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Isolation and Characterization of Bacteriophages Targeting Multidrug-Resistant *Escherichia coli* Isolated from Drinking Water Sources in Hyderabad, India

Keshapagu Sarita [1], Dr G Vikram[1]*, Seema[2], Dr Chand Pasha[2]

1. Department of Biotechnology, Bharatiya Engineering Science and Technology Innovation University (BESTIU), Gorantla, Andhra Pradesh, India
2. Department of Microbiology, Nizam College, Osmania University, Hyderabad. Telangana, India

Corresponding Authors**: Dr.G.Vikram**

Abstract: Problem: Microbial contamination of drinking water remains a significant global health challenge, particularly in developing countries like India, where multidrug-resistant (MDR) and biofilm-forming *Escherichia coli* persist despite conventional treatment methods, increasing the need for alternative, sustainable water-treatment solutions. **Approach:** Phages against MDR *E Coli* were isolated and purified through cesium chloride (CsCl) gradient centrifugation. Characterization included host inactivation of MDR *E. coli*, morphological examination using transmission electron microscopy (TEM), antibiofilm assay, host range assays, burst size determination, and stability testing under varied pH, temperature, ionic, and dilution conditions. **Findings:** Lytic bacteriophages were successfully isolated in the plaque assay. TEM revealed icosahedral heads (105nm) and contractile tails (120nm), classifying them under the *Myoviridae* family. Phages showed a burst size of approximately 110PFU per cell. They exhibited broad host ranges, infecting and inactivating 87% of MDR *E. coli* isolates while showing no infectivity towards non-*E. coli* strains. Optimal stability was maintained between pH 5-9 and temperature 30-45^{0}C. Calcium ions (Ca^{2+}) enhanced phage adsorption between 1-10mM range, while chloride ions (Cl^-) exhibited a modest effect up to 5mM, and plaque numbers decreased proportionally with each tenfold dilution. Biofilm assays revealed significant degradation, showing 2.0, 2.8, and 3.5-fold reductions in biomass after 24, 48, and 72hrs. **Conclusion:** The isolated bacteriophages demonstrate effective and environmentally stable biocontrol agents having high lytic activity, host specificity, and significant antibiofilm activity against MDR *E. coli* in water systems.

Keywords: Bacteriophage, *Escherichia coli*, Multidrug resistance, Biofilm, Drinking water, Hyderabad, Phage therapy

Introduction:

Water quality remains a critical global public health concern, as microbial contamination poses a serious health risk globally, especially in developing countries such as India. Millions of individuals suffer annually from illnesses linked to unsafe drinking water, including diarrhea, typhoid, and hepatitis. According to the World Health Organization (2022), waterborne diseases account for more than

one million deaths each year, while two billion people worldwide still lack access to safely managed drinking water, predominantly affecting populations in low- and middle-income countries such as India(Centers for Disease Control and Prevention, 2025). *Escherichia coli*, a common indicator bacterium for fecal contamination in water, has become increasingly resistant to multiple antibiotics and complicating treatment efforts. Waterborne *E. coli* contamination poses major health risks due to increasing multidrug resistance and biofilm formation, which protect them from disinfection and contribute to their persistence in water systems (Saffar et al., 2020). Our previous work (Sarita K et al., 2025) reported high levels of MDR, biofilm-forming *E. coli* in Hyderabad's drinking water, accompanied by poor physiochemical water quality, including high biochemical oxygen demand and metal ion concentrations exceeding safety limits. These findings demonstrate the complexity of bacterial survival and resistance in contaminated waters, highlighting the need for enhanced water treatment solutions.

