MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Expression of shrimp defense proteins genes

3.1.1 Collection of experimental animals

Healthy subadult *P. monodon* (approximately 10 weeks old) weighing 18-20 g were purchased from the local farm and acclimatized in tanks containing sea water with 30 ppt salinity under continuous aeration. Natural seawater collected from Arabian sea, near Someshwar, Mangalore was allowed to settle to remove sand and other suspended particles and then the seawater passed through sand filter, treated with ozone was used for the experiments. The animals were fed with pelleted feed (CP feed, Thailand). Temperature and pH were recorded daily. Salinity was measured using salinometer (Exma, Japan).

3.1.2 Preparation of bacterial culture for experimental infection

After one week of acclimatization, these shrimps were challenged with inactivated *Vibrio harveyi*. A virulent strain of *V. harveyi* isolated from moribund *P. monodon* larvae (Karunasagar *et al.*, 1994) and maintained in our culture collection in glycerol broth at -80 °C, was grown in Tryptone Soya Broth with 1 % NaCl (TSBS) and an aliquot was used for viable count determination by plating serial dilutions ranging from $10^{-4}$-10$^{-7}$ on Tryptone Soya Agar (TSA) with 1 % NaCl. After drawing out the sample for enumeration, the remaining *V. harveyi* culture was inactivated by heating at 60 °C for 1h and one ml aliquots were centrifuged at 10,000 x g for 5 min. The pellet was resuspended in 1 ml of glycerol broth and stored at -80 °C until use.

**Glycerol broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>70 ml</td>
</tr>
</tbody>
</table>

The medium was autoclaved at 110 °C for 10 min at 10 lb pressure.

**Tryptone Soya Agar (TSA)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>
Sodium chloride 1.0 g  
K$_2$HPO$_4$ 0.25 g  
Agar 2.0 g  
Distilled water 100 ml  

The medium was autoclaved at 121 °C for 15 min at 15 lb pressure.

3.1.3 Experimental inoculation of shrimps

Aliquot of inactivated *V. harveyi* culture stored at -80 °C was removed, pelleted by centrifugation and resuspended in Phosphate Buffer Saline (PBS, pH 7.4) to get final cell density of 10$^8$/ml. 200 µl of this suspension was injected intramuscularly between 3$^{rd}$ and 4$^{th}$ abdominal segment of the shrimp.

**Phosphate buffered saline**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>10 mM</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

3.1.4 Hemolymph collection

After 18 hrs post infection, haemolymph was collected using insulin syringe preloaded with one-tenth volume of anticoagulant (10% sodium citrate, pH 7) (Destoumieux-Garzon et al., 2001). This haemolymph was stored at -80 °C until use.

3.1.5 Extraction of total RNA from hemolymph

Total RNA from hemocytes was extracted using Trizol LS reagent (Invitrogen, USA) with minor modifications. Five hundred microliter of hemolymph was centrifuged at 2000 x g for 10 min and supernatant discarded. The resulting hemocytes pellet was suspended in 750 µl of Trizol LS reagent and volume was adjusted to 1 ml with diethyl pyrocarbonate (DEPC) treated water followed by incubation at room temperature (RT) (25 ± 2 °C) for 5 min. Then, 200 µl of chloroform was added, mixed by vigorous shaking by hand for 15 sec and incubated for 15 min at room temperature. The sample was then centrifuged at 12,000 x g for 15 min at 4 °C. The upper aqueous phase containing RNA was transferred to a fresh RNase free tube; 0.5 ml of isopropyl alcohol was added and incubated for 10 min at RT to precipitate the total RNA. The RNA precipitate was sedimented by centrifugation at 12,000 x g for 10 min at 4 °C. The
supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol by mixing and centrifuging at 7,500 x g for 5 min at 4 °C. After discarding the supernatant, the pellet dissolved in 20 µl of RNase free water and stored at -80 °C.

### 3.1.5.1 Quantification of RNA

Two microliters of the RNA sample were diluted to 1000 µl with MilliQ water (Millipore, USA) and the absorbance read at 260 and 280 nm. The RNA concentration was calculated as follows.

\[
RNA (\mu g/ml) = A_{260} \times D \times 40
\]

where,

- \( A_{260} \) - Absorbance at 260 nm
- \( D \) - Dilution factor
- 40 - Extinction coefficient of RNA

The purity of RNA was measured by calculating the ratio of \( A_{260} \) and \( A_{280} \). A ratio of 2 ± 0.15 was considered acceptable for further analysis.

### 3.1.6 cDNA synthesis of gene of interest

#### 3.1.6.1 Designing of oligonucleotide primers for various shrimp defense proteins

Nucleotide sequences encoding various shrimp defense proteins namely, lysozyme (LZ), ferritin (FER) and Intracellular fatty acid-binding protein (IFA) were retrieved from GenBank database. Primers were synthesized by Ms. Bioserve Biotechnologies (Hyderabad, India). For Histone (H2A), degenerate primers were synthesized with the help of FastPCR programme (www.primerdigital.com/index.php?page=36) from available partial N-terminal protein sequence of *Litopenaeus vannamei* H2A in GenBank database. Nucleotide/protein sequences for various shrimp defense proteins with their GenBank accession number, primer sequence, annealing temperature, expected amplicon sizes and cloning vector used for cloning the PCR products are given in Table 1.
Table 1. Primers used, annealing temperature, expected PCR product size and vector used for cloning the genes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>GenBank Accession number</th>
<th>PCR product size (bp)</th>
<th>Anneling temperature (ºC)</th>
<th>Vector used</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZ</td>
<td>AF425673</td>
<td>477</td>
<td>48</td>
<td>pEXP5-NT TOPO TA expression vector</td>
<td>F 5’ ATGAGGATCTTTCTCTTGGG 3’ R 5’ CTAGAATTCTGAAGACAGATTTT 3’</td>
</tr>
<tr>
<td>FER</td>
<td>AY955373</td>
<td>513</td>
<td>56</td>
<td>pQE30 UA expression vector</td>
<td>F 5’ ATGGGGATCTAAGTCCGCCAG 3’ R 5’ TTAGTGGAAATCTTATCAAC 3’</td>
</tr>
<tr>
<td>H2A</td>
<td>Q6PV61</td>
<td>369</td>
<td>47</td>
<td>pQE30 UA expression vector</td>
<td>F 5’ ATGTCNGGNGCNGGGAARGGGNGG 3’ R 5’ YTYYTTYTCNGTYTTYTNGG 3’</td>
</tr>
<tr>
<td>IFA</td>
<td>DQ459988</td>
<td>411</td>
<td>48</td>
<td>pQE30 UA expression vector</td>
<td>F 5’ ATGGCCAAGATCGAAGGAAG 3’ R 5’ TTATTCTAAACGGAGTAAAC 3’</td>
</tr>
</tbody>
</table>

3.1.6.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed to obtain single stranded complimentary DNA (cDNA) as per the protocol recommended by Fermentas Life Sciences (Fermentas International Inc., Canada) with minor modifications. Briefly, 9.5 µl of RNA was mixed with 2 µl of oligo d(T)18 primer, incubated at 70°C for 10 min and chilled at ice. Following this, 4 µl of 5X reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl2, 50 mM DTT), 1 µl of di-thiothreitol (DTT), 2 µl of 10 mM dNTP mix, 20 units of ribonuclease inhibitor (Fermentas Life Sciences, Canada), 100 units of Revert AidTM H Minus M-MuLV reverse transcriptase (Fermentas, Canada) were added. Final volume was adjusted to 20 µl by adding DEPC treated RNase free water. The reaction mixture was incubated at 42°C for 60 min followed by heating at 70°C for 10 min to inactivate reverse transcriptase and stored at -20°C until use.

The cDNA was subjected to PCR amplification using gene specific forward and reverse primers. The amplification of the shrimp protein genes were performed in a programmable thermocycler (M J Research, USA). PCR was carried out in 30 µl reaction mixture containing 2.0 µl template DNA, 10×assay buffer (10 mM Tris-HCl, pH 9.0; 1.5 mM MgCl2, 50 mM KCl, 0.01% Gelatin), 200 µM of each deoxyribonucleotide triphosphates, 10 pmol of each of forward and reverse primers and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore).
The optimized PCR programme consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at respective annealing temperatures shown in Table 1, 1 min extension at 72°C. The final extension was performed at 72°C for 10 min.

The PCR products were detected by agarose gel electrophoresis. 1.6 % (w/v) agarose gels were prepared in 1× TAE buffer. The molten agarose was cooled to below 65°C, ethidium bromide was added to a final concentration of 0.5 µg/ml, mixed and poured to gel mould and allowed to set. 10 µl of the PCR products were mixed with 4 µl of 6× loading buffer and loaded into the wells. 100 bp DNA ladder (Bangalore Genei, Bangalore) was used as a molecular weight marker. Electrophoresis was carried out at 100-120 V and the bands were visualized under UV transilluminator (Herolab, Germany).

