KOREAN J. FOOD SCI. TECHNOL. Vol. 51, No. 6, pp. 604~609 (2019)
https://doi.org/10.9721/KJFST.2019.51.6.604

KOREAN JOURNAL OF
한국식품과학회지
FOOD SCIENCE AND TECHNOLOGY

Isolation of bacteriophages having depolymerase and control of pathogenic *E. coli* O103 in biofilm on lettuce

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Abstract To control pathogenic *E. coli* in biofilm, bacteriophages were isolated from environmental samples. Seventeen isolates had depolymerase activities by translucent zones at the rims of plaques. To determine biofilm-forming ability, an abiotic plastic surface of polystyrene was used; *E. coli* O103 showed the highest biofilm formation at 30°C after 24 h. Moreover, biofilm by *E. coli* O103 on the biotic surface of lettuce was observed by a scanning electron microscope. The bacteriophage cocktail of ΦNOECP40 and ΦNOECP44 showing depolymerase activities was prepared to eliminate the *E. coli* inbiofilm. By organic acids, reduction of *E. coli* in biofilm was insignificant and almost undetectable. However, the abundance of *E. coli* in biofilm was reduced by 3 log CFU/mL from 7.3 log CFU/mL after 60 min with the bacteriophage cocktail. Therefore, we suggest that bacteriophages with depolymerase could be utilized to effectively control pathogenic *E. coli* in biofilm.

Keywords: non-O157 *E. coli*, biocontrol, biofilm, bacteriophage, depolymerase

Introduction

Hemolytic-uremic syndrome (HUS) causes acute renal failure in children and adults, followed by infection with shiga toxin-producing *Escherichia coli* (STEC) (Karmali et al., 1983). *E. coli* O157: H7 is one of the pathogenic *E. coli*, which produces shiga toxin after adhering to intestinal mucosa and causes hemorrhagic colitis, and is the predominant cause of HUS worldwide (Lee et al., 2015). Although *E. coli* O157: H7 is the most prevalent pathogenic STEC serotype and many studies on this serotype have been reported, non-O157 STEC serotypes, including O26, O103, O111, O113, and O121, are also commonly associated with this disease (Acheson, 2000). Illnesses linked to STEC serotypes other than O157:H7 appear to be on the rise worldwide, indicating that some of these organisms may be emerging pathogens (Mathusa et al., 2010). It is estimated that 20 to 50% of all STEC infections can be attributed to non-O157 strains, but the percentages differ greatly from country to country and among regions within a country (Johnson et al., 2006; Nataro et al., 1998). Estimation of the true percentage of infections caused by non-O157 STEC strains is difficult, because these strains are not routinely subjected to testing and previous research mainly focused on the O157 serotype *E. coli* (Nataro et al., 1998). Both O157 *E. coli* and non-O157 *E. coli* form a biofilm for survival, posing problems of contamination in the food industry that are increasing day by day (Lee et al., 2015; Park et al., 2018).

Biofilm matrix is composed of polysaccharides, proteins, nucleic acids, and lipids. The composition varies depending on the environmental conditions such as the microbial species, temperature, and the available nutrients (Davey and O'toole, 2000; Vu et al., 2009). However, a biofilm consists of mainly exopolysaccharides (EPS) released by microorganisms into extracellular spaces, which adhere to cell surfaces. Consequently, microorganisms surrounded by the produced EPS becomes a dense community (Donlan, 2002). Especially in the food industry, biofilm formation on food surfaces and drinking water distribution systems increases the risk of contamination of the related products (Rob et al., 2005).

