Chapter-3
MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

Standard culture of Klebsiella pneumoniae B5005 (01K2) obtained originally from Dr. M. Trautman was used in the present study. A summary of bacterial isolates used in the present study has been presented in table 3.1.

Table 3.1 Summary of bacterial isolates used in the present study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Characteristic</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae B5055</td>
<td>O1K2</td>
<td>M. Trautman³</td>
</tr>
<tr>
<td>Klebsiella pneumoniae 43816</td>
<td>O1K2</td>
<td>David P. Speert³</td>
</tr>
<tr>
<td>K1-K25</td>
<td>Clinical isolates from UTI⁶</td>
<td>DMC, Ludhiana</td>
</tr>
<tr>
<td>KPARV</td>
<td>Antibiotic resistant variant</td>
<td>Present study</td>
</tr>
<tr>
<td>KPVR</td>
<td>Bacteriophage resistant variant</td>
<td>Present study</td>
</tr>
<tr>
<td>Escherichia coli 25922</td>
<td>ATCC Type strain</td>
<td>ATCC³</td>
</tr>
<tr>
<td>DH5-a</td>
<td>endA1 hsdR17 (rk, mk,) supE44 th,-1 recA1 gyRA relA1_80d lacZ_M15 (lacZ YA-argF) U169</td>
<td>ATCC⁵</td>
</tr>
<tr>
<td>E1-E5</td>
<td>Clinical isolates from UTI</td>
<td>Clinical Lab., P.U.</td>
</tr>
<tr>
<td>Salmonella typhi Ty2</td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td>Salmonella typhimurium 98</td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PAO</td>
<td></td>
<td>Holloway et al1970</td>
</tr>
<tr>
<td>P1-P4</td>
<td>Clinical isolates</td>
<td>GMCH, Chandigarh</td>
</tr>
</tbody>
</table>

³ M. Trautman, Department of Medical Microbiology and Hygiene Ulm, Germany; ⁴ David P. Speert, Department of Pediatrics, University of British Columbia, Vancouver, Canada; ⁵ ATCC, American Type Culture Collection; ⁶ Microbial Type Culture Collection; ⁷ Urinary Tract Infections; ⁸ Dayand Medical college; ⁹ Govt. Medical College and Hospital, Sector-32, Chandigarh

3.2 GROWTH CONDITIONS

All bacterial isolates were grown in nutrient broth (HiMedia Laboratories, Mumbai, India) at 37°C. Bacteria were maintained on nutrient agar slants kept at 4°C while for long term storage glycerol stocks (60%; final concentration) were made and kept at -80°C. Before use, bacteria were subcultured twice in nutrient broth, followed by plating on nutrient agar plate and then picking up an isolated colony that was further processed. Susceptibility test of bacteria to various
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antimicrobials was performed in soyabean casein digest agar (HiMedia Laboratories, Mumbai, India). All bacterial and bacteriophage dilutions were made in sterile phosphate buffered saline (PBS; 0.1 M; pH 7.2).

3.3 BACTERIOPHAGE ISOLATION

*Klebsiella pneumoniae* B5055 specific bacteriophages, used in the present study, were isolated from the sewage water by the method of Cereveny et al (2002).

Method

3.3.1 Preparation of indicator bacterial cells

1) Two glass tubes (30.0 ml) containing 10.0 ml nutrient broth were inoculated each with isolated colony of *Klebsiella pneumoniae* B5055 and incubated at 37°C overnight.

2) From the growth in step 1, 2.5 ml was transferred to 250 ml of nutrient broth and incubated at 37°C under shaking conditions (150 rpm) for 4-6 h. The culture was centrifuged at 10,000 rpm for 15 min at 4°C.

3) Bacterial pellet from step 2 was given three washings in sterile PBS and ultimately suspended in same buffer and used immediately.

3.3.2 Processing of sewage sample

1) Sewage samples were collected in clean bottles. Collected samples were filtered through a coarse filter to remove any suspended matter.

2) Equal volume of sewage sample and nutrient broth was mixed. The mixture was inoculated with 5% (v/v) of bacterial culture and incubated overnight on a shaker (150 rpm) at 37°C.

3) Incubated sample was centrifuged at 10,000 rpm for 20 min at 4°C to remove the bacterial cell mass.
4) Pellet obtained after centrifugation was discarded and supernatant was filtered through 0.45 μm metafilter (Millipore). Phage titer in the filtered supernatant was assayed by spot assay (3.4.1).

3.3.3 Amplification of bacteriophages

1) To amplify the bacteriophages, filtered supernatant (3.3.2) was mixed with equal volume of nutrient broth to which 5% (v/v) bacterial growth (exponential phase) was added.

2) After overnight incubation at 37°C, mixture was centrifuged at 10,000 rpm for 20 min at 4°C.

3) Supernatant obtained was metafiltered (0.45 μm; Millipore) and activity in the filtrate was assessed by spot assay (3.4.1) and turbidity method (3.4.2).

3.4 DETECTION OF BACTERIOPHAGE ACTIVITY

Following methods were employed for the detection of bacteriophage activity in the phage preparation.

3.4.1 Spot assay (Chang et al 2005)

1) Soft agar (nutrient agar with 0.75% agar concentration) was dispensed (10.0 ml) in 30 ml glass test tubes. Soft agar was sterilized by autoclaving at 15 lbs for 15 min.

2) Sterile soft agar was maintained in molten state at 40-45°C in a water bath.

3) 100 μl of indicator bacterial culture was added to the molten soft agar. After thorough mixing, mixture was poured onto nutrient agar plate and allowed to solidify.

4) Plate containing bilayer of agar (lower layer of nutrient agar and upper of soft agar) was incubated at 37°C for 30 min, to evaporate the moisture from the surface.
**Materials and Methods**

5) 10 µl of supernatant containing bacteriophage was spotted onto dried plate. Plate was incubated at 37°C overnight.

6) Presence of clear area at the site of spot was indicative of the bacteriophage activity.

3.4.2 Turbidity method (Soothill 1992)

1) Two tubes containing 10.0 ml of sterile nutrient broth were labelled as 'test' and 'control'.

2) To both the tubes indicator bacterial culture was added and optical density (OD$_{600}$) was adjusted to 0.1.

3) To the tube 'test' 100 µl of bacteriophage suspension was added and mixed thoroughly. Both tubes, i.e. test and control, were incubated at 37°C.

4) OD$_{600}$ of the two tubes was measured at regular time intervals and decrease in OD$_{600}$, if any was noted.

3.5 BACTERIOPHAGE TITRATION (DOUBLE AGAR OVERLAY METHOD)

1) Soft agar (nutrient broth with 0.75% agar) was melted in boiling water bath and its temperature was maintained between 40-45°C in water bath.

2) Serial dilutions of the phage suspension to be titrated were made in sterile PBS (pH 7.2).

3) From each dilution of bacteriophage, 100 µl was added to soft agar (10 ml) to which 100 µl of indicator bacterial cells (10$^8$ cfu/ml) was also added.

4) Contents of tube were mixed thoroughly and poured over the previously prepared nutrient agar plate. Plate was gently swirled to ensure even distribution of soft agar on the plate.
5) After solidification, plate was incubated overnight at 37°C after which phage count was taken by counting the number of plaques that appeared on the plate. One plaque represented one bacteriophage. Titer of bacteriophage preparation was expressed as pfu (plaque forming units)/ml.

3.6 BACTERIOPHAGE PURIFICATION

Isolated bacteriophages were purified by single-plaque isolation method as described by Sambrook et al (1989).

1) Bacteriophage suspension was subjected to plaque separation by double agar overlay method as described in section 3.5.

2) From the isolated bacteriophages a single plaque was picked up by using a sterile spatula and transferred to nutrient broth containing 1% (v/v) indicator bacterial culture. Preparation was incubated at 37°C overnight under shaking conditions.

3) Preparation was centrifuged after incubation, at 10,000 rpm for 20 min at 4°C. Supernatant was removed and centrifuged again (10,000 rpm 20 min) with 1% (v/v) chloroform to remove any cell debris. After centrifugation aqueous phase was harvested and bacteriophage activity was titrated by double agar overlay method explained in section 3.5.

4) Number of plaques and morphology of the isolated plaque was documented. If homogenous plaque morphology was not detected, cycle of bacteriophage purification was repeated unless plaques with similar morphology were obtained.

3.7 PREPARATION OF HIGH TITER AND BACTERIOPHAGE STOCKS

For preparation of high titer and stocks, purified bacteriophage from single plaque was used.
Materials and Methods

3.7.1 Plate lysate method (Sambrook et al 1989)

Materials

A) SM Buffer

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.8 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>1M Tris-HCl (pH 7.5)</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>to 1.0 litre</td>
</tr>
</tbody>
</table>

B) Chloroform

Method

1) 100 μl of bacteriophage suspension (10⁸ pfu/ml) and 100 μl indicator bacterial suspension (10⁸ cfu/ml; exponential growth) were inoculated into molten soft agar (40-45°C) and after thorough mixing poured onto basal nutrient agar plate. Plate was swirled gently for even spreading.

2) Solidified plate was incubated at 37°C overnight.

3) After incubation, 5 ml of SM buffer was poured onto the plates containing plaques. Plates were reincubated at 37°C for 3-4 h.

4) After incubation SM buffer was collected into sterile tubes using sterile Pasteur pipette.

