CHAPTER 3.

MATERIAL & METHODS
Materials & Methods

3.1 Materials

Primers for VH and VL gene repertoire amplification are listed in Table 3.1.

(TABLE 3.1)

<table>
<thead>
<tr>
<th>Primary oligoes:</th>
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<tr>
<td>VH1FOR-2</td>
<td>TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC</td>
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<tr>
<td>VH1BACK</td>
<td>AGG TSM ARC TGC AGS AGT CWGG</td>
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<tr>
<td>MJK1FONX</td>
<td>CCG TTT GAT TTC CAG CTT GGT GCC</td>
</tr>
<tr>
<td>MJK2FONX</td>
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<td>MJK4FONX</td>
<td>CCG TTT TAT TTC CAA CTT TGT CCC</td>
</tr>
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<td>MJK5FONX</td>
<td>CCG TTT CAG CTC CAG CTT GGT CCC</td>
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<td>VK2BACK</td>
<td>GAC ATT GAG CTC ACC CAG TCT CCA</td>
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<th>PCR oligoes to make linker:</th>
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<tr>
<td>LINKFOR</td>
<td>TGG AGA CTG GGT GAG CTC AAT GTC</td>
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<tr>
<td>LINKBACK</td>
<td>GGG ACC ACG GTC ACC GTC TCC TCA</td>
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<table>
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<th>Oligoes for addition of restriction sites:</th>
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<tr>
<td>VH1BACKNcoI</td>
<td>GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC</td>
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<td>CAG GTS MAR CTG CAG SAG TCWGG</td>
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<td>JK1NOT10 GGT GCC</td>
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<td>JK2NOT10 GGT CCC</td>
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<tr>
<td>JK5NOT10 GGT CCC</td>
<td>GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT</td>
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<tr>
<td>R = A or G</td>
<td></td>
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<tr>
<td>S = G or C</td>
<td></td>
</tr>
<tr>
<td>W = A or T</td>
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Strains and vectors used in this study are listed in Table 3.2.

(Table 3.2)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
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<tr>
<td>BL21(DE3)</td>
<td>F ompT hsdS (Mφ80lacIq 21 lacIq 21 m15Δ7 lacZΔM15 lacY1 Δ(lac Pro +) gal dcm lon (DE3)</td>
<td>(NEB), USA.</td>
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<table>
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<th>Plasmids</th>
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<td>pET 22b</td>
<td>5.4 Kb, Amp', T7 promoter, lacO.</td>
<td>Novagen, USA</td>
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<tr>
<td>pET 28a</td>
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<td>Novagen, USA</td>
</tr>
<tr>
<td>pMAL-p2X</td>
<td>6.7 Kb, Amp', Ptac promoter,</td>
<td>NEB</td>
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</tbody>
</table>

Animals used in the study:

BALB/c mice (Female), 2-4 weeks of age were procured from National Institute for Nutrition, Hyderabad. Animals were kept in the Animal house, JNU, during the study.

3.1.1 Media

LB medium (Luria Bertani medium)

- Tryptone: 10 g
- Yeast extract: 5 g
- NaCl: 5 g
20 g of LB powder (Hi Media) was dissolved in double distilled water (ddH₂O) to make up the total volume to 1 litre. The media was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

**LB medium (Modified)**

<table>
<thead>
<tr>
<th>Ingredient</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>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>4.92 g</td>
</tr>
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</table>

20 g of LB powder (Hi Media), glucose and MgSO₄ was dissolved in double distilled water (ddH₂O) to make up the total volume to 1 litre. The media was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

**LB Agar plate**

<table>
<thead>
<tr>
<th>Ingredient</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>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

35 g of LB agar powder (Hi Media) was dissolved in ddH₂O to make up the total volume to 1 litre. The media was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in. Appropriate antibiotics were added as needed and poured in petri dishes. All plates were stored at 4°C.

**TB (Terrific Broth)**

<table>
<thead>
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<tbody>
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<td>Tryptone</td>
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<tr>
<td>Yeast Extract</td>
<td>24 g</td>
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<tr>
<td>Glycerol</td>
<td>4 ml</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.31 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>12.54 g</td>
</tr>
</tbody>
</table>


Phosphate salts were autoclaved separately and the total volume made up to 1L after mixing.

**RPMI 1640 (incomplete)**
RPMI 1640 media powder (Sigma), one vial for 1 liter, 10 mM HEPES, 2mM glutamine, 2mM pyruvic acid, 5X10^{-5} M β-mercaptoethanol, 2g/L sodium bicarbonate, 100 μg/ml streptomycin, 100 units/ml pencillin and 0.25μg/ml Amphotericin B.
Incomplete media is without the fetal calf serum (FCS) supplement. After dissolving all the components in ddH2O, volume was made up to one liter and media was filter (0.22μ filter) sterilized in aseptic conditions. This media was stored at 4°C.

**Antibiotics**

**Ampicillin**
The dry sodium salt of ampicillin was dissolved in sterile ddH2O to make the stock solution of 100 mg/ml. The stock solution was sterilised by filtration through a 0.22 micron disposable filter and stored at -20°C. The working concentration of the antibiotic was 100 μg/ml for broth and plates.

