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Comparison of Various Storage Conditions for The Stability of *Escherichia coli* O157:H7 Bacteriophage M8AEC16

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Abstract

In this study, bacteriophage M8AEC16, which is lytic to *Escherichia coli* O157:H7, isolated and characterized within the scope of our previous studies, was used to determine the stability during 6 months of storage at +4°C, -20°C and -85°C using glycerol, skimmed milk powder and carrageenan as cryoprotectants. For this purpose, bacteriophage M8AEC16, was enriched with *E. coli* O157:H7 ATCC 43895 and then phage suspension groups were prepared by adding three different cryoprotectants (test groups) in addition to those without cryoprotectant (control). Bacteriophage suspensions were stored at 3 different storage temperatures (+4°C, -20°C and -85°C) for six mount period. During storage, phage titers were determined on double-layer LB agar by making serial dilutions of phage suspensions on the 0th day and at the end of each month. As a result of the study, it was observed that stability was maintained at all temperatures for all groups during the first two months. However, in the third and fourth months, a decrease in the counts of bacteriophages were observed at -85°C in the glycerol added group. In addition to the control group, in the fifth month, carrageenan provided the highest preservation at all temperatures. In the sixth month, +4°C glycerol provided the best preservation, while carrageenan provided the highest preservation at other temperatures compared to the other test groups. In conclusion, the most successful result was obtained at +4°C for the preservation of *Escherichia coli* O157:H7 phage M8AEC16 considering the cost, usefulness and effectiveness.

Keywords: Bacteriophage, carrageenan, cryoprotectant, glycerol, preservation, skimmed milk powder.

INTRODUCTION

Bacterial pathogens are of serious importance for public health. According to the World Health Organization (WHO), it has been reported that approximately 600 million people become ill and 420 thousand people die every year as a result of consuming contaminated foods (WHO, 2023). These pathogens can contaminate foods at all stages of production such as slaughtering, milking, fermentation, packaging, and storage. Good Manufacturing Practices (GMP), Hazard Analysis Critical Control Points (HACCP) and other risk assessment practices may be insufficient in the control of pathogenic bacteria (Seçkin and Baladura, 2010).

The other issue with food-borne pathogens is antimicrobial resistance. Appropriate and extensive use of antimicrobials causes resistance in bacteria. In this case, existing antimicrobials may not be effective in treating these bacteria. Therefore, alternative control methods are needed to both protect public health and meet the demand of consumers who demand "minimally processed and chemical-free products". In this context, among these alternative methods, bacteriophages can be used for the biocontrol of bacterial pathogens effectively (Cufaoglu and Ayaz, 2019).

Bacteriophages (phages), which are obligate intracellular parasites, are defined as bacteria-killing viruses. It has been shown that they do not harm mammalian cells, but only infect specific bacterial cells. They can be found in all kinds of environments where bacteria can survive, such as human and animal skin, intestinal flora, fermented foods, sewage water, air and soil. Bacteriophages, like other viruses, multiply only in case of infection. They do not have their metabolic systems and ribosomes. Therefore, they cannot produce genetic information or synthesize proteins (Gökçe, 2010; Jończyk et al., 2011; Gümüştaş 2015).

There are two types of life cycles: lytic and lysogenic. The proliferation strategy followed by phages after the penetration stage determines their life cycle (Salmond and Fineran, 2015). The time it takes for bacteriophages to cause infection after encountering bacteria and the formation of new phages; is examined in four stages: adsorption, penetration, latent period and lysis period (Arda, 2011).

Phage therapy studies started with D'Herelle on patients with dysentery and plague. In the following years, phage cocktails such as “Pyophage” (contains phages targeting about 20 different pathogenic gastrointestinal bacteria) and “Intestiphage” (contains phages targeting *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus*, and *E. coli*) were used both as treatment and prophylactic against gas gangrene, and positive results were recorded (Abedon et al., 2011). In addition to their use for therapeutic purposes in humans and animals, bacteriophages have spread to a wide field of study by being used in the field of molecular biology, identification

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of bacteria, biotechnology studies, agricultural applications, biosanitation, bioprotection and biocontrol (Kasimoglu Dogru et al., 2017).