5) To the collected SM buffer 1% (v/v) chloroform was added that was then vortexed and centrifuged at 10,000 rpm for 10 min at 4°C. Aqueous phase was collected carefully. Bacteriophage titer of the aqueous phase was estimated by double layer agar overlay method.

3.7.2 Small scale liquid culture method (Sambrook et al 1989)

1) 20 ml nutrient broth in a 50 ml flask was inoculated using an isolated colony of indicator bacterial strain. Inoculated broth was incubated at 37°C overnight.
2) 10 ml of overnight bacterial growth was transferred to a 30 ml glass test tube to which 100 µl bacteriophage suspension (10^8 pfu/ml) was added.

3) Mixture was incubated at 37°C for 20 min to allow bacteriophage adsorption to occur, following which, 4.0 ml of prewarmed (37°C) nutrient broth was added to the tube and tube reincubated at 37°C for 12 h under shaking conditions (150 rpm).

4) After complete bacterial lysis, 200 µl chloroform was added to the suspension and incubation continued for another 15 min. Mixture was centrifuged at 10,000 rpm for 10 min at 4°C after incubation.

5) Supernatant was transferred to a sterile tube and 1 drop of chloroform was added and stored at 4°C.

3.8 PREPARATION OF LARGE SCALE LYSATE

1) A single colony of host bacterial culture was inoculated into 50 ml nutrient broth in 250 ml flask and incubated at 37°C, overnight.

2) To the 200 ml of prewarmed nutrient broth 5% (v/v) overnight bacterial growth was added and incubated at 37°C until the OD_{600} reached to a level where cell count was equivalent to 10^8 cfu/ml.

3) 100 µl of bacteriophage suspension (10^8 pfu/ml) was added to the bacterial culture and incubated at 37°C under shaking conditions (150 rpm) until bacterial culture got cleared.

4) After clearance of broth, bacterial culture (1% v/v) and an equal volume of prewarmed sterile nutrient broth was added and reincubated at 37°C. Procedure was repeated at least 3 times after which bacteria-bacteriophage suspension was ultimately incubated at 37°C overnight.

5) Next day, 1% chloroform was added to the bacteriophage suspension. It was centrifuged at 10,000 rpm for 30 min at 4°C. Aqueous phase
**Materials and Methods**

was collected carefully and bacteriophage titer was estimated by double agar overlay method explained in section 3.4.

6) High tittered bacteriophage suspension was stored at 4°C containing trace amounts of chloroform.

### 3.9 CONCENTRATION AND PURIFICATION OF BACTERIOPHAGE BY PRECIPITATION WITH POLYETHYLENE GLYCOL (PEG)

Concentration and purification of bacteriophage by precipitation with PEG was carried out using the method of Yamamoto et al (1970). The procedure involved the following steps.

1) To the high tittered bacteriophage lysate (described in section 3.7), NaCl (0.5 to 1 M, final concentration) was added and dissolved by continuous mixing, using a magnetic stirrer, at 4°C for 1 hour. The NaCl dissociates phage from bacterial debris and media components and improves the precipitation of phage by PEG.

2) After NaCl treatment, bacterial cell debris was removed by centrifugation (10,000 rpm; 10 min, 4°C), and the phage-containing supernatant was transferred to a clean flask.

3) Maintaining the sample at 4°C, PEG 8000 was added gradually, with constant stirring, to a final concentration of 8%–10%, w/v. The mixture was stored at 4°C for at least an hour, to allow the phage particles to form a precipitate.

4) The precipitate was sedimented by centrifugation (10,000 rpm for 10 min) at 4°C.

5) Using a sterile pipette the supernatant was removed carefully. Complete precipitation of bacteriophage was on the basis of absence of any bacteriophage activity in the supernatant, as determined by spot assay.
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6) To the sedimented bacteriophage pellet, 5 ml of SM buffer was added and allowed to stand at 4°C overnight. Next day suspension was mixed gently.

7) After suspending the phages, chloroform (100 µl) was added to the suspension and any remaining cell debris was removed by low-speed centrifugation (2500 rpm for 15 min) at 4°C.

3.10 STORAGE OF BACTERIOPHAGE

For long term storage, high titered bacteriophage was stored in SM buffer at -80°C containing 60% glycerol. For routine use bacteriophage was stored at 4°C in PBS (pH 7.2).

3.11 CHARACTERIZATION OF BACTERIOPHAGE

3.11.1 Host range

Bacterial isolates listed in Table 1 were used for determining the host range of bacteriophage.

Method

1) Stocks of bacterial isolates to be tested were thawed quickly. Bacterial cells were subcultured in tryptone soya broth (TSB) and colonies were isolated on tryptone soya agar.

2) A single isolated colony of each bacterial isolate was inoculated in separate 5.0 ml nutrient broth and incubated at 37°C.

3) All bacterial cultures were used at a cell density of 10⁸ cfu/ml.

4) 100 µl of bacterial culture was inoculated into the molten soft agar (0.7%). Soft agar was poured onto the basal nutrient agar. After solidification plate was incubated for an hour at 37°C.

5) After incubation, 10 µl bacteriophage suspension (1x10⁶ pfu/ml) was spotted onto the dried plate. Drop was allowed to dry and plate was incubated in inverted position at 37°C overnight.
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6) After incubation, according to the degree of clarity, spots were differentiated into three categories: clear, turbid and no reaction.

3.11.2 Effect of temperature

Effect of temperature was estimated on the stability of the bacteriophage and the optimum activity of the bacteriophage.

3.11.2.1 Stability of bacteriophage

1) 1.0 ml of bacteriophage suspension at a titer of $10^8$ pfu/ml was exposed to different temperatures viz. 25, 30, 37, 40, 56, and 60°C for 1 h.

2) Bacteriophage suspension (1.0 ml; $10^8$ pfu ml) held at 4°C for 1 h was used as control.

3) After incubation, various dilutions of the exposed bacteriophage suspensions (test and control) were prepared and phage titer was estimated by double agar overlay method (3.5). Results were reported as pfu/ml.

3.11.2.2 Optimum activity of bacteriophage

1) A single isolated colony of *Klebsiella pneumoniae* B5055 was inoculated into 5.0 ml nutrient broth and incubated at 37°C overnight.

2) From the overnight bacterial growth, OD$_{600}$ of bacterial culture was set equivalent to a cell density of $10^8$ cfu/ml.

3) Dilutions of the bacteriophage suspension were prepared.

4) 1.0 ml of bacterial suspension and 1.0 ml of bacteriophage ($10^6$ pfu/ml) was mixed to give a Mol (multiplicity of infection) of 0.01.

5) Mixture was incubated at 25, 30, 37, and 40°C for 1 h. After incubation bacteriophage sample was treated with 1% (v/v) chloroform and
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centrifuged at 10,000 rpm/ 5 min. Bacteriophage titer in the aqueous phase was estimated by double agar overlay method.

3.11.3 Stability of bacteriophage to UV light

1) Bacteriophage suspension was diluted to a phage density of $10^3$ pfu/ml.

2) 5.0 ml of diluted bacteriophage suspension was poured into sterile glass petri plate. Petri plate with bacteriophage suspension was exposed to UV light (Sankyo Denki, Japan; G3018, UV-C, $\lambda$ 253.7 nm).

3) At regular time intervals (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 min), 0.1 ml of bacteriophage suspension was removed.

4) Phage titer of the removed aliquot, at respective time point, was estimated immediately by plaque assay (section 3.5).

3.11.4 Effect of organic solvent on bacteriophage activity

1) 1.0 ml of bacteriophage suspension ($10^8$ pfu/ml) was taken in four tubes.

2) Three out of four tubes were labelled and equal volume of respective organic solvent i.e. chloroform, ether and ethanol were added. Fourth tube acted as control and nothing was added to it.

3) All the tubes were incubated at room temperature with intermittent shaking.

4) After 1 h tubes were centrifuged at 10,000 rpm for 10 min. Bacteriophage titer in the aqueous phase was estimated by double agar overlay method (section 3.5). Effect of organic solvent on the stability of bacteriophage was reported as percent inactivation in comparison to control tube.
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### 3.11.5 Effect of pH on bacteriophage stability

1) Different 50 ml flasks containing 20 ml nutrient broth were prepared.

2) The pH of nutrient broth in respectively labelled flasks was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 using 1M HCl or 1M NaOH.

3) From the flasks containing pH adjusted media, 9.0 ml of nutrient broth was added to each 30 ml glass tube. Tubes were plugged and autoclaved at 15 lbs for 15 min.

4) 1.0 ml of bacteriophage suspension, at 10^4 pfu/ml, was added to 9.0 ml of pH adjusted sterile nutrient broth to obtain a titer of 10^3 pfu/ml. Preparation was incubated at 37°C for 1 h. Bacteriophage suspension held at pH 7 was taken as control.

5) After incubation bacteriophage titer was estimated by double agar overlay method as described in section 3.5.

### 3.11.6 Evaluation of bacteriophage infectivity

#### 3.11.6.1 Adsorption rate

The adsorption rate was estimated according to the procedure of Adams (1959).

1) Bacteriophage having an initial titer of 10^6 pfu/ml was used.

2) 1.0 ml of titrated bacteriophage suspension was mixed with 1.0 ml of *Klebsiella pneumoniae* B5055 culture having a bacterial count of 10^8 cfu/ml to achieve an Mol of 0.01.