**TAE (Tris acetate EDTA) buffer (50×)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57 ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The solution was made up to a final volume of 1 liter with distilled water. The working solution (1000 ml of 1× TAE) was prepared by diluting 20 ml of 50× stock solution to 1000 ml with distilled water.

**Sample loading buffer (6x)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol blue</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Ethidium bromide (Sigma, U.S.A.)**

A stock solution was prepared by dissolving 5 mg of ethidium bromide in 1 ml of distilled water. The solution was either added to agarose gel during preparation or to the appropriate buffer (1×TAE buffer) to yield a final concentration of 0.5 µg/ml. In the latter case, the gel was allowed to stain in the solution for 20 min and destained in distilled water for 10 min to remove excess stain.
Healthy shrimp (*Penaeus monodon*).

Experimental infection and hemolymph collection

RNA extraction and reverse transcription

PCR amplification of desired genes by gene specific primer

Purification of PCR product

Ligation of purified PCR product in cloning vector

- **pQE30 UA vector**
  - (FER, H2A, IFA)
  - Transformation into *SG13009* competent cells
  - Screening of positive clone by PCR (gene specific primer)
  - Plasmid extraction of positive clone
  - Transformation into *BL21* competent cells
  - Expression of recombinant protein
  - Purification of recombinant protein

- **pEXP5-NT/TOPO TA vector**
  - (LZ)
  - Transformation into *TOP10* competent cells
  - Screening of positive clone by PCR (gene specific primer)

**Flow chart 1: Cloning, expression and purification of shrimp defense proteins**
3.1.7 Cloning of shrimp defense protein genes

3.1.7.1 Purification of PCR products

All the genes were PCR amplified in bulk (100 µl) using the primers and conditions as mentioned above. The PCR products were purified before ligation to remove contaminants like Taq DNA polymerase, primer dimers, remaining dNTPs which may interfere with subsequent processes, using QIAquick PCR purification kit (Qiagen).

To one volume of the PCR product, 5 volume of buffer PB was added, mixed and then transferred to QIAquick spin column placed in a 2 ml collection tube. The flow through was discarded after centrifugation at 10,000 x g for 1 min, washed by adding 0.75 ml buffer PE containing ethanol followed by centrifugation at 10,000 x g for 1 min. The DNA was eluted by adding 50 µl of elution buffer (10 mM Tris-Cl, pH 8.5) or distilled water to the QIAquick column placed in a fresh microcentrifuge tube and collected by centrifuging the column at 10,000 x g for 1 min (Fig 1).

Fig. 1. Purification of PCR products using QIAquick spin column
3.1.7.2 Ligation of PCR products into the expression vectors

The ligation reactions using different expression vectors were carried out according to the manufacturer’s protocols. Two expression vectors were used; pEXP5-NT/TOPO TA (Fig. 2a, Invitrogen, USA) and pQE30 UA (Fig. 2b, Qiagen, USA).

![Vectors used in this study. a. pEXP5-NT/TOPO TA linearized vector (Invitrogen, USA); b. pQE30 UA linearized vector (Qiagen, USA)](image)

For Invitrogen vector (pEXP5-NT/TOPO TA linearized plasmid DNA), ligation was performed using 4 µl fresh amplified DNA and 1 µl salt solution. One microlitre of vector (5-10 ng/µl) was added, mixed gently and incubated for 5-10 min at room temperature (22-23 °C).
For Qiagen vector (pQE30 UA linearized plasmid DNA), ligation was performed using 4 µl purified PCR product and 1 µl vector (50 ng/µl). Five microlitres of 2× ligation master mix was added, mixed briefly and incubated the tube at 16 ºC in a thermal cycler block for 2 hr.

3.1.7.3 Transformation

The ligation mix was transferred to tube containing 150 µl competent cells. In the case of pEXP5-NT/TOPO TA vector, *Escherichia coli* Top10 competent cells (supplied with the kit) were used, while in the case of pQE30 UA vector, *E. coli* SG13009/M15 competent cells were used. The contents were mixed by gentle tapping and incubated on ice for 30 min. The cells were subjected to heat shock at 42 ºC for 90 sec in a water bath. The tubes were then rapidly transferred to ice and allowed to chill for 1-2 min. 500 µl of LB broth was added to the tubes and incubated at 37 ºC for 1hr with vigorous shaking. The cells were centrifuged at 4000 x g for 10 min. After discarding the 300 µl supernatant, transformed cells were resuspended in remaining 200 µl broth and plated on two LB plates (100 µl/plate) containing antibiotics (100 µg/ml ampicillin and 25 µg/ml kanamycin for SG13009/M15 cells containing recombinant vector; 100 µg/ml ampicillin for Top10 cells containing recombinant vector). The plates were then incubated overnight at 37 ºC.

3.1.7.3.1 Preparation of competent cells

The procedure described by the suppliers (Qiagen, USA) was followed to prepare the competent cells. Aliquots of *E. coli* strains, SG13009 and BL21 were removed from the supplied vial with a sterile tooth pick, streaked on LB agar containing antibiotics (25 µg/ml kanamycin for SG13009 strain and 34 µg/ml chloramphenicol for BL21 strain) and incubated overnight at 37 ºC. Then single colony was inoculated into 10 ml of LB broth containing respective antibiotic and grown overnight at 37 ºC with constant shaking at 150 rpm. The following day, 1 ml of overnight grown culture was transferred into 100 ml of LB broth containing respective antibiotics and incubated for 90-120 min with vigorous shaking at 200 rpm at 37 ºC. Optical density at 600 nm (OD$_{600}$) was measured after every 30 min. When OD$_{600}$ reached 0.4-0.5, culture was chilled on ice, transferred to pre-chilled 50 ml polypropylene tubes and centrifuged at 4000 x g for 10 min using cooling centrifuge (Hearus, Germany). The supernatant was discarded and the cells pellet was suspended gently without damaging the cells in 30 ml of ice cold TFB1 buffer and the suspension was kept on ice for an additional 90 min and centrifuged at 2500 rpm at 4 ºC for 10 min. The supernatant was discarded carefully and the
cell pellet was resuspended in 4 ml of ice cold TFB2 buffer. About 150 µl of suspension of competent cells was aliquoted in 1.5 ml microfuge tubes, frozen and stored at -80 °C.

**Luria-Bertanii (LB) broth (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10</td>
</tr>
</tbody>
</table>

2.5 g of LB broth was dissolved in 100 ml of distilled water and sterilized at 121 °C for 15 min. To the cooled LB broth, antibiotics were added from the stock solutions to get the desired concentrations.

**Luria-Bertanii (LB) agar (Hi Media, Mumbai)**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
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<tbody>
<tr>
<td>Tryptone</td>
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</tr>
<tr>
<td>Yeast extract</td>
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<tr>
<td>Sodium chloride</td>
<td>10</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

4 g of LB agar was dissolved in 100 ml of distilled water and sterilized at 121 °C for 15 min. Then it was cooled to 50°C and antibiotics were added from the stock solutions to get the desired concentrations.

**Kanamycin stock solution (25 mg/ml)**

Stock solution was prepared by dissolving 0.0385 g of kanamycin powder (HiMedia, Mumbai) having the assay potency of 650 µg/mg in 1ml distilled water, filter sterilized and stored in aliquots at -20°C.

**Chloramphenicol stock solution (34 mg/ml)**

Stock solution was prepared by dissolving 0.0377 g of chloramphenicol powder (HiMedia, Mumbai) having the 90% purity in 1ml ice cold ethanol and stored in aliquots at -20°C.

**TFB1 buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RbCl</td>
<td>100 mM</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>50 mM</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>30 mM</td>
</tr>
</tbody>
</table>
Calcium chloride 10 mM
Glycerol 15%
pH 5.8

All the chemicals used were of molecular biology grade. pH was adjusted carefully to avoid formation of insoluble manganese precipitate. After adjusting the pH, the buffer was filter sterilized and stored at 4°C.

**TFB2 buffer**

- MOPS 10 mM
- RbCl 10 mM
- Calcium chloride 75 mM
- Glycerol 15%
pH 6.8

After adjusting the pH, the buffer was filter sterilized and stored at 4°C.

3.1.7.4 Screening of transformants

Transformants were randomly selected and screened for the presence of insert by preparing crude lysate of DNA (Dileep et al., 2003). Briefly, each of the selected colonies was inoculated to 2 ml of LB broth and incubated overnight at 37°C. To 50 µl of the culture, 450 µl of TE buffer was added and cells were lysed at 100°C for 10 min, snap chilled, centrifuged briefly for 5 min and 2 µl of supernatant was used as template for PCR using gene specific primers. Glycerol stock (30%) were made for all the positive clones and stored at -80°C.

3.1.7.4.1 Confirmation of the recombinant constructs

The orientation of the insert of the cloned shrimp protein genes were confirmed by using vector specific forward primers and gene specific reverse primer.

3.1.7.4.2 Sequencing of recombinant clones

Recombinant clones having genes of interest were selected and sequencing was done by Ms. Bangalore Genei, Bangalore/Ms. Bioserve Biotechnologies, Hyderabad.

