A Thesis for the Degree of Master of Science

Characterization of newly-isolated bacteriophages targeting *Staphylococcus aureus* and application of phages on bioactive food packaging films

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ABSTRACT

Staphylococcus aureus is a gram-positive, nosocomial pathogen that is associated with multidrug resistance. Methicillin-resistant *S. aureus* and vancomycin-resistant S. *aureus* are of particular concern as these 'superbugs' can no longer be treated with routine antibiotics. These concerns have led to continued efforts for discovering novel antibiotic-alternatives and bacteriophages (phages), viruses targeting specific host bacteria, have been actively studied. Phages have high potential as antibiotic alternatives for their abundance in the environment and no reported side effects. The present research isolated four *S. aureus* phages and characterized two phages with the highest lytic activities, HSA30 and HSA84, to evaluate their ability to control foodborne pathogens. Belonging to the *Myoviridae* family, HSA30 possessed a long-contractile tail with an icosahedral head. It demonstrated a broad host range against 22 out of 29 *S. aureus* strains tested. Moreover, it showed a strong lytic activity that was sustained for 19 hours upon divalent cation supplementation, highlighting its potential as an antibiotics alternative. Genomic analysis revealed that HSA30 genome had 140,358 bp-long nucleotides that encoded 224 coding DNA sequences and 3 tRNA's. Although no virulence factors nor drug resistance genes were identified in the genome, presence of recombinases suggest HSA30's potential to form lysogens. HSA84 was identified as a *Siphoviridae* and its genome encoded of *cro*- and *cI*- like repressors, implying that HSA84 may be a temperate phage. However, HSA84's ability to control 16 out of 29

tested *S. aureus* strains and strong host infectivity suggest its potential as an antimicrobial agent. Development of phage-immobilized food packaging materials suggested an alternative method to apply phages on food. Two virulent phages, SA11 (*Myoviridae*) and SA13m (*Siphoviridae*), were immobilized on two different cellulose media (cellulose membrane and nitrocellulose [NC] membranes). For enhanced immobilization efficiency, polyvinylamine (PVAm) was used to modify surface charge of the cellulose membrane. The amount of phages retained by the PVAm-coated cellulose membrane (PVAm membrane) was 4 log higher than that of the original membrane. Moreover, NC membrane was also evaluated as an immobilization medium and it achieved comparable immobilization efficiency as that of the PVAm membrane. Loss of infectivity was apparent in the membranes upon drying. To overcome this hurdle, Gum Arabic was coated on the bioactive packaging material. The overall result suggested an improved infectivity of the SA11-immobilized NC and PVAm membranes, and SA13m-immobilized PVAm membrane, but no bioactivity was observed in SA13m-immobilized NC membrane. When analyzed the functional properties (pH and storage stability) of the phage-immobilized membranes, SA11-immobilized NC membrane exhibited the highest stability against both pH stress and 2-day storage. It was able to achieve 3 log reduction of the host bacteria at all pH conditions. Moreover, this phage-membrane combination maintained its antimicrobial activity for 2 days upon storage at RH 85%.

Key words: Bacteriophage; *Staphylococcus aureus;* bioactive food packaging materials

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CHAPTER I.

Isolation and characterization of *S. aureus*-targeting bacteriophages

I-1. INTRODUCTION

Staphylcoccus aureus is one of the most well-known foodborne pathogens that accounted for the third highest (13%) number of foodborne illnesses cases from 2002 to 2016 in Korea (MFDS n.d.). These organisms are commonly found in the nose or on the skin of approximately 25-30% of the healthy individuals and produce heat-stable, protease tolerant enterotoxins that are responsible for foodborne illnesses and clinical symptoms (Adams, 2009). Moreover, *S. aureus* biofilm formation can be triggered by the exposure to environmental stresses (eg. Sodium chloride and glucose exposure, increase in temperature), which makes *S. aureus* more challenging to control in the food industry (Rode et al., 2007).

Antibiotics are often used as a treatment method for bacterial diseases, but drug overuse has resulted in an emergence of antibiotic resistant *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (Foster, 1996). For example, isolation of tetracycline-resistant *S. aureus* from the Netherlands was suggested to be linked to the use of oxytetracyclines in pig farms (Wulf and Voss, 2008). This finding suggests that antibiotic-resistant *S. aureus* is not only a threat in the healthcare setting, but it may also affect the community. A MRSA foodborne outbreak was reported in the United States (Jones et al., 2002) and isolation of MRSA from retail meat products (De Boer et al., 2009) confirm that MRSA may become a public health hazards due to food contamination. Therefore, alternative biocontrol

agents targeting *S. aureus* for the application in the food industry and healthcare facilities should be explored.

Bacteriophages (phages) are viruses that can lyse specific host bacteria with no effect on human cells, and they are ubiquitous in the environment (Clokie et al., 2011). Based on their life cycles, phages can be either virulent or temperate (Clokie et al., 2011). Virulent phages kill their host though rapid cell lysis and release their progeny, while temperate phages integrate their genome into the host chromosome until exposed to stress conditions (Clokie et al., 2011). Horizontal gene transfer of virulence and antibiotic resistance genes through lysogen formation makes temperate phage not ideal for foodborne pathogen control (Greer, 2005). Thus, virulent phages have been suggested as alternative biocontrol agents and several commercially-available products have received generally recognized as safe (GRAS) notifications by the US FDA (Andreoletti et al., 2011; Greer, 2005). Listex P100 (Micreos Food Safety B. V., Wageningen, the Netherlands), for example, was able to successfully control *Listeria monocytogenes* on catfish fillets (Soni et al., 2010), cooked ready-to-eat meat slices (Chibeu et al., 2013), and fruit slices (Oliveira et al., 2014). Moreover, *S. aureus* control using a cocktail of two lytic phages in fresh cheese suggest bacteriophages' potential as biocontrol agents (Bueno et al., 2012).

The research presented in this chapter will explore four newly-isolated bacteriophages targeting *S. aureus* by analyzing various characteristics including: morphology, host range, and bacterial growth inhibition. After comparing the

physiological properties of the isolated phages, genomic analysis will be conducted to evaluate their potential for food applications.

I-2. MATERIALS AND METHODS

I-2-1. Bacterial strains and bacteriophages

Total of 28 *S. aureus* strains (9 type strains and 18 isolated strains from various sources) were used for bacteriophage isolation (Table I-1). *S. aureus* animal isolate #130 (*S. aureus* #130) as used as the host bacteria for bacteriophage HSA30. For phages HSA 84, 85, and 86, *S. aureus* RN 4220 was used as the host. For the host range study, 17 staphylococcal strains, 3 other gram-positive bacteria, and 4 gram-negative bacteria were used (Table I-1).

All staphylococcal strains were stored in 15 % glycerol at -80˚C and working cultures were incubated at 37˚C for 12-18 hours on tryptic soy agar (TSA; Becton, Dickinson and Co., Franklin Lakes, NJ, USA). For the liquid culture preparation, a single *Staphylcococcus* colony from TSA was subcultured into tryptic soy broth (TSB; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and incubated overnight at 37˚C under shaking condition (220 rpm). Non-staphylococcal strains were cultured in the same manner as described above, except that Luria Bertani (LB) agar and broth were used as the growth medium. High-titer bacteriophage stocks were stored in glass vials with dialysis buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8), 10 mM $MgCl_2$) at 4˚C until further use.

I-2-2. Bacteriophage isolation

Samples were collected from wastewater treatment plants in Anyang and Gwacheon, Gyeonggi Province, South Korea as well as Seoul Grand Park Zoo, Gwacheon were used to screen for novel *S. aureus*-targeting bacteriophages.

First, solid samples were homogenized in 50 mL of sodium-magnesium (SM) buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$). Subsequently, 100 μL of chloroform was added to 50 mL of the samples and centrifugated at 15,000 rcf for 10 minutes at 4˚C. Subsequently, supernatant of each sample was filter sterilized using 0.22 μm pore sized filters (Millipore, Billerica, MA, USA). The first enrichment step of phages were performed by adding 5 mL of each sample solution to 5 mL 2X TSB broth, containing 1% overnight (ON) culture of the host bacteria, followed by incubating the mixture overnight at 37˚C with agitation (220 rpm).

After the enrichment step, the bacterial culture was centrifugated at 15,000 rcf for 10 minutes and the supernatant was filtered with 0.22μm pore size filter. The presence of bacteriophages in the supernatant was confirmed through spotting 10 μL of tenfold serial diluted filtrates on TSA covered with 5 mL of 0.4% semi-solid TSA containing 1% host bacteria. The agar plates were incubated at 37˚C overnight and were checked for plaque formation. Purification step was performed by picking a single plaque from an agar plate with a sterile 10 uL pipette tip and eluting in 500 μL of SM buffer. The eluted phages were then filtered and spotted again for single-plaque detection single. The purification step was repeated three times.

I-2-3. Bacteriophage propagation

Following the purification step, the isolated phages underwent propagation. The ON culture of the host bacteria was sub-cultured into fresh TSB (1:100 = ON culture: fresh liquid growth medium). The culture was incubated at 37˚C, 220 rpm until the growth phase reached the early exponential phase (OD_{600}=0.3~0.4). The host bacteria were infected with bacteriophage at the multiplicity of infection (MOI) of 0.1 and incubated at 37˚C for 5 hours, followed by centrifugation (15,000 rcf, 15 minutes) and filtration. The 10 mM of cation ($MgCl_2$, $CaCl_2$) was added to the culture if needed.

A high-titer phage stock was prepared by precipitating bacteriophages with polyethylene glycol 6000 (Junsei Chemical Co., Tokyo, Japan). Afterwards, the precipitated phages were concentrated using a CsCl-gradient (CsCl density=1.3, 1.45, 1.5, 1.7 g/mL) ultracentrifugation (Himac CP 100β; Hitachi, Tokyo, Japan) at 75,000 rcf for 2 hours at 4˚C. Obtained phage concentrate was dialyzed against dialysis buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$) for 2 hours at 4˚C. There was a buffer change halfway through the dialysis. Finally, titer of the bacteriophage stock was determined by overlaying 100 µL of ten-fold dilutions of phage stock on TSA covered with 5 mL of 0.4% semisolid TSA containing 100 µL host bacteria. Plates were incubated overnight in an upright position at 37˚C. Purified phages were stored in glass vials at 4˚C until further use.

I-2-4. Transmission electron microscopy

Four microliters of the purified bacteriophages (~ 10^{10} PFU/mL) were spotted on carbon-coated copper grids for 1 minute for phage adhesion. Subsequently, the phages were stained with 2% phosphotungstic acid (pH 7.5) for 10 minutes and rinsed with deionized water to remove excess staining solution. A transmission electron microscope (JEM-2100; 200 kV; JEOL Ltd., Akishima, Tokyo, Japan [TEM]) was used to take microscopic images of the isolated phages at the National Center for Inter-University Research Facilities, Seoul, South Korea. Morphological characteristics of the isolated phages were identified with the microscopic image, and they were used to classify the phages into one of the three bacteriophage families (*Myoviridae*, *Siphoviridae*, and *Podoviridae*) based on the guidelines provided by the International Committee on Taxonomy of Viruses (Fauquet 2005).

I-2-5. Host range determination

Host range of the phages HSA30, HSA84, HSA85 were determined by the spotting assay. 10^{10} PFU/mL of each phages were serially diluted by 10-folds, and 5 μL of the diluents were spotted on the test bacteria lawn supplemented with divalent cations (10 mM $CaCl_2$ and $MgCl_2$, each); a list of host bacteria used in this study in Table I-1. Bacteria lawn of the staphylococcal strains and non-staphylococcal strains were prepared in 0.4 % TSB soft agar and 0.4 % LB broth soft agar, respectively.

Once the spotted bacteriophage diluents were fully absorbed, the plates were incubated at 37˚C overnight and assessed plaque formation by the isolated phages.

I-2-6. Bacterial growth challenge assay

To confirm host growth inhibitory activities of the isolated phages, a bacterial growth challenge assay was conducted by observing the changes in the optical density of the host bacterial cells upon phage infection. ON culture of *S. aureus* RN4220 was sub-cultured to 30 mL TSB and incubated at 37˚C with aggitation until the cell growth reached an early-exponential phase ($O.D._{600}$=0.3~0.4). The host bacteria were then infected with bacteriophages HSA84, HSA85, and HSA86 at a MOI of 1 with divalent cation supplementation. The growth challenge assay was conducted at 37˚C with shaking for 24 hours and the OD_{600} values were measured every hour. In the case of HSA30, *S. aureus* #130 was used as the host bacteria. The treatment group without phage infection was used as a control group and the bacteriophage with the strongest lytic activity was selected for further characterizations.

