Chapter 6

Materials and Methods
6.1 Cultures, media and plasmids:

6.1.1 *E. coli* strains harboring plasmid constructs:

1) *E. coli* ECE 52 (DH5α) with recombinant plasmid pTZ19R-cry1Aa
2) *E. coli* ECE 53 (JM103) with recombinant plasmid pKK223-3-cry1Ac
3) *E. coli* ECE 54 (JM103) with recombinant plasmid pKK223-3-cry1Ab
4) *E. coli* ECE 127 (DH5α) with recombinant plasmid pTZ19R-cry1Ea
5) *E. coli* ECE 129 (DH5α) with recombinant plasmid pTZ19R-cry1Da
6) *E. coli* ECE 128 (DH5α) with recombinant plasmid pTZ-cry1Ba

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

1) *E. coli* DH5α strain was used as cloning host with blue-white selection
2) *E. coli* BL21 was used as host for expression of hybrid toxins from pET-30a vector

6.1.3 *Bacillus* reference strains:

1) *Bacillus thuringiensis* subsp. *kurstaki* HD1
2) *Bacillus thuringiensis* subsp. *kurstaki* HD73
3) *Bacillus cereus* ATCC 1457,
4) *Bacillus megaterium* QMB1551
5) *Bacillus sphaericus* WHO 2297
6) *Bacillus subtilis* 1012M15

All *E. coli* and *Bacillus* cultures were obtained from the Bacillus Genetic Stock Centre (BGSC), Ohio State University, USA while *Bacillus licheniformis* K125 (Suthar et al, 2008) was kindly provided by Dr. Harish Suthar, M. S University of Baroda.

*Btk* HD1 and *Btk* HD73 were used as reference strains for colony morphology, phase contrast microscopy, Cry protein analysis by SDS-PAGE and PCR analysis for *cry1* and *cry2* genes.
6.1.4 Culture growth conditions and maintenance:

*E. coli* strains were grown at 37°C overnight on plates or with 200 rpm rotary shaking condition in Luria-Bertani broth. *Bt* reference strain and *Bt* isolates were grown at 30°C 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 with ampicillin (100 ug/ml). Sub-culturing of each culture was performed every month. Reference *Bt* strains and *Bt* isolates 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 -20°C deep freezer overnight and next day transferred to -80°C for long term storage.

6.1.5 Insect sources and rearing

*H. armigera* and *S. spodoptera* eggs were kindly supplied by Mr. Hiren Patel, Anand Agriculture University, Anand, Gujarat, India. Eggs were kept in a jar tightly closed with a cloth. Larvae after hatching from eggs were provided with fresh castor leaves to *S. spodoptera* and cabbage leaves to *H. armigera*. *S. spodoptera* larvae after growing to 2nd instar were kept as five insects in one vial and *H. armigera* larvae were kept as single insect in one vial. Insect bioassay with the larvae was performed in the same vials by feeding them *Bt* isolates or Cry protein spread leaves.

6.1.6 Media:

Luria-Bertani medium (LB):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5%</td>
</tr>
<tr>
<td>pH</td>
<td>7.9</td>
</tr>
</tbody>
</table>

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LB plates were prepared by adding LB and agar powder at 2% 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 °C. 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 50ug/ml were used as per requirements.

**GYS (Glucose Yeast Extract) medium**

- Glucose: 0.1 gm%
- Yeast extract: 0.2 gm%
- K$_2$HPO$_4$: 0.5 gm%
- Ammonium sulfate: 0.2 gm%
- MgSO$_4$.7H$_2$O: 0.02 gm%
- MnSO$_4$.5H$_2$O: 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$_2$HPO$_4$ were added to medium with glucose and yeast extract, pH adjusted to 7.2 and autoclaved at 10 p.s.i. for 20 min. The K$_2$HPO$_4$ 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: 50 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°C.
6.1.7 Plasmids:
- pTZ57R/T vector: TA cloning of PCR products with blue/white selection (Fermentas, USA, Catalog no. #K1213)
- pET-30a vector: Expression vector with T7 promoter and IPTG based induction (Novagen, USA, Catalog no. 69909-3)
Plasmid preparations were stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at -20°C.

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 bromophenol 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 H₂O.
> SDS (10%): Dissolved 1 gm of SDS in 10 ml distilled H₂O.

