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W/O/W Emulsions Formula of Bacteriophages ϕPT1b as a Fruit Wash to Inhibit Pathogenic *Escherichia coli* Contamination

Icha Khairunnisa[1], Erlia Narulita[1,2*], Lina Winarti[3], Riska Ayu Febrianti[2], Kuswati[1], Ria Yulian[4]

[1]Biology Education Study Program, University of Jember, Jember 68121, Indonesia
[2]Laboratory of Molecular Medicine, CDAST, University of Jember, Jember 68121, Indonesia
[3]Faculty of Pharmacy, University of Jember, Jember 68121, Indonesia
[4]Environmental Microbiology Lab, Department of Agricultural Biological Chemistry, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, South Korea

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***Corresponding author:**
Erlia Narulita
email: erlia.fkip@unej.ac.id

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ABSTRACT

Pathogenic *Escherichia coli* (E. coli) infect fruit through irrigation with contaminated water. Bacteriophage ϕPT1b can infect E. coli and can act as food security. Bacteriophage formulations as emulsion preparations increase the efficiency of application and the stability of long-term storage, one of which is W/O/W emulsions. The objective of this study was to examine effect of cremophor RH 40, span 80 and their interaction on viscosity, droplet size and inhibition of bacteriophage ϕPT1b W/O/W emulsions against pathogenic E. coli. Factorial design method was used to get the optimum formula. The results showed that the cremophor RH 40, span 80, and their interactions increased the viscosity response, decreased the emulsion droplet size, and increased the inhibition against E. coli. FB is the optimum formula for ϕPT1b W/O/W emulsions which has 1% cremophor RH 40 and 4.5% span 80. The formula produced a viscosity of 29 dPas, an average emulsion droplet diameter size of 10.483, and an inhibition against E. coli of 8.65 m. The desirability index value of 0.905 indicates that FB meets 90.5% of the specified criteria. In conclusion, Cremophor RH 40, span 80, and their interactions increased the viscosity response and droplets size. FB fulfilled 90.5% of the criteria to obtain the optimum formula.

INTRODUCTION

Pathogenic bacteria infect fruit through irrigation water contaminated with sewage, soil, or animal dung as fertilizer (Djaafar and Rahayu, 2007). During the production process, irrigation water has become one of the most significant factors for food-borne pathogens contamination of fresh products (EFSA BIOHAZ Panel, 2014). One type of contaminant bacteria gained from irrigation water is *Escherichia coli* (E. coli) (Djaafar and Rahayu, 2007).

The conventional methods to overcome bacterial infections on food have some disadvantages. Temperature for inactivating E. coli is above 64.5 °C (D'aoust *et al*., 1988), which is inappropriate for fruit processing because it destroys its nutritional content. In addition, the NaOCl method also produces unhealthy by-products that caused carcinogenic or mutagenic effects (Artes-Hernandez *et al*., 2017). Also, utilizing antibiotics in the long term caused high levels of bacterial resistance (Castillo *et al*., 2018).

The application of bacteriophages can be an alternative solution to these problems. The use of bacteriophages as antibacterials is beneficial because they only attack the target pathogen, not all microorganisms or typical flora in the body (Hardanti *et al*., 2018). Bacteriophages also have negligible influence on food products, including physicochemical properties and food organoleptic (Pulido *et al*., 2015). Bacteriophage ϕPT1b is specific for infecting pathogenic E. coli that was isolated

