CRYSONES, the Cry1EC toxin encapsulated mesoporous SiO_2 nanoparticles, as an effective biopesticide against *Spodoptera litura*

Doctor of Philosophy

By

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07LAPH04

DEPARTMENT OF ANIMAL BIOLOGY

SCHOOL OF LIFE SCIENCES

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Thesis submitted to University of Hyderabad for the award of Ph. D. degree in the Department of Animal Biology

By

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(A central University established in 1974 by an Act of Parliament)

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DECLARATION

I, Swetha Kamireddy, hereby declare that this thesis entitled **"CRYSONES, the Cry1EC toxin encapsulated mesoporous SiO_2 nanoparticles, as an effective biopesticide against *Spodoptera litura*"** submitted by me under the guidance and supervision of Prof. Dayananda Siddavattam is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Name : Swetha Kamireddy

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Date :

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CERTIFICATE

This is to certify that the thesis entitled **"CRYSONES, the Cry1EC toxin encapsulated mesoporous SiO_2 nanoparticles, as an effective biopesticide against *Spodoptera litura*"** is a record of bonafide work done by Mrs. Swetha Kamireddy, for the Ph.D. programme in the Department of Animal Biology, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Prof. Dayananda Siddavattam
(Supervisor)

Head of the Department

Dean of the School

Dedicated

to

My Mother

Smt. Rama Devi. K

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Swetha Kamireddy

Contents

Introduction

Over the past five decades, crop protection has heavily relied on synthetic chemical pesticides. The uncontrolled use of the chemical pesticides, under the maxim, "if little is good, a lot more will be better" has played havoc with human and other life forms. In India, the use of the amount of chemical pesticides increased tremendously with time. The production of synthetic chemical pesticides dates back in India to 1952 with the establishment of manufacturing plant of DDT & BHC near Calcutta (Bhardwaj and sharma, 2013). At present, nearly 150 pesticides are registered, in India, legally. Currently India, is the largest producer of pesticides in Asia and ranks 12th in world for application of pesticides (Mathur, 2010).

Nearly, 9,000 species of insects and mites, 50,000 species of plant pathogens, and 8,000 species of weeds damage crops, worldwide. Statistical analysis show an estimated 14% of loss due to insect pests, 13% loss due to plant pathogens, and 13% loss by weeds (Lal and Pimentel, 2009). According to the reports, crop loss from pests declines by 35- 42% with the usage of pesticides (Zhang, 2011). Usage of pesticides is so critical in agriculture that about one-third of the field products are produced by applying pesticides (Liu et al., 2002). Despite their numerous benefits, the indiscriminate and unregulated usage of chemical pesticides has raised serious concerns about environment and human health. In India insecticides contribute to 76% of the pesticides used, as against 44% used worldwide (Mathur, 1999). Pesticides are mainly used for cotton crops (45%), followed by paddy and wheat, in India. The size of the Indian pesticide industry is about 2% of the total world market with an estimate of Rs.180 billion during 2010 (Statistics India Data 2011). However, when compared globally, the consumption of pesticides in India is low (around 500 g per ha) as against countries like Japan (12 kg per ha) and Germany (3 kg per ha). In India, problems resulting from unregulated and indiscriminate usage of chemical pesticides are quite frightening. This can be attributed to fragmented agricultural lands, low awareness among farmers about the

hazards of usage of pesticides, insufficient irrigation due to dependence on monsoons etc. Nearly, 25 million agricultural workers are suffering an episode of pesticide poisoning every year, in the developing countries (Bharadwaj and sharma, 2013). In the United States alone, there are about 67 thousands human pesticide poisonings per year (Zhang, 2011).

1.1. Use of chemical Pesticides:

Since 1970's, the insecticide market has been dominated by three chemical classes, organophosphates, carbamates and synthetic pyrethroids. The application of the synthetic organic pesticides has greatly protected and enhanced agricultural productivity. Worldwide, 4.6 million tons of chemical pesticides are sprayed into the environment, annually. There are about 500 pesticides used in mass applications which are highly poisonous to the environment. Pesticides are toxic by their very nature and are synthesized targeting the insects, weeds and rodents. Pesticides are released directly into the environment in order to exert their toxicity on the pests they control. Most of the pesticides applied are lost, due to evaporation, rain waste-out, photo-degradation, hydrolysis and other factors, before they could reach their targets (Sakamoto et al., 2004, Song et al., 2012). Only 1% of the sprayed pesticides reach their targets and 99% of pesticides applied are released into environment affecting non-target insects, polluting soils, water bodies and atmosphere, and finally absorbed by almost every organism.

As they are inherently toxic to living organisms, pesticides have severe impact on human health due to the exposure to residues in food and drinking water as well as on the environment by reaching non-target organisms by wind drift, leaching, runoff. Recently, high amounts of pesticides are detected on edible items like fruits and vegetables. Pesticide exposure severely effects the nervous system, manifested by a range of symptoms like deficits in neurological behavior, and abnormalities in nerve function (Keifer et al., 2010). The organophosphates (OPs)

and the carbamates block the action of acetylcholinesterase at peripheral nerves and in the central nervous system. The early symptoms of poisoning include headache, blurry vision, hypersecretion, bronchospasm, muscle twitching, nausea, and diarrhea. More severe poisoning can feature respiratory depression, loss of consciousness, and death during the acute stage. Pesticides may also have developmental, endocrine, and reproductive effects; this has been recognized for more than five decades. Epidemiological evidence suggests that pesticide exposure is also associated with increases in the risk of several cancers. These toxicity issues and environmental awareness have put restrictions on their uses and compelled scientific community to search for alternative pest control strategies.

1.2.***Bacillus thuringiensis*:**

Bacillus thuringiensis (Bt) was first discovered in the year 1901 by a Japanese bacteriologist, Shigetane Ishiwata in a deceased silk worm larvae while he was investigating the cause of sotto disease (sudden collapse disease). He named it as *Bacillus sotto*. Again in 1911, Berliner from Thuringia in Germany, isolated a related strain from a dead flour moth larvae causing flour moth disease and named the organism as *Bacillus thuringiensis*. Sporine, the first commercial insecticide based on Bt was used in France in 1938, and then in the USA in the 1958 (Ibrahim et al., 2010).

Bacillus thuringiensis (*Bt*) are gram-positive, non-capsulated, motile, spore-forming bacteria with proven insecticidal properties. *Bt* produces insecticidal proteins during the sporulation phase as parasporal crystals, which lie alongside the endospore. The production of these parasporal crystals is a biologically significant phenomenon that relieves stress physically by compensating water loss during spore formation and offers survival advantage by exerting lethal action on host insects (Ibrahim et al., 2010). Parasporal crystals are predominantly

comprised of Cry and Cyt toxins, also called δ-endotoxins. Cry proteins are parasporal inclusion (crystal) proteins from *Bacillus thuringiensis* that exhibit lethal effect on target insects. Similarly, Cyt proteins are parasporal inclusion proteins from *Bacillus thuringiensis* which exhibit hemolytic (cytolitic) activity. These toxins are highly specific to their target insect pests, are safe to humans, vertebrates and plants, and are completely biodegradable. Therefore, *Bt* is a viable alternative for the control of insect pests in agriculture and household (Perez et al., 2005).

Cry and Cyt toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT). Pore forming toxins are generally secreted as water-soluble molecules. They undergo multimerization upon recognition and binding to a specific receptor on the target membrane leading to conformational changes resulting in insertion into, or to translocate across cell membranes of their host (Ferdinand et al., 2013). PFTs are classified into (i) α-helical toxins (ii) the β-barrel toxins, based on the secondary structure of the protein regions that penetrate the host cell membrane. The α-helical toxins include toxins such as the colicins, exotoxin A, diphtheria toxin and also the Cry three-domain toxins where as β-barrel toxins include aerolysin, α-hemolysin, anthrax protective antigen, cholesterol-dependent toxins as the perfringolysin O and the Cyt toxins (Parker and Feil, 2005). Generally PFTs secreted by the host bacteria bind to the host cell surface receptors. On activation by the host proteases, the PFTs get triggered to form oligomeric structures which are insertion competent (Parker and Feil, 2005).

1.2.1. *B. thuringiensis* genome:

B. thuringiensis strains have a genome size ranging from 2.4 to 5.7 million bp (Carlson et al., 1994). On comparison of physical maps of both Bt and *B. cereus* chromosomes, it was observed that all chromosomes of two species have a similar organization near the replication origin with variability in the terminal half (Carlson et al., 1996). Several *B. thuringiensis* isolates

have more than one extra chromosomal elements, some of them circular and others linear (Carlson et al., 1994). High degree of genetic plasticity is observed in the Bt strains and toxins. Since long, it is known that genes encoding Cry toxins are often present on large plasmids (Gonzalez et al., 1981) of *B. thuringiensis*. The plasmids harboring *cry* genes were found to be conjugative in nature (Gonzalez et al., 1981). The *cry* genes can be exchanged between Bt strains by horizontal transfer between different strains, which results in potentially wide variety of Bt strains with different combinations of Cry toxins. Moreover, transposable genetic elements that flank genes also contribute to the variety of toxins produced naturally by Bt strains (Mahillon et al., 1994).

1.3. Cry toxins:

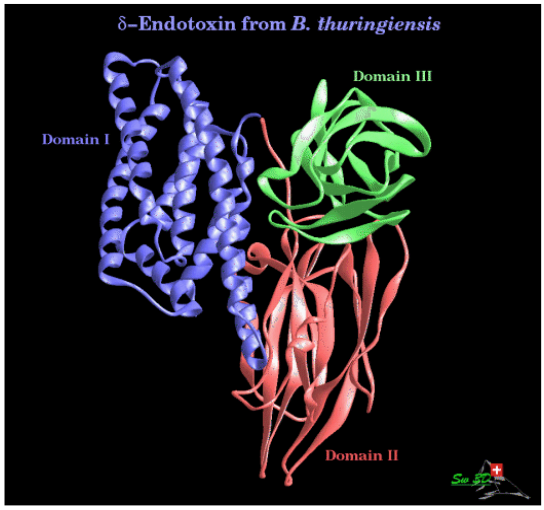

Fig 1-1. Three dimensional structure of delta endotoxin (from Prof. Ellar's home page).

Li et al., (1991) had resolved the three dimentional structure of Cry3A protein of *B. thuringiensis* subsp. tenebrionis by X-ray crystallography. Till date, eight Cry protein structures have been established by X-ray crystallography: Cry3Aa, Cry1Aa, Cry1Ac, Cry2Aa, Cry3Bb, Cry4Ba, Cry4Aa and Cry8Ea1. It was shown that all Cry toxins exhibit three dimensional structure and share high topological similarity. Domain I consists of a bundle of seven alpha helices among

which the central amphipathic alpha helix is well conserved among all the toxins and is involved in the insertion of toxin into the epithelial cell membrane. Domain II, consisting of three beta sheets and interacts with receptors, thereby contributing to toxin specificity and high-affinity binding (Dean et al., 1996; Schnepf et al., 1998). Domain III, also composed of beta sheets, contributes for toxin stability and binding specificity (Burton et al., 1999). It was reported that the Domain III of Cry1C was a major determinant of specificity for *Spodoptera exigua (*de Maagd et al., 2000).

1.3.1. Mode of action of Cry toxins:

The mode of action of Cry toxins in lepidopteran insects is well characterized. The Cry toxins primarily lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane (Aronson and Shai, 2001; de Maagd et al., 2001, Perez et al., 2005). The crystal inclusions ingested by the susceptible insects, get solubilised in the alkaline environment of the gut and are cleaved by mid gut proteases yielding 60–70 kDa active toxins which can resist proteases (Perez et al., 2005). The activated toxin binds to the specific receptors on the brush border membrane of the midgut epithelium (de Maagd et al., 2001; Perez et al., 2005), get oligomerized and later inserts into the membrane. Lytic pores are formed in microvilli of apical membranes due to the insertion of toxin (Aronson and Shai, 2001; Perez et al., 2005). Subsequently, the cells get lysed and disruption of the midgut epithelium releases the cell contents providing spores a germinating medium leading to a severe septicemia and finally insect death (de Maagd et al., 2001; Perez et al., 2005).

1.3.2. Expression and regulation of Insecticidal genes:

The formation of parasporal crystal proteins during sporulation is the fascinating aspect of *Bacillus thuringiensis*. Sporulation cycle in *Bt* is divided into 7 stages and the formation of parasporal crystals during sporulation is interlinked with the sporulation process. Cry toxins which are often referred to as δ- endotoxins are specific to the target insects. They are coded by genes belonging to different *cry* families. Cry proteins constitute 20-30% of the dry weight of the cell and generally accumulate in the mother cell from stage III to stage VII of sporulation. The high level expression of Cry proteins in coordination with sporulation is controlled by various mechanisms occurring at transcriptional, post-transcriptional and post-translational levels (Ibrahim et al., 2010). Major class of Cry toxins belong to *cryI* gene family where *cryI* genes express via two overlapping promoters (BtI and BtII). Transcription from the promoter, BtI, utilizes σ^E between stages II and VI, BtII utilizes σ^K from stage VI through the end of sporulation phase, thus ensuring the synthesis of the protoxins throughout the sporulation cycle. Cry *mRNA* stability plays a key role at the post transcriptional level, regulating the *cry* gene expression (Schnepf et al., 1998). The half life of *cry* mRNA is nearly ten minutes, which is almost five-fold greater than an average bacterial mRNA half life. The presence of putative transcriptional terminators at the 3' ends of *cry* mRNA, impairs the 3'- 5' exoribonuclease activity, thus resulting in the stability of *cry* mRNA. The post-translational regulation of Cry proteins involves the formation of stable parasporal crystals made of Cry proteins, which are resistant to the premature proteolytic degradation (Ibrahim et al., 2010).

1.3.3. Bt toxin classification:

Since the cloning and sequencing of the first *cry* gene, named *cry1A*a, more than 300 *cry* gene sequences encoding a variety of crystal proteins have been reported (Schnepf and Whiteley,

1981). Based on the *cry* gene sequences, host range and amino acid sequence similarity, different classifications of *cry* genes were proposed. Among those, the classification of *cry* genes proposed by Crickmore divides *cry* genes based on their insect host specificities into 6 major classes consisting 51 groups and subgroups (Ibrahim et al., 2010). Group 1— Cry1, Cry9 and Cry15 (lepidopteran); group 2— Cry2 (lepidopteran and dipteran); group 3— Cry3, Cry7 and Cry8 (coleopteran); group 4— Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cry20 (dipteran); group 5— Cry1I (lepidopteran and coleopteran); and group 6— Cry6 (nematodes).

1.3.4. Mechanism of action of Cry toxin:

The mechanism of action of the *B. thuringiensis* Cry toxins involves the solubilization of the crystal proteins in the alkaline environment of midgets of the target insects, proteolytic cleavage of the protoxins into activated toxins, binding of the activated Cry toxins to specific midgut receptors and finally insertion of the toxin into the apical membrane of midgut epithelial cells creating ion channels or pores (Schnepf et al., 1998). Each of the above mentioned steps have unique and critical role in eliciting toxicity and developing resistance towards certain Cry toxins (Oltean et al., 1999). This makes studying the mechanism of action of Cry toxins interesting. The solubility of crystal proteins depends on pH in the midgut of the susceptible insect and also on the composition of the protoxins. The secondary structure of the protoxins and the energy of the disulfide bonds present help in solubilizing the protoxins (Aronson et al, 1991). For most of the lepidopteran insects, protoxins are solubilized under the alkaline environment of the insect midgut. Many a times, differences in the extent of solubilization of the crystal toxins determine the degree of toxicity among different Cry proteins (Aronson et al., 1991; Du et al., 1994). Low level of solubilization of crystals is often speculated as the critical mechanism for resistance in the insect pests (McGaughey et al., 1998).

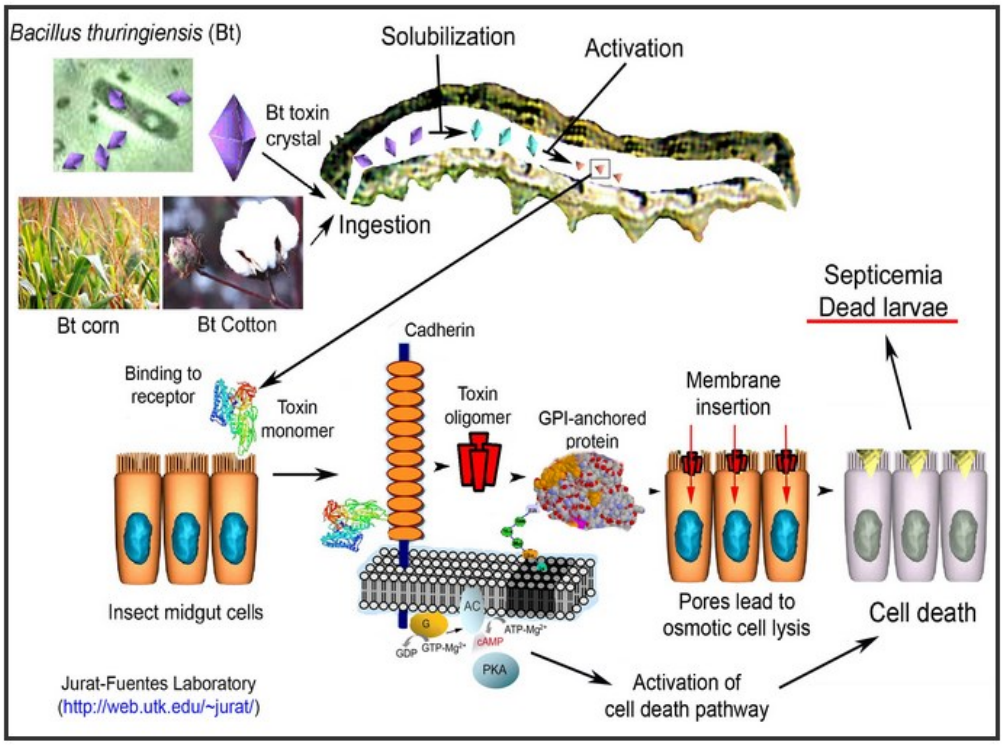

Fig 1-2. Mode of action of Cry toxins (by Dr. Juan Luis Jurat-Fuentes).

The solubilized crystal toxins undergo proteolytic cleavage in the midgut lumen of lepidopteran larvae by a variety of alkaline proteases, generating an active delta endotoxin. The midgut proteases mainly involve proteases belonging to serine protease class, having trypsin and chymotrypsin like protease activities (Broadway, 1997; Terra and Ferreira, 1994). The proteolytic cleavage of different Cry protoxins at both their N and C termini produce activated toxin with molecular weight of ≈ 65 kDa. The protoxin activation into active Cry toxin basically involves the proteolytic removal of 500 to 600 amino acid residues from the C terminus and 27 to 29 N-terminal residues (Rukmini et al., 2000; Schnepf et al., 1985). The cleaved fragments generated along with activated toxin, range between 10- to 35-kDa, are degraded into peptides and thus have no role in toxicity.

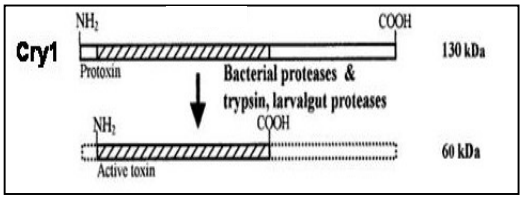

Fig 1-3. Activation of Cry1 toxin (Rukmini et al., 2000).

The proper proteolytic cleavage of ***B. thuringiensis*** delta endotoxin is the pre requisite for toxicity. An insufficient processing or over digestion of a toxin leads to its inactivation or loss of activity (Lightwood et al., 2000). For example, *Plodia interpunctella* (Indian meal moth) which is resistant to the delta endotoxins of ***B. thuringiensis*** sub sp. *entomocidus* HD-198 exhibited a lower protoxin activation rate than susceptible insects because of the decrease in the total proteolytic activity of the gut extract (Lightwood et al., 2000). The development of Bt resistance in *P. interpunctella* was reported due to the lack of major Bt protoxin activating gut protease (Oppert et al., 1997, Sayyed et al., 2005). Inagaki et al, (1992) demonstrated that in *Spodoptera litura,* overdigestion of the toxins of *Bacillus thuringiensis* HD1, is the cause of the lack of potency of HD-1 against this pest. The decreased activity of the Cry1C to *S. litura* larvae was also due to the degradation of toxin by proteases (Keller et al., 1996). The efficiency of midgut proteases possessed by insect is therefore a major determinant of toxin potency. Therefore, studies related to the gut protease activities of the individual insect are extremely important in order to understand the proteolytic activation of Bt toxins and its successful usage in pest management.

The activated toxin obtained after the proteolytic cleavage, perform two major functions- receptor binding and ion channel formation. The activated toxin binds to specific receptors on the apical membrane of the midgut epithelium of susceptible insects. Ligand blot analysis has been

employed to identify the toxin-binding proteins of brush border membrane vesicles (BBMVs) prepared from the midguts of the susceptible insects. Different types of molecules, i.e., membrane bound aminopeptidase N, cadherin-like protein (Gahan et al., 2001; Hara et al., 2003; Nagamatsu et al., 1999; Vadlamudi et al., 1995), anionic glycoconjugate (Valaitis et al., 2001), Glycolipids (Griffitts et al., 2005) and alkaline phosphatase (Fernandez et al., 2006; Jurat-Fuentes and Adang, 2004) in the insect midgut were found to serve as receptors for the Cry toxins.

1.4. Receptors involved in binding of Cry toxins:

1.4.1. Aminopeptidase N (APN)

APN is the most extensively studied receptor proteins identified and isolated from different lepidopteran insect pests. Membrane bound APNs are well-characterized ecto-enzymes that are widely distributed in animal tissues and have different functions like acting as receptors, cell adhesion molecules and signal transduction components (Taylor, 1993). They belong to the sub family of gluzincins of zinc binding metalloprotease/ peptidase super family. In the larval midguts, APNs along with the endo and carboxy peptidases present in the gut, help in digesting the proteins derived from insect diet (Pigott and Ellar, 2007). APNs play a major role as Cry toxin receptors. They are attached to the membrane by a glycosylphosphotidylinositol (GPI) anchor. Since it was first known that Cry1Ac binds to APNs, many APN receptors were isolated and characterized (Gill and Ellar, 2002). APNs have been classified into 5 classes whose sequence identity varies from 56% to 67% on an average. The 170 kDa APN from *H. virescens* and 120 kDa APN from *Manduca sexta* (belonging to Class I APNs) play a role in pore formation in membrane vesicles (Luo et al., 1997) and in channel formation in planar lipid bilayers (Schwartz et al., 1997), respectively. The interaction of 120 kDa APN from *Manduca sexta* with Cry1Ac is probably the best studied. The Cry1C interaction with APN receptors was established by gene silencing using a double stranded

RNA in *S. litura* (Rajagopal et al., 2002). Development of resistance in *S. exigua* towards Cry1Ca was attributed to the lack of APN gene expression (Herrero et al., 2005). Though 17 different APNs have been reported to bind to different Cry toxins, only 2 are shown to be involved in mediating toxin susceptibility. However, the *in- vivo* as well as *in-vitro* experiments prove that APNs play significant role in establishment of the toxic and lytic effect in the insect gut caused by Cry toxins (Gill and Ellar, 2002).

1.4.2. Alkaline phosphatase (ALP)

ALPs also act as Cry toxin receptors. Though, there are predictions on the involvement of alkaline phosphatase (ALP) in the Cry toxin action (Fernandez et al., 2006; Jurat-Fuentes and Adang, 2004), none of the ALP receptors have been characterized and shown to have any direct role in eliciting toxic effect on the susceptible insects. Cry1Ac was shown to interact with 65 kDa ALP in *M. sexta* (McNall and Adang, 2003) and 68 kDa ALP in *Heliothis virescens* (Jurat-Fuentes and Adang, 2004) and 65 kDa ALP interaction with Cry11Aa was shown in *Aedes aegypti*. ALP is attached to the membrane by a GPI- anchor, similar to APN (Eguchi, 1995).

1.4.3. Cadherin:

Cadherins are highly diverse group of proteins and are involved in variety of functions like cell adhesion, migration, cytoskeletal organization and morphogenesis (Pigott and Ellar, 2007). The role of cadherin-like protein as Cry toxin receptor has been confirmed by a number of studies. For example, disruption of a cadherin gene by a retrotransposon-mediated insertion has been associated with the high level resistance of *H. virescens* to Cry1Ac toxin (Gahan et al., 2001). Expression of a cadherin-like protein from *Bombyx mori* on the surface of Sf9 cells made these otherwise resistant cells, sensitive to Cry1Aa (Nagamatsu et al., 1999). Binding of Cry1Aa and Cry1Ac to BtR175 was inhibited after pretreatment with anti-BtR175 antibody, as this treatment

suppressed the lytic activity of the Cry toxins on collagenase-dissociated midgut epithelial cells of *B. mori* (Hara et al., 2003). Above reports suggest that the cadherin-like protein plays an important role in Cry toxin susceptibility and it is a functional Cry toxin receptor in the insect midgut at the genetic and cellular levels. Recently, a model of the sequential participation of cadherin receptor and APN leading to Cry1A toxin insertion into lipid rafts was proposed (Bravo et al., 2004). Competition binding studies demonstrated a correlation between insecticidal activity and toxin affinity (Van Rie et al, 1990). On contrary, Wolfersberger (1990) showed that Cry1Ab, despite exhibiting a relatively weaker binding affinity than Cry1Ac, was more active against gypsy moth larvae. Liang et al, (1995) confirmed that the affinity of toxin was not directly related to toxin activity and also proved a direct correlation between the toxicity and irreversible binding rate.

1.5.Transgenics:

The first transgenic plant expressing Cry toxin was tobacco, developed by Plant Genetic Systems (Belgium) in the year 1987 (Vaeck et al., 1987). Insecticidal property exhibited by Cry toxins of *Bacillus thuringiensis* (Bt) is a widely established phenomenon (Bravo et al., 2007). Since their inception into markets in 1996, *Bt* crops became an integral component of pest management practices in many countries (Sander et al., 2010). Plants engineered to express *cry* genes encoding the *B. thuringiensis* crystal proteins are the most widely cultivated transgenic crops. Many transgenic crops have been successful (Vaeck et al., 1987). In 2012, 170 million ha of genetically modified crops were grown worldwide out of which 58 million were contributed by Bt transgenic crops. Few examples of Bt transgenics which are commercially available are Bollgard cotton expressing Cry1Ac (Monsanto), Maize expressing Cry1Ab (Syngenta), Widestrike cotton expressing both Cry1F + Cry1Ac (Dow Agrosciences) and Bollgard II cotton expressing Cry1Ac + Cry2Ab2 (Monsanto). Cry1EC expressed in tobacco and cotton leaves demonstrated complete

mortality of *S. litura* and growth retardation in *Helicoverpa armigera* (Singh et al., 2004). Though transgenic brinjal, cabbage and other vegetable crops are engineered with insect resistance they are yet to get approval from DBT Govt. of India. Hence, the potential of Cry toxin is not completely exploited due to growing public concern on usage of genetically manipulated foods and also due to possible adverse influence of transgenic plants on environment, popular non-target insect species and human health (de Vendômois et al., 2010). This has led to exploration of new avenues in terms of ecological safety and toxicity.

1.6. Biopesticides:

The next alternative strategy employed was the use of Cry toxins as biopesticides. Since the use of Sporine, the first Bt product used in France in 1930's, the markets were flooded with variety of Bt insecticides containing mixture of spores and parasporal crystals. By 1999, Bt constituted 80% of all the bio-pesticides sold worldwide. Bt sprays were mostly targeted at the agriculture pests, mainly lepidopteran insects, infecting commercially and economically important crops. Bt sprays were used sporadically in crop fields as well as on the stored commodities to guard from pest infestation. The advantages of using Bt based products include their being natural products and being cost effective. Bt bioinsecticides have been successful under the traded names Dipel and Thuricide (Schnepf et al., 1998). *Bacillus thuringiensis kurstaki* HD1 became the basis for most of the Bt products and has been so far the, the greatest commercial success of agricultural pest control. The disadvantages of using Bt spray as an insecticide include 1) The accumulation of Cry toxins in the soil 2) their effect on non- target soil dwelling organisms 3) lack of effective Cry toxin content in formulations 4) Bt spray cannot be applied uniformly to all parts of the plant, 5) it cannot be delivered to pests that are inside plant tissues, and 6) Bt is susceptible to rapid degradation by UV light and removal by water runoff (Gatehouse et al., 2011). The effectiveness

of the Bt based bio-pesticide solely depends on the content and stability of the Cry protein. In the native host, *Bacillus thuringiensis*, these Cry toxins are synthesized in limited quantities only under certain physiological conditions (Bravo et al., 2007).

1.7. Expression of Cry toxins in GRAS organisms.

The increased resistance to chemical insecticides, growing concerns over the deleterious effects of chemicals on environment have provided a strong impetus for the development of alternative pest control strategies. Growing public grievance against engineering the edible crops and the impact of transgenic crops on popular non-target insect species have led to explore new avenues in terms of ecological safety and toxicity. The expression of the Cry toxins in GRAS (Generally Regarded As Safe) organisms like *E. coli*, *Pseudomonas fluorescens* would be a better alternative strategy for integrated pest management. *Pseudomonas fluorescens* has a number of traits that are advantageous for the fermentative production of recombinant proteins (Retallack et al., 2012). The usage of engineered bacteria has the advantage of having estimated and adequate activity required for the particular market. The bio insecticide thus produced will be consistent and is cost effective in terms of fermentation yields. This work aimed at the high level expression of active and soluble form of Cry toxins in GRAS organisms like *E. coli* and *Pseudomonas fluorescens.*

1.8. Use of Cry toxin encapsulated nanoparticles as Biopesticides:

The delivery of Cry toxins through nano-particles is the most possible alternative to alleviate the problems posed generally by Bio-pesticides like the issues of accumulation and persistence of Cry toxins in the environment and their stability. The high surface to volume ratio and surface energy of nano-pesticides facilitates the attachment and penetration of an effective agent on the surface of plant. As a result, the efficacy could be significantly increased by the

application of nano-pesticides. In some cases, controlled release technology can also contribute to the advantages of nano-pesticides. Therefore, it is believed that the production of nano-pesticides will result not only in the use of smaller amount but also fewer applications. Further, the nano-pesticides are environmentally friendly, provide new opportunities and have wide application prospects for pesticide research (Song et al., 2012). As the application of nanoparticles on the leaf and stem surface neither alter photosynthesis and respiration of crop plants, nor do they cause any alteration of gene expression in insect trachea, they are qualified as potential nano-biopesticides (Ghormade et al., 2011). The usage of amorphous silica is considered to be safe for humans by World Health Organization and US Department of Agriculture (Ghormade et al., 2011). The present work is therefore aimed to deliver Cry toxin through mesoporous silica nanoparticles so as to increase its sustained release to the target insects. In this project, the principles of nanomaterials is converged with the recombinant DNA technology, to generate 'CRYSONES' the mesoporous SiO_2 nanoparticles containing Cry toxin, to be used as an effective pest control agents. Mainly this research is focused on key pests affecting edible and commercially important crops, principally Lepidopteran insects, especially Spodoptera (Lewis et al., 1997).

1.9. *Spodoptera*

Currently, 90% of the microbial insect control products are based on *B. thuringiensis* (Escriche et al., 1995). At present, *B.thuringiensis* sp. *kurstaki* HD-1 is the most useful insecticidal strain because it exhibits toxicity against various *Lepidopteran* larvae. However, it is not effective against larvae of *Spodoptera* species (Bai et al., 1993) such as the beet armyworm, *S. exigua*, and the tobacco cutworm, *S. litura*, which affect many economically important crops (Moar et al., 1995; Kalman et al., 1995). But *B.thuringiensis* ssp. *kenyae* (Visser et al 1990; Masson et al., 1992), ssp. *Darmstadiensis*(Chambers et al., 1991), ssp. *Aizawai* (Sanchis et al.,1989) and ssp.

Galleriae (Kalman et al., 1993), all of which harbor either *cry1C* or *cry1E* gene, showed significant activity against *Spodoptera* species**.**

The genus *Spodoptera* contains five species (*litura, littoralis, exigua, frugiperda* and *exempta*), that occur throughout the world. They affect major crops like cotton, tobacco, castor, groundnut, a number of grain legumes and vegetable crops. Peanut (*Arachis hypogaea*) is an important oilseed crop. Its yield is vulnerable to a variety of abiotic and biotic stresses (Ghewande et al., 2002a). Among the biotic stresses, insect pests such as, *Spodoptera litura*, *Aproaerema modicella*, *Amsacta* spp., *Heliothis* spp., aphids, jassids, thrips and termites cause major yield losses. *S. litura* (tobacco cutworm/ tobacco caterpillar/ tobacco armyworm) causes the most serious insect damage to peanut crops in the southern part of India with yield losses up to 71% (Prasad and Gowda 2006). *S. litura* causes maximum damage at flowering and fruiting stages. Chemical, biological and mechanical methods are used either singly or in combination for pest management (Ghewande et al., 2002b). However, these strategies have limitations such as environmental safety, economic constraints and emergence of resistance in pests. The Cry toxins produced by Bt strains have been deployed to control *Spodoptera* sp. (Shelton et al., 2002; Naimov et al., 2003). Even at lower concentrations, the Cry1C has been reported to show significant toxicity to *Spodoptera* sp. (Hone´e et al., 1988; Hofte and Whiteley, 1989; Gill et al., 1992; Kalman et al., 1993). Transgenic plants expressing Cry1Ca at a high levels were reported to cause mortality and conferred protection against *Spodoptera* sp. (Strizhov et al., 1996). At relatively higher LC_{50} values, a few other proteins that resemble Cry1C (Visser et al., 1990; Kalman et al., 1993) and Cry1F (Chambers et al., 1991) have been reported to show toxicity to *Spodoptera* sp. The Cry1E is almost 70 % similar to Cry1C. It is not very effective against *S. exigua* (Visser et al., 1990; Masson et al., 1992; Bosch et al., 1994) but binds to receptors different from Cry1C. There

are not many reports on the toxicity of the *δ*-endotoxins to *S. litura* which is a common pest in warm and humid climates. A novel chimeric *δ*-endotoxin Cry1EC was designed specifically against *S. litura* by replacing amino acids 530–587 in a poorly active Cry1Ea protein with a homologous 70 amino acid stretch of Cry1Ca in domain III (Singh et al., 2004). The hybrid *δ*-endotoxin *cry1EC* expressed in tobacco has shown greater toxicity than the native toxins against *S. litura*.

1.10. Objectives of the present study:

1. Screening of *Bacillus thuringiensis* isolates from Directorate of Oilseed Research (DOR) culture collection center, Acharya N.G. Ranga Agricultural University, Hyderabad, for identification of strains producing Cry toxins effective against *Spodoptera litura.*
2. Construction of chimeric *cry1EC* gene and evaluation of chimeric Cry1EC toxicity against *Spodoptera litura*.
3. Heterologous expression of *cry1EC* gene in *Pseudomonas fluorescens* and evaluation of its efficacy to control *Spodoptera litura.*
4. Preparation and characterization of CRYSONES, Cry1EC toxin loaded mesoporous silica nanoparticles and their effectiveness on *Spodoptera litura*.

Materials and methods

2.1. Materials

2.1.1. Antibiotics

Name of the antibiotic	Name of the Supplier
Ampicillin sodium salt	HIMEDIA
Kanamycin Sulfate	HIMEDIA
Tetracycline hydrochloride	HIMEDIA
Chloramphenicol	HIMEDIA
Streptomycin	HIMEDIA
Gentamycin	HIMEDIA
Rifampicin	HIMEDIA

2.1.2. Chemicals

Name of the chemical	Name of the supplier
Absolute alcohol	HAYMAN
Acetic acid (Glacial)	Qualigens
Acrylamide	Merck
Agar	Himedia
Agarose	SeaKem
Ammonium persulphate	Sigma Aldrich Chemicals
Ammonium nitrate	Qualigens
Ammonium sulfate	Qualigens
Boric acid	Qualigens
Bovine serum albumin	GE Healthcare Lifesciences, USA
Bromophenol blue	GE Healthcare Lifesciences, USA
Butanol	Qualigens
Calcium chloride	Qualigens
Calcium nitrate	Qualigens
Chloroform	Qualigens

Coomassie Brilliant Blue G-250	SRL
Deoxynucleotide triphosphates	MBI Fermentas
Dimethyl sulfoxide (DMSO)	Sigma Aldrich Chemicals
Dipotassium hydrogen orthophosphate	Qualigens
N,N-Dimethylformamide	Merck
Ethidium bromide	SRL
EDTA (Ethylene diamine tetraacetic acid)	SRL
EGTA (Ethylene glycol tetraacetic acid)	SRL
Formaldehyde	Merck
Glutathione (GSH)	Sigma Aldrich Chemicals
Glycerol	Qualigens
Glycine	SRL
HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid))	USB
Hydrochloric acid	Qualigens
Imidazole	SRL
Isopropanol	SRL
Isopropyl thiogalactopyranoside (IPTG)	SRL
Lysozyme	Bangalore GeneI
Mannitol	Qualigens
Manganese chloride	SRL
Magnesium sulphate	SRL
Methanol	SRL
N,N'-Methylene bis acrylamide	SRL
Methylene blue	Sigma Aldrich Chemicals
β-mercaptoethanol	Sigma Aldrich Chemicals
Nickel chloride	Merck
Paraplast	Sigma Aldrich Chemicals
Peptone	HIMEDIA
Phenol Saturated	Bangalore GeneI

Piperazine-N, N'-bis[2-ethanesulfinic acid (PIPES)	SRL
Potassium chloride	Qualigens
Potassium dihydrogenortho phosphate	Qualigens
Potassium tellurite	Sigma Aldrich Chemicals
PMSF (phenylmethanesulfonylfluoride)	Sigma Aldrich Chemicals
Protein molecular weight marker	MBI Fermentas
Silver nitrate	SRL
Sodium acetate	Qualigens
Sodium benzoate	Sigma Aldrich Chemicals
Sodium carbonate	SRL
Sodium citrate	SRL
Sodium chloride	SRL
Sodium dihydrogen orthophosphate	Qualigens
Sodium dodecyl sulfate	SRL
Sodium hydroxide	SRL
Sodium Sulphate	Merck
Sucrose	Sigma Aldrich Chemicals
Tetra ethyl methylene diamine (TEMED)	Sigma Aldrich Chemicals
Tris-base	Sigma Aldrich Chemicals
Trichloroacetic acid	Sigma Aldrich Chemicals
Trifluoroacetic acid	Sigma Aldrich Chemicals
Tryptone	HIMEDIA
Tween-20	Bangalore GeneI
Xylene	Qualigens
X-gal (5-bromo-4-chloro-indolyl-β-D galactopyranoside)	Sigma Aldrich Chemicals
Yeast extract	HIMEDIA

2.1.3. Restriction enzymes and other DNA modifying enzymes.

Name of the Enzyme	Name of the Supplier
*Apa*I	MBI, Fermentas
*Bam*HI	MBI, Fermentas
*Bgl*II	MBI, Fermentas
*Dra*I	MBI, Fermentas
*Eco*RI	MBI, Fermentas
*Eco*RV	MBI, Fermentas
*Hind*III	MBI, Fermentas
*Not*I	MBI, Fermentas
*Pst*I	MBI, Fermentas
*Sma*I	MBI, Fermentas
*Xho*I	MBI, Fermentas
*Xmn*I	MBI, Fermentas
Klenow fragment	MBI, Fermentas
Proteinase K	MBI, Fermentas
RNase A	MBI, Fermentas
T4 DNA Ligase	MBI, Fermentas
Taq DNA polymerase	MBI, Fermentas
Pfu DNA polymerase	MBI, Fermentas

2.1.4. Bacterial strains used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference or Source
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Hanahan, 1983
E. coli BL21 DE3	*hsdS gal(λcIts857 ind1 sam7 nin5lacUV5 T7 gene 1*	Studier and Moffat, 1986

E. coli S 17- 1	*TpRSmRrecA, thi, pro, hsdR-M^{+}RP4: 2-Tc:Mu: Km Tn7 λpir.*	Simon, 1983
E.coli pir-116	*F^{-}mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galUgalK λ- rpsL (Str^{R}) nupG pir-116(DHFR)*	Epicenter^{R} Biotechnologies, USA
E.coli Arctic Express	*F^{-} ompThsdS(r$_{B}^{-}$ m$_{B}^{-}$) dcm+ Tetr gal λ(DE3) endA Hte [cpn10cpn60 Gentr], pLysS,Gm^{R}*	Stratagene
Pseudomonas fluorescens ATCC13525	Amp^{r}, Cm^{r}	Buch et al., 2009

2.1.5. Plasmids used in this study.

Plasmids	**Genotype or Phenotype**	**Reference or Source**
pTZ- 57 R	Ap^{r}, *lacZ*$^{+}$, TA cloning vector	Fermentas
pTZ-T7	*T7 polymerase* cloned in TA vector, Ap^{r}	This work
pTZ-tac	*tac* promoter cloned in TA vector, Ap^{r}	Our laboratory
pTZ-EC	*cry1EC* cloned in TA vector, Ap^{r}	This work
pGEMT-Gm	Gentamycin cassette cloned in TA vector, Gm^{r} and Ap^{r}	Our laboratory
pGEMT-Easy	Ap^{r}, *lacZ*$^{+}$, TA cloning vector	Promega
pGEX4T1	Ap^{r}, expression vector	Amersham
pSKS1	*cry1EC* cloned in pGEX4T1 as *Bam*HI fragment inframe with the N- terminal GST- tag.	This study

pUC19	Ap^r, expression vector	Amersham
pSKS2	*T7 polymerase* cloned as *Bam*HI and *Hind*III fragment in pUC19	This study
pSKS3	*tac* promoter cloned in pSKS2 as *Bam*HI and *Bgl*II fragment	This study
pSKS4	*Gentamycin* cassette cloned in pSKS3 as *Eco*RI fragment	This study
pSKS5	*phb* gene cloned in pGEMT- Easy vector	This study
pSKS6	Gentamycin-*T7 pol* cassette cloned in pTZ	This study
pSKS7	Gentamycin-*T7 pol* cassette cloned within *phb* gene in pSKS6	This study
pSUP202	Mob+, Amp^r, Cm^r, Tc^r and mobilizable suicidal vector	Simon et al., 1983.
pSKS8	Gentamycin-*T7pol* cassette along with *phb* flanking region taken as *Not*I fragment, made blunt and cloned in *EcoR*V digested pSUP202.	This study
pSKS9	Ampicillin gene inactivated in pSKS8 by digestion with *Pst*I, blunted and religated.	This study
pMMB206	Cm^r, broad host range mobilizable vector	Morales et al., 1991
pR6KS	Km^r, broad host range mobilizable vector with high copy number in *E. coli* pir116	This study
pR6KTS	Km^r, *T7* promoter based broad host range mobilizable vector	This study
pRSET A	Ap^r, expression vector	Invitrogen
pSKS10	*cry1EC* cloned in pRSETA as *Bam*HI and *Bgl*II fragment inframe with the N- terminal His- tag.	This study
pSKS11	*cry1EC* along with N terminal His tag was taken as *Bgl*II fragment and cloned in *Bam*HI digested pR6KS.	This study
pSKS12	*cry1EC* cloned in pR6KTS as *Bam*HI fragment inframe with the N- terminal His- tag.	This study

2.1.6. Primers used in this study.

Primers used for screening the *Bacillus thuringiensis* culture collection from DOR.

