CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

*Bacillus thuringiensis* subsp. *galleriae* (*B. t. g.*) strain used in the present study was selected for its high level insecticidal activity against cotton insect pest *Heliothis armigera* as well as the foliage semi-looper *Spodoptera littoralis*. It was maintained as spores in 50% glycerol at -20°C and was subcultured routinely on nutrient agar plates.

A strain of *E. coli* PR722 obtained from Dr. Paul Riggs, New England Biolabs, U.S.A was used as the cloning host.

2.2 CHEMICALS AND REAGENTS

Chemicals were procured locally, and from Sigma Chemical Company, St. Louis, U.S.A. and were of analytical grade. Restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from New England Biolabs, Beverly, USA, and Genei, Bangalore, India. Immunochemicals were from Jackson Immunoresearch Labs., USA and Genei, Bangalore, India. Radiochemicals were obtained from Bhabha Atomic Research Centre, Trombay, India. Buffers, reagents and media were prepared in glass distilled water. Solutions to be sterilised were autoclaved or constituted from sterile components and then filtered through sterile microfilters of 0.25 μm pore size.
2.3 CULTURE MEDIA

GY-medium: Glucose 3.4 g l\(^{-1}\), Yeast extract 1.0 g l\(^{-1}\), \((\text{NH}_4)\text{SO}_4\) 1.7 g l\(^{-1}\), \(\text{KH}_2\text{PO}_4\) 0.2 g l\(^{-1}\), \(\text{MgCl}_2\cdot\text{H}_2\text{O}\) 0.04 g l\(^{-1}\), \(\text{CaCl}_2\cdot\text{H}_2\text{O}\) 0.02 g l\(^{-1}\), \(\text{FeSO}_4\cdot7\text{H}_2\text{O}\) 0.1 mg l\(^{-1}\), \(\text{NaCl}\) 0.02 g l\(^{-1}\), \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\) 0.1 mg l\(^{-1}\), 100 \(\mu\text{l}\) of the stock solution of trace elements was added to the above medium. The composition of trace element stock solution was according to Kuhn et al (1979). The amount of amino acids supplemented in GY-medium were: Glutamic acid 40 mg l\(^{-1}\), Aspartic acid 40 mg l\(^{-1}\) and Threonine, Phenylalanine, Tryptophan, Glycine, Cysteine, Valine, Isoleucine each 20 mg l\(^{-1}\). This was designated as GYA medium. Luria broth from Hi-Media was used at a concentration of 20 g l\(^{-1}\), pH 7.2. Nutrient Agar (g l\(^{-1}\)): Peptone, 10; Yeast extract, 3; NaCl, 5; Agar 15 g l\(^{-1}\), pH 7.3. This was used as a plating medium for all the strains of Bacillus thuringiensis. Soy meal of a commercial grade was used for batch cultivation of Bacillus thuringiensis subsp. galleriae. The concentration of the components added to the medium (g l\(^{-1}\)): Soy meal, 5; NaCl, 2.5; pH was controlled at 7.2.

2.4 ANTIBIOTICS USED

Media for growing plasmid bearing E. coli were supplemented with 100 \(\mu\text{g}\) ml\(^{-1}\) ampicillin.

2.5 BIOREACTOR

All cultivations were carried out in a 2 l bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1.5 l or a 20 l Bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 15 l. Both the reactors were provided with provisions for control of pH, Temperature, Impeller speed, Foam, Aeration and Weight (2 l bioreactor only).
2.6 INOCULUM DEVELOPMENT

2.6.1 Shake flask

a) The heat-shocked spores of *Bacillus thuringiensis* subsp. *galleriae* were activated by plating on Nutrient agar plate, incubated at 30°C.

b) A single colony from the plate was transferred to a 500 ml Erlenmeyer flask containing 100 ml of GY or GYA medium. This was incubated overnight at 30°C under static conditions. During exponential phase the culture was transferred to 100 ml GY or GYA medium and incubated in the shaker at 30°C and 200 rpm.

c) The actively growing culture from Step b) (OD between 0.6 - 0.8) was then used as preinoculum.

