



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>
Journal DOI: [10.21474/IJAR01](https://doi.org/10.21474/IJAR01)

INTERNATIONAL JOURNAL
OF ADVANCED RESEARCH

RESEARCH ARTICLE

Isolation of bacteriophages from wastewater for possible utilization to combat multi-drug resistant *Pseudomonas* spp. isolated from public water pool.

***Asha Salunke¹, Hemal Makani¹, Archana Rasure¹ and Wisam Abdulkareem Hussein².**

1. BioEra Life Sciences Pvt. Ltd., BioEra Park, S. No. 125, Mumbai-Bangalore Highway, Tathawade, Pune – 411033, Maharashtra, India.
2. Alakaa High School, Ministry of Education, Thiqr, Suq al Shiuk, Iraq.

Manuscript Info**Manuscript History:**

Received: 15 May 2016
Final Accepted: 15 June 2016
Published Online: July 2016

Key words:

Pseudomonas, bacteriophage, phage therapy, multiple drug resistance, phage genome, phage proteome

***Corresponding Author**

Asha Salunke.

Abstract

A multi drug resistant *Pseudomonas* spp. was isolated and characterized from public water pools. In order to develop combat strategy to eradicate and prevent this opportunistic pathogen, bacteriophages were isolated from wastewater samples. Isolated bacteriophage showed large clear plaques indicating presence of lytic phages with higher infectivity and burst size of 36×10^7 PFU/ml. Genome size of the bacteriophage was approximately 30675 bp with 10 fragments yielded from EcoRI restriction and 6 fragments from HindIII restriction. Proteome analysis showed 10 well separated bands on SDS PAGE.

Copy Right, IJAR, 2016,. All rights reserved.

Introduction:-

Public water pools are rich source of variety of fecal and non-fecal organisms. Fecal contamination of the water pools may occur due to faeces released by bathers or contaminated source water or, in outdoor pools, may be due to direct animal contamination. Non-fecal human shedding like from vomit, mucus, saliva or skin is also a potential source of pathogenic organisms in swimming pools. Many bacteria can also form biofilms and pose health hazard in such environments. Thus swimming pools are often associated with outbreaks or incidents of waterborne infections and skin rash. (Papadopolou et al., 2008).

Though routine monitoring and disinfection are stringently followed for swimming pools, opportunistic pathogen *Pseudomonas aeruginosa* frequently contaminates this water bodies and poses a significant public health threat. (Fiorillo et al., 2001, Tate et al., 2003, Lumb et al., 2004).

Pseudomonas is a genus of Gram-negative, aerobic gammaproteobacteria, belonging to the family Pseudomonadaceae. Most *Pseudomonas* species, known to cause disease in humans, are associated with opportunistic infections. *Pseudomonas* is a non-fermentative aerobe that derives its energy from oxidation rather than fermentation of carbohydrates. This organism grows well at 25°C to 37°C, but can grow slowly or at least survive at higher and lower temperatures. (Catharine et al., 1977).

The ability to thrive in harsh conditions is a result of hardy cell walls of *Pseudomonas* that contain porins. *Pseudomonas* spp. are increasingly recognized as an emerging opportunistic pathogen of clinical relevance. One of its most worrying characteristics is low antibiotic susceptibility. Being Gram-negative bacteria, most *Pseudomonas* spp. are naturally resistant to Penicillin and the majority of related beta-lactam antibiotics, but are sensitive to Piperacillin, Imipenem, Ticarcillin, or Ciprofloxacin; Aminoglycosides such as Gentamicin and Amikacin are other choices for therapy. This resistance to most of the antibiotics is attributed to efflux pumps, which pump out some antibiotics before they are able to act. (Robert and David, 2000).

These limitations in the use of present antibiotic therapies lead to emergence of another thought process which theoretically explains means of controlling *Pseudomonas* infections using bacteriophages. Bacteriophages are bacterial viruses which attack the bacterial cells and kill them. The host specificity of bacteriophages makes them unique. This bacteriophage therapy though is in its developmental stages, holds great promise in future.