### Composition for SDS- PAGE

<table>
<thead>
<tr>
<th>Components</th>
<th>Resolving gel (10%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>2.8 ml</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>2.31 ml</td>
<td>0.33 ml</td>
</tr>
<tr>
<td>1.5M tris Cl (pH 8.8)</td>
<td>1.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>1M tris Cl (pH 6.8)</td>
<td>-</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>7.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>7.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.8 µl</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>

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. In case of RFLP analysis, post staining of gels was performed by gentle shaking with ethidium bromide 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 (\( \lambda = 254 \text{ nm} \)) and photographed by AlphaEase gel documentation system.

6.2.3 Vegetative whole cell protein profile by SDS-PAGE

1) 4ml of log phase culture (0.8-1.0 Absorbance unit at 600 nm) was transferred in sterile 2.0 ml microfuge tube.
2) Cells were pelleted down by centrifugation at 8000 RPM for 3 min and supernatant was discarded.
3) 50 µl of sterile D/W was added in the tube and mixed by vortex.
4) 10 µl of 0.5 M NaOH was added and incubated at RT up to 10 min for lysis of vegetative cells.
5) Centrifuged at 10,000 rpm for 2 min.
6) 10 µl 6X SDS loading buffer was added, mixed well and centrifuged again at 8000 RPM for 3 min.
7) Boiled for 2 min and 20 µl of sample was loaded onto 10% SDS PAGE gel and electrophoresis was carried out.

6.2.4 TA cloning

Ligation of PCR amplification products or eluted DNA fragments was done using TA cloning kit, catalog number #K1213, Fermentas, USA. 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.

**Ligation system**

<table>
<thead>
<tr>
<th>Buffer (10X)</th>
<th>2.0µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligase</td>
<td>1.0µL</td>
</tr>
<tr>
<td>T vector</td>
<td>2.0µL</td>
</tr>
<tr>
<td>PCR product</td>
<td>2.0µL</td>
</tr>
<tr>
<td>D/W</td>
<td>Upto 20µL</td>
</tr>
</tbody>
</table>
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6.2.5 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

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE buffer</td>
<td>Tris-Cl 10 mM (pH 8.0), 0.1M NaCl, 1mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>ALS-I</td>
<td>Tris-Cl 25mM (pH 8.0), EDTA 10 mM (pH 8.0), glucose 50mM</td>
</tr>
<tr>
<td>ALS-II</td>
<td>0.2N NaOH, 1% SDS</td>
</tr>
<tr>
<td>ALS-III</td>
<td>3M Potassium acetate, 5M glacial acetic acid</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10mM Tris-Cl, 1mM EDTA (pH 8.0)</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium iodide</td>
<td>6M</td>
</tr>
<tr>
<td>Glass solution</td>
<td>50% SiO₂</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>10m M Tris-Cl, 70% alcohol</td>
</tr>
</tbody>
</table>

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
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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.

6.2.7 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.

6.2.8 Competent cell preparation:
The protocol as described by Sambrook and Russel 2001 was used for preparation of competent *E. coli* DH5α using CaCl₂ and MgCl₂ with an
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efficiency of $10^6$ transformed colonies/µg of supercoiled plasmid DNA. A modification in mock transformation was done to achieve higher efficiency.

### Reagents & Composition

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂-CaCl₂ solution</td>
<td>80 mM MgCl₂, 20 mM CaCl₂</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1M</td>
</tr>
</tbody>
</table>

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 MgCl₂-CaCl₂ 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 CaCl₂ 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 CaCl₂ 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.

6.2.9 Transformation using CaCl₂:

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.
### 6.3 PCR systems and programs

#### 6.3.1 PCR systems and programs for various cry genes amplification

<table>
<thead>
<tr>
<th>Bt isolates</th>
<th>cry1</th>
<th>cry2</th>
<th>cry3, 7, 8</th>
<th>cry5, 12, 14, 21</th>
<th>cry11</th>
<th>cry13</th>
<th>cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10X)</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
</tr>
<tr>
<td>Primer F (2 µM)</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
</tr>
<tr>
<td>Primer R (2 µM)</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
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</tr>
<tr>
<td>dNTPs (250 µM each)</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
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</tr>
<tr>
<td>Taq DNA</td>
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<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Polymerase (3 U/µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total DNA</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
</tr>
<tr>
<td>Sterile D/W</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
</tr>
</tbody>
</table>

