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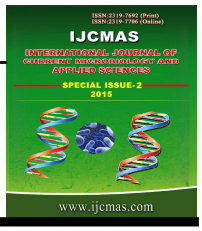

Original Research Article

Harnessing the Power of Bacteriophage for Pathogen Reduction in Wastewater

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K e y w o r d s

Bacteriophage, chloroform treatment, cocktail, *S. typhi*, *E. coli*, wastewater treatment

A B S T R A C T

Viruses that infect bacteria are known as bacteriophages. They can be isolated from all reservoirs where bacterial hosts are present, such as soil, sewage, sea water or even in the intestine of animals. As compared to viruses, phages can be detected by simple, inexpensive and rapid techniques. Phages can be used as bacterial killers and are very specific to their host. Phages against bacteria causing skin infection, like *Pseudomonas sps.* are used for external application as a treatment, also known as phage therapy. They can be used as biological control agent for plant pathogens. Bacteriophages can also be used for pathogen removal in waste water treatment. In this study we have isolated two bacterial strains from water sample taken from back side of Poona hospital, Mulla Muthariver, Pune. One bacteriophage against each of these bacterial strains was isolated by chloroform treatment method. Bacterial strains were identified till genus level and found to be *E. coli* and *Salmonella sps*. Titer for phage against isolate 1 was determined to be 2.5×10^{6}pfu/ml and for phage against isolate 2 was determined to be 1.9×10^{4}pfu/ml by soft agar overlay method. One step growth curve for both the phages PA1 and PA2 was performed. SEM imaging of PA1 was done and hexagonal symmetry of head was observed with size 100- 150 nm. TVC for the water sample was found to be 6.87×10^{6}cfu/ml, same water sample was treated with cocktail of phages for 14 hours and significant reduction in TVC was observed and was found to be 5×10^{5}cfu/ml. Hence, bacteriophages can be utilized as bio-control agent in wastewater treatment. Potential activity of bacteriophages against target bacteria was also studied by inoculating bacteriophages in actively growing host and monitoring reduction in cfu/ml till 12 hours. 100% reduction was observed at 9 hour for both *E.coli* and *Salmonella sps.*

Introduction

Bacteriophages are viruses which range from 24-200 nm in size; infect bacterial cells (Al-Mola *et al.,* 2010). These are the obligate intracellular parasites which infect bacteria, seize their replication machinery, replicate into thousands of new progenies and lyses the cell for escape (Gu *et al.,* 2012). Phages are the most abundant entities

on earth, (10^{30} to 10^{32}) and help in regulating microbial balance in environment. In 1971, a Canadian scientist, Felix d'Herelle at Pasteur Institute of Science accidentally came across phages as Fleming came across Penicillin (Lawrence Broxmeyer, 2004). Phages lack their own metabolic machinery and hence require bacterial host for replication. Phages are highly host specific and can infect a specific species or strain of bacteria. But there are some exceptions, like *listeria* A511, which can infect entire genus (Martin *et al.,* 1993).

Like all viruses, genome of bacteriophages consist of nucleic acid (RNA or DNA) surrounded by a protein coat, capsid. Capsid is made up of morphological subunits capsomeres. These capsomeres are comprised of protein subunit protomeres. Some phages also contain additional structures such as tail and fibres (Grabow, 2000). The receptors on the host cell are recognized by the phage tail fibers. After adsorption phage DNA will be incorporated into host cell, whereas capsid remains outside the cell. Host cell envelope is weakened by lysozyme activity of tail base plate of phages (HillaHadas *et al.,* 1997). Inside host cell, gene expression and morphogenesis occur. New virion progenies are produced and lyse the bacterial cell. Depending on the life cycle pattern, bacteriophages are of two types, lytic or lysogenic. Lytic phages typically proceed with replication immediately after infecting the host cell and new viruses are released in large numbers by lysis of the host cell. Lysogenic phages integrate their nucleic acid into the host cell where it remains until induced to become autonomous again and start replication and cell lysis (Raghu *et al.*, 2012).