In present study we isolated *Pseudomonas* spp. from public water pools. Isolation, enrichment of bacteriophages specific to *Pseudomonas* was done from river water samples. These phages were further studied for DNA size, restriction profile and protein profile.

Materials and method:-

Isolation of *Pseudomonas* spp:-

Approximately 250ml water samples were collected in sample collecting bottles, from 3 different public water pools in Pune, India. Collected water samples were transferred to the laboratory. 100µl samples were spread onto Sterile Cetrimide agar plates and plates were kept for incubation at 37°C for 24 hours. After incubation colony characters, Gram staining and motility test were performed to confirm presence of *Pseudomonas* spp. (Lowbury and Collins, 1955).

Antibiotic sensitivity test:-

Pseudomonas isolate was screened for antibiotic sensitivity by disc diffusion method. Overnight grown broth culture of *Pseudomonas* isolate was surface spread on a Cetrimide agar plate. Antibiotic impregnated discs were placed equidistantly on the agar plate. Discs impregnated with various antibiotics of standard concentrations were used for this analysis. The antibiotics used were Penicillin (10µg/ml), Ampicillin (20µg/ml), Tetracycline (30µg/ml), Streptomycin (10µg/ml), Erythromycin (15µg/ml), Gentamycin (10µg/ml). After placing the antibiotic discs the plates were incubated at 37°C for 24 hours (Bauer et al., 1966).

Enrichment of *Pseudomonas* phages:-

Approximately 250ml of river water samples were collected into the sample collecting bottle from different sites and transferred to the laboratory. Enrichment of phages was done by inoculating 100ml of each river water sample separately in 100ml of double strength LB media containing 1ml of overnight grown culture of *Pseudomonas*. The media was incubated at 37°C for 24 hours. After incubation medium was centrifuged at 5000rpm for 10 minutes at 4°C and 10ml. Supernatant was collected into a fresh vial. 0.2ml chloroform was added to the supernatant and mixed thoroughly; this was used as the enriched phage lysate (Gwyneth et al., 2006).

Isolation of phages using agar overlay method:-

Enriched Phage lysates were screened by double layer agar method. 25µl of enriched phage lysate was mixed with 0.2ml of overnight grown broth culture of *Pseudomonas* isolate (host culture) in 5 ml of cetrimide soft agar media and was poured on top of 10ml cetrimide hard agar plate. The plates were incubated at 37°C for 24 hours. Plaque forming units present per ml (PFU/ml) of the samples were calculated after observing the plates (Gwyneth et al., 2006).

One step growth curve of Bacteriophage:-

5ml of the overnight grown *Pseudomonas* culture and 5ml of enriched phage lysate were mixed and incubated for adsorption at 37°C for 20 minutes. After the incubation 90ml of sterile Luria Bertani broth was added to the mixture. Immediately 0.1ml of suspension was drawn from the mixture and centrifuged at 5000rpm for 10 minutes at 4°C and supernatant was collected into a fresh vial. 2µl chloroform was added to the supernatant and mixed thoroughly; this was used as the phage lysate and labeled as 0 minute lysate. The 0 minute phage lysate was further diluted to 10^{-4} . Double layer agar method was performed for the phage lysate to obtain PFU/ml of sample for 0 minute incubation. Similarly phage lysates were prepared after interval of every 30 minutes and PFU/ml calculated by double layer agar method. A graph of relative titers Vs time was plotted based on PFU/ml readings for various time interval phage lysate samples (Kokjohn et al., 1991).

Genome analysis:-

Bacteriophage DNA was extracted by phenol: chloroform method (Radhakrishnan and Ananthasubramanian 2012). Restriction analysis of isolated bacteriophage DNA was carried out using EcoRI and HindIII endonucleases. Whole DNA and restricted DNA were analyzed on 0.6% agarose gel with standard marker lane ranging from 20000 bp to 1000 bp.

Proteome analysis:-

Phage structural proteins were analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). 100µl of high titer phage suspension from the enriched phage lysate was boiled for 5 minutes with sample loading dye and was then electrophoresed on discontinuous SDS-PAGE gel (5% stacking, 12% resolving gel) by using the vertical slab gel electrophoresis system (BioEra, India). Phage protein samples along with the standard molecular weight marker were loaded on gel. Gel was stained using Coomassie blue staining solution. Molecular weight of phage proteins were estimated by comparing with standard molecular weight markers (Ngangbam and Nongmaithem, 2012).