2.6.2 Batch studies

a) The heat-shocked spores of *Bacillus thuringiensis* subsp. *galleriae* were activated by plating on Nutrient agar plate, incubated at 30°C.

b) A single colony from the plate was transferred to a 500 ml Erlenmeyer flask containing 100 ml of 0.5 % soy meal medium. This was incubated over night at 30°C under static conditions.

c) This was then transferred to 1 l of 0.5 % soy meal which was incubated in a shaker at 120 rpm for 3 hours which to be used as a preinoculum for batch experiments in bioreactors.
2.6.3 Chemostat studies

a) The heat-shocked spores of *Bacillus thuringiensis* subsp. *galleriae* were activated by plating on Nutrient agar plate, incubated at 30°C.

b) A single colony from the plate was transferred to a 500 ml Erlenmeyer flask containing 100 ml of GY or GYA medium. This was incubated in a shaker at 30°C and 200 rpm.

c) The actively growing culture from Step b) (OD between 0.6 - 0.8) was then transferred to the 2 l bioreactor containing prestertilized GY or GYA medium.

d) It was allowed to grow to sporulation. This sporulated culture was then given a heat shock at 80°C for 20 minutes. The reactor was drained to retain 100 ml and this was used as a preinoculum for further chemostat experiments.

2.7 CULTIVATION CONDITIONS

Cultivation of strains of *Bacillus thuringiensis* was carried out at 30°C and the impeller speed was maintained at 1800 rpm or 500 rpm. pH was controlled with 2N NaOH at pH 7.0 or pH 6.0 in the GY-medium supplemented with amino acids. The air flow rate was maintained at 50 l h⁻¹ or 5 l h⁻¹.

2.8 PULSE AND SHIFT TECHNIQUE

Pulse and shift methods were carried out according to Kuhn et al., (1979). A generalized schematics of the pulse and shift technique is presented in Figure 2.1. For pulse experiments the amino acids were grouped based on their general assimilation pathways are as follows: 1) Methionine, Aspartate, Threonine and
Isoleucine; 2) Proline, Glutamic acid, Arginine and Glutamine; 3) Histidine; 4) Phenylalanine, Tyrosine, and Tryptophan; 5) Glycine, Serine and Cysteine; 6) Alanine, Valine, Leucine, Isoleucine and Lysine. Steady states in dilution rate shift were identified based on gas exchange rates, substrate and biomass profile.

2.9 CONTAMINATION CHECK

This was performed by microscopic examination at frequent intervals. In chemostat studies, contamination check was carried out by plating of samples collected periodically and the culture was examined for morphological characteristics.

2.10 OFF-LINE ANALYSIS

2.10.1 Biomass dry weight

Dry weight was determined by passing 5 ml of culture broth through a 0.2 μm filter and drying at 105°C for 24 hours.

2.10.2 Bioassay

Bioassay was carried out by surface contamination method (Beegle, 1989). The toxicity was tested, against third or fourth instar larvae of *Bombyx mori*. The samples were serially diluted in sterile distilled water and sprayed evenly over the surface of carefully processed Mulberry leaves. Five larvae were placed per dip along with negative controls. All the assays were performed in triplicate. Larvicidal activity was periodically monitored for 120 hours.
2.10.3 Glucose

Glucose estimation was carried out either with a glucose analyser (Yellow Spring Instruments, Ohio, USA) or by an enzymatic kit (Autozyme, Accurex Biomedical, India) or by the standard Dinitro Salicylic Acid (DNS) method.

2.10.4 Spore count

Spore count was carried out by plating serially diluted, heat shocked, cultures on Nutrient agar plates. After overnight incubation at 30°C, the colony forming units were counted.

2.10.5 Variants

Periodically collected samples from the chemostat were plated on Nutrient agar plates and incubated at 30°C for 48 hours. The translucent colonies were selected as those of the variants while the pigmented colonies were counted as the wild type strain.

