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BACTERIOPHAGE AS BIOCONTROL AGENT FOR PATHOGEN REDUCTION IN WASTEWATER

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ABSTRACT

Many sewage wastewater treatment systems are aiming for complete pathogen removal without harming the environment which necessitates the search for new approaches. One such approach is the use of bacteriophage for pathogen removal. Bacteriophages can be used as bio-control agent against bacteria without interfering the natural micro-flora. Current study focuses on investigating the effect of bacteriophage against the pathogen present in wastewater by using bacteriophage as biocontrol agent. In this study five bacterial strains were isolated from sewage water collected from outlet connecting Mutha River, backside of Poona Hospital, Pune. The bacterial strains were identified till genus level based on biochemical characters and they may belong to *E.coli*, *Staphylococcus*, *Salmonella*, *Klebsiella*, *Pseudomonas* species.16srRNA gene sequencing was performed for one bacterial strain and it was identified as *Klebsiella veriicola* DSM 15968 (T) Titre for phage against Isolate 1, Isolate 2, Isolate 3, Isolate 4 and Isolate 5 were determined and found to be (PFU/ml) $1.7x10^4$, $1.2x10^6$, $1.98x10^6$, $2.1x10^4$ and $1.2x\ 10^4$ respectively. One step growth curve of PA1 was performed. FE-SEM of PA 4 was done and icosahedral head with tail was observed of 500nm size. TVC of wastewater was found to be 7×10^6cfu/ml. Reduction in total viable count of pathogen were determined by treating it with cocktail of phages, after the treatment with cocktail of phages for 18 hours significant reduction was observed and found to be $5.5x10^4$cfu/ml.

KEYWORDS: Wastewater, Pathogen reduction, Bacteriophages, Cocktail.

INTRODUCTION

Water is essential component of life, the world health organization (WHO) has been concerned with drinking water quality and its effect on the human health. India is densely populated country and therefore the present scenario of Indian rivers and other water bodies is really terrible due to fast growing urbanization, industrialization and modernization which leads to degradation of water quality in our country. Direct discharge of waste from domestic, agricultural, industrial into water bodies is suspected as major sources of water borne disease (Hutly 1990). On a global basis around 2 million death per year is due to water borne disease especially diarrhea in children. Wastewater containing pathogenic organism causes disease such as typhoid, salmonellosis, hepatitis, cholera dysentery etc. The frequent discharge of sewage containing pathogenic microbes into water resources extended the survival of these organisms to detectable level at higher concentrations. Bacteriophages i.e eaters of bacteria are found abundantly in biosphere as predator community. Some 23 years ago, a Norwegian group reported that aqueous system (ocean, lakes, rivers) contains high concentration of phages like particles its concentration varies with season and geo graphical locations (Berge etal 1989). In addition to this antimicrobial resistant pathogenic bacterial strain are ever emerging in the environment therefore bacteriophages have attracted in increased interest as alternative natural antimicrobial agent to fight bacterial disease. Today bacteriophages are applied against nuisance bacterial pathogens in fields like food, medical etc. The receptors on bacterial host is recognized by proteins on phage due to this specificity phages can be used as a biocontrol agent against certain pathogens. For successful application of phages as biocontrol agent, phages need to be isolated, enriched & should be produced in sufficient number for its application. Phage survival stability & infectivity is important criterion in order to use them as a biocontrol agent.

MATERIALS AND METHODS

Collection and characterization of Wastewater sample

Water sample was collected from sewage outlet connecting Mutha river back side of Poona Hospital, Pune, in pre-sterilized 1000ml capped flask from outermost end of the river. Water sample was filtered through coarse filter paper and Whatmann filter paper to remove any particulate matter. Collected sample were tested for pH, TDS, BOD and COD.

Bacteriological analysis of waste water sample

The sample was subjected to viable count studies by spreading 100ul of sample on sterile nutrient agar plate. Overnight incubation was done at 37°C. After 24 hours of incubation plates were examined. Plates which showed 40-200 colonies were used for determining the total viable bacterial count.

Isolation and characterization of target bacteria

Water sample was spread plate on specific media to isolate the target bacteria. Culture media used were 1. Eosin Methylene blue agar, 2. MSA (Mannitol salt agar) 3. Salmonella Shigella (ss) agar 4. MacConkey agar 5. Cetrimide agar. Bacterial isolates were subjected to further characterization and identification as per the standard procedure.

Drug resistant profile

0.3ml of each bacterial isolate were spread on Sterile Nutrient agar plates. Dodeca universal I DE001 (Hi Media) Disc was placed on it Incubation at 37° C for 24 hours.