Results and discussion:-**Isolation of Pseudomonas:-**

Pseudomonas was isolated from water sample obtained from Baner, Pune, India. White, circular with smooth margin and soft consistency colonies were observed on Cetrimide agar. Gram negative Rods were observed after Gram staining and the culture was found to be highly motile with hanging drop technique under microscope.

Antibiotic Sensitivity Test:-

Antibiotic sensitivity test was performed for *Pseudomonas* isolate obtained from water sample. It was found to be Resistant to Penicillin, Ampicillin, Tetracyclin and Streptomycin. It was intermediately resistant to Erythromycin and was Sensitive to Gentamicin. Results were concluded as per Standard Kirby-Bauer chart.

Water sample collection sites for isolation of Pseudomonas bacteriophage:-

Water sample sites selected for the isolation of *Pseudomonas* bacteriophages were contaminated river water sites. The possibility of presence of bacteriophages in such environment was greatest due to fecal and non-fecal contamination of sites and presence of host in larger numbers.

Phage enrichment, isolation and quantification:-

Three different types of plaques were observed upon enrichment of bacteriophages viz. clear plaques with small diameter, clear plaques with larger diameter and cloudy plaques with larger diameter. Clear plaques indicate the presence of lytic phages and the diameter of plaques directly indicates increased rate of infectivity and the burst size. Hence the lysate which gave larger clear plaques was selected for further analysis. The quantification of phages was done by calculating total number of plaques present on agar. Each phage gives rise to one plaque. Thus number of phages [Plaque Forming Units (PFU)] present per milliliter of the enriched phage lysate calculated by counting number of plaques observed upon incubation.

One step growth curve:-

The burst size was determined using one step growth curve. Burst size is a point on one step growth curve graph with maximum relative titer. The burst size for the isolated phage was found out to be 36×10^7 PFU/ml.

Gwyneth et. al. (2006) Studied and isolated bacteriophages infecting *Salmonella* species, they carried out single step growth curve analysis for the bacteriophages using optimal growth conditions for *S. Enteritidis* PT160. The experiment set up included use of enriched phage lysate and double layer agar overlay method at equal intervals of time upto 8 hr, to determine the phage adsorption and burst size. They found out that the phage infection resulted in a burst size of 139 PFU but was apparently inactive at a temperature typical of stored foods (5°C). Phage FGCSSa1 behaved unusually when grown on two *Salmonella* serotypes at 37°C in that the addition of phages appeared to retard growth of the host, presumably by the lysis of a fraction of the host cell population.

Genome analysis:-

The molecular analysis of phages was done to determine genome size and restriction profile. The molecular weight of standard marker was 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000 bp. DNA molecular size of phage was approximately 30675 bp.

Kwan et al. (2006) compared genome of 18 *Pseudomonas aeruginosa* bacteriophages and with exception of phages SD1 M and phiKZ approximately 281 kbp each, the remaining 16 phage genomes span a narrow size range from approximately 35 kbp to 72 kbp.

The restriction enzymes EcoRI and HindIII yielded restriction fragments of various sizes indicating many restriction site on the genome of *Pseudomonas* bacteriophage. EcoRI enzyme treated genome of bacteriophage yielded ten different fragments of sizes 8482 bp, 7467 bp, 6266 bp, 5104 bp, 4251 bp, 3527 bp, 2799 bp, 1948 bp, 1375 bp and 509 bp. HindIII restricted genome of bacteriophage gave six different fragments of sizes 11662 bp, 8711 bp, 7467 bp, 6373 bp, 5208 bp and 4498 bp.

Proteome analysis:-

The proteome analysis suggested presence of 10 different proteins present in *Pseudomonas* bacteriophage. The standard molecular weights of marker were 200 kD, 150 kD, 94 kD, 66 kD, 40 kD, 18 kD and 3 kD. The molecular weights of Phage proteins were found out to be 86 kD, 67 kD, 53 kD, 37 kD, 27 kD, 24 kD, 16 kD, 12 kD, 10 kD and 7 kD.

