deletion of certain residues of

amino acids at N-terminal of toxin to expose a hidden region of toxin corresponding to receptor binding (Mandal et al.2007; Morse et al. 2001), addition of specific proteolytic cleavage site (Hilder, et al.,1987; Walters et al.,2008); Cry toxin insecticidal activity enhanced by using other proteins such as serine protease inhibitor (Macintosh et al.,1990), promoting toxin-receptor binding interaction by contacting toxin with chitinase (Ding et al.,2008; Regev and Strizhov 1996), cyt toxin (Promdonkoy , 2000) or a small fragment of cadherin –like receptor have been reported. (Abdullah et al., 2009; Chen et al.,2007).

Domain swapping between Cry toxins is one of the successful strategy to enhance toxicity and broad specificity reported so far. For instance, chimeric Cry toxins with different domain combinations from a variety of Cry toxins have been constructed. Fusion of domain I-II of Cry1Ab toxin with domain III of Cry1C resulted into increased toxicity to *S. exigua* compared to the parental Cry toxins (de Maagd et al., 1996). Combination of domain I-II of Cry1Ia and domain III of Cry1Ba led to a change in specificity of the hybrid toxin towards order coleoptera (Naimov et al., 2001). Combination of domain I-II Cry1Ba with domain III Cry1Ca broadened the spectrum of activity towards *S. exigua* and *M. sexta* (de Maagd et al., 2000).Similar results were obtained with other hybrid toxins too (Karlova et al.,2005; Sakai et al.,2007). In some hybrid toxins, however decrease in toxicity was also observed (Karlova et al.,2005; Rang et al., 2001). Nevertheless, hybrid Cry toxins were successfully constructed to achieve the desired results of increase in toxicity, change of specificity as well as broadening the spectrum of action. Thus, there is need of novel toxins with improved toxicity and broad

spectrum of insecticidal activity. Moreover, lepidopteran specific Cry toxins like Cry1, Cry2 and Cry9 show often variation in insecticidal activity to target pests. For instance,

Cry1Ac has exhibited highest degree of toxicity to *H.armigera* (Liao et al., 2002; Chandrashekar et al, 2005; Li and Bouwer, 2012) while Cry9Aa shown lowest (Chakrabarti et al.,1998; Liao et al., 2002; Li and Bouwer, 2012) Also, Zhao et al., (2005) proposed that Cry toxins with substantial insecticidal activity against devastating crop pests with no cross resistance are important to manage insect resistance against transgenic Bt plants. Lepidopteran specific Cry9 toxin subtype such as Cry9A, Cry9B, Cry9C, Cry9D, Cry9E and Cry9F exhibits significant toxicity against agricultural crop pests but have not shown cross resistance to Cry1A toxins (Shu et al.,2013). Thus, Cry9 toxin can be a promising candidate for insect pest management. Here, Table 2.1 present some results of previous report on domain swapping between Cry toxins to improvement in Cry toxicity. N –terminal alpha helix (Figure 2.1) is cleavaged by protease to be proceeding and activation of Cry toxin. Currently, reports on insect resistance to Cry toxin (Table 2.2), deletion of alpha helix-1 could be act in enhancement in Cry toxin toxicity. Thus, the aim of present study was to construct a novel hybrid Bt toxin of *cry1Ac* and *cry9Aa* by domain swapping between domain I of *cry1Ac* and domain II & III of *cry9Aa* by overlap extension PCR and deletion of alpha helix-1 at N-terminal of recombinant Bt toxin to prepare *cry1Ac-cry9AaMod* toxin to enhance toxicity against lepidopteran insect pest *Helicoverpa armigera.* Domain III swapping between Cry toxins indicated that exchanging domain resulted to enhanced toxicity of toxins. Also, broaden specificity on toxin which helpful to targeting different insect pests.

Table 2.1: Effect of domain swapping on toxicity of Cry toxins

	Hybrid toxin & Reference	Details	Effect
1.	Cry1E - Cry1C Bosch et al, 1994	Domain I & II of Cry1E and domain III of Cry1C	Higher toxicity than parental Cry1Ca and comparable to Cry1Ea towards *S. exigua* and *Mamestra brassicae*
		Domain I & II of Cry1C and domain III of Cry1E	Toxic to *Manduca sexta* but no toxicity towards *S. exigua* and *M. brassicae*
2.	Cry1Ab - Cry1C de Maagd et al, 1996a	Domain I & II of Cry1Ab and domain III of Cry1C	Highly toxic towards *S. exigua* than Cry1Ab and Cry1C
		Domain I & II of Cry1C and domain III of Cry1Ab	Low activity to *S. exigua* and *M. sexta*, but bind strongly to BBMV
3.	Cry1Ac - Cry1C de Maagd et al, 1996b	Domain I & II of Cry1Ac and domain III of Cry1C	Did not bind to BBMV of *S. exigua*
		Domain I & II of Cry1C and domain III of Cry1Ac	Did not bind to BBMV of *S. exigua*
4.	Cry3Aa-Cry1Ac Carmona and Iberra 1999	Domain I-II of Cry3Aa and domain III of Cry1Ac	Hybrid showed 88% mortality to *L. texana*
5.	Cry1Ab - Cry1Ca de Maagd et al, 2000	Domain I-II Cry1Ab domain III Cry1Ca	Activity 26 fold higher than parental toxin Cry1Ab
6.	Cry`1Ac - Cry1Ca	Domain I-II Cry1Ac domain III Cry1Ca	Activity 25 fold higher than parental toxin Cry1Ac
7.	Cry1Ba - Cry1Ca	Domain I-II of Cry1Ba and entire domain III of Cry1Ca	Not more active against *S. exigua* but highly active against *M. sexta* than Cry1Ba and comparable to Cry1Ca.
		Domain I-II of Cry1Ba and domain III of Cry1Ca (has more C-terminal crossover point)	Significant activity against *S. exigua*, but less than Cry1Ca. Higher activity against *M. sexta* than Cry1Ba and comparable to Cry1Ca. (Broaden spectrum)

