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Research Article

Application of Bacteriophages as Biological Control Agents for *Escherichia coli* in Milk

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ABSTRACT

Bacteriophages have attracted attention due to their unique abilities in controlling microbial contamination and maintaining the quality of food products. The stability of phages against changes in pH and temperature plays a significant role in their applications in the food industry and public health. This study evaluates the pH and thermal stability of bacteriophage PhE8 and its role in reducing the microbial load in milk contaminated with *Escherichia coli* at two temperatures of 4°C and 25°C. The stability of bacteriophage PhE8 against pH and heat was measured. The activity of the bacteriophages was assessed using Plaque Forming Unit (PFU). To investigate the antimicrobial activity of PhE8, 5 ml of pasteurized milk was treated with 100 µl of *E.coli* (E8) bacteria along with 100 µl of phage suspension. The samples were stored for four days at 4°C and 25°C. Electron microscopy revealed that the isolated phage has a polyhedral head and a contractile tail. The latent period was 10 minutes. Phage PhE8 was stable within the pH range of 4 to 12 and remained stable at temperatures between 30°C and 60°C for 30 minutes. The studied phage demonstrated high potential in reducing the microbial load in milk at 4°C. The high stability of the phage at normal pH and its significant decrease in activity at high temperatures provide useful information for the practical use of phages in real-world conditions.

1. Introduction

Bacteriophages have been used for nearly a century to treat bacterial infections. With the discovery of antibiotics, the use of phages declined, but with the rise in antibiotic-resistant bacteria, their application has regained attention (Alisky et al., 1998). In recent years, the increasing prevalence of foodborne bacterial diseases, the emergence of new pathogenic bacteria, and the high resistance of pathogenic bacteria to conventional disinfectants have led researchers to extensively focus on phages as biological control agents for eliminating bacterial pathogens in the food industry (García et al., 2007). Although bacteriophages can halt the lactose fermentation process by starter cultures in the dairy industry, causing delays in production and changes in product quality, there has been a growing interest in using

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bacteriophages to control bacterial pathogens in dairy products in recent years (Brüssow et al., 2001). For instance, bacteriophages have been employed to control *Listeria monocytogenes* in chocolate-flavored milk and *Staphylococcus aureus* in both pasteurized and raw milk (Kim et al., 2007; O'flaherty et al., 2005; García et al., 2009). Infections caused by *E. coli* O157 are typically self-limiting, but certain toxin-producing strains can cause more severe symptoms and, in 10-15% of cases, lead to serious complications such as hemolytic uremic syndrome (Thung et al., 2017; Guh et al., 2010; De Schrijver et al., 2008; Ferguson et al., 2005). Phages, due to their unique characteristics like specific action, self-limiting replication, resistance systems, and the ability to induce gene mutations in bacteria, can be a viable alternative in inhibiting bacterial growth in food products (O'flynn et al., 2004; Oliver et al., 2009; Viazis et al., 2011). In recent years, the application of these viruses in treating bacterial infections (phage therapy) and enhancing food safety as bio preservatives in food products has gained widespread attention (García et al., 2007; Leotta et al., 2008; Raupach et al., 2005; Sakuma et al., 2006). This study aim to investigate the antimicrobial activity of bacteriophages isolated from sewage in controlling *E.coli* PTCC (1399) in milk.