In present study, *Pseudomonas* is isolated from Swimming pool water sample indicating potential health hazard and skin infection. *Pseudomonas* isolate was found out to be resistant to number of antibiotics which directly comments about the health threat. Bacteriophage isolated from wastewater sample is potentially active against the *Pseudomonas* isolate, and produced clear plaques indicating complete lysis of bacterial cells. Further analysis of bacteriophage lysate and clinical trial studies will help into developing a potential anti-*Pseudomonas* therapy and Cleaning of Swimming water pools using these enriched lysates.

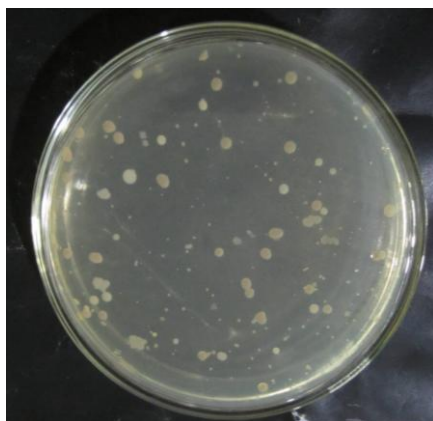


Figure 1:- *Pseudomonas* colonies on Cetrimide agar

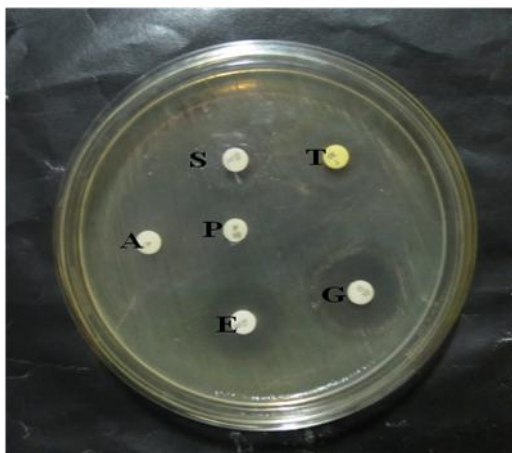


Figure 2:- Antibiotic Sensitivity Test

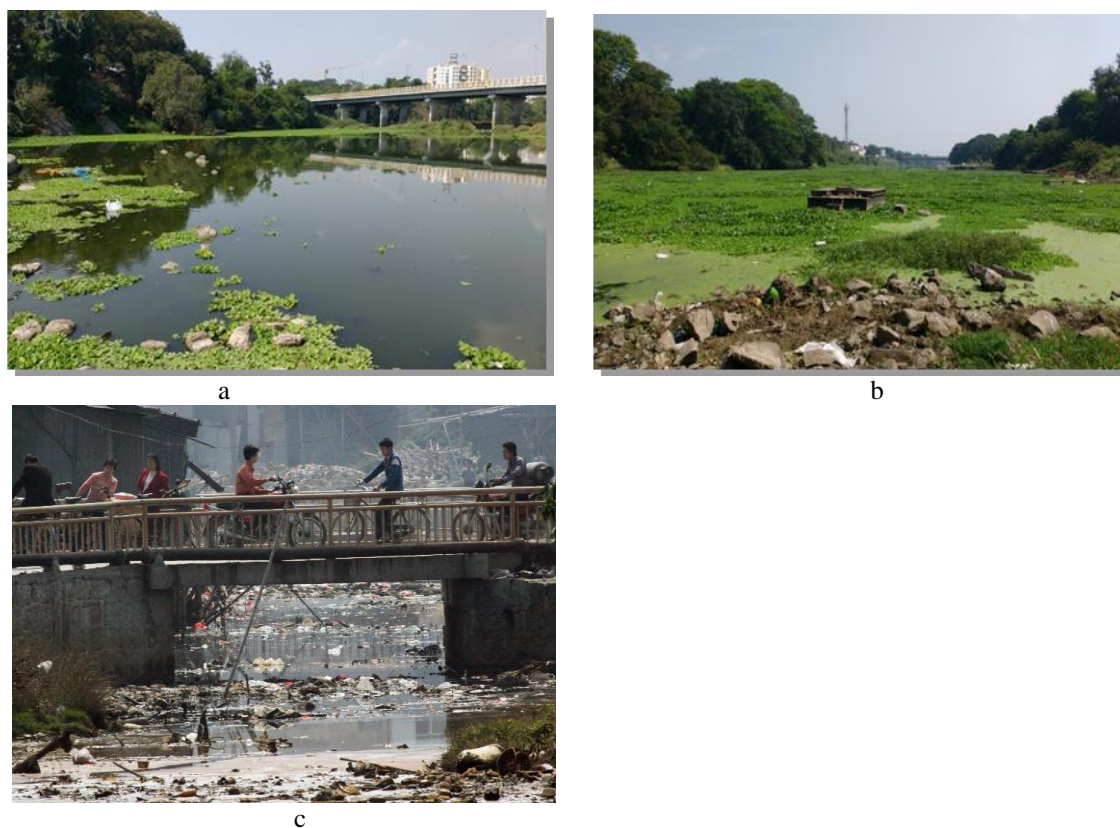


Figure 3 :- Phage sample collection sites (a) Mula River, Baner, Pune, India (b) Mula River, Aundh, Pune, India (c) Ambil Odha, Pune, India

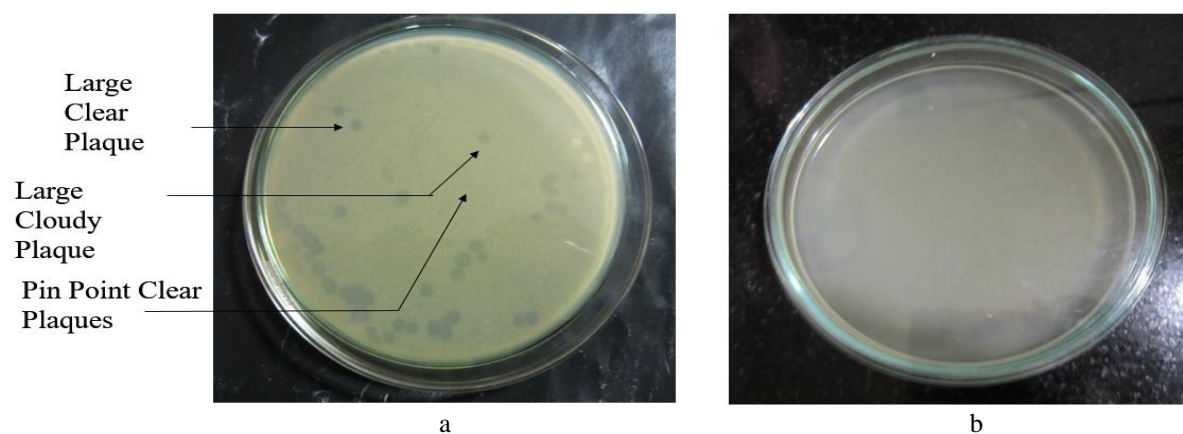


Figure 4:- Plaque assay on Cetrimide agar plates (a) Phage sample plate (b) Control plate

