Poll Res. 40 (1) : 153-162 (2021)

ISSN 0257–8050

BACTERIOPHAGE: AN ECO-FRIENDLY AND COST-EFFECTIVE GREEN TECHNOLOGY IN REUSE OF TREATED WASTEWATER FOR AGRICULTURE

PATWARDHAN RAJASHREE B.*[1], MACHCHHA POOJA K.[1], SONAWANE MADHURI P. [1] AND GORE SUNEETI S.[2]

[1]Haribhai V. Desai College of Arts, Science and Commerce, Pune 411 002, Maharashtra, India.
[2]Fergusson College, Pune 411 004, Maharashtra, India

(Received 6 June, 2020; accepted 11 August, 2020)

ABSTRACT

Broad spectrum of pathogenic microorganisms is present in treated wastewater where they survive for days, weeks and at times months in the soil and on crops that encounter with this treated water. These pathogens may enter the food chain and may cause health hazards to animals as well as human beings. To overcome this problem an eco-friendly approach has been designed to use bacteriophages to eliminate the pathogens from treated wastewater. This study focuses on isolation and characterization of bacterial strains *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli* and *Staphylococcus aureus*, and their specific bacteriophages from Pavana river water, Pune, Maharashtra, India. All the isolates obtained were found to be resistant to multiple antibiotics. Water samples were characterized with respect to pH, TS, TDS, TSS, DO and BOD and presence of heavy metals. Spot assay was performed as a confirmatory test for the bacteriophage and zone of clearance was observed. Phage titre was calculated, and morphology was studied. One step growth curve was performed and burst size was determined with respect to average number of progeny phage released per infected cell. The average burst size obtained were 52.13, 166, 205, 130 for *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Enterococcus cloacae*, respectively. Significant reduction was observed in TVC of the water sample after phage treatment. Phage treatment was also found to be effective against chlorine-resistant bacteria. Reusing the treated wastewater is an increasing global growth trend, which would be surely achieved by our novel approach by eliminating the pathogens with the help of bacteriophage treatment.

KEY WORDS : Wastewater, Bacterial pathogens, Bacteriophage, Agriculture

INTRODUCTION

There is growing fear that the world is moving towards a water crisis (Ursula *et al.*, 2000). Out of 100% of water content on the earth, 97.5% is sea water and only 2.5% is fresh water. Therefore, there is a need of reuse of wastewater to save the earth from water crisis. The reuse of wastewater in agriculture is one of the main options as a new source of water in regions where water is scarce. There are agronomic and economic benefits of wastewater used in agriculture. In addition to direct economic benefits that conserve natural resources, the fertilizer value of wastewaters is important. Food and agricultural organization estimated that typical wastewater effluent from domestic sources supplies all the nitrogen and much of the phosphorus and potassium that are normally required for agricultural crop production. In addition, micronutrients and organic matter also provide additional benefits. Contamination of soil can arise due to organic fertilizers like sewage sludge and manure, from the irrigation water, as well as from the ability of pathogens to persist and proliferate in vegetables. Waterborne bacterial diseases are the major contributors to diseases and

mortality in human beings (Hunter, 2003). Use of treated wastewater can persist potentially pathogenic bacteria, could spread resistant bacteria to plants which are normally absent in the intestine of humans, hence leading to infections in human beings (Falomir *et al.*, 2010). As Per WHO guidelines, 1989, water used to irrigate crops that are eaten raw should contain ≤ 10^3 faecal coliforms/100 mL and for irrigation of commercially processed and fodder crops the guideline limit is ≤ 10^5 faecal coliforms/100 mL. Most wastewater treatment plants are designed to reduce organic pollution of rivers and lakes and rarely are designed to remove all risks from pathogenic organisms. In wastewater treatment, three stages are followed: Primary treatment, Secondary treatment, and tertiary treatment. Chlorination is done in tertiary treatment. Chlorine is most used sanitizer, but poses health and environmental risks, and has variable behavior (Andrea, 2015). The chlorine efficacy decreases as the organic load increases. Its effectiveness and toxicity vary with pH and temperature. Therefore, regardless of the level of treatment provided, pathogenic organisms reach the agricultural fields and have the potential to contaminate both the soil and the crops. Capsulated or slime producing bacteria which escape the wastewater treatment process can cause serious infections in humans. Bacteriophages carry polysaccharide depolymerase enzymes (PDE) which are secreted during attachment of phage to the bacterial host (Adams and Park, 1956). The enzymes degrade the bacterial exopolysaccharide capsule (secondary receptor) allowing the phage to bind to primary10 receptor sites in the outer membrane (Hughes *et al*. 1998). Virus survival is enhanced in polluted waters, because of some protective effects that the viruses receive when they are adsorbed onto suspended solid particles or on the surface of bacteria present in the water. Bacteriophages the most abundant viral entity on the planet are omnipresent in all the ecosystems. Bacteriophages are obligate intracellular parasites that infect bacteria, reproduce by hijacking their host biosynthetic pathway and are host specific. Phages are classified as either lytic or lysogenic based upon their replication strategy. Bacteriophages have remarkable antibacterial activity against their specific bacterial host. Phage therapy involves the targeted application of bacteriophages that, upon encounter with specific pathogenic bacteria, can infect and kill them (Abedon *et al.*, 2011). Bacteriophage therapy has a tradition dating back almost a century, but interest in phage therapy reduced when antibiotics were discovered. With the emerging threat of infections caused by multidrug-resistant bacteria and scarce prospects of newly introduced antibiotics in the future, phages are currently being reconsidered as alternative therapeutics. The major characteristics of bacteriophages and phage-encoded proteins affecting their usefulness as antimicrobial agents had been shown by Drulis-Kawa *et al.* (2012). Ability of bacteriophages showing different advantages has attracted us to use them to kill the nuisance bacteria which escape the wastewater treatment process. In the present research investigation, we propose to use the phage therapy to encounter the pathogenic bacteria from treated wastewater which is regularly reused in the agricultural fields.