2. Materials and Methods

2.1. Isolation of Bacterial Strains

To isolate E. coli from food samples, 25 grams of the food (e.g., raw chicken meat or kebab) are added to 225 milliliters of sterile peptone water to create a homogenized suspension. Using a sterile pipette, 1 milliliter of the appropriate dilution of this suspension is transferred to a sterile plate. Approximately 15 milliliters of molten culture medium at around 45°C is then poured into the plate and allowed to solidify. The plates are incubated at 37°C for 24 hours to allow bacterial growth. After incubation, distinct colonies are observed and streaked onto selective media like Violet Red Bile Glucose Agar (VRBGA) and Eosin Methylene Blue (EMB) agar. A single colony is selected from these plates for further biochemical confirmation tests to identify E. coli.. Additionally, *E.coli* PTCC(1399) was obtained from the Iranian Research Organization for Science and Technology (IROST) for evaluating the application of isolated bacteriophages in food products. Various biochemical and differential tests were conducted to confirm the bacterial species under study. The microbial susceptibility of the bacterial strains was tested using the Kirby-Bauer disk diffusion method according to 2022 version of CLSI. The antibiotic disks used (from Pad Tan Teb Company, Iran) included ampicillin (10 μg), gentamicin (10 μg), amikacin (15 μg), ceftazidime (30 μg), and cefixime (5 μg) to determine antimicrobial sensitivity (Thung et al., 2017).

2.2. Isolation and Purification of Bacteriophages

Twelve wastewater samples were collected from hospitals located in Mazandaran Province to isolate bacteriophages specific to the isolated bacteria,. The samples were kept refrigerated for 24 hours to allow impurities to settle (Raupach et al., 2005). The samples were then centrifuged in two stages at 6000 g for 20 minutes (Eppendorf-Germany). Next, 10 milliliters of the filtered liquid, along with 5 milliliters of the bacterial sample in the mid-log phase, were added to 10 milliliters of Tryptic Soy Agar (Merck Germany). The mixture was incubated at 37°C for 24 hours (Pars Azma-Iran). The supernatant was carefully extracted using a sterile syringe and filtered through 0.22-micrometer filters. To further purify the bacteriophages, the double-layer agar method was employed. Initially, 100 microliters of the bacterial suspension along with 100 microliters of bacteriophage were added to semi-solid agar and then poured onto a solidified second layer containing Tryptic Soy Agar (Merck Germany) (Rasool et al., 2016; Thung et al., 2017).

2.3. Determination of Phage Lytic Range

The lytic activity of the isolated phage was assessed using the Spot Test method. Ten microliters of the bacterial suspension were cultured on LB agar (Merck Germany). Then, ten microliters of the phage lysate were added to three spots on the agar plate. The samples were incubated at 37°C (Wu et al., 2021).

2.4. Preparation for Transmission Electron Microscopy (TEM) Imaging

A drop of the purified phage solution was placed on a carbon- or diethylene chloride-coated grid. The sample underwent negative staining using a 2% uranyl acetate or phosphotungstic acid solution. The sample was then observed under a TEM (ZEISS, Germany) at 50 kV (Arivo et al., 2016; Litt Pushpinder et al., 2017).

2.5. One-Step Growth Curve

One milliliter of the bacterial suspension, with a final concentration of 10^6 CFU/mL, was combined with one milliliter of phage extract with a titer of 10^5 PFU/mL and incubated at 37°C for 15 minutes to allow the phage particles to adsorb. The mixture was then centrifuged at 12000 g for 1 minute, and the supernatant containing unabsorbed phages was discarded. The remaining pellet was cultured. Samples were collected every ten minutes for FU determination using the double-layer agar method (Bao et al., 2019).

2.6. Determination of Optimal MOI

To determine the optimal Multiplicity of Infection (MOI), bacterial samples with a concentration of approximately 10^7 CFU/mL were combined with bacteriophages at varying concentrations of 10^5, 10^6, $10^{7,}$ and 10^8 PFU/mL. The mixture was incubated at 37°C for 6 hours. Bacterial growth was monitored every 30 minutes for 60 minutes using an ELISA reader at OD600nm (ELX800, BioTek Instruments, USA) (Sakuma et al., 2006)

2.7. Assessment of Bacteriophage Stability Against Heat and pH

To assess heat stability, 100 microliters of bacteriophage with a titer of 10^9 PFU/mL were added to 900 microliters of TSB medium (Merck Germany) at various temperatures (30, 40, 50, 60, 70, and 80°C). The samples were incubated for 30 and 60 minutes. The phage titer was then determined using the double-layer agar method. To assess pH stability, 100 microliters of bacteriophage with a titer of 10^9 PFU/mL were added to 900 microliters of TSB medium adjusted to pH levels ranging from 2 to 13 (using NaOH or HCl) (El-Sayed et al., 2022; Guo et al., 2021; Li et al., 2021).

