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ORIGINAL RESEARCH

Antibiofilm Activity of Bacteriophage Isolated from Sewage-Polluted Water against *Escherichia coli*

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ABSTRACT

Objectives: This study aimed to explore bacteriophages, viruses that infect bacteria, as an alternative antibiofilm agent.

Methods: A laboratory-based, cross-sectional study was conducted at the Sainik Awasiya Mahavidyalaya Laboratory from February to July 2025. The water samples that were contaminated with effluents were collected from eight rivers and ponds across the Kathmandu valley. *E. coli* isolates were used as the host strain after being confirmed by biochemical tests. Phages were isolated and enriched from wastewater using centrifugation, filtration, and multiple cycles of incubation with log-phage host bacteria to gain high titres. The plaque assay, host range by spot assay, and Efficacy of Plating (EOP) were performed. Antibiofilm activity was evaluated using the microtiter plate crystal violet assay. The study compared biofilm formation in the isolated *E. coli* (E.C 8) with that of the standard *E. coli* (ATCC 8739). Statistical significance was determined using the t-test ($p \leq 0.05$).

Results: Bacteriophages were found in six of eight samples. Only Mulpani had a lytic phage with a titre of 7.5 PFU/ml, which was used for further testing. The phage exhibited moderate EOP, ranging from 0.28 to 0.60, and a moderate host range. The isolated phage showed selected antibiofilm activity, as it effectively reduced the biofilm of the isolated *E. coli* (22.8%).

Conclusion: This emphasizes the ability of lytic phages as antibiofilm agents.

Keywords: Effluent, Antibiotic Resistance, Biofilm, Extracellular Polymeric Substances, Bacteriophage

INTRODUCTION

Bacterial biofilms are a major contributor to antibiotic resistance, posing a major health concern. Biofilms are found in environmental settings, processing facilities, industrial settings, hospital settings, and natural environments. Most bacteria produce biofilms (Zhao et al., 2023). Biofilm is the accumulation of eukaryotic or prokaryotic cells, surrounded by the matrix of extracellular polymeric substances (EPS). EPS consists of a long chain of sugars, DNA, and other biological molecules (Harper et al., 2014). Biofilm formation is a five-stage developmental cycle determined by biochemical and mechanical adaptations. The first step is the initial attachment of planktonic cells to a surface, followed by adhesion and aggregation, which establishes the irreversible connection. Then, micro-colonies formation occurs by cell division and multiplication, causing the maturation stage where EPS matrix is produced, and structure is finalized via signalling. The cycle completes with dispersion, where cells are released into the environment to colonize new sites (Azeem et al., 2025). The biofilm matrix forms resistance to agents like antibiotics by creating impermeable barriers, which is the major cause of

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antibiotic resistance (Harper et al., 2014).

Antimicrobial resistance (AMR) currently causes the death of 700,000 deaths annually and is expected to increase to 10 million by 2050 (WHO, 2025). This growing challenge has driven interest in identifying alternative antimicrobial strategies. Bacteriophages show promising potential as antibiofilm agents. They are the viruses that affect the bacteria. They might be lysogenic phages, which coexist with hosts by inserting themselves into a bacterial genome, or lytic phages, which destroy themselves by replicating inside their hosts and releasing new phages to infect more bacteria (Harper et al., 2014). They produce enzymes such as depolymerase and lysin that break down EPS, disrupting the biofilms more effectively than the conventional antibiotics (Wiguna et al., 2022).

They can be used as biotechnological tools to combat harmful bacteria, including MDR organisms, *Shigella* spp, *Salmonella* spp, *Escherichia coli, Staphylococcus* spp, and *Proteus* spp. Because of their ability to infiltrate and destroy bacterial cells, lytic bacteriophages are primarily employed in phage therapy (Rogovski et al., 2021). Despite growing interest and its urgency, a significant gap in research and development. The challenge of effectively targeting mature and multispecies biofilms in common clinical and environmental settings remains largely unaddressed. The interaction between the phage and biofilm matrix is not understood. This gap limits the translation of phage therapy for biofilm-associated infections (Harper et al., 2014). Therefore, this study aimed to isolate the bacteriophages from river and pond waters collected from around Kathmandu Valley and evaluate their antibiofilm activity, exploring their potential to disrupt biofilms as an alternative to antibiotics.