from PST bacterial acquired from vegetable samples (Narulita *et al*., 2018). Bacteriophage formulations as emulsion preparations can improve the application efficiency and long-term storage stability. The emulsion can provide stability in long-term phage storage without a significant decrease in infectivity (Esteban *et al*., 2014). One method of emulsion application to foodstuffs is water-in-oil-in-water or W/O/W emulsions (double emulsions). The advantage of double emulsions method is protecting active ingredients during food processing (Garti and Aserin, 1996). The internal phase is able to carry hydrophilic active compounds. The entrapment in the internal phase by being coated by the external oil and air phase slows down the rate of time and the breakdown of the active components for a certain period of time (Sapei *et al*., 2012). The bacteriophage ϕPT1b W/O/W emulsions for washing fruit is more environmentally friendly compared to Sodium Lauryl Sulfate (SLS) and Sodium Laureth Sulfate (SLES). SLS and SLES form a foam on the washing liquid. When a lot of foam covers the surface of the water, the air and water contact is limited, so the amount of dissolved oxygen drops and causes the organisms to be deprived of oxygen and die (Ahsan *et al*., 2005). However, the W/O/W emulsions also lack stability because of several factors including the potential for interaction or emulgator competition adsorbed in internal water droplet interfaces or oil droplets in emulsions (Tania *et al*., 2020). This study aimed to examine effect of cremophor RH 40, span 80 and their interaction on viscosity, droplet size and inhibition of bacteriophage ϕPT1b W/O/W emulsions against pathogenic *E. coli.*

METHODS

Rejuvenation of *Escherichia coli*

E. coli isolate rejuvenated in Luria Bertani (LB) media with a composition of 1% peptone, 0.5% yeast, and 1% sodium chloride, and incubated at 37°C for 24 hours.

Propagation of Bacteriophage Particles by the Spot Test

The bacteriophage propagation method was conducted based on Narulita *et al*. (2018) with modifications. The medium used was LB double layer agar with bottom of 1% and top agar of 0.5%. The warm top agar (± 50°C) was vortexed with 300µl 4-hour-old *E. coli* isolate (log phase) in LB. The suspension of top agar and *E. coli* was poured over the bottom layer of the compacted media. The spot test was carried out by dripping 2µL of ϕPT1b suspension on the solidified media. Incubation was done for 24-72 hours at 37°C. The spot test results were shaken using SM buffer and then incubated at 4°C overnight. The suspension obtained was centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant obtained was filtered using a 0.2µm membrane filter.

Determination of Bacteriophage Concentration by Plaque Assay

Determination of bacteriophage concentration was carried out using the Yulinery and Triana (2016) method with modifications. The bacteriophage filtrate was diluted 10-1 to 10-6. As much as 0.5 ml of logarithmic bacterial culture was added to the bacteriophage filtrate tube and 0.1 ml of solution from the dilution series was added then incubated at 37°C for 10 minutes. Each Eppendorf tube was mixed into a warm top agar (0.5%). The mixture was then left to solidify and incubated at 37°C for 24 hours.

Production of ϕPT1b W/O/W Emulsions

The formula contains a mix of span 80 and Cremophor RH 40 with various concentrations according to Dwisari (2012) with modifications (Table 1). The emulsion production proceeded sequentially in two stages. Firstly, W/O primary emulsion production contained solvent of the Cremophor RH 40, bacteriophage ϕPT1b, and sorbitol into the aqueous phase (NaCl 0.05 M). Additionally, other ingredients were added in the next step which was homogenous mixing of Span 80 and isopropyl myristate. Both solutions were vortexed by magnet stirrer for 30 minutes at a speed of 2000 rpm. The second stage was the W/O/W emulsions production. The aqueous phase in the external phase is divided into 3 parts (2:1). Cremophor RH 40 was dissolved in the first aqueous phase (2 parts), xanthan gum was added to the second aqueous phase (1 part) and stirred until a homogeneous gel mass was formed. As much as 30% primary emulsion (W/O) was added gradually to the first external aqueous phase while continuously stirring using a magnetic stirrer at 600 rpm until homogeneous. Xanthan gum is added gradually into the emulsion until homogeneous.

The making of the in-emulsion used 2 surfactants, namely cremophor and span, while to make the out-emulsion used only cremophor. The amount of cremophor and span in each formula is different, but when adding all together, the ingredients used in a formula reached 100%.