-Cont-

8. Cry1Da – Cry1Ca	Domain I-II of Cry1Da and domain III of Cry1Ca	Both the hybrid toxins did not show any significant activity against *S. exigua* and *M. sexta*
9. Cry1Fa – Cry1Ca	Domain I-II of Cry1Fa and domain III of Cry1Ca	Hybrid toxin was 5.5 times more toxic than Cry1Fa and comparable to that of Cry1Ca against *S. exigua*
10. Cry1Ba – Cry1Ia Naimov et al, 2001	Domain I-II of Cry1Ia and domain III of Cry1Ba.	It was 2.5 times more toxic than Cry1Ia and 7.5 times more toxic than Cry1Ba against CPB (Change in specificity)
	Domain I-II of Cry1Ba and domain III of Cry1Ia	It showed very low activity against CPB
	Domain I & III of Cry1Ba and domain II of Cry1Ia	Showed highest activity against CPB from all three hybrid toxins
11. Cry1C - Cry1A Sakai et al, 2007	Domain I & II of Cry1C and domain III of Cry1A	Showed higher activity against cell line Sf9 than Cry1C and Cry1A
	Domain I & II of Cry1C and domain III of Cry4A	Showed higher activity than hybrid GST-CC1A, Cry1C and Cry4A toxins
12. Cry1Ba - Cry1Ac Karlova et al 2005	Domain I-II of Cry1Ba and domain III of Cry1Ac	Showed toxicity 16 fold higher than Cry1Ba to *H. virescens*
13. Cry1Ca - Cry1Ac	Domain I-II of Cry1Ca and domain III of Cry1Ac	115 times higher than Cry1Ca to *H. virescens*

Table 2.2: N terminal modification in Cry toxin to enhance toxicity against lepidopteran insect pests

No.	N-terminal modification in Cry toxin	Enhancement in toxicity	References
1	Cry1Acmod - remove 56 amino acids at their N terminus including all of helix α-1 of domain	2.1 times increases toxicity observed to pink ball warm	(Tabashnik et.al., 2013)
2	Cry1Abmod- remove 56 amino acids at their N terminus including all of helix α-1 of domain	Toxicity 1.6 times enhanced against pink ball warm	
3	Cry2Aa- Deletion of 42 amino acid residues from the N-terminal end	2.85 times enhancement seen in cotton leaf worm, 1.99 fold increase in case of cotton ball warm and 2.87 times enhanced in black cut worm pest compared to Cry2Aa toxin.	(Mandal et al., 2007)
4.	Lack of Helix α-1 of Domain I in Cry1Ab and Cry1Ac	53-fold higher compare to Cry1Ab and 11-fold higher to Cry1AcMod against cabbage looper	(Franklin et al., 2009)

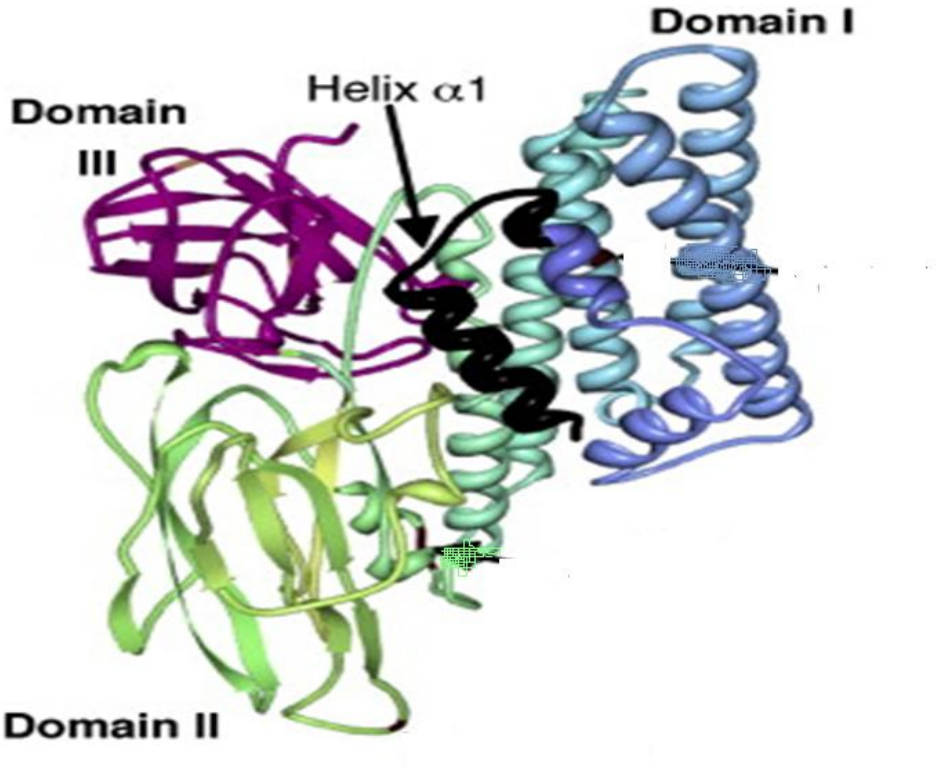

Figure 2.1: N –terminal region alpha helix coding region of Cry1A protein to be deleted to improve toxicity (Pardo Lopez et al., 2009)

2.2 Materials & Methods

2.2.1 Primer designing

Clustal ω (http://www.ebi.ac.uk/Tools/msa/clustalo/) tool was used to find out homology between *cry1Ac* and *cry9Aa* gene. Primers were designed from conserved block of Cry genes. Internal primers were designed from homologous sequences so as to generate overlapping ends in the amplified PCR product for recombination by OE-PCR.