**Kanamycin**
Kanamycin Stock solution (50 mg/ml) was prepared by dissolving the appropriate amount of the antibiotic powder in sterile ddH2O. It was filter sterilised and stored at -20°C. For liquid and solid medium, this stock solution was added to a final concentration of 50 μg/ml.
3.1.2 Stock Solutions of some common reagents

**T_{10}E_{1} buffer, pH 8.0**
Tris-Cl, pH 8.0  
10 mM
EDTA, pH 8.0  
1 mM
The above components were mixed and the pH adjusted to 8.0 with dilute HCl. Finally, the buffer was sterilised by autoclaving.

**2 N NaOH**
8 g of Sodium hydroxide was dissolved in 45 ml of ddH$_2$O. Finally, volume was made up to the 100 ml with ddH$_2$O. Solution was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

**10% Sodium dodecyl sulphate (SDS)**
100 g of SDS (electrophoresis grade) was dissolved in 700 ml of sterile ddH$_2$O and was heated at 55°C for 2 minutes. The final volume was made up to 1 litre with sterile ddH$_2$O.

**3 M Sodium acetate (pH 5.2)**
408 g of Sodium acetate was dissolved in water. pH was adjusted to the desired value with Acetic acid. ddH$_2$O was added to make 1 litre total volume. Solution was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

**DNase free RNase A**
Pancreatic RNase A was dissolved at a concentration of 10 mg/ml in 0.01 M Sodium acetate, pH 5.2, Boiled at 100°C for 15 minutes. Allowed to cool slowly at room temperature. pH was adjusted by adding 0.1 volumes of 1 M Tris HCl (pH 7.4). Dispensed into aliquots and stored at -20°C.
0.5 M EDTA
186.1 g of Na₂EDTA.2H₂O powder was dissolved in 700 ml of water. EDTA does not dissolve completely until the pH of the solution reaches 8.0. pH was adjusted to 8.0 with 10 M NaOH. Finally ddH₂O was added to 1 litre and solution was autoclaved.

1 M CaCl₂
147 g of Calcium chloride (CaCl₂.2H₂O) was dissolved in 1 litre of ddH₂O and solution was sterilised by filtration with a 0.22- micron filter membrane.

1 M Tris (pH 6.8, 7.2, 7.4, 8.0, 8.5, 8.8)
121.1 g Tris base was dissolved in 800 ml of ddH₂O. pH was adjusted to desired value by adding concentrated HCl. Final volume was made up to 1 litre with ddH₂O and sterilised by autoclaving.

50 % Glycerol
50 ml of pure glycerol was added to 50 ml of ddH₂O and mixed thoroughly. Finally solution was autoclaved for 15 minutes at 121°C/15 lb/sq in.

70 % Ethanol
70 ml of pure ethanol was mixed with 30 ml of sterile ddH₂O to make up the total volume to 100 ml and stored at 4°C.

1 M DTT
3.09 g of Dithiothreitol was dissolve in 20 ml of 0.01 M sodium acetate (pH 5.2) and stored at –20°C after filter sterilization with 0.22-micron filter.

30% Acrylamide (29:1)
- Acrylamide: 29 g
- N, N’-methylenebisacrylamide: 1 g
ddH₂O was added to make up the total volume of 100 ml. The solution was filtered and kept at 4°C.

**10% Ammonium persulphate**
10 g ammonium persulphate powder was added in 100 ml of sterile ddH₂O, mixed and solution was kept at 4°C.

**Ethidium bromide**
100 mg ethidium bromide tablet was dissolved in 10 ml of water. Left overnight on 37°C shaker to dissolve the tablet completely and stored in dark at room temperature.

**10 M Ammonium acetate**
385.4 g of ammonium acetate was dissolved in 150 ml of ddH₂O. Volume was made up to 500 ml. Solution was sterilized by autoclaving for 15 minutes at 121°C/15 lb/sq in.

**dNTP's mix (dATP, dCTP, dGTP, dTTP)**
25 mM each dNTP was prepared in T₁₀E₁ buffer, pH 7.5. All the four dNTP's were combined at a final concentration of 2.5 mM each and stored in small aliquots at -20°C.

**1M IPTG solution**
2.73 g of IPTG powder was dissolved in 8 ml of sterile ddH₂O. Volume was made up to 10 ml with ddH₂O. The solution was sterilised by filtration through a 0.22 - micron disposable filter and stored into small aliquots at -20°C.

**Phenol: Chloroform: Isoamyl alcohol**
25 parts (v/v) Phenol (previously equilibrated in 150 mM NaCl / 50 mM Tris HCl, pH 7.5 and 1 mM EDTA) was mixed with 24 parts (v/v) Chloroform and 1 part
(v/v) of Isoamyl alcohol. This solution was stored in a dark coloured glass bottle at 4°C.

5 M NaCl
292.2 g of sodium chloride was dissolved in 800 ml water and made up the total volume to 1 litre with water. The solution was sterilised by autoclaving.

Protease inhibitors
All the protease inhibitor solutions were made as 100 X stock. They were added to the pre-cooled solutions just before use. All the protease inhibitor solutions are active for 3-4 weeks at a storage temperature of -20°C. The required concentration of the solutions is 1X.

- Leupeptin 100 μg/ml in ddH₂O
- Aprotinin 100 μg/ml in ddH₂O
- Trypsin inhibitor 100 μg/ml in ddH₂O

100 mM Phenyl methyl sulphonyl fluoride (PMSF)
174 mg of PMSF powder was dissolved in 10 ml of isopropanol. The solution was divided in aliquots and stored at -20°C.

50x TAE buffer
Tris base 242 g
Glacial acetic acid 57.1 ml
Na₂EDTA.2H₂O 37.2 g
Sterile ddH₂O was added to make up the total volume to 1 litre.