Conventional water treatment practices, including chlorination, filtration, and UV irradiation, often struggle to efficiently eliminate multidrug-resistant (MDR) bacterial strains, particularly those embedded in biofilms or exhibiting antibiotic resistance. These limitations underscore the critical need for innovative, sustainable methods to combat resistant pathogens in water systems. Bacteriophages are selective viruses that infect and lyse bacteria, offering a complementary strategy or sustainable, eco-friendly alternative for controlling resistant bacteria in water (Soliman et al., 2023). Bacteriophages exhibit exceptional host specificity, targeting bacterial pathogens with precision while leaving beneficial microbiota intact. This specificity arises from the phage's ability to recognize and bind to unique surface receptors on bacterial cells, initiating infection only in susceptible hosts (Mohaisen et al., 2023). Unlike broad-spectrum disinfectants that indiscriminately eliminate microbial populations, phages can selectively target problematic bacteria such as multidrug-resistant *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Aeromonas* species commonly found in contaminated water sources (Mathieu et al., 2019). One of the most compelling advantages of bacteriophages biocontrol is the ability to disrupt and degrade bacterial biofilms (Mayorga-Ramos et al., 2024; Łusiak-Szelachowska et al., 2020). Recent research has confirmed the potential of phage biocontrol in reducing bacterial contamination and high lytic activity against MDR strains isolated from wastewater and surface water. Additionally, bacteriophages have been shown to remain stable and effective under diverse environmental conditions encountered in water systems, including variations in pH, temperature, salinity, and inorganic matter content, making them suitable for real-world applications (Silva et al., 2014).

This study continues from our earlier investigation into *E. coli* contamination and antibiotic resistance in Hyderabad's drinking water sources (Sarita K et al., 2025),aiming to isolate and characterize phages that can specifically target these resistant *E. coli*.

Materials and Methods

E. coli Host Strains

Previously isolated multidrug-resistant *E. coli* strains from Hyderabad drinking water sources were utilized for bacteriophage isolation. The bacterial isolates exhibited resistance against multiple antibiotics and demonstrated bio-film-forming ability. The *E. coli* was sub-cultured on Luria Bertani Agar plates and maintained at 4°C.

Bacteriophage Isolation

Sewage water samples were collected from local municipal wastewater treatment plants and nearby water bodies in Hyderabad. Bacteriophages were isolated by the enrichment method, taking 4ml of 0.2μ filtered sample waters and 1ml of 10×Luria broth, incubated with 1ml of exponentially growing *E. coli* cultures for 24 hrs at 37°C. The mixtures were vortexed, then centrifuged at 15000 RPM for 5mins and the supernatant was filtered by a 0.2μm syringe filter to obtain crude phage filtrate.

The filtered 100μl of bacteriophage suspension and 100μl of pure actively growing *E. coli* culture was taken in a sterile Eppendorf, kept at 37°C for 15min, and then it was mixed with 5ml low-melting LB agar (0.8%) and poured onto a Luria agar plate. These double agar plates were allowed to solidify at room temperature, and then plates were incubated at 37°C for 24hrs.

Bacteriophages Purification

An isolated plaque was taken using a sterile scalpel from the double agar plate and mixed with 500μl of phage buffer. The diluted phage suspension was mixed with an actively growing *E. coli* culture and incubated. From this mixture, 100μl was mixed with 5ml low-melting LB agar (0.8%) and poured onto Luria agar plates and incubated at 37°C for 24hrs. The single and isolated plaques were selected and purified by repeated plaque isolation until homogenous plaques were obtained. Further, the phage lysate was centrifuged at 15000 RPM for 15 min and filtered to remove cell debris. Then CsCl solution was layered into an ultra centrifuge tube to form a density gradient. The phage sample was placed on top and centrifuged at high speed (100,000 RPM for 1 hr). The bacteriophage particles form a visible band at the equilibrium position. This band was carefully extracted using a fine syringe and dialyzed against SM buffer [50mM Tris-HCl, 8mM $MgSO_4$, 10mM $CaCl_2$, 100mM NaCl, pH 7.5,50% glycerol (v/v)]to remove the toxic CsCl. Later, these phages are stored at 4 °C using SM buffer (Sambrook et al., 1989).

Host Inactivation studies

An actively growing (16hrs) 100ml pure *E. coli* culture was taken in a flask and kept at 37°C for 24hrs. Then to the flask 10ml of 0.2μ filtered phages were added and incubated at 37°C at 100RPM. The Samples were collected at hourly intervals for a total duration of 8 hrs. These hourly samples were individually plated on Luria agar to check the viability of *E. coli*. The viable count in the hourly samples was recorded by using the colony counter (Multilab India). LD90 is measured as the time required to kill 90% of cells.