Disc consist of following Antibiotics

Cefpodoxine (CPD), Chloramphenicol (C), Vancomycin (VA), Streptomycin (S), Rifampicin (RIF), Levofloxacin (LE), Ceftiaxone (CTR), Clindamycin (CD), Augmentin (AMC), Amikacin (AK).

Isolation of specific Bacteriophage against target bacteria

Isolation of Phages

a) Isolation of phages by chloroform method- Sample was filtered through coarse filter paper to remove any particulate matter. 10% chloroform was added to the filtrate and vigorously shaken for 30 mins. It was then transferred to sterile centrifuge tubes and centrifuge it at 8000rpm for 20 minutes at 4°C. After centrifugation supernatant was collected in the sterile centrifuge tubes and stored it as a lysate at 4^0c.

b) Isolation of bacteriophage by Membrane filters- The sample was subjected to centrifugation for 20mins at 8000rpm at 4°C and then it was filtered through membrane filters (0.22µ). Filtrate was stored as lysate at 4°C.

Enrichment of Phages

In 50ml nutrient broth each bacterial strains were grown overnight at 37°C to get organisms at log phase. 10 ml of sterile double strength Phage Broth and 10 ml lysate were added to

each five nutrient broth containing flask and incubated at 37°C for 48 hours. After incubation, respective treatment was given Stored at 4ºC as enriched lysate for spotting.

Conformational test for presence of Bacteriophages (By spot test)

0.1ml log phase host was spread on the sterile nutrient agar plate and 20µl lysate was spotted on the plate and incubated at 37°C for 24hrs.

Purification of bacteriophages and determination of pfu/ml

Lysate was serially diluted from 10^{-1} to 10^{-6}. 0.2µl of lysate mixed with 0.4µl of log phase host and incubated at 37°C for 30mins. Mixture was mixed in soft agar butt, 1ml of log phase host was added to the butt, vortexed and poured on the sterile basal NA plate Incubation at 37°C for 24hrs Number of plaques on plates were counted and PFU/ml was determined.

One step growth curve

0.9 ml of log phase host suspension and 0.1 ml of the phage lysate were mixed and incubated at 37°C. At precisely 10 min after starting the incubation 0.1 ml of mixture was diluted upto 10^{-5} dilution. At regular time intervals (00, 20, 30, 40, 50, 60, 70, 80 and 90 min), 0.1 ml of the 10^{-5} dilution and 0.1 ml of the host culture were mixed with soft agar and poured on sterile base agar plate. After incubation of 24 hours the number of plaques was counted and PFU/ml was determined.

Field emission scanning electron microscopy (FE-SEM)

Lysate was drop cast on silicon wafer, dried and examined by FE-SEM.

Utilization of Bacteriophages as Bio-Control Agent in Wastewater Treatment

A) In Luria broth-

T1- Luria broth with log phase host

T2- Luria broth with host and phages

B) In sterile waste water-

T1 - Sterile wastewater

T2 - Sterile wastewater with host

T3 - Sterile wastewater with phages

C) In wastewater-

T1-Wastewater

T2-Wastewater with cocktail of phages

RESULT

Characterization of Water Sample: Table 1: Physico-chemical Parameters of Water Samples.

pH	BOD (mg/L)	COD (mg/L)	TDS (mg/L)
8.2	165.2	520.1	180.2

Bacteriological analysis of water sample

Total viable count for the water sample was determined and found to be 7×10^6 cfu/ml.

Isolation and Characterization of Target Bacteria

Five bacterial strains were identified till genus level by referring to Bergey's Determinative Bacteriology. Isolates may belong to *E.coli*, *Staphylococcus*, *Salmonella*, *Klebsiella*, *Pseudomonas* species. Isolate 4 was identified till species level by 16SrRNA and was found to be *Klebsiella veriicola* DSM 15968 (T).

Drug resistant profile

Isolated *E. coli* species has shown reistance against 5 antibiotics, VA, RIF, CD, AMC, AK.

Isolated *Staphylococcus* species has shown resistance against 4 antibiotics, VA, RIF. CD, AMC.

Isolated *Salmonella* species has shown resistance against 7 antibiotics, C, VA, S, RIF, CD, AMC, AK.

Isolated *Klebsiella variicola* has shown resistance against 6 antibiotics, C, VA, RIF, LE, CD, AMC.

Isolated *Pseudomonas* species has shown resistance against 5 antibiotics, CPD, VA, S, RIF, AMC.

Isolation of specific bacteriophages against target bacteria: Conformational test for presence of bacteriophages: (By Spot Test).

Fig. 1: PA 1.

Fig. 2: PA 2.

Fig. 3: PA 3.

