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Vibrio parahaemolyticus ATCC 17802 inactivation by using methylcellulose films containing encapsulated bacteriophages

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Abstract: In the present study, it was aimed to inactivate *Vibrio parahaemolyticus* ATCC 17802, which is an important human pathogen, by making use of edible methylcellulose films containing encapsulated bacteriophages. For this purpose, the bacteriophages used as biocontrol agents specific for *V. parahaemolyticus* ATCC 17802 were isolated from raw bonito fishes (*Sarda sarda*) and then purified, and finally the stabilities of isolated bacteriophages were determined. The pure bacteriophage filtrates were encapsulated with sodium alginate by employing an extrusion method and the edible methylcellulose films were prepared. The bacteriophage stabilities of the prepared films were analyzed on the 0th, 4th, 7th, and 14th days of storage in a refrigerator (4 °C) under darkness and in illuminated room (22 °C) conditions. The methylcellulose films were determined to release bacteriophages to the environment and into raw fish fillets and antimicrobial effects of these films were detected against *V. parahaemolyticus*. In conclusion, a specific antimicrobial effect on *V. parahaemolyticus* ATCC 17802 was achieved by using edible methylcellulose films containing encapsulated bacteriophages. The results of microbial growth analysis showed an approximately 2.65 log difference in microbial levels between the control and the treatment samples. By maintaining the bacteriophage stability of prepared films, a new film that reinforces bacteriophage release was developed.

Key words: Encapsulation, bacteriophage, *Vibrio parahaemolyticus*, edible film, inactivation

1. Introduction

Active packaging materials are inert packaging materials, the formula of which might contain both antimicrobial polymers such as chitosan and encapsulated antimicrobial agents (1). When methylcellulose (MC) films containing encapsulated antimicrobial agents are employed in various food-packaging applications, they are considered a subclass of active packaging materials. The advantage of MC films is the use of agricultural biomaterials in packaging (2). Nowadays, many of the active packaging materials exhibit wide-spectrum antimicrobial properties that do not address a single specific pathogenic strain. It is very necessary to develop new antimicrobial packaging materials protecting the commensal flora and showing high specificity against only pathogenic organisms. The need for specificity in antimicrobial activity, especially against the pathogens that constitute a small portion of the total microorganism load on foodstuffs, is very important (3). The improved pathogen specificity of antimicrobial active packaging materials might increase the antimicrobial activity by reducing the interaction with nontargeted microorganisms (4). The present study differs from other studies in the literature as it uses pathogen-specific bacteriophages as biocontrol agents.

In all of the previous studies on biocontrol using bacteriophages, the microorganisms were inoculated on the surface of foodstuff, and the application of phages on the inoculated surface was performed by spraying the phages. As is known, the target pathogens might be spread across the surface or localized at any point, and they might also be located on one or more surfaces of the foodstuff. This reduces the effectiveness of phage therapy. In order to improve the use of phages beyond spraying the phages directly on the surface of foodstuffs, new material formulations having content such as encapsulated phages, which would improve the phage stability and ensure their release onto the targeted locations, are needed. Thus, the importance of phages as an additive in formulations of packaging materials will increase (5).

In the present study, the objective is to examine the phage encapsulation, stability, and releasing capabilities of MC films in order to develop bacteriophage-based edible antimicrobial films. The primary reason for choosing MC films as a model is the suitability of these films for the use of various antimicrobials such as essential oils, nisin, and lacto-peroxidase (6). In this study, it was aimed to meet the need for developing pathogen-specific edible films by

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combining edible films and bacteriophages having potential as biocontrol agents. The specific MC edible films were examined from the aspects of stability, dispersion, release, and antimicrobial effectiveness of phages. Moreover, the inactivation of *Vibrio parahaemolyticus*, which is an important human pathogen, will be performed by making use of edible MC films containing bacteriophages.

2. Materials and methods

2.1. Materials

The bacteria culture used in this study comprises the *Vibrio parahaemolyticus* ATCC 17802 strain. The raw fish samples (approx. 30 fish) required for phage isolation were obtained from fresh bonito fishes (*Sarda sarda*) sold in a Giresun fish market, and they were then immediately transferred to the laboratory under the necessary conditions (in iceboxes) for analyses within 24 h. The bonito fish were prepared as fillets of 100 g and then used in analyses.