2.10.6 Viable cell count

Viable cell counts were determined by plating serially diluted samples on nutrient agar plates and incubating at 30°C overnight.

2.11 ON-LINE ANALYSIS

2.11.1 Exhaust gas composition

On-line exhaust gas analyses were carried out for oxygen and carbon dioxide with paramagnetic O₂- (PMA 30; M&C, Instruments B.K., Bleiswijk) and infrared analyser (Binos-CO₂- analyser, Leybold-Heraeus, FRG) respectively.
2.11.2 pH

The pH was measured with a potentiometric sensor (Ingold AG, Urdorf, Switzerland). The sensor was calibrated before every cultivation with buffer solutions at pH 7 and 4 (T = 30°C).

2.12.3 pO₂

Oxygen partial pressure in the bulk liquid was measured amperometrically with a gas permeable sensor (Ingold AG, Urdorf, Switzerland). The zero was adjusted in the potentiometer and the 100% air saturation value was set with inoculated medium at the start of every run.

2.12.4 Temperature

Temperature was measured with Pt-100 sensor.

2.12.5 Weight

Liquid level in the bioreactor was measured by examining the weight of the bioreactor using a load cell and the controlled by a microprocessor.

2.13 CALCULATIONS

2.13.1 Biomass (Nomenclature attached)

\[ x = (F_f - F_i) \times 200 \]

2.13.2 Carbon dioxide production rate

\[ CPR = \frac{1}{V_R} \times (MAFR_{out}/V_L) \times (G_{co2\_out} - G_{co2\_in} \times (1 - G_{co2\_out})/(1 - G_{co2\_in} - G_{co2\_in}) \]
2.13.3 Dilution rate

\[ D = \frac{F}{V} \]

2.13.4 Economic yield coefficient

\[ Y_{eco} = \frac{x}{S0} \]

2.13.5 Maintenance coefficient

\[ \frac{1}{Yt} = \frac{1}{Yg} + \frac{m}{D} \]

2.13.6 Oxygen transfer rate

\[ \text{OTR} = \frac{1}{V_R} \frac{MAFR \text{ out}}{(1 - \text{Go}_2\text{out} - \text{Gco}_2\text{out})} \frac{(1 - \text{Go}_2\text{in} - \text{Gco}_2\text{in}) * \text{Go}_2\text{in} - \text{Go}_2\text{out})}{(1 - \text{Go}_2\text{in} - \text{Gco}_2\text{out})} \]

2.13.7 Respiratory quotient

\[ \text{RQ} = \frac{\text{OTR}}{\text{CPR}} \quad \text{(or)} \quad \frac{\text{qO}_2}{\text{qCO}_2} \]

2.13.8 Specific carbon dioxide production rate

\[ \text{qCO}_2 = \frac{\text{CPR}}{x} \]

2.13.9 Specific growth rate

\[ \mu = \frac{\mu_{\text{max}} S}{(Ks + S)} \]
2.13.10 Specific oxygen uptake rate

\[ q_{O2} = \frac{OTR}{x} \]

2.13.11 Specific substrate uptake rate

\[ qs = \frac{D (S_r - S)}{x} \]

2.13.12 Volumetric biomass productivity

\[ P = D \times X \]

2.13.13 Yield coefficient

\[ Y_{x/s} = \frac{(S_0 - S_t)}{(x - x0)} \]

2.14 CRYSTAL PURIFICATION

The sporulated culture from 1 l medium was harvested and washed with ice cold TE pH 7.2 (Tris-10 mM, EDTA-10mM) and the cell was disrupted by three passages through a French pressure cell at 15,000 lb/m^2. 5 ml portion of this suspension were layered on to 20 ml of a 48 % (W/V) sodium bromide (Baumann et al., 1985) solution in corex tubes and centrifuged at 10,000 rpm for 3 hours in a swing out rotor (Hitachi - Japan). Fractions of the gradient were examined under the microscope for the presence of crystals. Fractions judged as rich in crystals were pooled, washed with TE and resuspended in 0.5 ml TE and stored at -20°C.

