

Studies on Lytic, Tailed *Bacillus cereus*-specific Phage for Use in a Ferromagnetoelastic Biosensor as a Novel Recognition Element

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This study investigated the feasibility of the lytic, tailed *Bacillus cereus*-specific phage for use in a ferromagnetoelastic (FME) biosensor as a novel recognition element. The phage was immobilized at various concentrations through either direct adsorption or a combination of 11-mercapto-1-undecanoic acid (11-MUA) and [N-(3-dimethylaminopropyl)-N'-carbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS)]. The effects of time and temperature on its lytic properties were investigated through the exposure of *B. cereus* (4 and 8 logCFU/ml) to the phage (8 logPFU/ml) for various incubation periods at 22°C and at various temperatures for 30 and 60 min. As the phage concentration increased, both immobilization methods also significantly increased the phage density ($p < 0.05$). SEM images confirmed that the phage density on the FME platform corresponded to the increased phage concentration. As the combination of 11-MUA and EDC/NHS enhanced the phage density and orientation by up to 4.3-fold, it was selected for use. When various incubation was conducted, no significant differences were observed in the survival rate of *B. cereus* within 30 min, which was in contrast to the significant decreases observed at 45 and 60 min ($p < 0.05$). In addition, temperature exerted no significant effects on the survival rate across the entire temperature range. This study demonstrated the feasibility of the lytic, tailed *B. cereus*-specific phage as a novel recognition element for use in an FME biosensor. Thus, the phage could be placed on the surface of foods for at least 30 min without any significant loss of *B. cereus*, as a result of the inherent lytic activity of the *B. cereus*-specific phage as a novel recognition element.

Keywords: Ferromagnetoelastic biosensor, *Bacillus cereus*, recognition element, lytic tailed phage, optimization

Introduction

Bacillus spp. are gram-positive, motile, facultative anaerobic, and rod-shaped bacteria, with sizes between 0.5×1.2 mm and 2.5×10 mm [1, 2]. They are resistant to heat, dehydration, and other harsh physical stresses owing to their endospore formation ability [3]. Although the optimal temperature for their growth is known to be between 25°C and 37°C, some strains can grow in cold temperatures (as low as 3°C) or in high temperatures (up to 75°C) and across a relatively wide pH range (2.0–10.0) [1].

Owing to these physiological diversities, *Bacillus* spp. are isolated from a wide variety of habitats, including soils and hot springs [3]. Currently, there are 51 reported *Bacillus* species, which are divided into three groups on the basis of the morphology of the endospore and sporangium [4]. One of these species, *B. cereus*, is known as a major food-poisoning pathogens owing to its ability to produce enterotoxins, such as multiple hemolysins, phospholipases, and proteases [5].

B. cereus causes two types of food-poisoning syndromes (emetic or diarrheal), which may be involved in a variety of

infections, such as endophthalmitis, endocarditis, meningitis, periodontitis, osteomyelitis, wound infection, and septicemia [3, 6]. In the Netherlands, *B. cereus* is ranked as one of the top three food-poisoning pathogens (19%), after *Vibrio parahaemolyticus* and *Staphylococcus aureus* [2, 7]. In USA, more than 27,000 outbreaks of *B. cereus* occurred every year between 1993 and 1997, which made it the seventh most common foodborne pathogen [8]. In general, outbreaks of *B. cereus* are reported in approximately 1–20% of the world and the infective doses vary from 10^4 to 10^9 CFU/g in food [9, 10]. The implicated foods are spaghetti, pasta, rice, dairy, dried milk products, spices, meat, chicken, vegetables, fruits, grains, and seafood, as well as many fermented foods [11, 12].