I-2-7. Adsorption assay

After sub-culturing in 50 mL of TSB, the ON host culture was incubated under shaking condition at 37˚C and harvested when the OD_{600} reached 1.0. Subsequently, 1 mL of the harvested cells were suspended in 9 mL of fresh TSB along

with the phages (HSA30 and HSA84) and pre-incubated at 37˚C for 5 minutes. Phage infection was conducted at the MOI of 0.01 and the culture was incubated at 37˚C without agitation. Divalent cations (10 mM $CaCl_2$ and $MgCl_2$, each) were supplemented to the phage-bacteria mixture, if necessary. Sampling was conducted every 5 minutes after infection and the collected samples were immediately centrifuged at 15,000 rcf for 1 minute at 4˚C. Then, the supernatant was filtered using 0.22 μm syringe filters. Obtained supernatants underwent 10-fold serial dilution and overlaid on TSA plates to determine the titer of unadsorbed phages. For the control group, equivalent amount of phages were added to 10 mL of fresh TSB, and sampled at time 0 (before incubation at 37˚C) and 30 minutes after infection.

I-2-8. One-step growth curve

Once the cell density reached $O.D._{600}$ 0.3~0.4, subcultured host bacteria were harvested through centrifugation. Subsequently, HSA 30 and 84 were added to appropriate host bacteria at the MOI of 0.01 and incubated at RT for 5 minutes for adsorption. The bacteria-phage mixture was centrifuged and the supernatant was discarded to remove unadsorbed phages. Subsequently, the pellet was re-suspended in the equivalent volume of the fresh TSB and incubated at 37°C with agitation. Sampling was conducted every 5 minutes, where two sets of samples were immediately diluted and plated to determine the phage titer. For one of the samples, 1/100 volume of chloroform was added to release intracellular phages before plating.

This step indicated of the time required for bacteriophage assembly within the host bacteria (eclipse period). The lapsed time between the phage infection and cell lysis was denoted by the latent period. Finally, the burst size was quantified by the number of phage particles released upon cell lysis. These values were determined based on the PFU/mL obtained from the infection cycle (Ellis and Delbrück, 1939).

I-2-9. Bacteriophage genomic DNA purification

Phage genomic DNA was extracted as previously described by Wilcox and colleagues (Wilcox et al., 1996). To remove bacterial DNA and RNA, 10^{10} PFU/mL of the phage lysate was treated with 1 μg/mL of DNAseI and RNAseA for 30 minutes at room temperature. Then, 20 mM EDTA, 50 μg/mL proteinase K, and 0.5% of sodium dodecyl sulfate were added and incubated at 65˚C for 15 minutes to lyse the phages. The DNA was purified using the phenol-chloroform extraction method, and concentrated using ethanol precipitation method. Ethanol precipitation was conducted by using two volumes of 100 % ethanol and inverting the sample several times. Subsequently, phage DNA pellets were acquired by centrifucation at 21,000 rcf for 10 minutes at 4˚C and washed with 70 % ethanol. After the final washing step, the DNA sample was air-dried to remove residual ethanol and eluted with Tris-EDTA buffer (10 mM Tris-Cl, pH 8; 1 mM EDTA).

I-2-10. Full-genome sequencing of bacteriophage and bioinformatics analysis

The extracted phage gDNA was sequenced with Illumina Miseq platform (Illumina, San Diego, CA, USA) and assembled with CLC Genomics Workbench (Qiagen, Venlo, Netherlands) at Sanigen Inc., Gwacheon, South Korea. After the assembly, coding DNA sequnces (CDS) were predicted and annotated using the Rapid Annotations using Subsystems Technology (RAST) (Aziz et al., 2008). Subsequently, GeneMarkS was used to predict additional protein-coding genes that were not identified from the RAST predictions (Besemer and Borodovsky, 2005; Besemer et al., 2001). Finally, the GeneMarkS-predicted genes were manually annotated based on the Basic Local Alignment Serach Tool (BLAST) analysis results (Altschul et al., 1990). The ribosomal binding sites were predicted using the RBS-finder (J. Craig Venter Institute, Rockville, MD, USA). Transfer RNA were predicted by using tRNAscan-SE (Lowe and Eddy, 1997). After the genome annotation, circular genome maps were drawn using the GeneScene software (DNASTAR, Madision, WI, USA).

I-3. RESULTS AND DISCUSSION

I-3-1. Isolation and morphology of the newly isolated phages

Four bacteriophages that formed clear plaques on *S. aureus* RN 4220 and *S. aureus* #130 were isolated. HSA30 and 84 were isolated from the wastewater samples obtained from Seoksu and Gwacheon sewage treatment facilities, respectively. HSA85 was isolated from a soil sample obtained from the Sambar deer cage from Seoul Grand Park (Gwacheon, Gyeonggi Province, Korea). Finally, HSA86 was obtained from soil nearby a pond located at Seoul Grand Park.

Furthermore, morphology of each bacteriophages was analyzed using the TEM images. TEM analyses revealed that the isolated phages were either categorized into the *Siphoviridae* (HSA84, HSA85, and HSA86) or the *Myoviridae* (HSA30) families (Figure I-1). The morphological features of each phages are summarized in Table I-1.

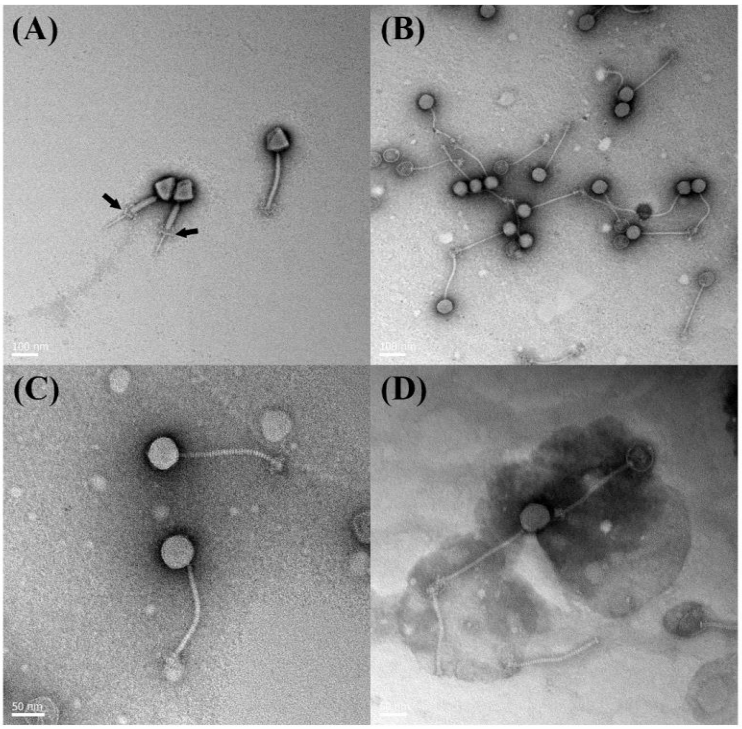

Figure I-1. Transmission electron microscopy images of *S. aureus* phages
a) HSA30, b) HSA84, c) HSA85, and d) HSA86

Table I-1. Morphological features of the isolated *S. aureus* phages

Bacteriophage	Family	Tail length (nm)	Capsid diameter (nm)
HSA30	*Myoviridae*	198 ± 10	76.1 ± 3.8
HSA84		195 ± 38	52.2 ± 3.4
HSA85	*Siphoviridae*	181 ± 17	55.2 ± 2.5
HSA86		170.5 ± 11	54.6 ± 3.1

I-3-2. Host range of the isolated bacteriophages

Antimicrobial spectrum of the newly-isolated phages, HSA 30, 84, 85, and 86, were compared by using 29 *S. aureus* strains (13 type strains and 16 isolated strains from Korea) and other strains from both Gram-positive and Gram-negative bacteria (Table I-1 and 2). All isolated phages showed lytic activity exclusively against *staphylococcal* strains. More specifically, HSA84, 85, and 86 demonstrated nearly identical host range, inhibiting the growth of six *S. aureus* strains including: MRSA CCARM 3089 and *Staphylococcus epidermidis* ATCC 35983 (Table I-2). HSA85 and 86 were also able to form turbid plaques in MRSA CCARM 3090, which showed resistance to HSA84.

HSA30 exhibited the broadest host range, forming single plaques in 11 out of 13 *S. aureus* type strains, where two were MRSA (Table I-2). It was also able to form single plaques against *Staphylococcus hominis* ATCC 37844 and *Staphylococcus warneri* ATCC 10209. Moreover, HSA30 showed the broadest host range against *S. aureus* isolates (Table I-2). 11 out of 16 isolated *S. aureus* strains (four animal isolates and seven clinical isolates) were susceptible to HSA 30 (Table I-2). HSA30's superior growth inhibitory spectrum against MRSA strains and clinical isolates suggest that this phage has a potential as an alternative antimicrobial agent to control *S. aureus*.

Table I-2. Host range of the isolated *S. aureus* phages

Bacterial host	Plaque formation[a]			
	HSA30	**HSA84**	**HAS85**	**HSA86**
S. aureus RN4220	T	C	C	C
S. aureus Newman	C	-	-	-
S. aureus ATCC 13301	T	-	-	-
S. aureus ATCC 23235	T	-	-	-
S. aureus ATCC 33586	T	-	-	-
S. aureus ATCC 33593	-	T	T	T
S. aureus KTCC 1916	T	C	T	T
S. aureus ATCC 6538	T	C	C	C
S. aureus ATCC 29213	T	C	T	T
S. aureus ATCC 12600	T	C	T	T
MRSA CCARM 3793	C	-	-	-
MRSA CCARM 3090	T	-	T	T
MRSA CCARM 3089	I	C	C	C
S. haemolyticus ATCC 29970	I	T	-	-
S. epidermidis ATCC 35983	-	T	T	T
S. hominis ATCC 37844	T	-	-	-
S. warneri ATCC 10209	C	-	-	-
***S. aureus* animal isolates**				
129	T	-	-	T
130	C	-	-	-
131	-	-	-	-
134	C	T	T	T
***S. aureus* clinical isolates**				
55	C	T	T	T
136	I	T	-	-
154	T	-	-	-
212	T	-	-	-
600	C	-	-	I
FMB-1	T	-	-	-
FMB-2	C	-	-	-
***S. aureus* human isolates**				
77	I	T	T	T
79	I	T	-	-
80	T	C	-	T
81	I	-	-	-
82	I	C	C	C
Other Gram-positive bacteria				
Enterococcus faecalis ATCC 29212	-	-	-	-
Bacillus cereus ATCC 14579	-	-	-	-
Bacillus subtilis ATCC 23857	I	-	-	-

Other Gram-positive bacteria				
Salmonella Typhimurium SL1344	-	-	-	-
Escherichia coli MG1655 ATCC 47076	-	-	-	-
Cronobacter sakazakii ATCC 29544	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-

[a] C, clear plaque; T, turbid plaque; -, no plaque; I, inhibition zone

I-3-3. Bacterial growth challenge assay

Antimicrobial effect of the isolated phages on *S. aureus* strains was evaluated by conducting a growth challenge assay, which was performed in a liquid culture. *S. aureus* RN 4220 was used as the host bacteria for HSA84, 85, and 86. However, a different strain, *S. aureus* #130, was used to evaluate the growth inhibitory effect of HSA30, because *S. aureus* RN 4220 showed no susceptibility to this phage (Table I-2).

All bacteriophages examined exhibited growth inhibitory activities against their respective host bacteria (Figure I-2a and b). HSA84 and 86 showed an identical growth inhibitory effect on *S. aureus* RN 4220, where a complete inhibition of bacterial growth was observed after 3 hours of infection (Figure I-2b). However, the inhibitory effect lasted for 6 hours and the bacterial growth resumed afterwards. Although HSA85 triggered complete inhibition of bacterial growth at 5 hours post-infection time, a similar growth inhibition pattern as that of HSA84 and HSA86 was observed. Interestingly, the resistant population emerged at the late stage of infection and displayed slow growth rate. It was implied that a mutation may have occurred at the genes responsible for optimal bacterial growth in the phage-resistant cells, resulting in a slower growth rate compared to that of the phage-sensitive cells.