The use of bacteriophages in the field of food safety is very important. Bacteriophages are used in the food industry for equipment and contact surface disinfection (biosanitation), decontamination (biocontrol) and shelf-life extension (bioprotection) of carcasses, processed foods, fresh vegetables, and fruits (Gencay et al. 2016; Kasimoglu Dogru et al. 2017; Kekillioglu et al., 2019). Bacteriophages can be applied by adding them into foods, applying them to the surface, immersing the food in phage-containing water, or attaching them to food packaging materials. The most preferred method is application to the surface as a spray, as it is easy to apply on foods such as cheese, sausage, and meat fillet (Pérez Pulido et al., 2016; Doğru et al., 2017).

Like all microorganisms, bacteriophages must first be preserved under appropriate conditions to be used in studies and applications. In order to choose the right preservation method, many criteria such as phage stability, population, genetic change, preservation of purity, frequency of use and cost should be evaluated. Many studies have been conducted and some methods have been recorded for long-term preservation of bacteriophages. These methods; freezing (Adams, 1950), drying (Prouty, 1953), lyophilization (freeze drying) (Harris, 1954) and encapsulation (Saygılı and Karagözlü, 2017; Malik, 2021). It has been reported that many factors such as storage temperature, storage time, preservatives (cryoprotectant) and phage density before processing affect stability when choosing appropriate storage conditions. It has also been stated that in addition to the existing methods, alternative methods such as hydrocolloids such as carrageenan and gelation techniques can be used in future studies (Malik, 2021).

In this study, the stability of *Escherichia coli* O157:H7 phage M8AEC16, which was isolated and characterized within the scope of previous studies, was observed during the storage at +4°C, -20°C and -85°C using cryoprotectants such as glycerine, skimmed milk powder and carrageenan.

MATERIALS AND METHODS

Activation and enrichment of phage culture:

This study aimed to determine the storage stability of lytic *E. coli* O157:H7 bacteriophage M8AEC16 which was classified in the family *Myoviridae* under the order *Caudovirales* with the A1 morphotype (Gencay et al., 2016). Bacteriophage M8AEC16 stored at -85°C was activated and enriched in the fresh culture of *E. coli* O157:H7 ATCC 43895 (ECO157) in Tryptic Soy Broth (TSB; Oxoid) for 24 hours at 37°C. The enriched phage suspension was centrifuged (10,000 x *g*) and then subjected to a 0.22 µm diameter millipore filter (Guo et al., 2019). The presence of the plaques was revealed with the double-layer agar method. For this purpose, 3-4 ml of Luria Bertani (LB; Sigma-Aldrich) Soft Agar (LB Broth + 0.75% agar) containing 200 µl of ECO157 (10^8 cfu/ml) was poured on LB agar as a second layer. Then filtered phage suspensions were inoculated on LB soft agar (Xie et al., 2015). Following the spot plating at room temperature, petri dishes were incubated at 37°C for 24 h. The next day, the plaques on the agars were observed (Xie et al., 2015).

Determination of titers of phage stock

Phage titers were detected by spot plating on double-layer LB agars. For this purpose, the log phase ECO157 as a host bacteria and M8AEC16 phage filtrate from which the phage was isolated were incubated in TSB at 37ºC overnight. At the end of the incubation, serial dilutions of the supernatant obtained were prepared by centrifuging at 10,000 x g for 5 minutes and passing it through a 0.22 µm diameter millipore filter. Then, 200 µl of log phase bacteria were added to 3 ml of LB soft agar and poured onto the previously prepared LB agar as a second layer. After the second layer was congealed at room temperature, 10 µl of each phage dilution was spotted. After incubation at 37ºC overnight clear plaques were calculated as plaque-forming unit (pfu/ml) (Kishi et al., 2018).

Preservation of phage groups and determination of titers during storage

In addition to the control phage suspension which didn't contain cryoprotectant and test phage groups, contained 20% of one of the cryoprotectant substances (glycerol, skimmed milk powder and carrageenan as a gelling agent) were stored under three different temperatures (+4°C, -20°C and -85°C).