As the removal of *B. cereus* from foods is not easy owing to its ubiquitous nature and heat-resistant endospores, many studies have focused on the development of rapid, sensitive, reliable, and practical biosensor methods as an intervention strategy [9, 13, 14]. Our research group developed a free-standing, phage-based ferromagnetoelastic (FME) biosensor composed of an FME platform and a specific phage for the recognition of target pathogens [15, 16]. The principle of the FME biosensor is based on the resonant frequency shift of the FME platform that is immobilized with a specific phage corresponding to the target pathogen. When the phage-immobilized FME platform is placed on food, if the target pathogen is present, specific bindings will occur between the phage and the pathogen. As the number of pathogens bound to the FME platform is directly proportional to the resonant frequency shift, the pathogens can be identified and quantified from the resonant frequency shift of the FME platform [17, 18]. Recent studies [19, 20] have demonstrated the potential application of the FME biosensor for *Salmonella* Typhimurium detection through the measurement of the resonant frequency, which did not require any tedious sample preparation. Furthermore, several previous studies [20–22] have already confirmed the rapidity, sensitivity, reliability, and practicability of the FME biosensor in comparison with a real-time quantitative PCR method. Thus, the FME biosensor could be employed for *B. cereus* detection to make use of the advantages mentioned above.

The search for a new recognition element that fits the target pathogen is a necessity for the approach. The required properties of the ideal recognition element are excellent selectivity (specificity), sensitivity, stability (durability), and cost efficiency, as well as ease and efficiency of immobilization [15, 23, 24]. Recently, phages have attracted attention owing to their excellent stability against harsh

temperatures, pH, and organic solvents, excellent selectivity against target pathogens (narrow selectivity), and cost-effectiveness [24, 25]. Many phages have been isolated and purified from the environment and foods for use predominately as biocontrol agents rather than as recognition elements [26, 27].

Our research group had already isolated and purified a novel *B. cereus*-specific phage (reported as BCP8-2) from fermented soybean products in Korea. The phage showed excellent selectivity against the *B. cereus* group only, among other *Bacillus* species [9]. The phage had an icosahedral head with a relatively long tail, as well as a lytic property, which followed the general property of almost all phages (approximately 96%) isolated from the environment and foods [28]. As indicated in Fig. 1, clear morphological differences were observed between the filamentous E2 phage commonly used in FME biosensors for *S. Typhimurium* detection and the *B. cereus*-specific phage. The E2 phage contains numerous binding sites on both sides of the E2 phage surface (Fig. 1A), whereas the tailed *B. cereus*-specific phage has only one binding site (at the end of the tail) for the target bacteria due to binding of its head on the sensor surface (Fig. 1B). Thus, the direct adsorption method used for immobilization of the filamentous E2 phage may not be appropriate for the stable and successful performance of the *B. cereus*-specific phage owing to its random, inappropriate, and poor orientation [29]. In addition, the lytic property of the *B. cereus*-specific phage is also problematic for its employment on the FME biosensor, because the principle of the FME biosensor is based on the mass change on the sensor. The lytic activity of the *B. cereus*-specific phage could cause bacterial lysis on the FME platform such that the loss of bacterial mass on the FME platform will decrease the resonant frequency shift. Finally,

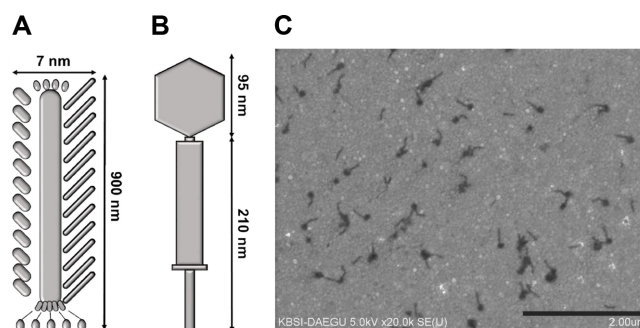


Fig. 1. Sketch images of the (A) E2 phage and (B) lytic, tailed *B. cereus*-specific phage, and (C) SEM image of the lytic, tailed *B. cereus*-specific phages on the ferromagnetoelastic platform. The bar in the SEM image indicates 2- μ m length.

the unexpected resonant frequency shift will influence the sensitivity and reliability of the FME biosensor method [30]. Therefore, these limitations must be overcome to employ this isolated lytic, tailed *B. cereus*-specific phage. The objectives of this study were to investigate (i) the optimum immobilization method of tailed *B. cereus*-specific phage on the FME platform, and (ii) the effects of incubation time and temperature on the lytic property of the *B. cereus*-specific phage prior to its employment on the FME biosensor.