HSA30 exhibited stronger growth-inhibitory effect against *S. aureus* #130. After a drastic suppression in the bacterial growth within 2 hours post-infection, the growth inhibition lasted for approximately 13 hours (Figure I-2a). Moreover,

supplementation of divalent cations resulted in a longer growth inhibitory effect as the cell growth was observed after 17 hours of infection. Despite of long duration of the bacterial growth inhibition, a drastic increase in the cell density was observed in *S. aureus* #130 after 19 hours of infection (Figure I-2a). Growth rates of the phage-resistant cells and the phage-sensitive cells were showed high similarity. It was thought that mutation of the HSA30-resistant population may have occurred at non-essential genes, suggesting the strain's physiological characteristics remained functional.

Considering the host range and the pattern of antimicrobial activity, it was concluded that HSA30 and one of HSA84, 85, 86 phages may result in synergistic biocontrol activity through broadening the spectrum of *S. aureus* growth inhibition and preventing the emergence of phage-resistant strain. Based on the experimental results from the growth inhibitory assay and the host range analysis, HSA30 and HSA84 were selected for further phage characterization. HSA30 was chosen for its unique phage morphology (it was the only *Myoviridae* isolated from this study) and its superior host inhibitory spectrum. HSA84 formed clear plaques against many host bacteria, suggesting stronger lytic activity compared to the other *Siphoviridae* phages from this research.

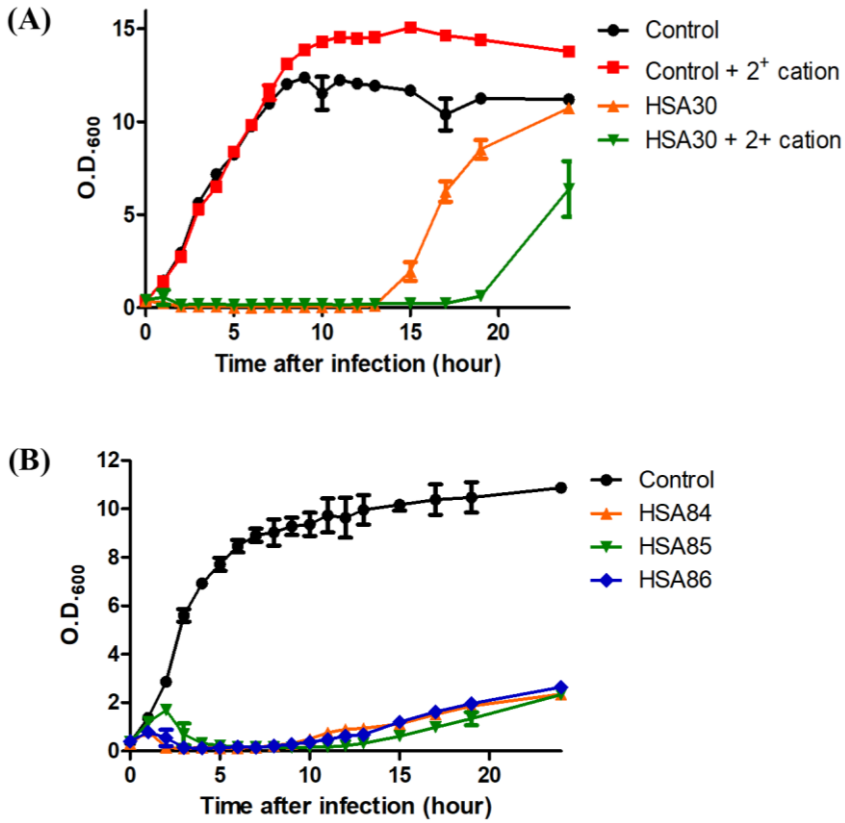

Figure I-2. Bacterial growth challenge assay of *S. aureus* phages Bacteriophages were used to infect a) *S. aureus* #130 and b) *S. aureus* RN 4220 (10^8 PFU/mL) at MOI of 1. SM buffer was used as a negative control. Data are the means from three independent replicate trials and the error bars represent the standard error of mean.

I-3-4. Determination of bacteriophage infection pattern

Adsorption efficiency of the phages HSA30 and 84 to the host bacteria was estimated by an adsorption assay. This assay was conducted by measuring the titer of the free phages after the host infection. With divalent cation supplementation, the percentage of free HSA30 decreased by 80% after 60 minutes of infection (Figure I-3a). However, adsorption efficiency of the same phage without cation treatment was reduced by 30% after 60 minutes. These findings suggest that divalent cations may enhance the infectivity of HSA30, resulting in a prolonged infection (Figure I-2b). HSA84 achieved faster adsorption efficiency than that of HSA30, where it took 20 minutes for 80% reduction in the HSA84 population (Figure I-3b). Thus, it was implied that HSA84 may have a better host adsorption efficiency than HSA30.

Moreover, one-step growth curve analysis was conducted to investigate the replication cycles of HSA30 and 84 (Figure I-4a, b). The replication cycle of HSA30 was illustrated in Figure I-4a. The eclipse and latent periods were 15 and 25 minutes, respectively. HSA30 resulted in a burst size of 80 phages particles per infected host bacteria after 60 minute-infection. In the case of HSA84, the eclipse and the latent periods were 15 and 30 minutes, respectively. Upon bacterial cell lysis, approximately 30 phages particles were released per infected host bacteria.

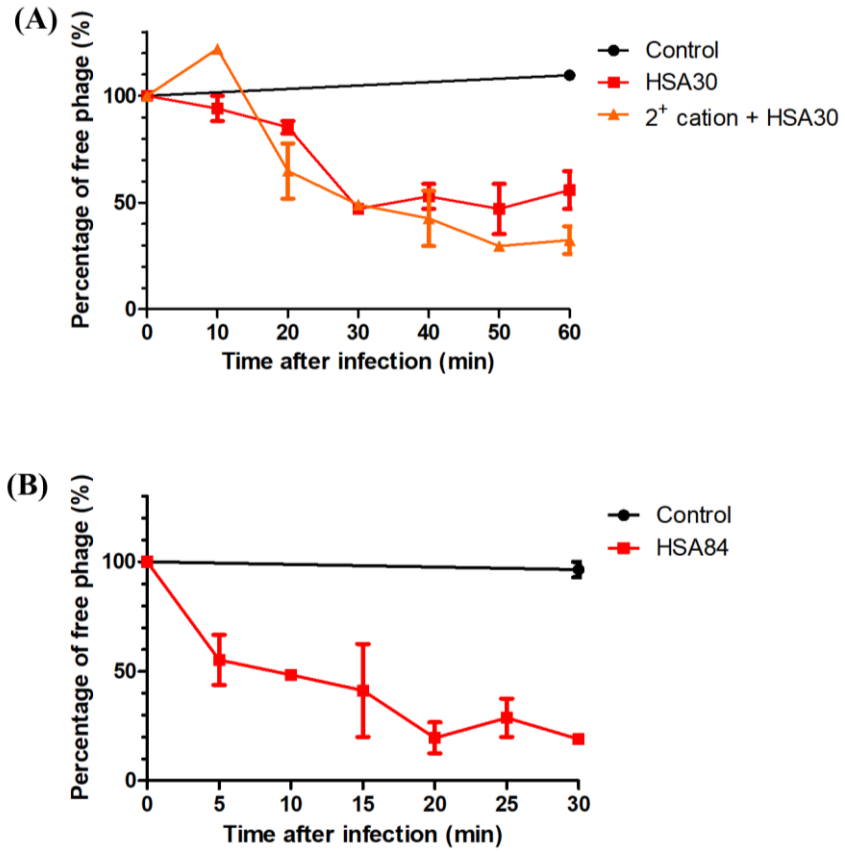

Figure I-3. Adsorption assay Exponentially growing *S. aureus* were infected with a) HSA30 and b) HSA84 (MOI of 0.01). After centrifugation and filtration of the phage + bacteria mixture, phage titer of the filtrate was measured using the overlay assay. The percentage of free phage was determined as: [(phage titer of the phage + bacteria suspension)/phage titer of the phage in TSB broth) X 100]. The control represents the phages inoculated in bacteria-free TSB broth. Data are the means from three independent replicate trials and the error bars represent the standard error of mean.

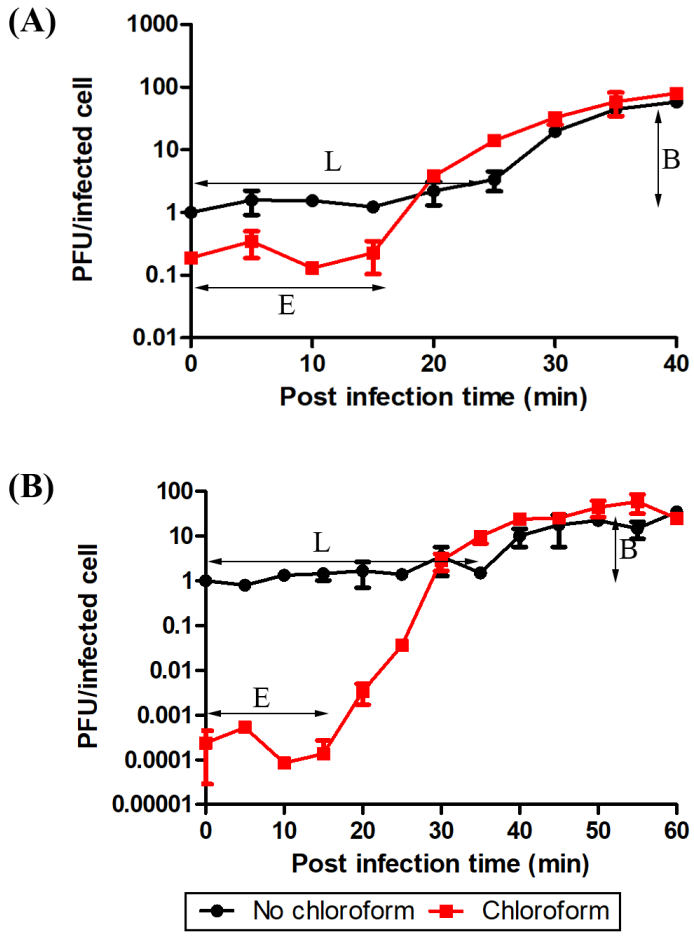

Figure I-4. One-step growth curve a) HSA30 and b) HSA84 E, eclipse period; L, latent period; B, burst size. Data are the means from three independent replicate trials and the error bars represent the standard error of mean.

I-3-5. Genomic analysis of bacteriophage

I-3-5-1. HSA30

The complete genome of *S. aureus* phage HSA30 consisted of 140,358 bp-long nucleotides with an overall G+C content of 30.2 %. A total of 224 open reading frames (ORFs) were predicted and 158 of them were given unknown function. Furthermore, this genome contained three tRNA-coding genes (Asp-tRNA, Phe-tRNA, and Ile-tRNA). HSA30 ORFs were categorized into five groups based on their functions: DNA packaging, phage structure, DNA regulation, cell lysis and additional function.

The genes encoding virion structures included all the necessary components for bacteriophage head assembly: putative head protease (HSA30_gp09), major capsid protein (HSA30_gp11) putative capsid protein (HSA30_gp19), and capsid/scaffold protein (HSA30_gp39, HSA30_gp41). Tail sheath protein (HSA30_gp18) distinguishes *Myoviridae* from other bacteriophage families, as it is responsible for giving *Myoviridae* its unique contractile tail machinery (Aksyuk et al., 2011). The presence of tail sheath protein and phage tail contraction observed in the TEM image (Figure I-1) confirmed that HSA30 belonged to the *Myoviridae* family.

The genes associated with DNA regulation and decision of phage life cycle were detected in the genome. Key components of the lytic/lysogenic decision gene cluster (*cI*- or *cro*-like repressors) were missing in the genome, suggesting that HSA30 may be a virulent phage. However, the presence of putative resolvase

(HSA30_gp53), integration host factor (HSA30_gp60), and recombinase (HSA30_gp65) suggests that HSA30 may have ability of lysogen formation (Matsuura et al., 1996; Oppenheim and Adhya, 2007; Thorpe and Smith, 1998).