2.8. Evaluation of Antimicrobial Activity of Isolated Bacteriophages in Milk

To eliminate any potential contamination, the milk samples were sterilized at 121°C for 15 minutes. Then, 100 microliters of a bacterial suspension with a final concentration of 10^5 CFU/mL were added to the milk samples. Subsequently, 100 microliters of phage lysate with a final concentration of 10^8 PFU/mL were added. A positive control containing the bacterial suspension and a negative control with sterile SM buffer (50 mM Tris-HCl, pH 7.5; 0.1 M NaCl; 8 mM MgSO4) were also prepared. The samples were stored at 4°C and 25°C for four days. Bacterial counts were performed at 0, 3, 12, 24, 48, 72, and 96 hours (El-Shibiny et al., 2017; Huang et al., 2018)

3. Results

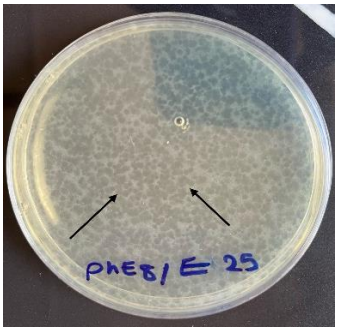
3.1. Isolation of Bacterial Strains and antibiotic resistance profiles

Eight *Escherichia coli* strains were isolated from food samples, including raw meat and pizza cheese. The antibiotic susceptibility of the studied bacteria to different antibiotic disks is as follows: all strains were resistant to ampicillin, two strain was resistant to ceftazidime, two strain was resistant to amikacin, five strains were resistant to cefixime, five strains were resistant to trimethoprim-sulfamethoxazole, and two strains were resistant to gentamicin (Table-1).

3.2. Isolation of Bacteriophage from Wastewater

Bacteriophage PhE8 was identified through its lytic activity against *E. coli* PTCC 1399, which was isolated from wastewater. The phage was detected by the formation of clear plaques on the bacterial culture, indicating successful lysis of the host bacterium. Distinct and clear plaques were selected for further study, and enrichment methods were used to achieve higher purity of the phage. Additionally, electron microscopy was employed to confirm the morphology and identity of the bacteriophage (Figure 1).

The lytic activity of the isolated bacteriophage on host bacteria was examined using the spot test method. The morphology of the bacteriophage was analyzed using an electron microscope. The results of the electron microscopy revealed that bacteriophage PhE8 has a polyhedral head with a diameter of approximately 34×27 nanometers and a contractile tail of approximately 13×164 nanometers (Figure 2).



Figurr 1.Bacteriophage phE8

3.3. Optimal MOI Determination

The antibacterial activity of bacteriophage PhE8 was examined at various MOIs (0.01, 0.1, 1, and 10). The results indicate that increasing the MOI significantly enhances the efficiency of bacteriophages in inhibiting bacterial growth. For example, at an MOI of 10, the bacterial density increased from 0.5 to 0.12 over six hours, compared to the positive control group, where the bacterial density significantly increased from 0.5 to 3.4. As the MOI decreases, the host bacterial density effectively increases. For instance, at an MOI of 0.1, the bacterial density increased from 0.5 to 0.16. These findings emphasize the importance of optimizing MOI in phage applications.