5 x Tris glycine buffer
Tris base 15.1 g
Glycine 72.0
SDS 5.0 g
ddH₂O was added to make up the total volume to 1 litre.
1 × Western transfer buffer
Tris base 3 g
Glycine 14.4 g
Methanol 200 ml
The volume was made up to 1 litre with ddH₂O.

6 × Gel loading buffer
Bromophenol blue 0.25% (w/v)
Xylene cyanol FF 0.25% (w/v)
Ficoll (Type 400) 15% (w/v)
ddH₂O was added to make up the total volume of 10 ml. Stored at room temperature.

Alternatively, the DNA loading dye was made with following components.
Bromophenol blue 0.25% (w/v)
Xylene cyanol FF 0.25% (w/v)
Glycerol 30% (v/v)
ddH₂O was added to make up the total volume of 10 ml. Stored at 4°C.

PBS (Phosphate Buffered Saline)
NaCl 100 mM
KCl 4.5 mM
Na₂HPO₄ 7 mM
KH₂PO₄ 3 mM
Water was added to make up the total volume and sterilised by autoclaving. The buffer was stored at 4°C.

Sodium carbonate/ Bicarbonate buffer pH 9.6
Na₂CO₃ Stock (A) 0.2M
NaHCO\textsubscript{3} Stock (B) 0.2 M

13 ml of stock A was mixed with 37 ml of stock (B) to get the desired pH and water was added to make the volume to 200 ml. stock solutions were autoclaved. The coating buffer pH9.6 and the stock solutions were stored at 4° C.

3.1.3 Recipes for Separating gel and Stacking gel

12% Separating gel 20.0 ml
30% Acrylamide mix 8.0 ml
(29% Acrylamide &
1% Bis-acrylamide)
1.5M Tris-Cl, pH 8.8 5.0 ml
10% SDS 0.2 ml
10% APS 0.2 ml
TEMED 0.008 ml
Distilled water 6.6 ml

Stacking Gel - 5.0ml
30% Acrylamide mix 0.83 ml
1.0 M Tris-Cl, pH 6.8 0.63 ml
10% SDS 0.05 ml
10% APS 0.05 ml
TEMED 0.005 ml
Distilled water 3.4 ml

2 X Sample buffer 100ml
Trizma base 1.52 gm
Glycerol 20.0 ml
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<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>SDS</td>
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<tr>
<td>β–Mercaptoethanol</td>
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**Electrophoresis buffer**

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<td>SDS</td>
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<td>pH adjusted to 8.3.</td>
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</table>

3.1.4 DNA modifying enzymes and reagents

The enzymes used for DNA manipulation were purchased from Boehringer Mannheim (Germany) and MBI Fermentas (USA). The oligonucleotides were obtained from Monica Talmor, Yale University Medical School (USA) and Microsynth (Switzerland).

3.2 Methods

3.2.1 Rapid isolation of plasmids form *E. coli* cells (colony preparations)

Preliminary screening of *E. coli* transformants to check the plasmid content and size, direct colony prep was done in order to screen large number of transformants. For this, colonies from petriplates were directly transferred to a microfuge tubes containing 25 μl of solution I (GTE; 50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0) and vortexed thoroughly. Equal volumes of freshly prepared solution B (30% glycerol, 0.25% bromophenol blue, 1% SDS,
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0.2 N NaOH) was added in the microfuge tube and vortexed. The mixture was incubated at \(-70^\circ C\) for 10 minutes and then loaded on the agarose gel and the bands were visualized under UV.

### 3.2.2 Plasmid isolation (mini preparations)

Mini preparation was done to obtain plasmid DNA in reasonable amounts for purification, restriction digestion and ligation. For this a total of 5 ml of the overnight grown culture was pelleted in turns in microfuge tubes at 12,000 x ‘g’ for 2 minutes. The supernatant was discarded and 250 µl of solution I (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0) was added to the cell pellet and resuspended by vigorous vortexing. 400 µl of freshly prepared solution II (0.2 N NaOH, 1 % SDS) was added and mixed gently by inverting the contents of the tube. Finally 350 µl of ice-cold solution III (5M potassium acetate, glacial acetic acid) was added, mixed well and stored on ice for 10 minutes. The microfuge tube was centrifuged at 12,000 x ‘g’ for 10 minutes at 4°C and the supernatant was transferred to a fresh tube. Equal volumes of P:C:I mix was added to the supernatant and mixed by vortexing. The mixture was centrifuged again for 10 minutes at 12,000 x ‘g’ and the upper aqueous phase was pipetted out into a new microfuge tube and 0.7 volumes of isopropanol was added at room temperature and mixed well. The mixture was centrifuged again at 12,000 x ‘g’ for 30 minutes at room temperature. The pellet was washed twice 100 µl 70% ethanol and dried in a dry bath. The dried pellet was finally suspended in 75 µl of T10 E1 and treated with RNase A.

### 3.2.3 DNA quantitation

The concentration of DNA was determined by spectrophotometry in UV range. Optical density (OD) of DNA solution was measured at 260 nm and 280 nm with appropriate blank of the solvent in which DNA was dissolved, using quartz cuvettes. An OD at 260 nm = 1 was considered equivalent to concentrations 50
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µg/ml for double stranded DNA and 20 µg/ml for single stranded oligonucleotides. The ratio of $\text{OD}_{260}/\text{OD}_{280}$ was determined to check the purity of the DNA preparation. The ratio of protein free pure DNA should be 1.8 to 2.0.