Nowadays multi-drug resistant bacteria are a serious problem in treatment of infections, main reason is persistent use of antibiotic in the past twenty years (Ibrahim *et al.*, 2012). Exposure to infections in hospitals and the practice among many doctors to prescribe strong antibiotics even for common infections and also the self-medication is fuelling resistance to antibiotics in organisms. The overuse of antibiotics that too targeted at incorrect bacteria, only strengthen the resistant one (TOI article 28/12/14). Phages can be an alternative to antibiotics in terms of multi-drug resistant bacteria. In polish and soviet studies phages were tested for phage therapy and applied tropically, orally and systemically. With few gastrointestinal and allergic side effects, 80 – 95% success rate were observed (Mathur *et al.*, 2003).

Enteric bacteria infect gastrointestinal tract of humans and animals (Rene *et al.*, 2007). For example, *Escherichia coli, shigellasps, salmonella sps, enterotoxigenic Escherichia coli (ETEC),* etc. (William *et al.*, 2008). Sewage and wastewater streams are consisting of these enteric pathogens (Rene *et al.*, 2007). The most common cause of worldwide food and water borne human diarrhea is *ETEC.* Due to *ETEC* in developing countries 650 million cases per year of enteric diseases occur which result in 800,000 deaths (Susan *et al.,* 2006). In developing countries *Escherichia coli* is 3rd of cases of childhood diarrhea (John Albert 1995). WHO estimates that due to diarrhea 5 million children die per year (John D. Snyder and Michael H. Merson, 1982). Phages isolated against pathogenic bacteria have the potential to eradicate their specific host and this method could be potential biocontrol mechanism to control pathogens in wastewater (Dhevagi Periasamy and Anusuya Sundaram, 2012).

The aim of this study was isolation and identification of *E. coli* and *Salmonella* sps.

from wastewater and isolation of lytic phages against these species. Investigation of their effect on decrease in viable count of the isolated microorganisms so that they can be used as bio-control agent in wastewater treatment. This technology could be used without causing any harm to non-pathogenic bacterial flora.

Material and Methods

Collection and characterization of wastewater sample

Samples were collected from two different locations during the month of January 2015 at MulaMutha River, Pune, in pre-sterilized 1000ml capped flasks from outer most end of the river. GPS coordinates of the location were recorded using the Android app GPS Test (version 1.3.2) and weather conditions were determined using http//www.weather. com and transported to the laboratory for further characterization. Collected samples were tested for pH, TDS, BOD and COD.

Bacteriological analysis of wastewater sample

All the samples were subjected to viable count studies by spreading 100µl of sample with 10^{-1} to 10^{-10} dilutions. Dilutions were prepared using sterile saline and spread on sterile nutrient agar plates. Overnight incubation was done at 37°C and plates showing 50 to 200 colonies were used for determination of the total viable bacterial count.

Isolation and characterization of target bacteria

The samples were also plated in selective media to isolate the target bacteria.Culture media used were 1.Eosin Methylene Blue agar, 2.Wilson Blair agar, 3.Salmonella Shigella (SS) agar. Bacterial isolates were subjected to further characterization and identification as per the standard procedures.

Isolation of specific bacteriophages against target bacteria

Isolation of phages

Sample was filtered through coarse filter paper to remove any particulate matter. 10% chloroform was added to the filtrate and vigorously shaken for 30 min. It was then transferred to sterile centrifuge tube and centrifuged at 8000 rpm for 20 min at4°C. After centrifugation supernatant was removed and stored as lysate at 4°C.

Enrichment of phages

In 100ml nutrient broth isolated bacterial strains were grown overnight at 37°C to get organisms at log phase. 20 ml of sterile double strength phage broth and 20 ml lysate were added to the same flask and incubated at 37°C for 48 hours. After incubation it was centrifuged at 8000 rpm for 20 min at4°C to remove the cell debris. 10% chloroform was added to centrifuged sample and shaken vigorously for 30 min followed by centrifugation at 8000 rpm for 20 min at4°C to remove chloroform. Pellet was discarded and supernatant was stored at 4°C as enriched lyaste for spotting.