Burst size determination

The Burst size of phages was determined by the method of Evseev et al.(2024) took 1ml of exponentially growing *E. coli* culture and 1phage plaque into a sterile Eppendorf. The suspension was gently mixed and incubated for 20min at 37°C. After incubation, aliquots of 100µl were collected and used for phage assay by double agar overlaying assay. Burst size was determined by quantifying the number of phages released per infected cell. The plaques formed were multiplied with dilution factor to get burst size.

Morphological Characterization

Phage particles were visualized using transmission electron microscopy (TEM) to study morphology and classify phage families. A single drop of phage suspension was applied to a copper grid with a carbon coated Formvar film and incubated for 10 min at 25^0C. The phages were negatively stained with 2% phosphotungstic acid at pH 7 and air-dried overnight. Imaging was performed using a Zeiss TEM900 transmission electron microscope (Carl Zeiss AG) operated at 80-120 kV to determine shape, size, and tail structure. The phage was observed with the Image SP software and a CCD camera.

Biofilm degradation by bacteriophages

The bacterial biofilm was developed in 96-well plates for 24, 48, and 72hrs to determine bacteriophage effect on *E. coli* bacterial biofilm (Cerca et al., 2007). The *E. coli* culture was grown overnight in tryptone soya broth (TSB) medium (Tryptone-1.7%, Soytone-0.3%, Dextrose-0.25%, Nacl-0.5%, K2HPO4-0.25%) and was diluted in a 1:10 ratio. After preparing dilutions, bacterial cultures were added to 96-well plates and incubated at room temperature for 24 hrs with shaking at 120RPM. Later, the bacteriophages were added to half of the wells and, the other half wells were taken as a control. The plate was incubated in a shaking incubator (120RPM) at room temperature, and the phage titer was collected the after 24 hrs. The wells were then washed with distilled water, after which 1% crystal violet solution was added and allowed to stain for 15mins. Then the crystal violet solution was removed from the wells, followed by washing with distilled water to remove unbound stain. The plates were then inverted and allowed to dry. Later, the OD of plates was taken at 600nm, and the experiments were repeated with 48 and 72hrs time intervals (Adnan et al., 2020).

Host Range Determination

The modified spot test reported by Cobián Güemes et al.(2023) was used for host range determination. Purified phage suspensions were spotted against a panel of 15 multidrug-resistant *E. coli* isolates, along with *K. pneumonia ,Salmonella*, *Staphylococcus aureus* and *Campylobacter* isolates. 1mlof pure *E. coli*, *K. pneumonia, Salmonella*, *Staphylococcus aureus,* and *Campylobacter* isolates were spread on selective agar plates. A 50µlof phage suspension was dropped on the plates individually. These plates were incubated at 37°C for 24 hrs. Plates were observed for plaques. Efficiency of plaque formation (EOP) was calculated to quantify infection

efficiency, defined as the ratio of plaque-forming units on each isolate to that on the original host strain.

Stability Assays: Effect of Temperature and pH

Thermal and pH stability of isolated phages were evaluated by the method of Kola et al. (2023), by incubating phages at varying temperatures and pH values, followed by titer estimation. Heat tolerance was evaluated by taking 10,000PFU/ml phage filtrate in sterile Luria broth and incubating at temperatures ranging from 4°C to 60°C for 1hr. The pH tolerance was checked by treating phage filtrate in tubes containing Luria broth of pH 1 to 12, and incubating at 37°C for 1hr. Then these bacteriophages treated with different temperatures and pH were overlayed using a double-layered plaque assay.

Effect of Calcium and Chloride Ions

The effect of calcium and chloride ions on phages was evaluated by incubating phages at varying concentrations of calcium and chloride ion. Concentration of 0, 1, 2, 4, 6, 8, 10, 12, 14, 15mM Calcium chloride ($CaCl_2$)was taken in consecutive tubes containing sterile Luria broth with 10,000PFU/ml phage filtrate and incubated at 37°C for 1hr (Chen et al., 2019). Different concentration of Sodium hypochlorite (NaOCl) 0, 1, 2, 3, 4, 5, 6, 8, 10mM were taken in tubes containing sterile Luria broth with phage filtrate and incubated at 37°C for 1hr (Brié et al., 2018). The bacteriophages treated with different concentrations of calcium and chloride ions were overlayed on agar for plaque assay.