MATERIALS AND METHODS

Media used : The soft agar concentration in the nutrient broth/Luria broth (Hi media) was 0.75%. For the adsorption of bacteriophages to host cells 0.7% of $CaCl_2$ was incorporated into the medium.

2. Isolation and characterization of the bacterial isolates: Wastewater samples were collected from five different places using sterile dark containers from Pavana river. These samples were filtered through coarse filter paper to remove debris. Filtrate obtained was serially diluted using 0.85% sterile saline and it was spread on the Cetrimide and Mac-Conkey's agar plates Plates were incubated at 37 °C for 48 hours. All the bacterial isolates were identified using Bergey's manual of Determinative bacteriology, 7th Edition.

Determination of antibacterial activity by disc diffusion: Antibacterial susceptibility testing was performed by the Kirby - Bauer disc diffusion method as per clinical laboratory standards Institute guidelines (CLSI). About 10^5 cells from overnight grown culture were spread on Luria agar plates. Polydiscs containing antibiotics (Don Whitley Scientific Equipment's, Mumbai, India) were placed on the plates. The zones of inhibition around the antibiotic discs were measured after incubation at 37°C for 24 hr. The cultures were assigned as resistant or sensitive by using CLSI standards (Patwardhan *et al.*, 2015; Periasamy *et al.*, 2013)

Characterization of Wastewater Samples: Wastewater samples were collected from Pavana

river in pre-sterilized 1000 mL capped flask. Collected water samples were tested for pH, Total Solids (TS), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Biological Oxygen Demand (BOD) and Dissolved oxygen (DO) accordingly stated by Mansura *et al.* (2015).

a) **pH:** pH of wastewater sample was monitored using pH meter.
b) **Total Solids (TS):** Total solids are calculated by weighing the clean and dry porcelain dish and weight were recorded as (W_1) g. 100 mL of well mixed water sample was taken in porcelain dish and evaporated at 103° to 105 °C for drying. After drying the dish was cooled and the weight was recorded in (W_2) grams. Weight of empty dish and weight of dish after drying was compared; the change in weight gave the total amount of solids (Fig. 9).
c) **Total Suspended Solids (TSS):** The weight of Whatman's filter paper No.1 was recorded as (W_3) grams. 100 mL of well mixed water sample was filtered by Whatman's filter paper No.1. The filter paper with residue was placed in oven, till drying. Dried filter paper with residue weight was recorded as (W_4) g. Weight of filter paper before and after drying was compared; change in weight gave the amount of total suspended solids (Fig. 9).
d) **Total Dissolved Solids(TDS):** Total dissolved solids were calculated by subtracting concentration of total solids from total suspended solids (Fig. 9)
e) **Biological Oxygen Demand (BOD):** BOD of wastewater sample was calculated by iodometric method.
f) **The determination of concentration for heavy metals in river water:** The heavy metal content of river water samples was determined by Flame Atomic Absorption Spectrometry (FAAS) (Radulescu *et al.*, 2014).

Isolation of Bacteriophage: Bacteriophages able to lyse the isolated bacterial isolates were isolated from the Pavana river. 100 mL of water sample was centrifuged, supernatant was filtered through 0.45 µm membrane filter. Filtrate obtained was stored as a source of bacteriophage lysate at 4 °C. Phages from the filtrate were isolated by double agar layer plaque assay technique stated by Ahiwale *et al.*, (2013).

Confirmatory test for presence of bacteriophages (spot test) : 100 µL bacterial hosts in the log phase were spread on sterile nutrient agar plate and 10 µL lysate was spotted on the plate and incubated at 37 °C for 24 hours.

Plaque Morphology: The mid log phase culture (1 mL) of respective bacterial host and an aliquot of 1ml filtrate (source of bacteriophages) were mixed and allowed to adsorb for 10 minutes. 0.1 mL of this mixture was spread onto pre-sterilized Nutrient agar plates. Plates were incubated at 37 °C and observed for plaques.

Lysate Preparation: Lysate was prepared by transferring single plaque of respective phage in flask containing 500 mL of Nutrient broth with mid log phase culture of respective host. Flask incubated on shaker at 100 rpm at 37 °C for 24 hrs. After incubation, broth was centrifuged at 8000 rpm for 10 mins. Supernatant was filtered through membrane filter assembly. The filtrate obtained was used for phage titre determination by using Agar overlay method as performed by Ahiwale *et al.* (2013).