Conformational test for presence of bacteriophages (by spot test)

100µl log phase host was spread on sterile nutrient agar plate and 20µl lysate was spotted on the plate and incubated at 37°C for 24 hours.

Purification of Bacteriophages and determination of pfu/ml.

The phage purification was done by repeated passaging of the plaques. The plaques

formed in the plaque assay were picked using a micropipette and inoculated in a fresh culture of the respective host and the cell lysate was prepared as mentioned earlier by chloroform treatment and passaged repeatedly till P4. By using soft agar overlay method pfu/ml of purified phages were determined.

Scanning Electron Microscopy (SEM)

Fixing was done with 2.5% glutaraldehyde (pH 7.2) for 1.5 h at 4°C. Samples were washed three times for 10 min with 0.01 M phosphate buffered saline (PBS). Dehydration steps were 10 min each in 50%, 70%, 80%, and 90% ethanol, a 1:1 mixture of 100% ethanol and tert-butanol, and pure tert-butanol. After lyophilization for approximately 4 h, samples were placed on the microscope platform, coated with a layerof heavy metal and examined.

Host range determination

Bacteriophages are highly specific, and can infect only a group of bacteria. This specificity was determined by infecting five different bacterial strains i.e. *E. coli, Staphylococcus aureus, Klebsiella pneumonia, Salmonella typhi* and *Pseudomonas*. The assay was done using phage spot test by spreading 100µl log phase host on nutrient agar plate. 20µl lysate was spotted on the plate and incubated at 37°C for 24 hours.

One step growth curve

Bacterial host used in this experiment were isolate1 and isolate2. 0.9 ml of log phase host suspension ($2x10^8$cfu/ml) and 0.1 ml the phage lysate ($4x10^6$pfu/ml) were mixed and incubated at 37^0C. At precisely 10 min after starting the incubation 0.1 ml of mixture was diluted upto10^{-5} dilution. At regular time intervals (00, 20, 25, 30, 40, 50, 60, 70 and 80 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.

Phage plaque visibility contrast was enhanced by incorporating 10 mM MgSo4 and 0.2% Maltose to the soft agar plate. Many bacteriophages require divalent cations such as Mg^{++} and Ca^{++} for attachment to bacterial host cells. Magnesium and Maltose facilitates the entry of phage particles into the cell (Dhevagi Periasamy and Anusuya Sundaram, 2012).

Drug resistant profile

For testing the antibiotic resistance profile of isolated bacteria we have used commercially available antibiotic rings i.e. Dodeca universal I DE001 (Hi Media). Disc was consisting of Cefpodoxime (CPD) 10µg, Chloramphenicol (C) 30µg, Vancomycin (VA) 30µg, Streptomycin (S) 10µg, Rifampicin (RIF) 5µg, Levofloxacin (LE) 5µg, Ceftriaxone (CTR) 30µg, Clindamycin (CD) 2µg, Augmentin (AMC) 30µg, Amikacin (AK) 30µg, Cefixime (CFM) 5µg, and Tetracycline (TE) 30µg. After spreading 0.3 ml of log phase bacterial culture on nutrient agar plate the discs were put on media and incubated at 37^0 C.

Activity of bacteriophages against target bacteria

Survival rate of target bacteria was assessed by inoculating phage in the actively growing log phase host. TVC of bacteria were calculated to know the activity of Bacteriophages. Isolate1 and Isolate2 were selected for the study.

Host organisms were inoculated in sterile nutrient broth, sterile waste water and non-

sterile waste water. The following are the treatment sets prepared for the study for each isolate 1 and 2 separately.