When a biofilm is formed, microorganisms become more resistant to the external environment and are physically protected from antibiotics or disinfectants (Stewart, 2001). Furthermore, microorganisms in biofilm are reported to be up to 1000 times more resistant to antibiotics (Niels et al., 2010). Methods that are used to remove biofilm are mechanical and manual cleaning, chemical cleaning, and the use of hot water (Chmielewski et al., 2003; Quan et al., 2017). It was observed that biofilm non-removed due to improper washing and disinfection increased bacterial resistance and appeared to be resistant to antibiotics (Sidhu et al., 2001, Simões et al., 2006). Moreover, residual biofilm caused chemical and biological reactions that led to metal corrosion and serious hygiene problems, as well as economic losses due to damage to food and equipment (Bremer et al., 2006; Gram et al., 2007).

Organic acid sanitizers that comply with the GRAS category decontaminators against foodborne pathogens have been used as antimicrobial treatments for food products (Akbas and Olmez, 2007; Lin et al., 2002; Raynaudi-massilia et al., 2009). However, new control strategies are constantly emerging in recent times (Simões et al., 2006). Bacteriophages are viruses that infects

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Received October 10, 2019; revised November 14, 2019; accepted November 25, 2019

bacteria and are widely distributed in nature. The special characteristics of the lytic bacteriophages; in particular, their target specificity, rapid bacterial killing, and ability to self-replicate, make them appropriate for applications to food protection (Denis et al., 2013); some studies also suggested that they might act as antimicrobial agents (Capparelli et al., 2007; McVay et al., 2007). In particular, bacteriophage-encoded depolymerase, an enzyme that degrades macromolecules, can easily have access to host bacteria by degrading polymers present on microbial surfaces such as EPS (Cornelissen et al., 2011; Yan et al., 2014). In addition, several studies have shown that the pure depolymerase was isolated from a bacteriophage suspension (Hernandez-Morales et al., 2018; Hughes et al., 1998), suggesting that the depolymerases may have a commercial potential. More than 7 reaction-types among 160 putative depolymerases were discovered from 143 phages infecting various bacteria (Latka et al., 2017; Pires et al., 2016). However, there are few reports on the mechanisms of action of pathogenic non-O157 STEC, which is one of the most deadly pathogens related to disease outbreaks in food industry in recent times.

Therefore, in this study, the bacteriophages having depolymerase were isolated and utilized to control pathogenic non-O157 *E. coli* in biofilm and to reduce bio-hazards posed to food.

Materials and Methods

Isolation of bacteriophage

Three non-O157 STEC, namely, *E. coli* NCCP 14018 (serotype O26), *E. coli* NCCP 13937 (serotype O103), *E. coli* NCCP 13979 (serotype O69), and two *E. coli* O157:H7 of *E. coli* NCTC 12079 and *E. coli* 505B were used as the host bacteria for bacteriophage isolation. These strains were subcultured on Eosin Methylene Blue agar (EMB, Becton, Dickinson and Company, NJ, USA) at 37°C for 24 h. Single colonies of the strains were cultured in 5 mL Luria Bertani (LB) broth with 10 mM $CaCl_2$ (LBC, Difco Laboratory, Detroit, MI, USA) at 37°C for 24 h. For the isolation of bacteriophages from the environment, 25 samples collected from animal feces, wastewaters, and soils were used as the environmental samples. Twenty-five milliliters of each sample was mixed with 225 mL of LBC broth, after which 1 mL of the host culture in stationary phase was inoculated and the sample was cultured at 37°C at 150 rpm (Jeio Tech, Daejeon, Korea) for 24 h. Later, the culture was centrifuged at 10,000×g for 10 min, and the supernatant was filtered with a 0.22 μm syringe filter (Millipore, Billerica, MA, USA). The plaque assay was performed by the double overlay agar method on LBC agar (Kropinski et al., 2009). Each sterile supernatant was mixed with 0.6% LBC soft agar and 100 μL host strain, and then overlayed on the LBC agar and cultured at 37°C for 24 h. The resultant plaque was separated and purified with SM buffer [100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), 0.01% gelatin], and a 10% glycerol stock was stored in a 80°C deep freezer.

