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Pilot-Scale Efficacy of Bacteriophage Cocktail For Reducing Enteric Bacteria In Domestic Graywater.

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Abstract : Domestic graywater reuse offers a sustainable approach to mitigate water scarcity. This study explores the potential of bacteriophages as a biocontrol agent for reducing the enteric bacteria in graywater. Graywater was initially quantified and characterised, followed by bacterial isolation and identified by API index via the Vitek system, revealing *Escherichia coli* (08) and *Enterococcus faecalis* (03). A total of 15 phages were successfully isolated and purified; among these, five—B28K7b, B28B27b, B28K1a, B28B1a, and B28K32c—were selected for further studies. Phages B28K7b and B28B27b exhibited the broadest host ranges and highest lytic activity compared to other phages, prompting their selection for the reduction study during the treatment process. Transmission Electron Microscopy (TEM) classified B28K7b as *Myoviridae* and B28B27b as *Siphoviridae* (order *Caudovirales*). In a 3000mL system of untreated graywater, phages applied at a multiplicity of infection (MOI) of 2 achieved 1.65 ± 0.05 $\log_{10}$ reductions in total heterotrophic counts and 2.01 ± 0.03 $\log_{10}$ reductions in *E. coli* (mean ± SD; $p < 0.05$) after 6 hrs. These results highlight bacteriophages as an effective, eco-friendly alternative for bacterial reduction in graywater, enabling safe non-potable reuse.

Keywords: Graywater, *E.coli, E. faecalis*, Bacteriophages, Pilot-scale treatment process.

INTRODUCTION

The rising urban population have intensified the demand for safe and clean water, while the limited availability of freshwater resources has enhanced the need for sustainable water management strategies (Birks & Hills, 2007). In India, the Central Pollution Control Board (CPCB) promotes graywater reuse—after primary treatment—for non-potable applications like toilet flushing and gardening, while the National Green Tribunal (NGT) reinforced this in its 2024 directives.As a result, there is growing interest in the reuse of domestic graywater for non-potable applications. However, its reuse is hindered by the presence of pathogenic bacteria and viruses; thus, there is a need to understand the graywater load before it can be safely reused (Fathy et al., 2024).

Since graywater is water generated from the kitchen and bathrooms, it may have an elevated load of easily degraded organic material, which can favour the growth of bacteria such as *Pseudomonas* spp., *Salmonella* spp., *E.coli, and Enterococcus* spp. etc., and thus load reduction needs to be studied before any attempt is made to reuse it (Ottoson et al., 2003). Conventional methods for microbial reduction depend on chemical or energy-intensive processes, which are often environmentally unsustainable and limit scalability in water-scarce urban areas. Treated graywater is a sustainable option for non-potable uses that conserves water and reduces environmental water pollution. When effectively managed, it can become an essential part of water resource management, especially in urban and semi-urban areas with water scarcity.

In this context, bacteriophages offer a potent, selective biological tool for safely reducing bacterial populations (Lee and Park 2015). Coliphages—viruses that specifically infect *Escherichia coli*, *Enterococcus faecalis*, have thus emerged as promising biological agents for bacterial control as they lyse target strains without affecting others (Sundar et al., 2009). Use of a phage cocktail can thus enhance the efficacy of the treatment against diverse contaminants typical in graywater (Kiruba et al., 2016).

Thus, the present study is a pilot-scale investigation into the use of phages as a means for reducing bacterial load in domestic graywater. The research aims to quantify graywater volumes, characterise microbial profiles, and testing phage efficacy and using phage cocktails against *Escherichia coli* and *Enterococcus faecalis* strains, to reduce its load in campus-derived graywater. By establishing phage-mediated treatment as a sustainable, safe alternative, this work aims to mitigate public health risks from enteric bacteria and promote graywater reuse practices.

II.MATERIALS AND METHODS

2.1 Study Sites and Graywater Sample Collection

Graywater samples were collected (1500mL each) within a period of 01year from two residential buildings within a college campus located in Andheri, Mumbai, in sterile HDPE bottles using the grab technique and analysed immediately or kept sealed and stored under cold and dry conditions (<4ºC) for not more than a day. To identify the selected area for sampling, assistance was taken from the Hostel authorities, and BMC floor plans were used to identify the different graywater collect basins for water arising from washbasins, kitchen sink, bathroom, and water used for washing clothes. Outlet pipes that were separated from the central wastewater collection system were shortlisted to collect the wastewater. The graywater samples were collected from 2 different point sources of the B28 building (Figure 1) labelled as

a. Point source B28K -which has wastewater from the Kitchen sink outlet.

b. Point source B28B- which has wastewater obtained from a combined outlet of washbasin and bathrooms.

c. The control sample was collected from the drinking water taps.

After collection, graywater samples underwent pretreatment via coarse screening (1 mm mesh) and sedimentation for 1–2 h, followed by pH and turbidity measurement of each sample.

Figure 1: Sampling site location (Source- Google Earth).

2.2 Microbiological Analysis of Graywater

2.2.1 Detection and Identification of bacterial load and enteric flora

Graywater samples collected were assessed for their total aerobic microbial load as well as the detection of enteric bacteria. The samples were analysed as per standard methods described in APHA 2005. 100mL of graywater samples were filtered through a 47-mm, 0.45-µm pore size mixed cellulose ester membrane filter[Merck, India] that retains the bacteria present in the sample. The filter pad was then placed on selective media such as Rapid HiColiform Agar[Himedia, India] and Bile Esculin agar [Himedia, India], respectively, while the total heterotropic count was determined by the standard plate count method using Plate Count Agar after an incubation for 24 to 48 hrs at 25±0.5°C. Isolates exhibiting typical colored colonies were further purified and presumptively identified based on biochemical reactions, and were identified as

per Bergey's Manual of Determinative Bacteriology and enumeration as CFU/mL. Confirmation of the isolates was undertaken through an Analytical Profile Index (API) based identification performed on a VITEK 2 system. This process validated the biochemical identifications across the broader set of environmental isolates. A single colony from each environmental bacterial isolates was inoculated into nutrient broth and incubated overnight, and were subsequently preserved as backup stocks by adding 30% glycerol and storing them at deep-freeze conditions.