METHODS

Study Design

The study was a laboratory-based, cross-sectional, analytical study. The numerous rivers and ponds across the Kathmandu valley, polluted with household and industrial effluents, served as the study location and were the sites of the sample collection. The water samples were collected from Jhaukhel, Siddhapokhari, Jorpati, Bramayani, Basbari, Kasan, Guhesowori, and Mulpani.

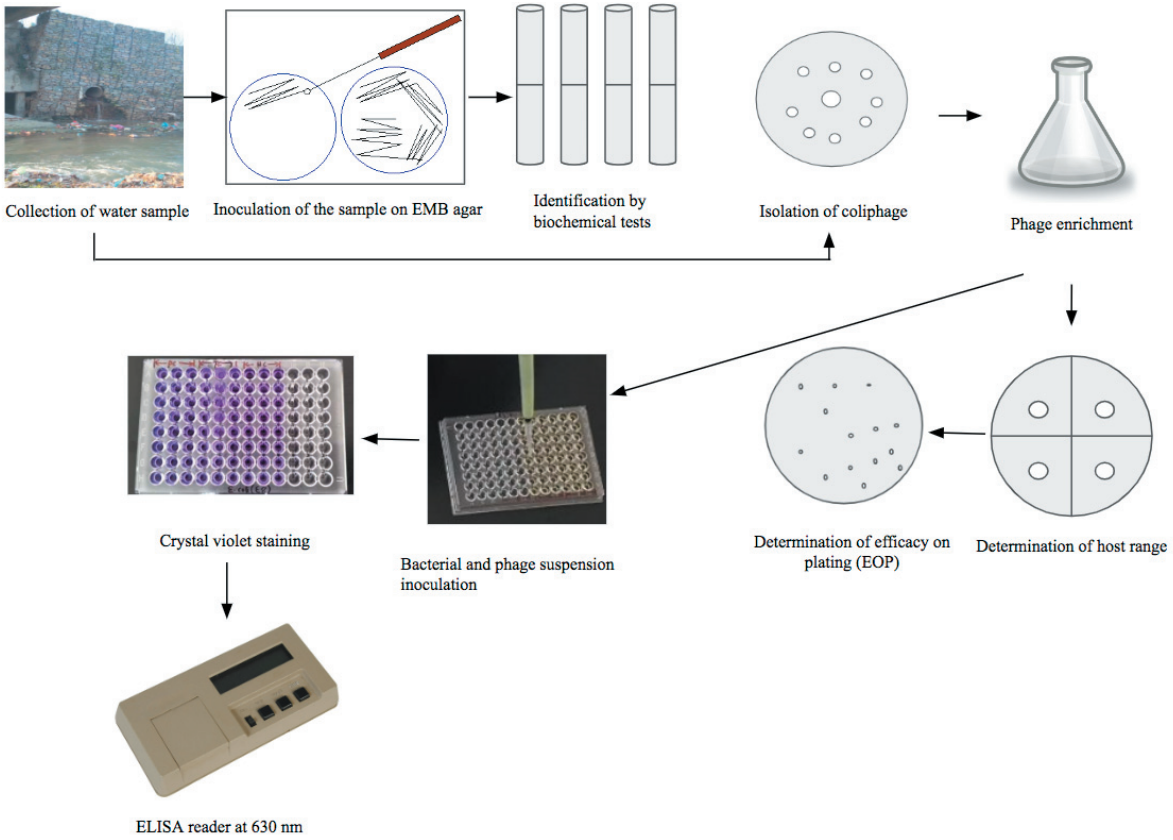


Figure 1: Showing the detailed method of phage screening and Antibiofilm Activity

Sample Collection

The contaminated waters were directly collected in plastic or glass containers. The sample bottles were closed until the time of the collection. While collecting the sample, the bottle was inserted into the water with its neck slightly below the surface and tilted upward. The opening was pointed in the direction of the water flow. The sample was stored at 4°C (US EPA, 2015).