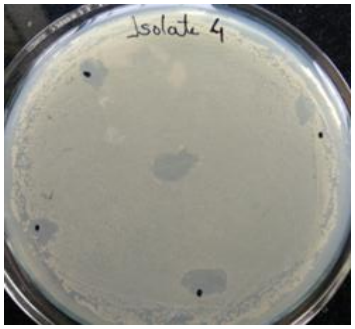

Fig. 4: PA 4.

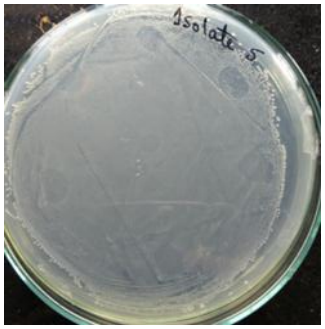

Fig. 5: PA 5.

Determination of PFU/ml: (By soft agar overlay method)

In the soft agar overlay method isolates showed plaques on the plate and their PFU/ml found to be

a. Table

Phages	PFU/ml
PA 1	1.7×10^{4}
PA 2	1.2×10^{6}
PA 3	1.98×10^{6}
PA 4	2.1×10^{4}
PA 5	1.2×10^{4}

One step growth curve of PA 1

b. Table

Time	Number of plaque
00	20
20	31
30	45
40	54
50	88
60	162
70	190
80	235

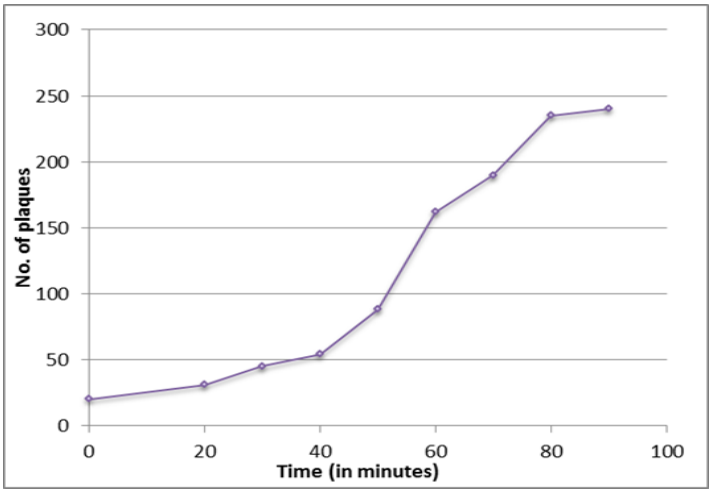

Fig. 6:

Characterization of bacteriophage by FE-SEM

Icosahedral head with tail was observed of 500 nm size. The size of head was 300 nm and tail was 200 nm.

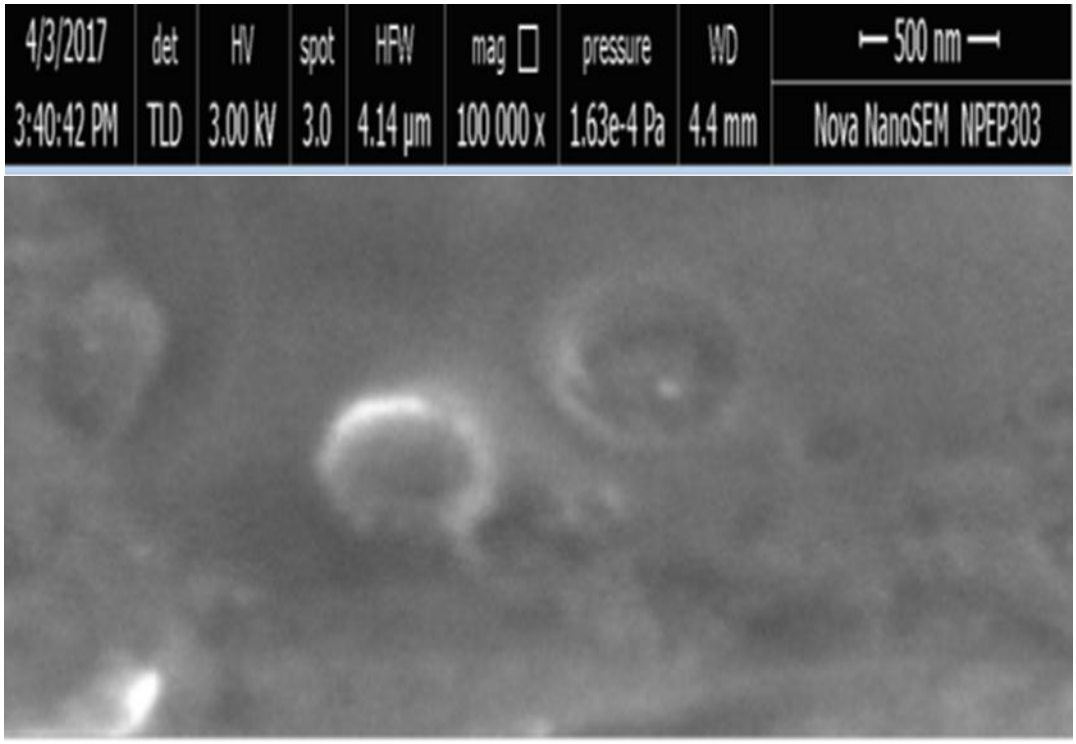

Fig. 8: Utilization of Bacteriophages as Bio-Control Agent in Wastewater Treatment.