Conservation of ORF of recombinant toxin was considered during primer designing. All primers were obtained from Xcelris, labs Limited, Ahmedabad.

2.2.2 PCR amplification of domain coding region

Standard E.*coli* strains ECE53 (*cry1Ac* cloned in plasmid pKK223-3) and ECE130 (*cry9Aa* cloned in plasmid pSB1402) were obtained from BGSC, Ohio, USA. Domain I of *cry1Ac* was amplified using forward primer Fp CGTAGCTAGCATG GATAACAATCCGAACATC (underlined shows NheI site) and reverse primer Rp CTACTCAACTGAAAATCTGTAACTGTTCGAATTGGATATCTT. Primers were designed based on published sequences of *cry1Ac* and *cry9Aa* available in BGSC website. Domain II & III of *cry9Aa* was amplified from ECE130 with forward primer Fp AAGATATCCAATTCGAACAGTTACAGATTTTCAGTTGAGTAG and reverse primer Rp GACTCTCGAGCTACTCTC GCGTCGGATTAACTGG (underlined show XhoI site). All domains were amplified with *Pfu* polymerase enzyme to avoid any sequence error in amplification. PCR products of domain I of *cry1Ac* (~0.8 kb) was confirmed by 2% AGE while domain II & III of *cry9Aa* (~1.1 kb) was confirmed by 0.8% agarose gel electrophoresis.

2.2.3 Construction of hybrid *cry1Ac-cry9Aa* gene by Overlap extension PCR

Hybrid *cry* gene was prepared by OE-PCR as per described by Heckman and Pease (2007). An equamolar concentration of PCR product of domain I of *cry1Ac* and domain II & domain III of *cry9Aa* were used as template in OE-PCR reaction system without primers. PCR program was as follow 1st denaturation 94 °C for 1min, 25 cycles of denaturation step at 94 °C for 30 sec, annealing step at 55°C for 20 sec and elongation

step 72°C for 2 min followed by finale extension at 72°C for 10 min. Sequencing of hybrid *cry1Ac-cry9Aa* was carried out to confirmed properly ORF of construct. Sequencing was out sourced at Xcelris, labs Limited, Ahmedabad, Gujarat. Amplification of hybrid gene was carried out by using flanking primers which used in OE-PCR.

2.2.4 Deletion of alpha helix-1 coding region of *cry1Ac-cry9Aa*

Alpha-helix of domain I was deleted using primer Fp CGTAGCTAGCGAATTTGTTC CCGGTGCTGGATT and Rp GACTCTCGAGCTACTC TCGCGTCGGATTAACTCC primers. Plasmid pBluescript KS (+) – *cry1Ac-cry9Aa* was used as template in the PCR reaction system. The underlined sequences represent restriction sites (NheI for forward primers and XhoI for reverse primer respectively). The PCR conditions were similar as described earlier. Amplified *cry1Ac-cry9AaMod* confirmed by performing to 0.8% agarose gel electrophoresis.

2.2.5 Cloning, Expression and Purification of hybrid Cry toxins

Hybrid construct *cry1Ac-cry9Aa* and *cry1Ac-cry9AaMod* were cloned at EcoRV site of pBluescript KS (+) plasmid (Figure 2.6 A & B) and transformed into *E. coli* (DH5 alpha). Desired clone was screened and confirmed by restriction digestion by releasing insert, subsequently sub cloned in pET- 28a (+) at XhoI and NheI restriction sites (Figure 2.7 A & B) followed by transformed to *E. coli* DH5α strain. pET- 28a (+) - *cry1Ac-cry9Aa* and pET- 28a (+) - *cry1Ac-cry9AaMod* were transformed into *E. coli* BL21 (DE3) plysS for expression analysis of hybrid toxins. Expression of N – terminal 6X His –tag of both hybrid toxins were carried out by induction of selected transformant when O.D. reached about 0.3-0.4 at 600 nm by using final conc. 1mM IPTG at 10° C for

12h. Induced *E. coli* cells treated with lysozyme (1 mg/ml) subsequently washed three times with wash buffer (0.1 M Tris, 1 mM EDTA, pH 8.0, 1 mM PMSF) and proceed for sonication. Then, lysed cells were centrifugation for 10 min at 10,000 RPM and pellet was solublised in 0.1 N Na_2CO_3 for 2h at 42 ℃. Hybrid toxins were purified under native condition by Ni-NTA agarose purification (Qiagen) system as per manufacturer's instructions. Subsequently, purified proteins concentration was estimated by SDS-PAGE gel electrophoresis using a calibration curve of BSA protein. Protein band density was estimated using ImageJ studio software to accurate dose of insect bioassay.

