Isolation and Characterization of *Escherichia coli*-infecting Bacteriophages

Thesis Submitted to Vidyasagar University for the Partial Fulfillment of the Degree of Bachelor of Medical Laboratory Technology (BMLT)

Submitted by

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CERTIFICATE

This is to certify that the project report entitled 'Isolation and Characterization of *Escherichia coli* -infecting Bacteriophages' submitted by **Chowdhury Aminul Islam,Kishor Kumar Pradhan, Koushik Ghosh, Koushik Ghosh, Manab Maity, Minarul SK, Mith Mondal, Mrinmoy Mondal, Nripen Chandra Sarkar, Roll – 1597766 No- 200267, Roll – 1597766 No- 200283, Roll – 1597766 No- 200284, Roll – 1597766 No- 200285, Roll – 1597766 No-200288, Roll – 1597766 No- 200289, Roll – 1597766 No-200290, Roll – 1597766 No- 200291, Roll – 1597766 No- 200293** to the Vidyasagar University, Midnapore, West Bengal, India, during the year of 2023 in partial fulfillment for the award of the degree of Bachelor of Medical Laboratory Technology (BMLT) is a bona fide record of project work carried out by her under my/our supervision. The contents of this report, in full or in parts, have not been submitted to any other Institution or University for the award of any degree.

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Declaration

I do hereby declare that the present bachelor thesis entitled '**Isolation and Characterization of *Escherichia coli*-infecting bacteriophages**' embodies the original research work carried out by me in the Department of Biological Sciences, Midnapore City College, Paschim Medinipur, West Bengal, India under the supervision of Dr. Kuntal Ghosh, Assistant Professor, Dept. of Biological Sciences, **MIDNAPORE CITY COLLEGE,** Kuturiya, Bhadutala, Pin-721129, Paschim Medinipur, West Bengal. No part there of has been submitted for any degree or diploma in any University. I shall not publish this work without consent of the supervisor.

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Abstract

Escherichia coli are one of the foods borne pathogen causing diarrhea, hemorrhageic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. In the lateritic West Bengal, the traditional fermented foods are very popular. However, their qualities are somehow questionable as they have been preparing in the household or small cottage industries using the traditional methods. Considering the emergence of antibiotic resistant bacteria, it is essential to find out the alternatives to control *E. coli*. Bacteriophage could be the alternative of the antibiotic. Therefore, our aim of this study was to isolate and partial characterization of *E. coli*-infecting bacteriophages. In this study, we have isolated an *E. coli*-infecting bacteriophage from the drain water. The isolated bacteriophage EP1 could lyse *E. coli* MTCC 433. In one step growth curve, it was observed that the latent period of EP1 was 20 min and burst size was 91. The bacteriophage also proved its ability to inhibit the growth of *E. coli* MTCC 433 in liquid culture and curd. Clearly, a detailed study is needed to prove its lytic ability in different food environment.

Keywords: *Escherichia coli*; bacteriophage; host range; lysis; biocontrol.

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Chapter 1: Introduction

1. Introduction

Escherichia coli has emerged as an important human pathogen (Riley et al., 1983). The infective dose can be as low as 10 cells, and symptoms of infection include diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura (Tarr, 1995). Outbreaks have been attributed to food, water, and person-to person and direct fecal contact (Duffy, 2003). *E. coli* may cause diarrheal disease by several mechanisms, and six categories or strains of diarrheagenic *E. coli* are now recognized (Willey et al., 2009):

a. enterotoxigenic *E. coli* (ETEC),
b. enteroinvasive *E. Coli* (EIEC),
c. enteroahemorrhagic *E. coli* (EHEC),
d. enteropathogenic *E. coli* (EPEC),
e. enteroaggregative *E. coli* (EAggEC),
f. diffusely adhering *E. coli* (DAEC).

In this lateritic West Bengal, traditional fermented foods such as haria, bori, idli, dosa, curd, paneer are very popular among the people. Most of the traditional fermented foods have been prepared in small cottage industries and household. Therefore, the qualities of the foods are sometimes questionable. It is already proved by many researchers that the traditional fermented foods contained different pathogenic bacteria such as *Bacillus cereus, Vibrio cholerae, Escherichia coli,* etc. (Roy et al., 2011; Roy et al., 2007).