T1 - (Control) LB with bacterial Isolate
T2 - Sewage water with bacterial Isolate
T3 - Sterile sewage water with bacterial Isolate
T4 - T1 and host specific bacteriophages
T5 - T2 and host specific bacteriophages
T6 - T3 and host specific bacteriophages

Utilization of Bacteriophages as Bio-Control Agent in Wastewater Treatment

T1 - wastewater as control.
T2 - wastewater inoculated with cocktail of bacteriophages.

After filtration 100 ml of wastewater sample was taken in pre-sterilized flask. The lysate of isolate 1 and isolate 2 specific phages at 10^9 PFU/ml were mixed and used for the treatment.

After inoculation initial and final TVC at 0 and 14 hour respectively was taken.

Results and Discussion

Characterization of Water Sample

Bacteriological Analysis of Wastewater Sample

The main objective was to determine the quality of waste water in terms of bacterial load. TVC for the water sample was determined and found to be 6.87×10^6cfu/ml.

Isolation and Characterization of Target Bacteria

Two bacterial strains were isolated and identified till genus level by referring Bergey's Determinative Bacteriology. Isolate1 found to be *E. coli* and Isolate2 found to be *Salmonella sps*.

Isolation of specific bacteriophages against target bacteria

Conformational test for presence of bacteriophages (By Spot Test)

100ul of lysate was spotted on plate spread by two isolated bacteria and all showed zone of lysis on plate

Determination of PFU/ml (By Soft Agar Overlay method)

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

Pahge against Isolate 1- 2.5×10^6 pfu/ml
Pahge against Isolate 2- 1.9×10^4pfu/ml

Host range determination

Host range of purified phages was determined using 7 different hosts. Phage PA1 showed plaques on lawns of isolated *E. coli* and PA2 showed plaques on lawns of isolated *Salmonella sps.* In the spot plaque assay no plaques were found on lawn of *E. coli, staphylococcus aureus, pseudomonas, salmonella typhi* and *Klebsiella*. Isolated bacterial strains were only found to be susceptible. No cross reactivity against another bacterial strain were found.

Drug resistant profile

Isolated *E. coli sps*. have shown resistance against 6 antibiotics, viz., RIF, LE, AMC, AK, S, CPD.

Isolated *Salmonella sps*. have shown resistance against 3 antibiotics, viz., CPD, VA, AM.

Table.1 Physico-chemical parameters of water samples

pH	BOD(mg/l)	COD(mg/l)	TDS(mg/l)
8.2	157.2	540.1	172.2

Table.2 Host range of the phage

		Phage Infectivity	
No.	**Culture**	**PA1**	**PA2**
1.	*E. coli*	-	-
2.	*Staphylococcus aureus*	-	-
3.	*Pseudomonas*	-	-
4.	*Salmonella typhi*	-	-
5.	*Klebsiella*	-	-
6.	Isolated *E.coli*	+	-
7.	Isolated *Salmonella typhi*	-	+

Table.3 TVC at 0 and 14th hour after PA1+PA2 phage cocktail inoculation

	TVC(cfu/ml)	TVC(cfu/ml)
Time	**Control**	**Test**
0 hour	6.87×10^{6}	6.87×10^{6}
14 hour	6.0×10^{6}	5×10^{5}

Table.4 Activity of PA1 against *E. coli*

Test	Time	0 Hour	3 Hour	6 Hour	9 Hour	12 Hour
T1		15	28	33	54	78
T2		11	18	139	157	UC
T3		08	122	127	208	48
T4		21	18	12	09	09
T5		18	14	12	00	00
T6		12	09	03	00	00

Table.5 Activity of PA2 against *Salmonella sps*

Test	Time	0 Hour	3 Hour	6 Hour	9 Hour	12 Hour
T1		34	49	57	14	00
T2		09	08	06	01	00
T3		04	00	00	03	03
T4		26	04	00	26	00
T5		03	05	01	00	00
T6		08	06	03	00	00

Fig.1 (A-B) Purification of the pahges, A – Passage 1 of coliphages, B – Passage 2 coliphages