Primers	Sequence	Purpose
I(-)	5’ MDATYTCTAKRTCTTGACTA 3’	To amplify *cryI* family specify gene region
I(+)	5’ TRACRHTDDBDGTATTAGAT 3’	To amplify *cryI* family specify gene region
IA	5’ CAATAGTCGTTATAATGATT 3’	To amplify *cryIA* gene specific region
IAa	5’ TTCCCTTTATTTGGGAATGC 3’	To amplify *cryIAa* gene specific region
IAb	5’ CGGATGCTCATAGAGGAGAA	To amplify *cryIAb* gene specific region
IAc	5’ GGAAACTTTCTTTTTAATGG 3’	To amplify *cryIAc* gene specific region
IAd	5’ ACCCGTACTGATCTCAACTA 3’	To amplify *cryIAd* gene specific region
IB	5’ GGCTACCAATACTTCTATTA 3’	To amplify *cryIB* gene specific region
IC	5’ ATTTAATTTACGTGGTGTTG 3’	To amplify *cryIC* gene specific region
ID	5’ CAGGCCTTGACAATTCAAAT 3’	To amplify *cryID* gene specific region
IE	5’ TAGGGATAAATGTAGTACAG	To amplify *cryIE* gene specific region
IF	5’ GATTTCAGGAAGTGATTCAT 3’	To amplify *cryIF* gene specific region
IG	5’ GGTTCTCAAAGATCCGTGTA 3’	To amplify *cryIG* gene specific region

The other primers used in this study

Primers	Sequence(Forward (F*)/ Reverse (R*))	Purpose
SDS01 SDS02	5' GAGCGAATTCATGGAGATAGTGAATAATCAGAA 3' 5' GCTCGGATCCTCGTGCATCCCTACTGGAAG 3'	To amplify 1588 bp of *cryIEa* gene
SDS03 SDS04	5' GAGCAGATCTGTTATAGTATTAACAGGAGCG 3' 5' GCTCGGATCCGCTACTAATAGAACCTGCACC 3'	To amplify 133 bp of *cryICa* gene

SDS05 SDS06	5' GAGCAGATCTGGTGAGCTTTATATAGATAAAATTGA 3' 5' GCTCGGATCCTCATTATAGCCCTAGTTGATTTGT 3'	To amplify 210 bp of *cryIEa* gene
SDS07 SDS08	5' GAGCGGATCCATGGAGATAGTGAATAATCAGAA 3' 5' GCTCGGATCCTCATTATAGCCCTAGTTGATTTGT 3'	To amplify *cryIEC* gene.
SDS09 SDS010	5' GAGACCCGGGGCTTCGTTAATACAGA 3' 5' TCTCCCCGGGTTAGAAAAACTCATCGAGCATC 3'	To amplify R6Kγ and *kanamycin* gene
SDS011 SDS012	5' GCTCGGATCCAATTTATCAGATCCAATAGGAGG 3' 5' TTACGCGAAGCTTAACGCGAAGTCCGACTCTAA 3'	To amplify *T7 polymerase* gene
SDS013 SDS014	5' GATCTCGATGGGCCCATTAATACGA 3' 5' ATCTACGAANNNNTTCTATTCGCTATTACGC 3'	To amplify *T7* promoter system
SDS015	5' GATCTATCTAGACGCCAGCCAGGACAGAAATGCC 3'	Forward primer of *gentamycin* gene
SDS016 SDS017	5' GATCAGTGCGGCCGCATGAAAACGCTAAAA 3' 5' ACTATCGCGGCCGCAGCAGGTCGACCAT 3'	To amplify 200 bp of *phb* gene
SDS018 SDS019	5' AACAAGATCTAGGGAGACCACAACGGTTTCCC 3' 5' CCTTAGATCTCTAGTTATTGCTCAGCGGTGGC 3'	To amplify *cryIEC* along with N terminal His-tag.
SDS020 SDS021	5' GAGCGGATCTTGACAATTAATC 3' 5' GAGCGGATCCGTATATCTCCTTCGAGC 3'	To amplify *tac* promoter region

2.1.7. Media components and antibiotics.

Luria Bertani (LB) broth

The LB medium was prepared by dissolving 10 g of peptone, 5 g of yeast extract and 10 g of NaCl in 500 ml of distilled water. The contents were stirred and finally made up to 1 litre with distilled water. The pH of the medium was adjusted to 7.6 with 1N NaOH and then sterilized by autoclaving. The LB agar plates were prepared by adding 1.5% agar to LB broth.

Ampicillin

Ampicillin stock solution was prepared by dissolving 250 mg of ampicillin in 10 ml of double distilled water and then filter sterilized. The sterilized solution was stored in aliquots at -

20°C. When required, ampicillin stock solution was added to the medium after cooling it to 45°C, to get the working concentration of 100 µg/ml.

Kanamycin

Kanamycin stock solution was prepared by dissolving 30 mg of kanamycin in 1 ml of double distilled water and then filter sterilized. The sterilized solution was stored in aliquots at -20°C. When required, kanamycin stock solution was added to the medium after cooling it to 45°C to get the working concentration of 30 µg/ml.

Gentamycin

Gentamycin stock solution was prepared by dissolving 20 mg of gentamycin in 1 ml of double distilled water and then filter sterilized. The sterilized solution was stored in aliquots at -20°C. When required, gentamycin stock solution was added to the medium after cooling it to 45°C to get the working concentration of 20 µg/ml.

Chloramphenicol

Chloramphenicol stock solution was prepared by dissolving 30 mg of chloramphenicol in 1 ml of absolute alcohol and then filter sterilized. The sterilized solution was stored in aliquots at -20°C. When required, chloramphenicol stock solution was added to the medium after cooling it to 45°C, to get the working concentration of 30 µg/ml.

Streptomycin

Streptomycin stock solution was prepared by dissolving 20 mg of streptomycin in 1 ml of double distilled water and then filter sterilized. The sterilized solution was stored in aliquots at -20°C. When required, streptomycin stock solution was added to the medium after cooling it to 45°C, to get the working concentration of 20 µg/ml.

Rifampicin

Rifampicin stock solution was prepared by dissolving 50 mg of rifampicin in 1 ml of isopropanol and stored in aliquots at -20°C. When required, rifampicin stock solution was added to the medium, after cooling it to 45°C to get the desired working concentrations.

2.1.8. Buffers and solutions

2.1.8.1. Solutions for plasmid isolation

Solution I

10 mL of 250 mM glucose, 6.25 mL of 0.2 M Tris pH 8.0, 1 ml 0.5 M EDTA (pH 8.0), were dissolved in 25 ml sterile millQ water and finally the volume was made up to 50 ml with sterile milliQ water. This solution was autoclaved for 20 min at 15 psi on liquid cycle and stored at 4^0C. Whenever required stock solution of DNase free RNAse A was added at a final concentration of 100 µg/ ml.

Solution II

Equal volumes of 0.4 N NaOH and 2% SDS solutions were freshly mixed and used as Solution II.

Solution III

To prepare Solution III, 24.61 g of sodium acetate was dissolved in 80 mL of sterile milliQ water and its pH was adjusted to 4.8 with glacial Acetic acid. Finally the volume of the solution was made upto 100 mL to get a final concentration of 3 M and stored at 4^0C.

Phenol chloroform solution

Phenol chloroform solution was prepared by mixing equal volumes of water saturated phenol and chloroform.

Chloroform: Isoamyl alcohol solution

96 mL of chloroform and 4 mL of Isoamyl alcohol were mixed and stored in amber colour bottle at 4^0C.

TE buffer

TE buffer was prepared by dissolving 121 mg of Tris and 37.2 mg of EDTA (pH 8.0) in 80 ml of distilled water. The pH of the buffer was adjusted to 8.0. Finally the volume was made up to 100 ml with distilled water to get the concentration of 10 mM Tris and 1 mM EDTA.

2.1.8.2. Solutions for Agarose gel electrophoresis

Tris Acetic acid EDTA (TBE) buffer

A stock solution of 10 X TBE buffer was prepared by adding 108 g of Tris, 55 g of boric acid and 40 ml of 0.5 M EDTA to 900 ml of distilled water and dissolved properly. The final volume was made up to 1000 ml with distilled water. A working stock for use was prepared by making up 100 ml of stock TBE buffer (10 X) to one litre with distilled water.

Sample Loading buffer (6X) for Agarose gel electrophoresis

5 mg of bromophenol blue, 25 mg of xylene cyanol and 4 g of sucrose were dissolved in few ml of distilled water and finally the volume was made up to 10 ml and stored at 4°C.

2.1.8.3. Solutions for transformation

IPTG

1M IPTG stock solution was prepared by dissolving 236.8 mg IPTG in 1 ml of autoclaved milli Q water and stored as aliquots of 100 µl at -20°C. When required the stock solution was thawed on ice bath and 100 µl of stock solution was added to 100 ml of cooled medium (45°C) to get 1 mM working concentrations of IPTG.

X-Gal

4% of X-gal stock solution was prepared by dissolving 40 mg of X-gal in 1 ml of N, N' -dimethylformamide. When required 100 µl of stock solution of 4% (w/v) X-gal was added to 100 ml of medium after cooling it to 45°C.

2.1.8.4. Solutions for SDS-PAGE

Acrylamide solution (30%)

100 ml acrylamide solution was prepared by dissolving 30 g acrylamide, 0.8 g N, N-methylene-bis-acrylamide in 70 ml of distilled water. The contents were then filtered and the solution was finally adjusted to 100 ml. The stock solution thus prepared was stored at 4ºC until further use.

Stacking gel buffer

Tris (3.93 g) was dissolved in 50 ml of double distilled water and the pH of the solution was adjusted to 6.8 using 1N HCl and finally the volume of the buffer was made up to 100 ml with distilled water.

Running gel buffer

Tris (59.93 g) was dissolved in 400 ml of distilled water and pH of the solution was adjusted to 8.8 using 1N HCl. Finally the volume of the buffer solution was made up to 500 ml using distilled water.

Tank buffer (pH 8.5)

Tank buffer (1X) was made by dissolving 3.03 g of Tris, 14.4 g of glycine and 1 g of SDS was dissolved in 500 ml of distilled water. Finally, the volume of the buffer was made up to 1000 ml. The buffer at 1X concentration contains 0.025M Tris, 0.192M glycine and 0.1% SDS.

Sample loading buffer for SDS-PAGE

1.2 ml of 0.5 M Tris (pH 8.0), 2 ml of 10% SDS, 1 ml of 10% glycerol, 0.5 ml of β-mercaptoethanol, 0.001 g of bromophenol blue were taken in a 10 ml reagent bottle. The contents were mixed well before the volume was made up to 10 ml and solution was stored at 4°C until further use. When necessary adequate amounts of loading buffer was used to prepare protein samples.

Staining solution

Coomassie brilliant blue (0.2 g) was dissolved in 30 ml of methanol. To this 10 ml of acetic acid was added and finally the volume was made up to 100 ml using distilled water. The contents were stored at room temperature in amber color bottle until further use.

Destaining solution

About 30 ml of methanol was mixed with 10 ml of glacial acetic acid before making up the volume to 100 ml using distilled water. The contents were freshly made and used to destain the SDS-PAGE gels.

Protein Markers

Low molecular weight protein markers supplied by MBI Fermentas were used. Size of the protein markers include, 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa.

2.1.8.5. Solutions for western blotting

Protein transfer buffer

Tris (3.03 g) and 14.4 g of glycine were dissolved in 650 ml of distilled water. To this, 200 ml of methanol was added and final volume was made up to 1000 ml with distilled water. The buffer was stored at 4^{o}C until further use.

TBS-T Buffer

Tris (2.42 g) and 8 g of sodium chloride were dissolved in 500 ml of distilled water. The pH of the solution was adjusted to 7.6 using 1N HCl before adding 1ml of 0.1% Tween-20. Finally the volume of the buffer solution was made up to 1000 ml with milli Q water.

Blocking Buffer

Blocking buffer was prepared by adding 10 g of skimmed milk powder to 10 ml of 1X Tris buffered saline with 0.1% Tween-20 and the contents were stirred well. The solution was prepared fresh before blocking the membrane.

Poncaeau

100 mg of Ponceau salt was dissolved in 100 ml of 5 % acetic acid.

Phosphate buffered saline (PBS) buffer

1 X PBS (pH 7.3) was prepared by dissolving the following compounds; 8.18 g of NaCl, 0.2012 g of KCl, 1.4196 g of Na_2HPO_4 and 0.2449 g of KH_2PO_4 in 800 ml of double distilled H_2O. The pH of the buffer was adjusted to 7.3 before adjusting the volume to 1000 ml. The buffer was sterilized by autoclaving and stored at room temperature.

Stripping solution

Stripping solution was prepared by dissolving 1.5 g of glycine, 0.1 g of SDS and 1 ml of tween- 20 in 100 ml of double distilled water. The pH of the solution was adjusted to 2.2 with 6 N HCl.

2.1.8.6. Solutions for BBMV preparation

MET buffer

Mannitol (10.93 g), 3.4 ml of 1 M Tris- Hcl (pH 7.5) and 2.2 ml of 0.45 M EGTA were dissolved and made upto 200 ml with double distilled water to give final concentrations of 300

mM mannitol, 17 mM Tris- HCl and 5 mM EGTA. The buffer was sterilized by autoclaving and stored at room temperature.

HBS-N buffer

HEPES (0.238 g) and 0.877 g of NaCl were dissolved in 80 ml of double distilled water. The pH of the buffer was adjusted to 7.4 with 0.5 N NaOH before adjusting the volume to 100 ml. The buffer was sterilized by autoclaving and stored at room temperature.

2.1.8.7. Solutions for histological and immunological studies

Bouin's fluid

Saturated picric acid, formaldehyde and acetic acid were mixed in 15:5:1 ratio respectively.

2.2. Methodology

2.2.1. Methods for DNA manipulations

2.2.1.1. Genomic DNA isolation

1.5 ml of overnight culture was centrifuged at 12000 rpm for 30 s. The cell pellet obtained was dissolved in 1 ml of 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mg/ml lysozyme and incubated for 1 h at 37°C. 0.5% SDS was added and incubated for 5 min. 100 µg/ml proteinase K was added, gently mixed and incubated for 6h or overnight at 50°C. The DNA was extracted by gentle inversion with an equal volume of phenol chloroform for 10 min at room temperature. The DNA was centrifuged at 8000 rpm at 10-12°C for 20min and the upper aqueous layer was removed without collecting the interface. One tenth volume of 3M NaOAc and 2 Volumes of absolute alcohol was added on the top of the DNA solution. Then the sample was centrifuged at 13000 rpm for 30 min at 10°C. The DNA was washed with 70% ethanol, dissolved in 5 ml of TE buffer and stored at 20°C for further use.

2.2.1.2. Plasmid Isolation by Alkaline lysis method

Plasmid DNA Isolation was carried out by the following procedures of Birnboim and Doly (1979), and Ish-Horowicz and Burke (1981) with slight modifications. A single bacterial colony carrying plasmid to be isolated was inoculated into 10 ml of LB medium containing appropriate antibiotic and was incubated overnight at 37°C with vigorous shaking (250 rpm). 1 ml of overnight culture was centrifuged at 6000 rpm for 10 min and supernatant was discarded. Bacterial pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) by vigorous vortexing. To the above bacterial suspension 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the contents were mixed by inverting the tube 5-6 times. Then 150 µl of ice-cold solution III (3M potassium acetate, pH 4.8) was added to the above bacterial lysate and mixed by inverting the tube 4-5 times. Then tube was kept on ice for 3-5 minutes. Precipitate formed in the above mixture was centrifuged by spinning the contents at 12000 rpm for 10 min. Then the supernatant was transferred into a fresh tube and equal volumes of phenol: chloroform mixture was added. The contents were mixed by vortexing and followed by centrifugation at 12000 rpm for 10 min. Aqueous phase was transferred to a fresh tube and $1/10^{th}$ volume of 3 M sodium acetate and 2 volumes of absolute alcohol were added and tubes were kept at –20°C for 30 min. Then the tubes were centrifuged at 12000 rpm for 10 minutes at 4°C in a microcentrifuge tube to pellet down the plasmid DNA. The DNA pellet was further washed with 70% ethanol to remove traces of salts associated with plasmid. Subsequently the plasmid DNA was dried before re-dissolving it in 50 µl of TE (pH 8.0).

2.2.1.3. Purification of plasmids using QIAgen Mini preparation kit method

Plasmids were purified using QIAgen mini preparation kit especially when used for cloning and sequencing reactions. A single bacterial colony carrying plasmid was inoculated into 3 ml of

LB broth containing appropriate antibiotic and was incubated overnight at 37 °C with vigorous shaking (~150 rpm). The overnight culture was centrifuged at 13000 rpm for 1 min and supernatant was discarded. The bacterial cell pellet was resuspended in 250 µl of buffer P1 and were lysed by adding 250 µl of buffer P2 prior to mixing of the tubes by inverting them 4-6 times. After lysis of the cells the contents were neutralized by adding 350 µl of buffer N3 prior to mixing of the contents by inverting immediately for 4-6 times. Then tubes were centrifuged at 13000 rpm for 10 min to pellet down the cell debris. After centrifugation the supernatant was directly transferred to a QIAprep column placed in a collecting tube. The entire assembly was placed in a microfuge and the supernatant was allowed to pass through the column for a minute by centrifuging at 13000 rpm. The column was then washed with 750 µl of buffer PE. To remove the residual wash buffer PE, column placed in the collection tube was centrifuged at 13000 rpm for 1 minute. Finally, plasmid DNA was eluted from the column by adding 50 µl buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O to the centre of QIA preparation column followed by brief centrifugation at 13000 rpm. The plasmid DNA was stored at –20°C until further use.

2.2.1.4. Polymerase Chain Reaction

PCR amplification reactions were performed in a 100 µl volume containing 3.0 mM$MgCl_2$, dNTP mix containing 200 µM each of dATP, dCTP, dGTP and dTTP mix(Fermentas), 20picomol of each forward and reverse primers, 1.0 Unit of *Taq* polymerase or *pfu* DNA polymerase, 40 ng of plasmid / genomic DNA used as a template. Amplifications were carried out in the thermal cycler (Biorad) by suitably adjusting the PCR programme depending on the expected size of the amplicon and Tm of the primers used. Amplified products were analyzed on 0.8 % agarose gel electrophoresis.

2.2.1.5. Purification of PCR products

After performing the polymerase chain reaction (PCR), the total PCR reaction mixture (100 µl) was taken into an eppendorf tube and the PCR product was purified using QIAgen PCR purification kit by following the manufactures protocol. The 100 µl PCR reaction mixture was mixed with 500 µl of buffer PB (binding buffer) and passed through Qia quick spin column fitted in a collecting tube. The flow through was discarded and the QIA quick spin column was washed with 0.75 ml of PE (wash buffer). The column was then placed in a new eppendorf tube and subjected for brief centrifugation to remove the traces of ethanol. The column was then placed in a 1.5 ml eppendorf tube and the DNA was eluted by adding 50 µl of buffer EB (10 mM Tris- Cl, pH 8.5).

2.2.1.6. Agarose gel electrophoresis

Agarose gel electrophoresis was performed by the following standard procedures described by Sambrook *et al*., 1989. Required amount of agarose was dissolved in TBE by heating in microwave oven. The solution was then cooled to 50 - 55°C and poured on a clean sterile gel tray fitted with combs. The gel was allowed to solidify at room temperature. After solidification the gel along with the gel tray was immersed in the TBE buffer poured in the electrophoretic tank. Buffer level was adjusted to cover the gel to a depth of about 1 mm. Appropriate amount of DNA was mixed with 4 µl of 10 X loading buffer (0.25 % bromophenol blue, 0.25% xylene cyanol, 40% sucrose) and loaded into the wells of submerged gel. Electrophoresis was carried out at 100 volts till the bromophenol blue reaches the end of the gel. The gel was then stained (0.5 µg/ml ethidium bromide in water) and destained (distilled water) before visualizing under UV transilluminator. The electrophoretic mobility of DNA was recorded by taking the photograph of the gel using gel

documentation system. The size of the unknown DNA fragments were determined by comparing their mobility pattern with that of the molecular size markers obtained from MBI, Fermentas.

2.2.1.7. Purification of DNA fragments from agarose gels

The DNA fragments were extracted from agarose gel by using QIAgen gel extraction kit. After performing agarose gel electrophoresis, appropriate portion of the gel containing desired DNA fragment was sliced, weighed and carefully taken into a microcentrifuge tube. Then 3 volumes (W/V) of buffer QG was added and incubated at 50^0C for 10 min to dissolve the gel. After the gel slice was completely dissolved, 1 volume(w/v) of isopropanol was added. Meanwhile the QIA quick column was placed in a collection tube and the contents were carefully transferred into the column. Immediately, the column fitted in collection tube was placed in a microfuge and the contents were centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was again washed with 0.75 ml of buffer PE (70 % alcohol). Finally the column was subjected to a brief spin to remove residual amount of ethanol found in buffer PE. The QIA quick column was then placed in a 1.5 ml eppendorf tube and the DNA was eluted in 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or Milli-Q water

2.2.1.8. DNA Ligation

The concentrations of vector and the insert were taken in 1:3 ratio in a reaction mixture containing 1U Ligase and 5X ligase buffer. The reaction mixture was incubated at 4˚C for 16 h for ligation. The ligated product was then transformed into appropriate cloning host or expression host.

2.2.1.9. Preparation of ultra-competent cells

Ultra Competent cells were prepared following the procedures of Inoue et al., (1990). *E. coli* cells required to make competent were grown at 18 ^{0}C in 250 ml of LB broth taken in 1 L

flask by inoculating with an initial inoculum of 1 % overnight culture. The cultures were allowed to grow (overnight) till the cell density reached to 0.4-0.5 OD at 600 nm. The culture was then chilled on ice for 10 minutes and centrifuged at 2500 g for 10 min at 4 ^{0}C to harvest the cells. The cell pellet was then suspended in 80 ml of ice cold Inoue transformation buffer [55 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 10 mM PIPES (0.5 M, pH 6.7)] and centrifuged at 2500 g for 10 min at 4 ^{0}C. The pellet was then resuspended into 20 ml of ice cold Inoue buffer and incubated on ice for10 minutes after adding 1.5 ml of DMSO. Working quickly, the cells were dispensed into sterile microfuge tubes in 100 µl aliquots and the tightly closed tubes were then snap frozen by placing them in a bath of liquid nitrogen. The tubes were stored at -70 0C until further use.

2.2.1.10. Transformation

Cells to be transformed were inoculated for overnight. 1% of overnight culture was inoculated for log phase. 1ml of this culture was taken and centrifuged at 6000 rpm for 5 min. Supernatant was discarded. Pellet was resuspended in 500 µl of ice cold 100 mM $CaCl_2$ and kept on ice bath for 30 min. It was then re-centrifuged at 6000 rpm for 5 min. Supernatant was discarded. Pellet was resuspended in 100 µl of 100 mM ice cold $CaCl_2$. Ligation product/ DNA was added into 50 µl of appropriate competent cells (cloning host or expression host). This mixture was then incubated on ice for 30 min. Heat shock was given at 42˚C for exactly 90 s and again kept on ice bath for 5 min. To this, 1 ml of LB broth was added and kept for growth in shaker incubator for 1 h. The cells were collected by centrifugation at 6000 rpm for 5 min and pellet was resuspended in 100 µl LB media. This was then spread on LB agar plate containing appropriate antibiotic.

2.2.1.11. Conjugation

Bi-parental Conjugation was performed essentially as described by Figruski and Helenski (1979). *E. coli* S17-1(donor) and *P. fluorescens* (recipient), were grown at 37^{O}C and 30^{O}C respectively, for log phase in LB media supplemented with appropriate antibiotics. Cultures grown to mid log phase were collected to harvest the cells. The cells were then thoroughly washed with saline and the donor and recipient cells were mixed in 3:1 ratio. The cells were pelleted by centrifugation at 8000 rpm. The cell pellets were then re-suspended in minimal amount of saline and plated on LB plates having no antibiotic. The plates were then incubated for 6- 8 h at 30^{O}C. The cell mass collected from LB plate was serially diluted and were independently plated on LB plates having appropriate antibiotics. Donor and recipient cells treated in similar manner, serving as negative controls, were also plated on the LB selection plates.

2.2.2. Expression of recombinant Cry1EC in *E. coli*

Overnight culture of *E. coli* having appropriate expression plasmid was taken and inoculated in 10 ml of LB medium containing appropriate antibiotics. The cultures were grown till mid log phase and induced by adding sterile IPTG to a final concentration of 1 mM. The induction was continued for 2-5 h and cells were harvested to analyze the expression of recombinant Cry1EC. When expressed protein was found in inclusion bodies, Arctic Express system was used. The induction in Arctic Express system was identical except that the induction was done at 12˚C for 24 h.

2.2.3. Expression of recombinant Cry1EC in *Pseudomonas fluorescens*

Overnight cultures of *Pseudomonas fluorescens* having appropriate expression plasmid was taken and inoculated in 10 ml of LB medium containing appropriate antibiotics. The cultures were grown till mid log phase and induced by adding sterile IPTG to a final concentration of 1

mM. The induction was continued for 5-10 h and cells were harvested to analyze the expression of recombinant Cry1EC by SDS- PAGE and western blotting.

2.2.4. Protein analysis

2.2.4.1. Estimation of protein by Bradford

An aliquot of sample was pipetted out into a 1.5 ml microfuge tube. The volume of the sample was adjusted to 0.1 ml with double distilled water. One ml of Bradford reagent (Bradford, 1976) was added and the contents were mixed by gentle inversion. After 15 min, absorbance at 595 nm was measured spectrophotometrically against a reagent blank prepared from 0.1 ml of appropriate buffer and 1 ml of Bradford reagent. Protein concentration of the sample was calculated from a standard curve drawn using known concentration of bovine serum albumin.

2.2.4.2. SDS-Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis for proteins was done by following the procedures of Laemmli (1970). 12.5% running acrylamide gel solution was prepared by mixing 3.6 ml of 30 % acrylamide solution, 4.5 ml of running buffer (0.98 M Tris-Cl, pH, 8.8), 0.9 ml of distilled water, 90 µl of 10% SDS, 4.5 µl of TEMED and 45 µl of freshly prepared 10% ammonium persulphate. The 7.5 % stacking acrylamide gel solution was prepared by taking 1.25 ml of 30 % acrylamide solution, 2.5 ml of stacking gel buffer (0.325 M Tris-Cl, pH, 6.8), 1.25 ml of water, 50 µl of 10 % SDS, 5 µl of TEMED and 50 µl ammonium persulphate. The resolving buffer was 50 mM Tris-Cl, 0.384 M glycine, 0.1 % SDS, pH 8.5. Protein samples were mixed with equal volumes of sample loading buffer (50 mM Tris HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM 2-mercaptoethanol) and kept in a boiling water bath for 5 min. The contents were then briefly centrifuged before loading the sample into the wells. The electrophoresis was carried at 150 V till the tracking dye reached the anode end of the gel. The

gels were stained with staining solution containing 0.2 % W/V coomassie brilliant blue in methanol: water: acetic acid (30:60:10) for 6-8 hrs. The gel was then destained in the destaining solution containing methanol: water: acetic acid (30:60:10) for another 6-8 hrs.

2.2.4.3. Silver staining

Silver staining of the gel was performed by following the procedure as described by Blum et al, 1987. The SDS- PAGE gel was fixed by incubating it in solution containing 50% methanol, 12 % acetic acid and 0.1 ml 37% formaldehyde for 1 hour. Further, the gel was washed 3 times (each 20 min) with 50% methanol. Sensitisation of gel was done by exposing it to 0.02 % sodium thiosulphate for exactly 1 min. Gel was rinsed rapidly with distilled water (each 20 s). Now the gel was incubated for 30 min in solution containing 0.2 % silver nitrate and 0.075 ml formaldehyde. After 30 min, the gel was rinsed three times (each 20 seconds) with double distilled water. Then, the gel was developed by keeping it in developing solution containing 6 % sodium carbonate, 4 ml of sodium thiosulphate and 50 µl of 37 % formaldehyde till the protein bands appear on the gel. After the appearance of protein bands, the gel was washed with distilled water two times (each 2 min) and immediately the stop solution containing 50 % methanol, 12 % acetic acid was added. The gel can be stored at 4^0C in 50 % methanol after washing it with 50 % methanol for 20 min.

2.2.4.4. Western Blot Analysis

PVDF membrane of the size same as that of the gel was cut and soaked in methanol for 1 min. It was then soaked in Towbin's buffer. Thin filterpaper was placed on the transfer instrument and air bubbles were removed by pressing with a glass rod. The PVDF membrane was placed on the thin filter paper and again bubbles were removed. Gel was placed over the PVDF membrane, marking the protein ladder side. Bubbles were gently removed and all sides were sealed using

parafilm wax strips. Thick filter paper was put on the gel and instrument was set for transfer. Transfer was done at 18 V for 30 min. After this, the membrane was put in 5% skimmed milk for 1 h for blocking non specific sites. Three washes of 10 min each was done using TBS-T and membrane was incubated for 2 h with primary antibody. Again, the membrane was washed three times as mentioned above. PVDF membrane was then incubated for 1 h with secondary antibody and developed using appropriate method (ECL detection /BCIP).

2.2.5. Protein Purification

2.2.5.1 Preparation of cell free extracts

The cell free extracts either from *E. coli* cells or *P. fluorescens* cells expressing desired protein were harvested and the cell pellet was washed twice in 20 mM TE buffer pH 8.0 and resuspended in the 7 volumes of 50 mM Tris buffer (pH 8.0) having 1 mM EDTA. After resuspension, the cells were disrupted by sonication for a period of 10 min with a pulse on and off cycle of 30s each at 4 ^{0}C. The resulting homogenate was centrifuged at 15,000 rpm for 30 min and the supernatant was again centrifuged at 45,000 rpm for 1 h at 4 ^{0}C. The supernatant thus obtained was considered as the cytoplasmic fraction and was used for purification of desired protein.

2.2.5.2. Glutathione affinity purification

The *E.coli* Arctic express cells transformed with an expression plasmid were sonicated after induction. After sonication, the cells were centrifuged at 8000 rpm for 10 min to remove the cell debris. The supernatant was taken and centrifuged at 12000 rpm for 30 min at 4˚C. The supernatant was applied on Glutathione sepharose column. Column was packed with 20 ml Glutathione agarose. Column was washed with Milli Q at the flow rate of 5 ml/min at 100% gradient and equilibrated with PBS. The sample was then applied onto the column at 0. 5 ml/ min

flow rate and washed up to 5- 10 times the packed volume with PBS. The protein was eluted with elution buffer at a flow rate of 1 ml/ min.

2.2.5.3. Ammonium sulfate fractionation

To the cytoplasmic fraction, ammonium sulphate was added slowly with constant stirring at 4^0C in order to bring the solution to 20 % saturation. Later, the solution was centrifuged at 15,000 rpm for 30 min to pellet out the precipitated proteins. Similarly, the supernatant obtained was further saturated to 40 %, 60 % and 80 %, respectively by adding appropriate amounts of ammonium sulphate. The proteins precipitated in each fraction were used for identification of desired protein.

2.2.5.4. Nickel Sepharose affinity purification

After ammonium sulphate precipitation the pellet was redissolved in the initial volume of binding buffer. This protein fraction was dialysed against the binding buffer to remove the ammonium sulphate salt. After dialysis, the sample was applied to the column pre-equilibrated with binding buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl). After sample loading, the column was washed with 10 volumes of wash buffer (binding buffer with 50 mM imidazole) and the bound proteins were eluted with linear gradient of elution buffer (binding buffer with 500 mM imidazole).

2.2.6. Antibody purification

Affinity chromatography was performed to purify IgG's for the serum collected from immunized rabbits. Initially, required amount (2 ml) of serum was taken and diluted it with equal volumes equilibration buffer (20 mM sodium phosphate buffer pH 7.0). The diluted serum was then applied on to a protein A column pre-equilibrated with the equilibration buffer at the flow rate of 1 ml/min. The column was then washed with 10 column volumes (CV) of equilibration

buffer and finally the IgG's bound to the column were eluted with elution buffer (0.1 M glycine-HCl, pH 2.7). The eluted IgG's were then stored at -20^{0}C in a 100 µl aliquots and used when necessary.

2.2.7. Preparation of BBMV membranes

The midguts were pulled gently from the larvae after both the last three abdominal segments and the head and thorax were removed. A small glass culture tube was rolled over the length of the gut to remove the gut contents. Dissected gut tissue was transferred to a centrifuge tube containing ice cold MET buffer (300 mM mannitol, 17 mM Tris- Hcl pH 7.5, 5 mM EGTA, Protease inhibitor), vigorously vortexed and briefly centrifuged for 5 min at 1000g to obtain clean midguts. Gut tissues were homogenized on ice in a tight fitting glass homogenizer in ice cold MET buffer (10 % w/ v). The homogenate diluted with an equal volume of ice cold 24 mM $Mgcl_2$, blended and held on ice for 15 min and centrifuged at 2500 g for 15 min at 4 ^{0}C. Supernatant was further centrifuged at 30000 g for 30 min at 4 ^{0}C. Pellet was resuspended in MET buffer and centrifuged again at 2500 g at 4 ^{0}C for 15 min. Again, the supernatant was centrifuged at 30000 rpm for 30 min at 4 ^{0}C. The resulting pellet, which corresponds to the BBMV preparation was resuspended in HBS-N buffer (10 mM HEPES, pH 7.4, 150 mM NaCl), flash frozen in liquid Nitrogen and stored at -80^{0}C until use.

2.2.8. Ligand blotting

BBMV preparations were heated in SDS sample buffer containing β-mercaptoethanol, resolved by SDS– 10% PAGE, and electrotransferred to nitrocellulose membrane. After being blocked with 3% BSA in TBS-T, the membrane was incubated with 1 µg of purified $Cry1EC_{NGST}$ per ml in TBST containing 0.2% BSA at room temperature for 1 h. The unbound toxins were removed by three washes with TBST. The membrane was then incubated with anti- GST- HRP-

conjugated antibody at room temperature for 1 h. After three washes with TBST, the blots were developed and analyzed.

2.2.9. Synthesis of Mesoporous Silica Nanoparticles

Mesoporous Silica Nanoparticles having ordered pores were synthesized by the surfactant-templated method. N-Cetyltrimethylammonium bromide (CTAB, 1.00 g, 2.74 mmol) was dissolved in 480 mL of water. Sodium hydroxide aqueous solution (2.00 M, 3.50 mL) was introduced to the CTAB solution and the temperature of the mixture was adjusted to 80°C. Tetraethoxysilane (TEOS, 5.00 mL, 22.4 mmol) was added dropwise to the surfactant solution under vigorous stirring. The mixture was allowed to react for 2 hrs to give a white precipitate. This solid crude product was filtered, additional water was then added into the reaction before leaving the solution overnight for stirring. After 24 hrs, the reaction solution was neutralized using hydrochloric acid solution (2 M). The sample was centrifuged and the pellet obtained was redispersed in ethanol. CTAB was removed from the pellet by acid extraction using an acetic acid/ethanol mixture (95/5 v/v). Further, the above sample was stirred for 30 min, before centrifugation to remove CTAB and acetic acid and then the pellet was dried at 85^{o}C for 2 hrs.

2.2.10. Protein loading on to silica nanoparticles

Prior to loading, the 100 mg of silica nanoparticles were washed with 20 mM Tris (pH 8.0) buffer 3-4 times, vacuum was applied to it and they were incubated with 130 µg of Cry1EC$_{N6HIS}$ toxin obtained from *P. fluorescens* expressing Cry1EC$_{N6HIS}$ and kept for overnight stirring under vacuum at 4 ^{0}C. Further, the silica nanoparticles were pelleted down and washed with 20 mM Tris (pH 8.0) buffer, twice. The final pellet obtained was re-dissolved in 400 µl of 20 mM Tris (pH 8.0) buffer. The protein loaded onto the nanoparticles was estimated by spectrophotometric method.

2.2.11. Insect rearing

Egg masses of *Spodoptera litura* were collected from castor fields of Directorate of Oilseeds Research, Hyderabad. Immediately after hatching, the neonates were transferred to a growth chamber and reared on fresh castor leaves (*Ricinus communis*) as diet under a photoperiod of 14:10 h (light: dark), 60-70% relative humidity at 27±2°C till pupation. The pupae were transferred onto moist sand in a container and allowed to develop into adults. The adults were then transferred to an aerated chamber and fed with 10 % honey solution supplemented with vitamin E as diet. The female adults deposited their eggs onto the surface of castor leaves in rearing chamber, which were then allowed to hatch into neonate larvae for maintenance of regular cultures. Under our rearing condition, a majority of *S.litura* larvae reached late third instar on sixth day post-hatching and the larva weighs around 0.2±0.03g. For toxicity bioassays and immunohistochemical studies 2nd instar and 3rd instar larvae were used, respectively.

2.2.12. Determination of toxicity of recombinant Cry1EC and CRYSONES

The castor leaves were circularly cut with an area of 12.5 sq cm. Different concentrations of the Cry1EC protein obtained through heterologous expression from *P. fluorescens* and *E. coli* were uniformly coated on to the castor leaves, along with proper controls in triplicates. Six *Spodoptera litura* 2nd instar larvae were allowed to feed on each leaf. The effect of the toxin on the insects was observed after 24 h and 48 h. After 48 h the alive insects were transferred to fresh leaves and monitored through their growth. CRYSONES containing equivalent CryIEC protein load were sprayed on the castor leaves and analyzed for their potential toxicity, as mentioned above. Mesoporous silica particles dissolved in 20 mM Tris (pH 8.0) were used as negative control.

2.2.13. Tissue isolation

The larvae were first narcotized by placing them on ice for 15-20 min. The larval cuticle were cut open from the ventral side of abdomen and cut-through the length of the larvae. Midguts were dissected out in ice cold insect Ringer solution. Midgut was cleared of food contents and peritrophic membranes. The tissues were used for histological studies and *in vivo* toxin-receptor interaction studies.

2.2.14. Hematoxylin-eosin staining

The insects were fed with the toxin and after 4 h, the insects were fixed in Bouin's fixative for 1- 2 h at room temperature and then were washed with series of increasing concentration of ethanol and kept in xylene. Further, the insects were embedded in wax containing xylene, and then transferred to pure wax. Finally, insects were embedded in paraplast without air bubbles. After that the embedded insects were blocked on to a wooden support for sectioning. Sections of 6µm thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and stained with hematoxylin–eosin following a series of dehydration,rehydration steps through graded concentrations of alcohol and xylene and mounted on slides using DPX mountant. Microscopic examinations and photography was done with a Nikon EFD-3 microscope fitted with a Nikon NFX-35 automatic camera (Nikon, Tokyo, Japan).