1) In Luria Broth and in sterile wastewater

10 fold reductions were observed after 18 hours of incubation at 37°C.

In wastewater

Reduction observed in TVC

Table 3:

Time	TVC (CFU/ml) T1 (Control)	TVC (CFU/ml) T2 (Test)
0 hours	7×10^6	7×10^6
18 hours	6.8×10^6	5.5×10^4

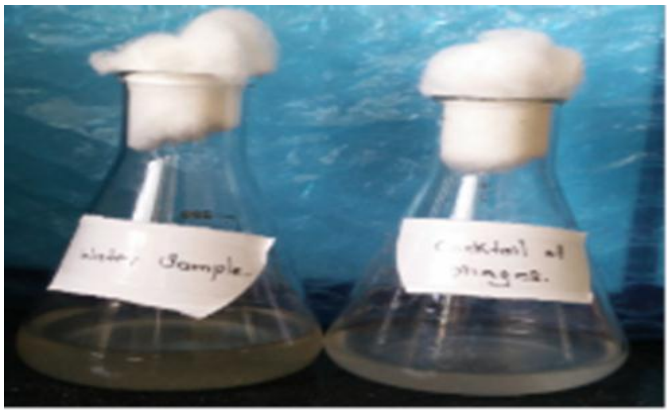

Fig. 9: (1) Wastewater (2) Wastewater with cocktail of bacteriophages.

DISCUSSION

Use of Bacteriophages for reducing pathogenic bacteria in sewage along with other standard methods like primary and secondary treatment could be considered as an effective and simple alternative for replacement of costly instruments and establishment of the old wastewater plants (Kevian et al). Many wastewater treatment systems are aiming for complete pathogen removal without harming the environment which necessitates the search for new approaches. One such approach is the use of bacteriophage for pathogen removal. Pathogen specific phages isolated from sewage have the potential to eliminate the dreadful pathogens (Periasamy and Sundaram et al 2013). The emergence of drug resistance shows the ability of microbes to evolve with each generation. Phages are thus being preferred because unlike broad-spectrum antibiotics, they are highly specific and do not illicit resistance from untargeted bacterial strains (Mahadevan M. Sundar, et al 2009). Phage cocktail, consisting of phages against E.coli and Salmonella typhi was inoculated in wastewater and 10 fold reduction was observed after 14 hours (Mansura S Mulani et al 2015).

This study focuses on isolation and characterization of bacterial species and their specific phages present in the same environment, to control pathogens present in wastewater We have isolated five bacterial strains and characterized them till genus level viz, *E.coli, Staphylococcus sps, Salmonella sps, Klebsiella sps, Pseudomonas sps.* Phages against each

isolates were isolated from the same sample viz; PA 1, PA 2, PA 3, PA 4, PA 5. Titre of each phages were determined and found to be $1.7x10^4$, $1.2x10^6$, $1.98x10^6$, $2.1x10^4$, $1.2x10^4$ respectively. A significant reduction in TVC was observed in wastewater after treatment with cocktail of phages. Characterization of PA 1 was done by FE-SEM and icosahedral head with tail was observed of 500nm size.

CONCLUSION

Based on our data and scanning of previous studies , pathogens present in wastewater have negative influence on the microbiological and physiochemical parameters on the environment, suggests that the activities of pathogens in wastewater in the environment is a major health and environmental threat. Traditional water purification methods viz; Chlorination, Radiation and Filtration are used for the reduction of pathogenic bacteria in the water systems, have many disadvantages. To reduce the risk of traditional antimicrobial resistant chemicals, an alternative strategy could be the use of bacteriophage.

Phages can settle in the natural water resources that constantly receives various types of wastes. Therefore, these phages can be used as potential biological disinfectant in the natural water bodies to bring about targeted killing. Pathogen specific phages isolated from wastewater had the potential to eliminate the dreadful pathogens. Thus indicating that phage based biocontrol could be a viable method of controlling pathogens in wastewater. Despite some of the hindrance to the phage treatment, the current awareness regarding phages indicate that phage application to wastewater treatment deserves attention.

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