Finally, putative tail lysin (HSA30_gp28), endolysin (HSA30_gp214), and holin (HSA30_gp215) were predicted to have cell lysis function. The BLASTP analysis results revealed that the endolysin (ORF 214) was composed of a cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) (PFAM05257) and an amidase_2 domain (PFAM01510). These findings suggest that HSA30 endolysin (LysHSA30), may exert host cell lysis through endopeptidase and amidase activity. In addition, a cell wall binding domain (CBD) (SH3_5) (SMART00287) was identified at the C-terminus of the endolysin and this domain is a common CBD found in the endolysins targeting *Staphylococcus* (Jarábková et al., 2015). Located next to the endolysin, holin (HSA30_gp215) consisted of a phage_holin_1 domain (pfam04531) and it allows the endolysin to reach the peptidoglycan by making the cell membrane permeable (Young et al., 2000). A putative tail lysin (HSA30_gp28) may serve as an additional cell wall degrading agent along with ORF 214 as it was predicted to contain a CHAP domain at the N-terminus.

With BLAST, sequences of various phage genomes were compared and it was apparent that HSA30 have high sequence similarity with *several S.* aureus-targeting phates including GH15 (JQ686190.1), philPLA-RODI (KP027446), P108 (KM216423.1), JD007 (JX87867.1), etc. Among them, Gutiérrez and colleagues

(2015) confirmed that a *Staphylococcus* phage vB_SauM_phiIPLA-RODI was a virulent phage even though it encoded resolvase. Therefore, although HSA30 showed high sequence similarity to many virulent phages, further experiments such as determining the frequency of bacteriophage-insensitive mutants and lysogen formation are required to elucidate HSA30 life cycle.

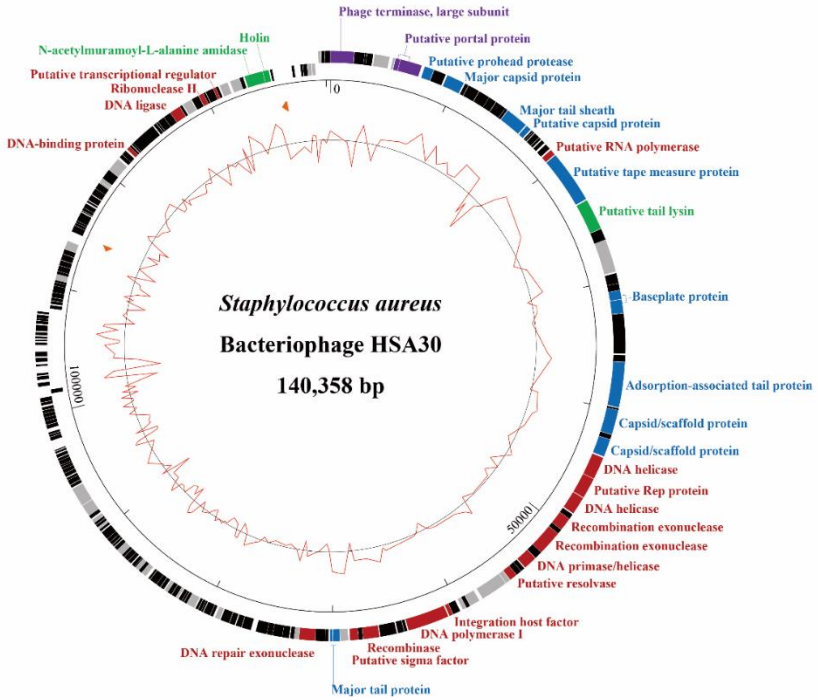

Figure I-5. Genome map of *S. aureus* phage HSA30 Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories such as phage structure (blue), DNA regulation (red), DNA packaging (purple), cell lysis (green) and additional function (grey). The inner circle with red line indicates the G+C content. Scale unit is base pair.

I-3-5-2. HSA84

HSA84 genome is a double-stranded circular DNA, consisting of 42,650 bp-long nucleotides with 60 ORFs and no tRNA. The overall G+C content (34.6 %) was similar to the average G + C content of 27 *S. aureus*-targeting bacteriophages (33.7 %) (Kwan et al., 2005). Based on the annotation results, 30 out of 60 predicted ORFs were assigned with various functional properties (Figure I-6). Predicted ORFs in the HSA84 genome were categorized into five groups (DNA packaging, phage structure, DNA regulation, cell lysis and additional function) based on the results obtained from the bioinformatics analysis.

Two N-acetyl-muramoyl-L-alanine amidases (HSA84-gp24; HSA84_gp28) and a holin (HSA84_gp27) represented the host cell lysis gene cluster in the HSA84 genome. The first amidase (HSA84_gp24) predicted in the genome was found adjacent to the tail fiber protein (HSA84_gp25), suggesting that this protein may be a tail lysin. HSA84 endolysin the second amidase (HSA84_gp28), was also composed of the CHAP, amidase_2, and SH3_5 domains. The holin (HSA84_gp27) also contained a phage_holin_1 domain (pfam04531), suggesting that this domain may be conserved in endolysins targeting *S. aureus*.

The genome arrangement of HSA84 is interesting, because *cI*-like (HSA84_gp31) and *cro*-like (HSA84_gp32) repressors were identified as well as an antirepressor (HSA84_gp34). These proteins play together to control the lytic-lysogenic switch and are commonly found in temperate bacteriophages (Kenny et al.,

2006). Lambda protein (CI) is responsible for maintaining the lysogenic cycle by promoting the transcription of genes required for developing and sustaining the lysogen. Cro protein, on the other hand, promotes the lytic life-cycle by suppressing CI expression (Schubert et al., 2007). Thus, the upstream and the downstream of the lytic-lysogenic switch region are likely to express proteins associated with cell lysis, structural proteins and DNA regulatory proteins, respectively. As expected, HSA84 genome consisted of genes promoting phage assembly and cell lysis in the upstream of the lytic-lysogenic switch region. On the opposite side of the genome mainly consisted of DNA regulatory genes. Specifically, Holliday junction resolvase (HSA84_gp49) may promote DNA recombination (Christiansen et al., 1996).

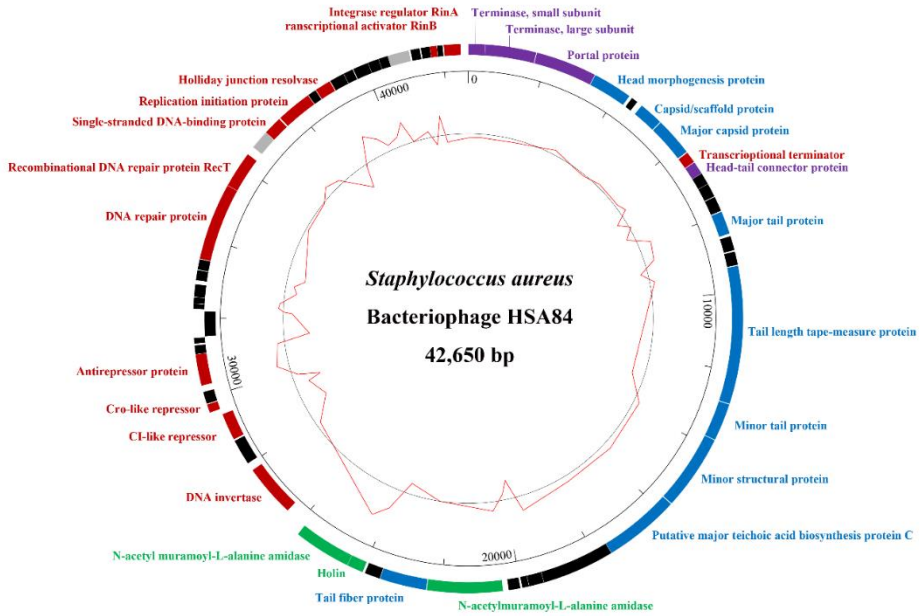

Figure I-6. Genome map of *S. aureus* phage HSA84 Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories such as phage structure (blue), DNA regulation (red), DNA packaging (purple), cell lysis (green) and additional function (grey). The inner circle with red line indicates the G+C content. Scale unit is base pair.

I-4. CONCLUSION

The first chapter of the present research characterized four *S. aureus*-targeting bacteriophages isolated from sewage water and soil samples, and evaluated their potential as antimicrobial agents. Among the phages characterized, HSA30 (isolated from sewage water sample from Seoksu Wastewater Treatment Facility) possessed the most desirable characteristics as a biocontrol agent. It demonstrated the broadest host range against both type and isolated *S. aureus* strains. Moreover, its ability to control 2 MRSA strains demonstrate HSA30's potential application in both healthcare and the food industry. Albeit HSA30's promising properties as a possible biocontrol agent, further work to identify HSA30's life cycle is necessary to use this phage in food applications.

HSA84 was also selected as another potential biocontrol agent. It demonstrated the second broadest host range with its ability to control 16 out of 29 *S. aureus* strains tested. Furthermore, HSA84 demonstrated a comparable host growth inhibition as it resulted in a slower phage-resistant bacteria growth. Because HSA30 and HSA84 had different host ranges, combining these two phages into cocktails may bring about a stronger *S. aureus* biocontrol agent that could be applied in the food industry.

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CHAPTER II.

Development of bacteriophage-based bioactive packaging material to control *Staphylococcus aureus*

II-1. INTRODUCTION

The food industry ensures food safety through employing various technologies including: thermal processing, freezing, dehydration, pickling, salting, and refrigeration to ensure food safety (Park et al., 2014). For example, dehydration of food lowers the moisture content of food to hinder pathogen growth within food. This technique, however, may exert negative quality attributes to the final product such as nutrient loss (eg. Vitamin C, carotenoids, and lycopene), colour, shape, and flavour changes (Clark et al., 2014; Sablani, 2006). Although conventional food processing techniques have assured the safety of food products for decades, an increase demand for fresh, minimally-processed food has put pressure on the academia and the food industry for better preservation techniques (Zink, 1997).

In recent years, bacteriophages have been commercialized as alternative agents to combat foodborne pathogens on food surfaces and the food processing facilities. However, commercially-available bacteriophages have limited application methods (dipping food product into a large volume of phage solution or incorporating phages into food batches), which makes them prone to cross contamination and reducing the working concentration of phages (Anany et al., 2011).

To overcome this limitation, phage-based bioactive food packaging materials have been developed to control various foodborne pathogens (Anany et al., 2011; Jabrane et al., 2011; Lone et al., 2016; Vonasek et al., 2014). Bioactive packaging materials are defined as food packaging materials that control spoilage or

pathogenic microorganism via active materials that are coated/formulated/or immobilized on the polymer and edible films (Lopez-Rubio et al., 2006). Phage-based bioactive materials may be applied to processed foods. Combining bacteriophage-based bioactive packaging material and vacuum packaging has lead to undetectable levels of *Listeria monocytogenes* on ready to eat turkey breast after 3 days of storage at 4˚C (Anany et al., 2011), suggesting that phage-based bioactive materials could be applied as a hurdle technology.

The second chapter of this research focused on the development of bacteriophage-based food packaging material. Virulent phages with different morphologies from the *Siphoviridae* and *Myoviridae* families were immobilized onto two types of cellulose-derived membranes (filter paper and nitrocellulose membranes) for the development of bioactive food packaging materials. Moreover, Gum Arabic (GA), sucrose, and trehalose were evaluated for their protective effect against inactivation of phage upon exposure to environmental stresses (eg. Desiccation). Finally, functional properties of the developed packaging materials were evaluated to postulate the spectrum of food they could be used.

II-2. MATERIALS AND METHODS

II-2-1. Bacterial strains and bacteriophages

Two *S. aureus*-targeting bacteriophages and their respective host bacteria used to develop phage-based bioactive food packaging materials are outlined in Table II-1. All staphylococcal strains were stored in 15 % glycerol at -80 ℃ and working cultures were incubated at 37 ℃ for 12-18 hours on tryptic soy agar. For the liquid culture preparation, a single *Staphylcococcus* colony from TSA was subcultured into TSB and incubated overnight at 37 ℃ under shaking condition (220 rpm). High-titer bacteriophage stocks were stored in glass vials with dialysis buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8), 10 mM $MgCl_2$) at 4 ℃ until further use.

Table II-1. Bacteriophages used for immobilization and their host bacteria

Phage	Family	Lifecycle	Host strain	Tail length (nm)	Source
SA11	*Myoviridae*	Virulent	*S. aureus* ATCC 13301	205	Kim and Myung, 2012
SA13m	*Siphoviridae*	Virulent	*S. aureus* RN 4220	150 ± 3	Chang, 2016

II-2-2. Bacteriophage propagation

Following the purification step, the isolated phages underwent propagation. Phage propagation was conducted by sub-culturing overnight (ON) culture of the host bacteria (1:100 = ON culture: fresh liquid growth medium) into fresh TSB. The culture was incubated at 37 ℃, 220 rpm until the growth phase reached the early exponential phase (OD_{600}=0.3~0.4). The host bacteria were infected with bacteriophage at the multiplicity of infection (MOI) of 0.1 and incubated at 37 ℃ for 5 hours, followed by centrifugation (15,000 rcf, 15 minutes) and filtration.

