CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS AND CHEMICALS

Chemicals of analytical grade were purchased from Sigma Chemical Company, St. Louis, USA and the components required for preparing the bacterial growth media were bought from HiMedia, Mumbai, Chennai. Reverse transcriptase enzyme, Restriction enzymes, T7 DNA polymerase and T4 DNA ligase were obtained from New England Biolabs, Beverly, MA, USA and taq polymerase was from Genecraft, Lüdinghausen, Germany and Genei, Bangalore, India. Oligonucleotide primers for PCR were synthesized from Microsynth, Balgach, Switzerland. QIAquick PCR purification kit from Quiagen Gmbh, Hilden, Germany was used to purify the PCR products and restriction enzyme digested plasmids and inserts DNA molecular weight markers were purchased from Genecraft, Lüdinghausen, Germany and Amersham Pharmacia Biotech, Piscataway, NJ, USA. The protein molecular weight marker was obtained from Genei, Bangalore, India. The chelating sepharose for purification of histidine tagged recombinant protein and Hybond- C nitro cellulose membrane for Western blotting were procured from Amersham Pharmacia Biotech, Piscataway, NJ, USA. 4-nitrophenyl stearate was purchased from Sigma Chemicals. The precipitatable substrate for HRP - DAB, the precipitatable substrates for ALP - NBT and BCIP and the soluble substrate for ALP - pNPP were obtained from Sigma, St.Louis, USA. Mouse anti-histidine monoclonal antibody was obtained from Amersham Pharmacia Biotech, USA.
2.2 BACTERIAL STRAINS AND PLASMIDS

DH5α strain of *E. coli* was obtained from Invitrogen, CA, USA. GJ1158 strain of *E. coli* was obtained from Genei, Bangalore, India. Genotypes of the *E. coli* strains that were used in this study are given in Appendix 1. *Serratia marcescens* strain isolated and characterized in CBT, Anna University, Chennai, India was used as template for genomic DNA isolation. T7 expression vector pRSET A was purchased from Invitrogen, CA, USA. The map and the restriction sites present in the MCS of pRSET A are shown in Appendix 1.

2.3 CULTURE MEDIA

Luria Bertani (LB) broth was used for the propagation of DH5α. The LB broth was prepared by dissolving 10 g of tryptone, 3 g of yeast extract and 5 g of sodium chloride in 1 litre of distilled water and the pH was adjusted to 7.2–7.4 with 1 N NaOH. GYE medium without NaCl in M9 salts mixture was used for the growth and induction of *E. coli* GJ1158. GYE medium comprising of 1.0% glucose, M9 salts, yeast extract, 2 mM magnesium sulphate and 1X trace metals mix. Stock solution comprised 1000X trace metal mixture containing 50 mM FeCl₃, 20 mM CaCl₂, 10 mM each of MnCl₂ and ZnSO₄, and 2 mM each of CoCl₂, CuCl₂, NiCl₂, Na₂MoO₄, and H₃BO₃ in Millipore water. To prepare solid medium, 2% agar was added to the LB or GYE broth. Media was supplemented with 100 μg / ml of ampicillin wherever required.

2.4 CLONING OF LIPASE AND MUTANT LIPASE GENES

Genomic DNA was isolated from wild type *Serratia marcescens* and used as a template for the amplification of 1842 bp lipase gene, 957 bp
N-terminal lipase domain and 885 bp C-terminal lipase domain. The amplified lipase gene fragment was cloned into pRSET A, a T7 expression vector at BamH1 and Hind III sites. Vector and PCR product were ligated using T4 DNA ligase. N-terminal and C-terminal gene fragments were also cloned similarly into pRSET A. The recombinant clones (named as pLNC615 – lipase, pLN320 – N-terminal lipase, pLC295 – C-terminal lipase) was transformed into DH5α strain of E.coli. Transformants were screened for presence of the insert by PCR using insert specific primers. Specific plasmids were extracted from the positive transformants and double digest using restriction enzymes to confirm the insert. The orientation of the insert was analysed by PCR using different combinations of T7 primers and insert specific primers.