Bacteriophage titre Determination (Plaques Assay): Bacteriophages were titrated to know the number of plaques formed against the respective host. Lysate as a source of bacteriophage was serially diluted using sterile Nutrient broth from 10^{-1} to 10^{-10} dilution. 0.1 mL of the each of the dilution was mixed with 0.2 mL of respective host. This mixture was placed in soft agar tube maintained at 40 °C. Soft agar was mixed well and poured on to pre-sterilized Nutrient agar plates. After settling of the soft agar, plates were kept inverted position in incubator at 37 °C for 24 hrs. After incubation plates were observed for visible plaques and the plaque number was enumerated. By agar overlay method pfu/mL of the various isolates was determined

10. Host specificity: Specificity of the phages for the bacterial strain were examined by following two methods

(i) 0.1 mL of the lysate was plated with each test bacterium and, after overnight incubation at 37 °C and the plates were examined for plaque formation.
(ii) Lysis or reduction in the turbidity of the medium was studied (Watanabe *et al.* 1970) by infecting the test bacteria with lysate (10^8 pfu/mL) in the liquid medium incubated overnight at 37 °C.

One step growth curve assay: The over all aspects of the infective cycle are most conveniently studied by means of one step growth curve experiment (Ellis and Delbruck, 1939). To clarify the growth characteristics of the phage, one step growth assay was performed. 0.9 mL bacterial culture (2×10^8cfu/mL) and 0.1 mL phage lysate (4×10^8pfu/ml) were added in tube and incubated at 37 °C for 5 minutes.

After the incubation 0.1 mL of the mixture was removed and transferred to 9.9 mL of sterile Nutrient Broth (10^{-2}). It was vortexed thoroughly, 0.1 mL mixture was removed after thorough mixing and was transferred to 9.9 mL of sterile Nutrient broth (10^{-4}), the tube was vortexed thoroughly. At regular time intervals (00, 10, 20, 30, 40, 50, 60, 70 and 80 min), 0.1 mL was withdrawn from 10^{-4} dilution and 0.1 mL of the host culture was mixed with soft agar and poured on sterile base agar plate. After overnight incubation at 37 °C, the plaques were counted.

Study of the effect of chlorine on the bacterial pathogens: Microbial resistance to chlorine was evaluated by adding 0.1 mL of a microbial suspension in 0.9 mL of a corresponding chlorine solution at 20 °C. Samples were collected after different treatment times ranging from 5 mins to 24 hrs at 5 ppm. After the desired contact time, appropriate serial dilutions were prepared, and colony count was determined.

Study of stability of liquid phage formulation: Each lysate was subjected to plaque assay technique on 15 nutrient agar plates to get the formation of plaques. The plates were flooded with sterile river water and kept at 4 °C. The soft agar was scraped and treated with chloroform and again kept at 4 °C. Supernatant was centrifuged at 10,000 rpm for 15 minutes and filtered through the membrane filter (0.2 µ). Each lysate was stored at 4 °C. The density of phages in each formulation was monitored for 3 months at the interval of 15 days.

RESULTS

Isolation and characterization of the bacterial isolates

The isolated and identified organisms from the water samples were *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli* and *Staphylococcus aureus*.

Determination of antibacterial activity by disc diffusion

Antibiotic resistance profile studied by polydisc diffusion method revealed that all bacterial isolates were found to be resistant to multiple antibiotics. All Gram-negative bacteria mentioned above were resistant to 25 different antibiotics tested. Gram Positive *S. aureus* was resistant to 21 different antibiotics except Ciprofloxacin, Imipenem, Norfloxacin and Piperacillin.

Characterization of wastewater Samples (Table 1): The pH of wastewater samples was found to be in the range of 6.4 to 8.8. The permissible limit of pH is 6.5-8.5 as per the standards of WHO. Total solids in surface water usually fall within the range of 20 mg/L to 500mg/L. Total solids of our sample were found to be higher than the standards (470 - 600 mg/L). As per standards TSS concentration < 20 mg/L is considered as clear water. TSS concentration of our water sample was found to be in the range of 18 – 22 mg/L, i.e. within the standard limits. As per Bureau of Indian Standards (IS: 10500) guidelines, 500 mg/L is the desirable limit for TDS. Our samples showed TDS values slightly higher than the standards. TDS of our water samples were in the range of 472 – 578 mg/L, which shows that higher amount of the organic matter is present in river water samples which must have favored the growth of pathogenic bacteria. There was no inhibitory effect of organic matter on bacteriophage. Presence of organic matter did not play a significant role on lytic activity of bacteriophages. Most pristine river have a 5-day carbonaceous BOD below 1 mg/L. Polluted water rivers have values of BOD in range of 2 to 8 mg/L. Our water sample BOD ranges from 3 to 5.2 mg/L, which indicates that the river is polluted. Metal concentration of Pavana river water at five different locations was found to be much higher than the permissible levels (Table 2).

Isolation of Bacteriophage: Fig. 1, 2, 3 and 4 shows bacteriophages isolated by double agar layer plaque assay technique.