Selection of bacteriophages having depolymerase

To obtain plaques with translucent zones from the isolated bacteriophages, 100 μL of the host strains cultured in LBC broth and 100 μL of bacteriophage suspension were mixed and incubated at 37°C for 1 h with shaking. Subsequently, it was mixed with 0.6% LBC soft agar, poured on the LBC agar, and cultured at 37°C for 24 h. The apperances of the plaques were confirmed and the bacteriophages showing translucent rims around clear plaques were selected (Cornelissen et al. 2011).

Biofilm formation of *E. coli* on polystyrene plastic surface and lettuce

An abiotic microtiter plate assay was performed to investigate the optimum conditions of biofilm formation of the five pathogenic *E. coli* strains. Each strain of *E. coli* was inoculated into 10 mL of Tryptic soy broth (TSB, Becton, Dickinson and Company) and cultured overnight at 37°C to prepare a bacterial suspension of 9 log CFU/mL. A diluted bacterial suspension of 7 log CFU/mL was also prepared by diluting the culture with TSB. The two suspensions were inoculated into each well of a 24-well tissue culture plate (SPL Life Sciences Co., Gyeonggi, Korea) to make the volume up to 2 mL. After incubation for 24 h at temperatures of 15, 20, 25, 30, 35, and 42°C, the bacteria and the culture solution were removed from the test plate. Wells were thoroughly washed with PBS buffer three times to remove materials other than the biofilm and dried completely at 60°C for 1 h to allow the biofilm to adhere to the well surface. After drying, the biofilm was stained with a crystal violet solution for 30 min and then washed twice with PBS to completely remove the crystal violet solution residue. Ninety-five percent (v/v) ethanol was added to elute the crystal violet stain from the biofilm, and the absorbance was measured at 580 nm (O'Toole, 2011).

To test the formation of *E. coli* biofilm in biotic food material, lettuce was selected as the target sample. The outer leaf of the lettuce was peeled, washed with water more than three times, cut into pieces of 2 cm by 2 cm, and sterilized with 70% ethanol for 15 min. Each sample along with 30 mL of TSB was placed in a sterile 50 mL conical tube and 100 μL of *E. coli* of about 9 log CFU/mL in TSB was inoculated and the sample cultured at 30°C for 24 h.

Later, the pieces of lettuce were removed from the tubes, placed in sterile 100 mL bottles, and washed with 50 mL of PBS buffer (pH 7.4) by shaking at 150 rpm for 5 min. The pieces were fixed with a fixative (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) at 4°C for 24 h. Pretreatment of samples for SEM was performed by using a modified method of Morris et al. (1997). After fixation, 1% osmium tetroxide was added and the pieces incubated at 4°C for 2 h before being washed with sterile distilled water. Further, the samples were dehydrated in serial dehydration solvents of 50, 70, 80, and 90% ethanol for 10 min. Finally, they were dehydrated twice using hexamethyldisilazane for 15 min. After platinum coating, the biofilm adhering to the vegetable pieces was observed using SEM (JSM-5410LV, Jeol Ltd., Tokyo, Japan).

Reduction of *E. coli* in biofilm on lettuce

To improve the biofilm-degrading ability of bacteriophages, two different phage suspensions of each 8 log PFU/mL were mixed

and the combination was used as a bacteriophage cocktail. The bacteriophage cocktail was composed of two different bacteriophages: ΦNOECP 40 (8 log PFU/mL) and ΦNOECP 44 (8 log PFU/mL). The bacteriophage cocktail was spotted onto *E. coli* NCCP 13937 present in biofilm on pieces of lettuce (2 cm×2 cm) and cultured at 37°C for 0, 5, 15, 30, and 60 min. LBC broth was used similarly to represent a negative control. After incubation, the lettuce pieces were washed three times in sterile saline to remove the phage cocktail solution, and homogenized in a stomacher bag with 25 mL of PBS buffer for 3 min. The suspension was recovered, diluted, and spread on LBC agar.