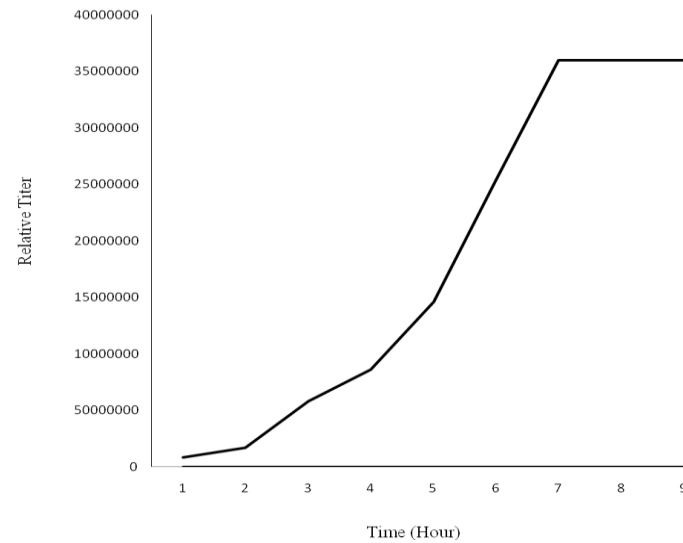


Figure 5:- One step growth curve of isolated *Pseudomonas* phage

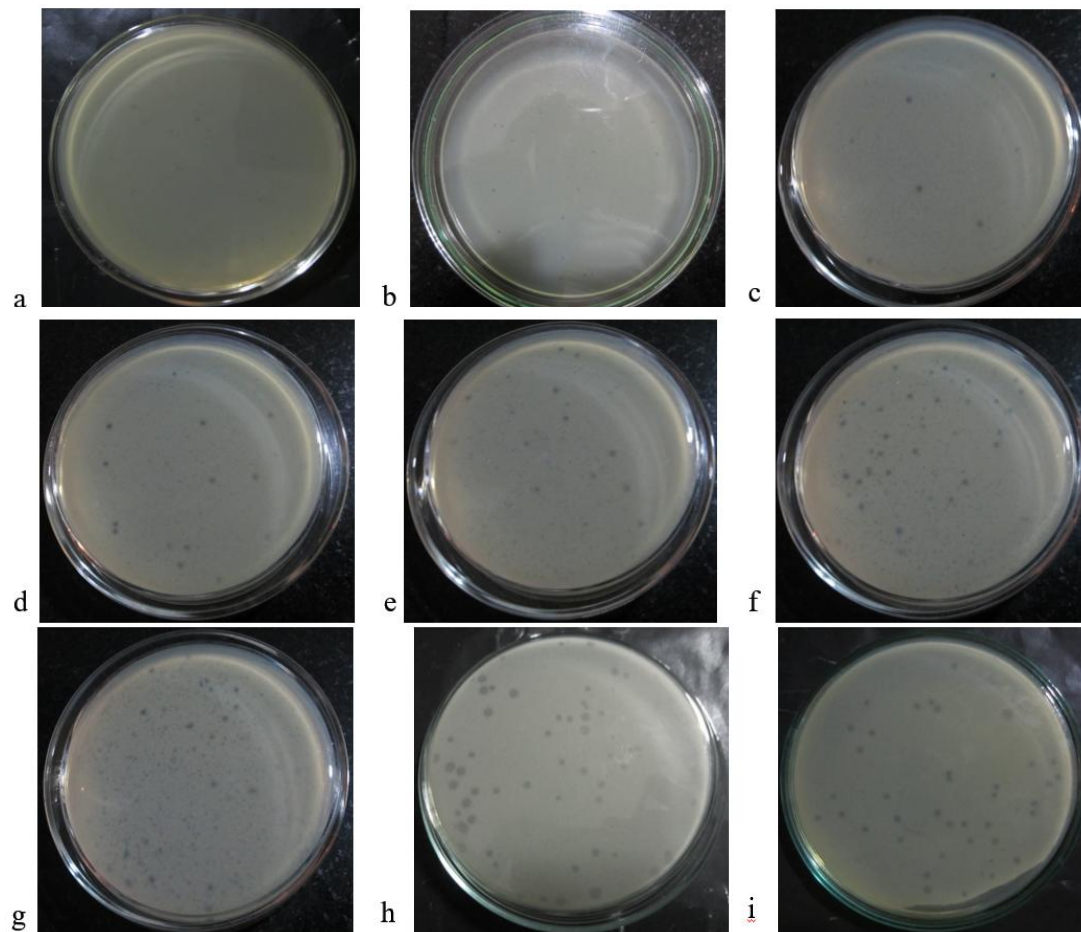


Figure 6:- One step growth curve (a) Lysate from 0 minute (b) Lysate from 30 minute (c) Lysate from 60 minute (d) Lysate from 90 minute (e) Lysate from 120 minute (f) Lysate from 150 minute (g) Lysate from 180 minute (h) Lysate from 210 minute (i) Lysate from 240 minute.