3) Mixture was incubated at 37°C.

4) At 1 min time intervals, till 10 min, 100 µl of bacteriophage suspension was removed into sterile eppendorf tubes. Preparation was chilled immediately to stop further bacteriophage adsorption.

5) All the aliquots were centrifuged at 10,000 rpm for 10 min at 4°C. Bacteriophage in the supernatant was titrated by double agar overlay method as described in section 3.5.
3.11.6.2 Single step growth curve


1) A single isolated colony of *K. pneumoniae* B5055 was inoculated into 5 ml nutrient broth and incubated at 37°C overnight.

2) Next day overnight growth was re-inoculated into fresh sterile 50 ml nutrient broth that was re-incubated for 6 h. From this exponential phase growth of bacteria, OD_{600} was adjusted to a bacterial count of 10^8 cfu/ml.

3) To the 5.0 ml of bacterial culture (10^8 cfu/ml) 5.0 ml of bacteriophage suspension (10^6 pfu/ml) was added to obtain a Mol of 0.01.

4) Preparation was allowed to stand for 5 min at room temperature for bacteriophage to get adsorbed.

5) The mixture was centrifuged at 10,000 rpm for 5 min at 4°C. Pellet thus obtained was re-suspended in 1.0 ml of nutrient broth and incubated at 37°C.

6) At 5 minutes intervals, upto 60 min, 100 μl of bacteriophage suspension was removed in duplicate and chilled (4°C) immediately.

7) One set was diluted immediately and titrated for bacteriophage content. To the other set, chloroform (1% v/v) was added and centrifuged at 10,000 rpm for 5 min. Bacteriophage concentration in the aqueous phase was estimated by double agar overlay method explained in section 3.5.

3.11.7 Electron microscopy

Transmission Electron Microscopy of the isolated bacteriophage was performed according to the method of Goodridge et al 2003 with some modifications.
Materials and Methods

Materials

1) Potassium phosphotungstic acid (PTA) (Himedia Laboratories, Mumbai, India): 4% solution was prepared in distilled water and stored at 4°C. Prior to use, solution was filtered through 0.45 μm metafilter (Millipore).

2) Copper grids. Carbon coated copper grids were purchased from Ted Pella Inc, UK.

Method

1) Bacteriophage preparation was purified by single plaque isolation method. High titer of purified bacteriophage was prepared.

2) High tittered bacteriophage preparation was subjected to ultracentrifugation at 100,000 rpm for 2 h (Sorvall M150GX, Newton, CT, USA). Bacteriophage pellet was suspended in PBS (pH 7.2).

3) Equal volume (100 μl each) of ultracentrifuged bacteriophage and PTA solution were mixed over a clean surface, in dust free environment. One drop of mixture was placed over the carbon coated copper grid and incubated at room temperature for 15 min.

4) After staining, excess stain was removed by blotting and grids were observed under transmission electron microscope (EM; Hitachi H 7500, Tokyo, Japan) at 80 kV at 150,000X.

3.11.8 Bacteriophage genome analysis

3.11.8.1) DNA isolation

Bacteriophage DNA was extracted following the standard protocol of Sambrook et al (1989).

Materials

EDTA (0.5 M, pH 8.0),
SDS (10%; w/v),
Pancreatic RNase (Bangalore Genei, Bangalore, India),
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Proteinase K (Bangalore Genei, Bangalore, India),
Phenol (Tris-saturated),
Chloroform
Ethanol,
Sodium acetate (3M, pH 5.5),
TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0),

**Method**

1) 600 μl of high titered bacteriophage suspension was taken in a sterile eppendorf tube.

2) Pancreatic RNase was added to a final concentration of 1 μg/ml and allowed to act on the bacteriophage suspension for 30 min at 37°C.

3) 0.5 M EDTA was added so as to obtain a final concentration of 20 mM.

4) Proteinase K was added to a final concentration of 50 μg/ml while SDS (10%) was added to a final concentration of 0.5%. The solution was mixed thoroughly and incubated at 56°C for 2.5 h.

5) After incubation, preparation was cooled to room temperature. An equal volume of tris-saturated phenol was added to the digestion mixture. By gentle mixing a homogenous emulsion of the aqueous and organic phase was obtained.

6) The phases were separated by centrifugation at 10,000 rpm for 10 min at 4°C. Aqueous phase was harvested and again subjected to another round of extraction using equal volume of tris-saturated phenol.

7) Again aqueous phase was collected into double autoclaved eppendorf tubes. To the harvested digestion mixture equal volume of tris-saturated phenol, chloroform and isoamyl alcohol in a ratio of [Chapter-3]
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25:24:1 was added. Mixture was shaken thoroughly and organic-aqueous phases were separated by centrifugation at 10,000 rpm for 10 min at 4°C.

8) Extraction of harvested aqueous phase was repeated using chloroform and isoamyl alcohol in a ratio of 24:1.

9) Ultimately the aqueous phase was extracted one time with equal volume of chloroform.

10) To the harvested aqueous phase, 2.5 volume of ice cold absolute ethanol was added. This was followed by the addition of sodium acetate (0.1 times the original volume).

11) Preparation was mixed thoroughly and kept at 4°C overnight for DNA precipitation. Next day precipitate was harvested by centrifugation at 10,000 rpm for 10 min. Pellet was washed three times with 70% ethanol. After washing, ethanol was drained and pellet was dried at 37°C by leaving the tubes open.

12) Dried DNA sample was suspended in 20 μl of TE buffer (pH 8.0) and kept at 4°C.

3.11.8.2 Restriction enzyme digestion of bacteriophage DNA

Materials

- Restriction enzymes:
  - EcoRI (Bangalore Genei, Bangalore, India),
  - Sau3AI (Bangalore Genei, Bangalore, India),
  - BsuRI (MBI Fermentas),
  - Hinfl (MBI Fermentas),

- Restriction enzyme buffer (of the respective manufacturer),
- Autoclaved double distilled water,
Materials and Methods

Method

1) For restriction enzyme digestion of isolated bacteriophage DNA, restriction cocktail was prepared as shown in Table 3.2.

Table 3.2 Ingredients of restriction cocktail

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Test (µl)</th>
<th>Control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Buffer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Isolated bacteriophage DNA</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

2) The digestion mixture was incubated at 37°C for 3 h. At the end of incubation, digestion mixture was placed at 4°C until processed further.

3.11.8.3 Agarose gel electrophoresis

Materials

Agarose (Himedia Laboratories, Mumbai, India)
Ethyedium bromide,
TAE buffer,
Mini (DNA) gel apparatus,
DNA sample to be electrophoresced,
DNA loading dye (6X),

Method

1) Agarose gel was prepared according to the recipe given in Table 3.3. Added the agarose and distilled water in a 500 ml flask. Heated the contents in a microwave oven for 2-4 min until the agarose dissolved.
**Materials and Methods**

Table 3.3 Ingredients of agarose gel

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>10X TAE</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>45.0 ml</td>
</tr>
<tr>
<td>Ethedium bromide (5mg/ml)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>

2) To the melted agarose, 10X TAE and ethedium bromide were added, mixed properly and poured onto the taped gel casting plate with casting combs in place. Gel was allowed to stand for 20-30 min at room temperature until gel solidified.

3) After solidification, tape and the comb were removed carefully. Gel with the tray was placed inside the horizontal electrophoresis apparatus.

4) After placing the gel, gently 1X TAE buffer was poured into the reservoir until the gel was just covered with the buffer.

5) In separate sterile eppendorf tubes one-tenth volume of 6X loading dye was mixed with each DNA sample and loaded into the wells.

6) The loaded gel was electrophoresed at 100V until the required separation was achieved.

7) Wearing gloves, tray containing the gel was picked up carefully and the DNA fragments were visualized by UV light. Picture of illuminated DNA bands was taken with a Polaroid camera.
3.11.9 SDS-PAGE ANALYSIS OF BACTERIOPHAGE PROTEINS (LAEMMLI 1970)

Materials

1) 30% Acrylamide solution
   Acrylamide 30.0 g
   N,N-methylene bis acrylamide 0.85 g
   Volume made to 100 ml distilled water.

2) Tris-HCl buffer (1.5 M, pH 8.8)
   Tris 18.15 g
   Distilled water 80.0 ml
   pH adjusted to 8.8 with HCl and final volume made to 100 ml with distilled water.

3) Tris-HCl buffer (0.5 M, pH 6.8)
   Tris 6.05 g
   Distilled water 80.0 g
   pH adjusted to 6.8 with HCl and final volume made to 100 ml with distilled water.

4) 10% Sodium dodecyl sulphate (SDS) solution
   Sodium Dodecyle Sulphate 10.0 g
   Distilled water 80.0 ml
   Volume made to 100 ml with distilled water.

5) 10% Ammonium persulphate (APS) solution
   Ammonium persulphate 0.5 g
   Distilled water 5.0 ml

6) Electrode buffer (running buffer)
   Tris-HCl 3.0 g
   Glycine 14.4 g
   10% SDS 10.0 ml
   Final volume made to 1000 ml with distilled water.
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7) **Temed (N, N, N, N-tetramethyle-ethylendiamine)**

8) **Sample solubilising dye**

- Tris buffer (0.5 M, pH 6.8) 1.2 ml
- 10% SDS 2.0 ml
- Bromophenol Blue (BPB) 0.005 g
- Glycerol 1.0 ml
- β-mercaptoethanol 500 μl

Final volume made to 10 ml with distilled water.