3.1.7.5 Expression of the recombinant proteins

The recombinant gene constructs containing gene for LZ was maintained in *E. coli* Top10 hosts. For expression studies, plasmids were extracted from these cells and transformed
to *E. coli* BL21 (DE3) pLysS. Recombinant gene constructs FER, H2A, and IFA were maintained and expressed in *E. coli* SG13009/M15 host cells.

To ascertain the expression, each of the positive clones was inoculated into 5 ml of LB medium containing antibiotics (100 µg/ml ampicillin and 34 µg/ml chloramphenicol for *E. coli* BL21(DE3) pLysS; 100 µg/ml ampicillin and 25 µg/ml kanamycin for *E. coli* SG13009 cells). Cultures were grown overnight at 37°C with constant agitation at 150 rpm. One ml of overnight cultures were inoculated into 10 ml of LB broth with specific antibiotics and incubated at 37°C with constant shaking until the OD$_{600}$ was 0.5-0.7. The cultures were induced with different concentrations of IPTG (1mM, 1.5 mM, 2 mM, 2.5 mM and 3 mM) and further incubated for 4 hr with the same conditions. Uninduced recombinant clone and *E. coli* strains (SG13009 and BL21 cells) were used as control. After incubation, 1 ml culture from each sample was centrifuged at 11,000 x g for 3 min and the supernatant was discarded. The pellet was resuspended in 30 µl distilled water and 30 µl of 2x sample buffer by continuous vortexing, lysed at 95°C for 5-10 min in a dry bath. Then, samples were loaded to the polyacrylamide gel for electrophoresis.

**Ampicillin stock solution (100 mg/ml)**

Stock solution was prepared by dissolving 0.1089 g of ampicillin powder (HiMedia, Mumbai) having 98% purity in 1 ml distilled water, filter sterilized and stored in aliquots at -20°C.

**Isopropylthio-β-D-galactoside (IPTG, 1 M)**

Stock solution was prepared by dissolving 2.3 g of IPTG in 10 ml of distilled water, filter sterilized and aliquots were stored at -20°C.

### 3.1.7.5.1 Plasmid DNA extraction

#### 3.1.7.5.1.1 Reagents supplied with kit (Eppendorf Fast Plasmid Mini Kit)

Lysis solution, RNase solution, Lysozyme, Wash buffer concentrate, Elution buffer and Fast Plasmid Spin Column Assembly.

#### 3.1.7.5.1.2 Plasmid extraction protocol

Young bacterial cultures at the logarithmic growth phase were used for plasmid DNA extraction. 1.5 ml of bacterial culture was centrifuged to sediment the cells to the bottom of the microfuge tube. The clear supernatant was discarded and to the pellet, 400 µl of ice cold
complete lysis solution was added. The pellet was mixed thoroughly and incubated at room temperature for 3 min. Subsequently, the lysate was transferred to the spin column assembly and centrifuged at maximum speed for 60 sec. 400 µl of diluted wash buffer was added to the spin column and centrifuged for 1 min. For elution, 50 µl of elution buffer was added to the spin column placed in a collection tube and centrifuged. The purified plasmid was collected in the collection tube and stored (Fig 3).
1.5 ml Bacterial Culture

Centrifuge for 1 minute to pellet the cells

Decant the media
Add 400 µl of ice-cold complete lysis solution
Vortex for a full 30 seconds at the highest setting

Incubate at room temperature for 3 minutes

Transfer the lysate to a Spin Column Assembly

Centrifuge for 30–60 seconds
Wash DNA with 400 µl of diluted wash buffer

Centrifuge for 30-60 seconds

Decant the filtrate from the waste tube and re-assemble the Spin Column Assembly

Centrifuge for 1 minute
Add 50 µl of Elution Buffer to the center of the Spin Column
Transfer the Spin Column into a collection tube

Centrifuge for 30-60 seconds

Purified Plasmid DNA

Fig. 3. Plasmid purification using Eppendorf fast plasmid mini kit
3.7.5.6 SDS-polyacrylamide gel electrophoresis

Recombinant proteins expressed in *E. coli* were analysed by the method of Laemmli (1970) using SDS-PAGE with some modifications. The various buffers and reagents used are as follows:

a) Acrylamide-bisacrylamide mixture

Twenty nine grams of acrylamide and 1.0 g of bisacrylamide (N, N’-methylene bisacrylamide) were dissolved in 80 ml of distilled water. The volume was made up to 100 ml and stored at 4°C.

b) Separating gel buffer (1.5 M Tris-Cl, pH-8.8)

181.7 g of Tris base was dissolved in 800 ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 liter with distilled water and stored at 4°C.

c) Stacking gel buffer (1 M Tris-HCl, pH-6.8)

121.1 g of Tris base was dissolved in 800 ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 liter with distilled water and stored at 4°C.

d) 10 % sodium dodecyl sulphate (SDS) solution

10 g of SDS was dissolved in 100 ml of distilled water and stored at room temperature.

e) 10% ammonium persulfate (APS) solution

1 g of APS was dissolved in 10 ml of the distilled water and stored at 4°C temperature. APS decomposes slowly and hence fresh solution was prepared after one week.

f) N,N,N’,N’-tetramethylethylene diamine (TEMED)

Electrophoresis grade TEMED (Bangalore Genei, Bangalore) stored at 4°C was used.

g) Electrode buffer (5× solution)

5× stock solution was prepared by dissolving 15.1 g of Tris base, 94 g of glycine (electrophoresis grade) in 900 ml distilled water and then 50 ml of 10 % SDS solution of electrophoresis grade was added and the final volume was made up to 1 liter with distilled
water and stored at room temperature. For running the gel, 1X buffer was prepared using 5× electrode buffer.

h) SDS gel loading buffer (2×)

2× gel loading buffer contained 100 mM Tris-Cl (pH 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 0.1% (w/v) bromophenol blue; 200 mM β-mercaptoethanol. Gel loading buffer lacking thiol reagent was stored at room temperature. 200 mM β-mercaptoethanol was added to buffer from 14 M stock mercaptoethanol just before use.

i) Staining solution

2.5 g of Coomassie Brilliant blue R250 (HiMedia, Mumbai), 450 ml of methanol and 100 ml of acetic acid were mixed, filtered and volume was made to 1 liter with distilled water. Solution was stored at room temperature.

j) Destaining solution

300 ml of methanol and 100 ml of acetic acid mixed with distilled water to make up 1 liter. Solution was stored at room temperature.

k) Standard protein molecular weight marker

A medium-range protein molecular weight marker ((PMW-M) Bangalore Genei, Bangalore) was used for determination of molecular weights of desired protein. The molecular weight protein standards included phosphorylase b (97,000 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soyabean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da).

3.1.7.5.6.1 Preparation of SDS-PAGE apparatus (Sambrook et al., 1989)

In this study, all recombinant proteins were analyzed on 15% resolving and 5% stacking gel. Glass plates were rinsed by absolute alcohol and cleaned using tissue paper. Spacers (1 mm) were placed on both sides between two plates in such a way that any bubbles could not move through that and seated in stand and checked by water. 10-15% resolving gel was prepared and poured up to 3/4 portion, and kept for solidification. After solidification, 5% stacking gel was prepared, poured and 1 mm comb was inserted into the staking gel. Gel was allowed to solidify. Comb was removed and the solidified gel with plates was fixed in gel running apparatus. Gel running tank was filled with 1X electrode buffer and prepared samples were loaded into wells. Samples were resolved by applying constant current of 20 mA for 2 hr.
After electrophoresis, the gel was transferred to a clean container and stained overnight at room temperature with shaking. After staining, the gel was destained until a clear background was obtained. Photographs of the gels were taken with Herolab gel documentation system (Germany).

Resolving gel mixture was prepared in a small beaker with a magnetic stirrer by mixing the components for a desired concentration of acrylamide according to the following chart (Harlow and Lane, 1998).

**Recipe for resolving gels (pH 8.8)**

<table>
<thead>
<tr>
<th>Solution component</th>
<th>15% gel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component Volumes (ml)</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Deionised water</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>1.3</td>
<td>2.5</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Immediately upon addition of APS and TEMED, the solution was poured into the assembled plates and overlaid with iso-butanol to prevent diffusion of oxygen into the gel and for obtaining a uniform margin of the gel. It was allowed to polymerize for about 15 min.

In a similar manner, stacking gel mixture with 5% acrylamide mix was prepared by mixing the components as below (Sambrook *et al.*, 1989).
Recipe for stacking gels (pH 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>1 ml</th>
<th>3 ml</th>
<th>5 ml</th>
<th>8 ml</th>
<th>10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>0.68</td>
<td>2.1</td>
<td>3.4</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>30% Acrylamide Mix</td>
<td>0.17</td>
<td>0.5</td>
<td>0.83</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>1.0 M Tris (pH-6.8)</td>
<td>0.13</td>
<td>0.38</td>
<td>0.63</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.001</td>
<td>0.003</td>
<td>0.005</td>
<td>0.008</td>
<td>0.01</td>
</tr>
</tbody>
</table>

3.1.7.5.6.2 Sample preparation for SDS-PAGE

One ml of test culture was pelleted by centrifugation at 11,000 × g for 3 min and supernatant was discarded. The pellet was resuspended in 30 µl distilled water and 30 µl of 2X sample buffer, vortexed and lysed at 95°C for 5-10 min in a dry bath. The samples were loaded to the SDS polyacrylamide gel.