Effect of dilutions

The isolated phage's stability was evaluated by incubating samples at different dilutions. Serial dilution of phage filtrate (10,000PFU/ml)was carried out in tubes of dilution factors 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, till 10^{-10}. These different phage dilutions were poured on LA for plaque assay.

Lytic Activity Assays

Phage lytic activity was determined by phage assay and plates incubation with and without exposing to ultraviolet (UV) light. Phage suspensions were first mixed with an actively growing E*. coli* culture and overlayed on two different plates using the double agar assay method. One agar plate was exposed to UV light for 30sec while the second plate was kept unexposed. Following treatment, both plates were incubated for 24hrs at 37°C. After incubation, plaque formation on the UV-treated and untreated plates was examined.

Results

Isolation of bacteriophages

In the plaque assay, after incubation, many clear plaques were isolated from the sewage enrichment method. Bacteriophage plaques were enumerated and quantified as plaque-forming units (PFU). Plaque diameters ranged from 0.5 to 3 mm.

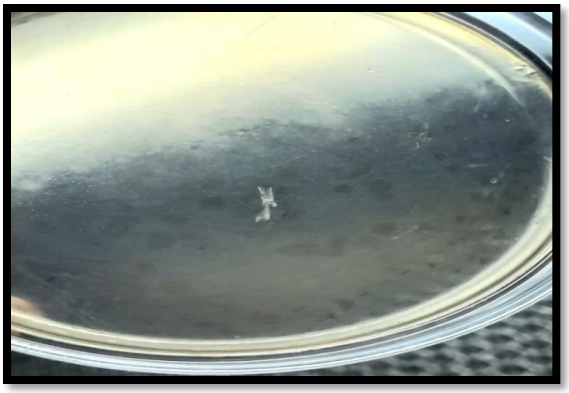

Fig. 1: Plaque assay

Bacteriophages Purification

The plaque was purified by repeated plaque isolation, followed by cesium chloride (CsCl) gradient centrifugation, and used in plaque assay with specificity to *E. coli*. The single plaque giving similar plaques was selected as the pure phage.

Host Inactivation Studies

The number of colonies in hourly samples was counted using the colony counter. The viable cell count was more till 1 hour, from the 2nd hour onward, a progressive decline in viability was observed. By the 5th hour, 90% of the initial *E. coli* cells were inactivated.

Burst size determination

The plaque exhibiting the largest burst size was identified as the bacteriophage with the highest effectiveness. The present phage has a burst size of 110PFU per cell.

Morphological Characterization

TEM analysis revealed phages belonging to the *Myoviridae* family with icosahedral heads (105 nm in diameter) and contractile tails (120 nm (Figure 2).

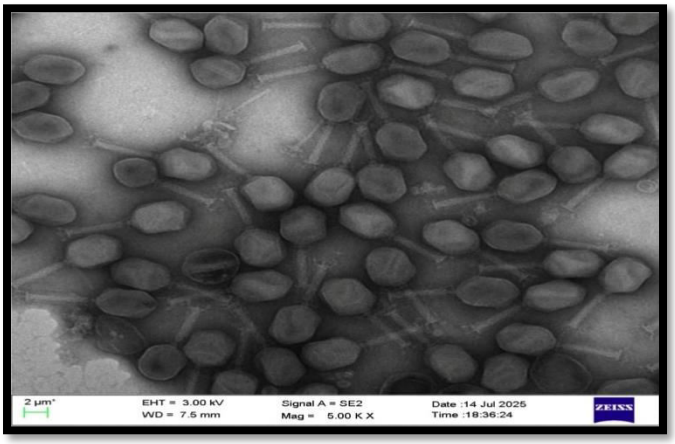

Fig. 2: Transmission electron microscopy (TEM) images of *E. coli* phage

Biofilm degradation by bacteriophages

Bacterial biofilm was degraded by bacteriophages. A significant reduction of 2.0-fold, 2.8-fold, and 3.5-fold in biofilm biomass of *E. coli* was observed for 24, 48, and 72hrs biofilms by phage in 24 hrs. A paired sample t-test revealed a significant difference between the treated biofilm and the control (phage-free).