2.2. Bacteriophage isolation and purification

Bacteriophage isolation and purification was done according to Kim (7) and Soykut (8). For the isolation of bacteriophages, 0.1 mL of host bacterium culture was added to the raw fish samples and they were incubated at 37 °C for 48 h for phage development. After the incubation, the samples were centrifuged (12,000 rpm; 10 min at 4 °C) and the phage supernatant obtained was passed through a 0.22-µm membrane filter. Then the filtrate was mixed with soft agar prepared by using equal amounts at 50 °C and then the mixture was dripped onto the prepared medium (5 g peptone, 3 g yeast extract, 5 g dissoluble starch, 10 mL 0.1 $MgSO_4$, 10 mL 0.1 M $CaCl_2$, 15 g agar agar, and 1000 mL distilled water, pH 7.2). The petri plate was checked in terms of plaque formation (lysis) after a night of incubation. A single plaque that was well separated was removed carefully from the agar by using a sterile loop and dissolved in prepared phage buffer (SM buffer solution; 5 g NaCl, 2 g $MgSO_4$, 50 mL 1 M Tris-HCl (pH 7.5), 5 mL 2% a/h gelatin solution, 1000 mL distilled water). In order to purify them, the phage stocks were incubated in ice with 10% polyethylene glycol for 1 h for precipitation of phage particles. For the removal of cell debris, the phage supernatants were centrifuged at 5000 rpm for 10 minutes at 4 °C and then mixed by adding them into preprepared SM buffer solution and chloroform making use of a vortex. The resultant supernatants were passed through a filter with 0.45-µm pores, and the pure filtrate was achieved by adding 10% chloroform (7,8).

2.3. Determining the bacteriophage specificity of *Vibrio parahaemolyticus*

The *V. parahaemolyticus* ATCC 17802 strain (10^6 CFU/mL) was added into TCBS agar medium (Merck 1.10263), and then the agar was allowed to harden. The phage filtrates (10^8–10^9 PFU/mL; 10 µL) were dropped onto the hardened agar, and then the petri plates were left for incubation. At the end of the incubation period (37 °C for 24 h), the formation (+) of clear plaque (lysis) at the points of dropping was accepted to indicate the bacterial sensitivity to the dropped phage, whereas the absence (-) of plaque formation was accepted to represent the resistance of the bacterium to the tested phage (8–10).

2.4. Preparation of methylcellulose films containing encapsulated bacteriophages

While preparing the film solution, 4 g of MC (Sigma Chemical; St. Louis, MO, USA) was diluted in 50 mL of distilled water and then heated to 90 °C. Glycerol (1.6 mL) was added to the dilution as a plasticizer. During stirring, 0.1 mL of antifoaming agent (Silicon Antifoam, Sigma; CN: 85390) was added in order to prevent the formation of air bubbles (11). The suspension containing 10^9 PUF/mL bacteriophages to be used in preparation of the film was added to the sterile coating material solution (0.5% a/h sodium alginate solution) at a 1/5 ratio. The obtained mixture was injected into 0.05 M $CaCl_2$ by using a 0.11-mm nozzle needle. The obtained capsules were left in the solution for 30 min in order to give them sufficient hardness, and then they were kept at 4 °C in sterile water containing 0.1% peptone after filtering (12,13).

After adding 10 mL of encapsulated phage suspension to the film solution, the volume was increased to 100 mL. The film solutions prepared were poured into sterile petri plates of 10 mL each, and then left for drying at 25 °C for 24 h at 50% relative humidity in order to obtain the antimicrobial films (5,11,14–16).

2.5. Determining the bacteriophage stability in methylcellulose films

The bacteriophage stability of MC films was tested under darkness and in refrigerator (4 °C) and illuminated room (22 °C) conditions. On the 0th, 4th, 7th, and 14th days of storage, the quartiles of round-shaped MC films were cut and added into 50 mL of distilled water by crumbling. In order to release the bacteriophages from the slivered film particles in distilled water, they were mixed at the room temperature (22 °C) for 8 h by using a mid-speed shaker. At the 4th, 6th, and 8th hours of shaking, the MC residuals were filtered and the filtrate was analyzed in order to determine the number of active bacteriophages particles that had been released. In order to compare the analysis results, 1 mL of 1×10^9 PFU/mL isolated and purified *V. parahaemolyticus* bacteriophages within the buffer was dried on a petri plate. For each of the storage periods, the phage-coated petri plates (2 petri plates for each storage period) were incubated in sterile water at 22 °C for 1 h in order to separate the phages from the surface. After the incubation, the water in the petri plates was removed and the residual was analyzed in order to determine the active bacteriophage particles (5).