3.1.7.5.7 Purification of recombinant proteins

For production of recombinant proteins in relatively large scale, 200 ml of LB broth was used. The cultures of the recombinant *E. coli* were grown and expressions were obtained under optimized conditions of IPTG induction and time course. The DNA inserts were expressed with 6 histidine residues as an N-terminal fusion protein. The metal binding domain in the fusion protein allows simple one step purification of immobilized metal affinity chromatography (IMAC). The following steps were used for purification of the recombinant proteins by Ni-NTA affinity chromatography.

Cells were harvested by centrifugation at 11,000 × g for 5 min after induction with IPTG for 4 hr. Cell pellets were resuspended in lysis buffer (pH 8.0) and stirred using magnetic beads for 1 hr at room temperature, sonicated on ice at 20-30 W with six 10 sec bursts. The lysates were centrifuged at 11,000 × g for 15 min at room temperature to pellet the cellular debris. The supernatant was added to 1 ml of 50% Ni-NTA agarose slurry and stirred gently on magnetic stirrer for 1 hr at 4 °C. The lysate-Ni-NTA mixtures were loaded into columns and
each column washed twice with 4 ml wash buffer (pH 6.3). The proteins were then eluted with 4 ml of elution buffer (pH 4.5), dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4 °C to remove guanidine hydrochloride and concentrated using vacuum concentrator and purity of these proteins were analyzed on 15 % SDS-PAGE. For lysozyme, disulfide bonds formed during denaturation were reduced by incubating the protein with 10 mM DTT (Dithiothreitol) at 4°C for 1 hr., followed by active refolding in refolding buffer (buffer #13: 55 mM Tris, pH 8.5, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, 264 mM NaCl, 11 mM KCl, 550 mM Gdn-HCl and 1 mM EDTA) (de-la-Re-Vega et al., 2004) by dialysis for 24 hrs at 4 °C.

Lysis buffer (pH 8.0)

- NaH₂PO₄ 100 mM
- Tris-Cl 10 mM
- Guanidine hydrochloride 6 M
- pH was adjusted to 8.0 using 5N NaOH

Wash buffer (pH 6.3)

- NaH₂PO₄ 100 mM
- Tris-Cl 10 mM
- Guanidine hydrochloride 6 M
- pH was adjusted to 6.3 using 1N HCl

Elution buffer (pH 4.5)

- NaH₂PO₄ 100 mM
- Tris-Cl 10 mM
- Guanidine hydrochloride 6 M
- pH was adjusted to 4.5 using 1N HCl.

3.1.7.5.8 Protein estimation

The protein content was measured by the method of Lowry et al. (1951). The procedure is based on quantitating the color obtained from the reaction of Folin Ciocalteau phenol reagent with tyrosyl residues of unknown protein and comparing this with a standard protein, usually bovine serum albumin (BSA). Five standard BSA (1 mg/ml in distilled water) samples were prepared in 0.1 N sodium hydroxide to give concentrations of 20, 40, 60, 80 and 100 µg / ml, respectively, in glass tubes in duplicates. Test samples were taken in 2 different dilutions of 20 µg and 40 µg along with neat. Equal volume of alkaline copper reagent was added to all tubes
and the mixture was incubated for 15 min at room temperature. Folin’s reagent (Sigma, USA) was added at a concentration of 50 µl/ml. The entire mixture was mixed well and incubated for another 30 min at room temperature. Optical density was measured at 690 nm. The protein concentration of the sample was obtained from the graph plotted for the standard BSA concentrations as optical density values.

**Copper sulphate (1%) solution**

- Copper sulphate: 0.1 g
- Distilled water: 50 ml

**Sodium potassium tartarate (2%) solution**

- Sodium potassium tartarate: 1 g
- Distilled water: 50 ml

**Sodium carbonate (4%) solution**

- Sodium carbonate: 2 g
- Distilled water: 50 ml

**Sodium hydroxide (0.1 N) solution**

- Sodium hydroxide: 0.2 g
- Distilled water: 50 ml

**Alkaline copper reagent (ACR)**

- Copper sulphate (1%): 1 ml
- Sodium potassium tartarate (2%): 1 ml
- Sodium carbonate (4%): 48 ml

**Bovine serum albumin (1%)**

- Bovine serum albumin: 0.01 g
- Distilled water: 1 ml

### 3.2 Study of activities/characteristics of recombinants proteins

#### 3.2.1 Lysozyme

#### 3.2.1.2 DNA sequencing and analysis

The plasmid from positive clone was purified using a Plasmid purification kit (QIAGEN). The cloned product was sequenced by M/s Bioserve, Hyderabad, India. The extent of similarity between the nucleotides and derived amino acid sequences to sequences in GenBank was analysed using the respective BLAST program available at NCBI.
The 3 dimensional structure of the protein was predicted based on the known structures of homologues proteins. 3Djigsaw programme was used for the prediction of 3D structure (http://www.bmm.icnet.uk/servers/3djigsaw/). The deduced amino acid sequence (Query lysozyme) was aligned with sequences of various c-type and i-type in GenBank Database. Phylogenetic tree was constructed from CLUSTAL generated alignment using Neighbor-joining method.

3.2.1.3 Activity of recombinant lysozyme against bacterial pathogens

3.2.1.3.1 Bacterial strains and media

Standard bacterial strains like *Vibrio parahaemolyticus* (AQ 4037), *V. vulnificus* (ATCC 27562), *V. cholerae* (ATCC 14035), *Salmonella Typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 10240) Escherichia coli (ATCC 25922) and *Listeria monocytogenes* (SLCC 5976) were maintained in glycerol broth at -80°C (Deep Freezer, Sanyo Corporation, Japan) and after reactivation, grown in Luria Bertani (LB) broth at 37 °C. *V. harveyi*, *V. alginolyticus*, *V. fischeri* used in the study were isolated from the environmental samples and characterized by a battery of biochemical tests as described earlier (Karunasagar et al., 1994; Otta et al., 2001).

3.2.1.3.2 Solid phase lysozyme assay

The activity of recombinant tiger shrimp lysozyme (TSL) against *Micrococcus luteus* and *Vibrio harveyi* was studied by solid phase and turbidimetric assays (Shugar, 1952). Bacterial cultures grown up to OD of 0.5 in TSB with 1% NaCl were used for both the assays. For solid phase assay bacterial lawn was prepared on TSB agar plates. After drying, 1.5 µg of purified recombinant lysozyme protein was added to a 3-mm well punched at the centre. Plates were incubated at 30°C and 37°C for *V. harveyi* and *M. luteus*, respectively. Standard hen egg white lysozyme (HEWL) was used as positive control for each culture. Cell lysates of non-recombinant, recombinant uninduced and recombinant induced *E.coli* were used as negative controls to confirm the activity of purified lysozyme protein.

Solid phase assay was also used to determine the lytic activity against various bacterial cultures which included *E. coli*, *L. monocytogenes*, *Salmonella Typhi*, *S. aureus*, *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus* and *V. fischeri*. HEWL (1.5 µg) was used as positive control and elution buffer as negative control in each case. All experiments were carried out in triplicate and diameter of the zone of inhibition was measured. To determine
whether there was a significant difference in activity of recombinant lysozyme against different bacterial pathogens, one way ANOVA was carried out \((p < 0.05)\). Based on ANOVA results, Duncan’s multiple range test was carried out to differentiate bacterial pathogens where lysozyme activity differed significantly \((p < 0.05)\).

3.2.1.3.3 Turbidimetric assay

For turbidimetric assay, 4 ml of each bacterial culture was centrifuged at 10,000 x g for 5 min and the pellet washed twice with PBS (pH 7.4). The bacterial pellet was resuspended in PBS to get an intial OD\(_{330}\) of 0.5. Recombinant lysozyme (3 µg/ml) was added and OD measurement at 530 nm was recorded every 40s over a 20 min period using a spectrophotometer. To confirm the relative bacteriolytic activity of lysozyme, initial and final viable counts were determined by spread plate method. Bacteriolytic activity was calculated as defined by de-la-Re-Vega et al. (2006)

\[
\text{Bacteriolytic activity} = \frac{\text{CFU}_{\text{initial}} - \text{CFU}_{\text{final}}}{\text{CFU}_{\text{initial}}}
\]

3.2.1.3.4 Determination of minimum inhibitory concentration (MIC)

To determine minimum inhibitory concentration (MIC) of lysozyme required for \(M. luteus\) and \(V. harveyi\), the method described by Miles and Amyes (1996) with modification was used. The entire experiment was performed in microtitre plates. Bacterial cultures were grown in TSB (1% NaCl) and compared with McFarland 0.5 turbidity standard. 10 µl of the cultures in duplicate was added to individual wells containing 170 µl of TSB and 20 µl of 2 fold dilutions (3 µg - 23ng) of recombinant lysozyme in PBS was added. A positive control consisting of 190 µl of TSB and 10 µl of culture and negative control consisting of 180 µl of TSB and 20 µl of recombinant lysozyme (highest dilution) was also used for each bacterial culture. The plate was incubated at 37°C for 17 h and OD read at 630 nm using Elix 800 Universal microplate reader (Biotek Instruments). All experiments were carried out in triplicate. Two-tailed student t-test was carried out to look for any significant difference between ODs at different lysozyme concentration and OD of negative control. MIC was recorded as the lowest concentration at which the OD was statistically similar to that in negative control \((t\text{-test}, p < 0.05)\).