2.2.15. Immunocytochemistry

The gut sections of *S. litura* were deparaffinised in xylene, rehydrated in successively lower graded concentrations of ethanol and then treated with 0.3-3% H_2O_2 for 10 min to prevent endogenous peroxidase reaction. The sections were washed twice with 0.1 M phosphate buffered saline (PBS), pH 7.4 for 10 min. The sections were then pre-incubated with 10% normal goat serum in 0.1 M PBS for 30 min at room temperature. After rinsing with PBS, the sections were

then incubated overnight at 4ºC with (1:5000) primary antibody solution (anti-His antibody). Later sections were washed thrice in PBS with 0.1% Tween 20 (PBST) for 10 min each and were then incubated with 1: 5000 dilution of secondary antibody (anti mouse horse radish peroxidise conjugated IgG) for two hrs. The sections were washed twice in PBS for 5 min each and then immunostain was developed using commercially available DAB reagent (3'3'-diaminobenzidine as chromogen and H_2O_2 as substrate for horse-radish peroxidise) and kept for 15 minutes at room temperature. The sections were washed twice with 1X PBS and dehydrated in graded ethanol, cleared in xylene and mounted using DPX mountant. Finally, the slides were left to dry for few hours and were observed under Light Microscope.

2.2.16. Enzymatic assays

2.2.16.1. ALP Assay

ALP enzyme activity in various tissue samples was examined as described by Walter and Schütt (1974). Assay was carried out with 2 and 4 µg each of midgut BBMV preparations at 37°C in assay buffer (903 mM diethanolamine, pH 9.8; 0.45 mM magnesium chloride) using 14 mM *p*-nitrophenyl phosphate (Sigma Aldrich, USA) as substrate. Absorbance was measured at 405 nm and the molar absorbance co-efficient of *p*-nitroanilide was taken as 18.5 mmol/L. The specific activity was expressed as µmol of *p*-nitrophenol released /min/mg of proteins.

Chapter 1
Results
&
Discussions

3.1. Background

Bacillus thuringiensis (Bt) is a gram +ve entomopathogenic bacteria and is known globally for its biotoxic properties. It was first isolated from lepidopteran insect larvae and since then found in different natural environments like soil (Martin and Travers, 1989), water bodies (Thanabalu et al., 1992), plants (Smith and Couche, 1991) and animal feces (Bernhard et al., 1997). The insecticidal crystal proteins produced during sporulation stage by Bt are comprised of one or more crystal proteins coded by *cry* and *cyt* genes (Yul, 2007). Over 140 types of Cry proteins and 9 types of Cyt proteins were discovered and more than 380 *cry* genes had been cloned and sequenced (Yul, 2007). The *cry* genes were found to be located mainly on plasmids. The conjugative nature of these plasmids harboring *cry* genes and the presence of transposable genetic elements had resulted in remarkable genetic plasticity among the *B. thuringiensis* sp. (Loguercio et al., 2001). This had resulted in different combinations of *cry* genes present in a wide variety of Bt strains.

The Bt strains belonging to *Bacillus thuringiensis* spp. *kurstaki* were found to be effective against many lepidopteran pests like *Helicoverpa, Plutella, Achaea janata*. Previous literature had shown that the Bt strains active against lepidopteran insects were rich in *cry* genes belonging to *cry*1, *cry*2 and *cry*9 families (Ibrahim et al., 2010). The toxicity of Bt strains against *Spodoptera,* a lepidopteran insect is very moderate. *Spodoptera litura* is a predominant pest in Indian subcontinent and crop loss due to *Spodoptera* infestation is extremely high. Therefore, the present study is undertaken to screen the native *Bt* isolates to identify Cry toxin coding genes that are effective against *Spodoptera litura*. Such novel *cry* genes are expected to provide an effective platform for developing Cry based technologies for controlling *Spodoptera* insects.

Directorate of Oil seed Research (DOR), Hyderabad has a vast collection of *Bacillus thuringiensis*. These Bt strains were isolated from local agricultural fields located from

southern part of India like Karnataka, Tamil nadu and Andhra Pradesh that are unexposed to any kind of Bt pesticides. The DOR has screened all of them to identify active *Bt* strains showing toxicity against different lepidopteran insects. However the DOR made no attempts to screen these effective strains for identification of *cry* content and to isolate novel *cry* genes. In this study the Bt strains that showed maximum toxicity towards lepidopteran insects like *Acheae janata*, *Helicoverpa* and *Spodoptera litura*, were obtained and all of them were screened for identification of *cry* gene content that contributed for toxicity.

3.2. Screening of native *Bacillus thuringiensis* isolates for their *cryI* gene content.

As stated before, about 115 native isolates (Table 3-1) were taken from the DOR *Bacillus thuringiensis* culture collection showing effectiveness against lepidopteran insects like *Acheae Janata*, *Helicoverpa* and *Spodoptera litura.* These cultures were then used for performing bioassays against 7 days old *Achaea janata*, 6 days old *Helicoverpa armigera* and 5 days old *Spodoptera litura* following methods described in materials and methods. These bioassays have shown that 69 isolates were effective against *A. janata* while 38 isolates were effective against *H. armigera.* In two to three days after application about 80 % mortality was recorded in case of *A. janata* and *H. armigera.* However, in case of *S. litura,* the mortality rate was low (60%- 70%) and also took prolonged period for eliciting such response (4 days). Based on this background these 115 strains of *Bacillus thuringiensis* were taken for further investigation. The molecular basis behind such differential toxicity was investigated.

S.No	Strain
1	KR-1
2	KR-2
3	KR-3
4	KR-4
5	KM-1
6	KM-2
7	K-1
8	K-2
9	K-3
10	K-4
11	K-6
12	K-7
13	K-8
14	K-9
15	K-10
16	K-11
17	K-12
18	K-13
19	K-14
20	K-16
21	K-17
22	K-18
23	K-19
24	K-20
25	K-21
26	K-22
27	K-23
28	K-24
29	K-25

S.No	Strain
30	K-26
31	K-27
32	K-28
33	K-29
34	K-30
35	D-1
36	D-2
37	D-4
38	D-5
39	D-6
40	D-7
41	D-8
42	D-9
43	D-10
44	D-19
45	D-12
46	D-13
47	D-14
48	D-15
49	D-16
50	D-17
51	D-18
52	D-20
53	D-21
54	D-22
55	D-23
56	D-24
57	B-1
58	G-1

S.No	Strain
59	G-2
60	G-3
61	G-4
62	G-5
63	G-6
64	G-7
65	G-8
66	G-9
67	G-10
68	G-11
69	G-12
70	G-13
71	G-14
72	G-15
73	G-16
74	G-17
75	G-18
76	G-20
77	G-21
78	G-22
79	G-23
80	G-24
81	G-25
82	G-26
83	G-27
84	G-28
85	G-29
86	G-30
87	G-31

S.No	Strain
88	G-32
89	G-33
90	G-34
91	G-35
92	N-2
93	N-3
94	N-4
95	N-5
96	N-6
97	N-7
98	N-8
99	N-9
100	N-10
101	N-11
102	N-12
103	N-13
104	N-14
105	N-15
106	W-1
107	W-2
108	W-3
109	W-4
110	W-5
111	DOR-5
112	DOR-34
113	HD1
114	K-5
115	N-1

Table 3-1. The *Bacillus thuringiensis* strains used in the present study. Serial numbers are indicated column 1. The column number 2 indicates strain code numbers given by DOR, Hyderabad.

3.3. Identification of *cry* genes

CryI toxins were known to be more effective against lepidopteran insects (Bravo et al., 2013). Since these Bt isolates have shown differential toxicity towards *Acheae Janata*, *Helicoverpa* and *Spodoptera litura* insects, all of them were screened to identify *cryI* toxin variants. While performing PCR amplification a slightly modified strategy developed by Ferrandis and his associates was used (Juarez-Perez et al., 1997). Two stage PCR was done for identification of novel CryI toxins in these strains. The strategy involved in two stage PCR is given below. Stage I involves a normal PCR meant for amplification of all *cryI* genes. The second PCR is done to identify *cryI* variants using *cryI* specific primers.

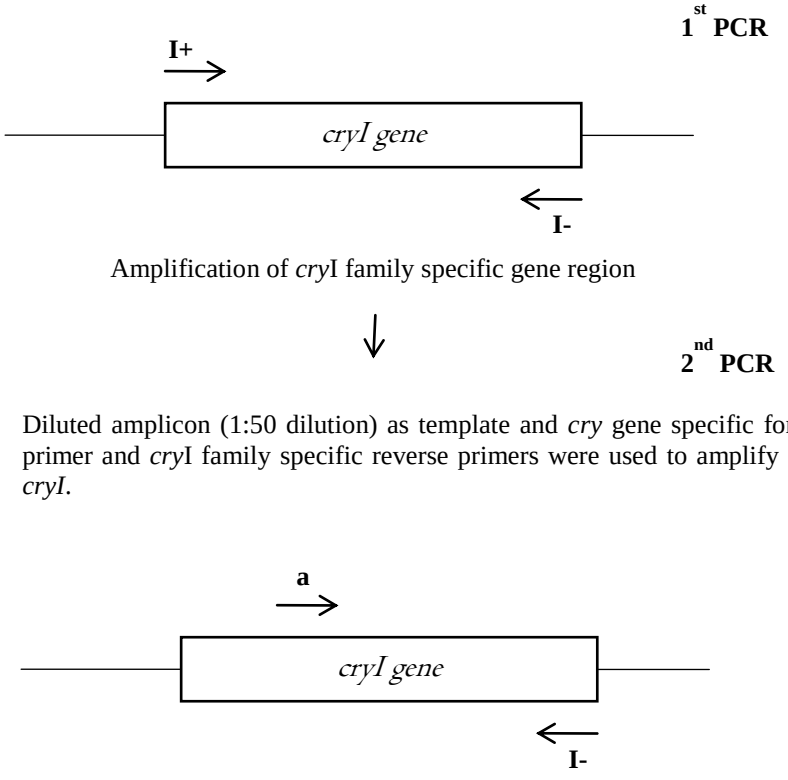

Fig 3-1. Strategy followed to amplify *cryI* family specific genes. Family primers are labelled I(+) and I(-). Small letter 'a' refers to gene specific primer.

As shown in Fig 3-1 *cry* family specific primers (I+, I-) were used. Amplification of 1.5-1.7 kb confirms the existence of *cryI* genes. Appropriate dilutions of these PCR product (1:50)

was used as template in second stage PCR along with type specific primers (IAa, IAb, IAc, IAd, IB, IC, ID, IE, IF, IG) and a family specific forward primer. The two stage PCR provide unique amplicon sizes. The length of the amplicon is indicative of the *cryI* type of gene present in the isolate (Juarez-Perez et al., 1997). The expected sizes of PCR amplicons for *cryIAa*, *cryIAb*, *cryIAc*, *cryIAd*, *cryIB*, *cryIC*, *cryID*, *cryIE*, *cryIF* and *cryIG* were 1286 bp, 1371 bp, 844 bp, 1212 bp, 1323 bp, 1176 bp, 1138 bp, 1137 bp, 967 bp and 1128 bp respectively. The method originally described by Juarez-Perez (1997) was used to detect *cryI* variants in the *Bacillus thuringiensis* strains isolated from Africa, Southern France and South Pacific islands (Juarez-Perez et al., 1997). In their study, they could detect a novel *cryIB* gene in a Bt strain 19 which differs by 16.1% from other members of the *cryIB* group with respect to the deduced protein sequence.

Primers	Sequence (5' --> 3')
I(-)	MDATYTCTAKRTCTTGACTA
I(+)	TRACRHTDDBDGTATTAGAT
IA	CAATAGTCGTTATAATGATT
IAa	TTCCCTTTATTTGGGAATGC
IAb	CGGATGCTCATAGAGGAGAA
IAc	GGAAACTTTCTTTTTAATGG
IAd	ACCCGTACTGATCTCAACTA
IB	GGCTACCAATACTTCTATTA
IC	ATTTAATTTACGTGGTGTTG
ID	CAGGCCTTGACAATTCAAAT
IE	TAGGGATAAATGTAGTACAG
IF	GATTTCAGGAAGTGATTCAT
IG	GGTTCTCAAAGATCCGTGTA

Table 3-2. Primers used in this study.

The two stage PCR has given interesting results. The first stage PCR was done for all the 115 strains of Bt isolates to check for the presence of *cryI* family band. Interestingly, out

of the 115 strains only 35 strains have showed the 1.5-1.7 kb *cryI* specific band. This result confirms the presence of *cryI* genes (Data not shown). This observation indicates that *cryI* genes were available in the DOR *Bacillus thuringiensis* culture collection. Till date, *Bacillus thuringiensis* spp. *kurstaki* HD-1 is the most useful strain which exhibits toxicity against lepidopteran insects. *Bacillus thuringiensis* spp. *kurstaki* HD-1 typically contains CryIAa, CryIAb, CryIAc and Cry2A proteins. In this chapter *Bacillus thuringiensis* spp. *kurstaki* HD-1, was taken as positive control for all the PCR amplifications with *cry* type specific primers (Fig. 3-2). The Bt formulations like Dipel, are products *Bacillus thuringiensis* spp. *kurstaki* HD-1 which typically contain CryIAa, CryIAb, CryIAc and Cry2A proteins and exhibit very low or no activity against *Spodoptera* sp. Fig. 3-2 shows the PCR amplification of *cryIAa*, *cryIAb* and *cryIAc* genes from the *Bacillus thuringiensis* spp. *kurstaki* HD-1. The other *cryI* genes i.e *cryIAd, cryIB, cryIC, cryID, cryIE, cryIF* and *cryIG* were absent in this strain. The *cryIE, cryIC* (Hofte and Whiteley, 1989) and *cryIF* (Chambers et al., 1991) which were found to be effective against *Spodoptera*, also belong to *cryI* gene family.

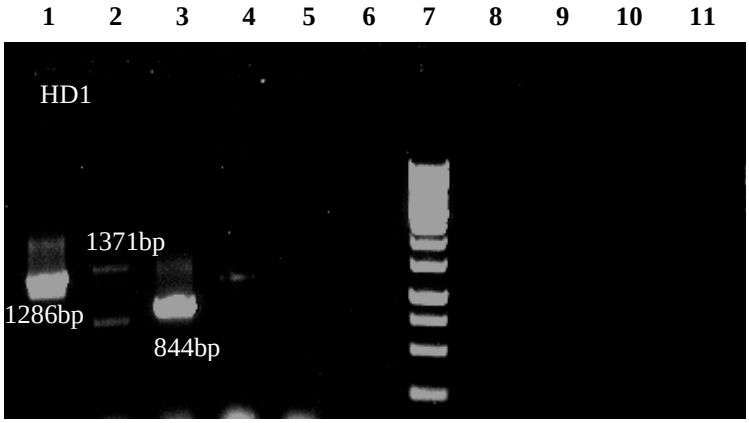

Fig 3-2: **Screening of *Bacillus thuringiensis* HD1 for identification of *cryI* gene variants through two stage PCR.** Lane1: Indicates the presence of *cry1Aa*, Lane 2: Indicates the presence of *cryIAb*, Lane 3: Indicates the presence of *cry IAc*. Lanes 4, 5, 6, 8, 9, 10 and 11 indicate the absence of *cryIAd, IB, IC, ID, IE, IF* and *IG* respectively.

Further, the 35 strains were subjected to second stage PCR where the *cry* type specific genes were amplified. In the second stage PCR, *cryI* gene specific primer was used along with the family specific reverse primer (I-). The *cryI* genes present in the 35 Bt strains were shown in Fig 3-3, 3-4 and listed in Table 3-3. Most of the strains showed *cryIAa*, *cryIAb* genes. Some showed existence of *cryIAc* and *cryIAd.* The *cryIC* and *cryIF* were seen only in strains G26 and K3. The *cryIA* type genes like *cryIAa*, *cryIAb*, *cryIAc* and *cryIAd* were known to be involved in toxicity against many lepidopteran insects except *S. litura* (Pardo-Lopez et al., 2009). The *cryIC* showed moderate toxicity towards *S. litura*, a troublesome lepidopteran insect (Agrawal et al., 2002). Similarly the *cryIF* was also involved in toxicity against lepidopteran insects (Gatehouse, 2008, Herman et al., 2002).

The predominant presence of *cryIA* genes in the screened *Bacillus thuringiensis* culture collection suggest the presence of Bt isolates active against lepidopteran insects, in the native soils. This might be contributing to the natural protection of the crops against lepidopteran insects like *Acheae Janata* and *Helicoverpa*. But the absence of a variety of *cryI* genes like *cryIB* (Bohorova et al., 2001), *cryID* (Polanczyk et al., 2000) and *cryIE* (Chang et al., 1998) limits the range of target insect pests. However, the *cryID* along with *cryIA* genes are known to have synergistic effect on *S. frugiperda* larvae (Polanczyk et al., 2000).The *cryIB* along with *cryIAb* also is known to elicit synergistic toxicity against southwestern corn borer, sugarcane borer and fall armyworm in transgenic tropical maize (Frutos et al., 2001). Though *cryIE* is not effective against lepidopteran insects, the gene fusion or domain swapping studies showed that it contributes to enhanced toxicity towards S. *litura* when combined with *cryIC* (Singh et al., 2004).

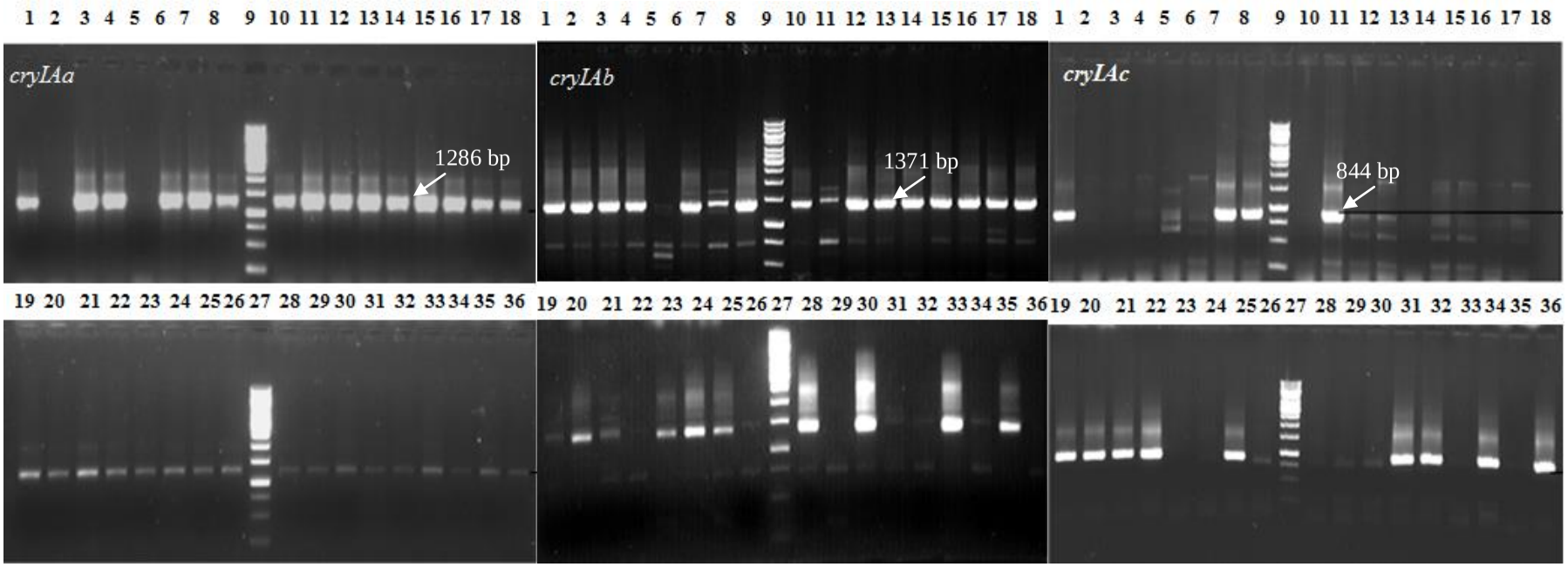

Fig 3-3. Screening of *Bacillus thuringiensis* strains for the presence of *cryIAa, cryIAb, cryIAc* by two stage PCR technique. Panel A, B and C correspond to the conserved domain amplicons of *cryIAa, cryIAb, cryIAc*, amplified from the genomic DNA isolated from *Bacillus thuringiensis* strains W2, N4, N1, G26, D23, D18, G14, N10, D19, D10, W5, N2, G23, K27, K18, K22, K3, D1, D5, G34, KM2, G31, N12, KR3, G4, G36, K14, N6, HD1, G13, K19, G11, D16 and KR1 respectively. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, 36 represent conserved domain amplicons of *Bacillus thuringiensis* strains W2, N4, N1, G26, D23, D18, G14, N10, D19, D10, W5, N2, G23, K27, K18, K22, K3, D1, D5, G34, KM2, G31, N12, KR3, G4, G36, K14, N6, HD1,G13, K19, G11, D16 and KR1 respectively. Lanes 9 and 27 represent 1 kb DNA ladder. The expected size of amplicon for *cryIAa* is 1286 bp. Existence of *cryIAa* is evident in all the 35 strains of *Bt* except in lane 2 and 5 which represent *Bt* strains N4 and D23. The expected size of amplicon for *cryIAb* is 1371 bp. Existence of *cryIAb* is evident in all 35 strains of *Bt* except in lanes 5, 11, 26, 29 and 36 which represent *Bt* strains D23, D10, G4, K14 and KR1. The expected size of amplicon for *cryIAc* is 844 bp. Existence of *cryIAc* is evident in lanes 1, 5, 7, 8, 11, 19, 20, 21, 22, 25, 31, 32, 34 and 36 which represent *Bt* strains W2, D23, G14, N10, D10, D1, D5, G34, KM2, KR3, HD1, G13, G11 and KR1.

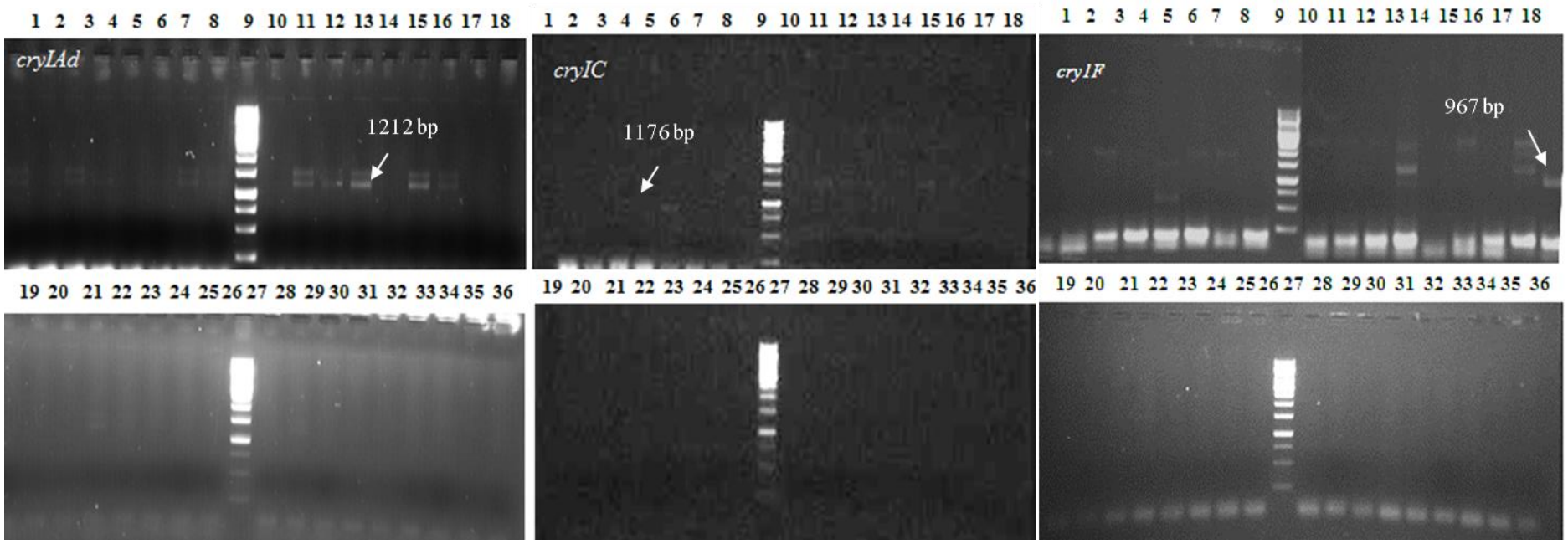

Fig 3-4. Screening of *Bacillus thuringiensis* strains for the presence of *cryIAd, cryIC, cryIF* by two stage PCR technique. Panel A, B and C correspond to the conserved domain amplicons of *cryIAd, cryIC, cryIF*, amplified from the genomic DNA isolated from *Bacillus thuringiensis* strains W2, N4, N1, G26, D23, D18, G14, N10, D19, D10, W5, N2, G23, K27, K18, K22, K3, D1, D5, G34, KM2, G31, N12, KR3, G4, G36, K14, N6, HD1, G13, K19, G11, D16 and KR1 respectively. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35 and 36 represent conserved domain amplicons of *Bacillus thuringiensis* strains W2, N4, N1, G26, D23, D18, G14, N10, D19, D10, W5, N2, G23, K27, K18, K22, K3, D1, D5, G34, KM2, G31, N12, KR3, G4, G36, K14, N6, HD1,G13, K19, G11, D16 and KR1 respectively. Lanes 9 and 27 represent 1 kb DNA ladder. The expected size of amplicon for *cryIAd* is 1212 bp. Existence of *cryIAd* was evident in Bt strains D10, W5, N2 and K27. The expected size of amplicon for *cryIC* is 1176 bp. *cryIC* was seen in lane 4 corresponding to *Bacillus thuringiensis* strain G26. The expected size of amplicon for *cryIF* is 967 bp respectively. *cryIF* was found in lane 18 corresponding to *Bacillus thuringiensis* strain K3.

S. No	STRAIN	IAa	IAb	IAc	IAd	IB	IC	ID	IE	IF	IG
1	W2	+	+	+	-	-	-	-	-	-	-
2	N4	-	+	-	-	-	-	-	-	-	-
3	NI	+	+	-	-	-	-	-	-	-	-
4	D18	+	+	-	-	-	-	-	-	-	-
5	G26	+	+	-	-	-	+	-	-	-	-
6	K14	+	-	-	-	-	-	-	-	-	-
7	G14	+	+	+	-	-	-	-	-	-	-
8	N10	+	+	+	-	-	-	-	-	-	-
9	D19	+	+	-	-	-	-	-	-	-	-
10	D10	+	-	+	+	-	-	-	-	-	-
11	W5	+	+	-	+	-	-	-	-	-	-
12	N2	+	+	-	+	-	-	-	-	-	-
13	G23	+	+	-	-	-	-	-	-	-	-
14	K27	+	+	-	+	-	-	-	-	-	-
15	K18	+	+	-	-	-	-	-	-	-	-
16	K22	+	+	-	-	-	-	-	-	-	-
17	K3	+	+	-	-	-	-	-	-	+	-
18	D1	+	+	+	-	-	-	-	-	-	-
19	D5	+	+	+	-	-	-	-	-	-	-
20	G34	+	+	+	-	-	-	-	-	-	-
21	HD1	+	+	+	-	-	-	-	-	-	-
22	G31	+	+	-	-	-	-	-	-	-	-
23	NI2	+	+	-	-	-	-	-	-	-	-
24	KR3	+	+	+	-	-	-	-	-	-	-
25	G31	+	+	-	-	-	-	-	-	-	-
26	G36	+	+	-	-	-	-	-	-	-	-
27	G4	+	-	-	-	-	-	-	-	-	-
28	D16	+	+	-	-	-	-	-	-	-	-
29	GI1	+	+	+	-	-	-	-	-	-	-
30	KM2	+	+	+	-	-	-	-	-	-	-
31	N6	+	+	-	-	-	-	-	-	-	-
32	G13	+	+	+	-	-	-	-	-	-	-
33	K19	+	+	-	-	-	-	-	-	-	-
34	KR1	+	-	+	-	-	-	-	-	-	-
35	D23	-	-	+	-	-	-	-	-	-	-

Table 3-3. The *cryI* gene profiles of the 35 *Bacillus thuringiensis* strains. Most of the strains showed *cryIAa, cryIAb* genes. Some showed *cryIAc and cryIAd.* The *cryIC and cryIF* were seen in strains G26 and K3.

As described earlier, out of all the *Bacillus thuringiensis* culture collection, only 8 strains showed 50-70% affectivity against *Spodoptera litura*. Further these 8 strains were once again screened to have a confirmed, in depth and clear analysis of *cryI* gene content. The details of *cry* gene amplification are shown in Fig. 3-5. It was observed that all the eight *Bacillus thuringiensis* strains contained *cryIAa*. Except *Bacillus thuringiensis* K14, all the other 7 strains contained *cryIAb*. The *cryIAc* was present only in *Bacillus thuringiensis* D5 and G34. *Bacillus thuringiensis* G26 (Fig. 3-5, panel D) and K3 (Fig. 3-5, panel B) contained *cryIC* and *cryIF*, respectively.

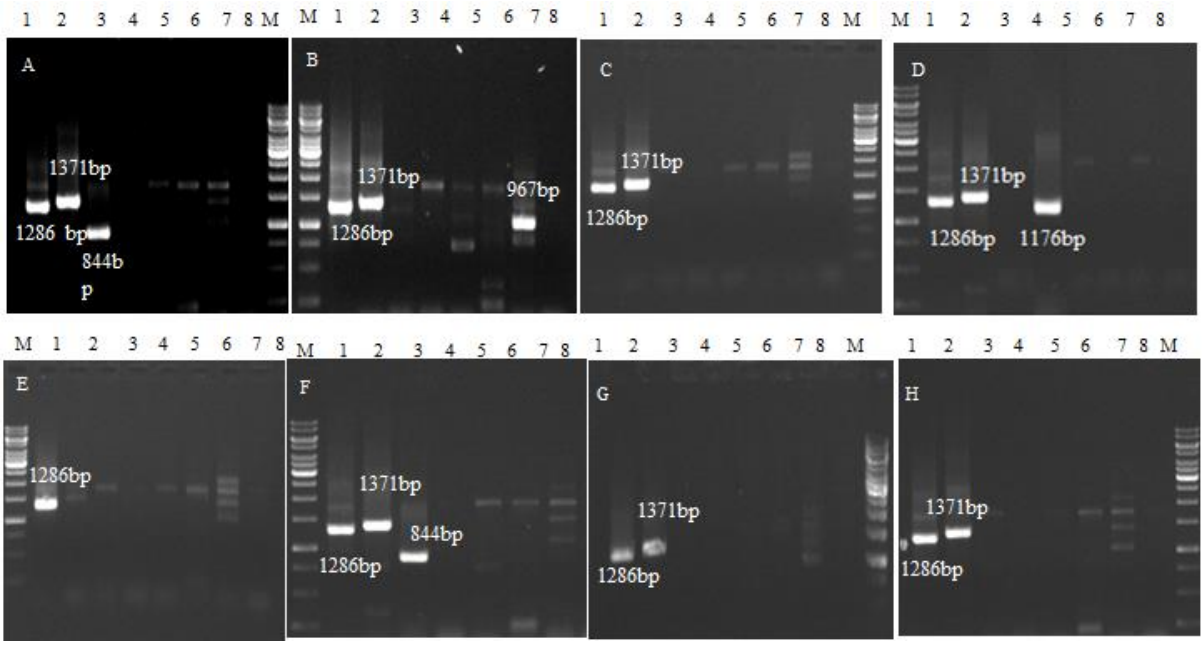

Fig 3-5. The *cryI* gene profile of *Bacillus thuringiensis* strains. Panels A- H represent *Bacillus thuringiensis* strains D5, K3, K19, G26, K14, G34, N6, and G31. Lanes 1 to 8 represent conserved domain amplicons of *cryIAa* , *cryIAb*, *cry IAc* , *cryIC* , *cryID* , *cryIE*, *cryIF*, *cryIG* genes respectively. *cryIC* and *cryIF* were present in *Bacillus thuringiensis* strains G26 and K3, respectively.

As discussed earlier, the commercially available bioinsecticides like Thuricide and Dipel are effective against many lepidopteran insects are ineffective against *S. litura*. *S. litura* being the pest of highly cultivated and economically important crop plants like ground nut, castor and cotton, makes it inevitable to develop an effective bio-control agent capable of controlling this insect pest. Many studies have been carried out on other *Spodoptera* species

like *S. frugiperda* (Rahman et al., 2012), *S. exigua* (de Maagd et al., 2000) but there are only few studies on *S. litura* (Singh et al., 2004). *S. litura* is the predominant pest in the Indian subcontinent. Domain swapping and shuffling techniques were adapted for increasing the toxicity and enhancing the susceptible host spectrum. Dr. Singh (2004) had shown that domain shuffling among the CryIC and CryIE toxins had resulted in the development of the transgenic tobacco resistant to *S. litura*. Using the information obtained in this chapter as molecular biology lead, we further proceeded with the construction of chimeric *cryIEC* gene to achieve our aim of developing an effective bio-pesticide formulation against *S. litura* larvae.

3.4. Conclusions:

1. Out of l15 *Bacillus thuringiensis* strains, the *cryI* gene profile of 35 strains was established.
2. Within 35 *Bt* strains, 33 strains had *cryIAa*, 30 showed *cryIAb*, 14 showed *cryIAc* , 4 strains had *cryIAd* .
3. *Bacillus thuringiensis* strain G26 showed *cryIC* along with *cryIAa* and *cryIAb.*

Chapter 2
Results
&
Discussions

4.1. Background

Biopesticides have gained lot of momentum over pesticides, owing to the contamination of natural resources and the increased resistance to the target pests (Singh et al., 2004). Cry toxins are lethal against insect species belonging to the orders Lepidoptera, Diptera, Coleoptera, Hemiptera, Hymenoptera and nematodes (Ibrahim et al., 2010, Bravo et al., 2012). Hence, Bt strains coding Cry toxins serve as an important reservoir of biopesticides. A number of Bt formulations, involving Bt spores, have been made and used as insecticides (Rosas-Garcia, 2009). Their use started in 1950's and continued till the emergence of recombinant DNA technology. Applying the gene manipulation techniques, insect resistant transgenic plants were developed by cloning the *cry* genes from a variety of Bt strains. The first insect resistant tobacco plant was developed in 1983 and since then a number of insect resistant crop plants were generated. Plants engineered to express *cry* genes encoding the *B. thuringiensis* crystal proteins are the most widely cultivated transgenic crops (Nguyen & Jehle, 2009, Singh et al., 2004). Cultivation of Bt transgenics had drastically reduced the usage of insecticides minimizing agricultural expenditure and damage to the environment (Kathage & Qaim, 2012, Kumar et al., 2011). In Indian scenario, the extent of transgenic crop cultivation is increasing mainly due to increased cultivation of Bt cotton (Kathage & Qaim, 2012). Cultivation of edible crops is discouraged mainly due to the public aversion for genetically modified foods and their possible effects on non target organisms and on the environment (Losey et al., 1999). Under such conditions, developing strategies to fully exploit the potential of Cry toxin mediated insect resistance is challenging.

The expression of the Cry toxins in GRAS (Generally Regarded As Safe) organisms like *E. coli*, *Pseudomonas fluorescens*, *Bacillus subtilis* would be a better alternative strategy for

agricultural pest management. The usage of engineered bacteria over the native isolates that contain novel toxic *cry* genes have several advantages. They contain more amount of toxin content and the quantity required can be scaled up using available fermentation and downstream processing technologies.

B. thuringiensis has evolved to produce large quantities and combinations of crystal proteins. Natural isolates of *B. thuringiensis* produce different types of crystal proteins to elicit toxic response on a variety of insect pests. Further, combination of Cry toxins have been shown to exhibit either synergistic or antagonistic effects (Schnepf et al., 1998). Hence, genetic manipulation techniques creating chimeric Cry toxins have gained popularity as one of the predominant strategies of insect pest management. The construction of hybrid Cry toxins which involves swapping of domain III has resulted in creation of novel toxins with a wider target range and higher toxicity (Pardo-Lopez et al., 2009). Cry1 toxins like Cry1Ab, Cry1Ac, Cry1Ba and Cry1Ea showed very low or no toxicity against *Spodoptera exigua*. However they became active toxins when their domain III is replaced with the similar domain of Cry1Ca (de Maagd et al., 1999). The Cry1Ab toxin is not toxic to *S. exigua* but the hybrid toxin containing domain III of Cry1C showed maximum toxicity against *S. exigua* and displayed 10 times more toxic effect than Cry1C toxin. Replacing 450– 612 amino-acids from domain III of Cry1Aa by those of Cry1Ac resulted in almost 300- fold increase in toxicity against *H. virescens* (Pardo-Lopez et al., 2009). The exchanges of domain III alter the efficiency with which the toxins bind to the insect membrane receptor proteins. Considering the importance of domain III, its shuffling has been proposed as an evolutionary threshold of insecticidal Cry toxins (Pardo-Lopez et al., 2009). *Bacillus thuringiensis* had established itself as an effective biotoxin globally. The Bt strains belonging to *Bacillus thuringiensis* spp *kurstaki* like *Bacillus thuringiensis* HD1 were found to

be effective against many lepidopteran pests like *Helicoverpa*, *Plutella*, *Achaea janata*. However, the toxicity of Bt strains against *Spodoptera* is very moderate. *Spodoptera* are predominant pests in Indian subcontinent and crop loss due to *Spodoptera* infestation is extremely high. Domain III of Cry1C was a major determinant of specificity for *Spodoptera exigua,* but its activity was too low to be used for field application with nearly no toxicity against third or fourth instar insect larvae (de Maagd et al., 1999, Xue et al., 2005). Cry1EC expressed in tobacco and cotton leaves demonstrated complete mortality of *S. litura* and growth retardation in *Helicoverpa armigera* (Singh et al., 2004). Despite of having such a promise, the transgenic plants containing either wild type or chimeric *cry* genes are permitted to grow in agriculture fields. Except Bt cotton, rest of them are confined to green houses justifying the claim that transgenic technology is a pot technology having no scope for seeing light. The present strategy is to exploit the Cry toxins as an effective pest management tools by combining recombinant DNA technology with nanotechnology. In this study, the expressions of either native or chimeric toxins were optimized. These recombinant toxins were then filled into the mesoporous nanoparticles to generate silicon nanoparticles filled with Cry toxins. These particles named as 'Crysones', were used to test their ability for controlling insect pests. The work is divided into four chapters and each one describes a particular aspect of the study.

4.2. Construction of chimeric *cryIEC* gene.

As described before, the chimeric Cry toxins are known to be more effective than native Cry toxins. The CryIC shows significant toxicity to *Spodoptera frugiperda* (Hofte and Whiteley, 1989). The Cry1E resembles the Cry1C subgroup to the extent of about 70%. It does not cause significant toxicity to *Spodoptera* sp and binds to receptors different from CryIC (Visser et al., 1990, Masson et al., 1992, Bosch et al., 1994) Not much data has been published on the toxicity

of the δ-endotoxins to *S. litura* (Prasad and Gowda, 2006). A novel chimeric δ-endotoxin CryIEC has earlier been designed specifically against *S. litura* by replacing amino acids 530–587 in a poorly active Cry1Ea protein with a homologous 70 amino acid stretch of CryICa in domain III (Singh et al., 2004). The hybrid δ-endotoxin CryIEC expressed in tobacco showed higher toxicity than the parental toxins against *S. litura* (Singh et al., 2004). In the present study the chimeric Cry protein CryIEC is chosen as model toxin to demonstrate Crysones effect on insect pests. Therefore the present chapter describes construction and expression of *cryIEC* in *E. coli.*

While construction of chimeric *cryIEC,* the desired regions of *cry* genes coding for various domains were amplified from *cryICa* and *cryIEa*, and generated inframe fusions as shown in Fig 4-1. The *cryICa* and *cryIEa* were taken from *Bacillus thuringiensis* strain G26, DOR, Hyderabad (*cryICa*) and ECE127 (*cryIEa*) clone from Bacillus Genetic Stock Centre, Ohio University, USA.

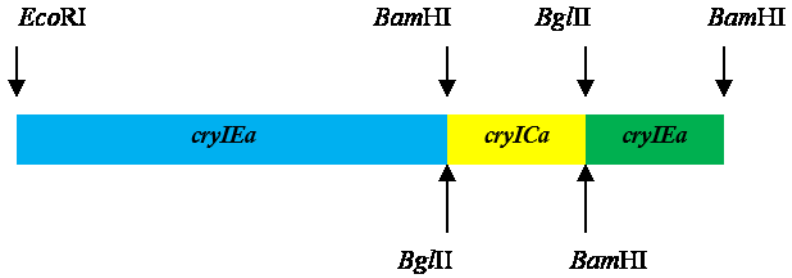

Fig 4-1. Construction of *cryIEC.*

The *cryIEa* gene 5' region of 1588 bp in size was amplified from *cryIEa* as *Eco*RI and *Bam*HI fragment using SDS01 (5' GAGCGAATTCATGGAGATAGTGAATAATCAGAA 3') and SDS02 (5' GCTCGGATCCTCGTGCATCCCTACTGGAAG 3') primers and the 133 bp of *cryICa*

gene coding for domain III was amplified as *Bgl*II and *Bam*HI fragment using SDS03 (5' GAGCAGATCTGTTATAGTATTAACAGGAGCG 3') and SDS04 (5' GCTCGGATCCGCTACT AATAGAACCTGCACC 3') primers. Finally 210 bp of *cryIEa* gene coding for C-terminal region of active CryIEa was amplified as *Bgl*II and *Bam*HI fragment using SDS05 (5' GAGCAGATCTGGTGAGCTTTATATAGATAAAATTGA 3') and SDS06 (5' GCTCGGATCC TCATTATAGCCCTAGTTGATTTGT 3') primers. All the three gene fragments were digested with the respective restriction enzymes and ligated by mixing in in 1:1 ratio. The ligation mix was then used as template to amplify full length *cryIEC* gene using SDS01 and SDS06 primers. The amplicon having expected size of *cryIEC* was gel extracted and cloned in TA vector and the recombinant plasmid was designated as pTZ-EC.

