6.1 Materials

6.1.1 *E. coli* strains harboring plasmids

1) *E. coli* ECE 130 (DH5α) with recombinant plasmid pTZ19R-*cry9Aa*
2) *E. coli* ECE 53 (JM103) with recombinant plasmid pKK223-3-*cry1Ac*

6.1.2 *E. coli* strains for cloning and expression:

1) *E. coli* DH5α strain with cloning plasmid pBluescript KS (+)was used as cloning host with blue-white selection
2) *E. coli* BL21 (DE3) plysS was used as host for expression of hybrid toxins from pET-28a (+) vector with T7 promoter and IPTG based induction (Novagen, USA, Catalog no. 69864-3).

6.1.3 *Bacillus* reference strains:

1) *Bacillus thuringiensis subsp. kurstaki* HD-1
2) *Bacillus thuringiensis subsp. kurstaki* HD-73

Btk HD1 and *Btk* HD73 were used as reference strains for colony morphology, phase contrast microscopy, Cry protein analysis by SDS-PAGE. All *E. coli* and *Bacillus* cultures were obtained from the Bacillus Genetic Stock Centre (BGSC), Ohio State University, USA

6.1.4 Culture growth conditions and maintenance:

E. coli strains were grown at 37 ºC overnight on plates or with 200 rpm rotary shaking condition in Luria-Bertani broth. *Bt* reference strain and *Bt* isolates were grown at 30ºC until large colony appear on plates (12-24 hours) or with 200 rpm rotary shaking condition in Luria-Bertani broth.

E. coli strains were maintained on Luria-Bertani plates. Sub-culturing of each culture was performed every month. Reference *Bt* strains and were maintained on Luria-Bertani plates without antibiotics.

All *Bt* strains and *E. coli* strains were also maintained as glycerol stocks prepared from freshly grown culture on plate. Single colony was mixed well with 20 % v/v glycerol in sterile D/W. Glycerol stocks were kept in -20ºC deep freezer overnight and next day transferred to -80ºC for long term storage.

6.1.5 Insect sources and rearing

H. armigera and *Spodoptera litoralis* larva were collected from Anand agricultural model farm, Baroda and from *C. cajan* grown field of Mr. Brijesh Patel located near to Savana tiles, Pvt.Ltd. At- Jambusar, Dist- Bharuch, Baroda. 2nd or 3 rd instar larva collected. Fed them with fresh leaves of cabbage till pupation. upon emergence of moths of *H.armigera*, kept a pair of opposite sex moth in a jar tightly closed with a cloth. Also, moths were fed with 0.1% honey. Care taken to prevent over exposure of sunlight. Keep observation for eggs laid. Larvae after hatching from eggs were provided with fresh and cabbage leaves to *H. armigera*. And castor leaves to *S. Spodoptera.*

6.1.6 Media:

Luria-Bertani medium (LB):

Tryptone - 1%

Yeast extract - 0.5%

NaCl - 0.5%

pH - 7.9

LB plates were prepared by adding LB and agar powder at 2.5 % w/v into D/W, autoclaved at 15 p.s.i. for 15 min and poured in petri dishes upon cooling up to 50-55 ºC. Similarly LB broth was made by mixing LB powder at 2% w/v into D/W and autoclaving. It was used for growing and maintenance of *E. coli* and *Bt* cultures. Ampicillin at a final concentration of 100 ug/ml & Kanamycin at a final concentration 30ug/ml were used as per requirements.

GYS (Glucose Yeast Extract) medium

Glucose	-	0.1 gm%
Yeast extract	-	0.2 gm%
K_2HPO_4	-	0.5 gm%
Ammonium sulfate	-	0.2 gm%
$MgSO_4.7H_2O$	-	0.02 gm%
$MnSO_4.5H_2O$	-	0.005 gm%
$CaCl_2$	-	0.008 gm%
pH	-	7.2

Stocks (10X) of each salt were separately made and autoclaved at 15 p.s.i. for 15 min for storage. During GYS preparation, stocks of salts except K_2HPO_4 were added to medium with glucose and yeast extract, pH adjusted to 7.2 and autoclaved at10 p.s.i. for 20 min. The K_2HPO_4 stock was added during inoculation aseptically. GYS medium was used for sporulation of *Bt* strains and isolation by *Bt* enrichment method.

6.1.7 Antibiotics stocks (1000X):

Ampicillin: 100 mg/ml in sterile D/W

Kanamycin: 30 mg/ml in sterile D/W

Antibiotics powder was dissolved in sterile D/W at concentrations stated above, filtered through a 0.22 µM filter and stored at -20 ºC.