3.2.1.3.5 Activity of recombinant lysozyme against \(V. harveyi\) in seawater

The activity of recombinant shrimp lysozyme (at different concentrations) against \(V. harveyi\) was assessed in seawater with and without EDTA. Seawater of salinity 35 ppt was
filtered and sterilized by autoclaving at 121°C for 15 min. *V. harveyi* culture was grown in TSB with 1% NaCl to an OD$_{600}$ of 0.5 and 50 µl of the culture was added to seawater and incubated for 5 min at 30°C. Recombinant tiger shrimp lysozyme (final concentrations of 0.5, 1.0 and 1.5 µg/ml) was added to medium. To study the effect of EDTA on *V. harveyi* and lysozyme activity, seawater containing *V. harveyi*, recombinant protein and 30 ppm of EDTA was also incubated. The mixture was incubated at 30°C and viable counts by surface spreading were determined at 0 min, 30 min, 1 h, 2 h and 3 h. Seawater with *V. harveyi* culture but without recombinant lysozyme and seawater with recombinant lysozyme but without culture were used as controls. All experiments were carried out in duplicate. Any significant difference between viable counts in recombinant lysozyme treated and untreated (culture control) *V. harveyi* in seawater was statistically evaluated by Mann-Whitney U test. Mann-Whitney U test was also carried out to determine whether there was a significant difference in antivibrio activity of recombinant lysozyme with and without EDTA.

### 3.2.2 Ferritin

#### 3.2.2.1 DNA sequencing and analysis

The cloned product was sequenced by M/s Genei Bangalore, India. The nucleotide and derived amino acid sequences were analyzed using BLAST program of NCBI ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). EsyPred3D programme was used to predict the 3 dimensional structure of the protein based on automated homology modeling using neural network ([http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/](http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/)) and iron (III) binding site was determined. The program Sequence Quickie-Calc version 5.0 software ([http://molecular_programming.com](http://molecular_programming.com)) was used for the prediction of molecular weight, pI and hydrophobic nature of the protein. Phylogenetic tree was constructed from CLUSTAL generated alignment using Neighbor-joining method.

#### 3.2.2.2 Mild acid treatment

The cleavage activity of the recombinant ferritin protein was studied as described by Andrews *et al.* (1987) with minor modifications. 2.5 mg/ml of recombinant protein was dissolved in 70% *v/v* formic acid and 5mM-2-mercaptoethanol. The sample was flushed with Nitrogen (N$_2$) vapour, sealed and incubated at 37°C for 60 h. The formic acid was evaporated under a stream of N$_2$. The digested protein was freeze-dried and analyzed on 15% SDS-PAGE.
3.2.2.3. Iron uptake studies

Iron uptake assay was performed as described by Levi et al. (1988). Briefly, freshly prepared ferrous ammonium sulfate (FAS, 50mM) was added to various amounts (0.05, 0.1 and 0.2 mg/ml) of recombinant ferritin protein in 0.1M Hepes buffer (for pH values below 6). The formation of amber iron products was followed in a spectrophotometer (Shimadzu UV-1601, USA) by monitoring the optical density at 310 (OD_{310}) nm at 0 and 10 min. Increase in OD_{310} values between 0 and 10 min was plotted against corresponding protein concentrations. Hepes buffer containing ferritin but without FAS and Hepes buffer with FAS but without ferritin were used as controls.

3.2.2.4. Activity of Ferritin against virulent V. harveyi

3.2.2.4.1 Virulence assay

The virulence of V. harveyi to shrimps (8-10 gm) was estimated and expressed as LD_{50} value (Reed and Muench 1938). An 18 h bacterial culture in brain heart infusion (BHI) broth was centrifuged and supernatant discarded. Ten fold serial dilution of the culture pellet was made in PBS and total viable count was determined by spread plate technique. Four consecutive dilutions of the culture in PBS (10^{4}-10^{7}) was injected intraperitoneally, each dilution to 10 shrimps and all the animals were observed for mortality upto 96 h.

Brain heart infusion broth

- Calf brain, infusion form: 20 g
- Beef infusion form: 25 g
- Proteose peptone: 0.1 g
- Dextrose: 0.2 g
- Sodium chloride: 0.5 g
- Disodium phosphate: 0.25 g
- Distilled water: 100 ml

The medium was autoclaved at 121 °C for 15 min at 15 lb pressure.

3.2.2.4.2 Effect on lethality of Vibrio harveyi for P. monodon

The virulence study estimated that 10^6 cfu/ml V. harveyi culture was lethal for P. monodon. Three groups of 10 shrimps (10-15 gm) each were acclimatized for 5 days in seawater with salinity of 35 ppt. In the experimental setup, one group was injected with purified protein (15 µg/gm of shrimp), a second group with the mixture of protein and virulent
V. harveyi culture at $10^6$ cfu/ml and a third group with V. harveyi culture ($10^6$ cfu/ml) alone. All the shrimps were observed for mortality up to 96 h.

### 3.2.2.4.3 Study of antimicrobial activity against V. harveyi

After protection studies, any direct antimicrobial activity of recombinant lysozyme against shrimp pathogenic V. harveyi was also studied. Solid phase assay at 15 µg protein concentration was carried out as described earlier. To study the effect on ferritin on viable count, 100 µl of overnight grown V. harveyi was added to sufficient number of test tube containing 4 ml of Luria-Bertani brood and allowed to grow until an OD$_{600}$ of 0.6. After this, 60 µl of recombinant ferritin (1 µg/µl in PBS) was added to these cultures in triplicate resulting in final concentrations of 15 µg ferritin/ml of culture. The mixtures were incubated at 30 °C and viable counts by surface spreading were determined at 0 min, 1 h and 2 h. V. harveyi cultures containing 60 µl of PBS were used as control.

### 3.2.3 Histone H2A

#### 3.2.3.1 Sequence analysis

The clones expressing recombinant H2A protein were sequenced by M/s Genei, Bangalore, India. The nucleotide sequence was translated to corresponding amino acid sequences by Transeq program ([www.ebi.ac.uk/emboss/transeq](http://www.ebi.ac.uk/emboss/transeq)). Molecular weight and isoelectric point (pI) of recombinant protein were calculated by Sequence Quickie-Calc version 5.0 software ([http://molecular_programming.com](http://molecular_programming.com)). Clustal generated multiple sequence alignment was performed to study the extent of similarity between P. monodon H2A protein and H2A proteins of other organism available in GenBank ([www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)). Multiple sequence alignment was also used to study the extent of similarity between P. monodon H2A and H2A derived antimicrobial peptide such as Buforin I (in *Bufo bufo*) and Parasin I (in *Parasilurus asotus*). Phylogenetic tree was constructed by neighbor-joining method.

#### 3.2.3.2 Study of antibacterial activity of recombinant histone H2A protein

Antibacterial activity of recombinant H2A (at different concentrations) was studied against various bacteria by solid phase assay. These bacteria included *M. luteus*, *E. coli*, *L. monocytogenes*, *Salmonella Typhi*, *S. aureus*, *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. fischeri*. Bacterial cultures were grown up to OD$_{600}$ of 0.5 in TSB with 1% NaCl. For solid phase assay, bacterial lawn was prepared on TSB agar plates. After drying, 1.5, 3.0, 5.0 and 10 µg of purified recombinant H2A protein were added to a 3-
mm wells punched into the lawns prepared for each bacteria, separately. Plates were incubated overnight at appropriate temperatures. 1.5 µg of standard hen egg white lysozyme (HEWL) was used as positive control for each culture. Cell lysates of non-recombinant, recombinant uninduced and recombinant induced E. coli were used as controls.

3.2.3.3 Treatment of recombinant H2A by pepsin enzyme

Any production of antimicrobial peptides from shrimp recombinant H2A protein was studied by pepsin mediated degradation of protein. For pepsin treatment of recombinant H2A, procedure described by Franceschi et al. (1988) was followed. Duplicate aliquots containing 300 µg of purified recombinant H2A were mixed with 100 µg of pepsin enzyme (Himedia, Mumbai) and dialyzed for 24 h against 0.5 M acetic acid in a 3.5 kDa cut-off dialysis membrane followed by a 12 h dialysis against solution containing 10 mM ammonium bicarbonate and 2 mM phenylmethylsulfonyl fluoride. These pepsin digestion and dialysis steps were carried out at 4 °C. Aliquots containing H2A protein but without pepsin and dialyzed similarly were used as control. After incubation, both controls and digested H2A were analyzed by Tricine SDS-PAGE to determine any production of peptides. Antimicrobial activity of pepsin treated and control H2A protein against various gram-positive and gram-negative bacteria was studied by solid phase assay, as described earlier.