Host Range Determination

Phages exhibited high efficiency in infecting 87% (13 of 15)of tested multidrug-resistant *E. coli* strains. The phages didn't infect *K. pneumonia, Salmonella*, *S. aureus,* and *Campylobacter* isolates

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Stability Assays

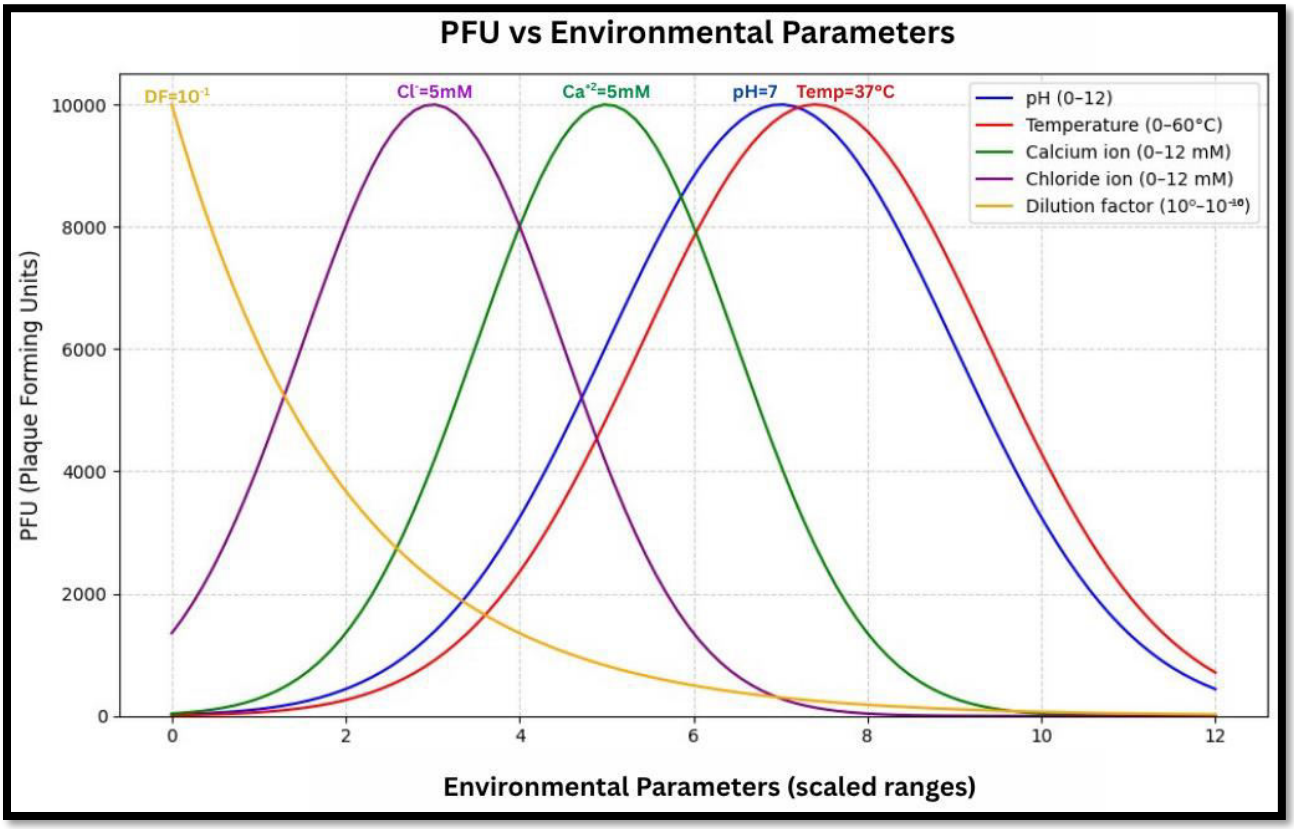

Fig. 3: of Graphical Representation PFU vs Environmental Parameters

Phages retained >80% of the initial titer after 1hr incubation at temperatures up to 45°C; viability declined sharply above 50°C. Optimal pH was found between pH 5 and pH 9, and reduced phage titers at acidic (pH 3) and alkaline conditions (pH 11).