Organic acid represented by 0.1% lactic acid was spotted onto the *E. coli* NCCP 13937 present in biofilm on pieces of lettuce for 0, 5, 15, 30, and 60 min. After the treatment, the lettuce pieces were washed three times in the sterile saline to remove the organic acid and homogenized in a stomacher bag with 25 mL of PBS buffer for 3 min. The PBS buffer was recovered, diluted, and spread on LBC agar.

Results and Discussion

Isolation of bacteriophages having depolymerase

The bacteriophages present in the samples were isolated from single plaques that showed translucent rims by using five *E. coli* hosts. Bacteriophages having depolymerase are distinguished by the presence of translucent zones at the rim of the clear plaques (Cornelissen et al. 2011). In the halo zone, phage replication stops or slows down as the bacteria enter a stationary phase; however, the tail spikes of the phage continue to depolymerize the bacterial EPS, thus increasing transparency at the rim of the clear halo zones (Cornelissen et al. 2012). Therefore, the presence of bacteriophages encoding depolymerase could be confirmed by clear plaques surrounded by a translucent halo.

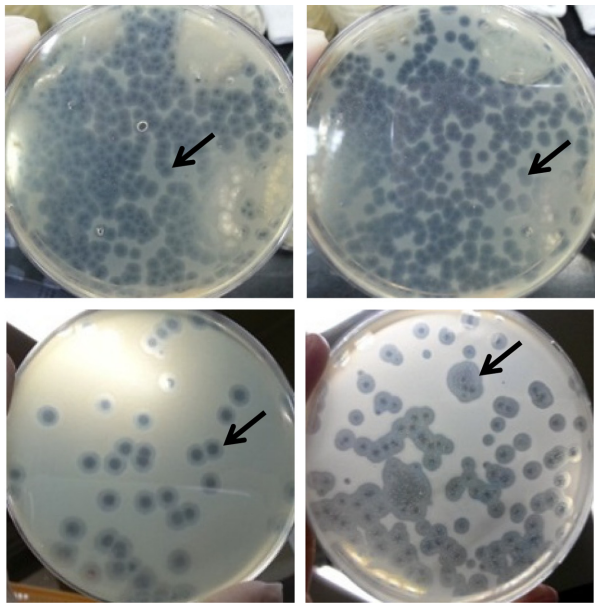

Fig. 1. Plaque shape of bacteriophages-encoded depolymerase. The clear plaques surrounding with the translucent halos (arrow) were observed on the plate with bacteriophage with *E. coli* strains. (A) Bacteriophages of ΦNOECP40 (left) and ΦNOECP44 (right) from *E. coli* NCTC 12079 (O157: H7 host). (B) Bacteriophages of ΦECP05 (left) and ΦECP06 (right) from by non-O157 *E. coli* NCCP13937 (O103)

A total of 17 bacteriophages with depolymerase activity were isolated from the plaques with the translucent halo zones when the hosts were cultured in a double-layer on LBC agar (Fig. 1). Among them, eight bacteriophages isolated from non-O157 *E. coli* host were named as ΦNOECP, and nine bacteriophages from *E. coli* O157: H7 host were named as ΦECP (Table 1). A procedure described by Hsu et al. (2013) was used to confirm the depolymerase

Table 1. Phages isolated from the different sources and the host strain of *E. coli*