Finally, a high-titer phage stock was prepared by precipitating bacteriophages with polyethylene glycol 6000 (Junsei Chemical Co., Tokyo, Japan). Afterwards, the precipitated phages were concentrated using a CsCl-gradient (CsCl density=1.3, 1.45, 1.5, 1.7 g/mL) ultracentrifugation (Himac CP 100β; Hitachi, Tokyo, Japan) at 75,000 rcf for 2 hours at 4 ℃. Obtained phage concentrate was undergone dialysis against dialysis buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$) for 2 hours at 4℃ with a buffer change halfway through the dialysis. Finally, titer of the bacteriophage stock was determined by overlaying 100 µL of ten-fold dilutions of phage stock on the surface of TSA covered with 5 mL of 0.4% semisolid TSA containing 100 µL host bacteria. Plates were incubated overnight in an upright position at 37 ℃. Purified phages were stored in glass vials at 4 ℃ until further use.

II-2-3. Preparation of cellulose membranes

Advantec Grade 5C quantitative filter paper (Toyo Roshi Kaisha, Tokyo, Japan) were cut into 23 mm diameter (4.15 cm^2) circle. The surface charge of the paper disks was modified based on the procedures outlined in a previous study (Anany et al., 2011). Pre-cut autoclaved paper disks were added to 0.5% (w/v) polyvinylamine (PVAm) polymer (Xelorex RS 1300, BASF, Ludwigshafen, Germany) dissolved in 5 mM NaCl at pH 10 for 10 minutes to produce paper disks possessing positively-charged surface. Subsequently, unadsorbed PVAm polymer was removed by rinsing the polymer-saturated disks in salt solutions with for 5 minutes.

Nitrocellulose (NC) membrane (Amersham™ Protan™ 0.2 μm NC; GE Healthcare, Chicago, IL, USA) was also considered an immobilization medium. The membranes were also cut into 23 mm diameter (4.15 cm^2) disks and were autoclaved for sterilization. Both immobilization matrices were stored at room temperature (RT) in Falcon tubes until further use.

II-2-4. Determination of the immobilization efficiency of the phage-immobilized membranes

Immobilization of phages on PVAm-treated cellulose membranes (PVAm membranes) and NC membranes was conducted as follows. Onto wet PVAm or NC membranes, 41.5 μL of high-titer phage solution (10^{11} PFU/mL) was spotted to achieve 10 μL/cm^2 and allowed to be completely absorbed by the membranes for 2

hours. Subsequently, phage-immobilized matrices were washed in 10 mL SM buffer eight times at 220 rpm, room temperature. Phage titer of each wash solution was determined using the standard overlay technique.

II-2-5. Determination of phage-immobilized membrane infectivity

Immobilization of phages on the immobilization matrices was conducted as outlined in Section II-2-4 with modified washing step. Instead of washing eight times in SM buffer, the number of wash cycles was reduced to three times. After washing, the immobilized membranes were subjected to 1.5-hour drying under a biosafety cabinet (Esco, Horsham, PA, USA).

The infectivity of phage-immobilized paper was evaluated by adding the bioactive paper to 10 mL TSB containing 10^8 CFU/mL of exponentially-growing *S. aureus* followed by incubation at 24 hours at 25 ℃ with shaking (220 rpm). The turbidity of the bacterial culture was measured every hour after phage infection at OD_{600} for 24 hours. An equal volume (41.5 μL) of bacteriophage stock solution and positively-charged cellulose discs were added to the bacterial culture as a positive control and negative control, respectively.

II-2-6. Selection of sugars to protect phages from environmental stresses

Trehalose (Sigma, St. Louis, MO, USA), sucrose (Sigma, St. Louis, MO, USA), and Gum Arabic (GA; Showa, Japan) were used to evaluate their potential of protecting

bacteriophages from environmental stresses. The experimental procedure was modified from a previous study (Anany et al., 2011). Briefly, 10 μL of SA11 phage (~10^9 PFU/mL) with and without the sugars (0, 25, 50, 75 mg/mL) was air-dried for 12 hours and reconstituted in 1 mL of SM buffer for 30 minutes. The phage titer of each reconstitutes was determined using the standard overlay assay and the phage counts were compared for analysis.

II-2-7. Gum Arabic treatment optimization

Before immobilization, GA was supplemented to the bacteriophage solution to enhance stability upon storage. Various GA concentrations were considered to maximize the infectivity of the bioactive paper with the minimal use of GA.

Preparation of the phage-immobilization was modified from the procedure outlined in Section II-2-4, where the high-titer phage stock was mixed with 15% (w/v) GA (final concentration: 0~120 mg/mL) to achieve the final phage titer of 10^{11} PFU/mL. Subsequently, 41.5 μL of the mixture was spotted onto the prepared membranes and incubated for 2 hours. After incubation, the phage-immobilized membranes were washed and dried for 1.5 hours. Prepared phage-immobilized papers were then used to infect the host bacteria (*S. aureus* ATCC 13301 and *S. aureus* RN4220 for SA11 and SA13m, respectively) in the early exponential growth phase ($O.D._{600}$ 0.3-0.4). The infected bacteria were incubated at 25°C, 220 RPM and optical density of each treatment conditions was measured for 24 hours to evaluate the

infectivity of the bioactive membranes.

II-2-8. Release of the phages from the bioactive packaging material after drying

Prepared bioactive packaing films were submerged in 10 mL SM buffer for 1 hour to determine whether phages were released after drying. After the incubation, papers were removed and the phage titer of the buffer was measured with the spotting assay. The experiment was performed in two independent replicates.

II-2-9. pH stability of the free phage and the phage-immobilized packaging materials

To determine the stability of bacteriophage-immobilized packaging materials under various pH conditions, prepared bioactive papers immobilized with SA11 and SA13m or equivalent amount of free phages were tested by exposing immobilized/free phages in 10 mL of acetate buffer (pH 5), phosphate buffer (pH 6, 7, 8), and Tris-Cl buffer (pH 9). After 1 hour of incubation at room temperature, infectivity of the immobilized papers exposed to various pH conditions were determined as previously described, and bacteria concentration after 24 hours of infection was evaluated to compare the treatment groups. Phage titer of the buffers after removing the phage-immobilized papers and the free phages exposed to various pH conditions were also determined. For the control group, phages were exposed to SM buffer.

II-2-10. Storage stability of the phage-immobilized packaging materials

The phage-coated packaging materials were stored in a closed container with relative humidity (RH) of 85% to evaluate the long-term storage effect on the infectivity of the bioactive packaging materials. The RH of the closed-container was controlled with saturated KCl and the temperature was maintained at 25 ℃. Also, the container was prepared one day before the day of experiment to ensure that the RH was equilibrated. Prepared phage-coated packaging materials were sampled after 1 and 2 days of storage in the closed chamber. Freshly-prepared packaging materials without any further storage were also tested as controls. To test the infectivity of the paper disks were tested in a similar manner as the procedure described in section II-2-4. In all storage stability assays, 120 mg/mL of GA was used.

II-2-11. Statistical analysis

The statistical analysis of the experimental data was accomplished with Graphpad Prism, version 5.01 (GraphPad Software Inc., La Jolla, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). A one-way analysis of variance (ANOVA) and a two-way ANOVA followed by Tukey's multiple comparison test (95% confidence interval) among more than two groups were performed. In all cases, statistical differences between the means were considered significant when $p \leq 0.05$.

II-3. RESULTS AND DISCUSSION

II-3-1. Immobilization efficiency of bacteriophage SA11 and SA13m on PVAm-treated cellulose membrane

The effect of PVAm on the immobilization efficiency of various bacteriophages on cellulose membranes were determined by washing bacteriophage SA11 and SA13m-treated cellulose membrane eight times and comparing the amount of phages released from each treatment condition. As previously mentioned, choosing virulent phages for food application is necessary to prevent horizontal gene transfer of virulence factors and antibiotic resistance genes (Greer, 2005; Hagens and Loessner 2007). This implied that utilizing HSA30 and HSA84 (newly isolated phages from Chapter 1) for the development of bioactive packaging material may not be appropriate as the life cycles of these phages are yet to be determined. Therefore, two virulent *S. aureus* phages, SA11 and SA13m were chosen for this study.

A continuous release of 5 to 8 log PFU/mL of SA11 from the unmodified cellulose membrane suggested that cellulose membrane was not an appropriate phage-immobilization medium (Figure II-1a). This was likely be affected by the interaction between the cellulose membrane and SA11. Bacteriophages were demonstrated to possess net-negative charge, where the phage heads were slightly more negatively-charged than that of the tail proteins (Anany et al., 2011; Serwer, 1987). Given that carboxyl groups provide paper surface a slight negative-charge (Cao et al., 2015), repulsive force between negatively-charged paper and bacteriophage may have exerted a negative effect on the phage immobilization

efficiency.

To overcome this limitation, PVAm was used to modify the surface charge of the paper to alleviate repulsive force between bacteriophage and the cellulose membrane. Using amine as its function group, PVAm was successfully used to modify surface charges of paper (Anany et al., 2011) and silica particles (Kirwan et al., 2008). Moreover, PVAm has been approved as an effective food contact substance in 2002 by the US FDA (US FDA 2017), making it appropriate to be used in food packaging materials. PVAm increased the phage retention by 4 log after the first wash (Figure II-1a). After washing three times, no phages were detected in the wash buffers of the PVAm membrane. Based on these findings, PVAm treatment improved the immobilization efficiency of bacteriophages on cellulose membrane. Consistent with the findings from the present study, Cademartiri and colleagues (Cademartiri et al., 2010) demonstrated that the immobilization efficiency of positively-charged silica particle was improved by 2-4 log than the native (anionic) silica particles. Moreover, the authors observed that the anionic phage head interacted with the cationic silica particle and the tails faced the external environment (Cademartiri et al., 2010). This finding suggests that immobilization of phages on a positive surface may maximize the infectivity by allowing the positively-charged phage tail to interact with the host cell.

SA13m also showed a similar immobilization efficiency pattern as that of SA11 (Figure II-1b). A continuous release of phages was observed in the unmodified

cellulose membrane and no phages were eluted from the fourth washing and onwards in the modified membranes.

To explore various cellulose-derived membranes for the phage application, phage immobilization efficiency on NC membranes was also tested. Because NC membranes are composed of cellulose with nitrate functional group (Costa et al., 2014), there was no need to modify the surface charge of NC. In addition, NC's application in western blots for protein immobilization implied that this membrane would retain bacteriophages well (Blake et al., 1984). As expected, approximately $10^4 \sim 10^5$ PFU/mL of SA11 and SA13m were eluted from the NC membrane after the first washing step (Figure II-2). This result was comparable to that of the PVAm membranes. Similar to the phages immobilized on the PVAm membranes, the amount of phages released from the NC membranes plateaued after the third washing step. Thus, it was determined that three wash cycles were sufficient to wash out poorly-bound bacteriophages for further studies.

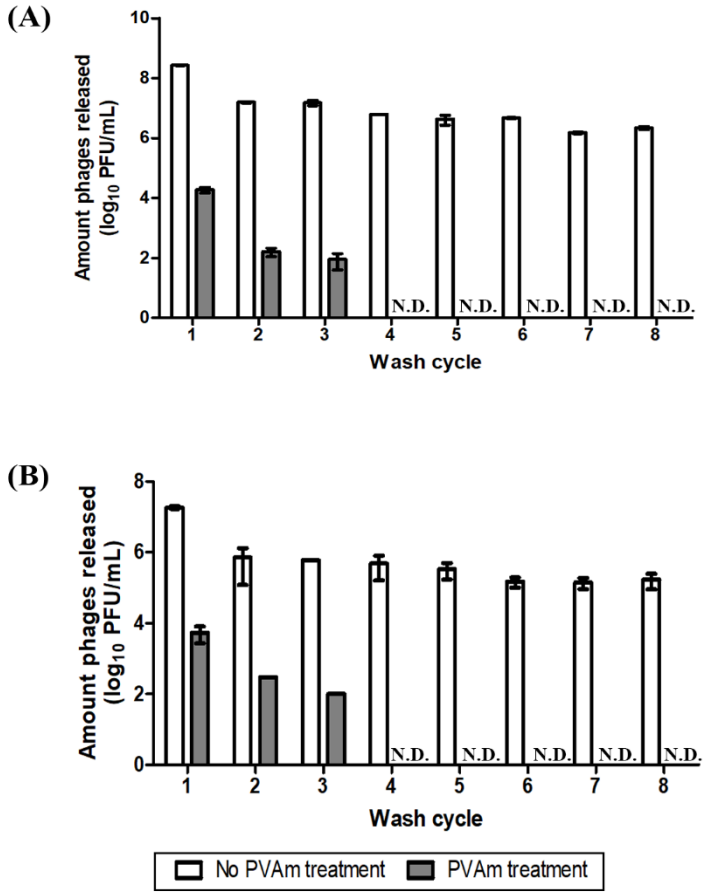

Figure II-1. Release of bacteriophages from cellulose membrane After the immobilization step, a) SA11 and b) SA13m-immobilized membranes were washed in SM buffer eight times. Subsequently, the phage titer of each buffer was measured using the standard overlay assay to estimate the amount of phages released from the immobilized membranes. Data are the means of two independent trials and the error bars indicate the standard error of mean.