3.2.4 Preparation of competent E. coli cells

The competent cells were prepared with a slight modification in the standard protocol (Sambrook et al. 1989). A glycerol stock of E. coli cells was streaked on LB agar plate using four-flame method. A single colony was picked and inoculated in 5 ml of LB broth and incubated overnight at 37°C with shaking. After 16 hours, 500 µl of the culture was used as inoculum (1% final concentration) for a 50 ml LB broth. Cells were grown to an OD of 0.3-0.5. The cells were chilled on ice and then transferred to a pre chilled sterile oakridge tube under aseptic conditions. The cells were centrifuged at 2,500 x ‘g’ for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 25 ml of chilled 100 mM CaCl₂ and incubated on ice for 30 minutes. The cells were centrifuged again at 2,500 x ‘g’ for 5 minutes at 4°C. The pellet was resuspended in 5 ml of 100 mM CaCl₂ and 50% glycerol was added to it to a final concentration of 15%. The cells were kept on ice for 2-3 hours and were finally stored at – 70°C as 200 µl aliquots. Next day one aliquot was used to transform with 10 ng DNA of a standard plasmid in order to check the efficiency of the competent cells as number of transformants per microgram of supercoiled plasmid DNA.

3.2.5 Transformation of E. coli cells

A 200 µl aliquot of competent cells was thawed on ice. 10 ng of plasmid DNA was added to the thawed cells and incubated on ice for 30 minutes. Then cells treated with heat shock by keeping the cells in water bath set at 42°C for 90 seconds. The cells were immediately transferred onto ice and 800 µl of
autoclaved LB broth was added to it. The cells were kept on incubator shaker set at 37°C, for 1 hour. Out of 1 ml culture, 25 μl of cells were plated on a LB agar plate containing an appropriate antibiotic for selection of transformants. The LB plate was incubated at 37°C for 12-16 hours for the emergence of colonies.

3.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel apparatus with 1X TAE as electrophoresis buffer. As per requirement, 1.0 to 1.5% agarose was dissolved in 1X TAE buffer by heating it in a boiling water bath. After allowing it to cool to 40 - 45°C, ethidium bromide was added to it to a final concentration of 1μg/ml. The gel was set by pouring it into a casting tray in which a comb of desired tooth size was inserted at one end to form wells. After the gel had set, the comb was removed and the gel was transferred to the gel tank filled with the electrophoresis buffer. Samples mixed with loading buffer were loaded into the wells. The gel was run at a constant voltage of 4-5 V/cm. The DNA bands were visualised under 260 nm UV light on a trans-illuminator.

3.2.7 Purification of MBP-GMCSF through amylose affinity column

We had decided to use periplasmic preparations of the fusion MBP-GMCSF protein for amylose column chromatography because periplasmic fractions of recombinant proteins are contaminated with less E. coli proteins and proteases. The yields of fusion protein are affected by a) the concentration of IPTG used to induce the cell cultures (recommended between 0.3mM and 0.5mM IPTG concentrations) as it affects the flux of fusion proteins to the periplasm, b) by the method used to prepare periplasmic fraction (periplasm is being prepared by using many methods which differ at the use of 5mM MgSO₄ solution volume). Hence we decided to optimize both for use of IPTG concentration and method to be used for periplasmic fraction preparation. Two, 100ml TB medium containing 1L flasks, inoculated with 1ml of an over night grown culture of cells containing
cells the fusion plasmid (pMAL-GMCSF) were cultured at 37°C with shaking at 220 rpm were induced with 0.3mM IPTG and and 0.5mM IPTG respectively. Induced cultures were harvested at 4th hour post induction by centrifugation at 4000 X g for 20 mins. Cell pellets were resuspended in 40ml of buffer (30mM Tris-HCl, 20% sucrose, pH 8.0) (80ml buffer for each gram of cells wet wt). EDTA was added to a final concentration of 1 mM and cell suspension was incubated for 5-10 mins. At room temperature with slow shaking. METHOD I: after centrifugation pellet was resuspended in 40ml of ice-cold 5 mM MgSO4, METHOD II: pellet was resuspended in120ml of ice-cold 5 mM MgSO4. To these periplasmic fractions 1M Tris HCl, pH 7.4 was added to make the final concentration of the periplasmic fraction to 20mM Tris HCl, equivalent to the column buffer. After this 5 ml of New England Biolabs amylose resin was pre-washed with wash buffer (20 mM Tris pH7.4, 200 mM NaCl, 10 mM β ME, 1mM EDTA) and the slurry loaded on a 25 ml column. Periplasmic fractions were then loaded onto wash buffer equilibrated amylose column. The unbound proteins were eluted from amylose resin by washing resin with 10 column volumes of 20 mM Tris pH7.4, 200 mM NaCl, 10mM β-ME, 1 mM EDTA. The bound MBP-GMCSF fusion protein was eluted with 10 fractions of 10 mM maltose (this is an optimized method, NEB-UK) in 20 mM Tris pH 8, 200 mM NaCl, 10 mM β ME. Results were confirmed on SDS-PAGE.

3.2.8 Factor Xa cleavage

To the eluted MBP-GMCSF, CaCl2 was added to a final concentration of 2 mM, then 1 unit of factor Xa per 50 mg of eluted fusion protein was added and incubated for 6 hours. Digestion was confirmed on SDS-PAGE.