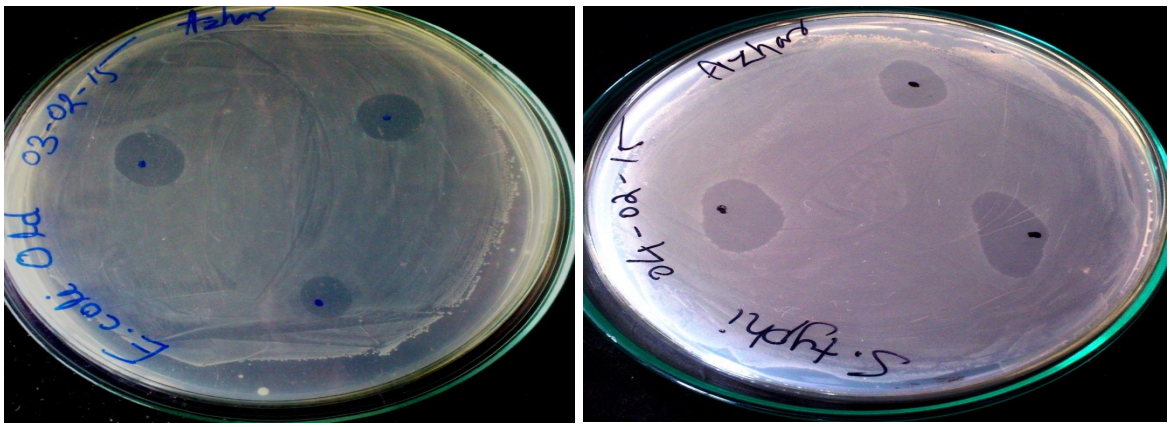


Fig.2

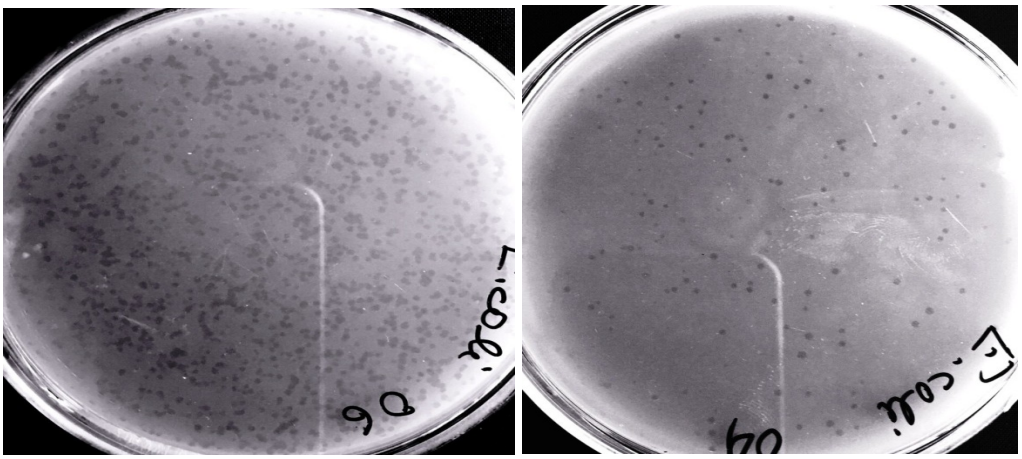


Fig.3 One-step growth curve for *E. coli* and *S. typhi*

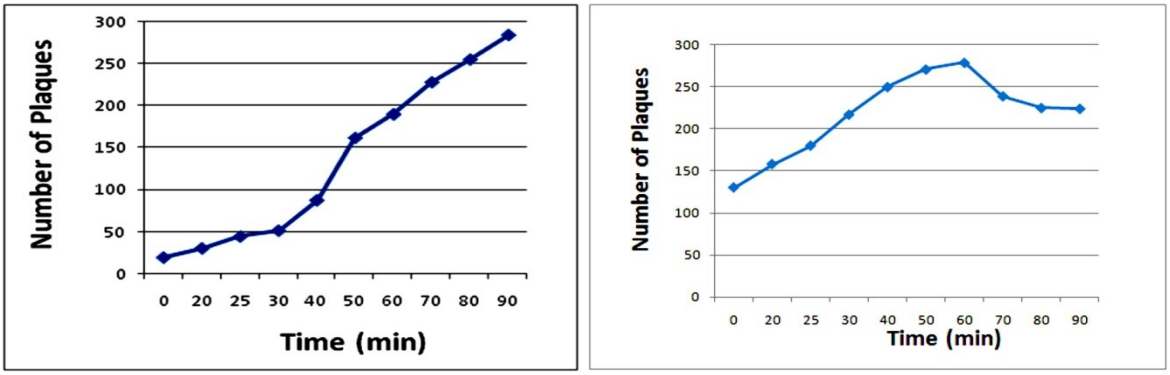