Materials and Methods

Preparation of *B. cereus*-Specific Phage and *B. cereus* Culture

Bacillus cereus JCM 2152 and *B. cereus*-specific phage suspension (10 logPFU/ml) in SM buffer were provided by Dr. Kim's laboratory in the Department of Food Science and Technology at Chonbuk National University (Korea). The *B. cereus*-specific phage was serially diluted in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgSO₄, pH 7.5) to adjust the phage concentration. *B. cereus* JCM 2152 was grown in tryptic soy broth (Difco Laboratories Inc., USA) for 16 h at 37°C with shaking at 110 rpm. After three washes in sterilized phosphate-buffered saline (PBS, pH 7.4; Life Technologies Co., UK) and centrifugation at 4,000 ×g for 4 min, the collected bacterial cells were resuspended in PBS. The concentration of bacterial cell suspension was adjusted to 10⁸ CFU/ml after measurement of the optical density at 640 nm, which was followed by serial dilution with PBS prior to use.

Preparation of the Ferromagnetoelastic Platform

METGLAS 2826MB alloy ribbon (Honeywell Inc., USA) was diced to 1 mm × 0.2 mm × 0.028 mm by an automatic micro-dicing saw (DAD 3220 Disco Automatic Dicing Machine; DISCO, Japan). After ultrasonic cleaning with acetone and methanol, the FME platform was annealed at 220°C for 2 h at 10⁻³ torr (Denton Vacuum, USA). Both sides of the annealed platform were sputtered with chromium (90 nm thick) and gold (150 nm thick) at 2.5 × 10⁻⁶ torr in order to provide corrosion protection and a biocompatible surface for bacteria binding [18].

Immobilization Studies of *B. cereus*-Specific Phage

To determine the optimum immobilization method of *B. cereus*-specific phage, a direct adsorption method and a combination of 11-mercapto-1-undecanoic acid (11-MUA; Sigma-Aldrich Co., USA) and (*N*-(3-dimethylaminopropyl)-*N'*-carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS; Sigma-Aldrich Co.) were used for comparison. For the direct adsorption method, an FME platform was placed directly in an Eppendorf tube containing 300 µl of *B. cereus*-specific phage suspension at 7, 8, 9, and 10 logPFU/ml and incubated at 22°C for 1 h with perpendicular agitation. Concurrently, the other group of FME platforms was modified by the combination of 11-MUA and EDC/NHS prior to immobilization of the *B. cereus*-specific phage. An FME platform

was placed in an Eppendorf tube containing 300 µl of 20 mM 11-MUA and incubated overnight at 22°C to form a self-assembled monolayer. After three washes with ethanol and filtered distilled water (FDW), consecutively, the 11-MUA-treated FME platform was immersed in an Eppendorf tube containing 150 µl of 50 mM EDC solution and 150 µl of 50 mM NHS solution for 1 h. After three washes with FDW, the EDC/NHS-treated FME platform was placed in an Eppendorf tube for *B. cereus*-specific phage immobilization by using the aforementioned procedures. Both phage-immobilized FME sensors were then washed three times with FDW and air dried prior to observation by using a scanning electron microscope (SEM).

SEM Observation

For the observation of the *B. cereus*-specific phage binding on a the FME platform, the FME platform was exposed to osmium tetroxide (OsO₄) (Sigma-Aldrich Co.) for 45 min. The FME platform was mounted on an aluminum stub and examined by using a SEM (S-4800; Hitachi Ltd., Japan) at 5.0 kV with 10,000× magnification. Twenty different locations on each FME platform were observed and the total number of phages was counted by using ImageJ software (National Institutes of Health, USA) to calculate the phage density.