Bacterial Isolation

Bacterial isolates were obtained from the processed polluted water sample in the laboratory. One ml of each sample was inoculated into the Nutrient Broth (NB) and incubated at 37°C for 24 hours. A loopful of enriched culture was inoculated on Eosin Blue Methylene (EMB) using the quadrant streaking technique and incubated at 37°C for 24 hours. Colonies exhibiting a distinctive metallic sheen were selected and subjected to a series of biochemical tests to confirm the presence of the bacteria (Fonteh, 2015). The isolated and biochemically identified *E. coli* was used as the host strain for the phage isolation (APHA, 1998; Cheesebrough, 2006).

Three clinical isolates and one from poultry faeces, available in the Sainik Awasiya Mahavidyalaya Microbiology laboratory, were also biochemically tested, which confirmed to be *E. coli* (APHA, 1998; Cheesebrough, 2006).

Plaque Assay

The samples were centrifuged at 8000 rpm for 10 minutes and followed by syringe filtration with a 0.22 µm filter to collect the supernatant (Wang et al., 2024). To increase the phage concentration, 20 ml of phage solution was added to 100ml of log-phase host bacteria. The mixture was incubated at 37°C in the shaking incubator at 200 rpm for 10 hours. Following incubation, it was centrifuged at 12000 rpm for 5 minutes to collect the supernatant, which was then filtered through a 0.2 µm syringe filter. The cycle was repeated three times to obtain a high-titre phage preparation. Phage detection was carried out using a plaque assay. The 50 µl host bacteria and 2 ml phage titre were mixed with 4 ml of top agar prepared and poured onto double-layer plates. It was incubated at 37°C, and plaque formation was observed (Wang et al., 2024).

Elution of Bacteriophage from the Plaque

The double-layer agar plates with plaques were chosen to recover bacteriophage. It was covered with a Salt of Magnesium (SM) buffer. After 30 minutes of incubation at room temperature, the top agar surface was scraped using the scraping tools. The top agar, along with the SM buffer, was collected in the sterile Falcon tube (Wang et al., 2024). Vortexing was used to mix it. The phage lysate suspension was centrifuged at 6000 rpm for 5 minutes. The supernatant was filtered through a 0.22µm filter and stored at 4ºC for further use (Phage Purification, 2023).

Phage Enrichment

The phage enrichment was performed with a slight modification of Quinones-Olvera. The 20µl bacteria were inoculated in the LB broth and incubated at 37ºC for 2 hours to obtain log phase bacteria. Subsequently, 2ml of phage suspension was added to it and incubated at 37ºC overnight for enrichment. After incubation, it was centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant was syringe filtered without disturbing the pellets, and the filtrate was transformed into the pre-labelled sterilised tubes. The enriched samples were stored at 4°C for further tests (Quinones-Olvera, 2023).

Determination of Host Range

For determining the host range, spot assay was performed using different bacterial strains, i.e., host bacteria (environmental isolates and clinical isolates) and reference bacteria *E. coli* (ATCC 8739 and ATCC 35218). The lawn was made on the LB agar using sterile cotton after being moistened with broth culture. In the marked area, 5 µl of each phage was spotted and allowed to dry before incubation at 37°C for 18-24 hours. The lysis zones were observed areas of the plates to note the effectiveness of the phage (Bhetwal et al., 2017).

Efficiency of Plating (EOP)

To assess the EOP, the plaque assay was conducted with a few modifications. The two sets of phage bacteria mixtures were prepared by combining 50µl of phage stocks with either 50µl of bacteria culture, i.e., host bacteria and reference bacteria *E. coli* (ATCC 8739), respectively, and incubated at room temperature for 10 minutes. The mixture was added to 4ml of soft agar and vortexed for a short period of time. The soft agar, maintained at around 50°C, was poured onto the bottom agar plates and allowed to solidify at room temperature. It was incubated at 37°C for 24 hours. Zone of lysis was observed, the number of plaques was counted, and EOP was calculated (Khan Mirzaei

& Nilsson, 2015).