Fig.4 (A-B) plaques on bacterial lawns of A- *E. coli* and B- *Salmonella typhi*

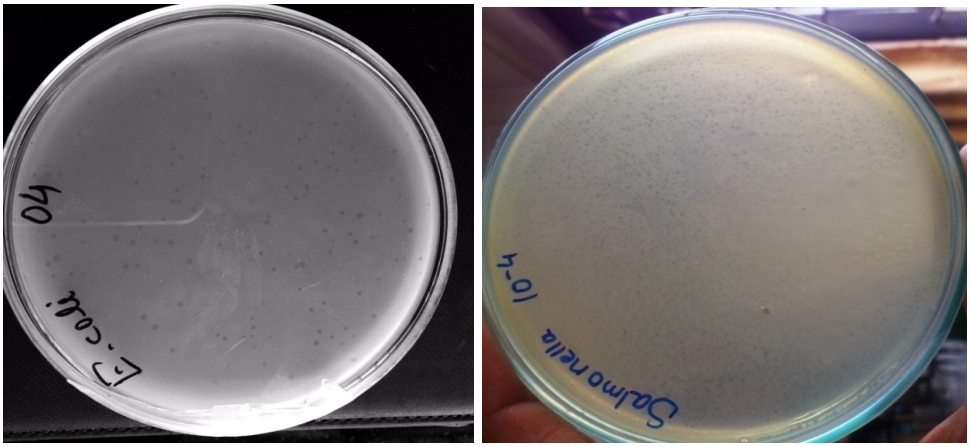


Fig.5 Scanning Electron Microscopy (SEM)

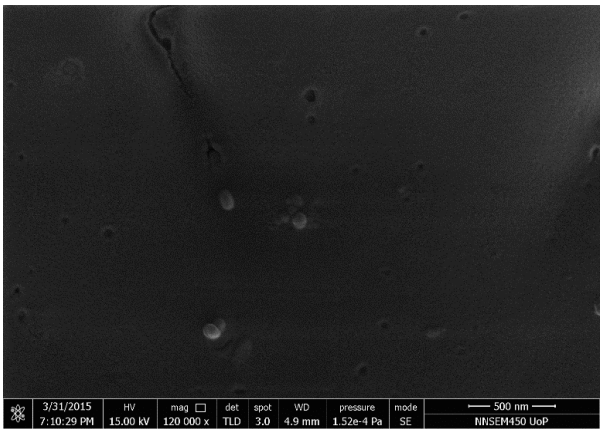
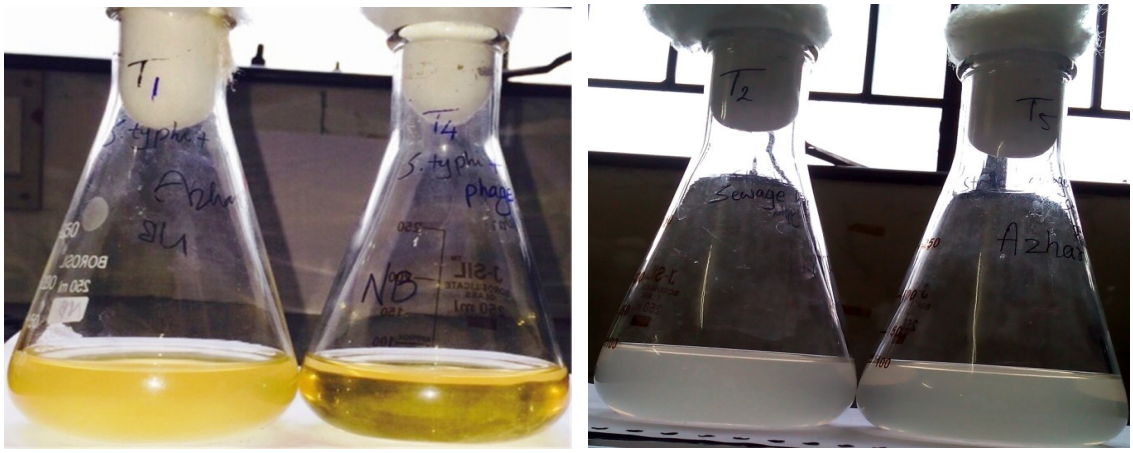