Calcium ions (Ca^{+2}) enhance phage adsorption at concentrations ranging from 1-10mM: at a 5 mM concentration, the plaque-forming unit counts were observed to be maximum, allowing optimal phage-host binding, indicating maximum infection

efficiency. A slight decline in PFU counts were observed at concentrations >5mM. A gradual decline in the PFU was observed at calcium ion concentrations exceeding 10mM,inactivating the phage particles, causing an ionic imbalance.

Chloride ions (Cl^-) has a modest effect on phage till 5mM concentration, the plaque-forming unit counts were observed as a control. A drastic decrease in PFU count was observed at concentrations exceeding 5mM.

In the phage dilution assay, a proportional drop in PFU count was observed with each successive tenfold dilution. The highest PFU count occurred at the 10^{-1} dilution, where plaques were abundant and well distributed across the plate. As the dilution factor increased from 10^{-2} to $10^{-5,}$ a gradual and more than consistent reduction in plaque numbers was observed.

Lytic Activity assay

The assay confirmed that the isolated phages undergo a lytic replication cycle. The same number of plaques was observed on both plates, indicating that these bacteriophages are strictly lytic, not lysogenic.

Discussion

This study reports the isolation and characterization of bacteriophages specific to multi-drug-resistant (MDR) *E.Coli* strains isolated from water samples (Sarita et al., 2025). The genetic and phenotypic diversity of *E. coli* strains, driven by mutation and horizontal gene transfer, significantly impacts bacteriophage susceptibility and requires phage efficacy testing across multiple isolates. Variations in IMViC profiles and antimicrobial resistance patterns reflect the genetic heterogeneity of environmental *E. coli* populations, which influences phage receptor accessibility and infection outcomes (Labrie et al., 2010). Despite this strain variability, the isolated phages exhibited a broad host range, which supports their potential application against heterogeneous bacterial populations.

The objective of this study was to isolate bacteriophages effective against MDR *E. coli* strains from sewage wastewater samples, as this source has consistently yielded effective phages in numerous previous studies (Chakraborty et al., 2018; Korf et al., 2019). The isolated bacteriophages showed consistent infectivity across the multiple tested MDR *E. coli* strains. Alexyuk et al. (2022) successfully isolated six lytic *E. coli* bacteriophages from wastewater samples collected from sewage systems in Kazakhstan, demonstrating that these phages could effectively eliminate various clinical *E.coli* strains within 4hrs at extremely low multiplicities of infection (MOI 10^{-7} to 10^{-8}). This observation highlights the potential applicability of these phages as biocontrol agents against genetically diverse *E..coli* populations.

Morphological examination by transmission electron microscopy (TEM) revealed that the isolated phages belong to the *Myoviridae* family, having icosahedral heads of ~105nm diameter and tails that are contractile with ~120nm length. Similarly, Fikadu et al. (2024) reported that phage VBO-*E. coli*4307 belongs to *Myovoridae,*exhibiting a typical icosahedral head (60-90nm diameter) and contractile tail(100-150nmlength). The burst size and lytic replication kinetics are critical determinants of bacteriophages' potential efficacy in biocontrol or

therapeutic applications targeting MDR *E. coli isolates*. The isolated bacteriophage in this study produced a burst size of 110(PFU) per cell. Similarly, Alexyuk et al. (2022) reported *E. coli* bacteriophages showed an average burst size of 117 PFU per cell collected from wastewater. Host inactivation studies assess the dynamics of bacterial population reduction following phage infection under controlled conditions, providing critical data on phage lytic efficiency and therapeutic potential. Costa et al. (2025) reported that phage cocktails effectively reduced bacterial loads of their *E. coli* hosts by 99.98 to 99.9997% in bacterial viability. In this study, viable *E. coli* cell counts remained relatively stable during the first hour, from 2nd hour a gradual decline in viability was observed, and 90% of the initial cells were inactivated by the fifth hour. Host range is a critical step in phage characterization, defining the spectrum of bacterial strains susceptible to productive infection and guiding the selection of phages suitable for biocontrol applications. The isolated phage exhibited broad host ranges, infecting and lyze 87% of the tested MDR *E. coli* isolates and did not infect *K. pneumonia, Salmonella*, *Staphylococcus aureus,* and *Campylobacter* isolates. Fikadu et al. (2024) reported phage VBO-*E. coli* 4307 and VBW-*E. coli* 4194 showed 50% lysis of the MDR *E. coli* clinical strains and not infected non-*E. coli* species (*K. pneumoniae, S.aureus, P. aeruginosa*).