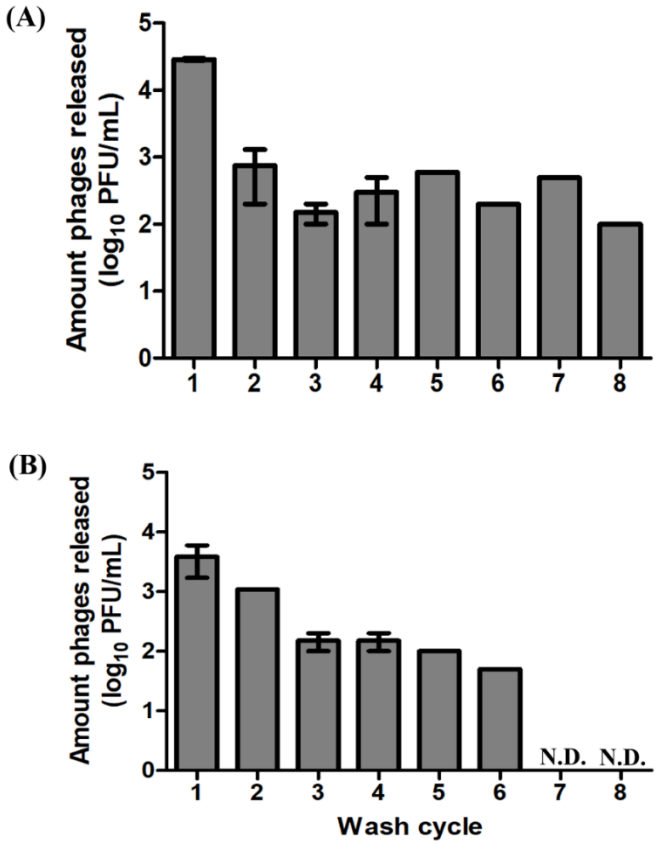

Figure II-2. Release of bacteriophages from NC membrane After the immobilization step, the a) SA11 and b) SA13m-immobilized membranes were washed in SM buffer eight times. Subsequently, the phage titer of each buffer was measured using the standard overlay assay. Data are the means from two independent trials and the error bars indicate the standard error of mean.

II-3-2. Infectivity of the phage-immobilized membranes

The infectivity of phage SA11 and SA13m-immobilized PVAm membranes were evaluated by conducting a bacterial growth challenge assay on *S. aureus* ATCC 13301 and RN 4220, respectively. Wet SA11-immobilized PVAm and NC membranes resulted in a decrease in the host cell density after 5 and 9 hours of infection, respectively (Figure II-3a, c). However, drying both membranes lead to a loss of infectivity. Interestingly, both wet and dried SA13m-immobilized PVAm/NC membrane showed no infectivity against *S. aureus* RN 4220 (Figure II-3b, d).

One of the common characteristics found in the phage-immobilized PVAm and NC membranes was that infectivity of the bioactive membranes was completely lost upon drying. Several papers made similar observations. For example, Rode and colleagues (Rode et al., 2011) also highlighted bacteriophage's susceptibility to desiccation through observing the stability of air-dried bacteriophage Stx2 on stainless steel. The authors observed 7-log reduction in the phage titer after desiccation stress. Anany and colleagues (2011) also addressed a similar problem associated with the loss of phage infectivity upon drying. Food packaging materials are typically stored at ambient temperature and humidity condition to prevent the loss of film performance (Siracusa et al., 2008). Therefore, addition of a protective agent on the bioactive packaging film would be necessary to prevent the loss of phage infectivity upon exposure to environmental stresses (eg. desiccation).

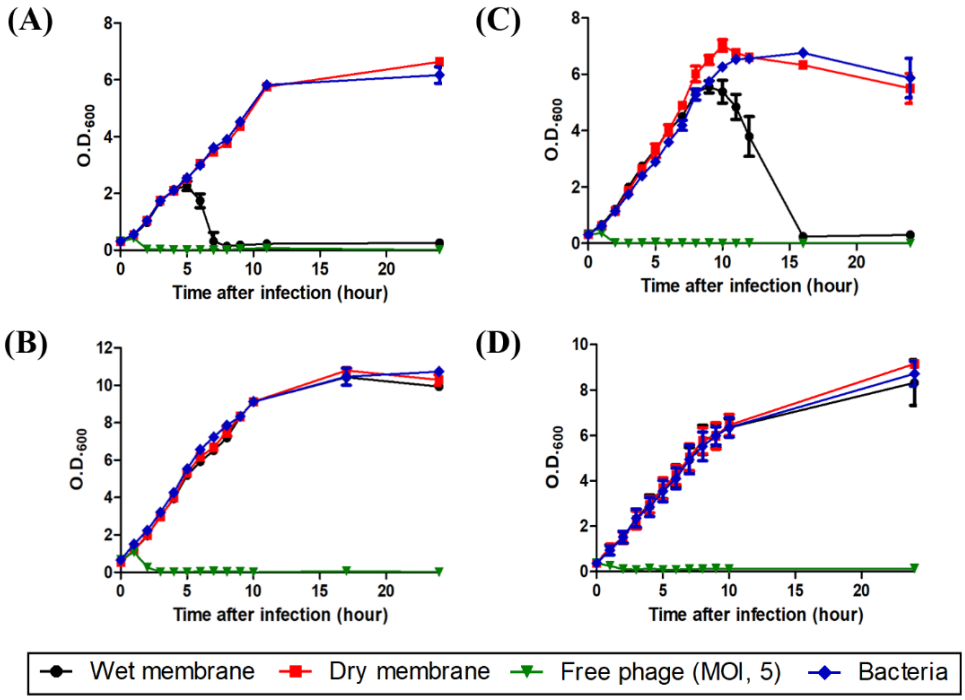

Figure II-3. Infectivity of the phage-immobilized membranes a) SA11, b) SA13m-immobilized PVAm membranes c) SA11, and d) SA13m-immobilized NC membranes *S. aureus* was infected with the dry/wet phage-immobilized membranes. Free phages (MOI, 5) and SM buffer were used as positive and negative controls, respectively. Data are the means from two independent replicate trials and the error are the standard error of mean.

II-3-3. Selection of sugars to protect bacteriophages from environmental stresses

Various sugars (GA, sucrose, and trehalose) were evaluated for their efficacy to reduce phages' sensitivity to desiccation stress. After 12 hours of desiccation at room temperature, the phages without sugar treatment completely lost their infectivity, but sugar treatment retained minimum 50% of the phage activity.

The protective effect of different sugars against SA11's susceptibility to desiccation stress was compared (Figure II-4). In comparison to the initial phage titer (10^{10} PFU/mL), approximately 3 ~ 4 log reduction in the infectivity was observed with the phages treated with sucrose and trehalose; no significant difference was observed amongst different sugar concentrations. However, 25 mg/mL of GA treatment resulted in approximately 2 log reduction in the phage titer after 12-hour desiccation. The use of trehalose (Uritani et al., 1995), GA (Sorokulova et al., 2012), sucrose (Puapermpoonsiri et al., 2010), and other sugars have been widely used for long-term storage of biological samples. For example, Puapermpoonsiri and colleagues (Puapermpoonsiri et al., 2010) indicated that the water content of a freeze-dried sample of bacteriophage with 0.1 M sucrose addition was 4-6 % and attained the highest lytic activity. Polar or ionic groups of sugar molecules can bind to water firmly, resulting in bound water (Vaclavik and Christian, 2008). As these bound water molecules would not be evaporated, a certain level of moisture content of the bioactive packiging material with sugar treatment would be retained albeit extensive drying. As GA exhibited the highest recovery of bacteriophages after desiccation, it

was selected as the protective agent for further studies.

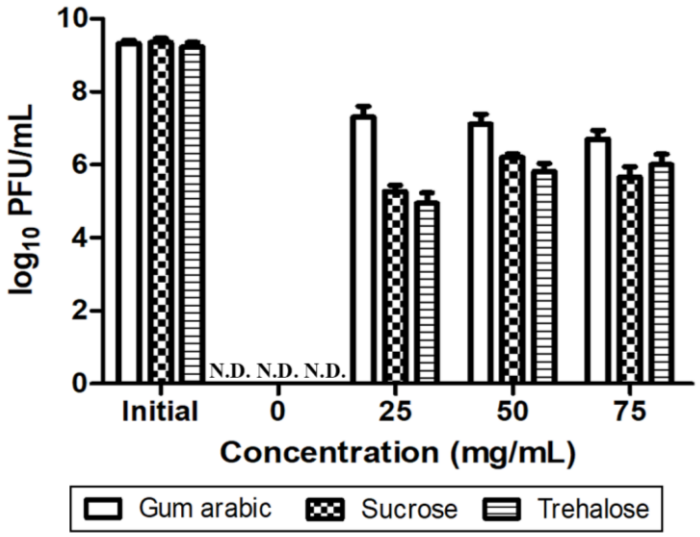

Figure II-4. Effect of Gum Arabic, sucrose, and trehalose on the phage infectivity SA11 phage was mixed with the sugars at various concentrations (25, 50, and 75 mg/mL) and air-dried for 12 hours at room temperature. Dried phages were reconstituted in SM buffer and the titer of each reconstituted phages was determined. Data are the means from three independent trials and the error bars indicate the standard error of mean.

II-3-4. Gum Arabic optimization for SA11 and SA13m-immobilized membranes

Minimum GA treatment condition to preserve the antimicrobial activity of SA11 and SA13m on dried bioactive packaging materials was determined through an OD-based inhibition test. As a result, 15-20 mg/mL of GA was sufficient for SA11-immobilized PVAm membrane to affect *S. aureus* ATCC 13301 growth, where no cells were detected after 8-hour of infection (Figure II-5a). However, SA13m-immobilized PVAm membrane required higher GA concentration to achieve host cell growth inhibition. A minimum of 40 mg/mL of GA was required to control the growth of *S. aureus* RN 4220 within 24 hours of infection (Figure II-5b).

GA also assisted the control of *S. aureus* using SA11-immobilized NC membrane. However, this bioactive membrane required a higher GA concentration for a successful control of the host bacteria. It was apparent that a minimum of 40 mg/mL of GA was needed to achieve a complete bacterial growth inhibition after 10 hours of infection, and 60 mg/mL of GA treatment resulted in a complete cell death within 6 hours of infection and the infectivity lasted for more than 18 hours (Figure II-5c). In the case of SA13m-immobilized NC membrane, GA did not enhance the antimicrobial activity against *S. aureus* RN 4220 (Figure II-5d). No further characterization on SA13m-immobilized NC membranes were conducted as there was no infectivity observed.

Upon immobilization, phages have limited motility and the only way for the phages to interact with the host is by exposing their binding receptors (located on

phage tails) to the external environment (Hosseinidoust et al., 2014). This implies that phage tails may play a key role in the infectivity of phage-based bioactive packaging materials. Indeed, the phages tested in the present research had different tail morphology and resulted in different levels of infectivity, where SA11 achieved faster host growth inhibition than SA13m. Belonging to the *Myoviridae* family, SA11 possesses a contractile, rigid tail, while SA13m, a *Siphoviridae*, has a non-contractile tail with flexibility (King et al., 2011). Because *Myoviridae* phage tails would likely to stay up right at all times, SA11-functionalized bioactive packaging materials would have higher chance of achieving successful host infection, given that these phages were immobilized in a desired orientation. This hypothesis should be confirmed by comparing the infectivity of phage-based bioactive packaging materials immobilized with multiple *Myoviridae* and *Siphoviridae* phages.