2.2.6 Insect Bioassay of Cry1Ac-Cry9Aa and Cry1Ac-Cry9AaMod hybrid Bt toxins

The bioassay of Cry1Ac-Cry9Aa and Cry1Ac-Cry9AaMod toxins were performed on laboratory reared second instar larvae of pest *Helicoverpa armigera* by leaf disc method. Recombinant toxins Cry1Ac-Cry9Aa and Cry1Ac-Cry9AaMod were spread on cabbage leaf with final concentration ranging from 0.1 - 10 ng per centimeter square then leaf were transferred into plastic vial. Laboratory reared single second instar larvae was placed into each vial and a tight plastic cover was used to confine larvae. Triplicate of twenty-four larva for each dose and five doses for each dose-response experiment were used. The bioassay experiment was performed in control environment condition (28 ± 1 ℃, 70 to 60% RH, and 12h Light: Dark regime).Then, mortality was recorded till a week. Parental Cry1Ac protein from plasmid pKK223-3-cry1Ac was purified as described by Ge et al. (1990) and used as control in similar manner to compare the toxicity. Mortality was calculated by probit analysis of SPSS software version.17.00 (Chicago, IL).

2.3 Results

2.3.1 Construction of *cry1Ac-cry9Aa* hybrid Bt toxin

Hybrid Cry toxin *cry1Ac-cry9Aa* of Bt was constructed by overlap extension PCR mediated recombination (Fig.2.3). A short overlapping sequence of six base pairs approach designed with one mismatch in homologous sequence to recombine the domain coding regions of *cry1Ac* and *cry9Aa* genes (Fig.2.4). The reverse primer for *cry1Ac* domain I coding region and the forward primer for *cry9Aa* domain II & III coding region were designed from the same homologous sequence from conserved block 2 to generate overlapping ends of six bps in the amplification products. OE-PCR mediated recombination of *cry1Ac* domain I coding region of size 802 bp (Fig.2.5 A) with *cry9Aa* domain II & III (Fig.2.5 B) coding region of size around 1107 bp yielded an expected recombinant gene *cry1Ac-cry9Aa* with size of ~1893bp (Fig.2.5 C).

2.3.2 Alpha helix deletion of *cry1Ac-cry9Aa* toxin

cry1Ac-cry9AaMod was prepared by deletion of 144 nucleotides corresponding to alpha helix-1 coding region from N terminal of *cry1Ac-cry9Aa* construct. PCR fragment was observed with size of approx 1757 bp by 0.8 % agarose gel electrophoresis (Fig.2.5 D).

2.3.3 Expression analysis of recombinant *cry1Ac-cry9Aa* toxin in BL21 (DE3) plysS

Cloned plasmid pET28a (+)-*cry1Ac-cry9Aa* was extracted from DH5 alpha and transformed into BL21(DE3) plysS for expression analysis of recombinant protein**.** Recombinant Cry1Ac-Cry9Aa was induced with 1mM IPTG at 10°C shaking condition for 6h. Expected band of ~74kD was observed on SDS-PAGE analysis (Fig2.8).

2.3.4 Purification of hybrid Bt toxins

His-tag purified hybrid toxin Cry1Ac-Cry9Aa showed a band of ~74 kDa toxin (Fig.2.9 A) and N terminal modified Cry1Ac-Cry9AaMod toxin revealed a band of ~ 68 kDa (Fig.2.9 B) after expression in *E. coli* BL21 (DE3) plysS strain.

2.3.5 Insect bioassay analysis

Native δ-endotoxin Cry1Ac from pKK223-3-*cry1Ac,* novel Bt toxin Cry1Ac-Cry9Aa and alpha helix deleted Cry1Ac-Cry9AaMod were tested against second instar larvae of *Helicoverpa armigera*. The result showed that Cry1Ac-Cry9Aa toxin exhibited a lower LC_{50} value 0.725 ng/cm^2 than control Cry1Ac toxin. Consequently, expressed hybrid Bt toxin Cry1Ac-Cry9Aa from pET- 28a (+)-*cry1Ac-cry9Aa* was 4.91 fold less LC_{50} value against devastating pest *H.armigera* than native Cry1Ac toxin from pKK223-3-*cry1Ac*. The Alpha helix-1 deleted mutant Cry1Ac-Cry9AaMod shown LC_{50} value 0.696 ng/cm^2 to *H. armigera* which was around 1.02 fold lower than the parental Cry1Ac-Cry9Aa toxin and 5.12 fold compared to parental toxin Cry1Ac (Table.2.3).

2.4 Discussion

Present investigation dealt with domain swapping between different *Bacillus thuringiensis* Cry toxins to enhance toxicity against *Helicoverpa armigera*. Recombination of domain coding region of *cry* gene could be performed successfully using six base pairs homologous sequences at 3'termini of genes. Since Cry1Ac is most toxic to *H. armigera* compare to other lepidopteran specific Cry toxins (Li and Bouwer,2012), its pore forming domain I was used to prepare *cry1Ac-cry9Aa* toxin with

cry9Aa domain II & III construct. Moreover, domain II of *cry9Aa* insecticidal protein has specificity

Table 2.3: Toxicity of recombinant Cry toxins against lepidopteran larvae *Helicoverpa armigera*

Toxins	LC_{50} (ng/cm^2)	95% Confidence limits		X^2 (Chi)	Toxicity enhancement factor (EF)[a]
		Lower	Upper		
Cry1Ac	3.564	1.822	3.780	1.763	_
Cry1Ac-Cry9Aa	0.725	0.493	1.620	1.650	4.915
Cry1Ac-Cry9AaMod	0.696	0.404	1.519	1.196	5.120

X^2: Chi square values for heterogeneity was less than tabular values ($p < 0.05$);

[a]: Toxicity enhancement factors of the hybrid toxins Cry1Ac-Cry9Aa and Cry1Ac-Cry9AaMod were determined relative to the Cry1Ac toxin used as a control.