2.4.1 Genomic DNA isolation

400 µl of Serratia marcescens culture grown overnight was vortexed with 5 µl of Nonidet P-40. Mixture was incubated at room temperature for 20 minutes and then centrifuged for 10 minutes at 10000 rpm. The supernatant was discarded and pellet was resuspended in 200 µl of high salt buffer (10mM HCL, pH 7.6; 10mM MgCl2 and 2mM EDTA, 0.4M NaCl). To the mixture 20 µl of 10% SDS was added. Suspension was mixed thoroughly by vortexing, and incubated for 10 minutes at 55°C, after which 125 µl of 5M NaCl was added and mixed well. Then the mixture was centrifuged at 10000 rpm for 5 minutes and the supernatant containing DNA was collected. The DNA was recovered by ethanol precipitation and dried. The dried DNA was suspended in 20 µl TE buffer.

2.4.2 Primers used for the amplification and cloning

Insert specific primers with appropriate restriction sites were designed for PCR-amplification of the Serratia marcescens lipase gene.
T7 promoter forward and reverse primers were also used to find out the orientation of the lipase gene fragment in the recombinant vectors. The sequence of the primers, their restriction sites and annealing temperature used for PCR amplification are given in Table 2.1.

### Table 2.1 Primers used for cloning lipase and its mutants into pRSET A

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Length</th>
<th>Annealing Temperature</th>
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<tbody>
<tr>
<td>pLNC615 (Forward)</td>
<td>CGCCGATCCGGCATCTTTAGCTATAAG</td>
<td>27</td>
<td>55 ºC</td>
</tr>
<tr>
<td>pLNC615 (Reverse)</td>
<td>CCCAAGCTTTTAGGCCAACACCACCTG</td>
<td>27</td>
<td>55 ºC</td>
</tr>
<tr>
<td>pLN320 (Reverse)</td>
<td>CCCAAGCTTTTACTGATAGAAGAACGG</td>
<td>27</td>
<td>55 ºC</td>
</tr>
<tr>
<td>pLC295 (Forward)</td>
<td>CGCGGATCCGATGGTCTGATGCGG</td>
<td>24</td>
<td>55 ºC</td>
</tr>
<tr>
<td>T7 promoter (Forward)</td>
<td>TAATACGACTCAGTTAGG</td>
<td>19</td>
<td>57 ºC</td>
</tr>
<tr>
<td>T7 promoter (Terminal)</td>
<td>TGCTAGTTATTGCTAGCGG</td>
<td>20</td>
<td>57 ºC</td>
</tr>
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</table>

### 2.4.3 PCR

The lipase gene, from *Serratia marcescens* was amplified by PCR on a MJ Minicycler, Watertown, Mass, USA for subcloning in T7 expression system. A reaction mixture containing

(i) 200µM of each dNTPs

(ii) 1X PCR buffer (50mM KCl, 10mM Tris.Cl, pH8.3)

(iii) 0.5M of each primer
(iv) 1 unit of Taq DNA polymerase and
(v) 20ng of template

were mixed. PCR was performed on a MJ Minicycler, Watertown, Mass, USA. The optimal annealing temperature of 55°C was used for all the primer sets. The PCR parameters used are as follows:

a) Initial denaturation 95°C 5 minutes
b) Denaturation 95°C 1 minute
   Annealing 55°C 1 minute
   Extension 72°C 1 minute

Steps in b were cycled for 35 times
c) Final extension 72°C 10 minutes

The amplified PCR products were analysed by agarose gel electrophoresis. For further manipulations the amplified PCR products were purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany). Analogous PCR was done for amplifying the N-terminal domain and C-terminal domain of lipase with pLNC615 as the template.

For screening transformants, a small portion of freshly grown transformant colony was picked using a sterile tooth pick and resuspended in 50µl of 0.1 X TE (1mM Tris and 0.1mM EDTA, pH 8.0) buffer. The cells were lysed by boiling for 10 minutes, snap-chilled on ice, centrifuged (10,000 rpm for 10 minutes) and 1µl of the supernatant was used as template in PCR.
2.4.4 Restriction digestion and ligation of DNA

Restriction enzyme digestions were performed using enzymes from Amersham Pharmacia Biotech Asia Pacific, Hong Kong in the manufacturers recommended buffers. Restriction enzyme digestions were performed as follows:

- DNA (3-4µg) 15µl
- Buffer (10X) 4µl
- Enzyme (2-3 units/mg of DNA) 5µl
- Deionised water 4µl
- BSA 3µl

The reaction mixture was incubated at 37°C for 4 hrs. The reaction was stopped either by heat inactivating the restriction enzymes or simply by the addition of loading dye that contains 50% glycerol. The restriction digestion was analyzed by agarose gel electrophoresis. For cloning, the restriction enzyme digested fragments were purified by QIAquick PCR purification kit and estimated by measuring the absorbance at 280nm and stored at -20°C till use.