9) **Stacking solution**

- 30% acrylamide solution 0.50 ml
- 0.5 M Tris-HCl buffer, pH 6.8 0.38 ml
- 10% SDS 0.03 ml
- 10% APS 0.03 ml
- TEMED 0.003 ml
- Distilled water 2.20 ml

10) **Separating gel (running gel)** of different concentrations was made according to recipe shown in table 3.4.

**Table 3.4 Composition of separating gel for electrophoresis**

<table>
<thead>
<tr>
<th>Ingredients (ml)</th>
<th>Separating gel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.9</td>
</tr>
<tr>
<td>30% Acrylamide solution</td>
<td>1.7</td>
</tr>
<tr>
<td>1.5 M Tris-HCl buffer, pH 8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS*</td>
<td>0.05</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*In native gel SDS was replaced with equal volume of distilled water
### Materials and Methods

#### Reagents for silver staining

11) **Fixing solution**
- Methanol: 40.0 ml
- Acetic acid: 10.0 ml
- Distilled water: 50.0 ml

12) **10% ethanol**
- Ethanol: 10.0 ml
- Distilled water: 90.0 ml

13) **Farmer's reagent**
- Potassium ferricyanide: 0.3 g
- Sodium thiosulphate: 0.7 g
- Sodium carbonate: 0.1 g
- Distilled water: to 250.0 ml

14) **Silver nitrate solution**
- Silver nitrate (AgNO₃): 0.2 g
- Distilled water: 200.0 ml

15) **Sodium carbonate solution (2.5%)**
- Sodium carbonate: 2.5 g
- Distilled water: 100.0 ml

16) **Developer**
- Sodium carbonate: 6.25 g
- Glacial acetic acid: 270 µl
- Distilled water: to 250.0 ml

17) **Stopper solution**
- Acetic acid: 2.0 ml
- Distilled water: 98.0 ml
**Materials and Methods**

**Method**

**A) Polymerization of gel**

1) Thin glass plates used for preparing the gel were cleaned thoroughly with alcohol, held vertically separated by spacers on both the sides and tightened in the assembly.

2) Running gel of desired percentage (Table 3.4) was poured into the space between the plates leaving 1.5 cm space for stacking gel at the upper end.

3) Gel was overlaid with distilled water and allowed to polymerize at 37°C for 40-50 min.

4) After polymerization of the running gel, water was removed by tilting the gel. Comb was inserted into the space over the separating gel and stacking gel was poured over the separating gel.

5) Gel was allowed to polymerize at 37°C for 30-45 min.

**B) Preparation of sample**

1) High titer of bacteriophage (10^{12-14} pfu/ml) was prepared.

2) Bacteriophage sample was placed inside the dialysing membrane and dialysed thoroughly against PBS (pH 7.2) for 24 h with change of buffer at every 6 h.

3) Protein concentration of the dialysed bacteriophage sample was estimated by Lowry’s method (section 3.16).

4) Sample buffer and bacteriophage sample were mixed in a ratio of 1:3 in an eppendorf tube. Bacteriophage sample was used at a protein concentration of 5-20 µg/ml.

5) After thorough mixing of sample and buffer, preparation was heated in boiling water bath for 3-5 min.

6) Immediately after heating, the sample was placed on ice until loaded into the gel.
Materials and Methods

C) Loading of sample and running the gel

1) After polymerization of the gel, the comb was removed carefully so that wells were not broken.

2) Each well was washed with distilled water and water was drained by tilting the gel.

3) Plates containing the polymerized gel were fitted in the electrode apparatus and the chamber was filled with electrode buffer, so that whole of gel was immersed in the buffer.

4) Sample, kept on ice, was loaded into the well made in the gel using a Hamilton syringe.

5) Gel apparatus was attached to a power supply and gel was run at a constant voltage of 50 V, until the sample buffer came into the separating gel after which voltage was increased to a constant value of 100V.

6) Gel was run until the sample buffer reached the bottom of the gel, after which gel was removed carefully and subjected to staining.

D) Silver staining method (Laemmli 1970)

1) The gel was kept in fixing solution for overnight.

2) Next day gel was given two washings with 10% ethanol for 10 min each.

3) Further gel was given three washings with sterile distilled water for 10 min each.

4) The gel was then dipped in Farmer’s solution for approximately 5 min.

5) The gel was again washed three times with distilled water for 5 min each.

6) Gel was then dipped in silver nitrate solution for 10-15 min after which solution was drained off.
**Materials and Methods**

7) Gel was then rinsed with 2.5% sodium carbonate solution for 5 min.

8) The gel was then dipped in developer solution until the protein bands appeared as dark brown bands against a clear background.

9) As soon as the bands appeared, the reaction was stopped by immersing the gel in 2% acetic acid solution.

10) Stained gel was stored in distilled water.

3.11.10 Bacteriophage kinetics in mice

1) High titre bacteriophage suspension (10^{12} pfu/ml) was poured into the prepared dialysing membrane. Bacteriophage suspension was dialysed extensively against PBS (pH 7.2) for 48 h at 4°C with a change of buffer at every 8 hrs.

2) After dialysis bacteriophage suspension was removed into a sterile tube and its titer was estimated by double agar overlay method as described in section 3.5.

3) Titer of the bacteriophage suspension was set at 10^8 pfu/ml by dilution.

4) Each animal in a group of 12 Balb/c mice weighing 20-25 g was injected intraperitonially (i.p.) with 1.0 ml of bacteriophage suspension.

5) Another group of 6 mice was injected i.p. with an equal volume of heat inactivated bacteriophage suspension. This group acted as negative (-ve) control.

6) Another group of 2 mice was injected i.p. with sterile normal saline (NS) and observed during the duration of the experiment.

7) At different time intervals i.e. 1, 6, 12, 24, 36 and 48 hrs, 2 mice each from test group, and 1 mouse from -ve control group were sacrificed by cervical dislocation.

8) Before sacrificing, mice (test and control) were examined thoroughly.

At each time point, rectal temperature of each mouse was recorded.
Also, general health characteristics such as lethargy, exudates from corners of eye, fur texture and any signs of illness were noted.

9) Various organs of the sacrificed mice i.e. blood, kidney, liver, urinary bladder and lungs were collected aseptically into sterile vials.

10) Harvested organs were homogenised aseptically in 1.0 ml sterile PBS.

11) Dilutions from the supernatant of the homogenised organs were made in PBS and bacteriophage titer was estimated by double agar overlay method as described in section 3.5.

12) All experiments were performed according to the guidelines of Animal Ethical Committee, Panjab University, Chandigarh, India.

3.12 PRODUCTION AND ISOLATION OF DEPOLYMERASE (DP)

3.12.1 Preparation of bacteriophage high titer

1) High titer of bacteriophage was prepared by the method explained in section 3.7.

3.12.2 Denaturation of bacteriophage suspension

Denaturation of bacteriophage suspension was carried out following the procedure of Rieger et al (1975).

Materials

Bacteriophage preparation

0.2 M HCl
0.4 M aqueous Tris
DNase (1mg/ml)
0.003 Mg++

Method

1) High titer of bacteriophage suspension was dialysed thoroughly against PBS (pH 7.2).
Materials and Methods

2) Titer of the dialysed bacteriophage suspension was estimated by double agar overlay method.

3) 50.0 ml of bacteriophage suspension was prewarmed to 37°C. To the prewarmed suspension, 0.2 M HCl was added dropwise with intermittent shaking until the pH of the solution dropped to 3.5.

4) Stirring and warming of the sample was continued for 5-8 min.

5) Mixture was neutralized by adding 0.4 M aqueous Tris, dropwise, to pH 7.0.

6) DNase I was added to a final concentration of 20 µg/ml along with 2 µl/ml of 0.003 M Mg++. Preparation was mixed thoroughly and incubated at 37°C for 3 h.

7) After incubation digested bacteriophage suspension was again poured into dialysing membrane and concentrated by negative pressure to a final volume of 2.5 ml. The preparation was labelled as crude enzyme.

8) Bacteriophage activity and depolymerase activity of the crude enzyme was estimated.

3.12.3 Ultracentrifugation

1) Crude enzyme preparation was subjected to ultracentrifugation at 100,000 rpm for 2 h at 4°C.

2) After centrifugation supernatant was harvested and its depolymerase activity and bacteriophage titer was estimated.

3.13 PURIFICATION OF DEPOLYMERASE

Materials

Crude depolymerase sample

0.2 M Tris-HCl buffer (pH 7.2)

Sepharose G-200
Materials and Methods

Diethylaminoethyl (DEAE)-Sepharose

1 M NaOH

NaCl (linear gradient 0.1 M to 1.0 M)

Method

3.13.1 Ion exchange chromatography

1) 4 g of Diethylaminoethyl (DEAE)-Sepharose slurry was suspended in 100 ml autoclaved distilled water and allowed to stand for overnight.

2) Next day supernatant was discarded and to the suspended slurry 0.2 M Tris-HCl buffer (pH 7.2) was added.

3) Slurry was loaded onto a glass column (15 X 1.0 cm internal diameter) carefully, avoiding entrapment of air bubbles.

4) Packed column was equilibrated with same buffer.

5) Column was regenerated with 1 M NaOH solution (three bed volumes).