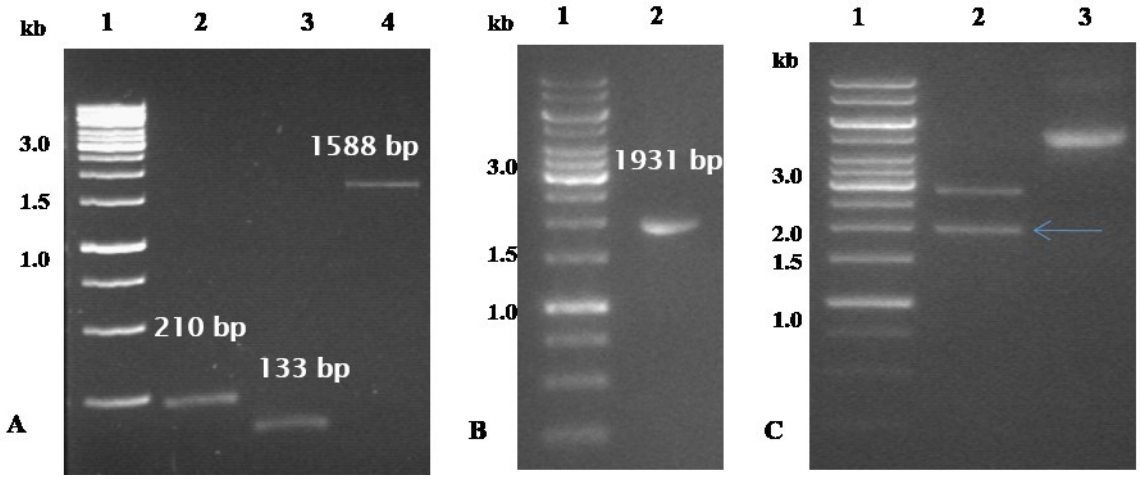

Fig 4-2. Construction of *cryIEC*. Panel A. Fig: Lane 1 represents 1 kb DNA ladder. Lanes 2 and 4 represent gene regions amplified from *cryIE* and lane 3 represent gene region amplified from *cryIC*. Panel B. Lane 1 represents 1 kb DNA ladder. Lane 2 represent *cryIEC* gene. Panel C. Lane 1 represents I kb DNA ladder. Lane 2 represent *Sac*I and *Bam*HI digested pTZ-EC and lane 3 represents uncut plasmid.

In order to check if the ligation of the 3 gene regions has resulted in generation of an inframe full length *cryIEC*, the chimeric *cryIEC* construct was sequenced (Fig 4-3). As shown in figure 4-3 the sequence created chimeric *cryIEC* gene, which codes for Domain I and II regions from

cryIEa gene and a part of Domain III is coded by *cryICa*. The deduced amino acid sequence coded by *cryIE* are shown in blue and green colour and the red colour sequence is cloned from *cry1C* gene. After confirming the sequence of *cryIEC,* experiments were performed to achieve its heterologous expression in GRAS (Generally Regarded As Safe) organisms. Initially *E. coli* expression system is used to express *cryIEC* gene (Fig 4-3).

```
ATGGAGATAGTGAATAATCAGAATCAATGCGTGCCTTATAATTGTTTAAATAATCCTGAAAATGAGATATTAGATATTGAAAGGTCAAATAGTACTGTAG 100
M  E  I  V  N  N  Q  N  Q  C  V  P  Y  N  C  L  N  N  P  E  N  E  I  L  D  I  E  R  S  N  S  T  V  A
CAACAAACATCGCCTTGGAGATTAGTCGTCTGCTCGCTTCCGCAACTCCAATAGGGGGGATTTTATTAGGATTGTTTGATGCAATATGGGGGTCTATAGG 200
  T  N  I  A  L  E  I  S  R  L  L  A  S  A  T  P  I  G  G  I  L  L  G  L  F  D  A  I  W  G  S  I  G
CCCTTCACAATGGGGTTTATTTTTAGAGCAAATTGAGCTATTGATTGACCAAAAAATAGAGGAATTCGCTAGAAACCAGGCAATTTCTAGATTAGAAGGG 300
 P  S  Q  W  G  L  F  L  E  Q  I  E  L  L  I  D  Q  K  I  E  E  F  A  R  N  Q  A  I  S  R  L  E  G
ATAAGCAGTCTGTACGGAATTTATACAGAAGCTTTTAGAGAGTGGGAAGCAGATCCTACTAATCCAGCATTAAAAGAAGAGATGCGTACTCAATTTAATG 400
I  S  S  L  Y  G  I  Y  T  E  A  F  R  E  W  E  A  D  P  T  N  P  A  L  K  E  E  M  R  T  Q  F  N  D
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  M  N  S  I  L  V  T  A  I  P  L  F  S  V  Q  N  Y  Q  V  P  F  L  S  V  Y  V  Q  A  A  N  L  H  L
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 S  V  L  R  D  V  S  V  F  G  Q  A  W  G  L  D  I  A  T  I  N  S  R  Y  N  D  L  T  R  L  I  P  I
TATACAGATTATGCTGTACGCTGGTACAATACGGGATTAGATCGCTTACCACGAACTGGTGGGCTGCGAAACTGGGTAAGATTTAATCAGTTTAGAAGAG 700
Y  T  D  Y  A  V  R  W  Y  N  T  G  L  D  R  L  P  R  T  G  G  L  R  N  W  V  R  F  N  Q  F  R  R  E
AGTTAACAATATCAGTATTAGATATTATTTCTTTTTTCAGAAATTACGATTCTAGATTATATCCAATTCCAACAAGCTCCCAGTTAACGCGGGAAGTATA 800
  L  T  I  S  V  L  D  I  I  S  F  F  R  N  Y  D  S  R  L  Y  P  I  P  T  S  S  Q  L  T  R  E  V  Y
TACAGATCCGGTAATTAATATAACTGACTATAGAGTTGGCCCCAGCTTCGAGAATATTGAGAACTCAGCCATTAGAAGCCCCCACCTTATGGACTCCTTA 900
 T  D  P  V  I  N  I  T  D  Y  R  V  G  P  S  F  E  N  I  E  N  S  A  I  R  S  P  H  L  M  D  S  L
AATAATTTGACCATTGATACGGATTTGATTAGAGGTGTTCACTATTGGGCAGGGCATCGTGTAACTTCTCATTTTACAGGTAGTTCTCAAGTGATAACAA 1000
N  N  L  T  I  D  T  D  L  I  R  G  V  H  Y  W  A  G  H  R  V  T  S  H  F  T  G  S  S  Q  V  I  T  T
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 F  F  R  R  S  E  N  I  T  P  T  L  G  I  N  V  V  Q  G  V  G  F  I  Q  P  N  N  A  E  V  L  Y  R
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S  R  G  T  V  D  P  L  N  E  L  P  I  D  G  E  N  S  L  V  G  C  S  H  R  L  S  H  V  T  L  T  R  S
CGTTATATAATACTAATATAACTAGCCTGCCAACATTTGTTTGGACACATCACAGTGCTACTAATACAAATACAATTAATCCAGATATTATTACACAAAT 1400
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TTTGTGTCTTTACAAGTCAATATTAACTCACCAATTACCCAAAGATACCGTTTAAGATTTCGTTATGCTTCCAGTAGGGATGCACGAGGATCTGTTATAG 1600
F  V  S  L  Q  V  N  I  N  S  P  I  T  Q  R  Y  R  L  R  F  R  Y  A  S  S  R  D  A  R  G  S  V  I  V
TATTAACAGGAGCGGCATCCACAGGAGTGGGAGGCCAAGTTAGTGTAAATATGCCTCTTCAGAAAACTATGGAAATAGGGGAGAACTTAACATCTAGAAC 1700
  L  T  G  A  A  S  T  G  V  G  G  Q  V  S  V  N  M  P  L  Q  K  T  M  E  I  G  E  N  L  T  S  R  T
ATTTAGATATACCGATTTTAGTAATCCTTTTTCATTTAGAGCTAATCCAGATATAATTGGGATAAGTGAACAACCTCTATTAGGTGCAGGTTCTATTAGT 1800
 F  R  Y  T  D  F  S  N  P  F  S  F  R  A  N  P  D  I  I  G  I  S  E  Q  P  L  L  G  A  G  S  I  S
AGCGGATCTGGTGAGCTTTATATAGATAAAATTGAACTTATTCTAGCAGATGCAACATTTGAAGAAGAATATGATTTGGAAAGAGCACAGAAGGCGGTGA 1900
S  G  S  G  E  L  Y  I  D  K  I  E  L  I  L  A  D  A  T  F  E  E  E  Y  D  L  E  R  A  Q  K  A  V  S
GTGCCCTGTTTACTTCTACAAATCAACTAGGGCTATAATGA 2000
  A  L  F  T  S  T  N  Q  L  G  L  *  *
```

Fig 4-3. The nucleotide and the deduced amino acid sequence of chimeric *cryIEC.*

4.3. Construction and Expression of CryIEC$_{NGST}$ and CryIEC$_{N6HIS}$ in *E. coli* BL21.

Crystal genes were expressed successfully into GRAS organisms like *E. coli*, *B. subtilis*, *B. megaterium*, and *Pseudomonas fluorescens* (Schnepf et al., 1998). The engineered forms of Cry proteins showing improved potency or yield, expressed in GRAS organisms can make Cry

biopesticides a more efficient and practical alternative to synthetic chemical control agents and Bt transgenics. Here, *E. coli* was chosen as the preliminary host for the expression of chimeric *cryIEC* as it was easy to manipulate. Also, Cry proteins like CryIAa were successfully expressed and shown to be active when expressed in *E. coli* (Schnepf and Whiteley, 1981).

In the present study CryIEC was expressed with both N-terminal GST tag and C-terminal His-tag. The *cry1EC* was amplified using SDS07 (5' GAGCGGATCCATGGAGATAGTGA ATAATCAGAA 3') and SDS08 (5' GCTCGGATCCTCATTATAGCCCTAGTTGATTTGT 3') primers and plasmid pTZ- EC, containing *cryIEC* as template. As *Bam* HI sites were incorporated in both the forward and reverse primers, the *cry1EC* amplicon was digested with *Bam* HI and cloned in *Bam* HI digested pGEX 4T1. The resulting plasmid having insert in right orientation was designated as pSKS1.

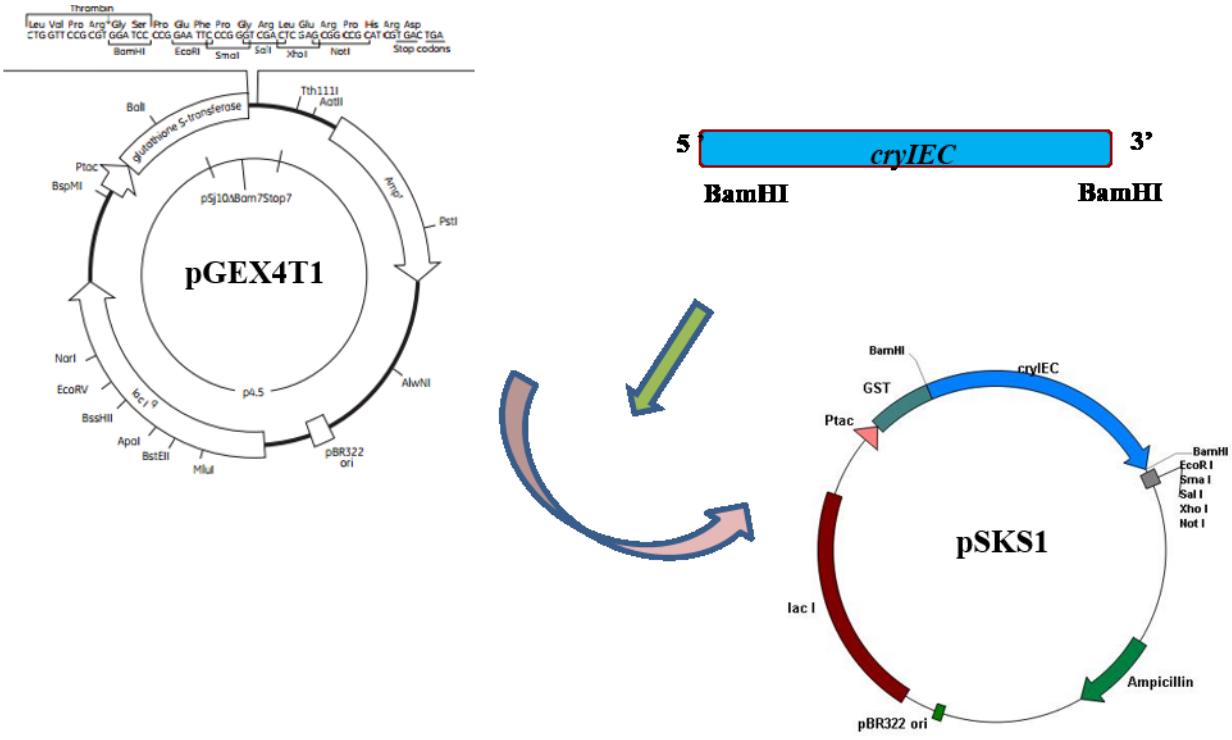

Fig 4-4. Schematic representation of construction of pSKS1.

Figure 4-4 describes the cloning strategy used for cloning *cryIEC* gene as *Bam*HI fragment. Before proceeding for induction its orientation was confirmed by digesting with *EcoRV* (present in insert) and *Sal* I (present in vector), as these are unique either to insert or vector. The release of 1.6 kb *Eco*RV /*Sal*I fragment confirmed existence of *cry1EC* is in right orientation (Fig 4-2B). The recombinant plasmid pSKS1, when induced codes for $CryIEC_{NGST}$

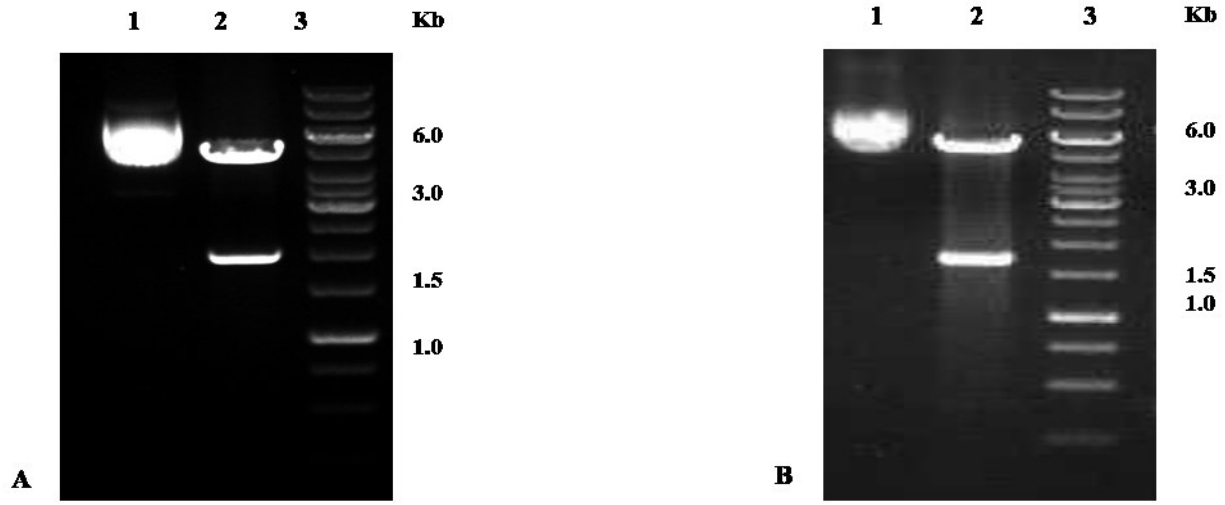

Fig 4-5. Cloning of *cry1EC* in pGEX 4T1. Panel A indicates undigested recombinant plasmid (lane 1), the recombinant plasmid pSKS1 digested with *Bam*HI (lane 2) and kb ladder (lane 3). Release of 2 kb fragment is seen in lane 2. Panel B indicates the orientation of cloned *cry1EC* gene in pGEX4T1. Release of 1.6 kb fragment when digested with *EcoR* V and *Sal* I in lane 2 confirms the orientation of *cry1EC* gene. Lane 1 represents the undigested recombinant plasmid and lane 3 represents kb ladder.

While generating the clone to code for $CryIEC_{N6HIS,}$ pRSET-A was choosen as expression vector. Cloning of *cryIEC* in pRSET-A places it under the control of *T7* promoter and fuses its coding sequence in frame with the N terminal His tag of pRSETA. Initially the *cry1EC* was amplified using primer set SDS07 and SDS08 and pTZ- EC as template. As *Bam*HI sites were incorporated in both the forward and reverse primers, the *cry1EC* amplicon was digested with *Bam*HI and cloned in *Bam*HI and *Bgl*II digested pRSETA. The resulting plasmid was designated as pSKS10. The cloning strategy followed was shown in Fig 4-6.

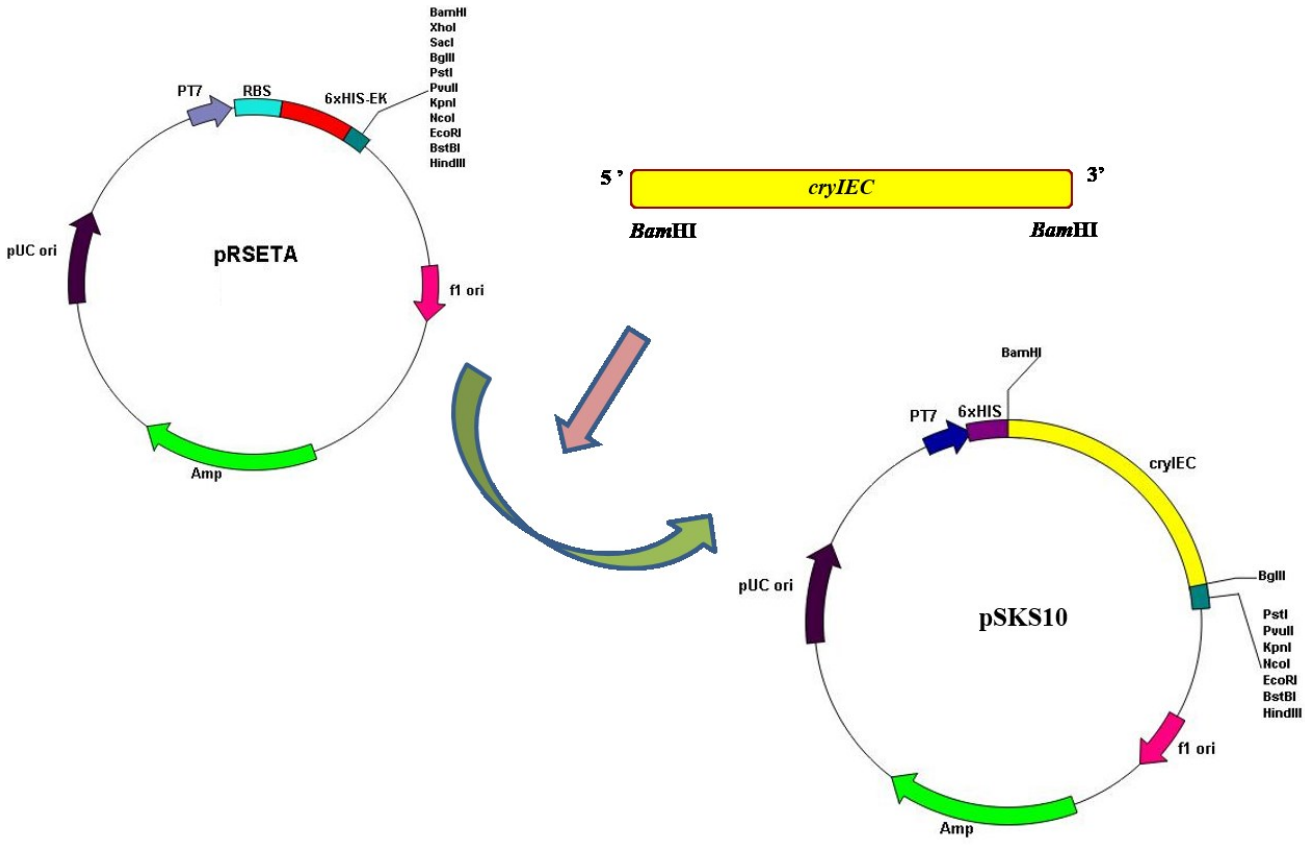

Fig 4-6. Schematic representation of construction of pSKS1.

As the *cry1EC* digested with *Bam*HI was cloned in *Bam*HI and *Bgl*II sites of pRSETA, the *Bam*HI- *Bgl*II site is lost i.e it can neither be cut with *Bam*HI nor with *Bgl*II. So *Pst*I was chosen for digestion to show the release of the insert. The orientation of the *cry* gene was confirmed by digesting with *Pvu*II (present in insert) and *Dra*I (present in vector) as digestion with these enzymes facilitates release of 1.6 Kb fragment (Fig 4-7B).

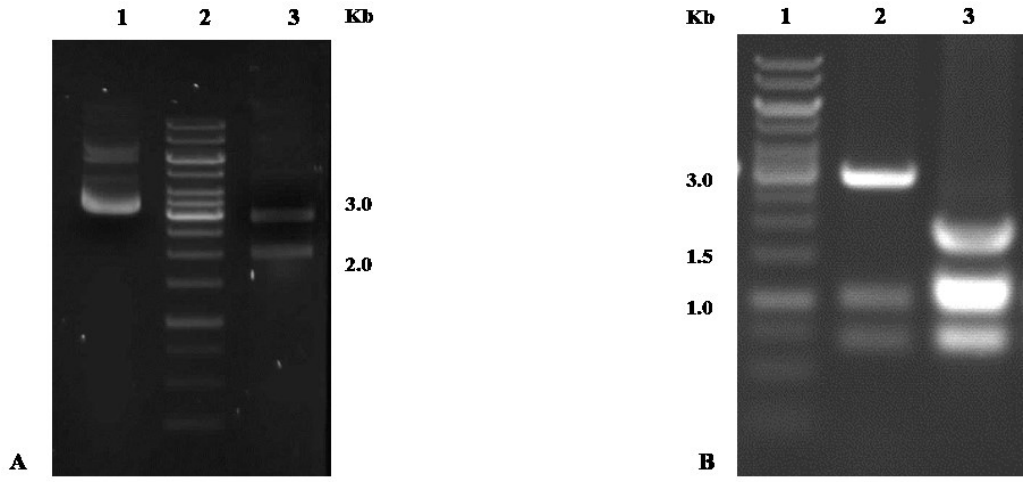

Fig 4-7. Cloning of *cry1EC* in pRSET A. Panel A indicates undigested recombinant plasmid (lane 1), kb ladder (lane 2) and the recombinant plasmid pSKS10 digested with *Bam*HI and *Pst* I (lane 3). Release of 2 kb fragment is seen in lane 3. Panel B indicates the orientation of cloned *cry1EC* gene in pRSET A. Release of 1.6 kb fragment when digested with *Dra* I and *Pvu* II in lane 3 confirms the orientation of *cry1EC* gene. Lane 2 represents the pSKS10 clone with wrong orientation and lane 1 represents kb ladder.

The recombinant plasmids, pSKS1 and pSKS10 were independently transformed into *E. coli* strains DH5α and BL21. The mid log phase cultures containing the expression plasmids were induced as described in materials and methods. After induction, proteins expressed from control and test cultures were analysed on 12.5% SDS- PAGE. As the expression levels were low, a faint band corresponding to the size of 98 kDa was seen in *E. coli* BL21 cell lysate harboring pSKS1. The size of the protein coincided with the size of CryIEC$_{NGST}$ (26 kDa + 72 kDa). To gain further insights into the expressed protein, the gel was blotted and western analysis was performed using anti- GST antibody. As shown in Fig 4-8B (lane 5), the 98 kDa size protein gave a positive signal. No such signal was seen either in uninduced cultures or in the cultures with no expression plasmids. This confirms the expression of GST- Cry1EC in *E. coli* BL21 (pSKS1). In a similar experiment, the *E. coli* BL21 containing pSKS10 was induced to express Cry1EC$_{N6HIS}$ from *T7* promoter. The proteins expressed in induced cultures and controls were analysed on SDS- PAGE to identify Cry1EC$_{N6HIS}$ (Fig 4-9A). Relatively low expression

was noticed in the induced culture (Fig 4-9B, lane 5). This infers, pGEX derivative, pSKS1 was a better expression plasmid for expressing Cry1EC$_{N6His}$ protein. Therefore, further experiments were done using expression plasmid pSKS1.

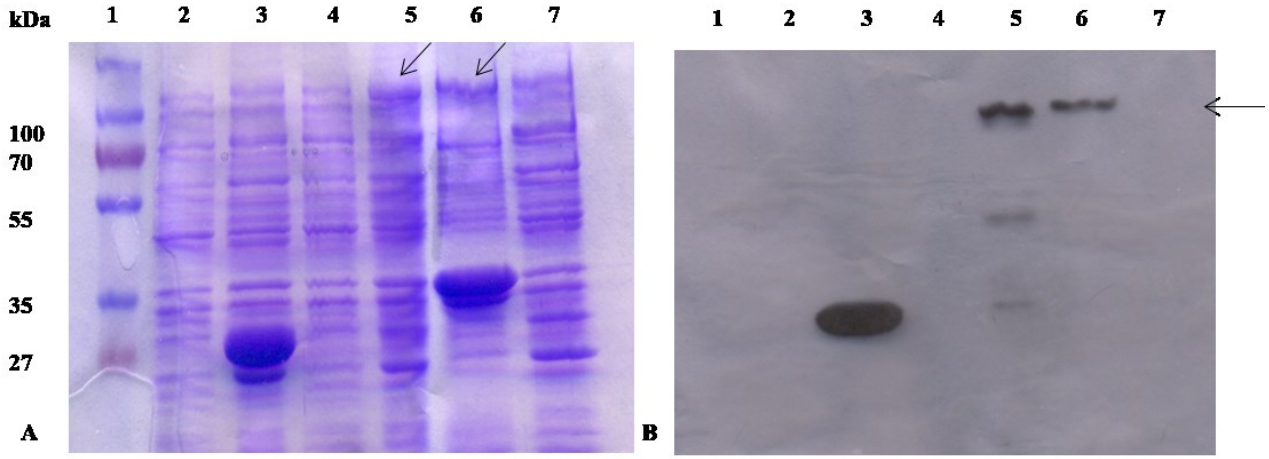

Fig 4-8. Subcellular fractionation and detection of Cry1EC$_{NGST}$ in *E. coli* BL21 containing expression plasmids. *E. coli* BL21 containing expression plasmid pSKS1 was induced and fractionated into particulate and soluble fraction by centrifuging the lysate at 12000 rpm. Panel A) Lane 1: Prestained protein marker; lanes 2 and 3 represent uninduced and induced cell lysate of BL21 expressing GST; lanes 4 and 5 represent uninduced and induced cell lysate of *E. coli* BL21 (pSKS1); lane 6 and 7: particulate and soluble fraction of the induced *E. coli* BL21 (pSKS1); Panel B) Corresponding western blot showing signals with anti GST- HRP antibody. Expression of Cry1EC$_{NGST}$ (98 kDa) was seen in lanes 5 and 6.

Further, the cell lysate of induced *E. coli* BL21 cells with expression plasmid pSKS1 was sonicated and the soluble and insoluble fractions were analysed on SDS- PAGE (Fig 4-8A) to check the localization of protein. Western blot analysis was done to confirm the results (Fig 4-8B, Lanes 6 & 7). However when further experiments were done to see if the expressed protein is in soluble form, most of the protein was found in inclusion body fraction (particulate fraction) (Fig 4-8B, Lane 6). Very low amounts of expressed protein was found in soluble form (Fig 4-8B, Lane 7). As the protein concentration in the soluble fraction is low and the protein in the inclusion bodies fail to elicit toxic response towards the targeted insects, the chimeric *cry1EC*

was further expressed in *E. coli* Arctic Express system to obtain the recombinant CryIEC$_{NGST}$ in soluble form.

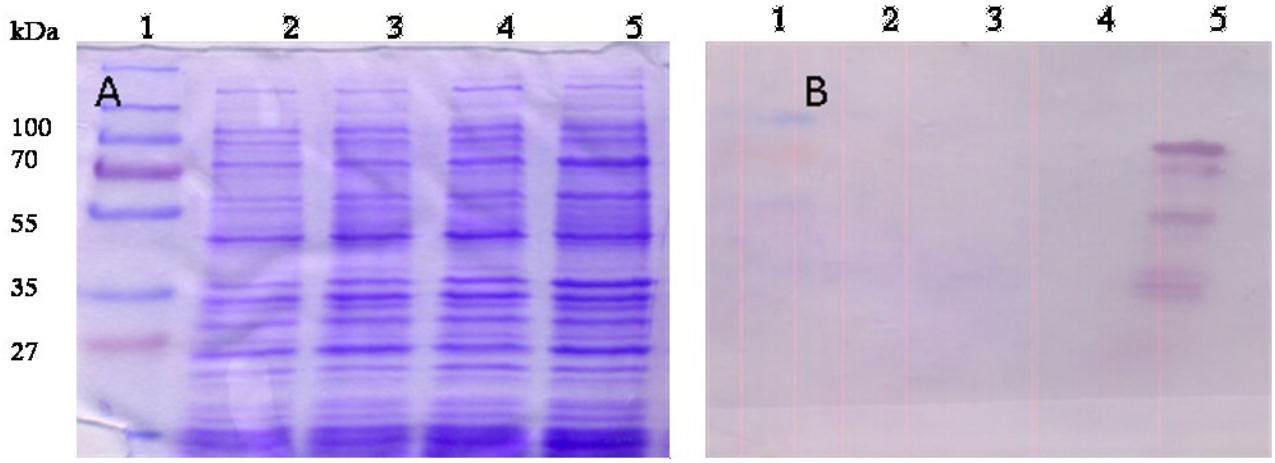

Fig 4-9. Expression of Cry1EC under *T7* promoter in *E. coli* BL21: Panel A) The SDS- PAGE indicates prestained protein molecular weight marker (lane 1), lanes 2 and 3 represent protein extracts prepared from *E. coli* BL21 cells containing expression vector pRSET A, protein extracts prepared from *E. coli* BL21(pSKS10) uninduced (lane 4) and induced cultures (lane 5). Panel B represents corresponding western blot probed by using anti His antibody. Expression of Cry1EC$_{N6HIS}$ (78 kDa) was seen in lane 5.

4.4. Expression of CryIEC$_{NGST}$ in *E. coli* Arctic Express:

E. coli Arctic Express expresses chaperonins, *cpn60* and *cpn10*, from the Antarctic isolate, *Oleispira antarctica.* Chaperonins Cpn60 and Cpn10, show high protein refolding activities at temperature range of of 4-12^{0}C. The recombinant proteins were expressed by inducing with IPTG at 12^{0}C for 24 hrs. The *cry1*EC cloned in pGEX 4T1 (pSKS1) was expressed in *E. coli* Arctic express by induction with 1mM IPTG at 12˚C for 24 h.

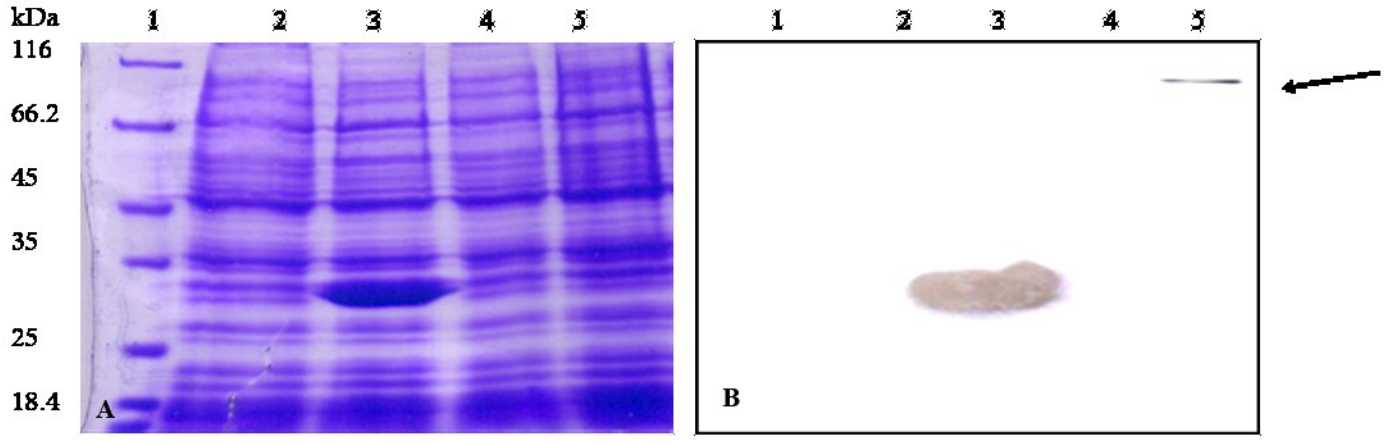

Fig 4-10. **Expression of *cry1EC* in *E. coli* Arctic Express.** Panel A) The SDS- PAGE indicates protein extracts prepared from *E. coli* Arctic Express (pGEX- 4T1) uninduced (lane 2) and induced cultures (lane 3). Lanes 4 and 5 represent similar extracts prepared from *E. coli* Arctic Express cells containing expression plasmid pSKS1; lane 1 represents unstained protein marker. Panel B represents corresponding western blot probed by using anti GST antibody. Expression of CryIEC$_{NGST}$ (98 kDa) was seen in lane 5.

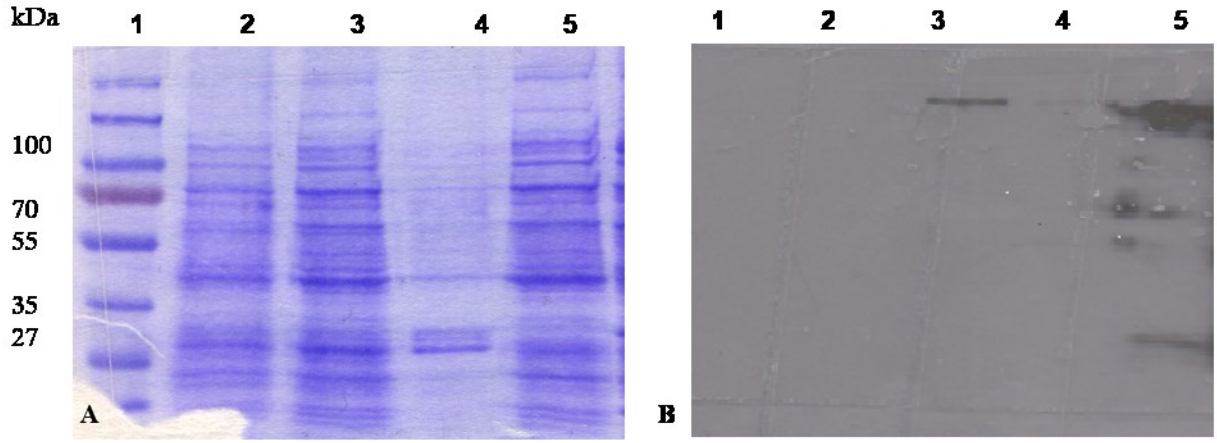

Fig 4-11. **Subcellular fractionation and detection of Cry1EC in *E. coli* Arctic Express containing expression plasmids**. *E. coli* Arctic Express containing expression plasmid pSKS1 were induced and fractionated into particulate and soluble fraction by centrifuging the lysate at 15000 rpm. Panel A) Lane 1 represents Prestained protein marker; lane 2 represents uninduced Arctic Express cells having pSKS1; lanes 3 and 4 represent soluble and particulate fractions of induced *E. coli* Arctic Express cells containing pSKS1; lane 5 represents induced cell lysate of *E. coli* BL21 with *cry1EC* which serves as a positive control; Panel B represents corresponding western blot probed by using anti GST antibody. Expression of CryIEC$_{NGST}$ (98 kDa) was seen in lane 3, 4 and 5.

The expression of the protein was analysed by SDS- PAGE (Fig 4-10A) and confirmed by corresponding western blot analysis using anti- GST HRP conjugate antibody (Fig 4-10B). The expected size of the protein (72 kDa) along with GST (26 kDa) was 98 kDa. Most of the protein was found to be present in the soluble fraction (Fig 4-11, lane 3) suggesting that the protein was properly folded when expressed in *E. coli* Arctic Express.

4.5. Purification of Cry1EC$_{NGST}$:

After achieving soluble expression of CrylEC$_{NGST}$ in *E. coli* Arctic Express, an attempt was made to purify the recombinant Cry1EC$_{NGST}$ using GST affinity chromatography (Glutathione Sepharose Fast Flow column). The 24 h induced *E. coli* Arctic Express cells expressing Cry1EC$_{NGST}$ protein were pelleted, lysed by sonication in presence of 0.15 mM PMSF and the soluble fraction of cell lysate was obtained by centrifugation at 15000 rpm for 30 min. This soluble fraction which contains Cry1EC$_{NGST}$ protein, was incubated with 1% triton X-100 on ice for 10 min and was subjected to GST affinity purification (described in Materials and methods). It was observed that Cry1EC$_{NGST}$ protein was expressed at low levels in *E. coli* Arctic Express when compared to *E. coli* BL21. Cry1EC$_{NGST}$ was not stable when the induced cell pellets were stored in -80°C for more than 3 days. So, care was taken to process the protein immediately after induction. As seen in SDS-PAGE done for affinity purified protein, considerable amount of protein is degraded. There is quite a good amount of free GST. Its not known if its generated during purification process or its due to incomplete translation of chimeric mRNA due to difference in codon usage of vector specified GST region and Bt derived Cry region. However considerable amount of fusion protein was found in affinity purified fractions and hence used these protein for performing toxicity and ligand binding assays.

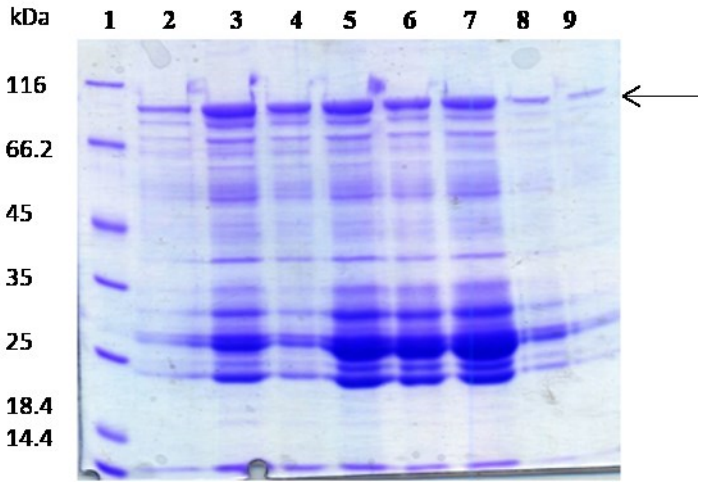

Fig 4-12. **Purification of Cry1EC$_{NGST}$**. Lane 1: unstained protein molecular weight marker; lanes 2-9 represent elution fractions of CryIEC$_{NGST}$ protein collected after applying soluble fraction of induced *E. col*i Arctic Express cells with expression plasmid, pSKS1 onto GST affinity column. The CryIEC$_{NGST}$ protein of 98 kDa was seen in all the 8 fractions.

4.6. Interaction of CryIEC$_{NGST}$ toxin with gut receptor proteins of *S. litura*

Having obtained soluble expression of CryIEC$_{NGST,}$ its true application depends on its ability to interact with the receptors found in insect gut. Its indeed the prerequisite for eliciting the toxic response in insects. Therefore ligand binding assay was performed by following standard protocols (Agrawal et al., 2002). The active Cry toxin binds to different types of receptors present in the midgut epithelium of susceptible insects. Membrane bound aminopeptidase N, cadherin-like proteins, anionic glycoconjugates, Glycolipids and alkaline phosphatase serve as receptors for the Cry toxins in the insect midgut (Pigott & Ellar, 2007). Previous studies showed that Cry1C shows moderate toxicity against *Spodoptera litura* due to its capacity to bind to the aminopeptidase receptors in the mid gut of the insect (Avisar et al., 2004, Agrawal et al., 2002).

4.6.1. Preparation of Brush Border Membrane Vesicles (BBMV) from *Spodoptera litura*.

Membrane bound aminopeptidase receptors were known to be involved in the binding to the Cry toxins and eliciting toxicity on susceptible insects (Budatha et al., 2007, Agrawal et al., 2002).

For studying the interaction of Cry1EC$_{NGST}$ toxin with the gut receptors, especially APNs, BBMV proteins were isolated using the protocol described in Materials and Methods. Alkaline phosphatase assay was done to check for the purity of BBMV preparation, using *p*- nitrophenyl phosphate as substrate (Walter and Schutt, 1974). The midgut BBMV showed high specific activity of 160 X 10^2 µmol of *p*-nitroanilide released/min/mg of protein. This BBMV preparation was further analysed for APN receptors with western blotting by probing with anti- APN antibodies. Anti- APN antibody was purified from anti- sera containing anti- APN antibodies using Protein A Sepharose chromatography according to standard protocols (Fig 4-13). Both western blot analysis (Fig 4-14) and alkaline phosphatase activity (data not shown) indicated isolation of BBMV proteins without having any cross contamination.