Table 1. Characterization of Wastewater samples

Sample	pH	TS (mg/L)	TSS (mg/l)	TDS (mg/L)	BOD (mg/L)
WWS -1	7.7	550 mg/L	20mg/L	530 mg/L	4.1 mg/L
WWS-2	6.4	470 mg/L	18 mg/L	472 mg/L	3 mg/L
WWS-3	8.8	600 mg/L	22 mg/L	578 mg/L	5.2 mg/L
WWS-4	7.5	520 mg/L	15 mg/L	552 mg/L	3.7 mg/L
WWS-5	8.4	450 mg/L	19 mg/L	425 mg/L	4.6 mg/L

WWS: - wastewater sample

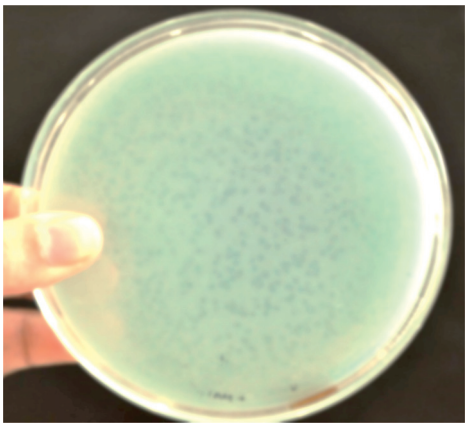

Fig. 1. Plaques of *Pseudomonas aeruginosa*

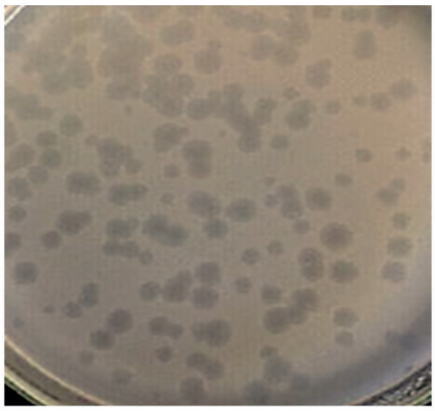

Fig. 3. Plaques of *Proteus vulgaris*

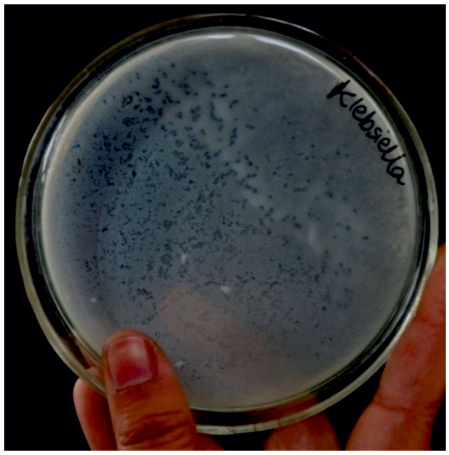

Fig. 2. Plaques of *Klebsiella pneumoniae*

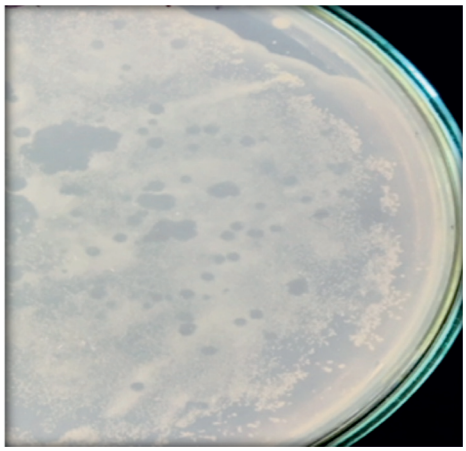

Fig. 4. Plaques of *Acinetobacter baumannii*

Spot assay: Spot assay showed zone of clearance which confirmed the presence of bacteriophages against the bacterial pathogens from wastewater samples.

Plaque assay (Table 3): Plaque assay was performed to determine the titer of the host specific bacteriophage as well as to study the ability of the bacteriophage to replicate within the susceptible host cell. The results (Fig. 5) reveal that as the lysate was diluted the concentration of the bacteriophages goes on decreasing.

Host specificity

The bacteriophages were found to be specific to host by both the methods. Reduction in the turbidity of culture was observed by overnight incubation of the phage with host bacterial culture.

One step growth curve

Multiplicity of infection (MOI) was calculated as Burst size measures the average progeny of the phage particle released per infected bacterial cell. The average burst size obtained were 52.13, 166, 205,

Table 2. Mean concentration of heavy metals in Pawana river water samples

Sr No	Location	Pb (ppm)	Ni (ppm)	Cr (ppm)	Cd (ppm)
1	Pimpri	0.262 ± 0.3	0.513 ± 0.6	0.109 ± 0.1	0.081 ± 0.06
2	Chinchwad	0.671 ± 0.5	0.361 ± 0.2	0.074 ± 0.06	0.079 ± 0.05
3	Dapodi	0.487 ± 0.34	0.853 ± 0.7	0.091 ± 0.08	0.076 ± 0.04
4	Dehu	0.321 ± 0.4	0.921 ± 0.8	0.210 ± 0.03	0.052 ± 0.05
5	Wakad	0.712 ± 0.8	0.831 ± 0.6	0.187 ± 0.1	0.064 ± 0.04

Permissible values: Pb: 0.01ppm, Ni: 0.02 ppm, Cr: 0.05 ppm, Cd: 0.003 ppm

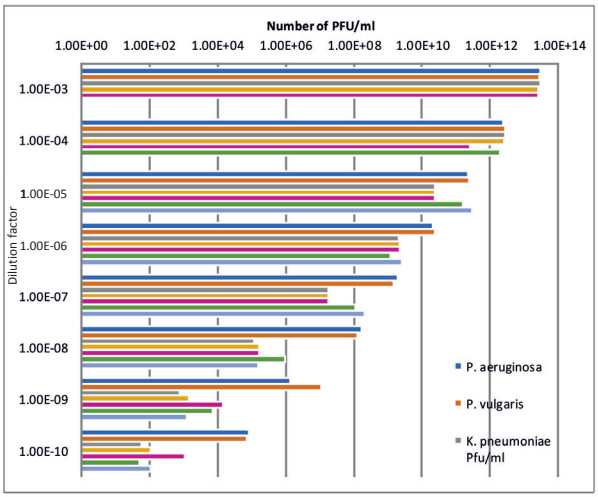

Fig. 5. Plaque Assay to study number of pfu/mL

130 for *Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumoniae, and Enterococcus cloacae respectively* (Table 4, Fig. 6). Burst size was estimated from triplicate experiments using the equation explained byJiang *et al*. (1998).