It is calculated as the ratio of PFU/ml of test host to PFU/ml of reference host. EOP values were interpreted as >0.5 (high efficiency), 0.1-0.5 (moderate), 0.001-0.1 (low), <0.001 (no infection) (Khan Mirzaei & Nilsson, 2015).

Antibiofilm Activity of Phage

The biofilm-forming capacity of the host bacteria strain was tested in triplicate using the crystal violet assay with a minor modification. The test was done using the Microtiter Plate method. One set of the test contained three controls, viz., positive control, negative control, and phage-treated. The positive control test tube contained only bacterial isolates, the negative control test tube contained only LB broth, and the treated test tube contained bacterial isolates with phage. Three to five similar colonies were sub-cultured in 5 ml LB broth and incubated for 24 hours. After vortexing, 2 µl of the suspension was inoculated into 200 µl LB broth. For the phage activity, 2 µl bacterial suspension with 50 µl stock phage suspension were inoculated in 150 µl LB broth. They were aerobically incubated at 37°C for 24 hours ± 30 minutes. After washing each tube with 300 µl sterile saline, they were heat-fixed at 60°C for 1 hour. Staining was performed with 200 µl 0.1% crystal violet for 15 minutes, followed by washing with tap water and air drying. After homogenous resolubilization of the dye with 1 ml of 95% ethanol, the optical density (OD) of the microtiter plate was measured at 630 nm using an ELISA reader (Plota et al., 2021). The same set of tests was performed for *E. coli* ATCC 8739.

Statistical Analysis

The statistical analysis was performed using Excel. To interpret the effectiveness, the Mean OD, Standard Deviation, and % biofilm inhibition were calculated. Two-sample t-test was performed to compare the dependent variable, Mean OD, between two specific groups (the independent variable). Group 1 (control) was the mean OD of the *E. coli* without phage, and group 2 (treatment) was the mean OD of the *E. coli* with phage. The values were interpreted as "Significant" or "Not Significant" by comparing the p-value, such that if the p-value ≤ 0.05, the value is significant, otherwise not significant.

RESULTS

The *E. coli* (E.C 8) that was isolated from sample no. 8 was used as the host strain to isolate bacteriophage. Among the water samples collected from eight rivers and ponds, bacteriophage presence was detected in six samples. Notably, samples from Jorpati, Mulpani, and Siddhapokhari exhibited the highest plaque counts. No plaque formation was detected in samples from Jhaukhel and Bramayani, suggesting the absence of detectable phage.

The water samples collected from Mulpani exhibited lytic phage activity, forming 15 plaques with a calculated titer of 7.5 PFU/ml, as shown in *Table* 2. The plaques were spherical, smooth, transparent, colorless, and lytic in nature. In contrast, plaques observed from the remaining five sites were lysogenic in nature.

Table 1: Distribution of water samples used for screening of Coliphage

S.N.	Sample No.	Sample Site	Plaque Formation	No. of Plaque
1	E8	Mulpani	+	15
2	E7	Guheswori	+	7
3	E6	Kasan	+	10
4	E5	Basbari	+	13
5	E4	Bramayani	-	0
6	E3	Jorpati	+	30
7	E2	Siddhapokhari	+	18
8	E1	Jhaukhel	-	0