6) After regeneration, column was again equilibrated with 0.2 M Tris-HCl buffer (pH 7.2).

7) Dialysed protein was applied directly onto the regenerated column.

8) To remove the unbound protein, column was again washed with 0.2 M Tris-HCl (pH 7.2) until the OD$_{280}$ of the flow through became zero.

9) After washing, bound protein was eluted with a linear gradient of NaCl from 0.1 M to 1.0 M made in the same buffer. The flow rate was 1ml/min and 3.0 ml fractions were collected.

10) Depolymerase activity and the absorbance at 280nm ($A_{280}$) was recorded for each fraction. Fractions showing depolymerase activity were pooled and concentrated by negative pressure dialysis.
11) Protein estimation of the pooled fractions was done by Lowry's method.

3.13.2 Size exclusion chromatography

1) Concentrated depolymerase enzyme was filtered through 0.45 syringe filter.

2) Sepharose G-200 column (90 X 1 cm internal diameter) was equilibrated with 0.2 M Tris-HCl buffer (pH 7.2).

3) Concentrated and filtered depolymerase was loaded onto equilibrated column that was eluted using the same buffer at a flow rate of 20ml/h. 3.0 ml fractions were collected.

4) Protein concentration of the collected fractions was estimated by Lowry method and depolymerase activity of the collected fractions was also estimated (section 3.14).

5) Fractions showing depolymerase activity were pooled and subjected to acrylamide gel electrophoresis.

3.14 ENZYME ASSAY

Assay for depolymerase activity was performed by employing two methods as explained below.

3.14.1 Spot method

1) *Klebsiella pneumoniae* B5055 was grown to mid exponential phase growth phase at 37°C for 6 h.

2) To the molten (40°C) soft agar 100 µl of bacterial culture was added. After mixing, soft agar was poured onto nutrient agar plate that was allowed to solidify and incubated at 37°C for 48 h.

3) Purified depolymerase was diluted serially in sterile sodium phosphate buffer (pH 7.0). Various dilutions of depolymerase enzyme were spotted onto bacterial lawn of 48 h.
4) Spotted plates were incubated at 37°C overnight after which enzyme reaction was noted.

5) Reciprocal of the highest dilution of the enzyme preparation giving a perceivable clearance of the bacterial lawn was taken as the depolymerase titer.

3.14.2 Liberation of reducing sugar (Bartell and Orr 1968)

1) Capsular polysaccharide (CPS) was isolated from the planktonic culture of *Klebsiella pneumoniae* B5055. Isolated CPS was used as enzyme substrate.

2) Concentration of CPS was adjusted to 1 mg/ml of capsular sugar using sterile distilled water and its temperature was brought to 37°C.

3) Enzyme depolymerase held at 4°C was mixed with equal volume of diluted and pre-incubated CPS.

4) The reaction mixture was kept at 37°C and at 5 min intervals till 60 min of reaction, 500 μl of reaction mixture was withdrawn into separate sterile tube.

5) Withdrawn sample was immediately chilled by keeping over crushed ice to stop the enzyme reaction.

6) Amount of reducing sugar produced was estimated as described in section 3.15.

7) Enzyme activity was defined as the number of nM of reducing sugar produced by the enzyme per mg of substrate per minute of reaction duration.

3.15 ESTIMATION OF LIBERATED REDUCING SUGAR

Reducing sugar in the reaction mixture was estimated by the method of Rondle and Morgan (1955) as modified by Boas (1953).
**Materials and Methods**

**Materials**

1) Acetylacetone reagent (should be prepared fresh and can be used upto 18 h after preparation, if kept at 4°C)

   Acetylacetone 1.0 ml  
   0.5 N Na₂CO₃ 50 ml

2) Aldehyde reagent (the reagent should be prepared fresh, should be pale yellow and free from any sediments)

   p-dimethylaminobenzaldehyde 0.8 g  
   Absolute ethanol 30 ml  
   Conc. HCl 30 ml

3) Absolute ethanol

**Method**

1) 500 μl of reaction mixture withdrawn at 5 min intervals, for duration of 1 h, was processed as test sample. Standard curve was plotted by taking different concentrations (5-50 μl/ml) of galactosamine and processed similarly along with the test.

2) 1.0 ml acetylacetone reagent was added to each tube.

3) This was followed by addition of 0.4 ml of distilled water to each tube.

4) Contents of tubes were mixed thoroughly and heated in boiling water bath for 20 min.

5) After this, tubes were brought to room temperature.

6) To each tube, 2.5 ml of absolute ethanol was added followed by 1.0 ml of aldehyde reagent.

7) Contents were mixed thoroughly by shaking and 1.0 ml of absolute ethanol was added.
8) Mixture was heated at 65-68°C for 10 min in a water bath after which it was cooled to room temperature.

9) Optical density (OD) of the mixture was taken at 530 nm.

### 3.16 PROTEIN ESTIMATION

Protein content in the samples was assayed according to the modified method of Lowry et al (1951).

#### Materials

- 2% Na$_2$CO$_3$ in 0.1 N NaOH (A)
- 1% CuSO$_4$.5H$_2$O (B)
- 2% Sodium Potassium Tartarate (C)

Working solution: 98 ml of A + 1 ml of B + 1 ml of C

Folin-Ciocalteu’s reagent: 2 N reagent (diluted with distilled water to 1 N)

Bovine serum albumin (BSA): 10-100 µg/ml as standard

#### Method

1) 2.5 ml of working solution was added to 0.1 ml of protein sample and incubated at room temperature for 10 min.

2) After incubation, 0.25 ml of 1 N Folin-Ciocateu’s reagent was added and mixed immediately.

3) Tubes were incubated at 37°C for 30 min at room temperature. Optical density was taken at 660 nm.

4) Blank containing distilled water, instead of protein solution, was also processed in a similar manner.

5) Protein quantitation was done using a standard curve, which was prepared using graded concentrations of bovine serum albumin.
Materials and Methods

3.17 EXTRACTION OF CAPSULAR POLYSACHCHARIDE (CPS)

Capsular polysaccharide from bacterial culture was extracted by the modified method of Hanlon et al (2001).

Materials

Absolute ethanol
Nutrient agar made in Roux bottles
Sterile distilled water

Method

1) 160 ml nutrient agar was prepared in each Roux bottle and autoclaved.
2) 5 ml nutrient broth was inoculated with an isolated colony of Klebsiella pneumoniae B5055 and incubated at 37°C overnight.
3) Nutrient agar in Roux bottles was inoculated with 1.0 ml of overnight bacterial growth.
4) Inoculated bacterial culture was spread evenly onto whole surface of nutrient agar by tilting the bottle. If the media in Roux bottles was dry, 1.0 ml of normal saline was also added along with the bacterial culture. Incubation was done at 37°C for 48 hrs.
5) After incubation bacterial growth was harvested into a sterile, screw capped, 30 ml centrifugation tube.
6) To the scrapped growth, two volume of distilled water was added and tube vortexed 3 times for 1 min each and then centrifuged at 10,000 rpm for 1 h at 4°C.
7) Supernatant containing extracellular exopolysaccharide was removed and the pellet was discarded.
8) Three volumes of cold absolute ethanol were added to the supernatant for precipitation of exopolysaccharide.
9) Preparation was again centrifuged at 10,000 rpm for 20 min at 4°C. Precipitated CPS was dissolved in sterile distilled water to produce a viscous solution.

10) Isolated CPS was stored at -20°C until used.

3.18 ESTIMATION OF POLYSACCHARIDE SUGAR

Sugar content of the purified CPS was estimated by the method of Dubois et al (1956).

Materials

5% phenol in distilled water

Conc. H$_2$SO$_4$

Glucose (10-200 µg /ml) as standard

Method

1) 1 ml of 5% phenol solution was added to 0.1 ml of polysachharide solution and mixed properly.

2) To the mixed solution, 2.5 ml of conc. H$_2$SO$_4$ was added carefully.

3) Very carefully preparation was mixed by turning over the tube and incubated at room temperature for 30 min.

4) Optical density at 488 nm was recorded.

5) Blank containing distilled water, instead of polysachharide solution was also included and processed in a similar manner.

6) Amount of polysaccharide in the preparation was determined from the standard curve prepared using graded concentrations of glucose.

3.19 CHARACTERIZATION OF DEPOLYMERASE

Purified depolymerase enzyme was studied for following parameters.
3.19.1 Optimum substrate concentration

1) Stock solution of enzyme substrate i.e. CPS kept at -20°C was thawed.

2) From the stock solution various dilutions of substrate were made. Concentration of CPS in various dilutions ranged from 0.05 mM to 10 mM.

3) Appropriately diluted substrate was pre-incubated at 37°C after which equal volume of depolymerase was added and mixed properly.

4) Reaction mixture was incubated at 37°C. At 20 min after incubation 500 µl reaction mixture was withdrawn and chilled immediately to stop the enzyme action.

5) Depolymerase activity was estimated by determining the amount of reducing sugar produced, as per the method explained in section 3.15.

3.19.2 Optimum temperature for activity

1) Equal quantities of depolymerase and CPS were mixed in sterile eppendorf tube.

2) Reaction mixture was incubated at 22, 30, 37, 40 and 45°C.

3) 10 min after incubation, 500 µl of reaction mixture was withdrawn into sterile glass tube and chilled immediately.

4) Depolymerase activity in the samples was estimated by the amount of reducing sugar liberated.