During storage, titer determination was performed on double-layer LB agar by making serial dilutions of phage suspensions on the 0^{th} day and 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} months. The results obtained were calculated as pfu/ml and compared with the initial titers. Then the reduction in the number of phages and the stability of the phages under storage conditions were investigated. These phage counts were carried out in three repetitions.

Statistical Analysis

In order to determine the most effective preservation method, control (without cryoprotectant) and test phage groups (with cryoprotectant: carrageenan, skimmed milk powder or glycerol) bacteriophages were stored at +4°C, -20°C and -85°C for 6 months. In order to determine the effect of cryoprotectants, statistical analysis were carried out with the Greenhouse-Geisser test and the Sphericity Assumed test to determine whether the change of concentration at constant temperature according to the elapsed time is significant and whether the change of the concentration according to the elapsed time is significant when keeping the cryoprotectant constant and comparing the results at different storage temperatures (Alpar, 2010).

RESULTS

In the study, the stability of M8AEC16 bacteriophage, which is lytic to *E. coli* O157:H7, in the presence of glycerol, skimmed milk powder, or carrageenan cryoprotectants during 6 months of storage at +4°C, -20°C and -85°C was examined, and the analysis results are shown in Table 1.

When the results of the study were examined, no significant decrease (0.78 log pfu/ml) ($p>0.05$) was observed in the number of control bacteriophages stored at +4°C without cryoprotectant for 6 months. In parallel, no decrease in phage stocks was observed in the counts between the 0th day and the 3rd month in the cryoprotectant groups, and it was noted that the phages maintained their stability at storage temperatures (+4°C, -20°C, and -85°C).

In the 4th month of storage at +4°C, a decrease of 0.75 to 1.31 log pfu/ml was observed in all cryoprotectant-added phage stocks, and the least decrease in the number of bacteriophages occurred in the glycerinated phage stock, and the highest decrease was in the carrageenan added stock. In addition, while a 1 log pfu/ml decrease was recorded in skimmed milk powder and glycerol phage stocks at -20°C, no significant decrease was observed in carrageenan phage titer ($p>0.05$). On the other hand, at -85°C, a decrease of approximately 1 log pfu/ml was observed in all cryoprotectant-added phage stocks. When the counts of the storage for the 4th month were examined, in addition to the control phage stock, the highest stability values were observed in the carrageenan-added phage stock stored at -20°C.

Table 1. Counts of bacteriophages detected under storage conditions (log pfu/ml).

Storage period	Number of bacteriophages (log pfu/ml)									
	+4°C		+4°C		-20°C			-85°C		
	Control	C	SMP	G	C	SMP	G	C	SMP	G
0th day	17,78	16,78	16,95	16,70	16,78	16,95	16,70	16,78	16,95	16,70
1st month	17,70	16,70	16,95	16,60	16,70	16,93	16,47	16,70	16,68	16,00
2st month	17,70	16,70	16,95	16,30	16,70	16,75	16,47	16,70	16,07	16,00
3st month	17,60	16,70	16,90	16,30	16,70	16,70	16,47	16,60	16,00	15,94
4st month	17,60	15,47	15,84	15,95	16,70	15,95	15,70	15,70	15,84	15,70
5st month	17,78	14,00	14,84	15,95	15,70	15,30	15,70	15,60	15,30	15,47
6st month	17,00	14,00	14,60	15,78	15,00	15,30	15,82	15,60	15,30	15,47

C: Carrageenan, **SMP:** Skimmed Milk Powder, **G:** Glycerol

In the 5th month of the study, a decrease was observed in all cryoprotectant-added phage stocks at +4°C. However, the most serious decrease compared to the previous month was in the phage stocks with added carrageenan (1.47 log pfu/ml) and skimmed milk powder (1 log pfu/ml), while no decrease was observed in the glycerol stock. In the study, a decrease was observed in all phage stocks with cryoprotectant added at -20°C and -85°C, but the only phage stock that did not show a decrease compared to the previous month was the one containing glycerol at -20°C.