2.15 SOLUBLIZATION OF THE CRYSTAL

Purified crystal was suspended in 0.25 ml of distilled water and the pH of the suspension was brought to 12.0 with 1 N NaOH (Baumann et al., 1985). The
sample was incubated at 30°C for 3 hours and the pH was monitored periodically and adjusted with 1N NaOH as and when needed. The pH was lowered by adding 0.2 ml of 1M Tris-HCl (pH 7.5) after the incubation period was over, the suspension was centrifuged at 15,000 rpm for 30 minutes. The supernatant was stored at -20°C and referred to as alkali solubilized toxin or crude cell extract.

2.16 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins present in cell extracts were analysed by SDS-PAGE according to the method of Laemmli [1970] with some modifications. The compositions of the buffers used are described below.

2.16.1 Buffers, reagents and gel composition for SDS PAGE:

a) Monomer solution: 20% acrylamide and 0.8% N, N'-methylene bisacrylamide. The solution was filtered through Whatman 1 mM paper and stored at 4°C.
b) Stacking gel buffer: 0.5 M Tris HCl, pH 8.0
c) Separating gel buffer: 1.5 M Tris HCl pH 8.8.
d) SDS: 10% SDS
e) Ammonium persulfate: 140 mg/ml; prepared fresh.
f) Electrophoresis buffer: 0.025 M Tris HCl, 0.192 M glycine, 0.1%SDS; pH 8.3.
g) Sample buffer: 10%SDS, 10% v/v 2-mercaptoethanol, 50% sucrose, 0.025% Bromophenol blue in 1/4x stacking gel buffer. For a 10% (w/v) separating gel, 15 ml monomer solution, 4.5 ml separating gel buffer, 10.5 ml distilled water, 300 µl SDS, 150 µl ammonium persulfate, and 15 µl TEMED (n,N,N',N'tetramethylene diamine) were used. For 7.5% gels 11 ml of the monomer solution and 4.5 ml of the separating gel buffer was used and the rest of them were the same as that for the 10% gels. For a 4% (w/v) stacking gel, 2 ml monomer solution, 2.4 ml stacking gel buffer, 150 µl SDS, 5.6 ml distilled water, 5 µl TEMED were used.
The protein estimations were carried out as per the protocol of Lowry et al. [1951].

Electrophoresis was performed at room temperature at a constant current of 30 mA. When the bromophenol blue dye reached 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from the glass plates and soaked in staining solution (0.25 g Coomassie brilliant blue R250 in 45 ml methanol, 10 ml glacial acetic acid and 45 ml distilled water). After 3 hours the gel was rinsed briefly to remove excess stain and then immersed in destaining solution which was the staining solution minus the stain. Destaining was stopped when the gel background was colourless.

2.17 WESTERN TRANSFER

After the electrophoresis was complete, the gel was incubated for 10-15 minutes in the transfer buffer to eliminate swelling. In the mean time, the NCP, cut to the desired size was incubated for 5-10 minutes in transfer buffer (Tris 25 mM, glycine 192 mM, methanol, 20% and SDS 0.1%). The nitrocellulose was overlaid on the gel (by avoiding air bubble) and sandwiched between the filter paper and scotch Brite pads. The gel was placed 'cathodic' to the NCP. The transfer was carried out at 300 mA for 4 hours in the cold room by using LKB transfer 2005 electroblotting apparatus. After the transfer was complete, the molecular weight marker lane was stained with amido black (100 mg amido black in 45 ml of methanol and 10 ml of acetic acid, made up to 100 ml of distilled water) for 2-3 minutes and then destained with several changes with destainer until the background stain was eliminated. The rest of the NCP was blocked for 1 hour at room temperature with 3% skim milk in PBST (PBS with 0.3% Tween-20). The NCP was washed in wash buffer for three times of five minutes duration. The NCP was incubated for one hour with the desired primary antibody diluted in PBST. After intensive washing in the wash buffer, the NCP was incubate for another 1 hour with secondary antibody (1/1000 dilution) conjugated with horse radish peroxidase or alkaline phosphatase. In the case of monoclonal antibodies
after primary sera, the incubation was carried out with the monoclonal antibody at 37°C for 2 hours followed by 4°C overnight.