**PCR program**

<table>
<thead>
<tr>
<th>First denaturation</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
<th>95 °C for 4 min</th>
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</thead>
<tbody>
<tr>
<td>PCR for 30 cycles:</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
<tr>
<td>Ta at 30 sec</td>
<td>Ta = 49.5 °C</td>
<td>Ta = 50 °C</td>
<td>Ta = 50 °C</td>
<td>Ta = 51 °C</td>
<td>Ta = 51 °C</td>
<td>Ta = 47 °C</td>
<td>Ta = 55 °C</td>
</tr>
<tr>
<td>72 °C for Ta</td>
<td>Ts = 25 sec</td>
<td>Ts = 1 min 40 sec</td>
<td>Ts = 50 sec</td>
<td>Ts = 40 sec</td>
<td>Ts = 25 sec</td>
<td>Ts = 40 sec</td>
<td>Ts = 40 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Note: Ta = Annealing temperature, Ts = Annealing time
### 6.3.2 PCR systems and programs for domain coding region amplification

<table>
<thead>
<tr>
<th>Genes/PCR system and program</th>
<th><em>cryIAc</em> domain I</th>
<th><em>cryIAc</em> domain II-III</th>
<th><em>cryIAb</em> domain II-III single strands</th>
<th><em>cryIAc</em> domain II-III</th>
<th><em>cryIAc</em> domain I-III single strands</th>
<th><em>cry1Ea</em> domain III</th>
<th><em>cry1Ea</em> deg II-III F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR systems</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
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<tr>
<td>Primer F (2 µM)</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
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</tr>
<tr>
<td>Primer R (2 µM)</td>
<td>3 µL</td>
<td></td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
<td>3 µL</td>
</tr>
<tr>
<td>dNTPs (250 µM, each)</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td><em>Pfu</em> DNA</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Polymerase (3 U/µL)</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~25 ng</td>
<td>~5 ng</td>
<td>~5 ng</td>
<td>~25 ng</td>
</tr>
<tr>
<td>Template DNA</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
</tr>
<tr>
<td>Sterile D/W</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
<td>Up to 30 µL</td>
</tr>
<tr>
<td><strong>PCR program</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First denaturation</td>
<td>95 °C for 4 min</td>
<td>95 °C for 1 min</td>
<td>95 °C for 4 min</td>
<td>95 °C for 1 min</td>
<td>95 °C for 4 min</td>
<td>95 °C for 1 min</td>
<td>95 °C for 4 min</td>
</tr>
<tr>
<td>30 cycles:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing step</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
<tr>
<td>72°C for Time</td>
<td>1 min</td>
<td>1 min</td>
<td>3 min</td>
<td>3 min</td>
<td>1.5 min</td>
<td>1.5 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
</tr>
</tbody>
</table>
6.3.3 PCR system and program for ssOE-PCR of *cry1Ac* and *cry1Ea* genes

<table>
<thead>
<tr>
<th>PCR system</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer (10X)</strong></td>
<td>Initial denaturation 94°C for 2min</td>
</tr>
<tr>
<td><strong>cry1Ea Domain III</strong></td>
<td><strong>PCR for 15 Cycles</strong></td>
</tr>
<tr>
<td>8 µl (300 ng)</td>
<td><strong>Denaturation</strong> 94°C for 30sec</td>
</tr>
<tr>
<td><strong>cry1Ac Domain I-II</strong></td>
<td><strong>Annealing</strong> 50°C for 30sec</td>
</tr>
<tr>
<td>4 µl (200 ng)</td>
<td><strong>Extension</strong> 72°C for 3 min</td>
</tr>
<tr>
<td><strong>dNTPs mix</strong></td>
<td><strong>Final extension</strong> 72°C for 10min</td>
</tr>
<tr>
<td>1.5 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Pfu DNA polymerase</strong></td>
<td></td>
</tr>
<tr>
<td>0.5 µl (1.5 U)</td>
<td></td>
</tr>
<tr>
<td><strong>D/W</strong></td>
<td>Up to 30 µl</td>
</tr>
</tbody>
</table>