2.6. Determining the bacteriophage release from methylcellulose films into the environment

In order to determine the phage release profile of MC films within the aqueous solution, disks of 1.5 cm were randomly cut from the films. The disk films that were cut were then placed into distilled water that was not mechanically shaken. The films were taken from the water at the 1st, 2nd, 3rd, 4th, and 5th hours and the resultant solution was analyzed for PFU counting in order to determine the number of phages released into the water (5,17).

In order to determine the phage release in raw fish fillets, the raw fish fillets were divided into small pieces that could contact the 1.5-cm disks. In order to facilitate the contact between bacteriophage-containing film disks and raw fish fillets, the raw fish fillets were washed with 10 mL of sterile water. At the 1st, 2nd, 3rd, 4th, and 5th hours, the raw fish fillets were separated from the disk films. Then the disk films were placed into 9 mL of water and stirred at 250 rpm for 10 min. The washing water was taken as a sample in order to count the active phages released from films and the number of released bacteriophages was determined (5).

2.7. Determining the antimicrobial effect of methylcellulose films containing bacteriophages

Bacteriophage-containing MC (B-MC) films and the control films containing no bacteriophages were placed in the wells of cell-culture test plaques having flat bottoms for determining the antimicrobial effect in vitro. *V. parahaemolyticus* culture (1 mL) with diluted alkali peptone medium was added to each of the wells at 3 log CFU/mL concentration. In order to ensure the vaporization of medium, the tops of the cell-culture test plaques were closed and then kept at room temperature (22–25 °C) for 24 h. The beginning inoculum samples taken at the 0th hour and the inoculum samples taken at the 24th hour were disseminated on TCBS agar medium (Merck) and incubated for 18 h at 35 °C. After they became visible (small blue-green centered colonies), the colonies were counted and expressed in log CFU/mL (5,18,19).

In order to determine the antimicrobial effects of B-MC films on *V. parahaemolyticus* in raw fish fillets, *V. parahaemolyticus* was inoculated on raw fish fillet samples at 10^4–10^5 CFU/cm^2 (11). The prepared samples, after inoculation was completed, were placed between same-sized films and then wrapped carefully. All the prepared samples were packaged with flexible and low-density polyethylene film, and the packages were closed using a pyrolytic plating device. The samples were stored in the refrigerator and the analyses were performed during the storage period (15,20). All analyses were carried out in 2 parallels and 3 repeats in accordance with the FDA Bacteriological Analytical Manuel using TSCB agar (21).

2.8. Statistical analyses

The statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Unilateral variance analysis (ANOVA) was used in comparing the significance of experiments, whereas the Duncan multiple comparison test was employed in comparing the differences of groups ($P \leq 0.05$) (22).

3. Results

3.1. Bacteriophage stability of methylcellulose films

The bacteriophage stability results of the control group (plastic surface) and B-MC films at the 0th, 4th, 7th, and 14th hours of days during the 14-day storage period under darkness and in a refrigerator are presented in Figure 1. The bacteriophage stability of B-MC films and the control group (plastic surface) under room (22 °C) and illumination conditions on the 0th, 4th, 7th, and 14th days of storage are presented in Figure 2.

3.2. Bacteriophage release of methylcellulose films

In order to investigate the phage release from B-MC films, the films samples were interacted with water and raw fish fillets. In Figure 3, the release of phages from water and raw fish fillet surfaces is presented.

3.3. Antimicrobial inhibition of methylcellulose films

The antimicrobial effects of B-MC films on *V. parahaemolyticus* under in vitro conditions are presented in Table 1. The antimicrobial effect of B-MC films on *V. parahaemolyticus* in raw fish fillets was determined and is presented in Table 2.

4. Discussion

One of the main difficulties in developing bacteriophage-based packaging materials is the difficulty of ensuring the stability of phages in packaging formulations. The phage stability in active packaging materials is a critical necessity for the successful integration of phages in packaging materials and food systems (5). From the aspect of bacteriophage stability, the difference between a refrigerator (4 °C) and dark and lighted room conditions (22 °C) during the storage period was statistically significant ($P \leq 0.05$). Under all conditions, the most significant decrease in encapsulated bacteriophage stability was observed on the 7th and 14th days. Although the data obtained in this study showed similarities with those obtained by Vonasek et al. (5), the bacteriophage stability was found to be lower at 22 °C in the lighted environment in this study. In the work of Vanosek et al. (5), only 1 log unit decrease was observed in films prepared with T4 encapsulated bacteriophages (host strain: *E. coli* BL21) and whey during a 5-week storage at 22 °C and in a lighted environment, whereas a decrease of 4 log units was observed in the present study. In order to evaluate the effectiveness of MC film for stability of bacteriophages more accurately, the