The current technologies employed to inactivate bacterial pathogens in foods are not infallible, as proved by the continuous increase in several foodborne diseases caused by pathogens, such as *Salmonella*, *Campylobacter*, *Escherichia coli*, *Listeria* and others that have an enormous impact on public health (DuPont, 2007). Contaminating bacteria can get access to food during slaughtering, milking, fermentation, processing, storage or packaging (Garcia et al., 2008). Over the last few years, a number of strategies to minimize the microbial load of raw products have been explored as the use of antibiotics is restricted due to the negative impact on human antimicrobial therapies. Problems of acceptability and deterioration of the organoleptic properties have been described after physical treatments such as steam, dry heat and UV light (Garcia et al., 2008). Moreover, the extensive use of

sanitizers has led to the development of resistant bacteria rendering these procedures less effective (Garcia et al., 2008). On the other hand, some approaches often used in the food industry to reduce contamination by foodborne pathogens cannot be directly applied to fresh fruits, vegetables and ready-to-eat products (Garcia et al., 2008). Hence, despite recent advances to avoid transmission of bacterial pathogens throughout the food chain, novel strategies are still required to fulfill consumer demands for minimally processed foods with fewer chemical preservatives.

The exploitation of bacteriophages as a realistic approach to the control of pathogens has attracted considerable interest in recent years because of the emergence of antibiotic-resistant bacteria. Bacteriophages are viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause the bacterium to lyse. Felix d'Herelle (1919) was probably the first scientist who used bacteriophages as a therapy to treat severe dysentery. The current threat of antibiotic-resistant bacteria has renewed the interest in exploring bacteriophages as biocontrol agents in Western countries (Matsuzaki et al., 2005). In fact, some products based on bacteriophages are already commercially available ('PhageBioderm', 'Bacteriophagum Intestinalis Liquidum', 'Pyobacteriophagum Liquidum') (Garcia et al., 2008).Bacteriophages are found to have a wider range of application such as in food processing industry, against plant pathogens, and in water treatment plant, as a disinfecting for diagnostic tools and in therapy to treat bacterial infection.

Phages are typically found, transferred in terrestrial and aqueous environments, even they can be retrieved in any type of environment where their bacterial hosts are arrived (Weinbauer, 2004). Bacteriophages target specifically *Escherichia coli* strains have been used as indicator for treat coliforms and enteric viruses for water pollution (International organization for standardization, 2000). Phages are part of both first one is gastrointestinal and second one is environmental ecosystem and are among the simplest and most abundant organisms on earth (Carlton, 1999; Sulakvelidze et al., 2001). Among of these lytic phages are suitable for phages therapy in opposition to temperate phages (Karma, 1994). Additionally, some care centres are particularly specialized in phage therapy (for example Southwest Regional Wound Care Centre, Texas, http://www.woundcarecenter.net).

In our previous study we observed that the curd samples contained 10^2 to 10^6 CFU/g of *Escherichia coli*. Therefore, this should be matter of concern as it may cause different diseases in humans. Considering that, our aim of this study is to isolation and Partial characterization of *E. coli*-infecting bacteriophages and its successful application in the curd preparation.

Bacteriophage

The initiated extensive studies of the highly symmetrical head, tails and baseplate using primarily E.M. (Kaper et al., 2004). The phages are different size and shape. The most observed group suggested that the phages have ds-DNA genome. The tailed phages have three major components: the genome packed capsid, a tail that served as a pipe during infection to source transfer of genome into host cell and a major tacky system at the of the tail that will recognize the host cell and penetrate its wall for the reproduction (Helen et al., 2019).

Life cycle of bacteriophage

On the earth, the bacteriophages are the most richly found. The bacterial viruses have genetic material in the form of DNA or RNA, which is coated by protein (Clark and March, 2006). This protein coat is known as capsid. The capsid is connected with a tail which has fibers. This tail used for attachment to receptors on bacterial cell surface. Except filamentous phages almost all the phage has polyhedral capsid (Ackerman, 1998). Phages infect bacteria and can propagate in two possible way, lytic cycle and lysogenic life cycle. When phages multiply vegetatively they kill their host and the life cycle is referred to lytic cycle (Inal, 2003). If supposition to some harsh condition like ultraviolet ray occurs then the prophage will escape via lysis of bacteria (Summers, 1999). The bacteriophage infects and complete their life cycle inside *E. coli* in the following steps:

Attachment

The bacteriophage attaches itself on the surface of bacteria. The process will know as absorption. The tip of the ten fibers attach to the specific receptor on the surface of the bacterial cell.