3.2.3.3.1 Tricine SDS-PAGE (Schagger, 2006)

Separation of peptides and proteins under 10 to 15 kDa is not possible in the traditional Laemmli discontinuous gel system because the co-migration of SDS and smaller proteins obscure the resolution. The Tricine SDS-PAGE method (Schagger, 2006) uses a modified buffer to separate the SDS and peptides, thus improving the resolution. The various buffers and reagents used in electrophoresis are as follows:

a) Acrylamide-bisacrylamide mixture

Twenty nine grams of acrylamide and 1.0 g of bisacrylamide (N, N’-methylene bisacrylamide) were dissolved in 80 ml of distilled water. The volume was made up to 100 ml and stored at 4 ºC.

b) Tris-Cl/SDS buffer (pH-8.45)

182 g of Tris base was dissolved in 300 ml of distilled water and pH was adjusted to 8.45 using 1N HCl. The final volume was made up to 500 ml with distilled water,
filtered through a 0.45 µm filter and 1.5 g of SDS was added. This solution was stored at 4°C.

  c) Anode buffer

    121.1 g of Tris base was dissolved in 500 ml of distilled water and pH was adjusted to 8.9 with concentrated HCl. The final volume was made up to 5 liters with distilled water and stored at 4°C.

  d) Cathode buffer

    12.11 g of Tris base, 17.92 g of tricine and 1 g SDS were dissolved in distilled water. The final volume was adjusted to 1 liter with distilled water and stored at 4°C.

  e) 10% ammonium persulfate (APS) solution

    1 g of APS was dissolved in 10 ml of the distilled water and stored at 4°C temperature. APS decomposes slowly and hence fresh solution was prepared after one week.

  f) N,N,N′,N′-tetramethylethylenediamine (TEMED)

    Electrophoresis grade TEMED (Bangalore Genei, Bangalore) stored at 4°C was used.

  g) 2x Tricine sample buffer

    2x Tricine sample buffer consisted of 0.08 M Tris-Cl/SDS (pH 6.8), 24% (v/v) glycerol, 8% (w/v) SDS, 0.2 M DTT and 0.02% (w/v) coomassie blue G-250.

  h) Staining solution

    2.5 g of Coomassie Brilliant blue R250 (HiMedia, Mumbai), 450 ml of methanol and 100 ml of acetic acid were mixed, filtered and volume was made up to 1 liter with distilled water. Solution was stored at room temperature.

  i) Destaining solution

    300 ml of methanol and 100 ml of acetic acid mixed with distilled water to make up 1 liter. Solution was stored at room temperature.

  j) Standard protein molecular weight marker

    An ultra low range molecular weight marker (M3546, Sigma, USA) was used for determination of molecular weights of desired protein. The molecular weight protein standards included triosephosphate isomerase from rabbit muscles (26,600 Da), myoglobin from horse heart (17,000 Da), α-lactalbumin from bovine milk (14,200 Da),
aprotinin from bovine lung (6,500 Da), insulin chain B (3,496 Da) and bradykinin (1,060 Da).

3.2.3.3.2 Preparation of polyacrylamide gel and electrophoresis

The PAGE apparatus was assembled as described earlier and gel was prepared according to the recipe given below. The solidified gel with plates was fixed in gel running apparatus. The upper chamber of gel running apparatus was filled with anode buffer and lower chamber with cathode buffer. For sample preparation, 50 µl of protein was mixed with 50 µl of 2x tricine sample buffer and incubated at 40 °C for 60 min. After loading 50 µl samples in wells, electrophoresis was carried out at 30 V (constant voltage) for 1 h followed by at 150 V (constant voltage) for 4-5 h. After electrophoresis, the gel was transferred to a clean container and stained overnight at room temperature with shaking. After staining, the gel was destained until a clear background was obtained. Photographs of the gels were taken with Herolab gel documentation system (Germany).

Recipe for resolving and stacking gels

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Resolving gel (ml)</th>
<th>Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide mix</td>
<td>9.80</td>
<td>1.62</td>
</tr>
<tr>
<td>Tris-Cl/SDS, pH 8.45</td>
<td>10.00</td>
<td>3.10</td>
</tr>
<tr>
<td>Deionised water</td>
<td>7.03</td>
<td>7.78</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.17</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>0.05</td>
<td>0.025</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.005</td>
</tr>
</tbody>
</table>

3.2.4 Intracellular fatty acid binding protein

3.2.4.1 Sequence analysis

The cloned product was sequenced by M/s Genei Bangalore, India. The nucleotide sequence was analyzed using BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov). The nucleotide sequence was translated to corresponding amino acid sequences by Transeq program (www.ebi.ac.uk/emboss/transeq). EsyPred3D programme was used to predict the 3 dimensional structure of the protein based on automated homology modeling using neural network (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/). The program
Sequence Quickie-Calc version 5.0 software (http://molecular_programming.com) was used for the prediction of molecular weight, pI and hydrophobic nature of the protein. Phylogenetic tree was constructed from CLUSTAL generated alignment using Neighbor-joining method.

3.3 Study of gene expression in shrimp in response to WSSV challenge

3.3.1 Suppression subtractive hybridization (SSH)

For SSH, PCR-Select cDNA subtraction kit (Clontech, USA) was used.

3.3.1.1 Collection of experimental animals

Healthy subadult *P. monodon* (approximately 10 weeks old) weighing 18-20 g were purchased from the local farm and acclimatized and maintained as described earlier. Shrimp were randomly screened for the presence of WSSV by PCR as described by Otta *et al.* (2003). Healthy and WSSV negative animals were used in this study. During random screening some samples found positive for WSSV infection were used for virus purification.

3.3.1.1.1 Purification of WSSV

For purification of WSSV, the method described by Xie *et al.* (2005) was followed with minor modifications. The tissues of WSSV infected shrimp excluding hepatopancreas were used for WSSV purification. Twenty grams of infected tissues were homogenized in 500 ml TNE buffer (50 mM Tris-Cl, 400 mM NaCl, 5 mM EDTA; pH 8.5) containing a combination of protease inhibitors (1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM Na₂S₂O₅), and centrifuged at 3500 x g for 5 min at 4°C. After filtering through a nylon net (400 μm), the filtrate was centrifuged at 23,000 x g for 30 min at 4°C. The upper loose pellet was removed carefully, and the lower white pellet was suspended in 10 ml TM buffer (50 mM Tris-HCl, 10mM MgCl₂, pH 7.5). After centrifugation at 3500 x g for 5 min, the virus particles were pelleted by centrifugation at 23,000 x g for 20 min at 4°C, resuspended in 10 ml TM buffer containing 0.1% NaN₃ and aliquoted 1 ml in 1.5 ml microcentrifuge tubes and stored at -80°C.

3.3.1.2 WSSV challenge and hemolymph collection

Experimental animals were challenged with purified WSSV by injecting 50 µl of purified virus suspension intramuscularly between 3rd and 4th abdominal segment of the shrimp. Third day post infection, hemolymph was collected as described earlier. Hemolymph from
unchallenged shrimp was used as control. WSSV infection in shrimps was also confirmed by polymerase chain reaction (PCR).

3.3.1.2.1 Molecular diagnosis of WSSV

The WSSV infection in shrimps was confirmed by PCR using the primers described by Otta et al. (2003). Approximately 100 mg of tissues like gills, muscle and cuticle were removed aseptically from the shrimps. The tissues were then transferred to a sterile disposable plastic pouch and homogenized. 800 μl of digestion buffer (10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% Triton-X100; 6 M Guanidine hydrochloride; 0.1 M Sodium acetate) was added. The homogenate was incubated at room temperature (28 ± 1 °C) for 30 min.

Following incubation, the homogenate was transferred to a 1.5 ml microfuge tube and centrifuged at 6,000 x g for 10 min. The supernatant was transferred to a fresh tube and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed and then centrifuged at 10,000 x g for 10 min. The aqueous phase containing DNA was transferred to a fresh tube followed by precipitation by adding 2-3 volumes of 100% ethanol and centrifuged at 14,000 x g for 20 min. After ethanol precipitation, the pellet was washed twice with 1 volume of 95% ethanol and then vacuum dried. Finally, the DNA pellet was dissolved in 100 μl of 1× TE buffer or 100 μl of distilled water. The extracted DNA was stored at -20 °C till further use. Two DNA oligonucleotide primers designed to amplify 310 bp sequence of WSSV-DNA by Otta et al. (2003) were used to detect WSSV infection in preliminary screening and experimentally infected shrimps.