Finally, in the 6th month counts, no significant decrease in phage stocks (0 - 0.70 log pfu/ml) was observed compared to the previous month, and the highest decrease occurred in the phage stock with carrageenan at -20°C at the level of 0.70 log pfu/ml. On the other hand, considering the six-month storage period, the decrease in the number of bacteriophages in the control group was recorded in the range of 0.78 to 2.78 log pfu/ml. Accordingly, the least decrease in the number of bacteriophages following the control group was detected in phage stocks containing glycerol at -20°C and +4°C, with 0.88 and 0.92 log pfu/ml, respectively. On the other hand, the highest decrease in bacteriophage titer was 2.35 and 2.78 in stocks containing skimmed milk powder and carrageenan stored at +4°C, respectively.

When the temperature was kept constant and the effect of cryoprotectants was examined, the measurements differed over time when the change in concentration according to the elapsed time was determined whether it was significant or not. ($p<0.001$). However, the interaction between temperature and time was not significant. In other words, the change over time did not differ at different temperatures. ($p=0.460$; >0.05). It was statistically determined that the difference between temperature groups was not significant. ($p>0.05$). When we kept the cryoprotectant constant and compared the results at different storage temperatures and examined whether the change in concentration over time was significant, the measurements differed over time ($p<0.001$). Additionally, the interaction between group and time was significant ($p=0.039$; <0.05). In other words, it was statistically determined that the difference between cryoprotectant substances was significant.

The change in bacteriophage stock counts depending on temperature and cryoprotectant variables during 6 months of storage are shown in Figures 1 to 6.

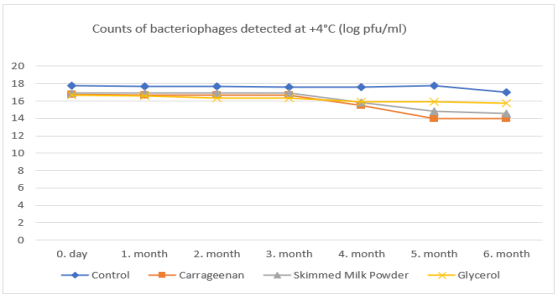


Figure 1. Counts of bacteriophages detected at +4°C.

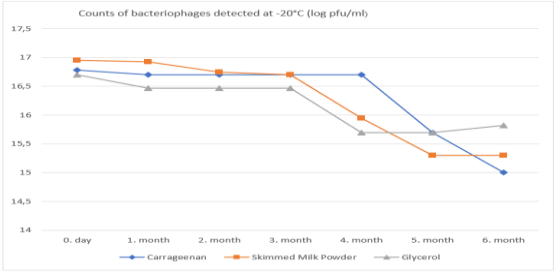


Figure 2. Counts of bacteriophages detected at -20°C.

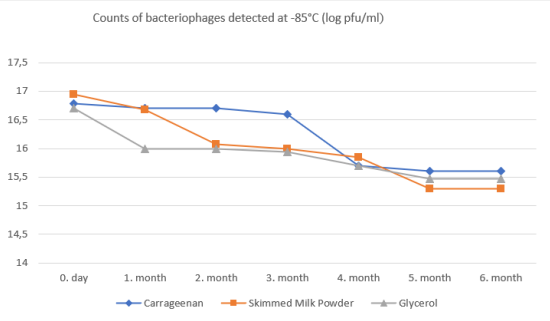


Figure 3. Counts of bacteriophages detected at -85°C.

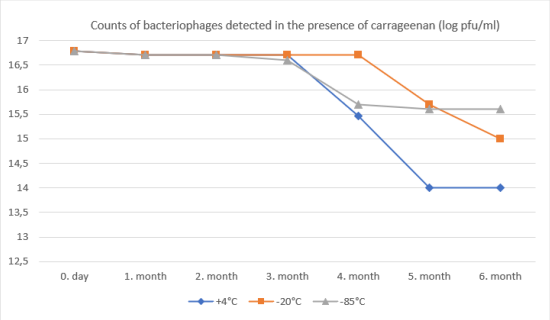


Figure 4. Counts of bacteriophages detected in the presence of carrageenan.