Classification	Name	Source	Host strain	Depolymerase activity
E. coli Non-O157 Bacteriophage (NCCP14018, O26; NCCP13939, O103)	ΦNOECP07	Pig feces	*E. coli* NCCP 14018	++*
	ΦNOECP12	Pig feces	*E. coli* NCCP 14018	++
	ΦNOECP17	Pig feces	*E. coli* NCCP 14018	++
	ΦNOECP20	Pig feces	*E. coli* NCCP 14018	++
	ΦNOECP28	Pig feces	*E. coli* NCCP 14018	++
	ΦNOECP40	Sewage	*E. coli* NCCP 13937	+++
	ΦNOECP44	Sewage	*E. coli* NCCP 13937	+++
	ΦNOECP55	Sewage	*E. coli* NCCP 13979	+
E. coli O157:H7 Bacteriophage	ΦECP01	Cattle feces	*E. coli* 505B	+
	ΦECP03	Cattle feces	*E. coli* 505B	+
	ΦECP05	Soil	*E. coli* NCTC 12079	++
	ΦECP06	Soil	*E. coli* NCTC 12079	+
	ΦECP07	Soil	*E. coli* NCTC 12079	+
	ΦECP08	Soil	*E. coli* 505B	++
	ΦECP09	Soil	*E. coli* 505B	++
	ΦECP10	Soil	*E. coli* 505B	++
	ΦECP14	Sewage	*E. coli* NCTC 12079	++

*Symbols indicated the relative sizes of the translucent zone at the rim of the clear plaque

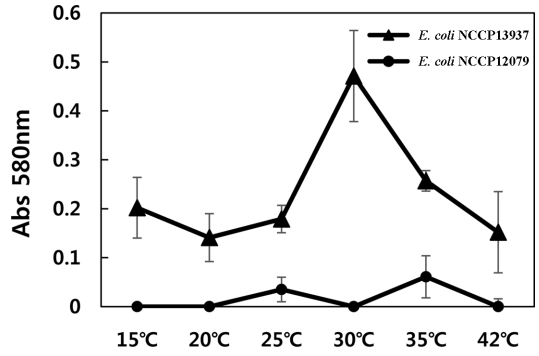

Fig. 2. Biofilm formation activities of *E. coli* NCCP 13937 on the abiotic plastic plates. Biofilm formed by *E. coli* NCCP 13937 at 30°C was stained with crystal violet and eluted using ethanol and then determined by absorbance at the various temperatures.

activity of the isolated phages. LB agar in a 6- or 24-well plate was overlaid with the top agar that had been inoculated with 100 mL of a fresh bacterial culture, and the phage suspension was spotted onto the plate after the top agar had solidified. After overnight incubation at 37°C, phage 0507-KN2-1 produced plaques with small clear centers surrounded by hazy rings (halos), suggesting the production of soluble phage enzymes, such as exopolysaccharide depolymerases. According to Lin et al. (2014), the clear plaques surrounded by translucent halos were observed on the plate on which the phage filtrate had been incubated with *Klebsiella pneumoniae* NTUH-K2044. The gene encoding depolymerase was confirmed by the analysis of the phage ORFs.

Therefore, in this study, 17 bacteriophages were isolated from the translucent rings around the clear plaques as previously reported. Among the isolates, two phages, ΦNOECP 40 and ΦNOECP 44, showed the highest depolymerase activities and were utilized as a phage cocktail for further studies.

Biofilm formation of *E. coli* on various surfaces

Formation of *E. coli* biofilm on an abiotic polystyrene plastic surface: A cultured bacterial suspension of 9 log CFU/mL in TSB and a diluted one of 7 log CFU/mL were inoculated on 24-well plastic plates, and biofilm formed at various temperatures were observed. *E. coli* NCCP 13937 showed the highest biofilm-forming ability at 30°C (Fig. 2). The absorbance at 580 nm for *E. coli* NCCP 13937 of 9 log CFU/mL was 0.218 at 15°C and 0.209 at 30°C, whereas the absorbance at 580 nm of *E. coli* NCCP 13937 of 7 log CFU / mL was 0.471 at 30°C, which indicated a high biofilm-forming ability. However, *E. coli* 505B, *E. coli* NCTC 12079, *E. coli* NCCP 14018, and *E. coli* NCCP 13979 showed a generally low biofilm-forming ability at 580 nm (below 0.1) at various temperatures (data not shown here). According to Kim (2014), *E. coli* KCCM 11234 was found to have the highest biofilm-forming ability at 35°C among the temperature conditions of 4, 20, and 35°C with 7.17±0.08 log CFU /coupon of the plastic. Furthermore, Mohammad et al. (2017) reported that all the three *E. coli* O157: H7 strains had a high biofilm-forming ability at 30°C rather than 10°C. Therefore, from our studies and from those of others, the optimal temperature for the formation of *E. coli* biofilm was verified to be ranging between 30-35°C.