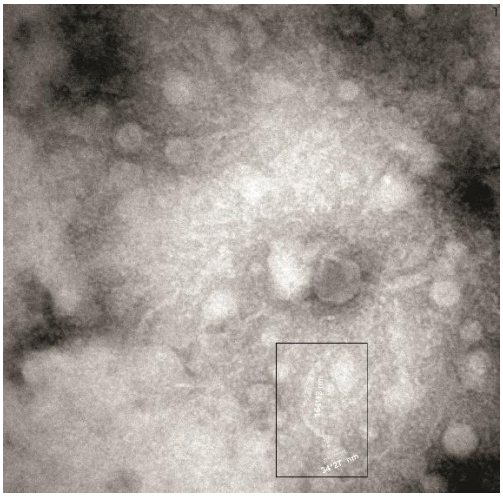


Figure 2. TEM micrograph of bacteriophage PhE8: approximate head diameter: 34×27 nm in sample A, and approximate tail length: 13×164 nm.

Table.1 Antibiotic resistance profiles of six different salmonella strains

Strains	Antibiotics					
	1	2	3	4	5	6
E2	R	R	R	S	S	S
E02	R	S	S	I	S	I
E03	R	S	I	R	I	S
E8	R	I	R	I	R	I
E10	R	R	R	R	R	R
E19	R	R	R	S	S	S
E25	R	S	S	S	S	R
E26	R	S	S	S	S	S
E27	R	S	R	I	I	S

: Intermediate; R: Resistance. ; S: Susceptible

1-Ampicilin, , 2- sulfamethoxazole, 3- Cefixime, 4- Amikacin, 5- Ceftazidime, 6- Gentamicin

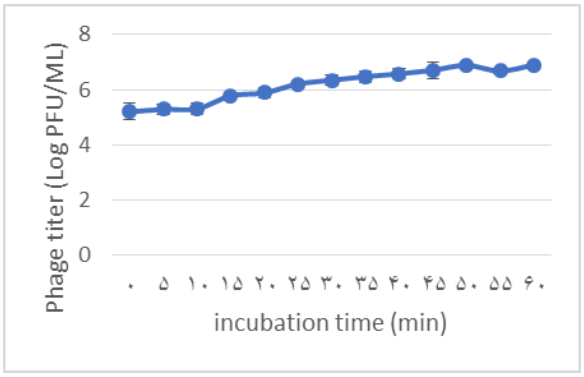


Chart 1. One-step growth curve of bacteriophage PhE8

3.4. Evaluation of Bacteriophage Stability Against Heat and pH

The thermal stability of phage PhE8 was examined across a temperature range of 30 to 80 degrees Celsius, with incubation times of 30 and 60 minutes. The study showed that after exposure to temperatures between 30 to 60 degrees Celsius for 30 minutes, the phage titer did not decrease significantly. However, at temperatures above 60 degrees, up to 80 degrees, a significant reduction in phage titer was observed, from log PFU/mL 9 to log PFU/mL 7.12. After 60 minutes at temperatures between 30 to 60 degrees Celsius, the phage titer decreased from log PFU/mL 9 to log PFU/mL 7.65. At temperatures of 70 degrees and above, no bacteriophage activity was detected (Chart-2). The evaluation of phage PhE8 stability against pH showed that it remains active across a wide pH range (4-12). However, when exposed to strong acidic or alkaline conditions (pH ≤ 3 or pH ≥ 13), its activity dropped below the detection limit. The highest phage titer was observed at pH 7 (log PFU/mL 7.52) (Chart-2).