Number of bacteria (TVC) from wastewater before and after the phage treatment

There was great reduction in the TVC of respective bacteria before and after the bacteriophage treatment (Table 5, Fig. 7).

Study of stability of liquid phage formulation: Phage formulation of each bacterial pathogen was stable for 3 months at 37 °C. The phage titre in the lysate was 100% in comparison with the initial phage titre for each bacterial pathogen.

Table 3. Plaque Assay to study number of pfu/mL

Dilution	*P. aeruginosa* Pfu/mL	*P. vulgaris* Pfu/mL	*K. pneumoniae* Pfu/mL	*E. cloacae* Pfu/mL	*E. coli* Pfu/mL	*S. aureus* Pfu/mL	*Acinetobacter* Pfu/mL
10^{-3}	2.95×10^{13}	2.85×10^{13}	2.98×10^{13}	2.60×10^{13}	2.60×10^{13}	TNC	TNC
10^{-4}	2.44×10^{12}	2.70×10^{12}	2.75×10^{12}	2.52×10^{12}	2.52×10^{11}	1.89×10^{12}	TNC
10^{-5}	2.22×10^{11}	2.44×10^{11}	2.34×10^{10}	2.41×10^{10}	2.41×10^{10}	1.56×10^{11}	2.90×10^{11}
10^{-6}	2.14×10^{10}	2.32×10^{10}	2.06×10^{9}	2.30×10^{9}	2.30×10^{9}	1.22×10^{9}	2.55×10^{9}
10^{-7}	1.95×10^{9}	1.44×10^{9}	1.74×10^{7}	1.85×10^{7}	1.85×10^{7}	1.10×10^{8}	2.12×10^{8}
10^{-8}	1.74×10^{8}	1.23×10^{8}	1.22×10^{5}	1.66×10^{5}	1.66×10^{5}	0.95×10^{6}	1.61×10^{5}
10^{-9}	1.38×10^{6}	1.1×10^{7}	0.75×10^{3}	1.45×10^{3}	1.45×10^{4}	0.72×10^{4}	1.30×10^{3}
10^{-10}	0.86×10^{5}	0.7×10^{5}	0.6×10^{2}	1.1×10^{2}	1.1×10^{3}	0.52×10^{2}	1.13×10^{2}

TNC -Too numerous to count

Table 4. One Step Growth Curve to study number of pfu/ml released in minutes

Time(mins)	*P. aeruginosa*	*P. vulgaris*	*K. pneumoniae*	*E. cloacae*	*E. coli*	*S. aureus*	*A.baumannii*
0	0.87×10^{9}	0.74×10^{9}	0.75×10^{9}	0.70×10^{9}	0.70×10^{9}	0.69×10^{9}	0.80×10^{9}
10	1.30×10^{9}	1.5×10^{9}	1.22×10^{9}	1.87×10^{9}	1.87×10^{9}	0.72×10^{9}	0.94×10^{9}
20	1.53×10^{9}	2.4×10^{9}	1.74×10^{9}	1.10×10^{9}	2.10×10^{9}	0.85×10^{9}	1.25×10^{9}
30	2.91×10^{9}	2.68×10^{9}	2.06×10^{9}	1.23×10^{9}	2.23×10^{9}	0.93×10^{9}	1.81×10^{9}
40	2.8×10^{9}	2.35×10^{9}	2.34×10^{9}	2.44×10^{9}	2.34×10^{9}	0.98×10^{9}	2.09×10^{9}
50	2.45×10^{9}	1.98×10^{9}	2.04×10^{9}	2.32×10^{9}	2.42×10^{9}	1.20×10^{9}	2.23×10^{9}
60	2.44×10^{9}	1.74×10^{9}	1.98×10^{9}	1.22×10^{9}	2.62×10^{9}	1.24×10^{9}	2.22×10^{9}
70	2.44×10^{9}	1.66×10^{9}	1.96×10^{9}	1.12×10^{9}	2.85×10^{9}	1.35×10^{9}	2.22×10^{9}
80	2.44×10^{9}	1.65×10^{9}	1.94×10^{9}	1.12×10^{9}	2.85×10^{9}	1.35×10^{9}	2.21×10^{9}

Table 5. Reduction in number of bacteria from wastewater by bacteriophage formulation

Bacterial Pathogen	TVC before phage treatment	TVC after phage treatment
Klebsiella pneumoniae	6.87×10^{6}	4.18×10^{4}
Enterobacter cloacae	5.32×10^{6}	5.45×10^{4}
Pseudomonas aeruginosa	7.28×10^{6}	2.36×10^{5}
Proteus vulgaris	8.16×10^{6}	9.16×10^{4}
Esherichia coli	5.32×10^{6}	3.35×10^{4}
Acinetobacter baumannii	5.76×10^{6}	2.11×10^{5}
Staphylococcus aureus	9.0×10^{6}	2.0×10^{4}