The ligation mixtures, one with both the vector and the insert and the other with the vector alone as the control were prepared. In the control ligation instead of PCR product, deionized water was added. The ligation reaction was set up for 20 µl volume as follows:

- Restricted pRSET A 2 µl
- PCR product 8 µl
ligations were performed overnight (16 hrs) at 4°C. Molar ratios of 1:4 of vector to insert were used in the ligation reactions.

### 2.4.5 Confirming the orientation of the insert

The orientation of the insert in the recombinant plasmid, pLNC615, pLN320 and pLC295 were analyzed by PCR using different combinations of T7 primers and insert specific primers. The sizes of the PCR products obtained were compared with pRSET A map to find out the orientation of the insert.

### 2.5 EXPRESSION OF RECOMBINANT PROTEIN IN E.COLI

For storage and maintenance purposes, the recombinant plasmids pLNC615, pLN320 and pLC295 were transformed into an *E.coli* host DH5α which lacks T7 RNA polymerase and hence nonexpressing. For expression studies the recombinant plasmid was transformed into *E.coli* strain GJ1158. The T7 RNA polymerase gene is present as a chromosomal copy under the control of pro U promoter in the *E.coli* strain GJ1158, enabling the expression of the genes under the control of T7 promoter with the use of cheap and non toxic inducer NaCl (Bhandari and Gowrishankar 1997). The expected molecular weight of the expressed pLNC615, pLN320 and pLC295 along with the N-terminal fusion tag is 65 kDa, 35.2 kDa and 33.5 kDa respectively.
2.5.1 Expression kinetics

A single recombinant *E. coli* colony (pLNC615 in GJ1158) was picked using a sterile loop and resuspended in 50ml of GYE medium. The culture was grown at 37°C at 150 rpm. When the $A_{600}$ of the culture reached 0.4, the expression of the recombinant protein was induced by the addition of NaCl (0.4 M Nacl, final concentration) and grown for a further period of 2 hrs at 37°C. The cells were harvested by centrifugation (4,000 rpm, 20 minutes) and stored at -20°C till further use.

The cell pellets were solubilised in 1X SSB, electrophoresed on a 12% SDS-PAGE, followed by staining with Coomassie Brilliant Blue dye.

2.6 PURIFICATION OF INCLUSION BODIES

Recombinant protein was expressed as inclusion bodies in all three clones. 25 mg wet weight of cell pellet was suspended in 3ml of 50 mM Tris (pH 8.5), this mixture was subjected to sonication at 30 second pulse for 5 minutes. Sonicated sample was centrifuged (10000 rpm, 15 minutes at 4°C) and the supernatant decanted. The pellet was resuspended in 3 ml 50mM Tris (pH 8.5) and 1% sodium deoxy cholate solution. Mixture was again sonicated at 30 second pulse for 3 minutes. Further, sonicated sample was centrifuged (10000 rpm, 15 minutes at 4°C). The pellet obtained was washed and suspended in 3ml of 50 mm Tris (pH 8.5) and incubated at room temperature for 30 minutes. The incubated sample was centrifuged (10000 rpm, 15 minutes at 4°C), and the pellet was washed in Milli Q water. Washed pellet contained proteins in the form of purified inclusion bodies. Purified inclusion bodies were solubilized by stirring for 60 minutes in 20mM NaH$_2$PO$_4$, 0.5M NaCl, pH 7.0, 6M urea. The solution of purified solubilized inclusion bodies
was clarified by centrifugation at 10000Xg for 15 min at room temperature (Patra et al 2000).

2.7 IMAC

The solubilized inclusion body was purified by IMAC. The following procedure was used:

(a) Chelating sepharose column was packed by removing the alcohol content from it and washing 5-6 times with MilliQ water.

(b) The column was charged with 6% nickel chloride (NiCl₂) by passing the nickel chloride solution for 4 times each with 3 column volumes.