Table 1. Formulation of W/O/W emulsions used in this study

Contents	Formula (% w/w)			
	F1	FA	FB	FAB
ϕPT1b	5.00	5.00	5.00	5.00
Isopropyl Myristate	18.80	18.30	18.15	17.65
Cremophor RH40 (primary emulsion)	1.00	1.50	1.00	1.50
Span 80	3.85	3.85	4.50	4.50
Sorbitol	1.35	1.35	1.35	1.35
Xanthan Gum	0.80	0.80	0.80	0.80
Cremophor RH40 (W/O/W emulsions)	3.50	3.50	3.50	3.50
NaCl 0.05 M	33.15	33.15	33.15	33.15

Physiochemical Characterization and Evaluation of ϕPT1b W/O/W Emulsions
Organoleptic Test

ϕPT1b W/O/W emulsions were observed visually to evaluate texture, color, odor, and consistency. Emulsions are generally white in color and have an aroma from the oil used.

pH Evaluation

The pH measurement of ϕPT1b emulsions was carried out using a digital pH meter (Amtast Amt20). The pH measurements represent the mean ± standard deviation (SD) of three replicates.

Viscosity

The ϕPT1b emulsions was measured with a Brookfield Spindle Viscometer #2. Viscosity of 2000 - 4000 cps of W/O/W emulsion was determined as a criterion in determining the optimum formula. One mL from each prepared fruit wash emulsion was filled into the measurement chamber. The chamber was capped for 60 s until it was stable, and then the data were recorded. The results represent the mean ± SD of three replicates.

Globule Morphology and Diameter

The average globule diameter was measured using a calibrated microscope and micrometer. The W/O/W emulsions preparations were placed on a slide and covered with a cover slip and observed with a magnification of 40 x 10. The diameter of the emulsion was determined by observing 50 droplets to calculate the average diameter. The visible image was then photographed to observe its morphology. The globule with the smallest average diameter was the most optimum.

Inhibition Test against *E. coli*

The antibacterial activity test of the formula was carried out using spot test based on Narulita *et al*. (2018) with modifications. The media used was double layer agar of LB (Luria Bertani). Petri dishes were divided into 9 parts for prepared ϕPT1b emulsions, prepared W/O/W emulsions with no ϕPT1b (negative control), and suspension of ϕPT1b in SM buffer (positive control), each for three replications. The formula with the largest lysis zone was the most optimum formula. The results represent the mean ± SD of three replicates.

Statistical Analysis

The results of the measured pH, viscosity, size, and zone of inhibition were presented by the mean and SD of at least three replicates. The mean comparison of the inhibition activity of the prepared ϕPT1b emulsions, negative control, and positive control were performed by T-test, and the p-value of < 0.05 was set as a criterion for statistically significant difference. The program to analyze optimum preparations of the ϕPT1b emulsions was Design Expert v12. The most significant desirability index value chose the optimum recipe from the output.

RESULTS AND DISCUSSION

Characterization and Evaluation of ϕPT1b W/O/W Emulsions. The manufacture of double emulsions is generally divided into 2 stages, namely the manufacture of primary emulsions and the manufacture of secondary emulsions. Primary emulsion with W/O type (water in oil) is made by emulsifying a mixture of isopropyl myristate (IPM) as oil phase, span 80 and Cremophor RH40 as emulsifier or surfactant, sorbitol as cosurfactant, and PT1b in SM buffer as water phase. The mixture was homogenized using a magnetic stirrer at a speed of 2000 rpm for 30 minutes. Sorbitol is added to the double

emulsion to reduce the size of the emulsified water droplets. Sorbitol is able to reduce the attractive energy between droplets by lowering the surface tension between the water and oil phases. The droplet size of the emulsion needs to be reduced because the smaller the droplet size, the greater the stability of the primary emulsion. When the stability of the primary emulsion increases, it will increase the stability of the double emulsion.

The water dispersion causes the milky white color of the emulsion in oil with macro size. The characteristic odor of oil in primer emulsions was due to isopropyl myristate, which was the most dominant ingredient. Consistency differences between the primary and double emulsion were due to cremophor RH 40 and xanthan gum. The emulsion consistency relates to the viscosity where a thick solution has a higher density than a liquid solution. The viscosity test shows that Cremophor RH 40 positively affects viscosity, increasing the thickness of multiple emulsions. Furthermore, adding xanthan gum in a double emulsion also increases the density (Dwisari, 2012).

The organoleptic observations showed that all four formulas (F1, FA, FB, and FAB) have an odorless and creamy milky white color formation. There were no visual quality differences among the formulas on the primary emulsion and on W/O/W emulsion (Table 2).