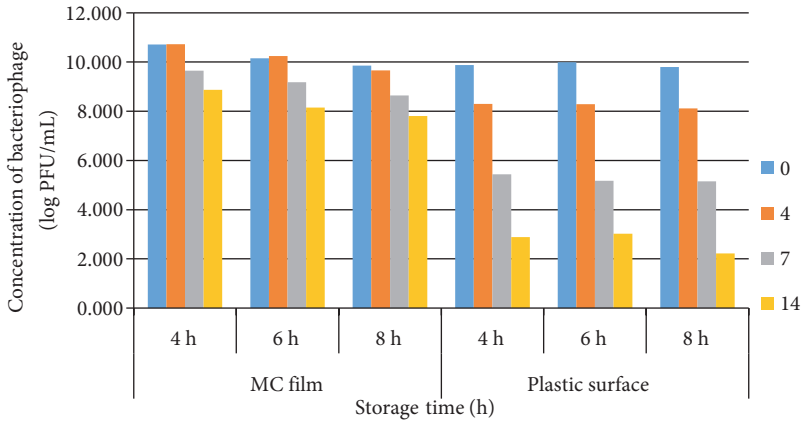

Figure 1. Bacteriophage stability of methylcellulose films containing encapsulated bacteriophages (4 °C, in darkness).

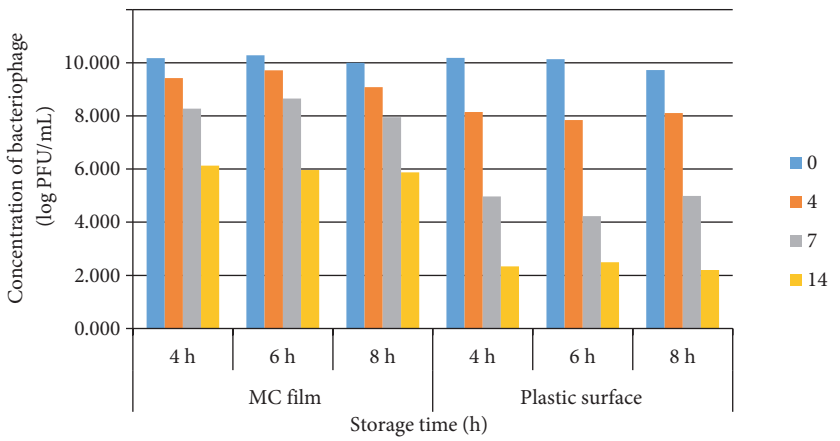

Figure 2. Bacteriophage stability of methylcellulose films containing encapsulated bacteriophages (22 °C, illuminated room conditions).

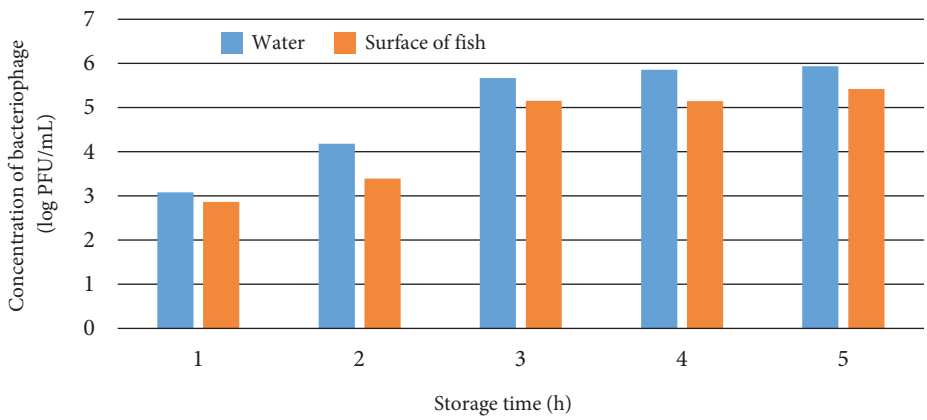

Figure 3. Bacteriophage concentration released to water and fish surfaces from methylcellulose films (log PFU/mL).

phages from the control polystyrene plastic surface were collected. Polystyrene was chosen because it is a popular packaging material. According to the analysis results, the storage of phages precipitated on a plastic surface under room and refrigerator conditions led to significant phage loss.