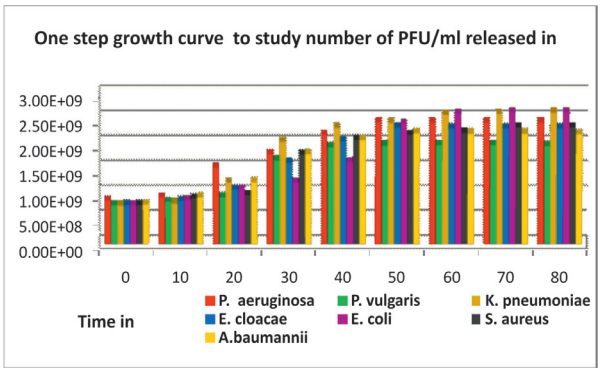

Fig. 6. One Step Growth Curve to study number of pfu/mL released in minutes

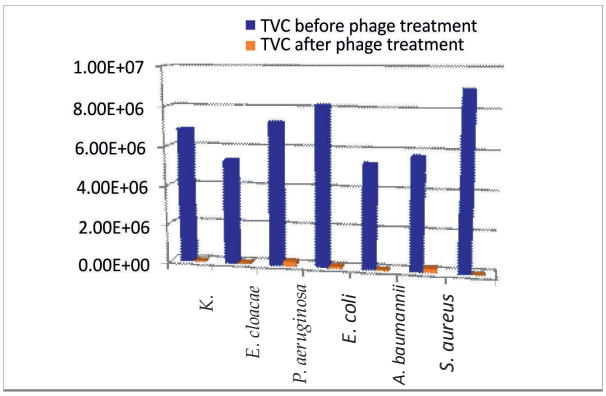

Fig. 7. Reduction in number of bacteria from wastewater by bacteriophage formulation

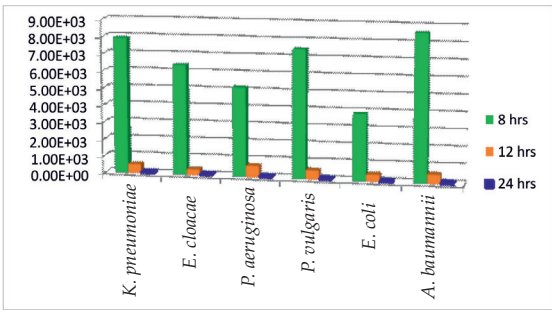

Fig. 8. Study of the effect of chlorine at 5 ppm on the bacterial pathogens

DISCUSSION

The present study indicates that the Pavana River which carries industrial and domestic waste is highly polluted. Different pathogenic microorganisms isolated and identified as *Pseudomonas aeruginosa, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Proteus vulgaris,* and *Staphylococcus aureus* which cause many diseases in human beings were found to be highly resistant to more than 20 different antibiotics which is life threatening. The rapid rise of multidrug-resistant bacteria in river water warrants infection control programs to limit the emergence and spread of antimicrobial-resistant pathogens (D'Agata, 2004).

In an era where antibiotic-resistant bacterial infections are on the rise, bacteriophages provide numerous advantages (Catherine and Stephen, 2011). In the present investigation phages were isolated against each pathogen from Pavana river which were characterized by Scanning Electron Microscopy. Spot Assay was performed as a confirmatory test for bacteriophages. To determine the titre of phages, plaque assay was performed which indicated that count of plaques was decreased with increasing dilutions.

Characterization of wastewater gives the idea about different components present in water as well as it indicates the resistance of pathogens and bacteriophages to different components present in water such as heavy metals, chemicals, and toxic components. Various parameters of water were studied in the present study such as pH, total solids, total suspended solids, total dissolved solids, and BOD (Biochemical oxygen demand) which revealed heavy load of microorganisms in water. Metal concentration of Pavana river water at five different locations was found to be much higher than the permissible levels. Phages against all the bacterial

Table 6. Study of the effect of chlorine at 5 ppm on the bacterial pathogens

Bacterial Pathogen	Time of exposure to chlorine at 5 ppm		
	8 hrs	12 hrs	24 hrs
Klebsiella pneumoniae	7.9×10^3	5.42×10^2	96
Enterobacter cloacae	6.4×10^3	3.5×10^2	82
Pseudomonas aeruginosa	5.2×10^3	6.82×10^2	79
Proteus vulgaris	7.5×10^3	5.42×10^2	87
Esherichia coli	3.9×10^3	4.21×10^2	69
Acinetobacter baumannii	8.6×10^3	5.42×10^2	95
Staphylococcus aureus	4.35×10^3	5.32×10^2	73

pathogens were also isolated from same locations which confirms the resistance of bacteriophages to heavy metals like Pb. Cr, Ni and Cd. So, metal resistant specific bacteriophages can be used as biocontrol agents for killing of metal resistant bacteria present in the water. Thus, phage formulation can stay alive in the water resources that constantly receives various types of pollutants like heavy metals.