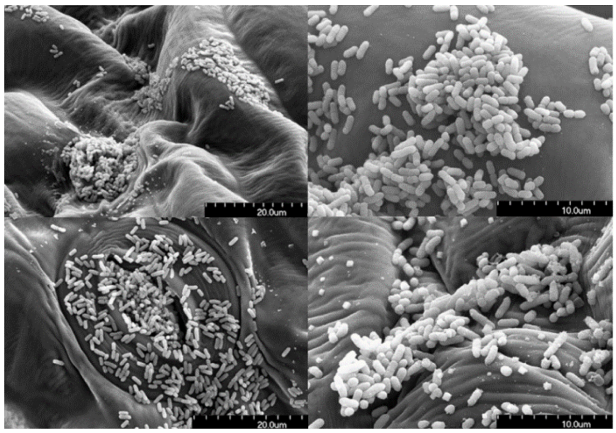

Fig. 3. SEM images of *E. coli* NCCP 13937 in biofilm on the surface of lettuces. *E. coli* sells formed biofilm near stoma of the lettuce. Figure on left upper; 20000× magnification, Figure on left lower; 25000× magnification, Figure on right; 50000× magnification.

Formation of *E. coli* biofilm on the biotic surface of lettuce: To observe formation of biofilm on food items, lettuce pieces were treated with *E. coli* NCCP 13937 and were incubated at 30°C, 24 h; the biofilm thus formed was observed by SEM (Fig. 3). Typical dense bacterial community adhering to the surface of the lettuce pieces was seen even after intense washing, thereby confirming that *E. coli* could form a biofilm on the vegetable surfaces. Similarly, Mohammad et al. (2017) studied the formation of biofilm by treating lettuce leaves with *E. coli* O157: H7 by using SEM and reported that colonization occurred around the leaves and pores after 24 h. Taken together, our results suggest *E. coli* NCCP 13937 forms biofilm on biotic (e.g. lettuce) as well as on abiotic surfaces (e.g., plastic surface).

Reduction of *E. coli* in biofilm on lettuce

Lactic acid treatment: Among the many chemical disinfectants used, organic acids have been preferred as food antimicrobial treatments because they are included in the GRAS category against foodborne pathogens. The results of treatment of control serotype, O103 *E. coli* NCCP 13937, with 0.1 % lactic acid in biofilm as a chemical sanitizer in lettuce are shown in Fig. 4A. The rate of reduction was similar to that of the negative control until the treatment time of 15 min, and the difference was less than 0.5 log CFU/g after 30 min of treatment. It was also confirmed that the control *E. coli* in biofilm formed on lettuce treated with organic acid sanitizers such as acetic acid and citric acid was insignificant and the treatment was almost ineffective (data not shown). According to Kim (2013), 0.1% lactic acid was reported to show a reduction rate of about 64% for *E. coli* when cultured in the planktonic culture broth without biofilm formation. Maximum reduction by lactic acid for *E. coli* was about 2 log CFU/g in the lettuce incubated at 4°C for 24 h (Akbas and Olmez, 2007), and inactivation of the pathogen by malic acid was more than 5 log cycles after 24 h at 5°C (Raynaudi-Massilia et al., 2009). There appeared to be a contradiction between the results of *E. coli* in