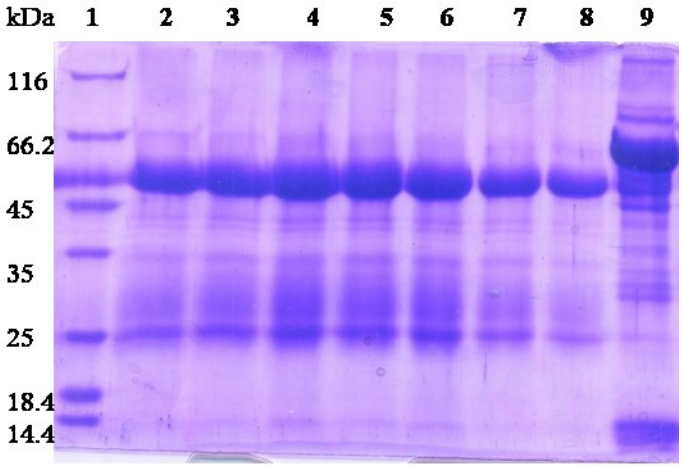

Fig 4-13. **Purification of anti- APN antibody:** Lane 1 represents unstained protein molecular weight marker. Lane 2-8 represent different elution fractions of purified anti- APN antibody and lane 9 represents the flow through fraction

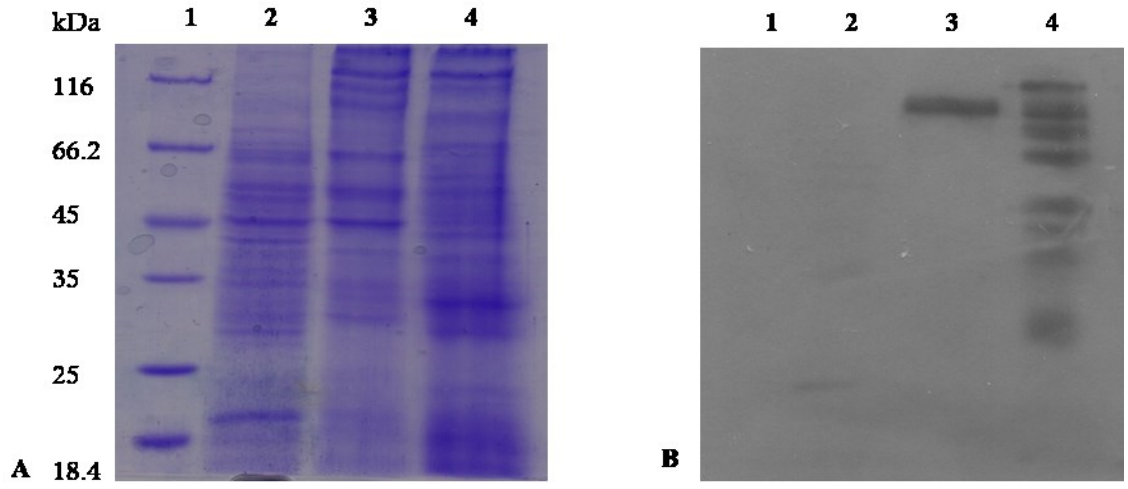

Fig 4-14. **Analysis of BBMV membrane preparation of *Spodoptera litura*.** Panel A: Lanes 1 represents unstained protein molecular weight marker, lane 2 represent *E. coli* membrane preparation, lanes 3 and 4 represent BBMV preparation from *Spodoptera litura* and *Achaea janata* respectively. Panel B represents the corresponding western blot probed with anti APN antibodies. Lane 3 shows 108 kDa APN protein.

4.6.2. Ligand Binding Assay- CryIEC$_{NGST}$ receptor interaction

Ligand blotting was done according to the procedure described in methodology, to establish the toxin- receptor interaction (Agrawal et al., 2002). The BBMV membrane preparation run on 10% SDS- PAGE was transferred to nitrocellulose membrane, incubated with Cry1EC$_{NGST}$ toxin. and analysed by western blotting after probing with anti- GST HRP conjugated antibody. There was a clear signal at 108 kDa size which corresponds to the APN receptor (Fig 4-15, Panel B, lane 3). Thus, the result shows that Cry1EC has good binding affinity with the APN receptor present on the gut of *S. litura.* The blot was stripped and re-probed with anti- GST HRP conjugated antibody without prior incubation with the Cry toxin. The absence of 108 kDa signal confirms the specific interaction of the Cry toxin and the APN receptor (Fig 4-15, Panel C, lane 3). The blot was again stripped and probed with anti- APN antibody with prior incubation with the Cry

toxin. The presence of 108 kDa signal confirms the size of APN receptor of *Spodoptera litura* (Fig 4-15, Panel D, lane 3).

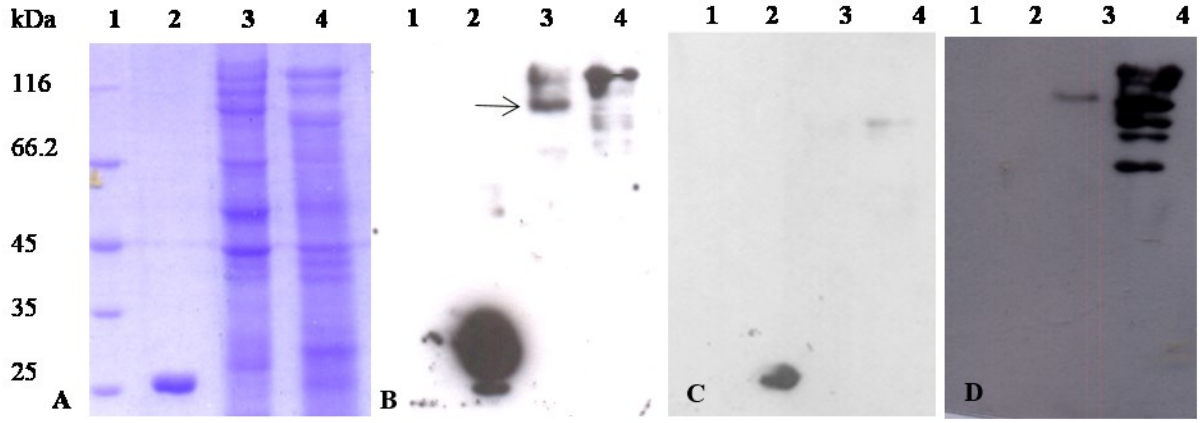

Fig 4-15. Ligand blot analysis. Panel A: Lane 1 represents unstained protein molecular weight marker; lane 2 represents pure GST protein; lane 3 represents BBMV preparation from *S. litura* and lane 4 represents BBMV preparation from *Achaea janata.* Arrow mark indicates APN protein of *S. litura.* Panel B represents corresponding western blot probed with anti- GST antibodies after incubation with Cry1EC toxin. Panel C represents corresponding western blot probed with anti- GST antibodies after incubation with pure GST protein. Panel D represents western blot probed with anti- APN antibodies.

4.7. CryIEC$_{NGST}$ Toxin Bioassay against 2nd instar larvae of *Spodoptera litura*

After establishing the interaction of CryIEC$_{NGST}$ toxin with the gut receptors of *S. litura*, the toxicity of CryIEC$_{NGST}$ was analyzed against 2nd instar larvae of *S. litura.* After purification of the Cry1EC$_{NGST}$ protein an attempt was also made to evaluate if it was folded properly and the recombinant plasmid retained the toxic effect. While analyzing these activities, the pure Cry1EC$_{NGST}$ was uniformly applied at a concentration of 150 ng/ sq cm on 12.5 sq cm castor leaf used for feeding *Spodoptera litura* larvae. Six second instar larvae were allowed to feed on each

castor leaf coated with Cry1EC$_{NGST}$ toxin and pure GST protein. The experiment was done in triplicates where pure GST protein serves as negative control.

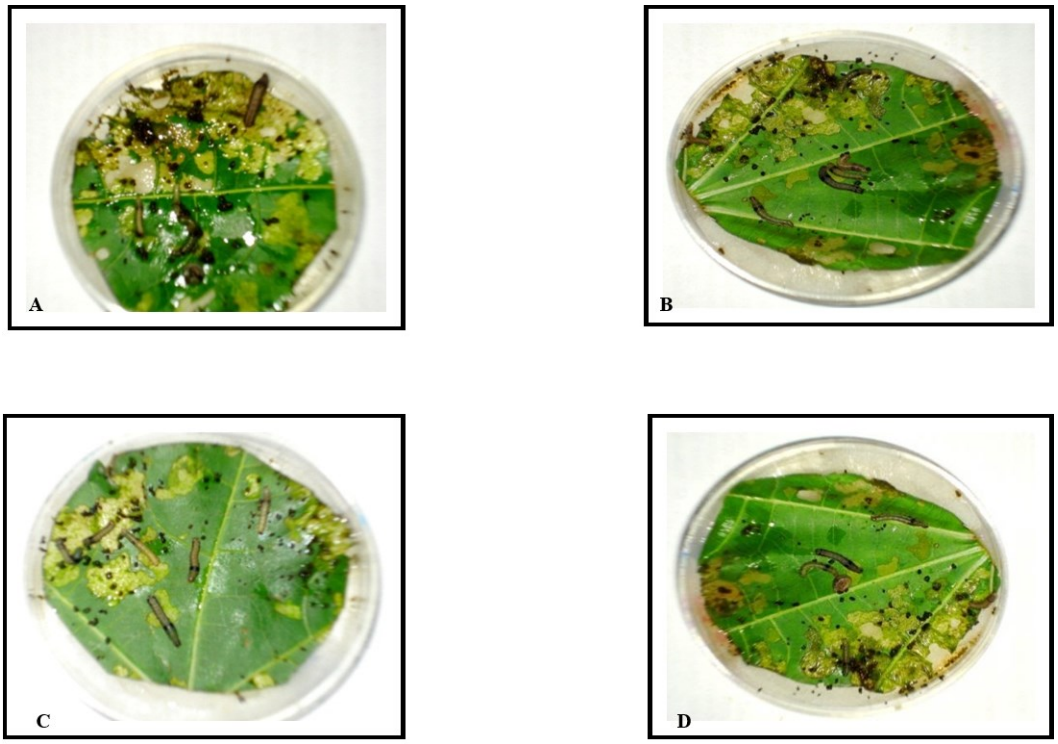

Fig 4-16. Bio assay of recombinant Cry1EC$_{NGST}$. Panel A, B, C, D represent feeding behavior and toxicity of Cry1EC$_{NGST}$ towards *Spodoptera litura* larvae on castor leaves coated with pure GST protein (Panel A) and with Cry1EC$_{NGST}$ protein in triplicates (Panel B, C, D).

As shown in Fig 4-16, the *S. litura* larvae feeding on recombinant Cry1EC$_{NGST}$ showed anti-feeding and retarded growth. However no mortality was observed. These results clearly indicate that the heterologously expressed Cry1EC$_{NGST}$ toxin, though binding to receptors is not eliciting similar toxicity like wild type Cry proteins. Initially it was assumed that the GST tag was reducing the toxicity. However, experiments repeated using CryIEC$_{N6His}$ gave similar results. Almost identical toxicity was seen when CryIEC$_{NGST}$ and CryIEC$_{N6His}$ were used as toxins in

bioassays. Explanation for these unusual results requires further experimentation. Primarily post translational modifications and misfolding are primarily shown to be responsible when recombinant proteins show either lost or reduced biological activity (Ibrahim et al., 2010). Supporting our observations studies conducted elsewhere have shown similar observations (Singh et al., 2004). Change of expression host helped in producing active recombinant proteins. The inactive recombinant proteins became active once expressed in hosts having considerable taxonomic relationship with the organism from where the gene is sourced (Binda et al., 2013). Therefore, we have generated expression system using *Pseudomonas fluorescens* to clone and express recombinant CryIEC toxins. These strategies are described in the next chapter.

4.8. Conclusions.

1. Chimeric *cry1EC* gene was constructed.
2. $CryIEC_{NGST}$ was expressed in *E. coli* BL21 and *E. coli* Arctic Express cells.
3. $CryIEC_{NGST}$ was purified using Glutathione Affinity Chromatography.
4. Ligand blotting showed the interaction of $Cry1EC_{NGST}$ toxin with the gut membrane proteins of *Spodoptera litura*.
5. $CryIEC_{NGST}$ was tested for its toxicity against 2^{nd} instar larvae of *Spodoptera litura* which showed no mortality of *S. litura*, though there was considerable size reduction and effect on growth of the insects.

Chapter 3
Results
&
Discussions

5.1. Background

Expressing a recombinant protein in its native and active form is the major bottleneck when it is expressed in heterologous systems (Davis et al., 1999). The major advantage of using the prokaryotic expression systems like *E. coli*, *Bacillus* sp and *Pseudomonas* sp. is that they facilitate rapid production and purification of recombinant proteins. The low production costs make them very attractive among the available expression systems (Davis et al., 1999; Baneyx & Mujacic, 2004).

Several studies have shown that Cry toxins produced in *E. coli* accumulate as inclusion bodies and generally are not as active as the native ones (Singh et al., 2004). Other alternative hosts for production of Cry toxins include *Pseudomonas fluorescens*. *Pseudomonas fluorescens* is believed to be a very suitable bacteria for heterologous expression of Cry toxins for a variety of reasons (Rodriguez et al., 2006). *Pseudomonas fluorescens* strains are often found in the natural environment. Some of them colonize roots of certain plants, promote their growth and protect them from fungal diseases (Selinger et al., 1998). *P. fluorescens* exhibit anti-fungal activity and the organism does not produce toxins that harm non-target species. Therefore, *Pseudomonas fluorescens* is a considered as one of the GRAS (Generally Regarded as Safe) organisms. Besides being a GRAS organism, it has a number of advantageous traits for the fermentative production of recombinant proteins (Rettallack et al., 2011). *P. fluorescens* is capable of growing by simple shaking in a temperature-controlled air chamber without oxygen supplementation. It lacks the physiological apparatus to switch to anaerobic growth, which produces organic acids like *Escherichia coli* that limit a bacterial culture's growth, under microaerophilic conditions. *P. fluorescens* continues growing as long as nutrients and oxygen are available. This results in accumulation of a significant amount of biomass nearly 30–50 OD units at A_{595}. High biomass

availability leads to significant amount of expressed protein (Squires, 2011). These advantages make *P. fluorescens* have an edge over other hosts making it a preferential host for expression of insecticidal proteins (Lang et al., 2014).

The high level expression of an active recombinant protein in a heterologous system is a hectic task as the transcript competes with the host transcription and translational machinery leading to the production of low amounts of recombinant proteins. Several regent studies have shown usage of T7 polymerase based expression system (Studier and Moffat, 1986, Gamer et al., 2009). The T7 promoters are dependent for transcription on T7 polymerase and not on host RNA polymerase. Moreover, T7 RNA polymerase is resistant to rifampicin. If T7 polymerase based expression system is induced after the host cell reaches to mid log phase, addition of rifampicin inhibits host RNA polymerase. Such inhibition makes the hosts transcription and translational machinery available exclusively for the production of recombinant proteins (Tabor, 2001). Using T7 polymerase based expression system, in certain cases, the recombinant protein expression levels were achieved to the extent of 30% of total soluble proteins (Gamer et al., 2009). However, this system is confirmed in *E.coli* host (Studier and Moffat, 1986), *Bacillus* sp (Gamer et al., 2009) and its potential is not extended to other GRAS organisms. This chapter describes strategies used for the development of T7 polymerase based expression system in *Pseudomonas fluorescens*.

Development of T7 polymerase based expression system in *Pseudomonas fluorescens*, requires 1. Mobilizable broad host range T7 promoter based expression vector 2. Modification of host to code for RNA polymerase system. These two critical steps were successfully completed and an effective T7 polymerase based expression system was developed to achieve heterologous expression of cloned genes in *P. fluorescens*.

5.2. Construction of broad host range mobilizable shuttle vector.

Before proceeding to construct T7 polymerase based expression vector suitable for expression of cloned genes in *P. fluorescens*, initial studies were performed by using *P. fluorescens* ATCC 13525, obtained from HIMEDIA, Mumbai, India. A broad host range mobilizable *Inc* Q replica based expression vector, pMMB206 (Morales, et al 1991) was used as a source plasmid for the generation of a vector capable of expressing cloned genes in *P. fluorescens*. While using it as source plasmid, the plasmid pMMB206 is modified to facilitate its transformation and cloning. The vector pMMB206, used as source plasmid is a low copy number plasmid and it often creates problems while cloning genes to be expressed in heterologous host. Therefore a vector is generated to facilitate increase of copy number without compromising its replication ability in *P. fluorescens*. The Pir protein dependent *R6Kγ* replication origin facilitates to increase the copy number of plasmid in pir+ *E. coli* strains. Such origin of replication along with kanamycin resistance gene was taken from Ez- Tn5< *R6Kγ* ori/ KAN-2> transposon (Epicentre Biotechnologies). The cloning strategy is described in Fig 5-1A. Initially, kanamycin cassette and R6K*γ* were taken as blunt ended fragment by amplifying with SDS09 (5' GAGACCCGGGGCTTCGTTAATACAGA 3') and SDS010 (5' TCTCCCCGGGT TAGAAAAACTCATCGAGCATC 3') primer set. As *Sma*I sites were incorporated in both the forward and reverse primers, the R6kγ– kanamycin amplicon (Fig 5-1B) was digested with *Sma*I and cloned in *Dra*I site of pMMB206. Digestion of pMMB206 with *Dra*I eliminated chloramphenicol gene. The resulting clone was designated as pR6KS and was confirmed by digestion with *Bam*HI (present in the vector backbone) and *Xho*I (present in *R6Kγ* kanamycin cassette).

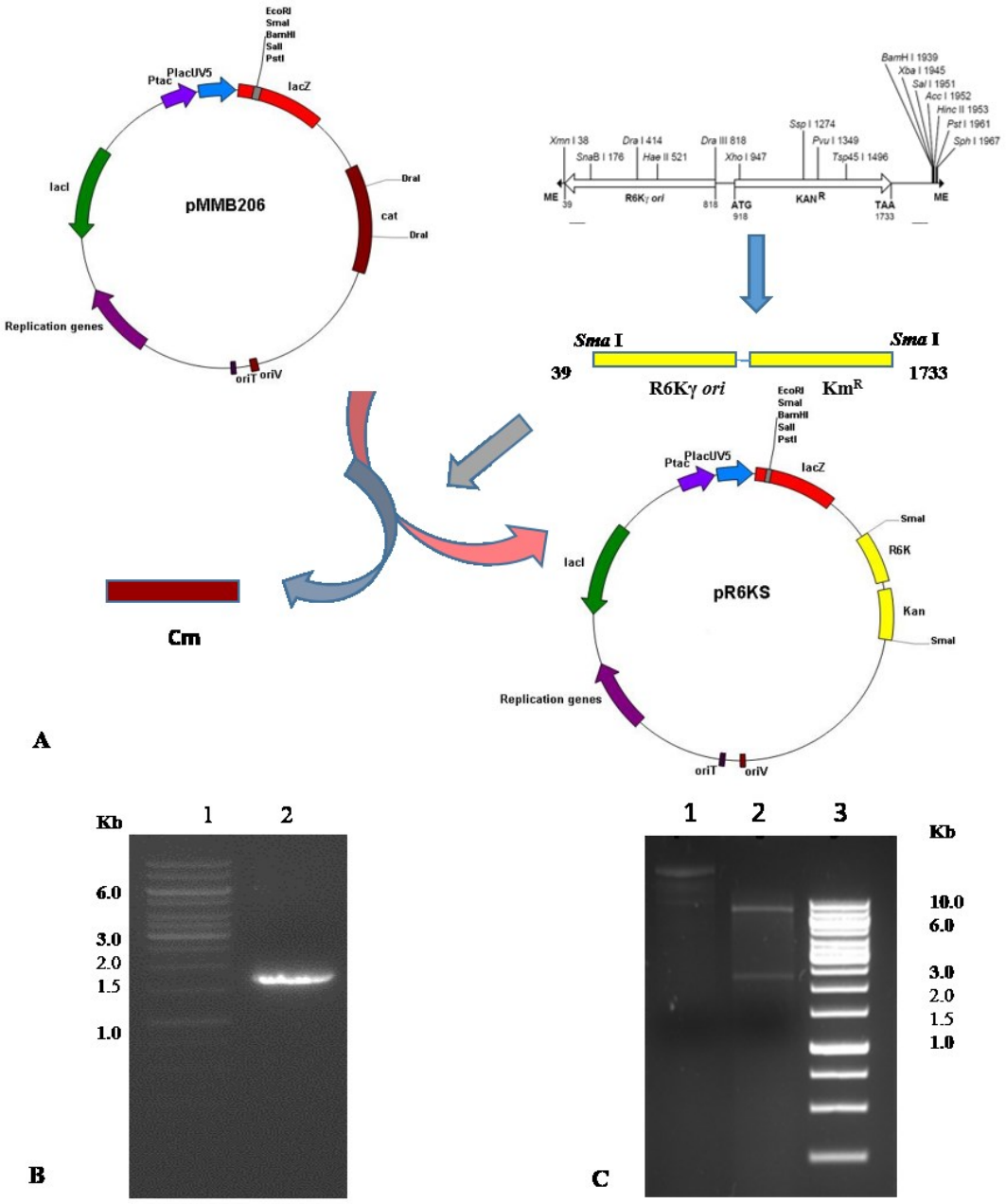

Fig 5-1. Construction of broad host range mobilizable shuttle vector pR6KS. Panel A. Schematic representation of construction of broad host range mobilizable shuttle vector pR6KS. Panel B. Lane 1 represents 1 kb DNA marker and lane 2 represents R6kγ amplicon amplified as *Sma* I fragment from EZ-Tn5 <R6K γ *ori*/KAN-2> vector. Panel C. Lane 1 represents uncut pR6KS; lane 2 represents pR6KS digested with *Bam* HI and *Xho* I. Lane 3 represents 1 kb DNA ladder. Release of 2.3 kb fragment confirms the cloning of R6k *ori* and kanamycin gene into pMMB206 vector backbone.

A release of 2.3 kb fragment confirmed the construction of pR6KS (Fig 5-1C). The vector pR6KS replicates in *P. fluorescens* as low copy number using *IncQ* replicative origin and the same plasmid can be obtained as high copy number plasmid in *E. coli* pir116 by inducing Pir protein, due to existence of R6K*γ* replication origin.

5.3. Cloning and expression of CryIEC$_{N6HIS}$ in *Pseudomonas fluorescens*

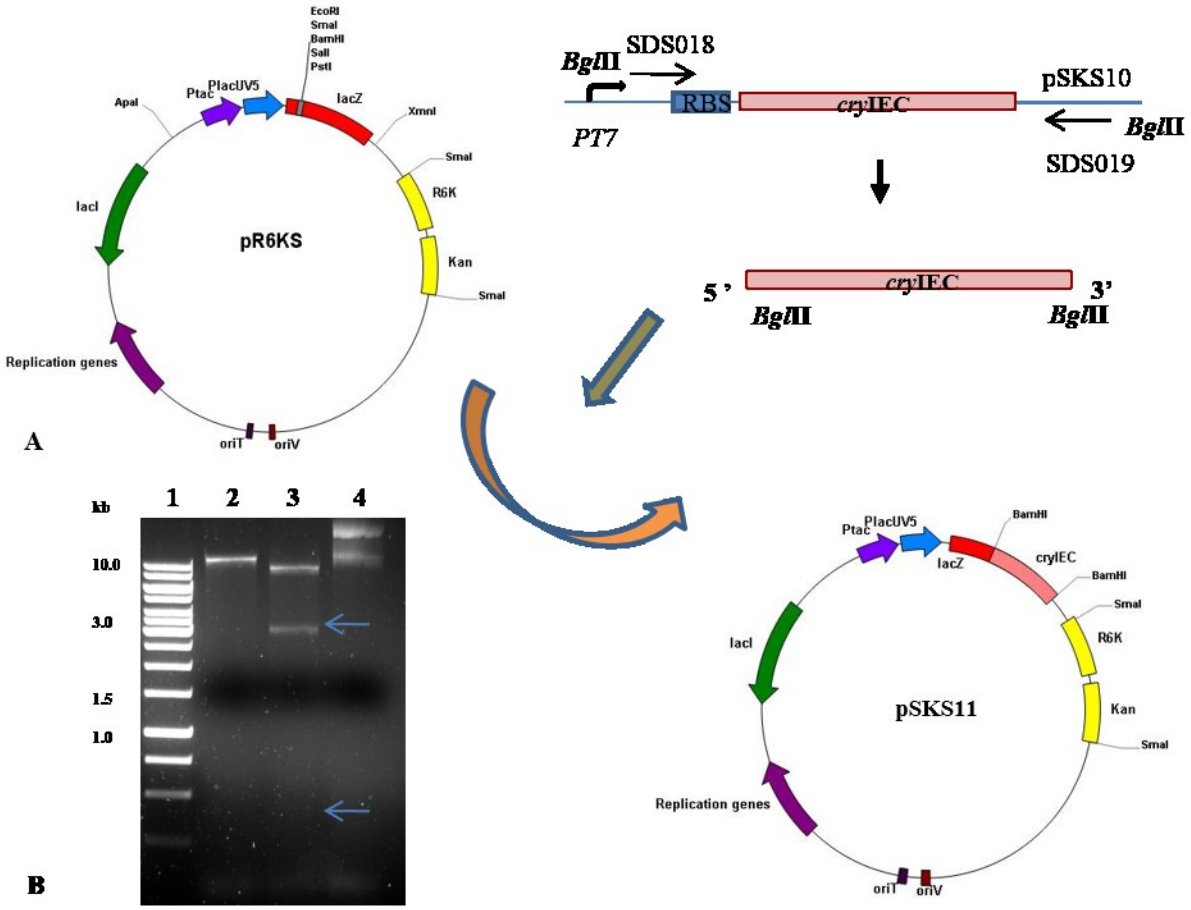

Fig 5-2. Cloning of *cry1EC* in pR6KS. Panel A. Schematic representation of construction of pSKS11. Panel B. Lane 1 represents 1 kb ladder; lane 2 represents pSKS11 recombinant plasmids digested with *Apa* I and *Xmn* I and lane 3 represents uncut plasmid. Lane 2 confirms the clone and its correct orientation.

In chapter 2, the cloning strategies and expression of chimeric *cry1EC* were described. Inorder to obtain CryIEC, the *cry1EC* was amplified along with its N-terminal His tag, using SDS018 (5' AACAAGATCTAGGGAGACCACAACGGTTTCCC 3') and SDS019 (5' CCTTAGATCTCTAG TTATTGCTCAGCGGTGGC 3') and pSKS10 was used as template. As *Bgl*II sites were appended in both forward and reverse primers respectively, the *cry1EC* amplicon was digested with *Bgl*II and cloned in *Bam*HI digested pR6KS, thus constructing pSKS11. The clone was further confirmed by digestion with *Apa*I and *Xmn*I. Release of 3015 bp and 437 bp confirmed both the cloning and the correct orientation of *cry1EC* in pR6KS. This recombinant plasmid was designated as pSKS11 (Fig 5-2).

5.3.1. Mobilization of pSKS11 in *P. fluorescens*.

The expression plasmid pSKS11 was introduced into *P. fluorescens* by performing biparental mating. Initially, the *E. coli* S17-1 cells were transformed with plasmid pSKS11 and the transformed *E. coli* S17-1 (pSKS11) was used as donor. The conjugation protocol described in materials and methods section was followed. The mid log phase culture of *P. fluorescens* (pSKS11), was induced as described in methods. After induction, proteins expressed from control cultures were analysed on 12.5 % SDS- PAGE. As the expression levels were low, a faint band corresponding to the size of 78 kDa was seen in *P. fluorescens* cell lysate having pSKS11. The size of the protein coincided with the $Cry1EC_{N6HIS}$. Further insights into the expressed protein were gained by performing western blot. The gel was blotted and western analysis was performed using anti- His antibody. As shown in Fig 5-3, the 78 kDa size protein gave a positive signal. No such signal was seen either in uninduced cultures or in the cultures with no expression plasmids. This confirms the expression of $CryIEC_{N6HIS}$ in *P. fluorescens* (pSKS11) and usefulness of constructed expression vectors to express cloned genes in *P. fluorescens*.

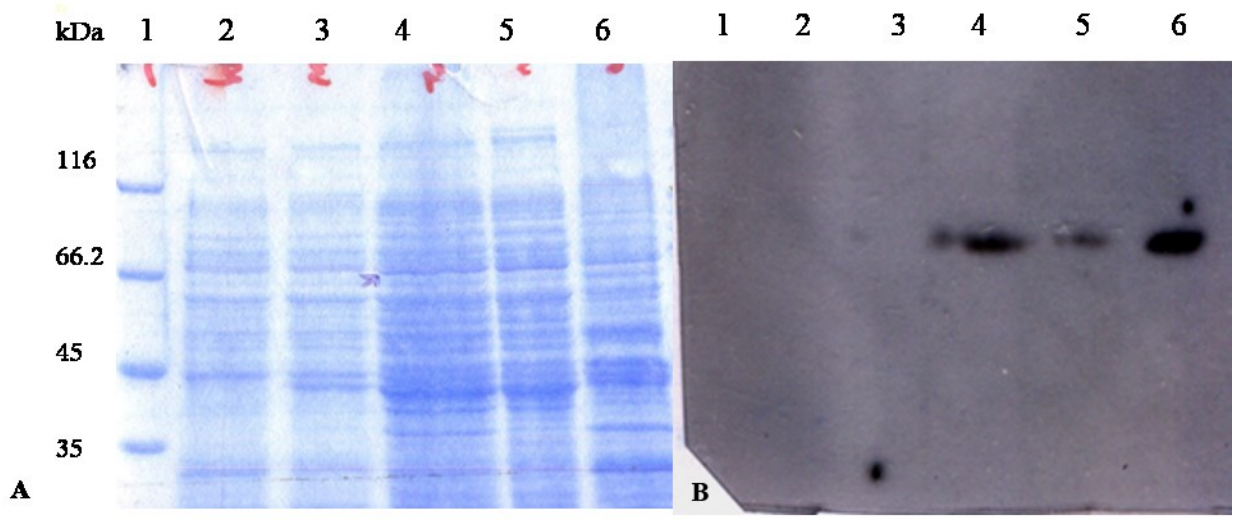

Fig 5-3. Expression of Cry1EC in *P. fluorescens.* Lane 1 represents: Protein molecular weight marker; lane 2 represents uninduced cell lysate fraction of wild type *P. fluorescens,* lane 3 represents uninduced cell lysate fraction of *P. fluorescens* containing pSKS11, lanes 4-6 represent induced, particulate and soluble fractions of *P. fluorescens* (pSKS11). Panel B represents corresponding western blot probed using anti- His antibody. A signal at 78 kDa confirms the expression of $Cry1EC_{N6HIS}$ in *P. fluorescens.* Most of the expressed $Cry1EC_{N6HIS}$ was found in soluble form (Panel B, lane 6)

5.4. Toxicity bioassay

As described in the earlier chapter, CryIEC expressed in *E. coli* showed no mortality of *Spodoptera litura* larvae. Therefore, the *P. fluorescens* expressing $CryIEC_{N6HIS}$ was preliminarily analysed for its toxicity against *Spodoptera litura* larvae. The cell lysate fraction of *P. fluorescens* expressing $CryIEC_{N6HIS}$ was coated at 25 µg/ sq cm and 50 µg/ sq cm concentrations on 12.5 sq cm castor leaves. The cell lysate fraction of wild type *P. fluorescens* was used as control. The experiment was done in triplicates to ensure the results obtained in the toxicity bioassays. It was observed that the toxicity bioassays with cell lysates (50 µg of total protein/sq cm of castor leaf) of *P. fluorescens* expressing $CryIEC_{N6HIS}$ showed 65 % mortality within 12 h (Fig 5-4). This clearly indicates that the $CryIEC_{N6HIS}$ toxin expressed in *P. fluorescens* was more toxic than its counterpart produced in *E. coli*.

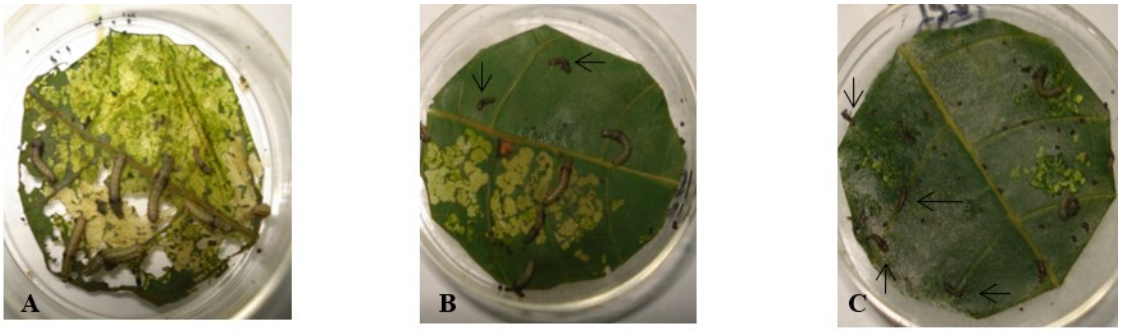

Fig 5-4. Bio assay of recombinant Cry1EC$_{N6HIS}$. Panel A represents *Spodoptera litura* larvae on castor leaves coated with cell lysate of wild type *Pseudomonas fluorescens* (50 µg/ sq cm of total protein). Panels B, C represent *Spodoptera litura* larvae on castor leaves coated with cell lysate fraction of *Pseudomonas fluorescens* expressing CryIEC$_{N6HIS}$ at total protein concentrations of 25 µg/ sq cm and 50 µg/ sq cm respectively. The effect on feeding behavior and toxicity of Cry1EC$_{N6HIS}$ towards *Spodoptera litura* larvae on castor leaves was observed. Arrow marks indicate dead insects.

It is really an interesting observation. The CryIEC$_{N6HIS}$ toxin expressed in *E. coli* was found in soluble form to show that the toxin produced was not mis-folded. Supporting this observation, the *E. coli* expressed Cry toxin bound to receptors like wild type Cry toxins. Considering this observation, the differential toxicity remains an unsolved puzzle. Based on the present work, the *P. fluorescens* expressed toxin is active and effectively controls *S. litura* larvae. The reason for the heterologously expressed CryIEC$_{N6HIS}$ toxin could be due to the post translational modifications. The *P. fluorescens* expressed CryIEC$_{N6HIS}$ toxin may be post translationally modified to be an effective toxin. Alternatively, *P. fluorescens* coded host protein might be acting along with CryIEC$_{N6HIS}$ to enhance the toxicity of *P. fluorescens* produced CryIEC$_{N6HIS.}$ The affinity purified CryIEC$_{N6HIS}$when analyzed on SDS-PAGE gives an additional band of 70 kDa (Fig. 5-18). It remains to be established if this co-eluted protein has any role in enhancing the toxicity of CryIEC$_{N6HIS}$ produced in *P. fluorescens*.

5.5. Development of *T7* polymerase based Expression System in *Pseudomonas fluorescens*

Having observed such increased toxicity of the CryIEC$_{N6HIS}$ toxin towards *S. litura* larvae, further studies were targeted at enhancing the expression levels of CryIEC$_{N6HIS}$ toxin in *P. fluorescens*. For this, the T7 polymerase expression system was exploited inorder to enhance the CryIEC$_{N6HIS}$ production levels. T7 polymerase expression system was successfully exploited in *E. coli* BL21 but was never tried in *P. fluorescens*.

Initial studies conducted to express CryIEC$_{N6HIS}$ gave positive results. The *P. fluorescens* expressed CryIEC$_{N6HIS}$ was highly toxic to *S. litura* larvae. Therefore an attempt was made to introduce T7 polymerase driven expression system into *P. fluorescens*. In order to achieve this objective, a T7 promoter based expression vectors had to be designed along with modification of host organism to code for T7 RNA polymerase. The overall strategy followed to develop T7 polymerase based expression system in *P. fluorescens* is shown in Fig 5-5. These two objectives were successfully completed and T7 polymerase based expression system was designed.

5.5.1. Construction of *T7* polymerase based mini transposon to be integrated into the genome of *P. fluorescens:*

Initially, *T7 polymerase* gene was amplified with SDS011 (5' GCTCGGATCCAATTT ATCAGATCCAATAGGAGG 3') and SDS012 (5' TTACGCGAAGCTTAACGCGAAGT CCGACTCTAA 3') using pGP1-2 plasmid as template. The amplified *T7 polymerase* gene (Fig 5-6B) was cloned into TA vector and designated as pTZ-T7. As *Bam*HI and *Hind*III sites were appended to both the forward and reverse primers, respectively, the pTZ-T7 plasmid was digested with *Bam*HI and *Hind*III. The 2.8 kb fragment corresponding to *T7 pol* gene was gel extracted and cloned in pUC19 digested with the same enzymes. The resulting recombinant plasmid was

designated as pSKS2 (Fig. 5-6A). In plasmid pSKS2 the ORF coding for T7 polymerase alone was cloned. It does not contain its indigenous promoter. Therefore an appropriate inducible promoter could be placed before *T7 pol.*

5.5.2. Host manipulation.

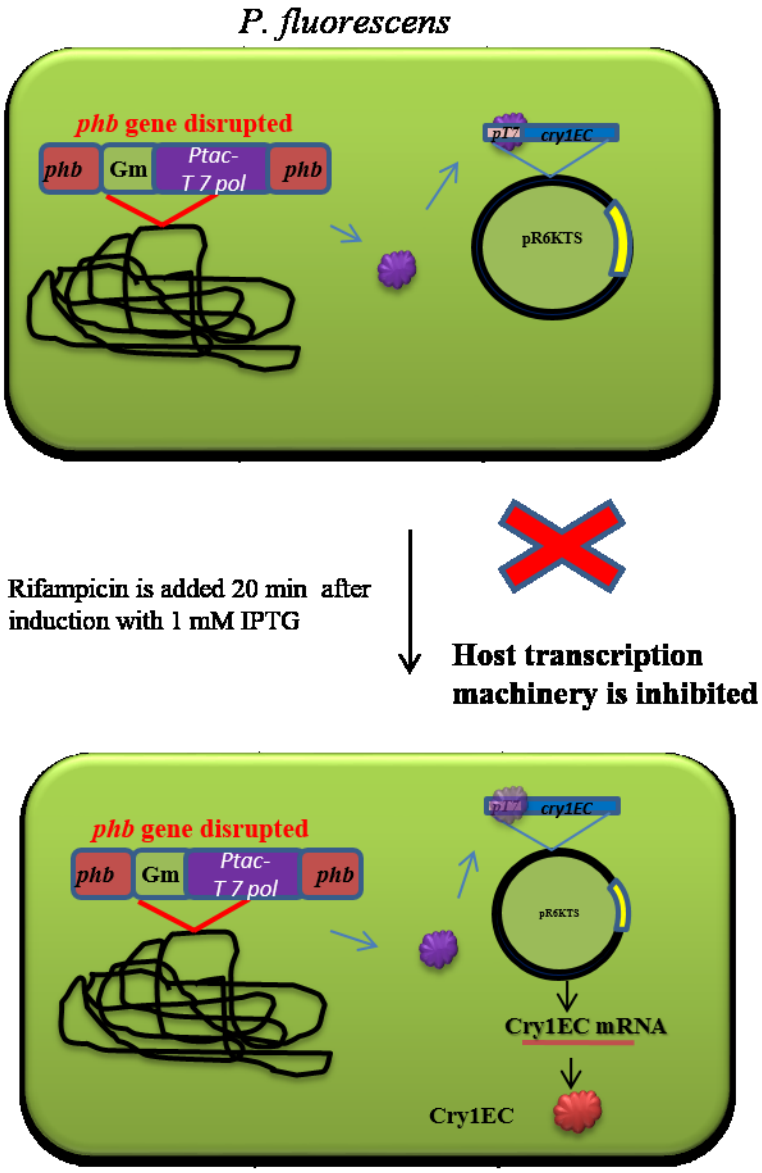

Fig 5-5. Schematic representation indicating the integration of *T7 pol* in the genome of *Pseudomonas fluorescens.*

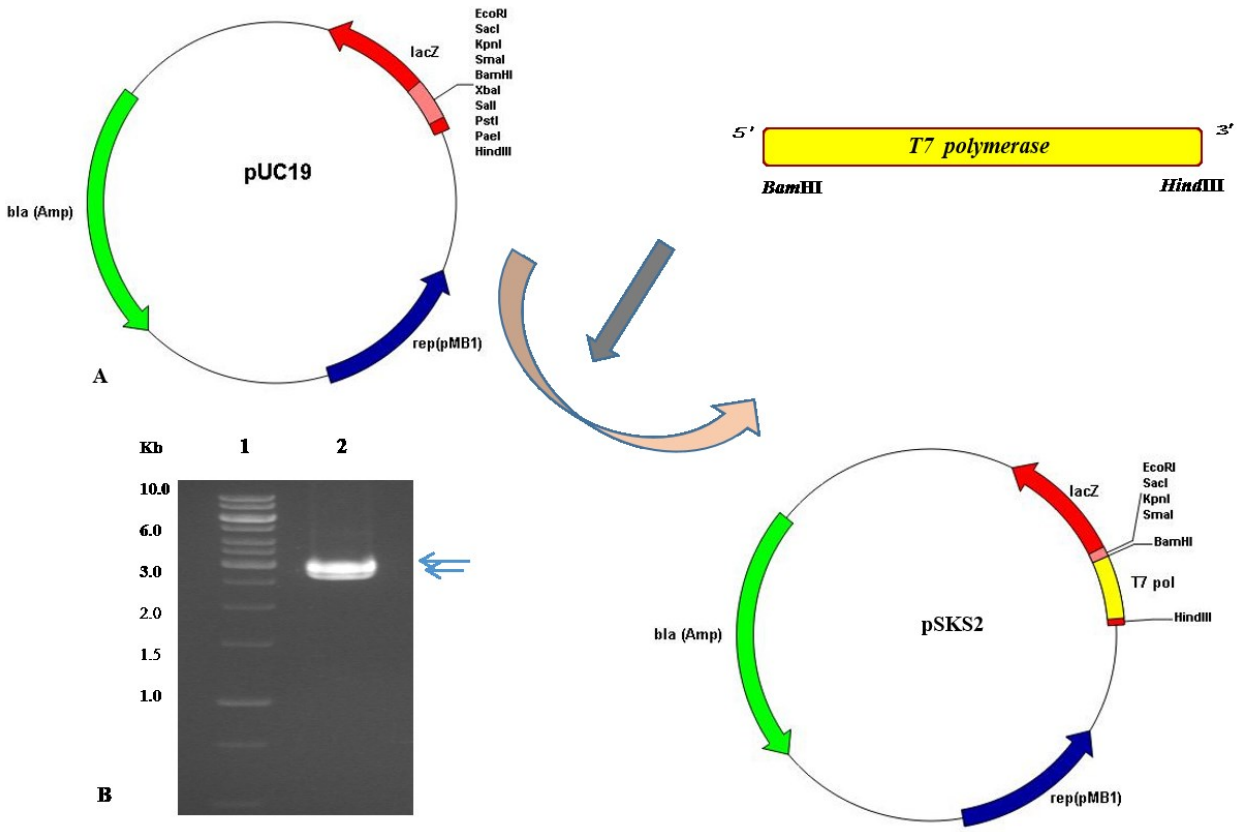

Fig 5-6. Cloning of *T7 pol* in pUC19. Panel A. Schematic representation of construction of pSKS2. Panel B. Lane 1 represents 1 kb DNA marker; Lane 2 represents pSKS2 clone confirmed by digestion with *Bam* HI and *Hind* III. A 2.8 kb release confirms the cloning of *T7 pol* in pUC19 vector.