to lepidoptera (Bravo, 1997). To improve toxicity of Cry9Aa toxin, its domain I coding region was selected to exchange with domain I of *cry1Ac*. It results in a remarkable 4.9 fold enhanced toxicity to *H. armigera*. There are reports of construction of biologically active Cry toxins by domain shuffling approach which had enhanced toxicity and broaden specificity. For instance, Karlova et al.,(2005) reported that domain I-II of Cry1Ba and domain III of Cry1Ac showed toxicity 16 fold higher than Cry1Ba to *H. virescens*. Also, domain I-II of Cry1Ca and domain III of Cry1Ac have seen 115 times higher toxicity than Cry1Ca to *H. virescens.* Naimov et al.,(2001) reported hybrid toxin constructed by

domain I-II of Cry1Ia and domain III of Cry1Ba had 2.5 times more toxicity than parental Cry1Ia.In contrast to the above reports, Rang et al.,(2001) observed decrease in toxicity of four hybrids Cry1 toxin containing domain I from other toxins. Hence, it is believed that exchange of domain I has important role in toxicity.

Though Cry9 type δ-endotoxinsof *Bacillus thuringiensis* are known to be insecticidal against several lepidopteran insects like *Plutella xylostella, Exorista larvarum, Spodoptera exigua* (Marchettia, 2009), Cry9Aa has showed poor larvicidal activity against *Helicoverpa armigera.* The LD_{50} value of Cry9Aa was found 4000 fold higher than Cry1Ac (Li and Bouwer, 2012). Moreover, phylogenetic analysis of amino acid sequence of three domains of Cry9Aa to other members of Cry9 family revealed that domain I was evolved independently (de Maagd et al., 2001) (Fig. 2.2) and might developed into a potent pore forming activity while domain II & III of Cry9Aa and Cry9 family members belong to same ancestor.(Schwartz, 1997) reported that domain III of CryIAa toxin participate in membrane channel formation in artificial lipid membrane. Thus, we speculate domain III of *cry9Aa* play an important role in toxicity or pore formation. The enhancement in toxicity of hybrid toxin due to domain I swapping between *cry1Ac* and *cry9Aa* caused biologically potent insecticidal toxin. Therefore, our results support the hypothesis of Fang et al.,(2016) that Cry9Aa insecticidal toxin acts as a good biocontrol agent.

Deletion of alpha helix-1 of N terminal of Cry toxin can causes oligomerization of the toxin and subsequent pore formation even in the absence of CAD receptor (Soberón et al., 2007). Similarly, (Mandal et al., 2007) demonstrated that removal of alpha helix of domain I of Cry2Aa caused significant increase in toxicity against insect larvae of

Helicoverpa armigera, Spodoptera litoralis and *Agrotis ipsilon* due to exposure of hydrophobic region of domain-1 which facilitate the receptor binding on surface of epithelial membrane. Moreover, (Tabashnik et al., 2013) reported alpha helix-1 deleted

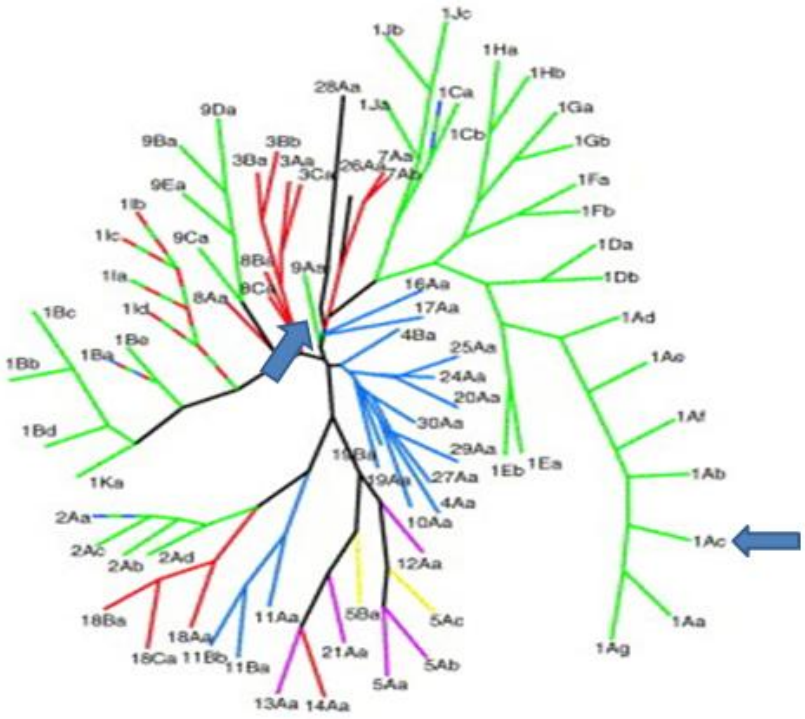

Figure 2.2.: Phylogenetic analysis of amino acids sequence of domain I of different Cry proteins (de Maagd et al.,2001)

mutants Cry1AcMod and Cry1AbMod toxins have shown 43 and 68 times more toxicity respectively compared to wild type Cry1Ac toxin against resistance strain (BX-R) of *Pectinophora gossypiella*. Considering these facts in mind, alpha helix-1 of domain-I of Cry1Ac-Cry9Aa hybrid toxin was deleted and around one fold further enhancement in toxicity achieved against *Helicoverpa armigera* pest. Our speculation for enhanced toxicity of Cry1Ac-Cry9AaMod toxin would be either toxin could form oligomer by

omitting binding to mutated CAD receptor located on insect gut membrane or deletion of alpha-heix1 revealed hidden region of toxin corresponding to the binding of receptors.