Effects of Incubation Time and Temperature on the Lytic Activity of *B. cereus*-Specific Phage

An aliquot of *B. cereus*-specific phage suspension (8 logPFU/ml) was mixed with an equal volume of the overnight culture of *B. cereus* JCM 2152 (4 or 8 logCFU/ml). To investigate the effect of time, 1 ml of the bacteria and phage mixture was placed in an Eppendorf tube and agitated slowly on a rotary shaker at 22°C for 0, 15, 30, 45, and 60 min. To investigate the effect of temperature, 1 ml of bacteria and phage mixture was incubated on a rotary shaker at various temperatures (4°C, 15°C, 22°C, and 37°C) for 30 and 60 min, respectively. The incubated mixture was passed through a nuclepore polycarbonate membrane paper (pore size 0.2 µm; Whatman Inc., USA) to remove excess *B. cereus*-specific phage. The polycarbonate membrane paper was placed in a tube containing 2 ml of PBS buffer and vortexed vigorously to collect *B. cereus* JCM 2152 from the polycarbonate membrane paper. The collected bacterial suspension was serially diluted with PBS buffer, and the viable number of *B. cereus* JCM 2152 was enumerated by the plate count method by using mannitol egg yolk polymyxin agar (Difco Laboratories Inc.). The survival rate of *B. cereus* in the mixture was calculated by dividing of the bacterial number after exposure to *B. cereus*-specific phage at the specified conditions by that of unexposed samples.

Statistical Analysis

All experiments were performed at least in triplicate and the data were expressed as the mean ± standard deviation. Student's paired *t*-test for two groups and one-way analysis of variance for more than two groups were used to compare the means by using

GraphPad and InStat V.3 software (GraphPad, USA). Differences were considered to be statistically significant at $p < 0.05$.

Results and Discussion

Immobilization Study of *B. cereus*-Specific Phage Using Direct Adsorption and a Combination of 11-MUA and EDC/NHS

Unlike the morphology of the filamentous E2 phage (Fig. 1), the *B. cereus*-specific phage has an icosahedral head (95 nm in length) and a relatively long tail (210–220 nm) [9]. On the basis of previous studies [27, 31–33], the ideal immobilization of lytic phage has been determined by phage density and orientation on the sensor surface, meaning that the lytic phage needs to be kept in position as “head-down” and “tail-up” with an appropriate distance to prevent steric hindrance between adjacent tails. Although the direct adsorption method is popular and has been commonly used for the immobilization of filamentous E2 phage owing to its simplicity and practicality, the relatively weak bindings and poor orientation were problematic on the sensor surface [26, 30]. To overcome the limitations of the direct adsorption method, a combination of 11-MUA and EDC/NHS was introduced owing to its recognition as the most efficient immobilization method for phages [34]. In addition, the concentration of the lytic, tailed phage is an important factor not only to allow the appropriate phage orientation with certain distances but also to avoid or minimize phage aggregation. Thus, two representative immobilization methods (direct adsorption and the

combination of 11-MUA and EDC/NHS) and various concentrations of *B. cereus*-specific phage were employed to determine the optimum immobilization of phage.

As shown in Fig. 2, various concentrations of *B. cereus*-specific phage were immobilized directly on the FME platform by continuous and perpendicular movement inside of the phage suspension in order to prevent and/or minimize the aggregation of phage on the FME surface. As the phage concentration increased, the immobilized phage density also increased significantly ($p < 0.05$) (Fig. 2A). The immobilized phage density on the FME platform was 21 ± 3 , 57 ± 4 , 112 ± 8 , and 186 ± 9 phages/ $100 \mu\text{m}^2$ at 7, 8, 9, and 10 logPFU/ml, respectively. The SEM images (Figs. 2B–2E) also confirmed that the binding of the phage increased correspondingly with increased phage concentration. Although the highest concentration (10 logPFU/ml) of the phage enhanced the coverage of sensor surface, the orientation of the phage was not uniformly distributed. In addition, aggregated spots were clearly observed at 10 logPFU/ml (Fig. 2E). Based on our previous experience, the aggregation on the sensor surface could lead to a decrease in the binding efficiency of the phage owing to the steric hindrance and the interaction of neighboring phages tails [31, 34–36]. As the tail length (approximately 210–220 nm) of *B. cereus*-specific phage was relatively longer, it was assumed that the longer tails were more likely to aggregate with each other.