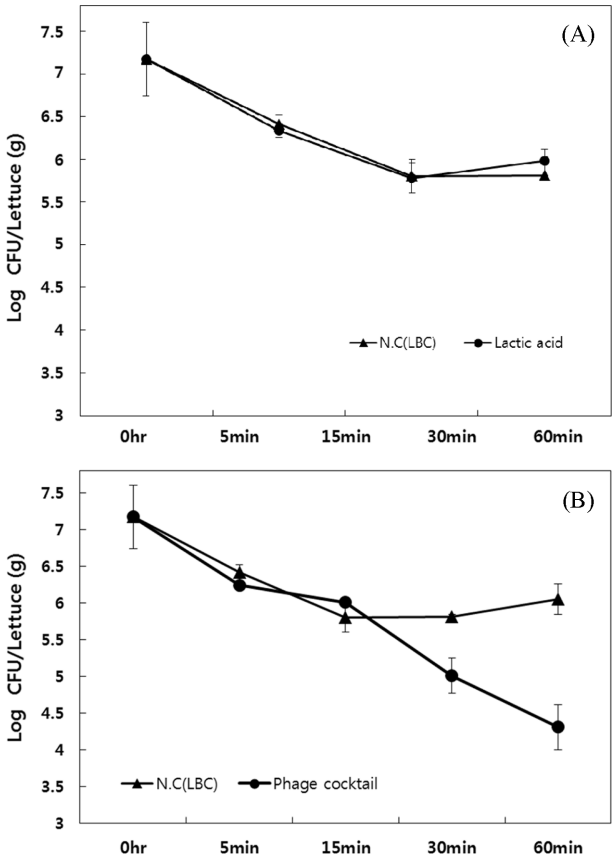

Fig. 4. Reduction of *E. coli* NCCP 13937 in biofilm on lettuce using the bacteriophage cocktail. Symbols: (A), Lactic acid, *E. coli* NCCP 13937 with lactic acid of 0.1%; N.C (LBC), *E. coli* NCCP 13937 without lactic acid in LB broth: (B), phage cocktail, *E. coli* NCCP 13937 with ΦNOECP40+ΦNOECP44 of 9-10 pfu/mL; N.C (LBC), *E. coli* NCCP 13937 without the phages at LB broth

biofilm and *E. coli* in planktonic conditions, and biofilm seemed to pose a barrier for eradicating the pathogens.

Bacteriophage treatment: The efficacy of removal of serotype O103 *E. coli* NCCP 13937 in biofilm formed on lettuce using the bacteriophage-origin depolymerase was confirmed in Fig. 4B. *E. coli* in biofilm on lettuce was not almost reduced by the bacteriophages until 15 min, which was the same with the negative control without the bacteriophages. However, at 60 min of bacteriophage treatment, *E. coli* decreased to about 4.3 log CFU/g with a 40% log scale reduction rate. In Kim's report (2013), *E. coli* biofilm-adhered green vegetables showed a reduction of about 1 log CFU/g after 1 h (a reduction rate of 13%), when individual bacteriophages were used. According to Mohammad et al. (2017), the bacteriophage cocktail treatment of *E. coli* O157: H7 in biofilm adhered to lettuce showed a reduction of about 2 log CFU /cm after 30 h (30% reduction). It was confirmed that the reduction of *E. coli* in biofilm formed on lettuce was higher with the use of a bacteriophage cocktail rather than with individual bacteriophages possessing depolymerase activity. In addition, it was proven that treatment with the bacteriophage having depolymerase was more effective in controlling non-O157 *E. coli* biofilm formed on lettuce than above other reports.

Consequently, our results suggest that bacteriophages showing depolymerase activity could be applied as effective biofilm controlling agents. Enzymatical and biotechnological researches on the depolymerase need to be conducted in the further study.

Acknowledgments

This research was supported by high value-added food development project (117060-03-2-HD020) of Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry funded by Ministry of Agriculture, Food and Rural Affairs.

Conflict of Interest

The authors have no financial conflict of interest to declare.

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