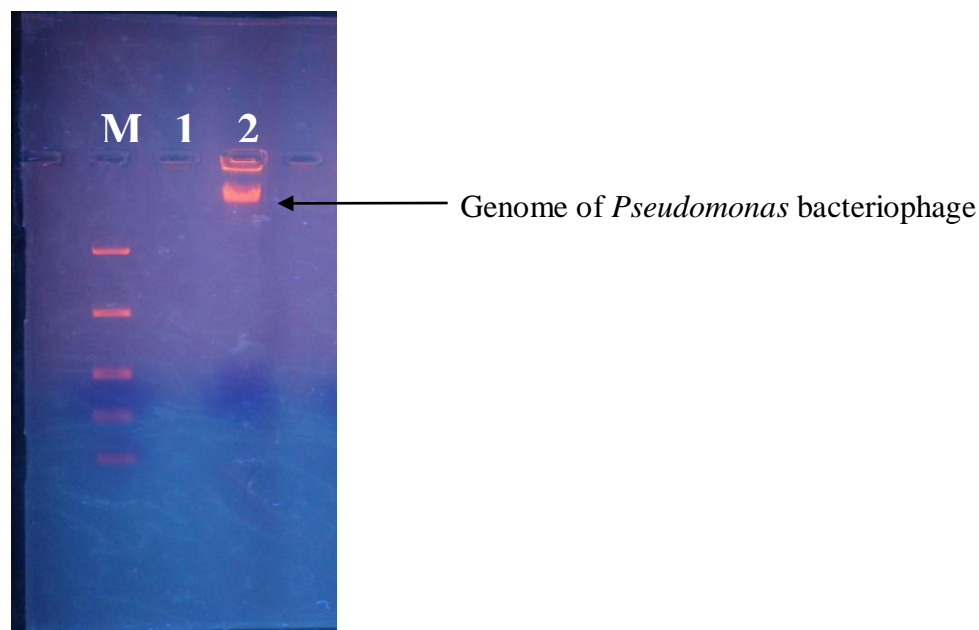


Figure 7:- Agarose gel electrophoresis

M: Standard Molecular weight marker lane with molecular weight 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000 bp

Lane 2: *Pseudomonas* bacteriophage genome with molecular weight approximately 30675 bp

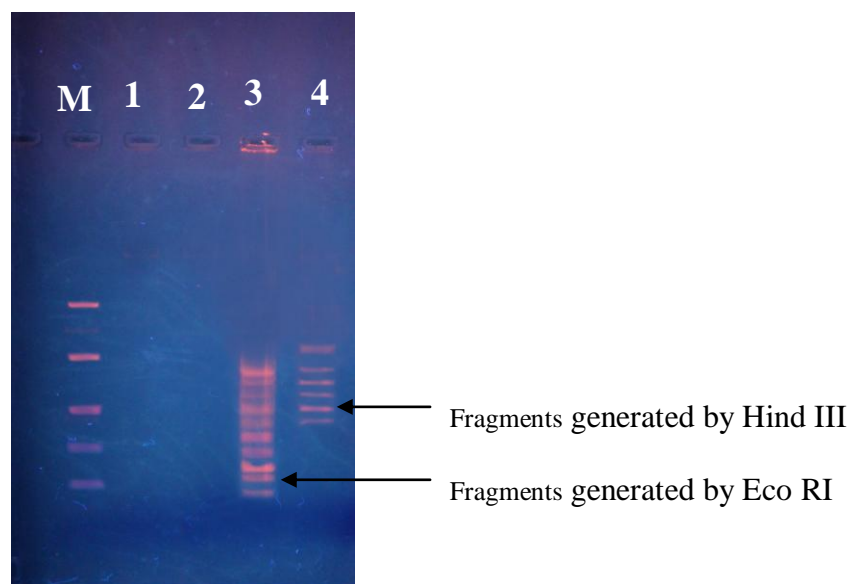


Figure 8:- Restriction digestion of *Pseudomonas* bacteriophage genome

M: Standard Molecular weight marker lane with molecular weight 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000 bp