Penetration

The base plate and the tail fibers are attached firmly to the bacterial cell. The phage muramidase (lysozyme) weakens a part of the cell wall and the hollow core is the pushed downwards through it. The DNA is injected inside the bacterial cell.

Synthesis

The component of new virus particles are produced after the nucleic acid is released into the cell. The subunits of phage head, tail and late protein then appeared. The synthesized is carried out by specific enzyme called early protein. The nucleus and cytoplasm also contain the component of phage.

Maturation and assembly

On maturation, the head and tail protein of phage DNA assemble and each component of phage DNA is surrounded by a protein coat. Ultimately, the tail structure are added forming a virion.

Release or lysis

The infected bacterial cell is lysed releasing the progeny phages. The phage enzymes weaken the cell wall of bacteria during replication. In this phase mature viruses burst out of the host cell in a process called lysis and the progeny viruses are liberated into the environment to infect new cells.

Bacteriophages are among the most common and diverse entities in the biosphere. Bacteriophages are ubiquitous viruses, found wherever bacteria exit. They can be found in soil and seawater, oceanic and terrestrial surfaces and extreme environments, such as those characterized by very high or very low temperatures. Moreover, they have been detected in hospitals, in wastewater and where bacteria can live, including animal and human tissues (Clokie et al., 2011).

Bacteriophage for biocontrol of pathogens in food

Bacteriophage based bio-control measurements have a great potential to enhance microbiological safety based, namely on their long history of safe use relatively easy handing and their high and specific antimicrobial activity (Garcia et al., 2008). To prevent or reduced colonization and disease on the base of phage therapy and also depurate carcasses and other row product fresh fruit and vegetables. Bacteriophages also use in hurdle technology in combination with different prevention methods (Leverentz et al., 2003).

Chapter 2: Literature Review

2. Literature Review

Yazdi et al. (2020) observed that VB_EcoS-Golestan is a virulent phage that belongs to *Kagunavirus* genus of *Guernseyvirinae* subfamily, *Siphoviridae* family. This lytic bacteriophage had a broad host range specificity against both antibiotic sensitive and multidrug-resistant UPEC isolates, a rapid adsorption time, and large burst size, and high stability at a wide range of pH and temperatures, which makes it a promising agent against *E. coli* infections. Moreover, annotation of its whole genome sequence confirmed that there is no virulence factor in its genome including, toxin, lysogenic or antibiotic resistance genes. Therefore, VB_EcoS-Golestan is a potential agent for phage therapy of UTI caused by *E. coli*.

Hyman (2019) suggested that most phages, especially those intended for phage therapy, are isolated using slight variations on the classic enrichment protocol. A subset of phages are isolated using a mixture of hosts with the intent of obtaining broader host range phages and several protocols have been developed specifically to select phages with increased host range or polyvalent phages. These latter protocols are not in wide use currently. Instead, host range is characterized after isolation and a diversity of host ranges are seen. While most groups use methods that rely on phage growth to test host range, there are still some who rely on less accurate, but easily performed, spot testing alone. It also suggests that the strains used, to the extent possible, should mirror the strains infecting patients, which means using clinical isolates and considering the location in which hosts were isolated as well as their identity. This is more extensive testing than is sometimes done when initially characterizing a Pharmaceuticals 2019, 12, 35 15 of 23 phage's host range but it should increase the likelihood that a phage therapy product will be successful in treating many cases of the target pathogen infection.

Vahedi et al. (2018) suggested that isolated and identifying a specific bacteriophage against EPEC and characterize bacteriophage *in vitro* and *in vivo*. The increasing resistance of enteropathogenic *Escherichia coli* (EPEC) to commonly used antibiotics has made it difficult to choose the best treatment option. Bacteriophage therapy could be a potent alternative to antibiotic therapy for antibiotic-resistant bacteria. The specific bacteriophage was isolated, and the effect of phage therapy on 48 mice (Balb/c) was investigated. The effect of the phage on bacteria *in vitro* was investigated and it was observed that by adding the phage to EPEC-infected HEp-2 cells (10^6 pfu/ ml), the bacteria were completely demolished and the phage

was very effective.