(c) The Ni²⁺ charged column was washed with MilliQ water for 4 times and was equilibrated with the binding buffer, pH 8.0 by passing the buffer through the column for 4 times each with 3 column volumes.

(d) The solubilized inclusion body was added to the column and allowed to bind with the matrix, overnight at 4°C. The volume of protein load was calculated based on the protein binding capacity of the sepharose matrix and the concentration of the purified inclusion body.

(e) The column was washed 10 times, each time with 3 column volumes with binding buffer containing 20 mM imidazole for removing non-specific binding.
Imidazole gradient of 0.5 to 2.5 M was used to optimize the elution and the recombinant protein was found to be eluting at 1M Imidazole concentration.

The eluted protein was refolded by pulse dilution.

The refolded protein was dialyzed overnight at 4°C against several changes of 0.1X PBS.

The purity of the eluted protein was analyzed on SDS-PAGE and it was stored at -80°C for further use.

2.8 LIPASE ASSAY

2.8.1 Procedure

The substrate 4-nitrophenyl stearate (1 mM) was dissolved in 50 µl undecane forming the hydrophobic phase and 50 µl of the aqueous phase containing lipase was taken in 0.5 ml eppendorf. The reaction was carried out at 37°C by shaking the mixture at 180-rev/min using gyrorotatory shaker for 45 minutes. The hydrolysis of the substrate took place at the aqueous-organic interface and the product 4-nitrophenol formed was transferred to borate buffer (prepared by adding 50 ml of 0.025M disodium tetraborate to 23.3 ml of 0.1M NaOH and adjusting the final volume to 100ml with double distilled water) of pH 10.6 and the liberated 4-nitrophenol was quantified spectrophotometrically at 400 nm as 4-nitrophenolate (Lakshmi et al 1999).

2.8.2 Lipase Activity

One unit of lipase activity is defined as the amount necessary to produce 1 micromole of 4-nitrophenol per minute at 37°C using 4-nitrophenyl stearate as substrate.
2.8.3 Specific Activity

Lipase specific activity is defined as lipolytic activity Units (U l\(^{-1}\)) per milligram (mg l\(^{-1}\)) of the total extracellular protein.

2.8.4 Productivity

Productivity is defined as lipolytic activity units (U ml\(^{-1}\)) per A\(_{620}\).

2.8.5 The Molar Absorptivity (\(\varepsilon\))

Dissolving 4-nitrophenol in borate buffer of pH 10.6 and plotting a standard graph calculated the molar absorptivity of 4-nitrophenolate. The \(\varepsilon\) was found to be 10.12 x 10\(^4\) L mol\(^{-1}\) cm\(^{-1}\) (Figure 2.1). The same assay procedure was followed for assaying the activity of the unknown enzyme sample.

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<th>Regression Coefficients</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>Std. Coeff.</th>
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<th>P-Value</th>
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<td>.043</td>
<td>.009</td>
<td>.211</td>
<td>.8378</td>
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<td>.627</td>
<td>.983</td>
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![Regression Plot](image)

**Figure 2.1 Standard graph for 4-nitrophenolate measured at 400 nm**
2.9 KINETICS

To measure the effect of temperature on the enzyme, purified and refolded protein was subjected to kinetic analysis at different temperature range. 0.5mM to 2.5mM of 4-nitrophenyl stearate was dissolved in 50 µl undecane forming the hydrophobic phase and 50 µl of purified protein was added to it. The reaction was carried out at temperatures ranging from 4°C to 65 ºC by shaking the mixture at 180-rev/min using gyrorotatory shaker for 45 minutes. The hydrolysis of the substrate took place at the aqueous-organic interface and the product 4-nitrophenol formed was transferred to borate buffer (prepared by adding 50 ml of 0.025M disodium tetraborate to 23.3 ml of 0.1M NaOH and adjusting the final volume to 100ml with double distilled water) of pH 10.6 and the liberated 4-nitrophenol was quantified spectrophotometrically at 400 nm as 4-nitrophenolate. Km and Vmax were obtained from Linewaver-Burk plots.