Another factor influencing different host infectivity level may be the amount of phages eluted from the bioactive membranes during infection. Figure II-6 illustrates the amount of phages released from various membranes. The data revealed that GA promoted the elution of bacteriophages from the dried immobilized membranes. For example, 7.5×10^3 PFU/mL of phages were eluted from the SA11-immobilized PVAm membrane treated with GA while no GA treatment group did not induce the release of any phage particles (Figure II-6). In addition, the amount of phages released from the membranes treated with GA or not was proportional to the infectivity of phage-immobilized membranes. For example, relatively small amount

of GA was sufficient to kill bacteria with SA11-immobilized PVAm membrane (Figure II-5), which released highest amount of phages (Figure II-6). These findings imply that GA may enhance the infectivity of the phage-immobilized bioactive packaging materials through protecting bacteriophages from desiccation, and promoting the release of bacteriophages.

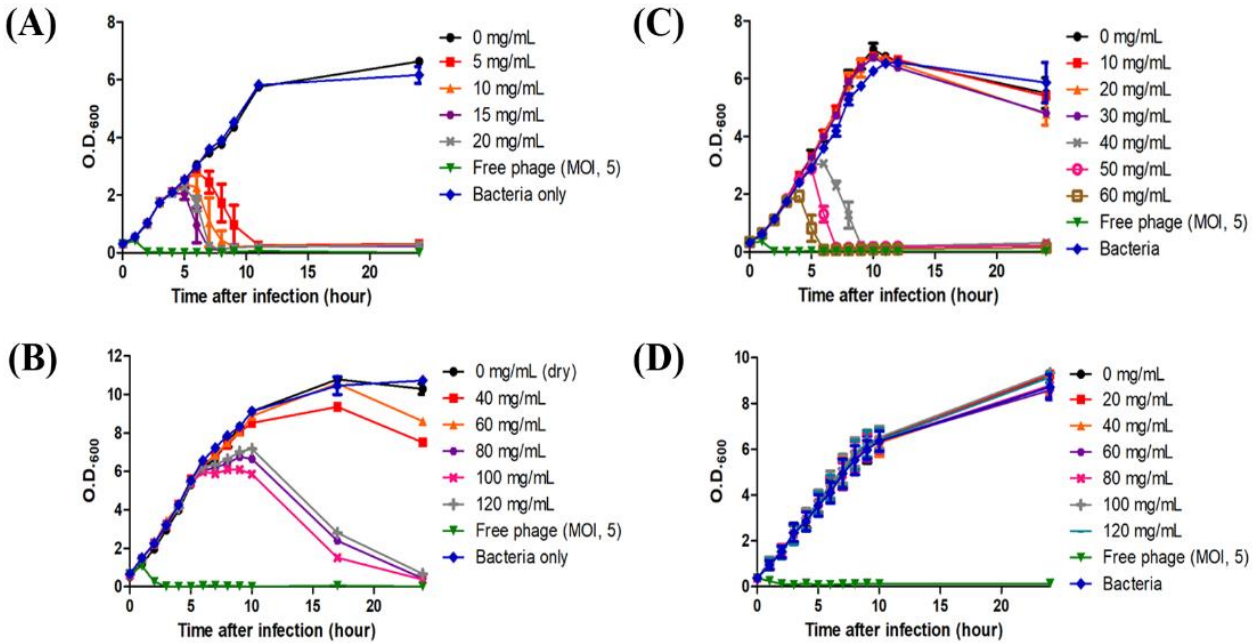

Figure II-5. Gum Arabic treatment optimization a) SA11, b) SA13m-immobilized PVAm membranes. c) SA11, d) SA13m-immobilized NC membranes Dry phage-immobilized membranes with GA treatment were added to *S. aureus* at the early-exponential growth phase and the changes in the cell density was detected to observe the infectivity of the bioactive membranes. Free phages (MOI, 5) and SM buffer were used as positive and negative controls, respectively. Data are the means from two independent replicate trials and the error are the standard error of mean.

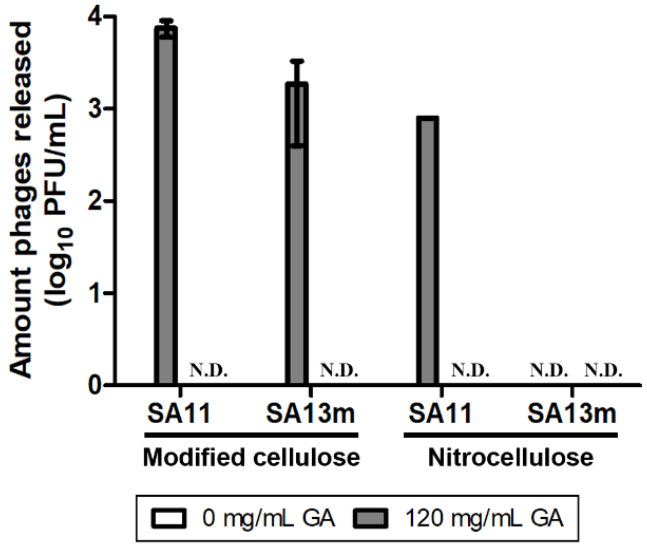

Figure II-6. Elution of phages from the dried phage-immobilized membranes Phage-immobilized membranes were submerged in SM buffer for 1 hour to determine whether phages are released from the membranes after drying. Subsequently, phage titer of the buffer solution was measured to quantify the amount of phages released from the bioactive membranes. Data are the means from two independent replicate trials and the error are the standard error of mean.

II-3-5. Effect of pH on the stability of free SA11 and SA13m

Stability of bacteriophage SA11 and SA13m at various pH conditions were evaluated. After exposure to pH adjusted buffers (pH range: 5.0 ~ 9.0), SA11 exposed to all pH conditions maintained its titer (Figure II-7a). In contrast, there was approximately 10^1~10^2 PFU/mL reduction in the lytic activity of SA13m exposed to pH 5.0 (acetic acid) compared to the other treatment groups (Figure II-7b).

Findings from this study suggest that free SA11 and SA13m are stable at pH 5-9. Considering *S. aureus* is a commonly occurring pathogen in cheese (do Carmo et al., 2002), meat (Jones et al., 2006), milk (Asao et al., 2003), and deli-meat (Richards et al., 1993), SA11 and SA13m's stability at a broad host range make them appropriate for the pathogen control in food products commonly associated with *S. aureus* outbreaks. This is because all food products mentioned above fall within the pH range of 5 to 9 (Table II-2). Although the present study exposed the bacteriophages to various pH conditions for one hour, *S. aureus* lytic phage Stau2's stability at pH 5 to 13 (which was exposed to the stress condition for 24 hours) suggest SA11 and SA13m may also withstand long-term exposure to extreme pH conditions (Hsieh et al., 2011).

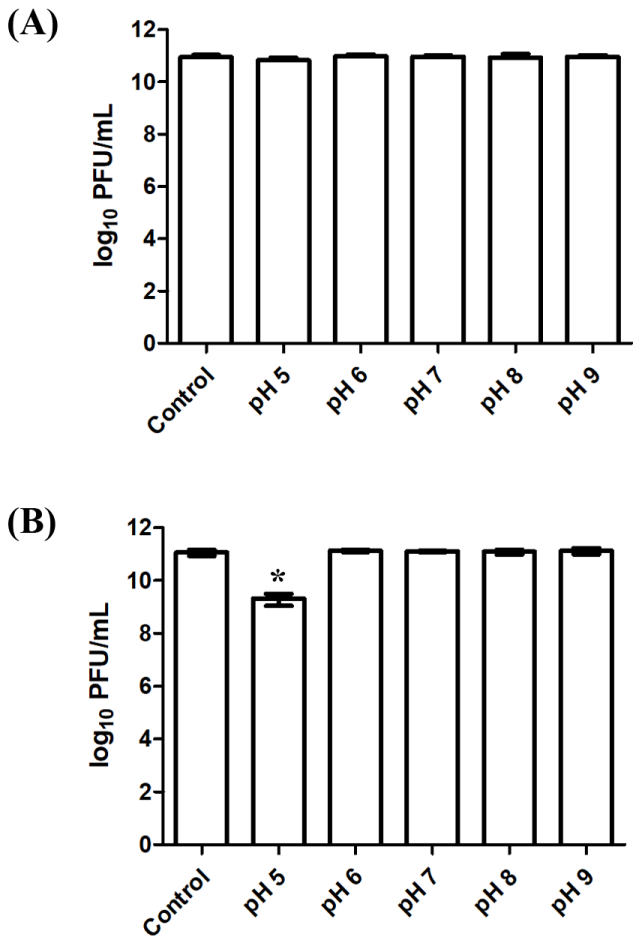

Figure II-7. The effect of pH on the stability of free phages Stability of a) SA11 and b) SA13m against various pH conditions were tested by exposing the phages to pH adjusted TSB for 1 hour. After the exposure, the phage titer was measured using the overlay assay. Data are the means from two independent replicate trials and the error are the standard error of mean. Treatments denoted by astricks (*) were significantly different $p \leq 0.05$.

Table II-2. pH values of foods associated with *S. aureus* foodborne illnesses

Food product	pH range[a]
Milk	6.3-8.5
Cheese, Cheddar	5.9
Beef, Ground	5.1-6.2
Ham	5.9-6.2

[a] US FDA, 2012

II-3-6. Effect of pH on the infectivity of the free/immobilized SA11 and SA13m

Phages that had been exposed to various pH conditions were used to infect the host bacteria (*S. aureus* ATCC 13301 and *S. aureus* RN 4220) to further investigate the effect of pH on phage infectivity. It was anticipated that SA11's infectivity against *S. aureus* ATCC 13301 would not be compromised by the exposure to various pH condition, because pH did not have any impact on the titer of SA11 (Figure II-7a). As expected, SA11 achieved about 2 log reduction of *S. aureus* ATCC 13301 in all treatment groups (Figure II-8a). An interesting observation of the SA13m infectivity was observed (Figure II-8b). It was demonstrated that the infectivity of SA13m was not compromised after being exposed to the buffer at pH 5, despite the fact that the phages recovered in the buffer solution was 2 log lower than the rest of the treatment conditions (Figure II-7b). There were no significant relationships between the infectivity of SA13m and the pH of buffer solution (Figure II-8b; $p > 0.05$).

Effect of pH on the infectivity of immobilized SA11 and SA13m was also determined. At all pH conditions and immobilization media, the infectivity of SA11 remained constant, maintaining infectivity with 2 – 3 log reduction of host bacteria. Interestingly, the infectivity of SA11-immobilized NC membrane showed significantly enhanced antimicrobial effect than that of free phage (Figure II-8a; $p \leq 0.05$), suggesting the immobilization of this phage onto NC membrane has a potential for food application. Unlike SA11-immobilized bioactive packaging materials,

SA13m on PVAm membranes demonstrated very little infectivity (log reduction value of 0.71 to 1.15) against *S. aureus* RN 4220 (Figure II-8b). Considering the average log reduction achieved by free SA13m was 3.47, the infectivity of the immobilized phages was significantly reduced by 2.5 log ($p \leq 0.05$). This finding implied that SA13m-immobilized PVAm membrane may not be suitable for controlling *S. aureus* on food surfaces.

Therefore, the findings from this study suggest that SA11-immobilized bioactive packaging materials would be more applicable as food packaging material as their infectivity were either preserved or enhanced upon immobilization.