3.2.9 Inclusion body isolation & purification

The pET28a-GM-CSF / BL21 (DE3) cells were harvested after four hours of post-induction. Cell pellet from 1L of induced culture was used for further purification
steps to generate purified inclusion bodies. The cell pellet was resuspended in 50 ml of sonication buffer (50 mM Tris buffer, 5mM EDTA, 1mM PMSF pH 7.4) and sonicated to ensure complete lysis. The lysate was centrifuged at 14,000 x 'g' for 20 minutes. The pellet was resuspended in 15 ml of 50 mM Tris buffer pH 8.0, containing 1% Deoxycholic acid, known to dissolve membrane proteins (Marston, 1986), and incubated for one hour on a rotary shaker. Optimum deoxycholic acid concentration was found out to be 0.5%, but it gave high background of contaminating bands hence 1% concentration which tend to dissolve the GM-CSF IB also but gave fairly pure GM-CSF band, was chosen (seen in the pilot experiment, data not shown). The suspension was centrifuged as earlier and the supernatant was discarded. This step was repeated once. The pellet was washed twice with 50mM Tris buffer pH 8.0 and then twice with ddH$_2$O. Fractions collected at various steps were analysed on a 12% SDS-PAGE gel. A fairly pure protein band with very less contamination was seen on the gel.

A fraction of the inclusion bodies was used to do a pilot experiment to find the concentration of urea required to fully dissolve the GM-CSF inclusion bodies. Same amount of inclusion bodies (IB) was resuspended in five different denaturing buffers: 2M, 4M, 6M, 8M urea and 5M guanidinium hydrochloride in 50mM Tris buffer, pH 8.0. These GM-CSF IBs suspensions were kept on shaking for 30min.s and then centrifuged. Both supernatant and pellet samples were prepared and run on 15% SDS-PAGE. 8M urea and 5M GdmHCl showed complete IB dissolution. In our further experiments we used 8M urea as the IB denaturant instead of GdmHCl because GdmHCl precipitates more often than urea, making its use cumbersome.

### 3.2.10 Anion-exchange chromatography purification of GM-CSF IBs and partial refolding

GM-CSF IBs were subjected to anion exchange chromatography. IBs were resuspended in denaturation buffer 50mM Tris, 8 M urea, pH 8.0 (the PI of the asparagenase signal sequence containing GM-CSF is 5.8). The column purification was done in the denatured conditions. 9 ml of the MonoQ HR 5/5
anion exchanger slurry (50% slurry, sigma) was packed up into a column. Column was equilibrated with the same denaturing buffer used to denature the IBs. Protein sample was loaded onto the column; flow-through was collected and again loaded onto the column to ensure proper protein loading. Column was washed with 120 ml of the denaturation buffer. The bound GM-CSF was eluted using elution buffer (50 mM Tris, 8M Urea, 400mM NaCl). The eluting NaCl concentration was determined in a pilot experiment performed with 2 ml column volume. The bound GM-CSF was subjected to step gradient elution with NaCl concentration ranging from 100mM, 200mM......1M NaCl in 50 mM Tris buffer. One column volume of each NaCl concentration was passed and eluted fractions were collected in the volume of 0.5 ml. Eluted samples were run on 12% SDS-PAGE. Our protein started eluting with 300mM NaCl and peaked with 400mM NaCl. Preliminary visual protein estimation was done on 12% SDS-PAGE and then with GM-CSF ELISA kit (Duo ELISA kit, R & D). Single protein band was observed on the protein gel. Partially purified protein sample in denatured state was then subjected to dilution renaturation in the presence of glutathione redox system (Lilie, et al. 1998; Ahmed, et al. 1975; Wetlaufer, et al. 1987) with renaturation buffer [50mM Tris, 2M urea, 10% sucrose, 1mM EDTA, 1mM PMSF, 3mM Glutathione (reduced), 0.5mM (oxidized), 0.4M NaCl, 0.5M Arginine, pH 8.0]. Pulse dilution (Rudolph, 1990) was performed at 4° C, solution being stirred slowly overnight.

3.2.11 Mice immunizations and spleen lymphocyte isolation

BALB/c mice of 2-4 weeks of age were immunized with rhGM-CSF to illicit an immune response. Initial immunization: 25ug antigen / mouse, subcutaneously in CFA (Freund’s complete adjuvant) (sigma immunoChemicals), 0.1ml / mouse. Equal volumes of antigen in PBS and adjuvant were mixed and emulsified by passing through a 18 gauge needle several times till non dispersible drop of the emulsified antigen is formed in water/ saline. Mice were bled from tail vein to collect preimmune serum. 1st booster after two weeks: 20ug / mouse,
intraperitonealy, in IFA (Freund’s incomplete adjuvant) (sigma), 0.1ml / mouse. After one week mice were again bled to collect immunized sera, to test antibody response in the mice. ELISA against rhGM-CSF was performed. Sera gave positive signal. 2nd booster was given on 4th week: 15ug / mouse, intravenously, in PBS, 0.1ml / mouse. After four days of the last booster mice were sacrificed for their spleen tissue. Spleen tissue was reduced to single cell suspension by disruption and homogenization of the tissue material with the frosted slides in IRPMI 1640 (incomplete) media. Spleen tissue contains RBCs (red blood cells) in abundance. Hence spleen single cell suspension was subjected to Ficoll-Paque density gradient separation of dead cells and RBCs. Dead cells and RBCs pass through the high density solution of Ficoll during centrifugation and live cells remain floating on the top of the high density material. Single cell suspension was diluted with the IRPMI and was layered over the Ficoll solution in 15 ml centrifuge tubes such that two solutions boundaries did not mix (Ficoll to cell suspension ratio is 1:3). This set up was then centrifuged at 1500 rpm (800 X g) in swing- bucket rotor mini-centrifuge for 25 mins at room temperature. Lymphocyte were recovered from the top of the ficoll solution layer, cells were pelleted and washed with IRPMI and finally resuspended in 1 ml of IRPMI. Cells were counted using hemocytometer.