In general, the pH of all formulas tends to be acidic with a range of 5.8 to 6.2 (Table 3). Brookfield viscometer with a two-spindle conducts the measurement of F1, FA, FB, and FAB viscosity (Table 3). It shows the influence of Cremophor RH 40 and Span 80 with also their interactions on the viscosity response. All three factors produce positive results.

The acidic pH is due to the presence of NaCl (Leela and Sharma, 2000). The hydrolysis reaction causes the oil breakage into free fatty acids and glycerol (Mahargiani, 2002). Then, the NaCl chelates the glycerol, but free fatty acids as weak acids will increase. Thus, the results suggested that the preparation pH depends on the acidity Constanta and the degree of fatty acids ionization. The pH test indicated an increase of the pH average from F1 to FAB (Table 3), which is relates to the composition of Span 80 (pH ≤8) (Leela and Sharma, 2000). F1 and FA contain 3.8 5% of Span 80, while FB and FAB were higher (4.5%). Therefore, FB and FAB were chosen due to a neutral pH is more suitable for long-term phages storage (Rowe *et al*., 2006).

The morphology and size of the droplet are presented at Fig. 1A. The droplets appear round and some droplets show the presence of 1 to more than 10 smaller (W/O) droplets within the (O/W) droplets. Smaller O/W droplet sizes result in fewer W/O droplets (Fig. 1B).

Table 2. Organoleptic primary and W/O/W emulsions

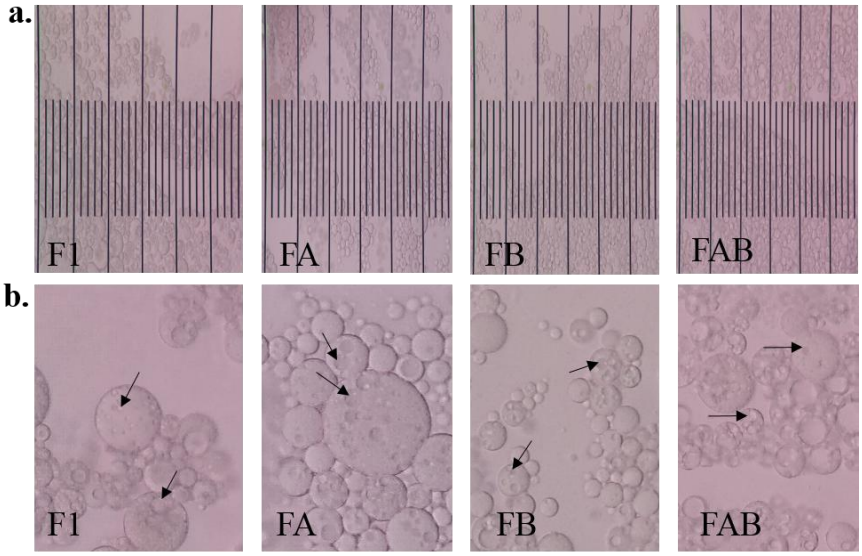
Formula	Organoleptic Primary Emulsions			Organoleptic W/O/W Emulsions		
	Color	Smell	Consistency	Color	Smell	Consistency
F1	Milky white	The special smell of oil	Liquid	Milky white	Odorless	Thick
FA	Milky white	The special smell of oil	Liquid	Milky white	Odorless	Thick
FB	Milky white	The special smell of oil	Liquid	Milky white	Odorless	Thick
FAB	Milky white	The special smell of oil	Liquid	Milky white	Odorless	Thick

F1 (formula with Cremophor factor RH 40 1% and Span 80 3.85%); FA (formula with Cremophor factor RH 40 1.5% and Span 80 3.85%); FB (formula with Cremophor factor RH 40 1% and Span 80 4.5%); FAB (formula with Cremophor factor RH 40 1.5% and Span 80 4.5%).