(+): Plaque formation (-): No plaque formation

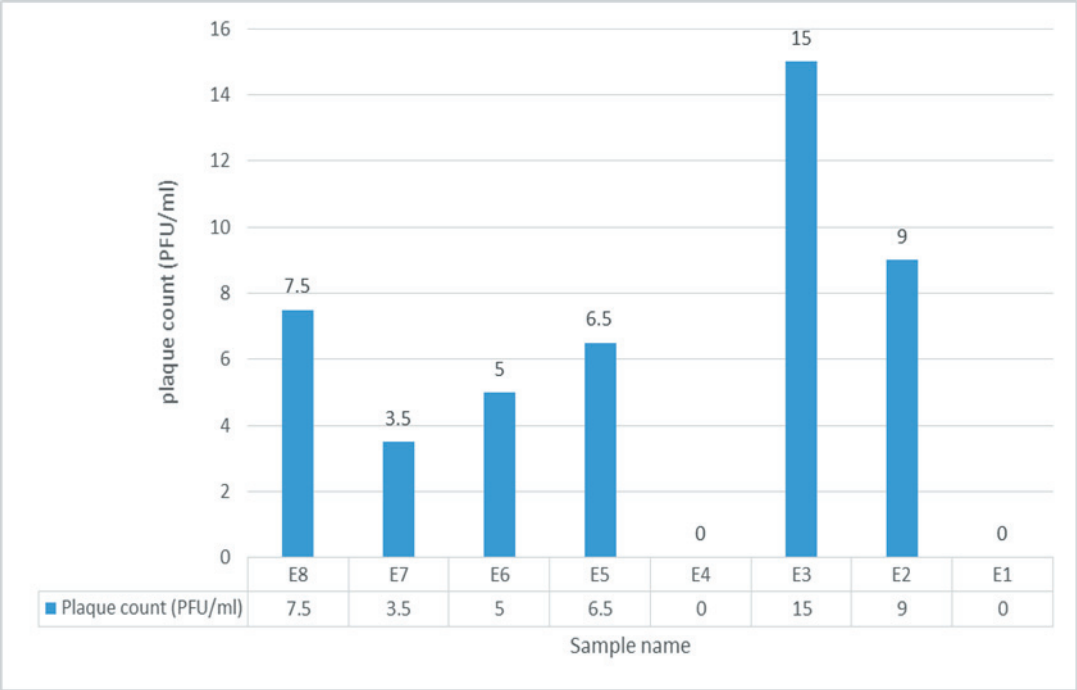


Figure 2: Bar Graph showing PFU/ml of the Phage Isolated from Different Sample Sites

Table 2: Plaque Morphology Table

SN	Sample	Name of Bacteria	Plaque Morphology					
			Size (in mm)	Shape	Margin	Opacity	Color	Remarks
1	E8	*E. coli*	3	Sphere	Smooth	Transparent	Colorless	Lytic
2	E7		1	Sphere	Smooth	Opaque	Colorless	Lysogenic
3	E6		3	Irregular	Smooth	Opaque	Colorless	Lysogenic
4	E5		5	Sphere	Smooth	Opaque	Colorless	Lysogenic
5	E4		-	-	-	-	-	-
6	E3		1	Sphere	Smooth	Opaque	Colorless	Lysogenic
7	E2		3	Irrigular	Smooth	Opaque	Colorless	Lysogenic
8	E1		-	-	-	-	-	-

(-): No plaque formation

The determination of the host range was performed via spot assay. Moderate lysis was observed on the isolated E. *coli* (E.C 8), but no zone was observed in E.C 2 by the coliphage E8 in Table 3. By comparing the EOP value (0.1≤ EOP ≤ 0.5), the strains showed a moderate host range on average. *Table 4* shows the host range of *E. coli* that were clinical and poultry fecal isolates via spot assay. Zone formation was not observed on any of the strains. Similarly, *Table 5* shows the host range of standard *E. coli*. ATCC 8739 showed a clear lysis, whereas ATCC 35218 didn't show zone formation.

Table 3: Determination of Host Range of *E. coli* (Environmental isolates)

SN	Method	Volume Spotted	Bacterial Strain (EC2)	Bacterial Strain (EC8)
1	Spot Assay	10µl	-	+
2			-	+
3			-	+
4			-	+
5			-	+
6			-	+

(++): Clear zone formation; (+): Moderate zone formation; (-): No zone formation

Table 4: Determination of Host Range of *E. coli* (Clinical and Poultry feces isolates)

SN	Method	Volume Spotted	Bacterial Strain (EC.I)	Bacterial Strain (EC.II)	Bacterial Strain (EC.III)	Bacterial Strain (EC.IV)
1	Spot Assay	10µl	-	-	-	-
2			-	-	-	-
3			-	-	-	-
4			-	-	-	-
5			-	-	-	-
6			-	-	-	-