2.2.2 Isolation and Purification of phages

Graywater samples were collected and processed for phage recovery within 24 hrs of collection in the laboratory. The double enrichment process was carried out using standard USEPA1601/1602 methods. A 1000 mL sample was filtered through a 0.45 µm membrane filter to remove bacteria, allowing phages to pass through and be retained in the filtrate. Phages were enriched from the filtrate using standard host strains, including *E. coli* 15597, *E. coli 10536*, *E. faecalis 29212,* and various environmental isolates isolated from the graywater samples. For enrichment, 50 mL of the filterate sample was mixed with 50 mL of 2X Nutrient Broth [HiMedia, India] supplemented with 2 mM $CaCl_2$ and 5 mL of a 24-hour-grown culture of the specific host strain, against which coliphages were to be enriched. The flasks were incubated at 37°C for 24 hrs. After incubation, the content was transferred into sterile 50 mL centrifuge tubes and centrifuged at 10,000 × g for 10-15 minutes under cold conditions. The upper aqueous layer containing the phage particles was collected, while the cell debris settled at the bottom of the tube was discarded. The process was repeated in triplicate to obtain purified phage lysate. The phage lysate was further enriched by the addition of 1:1 volume of chloroform, followed by vigorous shaking that helped to lyse any bacterial cells that were present in the aliquot. The upper aqueous layer containing the inert phage particles was collected and stored at 4°C.

2.2.3 Detection and enumeration of phages

Following enrichment, phages were detected using the spot test technique by spotting 10 µL of lysate onto a lawn of specific host bacteria. Zones of clearance after 24 hrs at 25°C indicated the presence of phages. Samples showing positive results were then enumerated using the conventional double agar overlay (DAL) method. All assays were performed in triplicate. Plaques with varied morphologies suggested the presence of multiple phage clones. Thus, single plaques with unique morphologies were picked using a sterile borer and inoculated in Nutrient broth containing 2mL of log-phase host cultures, and purified through repeated plaque assays until single-morphology plaques were achieved. Purified high-titer lysates were stored at 4°C for further analysis.

2.2.4 Characterisation of phages

2.2.4.1 Transmission Electron Microscopic analysis of Phages.

Purified lysates were added to a sterile 100mL screw cap bottle containing 10% Polyethylene glycol (PEG) 6000 [Sigma Aldrich, USA] and 1.02gm of sodium chloride (NaCl)[SRL, India], mixed, and transferred to a shaker for 01 hour. The lysate bottles were kept in a refrigerator overnight, and the liquid was then centrifuged at 10,000x g for 10-15 mins at 4°C to obtain the phage pellet. Supernatant was discarded, and the pellet was resuspended in 2mL Phosphate Buffered Saline (PBS) and vortexed. The concentrate was then again filtered through a 0.22µm filter to remove any debris, and the filtrate was stored at 4°C until further analysis (Loh et al. 2021)

The morphological features of selected phages were examined by transmission electron microscopy (TEM: FEI Tecnai 12/003 transmission electron microscope; Carl Zeiss) at an 80-kV accelerating voltage. The phage suspensions ($1x10^8$ PFU/mL) were concentrated and were negatively stained using 10µL of 2% (w/v) uranyl acetate on carbon-coated grids and observed at a magnification of × 30,000 under an electron microscope (S. Samir et al., 2022). Phages were classified according to the International Committee on Taxonomy of Viruses (ICTV) classification.

2.2.4.2 Host Range Determination and Efficiency of Plating (EOP)

The host range of the isolated phages was initially assessed by spot test assay. Briefly, mid-log phase cultures of reference host bacteria (*E. coli* ATCC 10536, *E. coli* ATCC 15597, and *E. faecalis* ATCC 29212) and their environmental isolates were mixed with soft agar (0.7%) and overlaid onto nutrient agar plates.10 µL of phage lysate was spotted onto the bacterial lawns, and plates were incubated at 37 °C for 18–24 h.

Zones of clearance after 24 hrs at 25°C indicated the lytic activity of phages against host bacteria. Positive isolates were tested for plaque-forming ability by using the conventional double agar overlay (DAL) method.

Phages showing high lytic activity in the spot assay were further analysed for efficiency of plating (EOP) using the double-layer agar method. Serially diluted phage suspensions were mixed with host cultures, overlaid onto agar plates, and incubated at 37 °C. Plaques were counted, and EOP was calculated as the ratio of PFU/mL on the test host to that on the reference host. Experiments were performed in triplicate and results expressed as mean ± SD. The ability of the phage lysates to target multiple strains was shortlisted and further used for the treatment process.

2.2.4.3 Stability Analysis of phages

Phages were resuspended in Sodium chloride-Magnesium sulfate buffer (SM buffer) and stored at 4°C for phage stability analysis. High titre of phage lysates (10^8PFU/mL) were incubated at different temperature range-25°C, 37°C, and 52°C for 1 hour, while for stability analysis at varied pH, various pH values of 5, 7, and 9 were prepared and phages were suspended in them for 1 hour at 25 °C. and the surviving phage lysates were enumerated by the double agar layer method. The initial phage titre was considered as the control, and the log reduction value was calculated accordingly; and all the assays were conducted in triplicate.

2.2.4.4 Adsorption, latent period, and phage burst size.

Experiments were conducted as described in a previous study (Gallet et al. 2009), with some modifications. The host bacterium was grown to mid-log phase, and each phage was added separately to the suspension at a multiplicity of infection (MOI) of 0.01. At predetermined time - 0, 5, 10, 15, 30, 60 minutes, 100µL of aliquots were withdrawn in 900 µL ice-cold phage buffer (50 mM Tris, 10 mM EDTA, pH 7.5), and 0.5mL of chloroform was also added. The samples were centrifuged to sediment bacterial cells and the adsorbed phage particles.The free phages in the filtrate was determined using the plaque assay. To enumerate unabsorbed phage particles, the double agar layer method was performed.