The density of the *B. cereus*-specific phage and SEM images after immobilization of the phage by using the combination of 11-MUA and EDC/NHS method are shown

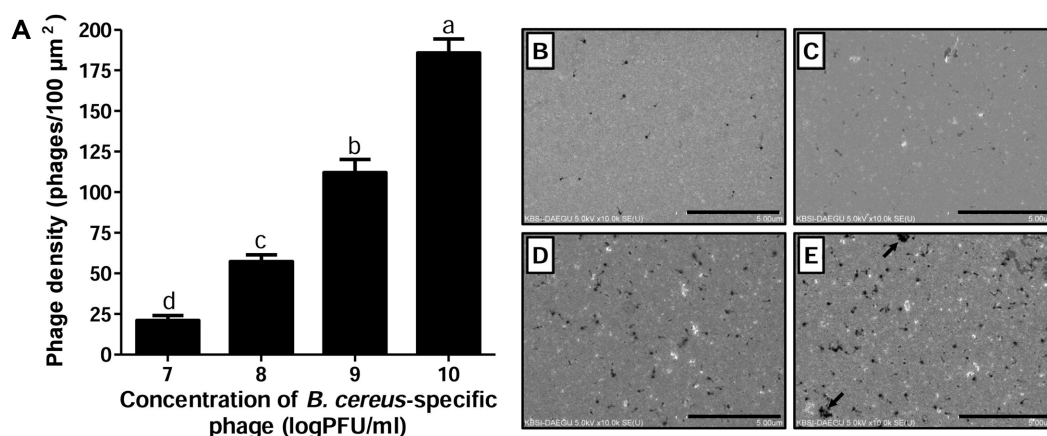


Fig. 2. Immobilization of *B. cereus*-specific phages onto a ferromagnetoelastic platform by direct adsorption.

(A) Histogram plot of *B. cereus*-specific phage density. The letters (a, b, c, and d) indicate significantly different means among the groups at $p < 0.05$. SEM images of *B. cereus*-specific phage immobilized on ferromagnetoelastic platform with a concentration of (B) 7 logPFU/ml, (C) 8 logPFU/ml, (D) 9 logPFU/ml, and (E) 10 logPFU/ml by using a direct adsorption method. The arrows in (E) indicate aggregations, and the bars in the SEM images indicate 5- μm length.