Unfortunately, these pathogenic antibiotic resistant bacteria do not respond to the conventional water purification methods. Therefore, there is a necessity of environmentally friendly approaches to overcome the problems associated with the antimicrobial resistant bacterial pathogens. Chlorination is involved in wastewater treatment for disinfection. The existence of chlorine resistant bacteria in wastewater has been considered as an important potential health problem (Mohammad *et al.*, 2004). In the present investigation the pathogenic organisms isolated from the water sample were resistant to the chlorination even after 24 hours at 5 ppm of chlorine (Table 6, Fig. 8). According to previous study, bacteriophages were found more resistant to chlorination than bacteria. Usefulness of bacteriophages as model micro-organisms had been shown by Durán *et al.,* (2003) for evaluating chlorination treatment of water. In sewage effluent, the chlorine combines amazingly fast with ammonium and organic matter to give chloramines. Reduction in the number of bacterial pathogens may be due to combine effect of chlorine and monochloramine. These chloramines are toxic to human health and cause different health problems like skin problems, respiratory diseases, and kidney problems. Comparison of the chlorine treatment and phage therapy proved that the phages were more efficient in inactivation of pathogens than chlorine. Bacteriophages are host dependent for replication. Due to host specificity of phagesthere is no harm to other microbial community and human health. The present investigation has developed a new methodology for the control of chlorine resistant bacterial pathogens present in treated wastewater which is used for agriculture. Phage therapy is the application of phages to the environment in the destruction of pathogenic bacteria. In this investigation bacteriophages retained their ability to lyse bacterial pathogens which proves that organic matter does not inhibit bacteriophage activity. Therefore, bacteriophages can be utilized as eco-friendly approach in wastewater treatment. Bacteriophage treatment has been Generally Recognized As Safe (GRAS) (Andrea, 2015). Mathur *et al.* (2003) reported the efficacy of phages against *Escherichia coli, Acinetobacter spp., Pseudomonas spp,* and *Staphylococcus aureus.* The goal of the present investigation was to assess how bacteriophage can be a control strategy in preventing bacterial pathogens in contaminating crops. In the present study, bacteriophages were found effective in controlling *Pseudomonas aeruginosa, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Proteus vulgaris, and Staphylococcus aureus.* The application of phages to bacteria-containing environments like water makes a quantitative difference, not a qualitative one. Since phage replication can only occur within phage-susceptible bacteria, and the consequence of phage therapy is the destruction of phage-susceptible bacteria, phage populations tend to decline following phage therapy. The phage therapy is simply another form of biological control; the use of one organism to suppress another; and like other biological controls, the application of phage therapy holds a potential to reduce the usage of anti-pest chemicals. The potential application of phage therapy in wastewater treatment systems to improve effluent and sludge emissions into the environment has been previously discussed (Withey *et al*., 2005); Kutter, 2005). Bacteriophages are highly abundant in the aquatic environment ranging from 10^4 mL-L to in excess of 10^8 mL/L. (Bergh *et al.,* 1989). Numbers are typically 3-10 times greater than the bacterial counts, although there is substantial variation between ecosystems (Weinbauer, 2003). Environmental and health risks are associated with reuse of wastewater for irrigation (Shakir *et al*., 2017). This bacteriophage therapy targets and kills the pathogenic bacteria in treated wastewater which may enter the food chain during various crop processing steps. Ahiwale *et al.* (2012) have discussed the potential application of bacteriophage technologyto eliminate human bacterial pathogens in different water systems including river. Development of phages in the polluted river water will provide long term and cost-effective control of potentially pathogenic bacteria. At present water shortage is a growing problem in the whole area and wastewater reclamation and reuse is one of the main key factors for its sustainable development (Cooper and Olivieri, 1998). Bacteriophages can be used as potential biological control agents in the natural water bodies to bring about targeted killing of

bacterial pathogens (Ahiwale *et al.,* 2016). Our research investigation has proved the Phage therapy for treated wastewater as a novel antibacterial agent against bacterial pathogens. Liquid phage formulations could be an alternative weapon to kill the antibiotic resistant bacterial pathogens.

In the present investigation, highly effective lytic bacteriophages against multidrug-resistant bacteria were isolated from the Pavana river water. Formulations of these bacteriophages were prepared. The phages were stable for 3 months at 37 °C. Results strongly support that the phage formulation in water could be used as an useful biological control therapy for pathogenic bacteria in treated wastewater to prevent their entry in the food chain during various crop processing steps.

ACKNOWLEDGEMENT

Authors are thankful to Haribhai V. Desai College, Pune for constant support in completion the present research work.

REFERENCES

Abedon, S. T., Kuhl, S.J., Blasdel, B.G. and Kutter, E.M. 2011. Phage treatment of human infections. *Bacteriophage*. 1 (2) : 66-85.

Adams, M.H. and Park, B. H. 1956. An enzyme produced by a phage-host cell system. II. The propertiesof the polysaccharide depolymerase. *Virology*. 2 : 719-736.

Ahiwale, S.S. and Kapadnis, B.P. 2016. Stability and infectivity of bacteriophages under different environmental conditions. *Int. J. Res. Biosciences*.5 (1) : 55-71.

Ahiwale, S., Koparde, P., Deore, P., Gunale, V. and Kapadnis, B.P. 2012. Bacteriophage Based Technology for Disinfection of Different Water Systems. In: Satyanarayana T., Johri B., Anil Prakash (eds) *Microorganisms in Environmental Management*. Springer, Dordrecht.

Ahiwale, S., Tagunde, S., Khopkar, S., Karni, M., Gajbhiye, M. and Kapadnis, B. 2013. Liquid based formulations of bacteriophages for the management of waterborne bacterial pathogens in water microcosms. *Indian J Exp. Biol*. 51 (11) : 1038-1045.