3.2.12 Total RNA isolation

Isolation of total RNA from spleen cells was done by using RNeasy Mini Kit (Qiagen). Following the standard protocol cell lysis was accomplished by incubation of the sample in a special lysis / - binding buffer. At the same time, RNases were inactivated. In the presence of chaotropic salt (guanidinium hydrochloride) nucleic acids bind specifically to the surface of glass fibers. The binding reaction occurs within seconds due to the disruption of the organised structure of water molecules and the interaction with nucleic acids. Thus, adsorption to the glass fiber fleece is favoured. The binding process is specific for nucleic acids, but the binding conditions are optimised for RNA. Rest of
contaminating DNA was digested by DNase I, applied directly on the glass fiber fleece. The bound RNA was purified from salts, proteins and other cellular impurities by simple washing steps and was eluted in sterile RNase free water. Total RNA was estimated spectrophotometrically at 260 nm. An O.D. of 1 measured at 260nm corresponds to 40μg of RNA per ml.

3.2.13 cDNA synthesis and generation of VH and VL gene repertoire

Total RNA generated above contains maximum population of mRNAs. It was used to generate cDNA using oligo dT primer provided with the RT-PCR kit (Qiagen). RNA was incubated at 65°C for 10 min., immediately was cooled on ice and within 2 min was used in the following RT reaction.

RNase free H₂O 54 μl
10 mM dNTP 5 μl
10X RT buffer 10 μl
mRNA (2.5μg) 10 μl
oligo dT primer (10μM) 10 μl (Final conc.1μM)
0.1M DTT solution 10 μl
Reverse transcriptase enz. (40U/μl) 1 μl

Above reaction mixture was incubated at 37°C for one hour. This experiment generated mRNA:cDNA (first strand mix) hybrid which was used to prime the PCR amplification of VH and VL gene repertoires.

Above reaction was divided into two tubes named VH and VL (Table 3.3)
Table 3.3: PCR reaction mixture for VH and VL gene repertoire amplification.

<table>
<thead>
<tr>
<th>VH amplification</th>
<th>VL amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>First strand mix</td>
<td>First strand mix</td>
</tr>
<tr>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>10X taq buffer</td>
<td>10X taq buffer</td>
</tr>
<tr>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>10mM dNTPs</td>
</tr>
<tr>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>VH1 FOR2 primer</td>
<td>MJK mix primer</td>
</tr>
<tr>
<td>(25pmol/µl)</td>
<td>(25pmol/µl)</td>
</tr>
<tr>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>VH1 BACK primer</td>
<td>Vx2 BACK primer</td>
</tr>
<tr>
<td>(25pmol/µl)</td>
<td>(25pmol/µl)</td>
</tr>
<tr>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Taq enz. (10U/µl)</td>
<td>Taq enz. (10U/µl)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>sterile H₂O</td>
<td>sterile H₂O</td>
</tr>
<tr>
<td>79.5 µl</td>
<td>79.5 µl</td>
</tr>
</tbody>
</table>

(MJK mix is equimolar mixture of MJK1FONX, MJK2FONX, MJK4FONX, MJK5FONX primers)

This PCR mixture was subjected to amplification according to the following PCR cycle (Table 3.4).

Table 3.4: PCR reaction conditions for amplification of the VH and VL gene fragments

<table>
<thead>
<tr>
<th>1 cycle</th>
<th>30 cycles</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C, 5 min.</td>
<td>94°C, 30 sec.</td>
<td>72°C, 7 min.</td>
</tr>
<tr>
<td>50°C, 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72°C, 1 min.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.14 PCR amplification of linker molecule

Linker molecule sequence was synthesized from microsynth (USA). The sequence is:

5’ GGC ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GCC GGT GCC GGA TCG GAC ATC GAG CTC ACT CAG TCT CCA 3’.

This linker sequence creates a 15 amino acid (Gly₄Ser)₃ sequence between the heavy and light chain variable domains. Linker molecule was PCR amplified
using LINK FOR and LINK BACK primers listed in table 3.1, according to the PCR programme described in the Table 3.4.

3.2.15 Gel elution of DNA from agarose gels

DNA was extracted from agarose gel using Agarose Gel DNA Extraction Kit (Novagen, USA). Nucleic acids bind specifically to the surface of glass or silica materials in the presence of a chaotropic salt (sodium perchlorate). The binding reaction occurs due to the disruption of the organised structure of water molecules and the interaction with the nucleic acids. Thus the adsorption to the specifically pretreated spherical silica matrix is favoured. Since the binding process is specific for nucleic acids, the bound material can be separated and purified from impurities e.g. salts and proteins, by a simple washing step. Nucleic acids elute from the matrix in a low salt buffer or water.

3.2.16 Assembly of single chain Fv antibody fragments

\( V_H, V_L \) and linker gene fragments were assembled using PCR reaction. Equimolar amounts of all three components were mixed. This reaction has two PCR steps: a) assembly PCR and b) restriction site addition PCR.