3.19.3 Stability at various temperatures

1) 1 ml depolymerase enzyme at a concentration of 37 µg/ml was suspended in sterile eppendorf tube.

2) Tubes were exposed to various temperatures viz. 4, 22, 30, 37, 40, 56, and 65°C.
3) At 30 min and 60 min after exposure, enzyme sample was withdrawn and mixed with equal quantity (500 μl) of CPS and incubated at 37°C for enzyme reaction.

4) Enzyme activity in the samples, withdrawn at different time intervals was determined by estimating the concentration of reducing sugar liberated in reaction mixture, by the method described in section 3.15.

3.19.4) Optimum pH for activity

Materials

- 0.2 M Sodium acetate buffer (pH 3.0 to 6.0)
- 0.2 M Sodium phosphate buffer (pH 7.0 to 8.0)
- 0.2 M Tris-HCl buffer (pH 9.0 to 11.0)

Method

1) Respective pH of depolymerase enzyme was adjusted using various buffers.

2) To the pH adjusted enzyme sample equal volume of CPS was added and incubated at 37°C.

3) 10 min after incubation enzyme reaction was stopped by chilling.

4) Depolymerase units were estimated by estimation of reducing sugar in the sample.

3.19.5 Stability of DP at various pH values

1) 250 μl of depolymerase at a concentration of 37 μg/ml was added to 250 μl of buffers with different pH in sterile eppendorf tubes.

2) Tubes were held at room temperature and samples were withdrawn at 10, 20, 30, and 60 min.

3) To the enzyme sample at each time point and pH value, equal volume of CPS was added incubated further at 37°C for 10 min.
**Materials and Methods**

4) After incubation reaction was stopped by chilling and enzyme activity was estimated by estimating the concentration of reducing sugar liberated, by the method explained in section 3.15.

**3.19.6 Effect of metal ions**

**Materials**

<table>
<thead>
<tr>
<th>Iron Sulphate</th>
<th>Copper Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt Chloride</td>
<td>Zinc Chloride</td>
</tr>
</tbody>
</table>

**Method**

1) 500 µl of enzyme at a protein concentration of 37 µg/ml was added into sterile eppendorf tubes.

2) Each metal ion, enlisted above, was added to different eppendorf tubes containing depolymerase to achieve a concentration range of 0.2, 2, 20 and 200 mM.

3) After thorough mixing, preparation was held at room temperature for 1 h.

4) To each tube, 500 µl of CPS was added and incubated at 37°C.

5) Enzyme activity of the exposed depolymerase was estimated at 20 min after incubation by determining the reducing sugar in each tube.

6) A tube containing only enzyme without any added metal ion was also processed that acted as control.

**3.19.7 Effect of chemical compounds**

**Materials**

<table>
<thead>
<tr>
<th>EDTA</th>
<th>PMSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate</td>
<td>Tween-20</td>
</tr>
<tr>
<td>Tween-80</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>Sodium dodecyl Sulphate (SDS)</td>
<td></td>
</tr>
</tbody>
</table>

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*Chatper-3* 82
Method

1) 500 µl of enzyme at a protein concentration of 37 µg/ml was added into sterile eppendorf tubes.

2) The chemical compounds, enlisted above, were added to achieve a final concentration of 10 mM.

3) After thorough mixing, preparation was held at room temperature for 1 h.

4) To each tube, 500 µl of CPS was added and incubated at 37°C.

5) Enzyme activity of the exposed depolymerase was estimated at 20 min after incubation by quantifying the reducing sugar in each tube.

3.19.8 Molecular weight determination

1) For checking the purity of enzyme preparation and determining the molecular weight of depolymerase, SDS-PAGE of the purified enzyme was carried out by the method as described in section 3.11.9.

3.19.9) Determination of host range

Materials

Bacterial cultures used for determining the host range of enzyme are listed in Table 3.1.

Method

1) An isolated colony of the respective bacterial cultures was inoculated into 5.0 ml sterile nutrient broth.

2) Using the double agar overlay technique 72 hour old bacterial lawn was prepared for each strain.

3) 10 µl of depolymerase preparation (37 µg/ml) was spotted onto bacterial lawn of each organism.
4) Spotted enzyme was allowed to dry and then plates were incubated at 37°C overnight.

5) Appearance of any clear area was noted.

3.20 BIOFILM PRODUCTION

1) An isolated colony of *Klebsiella pneumoniae* B5055 was inoculated in 50 ml nutrient broth and incubated at 37°C overnight.

2) Next day bacterial culture was pelleted by centrifugation at 8,000 rpm for 15 min.

3) Supernatant was discarded and to the pellet 5 ml normal saline was added.

4) Pellet was given three washings by repeating the same procedure.

5) Ultimately, bacterial pellet was suspended in NS and OD_{600} was adjusted to 0.3, which is equivalent to 10^8 cfu/ml, (determined separately).

6) A sterile 96 well microtiter plate was opened under sterile conditions.

7) 100 μl sterile nutrient broth was added to each selected well of microtiter plate.

8) 100 μl bacterial culture (10^8 cfu/ml) was added to each well to which nutrient broth was added. Utmost care was taken so that droplets were not produced that might contaminate the control wells.

9) To certain number of wells, 200 μl of nutrient broth was added. These wells acted as plate sterility control.

10) Plate was incubated at 37°C for desired period of time.

11) Every 18 h (overnight), spent media in the wells was replaced by fresh nutrient broth.

12) The process was continued up to 7 day.
Materials and Methods

3.21 PROCESSING OF BIOFILMS GROWN IN MICROTITER PLATE WELL

1) At appropriate time intervals contents of desired number of wells were removed.

2) To the emptied wells 200 µl NS was added and using micro-pipette contents were aspirated to remove any loosely bound bacteria.

3) The procedure of washing was repeated three times.

4) Biofilm formed on the well surface was scraped using a sterile spatula. Scraped biofilm was suspended in 100 µl NS.

5) Suspended biofilm was vortexed three times for 1 min each.

6) Appropriate dilutions were made and viable cell count estimated.

3.22 MINIMUM INHIBITORY CONCENTRATION (MIC) ESTIMATION

1) OD₆₀₀ of bacterial culture was set to 0.3, equivalent to cell density of 10⁸ cfu/ml.

2) 1.0 ml each of Tryptone soya broth (TSB) was added in 10 different glass tubes.

3) A stock solution of ciprofloxacin was made to have a final concentration of 2048 µg/ml.

4) From the stock solution, 1.0 ml was added to the first tube containing 1.0 ml TSB.

5) Serial dilutions from the first tube through to the last tube were made by transferring 1.0 ml serially.

6) To the tubes containing graded concentrations of ciprofloxacin, 100 µl bacterial culture (10⁸ cfu/ml) was added.

7) Controls were also put alongside. One tube containing only the growth medium and the antibiotic acted as antibiotic sterility control. Another tube containing TSB and bacterial culture indicated the growth supporting properties of medium.
Materials and Methods

8) All tubes (test+control) were incubated at 37°C overnight.

9) Highest dilution of the antibiotic that visibly inhibited the bacterial growth after incubation was taken as MIC of ciprofloxacin.

3.23 OPTIMUM MULTIPLICITY-OF-INFECTION (MOI) DETERMINATION

3.23.1 Against planktonic cells

1) High titer of bacteriophage was prepared (section 3.3.6.2).

2) 3.0 ml of bacterial culture (10^8 cfu/ml) was added to different glass tubes.

3) To the tubes containing bacterial culture appropriate dilution of the bacteriophage suspension was added to obtain the desired MOI, ranging from 0.01 to 100.

4) Tubes were incubated at 37°C overnight. After incubation viable cell count in the treated bacterial culture was estimated.

3.23.2 Against biofilm cells grown in microtiter plate

1) Biofilms were grown in 96-well microtiter plate as described in section 3.20.

2) To the washed biofilm in wells appropriate dilution of the bacteriophage suspension was added to obtain the desired MOI.

3) Sterility control (only media) and positive control (growth media+bacterial culture) were also included in each assay. Incubation was done at 37°C overnight after which bacterial load was estimated by viable cell count.

3.24 ANTIMICROBIAL TREATMENT OF BIOFILM

3.24.1 Antibiotic treatment

1) Biofilm was grown in wells of 96-well microtiter plate as described in section 3.20.
2) On each day, to the appropriately washed wells, ciprofloxacin at a concentration of 10 µg/ml was added. In rest of the wells spent media was replaced with fresh sterile nutrient broth.

3) Plate was incubated at 37°C for 3, 6 and 24 h.

4) At the end of each incubation time, contents of wells, to which antibody was added, were removed and biofilm in well was processed as described in section 3.21.

5) Bacterial load of the biofilm in the processed well was estimated by the viable cell count method.

3.24.2 Bacteriophage treatment

1) Biofilm was grown in wells of 96-well microtiter plate as described in section 3.20.

2) Biofilm on each day (1-7) was exposed to bacteriophage at a Mol of 1.0 for 3, 6 and 24 h at 37°C.

3) After incubation, wells were emptied and biofilm processed by the method described in section 3.21.

4) Bacterial count of the treated biofilm was estimated by viable cell count method and reported as \( \log_{10} \) reduction against the untreated biofilm of similar time duration.

3.24.3 Combined treatment (bacteriophage + ciprofloxacin)

1) Biofilm was grown in the microtiter plate well (section 3.20) for varying time periods (1-7 days).