The plasmid pSKS2 contains *T7 pol* gene without its indigenous promoter. Therefore, the *Ptac* promoter was taken from pMMB206 as *Bam*HI and *Bgl*II fragment by performing PCR amplification using primer set (SDS020 (5' GAGCGGATCTTGACAATTAATC 3') and SDS021 (5' GAGCGGATCCGTATATCTCCTTCGAGC 3')). The 80 bp *Ptac* promoter fragment was then cloned upstream of pSKS2 by digesting with *Bam*HI. The plasmid with right orientation of promoter is selected by performing PCR using promoter specific forward (SDS020) and *T7 pol* gene specific reverse (SDS012) primers (Fig 5-7A). Further, sequencing was also performed to get

the orientation of the promoter motif. The resulting recombinant plasmid was designated as pSKS3.

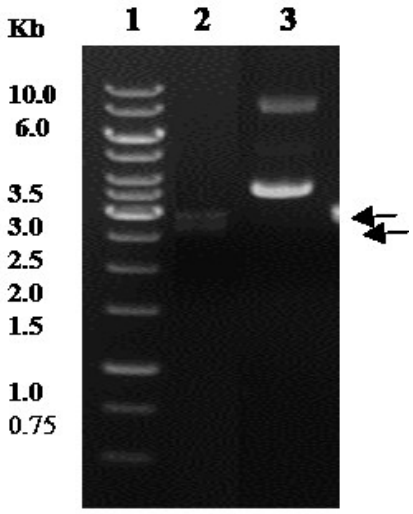

Fig 5-7: **Cloning of *tac* promoter preceding *T7 pol* gene in pSKS2.** Panel B. Lane 1 represents 1 kb DNA ladder; lane 2 represent *Bam* HI- *Hind* III digested pSKS3 plasmid. Lane 3 represents uncut plasmid pSKS3 plasmid. As showing the release of *tac* promoter (80 bp) is difficult, the pSKS3 was digested with *Bam* HI and *Hind* III which releases *T7 pol* gene. *tac* promoter (80 bp) region gets added up to the pUC19 backbone with a size of 2786 bp.The clone was further confirmed by sequencing.

5.5.3. T7 Polymerase/ promoter system for *P. fluorescens*.

In order to integrate *T7 pol* gene into the genome of *P. fluorescens*, a genetic cassette was generated by placing gentamycin resistant gene upstream of *Ptac-T7pol* gene. This was to facilitate selection of transformants with *T7 pol* gene integrated into the genome. In our previous studies our laboratory generated a plasmid by cloning gentamycin resistant gene in pGEMT- Easy vector (our laboratory). The gentamycin resistant gene was then excised from the plasmid pGEMT-Gm as *Eco*RI fragment and cloned in *Eco*RI site of pSKS3. The generated plasmid was designated as pSKS4, which contains gentamycin resistance gene upstream of *PtacT7 pol* gene (Fig 5-8).

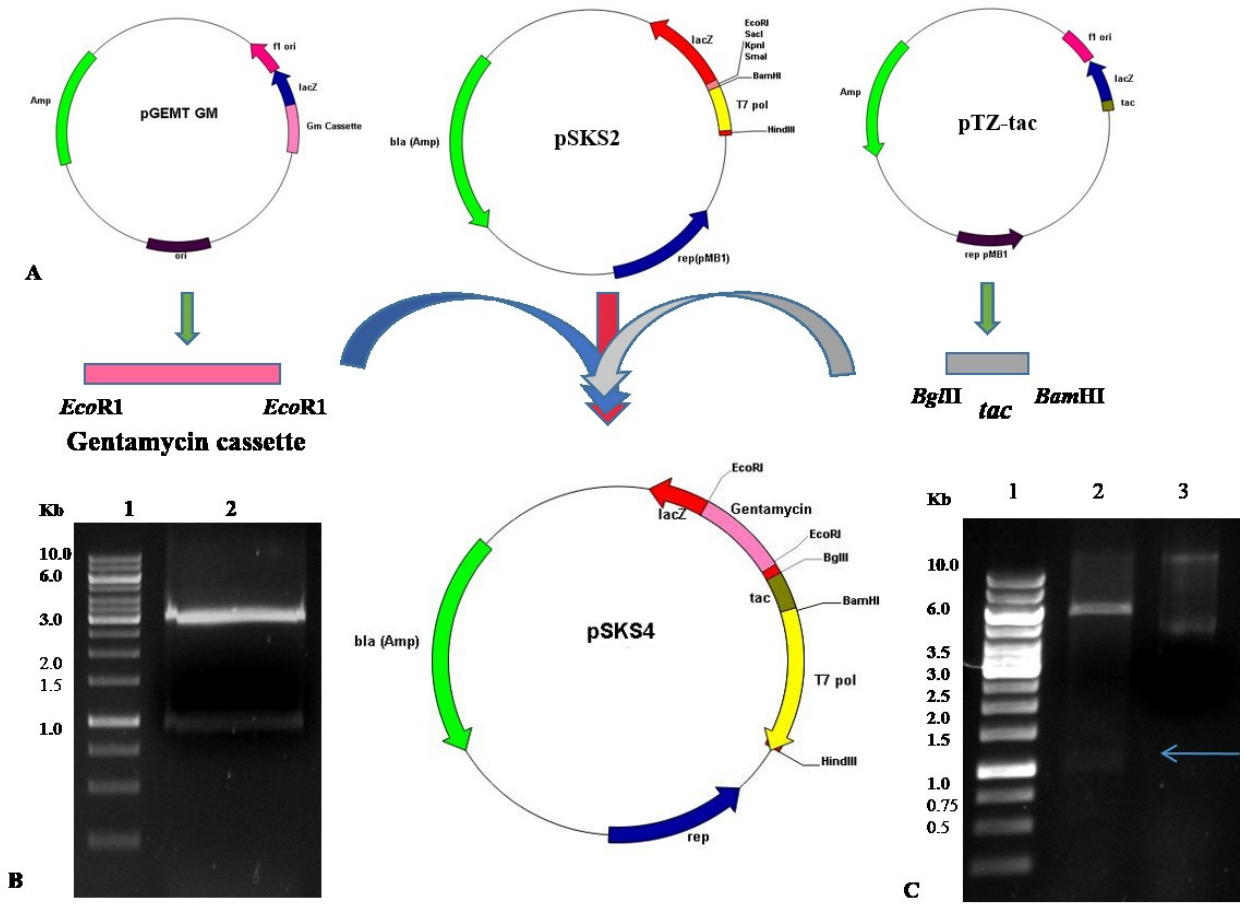

Fig 5-8. Construction of pSKS4 recombinant plasmid. Panel A. Schematic representation of construction of pSKS4. Panel B. Lane 1 represents 1 kb DNA ladder; lane 2 represents pGEMT- Gm plasmid digested with *EcoR* I. 1kb release seen in lane 2 corresponds to Gentamycin Cassette. Panel C. Lane 1 represents 1 kb DNA ladder; lane 2 represents pSKS4 plasmid digested with *EcoR*I. Lane 3 represents uncut pSKS4 plasmid. Release of 1 kb gentamycin cassette upon digestion with *Eco*RI confirms cloning of Gentamycin cassette into pSKS3 plasmid (pSKS4).

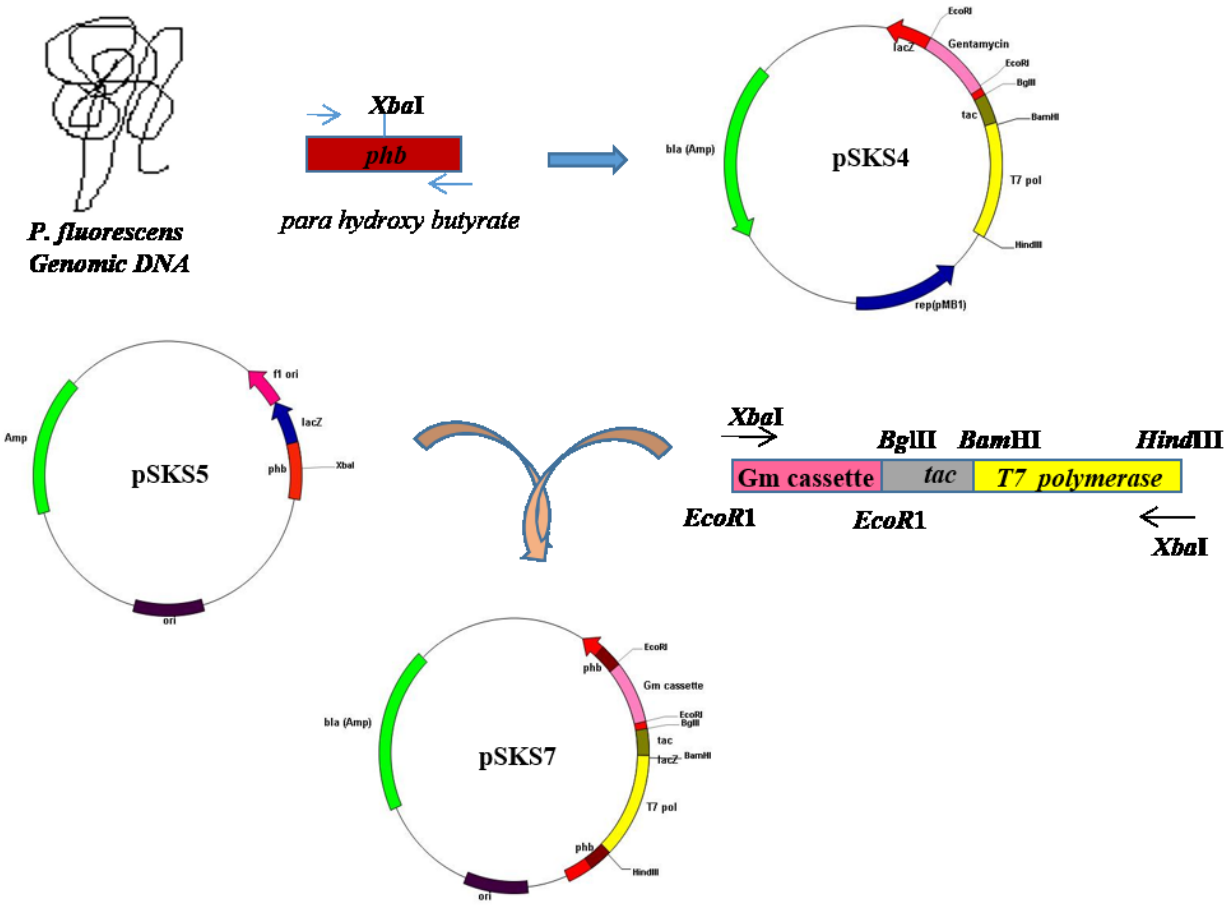

Fig 5-9. Schematic representation of the construction of pSKS5 and pSKS7.

In order to facilitate integration of gentamycin-*T7 pol* gene cassette, it has to be flanked by DNA regions homologous to *P. fluorescens* chromosomal DNA. The *phb* gene, coding for *para*-hydroxyl butyrate, is not essential for the survival of the host. Therefore, 200 bp of *phb* gene was amplified using SDS016 (5' GATCAGTGCGGCCGCATGAAAACGCTAAAA 3') and SDS017 (5' ACTATCGCGGCCGCAGCAGGTCGACCAT 3') primers, *P.fluorescens* genome as template and cloned in TA vector. The resulting recombinant plasmid was designated as pSKS5 (Fig 5-9). As *Not*I site was appended on both SDS016 and SDS017 primers, a release of 200 bp when digested with *Not*I confirmed the cloning of *phb* in pGEMT, pSKS5 (Fig 5-10B).

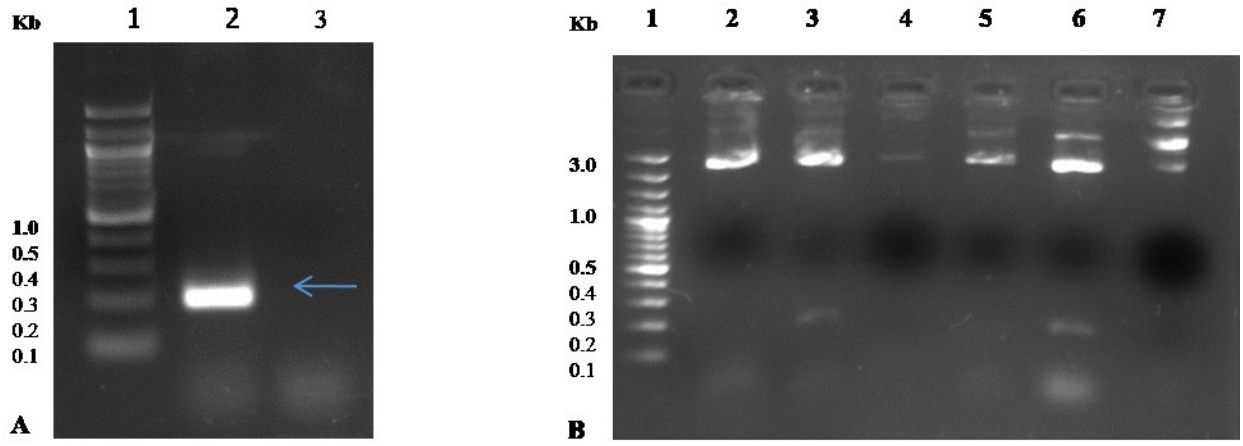

Fig 5-10. Amplification and cloning *of p- hydroxyl butyrate* gene. Panel A. Lane 1 represents 100 bp DNA ladder; lane 2 represents *phb* gene amplified with SDS016 and SDS017 primers using *P. fluorescens* genome as template. Panel B. Lane 1 represents 100 bp ladder; lanes 2- 6 represent pSKS5 plasmids digested with *Not* I and lane 7 represents uncut pSKS5 plasmid. A release of 200 bp in lanes 3 and 6 confirms the construction of pSKS5.

The DNA fragment containing partial *phb* gene has unique *Xba*I site at the central region. If Gentamycin T7 polymerase gene cassette is taken as *Xba*I fragment and cloned in the unique *Xba*I site of *phb* gene, it places 100 bp of *phb* gene on both the sides of Gentamycin T7 polymerase cassette. While cloning Gentamycin - *T7 polymerase* cassette at the *Xba*I site of *phb* gene, it was amplified from pSKS4 plasmid using SDS015 (5' GATCTATCTAGACGCCAGCCAGGACAGAAATGCC 3') and SDS012 primers appended with *Xba* I site. In the Gentamycin - *T7 polymerase* cassette there is no *Xba*I site. Therfore, its amplification facilitates cloning of the cassette at the unique *Xba*I site of *phb* gene. The Gentamycin - *T7 polymerase* cassette amplified from pSKS4 was gel extracted and cloned into *Xba*I digested pSKS5. The resultant recombinant plasmid was designated as pSKS7 (Fig 5-11). The *phb* gene was amplified as *Not*I fragment. There are no *Not*I sites in the entire *T7 pol* gene cassette.

Therefore, the Gentamycin - *T7 polymerase* cassette inserted in the *phb* gene can be excised as *Not*I fragment along with the flanking *phb* sequence. The *Not*I fragment excised from pSKS7 plasmid was made blunt by performing end-filling reaction and cloned in *Eco*RV site of suicidal vector, pSUP202. The resulting recombinant plasmid was designated as pSKS8. As *P. fluorescens* is resistant to ampicillin, the *bla* gene coding for ampicillin has to be inactivated in pSKS8 in order to facilitate its mobilization and selection in *P. fluorescens*. The gene coding for ampicillin in pSKS8 was inactivated and designated as pSKS9 (Fig 5-12). Existence of Gentamycin - *T7 polymerase* cassette was confirmed by digestion with *Xba*I. As shown in restriction map, the *Xba*I digested plasmid pSKS9 releases 4 kb fragment showing proper cloning of Gentamycin - *T7 polymerase* cassette in the suicidal vector.

5.5.4. Gentamycin - *T7 polymerase* cassette mobilization and homologous recombination.

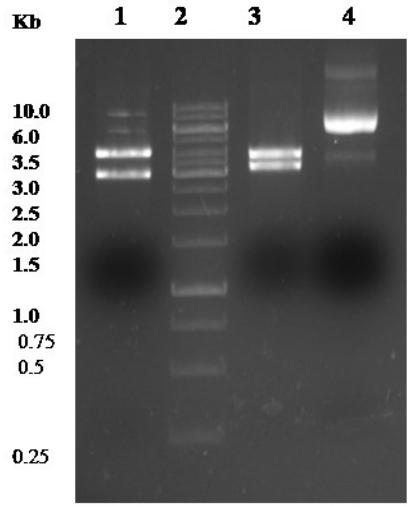

Fig 5-11: **Cloning of Gentamycin- *T7* polymerase cassette in pSKS5.** Lane 1 represents pSKS7 digested with *Not*I; lane 2 represents 1 kb DNA ladder; lane 3 represents pSKS7 digested with *Xba*I and lane 4 represents uncut pSKS7. A release of ~ 4.2 kb in lane 1 and ~ 4 kb in lane 3 confirms the construction of pSKS7.

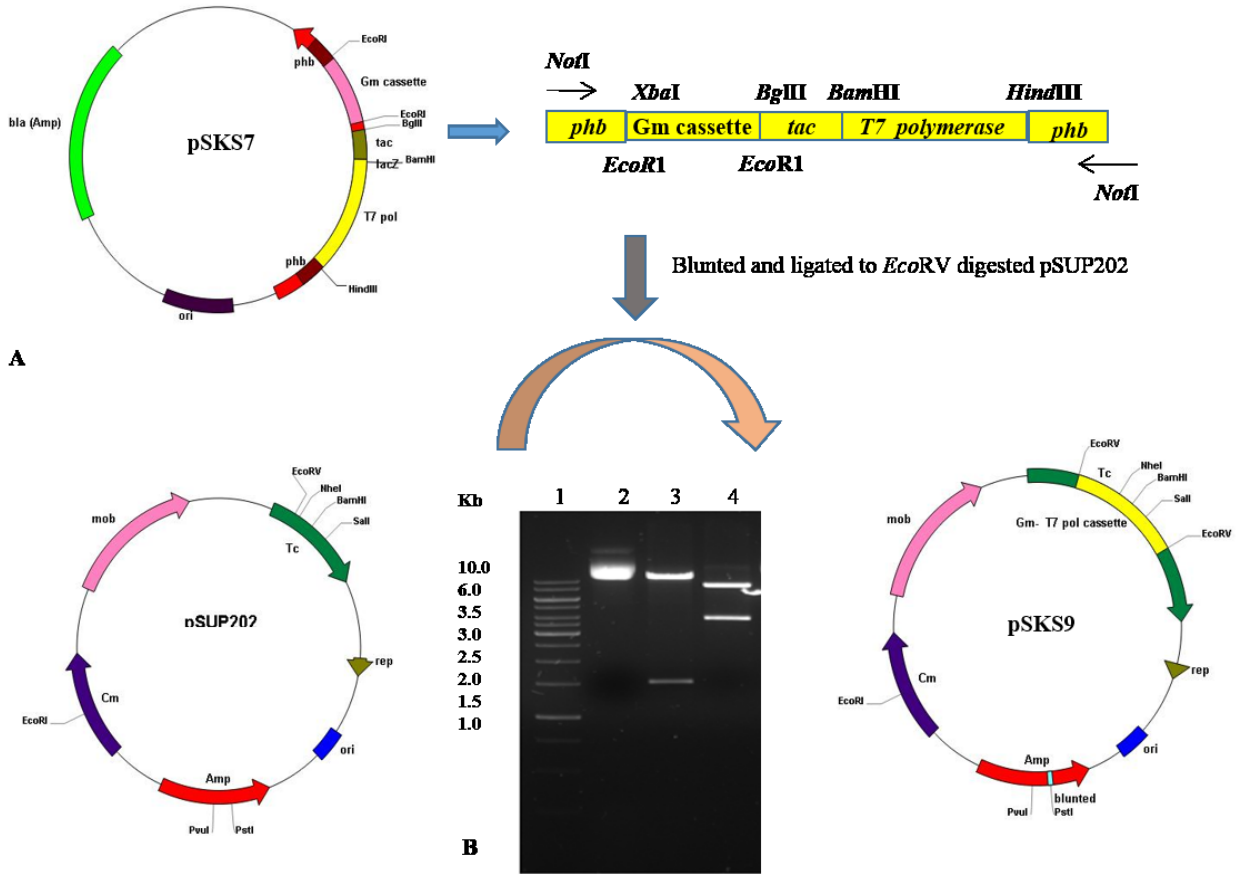

Fig 5-12. Cloning of T7 pol:: Gm cassette along with *phb* flanking regions in pSUP202. Panel A. Schematic representation of construction of pSKS9. Panel B**.** Lane 1 represents 1 kb DNA marker, lane 2 represents uncut pSKS9 DNA, lanes 3 and 4 represent pSKS9 digested with *BamH*I and *Xba* I respectively.

Strategies followed to mobilize Gentamycin - *T7 polymerase* cassette are described in materials and methods section. As plasmid pSKS9 is the derivative of the suicidal plasmid pSUP202, it fails to replicate in *P. fluorescens*. After bi-parental mating between *E. coli* S17-1 (pSKS9) and *P. fluorescens*, the ex-conjugants were selected on LB plates containing Gm and Amp. Initially the genomic DNA was isolated from ex-conjugants and PCR was performed. The forward primer is specific to gentamycin gene (SDS015) while reverse primer is specific to *phb* gene (SDS017). Amplification of 4 kb fragment indicated precise integration of *T7 pol* gene at *phb* locus (Fig 5-13). The resulting *P. fluorescens* strain was designated as *P. fluorescens* DS003.

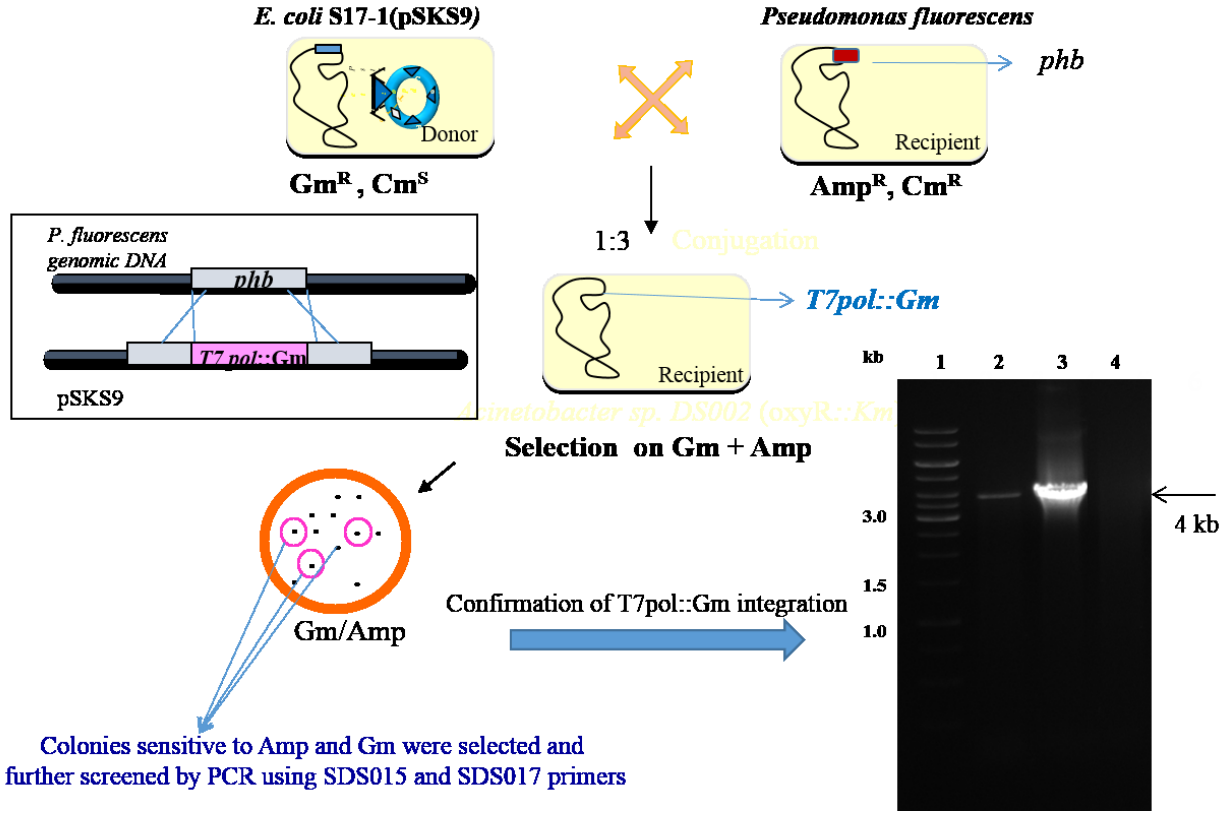

Fig 5-13: **Mobilization and integration of *T7 pol* cassette into the genome of *P. fluorescens***. Panel A. Construction of *T7 pol* based expression system in *P. fluorescens*: Panel B. Lane 1 represents 1 kb ladder; lane 2 represents Gentamycin *T7 pol* cassette amplified using SDS015 and SDS017 with engineered *P. fluorescens* genomic DNA as template; lane 3 represents Positive control; lane 4 represents –ve control. Arrow mark indicates a ~ 4 kb amplification in lane 2, as expected

5.5.5. Expression of T7 polymerase in *P. fluorescens* DS003.

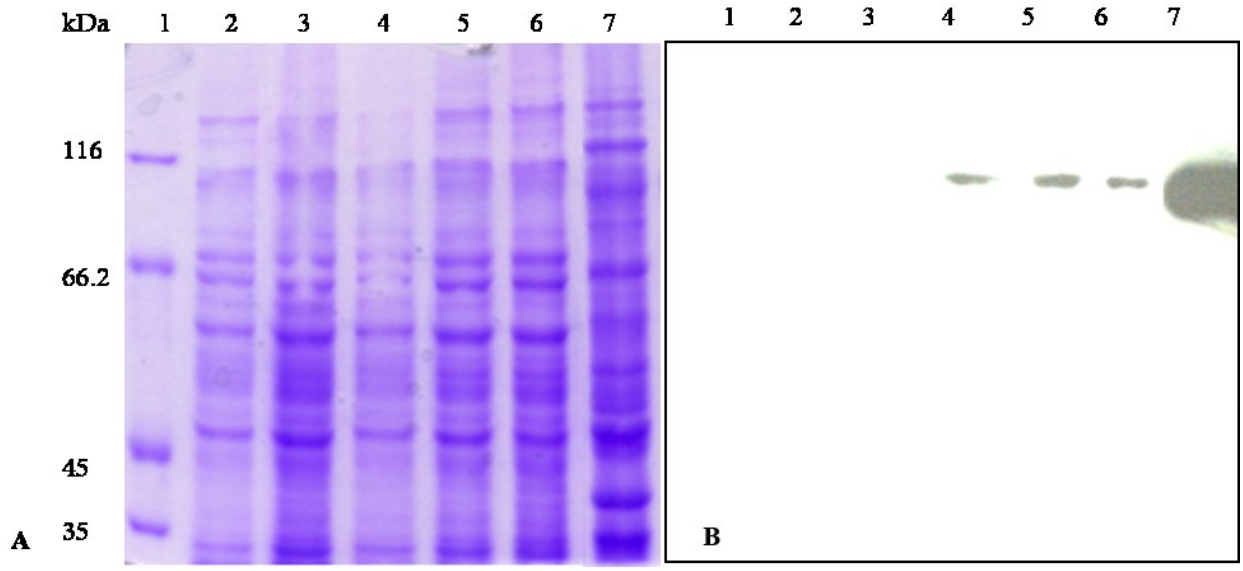

Fig 5-14. Expression of T7 polymerase. Panel A. Expression of *T7* polymerase. Lane 1 represents unstained protein molecular weight marker, lane 2 and 3 represent uninduced and induced cell lysates of wild type *P. fluorescens*, lanes 4-6 represent uninduced (lane 4) and induced cell lysates of engineered *P. fluorescens* at 2.5 h (lane 5), 3.5 h (lane 6) andlane 7 represents induced *E. coli* BL21. Panel B represents corresponding western blot with anti-T7 pol antibody. A signal corresponding to T7 polymerase (98 kDa) was seen in lanes 4, 5 and 6.

In order to assess the expression of T7 polymerase in *P. fluorescens* DS003, 1% of overnight culture of exconjugate that gave positive amplification, was taken and inoculated in 10 ml of LB medium containing ampicillin and gentamycin. The cultures were grown till mid log phase and induced by adding sterile IPTG to a final concentration of 1 mM. The induction was continued for 2- 6 h and cells were harvested at 2 h and 3.5 h time points. The expression of *T7* polymerase was analyzed by SDS- PAGE (Fig. 5-14A) and western blotting(Fig. 5-14B) by probing with anti-T7 antibodies (Promega). The western blot analysis with anti-T7 antibodies suggested the expression of *T7* polymerase in both the uninduced and induced cell lysate fractions. The engineered *P.*

fluorescens strains having *T7 pol* gene were designated as *P. fluorescens* DS003 and used as host for expression of CryIEC$_{N6HIS}$ toxin.

5.5.6. Construction of *T7 polymerase* based Broad host range mobilizable shuttle expression vector.

As described earlier, to express CryIEC$_{N6HIS}$ in the *P. fluorescens* DS003, the *cryIEC* gene need to be cloned under the control of *T7* promoter. Further, the host *P. fluorescens* DS003 cannot be made competent to take the recombinant DNA via transformation. Therefore, suitable vector system with T7 promoter motif had to be designed. Strategy designed to develop *T7* promoter dependent vector system is shown in Fig 5-15. In plasmid pR6KS (Fig 5-1) there are unique *Apa*I and *Xmn*I sites. If digested with these two enzymes, it eliminates the *lac* promoter system from the plasmid backbone and retains replicative origin and origin of transfer, *ori*T. Using pRSETA (Invitrogen) as template, the T7 promoter and multiple cloning site were taken by amplification using primer set [SDS013 (5' GATCTCGATGGGCCCATTAATACGA 3') and SDS014 (5' ATCTACGAA NNNNTTCTATTCGCTATTACGC 3')] appended with *Apa*I and *Xmn*I, respectively. This amplicon (Fig. 17B) was ligated into *Apa*I- *Xmn*I fragment of pR6KS. The resulting recombinant plasmid is designated as pR6KTS (Fig 5-15). Since pR6KTS has *ori*T and broad host range replicative origin, it can be mobilized in *P. fluorescens* DS003 and the cloned genes can be induced following induction of T7 polymerase adapting conventional methods.

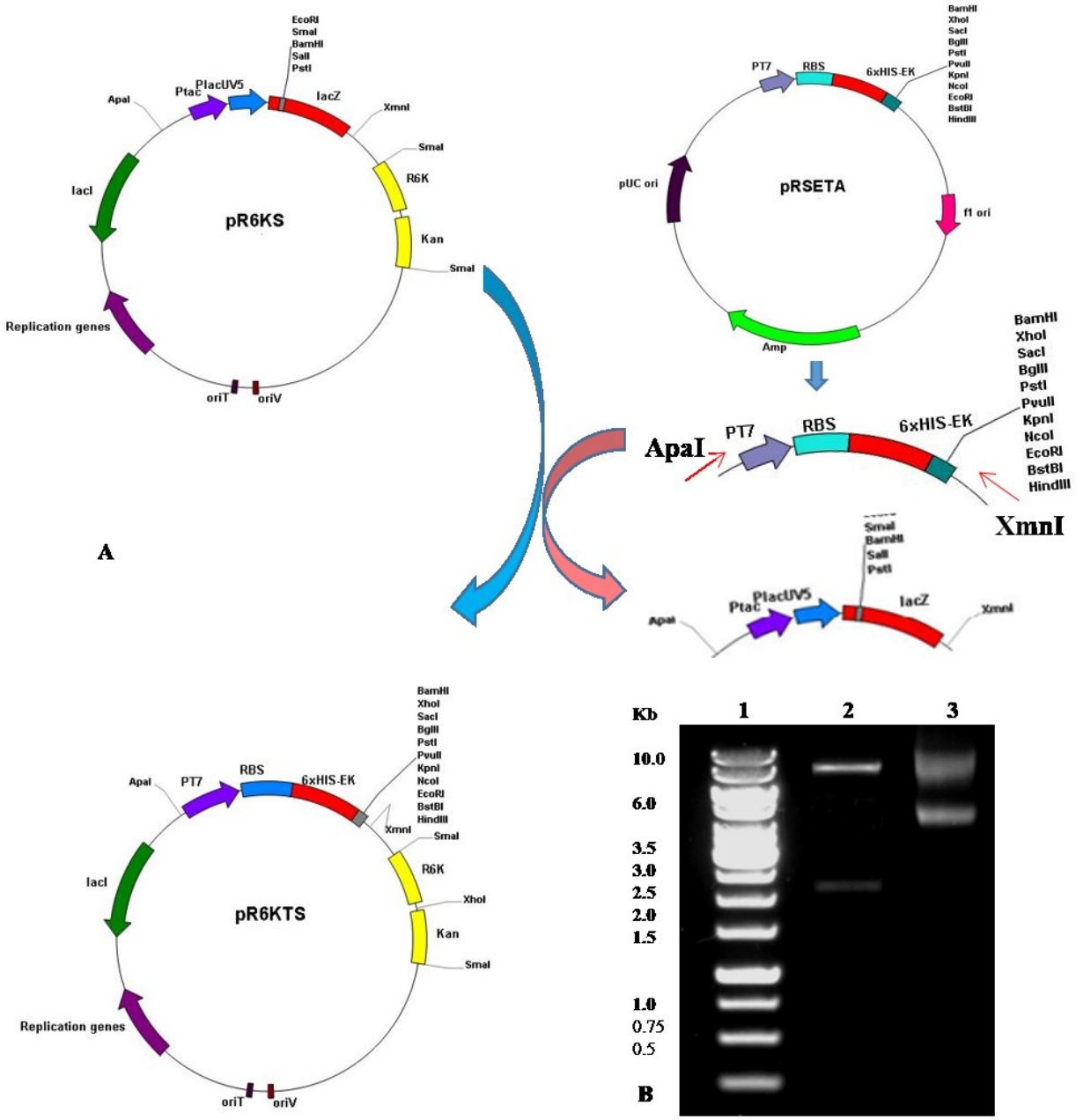

Fig 5-15. Construction of *T7* promoter based broad host range mobilizable vector. Panel A. Schematic representation of the construction of *T7* promoter based broad host range mobilizable shuttle vector, pR6KTS. Panel B. Lane 1 represents 1 kb DNA ladder; lane 2: *Xho* I digested pR6KTS; lane 3: uncut plasmid. *Xho* I is present in the *T7* promoter based expression cassette and in the vector backbone of pR6KS. So, a release of 2.3 kb fragment in lane 2 confirms the construction of pR6KTS.

5.6. Expression of CryIEC$_{N6HIS}$ in *Pseudomonas fluorescens* DS003.

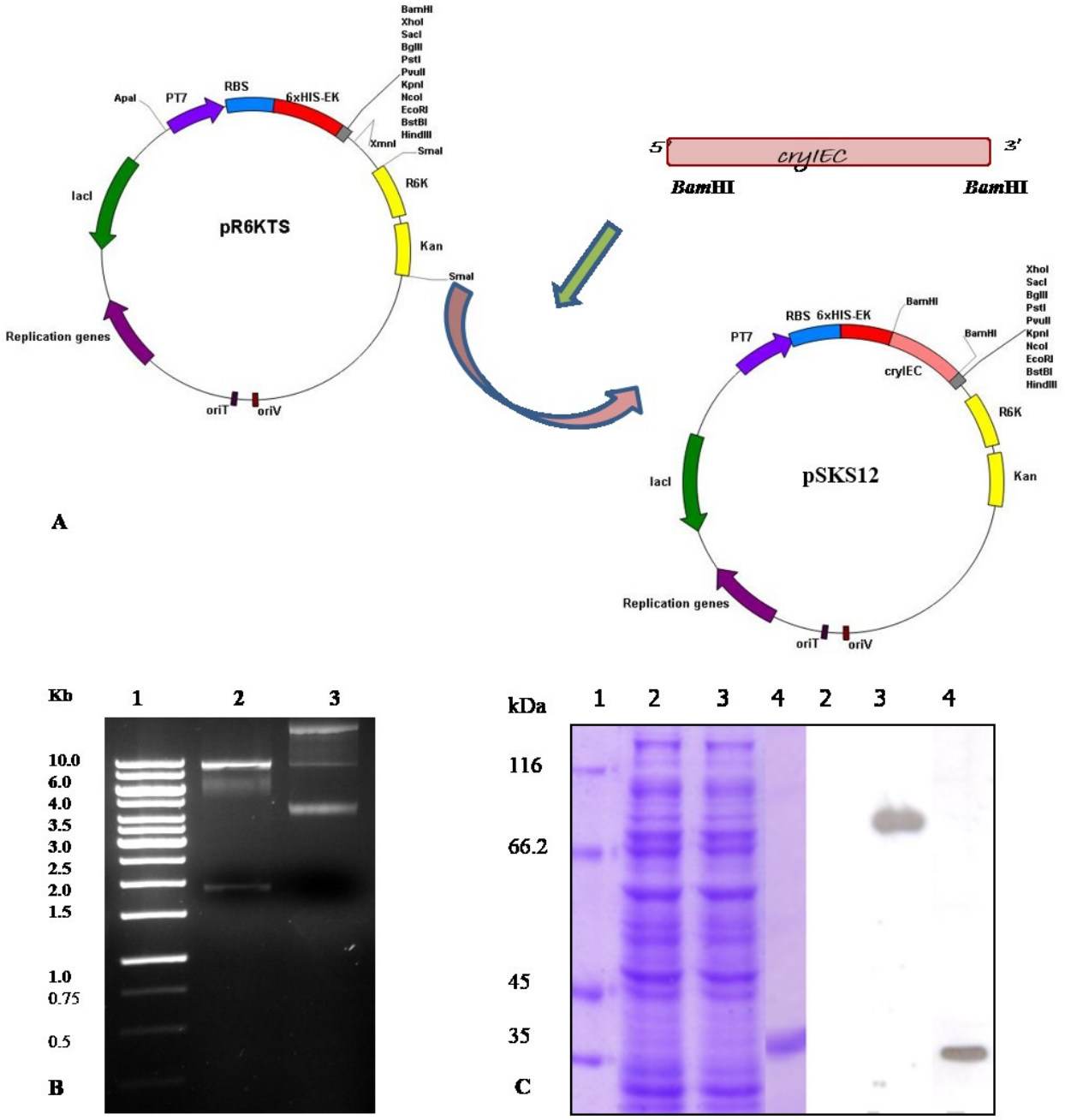

Fig 5-16. **Construction and expression of pSKS12**. Panel A represents schematic representation of pSKS12. Panel B.Lane1 represents 1 kb DNA ladder; lane 2 represents *BamH* I digested pSKS12 and lane 3 represents uncut plasmid. Panel**C.** Lane 1 represents Protein molecular weight marker; Lane 2: uninduced cell lysate fraction of the engineered *P. fluorescens* (pSKS12). Lane 3: Induced soluble fraction of engineered *P. fluorescens* containing expression plasmid, pSKS12. Lane 4: Positive control. Panel B represents corresponding western blot probed using anti- His antibody. A signal corresponding to CryIEC$_{N6HIS}$ (78 kDa) is seen in lane 3.