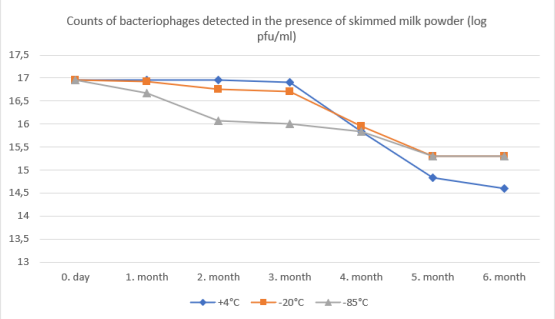


Figure 5. Counts of bacteriophages detected in the presence of skimmed milk powder.

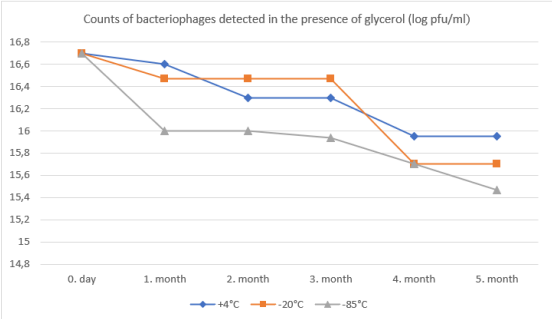


Figure 6. Counts of bacteriophages detected in the presence of glycerol.

DISCUSSION AND CONCLUSION

In a study investigating the long-term preservation of bacteriophages with standard cooling without a decrease in phage activity; the stability of 5 different bacteriophages (JHP, RLP, RSP, SaPL, IPL) were examined one year after being frozen at different temperatures (-20°C, -80°C, -196°C). In the study, 50% glycerol was used for preservation at -20°C and -80°C. When bacteriophages stored at 4°C were checked after 1 year, it was observed that there was no decrease in their titers (Clark et al., 1962; Clark and Geary., 1973; Verbeken, 2015; Alvi et al., 2018). According to the study, An 84-100% decrease (from 8 log units to 17 log units) was recorded in the titers of phages stored at 37°C. Similarly, a 69-100% decrease (from 8 log units to 15 log units) was observed in the titers of phages stored at 25°C. While a decrease from 5 log units to 12 log units (between 25-70%) was recorded at -20°C, a decrease between 45-100% was observed at -80°C (Alvi et al., 2018). In the study, a loss of stability was noted in freezing conditions at -20°C and -80°C, while at 25°C and 37°C, very little recovery was achieved after 12 months. As a result, the most reasonable temperature for storing tailed bacteriophages was reported as +4°C which is compatible with the findings of our study.

In another study conducted on the long-term preservation of bacteriophages, the effect of the structure of the phage on stability was investigated (Ackerman et al., 2004). Lysates were maintained at +4°C and -80°C (in liquid nitrogen) with glycerol. Lysogenic phages were stored at -80°C with 15% glycerol. According to studies, it has been stated that lipid-free, tailed cubic structured, or filamentous phage lysates can be stored for 5-10 years, but the titers will decrease by 1 log per year (Vieu and Croissant 1966; Steele, 1976), while it has been stated that more meticulous studies are required for lipid-containing phages. It has been stated that chloroform should not be preferred for storage because it inactivates one third of tailed and lipid-containing filamentous phages (Ackherman et al., 2004). Unlike our study findings, it has been stated that lysates should not be stored alone at +4°C and should be supported by lyophilization and deep freezing. In the study, the survival of the phages was not guaranteed even in deep freezing at -80°C or in liquid nitrogen (Ackhermann et al., 2004).