Bacteriophages showed promising anti biofilm efficacy, targeting MDR *E. coli* strains. Bacterial biofilms were effectively degraded by bacteriophages in 24 hrs with significant reductions in *E. coli* biofilm biomass of 2.0, 2.8, and 3.5-fold in 24, 48, and 72 hrs, respectively. These findings are consistent with numerous studies demonstrating the antibiofilm efficacy of *E. coli* phages. Adnan et al. (2020) reported that 6 hrs of treatment with bacteriophage MA-1 for 24, 48, and 72 hrs significantly reduced 2.0-fold, 2.5-fold, and 3.2-fold in biofilm biomass of MDR *P.aeruginosa* were observed. Gu et al. (2019) found that phage vB_EcoP-EG1 significantly reduced the biomass of the *E. coli* strain MG1655 biofilm and clinical strain 390G7 compared to the control, with moderate reductions of 60% and 50%, after 24hrs of treatment. Linkages between biofilm formation and antimicrobial resistance underscore the importance of developing integrated phage-biofilm disruption approaches to improve lytic effectiveness (Fu et al., 2010).

Environmental tolerance assays demonstrated the stability of the isolated bacteriophages across a spectrum of physicochemical conditions, including fluctuations in pH, temperature, calcium, and chloride ion concentrations, and serial dilutions typically encountered in local aquatic environments. The isolated bacteriophages exhibited optimal stability and infectivity around neutral to mildly alkaline pH (5-9)and at temperature between 30°C to 45°C, with a decline in titer observed beyond these ranges, highlighting the importance of maintaining favorable physicochemical conditions for maximum biocontrol efficacy. Hu et al. (2024) reported that phage PGX1 targeting multidrug-resistant entero toxigenic *E. coli* demonstrated strong stability across pH (4-10) and temperature between 4 °C to 40°C. Calcium ions showed a dose-dependent reduction in phage titer above 10mM, suggesting that ionic interactions influence *E. coli* phage structural integrity and adsorption dynamics. Nawaz et al. (2024) found that calcium chloride shortened the absorption time and latency period of *E. coli* phage ES10 and significantly inhibited

biofilm formation of host bacteria, a significant increase in phage titer of approximately 2 log units(p<0.05) was recorded on exposure to 5 mM CaCl2. In another experiment, Chhibber et al. (2014) reported that calcium ions of 5mM final concentration were supplemented on agar, gradually increasing phage titer for a broad-spectrum MRSA bacteriophage, with the exposure to calcium ions increasing the rate of adsorption and participating in the process of phage genome penetration into the cytoplasm of the host. Chloride ions exhibited phage reduction above 5mM concentrations. Briéet al. (2018) investigated the impact of free chlorine on MS2 phage, demonstrating that chlorine exposure led to modifications in charge density and potential alterations in capsid protein structure. A more than proportional drop in PFU count with each tenfold dilution was recorded in this study, confirming the loss of phage structure at higher dilutions. Glonti et al. (2022) stated that the agar plate was spread with clear individual plaques, particularly in high dilutions, while lower dilutions may not show the expected results, demonstrating that the phage particles remain viable and infectious across the dilution series, which is essential for reliable phage quantification and characterization. Such environmental adaptations must be systematically considered during the design and implementation of bacteriophage-based applications to preserve phage infectivity, stability, and therapeutic efficacy across heterogeneous and potentially fluctuating environmental conditions.

Conclusion

This study demonstrated the successful isolation and characterization of bacteriophages having strong Lytic and anti biofilm potential against MDR *E. coli* strains from sewage waste water, underscoring their potential as a sustainable alternative and environmentally stable biocontrol agents. Overall, these findings support the integration of phage-based approaches into water treatment systems to reduce microbial contamination, antibiotic resistance dissemination, and biofilm persistence.

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