The *cryIEC* was taken as a *Bam*HI fragment from pSKS1 (described in chapter 2) and cloned in *Bam*HI digested pR6KTS. This cloning strategy places *cryIEC* under the T7 promoter in frame with the vector coded N- terminal His tag (Fig 5-16A). The clone obtained was designated as pSKS12 and was confirmed by a release of 1.9 kb upon digestion with *Bam*HI (Fig 5-16B).

Further, pSKS12 was transformed into *E. coli* S17-1 and later mobilized into *P. fluorescens* DS003 as described in materials and methods. The expression of CryIEC$_{N6HIS}$ in *P. fluorescens* DS003 expressing CryIEC$_{N6HIS}$ from T7 promoter system was analyzed by inducing the mid log phase culture with 1 mM IPTG at different time intervals. At 4h after induction, maximum CryIEC$_{N6HIS}$was produced (Fig 5-16C). Most of the expressed protein was found in soluble form.

5.7. Influence of Rifampicin on expression of CryIEC$_{N6HIS}$ in *P. fluorescens* DS003.

As seen in *E. coli*, described earlier, the addition of rifampicin is expected to enhance the transcription and translation of cloned genes if they are cloned under the control of *T7* promoter (Studier and Moffat, 1986). The overnight culture of *P. fluorescens* DS003 (pSKS12) was inoculated in 10 ml of LB medium containing ampicillin, gentamycin and kanamycin. The cultures were grown till mid log phase and induced by adding sterile IPTG to a final concentration of 1 mM. Rifampicin was added at a concentration of 200 µg/ ml, 20 min after induction. The induction was continued for 1-5 hrs and cells were harvested at 1h, 2 h and 3.5 h time points and the expression of recombinant CryIEC$_{N6HIS}$ was analyzed by SDS- PAGE and western blotting (Fig. 5-17). The increased production of CryIEC$_{N6HIS}$ with time was observed. As seen in SDS- PAGE and corresponding western blot, there was no significant rise in production of CryIEC$_{N6HIS}$. It may be due to several reasons. The Rifampicin at the added concentration showed no effect of host

RNA polymerase. One reason for such resistance is low permeability of rifampicin at low concentrations (Piddock et al., 2000, Stubbs et al., 2014). In the absence of rifampicin dependent inhibition of RNA polymerase in *P. fluorescens* DS003, it is not possible to enhance the expression of cloned genes under the control of T7 promoter. Alternatively, the *P. fluorescens* DS003 is a derivative of *P. fluorescens* ATCC13525. It is a wild type strain and positive for several proteases which could cleave excess protein made during induction. In the absence of protease negative *P. fluorescens* DS003, it is not possible to enhance the production of recombinant proteins in *P. fluorescens* DS003.

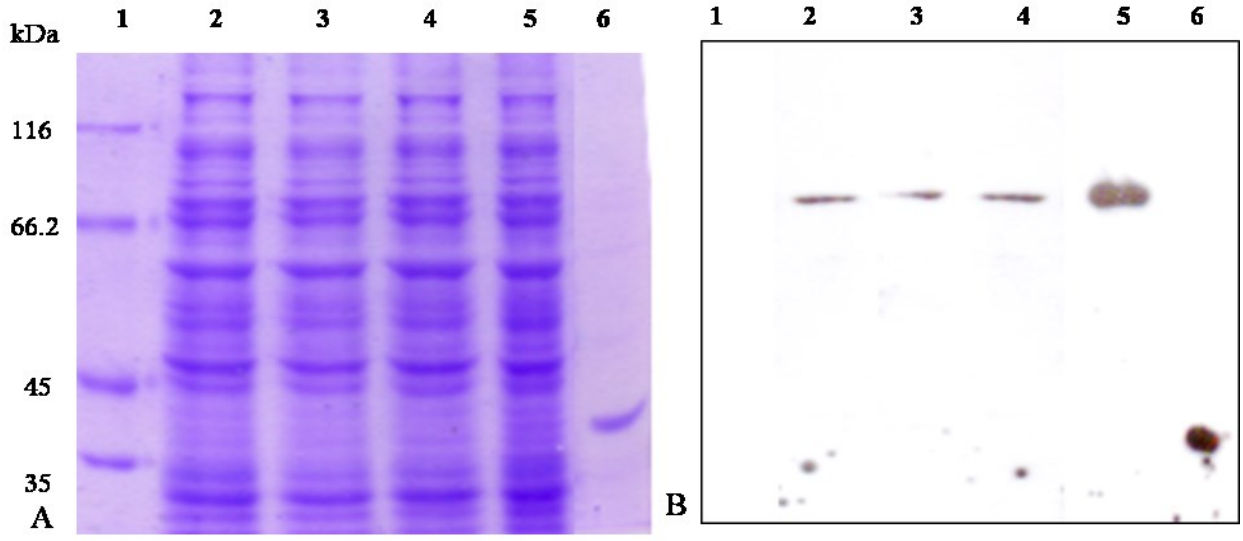

Fig 5-17. **Expression of CryIEC$_{N6HIS}$**. Panel A. Lane 1 represents unstained protein molecular weight marker, lane 2-5 represent uninduced cell lysate fraction of *P. fluorescens* DS003 (pSKS12), rifampicin treated (200 µg/ ml) induced cell lysate fractions of *P. fluorescens* DS003 expressing CryIEC$_{N6HIS}$ from T7 promoter at 1 h (lane 3), 2 h (lane 4) and 3.5 h (lane 5) after induction. Lane 6 represent positive control. Panel B represent corresponding western blot analysis with anti-His antibody.

5.8. Purification of CryIEC$_{N6HIS}$ toxin from *P. fluorescens* DS003.

Having obtained soluble and relatively increased level of expression of CryIEC$_{N6HIS}$ in *P. fluorescens* DS003, an attempt was made to purify CryIEC$_{N6HIS}$ using Nickel Sepharose affinity column. There were problems with the purification of CryIEC$_{N6HIS}$. The high viscosity of the

cytosolic fraction and existence of pigmented proteins prevented the free flow of cytoplasmic fraction through the column. which had resulted in the clogging of the column. Therefore, the soluble fraction containing CryIEC$_{N6HIS}$ was fractionated by ammonium sulphate. Most of the CryIEC$_{N6HIS}$ protein was found at 40% saturated fraction. Therefore this 40% fraction was dialyzed and was then used for further studies. The pure protein when analysed on SDS-PAGE gave two protein bands. One of them matched perfectly with CryIEC$_{N6HIS}$. The second band approximately at 70 kDa is unknown. Further studies are in progress to establish the identity of the protein (Fig. 5-18) and to ascertain if the enhanced toxicity is due to synergic effect of CryIEC$_{N6HIS}$ and the unidentified protein.

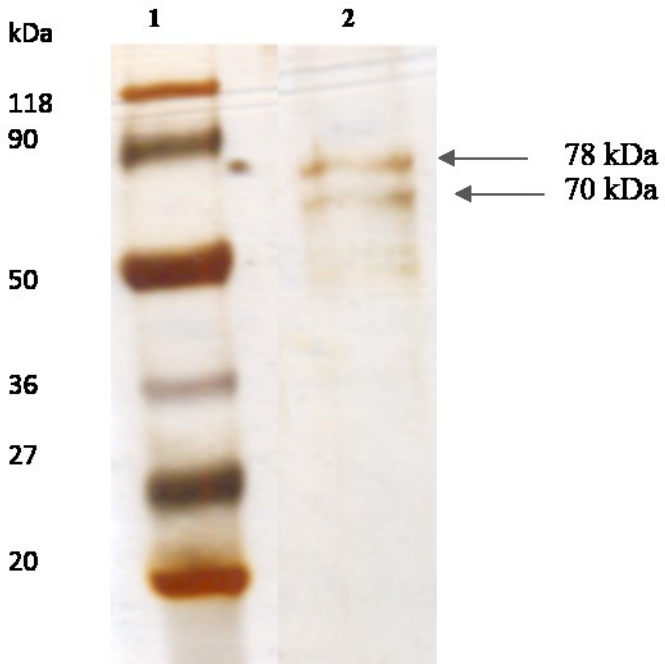

Fig 5-18. Purification of CryIEC$_{N6HIS}$ using Nickel Sepharose Affinity chromatography. Lane 1 represents Protein molecular weight marker, lanes 2 represents Affinity purified His tagged Cry1EC protein. Arrow marks indicate Cry1EC protein and the co-eluted protein of unknown identity (70 kDa).

5.9. Conclusions.

1. Broad host range mobilizable shuttle vector, pR6KS was constructed to facilitate the expression of CryIEC$_{N6HIS}$ in *P. fluorescens.*

2. Cry1EC$_{N6HIS}$ was successfully expressed in *P. fluorescens.* Toxicity bioassays with cell lysates (50 µg of total protein/sq cm of castor leaf) of *P. fluorescens* expressing CryIEC showed 65 % mortality within 12 h.

3. *T7* polymerase based expression system was developed in *P. fluorescens* ATCC 13525 and the modified host was designated as *P. fluorescens* DS003.

4. Rifampicin activity on induced *P. fluorescens* DS003, expressing CryIEC$_{N6HIS}$, was analysed. Using the *T7* RNAP-dependent gene expression system, *P. fluorescens* expressing Cry1EC$_{N6HIS}$ has shown increased toxin expression with increased time period, when compared to the non-rifampicin treated cultures.

5. Cry1EC$_{N6HIS}$ was expressed and purified in engineered *P. fluorescens.*

Chapter 4
Results
&
Discussions

6.1. Background

The organization of materials into functional systems at the nanoscale levels was first envisioned by Richard Feynman in the 1960s. The major characteristics of nanomaterials include their size, structure, surface properties, aggregation state, solubility and chemical composition (Sonkaria et al., 2012, Yanes & Tamanoi, 2012). Miniaturization results in significant changes in the surface to volume ratio of nanoparticles which in turn govern the availability of active sites, affecting their overall property (Sonkaria et al., 2012). Generally nanoparticles range from 1-100 nm in size. Their physical and chemical attributes and their long proven potential for highly innovative and marketable technologies make them pioneers in drug delivery, environmental, information technology, engineering, defence and security, energy, electronics, chemical and aerospace industries etc., (Sonkaria et al., 2012).

Functional nanoparticles of silica, especially Mesoporous silica nanoparticles (MSNs) have many applications in medicine as drug delivery vehicles and as nano-pesticides (Barik et al., 2008, Barik et al., 2012). The mechanism of action of silica nanoparticles as carrier is based on their ability to penetrate cuticular lipid barrier of insect pests. Cuticular lipid barrier of insects helps them in protecting their water barrier and thereby prevent death from desiccation. When applied on leaves and stem surfaces, silica nanoparticles get absorbed into the cuticular lipids by physiosorption and cause death of insects purely by physical means (Barik et al., 2008, Rai & Ingle, 2012). The usage of nano-pesticides is highly advantageous over chemical pesticides and transgenics.

6.1.1. Use of Cry toxin encapsulated nanoparticles as Biopesticides:

The delivery of Cry toxins through nanoparticles is the most plausible alternative to alleviate the disadvantages / problems posed generally by biopesticides. The increase in the surface to volume

ratio and surface energy of nanopesticides facilitates the penetration and attachment of an effective agent on the surface of plant (Song et al., 2012). As a result, the efficacy is significantly increased if conventional biopesticides are converted to nanopesticides. Further the controlled release of active molecules loaded in nanoparticles turns out to be the major advantage of nanopesticides. Such controlled release brings necessary effect with substantially low amounts of bioactive molecules. Further, the nanopesticides are environmentally friendly, believed to provide new opportunities and wide application prospects for pesticide research (Song et al., 2012). Application of nanoparticles on the leaf and stem surface neither alter photosynthesis and respiration of crop plants, nor do they cause alteration of gene expression in insect trachea, hence they are qualified as potential nano-biopesticides (Ghormade et al., 2011). The World Health Organization and US Department of Agriculture have approved usage of amorphous silica in health and agricultural practices (Ghormade et al., 2011). Nanoparticles are preferentially employed as bio-pesticides as they offer a greater surface area and circulate easily in lepidopteran systems. Moreover, they act as carriers and are removed naturally from the insect body within 24 h (Ulrichs et al., 2006, (Barik et al., 2008). Keeping in view of such great advantages an attempt is made to deliver Cry1EC$_{6His}$ produced in *Pseudomonas fluorescens* through mesoporous silica nanoparticles. This route of administration of Cry toxin is expected to create a sustained release to the target insects. This Chapter is exclusively devoted to describe the experiments conducted to load Cry1EC$_{N6His}$ onto mesoporous silica nano-particles to generate "CRYSONES" and to test their efficacy to control *S. litura* insect larvae. This interdisciplinary effort is made by converging the principles of nanomaterials with the recombinant DNA technology. The generated 'CRYSONES' the mesoporous SiO_2 nanoparticles containing Cry toxin, were tested for their

ability to control *S. litura* larvae, that affect commercially important crops of Indian subcontinent (Lewis et al., 1997).

6.2. Large scale purification of Cry1EC$_{N6HIS}$:

Recombinant Cry toxin Cry1EC$_{N6HIS}$ used to generate CRYSONES was purified from *P. fluorescens* DS003 (Chapter 3). The protocols pertaining to its induction and purification were described elsewhere in the theses. Since more amount of Cry1EC$_{N6HIS}$ was needed we have purified the protein from the 5 l of induced culture of *P. fluorescens* DS003 (Chapter 3). Finally about 6 mg of Cry1EC$_{N6HIS}$ could be achieved . The purified toxin was stored at 4°C until further use.

6.3. Preparation and Characterization of Silica nanoparticles:

The mesoporous silica nanoparticles were prepared as described in materials and methods section. The size of the mesoporous silica was characterized by observing the nanoparticles under Scanning Electron Microscopy (SEM). The SEM images were obtained with a Hitachi S 3400M at an accelerating voltage of 3- 30 kV at 15 kV max. Samples for SEM observation were prepared by spin coating of the dispersions of nanoparticles onto silicon substrates followed by sputter deposition of Au for 20 s in an argon atmosphere with a Hitachi E-1010 ion sputter. The images taken in SEM at three different magnifications (1 µm, 3 µm and 5 µm) suggest the size of nanoparticles are between 200- 300 nm range (Fig 6-1). The mesoporous silica nanoparticles were further observed under TEM (FEI TECNAI G2 make) to observe the stability of the particles and to check for the porosity (Fig 6-2). TEM analysis further confirmed the porosity and estimated the size of the mesoporous nanoparticles to be around 200 nm (Fig 6-2).

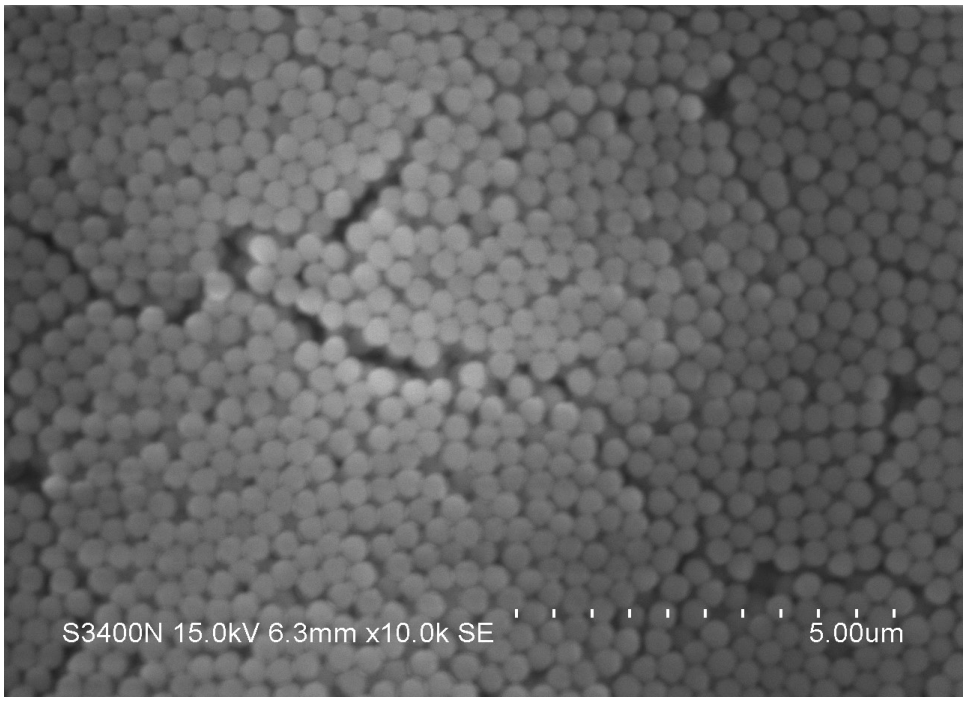

Fig 6-1. SEM image of mesoporous silica nanoparticles particles.

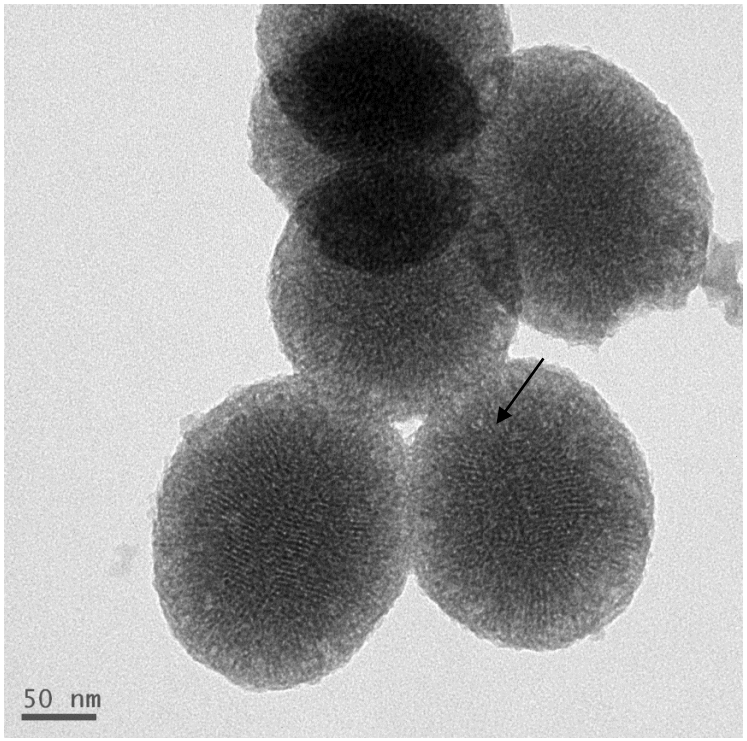

Fig 6-2. TEM image of mesoporous silica nanoparticles. The arrow mark indicate the pores (Paik et al., 2012).

6.4. Synthesis of CRYSONES

After preparing the mesoporous nanoparticles, the next aim was to couple these MSNs with CryIEC toxin. The adsorption of Cry toxins to silica nanoparticles is governed by electrostatic and vanderwaal interactions. Earlier studies on CryIAb adsorption onto silica nanoparticles had shown that the sum of electrostatic and vanderwaal interactions of CryIAb to negatively charged surfaces is weak at pH>5 and resulted in reversible adsorption (Madliger et al., 2004). Reversible Cry1Ab-SiO2 interactions suggested that Cry1Ab toxin had not undergone extensive conformational changes on SiO_2 and was stable (Sander et al., 2010). CryIAb adsorption to SiO_2 was shown to be driven by patch- controlled electrostatic attraction which means that domains II and III with high Iso-electric points (IEPs) were oriented towards SiO_2 (pH 2.0) (Sander et al., 2010). The same mechanism can be attributed to $Cry1EC_{N6HIS}$ where in buffer with a pH higher than its IEP ($IEP_{Cry1ECN6HIS}$ – 5.8), the toxin with its positively charged domains II and III, is attracted towards the negatively charged SiO_2 particles. This would result in adsorption and also partial insertion of the domains into the pores of mesoporous silica nanoparticles. While loading $Cry1EC_{N6HIS}$ to silicon nanoparticles about 1 mg of mesoporous silica nanoparticles were taken and washed with 20 mM Tris buffer (pH 8.0) and kept for overnight in stirring with 20 mM Tris buffer (pH 8.0), for equilibration. The next day, the mesoporous silica nanoparticles were pelleted down and incubated overnight at 4^0C with 130 µg of $CryIEC_{N6HIS}$ with constant stirring. Later, the nanoparticles were pelleted down, washed twice with 20 mM Tris buffer (pH 8.0) to remove any unbound proteins. These protein coupled silica nanoparticles (CRYSONES) were estimated spectrophotometry. It was observed that only 10 µg of $CryIEC_{N6HIS}$ were loaded per 1 mg of mesoporous silica nanoparticles. The remaining 120 µg of $CryIEC_{N6HIS}$ was detected in the supernatant. The $Cry1EC_{N6HIS}$ loaded nanoparticles

"CRYSONES" were repeatedly washed till no protein was detected in the wash fractions. This shows the stable adsorption of $CryIEC_{N6HIS}$ to the mesoporous silica nanoparticles.

In order to improve the protein load on to the nanoparticles, mesoporous silica nanoparticles along with the protein fraction were subjected to vacuum, in and out, at least three times in 6 hrs time period. This was done to make sure that the proteins were well encapsulated in the nanoparticles. Further, the nanoparticles were left overnight in stirring with the protein fraction at 4^0C. Later, the nanoparticles were pelleted down, washed twice with 20 mM Tris buffer (pH 8.0) to remove any unbound proteins. These protein coupled silica nanoparticles (CRYSONES) was quantified to estimate the protein load in the nanoparticles. It was observed that 12.4 µg of $CryIEC_{N6HIS}$ was loaded per 1 mg of mesoporous silica nanoparticles. This was considered as a significant increase in the protein load in the mesoporous silica nanoparticles. These heavily loaded CRYSONES were further checked for their integrity under SEM (Fig 6-3) and TEM (Fig 6-4). A dense layer corresponding to the coat of protein was observed on the surface of CRYSONES. A slight increase in the size was observed for the nanoparticles adsorbed with the CRYSONES in comparison with the nascent mesoporous silica nanoparticles (Fig 6-4).

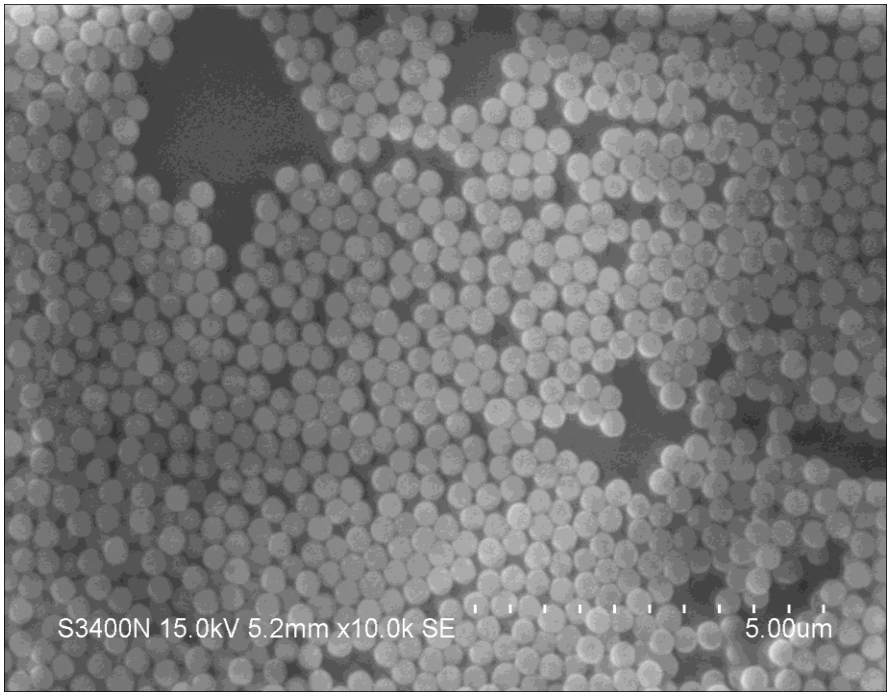

Fig 6-3. SEM image of CRYSONES having 12.4 µg of $Cry1EC_{N6HIS}$ per 1 mg of mesoporous silica nanoparticles.

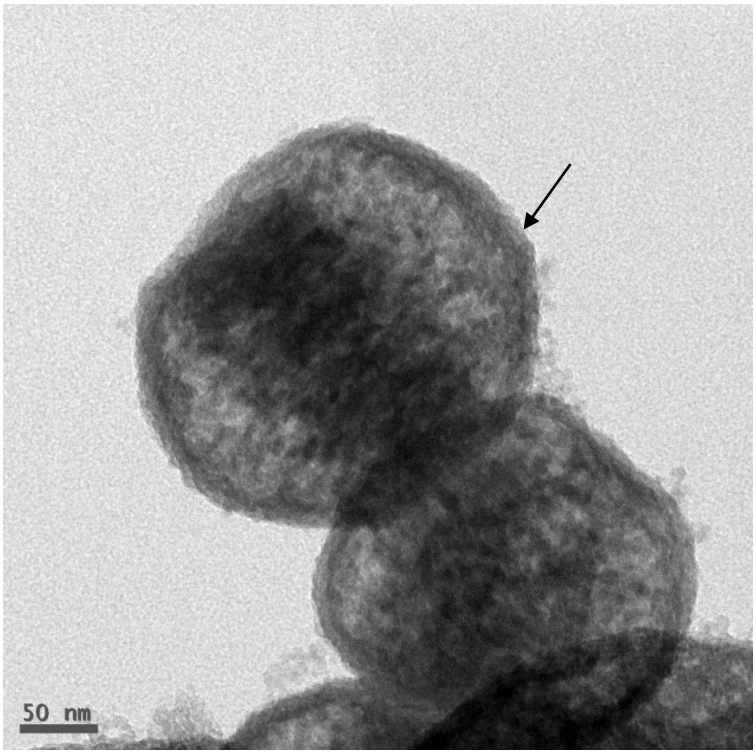

Fig 6-4. TEM image of CRYSONES. Arrow mark indicates the $CryIEC_{N6HIS}$ protein adsorbed to the silica nanoparticles.

6.5. CRYSONES interaction with *S. litura* gut receptor proteins:

After having established the integrity of CRYSONES, the effect of these CRYSONES on *S. litura* larvae was analysed, in comparison with the pure CryIEC$_{N6HIS}$ toxin. The CryIEC$_{N6HIS}$ and CRYSONES were coated onto either side of 12.5 sq cm^{-1} castor leaf at a concentration of 500 ng sq cm^{-1}. Similar leaves coated with buffer and nascent mesoporous silica nanoparticles were used as controls. The 3rd instar larvae of *S. litura*, were left for starving for 12 h before allowing to feed on the corresponding leaves for 6- 8 hrs. Three 3rd instar larvae of *S. litura* were allowed to feed on each leaf. It was observed that the insects were reluctant to eat in the initial hours but later fed on the corresponding leaves. After 6-8 h, the insects were fixed in bouin's fixative and their guts were pulled out and processed as described in materials and methods. The effect of CryIEC$_{N6HIS}$ and CRYSONES on *S. litura* larvae was established by by performing both histological staining and immunohistochemistry experiments.

6.5.1. Histological studies:

Hematoxylin and eosin (H&E) staining was adopted to investigate the effect of CryIEC$_{N6HIS}$ toxin on the gut morphology of *S. litura* larvae. Histological sections from gut of the CryIEC$_{N6HIS}$ and CRYSONES treated larvae revealed the presence of cellular debris in the lumen and irregular arrangement of Goblet as well as columnar cells in the gut epithelium (Fig 6-5 C & D). Extensive nuclear fragmentation and presence of large vacuolar regions were also seen in the gut membrane (Fig 6-5D). The mid- gut epithelium was much thinner in Cry toxin and CRYSONES treated insects (Fig 6-5 C & D) compared to that of control insects (Fig 6-5 A & B). Upon analyzing the data obtained from histological studies, it was observed that the effect of CRYSONES was slightly more intense in comparison with CryIEC$_{N6HIS.}$ It was speculated that

the effect of CRYSONES would be still higher in natural conditions i.e if the insect was not fixed in short duration as 6- 8 h, because the complete release of Cry toxin from mesoporous silica nanoparticles would enhance the lethal effects. Further studies related to the release kinetics of Cry toxin from the CRYSONES had to be conducted.

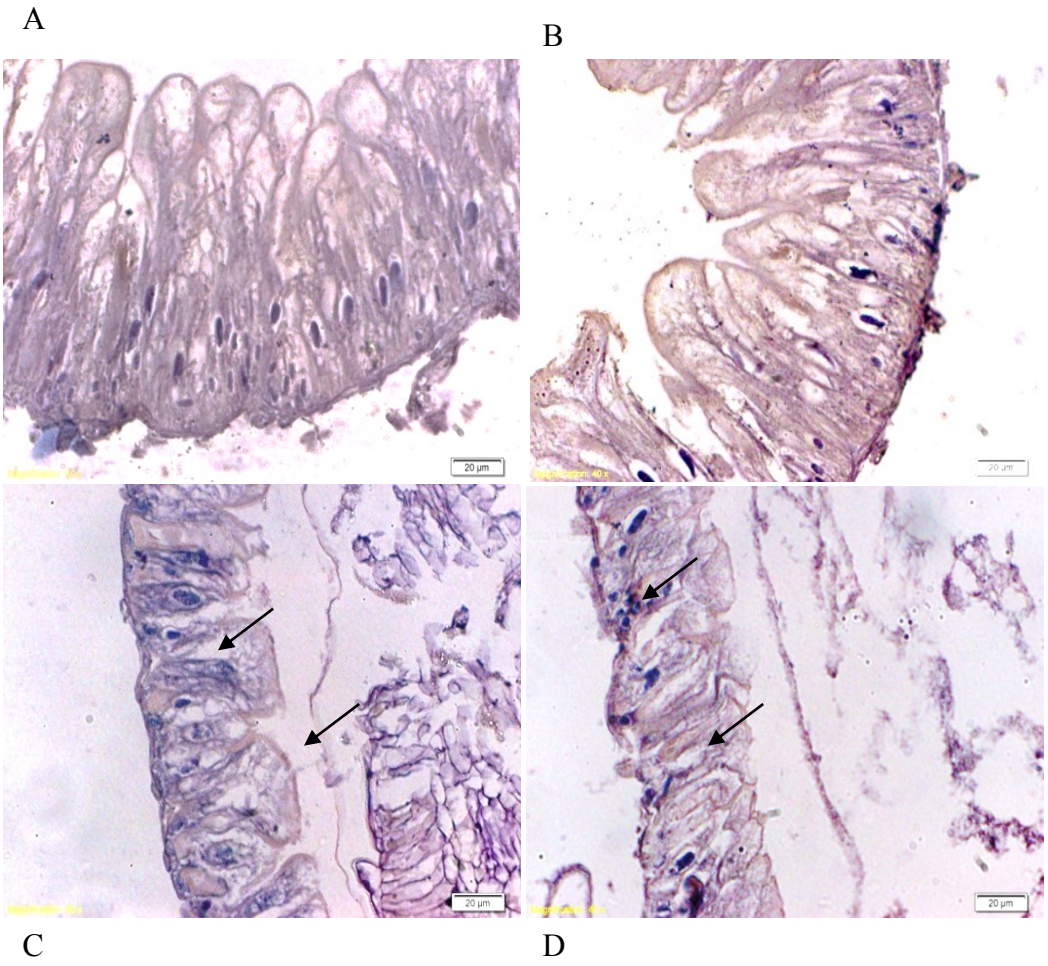

Fig. 6-5. The Hematoxylin- Eosin stained midgut sections of *S. litura* larvae when fed with castor leaves coated with Buffer (panel A), Silica (panel B) CryIEC$_{N6HIS}$ toxin (panel C), CRYSONE S (panel D). The damaged portions in free toxin /CRYSONES treated larvae are shown with arrows . High vacuole formation, disruption of gut membrane and severe decrease in the thickness of the mid gut epithelium was observed. The diseffects were more intense in the larvae treated with CRYSONES (panel D) in comparison with those treated with only toxin (panel C)

6.5.2. Immunohistochemistry:

Interaction of Cry toxin/ CRYSONES with the gut receptors was investigated by adopting immunohistochemistry. The aminopeptidase (APN) receptors present in the gut membrane of *Spodoptera litura* are known to interact with Cry toxins like CryIC, CryIA, CryIF (Avisar et al., 2004, Pigott & Ellar, 2007). In-vitro studies using ligand blotting (described in chapter 2) had revealed the interaction of CryIEC$_{NGST}$ toxin with the APN receptor proteins present in the brush border membrane vesicles of *S. litura* larvae. In this chapter, we further analysed the *in-vivo* interaction of the CryIEC$_{N6HIS}$ toxin with the gut receptor proteins using immune-histochemistry.

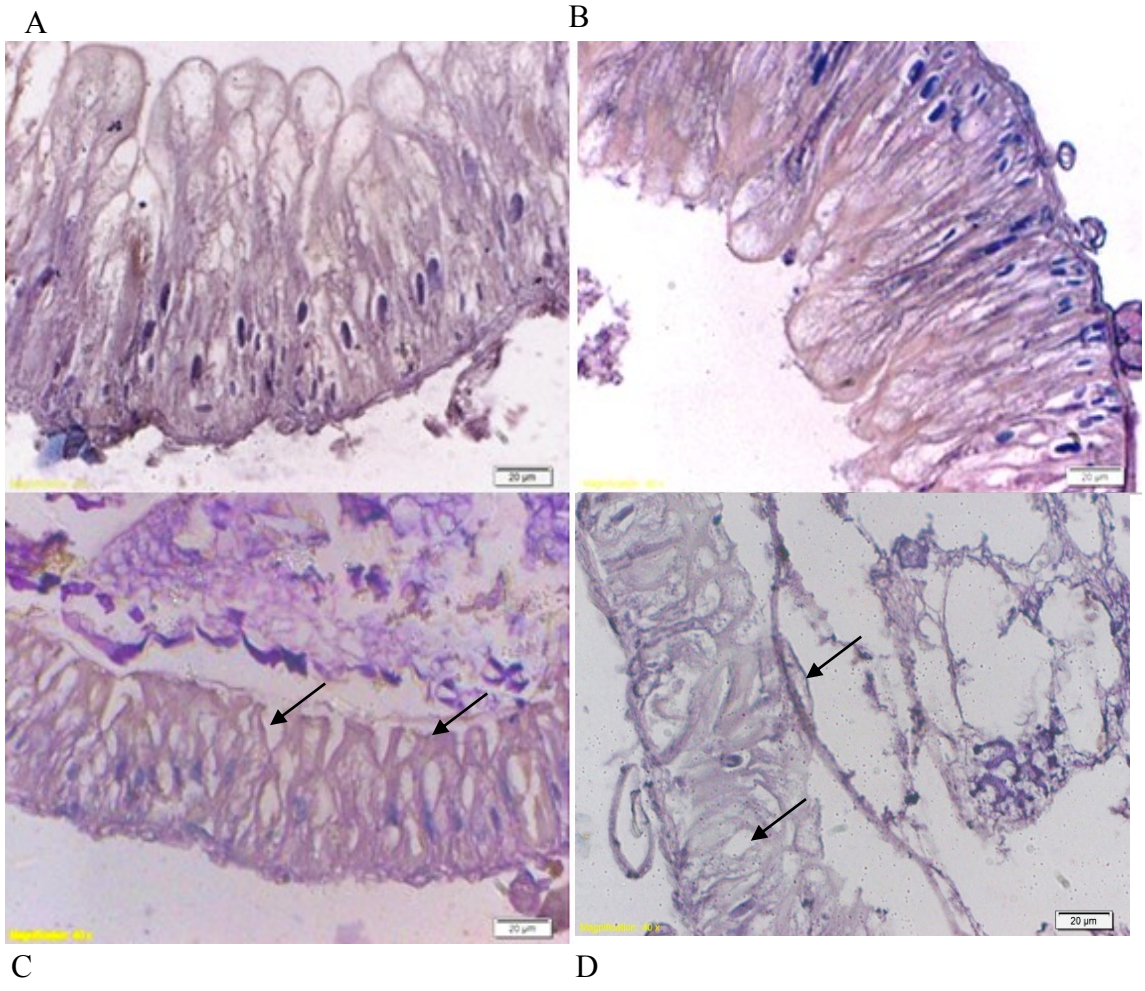

Fig 6-6. Immunohistochemistry. Immunostained midgut sections of *S. litura* larvae when fed with castor leaves coated with Buffer (panel A), Silica (panel B) CryIEC$_{N6HIS}$ toxin (panel C), CRYSONES (panel D). High vacuole formation, disintegration of the gut tissue and the immune-stained regions are shown with arrow marks.

While performing these interactions, the 3rd instar *S. litura* larvae were fed with the CryIEC$_{N6HIS}$ or sprayed with CRYSONES, in similar manner as described earlier. The slides containing the gut sections of the insects exposed to CRYSONES / CryIEC$_{N6HIS}$ along with the proper controls were processed accordingly and subjected to Immunostaining (described in materials and methods). The processed mid-gut sections were then probed with anti-His 1° antibody succeeding with anti-mouse horse radish peroxidase conjugated IgG 2° ab and developed by DAB reagent. The darkly stained portions (brown in colour) seen in the Fig 6-6 (Panels C& D), prove the interaction of Cry1EC$_{N6HIS}$ toxin to the gut receptors. Such stained regions were absent in the control sections (Fig 6-6 A& B). It was observed that the effect of CRYSONES on *S. litura* larvae was more intense as more nuclear fragmentation and disintegration of gut tissue was observed when compared to those treated with only Cry1EC$_{N6HIS}$ toxin.

6.6. Effect of CRYSONES on mortality of *S. litura* larvae.

After having confirmed the interaction of CRYSONES with the gut receptors, the activity of CRYSONES was assayed against the larvae of *S. litura*. CRYSONES, mesoporous nanoparticles carrying CryIEC$_{N6HIS}$ toxin, were used for bioassay against *Spodoptera litura*. 1 mg of silica (containing 12.4 µg of CryIEC$_{N6HIS}$) was dissolved in 800 µl of 20 mM Tris buffer (pH 8.0). CRYSONES were applied on each side of the 12.5 sq cm castor leaf at the concentrations of 10 ng, 20 ng, 30 ng, 40 ng, 50 ng, 60 ng and 80 ng per sq cm of castor leaf, respectively. Similarly, Cry1EC toxin of similar concentrations (10 ng, 20 ng, 30 ng, 40 ng, 50 ng, 60 ng and 80 ng per sq cm) were coated on to the castor leaves along with the only buffer and mesoporous silica nanoparticles controls. The leaves applied with toxin and CRYSONES were shade dried and placed on moist cotton in petri plates. Six 2nd instar *Spodoptera litura* were allowed to feed on each leaf. The experiment was done in triplicates and was also repeated once more

independently. The mortality was recorded every 12 h interval for 2 days and LC_{50} was calculated after 48 h post-feeding by using probit analysis (Finney, 1952). The CRYSONES showed a higher lethal activity against *Spodoptera litura* when compared to $CryIEC_{N6HIS}$ (Table 6-1). The LC_{50} value for CRYSONES could be much lower in natural conditions than recorded in the laboratory as the CRYSONES increase the stability of $CryIEC_{N6HIS}$ protein. Initial observations showed that the $CryIEC_{N6HIS}$ which would degrade within a week at 4°C was found to be stable till a month in the form of CRYSONES at 4°C. This increase in stability of the toxin along with the adsorption capability of silica nanoparticles to the plant surfaces would increase the availability of the toxin to the susceptible insects for longer durations and thus contributing for the enhanced toxicity (Bhattacharyya et al., 2010). The high stability of the CRYSONES and their prolonged effects were evident when no *S. litura* larvae emerged into adults after exposing the larvae to CRYSONES for 48 h and then maintaining them on fresh leaves (Fig 6-8, panel B).

Cry Toxin	**Toxicity bioassay** **LC_{50} (ng/cm^2 of leaf surface)**
Purified $CryIEC_{N6HIS}$ toxin	50 ng/ sq cm
CRYSONES	40 ng/ sq cm

Table 6-1. Evaluation of insecticidal activity of CRYSONES and $CryIEC_{N6HIS}$ toxin.

Further, the body weights of the insects which survived the toxicity were taken after 24 h of feeding with respective toxins. The weight of each larvae of control silica group reached to 45

± 0.8 mg, weight of each larvae of the control buffer group reached to 46 ± 0.9 mg, the weight of each larvae of the CRYSONES (50 ng/ sq cm and 40 ng/ sq cm) group reached to 12 ± 0.7 mg and 15 ± 0.8 mg and weight of each larvae of the toxin group reached to 18 ± 0.8 mg (Fig 6-7). This data reveals that the activity of CRYSONES resulted in much lower body mass of the insects with 12- 15 mg when compared to the control insects with 45-46 mg of body weight.