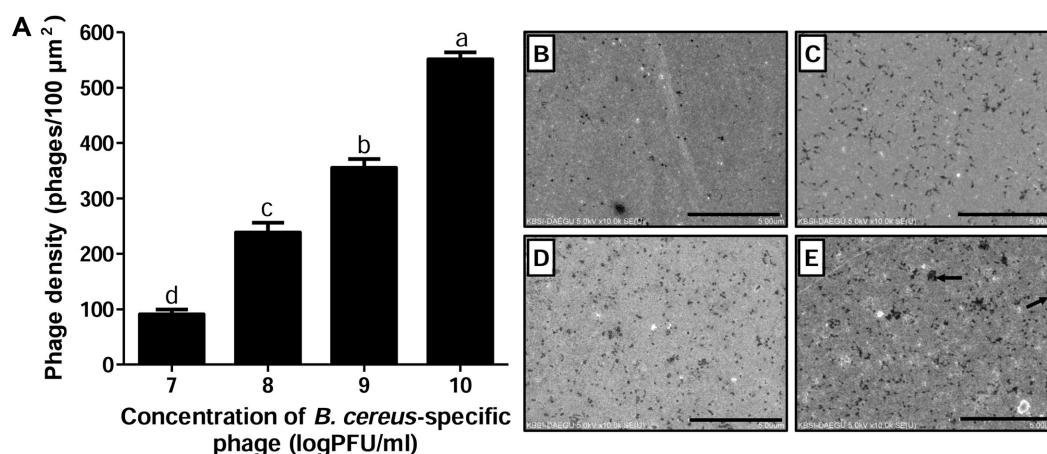


Fig. 3. Immobilization of *B. cereus*-specific phages onto a ferromagnetoelastic platform by a combination of 11-MUA and EDC/NHS. (A) Histogram plot of *B. cereus*-specific phage density. The letters (a, b, c, and d) indicate significantly different means among the groups at $p < 0.05$. SEM images of *B. cereus*-specific phage immobilized on ferromagnetoelastic platform with a concentration of (B) 7 logPFU/ml, (C) 8 logPFU/ml, (D) 9 logPFU/ml, and (E) 10 logPFU/ml by using a combination of 11-MUA and EDC/NHS. The arrows in (E) indicate aggregations, and the bars in the SEM images indicate 5- μm length.

in Fig. 3. As the phage concentration increased, more phages were immobilized on the FME sensor surface. The immobilized phage density on the FME platform was 91 ± 9 , 238 ± 18 , 355 ± 16 , and 551 ± 13 phages/100 μm^2 at 7, 8, 9, and 10 logPFU/ml, respectively. The increased phage density caused by the increased phage concentration was statistically significant ($p < 0.05$) and confirmed by SEM images (Figs. 3B–3E). Similar to the direct adsorption method, the phage density was the greatest at 10 logPFU/ml; however, the greatest phage binding was partially derived from some aggregations on the sensor surface, rather than from uniform distribution of the phage.

When both methods were compared at each concentration, significant differences were found between the two different immobilization methods ($p < 0.05$). As shown in Fig. 4, the phage density immobilized with the combination of 11-MUA and EDC/NHS was significantly greater than that immobilized with the direct adsorption method at every concentration ($p < 0.05$). Overall, the phage density obtained by using the combination of 11-MUA and EDC/NHS was approximately 3.0–4.3-fold greater than that obtained by using the direct adsorption method. Thus, it was concluded that the covalent attachment of the phage using the combination of 11-MUA and EDC/NHS enhanced the stable anchoring and uniform orientation of the phages on the FME platform. So far, only a limited number of studies regarding phage immobilization have been attempted by using sugars (glucose and sucrose) and amino acids (histidine and cysteine), a self-assembled monolayer with

dithiobis-succinimidyl propionate, and a combination of 11-MUA and EDC/NHS [26, 30]. The phage density of a tailed *E. coli*-specific phage [36] with a similar size (200 nm in tail length and 90 nm in head length) was improved by using glucose (2.4 ± 1.3 phages/ μm^2), sucrose (3.67 ± 1.1 phages/ μm^2), histidine (1.02 ± 0.06 phages/ μm^2), and cysteine (3.38 ± 0.1 phages/ μm^2); these values were greater

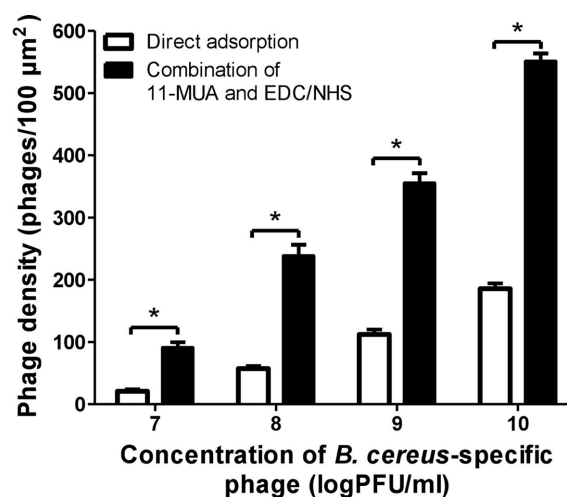


Fig. 4. Comparison of the immobilized density of the *B. cereus*-specific phage bound on a ferromagnetoelastic platform by using direct adsorption and a combination of 11-MUA and EDC/NHS.