In case of dot-blot ELISA individual samples were spotted using S&S Mini-dot blot apparatus and the NCP sheets were dried at room temperature and processed as in western blot. In the case of horse radish peroxidase, the colour development was carried out by using diaminobenzidine 15 mg/10 ml of PBS and 0.1% of 30% hydrogen peroxidase.

For alkaline phosphatase staining, the blots were incubated in predetection buffer (100 Tris HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl) for 10 minutes and the colour development was carried out by using 30 μl of bromo-chloro-indolyl phosphate (50 mg/ml in 100% diethylformamide) and 16.5 μl of nitroblue tetrazolium (50 mg/ml in 70% diethyleformamide).

2.18 PREPARATION OF PLASMID DNA

Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly [1979] as described below. Composition of buffers and reagents for preparation of plasmid DNA:

a) TEG 50 mM glucose, 25 mM Tris HCl and 10 mM EDTA, pH 8.0.

b) Alkaline SDS 1% (w/v) SDS in 0.2 mM NaOH prepared fresh

c) Sodium acetate 3 M Sodium and 5 M acetate, pH 4.8

d) Distilled ethanol

e) TE 10 mM Tris HCl, 1 mM EDTA, pH 8.0.
2.18.1 Small scale preparation of plasmid DNA

Procedure

(All centrifugation steps in this procedure were performed in an Eppendorff 5414S centrifuge at 12000 rpm and at room temperature).

a) A 1.5 ml culture of a plasmid bearing strain of *E. coli* was grown in LB broth containing antibiotics at 37°C with constant agitation till the stationary phase. The culture was transferred to a 1.5 ml microtube and centrifuged for 45 sec. The medium was decanted and centrifuged again for 10 sec. The residual medium was removed by aspiration.

b) The cell pellet was resuspended in 100 μl of TEG buffer and vortexed thoroughly.

c) Alkaline SDS (200 μl) was added to the cells which was inverted gently ten times and then placed in an ice water bath for 5 min.

d) Sodium acetate (150 μl) was added and the contents were mixed gently. The samples were incubated at 70°C for 30 min, thawed, mixed thoroughly and spun for 10 min.

e) The supernatant was transferred to a fresh tube and 1 ml ethanol was added, mixed by inversion and incubated at room temperature for 10 min.

f) The sample was spun for 5 min and the supernatant removed as in step a). The pellet which contained plasmid DNA, contaminating chromosomal DNA and RNA was dissolved by vortexing in 50 μl TE. Ethanol (150 μl) and 10 μl sodium acetate (step d)) were added to the tube and incubated at room temperature for 10 min.

g) The sample was centrifuged for 5 min and the supernatant was discarded. The precipitation by ethanol was repeated twice and the final pellet was dissolved by adding 100 μl sterile TE buffer. Chloroform (25 μl) was added as preservative and the DNA stored at 4°C.
Note:

Incubating the solution (of step (f)) with a 1/10 volume of sodium acetate and two volumes ethanol at room temperature for 5 min was sufficient to precipitate all the nucleic acid present but at occasions, especially when the nucleic acid concentration was low or if the volumes being handled were large, the incubation time was prolonged and the temperature of incubation was lowered to -70°C to precipitate all the DNA. The subsequent centrifugation time was also increased to 15 min.