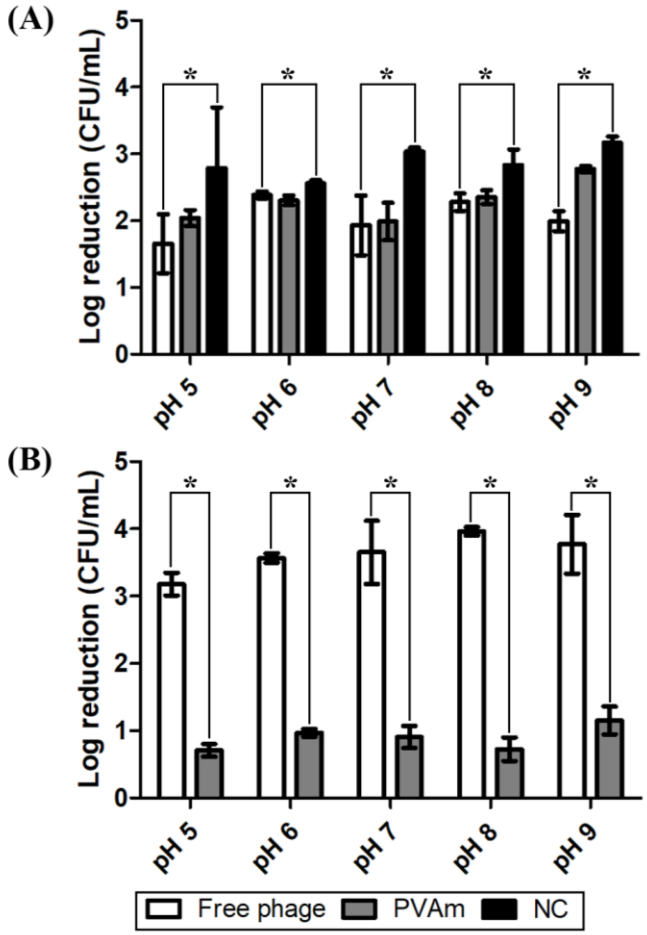

Figure II-8. The effect of pH on the infectivity of free/immobilized phages After the 1-hour exposure to various pH conditions, free/immobilized a) SA11 and b) SA13m were used to infect the host bacteria. Log reduction of the *S. aureus* cells after the 24-hour infection to the phages were determined. Data are the means from two independent replicate trials and the error are the standard error of mean. Treatments denoted by astricks (*) were significantly different $p \leq 0.05$.

II-3-7. Storage stability of SA11 and SA13m-immobilized membranes

Stability of the phage-immobilized membranes upon long-term storage was evaluated trough the bacterial growth challenge assay. Prepared membranes were incubated in a closed container with controlled RH at 85% for two days and the infectivity of the stored membranes were evaluated for three days. Unexpectedly, SA11-immobilized PVAm membrane showed significant reduction in activity with long-term storage as no infectivity was observed even after 1 day of storage (Figure II-10a). Due to poor antimicrobial effect of SA11-immobilized on PVAm membrane in the storage stability test, it was anticipated that the loss of infectivity may be associated with SA11's susceptibility to long-term desiccation. SA11 on NC membrane, however, demonstrated that the infectivity was maintained after 2 days of storage in the controlled chamber (Figure II-10c).

Interestingly, SA13m-immobilized PVAm membrane showed higher stability than SA11 in long-term storage, although SA13m showed poor infectivity upon immobilization (Figure II-10b). Unlike the SA11-immobilized PVAm membrane, a decrease in the cell density was observed with the SA13m-immobilized PVAm membrane that was stored in a controlled RH chamber for 1 day.

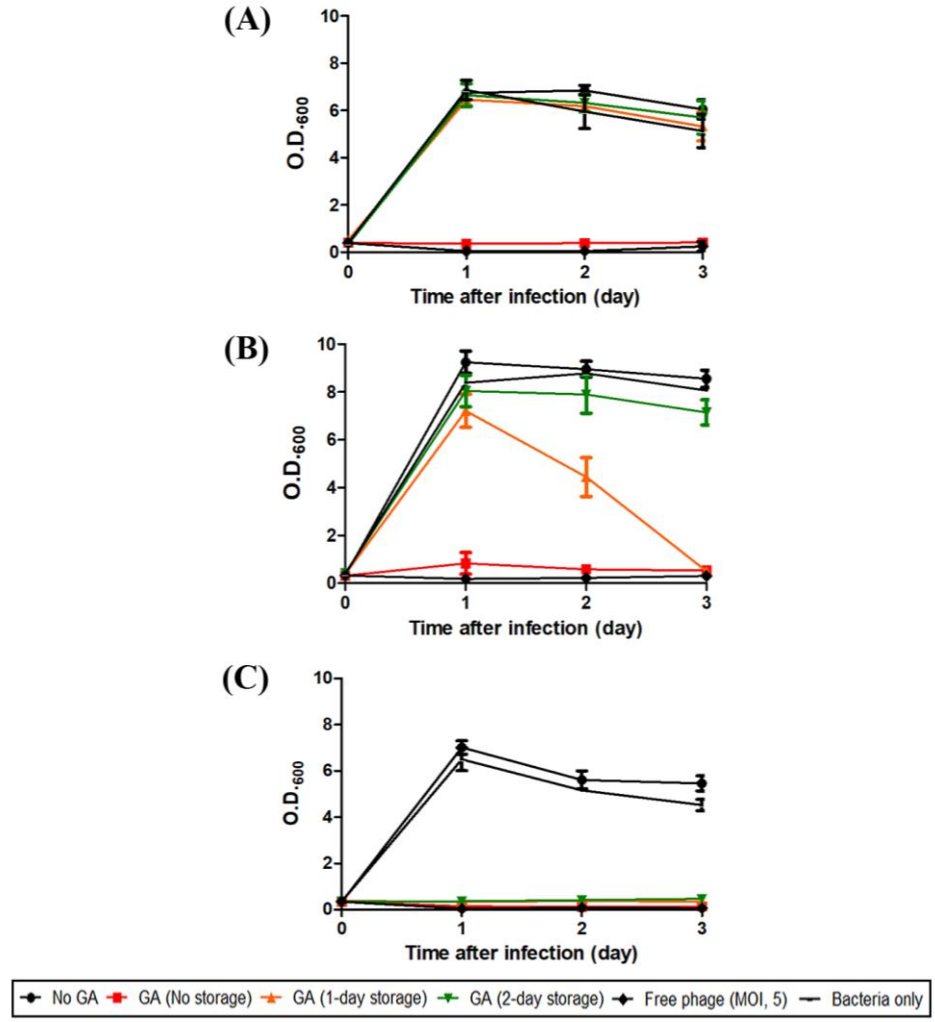

Figure II-9. The effect of storage at 85 % RH on the infectivity of the immobilized bacteriophages a) SA11, b) SA13m on PVAm membrane and c) SA11 on NC membrane Dried phage-immobilized papers (phage titer: 10^9 PFU/cm^2) were stored inside a leak-proof container with controlled relative humidity (85% RH; saturated KCl) at ambient temperature up to two days. Log reduction of *S. aureus* ATCC 13301 after a 3-day infection were determined. Data are the means from three independent replicates and the error are the standard error of mean.

II-4. CONCLUSION

In this chapter, bacteriophage-immobilized bioactive packaging materials were developed and characterized to evaluate their potential as novel antimicrobial agent. Two phages, SA11 (*Myoviridae*) and SA13m (*Siphoviridae*), were immobilized on two different cellulose media (cellulose membrane and nitrocellulose [NC] membranes). It was confirmed that modification of the surface charge of cellulose membrane using polyvinylamine (PVAm) enhanced the immobilization efficiency of both phages. In the case of NC membranes, charge conversion was not required and exhibited comparable immobilization efficiency as that of PVAm-treated cellulose membrane.

Despite enhanced immobilization efficiency, drying the bioactive membranes resulted in a complete loss of infectivity, which is not a desirable trait in an antimicrobial agent. To overcome this hurdle, Gum Arabic (GA) was supplemented to preserve the bioactivity of the membranes exposed to desiccation. Overall result suggested an improved infectivity of the SA11-immobilized NC and cellulose membranes, and SA13m-immobilized cellulose membrane, but no bioactivity was observed in SA13m-immobilized NC membrane.

Functionality (pH stability and storage stability) of the GA-treated bioactive membranes were evaluated to determine their potential to be applied in the food industry. It was apparent that pH did not appear to have a significant impact on the bioactivity of SA11-immobilized NC and cellulose membrane, but SA13m

demonstrated lower infectivity than free SA13m. Furthermore, storage stability test confirmed that SA11 on NC membrane maintained its activity for 2 days upon storage at RH 85%. These findings suggest that SA11 on NC membrane has the highest potential as a novel antimicrobial food packaging material. However, further optimization of the functional properties would be necessary to commercialize this bioactive packaging material as this research was a preliminary step of the phage-based bioactive packaging material development. Optimization should be taken based on the findings from additional studies elucidating its mechanical (eg. Tensile strength, seal and peel tests, etc) and physical (moisture/gas permeability, thermal stability, etc) properties of the packaging material.

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국문 초록

황색포도상구균은 그람 양성의 고병원성 식중독균으로서, 항생제 내성균발생 비율이 매우 높은 것으로 알려져 있다. 특히 메티실린 및 반코마이신 저항성 황색포도상구균 등의 항생제 내성 황색포도상구균은 현존하는 대부분의 항생제로 치료가 불가능하기 때문에 의료 분야에서 매우 심각한 문제를 일으키고 있다. 따라서 기존 항생제를 대체할 수 있는 새로운 식중독균 제어방법이 다수 연구되어 왔으며, 그 일환으로 박테리오파지에 대한 연구가 진행중에 있다. 박테리오파지는 자가증식, 높은 숙주 특이성 등의 특징을 갖고 있으며, 인체에 대한 부작용은 알려진 바가 없고, 그 다양성이 대단히 높아 새로운 항생제 대체재로 각광을 받고 있다. 본 연구에서는 황색포도상구균을 사멸할 수 있는 4종의 박테리오파지를 분리 하고, 그중 숙주 용해능이 우수한 HSA30과 HSA84에 대한 생물학적 특성을 분석하여 이들의 식중독균 저해 능력을 확인하였다. 먼저 HSA30은 긴 수축성 꼬리와 정이십면체의 머리를 지니고있는 *Myoviridae*과에 속함을 알 수 있었고, 2가 양이온 처리시 황색포도상구균의 증식을 19시간동안 억제할 수 있었으며, 본 연구에서 검사한

29 종의 황색포도상구균 중 22 종에 대해 사멸능을 보였다. 유전체 분석 결과, HSA30의 유전체는 140 kb 크기의 이중나선 DNA로 이루어져 있었으며, 224개의 코딩 유전자와 3개의 tRNA 유전자가 예측되었다. 또한 HSA30의 유전체에서는 독성인자 및 항생제 내성 유전자가 발견되지 않아, HSA30을 이용하였을 때 발생할 부작용은 예측되지 않았다. 하지만 재조합 효소 등 용원성 파지의 lysogeny 형성에 관여하는 유전자가 예측되어, HSA30 역시 이러한 용원성 파지일 가능성이 있음을 확인하였다. HSA84는 *Siphoviridae*과에 속해있으며, 유전체에 용균/용원 결정 유전자가 존재하는 것을 보아 이 파지는 용원성 파지로 예측되었다. 하지만, 다양한 포도상구균 사멸능과 우수한 숙주균 저감 효과로 보아 HSA84 역시 생물학적 방제제로서의 가능성을 보임을 확인하였다. 또한 본 연구에서는 박테리오파지를 기반으로 한 새로운 식품 포장재 개발을 진행하고, 그 기능을 향상시키기 위한 방법을 제시하였다. *Myoviridae* 과에 속하는 SA11과 *Siphoviridae*인 SA13m을 셀룰로오스막과 니트로셀룰로오스막에 각각 흡착시키고 이들의 황색포도상구균 사멸능을 비교하였다. 우선 음전하를 갖는 셀룰로오스막에 양전하를 띄는 폴리비닐아민 (polyvinylamine; PVAm)을 코팅하여 줌으로써 셀룰로오스막의 파지 고정화 효율을 약 10,000배 가량 크게

향상시킬 수 있음을 확인하였다. 니트로셀룰로오스막 또한 PVAm이 처리된 셀룰로오스막과 비슷한 파지 고정 효과를 보이며 새로운 포장재로서의 가능성을 확인하였다. 하지만 파지 고정 포장재를 건조한 후에는 황색포도상구균 사멸능이 급격히 감소함을 확인하였고, 이를 보완하기 위해 포장재에 여러 가지 안정제를 처리하여 주었다. 여러 종류의 안정제 중에서 아라비아검의 효율이 가장 높은 것을 확인하였고, SA13m이 고정된 니트로셀룰로오스막을 제외한 다른 포장재는 건조 후에도 황색포도상구균 사멸능이 복구됨을 확인하였다. 식품 포장재로서의 기능성을 확인하기 위해, 새로 개발된 파지 기반 식품포장재가 여러 pH에 대해 얼마나 안정적으로 활성을 유지하는지, 또 장기간 보관에 따른 활성 감소가 발생하는지 확인하기 위한 실험을 진행하였다. 그 결과 SA11이 고정된 니트로셀룰로오스는 모든 pH 조건에서 99.9%의 숙주균을 저감 하였고, 이틀동안 85% 습도에 보관되어도 활성을 잃지 않는 것을 확인하여 식품 포장재로서 높은 가능성을 보임을 확인하였다.

주요어: 박테리오파지, 황색포도상구균, 항균성 식품 포장재

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