To determine the phage latent period and burst size, a one-step growth curve experiment was conducted as described in a previous study (Lee and Park 2015), and a graph was plotted between the released phage number and time. The latent period and the burst size of the phage were calculated from the curve.

2.2.4.5 Determining the optimal Multiplicity of Infection (MOI)

The lytic activity of the phages were evaluated at different MOIs. The host bacteria were seeded in a sterile graywater sample with serially diluted phage at different MOIs (0.1,1,2 and 3) in 20 mL Sterile Nutrient broth. As a control, bacterial cultures without phage were used. The mixer was incubated at 37°C, and the supernatant was collected every 3 hrs and centrifuged at 10,000x g for 10-15 mins at 4°C, and the titre of non-absorbed phages was estimated by the double agar overlay (DAL) method (Runa et al. 2021). The proportion of phage concentration to host cells was calculated according to the formula:

MOI=Number of phages (PFU/mL)/Number of host cells (CFU/mL).

2.3 Pilot-scale study for effective bacterial reduction in graywater using selected phage cocktail.

A total of 2 pilot-scale setup was studied for bacterial reduction in graywater effluent samples from the B28 building were carried out in a 3000mL system in PVC reactors with lids and taps to collect the treated graywater at regular intervals. The respective host bacteria in the test system were at a concentration of $1x10^4$-$1x10^5$ CFU/mL. One reactor served as the test system (graywater + phage cocktail at MOI 2.0, achieved by adding 3 mL of phage cocktail to the system, while the control contained untreated graywater; both were maintained at 25°C with periodic mixing. Phage cocktail prepared as 60:40 (v/v) mixture of B28K7b ($6×10^8$ PFU/mL) and B28B27b ($4×10^7$ PFU/mL). Samples were analysed, and their average bacterial load before and after the treatment process was determined. In addition to assessing bacterial reduction, pH and turbidity of the graywater samples were monitored to examine whether these physicochemical parameters have any influence on phage activity.

Sample aliquots (01mL) were withdrawn at 0 hr, 3 hr,6 hr, and 9 hr, serially diluted from both the system, and plated in triplicate on Plate Count Agar (total heterotrophs) and Rapid HiColiform Agar (HiMedia, India; *E. coli*) following standard protocols. The log reduction value for each time interval was calculated for both systems.

The Log Reduction value (LRV) formula =log10(N_0 / N_t) where:

- N_0 = concentration of bacteria before treatment process (CFU/mL)
- N_t= concentration of bacteria after treatment process(CFU/mL)

2.4 Statistical data analysis

All the experiments were performed in triplicate. The effect of pH and temperature was analysed by one-way ANOVA. Bacterial reduction study of the graywater system was done by a paired t-test (two-tailed). Data are expressed as means ± standard deviation (SD). The results were analysed by one-way ANOVA (analysis of variance) in SPSS software version 23.0.(IBM Corp., Armonk, NY, USA).Statistical significance was determined at $p < 0.05$.

III. RESULTS AND DISCUSSION

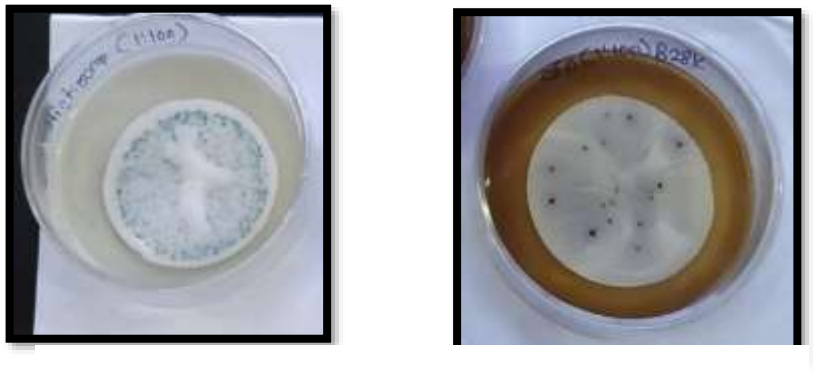
A total of 30 graywater samples were collected from two selected point sources (B28K and B28B) of discharge release from building B28. The graywater samples were collected early in the morning, as variation in graywater production was observed with peak flows achieved during 8 -10 am, and the least discard was being released between 4-6 pm. This was confirmed by the flow rate observed over a period of 03 months. In the previous papers, researchers reported that approximately 60–65% of water supplied by Thames Water was converted to graywater that was released in the morning time interval (Birks and Hills 2007).

pH and turbidity of each sample were measured before microbial quantification. Post sedimentation process, it was noted that pH was in the range of 7.2±0.6 and turbidity was 11.5 ± 10 NTU. Then the supernatant sample was used for microbial analyses and the phage treatment process.