In a study on four different *Staphylococcus* bacteriophages, the effect of storage at different temperatures using the lyophilization method and different stabilizers on stability was compared. In the study, philPLA35 and philPLA88, which belong to the *Siphoviridae* family, and philPLA-RODİ and philPLA-CIC bacteriophages, which belong to the Myoviridae family, were used. Phages were preserved in the presence of trehalose, sucrose, glycerol, and skim milk. In the study, phages were stored at 20-25°C for 6 months, at 4°C, -20°C, and -80°C for 24 months, and at -196°C for 12 months. Stability was lost in phages after 6 months at 20-25°C. In *Siphoviridae*, stability was preserved at -20°C for all stabilizers (there was a decrease in infected philPLA88). This finding is different from our study findings since a decrease was detected in all cryoprotectant added phage (*Myoviridae*) groups at -20°C after 6 months of storage. It is thought that this difference may be due to the classification of the tested phages in different families. Similarly in the study, *Myoviridae* phages, in which sucrose and trehalose were used as stabilizers, could not maintain their stability at -20°C and a decrease in titer was observed. No serious loss of stability was observed for all phages in the presence of all stabilizers at -80°C. For *Siphoviridae* and *Myoviridae*, decreases of less than 1 log unit were observed after 24 months. Similar results were recorded after 12 months in samples stored at -196°C in

liquid nitrogen (Gonzales-Menendes et al., 2018). In this study, it was clearly observed that the results of *Siphoviridae* phages were different from our study, but similar findings were obtained with *Myoviridae* phages.

In another study, the effects of preservatives and storage temperature on the stability of phages preserved using the lyophilization method were compared. Three different phages used in this study (ECP311 against *Escherichia*, KPP235 against *Klebsiella* and ELP140 against *Enterobacter*) were tested on the 0th, 1st, 5th, 10th, 15th, and 20th day at +4°C and 37°C in the presence of glucose, sucrose, mannitol, gelatine, sorbitol, and polyethylene glucose as cryoprotectant substances. As a result of the study, it was observed that sucrose, gelatin, and their combinations were successful in preserving phage viability after lyophilization. In the study, while viability was preserved for up to 20 months at 4°C, a decrease in activity was noted after 10 months at 37°C (Manohar and Ramesh, 2019).

In a different study which was conducted comparing the effects of encapsulation and microencapsulation on stability, and it was observed that microencapsulation provided better stability for phages. Alginate encapsulation and microencapsulation methods were used in the study. In the study, 4 different bacteriophages stored at +4°C and +20°C were checked in the 1st, 2nd, and 3rd months. Only philPLA-RODI maintained its stability when encapsulation was performed at +4°C. All bacteriophages lost their stability at +20°C. In microencapsulation application, while all phages preserved their hyphens at +4°C, stability losses occurred at -20°C (Gonzales-Menendes et al., 2018). These results showed that cold storage (+4°C) can be used effectively for the preservation of these types of phages instead of frozen storage (-20°C or -85°C), which requires higher energy costs and special devices.Similar to these studies; it was observed that stability was maintained at all tested temperatures (+4°C, -20°C, and -85°C) and with all tested cryoprotectants (glycerol, skimmed milk powder, and carrageenan) during the first two months. However, in the third and fourth months, a decrease in glycerol was observed at -85°C. In the fifth month, carrageenan provided the highest preservation at all temperatures. In the sixth month, +4°C glycerol provided the best preservation, while carrageenan provided the highest preservation at other temperatures.

As a result, when the effects of storage temperatures on the stability of the lytic M8AEC16 phage against *Escherichia coli* O157:H7 were evaluated, the most successful result was obtained in storage without cryoprotectant at +4°C. In the cryoprotectant groupsthe best preservation was determined at +4°C and -20°C in the presence of glycerol. In addition to this study, when the results of the published articles were examined, it was observed that there is no correlation with the findings of the studies that used similar conditions for the storage of bacteriophages. It is thought that these differences are mostly due to the type of bacteriophage. For this reason, it was concluded that it is important to determine the appropriate storage conditions for each phage included in the culture collection and stocked for use as a biocontrol agent.

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Conflict of Interest
The authors declared that there is no conflict of interest.

Authorship Contributions
Concept: N.F.A, N.D.A, Design: N.F.A, N.D.A, Data Collection or Processing: N.F.A, N.D.A, Analysis or Interpretation N.F.A, N.D.A, Literature Search N.F.A, Writing: N.F.A, N.D.A.

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