Peng et al., (2018) isolated a phage that might be used for pathogenic *E. coli* control. Genomic analysis of the phage revealed that the phage had undergone at least three rounds of genome recombination events during the evolutionary process. The finding of this study not only provides resources for developing phage therapy against *E. coli*, but also showed the highly variable genome structure of the phage.

Ahmed et al. (2018) suggested that ECa1 phage had higher merit for the inactivation of multi drug resistant *E. coli* O168 than the ECb1 and ECc1 phages. The higher burst size and a shorter latent period of ECa1 compared to the other two phages improved the efficiency of phage therapy. Also, the inactivation of *E. coli* O168 by the phage cocktails ECa1, ECb1, ECc1 was the most effective one due to the reductions by 3.5 log after 4 h of phage treatment. Therefore, phage cocktails would be considered as biological control to treat diarrheal duckling owing to specify of those phages to their host cells. This study confirmed the higher efficacy of phage cocktail in controlling the infection of duckling with multidrug resistant *E. coli* O168 serotype. These bacteriophages will reduce mortality in duckling, and also protect human health from adverse side effects of antibiotic residues.

Manohar et al. (2018) suggested thatthe isolated *Escherichia* phage myPSH1131 against pathogenic *E. coli* was found to be effectively eliminating or reducing the bacteria in both *in vitro* and *in vivo*. The *Escherichia* phage myPSH1131 was found to have broad host range infectivity against five different pathotypes of *E. coli* which is one of the important aspects for the selection of phages during therapy. Lyophilization studies showed that the use of sucrose and the combination of sucrose plus gelatin can restore the viability of *Escherichia* phage myPSH1131 during long-term preservation at 4˚C. *In vivo* studies using *G. mellonella* as a model organism showed promising results that can be considered in the future for testing in murine models and for clinical trials. Antibiotic resistance has attained a dangerous level to the humankind that needs to be addressed with an alternative option such as phage therapy. Relentless efforts are being taken to study the science behind phage therapy and our study is one such an effort to improve the understanding behind the use of bacteriophages as a therapeutic tool.

Drulis-Kawa et al. (2012) suggested that the major characteristics of bacteriophages and phage-encoded proteins affecting their usefulness as antimicrobial agents. It is noteworthy that the use of only modified phages would also help to reduce the use of antibiotics. It seems

that the application of phage-encoded proteins instead of environmentally isolated phages is more promising in terms of a broader activity spectrum, better tissue penetration, lower immunogenicity and low probability of bacterial resistance. Due to all these properties, lysins, for example, are effective in specific bacteria diagnostics and detection, phytopathogens elimination and in food preservation.

López-Cuevas et al. (2011) isolated four phages from cattle and poultry feces and analyzed for their ability to lyse *Salmonella* serotypes and *Escherichia coli* O157:H7. The phage one-step growth curves, morphology, and genetic characteristics were determined. All phages showed a lytic effect on various *Salmonella* serotypes and *E. coli* O157:H7, which lysed at least 70% of the 234 strains tested. In vitro phage challenge showed a total reduction of *E. coli* O157:H7, *Salmonella Typhimurium* and Saintpaul counts at 2 h, whereas for *Salmonella* Montevideo a reduction and retardation growth, at a multiplicity of infection (MOI) of 100, was observed; however, under a MOI of 10 000, no viable cells were detected after 4 h. The wide host ranges of these phages suggested they could be used for simultaneous biocontrol of some *Salmonella* serotypes and *E. coli* O157:H7. SIn this investigation, bacteriophages with lytic effect on numerous Salmonella serotypes and *E. coli* O157:H7 were isolated and characterized. The wide host ranges of these phages suggested that they could be used for simultaneous biocontrol of some of the Salmonella serotypes and *E. coli* O157:H7.

Oliveira et al. (2008) told that isolated and characterized bacteriophages, evaluate its lytic performance against avian pathogenic *Escherichia coli* (APEC) strains with high patterns of antibiotic resistance, in order to select phages for a therapeutic product to treat coli bacillosis in chickens. 70.5% of the tested E. coli strains were sensitive to a combination of three of the five isolated phages that seemed to be virulent and taxonomically belong to the Caudovirales order. Two of them look like 16–19, T4-like phages (*Myoviridae*) and the third is a T1-like phage and belongs to *Siphoviridae* family. All of them are genetically different. It was possible to obtain a combination of three different lytic bacteriophages with broad lytic spectra against the most prevalent O-serotypes of APEC.