3.1 Characterisation and identification of Enteric bacteria predominantly observed in Domestic graywater

Enumeration studies revealed that *E. coli* population formed about approximately 10 %(2.01±0.05 x10^3CFU/mL) and *E. faecalis* formed 4 % (1.26 ±0.03x10^2 CFU/mL) of the total heterotrophic load (2.57±0.06 x10^5 CFU/mL) in the B28B graywater samples while in the B28K graywater sample *E. coli* population formed about approximately 7 % (1.31 ±0.03x10^3 CFU/mL)and *E. faecalis* formed 2 %(1.02 ±0.05 x10^2 CFU/mL) of the total heterotrophic load (3.21 ±0.02 x10^5 CFU/mL)as shown in (Figure 2). These baseline levels of indicator organisms *E. coli* and *E. faecalis,* in the representative samples, help in subsequent phage reduction studies. The presence of *E. coli* and faecal coliforms showed faecal contamination, indicating the possible presence of bacterial pathogens. This is similar to the earlier studies conducted by (Birks et al. 2007)and (Ottoson et al. 2003), they also detected a higher number of total coliform and *Escherichia coli* in their graywater samples, such that 14 out of 32 environmental isolates (46%) and 06 (18%) were biochemically identified as *E. coli*, *E. faecalis,* respectively. This data is similar to earlier studies conducted by (Fathy et al. 2024) and (Sundar et al. 2009). This substantiates the emphasis on targeting *E. coli* and *E. faecalis* as an indicator for faecal contamination. To validate biochemical identifications, a representative subset of 11 putative environmental isolates (*E. coli* [n=8]and *E. faecalis*[n=3], selected across samples for morphological diversity) underwent confirmatory Analytical Profile Index (API) analysis on the VITEK 2 system (Table 1). All confirmed ≥95% species-level identity, ensuring the biochemical profiling reliably represented the dominant populations targeted in phage studies.

Graywater samples (n=30) collected over one year revealed significant seasonal variation in both *E. coli* and *E. faecalis* concentrations (Table 2). Summer samples from both sources (B28K, B28B) exhibited peak mean concentrations of 1.91 × 10^3 CFU/mL for *E. coli* and 1.26 10^2 CFU/mL for *E. faecalis* than winter and rainy season levels. These elevated summer loads ($\log_{10}$ ≈ 3.28 CFU/mL for *E. coli* and $\log_{10}$ 2.91≈ CFU/mL for *E. faecalis*) reflect increased domestic water usage and warmer temperatures that favour enteric bacterial proliferation.



(a) *(b)*

Figure 2 (a), (b):Typical colony characteristics on selective agar media- Rapid HiColiform Agar and Bile Esculin agar.

Table 1: API based Identification of Selected Isolates obtained from graywater samples.

Sr No.	Graywater samples	Presumptive ID	No. of Isolates Obtained	Selected Isolates (n)	VITEK ID (% Confidence)
1	B28K	*E. coli*	6	8	*E. coli* (97-99%)
2	B28B		7		
3	B28K	*E. faecalis*	3	3	*E. faecalis* (95-97%)
4	B28B		2		

3.2 Isolation and Purification of phages against enteric organisms-*E.coli* and *E. faecalis*

Phage lysates obtained after enrichment were tested against the reference host and environmental isolates for its lytic activity, detected by the zone of clearance, initially by spot test and then by the Double agar layer method (DAL) (Figure 3). A total of 21 coliphages were initially screened; 15 were selected for further characterisation based on lytic activity and host range. Among these, 13 phage lysates demonstrated infectivity against both the standard ATCC *Escherichia coli* strains and the environmental *E. coli* isolates, whereas two phage lysates were capable of infecting the standard ATCC *Enterococcus faecalis* strain as well as its corresponding environmental isolates. Purified high-titre phage lysates were kept at 4°C. Some phage lysates demonstrated the ability to infect *E. coli* and *E. faecalis*, as well as other bacterial genera(Table 3). Concentration of the purified lysate against *E. coli* was in the range of 3.2×10^{8}-4.0×10^{8}PFU/mL, and purified lysate against *E.faecalis* the range of $1.5x10^{5}$-$2.1\ x10^{5}$PFU/mL (Figure 4). The present study is in accordance with the earlier report of (Shende et al. 2017), who also found predominant of *E. coli* & *E. faecalis* in animal waste disposal as well as in domestic samples.

05 phages out of the selected 13 phages exhibited lytic activity and were selected for further analysis (B28K1a, B28K7b, B28K32a, B28B27b, and B28B1a). B28K1a and B28K7b phages produced visible, clear plaques of a diameter ranging from 1-3mm, while B28K32a and B28B27b phages produced clear plaques of a diameter ranging from 3-4 mm. The plaque morphology of B28B1a coliphage appeared as pinpoint plaques of a diameter less than 1mm. Rest all the plaques produced, cloudy plaques of diameter 3-5 mm. Each plaque was chosen for the phage purification process. Successive purification steps were performed to ensure that the lysate gave plaques that were all of similar morphology and size, and selected for further analysis.

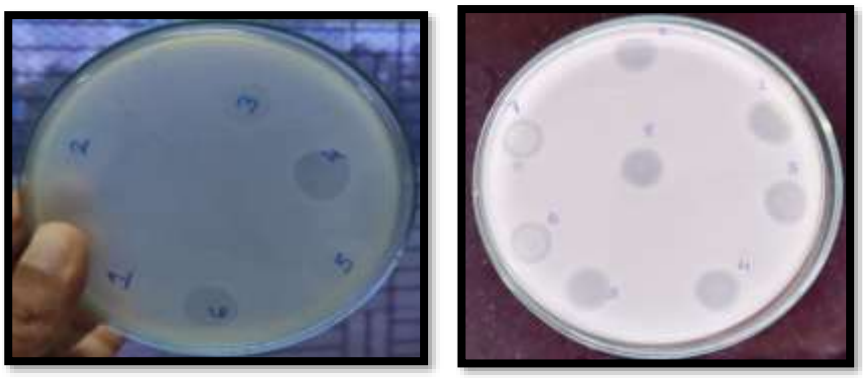


Figure 3: Spot test- Phage Lysates showing clear and turbid clearance when spotted on host *a) E. coli* ATCC 15597 b) EcEI1B.