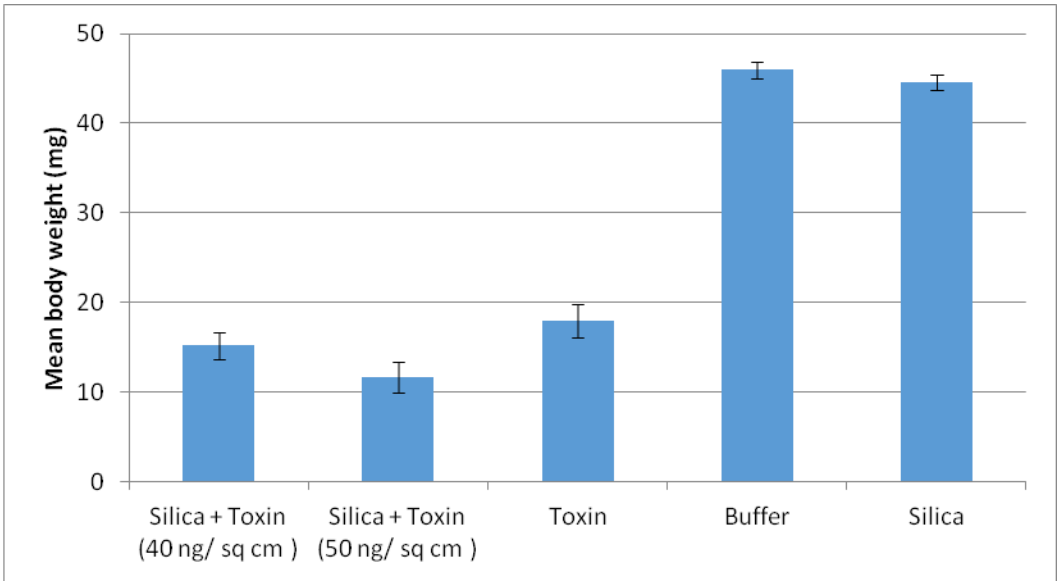

Fig 6-7. The effect of CRYSONES on the body weight of *S. litura* larvae.

6.7. Effect of CRYSONES on growth of *S. litura* larvae.

The effect of CRYSONES was assayed even at a concentration as low as 12 ng of sq cm^{-1}. The assay was done in duplicates where four 3rd instar larvae were allowed to feed on each 12.5 sq cm^{-1} castor leaf. Not even a single larvae reached the adult stage. The toxicity assay analysis revealed that nearly 15 % of the larvae were dead within 48 h of feeding. The remaining alive insects were shifted to fresh leaves and monitored through their growth stages. About 45 %

insects did not undergo metamorphosis into pupae stage and died eventually. 40 % insects molted into pupae but could not emerge into adults (Fig 6-8). The control insects treated with silica, as control, were also monitored throughout the developmental stages and were found to be healthy and emerged into normal adults (Fig 6-8).

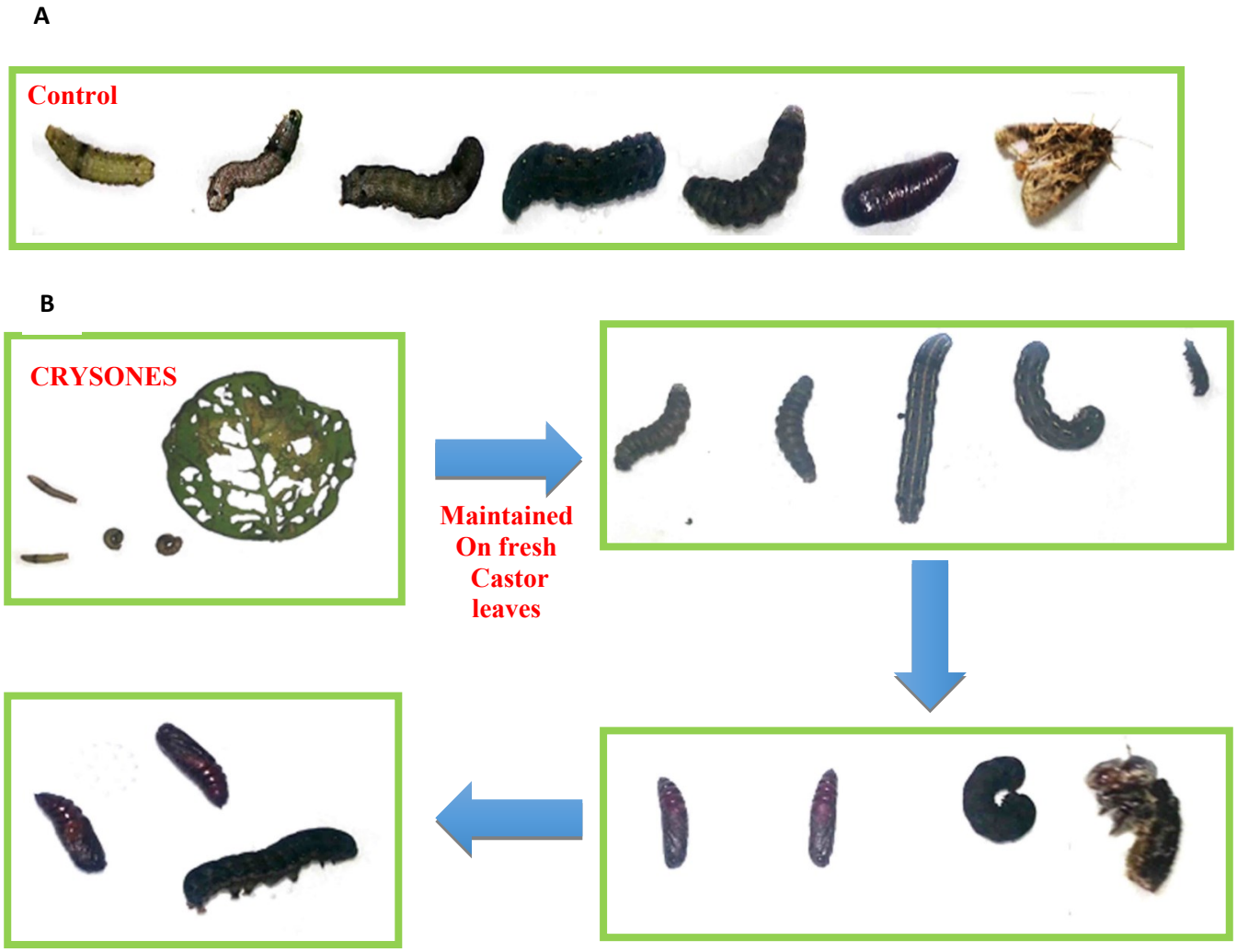

Fig 6-8. Effect of CryIEC$_{N6HIS}$ toxin on the growth of *Spodoptera litura*. Panel A represents the developmental stages of the control *S. litura* larvae which were treated with only buffer. The control larvae had undergone metamorphosis from 3rd instar to 4th and 5th instar, converted into pupae and finally emerged as adults. Panel B represents the fate of the *S. litura* larvae exposed to CRYSONES for 48 h and then shifted and maintained on fresh castor leaves. Few larvae did not get moulted and died eventually. Few survived till fifth instar, developed abnormalities and perished and some developed into pupae but could not emerge into adults. It was observed that no larvae exposed to CRYSONES could develop into healthy adult and all of them perished during their developmental stages.

Further, it was also observed that the exposure of larvae to CRYSONES resulted in retarded larval growth and reduced the larval weights significantly (Fig 6-9, A, B). The feeding behavior of the *S. litura* larvae was analysed and it was found that the larvae treated with CRYSONES fed very little compared to the control insects (Fig 6-9).

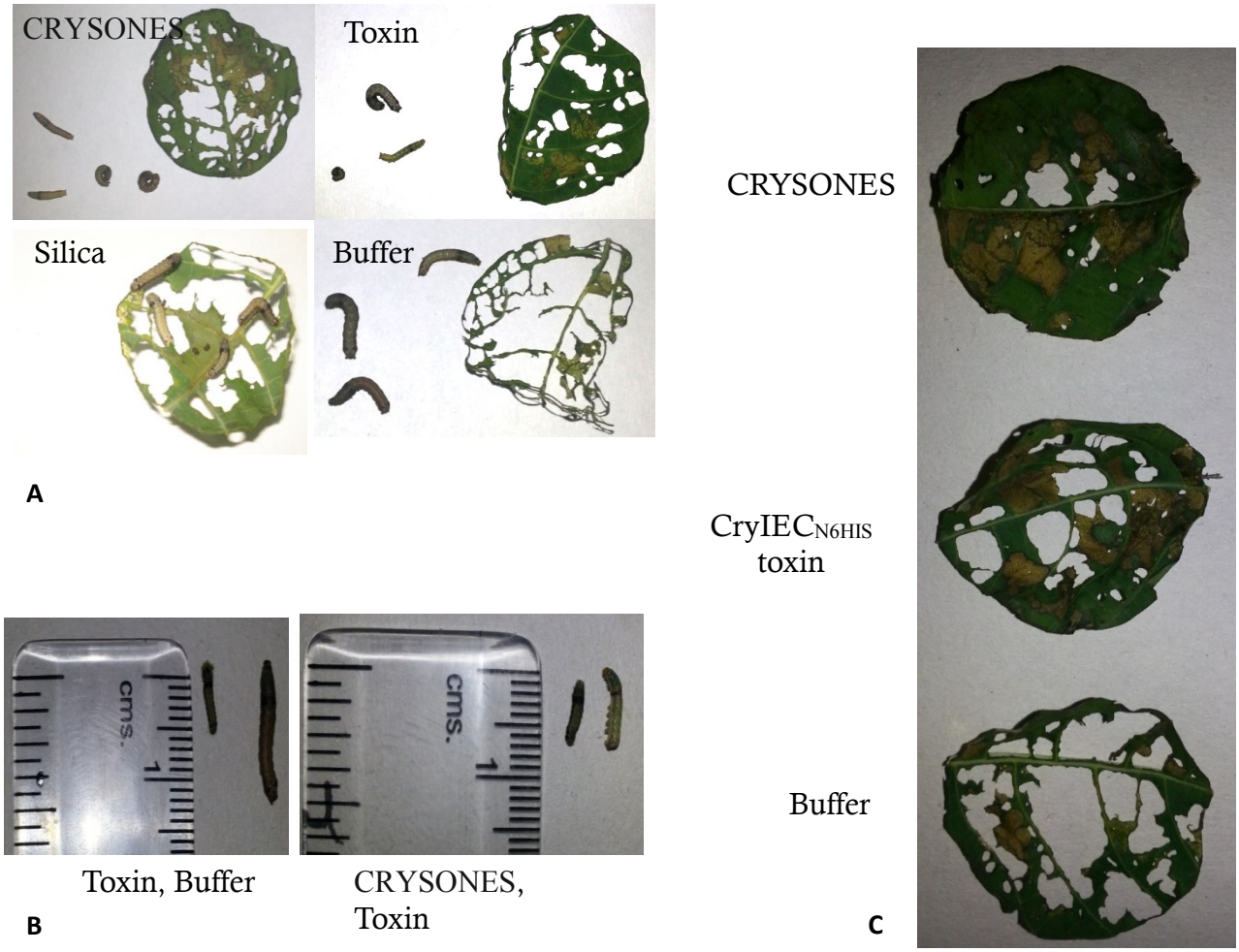

Fig 6-9. Effect of CRYSONES on *S. litura* larvae at a low concentration of 12 ng sq cm^{-1} of leaf. Panel A represents the toxicity bioassay against *S. litura* larvae after 24 h. Panel B represents the comparison of the size of the insects exposed to CRYSONES and CryIEC$_{N6HIS}$ toxin with the control. Panel C shows the feeding behavior of the insects exposed to CRYSONES and CryIEC$_{N6HIS}$ toxin in comparison with the control

The data generated in this chapter through the histological, immunohistochemistry, toxicity bioassay and growth behaviour studies, clearly shows that CRYSONES are very promising biopesticides against the *Spodoptera litura* and the data generated suggests to be a viable lead for further improvement of the technology.

6.8. Conclusions:

1. Mesoporous silica nanoparticles were synthesized.
2. Silica nanoparticles were loaded with pure CryIEC$_{N6HIS}$ toxin and further referred to as CRYSONES.
3. SEM and TEM images of mesoporous silica particles and CRYSONES were observed for their integrity.
4. *In-vivo* analysis of the effect of CRYSONES and CryIEC$_{N6HIS}$ on *S. litura* gut tissue was done by histological staining. It showed high vacuole formation and deformation of gut tissue when 3rd instar *S. litura* larvae were fed with CRYSONES in comparison with CryIEC$_{N6HIS}$.
5. Immunohistochemistry revealed the CRYSONES interaction with gut receptor proteins of 3rd instar larvae of *Spodoptera litura.*
6. Toxicity bioassays showed very good activity of CryIEC toxin and CRYSONES against *S.litura* with a LC_{50} value of 50 ng/ sq cm and 40 ng/ sq cm, respectively.

References

1. Agrawal N, Malhotra P, Bhatnagar RK: **Interaction of gene-cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C.***Appl Environ Microbiol*2002, **68:**4583-4592.

2. Aronson AI, Han ES, McGaughey W, Johnson D: **The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects.***Appl Environ Microbiol*1991, **57:**981-986.

3. Aronson AI, Shai Y: **Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action.***FEMS MicrobiolLett*2001, **195:**1-8.

4. Avisar D, Keller M, Gazit E, Prudovsky E, Sneh B, Zilberstein A: **The role of *Bacillus thuringiensis* Cry1C and Cry1E separate structural domains in the interaction with *Spodoptera littoralis* gut epithelial cells.***J BiolChem*2004, **279:**15779-15786.

5. Bai C, Degheele D, Jansens S, Lambert B: **Activity of insecticidal proteins and strains of *Bacillus thuringiensis* against *Spodoptera exempta*.** *J Invertebr. Pathol* 1993, **62:**211–215

6. Baneyx F, Mujacic M: **Recombinant protein folding and misfolding in *Escherichia coli.***Nat Biotechnol*2004, **22:**1399-1408.

7. Barik TK, Kamaraju R, Gowswami A: **Silica nanoparticle: a potential new insecticide for mosquito vector control.***Parasitol Res* 2012, **111:**1075-1083.

8. Barik TK, Sahu B, Swain V: **Nanosilica-from medicine to pest control.***Parasitol Res* 2008, **103:**253-258.

9. Bernhard K, Jarrett P, Meadows M, Butt J, Ellis DJ, Roberts GM, Pauli S, Rodgers P, Burges HD: **Natural isolates of Bacillus thuringiensis: Worldwide distribution, characterization, and activity against insect pests.** *J. Invertebr. Pathol* 1997, **70:** 59-68.

10. Bhardwaj T, Sharma JP: **Impact of Pesticides Application in Agricultural Industry: An Indian Scenario.** *Int J Agriculture and Food Science Technology* 2013, **4:** 817-822

11. Bhattacharyya A, Bhaumik A, Rani PU, Mandal S, Epidi TT: **Nano-particles - A recent approach to insect pest control.** *Afr J Biotechnol*2010, **9:** 3489-3493.

12. Binda E, Marcone GL, Berini F, Pollegioni L, Marinelli F: **Streptomyces spp. as efficient expression system for a D,D-peptidase/D,D-carboxypeptidase involved in glycopeptide antibiotic resistance.***BMC Biotechnol*2013, **13:**24.

13. Birnboim HC, Doly J: **A rapid alkaline extraction procedure for screening recombinant plasmid DNA.***Nucleic Acids Res* 1979, **7:**1513-1523.

14. Blum H, Beier H, Gross HJ: **Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels**. *Electrophoresis*1987, **8:** 93–99.al

15. Bohorova N, Frutos R, Royer M, Estañol P, Pacheco M, Rascón Q, McLean S, Hoisington D: **Novel synthetic *Bacillus thuringiensis cry1B* gene and the cry1B-cry1Ab translational fusion confer resistance to southwestern corn borer, sugarcane borer and fall armyworm in transgenic tropical maize**. *Theor and Appl Genet*2001, **103:** 817-826

16. Bosch D, Schipper B, van der Kleij H, de Maagd RA, Stiekema WJ: **Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management.***Biotechnology (N Y)* 1994, **12:**915-918.

17. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.***Anal Biochem*1976, **72:**248-254.

18. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle ofprotein-dye binding**. *Anal. Biochem*1976.

19. Bravo A, Gill SS, Soberon M: **Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control.***Toxicon*2007, **49:**423-435.

20. Bravo A, Gomez I, Conde J, Munoz-Garay C, Sanchez J, Miranda R, Zhuang M, Gill SS, Soberon M: **Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains.***BiochimBiophysActa*2004, **1667:**38-46.

21. Bravo A, Gomez I, Porta H, Garcia-Gomez BI, Rodriguez-Almazan C, Pardo L, Soberon M: **Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity.***MicrobBiotechnol*2013, **6:**17-26.

22. Bravo A, Gomez I, Porta H, Garcia-Gomez BI, Rodriguez-Almazan C, Pardo L, Soberon M: **Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity.***MicrobBiotechnol*2012.

23. Broadway RM: **Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors.***J Insect Physiol*1997, **43:**855-874.

24. Buch AD, Archana G, Kumar GN: **Enhanced citric acid biosynthesis in *Pseudomonas fluorescens* ATCC 13525 by overexpression of the Escherichia coli citrate synthase gene.***Microbiology* 2009, **155:**2620-2629.

25. Budatha M, Meur G, Kirti PB, Dutta Gupta A: **Characterization of *Bacillus thuringiensis* Cry toxin binding novel GPI anchored aminopeptidase from fat body of the moth Spodoptera litura.***BiotechnolLett*2007, **29:**1651-1657.

26. Burton SL, Ellar DJ, Li J, Derbyshire DJ: **N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin.***J MolBiol*1999, **287:**1011-1022.

27. Carlson CR, Caugant DA, Kolsto AB: **Genotypic Diversity among *Bacillus cereus* and *Bacillus thuringiensis* Strains.***Appl Environ Microbiol*1994, **60:**1719-1725.

28. Carlson CR, Johansen T, Kolsto AB: **The chromosome map of *Bacillus thuringiensis* subsp. canadensis HD224 is highly similar to that of the Bacillus cereus type strain ATCC 14579.***FEMS MicrobiolLett*1996, **141:**163-167.

29. Chambers JA, Jelen A, Gilbert MP, Jany CS, Johnson TB, Gawron-Burke C: **Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. aizawai.***J Bacteriol* 1991, **173:**3966-3976.

30. Chang JH, Roh JY, Je YH, Park HW, Jin BR, Woo SD, Kang SK: **Isolation of a strain of *Bacillus thuringiensis* ssp. *kurstaki* HD-1 encoding delta-endotoxin Cry1E**.*LettApplMicrobiol*1998, **26:**387-390.

31. Damgaard PH, Hansen BM, Pedersen JC, Eilenberg J: **Natural occurrence of *Bacillus thuringiensis* on cabbage foliage and in insects associated with cabbage crops.***J ApplMicrobiol*1997, **82:**253-258.

32. Davis GD, Elisee C, Newham DM, Harrison RG: **New fusion protein systems designed to give soluble expression in *Escherichia coli*.***BiotechnolBioeng*1999, **65:**382-388.

33. de Maagd RA, Bakker P, Staykov N, Dukiandjiev S, Stiekema W, Bosch D: **Identification of *Bacillus thuringiensis* delta-endotoxin Cry1C domain III amino acid residues involved in insect specificity.***Appl Environ Microbiol*1999, **65:**4369-4374.

34. de Vendomois JS, Cellier D, Velot C, Clair E, Mesnage R, Seralini GE: **Debate on GMOs health risks after statistical findings in regulatory tests.***Int J BiolSci*2010, **6:**590-598.

35. Dean DH, Rajamohan F, Lee MK, Wu SJ, Chen XJ, Alcantara E, Hussain SR: **Probing the mechanism of action of *Bacillus thuringiensis* insecticidal proteins by site-directed mutagenesis--a minireview.***Gene* 1996, **179:**111-117.

36. deMaagd RA, Bravo A, Crickmore N: **How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world.***Trends Genet* 2001, **17:**193-199.

37. deMaagd RA, Kwa MS, van der Klei H, Yamamoto T, Schipper B, Vlak JM, Stiekema WJ, Bosch D: **Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition.***Appl Environ Microbiol*1996, **62:**1537-1543.

38. deMaagd RA, Weemen-Hendriks M, Molthoff JW, Naimov S: **Activity of wild-type and hybrid *Bacillus thuringiensis* delta-endotoxins against Agrotisipsilon.***Arch Microbiol*2003, **179:**363-367.

39. deMaagd RA, Weemen-Hendriks M, Stiekema W, Bosch D: ***Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specificity determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids.***Appl Environ Microbiol*2000, **66:**1559-1563.

40. Du C, Martin PA, Nickerson KW: **Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal *Bacillus thuringiensis* Protein Crystals.***Appl Environ Microbiol*1994, **60:**3847-3853.

41. Eguchi M: **Alkaline phosphatase isozymes in insects and comparison with mammalian enzyme.***Comp BiochemPhysiol B BiochemMolBiol*1995, **111:**151-162.

42. Escriche B, Tabashnik B, Finson N, Ferre J: **Immunohistochemical detection of binding of CryIA crystal proteins of *Bacillus thuringiensis* in highly resistant strains of *Plutella xylostella* (L.) from Hawaii.***BiochemBiophys Res Commun*1995, **212:**388-395.

43. Fernandez LE, Aimanova KG, Gill SS, Bravo A, Soberon M: **A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae.***Biochem J* 2006, **394:**77-84.

44. Figurski DH, Helinski DR: **Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans**. *ProcNatlAcadSci*1979, 1648-1652.

45. Finney DJ: **On the precision of biological assays.***ActaPharmacolToxicol (Copenh)* 1952, **8:**55-70.

46. Gahan LJ, Gould F, Heckel DG: **Identification of a gene associated with Bt resistance in *Heliothis virescens*.***Science* 2001, **293:**857-860.

47. Gamer M, Frode D, Biedendieck R, Stammen S, Jahn D: **A T7 RNA polymerase-dependent gene expression system for *Bacillus megaterium*.***ApplMicrobiolBiotechnol*2009, **82:**1195-1203.

48. Gatehouse JA: **Biotechnological prospects for engineering insect-resistant plants.***Plant Physiol*2008, **146:**881-887.

49. Ghewande MP, Desai S, Basu MS: **Diagnosis and management of major diseases of groundnut**. Bulletin, *National Research Centre for Groundnut*2002a

50. Ghewande MP, Nandagopal V, Desai S, Basu MS: **Integrated pest management in groundnut.** Bulletin, *National Research Centre for Groundnut*2002b

51. Ghormade V, Deshpande MV, Paknikar KM: **Perspectives for nano-biotechnology enabled protection and nutrition of plants.***BiotechnolAdv*2011, **29:**792-803.

52. Gill M, Ellar D: **Transgenic Drosophila reveals a functional in vivo receptor for the *Bacillus thuringiensis* toxin Cry1Ac1.***Insect MolBiol*2002, **11:**619-625.

53. Gill SS, Cowles EA, Pietrantonio PV: **The mode of action of *Bacillus thuringiensis* endotoxins.***Annu Rev Entomol*1992, **37:**615-636.

54. Gonzalez JM, Jr., Dulmage HT, Carlton BC: **Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*.***Plasmid* 1981, **5:**352-365.

55. Griffitts JS, Haslam SM, Yang T, Garczynski SF, Mulloy B, Morris H, Cremer PS, Dell A, Adang MJ, Aroian RV: **Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin.***Science* 2005, **307:**922-925.

56. Hanahan D: **Studies on transformation of *E. coli* with plasmids.** *J Mol Biol*1983, **166:** 557.

57. Hara H, Atsumi S, Yaoi K, Nakanishi K, Higurashi S, Miura N, Tabunoki H, Sato R: **A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae.***FEBS Lett*2003, **538:**29-34.

58. Herman RA, Wolt JD, Halliday WR: **Rapid degradation of the Cry1F insecticidal crystal protein in soil.***J Agric Food Chem*2002, **50:**7076-7078.

59. Herrero S, Gechev T, Bakker PL, Moar WJ, de Maagd RA: ***Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes.***BMC Genomics* 2005, **6:**96.

60. Hofte H, Whiteley HR: **Insecticidal crystal proteins of *Bacillus thuringiensis*.***Microbiol Rev* 1989, **53:**242-255.

61. Honee G, van der Salm T, Visser B: **Nucleotide sequence of crystal protein gene isolated from *B. thuringiensis* subspecies *entomocidus* 60.5 coding for a toxin highly active against *Spodoptera* species.***Nucleic Acids Res* 1988, **16:**6240.

62. Ibrahim MA, Griko N, Junker M, Bulla LA: ***Bacillus thuringiensis*: a genomics and proteomics perspective.***Bioeng Bugs* 2010, **1:**31-50.

63. Inoue H, Nojima H, Okayama H: **High efficiency transformation of *Escherichia coli* with plasmids.***Gene* 1990, **96:**23-28.

64. Ish-Horowicz D, Burke JF: **Rapid and efficient cosmid cloning.***Nucleic Acids Res* 1981, **9:**2989-2998.

65. Juarez-Perez VM, Ferrandis MD, Frutos R: **PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes.***Appl Environ Microbiol*1997, **63:**2997-3002.

66. Jurat-Fuentes JL, Adang MJ: **Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae.***Eur J Biochem*2004, **271:**3127-3135.

67. Kalman S, Kiehne KL, Cooper N, Reynoso MS, Yamamoto T: **Enhanced production of insecticidal proteins in *Bacillus thuringiensis* strains carrying an additional crystal protein gene in their chromosomes.***Appl Environ Microbiol*1995, **61:**3063-3068.

68. Kalman S, Kiehne KL, Libs JL, Yamamoto T: **Cloning of a novel cryIC-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*.***Appl Environ Microbiol*1993, **59:**1131-1137.

69. Kathage J, Qaim M: **Economic impacts and impact dynamics of Bt (*Bacillus thuringiensis*) cotton in India.***ProcNatlAcadSci U S A*2012.

70. Keifer M, Gasperini F, Robson M: **Pesticides and other chemicals: minimizing worker exposures.***J Agromedicine*2010, **15:**264-274.

71. Keller M, Sneh B, Strizhov N, Prudovsky E, Regev A, Koncz C, Schell J, Zilberstein A: **Digestion of delta-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC.***Insect BiochemMolBiol*1996, **26:**365-373.

72. Kumar S, Misra A, Verma AK, Roy R, Tripathi A, Ansari KM, Das M, Dwivedi PD: **Bt brinjal in India: a long way to go.***GM Crops* 2011, **2:**92-98.

73. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.***Nature* 1970, **227:**680-685.

74. Lal R, Pimentel D: **Biofuels: beware crop residues.***Science* 2009, **326:**1345-1346; author reply 1346.

75. Lang K, Zierow J, Buehler K, Schmid A: **Metabolic engineering of *Pseudomonas* sp. strain VLB120 as platform biocatalyst for the production of isobutyric acid and other secondary metabolites**. *Microb Cell Fact* 2014, **13:**2.

76. Lewis WJ, van Lenteren JC, Phatak SC, Tumlinson JH, 3rd: **A total system approach to sustainable pest management.***ProcNatlAcadSci U S A*1997, **94:**12243-12248.

77. Li JD, Carroll J, Ellar DJ: **Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution.***Nature* 1991, **353:**815-821.

78. Liang Y, Patel SS, Dean DH: **Irreversible binding kinetics of *Bacillus thuringiensis* Cry1A δ–endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity.** *J. Biol. Chem*1995, **270:** 24719-24724.

79. Lightwood DJ, Ellar DJ, Jarrett P: **Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin.***Appl Environ Microbiol*2000, **66:**5174-5181.

80. Liu TX, Sparks AN, Jr., Chen W, Liang GM, Brister C: **Toxicity, persistence, and efficacy of indoxacarb on cabbage looper (Lepidoptera: Noctuidae) on cabbage.***J Econ Entomol*2002, **95:**360-367.

81. Loguercio LL, Santos CG, Barreto MR, Guimaraes CT, Paiva E: **Association of PCR and feeding bioassays as a large-scale method to screen tropical *Bacillus thuringiensis* isolates for**

a cry constitution with higher insecticidal effect against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae.*LettApplMicrobiol*2001, **32:**362-367.

82. Los FC, Randis TM, Aroian RV, Ratner AJ: **Role of pore-forming toxins in bacterial infectious diseases**.*MicrobiolMolBiol Rev* 2013, **77:**173-207.

83. Losey JE, Rayor LS, Carter ME: **Transgenic pollen harms monarch larvae.***Nature* 1999, **399:**214.

84. Luo K, Sangadala S, Masson L, Mazza A, Brousseau R, Adang MJ: **The heliothisvirescens 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A delta-endotoxin binding and pore formation.***Insect BiochemMolBiol*1997, **27:**735-743.

85. Madliger M, Sander M, Schwarzenbach RP: **Adsorption of transgenic insecticidal Cry1Ab protein to SiO2. 2. Patch-controlled electrostatic attraction.***Environ SciTechnol*2010, **44:**8877-8883.

86. Mahillon J, Rezsohazy R, Hallet B, Delcour J: **IS231 and other *Bacillus thuringiensis* transposable elements: a review.***Genetica*1994, **93:**13-26.

87. Martin PA, Travers RS: **Worldwide Abundance and Distribution of *Bacillus thuringiensis* Isolates.***Appl Environ Microbiol*1989, **55:**2437-2442.

88. Masson L, Moar WJ, van Frankenhuyzen K, Bosse M, Brousseau R: **Insecticidal properties of a crystal protein gene product isolated from *Bacillus thuringiensis* subsp. kenyae.***Appl Environ Microbiol*1992, **58:**642-646.

89. Mathur SC: **Future of Indian pesticides industry in next millennium**. *Pesticide Information*2010, **24:** 9–23.

90. McGaughey WH, Gould F, Gelernter W: **Bt resistance management.***Nat Biotechnol*1998, **16:**144-146.

91. McNall RJ, Adang MJ: **Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in Manducasexta midgut through proteomic analysis.***Insect BiochemMolBiol*2003, **33:**999-1010.

92. Moar WJ, Pusztai-Carey M, Van Faassen H, Bosch D, Frutos R, Rang C, Luo K, Adang MJ: **Development of *Bacillus thuringiensis* CryIC Resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae).***Appl Environ Microbiol*1995, **61:**2086-2092.

93. Morales VM, Backman A, Bagdasarian M: **A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants**. *Gene1991*, **97:** 39-47.

94. Nagamatsu Y, Koike T, Sasaki K, Yoshimoto A, Furukawa Y: **The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin.***FEBS Lett*1999, **460:**385-390.

95. Naimov S, Dukiandjiev S, de Maagd RA: **A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato.***Plant Biotechnol J* 2003, **1:**51-57.

96. Nguyen HT, Jehle JA: **Expression of Cry3Bb1 in transgenic corn MON88017.***J Agric Food Chem*2009, **57:**9990-9996.

97. Oltean DI, Pullikuth AK, Lee HK, Gill SS: **Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA.***Appl Environ Microbiol*1999, **65:**4760-4766.

98. Oppert B, Kramer KJ, Beeman RW, Johnson D, McGaughey WH: **Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins.***J BiolChem*1997, **272:**23473-23476.

99. Paik P, Mastai Y, Kityk I, Rakus P, Gedanken A: **Synthesis of amino acid block-copolymer imprinted chiral mesoporous silica and its acoustically-induced optical Kerr effects.** *J Solid State Chemistry*2012, **192:** 127–131.

100. Pardo-Lopez L, Munoz-Garay C, Porta H, Rodriguez-Almazan C, Soberon M, Bravo A: **Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*.***Peptides* 2009, **30:**589-595.

101. Pardo-Lopez L, Soberon M, Bravo A: ***Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection.***FEMS Microbiol Rev* 2013, **37:**3-22.

102. Parker MW, Feil SC: **Pore-forming protein toxins: from structure to function.***ProgBiophysMolBiol*2005, **88:**91-142.

103. Perez C, Fernandez LE, Sun J, Folch JL, Gill SS, Soberon M, Bravo A: ***Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor.***ProcNatlAcadSci U S A*2005, **102:**18303-18308.

104. Piddock LJ, Williams KJ, Ricci V: **Accumulation of rifampicin by *Mycobacterium aurum, Mycobacterium smegmatis* and *Mycobacterium tuberculosis*.***J AntimicrobChemother*2000, **45:**159-165.

105. Pigott CR, Ellar DJ: **Role of receptors in *Bacillus thuringiensis* crystal toxin activity.***MicrobiolMolBiol Rev* 2007, **71:**255-281.

106. Polanczyk RA, Pires da Silva RF, Fiuza LM: **Effectiveness of *Bacillus thuringiensis* strains against *Spodoptera frugiperda* (Lepidoptera: Noctuidae)**. *J. Microbiol*2000, **31:**165–167.

107. Prasad MNR, Gowda MVC: **Mechanisms of resistance to tobacco cutworm *(Spodoptera litura*) and their implications to screening for resistance in groundnut.** *Euphytica*2006,**149:** 387–399.

108. Rahman K, Abdullah MA, Ambati S, Taylor MD, Adang MJ: **Differential protection of Cry1Fa toxin against *Spodoptera frugiperda* larval gut proteases by cadherin orthologs correlates with increased synergism.***Appl Environ Microbiol*2012, **78:**354-362.

109. Rahman M, Glatz R, Roush R, Schmidt O: **Developmental penalties associated with inducible tolerance in *Helicoverpa armigera* to insecticidal toxins from *Bacillus thuringiensis.***Appl Environ Microbiol*2011, **77:**1443-1448.

110. Rai M, Ingle A: **Role of nanotechnology in agriculture with special reference to management of insect pests.***ApplMicrobiolBiotechnol*2012, **94:**287-293.

111. Rajagopal R, Sivakumar S, Agrawal N, Malhotra P, Bhatnagar RK: **Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor.***J BiolChem*2002, **277:**46849-46851.

112. Retallack DM, Jin H, Chew L: **Reliable protein production in a *Pseudomonas fluorescens* expression system.***Protein Expr Purif*2012, **81:**157-165.

113. Roh JY, Choi JY, Li MS, Jin BR, Je YH: ***Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control**.*J MicrobiolBiotechnol*2007, **17:**547-559.

114. Rosas-Garcia NM: **Biopesticide production from *Bacillus thuringiensis*: an environmentally friendly alternative.***Recent Pat Biotechnol*2009, **3:**28-36.

115. Rukmini V, Reddy CY, Venkateswerlu G: ***Bacillus thuringiensis* crystal delta-endotoxin: role of proteases in the conversion of protoxin to toxin.***Biochimie*2000, **82:**109-116.

116. Sakamoto N, Saito S, Hirose T, Suzuki M, Matsuo S, Izumi K, Nagatomi T, Ikegami H, Umeda K, Tsushima K, Matsuo N: **The discovery of pyridalyl: a novel insecticidal agent for controlling lepidopterous pests.***Pest ManagSci*2004, **60:**25-34.

117. Sambrook J, Fritsch EF, Maniatis T: **Molecular cloning: a laboratory manual**, 2nd ed. Cold Spring Harbor Laboratory Press, ColdSpring Harbor, N.Y1989.

118. Sanchis V, Lereclus D, Menou G, Chaufaux J, Guo S, Lecadet MM: **Nucleotide sequence and analysis of the N-terminal coding region of the *Spodoptera*-active delta-endotoxin gene of *Bacillus thuringiensis aizawai*.***MolMicrobiol*1989, **3:**229-238.

119. Sander M, Madliger M, Schwarzenbach RP: **Adsorption of transgenic insecticidal Cry1Ab protein to SiO2. 1. Forces driving adsorption.***Environ SciTechnol*2010, **44:**8870-8876.

120. Sayyed AH, Attique MN, Khaliq A, Wright DJ: **Inheritance of resistance and cross-resistance to deltamethrin in Plutellaxylostella (Lepidoptera: Plutellidae) from Pakistan.***Pest ManagSci*2005, **61:**636-642.

121. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH: ***Bacillus thuringiensis* and its pesticidal crystal proteins.***MicrobiolMolBiol Rev* 1998, **62:**775-806.

122. Schnepf HE, Whiteley HR: **Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*.***ProcNatlAcadSci U S A*1981, **78:**2893-2897.

123. Schnepf HE, Wong HC, Whiteley HR: **The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence.***J BiolChem*1985, **260:**6264-6272.

124. Schwartz JL, Lu YJ, Sohnlein P, Brousseau R, Laprade R, Masson L, Adang MJ: **Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors.***FEBS Lett*1997, **412:**270-276.

125. Selinger LB, Khachatourians GG, Byers JR, Hynes MF: **Expression of a *Bacillus thuringiensis* delta-endotoxin gene by *Bacillus pumilus*.***Can J Microbiol*1998, **44:**259-269.

126. Shelton AM, Zhao JZ, Roush RT: **Economic, ecological, food safety, and social consequences of the deployment of bt transgenic plants.***Annu Rev Entomol*2002, **47:**845-881.

127. Simon R, Priefer U, Puhler A: **A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria**. *Biotechnology1983*, **1:** 784.791.

128. Singh PK, Kumar M, Chaturvedi CP, Yadav D, Tuli R: **Development of a hybrid delta-endotoxin and its expression in tobacco and cotton for control of a polyphagous pest *Spodoptera litura*.***Transgenic Res* 2004, **13:**397-410.

129. Smith RA, Couche GA: **The Phylloplane as a Source of *Bacillus thuringiensis* Variants.***Appl Environ Microbiol*1991, **57:**311-315.

130. Song MR, Cui SM, Gao F, Liu YR, Fan CL, Lei TQ, Liu DC: **Dispersible silica nanoparticles as carrier for enhanced bioactivity of chlorfenapyr.** *J.Pesticide Science*2012, **37:** 258-260

131. Sonkaria S, Ahn SH, Khare V: **Nanotechnology and its impact on food and nutrition: a review.***Recent Pat Food NutrAgric*2012, **4:**8-18.

132. Squires C: ***Pseudomonas fluorescens* Expression Technology for Subunit Vaccine Production and Development**. *BioProcess Int*. 2011, S22–S26

133. Strizhov N, Keller M, Mathur J, Koncz-Kalman Z, Bosch D, Prudovsky E, Schell J, Sneh B, Koncz C, Zilberstein A: **A synthetic cryIC gene, encoding a *Bacillus thuringiensis* delta-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco.***ProcNatlAcadSci U S A*1996, **93:**15012-15017.

134. Stubbs TL, Kennedy AC, Skipper HD: **Survival of a Rifampicin-Resistant *Pseudomonas fluorescens* Strain in Nine Mollisols**. *Appl and Environ Soil Science* 2014

135. Studier FW, Moffatt BA: **Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.***J MolBiol*1986, **189:**113-130.

136. Tabor S: **Expression using the T7 RNA polymerase/promoter system**.*CurrProtocMolBiol*2001, **16:**12.

137. Taylor A: **Aminopeptidases: structure and function.***FASEB Journal* 1993, **7:**290-298.

138. Terra WR, Ferreira C: **Insect digestive enzymes: properties, compartmentalization and function.***Comp BiochemPhysiol*1994, **109B:**1-62.

139. Thanabalu T, Hindley J, Brenner S, Oei C, Berry C: **Expression of the mosquitocidal toxins of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae.***Appl Environ Microbiol*1992, **58:**905-910.

140. Ulrich C, Mewis I, Goswami A, Chatterjee SD, Banerjee SP, Adhikary S, Bhattacharyya A: **Biodiversity-Macro and Micro: To be Nano or Not to be!.** *Everyman's Science* 2006, **11:** 433-443.

141. Vadlamudi RK, Weber E, Ji I, Ji TH, Bulla LA, Jr.: **Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*.***J BiolChem*1995, **270:**5490-5494.

142. Vaeck M, Reynaerts A, Höfte H, Jansens S, Beuckeleer MD, Dean C, Zabeau M, Montagu MV, Leemans J: **Transgenic plants protected from insect attack**. *Nature1987*, **328:** 33.37.

143. Valaitis AP, Jenkins JL, Lee MK, Dean DH, Garner KJ: **Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity.***Arch Insect BiochemPhysiol*2001, **46:**186-200.

144. Van Rie J, Jansens S, Hofte H, Degheele D, Van Mellaert H: **Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins.***Appl Environ Microbiol*1990, **56:**1378-1385.

145. Visser B, Munsterman E, Stoker A, Dirkse WG: **A novel Bacillus thuringiensis gene encoding a *Spodoptera exigua*-specific crystal protein.***J Bacteriol* 1990, **172:**6783-6788.

146. Walter K, Schütt C: **Methods of Enzymatic Analysis** (Bergmeyer, H.U. ed) 2nd ed., Academic Press, Inc., NY1974, **2:** 860-864.

147. Wolfersberger MG: **The toxicity of two *Bacillus thuringiensis* delta-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins.***Experientia*1990, **46:**475-477.

148. Xue JL, Cai QX, Zheng DS, Yuan ZM: **The synergistic activity between Cry1Aa and Cry1c from *Bacillus thuringiensis* against *Spodoptera exigua* and Helicoverpaarmigera.***LettApplMicrobiol*2005, **40:**460-465.

149. Yanes RE, Tamanoi F: **Development of mesoporous silica nanomaterials as a vehicle for anticancer drug delivery.***TherDeliv*2012, **3:**389-404.

150. Zhang WJ, Jiang F, Ou JF: **Global pesticide consumption and pollution: with China as a focus**. *Proceedings of the International Academy of Ecology and Environmental Sciences*2011, **1:**125-144.