Brussow (2005) have been proposed that phages as a natural antimicrobial agent to fight bacterial infections in humans, in animals or in crops of agricultural importance. Phages have also been discussed as hygiene measures in food production facilities and hospitals. These proposals have a long history, but are currently going through a kind of renaissance as documented by a spate of recent reviews. This review discusses the potential of phage therapy with a specific example, namely *Escherichia coli*.

O'flynn et al. (2004) reported that the exploitation of bacteriophages as biocontrol agents to eliminate the pathogen *E. coli* O157:H7. Two distinct lytic phages (e11/2 and e4/1c) isolated against a human strain of *E. coli* O157:H7, a previously isolated lytic phage (pp01), and a cocktail of all three phages were evaluated for their ability to lyse the bacterium *in vivo* and *in vitro*. However, bacteriophage-insensitive mutants (BIMs) emerged following the challenge. All tested BIMs had a growth rate which approximated that of the parental O157 strain, although many of these BIMs had a smaller, more coccoid cellular morphology. In addition, BIMs commonly reverted to phage sensitivity within 50 generations. *E. coli* O157:H7 is a prevalent pathogen with a low infectious dose. This study indicates that phage therapy could be a viable method of controlling this pathogen. The frequency of BIM formation is low, and all mutants revert to phage sensitivity; therefore, we expect that BIMs should not hinder the use of these phages as biocontrol agents of the pathogen *E. coli* O157: H7.

Hagens et al., (2004) suggested that bacteriophages may act as vectors of undesirable traits (virulence and antibiotic-resistance genes) and temperate phages mediate lysogenic conversion that have raised safety concerns. Recent advance in genomics and phylogenic studies make it possible to understand gene flow among phages and hosts and potentially harmful bacteriophages could be avoided or re-designed without undesirable traits and lacking any gene dissemination systems. As an example, bacteriophages could be genetically engineered to block phage replication once the host has been killed. This would prevent the release of large numbers of phages in a particular environment.

Chapter 3: Aims and Objectives

3. Aims and Objectives

The aim of the study was to use *E. coli* infecting bacteriophage as biocontrol agent.

The objectives are:

i. Isolation of *Escherichia coli*-infecting bacteriophages.

ii. Partial characterization of *Escherichia coli*-infecting bacteriophages.

iii. Application of bacteriophage to control *Escherichia coli* in fermented food.

Chapter 4: Materials and Methods

4. Materials and Methods

***4.1. Samples*:** Different types of environmental samples (water and soil) were used for *Escherichia coli*-infecting bacteriophage isolation.

4.2. Propagation of phages: Ten gram or milliliter of sample was mixed with 20 ml of Nutrient broth and 1 ml of each overnight-grown bacterial culture (*Escherichia coli* strains) and then incubated at 37 °C with agitation (160 rpm) for 12-18 h. After incubation, samples were centrifuged at 13,000 RPM for 10 min and the supernatant was used for phageisolation. Positive samples (phage containing) was selected based on the dotting assay and the single plaque was isolated.

4.3. Host range analysis: Five microliter of virions (1×10^8 PFU [plaque forming units]/ml) was dotted onto the TSA soft agar overlayer containing the test bacteria. The plates were incubated at 37 °C and bacterial growth inhibition was monitored.

***4.4. One-step growth assay*:** A one-step growth assay of phages was performed as previously described Ellis and Delbrück (1939). To summarize, the culture of *E. coli* was grown overnight, added to 25 mL of fresh nutrient broth media (1% inoculum) and incubated at 37°C. When the optical density at 600 nm (OD_{600}) will be reached at 0.3 (approximately 1 $\times 10^7$ CFU/ml), the bacteriophage was added at MOI of 0.01 and incubated for 5 min, followed by centrifugation at 8000 rpm for 10 min. The pellet was resuspended in the same volume of fresh nutrient broth. Then 1 mL of this mixture was aliquoted into micro-centrifuge tubes (1.5 ml) followed by incubation at 37°C with shaking (160 rpm). At every 10 min, one tube was taken out from the incubator and centrifuged at 8000 rpm for 3 min. The supernatant was then collected and used for PFU counting by using TA soft agar.