2) On each day ciprofloxacin (10 µg/ml; final concentration) and bacteriophage (Mol=1.0) was added to the same well containing the biofilm. For this treatment different rows in the microtiter plate were labelled from 1 to 7, corresponding to the age of the biofilm. 1 day old biofilm in wells in row 1 were exposed to antibiotic+bacteriophage treatment for 3, 6 and 24 hours. Spent media in rest of the wells was
Materials and Methods

replaced with fresh growth medium. Similarly on 2nd day 2 day old biofilm in row 2 was exposed to combined treatment. In same fashion other rows were treated on different days.

3) After each exposure, plate was incubated at 37°C for 3, 6 and 24 h after which wells were emptied using micro-pipette and exposed biofilms were processed as the method explained in section 3.21.

4) Viable cell count of the treated biofilm was estimated and results were reported as log_{10} reduction in comparison to untreated control.

3.25 BIOFILM TREATMENT WITH DEPOLYMERASE

1) Biofilm was developed for 7 days in wells of microtiter plate as explained in section 3.20.

2) On each day, as described in section 3.24, to the appropriate number of wells containing biofilm, 20, 37, and 42 µg/ml of depolymerase protein was added and incubated at 37°C.

3) At 15, 30, 60, 120 min after incubation well contents were removed and bacterial load in the biofilm was estimated by viable cell count. Results were reported as log_{10} reduction in comparison to untreated biofilm of respective day.

3.26 BIOFILM TREATMENT WITH DEPOLYMERASE AND CIPROFLOXACIN

3.26.1 Concomitant application of depolymerase and ciprofloxacin

1) Biofilms of *Klebsiella pneumonia* B5055 were grown in the wells of 96-well microtiter plate for various days (1-7).

2) After appropriate washing of biofilm in the microtiter plate well, on each day depolymerase (37 µg/ml) and ciprofloxacin (10 µg/ml) were added concomitantly to same well and incubated at 37°C for 6 hours.
3) After incubation, contents were removed and wells were washed to remove adherent cells. Biofilm from the washed wells was scraped and suspended in NS and its viable count was estimated.

3.26.2 Sequential application of depolymerase and ciprofloxacin

1) Biofilm in the microtiter plate well was grown for 7 days.

2) On each day washed biofilm was first exposed to 37 μg/ml of depolymerase for 60 min followed by 6 hour treatment with ciprofloxacin. Incubations were done at 37°C.

3) Treated biofilm was washed, scraped and suspended in sterile NS. Bacterial load of the treated biofilm was estimated by estimation of viable cell count.

3.27 DEVELOPMENT OF ANTIMICROBIAL RESISTANT VARIANTS

3.27.1 Antibiotic resistant variants (KPARV) (Nilsson and Soren, 1986)

1) Using 6 h old exponential phase bacterial growth, OD_{600} of 5 ml of nutrient broth was adjusted to 0.3 (=10^8 cfu/ml).

2) To this bacterial growth, ciprofloxacin was added to achieve a final concentration of 0.625 μg/ml.

3) After thorough shaking, mixture was incubated at 37°C for 48 h.

4) After incubation, dilutions of the incubated mixture were made and plated onto nutrient agar plates that were incubated at 37°C overnight.

5) After incubation, randomly a colony was picked up from the plate and inoculated into fresh nutrient broth that was incubated at 37°C overnight.

6) Procedure was repeated at least three times when finally a colony was selected and its susceptibility to the antibiotic was checked by disk diffusion method of Bauer et al (1966).
**Materials and Methods**

7) Bacterial colony showing resistance to antibiotic was chosen and labelled as KPARV.

8) Glycerol (60%; final concentration) stocks were prepared that were kept at -80°C and for routine use KPARV was maintained on nutrient agar containing ciprofloxacin (0.625 μg/ml).

**3.27.2 Development of bacteriophage variants**

1) Biofilm in the wells of microtiter plate was grown for 24 h.

2) 1 day old biofilm was exposed to bacteriophage treatment at a Mol of 1.0 at 37°C overnight.

3) From the overnight incubated culture, 100 μl was spread plated onto nutrient agar and incubated at 37°C overnight.

4) From the isolated colonies that were obtained after overnight incubation, a single colony was picked up randomly and inoculated into 3.0 ml sterile nutrient broth and incubated at 37°C.

5) Once the OD$_{600}$ was reached to 0.3 (=10³ cfu/ml), planktonic bacterial culture was incubated with bacteriophage suspension having a bacteriophage titer of 10¹⁰ pfu/ml (Mol of 100) at 37°C overnight.

6) Next day 100 μl bacterial culture was again spread plated onto nutrient agar and incubated at 37°C overnight.

7) Finally a colony was selected randomly, grown into planktonic culture in nutrient broth and labelled as KPØRV, the bacteriophage resistant variant.

8) Bacteriophage sensitivity of KPØRV was tested against respective bacteriophage and antibiotic.

9) KPØRV was confirmed to be *Klebsiella pneumoniae* by biotyping.

10) Glycerol stocks of KPØRV were prepared and stored at -80°C.
Materials and Methods

3.28 SCANNING ELECTRON MICROSCOPY (SEM) OF ANTIBIOTIC AND BACTERIOPHAGE RESISTANT VARIANTS

1) OD$_{600}$ of respective bacteria (KPB5055, KPØRV and KPARV) was adjusted in NS to 0.3.

2) Using fine coat ion sputter JFC-1100, bacteria were sputtered with gold dust.

3) Electron micrographs of bacteria were taken at 25 kV using Jeol JSM-6100 scanning electron microscope.

3.29 PHAGOCYTIC UPTAKE AND KILLING

Method of Hampton et al (1999) was followed to check the phagocytic ability of KPB5055 and its bacteriophage resistant (KPØRV) and antibiotic resistant (KPARV) variants.

3.29.1 Isolation of mouse peritoneal macrophages

Materials

- Mouse (Balb/c; either sex; 20-25 g)
- RPMI (ice cold)
- Fetal Calf Serum (FCS; 10%)
- 5 ml syringe fitted with 20 gauge needle
- Sterile glass Petri plate
- Phosphate buffered saline (10mM; pH 7.2)

Method

1) Mice were sacrificed by cervical dislocation.

2) Killed mice were spread over wax tray to expose their dorsal region.

3) Fur of mice was swabbed with ethanol and removed using scissors, without disturbing the peritoneal covering.

4) Using a 20 gauge needle 5-8 ml ice cold RPMI, with added 10% FCS was injected into the peritoneal cavity.
**Materials and Methods**

5) Peritoneal cavity was massaged carefully for 3-5 min after which injected fluid was sucked back using the same syringe.

6) Contents of syringe were de-loaded into a sterile glass petri plate and incubated at 37°C for 1 h in 5% CO₂.

7) After incubation contents of plate were removed by decanting.

8) Adhered cells were washed twice with chilled RPMI.

9) After washing adhered macrophages were scraped into ice cold sterile PBS. Two washings were given in the same buffer after which cells were suspended in the same buffer.

10) Macrophage count was adjusted to 1X10⁶/ml using haemocytometer.

3.29.2 Opsonisation of bacteria

**Materials**

Hank’s Balanced Salt Solution (HBSS; 10 mM, pH 7.2, containing 1 mM CaCl₂, 0.5 mM MgCl₂, and 1 mg/ml glucose)

**Method**

1) 10 ml of 6 h old bacterial growth in nutrient broth was centrifuged at 5000 rpm for 7 min.

2) Supernatant was discarded and pellet was resuspended in HBSS. Two washings were given using the same buffer.

3) Ultimately pellet was suspended in HBSS and OD₆₀₀ adjusted to 0.1.

4) To the bacterial culture, normal mouse serum (NMS; 10% v/v) was added and incubated at 37°C for 20-30 min with intermittent shaking.

5) Bacteria were re-centrifuged at 5000 rpm for 7 min and pellet was resuspended in original volume of HBSS.

6) Opsonised cells were used immediately.
3.29.3 Phagocytic assay

Materials

Opsonised bacteria (10^8 cfu/ml)
Normal Mouse Serum (NMS)
Macrophages (10^6/ml)
HBSS (10 mM; pH 7.2)
PBS (ice cold; 10 mM)

Method

1) The following ingredients were added to two tubes, each tube labelled as 'test' and 'control'.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Test (μl)</th>
<th>Control (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opsonised bacteria</td>
<td>750</td>
<td>750</td>
</tr>
<tr>
<td>NMS</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Macrophages</td>
<td>835</td>
<td>-</td>
</tr>
<tr>
<td>HBSS*</td>
<td>-</td>
<td>835</td>
</tr>
</tbody>
</table>

*Hank’s Balance Salt Solution

2) Both the tubes were incubated at 37°C.

3) From 'test' tube 250 μl samples were withdrawn at 10, 20, 30, 60, and 90 min interval while from tube labelled as 'control' 250 μl sample was withdrawn at 0, 10, 20, 30, 60, and 90 min.

4) To the withdrawn sample equal volume of ice cold PBS was added and centrifuged at 1800 rpm for 5 min.

5) Supernatant was harvested.
Materials and Methods

6) Three washings were given to the pellet using ice cold PBS. The supernatant from each washing was pooled.

7) Ultimately, pellet was suspended in ice cold PBS and 20 µl 0.5% Triton-X was added and incubated at room temperature for 30 min.