First PCR was set in 50 \( \mu l \) reaction volume in a separate chamber that was used for PCR exclusively to prevent contamination. Approximately 50 ng of \( V_H \) DNA, 50 ng \( V_L \) DNA and 10 ng of the linker gene fragments were mixed. Final concentration of dNTP’s and MgCl\(_2\) was kept 20 mM each and 1.75 mM respectively. Commercially available stock of reaction buffer was added such that final concentration was 1X in reaction. 3.0 unit of Taq DNA polymerase enzyme was used depending on the fidelity desired. The volume was made up with sterile milli Q water. The thermal cycler (Perkin-Elmer) was programmed according to the following programme (Table 3.5 a).
Table 3.5(a)

<table>
<thead>
<tr>
<th>7 cycles</th>
<th>94°C for 1 min.</th>
<th>63°C for 4 min.</th>
</tr>
</thead>
</table>

In the second PCR, the assembled scFv DNA is amplified and restriction sites are added. A bulk primer mix was prepared by adding the following components.

**Bulk primer mix.**
- 10X taq buffer: 5 µl
- 10 mM dNTPs: 1 µl
- JK mix primer (25pmol / µl): 2 µl
- VH1 BACKNco1 primer (25pmol / µl): 2 µl
- Taq enz. (10U/ µl): 0.5 µl
- sterile H₂O: 39.5 µl

(JK mix primer is equimolar mix of JK1NOT10, JK2NOT10, JK4NOT10, JK5NOT10 primers).

This bulk primer mix was added to the assembled scFv reaction and second PCR reaction was run according to the following programme (Table 3.5b).

Table 3.5(b)

<table>
<thead>
<tr>
<th>1 cycle</th>
<th>30 cycles</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C, 5 min.</td>
<td>94°C, 30 sec.</td>
<td>55°C, 1 min.</td>
</tr>
</tbody>
</table>

3.2.17 Restriction digestion of DNA

DNA samples were digested with restriction endonucleases in the appropriate buffers supplied by the manufacturer (MBI fermentas) at the recommended conditions. For routine analysis 1µg to 3 µg of DNA was used, all the reactions were set up in 20 µl for restriction analysis. Restriction digestion for ligation
purposes was done in 50 µl of final volume. The reaction mix after completion of digestion was heat inactivated at 65°C for 20 minutes. In cases where double digestion was required, DNA molecules were digested simultaneously in 1X/2X Y-Tango buffers supplied by manufacturer (MBI fermentas).

3.2.18 Ligation of DNA fragments

The DNA fragments digested with restriction endonuclease(s) were mixed with the vector digested with the same restriction enzyme(s) or vector having compatible ends. Usually 30 to 50 ng of vector DNA, 3 to 6 fold molar excess of insert and 0.5 U of T4 DNA ligase were used in ligation set up. The reaction was set up in final volume of 10 µl and the reaction mix was incubated at 22°C for 16 hours. The reaction mixture was heat inactivated at 65°C for 20 minutes prior to transformation of ligase mix in high efficiency competent cells.

3.2.19 DNA sequencing

The sequence of the DNA fragments of scFv cloned in pET22bvector (Clone 4, 5, 56, and 196) were confirmed by sequencing of the constructs by automated sequencing at the DNA sequencing facility available at School of Life Sciences (SLS), JNU, New Delhi.

3.2.20 Protein gel electrophoresis

Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method described by Laemmli (1970). The stacking gel containing 4% acrylamide, 0.106% N, N’-methylene bisacrylamide, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8) were mixed and polymerized. The separating gel had 12% or 15% acrylamide depending on the case and 0.1% SDS. Running buffer consisted of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 containing 0.1% SDS. The protein samples were prepared in
sample buffer [0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and 5% β-mercaptoethanol (Laemmli, 1970)] and immersed in a boiling waterbath for 3-5 minutes. Standard marker protein mixture (Boehringer, Bangalore Genei) was run simultaneously to calculate the subunit molecular size of the proteins.

3.2.21 Coomassie Brilliant Blue staining

SDS-Polyacrylamide gels containing more than 200 ng protein concentration were visualised by standard Coomassie Brilliant Blue R 250 (CBB R 250) staining solution {0.1% (w/v) CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water}, followed by de-staining in 25% (v/v) methanol and 10% (v/v) acetic acid in water.

3.2.22 Western Blotting of proteins

The proteins were separated on a 12.5% SDS-PAGE and transblotted onto a nitrocellulose (NC) membrane in a buffer containing 25 mM Tris HCl pH 8.3, 192 mM Glycine and 20% methanol (Towbin et al. 1979). After the transfer, the NC membranes were incubated in PBST (10 mM) containing 2% BSA for 90 minutes to block additional protein binding sites. After a brief wash with PBST, the membrane was incubated with anti-GM-CSF/anti 6 X His tag monoclonal antibodies (mouse IgG, 2µg/ml and 1:5,000 dilution respectively) for 1 hour at RT with gentle rocking. Then the membrane was washed 3 X 10 minutes in PBST and incubated in anti-mouse IgG conjugated to Horse Radish Peroxidase (HRPO; 1:10,000 dilution) at RT for 1 hours with gentle rocking. Finally, the membrane is washed 3 X 10 minutes in PBST and the immunoreactive bands were visualized by 10 mg DAB and 20 µl H₂O₂ solution in 10 ml of 100 mM Tris HCl, pH 7.6, until the bands develop to the desired intensity.
3.2.23 ELISA Sample preparation of expressed scFvs

In a pilot experiment it was established that scFv cloned in pET22b expression vector, upon induction with IPTG (1mM), forms inclusion bodies inside E. coli cytoplasm. To screen the GM-CSF scFv among the whole expressed library clones, small scale, rapid sample preparation method was devised. 1ml pellets of the expressed clones were resuspended in 2% SDS (sodium dodecyl sulphate) solution, in 100μl volume. This cell suspension was then incubated in boiling hot water bath for 30 sec. This makes the suspension a clear solution. 2N SDS solubilizes the inactive proteins (Tanford, 1968). These samples were then diluted by a factor 10^3, thereby reducing the SDS concentration below its effective denaturation and on the other hand partially renaturing the solubilized, denatured proteins to their active form by simple air oxidation (Anfinsen, et al. 1961).