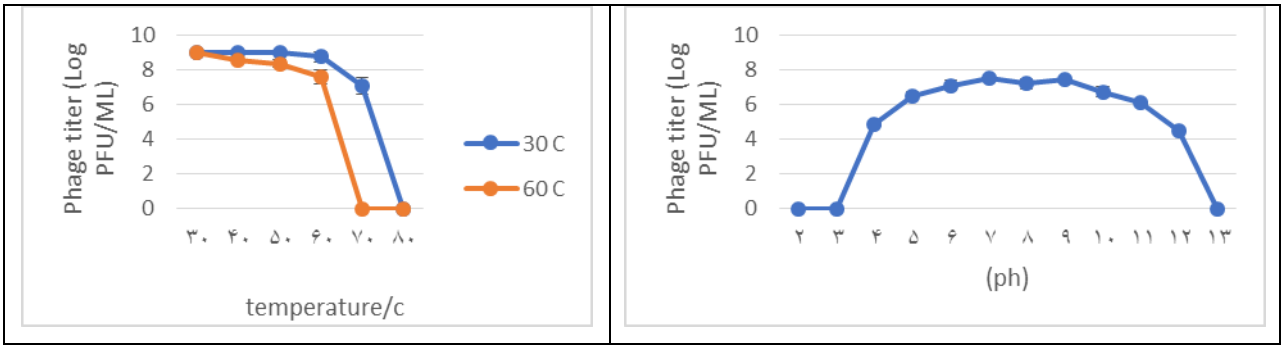


Chart 2. Evaluation of Bacteriophage PhE8 Stability Against A: pH B: Heat at 30 and 60 Degrees Celsius

3.5. Evaluation of Antimicrobial Activity of Bacteriophage PhE8 in Milk

The bacterial count of *Escherichia coli* (E8) in milk treated with bacteriophage PhE8 at 25 degrees Celsius indicated that the number of viable bacterial cells was log CFU/mL 3.77 at time zero, but after 3, 12, and 24 hours, it decreased to log CFU/mL 3.35, log CFU/mL 2.92, and log CFU/mL 1.95, respectively. This represents a significant reduction of log CFU/mL 1.84 compared to the initial time. However, after 48 hours, there was a resurgence of bacterial cells. Nevertheless, compared to the control group at the same time point and after four days, the number of viable bacterial cells in the sample treated with bacteriophage was log CFU/mL 2.95 lower. The examination of milk artificially contaminated with E8 bacteria at 4 degrees Celsius showed that after treating the sample with PhE8, the viable bacterial cells decreased from log CFU/mL 3.72 at time zero to log CFU/mL 2.1 after 3 hours. After 12 hours of phage treatment at 4 degrees Celsius, the bacterial cells were undetectable. Even after 96 hours, no bacterial cells were observed. In contrast, in the control group, the number of viable bacterial cells was log CFU/mL 5.35 after 96 hours. This indicates that the bacterial cells were completely eradicated by the bacteriophage (Chart-3).

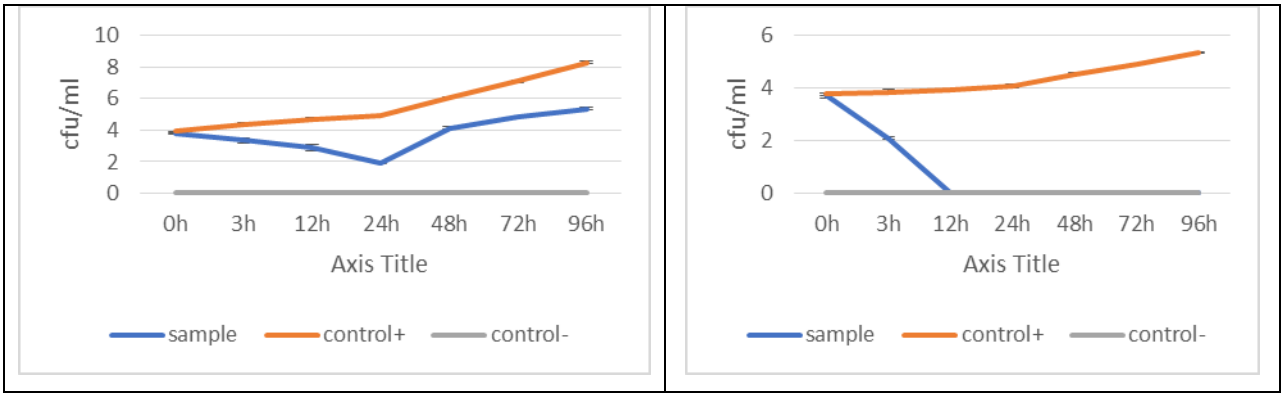


Chart 3. Evaluation of the Antimicrobial Role of Bacteriophage PhE8 in Milk Contaminated with E8 Bacteria
A: at 4 Degrees Celsius
B: at 25 Degrees Celsius