Table 3. Double Emulsion Formula Evaluation

Formula	Cremophor RH40 (%w/w)	Span 80 (%w/w)	pH	Viscosity (dPas)	Droplet Size (µm)	Inhibition Size (mm)
F1	1.00	3.85	5.80 ± 0.14	38.33±2.35	15.74±0.72	7.40±0.24
FA	1.50	3.85	5.83 ± 0.12	28.67±0.94	17.21±2.33	7.98±0.13
FB	1.00	4.50	6.00 ± 0.14	29.00±2.82	10.45±0.43	8.65±0.36
FAB	1.50	4.50	6.20 ± 0.00	46.00±1.41	14.91±1.09	7.01±0.51

F1 (formula with Cremophor factor RH 40 1% and Span 80 3.85%); FA (formula with Cremophor factor RH 40 1.5% and Span 80 3.85%); FB (formula with Cremophor factor RH 40 1% and Span 80 4.5%); FAB (formula with Cremophor factor RH 40 1.5% and Span 80 4.5%).



(a) (b)

Figure 1. A. Emulsion droplets under the microscope with a 40 x 10 magnification; **B.** Emulsion droplets (zoom in); F1 (formula with Cremophor RH 40 1% and Span 80 3.85%); FA (formula with Cremophor RH 40 1.5% and Span 80 3.85%), FB (formula with Cremophor RH 40 1% and Span 80 4.5%), and FAB (formula with Cremophor RH 40 1.5% and span 80 4.5%).

Table 4. Inhibitory Diameter

Formula	Inhibitory Diameter (mm)
F1	7.40 ± 0.24
FA	7.98 ± 0.13
FB	8.65 ± 0.36
FAB	7.01 ± 0.51
N1	6.28 ± 0.59
NA	5.46 ± 0.62
NB	6.23 ± 0.40
NAV	6.03 ± 0.12
K +	10.48 ± 0.89

F1 (formula with Cremophor factor RH 40 1% and Span 80 3.85%); FA (formula with Cremophor factor RH 40 1.5% and Span 80 3.85%); FB (formula with Cremophor factor RH 40 1% and Span 80 4.5%); FAB (formula with Cremophor factor RH 40 1.5% and Span 80 4.5%); N1 (F1 without bacteriophages); NA (FA without bacteriophages); NB (FB without bacteriophages); NAB (FAB without bacteria); and K+ (ΦPT1b bacteriophage in SM buffer).

A total of 50 droplets were measured randomly under a light microscope with a 40 x 10 magnification using a micrometer. Table 4 illustrates all the diameters of the droplet. The particle size counts by Polydispersity Index (PI) (dividing standard deviation against the average). PI values of F1, FA, FB, and FAB were 0.04; 0.13; 0.04; and 0.07. PI values ranging from 0.01 to 0.5-0.7 indicate uniformly distributed emulsion droplets (Reymundo *et al*., 2002).

The span 80 factor produces a positive effect on increasing the inhibitory response. While the cremophor factor and the interaction of the two factors have a negative effect on the inhibitory response. The most dominant factor on viscosity response is the interaction of the two emulgators. The results of ANOVA analysis showed that the cremophor RH 40 and span 80 factors gave an insignificant effect on the inhibition response with a significance value of 0.0636 and 0.5774, respectively. While the interaction factor of the two emulgators gave a significant effect on the response of emulsion droplet size with a significance value of 0.0019.

The Cremophor RH 40 and the interaction between both factors (Cremophor RH 40 and Span 80) positively affect the droplet size. Whereas Span 80 gives a negative effect that decreases the emulsion droplet's response size. The Cremophor RH 40 and Span 80 factors significantly affect the response of the emulsion droplet size (p-values 0.0147 and 0.0042). In contrast, the interaction of the two emulators had a no significant effect (p-value of 0.1568).

The thickness of the emulsions depends on the emulsifier concentration; the higher the concentration, the higher the viscosity (Jonczyk *et al*., 2011). FAB with high concentration reaches the most excellent consistency among all formulas. High viscosity will reduce the ability of the emulsion to spread on the fruit surface but can increase the stability of the emulsion. The thick emulsion is also more stable, although it produces bigger droplets than on primary emulsion (Syukri *et al*., 2009). Bigger droplets are formed because in a double emulsion droplet contains several primary droplets. In general, small droplets will move more slowly than large droplets. This is why coalescence between double emulsions is more likely to occur than coalescence between primary emulsions. This possibility can be reduced by the high viscosity of the emulsion. However, according to the desired goal, for making the fruit-washing emulsion, the viscosity criteria are determined from 20 dPas to 40 dPas (2000 cps-4000 cps). Based on these criteria, it appears that the FAB did not meet the viscosity criteria because it had an average of 46 dPas.