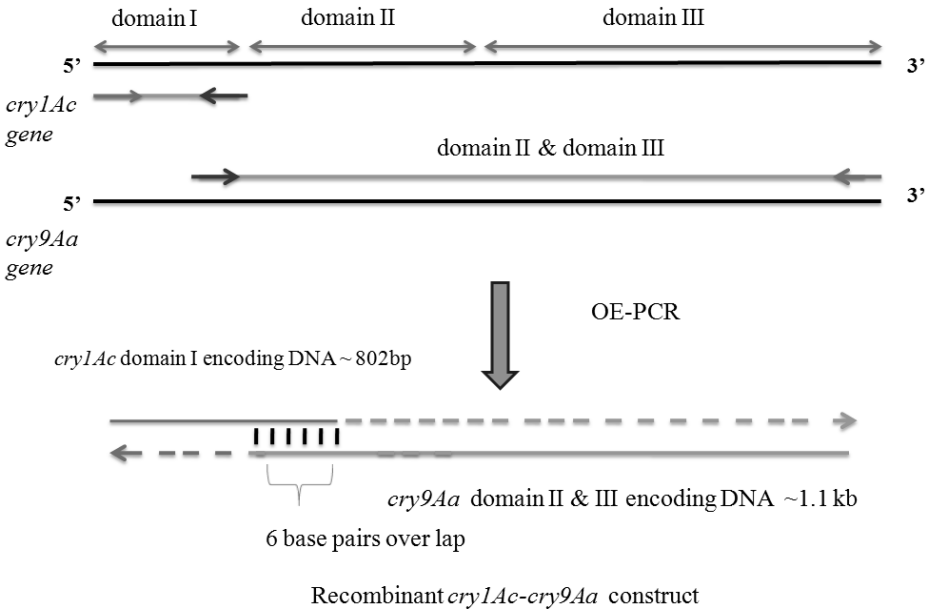

Figure 2.3: Schematic representation of primer positions, domain regions and strategy used to generation of *cry1Ac-cry9Aa* hybrid gene

```
Cry1Ac1 746 TTATGATAGTAGAAGATATCCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTAT 804
Cry9Aa1 854 TCTTGATATTACTAATTACCCAATAGAAACAGATTTTCAGTTGAGTAGGGTCATTTAT 912
            * :***** ** :*.:** *****: .*****:**  **.**.* :**.*: ******
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Figure 2.4: Overlap base pairs showing six base pairs homologous with 1 mismatch

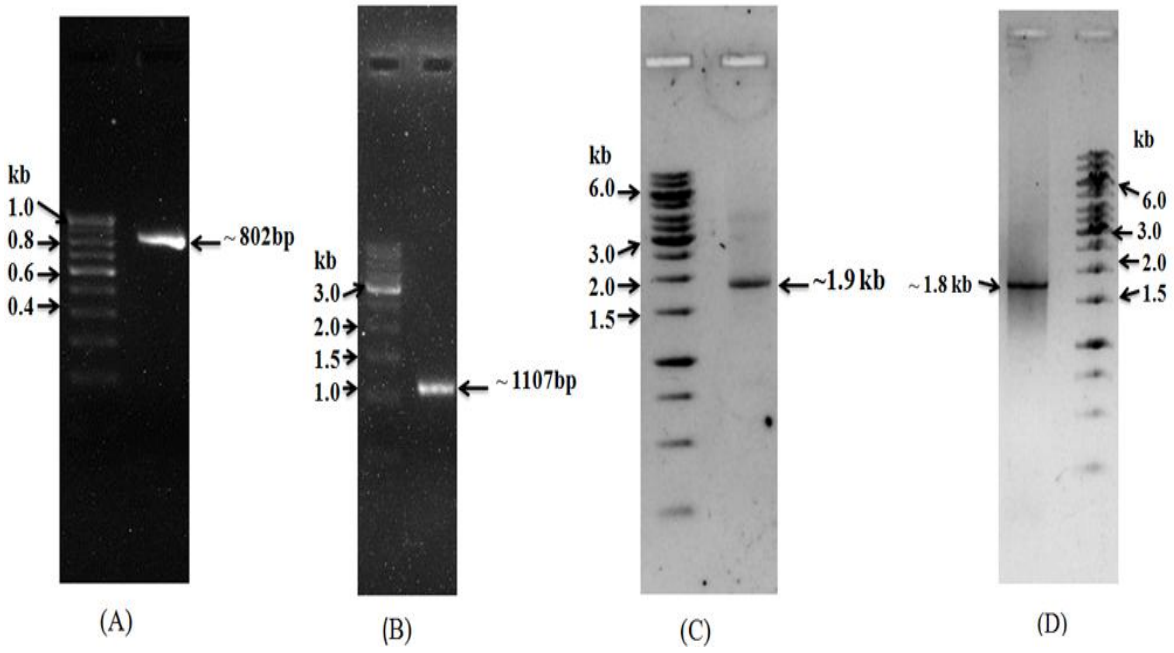

Figure 2.5: Construction of chimeric *cry1Ac- cry9Aa* gene (A) PCR amplification of domain coding regions; lane 1: 100bp step up ladder, lane 2 : PCR amplification product of *cry1Ac* domain I coding region, (B) PCR amplification of domain coding regions, lane 1: 1Kb ladder, lane 2: PCR amplification products of *cry9Aa* domain II & III coding region (C) Hybrid *cry1Ac- cry9Aa* gene; lane 1: 1Kb ladder , lane 2: OE-PCR products of *cry1Ac* domain I and *cry9Aa* domain II & III coding regions (D) Alpha helix deletion in domain I of *cry1Ac- cry9Aa;* lane 1: PCR product of *cry1Ac-cry9AaMod*, Lane 2: 1Kb ladder.

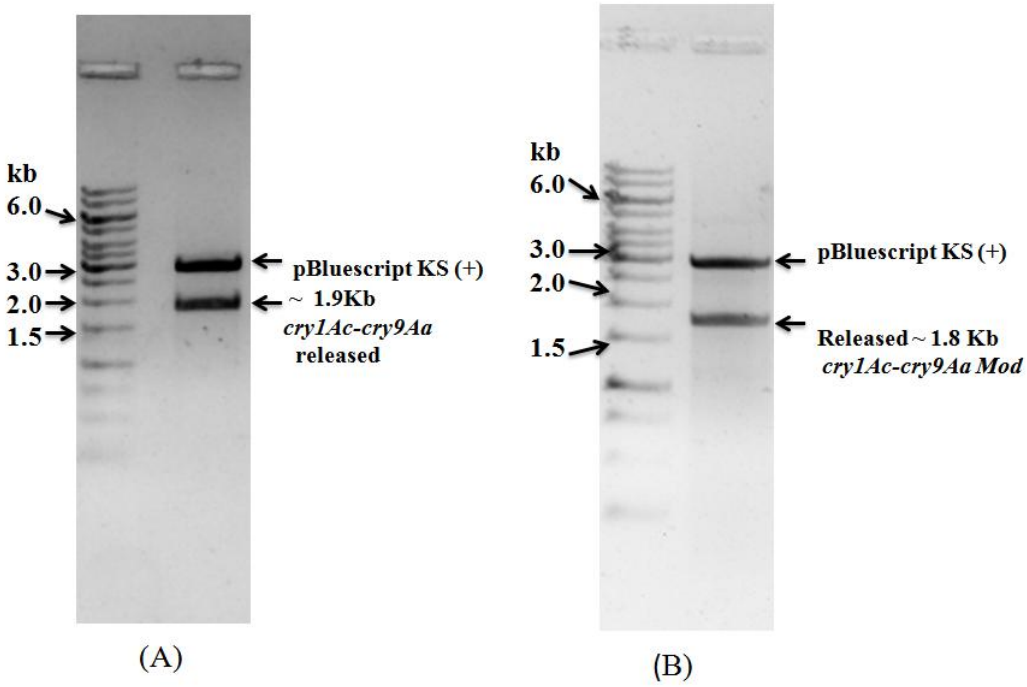

Figure 2.6 : (A) Lane 1- 1Kb ladder ; Lane 2- Release of insert *cry1Ac- cry9Aa* from pBluescript $KS_{(+)}$vector backbone by digestion with XhoI and NheI, (B) Lane 1:1Kb ladder ;Lane 2- Release of insert *cry1Ac- cry9AaMod* from pBluescript $KS_{(+)}$vector backbone by digestion with XhoI and NheI.

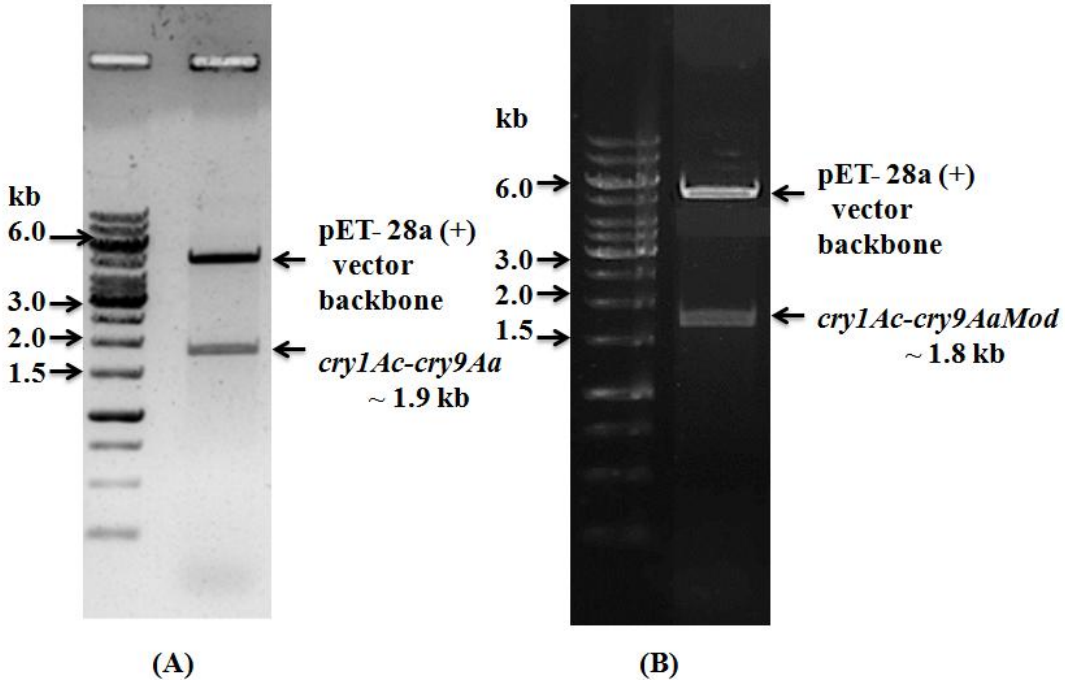

Figure 2.7: (A) 0.8% Agarose gel electrophoresis for sub clone confirmation of chimeric *cry1Ac- cry9Aa*: Lane 1- 1kb ladder ; Lane 2- release of insert *cry1Ac- cry9Aa* from pET-28a (+) vector backbone by digestion with XhoI and NheI. (B) Sub-clone confirmation of alpha-helix deleted *cry1Ac- cry9Aa* construct. Lane 1- 1kb ladder ; Lane 2- release of insert *cry1Ac- cry9Aa* from pET- 28a (+) vector backbone.

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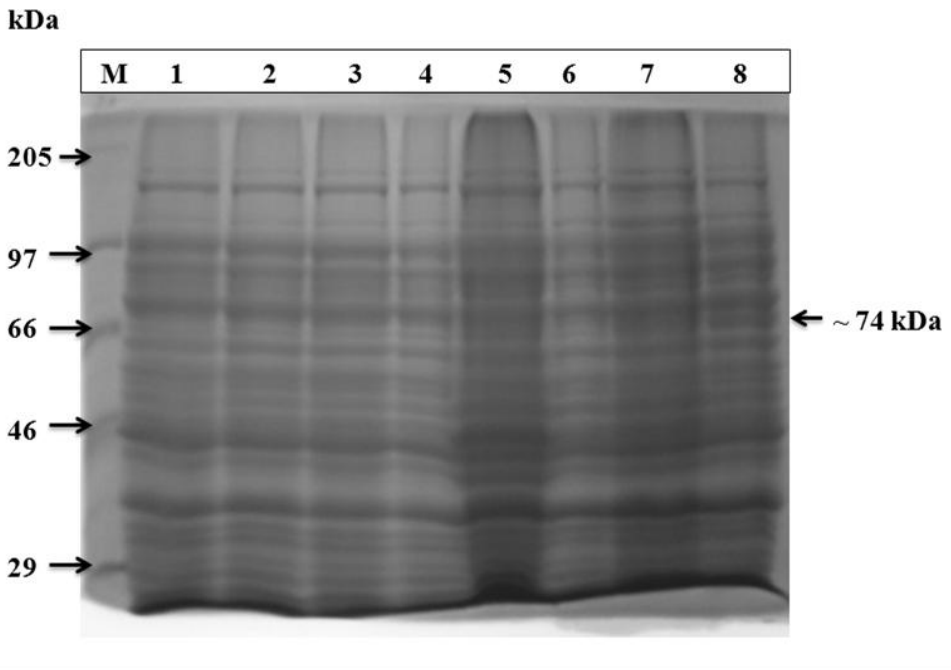