2.10 HEMOLYSIN ASSAY

A quantitative hemolysin assay was performed for purified protein as described previously, with little modification (Scheffer et al 1985). Briefly, 200µl of purified protein was incubated at 37°C for 60 min, in 10mM Tris buffer (pH 7.4) with 20mM CaCl₂, 100Mm NaCl and 2% whole blood in a final volume of 1ml. The unlysed erythrocytes were removed by centrifugation (1 minute in an eppendorf microfuge). The extent of hemolysis was determined by measuring the A₄₂₀ of the supernatant and comparing with a sample incubated with water, taken as 100% value.
2.11 GENERAL METHODS

General techniques in molecular biology such as protein estimation, plasmid DNA extraction, agarose and SDS-PAGE, transformation and western blotting which were used in this study are described in the following sections:

2.11.1 Protein estimation

The estimation of protein was carried out in the microtitre plate by modified Bradford’s (Bradford 1976) method.

2.11.1.1 Bradford’s method

(a) Standard BSA solution- 0.3 to 1.5 µg concentration in 3-15 µl volume made up with 1X PBS was added in duplicates to the wells of a microtitre plate. The volume in the wells was made up to 100 µl with water. Wells with 100 µl of water alone served as reagent blank.

(b) The samples in which the concentration of protein was to be measured were taken in different volumes ranging from 1–5 µl.

(c) 25 µl of Bradford’s reagent (100 mg CBB G-250 in 50 ml of 95% ethanol, 100 ml of 85% (w/v) phosphoric acid, diluted to 1 litre) was added to all the wells.

(d) The formation of blue coloured complex was measured at 595 nm. The concentration of protein present in the unknown solution was calculated from the standard graph.
2.11.2 Plasmid DNA extraction

(a) Plasmid DNA extraction from recombinant *E.coli* was based on the method of Birnboim and Doly (1979). All the centrifugation steps in this procedure were performed in a microfuge at 12000 g.

(b) A 3ml culture of plasmid grown overnight bearing *E.coli* was centrifuged for 5 minutes and the supernatant was discarded. The residual medium was removed by brief centrifugation followed by aspiration.

(c) The cell pellet was resuspended in 120 μl of TE buffer (50 mM Tris-HCl, pH 8.0 and 10 mM EDTA).

(d) RNase was added to a final concentration of 0.5 μg/μl to the 120 μl and mixed by pipetting and incubated at 37°C for 30 minutes.

(e) Freshly prepared 120 μl of alkaline-SDS (1% SDS in 0.2 N NaOH) was added followed by the same volume of 3.2 M potassium acetate solution, pH 5.2.

(f) The contents were mixed gently and centrifuged at 4°C for 30 minutes.

(g) The supernatant was carefully transferred into a fresh tube. The sample was extracted once with equal volume of tris buffered phenol: chloroform: iso-amyl alcohol (25:24:1) and once with equal volume of chloroform: iso-amyl alcohol (24:1).

(h) The plasmid DNA in the aqueous phase was precipitated by adding 2.5 volumes of ethanol or equal volume of
isopropanol for 30 minutes at -20°C and pelleted by centrifugation for 30 minutes at 4°C.

(i) The supernatant was discarded and the pellet was washed using 0.5 ml of 70% ethanol by centrifugation at 4°C for 10 minutes. The pellet was dried under a light source and resuspended in 30 µl of sterile H₂O.

2.11.3 Agarose gel electrophoresis

Horizontally submerged gels were used to separate the DNA fragments (Sambrook et al 1989). 0.5X Tris-Borate EDTA buffer of pH 8.3 (44.5 mM Tris, 44.5 mM Boric acid and 1 mM EDTA) was used. The electrophoresis was performed at constant 100 volts at room temperature. The gel loading buffer contained 0.2% Orange-G in 50% glycerol and TBE.

1% agarose gels were employed for checking plasmids and their restriction digestion products, whereas for checking the PCR product 1.2% gels were used. Gels were stained with 0.5 µg of ethidium bromide per ml of the gel-casting buffer and viewed under the UV transilluminator in the Chemiimager Gel documentation system. 1 Kb ladder was used as molecular weight marker.

2.11.4 Transformation of E.coli with plasmid DNA

The following procedure (Sambrook et al 1989) was used for the transformation of plasmid DNA to bacterial strains.

(a) A single colony of freshly revived E.coli culture was inoculated into 3 ml of LB and grown at 37°C overnight.
(b) 150 µl of overnight culture was inoculated into 15 ml of LB medium in a conical flask and allowed to grow at 37°C till the OD$_{600}$ reached close to 0.6.