***4.5. Liquid culture inhibition assay*:** Growth inhibition of *E. coli* MTCC 433 by bacteriophage in liquid culture was performed as described by Shin et al. (2011) with some modification. An overnight culture of *E. coli* MTCC 433 (1% inoculum) was added to fresh nutrient broth media and incubated at 37 °C with shaking (160 rpm). After 3 h when OD_{600} was reached approximately 0.3, bacteriophage was added at MOI of 1 and 0.1 levels. The samples were collected at every 1 h and OD_{600} was measured using spectrophotometer.

***4.6 Stability testing*:** The bacteriophages' stability was tested at broad pH range (pH 5-9), high alkanity, and broad salinity (0-40 ppm) by the standard methods (Ibrahim et al., 2017). Briefly, SM buffer was calibrated using 1 M HCl to pH 3, 5, 6, 7, 8, and 9 and 900 µl of each

calibrated solution was aliquoted into 1.5 ml vials. The bacteriophage stock was added to the calibrated buffer solutions to the final titer of 10^8 PFU/ ml. The vials were gently inverted to mix, and left to stand at room temperature for 60 min. After 60 min, 100 µl of each solution were sampled and mixed with 100 µl of exponentially growing *E. coli*. After 12 h incubation at 35 °C, the pH resulted in the highest number of plaque (titer) was considered optimum.

4.7 Application of E. coli phage for controlling of E. coli in fermented food: Three conical flasks containing 50 ml milk (pasteurized) was inoculated with *Lactobacillus* sp. (10^3 CFU/mL). E2. One conical flask served as control where no *E. coli* and bacteriophage was added. Next one served as infected group where *E. coli* (10^4 CFU/mL) was artificially inoculated. Last conical flasks contained *E. coli* (10^4 CFU/mL) and phages (10^4 or 10^5 PFU/mL). All of these flasks were incubated at 37 ^{o}C for 24 h. After fermentation, *E. coli* and Lactobacillus sp. were enumerated on MacConkey agar and Rogosa SL agar by spread plating technique.

4.8 Statistical analysis: All of the experiments were carried out three times. The data are represented as the mean ± Standard Deviation (SD).

Chapter 5: Results and Discussion

5. Results and Discussion

5.1. Isolation of bacteriophage

Bacteriophage EP1 was isolated from an environmental sample by using *E. coli* MTCC 433 as a host. This bacteriophage showed clear plaques after 12 hours incubation at 37°C. Plaque was purified three times after isolation.

5.2. Host range analysis of bacteriophage

For host range analysis *E. coli* MTCC 433 *Vibrio harveyi* ATCC 14126, *Lactobacillus* sp, *Aeromonas hydrophila* MTCC 1739 were used as hosts. The phage could infect *E. coli* MTCC 433, whereas, it could not infect the other tested strains (Table 1).

Table 1. Host range analysis of isolated phage.

Phage name	**Bacteria name**			
	E. coli MTCC 433	*Vibrio harveyi* ATCC 14126	*Aeromonas hydrophila* MTCC 1739	*Lactobacillus* sp.
EP1	+	-	-	-

5.3. One step growth curve of bacteriophage EP1

The one step growth curve of bacteriophage EP1 showed that the latent period was 10 min. The rise phase (start of phage release from infected host until no more phages were released from its infected host) was estimated between 10 min to 40 min. The brust size was approximately 91 on the propagation stain *E. coli* MTCC 433. In the study of Necel et al. (2020), similar burst size was observed for the phage vB_Eco4M-7. However, there are some *E. coli* phages whose burst sizes were more than 100. Clearly, more isolation of phages is needed.

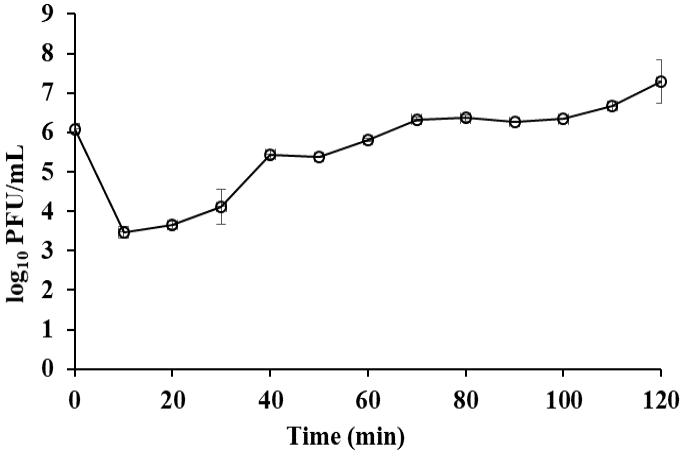

Fig. 1. One step growth curve of *E. coli* bacteriophage EP1.