(-): No zone formation

EC.I, EC.II and EC.III= Clinical isolate

EC.IV=Poultry faeces isolate

Table 5: Determination of Host Range of Standard *E. coli*

SN	Method	Volume Spotted	Bacterial Strain E.coli ATCC 8739	Bacterial Strain E.coli ATCC 3518
1	Spot Assay	10µl	++	-
2			++	-
3			++	-
4			++	-
5			++	-
6			++	-

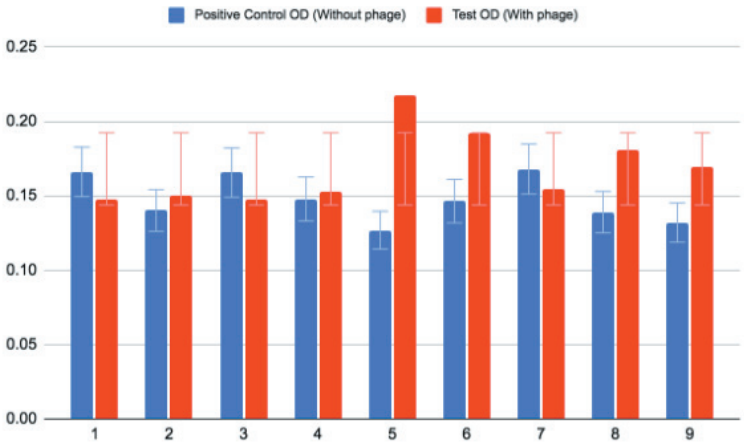
(++): Clear zone formation; (-): No zone formation

The EOP value ranged from 0.28 to 0.60, as shown in *Table 6*, indicating that the strain exhibits moderate phage infectivity related to the standard host.

Table 6: Efficacy of Plating (EOP) Coliphage against test isolates and ATCC reference strains.

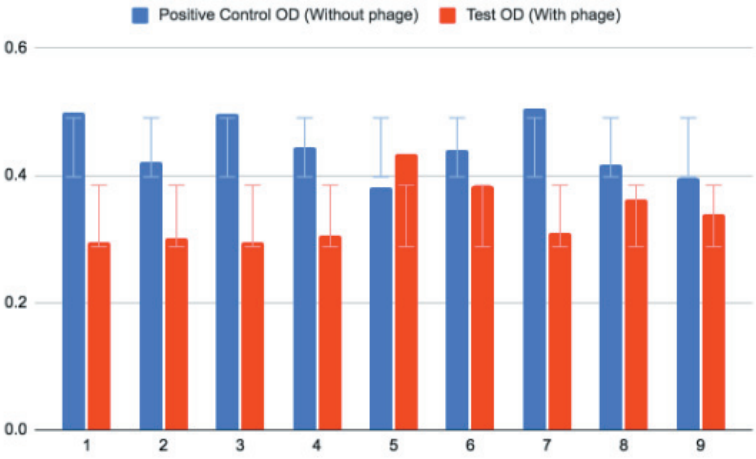
SN	Host Strain (Test Host)	Host Strain (Standard Host)	Titer (PFU/ml) of Test strain	Titer (PFU/ml) of Standard strain	EOP Value	Interpretation
1	*E. coli*	*E. coli* ATCC 8739	2000	6000	0.33	Moderate
2			1466.666	2466.66	0.59	High
3			1200	2000	0.60	High
4			2000	4400	0.45	Moderate
5			1333.33	4666.66	0.28	Moderate
6			1200	3200	0.37	Moderate

Antibiofilm activity of the bacteriophage was assessed using a microtiter plate reader. The mean Optical Density (OD) values for the positive control (untreated bacteria) and the test control (phage-treated bacteria) for the isolated and the standard *E. coli* were calculated. The respective mean OD of the isolated *E. coli* was found to be 0.444 and 0.336 (p-value < 0.05), whereas that of the standard *E. coli* was found to be 0.148 and 0.168 (p-value >0.05). These OD values were then used to calculate the percentage of biofilm inhibition. The outcome suggests that the bacteriophage has a reducing effect on biofilm formation by isolated *E. coli*, as indicated by an average inhibition percentage of 22.8%. However, the standard *E. coli* (ATCC 8739) displayed a negative inhibition value of -15.73%.