PCR was carried out in 30 μl reaction mixture containing 2.0 μl template DNA, 1× assay buffer (10 mM Tris –HCl, pH 9.0; 1.5 mM MgCl₂, 50 mM KCl, 0.01% Gelatin), 200 μM of each the four deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, dGTP), 10 pmol of each primers and 0.9 U of Taq DNA polymerase (Bangalore Genei, Bangalore). The primers used and the cycling conditions were essentially same as described previously by Otta et al. (2003) consisting of 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 30 sec extension at 72°C. An initial denaturation for 5 min at 94°C and final extension at 72°C for 5 min were provided. The PCR products were detected by agarose gel electrophoresis as described earlier.
3.3.1.3 Extraction of total RNA

Total RNA was extracted using Trizol LS (Invitrogen, USA) reagent by protocol described earlier. Total RNA was used separately from infected and control hemolymph, designated as tester and driver, respectively. Total RNA was quantified spectrophotometrically.

3.3.1.4 Purification of poly A⁺ RNA (mRNA) from total RNA

mRNA was separately purified from tester and driver total RNA using Oligotex mRNA Maxi kit (Qiagen, USA). The procedure described by the suppliers was followed to purify mRNA. Two mg of total RNA was pipetted into an RNase free 1.5 ml microcentrifuge tube, and final volume adjusted to 650 µl with RNase free water. To this tube 650 µl of buffer OBB and 115 µl Oligotex suspension was added followed by repeated pipetting to mix thoroughly the contents. Samples were incubated at 70 °C for 3 min followed by at 20 to 30 °C for 10 min. The samples were centrifuged at 14,000 x g for 2 min and supernatant was carefully removed by pipetting. The resulting oligotex:mRNA pellet was resuspended in 600 µl of buffer OW2 and transferred to large spin column (supplied with the kit) followed by centrifugation at 14,000 x g for 1 min. The spin column was transferred to a new RNase free 1.5 ml microcentrifuge tube and 600 µl of buffer OW2 was added. This assembly was centrifuged at 14,000 x g for 1 min and flowthrough discarded. After centrifugation, 50 µl of hot (70 °C) buffer OEB was added to the column and mixed by pipetting up and down 3 or 4 time followed by centrifugation at 14,000 x g for 1 min. The flow through containing purified mRNA was collected and quantified spectrophotometrically. The quality of mRNA was analyzed by formaldehyde-agarose gel electrophoresis (Sambrook et al., 1989).

3.3.1.4.1 Formaldehyde agarose gel electrophoresis

mRNA was resolved on 1.2 % agarose gel prepared in 1 × MOPS electrophoresis buffer. The molten agarose was cooled to below 65 °C followed by addition of 1.8 ml of 37% formaldehyde per 100 ml of gel and ethidium bromide to a final concentration of 0.5 µg/ml. After mixing, gel was poured to a mould and allowed to set for 1 h at room temperature. During analysis, 4 µl of 1:10 diluted mRNA was mixed with 1µl of 5 × RNA loading buffer and incubated at 65 °C for 5 min followed by chilling at ice for 10 min. The prepared RNA samples were loaded into agarose wells. Electrophoresis was carried out 50 V for 30-60 min and mRNA was observed under UV transilluminator (Herolab, Germany).

10 × MOPS (3-[N-morpholino] porpanesulfonic acid) electrophoresis buffer
41.8 g of MOPS was dissolved in 700 ml of sterile DEPC-treated water and pH was adjusted to 7.0 with NaOH pellets. To this solution, 3.42 g of sodium acetate and 20 ml of DEPC treated 0.5 M EDTA (pH 8.0) were added and final volume was adjusted to 1 liter with DEPC treated water. 100 ml this 10 × buffer was mixed with 20 ml of 37 % formaldehyde 880 ml of DEPC treated water to make 1 × MOPS electrophoresis buffer.

**5 × RNA loading buffer**

- 10 × MOPS electrophoresis buffer: 4 ml
- Formamide: 3.084 ml
- 100 % Glycerol: 2 ml
- 37 % formaldehyde: 720 µl
- 0.5 M EDTA: 80 µl
- Saturated aqueous bromophenol blue: 16 µl

3.3.1.5 Oligonucleotides

cDNA synthesis primer

5’ -TTTTGTACAGCTT30N1N- 3’

Adaptor 1

5’ –CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT- 3’
3’ -GGCCCGTCCA- 5’

Adaptor 2R

5’ –CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT- 3’
3’ -GCCGCTCCA- 5’

PCR primer 1

5’-CTAATACGACTCACTATAGGG- 3’

Nested PCR primer 1

5’ –TCGAGCGGCCGCCCGGGCAGGT- 3’

Nested PCR primer 2R

5’ –AGCGTGGTCGCGGCCGAGGT- 3’
M 13 forward primer  
5’ –GTAAAACGACGGCCAG- 3’

M 13 reverse primer  
5’ –CAGGAAACAGCTATGAC- 3’

β-actin forward primer  
5’ –CCCTGTTCCAGCCCTCATT- 3’

β-actin reverse primer  
5’ –GGATGTCCACGTCGACTT- 3’

3.3.1.6 Synthesis of first-strand and second strand cDNA

Both tester and driver mRNAs were separately reverse transcribed to first-strand cDNA. Four microliter of mRNA (tester/driver) was mixed with 1 µl of 10 µM cDNA synthesis primer and incubated at 70 °C for 2 min. The samples were chilled on ice for 2 min and centrifuged briefly. Following this 2 µl of 5x first-strand buffer buffer (250 mM Tris-HCl (pH 8.5), 40 mM MgCl₂, 150 mM KCl, 5 mM DTT), 1 µl of each dNTP (10 mM), 1 µl of RNase free water, 1 µl of AMV reverse transcriptase (20 units/µl) were added. The reaction mixture was vortexed and centrifuged briefly followed by incubation at 42 °C for 1.5 h in a thermal cycler. After incubation, reaction was terminated by incubating the samples on ice for sometime.

Second strand synthesis was carried out immediately after the completion of first strand synthesis. Ten microliter of first strand reaction was mixed with 48.4 µl of sterile H₂O, 16 µl of 5x second-strand buffer (100 mM Tris-HCl (pH 7.5), 500 mM KCl, 25 mM MgCl₂, 0.75 mM β-NAD, 0.25 mg/ml BSA), 1.6 µl of 10 mM dNTP mix and 4.0 µl of 20x second-strand enzyme cocktail (DNA polymerase I, 6 units/µl; RNase H, 0.25 units/µl; E. coli DNA ligase, 1.2 units/µl). The reaction mixture was briefly centrifuged and incubated at 16 °C for 2 h in a thermal cycler. After 2 h, 2 µl (6 u) of T4 DNA polymerase was added and sample tube was incubated at 16 h for 30 min to generate blunt ended DNA. After incubation, the reaction was terminated by addition of 4 µl of 20x EDTA/glycogen mix. The reaction tube was stored at -20 °C until further use.
3.3.1.7 Purification of second-strand cDNA

Second-strand cDNA was purified from reaction mixture by phenol:chloroform extraction. Briefly, 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube containing second-strand reaction mixture. This tube was vortexed thoroughly and centrifuged at 14,000 rpm for 10 min to separate phases. Top aqueous layer was carefully collected and transferred to a fresh 0.5 ml microcentrifuge tube. To this tube, 100 µl of chloroform:isoamyl alcohol (24:1) was added followed by brief vortexing and centrifugation at 14,000 rpm 10 min. Upper aqueous phase was transferred to a fresh 0.5 ml centrifuge tube followed by addition of 40 µl of 4M ammonium acetate and 300 µl of 95% ethanol to facilitated the precipitation of cDNA. The mixture was vortexed briefly and centrifuged at 14,000 rpm for 20 min at room temp. The pellet was washed with 500 µl of 80% ethanol and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and pellet was air dried for about 10 min to evaporate residual ethanol. The dry pellet was dissolved in 50 µl of sterile water and stored at -20 °C until Rsa I digestion.

3.3.1.7 Rsa I digestion

Both tester and driver purified double stranded cDNA (ds-cDNA) were separately digested with Rsa I restriction enzyme. This step generates shorter, blunt-ended ds-cDNA fragments which are optimal for subtraction and required for adaptor ligation during later stages. In a 200 µl microcentrifuge tube, 43.5 µl of ds-cDNA was mixed with 5.0 µl of 10x Rsa I digestion buffer (100 mM Bis Tris Propane-HCl (pH 7.0), 100 mM MgCl₂, 1 mM DTT) and 1.5 µl of Rsa I restriction enzyme (10 units/µl). After mixing and brief centrifugation, the tube was incubated at 37 °C for 1.5 h. After incubation, the digestion reaction was terminated by adding 2.5 µl of 20x EDTA/glycogen. Digested ds-cDNA (tester/driver) was purified from reaction mixture by phenol:chloroform extraction as describe earlier and final pellet was dissolved in 5.5 µl of sterile water.