8) Bacterial count in the pooled supernatant and the treated pellet was estimated by viable cell count method.

3.30 EXTRACTION OF OUTER MEMBRANE PROTEINS (OMP)

The method of Filip et al (1973) was followed using the Sarkosyl preparation.

Materials

HEPES buffer (10 mM)

1% N-lauroylsarcosine

Method

1) An isolated colony of bacterial isolate was inoculated into 5 ml nutrient broth and incubated at 37°C for 2.5 h under shaking conditions.

2) Bacteria were pelleted by centrifugation at 8000 rpm for 10 min.

3) Supernatant was discarded and to the pellet 200 µl of 10 mM HEPES was added. Cells were sonicated (400 kW; 10 cycles of 1 min each with 1 min rest).

4) Sample was again centrifuged at 8000 rpm for 10 min to remove any unbroken cells.

5) Supernatant was ultracentrifuged (Sorvall M150 GX, Newton, CT, USA) at 150,000 g for 60 min at 4°C.

6) To the pellet 1% N-lauroylsarcosine was added and mixed properly by pipette.

7) Sample again was ultracentrifuged at 120,000 g for 40 min at 4°C.
8) Supernatant containing inner membrane proteins was discarded while pellet was harvested.

9) OMPs were analysed on 12.5% SDS-PAGE (20 μg protein/lane).

3.31 **ESTIMATION OF MUTATION FREQUENCY**

For estimation of mutation frequency in bacterial culture method of Drago et al (2005) was followed.

3.31.1 For planktonic cells against antibiotic

1) OD₆₀₀ of bacterial culture was adjusted, equivalent to 10⁸ cfu/ml.

2) 1 ml of this bacterial culture was exposed to 0.625 μg/ml of ciprofloxacin, overnight.

3) Next day 100 μl of exposed bacterial culture was plated onto nutrient agar and antibiotic containing nutrient agar plates.

4) Plates were incubated at 37°C for 48 h and mutation frequency calculated as number of resistant colonies/ml.

3.31.2 For planktonic cells against bacteriophage

1) 1.0 ml of bacterial culture (10⁸ cfu/ml) was mixed with 1.0 ml of bacteriophage suspension (10⁸ pfu/ml). Mixture was incubated at 37°C, overnight.

2) Next day bacterial suspension was plated onto nutrient agar.

3) Colonies with altered morphology were counted and checked for their bacteriophage susceptibility. Results were reported as cfu/ml.

3.31.3 For planktonic culture against combination of bacteriophage and ciprofloxacin

1) To 1.0 ml of bacterial culture (10⁸ cfu/ml) ciprofloxacin (0.626 μg/ml) and bacteriophage (Mol=1.0) was added simultaneously.

2) Mixture was incubated at 37°C overnight.
Materials and Methods

3) From the incubated bacterial culture, 100 μl was spread plated onto normal and ciprofloxacin containing nutrient agar plates.

4) After incubation at 37°C for 48 h bacterial colonies that appeared on the two media were counted and results reported as cfu/ml.

3.31.4 For biofilm against antibiotic

1) 36 h old biofilm was exposed to 10 μg/ml of ciprofloxacin and incubated at 37°C for 3 h.

2) After incubation biofilm was scraped and cells were suspended in NS.

3) NS suspended cells were vortexed (3 times for 1 min each with a gap of 1 min) and 100 μl was plated onto normal nutrient agar and ciprofloxacin containing (10 μg/ml) nutrient agar.

4) Plates were incubated at 37°C. Colonies appearing after 48 h were counted. Results were reported as cfu/ml.

3.31.5 For biofilm against bacteriophage

1) 36 h old biofilm in microtiter plate was exposed to a bacteriophage suspension at a Mol of 1.0. Plate was incubated at 37°C for 3 h.

2) After incubation, remaining biofilm was scraped and its bacterial load enumerated by plating 100 μl on nutrient agar plates.

3) Bacteriophage resistant colonies with altered morphology were counted and results reported as cfu/ml.

3.31.6 For biofilm against combination treatment

1) Biofilm (36 h) was exposed to ciprofloxacin (10 μg/ml) and bacteriophage (Mol=1.0).

2) After incubation at 37°C for 3 h, biofilm was scraped and vortexed. Bacterial culture was plated (100 μl) onto antibiotic containing and plain nutrient agar plates.

3) Plates were incubated at 37°C for 48 h after which colonies were counted and results reported as cfu/ml.
3.32 DEVELOPMENT OF BIOFILM ON GLASS COVERSILP

Biofilm were grown on air-liquid interface using batch culture model explained by Hughes et al 1998.

1) A yellow tip box was suitably modified and converted into a batch culture vessel.
2) Inner tip holder was suitably modified to hold glass coverslips vertically.
3) The tip holder was placed in inverted position in the outer box with coverslips in place.
4) Whole system was autoclaved carefully at 15 lbs/15 min, so that coverslips did not move from their place.
5) 200 ml nutrient broth was autoclaved separately at 15 lbs/15 min.
6) To the cooled nutrient broth 100 μl bacterial culture (10^8 cfu/ml) was added.
7) Inoculated nutrient broth, very carefully, taking all precautions, was poured into the tip box batch culture vessel and incubated at 37°C.
8) Each day desired number of coverslips was removed aseptically and placed inside wide-mouthed, 30 ml centrifugation tubes (Corning) containing 1 ml NS.
9) Using a sterile homogenizer, coverslip was crushed and vortexed to break bacterial clumps.
10) Appropriate dilutions were made and plated onto nutrient agar and incubated at 37°C overnight.

3.33 BIOFILM TREATMENT ON GLASS COVERSILPS

3.33.1 Treatment with antibiotic, bacteriophage and combination
1) Biofilm of desired day (1to 7) were removed aseptically from the tip box.
**Materials and Methods**

2) Removed coverslip was rinsed thoroughly with sterile NS and placed in either ciprofloxacin (10 μg/ml) solution, bacteriophage suspension (M0l=1.0) or solution containing both at respective concentrations, for 1 h.

3) After respective treatment biofilm laden coverslip was washed in sterile PBS and placed inside wide mouthed centrifugation tube containing 1.0 ml PBS.

4) Coverslip was crushed and suspension was vortexed for 3 min.

5) Bacterial load of the vortexed suspension was estimated by plating various dilutions of suspension on nutrient agar plates.

3.34 CONFOCAL SCANNING LASER MICROSCOPY (CSLM) OF BIOFILM

3.34.1 DAPI labelling of bacteriophage

1) 25 ml of nutrient broth in flask was inoculated with bacterial culture and incubated at 37°C for 6 h.

2) A single plaque cut from a plate containing isolated bacteriophage plaques was inoculated into log bacterial culture.

3) Simultaneously, 125 μg of DAPI was added to infected bacterial culture to attain a final concentration of 5 mg/L.

4) Mixture was incubated at 37°C for 6 h under shaking conditions.

5) After incubation, mixture was loaded onto 20-70% CsCl step-gradient in 5 ml tubes and ultracentrifuged for 20 min at 150,000 g at 20°C.

6) After centrifugation bright red band at 50-60% CsCl concentration, depicting intact bacteriophage was harvested.

7) Bacteriophage titer of the retrieved labelled bacteriophage was estimated by double agar overlay method as described in section 3.5.

8) Titer was expressed in pfu/ml.
Materials and Methods

3.34.2 FITC labelling of biofilm
1) Biofilms were stained in-situ.
2) For this 1 mg/ml stock solution of FITC was prepared in acetone.
3) From the stock solution 25 µg/ml of working solution was prepared in 1 mM phosphate buffer.
4) 50 µl of working solution was applied to the biofilm on coverslip and allowed to react for 10 min in dark at room temperature.
5) Coverslip was rinsed gently by submerging in a beaker containing sterile filtered phosphate buffer.

3.34.3 Interaction of stained biofilm with labelled bacteriophage
1) Biofilm was stained as described in section 3.34.2.
2) Labelled bacteriophage was applied to the stained biofilm at Mol of 1.0.
3) Interaction was allowed to occur for 1 h in dark.
4) Afterwards coverslip was washed in sterile phosphate buffer and visualized under Confocal Scanning Laser Microscope (CSLM) (LSM 510 Meta, Carl Zeiss, Germany).
5) Coverslips carrying treated biofilm were also crushed in sterile phosphate buffer and bacterial load enumerated by viable cell counting.

3.35 CRYSTAL VIOLET STAINING OF BIOFILM
1) For staining of biofilm on coverslip, biofilm laden coverslip was placed directly into 0.1% crystal violet solution (w/v) made in distilled water.
2) Preparation was allowed to stand for 10 min.
3) Stained biofilm was washed with sterile NS after which it was placed over a blotting paper and allowed to dry.
4) Stained biofilm on coverslip was visualized using a compound light microscope under oil-immersion objective.
3.4 STATISTICAL ANALYSIS

Data are expressed as means ± standard deviation (SD) of mean. Statistical analysis were performed using Microsoft Excel Programme. Data were grouped on basis of different treatments, done on different days and compared with the untreated control of respective day. Log reduction values were calculated by the method of Anderl et al (2000). Positive log reduction value depicted decrease in microbial count while negative value showed increase in microbial count. Comparison was made using single- tailed ANOVA. P ≤ 0.05 was considered significant.