Positive Control OD (without phage)	0.1661	0.1401	0.1659	0.1479	0.1269	0.1464	0.168	0.139	0.132
Test OD (with phage)	0.1477	0.1505	0.1475	0.1531	0.2172	0.192	0.1545	0.1808	0.1698

(i)



Positive Control OD	0.4983	0.4203	0.4977	0.4437	0.3807	0.4392	0.5043	0.4176	0.3954
Test OD	0.2954	0.301	0.295	0.3062	0.4344	0.384	0.309	0.3616	0.3396

(ii)

Figure 3: Bar graph showing the antibiofilm activity of E8 against (i): *E. coli* ATCC 8739, and (ii): isolated *E. coli* by the microtiter plate method

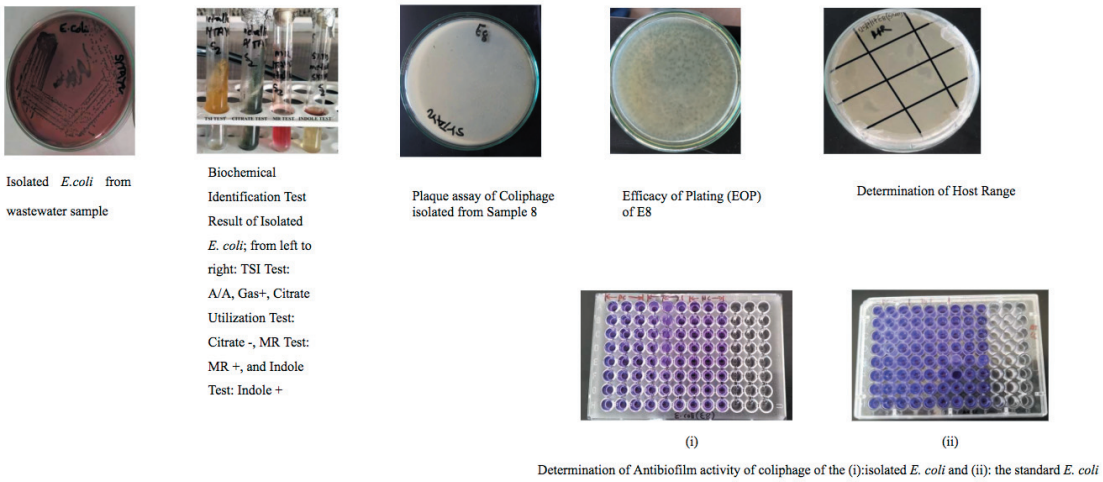


Figure 4: Photographs show the results of Coliphage isolates and Microtiter assay for Antibiofilm Activity

DISCUSSION

This study found the lytic bacteriophage was recovered only from Mulpani, which is because the relationship between phage and bacteria is host-dependent. This agrees with Ballesté et al., (2022), who noted that if the wastewater has a low population of bacteria or fewer phage-susceptible strains, the numbers of that phage can also be low or absent. The isolation of lytic bacteriophage from the wastewater was confirmed by the formation of round plaques, which are capable of infecting and lysing the host-specific bacteria immediately, consistent with the observation of Doekes et al., (2021). In a study done by Fathy et al., (2024) and Sivakumar et al., (2025), lytic phages were found in their respective wastewater samples, indicating that the wastewater acts as a reservoir of phages. The lysogenic phages should be excluded because of their low bactericidal effect (Gordillo & Barr, 2021). They can integrate their genome into a bacterial cell chromosome, which is replicated and passed on to the daughter cell without killing them (Kasman & Porter, 2022). This underscores that they are not useful as a biocontrol or therapeutic means as a lytic phage.