3.3.1.8 Adaptor ligation

Adaptors were ligated to only double stranded tester cDNA and not to the driver cDNA. One microliter of purified tester cDNA was diluted with 5 µl of sterile water. Two microliter of diluted tester cDNA was transferred to two separate tubes and designated as tester 1-1 and tester 1-2, respectively. Ligation master mix for each reaction was prepared by adding 3 µl of sterile water, 2 µl of 5x ligation buffer (250 mM Tris-HCl (pH 7.8), 50 mM MgCl₂, 10 mM DTT, 0.25 mg/ml BSA) and 1 µl of T4 DNA ligase (400 units/µl) in microcentrifuge tube. Two
different types of adaptors were ligated to tester 1-1 (Adaptor 1) and tester 1-2 cDNA (adaptor 2R). Final reactions were prepared as per the Table 2. After mixing,

**Table 2. Setting up the ligation reactions.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Tester 1-1</th>
<th>Tester 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted tester cDNA</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Adaptor 1 (10 µM)</td>
<td>2 µl</td>
<td>-</td>
</tr>
<tr>
<td>Adaptor 2R (10 µM)</td>
<td>-</td>
<td>2 µl</td>
</tr>
<tr>
<td>Ligation master mix</td>
<td>6 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

the tubes were centrifuged briefly. In a fresh centrifuge tube, 2 µl of tester 1-1 and tester 1-2 were mixed. This was used as **unsubtracted tester control**. All the tubes were incubated overnight at 16°C. One microliter of 20x EDTA/glycogen mix was added to sample tubes followed by heating at 72°C for 5 min to inactivate the ligase. After ligation, 1 µl of unsubtracted tester control was diluted into 1 ml of sterile water and used as template during PCR amplification. All the samples were stored at -20°C.

**3.3.1.9 First hybridization**

During first hybridization process, an excess of driver cDNA was added to each tester cDNA, samples were heat denatured, and allowed to anneal. The remaining single stranded cDNAs (available for the second hybridization) were dramatically enriched for differentially expressed sequences because non target cDNAs present in tester and driver cDNA formed hybrids.

During hybridization both tester cDNAs (tester 1-1 and tester 1-2) were mixed with excess of driver cDNA as per Table 3.

**Table 3. Setting up the first hybridization**

<table>
<thead>
<tr>
<th>Component</th>
<th>Tester 1-1</th>
<th>Tester 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsa I digested driver cDNA</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Adaptor 1 ligated tester 1-1</td>
<td>1.5 µl</td>
<td>-</td>
</tr>
<tr>
<td>Adaptor 2R ligated tester 1-2</td>
<td>-</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>4× hybridization buffer</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td>4.0 µl</td>
<td>4.0 µl</td>
</tr>
</tbody>
</table>
After mixing and brief centrifugation, samples were incubated at 98°C for 1.5 min followed by 68°C for 8 h.

3.3.1.10 Second hybridization

During second hybridization, the two samples from first hybridization were mixed together, and fresh denatured driver cDNA was added to further enrich for differentially expressed sequences. New hybrid molecules were formed which were differentially expressed cDNAs with different adaptors on each end.

In a microcentrifuge tube, 1 µl of driver cDNA, 1 µl of 4× hybridization buffer and 1 µl of sterile water was added. This tube was incubated at 98°C for 1.5 min in thermal cycler followed by addition of tester 1-1 and tester 1-2 cDNA from first hybridization. After brief centrifugation, this tube was incubated overnight at 68°C. To this subtracted DNA, 200 µl of dilution buffer (pH 8.3) (20 mM HEPES pH (6.6), 20 mM NaCl, 0.2 EDTA (pH 8.0)) was added followed by heating at 68°C for 7 min in a thermal cycler. Diluted subtracted DNA was stored at -20°C until PCR amplification.

3.3.1.10.1 Analysis of subtraction efficiency

Efficiency of subtraction reaction was evaluated by comparing the relative amount of constitutively expressed β-actin gene in unsubtracted and subtracted cDNA. Forward and reverse primer were designed from P. monodon β-actin sequence available in GenBank (Accession no. EF087977). 10 pmol of β-actin forward and reverse primers and 2 µl of template DNA (diluted subtracted tester control/diluted subtracted DNA) was added to 30 µl PCR reaction mixture containing other components, as described earlier. The optimized PCR programme consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 60°C, 30 sec extension at 72°C. The final extension was performed at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis on 2% gel.

3.3.1.11 PCR amplification

Differentially expressed cDNAs were selectively amplified by the two step PCR. Prior to thermal cycling, the missing strands of the adaptors were filled in by a brief incubation at 75°C resulting in formation of binding sites for PCR primer 1. In the first step PCR, only
cDNAs with different adaptor sequences on each end were exponentially amplified. In the second step, nested PCR was used to further reduce background and enrich for differentially expressed sequences.

3.3.1.11.1 First step PCR

The amplification of differentially expressed genes in subtracted DNA was performed in a programmable thermocycler (MJ Research, USA). PCR was carried out in 24 µl reaction mixture containing 1.0 µl template DNA (diluted subtracted), 10× assay buffer (10 mM Tris-HCl, pH 9.0; 1.5 mM MgCl₂, 50 mM KCl, 0.01% Gelatin), 0.5 µl of 10 mM dNTPs mix, 1 µl of 10 µM PCR primer 1 and 0.5 µl of 50× Advantage cDNA polymerase mix.

The optimized PCR programme consisted of initial incubation at 75°C for 5 min, initial denaturation at 95°C for 5 min, followed by 30 cycles of 10 sec denaturation at 95°C, 30 sec annealing at 66°C, 1.5 min extension at 72°C. The final extension was performed at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis on 2% gel as described earlier.

3.3.1.11.2 Second step PCR (Nested PCR)

Three microliter of primary PCR products were diluted in 27 µl of sterile MilliQ water and used as template for nested PCR. PCR master mix containing 1 µl each of 10 µM nested PCR 1 and 2R, instead of PCR primer 1 was prepared as described earlier. One microliter of diluted template DNA was added to master mix. The optimized PCR programme consisted of initial denaturation at 95°C for 5 min, followed by 20 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 68°C, 1.5 min extension at 72°C. The final extension was performed at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis on 2% gel, as described earlier.

3.3.1.12 Cloning of amplified PCR products from subtracted cDNA

3.3.1.12.1 Purification of PCR products

Subtracted PCR product was obtained in bulk (100 µl) using the primers and condition described in nested PCR. The PCR products were purified before ligation using QIAquick PCR purification kit (Qiagen, USA).
3.3.1.12.2 Ligation of PCR product into cloning vector

Purified PCR product was cloned in pSC-A PCR cloning vector (StrataClone, USA). Ligation reaction was performed in a tube containing 3 µl of StrataClone cloning buffer, 2 µl of fresh PCR product and 1 µl of Strataclone vector mix (5 ng/µl). After mixing gently with repeated pipetting, the tube was incubated at room temperature (22-23°C) for 5 min. After ligation the tube was stored at -20°C until transformation.

3.3.1.12.3 Transformation

The ligation mixture was transferred to the tube containing 50 µl of StrataClone SoloPack competent cells. Transformation was carried out by heat-shock at 42°C for 45 sec. 250 µl of pre-warmed SOC medium was added to the transformation reaction mixture and incubated at 37°C for 1 h with agitation. After incubation, 25, 50, and 100 µl of transformation mixture were plated on LB-ampicillin-X-gal plates (containing 100 µg/ml ampicillin and 40 µl of 2% X-Gal). After overnight incubation at 37°C, recombinant white colonies were selected for screening by PCR.

**SOC broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

The above ingredients were added to 100 ml of distilled water and autoclaved at 121°C for 15 min. After cooling, 1 ml each of filter sterilized 1 M MgCl₂ and 1 M MgSO₄ was added; and 99 ml of this medium was mixed with 1 ml of 2 M filter sterilized glucose, just prior to use.

**2% X-Gal**

0.2 g of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was dissolved in 10 ml of dimethylformamide (DMF) and stored at -20°C.

3.3.1.12.4 Screening of recombinant clones

Transformants were selected and screened for the presence of insert by preparing crude lysate of DNA (Dileep et al., 2002), as described earlier. In a 30 µl reaction master mix, 2 µl of crude lysate was added as template for PCR with 10 pmol each of M13 F and R primers. The optimized PCR programme consisted of initial denaturation at 95 °C for 5 min, followed by 30
cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, 2 min extension at 72 °C. The final extension was performed at 72 °C for 10 min. Glycerol stock (30%) were made for all the positive clones and stored at -80 °C.

3.3.1.13 Sequence analysis

Recombinant clones were selected and sequenced by Ms. Bangalore Genei, Bangalore and Ms. Bioserve Biotechnologies, Hyderabad. A total of 250 clones were sequenced. Various bioinformatics tools were used to analyze the nucleotide sequences. The vector sequences were removed using VecScreen (http://www.ncbi.nlm.nih.gov/-VecScreen/VectScreen.html) and any presence of open reading frame (ORF) was determined by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Nucleotide sequences were translated by use of Transeq programme (http://www.ebi.ac.uk/emboss/transeq/). The extent of similarity between the nucleotides and derived amino acid sequences to known sequences in GenBank was analysed using the respective BLAST programs available at NCBI (http://www.ncbi.nlm.nih.gov/). All the sequences obtained in this study were submitted to GenBank and Accession no. obtained.