4. Discussion

The results of this study indicate that bacteriophages isolated from wastewater can effectively reduce *Escherichia coli* populations in dairy products such as milk. Milk samples inoculated with these phages showed a significant reduction in bacterial counts compared to control samples. These findings suggest the potential of bacteriophages as a tool for controlling microbial contamination in dairy products. In terms of affordability, the use of bacteriophages isolated from wastewater for controlling Escherichia coli in dairy products like milk offers a cost-effective alternative to traditional antimicrobial treatments. Wastewater is an easily accessible and low-cost source for phage isolation, reducing the overall cost of production. Additionally, phages can be produced in large quantities using simple culturing methods, which further reduces expenses. Since bacteriophages specifically target bacterial pathogens without affecting the food product or beneficial microorganisms, their application can be more economically viable compared to chemical preservatives or antibiotics. This makes phage therapy an affordable and sustainable solution for microbial contamination in the dairy industry. This result is consistent with a study conducted by (Gutiérrez et al., 2019), where the use of phages in milk effectively reduced pathogen levels while maintaining milk quality. These results indicate that phages can be a practical method for reducing microbial contamination in dairy products. The findings showed that phages could effectively kill the target bacteria and exhibit strong lytic activity. This lytic activity remained stable under various temperature and pH conditions, indicating high resistance and usability of these phages in different environmental settings. A similar study demonstrated that bacteriophages could effectively eliminate pathogenic bacteria in various environments. In evaluating antibiotic resistance, various *Escherichia coli* strains were isolated from different food sources and tested for antimicrobial sensitivity using antibiotic discs (Zhou et al., 2022). The results showed that some strains exhibited high antibiotic resistance, highlighting the need for alternative methods, such as bacteriophages, for controlling microbial contamination. A similar study also demonstrated that certain *Escherichia coli* strains had high antibiotic resistance, and using phages could be a suitable solution for controlling these bacteria . Phage stability under different temperature and pH conditions was also investigated. The results showed that phages remained stable across a temperature range of 30 to 60 degrees Celsius and a pH range of 4 to 12, maintaining their lytic activity. These findings suggest the high resistance and applicability of these phages in various environmental conditions. A similar study also found that bacteriophages can remain stable and retain their activity under different temperature and pH conditions (Guo et al., 2021). In this study, the bacteriophage isolated from wastewater effectively eliminated the target bacteria. These results indicate the potential of phages as an effective method for controlling microbial contamination. A similar study showed that bacteriophages isolated from

wastewater could effectively kill pathogenic bacteria (Liu et al., 2020). The use of bacteriophages in food products not only helps reduce microbial contamination but also positively affects the quality of the product. This study's results showed that phages could help preserve milk quality and extend the shelf life of these products. A similar study also demonstrated that using phages in food products could help maintain product quality and extend shelf life. Given the positive results of this study and similar studies, using bacteriophages as an alternative and complementary method for controlling microbial contamination in food products should be considered (Cieplak et al., 2018). Future research should further explore phage applications under various conditions and evaluate the long-term effects of their use. Improving phage isolation and purification techniques and conducting genetic research to enhance their efficiency are also important topics for future research.

Conclusion

Given the rise of multidrug-resistant bacteria and the high prevalence of foodborne diseases, the need for alternative and effective methods to reduce spoilage-causing bacteria in the food industry is more pressing than ever. Bacteriophages, due to their abundance in the environment and highly specific action against pathogenic bacteria, can be used as biocontrol agents while preserving the flavor and organoleptic properties of food products in the industry. Genomic sequence analysis and molecular analysis of isolated phages can further enhance their application in the industry.

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Refereces

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