2.19 LARGE SCALE PREPARATION OF PLASMID DNA

The cells were harvested by centrifugation at 5000 rpm in the RPR12-2 rotor of a Hitachi HIMAC SCR20BA centrifuge at 4°C. The pellet obtained from an one litre culture was resuspended in 10 ml of TEG buffer. The volumes of other reagents were appropriately scaled up and the centrifugation parameters modified. Each spin was performed at 4°C and 12000 rpm for 10 min. The final pellet was dissolved in 1 ml TE buffer.

2.20 PURIFICATION OF PLASMID DNA BY ULTRACENTRIFUGATION

Recombinant plasmids extracted by the procedures described above were sufficiently pure for most purposes including restriction and subcloning. However, pPR683 used for the initial cloning experiments were purified by centrifugation in gradients of caesium chloride containing ethidium bromide [Sambrook et al, 1989].

Procedure

a) The plasmid preparation obtained from a one litre culture was diluted to 2.5 ml with TE buffer in a 5 ml ultracentrifuge tube and 2.5 g caesium chloride was added to the solution. The tube was inverted repeatedly till
the salt dissolved. Ethidium bromide (0.4 ml of a 10 mg ml\(^{-1}\) solution in distilled water) was added and the tube was filled nearly to capacity with a solution of caesium chloride in TE (pH 8) prepared by dissolving 10 g of caesium chloride in 10 ml TE.

b) The samples were centrifuged for 24 h in the RPV65T rotor of a Hitachi HIMAC SCP70H ultracentrifuge at 40000 rpm and 20°C.

c) The tube was illuminated by long wave ultra-violet light and the lower of the two fluorescent bands was recovered by puncturing the centrifuge tube just below the band and withdrawing most of the fluorescing material in that band.

d) Ethidium bromide was removed from this solution by three extractions with water saturated n-butanol. The solution was then diluted three fold, dialysed extensively with TE and the plasmid DNA was precipitated with ethanol. After two precipitations, the plasmid DNA was stored as a solution in TE over chloroform.

e) The concentration of DNA was then estimated by measuring the absorbance at 260 nm of an appropriately diluted sample. (Double-stranded DNA at a concentration of 50 \(\mu g/ml\) gives an absorbance of 1). The ratio of absorbances at 260 and 280 nm should be close to 1.8 for a DNA preparation free of significant contamination by protein [Sambrook et al, 1989].

2.21 DIGESTION BY RESTRICTION ENZYMES

The restriction enzymes used were Pst I, Bam HI, Sac I and Dra I. For all these enzymes the restriction temperature was 37°C. All the restriction enzymes and T4 DNA ligase, the buffers were provided by the manufacturer namely New England Biolabs, Beverley, USA.
2.21.1 NEB buffer composition (10 X stock)

Buffer 1: 10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1mM DTT (pH 7.0 at 25°C)
Buffer 2: 100mM Tris- HCl (pH 7.9 at 25°C), 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT.
Buffer 3: 50 mM Tris-HCl (pH 7.9 at 25°C), 10 mM MgCl₂ , 100 mM NaCl, 1 mM DTT.
Buffer 4: 20 mM Tris-acetate (pH 7.9 at 25°C), 10 mM Magnesium acetate, 50 mM Potassium acetate, 1 mM DTT.
BSA (Bovine serum albumin) (10 mg ml⁻¹) (100 x stock): To be used as 100 μg ml⁻¹ final concentration.

Procedure

a) DNA ( 1 μg /10 μl), 7 μl of distilled water and 2 μl of appropriate 10 x buffer were mixed in a microtube.
b) One unit of restriction enzyme was added to the tube, the tube contents were mixed gently and the tube was incubated at 37°C for 2 hours. The completion of digestion was monitored by agarose gel electrophoresis.