Fig.6 (A-B)Phage therapy, A - positive control T1, growth of *E. coli* in Nutrient broth, B –T4, mixture of *E. coli* and its specific phages. After incubating 12 hours at 370C activity of phages can be seen clearly



One-step growth curve

Lytic life cycle of bacteriophage was characterized using their specific host that is *E. coli* and *Salmonella typhi.*

Utilization of bacteriophages as bio-control agent in wastewater treatment

Use of Bacteriophages for reducing pathogenic bacteria in sewage along with other standard methods like activated sludge could be considered as an effective and simple alternative for costly replacement of instruments and establishment of the old wastewater treatment plants (Keivan Beheshti Maal *et al.,* 2014). Phage mediated bacterial mortality has the capacity to influence treatment performance by controlling the abundance of key functional groups (Dhevagi Periasamy and Anusuya Sundaram, 2012). In a in-vitro experiment a small amount of phage cocktail can proliferate quickly, resulted in the fast elimination of bacteria in infected mice and lower the development of phage resistance (Gu *et al.,* 2012). Phage cocktail, consisting of phages against *E. coli* and *S. typhi* was inoculated in waste water, and 10 fold reduction was observed after 14 hours.

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 treatment plants (Keivan Beheshti Maal *et al.,* 2014). Phage mediated bacterial mortality has the capacity to influence treatment performance by controlling the abundance of pathogenic bacteria (Dhevagi Periasamy and Anusuya Sundaram, 2012). In a in-vitro experiment a small amount of phage cocktail can proliferate quickly, resulted in the fast elimination of bacteria in infected mice and lower the development of phage resistance (Gu *et al.,* 2012). Many wastewater treatment plants aim for the complete pathogen removal during treatment by using disinfectants like chlorine which can harm the environment. Hence development of cheap and ecofriendly approaches is necessary. Pathogen specific phages isolated from sewage have the potential to eliminate the dreadful pathogens (Periasamy and Sundaram *et al.,* 2013).

This study focuses on isolation and characterization of bacterial species and their specific phages present in the same environment to develop new strategy for generalized control of bacterial populations, especially pathogens in waste water. We have isolated two bacterial strains and characterized them till genus level viz. *E. coli* and *Salmonella sps.* Phages against both the isolates were isolated from the same sample viz. PA1 and PA2. Titre of PA1 and PA2 was found to be 5×103 pfu/ml and 1.9×104 pfu/ml respectively. Twelve fold reduction in TVC was observed in wastewater by treating it with cocktail of phages. Potential activity of bacteriophages against target bacteria has shown 100% reduction in cfu/ml for both *E. coli* and *Salmonella* sps. at 9th hour

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