This sample preparation was used for the very first ELISA screening where hundreds of the samples were handled. Later for the few selected scFv clones, another methodology was used. Here we first prepared scFvs inclusion bodies (as in section 3.2.7) and then subjected them to denaturation, followed by dilution renaturation. Four hour induced 10 ml cultures of scFv were harvested. cell pellets were resuspended in sonication buffer (50mM Tris-Cl, 5mM EDTA, 1mM PMSF, pH 8.0) and sonication was performed at 4°C (30 seconds burst / 30 seconds cooling / 200-300 W) for 5 cycles. Inclusion bodies were pelleted and washed. The 5μg of each scfv protein was resuspended in 100μl of denaturation buffer (50mM Tris, 8M Urea, 1.5mM DTT) and incubated at room temperature for half an hour and then 5μg/100μl protein was diluted to 0.5μg/ml in PBS. 0.5μg/ml concentration of every scFv was used in ELISA assay against rhGM-CSF.

3.2.24 ELISA

The microwells were coated by incubating 100μl of antigen (mouse anti-human GM-CSF) at 5μg/ml concentration in coating buffer (carbonate/ bicarbonate buffer, pH 9.6) in each well of a 96 well micro ELISA plate at 4°C O/N.
plates were washed thrice by filling each well with buffer PBS-Tween20 (400μl) using a squirt bottle. Then the coated plates were blocked with 3% BSA (300μl/well) for 2hr. at RT in a humid chamber. After washing, 100μl / well of primary antibody (diluted in PBS-Tween20) was added to all wells excepting in the control wells and incubated for 2h at RT in the humid chamber. After this the plates were again washed thrice with PBS-Tween20. 100μl of detection antibody (biotinylated mouse anti-human-GM-CSF) or secondary antibody (HRP linked anti His tag antibody) at a concentration of 0.5 μg/ml or 1:10000 dilution respectively was added to the wells and incubated at RT. In case of the biotinylated antibody, 100μl / well Streptavidin-HRPO at 1/200 was added to each well and incubated for 20 minutes at RT. The plates were again washed thrice with PBS-Tween20. Colour was developed by adding 100μl of substrate solution (20X TMB-H₂O₂ solution, Bangalore Geni, India) at 1:20 per well and incubating in the dark for 20 minutes. Stop solution (2N H₂SO₄) was added and the absorbance was read at 450nm.

3.2.25 Growth media and conditions for expression studies of scfvs

For expression studies primary batch cultures were grown overnight in LB medium (10 ml media in 100ml flasks) with ampicillin (100 μg/ml) at 37°C with shaking at 220 rpm. 1% of the overnight grown culture was inoculated in Luria Bertani (LB) media, modified LB media and buffered Terrific Broth (TB) media (yeast extract 24 gm, tryptone 12 gm, 0.4% glycerol), cells were cultivated in 500 ml flasks with 50 ml media. Cells were grown to an OD₆₀₀ of 0.8 - 2.5 (in LB and TB respectively) and induced with 1mM IPTG (final concentration). Cells were cultivated for 24 hours post induction, samples were collected and the cells were harvested by centrifugation at 4000 x ‘g’ for 10 minutes at 4°C. Cytoplasmic soluble, cytoplasmic insoluble and periplasmic fractions were prepared from the collected samples and analyzed for expression.
3.2.26 Periplasmic localization

The pellet from 1 ml of culture was resuspended in 500 μl of 30 mM Tris HCl, pH 8.0, 20% sucrose and 1 mM EDTA pH 8.0 and incubated on ice for 15 minutes. Centrifugation was done at 8,000 x ‘g’ at 4°C for 10 minutes, supernatant was discarded and the pellet was resuspended in 500 μl ice cold 5 mM MgSO₄. Sample was incubated on ice for 15 minutes and centrifugation was done at 10,000 x ‘g’ at 4°C for 10 minutes. Supernatant (periplasmic extract) was collected.

3.2.27 Cytosolic localization

The pellet from 1 ml culture was resuspended in 0.4 ml of sonication buffer (25 mM Tris HCl, pH 8.0). Sonication was done at 4°C (30 seconds burst / 30 seconds cooling / 200-300 W) for 5 cycles. Centrifugation was done at 10,000 x g for 15 minutes at 4°C. The supernatant was collected and the pellet was saved for the localisation of the protein in inclusion bodies.

3.2.28 Inclusion body localization

The pellet from the cytoplasmic fraction was resuspended in 8 M urea, 25m M Tris HCl, pH 7.8 and incubated at 37°C for 45 minutes. Centrifugation was done at 10,000 x g for 30 minutes at room temperature and the supernatant (inclusion bodies) was collected.