6.3.4 PCR system and program for ssOE-PCR of *cry1Ac* and *cry1Ab* genes

<table>
<thead>
<tr>
<th>PCR system</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer (10X)</strong></td>
<td>Initial denaturation 94°C for 5min</td>
</tr>
<tr>
<td><strong>cry1Ab domain II-III</strong></td>
<td><strong>PCR for 15 cycles</strong></td>
</tr>
<tr>
<td>5 µl (540 ng)</td>
<td><strong>Denaturation</strong> 94°C for 30sec</td>
</tr>
<tr>
<td><strong>cry1Ac domain I</strong></td>
<td><strong>Annealing</strong> 55°C for 45sec</td>
</tr>
<tr>
<td>3 µl (150 ng)</td>
<td><strong>Extension</strong> 72°C for 4 min &amp; 30 sec</td>
</tr>
<tr>
<td><strong>dNTPs mix</strong></td>
<td><strong>Final extension</strong> 72°C for 5min</td>
</tr>
<tr>
<td>1.5 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Pfu DNA polymerase</strong></td>
<td></td>
</tr>
<tr>
<td>0.5 µl (1.5U)</td>
<td></td>
</tr>
<tr>
<td><strong>D/W</strong></td>
<td>Up to 30 µl</td>
</tr>
</tbody>
</table>
6.3.5 PCR system and program for traditional OE-PCR of cryIAc and cryIAb genes

**PCR system**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10X)</td>
<td>3 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1.8 µl</td>
</tr>
<tr>
<td>cryIAc domain I</td>
<td>9 µl (150 ng)</td>
</tr>
<tr>
<td>cryIAb domain II-III</td>
<td>15 µl (540 ng)</td>
</tr>
<tr>
<td>Pfu DNA Polymerase</td>
<td>0.5 µl (1.5U)</td>
</tr>
<tr>
<td>D/W</td>
<td>Up to 30 µl</td>
</tr>
</tbody>
</table>

**PCR Program**

Initial denaturation: 94°C for 5 min

30 cycles of PCR: 94°C for 30 sec

50°C for 45 sec

72°C for 4 min

Final extension: 72°C for 10 min

6.4 Ligation of hybrid genes with pET30a vector:

Ligation of hybrid gene cryIAcAcEa was done using Bam HI and Sal I pET30a. Following ligation system was set up at 22°C overnight on ligation bath.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Insert</td>
<td>11.5 µl (~160 ng)</td>
</tr>
<tr>
<td>Vector</td>
<td>1 µl (~50 ng)</td>
</tr>
<tr>
<td>Ligase</td>
<td>1 µl (10 units)</td>
</tr>
<tr>
<td>Total volume</td>
<td>Up to 15 µl</td>
</tr>
</tbody>
</table>
Chapter 6: Materials and Methods

Ligation of hybrid gene cry1AcAbAb was done with pET30a. Following ligation system was set up at 22°C overnight on ligation bath.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>1.5 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert</td>
<td>10 µl (160 ng)</td>
</tr>
<tr>
<td>Vector</td>
<td>1 µl (50 ng)</td>
</tr>
<tr>
<td>Ligase</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>Up to 15 µl</td>
</tr>
</tbody>
</table>

6.5 Colony PCR

In order to allow rapid screening of E. coli DH5α clones and E. coli BL21 transformants, colony PCR was carried out. Half of a colony directly from the plate was picked by sterile toothpick and mixed with the PCR reaction. PCR amplification was performed and reaction was checked by agarose electrophoresis to observe the expected band and confirm the clone or transformant.

6.6 Sequencing of PCR amplification product

For sequencing purpose, cloning of partial gene amplification product was performed by InsTA clone PCR cloning kit, Fermentas, as per the manufacturer's instruction. PCR amplification product without elution was directly used in ligation reaction as followed.

<table>
<thead>
<tr>
<th>Ligation system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation buffer</td>
</tr>
<tr>
<td>Vector</td>
</tr>
<tr>
<td>(pTZ57R/T)</td>
</tr>
<tr>
<td>Insert</td>
</tr>
<tr>
<td>Ligase enzyme</td>
</tr>
<tr>
<td>Sterile D/W</td>
</tr>
</tbody>
</table>
Ligation reaction after through mixing was kept at 22°C overnight, followed by transformation using 10 µL 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. Plasmid extraction from white colonies was carried out, confirmed by restriction analysis with Bam HI and sent for sequencing. Sequencing service was provided by Bangalore Genei Pvt. Ltd., Bangalore, India.