(c) The culture was kept on ice for 30 minutes and centrifuged at 3500 g for 5 minutes at 4°C.

(d) The cell pellet was resuspended in 4 ml of ice cold 0.1 M CaCl$_2$ (calcium chloride) and incubated on ice for 15 minutes.

(e) Cells were pelleted by centrifugation as in step c and the pellet was resuspended in 8 ml of 0.1 M CaCl$_2$ and incubated on ice for 30 minutes.

(f) Cells were pelleted by centrifugation as in step c and the cell pellet was resuspended in 500 µl of 0.1 M CaCl$_2$. Approximately 1 µg of plasmid DNA was added to 100 µl of the above cells and further incubated for 30 minutes on ice.

(g) A heat shock at 42°C was given to the cells for 90 seconds and the cells were then chilled on ice for 5 minutes.

(h) 400 µl of fresh LB medium was added to this tube, the cells were allowed to grow for 45 minutes at 37°C with constant shaking and 100 µl of cells were plated on to LB agar plates supplemented with ampicillin.

(i) A positive control plasmid, (pRSET A) was used in all the experiments to verify the transformation efficiency. Cells with no plasmid DNA served as negative control to check for contamination.
2.11.5 SDS-PAGE

Proteins extracted from recombinant *E.coli* were analyzed by the method of Laemmli (1970) with minor modifications. The various buffers used are as follows:

(a) Monomer solution: 29.2% acrylamide and 0.8% N, N’- methylene bis acrylamide in distilled water. The solution was filtered through Whatman filter paper and stored in amber color bottles at 4°C.

(b) Separating gel buffer: 1.5M Tris-Cl, pH 8.3

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

(d) Electrophoresis buffer: 0.025 M Tris-Cl, 0.192 M glycine, 0.1% SDS, pH 8.3.

(e) SSB (3X): (6% SDS, 30% glycerol, 16% (v/v) β-mercaptoethanol, 0.06% bromophenol blue in stacking gel buffer.

Depending on the size of the proteins to be separated, 12-15% separating gels and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed in microtitre plate using Bradford’s protocol and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 20 mA. The gels were stained either with Coomassie Brilliant Blue R-250 (0.25 g of CBB R-250 in 45% methanol and 10% acetic acid) for 2 hrs and destained with 45% methanol and 10% acetic acid until a clear background was obtained on the gel, or with Coomassie Brilliant Blue G-250. In G-250 staining, the gels were treated with the fixer (25% isopropanol and 10% acetic acid) for 30 minutes, followed by staining
the gel with G-250 staining solution (0.006% CBB G-250 in 10% acetic acid) till the clear protein bands were obtained. The gels were documented in a Chemi-Imager Gel documentation system.

2.11.6 Western blotting

After electrophoresis, the SDS-PAGE gel was incubated for 15 minutes in the transfer buffer (48 mM Tris base, 39 mM glycine, 20 % methanol, 0.037 % SDS) as described by Towbin et al (1979) with slight modifications in the protocol. Nitrocellulose membrane was cut into the exact size of separating gel and was incubated for 15 minutes in the transfer buffer. Without trapping air bubbles the gel was overlaid on the membrane and sandwiched between filter papers. Electrophoretic transfer was carried out at 20 volts, 120 mA for 90 minutes using the Hoefer Semiphor semi dry blotting apparatus (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After the transfer, the membrane was stained with ponceau stain (0.5% ponceau in 1% acetic acid) to ensure the transfer of the proteins. The membrane was washed with 1X PBS and the molecular weight marker lane alone was cut and stained with amidoblack (100 mg amido black in 45% methanol, 10 % acetic acid). The rest of the NCM was blocked in 5% skimmed milk powder prepared in 1X PBS, at 37°C for 2 hours.

The membrane was washed with PBS 5 times, 10 minutes each, and then incubated with appropriately diluted primary antibody for overnight at 4°C or 3 hours at 37°C. After washing the membrane with PBS, it was incubated for 1 hour and 30 minutes with recommended dilution of secondary antibody conjugated either with ALP or HRP. After washing, the membrane was developed with NBT and BCIP in case of ALP and with DAB in case of HRP. After the appearance of bands, the reaction was stopped by washing the membrane with 1X PBS.