5.4. Liquid culture inhibition of bacteriophage EP1

E. coli infecting bacteriophage EP1 effectively inhibited the growth of *E. coli* MTCC 433 in liquid culture. Clear lysis was observed after 3 h of post infection for MOI 0.1 and MOI 1 whereas the OD_{600} of the control (no bacteriophage addition) was 1.309 after 6 h (corresponds to 2 h of post infection) (Fig. 2). Similar lysis patterns of *E. coli* phages were also observed (Yazdi et el., 2020). As the phage could clearly lysed its host bacteria, the phage might have strict lytic activity and it could be used as biocontrol agent.

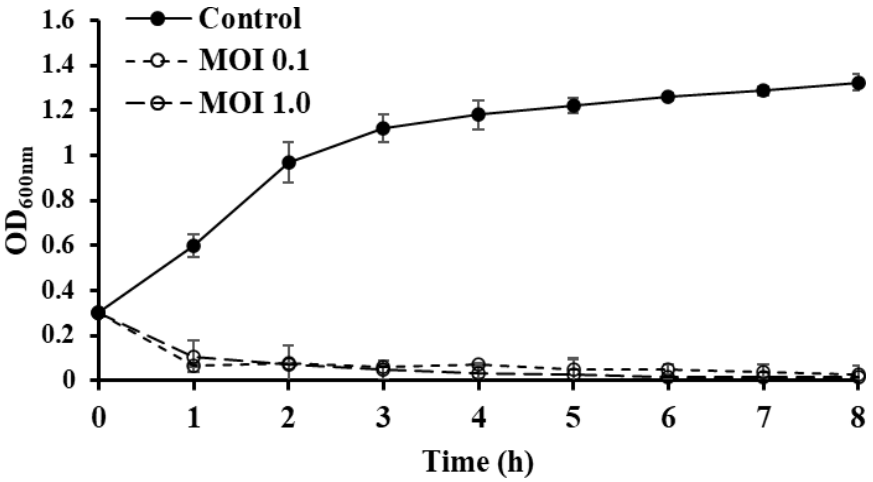

Fig. 2. Liquid culture inhibition of *E. coli*-infecting bacteriophage EP1.

5.5. pH stability of bacteriophage EP1

The sensitivity of *E. coli*- infecting bacteriophage ECP1 at different pH was shown in Fig. 3. The survival rates at pH 3, 5, 6, 7, 8, and 9 were 97.40%, 97.75%, 97.86%, 98.93%, 99.76%, and 97.63% after 1 h, respectively. Similarly, 96.34%, 96.69%, 96.89% 97.52%, 97.58%, and 95.86% survivability after 6h; and 95.40%, 96.60%, 96.73% 96.23%, 97.87% and 94.21% survivability were observed after 24 h. for pH 3, 5, 6, 7, 8, and 9, respectively (Fig. 3). Abdelrahman et al. (2022) also observed that their isolated phages were also stable at broad pH range. Hence, it is clear that the phage could survive in extremeacidic and alkaline condition.

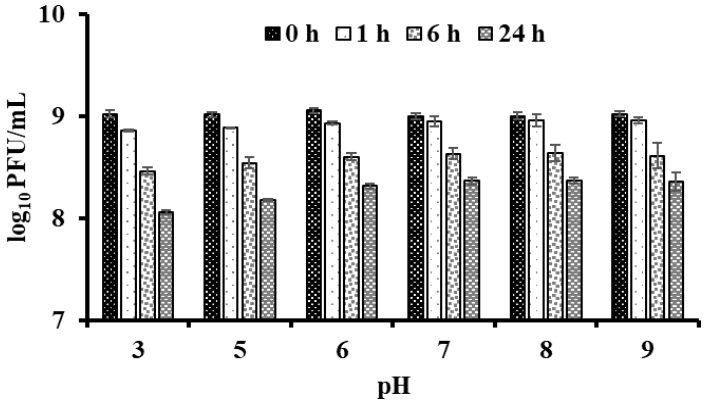

Fig. 3. pH stability of *E. coli*-infecting bacteriophage EP1.