Andrea, W.Y.L. 2015. Application of Bacteriophage Cocktail in Leafy Green Wash Water to Control *Salmonellaenterica* a master's thesis Presented, University of Massachusetts - Amherst Scholarworks@Umass Amherst.

Bergh, O., Borsheim, K. Y., Bratbak, G. and Heldal, M.1989. High abundance of viruses found in aquatic environments. *Nature*. 340 : 467-468.

Catherine, L.C. and Stephen, T.A. 2011. Pros and cons of phage therapy. *Bacteriophage*. 1 (2) : 111-114.

Cooper, R. C. and Olivieri, A. W. 1998. Infectious disease concerns in wastewater reuse. In: Asano T, ed. *Wastewater Reclamation and Reuse.* Lancaster, PA, Technomic Publishing. 489-520.

D'Agata, M. C. D. 2004. Rapidly Rising Prevalence of Nosocomial MultidrugResistant, GramNegative Bacilli: A 9Year Surveillance. *Study Infection Control and Hospital Epidemiology*. 25(10) : 842-846.

Drulis-Kawa, Z., Majkowska, S. G., Maciejewska, B., Delattre, A.S. and Lavigne, R. 2012. Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Curr Protein Pept Sc.* 13 : 699-722.

Durán, A. E., Muniesa, M., Mocé-Llivina, L., Campos C., Jofre, J. and Lucena, F. 2003. Usefulness of different groups of bacteriophages as model micro-organisms for evaluating chlorination. *J. Appl. Microbiol.* 95 : 29-37.

Ellis, E.L. and Delbrück, M. 1939. The growth of bacteriophage. *J Gen Physiol*. 22 (3): 365-384.

Falomir, M.P., Gozalbo, D. and Rico, H. 2010. Coliform bacteria in fresh vegetables: from cultivated lands to consumers. Current Research, Technology and Education Topics In: *Applied Microbiology and Microbial Biotechnology*. A. Méndez-Vilas (Ed.) 1175-1181.

Hughes, K.A., Sutherland, I.W. and Jones, M.V. 1998. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology*. 144 : 3039-3047.

Hunter, P. R. 2003. Climate change and waterborne and vector-borne disease. *J. Appl. Microbiol.* 94 Suppl : 37S-46S.

Jiang, S. C., Kellogg, C. A. and Paul, J. H. 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Oahu, Hawaii. *Appl. Environ. Microbiol.* 64 : 535-542.

Kutter, E. and Sulakvelidze, A. 2005. Bacteriophages: Biology and Applications. *CRC Press*, Florida.

Mansura S. Mulani, Syed Azhar, Shaikh Azharuddin and Shilpa Tambe, 2015. Harnessing the Power of Bacteriophage for Pathogen Reduction in Wastewater. *Int J Curr Microbiol Appl Sci.* 2 : 152-161.

Mathur, M. D., Vidhani, S. and Mehndiratta, P. L. 2003. Bacteriophage Therapy: An Alternative to Conventional Antibiotics, National staphylococcal phage typing Centre. *Department of Microbiology.* 51.

Mohammad, I., Al-Berfkani, Anies, I. Z. and Bayazed H. 2014. Assessment of chlorine resistant bacteria and their susceptibility to antibiotic from water distribution system in Duhok province. *J Appl Biol Biotechnol*. 2 (06) : 010-013.

Patwardhan, R. B., Shinde, P. S., Chavan, K. R. and Devale, A. 2015. Reversal of Plasmid Encoded Antibiotic Resistance from Nosocomial Pathogens by Using *Plumbago auriculata* Root Extracts. *Int J Curr Microbiol Appl Sci*. 2 : 187-198.

Periasamy, D. and Sundaram, A. 2013. A novel approach for pathogen reduction in wastewater treatment. *J Environ Health Sci. Eng.* 11 (1) : 12.

Radulescu, C., Dulama, I.D., Stihi, C, Ionita, I, Chilian, A., Necula, C. and Elena, D. C. 2014. Determination of heavy metal levels in water and therapeutic mud by atomic absorption spectrometry. *Rom. Journ. Phys*. 59 (9-10) : 1057-1066.

Shakir, E., Zahraw, Z., Hameed, A. and Al-Obaidy, M.J. 2017. Environmental and health risks associated with reuse of wastewater for irrigation. *Egyp J Petroleum*. 26 (1) : 95-102.

Ursula, J., Blumenthal, D., Duncan, M., Anne, P., Guillermo, R. P., Rebecca, S. 2000 Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines. *Bulletin of the World Health Organization.* 78 (9).

Watanabe, K., Takesue, S., Jin-Nai, K. and Yoshikawa, T. 1970. Bacteriophage active against the lactic acid beverage producing bacterium *Lactobacillus casei*. *Applied Microbiology*. 20 (3) : 409-415.

Weinbauer, M.G. 2003.Ecology of prokaryotic viruses. *FEMS Microbiol Rev*. 28 : 127-181.

WHO Health guidelines for the use of wastewater in agriculture and aquaculture. Report of a WHO Scientific Group. Geneva, World Health Organization, 1989 (WHO Technical Report Series, No. 778.

Withey, S., Cartmell, E., Avery, L. M. and Stephenson, T. 2005. Bacteriophages - Potential for Application in Wastewater Treatment Processes. *Science of the Total Environment*. 339 (1-3) : 1-18.