The asterisk (*) indicates significant differences between the direct adsorption and the combination methods at $p < 0.05$.

than that obtained by the direct adsorption method (0.49 ± 0.15 phages/ μm^2) in their study. Based on these results, our combination of 11-MUA and EDC/NHS provided a better phage density (5.5 ± 0.1 phages/ μm^2). In addition, our results also partially agreed with Tawil *et al.*'s [34] study, which found that the combination of 11-MUA and EDC/NHS enhanced the phage density (data were not provided) and finally improved the binding of the target, *Staphylococcus aureus*, onto the sensor surface. Therefore, the combination of 11-MUA and EDC/NHS could be an effective method for the immobilization of *B. cereus*-specific phages. The optimum concentration of the phage was determined to be 9 logPFU/ml after considerations of phage density, phage aggregation, uniform orientation, and cost-effectiveness (phage concentration).

Effects of Incubation Time and Temperature on the Lytic Activity of *B. cereus*-Specific Phage

In a previous study [9], the *B. cereus*-specific phage was shown to have lytic properties so that the phage may lead to bacterial lysis on the FME surface. To initiate the lytic property of the phage against the target bacteria, the phage needs to bind with bacterial cells. This binding is affected by incubation temperature and time, pH, ionic strength in solution, and organic cofactors, as well as bacteria and phage concentrations [18, 37, 38]. As the incubation time and temperature are the most important factors for the performance of FME biosensors owing to direct employment of the FME platform on the food surface, these two factors were considered in this study.

To investigate the effect of time on the lytic activity of *B. cereus*-specific phage (Fig. 5), two different concentrations of bacteria were mixed with the same concentration of the phage at the same ratio and incubated at various incubation times. If the lytic property was expressed or enhanced by a longer incubation time, the survival rate of *B. cereus* would be decreased. At the incubation time of 30 min, no significant differences were found between survival at 0, 15, and 30 min. However, the survival rate of *B. cereus* started to decrease significantly when the incubation time reached 45 and 60 min ($p < 0.05$). At 60 min, the survival rate of *B. cereus* was decreased by up to $88.0 \pm 1.0\%$ and $95.0 \pm 0.6\%$ for the bacterial concentrations of 4 and 8 logCFU/ml, respectively. Up to 30 min, no significant differences in survival rate between the two different concentrations of *B. cereus* were observed, even though the lower concentration of *B. cereus* (4 logCFU/ml) was exposed to 10,000-fold higher concentration of phage (8 logPFU/ml), presumably

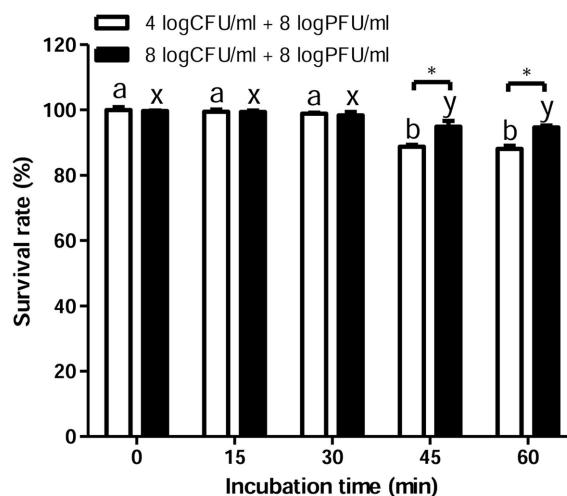


Fig. 5. Effect of incubation time on the lytic activity of *B. cereus*-specific phage against *B. cereus*.