5.6. Stability of bacteriophage EP1 to temperature

Bacteriophage EP1 survivability were decreased as temperature dependent manner. The survival rates of bacteriophage EP1 after 30 min were 98.29%, 98.08%, 97.97%, 93.82%, after 60 min 97.13%, 97.12%, 96.91%, 59.23% after 90 min 96.59%, 96.48%, 96.37%, 12.56% and after 120 min 96.06%, 94.57%, 93.39%, 0% at 4º, 28º, 37º, 60º C respectively (Fig. 4). The *E. coli* bacteriophage ZCEC11 was also found to be stable at 4º to 37º C temperature (Abdelrahman et al., 2022).

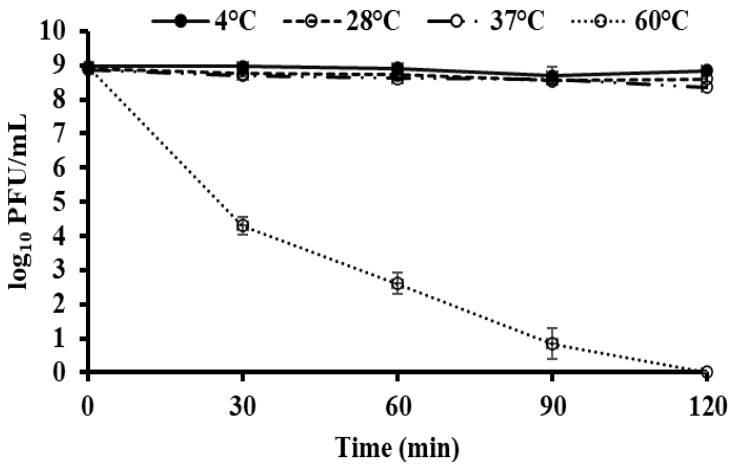

Fig. 4. Temperature stability of *E. coli* bacteriophage EP1.

4.7 Application of E. coli phage(s) for controlling of E. coli in fermented foods

After 48 h incubation, we observed that the number of *E. coli* in curd was reduced 3.9 log CFU/mL from the infected group (Fig. 5). Ahmed et al. (2018) suggested that phage cocktails using ECa1, ECb1, and ECc1 were effective to control *E. coli* O168 in curd preparation. Tabla et al. (2022) also revealed a reduction in *E. coli* growth at the beginning of milk fermentation with phage treatment. Clearly, more study is needed to evaluate the isolated phage's ability in different environment including food.

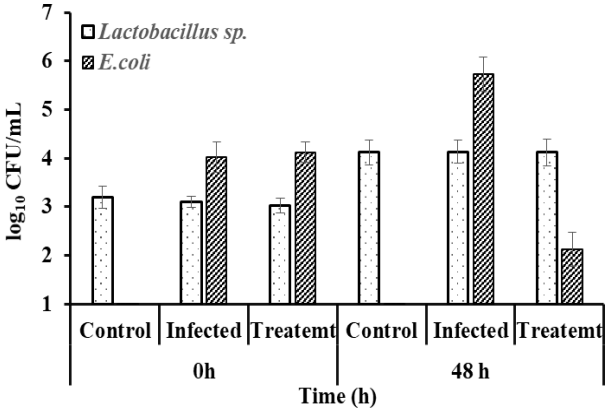

Fig. 5. Application of *E. coli* bacteriophage (EP1) for controlling of *E. coli* in curd.

Chapter 6: Conclusion

6. Conclusion

In this study, we have isolated an *E. coli*-infecting bacteriophage from the sewage water. The isolated bacteriophage EP1 could lyse *E. coli* but unable to infect other tested bacteria.. The bacteriophage also proved its ability to inhibit the growth of *E. coli* in liquid culture and had broad pH and temperature stability. Moreover, the bacteriophage successfully inhibited the growth of *E. coli* in curd preparation. Hence, it can be concluded that the bacteriophage might be used as a biocontrol agent. Clearly, a detailed study is needed to prove its lytic ability in different environmental condition.

7. Future Scope

7. Future Scope

There is an enough scope to prove the ability of the isolated bacteriophage to control *E. coil* in different food environment. Moreover, the genome annotation of the bacteriophage would enrich the classification scheme of the *E. coil*-infecting bacteriophages. Furthermore, immobilization of the bacteriophages may help to increase their viability.

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