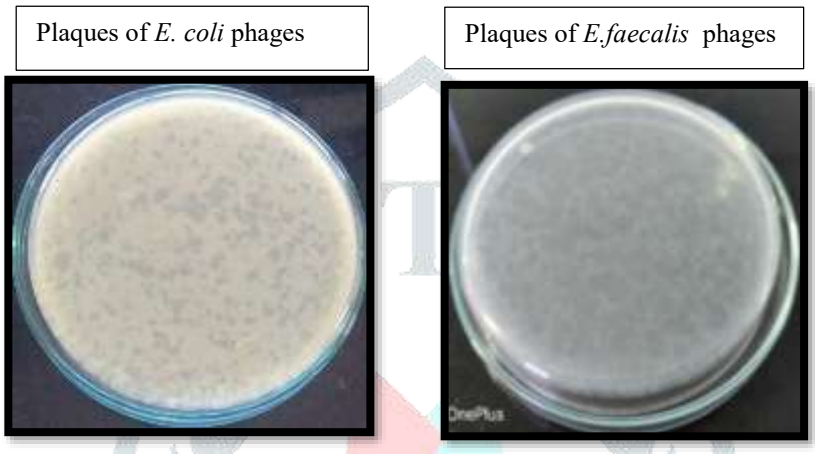


Figure 4: Plaques exhibiting diverse morphologies were observed post-enrichment and selected for purification.

3.3 Characterisation of phages

3.3.1 Phage morphology by Transmission Electron Microscopy(TEM) analysis

TEM analysis of both the phages(B28K7b, B28B27b revealed that. B28K7b particles exhibit an icosahedral head and an extended contractile tail, with an overall length of approximately 255 nm (Figure 5 a). Morphological analysis suggests that B28K7b belongs to the Myoviridae family. Phage B28B27b particles exhibit an icosahedral head and an extended non-contractile tail, with an overall length of approximately 150 nm(Figure 5 b). Morphological analysis suggests that B28B27b belongs to the Siphoviridae family. According to the ICTV (International Committee on Taxonomy of Viruses) both phage family belongs in the order Caudovirales.

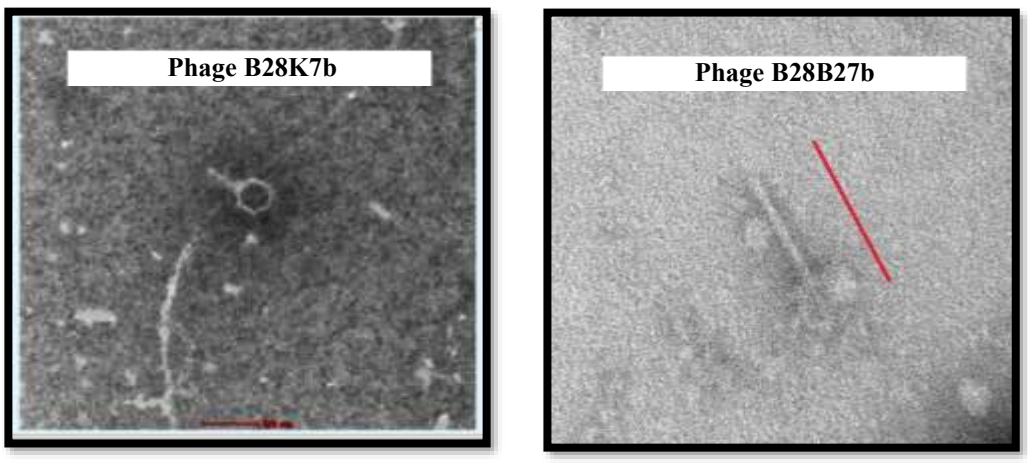


Figure 5: TEM image of a) Phage B28K7b(50nm) and b) Phage B28B27(50nm).

3.3.2 Host range determination of phages

The host range of all 05 phages was checked against *E.coli* (08) and *E.faecalis* (03) environmental isolates to evaluate the host specificity of each phage by spot test assay. Overall, *E. coli* isolates were susceptible to 04 phage lysates (B28K1a, B28K7b, B28K32a, B28B27b), among the selected phage lysates, demonstrating that the phages are highly susceptible against a wide range of *E.coli* strains, i.e., polyvalent phages, while *E. faecalis* isolates were susceptible to only 01 phage lysate (B28B1a) exhibited a limited host range, i.e., monovalent phages.

Phage B28B27b exhibited the broadest host range, showing approximately 95% infectivity against the other reference ATCC strains, whereas B28K32c demonstrated lytic activity against only a single bacterial genus. Phage B28K7b displayed moderate to high host range activity (75–90%), while none of the tested bacterial genera were sensitive to phage B28K1a. Phage B28B1a showed lytic activity limited to one genus of environmental isolates. Consequently, only two coliphages, B28K7b and B28B27b, demonstrated broad host range susceptibility and comparatively higher EOP values (Table 3). The wide host range observed for these phages is consistent with previous reports (S. Samir et al. 2022; Shende et al. 2017), which indicate that bacteriophages are not always genus-restricted. Based on host range, plaque clarity, phage titre, environmental stability, and EOP, phages. Host range screening identified B28B27b (95%) and B28K7b (75-90%) as polyvalent coliphages with complementary spectra, achieving near-complete coverage of environmental *E. col*i isolates. B28K7b and B28B27b were shortlisted for further characterisation. In addition, environmental isolates EcEI1B and EcEI1C, which exhibited high EOP values, were selected for subsequent stability studies.

Table 2: Determination of host range of phages against standard reference stains.

Sr No.	Phage Lysate	Target Host Strain	Host Range against standard *S. typhi* 1331, *P.aeruginosa15442, K. pneumonia 10031* (%)
1	B28K1a	*E. coli* 10536	00 %
2	B28K7b	*E. coli* 15597	75 %
3	B28B27b	*E. coli* 15597	95 %
4	B28B1a	EcEI1C	05 %
5	B28K32c		50 %
6	B28K7b	EcEI1C	90 %
7	B28B27b	EcEI1B	95 %
8	B28K7b	*E. faecalis* 29212	25 %
9	B28B27b		00 %
10	B28K7b	EfEIIA, EfEIIC	05 %
11	B28B27b		00 %

Key-EcEI1-Environmental isolates of *E.coli*
EfEI-Environmental isolates of *E. faecalis*

Table 3 :Efficiency of plating of 02 selected phage for the host range test.