The letters (a, b and x, y) indicate significantly different means among the groups within the same concentration of phage at $p < 0.05$. The asterisk (*) indicates that there were significant differences between the mixture of *B. cereus* at 4 logCFU/ml and the phage at 8 logPFU/ml, and the mixture of *B. cereus* at 8 logCFU/ml and the phage at 8 logPFU/ml.

due to the necessity of a latent time period (time interval for phage adsorption and the onset of the first burst) ($p > 0.05$). However, when the incubation time reached to 45 and 60 min, the survival rates at the lower bacterial concentrations were significantly lower than those at the higher bacterial concentration ($p < 0.05$). Theoretically, this experimental condition would provide the best status for the phage to interact with target bacteria, because they were agitated continuously in the liquid solution. The continuous slow agitation in liquid solution will enhance the probability of interaction between the phage and the target bacteria. However, as the phage-immobilized FME platform will be placed on the surface of foods contaminated with *B. cereus*, this status may not provide the optimum condition for the phage binding with *B. cereus*. Thus, a contact time of 30 min of *B. cereus*-specific phage on the FME platform with the target will not present any chance for bacteria lysis as a result of the inherent lytic property of the *B. cereus*-specific phage.

To investigate the effect of temperature on the lytic activity of the *B. cereus*-specific phage (Fig. 6), the mixture of bacteria and the phage was incubated at various temperatures for 30 and 60 min, respectively. As shown in Fig. 6A, there were no significant differences in the survival

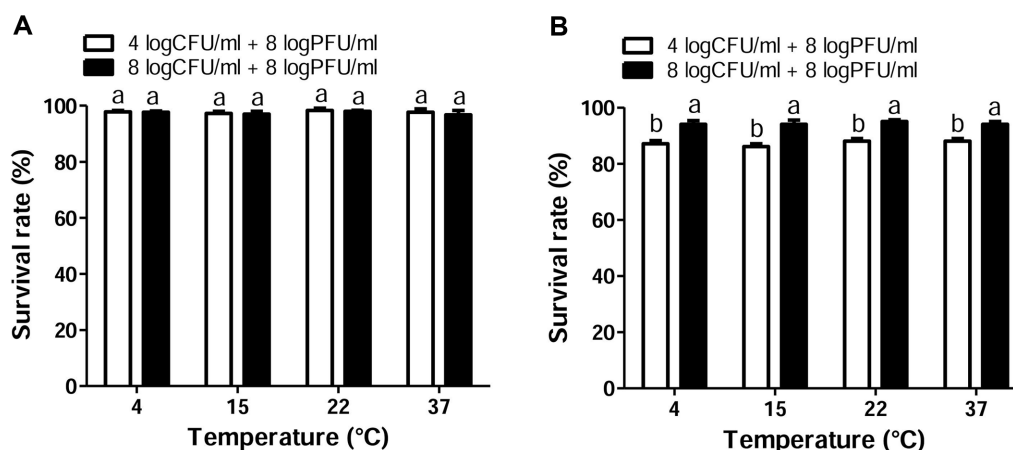


Fig. 6. Effect of temperature on the lytic activity of the *B. cereus*-specific phage after incubation with *B. cereus* for (A) 30 min and (B) 60 min.

The letters (a and b) indicate significantly different means among the groups at $p < 0.05$.

rates of *B. cereus* at each tested concentration when exposed to various different temperatures for 30 min ($p > 0.05$). Even though the incubation time was increased to 60 min, the survival rate of *B. cereus* was not affected by temperature at each concentration. However, there were significant differences observed between the mixture of *B. cereus* at 4 logCFU/ml and the phage at 8 logPFU/ml, and the mixture of *B. cereus* at 8 logCFU/ml and the phage at 8 logPFU/ml ($p < 0.05$); differences of approximately 6–7% between the two mixtures were found in the survival rate. The relatively greater MOI (the ratio of phage to bacteria) showed greater bacterial reduction, as expected. However, there was a negligible bacterial reduction (approximately 0.4 logCFU/ml) observed at over 60 min incubation. Overall, as the lytic activity of the *B. cereus*-specific phage was not influenced or accelerated by different temperature conditions, the *B. cereus*-specific phage could be employed on the FME biosensor as a novel recognition element suitable for temperatures in the range of 4–37°C. In conclusion, this study has demonstrated the feasibility of the lytic, tailed *B. cereus*-specific phage as a novel recognition element for use in an FME biosensor. Further studies will be needed to investigate the application of the FME biosensor with the novel *B. cereus*-specific phage for food testing.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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