2.22 AGAROSE GEL ELECTROPHORESIS OF DNA

Horizontal submerged gels were employed in this study. The buffer for electrophoresis was TEB (89 mM Tris, 89 mM Boric acid and 2 mM EDTA,pH 8.3). The gel loading buffer was a solution of 20% sucrose and 0.01 % Bromophenol blue in TE. Samples containing an appropriate quantity of DNA in TE, and previously treated with pancreatic RNase A, were mixed with a 1/4 volume of a gel loading buffer, heated for 5 min at 65°C and then chilled on ice (RNase treatment of samples was routinely done to prevent RNA from obscuring DNA fragments of 0.75 KB and
smaller during subsequent visualisation). The heating step helped to dissociate proteins such as restriction enzymes from DNA.

Depending on the size of fragments to be separated, 0.7-1.2% agarose gels were used. The gels were 10 cm long and 3 mm thick. Electrophoresis was performed at 10 V/cm in a cold room and stopped when the dye reached 1 cm from the bottom of the gel. Gels were stained in a 1 µg/ml solution of ethidium bromide in water for 5 min and viewed under illumination of 300 nm. Photographs were taken with a Polaroid MP-4 camera and Polaroid type 665 film; a red filter (wratten No.22A) was used to reduce background.

2.23 RESTRICTION AND ELUTION OF pES 1 DNA

About 50 µg of recombinant pES 1 plasmid was suspended in TE (pH 8.0) to give a final volume of 170 µl. 20 µl of 10 X restriction buffer (Dra I Buffer 4) was added and a three fold excess of Dra I enzyme was used and incubated for 1 hour at 37°C. When the digestion was complete, EDTA was added to a final concentration of 5 mM and extracted once with phenol-chloroform and isoamyl alcohol mixer and once with chloroform.

The restricted DNA was run on 0.7 % preparative agarose gel and was stained with ethidium bromide to visualise the DNA under ultra violate radiation. The Dra I cut pES 1 plasmid DNA was incepted on the gel with a strip of DEAE (NA-45, S and S) membrane which was already activated by 10 mM EDTA (10 min) and 0.5 M NaOH ( for 5 min) and was rinsed with distilled water before use. On interception with strip, the DNA band to be eluted was run onto the strip and over it. The electrophoresis was stopped and the DNA bound membrane was washed in NET buffer (0.15 M NaCl, 0.1 mM EDTA , 20 mM Tris pH 8.0) 45 min at 65°C with occasional swirling. The buffer was collected and extracted with 3 volumes of water saturated n-butanol to remove ethidium bromide and the DNA was initially precipitated with 2.5 volume of ethanol (5 hours at 20°C) and was reprecipitated using
0.3 M sodium acetate to remove any NaCl residues (Winberg and Hammers Jold 1988).

2.24 LIGATION OF VECTOR AND PASSENGER DNA

The 10X buffer used for ligation contained; 0.5 M Tris HCl (pH7.4), 0.1 M Magnesium chloride, 0.1 mM DTT, 10 mM spermidine, 10 mM ATP, 1 mg/ml nuclease free bovine serum albumin (Sambrook et al, 1989).

Procedure:

a) appropriate amounts of passenger and vector DNA, 2 µl of 10X ligase buffer and distilled water to 19 µl were mixed in a microtube.

b) T4 DNA ligase (1 unit) was added and the contents were gently mixed. The tube was incubated for 18 h at 12°C for EcoRI termini.

c) At the end of the incubation period the ligated DNA was used directly for transforming competent *E. coli* cells.

2.25. TRANSFORMATION OF *E. coli* BY PLASMID DNA

Transformation of *E. coli* PR722: The procedure for rendering cells of *E. coli* competent for transformation is based on that described by Sambrook *et al.* (1989) with some modifications as described below:

a) *E.coli* PR722 was inoculated from a glycerol stock into 10 ml LB broth and was incubated static for 12 h at 37°C.

b) One ml of this preculture was used to inoculate 50 ml prewarmed LB broth contained in a 500 ml Erlenmeyer flask. The flask was immediately placed on a rotary shaker operated at 350 rpm and maintained at 37°C.
c) At an optical density of 0.2 at 600 nm, the flask was removed from the shaker and transferred to an ice water bath in which it was agitated so as to cool its contents rapidly.