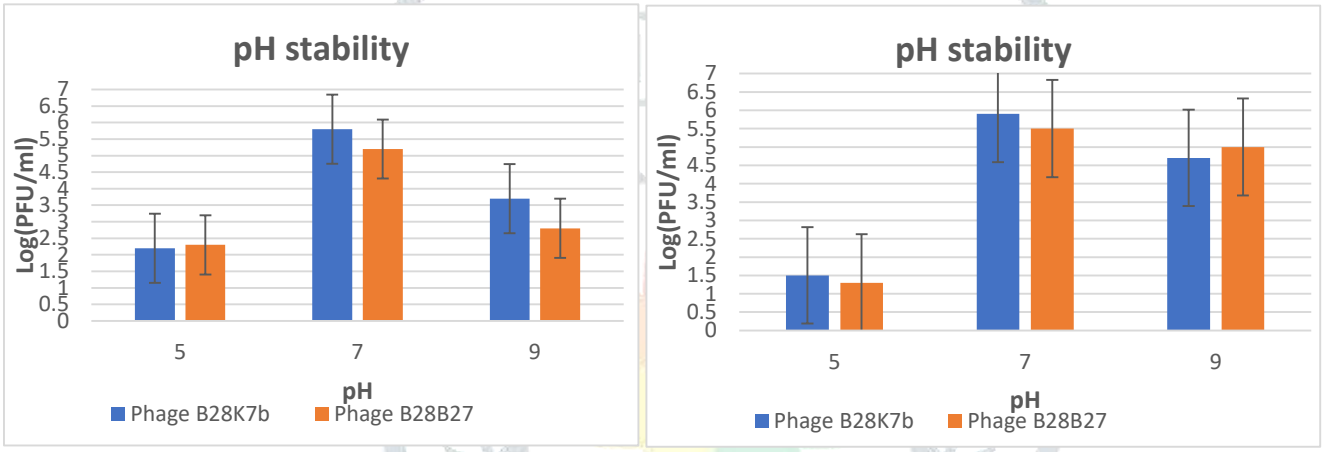
Phages	Reference host bacteria	Environmental isolates	No of isolates showing positive in spot test(%)	EOP±SD	Type of EOP
B28K7b	*E. coli* 15597	*Ec*EI1A	04/08	0.12±0.02	Moderate
		*Ec*EI1B,	(50%)	0.64±0.09	**High**
		*Ec*EI1D,		0.14±0.03	Moderate
		*Ec*EI1E		0.09±0.05	Low
B28B27b	*E. coli* 15597	*Ec*EI1C	03/08	0.66±0.02	**High**
		*Ec*EIF	(0.37%)	0.16±0.03	Moderate
		*Ec*EI1G		0.11±0.01	Moderate

3.3.3 Stability Analysis of phages

Phages B28K7b and B28B27 exhibited thermal stability at 25°C and 37°C against *E. coli* hosts EcEI1C and EcEI1B ($p > 0.05$; Figure 7), with minor differences between temperatures, but showed significant titre reductions at 52°C ($p < 0.01$). Similarly, both phages remained stable across pH 7.0–9.0 at 25°C ($p > 0.05$; Figure 6), with optimal activity at neutral pH, while pH 5.0 markedly reduced viability ($p < 0.001$).

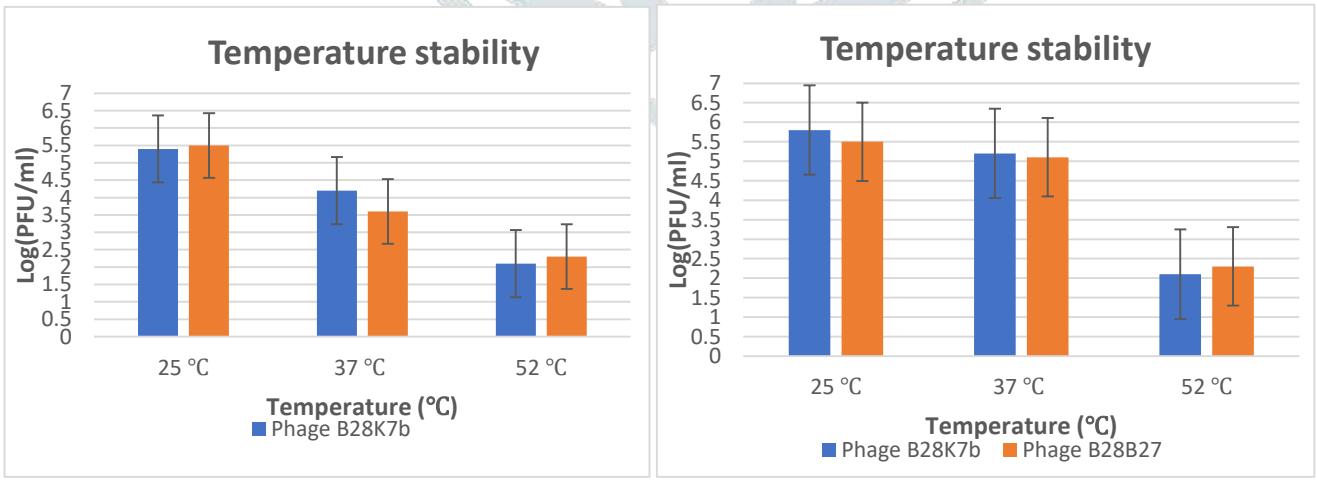
Initial phage count of both the phage B28K7b and B28B27 were 10^8PFU/mL. A similar study was done and reported by(Kiruba et al. 2016) that *Camplylobacter* phages are also active at neutral and high pH rather than low pH conditions. In accordance to our study (Runa et al. 2021), also reported that coliphages and *Bacillus* phages viability was observed more in neutral pH and temperature range from 30-45°C. Likewise present study, many other researchers such as (Kim et al. 2011; Samir et al. 2022) have obtained phage stability at neutral pH and room temperature (25°C).