6.2 Protocols:

6.2.1 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Sambrook and Russel 2001

- Acrylamide solution: 29% acrylamide with 1% bisacrylamide was added slowly to 60 ml of sterile D/W and mixed by magnetic stirrer for four hours. Final volume was

made to 100 ml and filtered through Whatman no. 1 paper. Solution was stored at 4°C.

- Resolving gel buffer (pH 8.8) (100 ml): 22.7 gm of Tris-Cl was dissolved in distilled water, adjusted the pH to 8.8 with concentrated HCl and final volume was made up to 100ml with sterile D/W.
- Stacking gel buffer (pH 6.8) (100 ml): 7.26 gm of Tris-Cl was dissolved in D/W, adjust pH to 6.8 with concentrated HCl and final volume made up to 100 ml.
- Running buffer: 15.1 gm of Tris base, 94 gm of glycine and 5 gm of SDS were dissolved in 800 ml sterile D/W and final volume was adjusted to 1 liter.
- Sample buffer (Laemmli's buffer 3x): 3.65 gm of Tris-Cl (pH 6.8), 0.5 gm of SDS, 5 gm of sucrose, 0.25 ml of β-mercaptoethanol, and 125 mg of bromo phenol blue dye were mixed well in 40 ml sterile D/W and final volume was made up to 50 ml.
- 10% Ammonium per sulphate (10% APS): Dissolved 100 mg of APS in 1 ml distilled H_2O.
- SDS (10%): Dissolved 1 gm of SDS in 10 ml distilled H_2O.

Composition for SDS- PAGE

Components	**Resolving gel (10%)**	**Stacking gel (5%)**
	(7 ml)	**(2 ml)**
D/W	1.9 ml	1.4 ml
30% Acrylamide	1.7 ml	0.33 ml
1.5M Tris (pH 8.8)	1.3 ml	-
1M tris Cl (pH 6.8)	-	0.25 ml
10% SDS	50.0 µl	20.0 µl
10% APS	50.0 µl	20.0 µl
TEMED	4.0 µl	3.0 µl

SDS-PAGE was carried out at constant 50 volts initially during migration in stacking gel and at constant 80 volts when bromophenol blue dye front enters resolving gel.

6.2.2 Agarose gel Electrophoresis

DNA fragments were separated by using 0.8% to 2.0% w/v agarose gels as described by Sambrook and Russel 2001.

- Loading dye: 40 % (v/v) glycerol and 0.25% (w/v) bromo phenol blue dye were mixed well in sterile D/W.
- 50 X TAE Buffer stock solution: 242 gm of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were mixed in 800 ml of D/W and final volume made to 1 liter.

Agarose gels were casted with 1X TAE buffer and ethidium bromide (0.5 μg/ml) and electrophoresis was carried out in 1X TAE buffer at ~5V/cm of gel solution (0.5 μg/ml) for half an hour and washed with sterile D/W for 15 min. DNA bands were visualized under short wavelength UV light (λ = 254 nm) and photographed by AlphaEase gel documentation system.

6.2.3 Blunt end cloning in pBluescript KS (+)

Ligation of PCR amplification products or eluted DNA fragments was done pBluescript KS (+). Amplicons were cloned at EcoRV site of pBluescript KS (+). Concentration of vector to insert was kept in 1:3 molar ratios. Ligation system, as mentioned below was set up at 22°C overnight in ligation bath. Transformation performed by taking of ligation mixture with 200 μL competent cells. Transformed DH5α cells were plated on X-GAL (20 mg/ml) spread L.B. plates for blue white selection of clones.

Ligation system

Buffer (10X)	2.0µL
Ligase	1.0µL
pBluescript KS (+)	2.0µL
PCR product	2.0µL
D/W	Upto 20µL

6.2.4 Plasmid extraction

Plasmid extraction was done by modified Alkaline Lysis (Miniprep) method as described by Sambrook and Russell 2001. The protocol was modified to work with higher volume of culture which gives comparable/higher yields of plasmid as compared to standard protocol of Miniprep. The total volume of alkaline lysis solutions used was increased as per the increase in culture volume.

Reagents & Composition

Reagents	**Composition**
STE buffer	Tris-Cl 10 mM (pH 8.0),0.1M NaCl, 1mM EDTA(pH 8.0)
ALS-I	Tris-Cl 25mM (pH 8.0), EDTA 10 mM (pH 8.0), glucose 50mM
ALS-II	0.2N NaOH, 1% SDS
ALS-III	3M Potassium acetate, 5M glacial acetic acid
TE buffer	10mM Tris-Cl, 1mM EDTA (pH 8.0)