Lane 3: gDNA digested with EcoRI with molecular weight 8482 bp, 7467 bp, 6266 bp, 5104 bp, 4251 bp, 3527 bp, 2799 bp, 1948 bp, 1375 bp and 509 bp

Lane 4: gDNA digested with HindIII with molecular weight 11662 bp, 8711 bp, 7467 bp, 6373 bp, 5208 bp and 4498 bp



Figure 9:- Proteome analysis by SDS PAGE

M: Standard molecular weight marker lane with molecular weights 200 kD, 150 kD, 94 kD, 66 kD, 40 kD, 18 kD and 3 kD

Lane 1: Proteins extracted from Pseudomonas bacteriophage with molecular weights 86 kD, 67 kD, 53 kD, 37 kD, 27 kD, 24 kD, 16 kD, 12 kD, 10 kD and 7 kD

References:-

1. **Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M. (1966):** Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45: 493-496.
2. **Catharine, B.S., Kathleen, S., Ian, A.H., (1977):** Experimental Studies on the Pathogenesis of Infections Due to *Pseudomonas aeruginosa*: Direct Evidence for Toxin Production during *Pseudomonas* Infection of Burned Skin Tissues. *The Journal of Infectious Diseases.* 136 (4): 555-561.
3. **Fiorillo, L., Zucker, M., Sawyer, D., Lin, A.N. (2001):** The *Pseudomonas* hot-foot syndrome. *N. Engl. J. Med.* 345: 335-338.
4. **Gwyneth, V., Carey, S., Billington, C., Cornelius, A.J., Hudson, J.A., Heinemann, J.A. (2006):** Isolation and characterization of bacteriophages infecting *Salmonella* spp. *FEMS Microbiol Lett.* 258: 182-186.
5. **Kwan, T., Liu, J., DuBow, M., Gros, P., Pelletier, J. (2006):** Comparative Genomic Analysis of 18 *Pseudomonas aeruginosa* Bacteriophages. *Journal of Bacteriology.* 188(3): 1184-1187.
6. **Kokjohn, T. A., Sayler, G. S., Miller, R. V. (1991):** Attachment and replication of *Pseudomonas aeruginosa* bacteriophages under conditions simulating aquatic environments. *Journal of General Microbiology.* 137: 661 – 666
7. **Laemmli, U.K. (1970):** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
8. **Lowbury, E.J., Collins, A.G. (1955):** The use of a new cetrimide product in a selective medium for *Pseudomonas pyocyanea*. *J. Clin. Pathol.* 8: 47-48.
9. **Lumb, R., Stapledon, R., Scroop, A., Bond, P., Cunliffe, D., Goodwin, A., Doyle, R., Bastian, I. (2004):** Investigation of spa pools associated with lung disorders caused by *Mycobacterium avium* complex in immunocompetent adults. *Appl. Environ. Microbiol.* 70: 4906-4910.
10. **Ngangbam, A.K., Nongmaithem, B.L. (2012):** Molecular Characterization of *Salmonella* Bacteriophages Isolated From Natural Environment and Its Potential Role in Phage Therapy. *Journal of Agriculture and Veterinary Science.* 1(6): 701-708.
11. **Papadopoulou, C., Economou, V., Sakkas, H., Gousia, P., Giannakopoulos, X., Dontorou, C., Filioussis, G., Gessouli, H., Karanis, P., Leveidiotou, S. (2008):** Microbiological quality of indoor and outdoor swimming pools in Greece: Investigation of antibiotic resistance of the bacterial isolates. *International Journal of Hygiene and Environmental Health.* 211: 385-397.
12. **Radhakrishnan, A., Ananthasubramanian, M. (2012):** Characterization and lytic activity of *pseudomonas fluorescens* phages from sewage. *Brazilian Journal of Microbiology.* 43(1): 356-362.
13. **Robert, E.W., David, P.S. (2000):** Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resistance Updates.* 3: 247-255.
14. **Tate, D., Mawer, S., Newton, A. (2003):** Outbreak of *Pseudomonas aeruginosa* folliculitis associated with a swimming pool inflatable. *Epidemiol. Infect.* 130: 187-192.