In our study, both the phage lysates B28K7b and B28B27b remain viable against both the host cultures at all the given temperatures, while the pH range showed a decrease in phage activity at lower pH. Temperature and pH are the important environmental factors that influence the survival and activity of phages. The pH and temperature range of domestic graywater were in the range of 20-42°C and pH 6.0-8.5. Therefore, it can be reasonably assumed that the efficacy of phages in reducing bacterial populations would remain unaffected under these conditions.



(a) Host culture - EcEI1C (b) Host culture - EcEI1B

Figure 6 :Effect of pH on phage activity (Mean±SD).



(a) Host culture - EcEI1C (b) Host culture - EcEI1B

Figure 7: Effect of temperature on phage activity (Mean±SD).

3.3.4 Adsorption, latent period, and phage burst size.

A maximum of 75% both the phage adsorption was documented within 20 minutes of incubation. For B28K7b phage, most particles are adsorbed within 6 minutes, and for B28B27b phage, most particles are adsorbed within 10 minutes, thus indicating a higher probability of encounter between the phage and host and thus higher adsorption rate, this correlates with the previous study that a stage that depends on

the specific phage receptors of the host cell . The adsorption curves of B28K7b and B28B27b are similar (**Figure *8***). One-step growth curves of B28K7b and B28B27b phages. Host bacteria, such as EcEIB and EcEIC exhibited almost similar burst size i.e B28K7b exhibited shorter latent period (18 min) and higher burst size (100 PFU/cell) compared to B28B27b (23 min, 80 PFU/cell)(Figure 9).

3.3.5 Determining the optimal Multiplicity of Infection (MOI)

MOI was investigated to determine the phage-host concentrations for optimum phage activity. Host bacteria, such as EcEIB and EcEIC, were infected with both phages B28K7b and B28B27b phages at different MOIs of 0.1, 1, 2, and 3. The optimum MOI of both the phages were found to be 2, indicating that the concentration of phages must be double the host strain concentration to obtain the highest titer of infectivity.

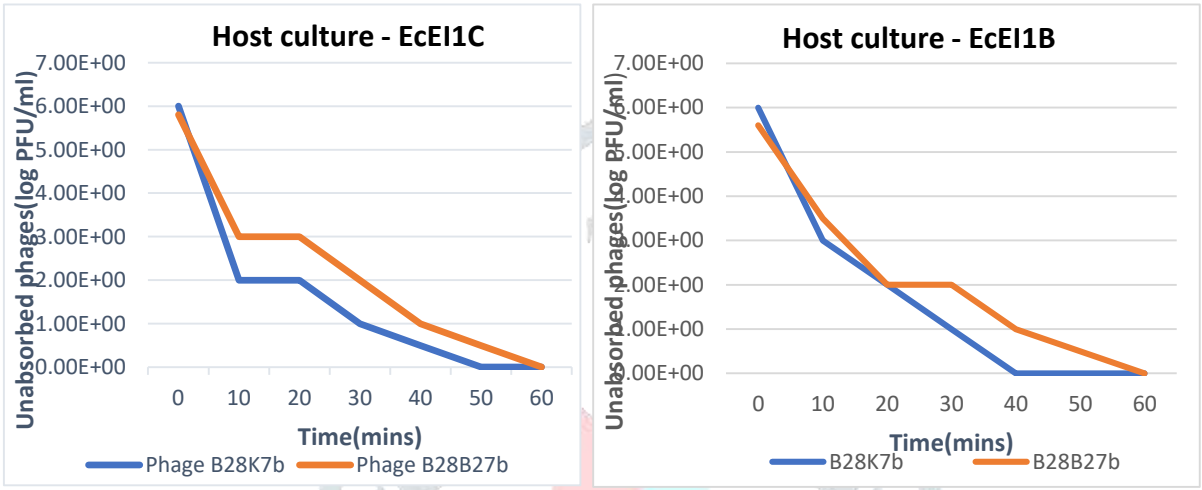


Figure 8: Adsorption time of phage B28K7b and phage B28B27b.

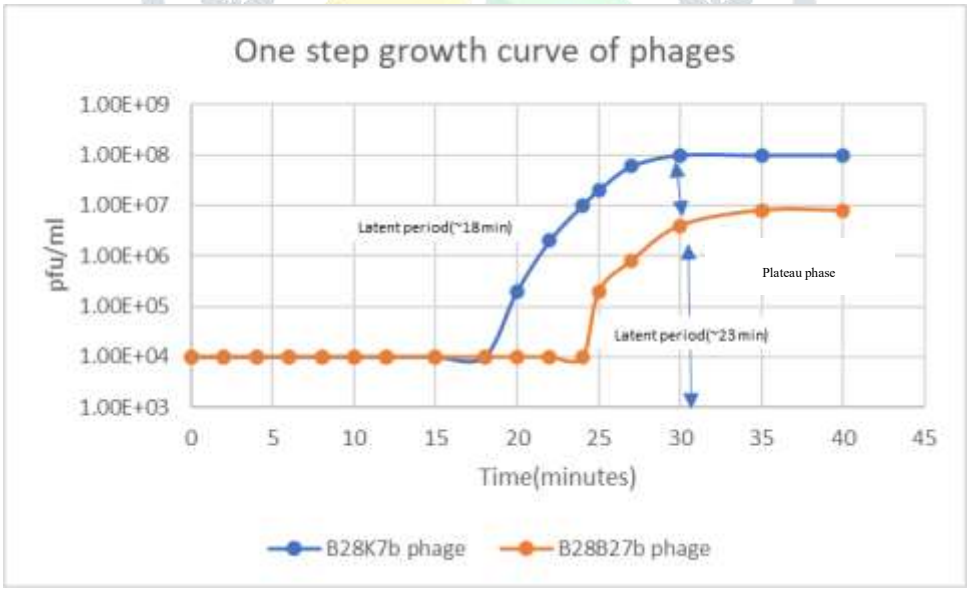


Figure 9*:* One-step growth curve of phage B28K7b and phage B28B27 against EcEIB and EcEIC