The spot assay of the phage showed a moderate host range and when compared with the average value of EOP (average EOP = 0.436), the strain was found to have moderate host range as well. The moderate host range was observed only in the isolated *E. coli* (E.C 8), while the clinical isolates and poultry faeces isolate, *E. coli*, had no host range, which shows that the coliphage had only intraspecific host range, but not interspecific. The EOP values in our study range from 0.28 to 0.6, which fall under the moderate range; they indicate reduced infectivity in the host strain compared to the standard strain. These values are lower in comparison to previous findings (1.2-1.4) by Sada & Tessema (2024). In the experiment conducted by Fathy et al., (2024), the isolated phages, particularly S3 and F3 coliphages, exhibited significant lytic potential against diverse *E. coli* strains of 66.6% and 41.6% respectively. Khan Mirzaei & Nilsson (2015) found that the bacterial strain in their study had high productive infection (EOP ≥ 0.5), which was lower than the results of the spot tests. They compared both the spot test result and EOP value for determining host range, as the spot assay is a qualitative test while EOP is a quantitative measure. In the study done by Bhetwal et al., (2017), almost all the coliphages showed very high host range against the *E. coli* strain, suggesting it is highly effective against a variety of *E. coli* strains. This finding is consistent with our result. Even the same bacterial strain can have a wide range in phage activity due to bacterial mutation, which causes the variation in Efficacy of Plating (EOP) levels (Bull et al., 2014).

The antibiofilm activity against isolated *E. coli* by the coliphage showed 22% biofilm inhibition. The biofilm's EPS consists of exopolysaccharide, extracellular DNA, and protein (Archell et al., 2025). Bacteria produce biofilms to protect themselves from external pressures (Archell et al., 2025). The bacteriophage encodes enzymes like lipase, depolymerase, or hydrolase to disrupt the EPS (Archell et al., 2025). These enzymes

are produced after bacteriophages infect bacterial cells within the biofilm, and when phages are released from the lysed bacteria, they degrade the biofilm (Archell et al., 2025). Our result can be supported by the experiment conducted by González-Gómez et al., (2021), which found that certain bacteriophages had an adhesion ability of 10% and 20%. They suggested that less biofilm inhibition may be due to EPS producing strains and a lower adhesion ability.

Our results on antibiofilm activity were consistent with Shivakumar et al., (2025) and Pérez et al., (2024). In the study conducted by Shivakumar et al., (2025), the phage was an efficient antibiofilm agent against *E. coli* (ATCC 25922), with 1.0 MOI, which was the optimal concentration for gaining maximum initial inhibition. The greatest dramatic change in biofilm-forming ability was seen in the phage-sensitive *E. coli* WG5, as reported by Pérez et al., (2024). *E. coli* WG5 showed a significant reduction in biofilm formation (53.8%, $p < 0.05$) at a phage titre of 105 PFU/mL, reaching 100% suppression when the cells were treated with 109 PFU/mL of SOM7 coliphage (Pérez et al., 2024).

In the study conducted by Bràs et al., (2024), a decrease in biofilm after phage treatment. Phage may be unable to inhibit the biofilm of the standard ATCC strain due to a stronger polysaccharide layer or the formation of more biofilm layers as a defence mechanism (Adeyemo et al., 2022). These findings align with those showing that standard *E. coli* ATCC 8739 exhibited -15.73% inhibition. The production of biofilm in the presence of bacteriophage has also been observed in the experiment done by Mangieri et al., (2021). They suggested that phage predation can be the cause of an increase in the biofilm levels in bacteria.

A limitation of the study is that only a few *E. coli* isolates and ATCC strains were used. This limits the ability to assess the phage's lytic range and specificity. The host range determination did not include the other members of Enterobacteriaceae, restricting the broader applicability of the phage. Further studies should include diverse bacterial isolates, multiple phage isolates and high-quality replicates to determine the phage lytic potential and bacterial host specificity.

CONCLUSION

In this study, lytic and lysogenic phages were isolated from the 8 wastewater samples; however, lytic phages were recovered only from Mulpani and were further processed for the study. This suggested a varying distribution of phages due to ecological and microbial factors such as the availability of specific hosts, pH, temperature, and nutrient levels. The antibiofilm activity of coliphage was studied by using a crystal violet assay in a 96-well microtiter plate. The coliphage showed selective efficacy, demonstrating a good biofilm inhibition against the isolated *E. coli* but no inhibition of biofilm in the standard *E. coli* (ATCC 8739).

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CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest regarding the publication of this paper.

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