d) After 10 min, 40 ml of the chilled culture was transferred to a sterilised and chilled screw cap centrifuge tube and the cells pelleted at 3500 rpm in the RPR 20-2 rotor of a Hitachi HIMAC SCR20BA high speed centrifuge for 10 min and at 10°C.

e) The supernatant was discarded and the pellet was gently resuspended in 20 ml ice cold sterile 50 mM calcium chloride. The tube was kept in an ice water bath for 15 min and centrifuged as before. This time the pellet was resuspended in 2.5 ml ice cold sterile calcium chloride and the tube was toured in ice. The cells were now competent for transformation.

f) Glass test tubes containing the DNA samples were placed in ice. After the tubes cooled, 200 µl of the competent cell suspension was added rapidly to each tube and also to a tube not containing DNA; this tube served as a negative control. The tubes were incubated at 0°C.

g) After 45 min, the tubes were transferred for 2 min to a 42°C water bath.

h) Then, 0.8 ml prewarmed LB broth was added to each tube and the tubes incubated for 90 min at 37°C.

i) The tube contents, in 50 µl volumes (for samples of ligation mixtures) were plated onto LB agar plates containing suitable antibiotics.

j) The plates were incubated at 37°C for 18 hours by the time colonies arising from transformed cells appeared on the plates whereas plates receiving control cells remained sterile.

2.26 NICK TRANSLATION

Nick translation of the DNA fragment was carried out by using BRL Nick translation systems (Bethesda Research Lab), kits and the protocol as described by the manufacturer; 500 - 700 ng of test DNA was nicktranslated by using alpha-32 P dATP (specific activity 3000 curie/mmol).
2.27 HYBRIDISATION OF DNA

The Hybond-N membrane with DNA was pre-hybridised with 6x SSC (20xSSC: 175.3 g of NaCl and 88.2 g of Sodium citrate in 800 ml of water, the pH was adjusted to 7.0 with a few drops of 10 N NaOH and the volume made up to one litre), 0.5% SDS, 5x Denhardts solution (Ficoll 5 g, polyvinylpyrrolidone 5 g, BSA 5 g and water to 500 ml) and 100 μg/ml denatured Solmon sperm DNA and incubated for 4 hours submerged in a water bath at 68°C. After pre-hybridisation, the nick translated DNA probe (denatured) and EDTA to a final concentration of 0.01 M was added and incubated at 68°C for 12 hours.

After hybridisation the filter was removed and immediately submerged in a tray containing 2x SSC and 0.5% SDS at room temperature. After 5 min, the filter was incubated in a solution containing 2x SSC and 0.1% SDS and incubated for 15 min at room temperature with gentle agitation. The filter was further incubated at 68°C for 2 hours with 0.1x SSC and 0.5% SDS. The filter was dried at room temperature and wrapped in between saran wrap and exposed for autoradiography.

2.28 SOUTHERN TRANSFER

After staining the gel, the DNA was nicked by exposing the gel to short wave UV-light for 15 min. The gel was placed in 500 ml of denaturation buffer and was shaken gently for 30 min. The buffer removed and the neutralisation buffer was used for incubation for 30 min. (Wicks were cut from Whatman 3MM filter paper :Nitrocellulose sheet of the same size as the gel). The nitrocellulose paper (NCP) was initially dipped in distilled water and then in 10xSSC. (Paper towels were cut having the same size as the gel). The wicks were wetted using 10x SSC and was centred over the gel support placed in the blotting tray. The denatured and the renatured gel was gently placed on top of the wicks and was centred without air bubbles. Paper towels were placed on top making a stack over which a glass plate was placed with 500 ml
bottle of water. The transfer occurred well when done overnight. When the transfer was complete, the NCP was washed with 3xSSC for 15 min. The NCP was placed on an aluminium foil, with the transferred side facing up. The NCP was blotted to remove the moisture. The NCP was baked in a vacuum oven at 80°C for 2 hours. The NCP was now hybridised.