Figure 2.8. (A) Expression analysis of Cry1Ac-Cry9Aa toxin ; Lane M: Protein molecular weight marker; Lane 1: DE3 (plys S); Lane 2: DE3(plysS) pET-28a (+) w/o induction ;Lane 3: DE3(plysS) pET-28a (+) -*cry1Ac- cry9Aa* w/o induction at 3h; Lane 4: DE3(plysS) pET-28a(+)- *cry1Ac- cry9Aa* w/o induction at 6h; Lane 5: DE3(plysS) at 6h; Lane 6: DE3(plysS) pET-28a (+) at 6h induction; Lane 7: DE3(plysS) pET-28a(+) - *cry1Ac- cry9Aa* induction at 3h; Lane 8: DE3(plysS) pET-28a(+) - *cry1Ac- cry9Aa* induction at 6h.

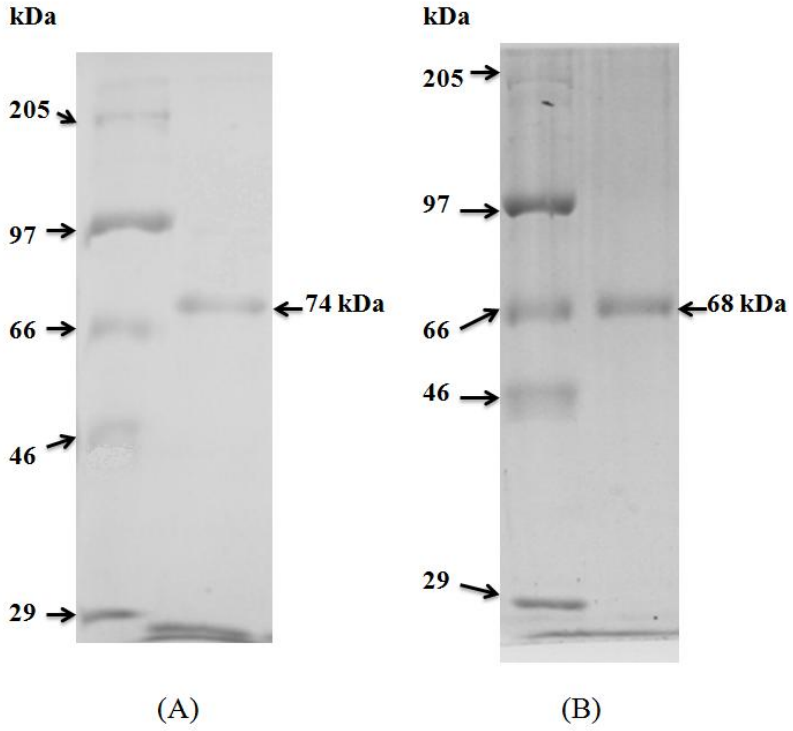

Figure 2.9: SDS-PAGE analysis of hybrid Cry toxins

(A) SDS-PAGE analysis of purified chimeric Cry1Ac-Cry9Aa; lane 1: Protein molecular weight marker, Lane 2: purified Cry1Ac-Cry9Aa toxin with size of ~74 kDa

(B) SDS-PAGE analysis of purified hybrid Cry1Ac-Cry9AaMod; lane 1: Protein molecular weight marker, Lane 2: purified Cry1Ac-Cry9AaMod hybrid toxin size of ~ 68 kDa

2.5 References

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