1) A single colony of *E. coli* was inoculated in 5 ml of LB broth with proper antibiotic. The culture was incubated overnight at 37 °C with vigorous shaking.
2) The 2 ml culture was centrifuged at maximum speed for 5 min in a 2 ml microfuge tube. Supernatant was discarded and again 2 ml culture was centrifuged.
3) The pellet was washed with 2ml of STE, vortexed & centrifuged at maximum speed for 5 min.
4) The supernatant was discarded & 250 µl of ice cold ALS-I was added to resuspend the pellet by vortexing.
5) An aliquot of 500 µl of ALS-II was added, mixed gently by inverting 5 times and the tube was stored on ice for 10min.
6) An aliquot of 375 µl ALS-III was added & mixed by inverting the tubes 5 times, tube was stored on ice for 5 min.
7) Centrifugation was done at maximum speed for 5 min, supernatant (~1 ml) was transferred to a fresh 1.5 ml microfuge tube, 0.6 volume of Isopropylalcohol (0.6 ml) was added to supernatant and mixed well. The tube was incubated at R.T. for 30 min.
8) Centrifugation was done at maximum speed for 10 min. The supernatant was carefully discarded, 1 ml of 70% alcohol was added, inverted gently 2 times, centrifuged for 2 min and finally the alcohol was removed carefully.
9) The microfuge tube was kept in an inverted position to completely remove last traces of alcohol. An aliquot of 80-100 µl TE or D/W was added and kept at 68°C on dry bath for 15 min to dissolve DNA. The efficiency of plasmid preparation was checked by agarose gel electrophoresis.

6.2.5 Elution by glass solution method:

DNA bands from agarose gels were eluted by glass solution method which was used further for ligation reaction.

Reagents & Composition

Reagent	Composition
Sodium iodide	6M
Glass solution	50% SiO_2
Wash Buffer	10m M Tris-Cl, 70% alcohol

1) DNA band of interest was excised from ethidium bromide stained agarose gel with a sharp blade. Gel piece was transferred to 2.0 ml microfuge tube and 2.5 volumes of 6 M sodium iodide solution was added. In case of solution containing DNA, 2.5 volumes of sodium iodide solution was added before proceeding to step 3.
2) To solubilize the gel, the solution was incubated at 45 °C – 55 °C for 10 min in water bath, the contents were mixed thoroughly and incubated for a further 5 min. The agarose gel piece was observed to be completely dissolved.
3) The glass solution was mixed by vortexing, until it formed a homogenous mixture. An aliquot of 15 µl of glass solution was added to the sample containing 5 µg or less of DNA. The contents were mixed thoroughly & kept at room temperature for 10 min and then on ice for 20 min with occasional mixing. This allowed adsorption of DNA molecules to the glass beads.
4) The tube was centrifuged at 5000 rpm for 1 min and the supernatant was discarded. DNA bound to the glass formed a hard pellet.

Note: If the DNA is very important, save the supernatant at this point. If in case the entire DNA has not bound to the glass solution, fresh glass solution can be added to the supernatant and the procedure may be repeated.

5) Wash buffer (200 µl per 15 µl of glass solution) was added, vortexed and centrifuged at 12000 rpm for 30 sec and the supernatant was discarded.

6) Step 5 was repeated twice. To remove traces of wash buffer after the final wash, the tube was kept at 37 °C for 10 min. Incubation was continued if smell of alcohol was observed.

7) For elution of DNA, 30-40 µl of water or 1X TE buffer was added to the pellet and the pellet was resuspended by mild vortexing and incubated at 45-55°C for 10 minutes. The lack of high concentration of salt facilitates DNA to release from the glass and elute in water or 1X TE buffer.

8) Centrifugation was done at 12000 rpm for 30 sec and the supernatant was collected in a fresh tube. A second elution (20 µl) helps in recovering DNA that is not eluted. The supernatant was pooled together. A final spin for a few seconds was done to remove traces of glass that might be present in the sample.

9) The efficiency of elution was checked on agarose gel. Generally the yield was more than 60-70%.

10) After elution the DNA was ready for ligation.

6.2.6 Concentration of DNA by alcohol precipitation:

Alcohol precipitation was done to concentrate the DNA from PCR reaction which was used further for the ligation reaction.

1) Around 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol was added to sample containing DNA in a microfuge tube. The mixture was incubated for 30 minutes at -20°C.

2) The precipitates of DNA were collected by centrifugation at 10, for 15 minutes at 4°C. The supernatant was discarded and pellet was washed with 70% alcohol. After centrifugation at 10,000 rpm for 5min supernatant was discarded and the tube was kept open on the bench for a few minutes to allow ethanol to evaporate.
3) The damp pellet of DNA was dissolved in a small volume (20-30 µl) of TE buffer (pH8.0). The mixture was incubated for 1 hour at room temperature.
4) Concentration of DNA was checked by agarose gel electrophoresis with molecular markers having known amount of DNA in bands.