Table 1. The antimicrobial effects of the control and bacteriophage-containing methylcellulose films in vitro (log CFU/mL).

Storage period (h)	Control MC film*	MC film + bacteriophage*
0	3.39 ± 0.105^{aA}	3.39 ± 0.105^{bA}
24	6.11 ± 0.344^{bB}	2.12 ± 0.187^{aB}

*Mean and standard deviation; mean values with different lowercase letters indicate significant differences ($P \leq 0.05$) within a column; mean values with different uppercase letters indicate significant differences ($P \leq 0.05$) within a row.

Table 2. The antimicrobial effects of the control and bacteriophage-containing methylcellulose films in raw fish fillets.

Storage period (day)	Control MC film*	MC film + bacteriophage*
0	4.63 ± 0.183^{aA}	4.62 ± 0.172^{dA}
4	5.20 ± 0.197^{bB}	2.62 ± 0.581^{cB}
7	7.20 ± 0.133^{cC}	1.77 ± 0.129^{aBC}
14	8.43 ± 0.216^{dD}	1.97 ± 0.078^{bC}

*Mean and standard deviation; mean values with different lowercase letters indicate significant differences ($P \leq 0.05$) within a column; mean values with different uppercase letters indicate significant differences ($P \leq 0.05$) within a row.

Bacteriophages cannot survive for a long period without any host cells, because they do not have their own metabolisms and are host-specific intracellular parasites (23). The possible reason for the loss of bacteriophage stability on films is that the films were not in contact with foodstuff and thus there was no contact between bacteria and bacteriophages. This is the main reason for the decrease in the number of bacteria under room and refrigerator conditions during the study. Despite that, the main reasons for B-MC films to offer higher bacteriophage stability under room and refrigerator conditions is thought to be the use of glycerol (plasticizer) and sodium alginate in bacteriophage encapsulation in our study. It is believed that sodium alginate is useful in stabilizing the capsids in drying the MC films. The previous studies showed the importance of the use of proteins, surfactants, and organic materials for maintenance of viral stabilization (24–26). In the present study, the release of phages was 1.5×10^5 PFU/mL during the first 30 min and in total 1.6×10^7 PFU/mL at the end of 5 h of incubation, similar to the results obtained by Ma et al. (27), who reported bacteriophage release from alginate macromolecules. Again, there is a similarity with the study of Vonasek et al. (5), reporting the release of T4 bacteriophages from whey isolate films. In order to optimize the phage stabilization properties of MC films, more studies are needed on this subject.

As a result of the analyses of the antimicrobial effect on *V. parahaemolyticus* under in vitro conditions, no antimicrobial effect was observed in control films. When comparing the 0th hour to the 24th hour, there was an increase by 2.72 log units. The B-MC films yielded a decrease by 1.27 log units in the number of *V. parahaemolyticus* at the end of the 24-h period ($P \leq 0.05$). As presented in Table 2, no antimicrobial effect was achieved from control films against *V. parahaemolyticus* and the number of *V. parahaemolyticus* on the 14th day of storage at refrigerator temperature increased by approximately 3.80 log units. A gradual decrease was determined in the number of *V. parahaemolyticus* during storage using B-MC films for the fish fillets. On the 14th day of storage, a decrease by approximately 2.65 log units was found. By using the B-MC films, an effective inactivation could be achieved against *V. parahaemolyticus* at the end of 14 days of storage. Given the fact that the samples were stored at refrigerator temperature, it can be seen that the inactivation effect obtained at room temperature (the inactivation obtained in the first 24 h under in vitro conditions) was higher. In harmony with the literature, this can be explained by the higher lysis activity provided by bacteriophage activity at optimum development temperature of the host bacterium. Storage at 4 °C inactivates the development of *V. parahaemolyticus*, and it is also a factor that limits bacteriophage activity.

In conclusion, the B-MC films yielded successful inactivation of *V. parahaemolyticus* 17802 during 14 days of storage. Moreover, it should be noted that the inhibition effect that was obtained was a pathogen-specific effect. The antimicrobial edible films are not expected to have inhibition effects on spontaneous saprophytic flora and other pathogen strains that might develop due to the food processing and storage conditions. The finding that the obtained films have antimicrobial effect is not solely enough for the appropriateness of their use as packaging material. The physicochemical properties of the manufactured films (thickness, tensile-prolongation strength, water permeability value, oxygen permeability value, etc.) should be determined and the films should be characterized.

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