3.4 Pilot-scale study for effective bacterial reduction in graywater using selected phage cocktail.

The test systems (3000 mL graywater + phage cocktail at MOI 2) and a control (3000 mL graywater only) were set up .From 1.3 × 10^3 CFU/mL initial load, phage treatment reduced *E. coli* to 1.0× 10^1 CFU/mL (2.1-log reduction), and total heterotropic count was reduced to 2.0x10^3 CFU/mL from initial count of 2.5 × 10^5 CFU/mL, significantly outperforming controls within 6–9 h. (Figure 10). pH and turbidity of the graywater were also studied, post sedimentation process, and it was noted that the pH of graywater was in the range of 7.4±0.3 and turbidity 15 ± 3 NTU. After the treatment process pH of graywater was in the range

of 7.0±0.3, and the turbidity was 5 ± 5 NTU. This shows treatment doesn't alter pH significantly while turbidity drops alongside bacterial reductions. Pilot reduction studies were carried out in triplicate.

Mean obtained in this study was 1.65±0.05(Mean ± SD; $p = 0.0003$) for total heterotrophic count and 2.01±0.03 (Mean ± SD; $p = 0.0002$) for *E.coli* with the effluent graywater system. These results meet CPCB standards for non-potable reuse and are also in accordance with USEPA, demonstrating that phage biocontrol effectively bridges the gap between raw graywater and safe reuse quality. Although the log reduction in the laboratory environment is smaller, given the number and load of microorganisms in graywater, it laid the scope for further analysis in this direction.

Prior studies (Ottoson et al. 2023) reported that with electron beam irradiation was able to proceed with 8 log reduction in wastewater, whereas (Jofre et al. 2016) suggested that the treatment efficiency of a similar system was less than 1 log reduction in bacteria. These findings highlight the rapid and effective lytic action of the phage treatment under simulated and real graywater conditions. In a multi-barrier approach, phage treatment is complemented by pre treatment of coarse filtration and followed by terminal disinfection (chlorination at 0.5–1 mg/L achieving >5-log total bacterial reduction to meet non-potable graywater reuse standards(CPCB and USEPA for urban irrigation).

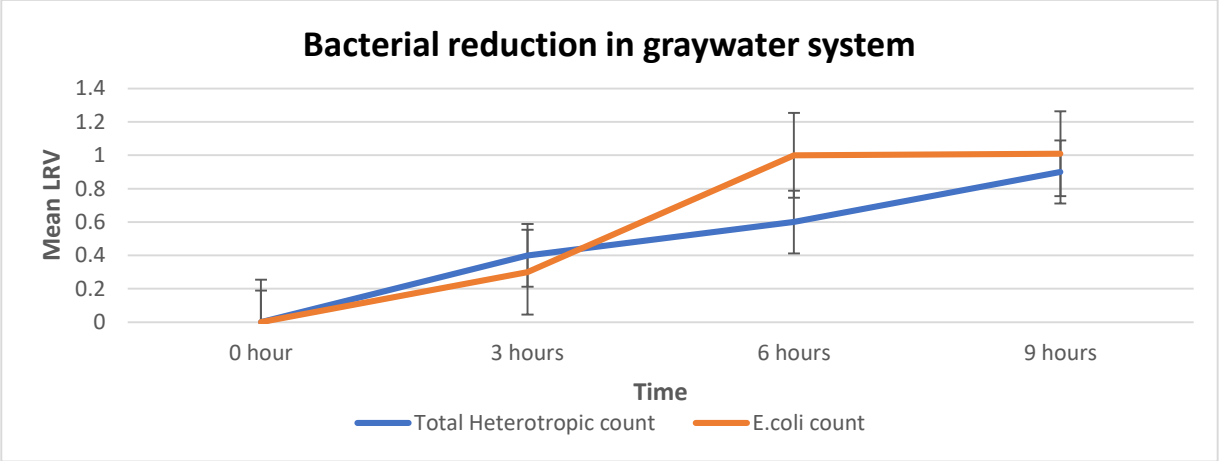


Figure 10 :Log reduction value(LRV) of total heterotrophic organisms and E.coli by phage cocktail in graywater.

CONCLUSION

This pilot-scale study demonstrates bacteriophages as an effective tool for the reduction of enteric bacteria, particularly *Escherichia coli* and *Enterococcus faecalis*, in domestic graywater. Seasonal variation in microbial load highlights the critical role of environmental factors in developing graywater treatment system. The isolation and characterisation of distinct phages, particularly B28K7b and B28B27b, demonstrated strong lytic activity and stability under various environmental conditions. The log reduction value (LRV) using phage cocktail in the present study demonstrated a 2-log reduction within 6–9 hrs at an effective MOI of 2, in graywater systems. Transmission Electron Microscopy (TEM) confirmed their classification within the *Myoviridae* and *Siphoviridae* family of order Caudovirales. The research supported phage-based treatment as a sustainable, low-impact alternative for enhancing graywater quality and promoting safe non-potable reuse. This kind of study helps to determine that coliphages can serve as reliable microbial indicators in the context of domestic graywater treatment and reuse. It supports public health risk assessment and helps to optimise low-cost water recycling technologies, especially in water-scarce regions.

Pilot-scale validation confirms phage biocontrol scalability. 2-log *E. coli* reduction within CPCB limits complements chlorination for 6-log total safety. Phage treatment offers sustainable graywater reuse (toilet flushing, irrigation) in water-scarce regions. Although this study demonstrates the lytic potential of isolated phages, safety and regulatory aspects relevant to reuse applications must be considered. The potential impacts on downstream water quality were not assessed and represent limitations of the present study. Future work requires molecular detection methods such as whole-genome sequencing, water quality assessment and regulatory validation.

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