6.2.7 Competent cell preparation:

The protocol as described by Sambrook and Russel 2001 was used for preparation of competent *E. coli* DH5α using $CaCl_2$ and $MgCl_2$ with an efficiency of 10^6 transformed colonies/µg of supercoiled plasmid DNA. A modification in mock transformation was done to achieve higher efficiency.

Reagents & Composition

Reagents	Composition
$MgCl_2$-$CaCl_2$ solution	80 mM $MgCl_2$, 20 mM $CaCl_2$
$CaCl_2$	0.1M

1) A single colony of *E. coli* DH5α was inoculated in 5 ml of sterile LB broth. The culture was incubated overnight at 37°C with vigorous shaking.
2) 1 ml of overnight grown culture was transferred in 100 ml sterile LB broth and incubated at 37°C with vigorous shaking till the OD_{600} reaches 0.4.

3) The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes (Sorval tubes). Culture was kept at 0°C for 10 min.

4) Cells were recovered by centrifuging at 4500 rpm for 10 min at 4°C.

5) The supernatant was decanted from cell pellet and tubes were placed in an inverted position in laminar air flow for 1 min to allow last traces of the media to drain away.

6) Pellet was resuspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution.

7) Cells were recovered by centrifuging at 4500 rpm for 10 min at 4°C.

8) Medium was decanted from cell pellet and tubes were placed in an inverted position for 1 min in laminar air flow to allow last traces of the media to drain away. Pellet from each tube was resuspended by swirling or gentle vortexing in 1 ml of 0.1 M $CaCl_2$ and 1ml of 40% glycerol. The cells were directly used for transformation for mock transformation.

9) Mock transformation was performed by adding aliquots of 5 µL, 10 µL, 15 µL and 20 µL of 0.1 M $CaCl_2$ to each of 200 µL cells along with 5 ng of supercoiled plasmid. The dilution yielding highest number of transformants was selected for this lot of cells.

10) Remained cells were kept at 16°C overnight. Next day cells were dispensed into aliquots of 200 µL and frozen at -80°C.

6.2.8 Transformation using $CaCl_2$:

1) DNA sample (plasmid or ligation reaction) was added to the 200 µL of cells and the contents mixed by swirling gently. The tube was stored on ice for 30 min.
2) The tube was transferred to a rack placed in preheated 42°C water bath and incubated exactly for 90 sec without shaking.
3) The tube was rapidly transferred to an ice bath for 2 min.
4) An aliquot of 800 µl of LB medium was added to the tube and incubated for 45 min at 37°C to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
5) Appropriate volume of transformed competent cells was plated onto LB plates with appropriate antibiotic and or X-GAL.
6) The plates were incubated at 37 °C for 12-16 hours.

6.2.9 PCR system and program for OE-PCR of *cry1Aa* and *cry9Aa* genes

PCR system

Buffer (10X)	3 µl
dNTPs (2mM)	2.8 µl
cry1Ac domain I	9 µl (150 ng)
cry9Aa domain II-III	15 µl (540 ng)
Pfu DNA Polymerase	0.5 µl (1.5U)
D/W	Up to 30 µl

PCR Program

Initial denaturation: 94°C for 5 min

30 cycles of PCR : 94°C for 30 sec

55°C for 20 sec

72°C for 2 min

Final extension : 72°C for 10 min

6.2.10 Ligation of hybrid genes in pET- 28a (+)

Ligation of hybrid gene *cry1Ac-cry9Aa* and *cry1Ac-cry9AaMod* was done using sites XhoI and NheI of pET28a (+). Following ligation system was set up at 22°C overnight on ligation bath.

Buffer	1.5 µl
Insert	11.5 µl (~160 ng)
Vector	1 µl (~50 ng)
Ligase	1 µl (10 units)
Total volume	Up to 15 µl

Ligation of hybrid gene *cry1Ac-cry9AaMod* was done with pET28a (+). Following ligation system was set up at 22°C overnight on ligation bath.

Buffer	1.5 µl
Insert	10 µl (160 ng)
Vector	1 µl (50 ng)
Ligase	1 µl
Total volume	Up to 15 µl

6.2.11 Acetone based co-precipitation method for spore crystal aggregate preparation

of *Btk HD-1*. (Dulmage et al. 1970).

100 ml (~1×10^{15} spores/ml) sporulated culture of *Btk HD-1* was centrifuged at 8000 RPM for 10 min. Pellet was resuspended in 9 ml 5 % lactose. Prepared suspension stirred till 30 min for even distribution.36 ml acetone added by gradually in 10 min interval. Mixer allowed to stand for 10 min. Subsequently, excess acetone filter out with suction pump. Residues of spore-crystals aggregate kept overnight at RT for drying.