A smaller O/W droplet brings fewer W/O droplets. In deduction, the smaller the droplet size, the better the stability of the emulsion. Oil droplets with a diameter of 0.1 µm move 100 times slower than oil droplets with a diameter of 1 µm (Reymundo *et al*., 2002). When the droplet moves slowly, the aggregation process will take longer. The aggregation process causes the droplets to merge and produce a phase separation in the emulsion. Likewise, with multiple emulsions, the smaller the number of primary emulsion droplets in a double emulsion, the less the aggregation of a droplet.

The spot test revealed the presence of bacteriophages (Fig. 2), while inhibition diameter that is measured using calipers presented in Table 4. Cremophor RH 40 is a common additive substance used for increasing drug release. Using it in primer emulsions will increase the release of active ingredients. The active ingredient diffuses gradually, decreasing the emulsion density. A droplet with a lower density moves upward quickly due to the force of gravity (Taherian *et al*., 2008). In surface area, many droplets aggregate each other become macro droplets. Span 80 is a stabilizer that keeps the droplets from reuniting (Rahmawanty *et al*., 2015). Lipophilic characters of it influence the ability to bind oil (Estiasih *et al*., 2015). A low oil binding ability forms macro droplets more easily.

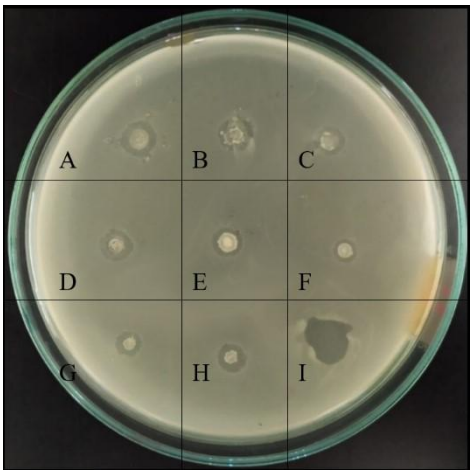


Figure 2. Inhibition of W/O/W emulsion against *E. coli*; **A** (F1, formula with Cremophor RH 40 1% and Span 80 3.85%); **B** (FA, formula with Cremophor RH 40 1.5% and Span 80 3.85%), **C** (FB, formula with Cremophor RH 40 1% and Span 80 4.5%), and **D** (FAB, formula with Cremophor RH 40 1.5% and span 80 4.5%); **E** (F1 non-bacteriophage (N1)); **F** (non-bacteriophage FA (NA)); **G** (non-bacteriophage FB (NB)); **H** (non-bacteriophage FAB (NAB)); **I** (K + / non-formulation PT1b).

Table 5. Solutions offered by factorial designs

Cremophor RH 40	Span 80	Viscosity	Size of Emulsion Droplet	Inhibition	Desirability	
1,000	4,500	29,000	10,483	8,650	0.905	Selected
1,004	4,500	29,150	10,522	8,636	0,900	
1,008	4,500	29,277	10,555	8,623	0,896	
1,000	4,461	29,567	10,842	8,574	0,872	
1,044	4,500	30,509	10,871	8.505	0,855	
1,067	4,500	31,280	11,068	8,431	0,829	
1,500	3,850	28,667	16,900	7,983	0,442	

K+ has a wider inhibition than the emulsion formula with bacteriophage. This is because in the emulsion formula there are layers of internal oil phase and external water phase that slow down the release rate of active ingredients (bacteriophages) for a certain period of time. In addition, hydrocolloids in the form of xanthan gum in the emulsion can reduce the mobility of active ingredients (bacteriophages) in the emulsion. Because of this, the emulsion formula with bacteriophages requires a longer incubation time compared to bacteriophages in buffered SM (K+).

It was also found that the formula without bacteriophage also had inhibition against E. coli. This is thought to be due to the presence of emulgators/surfactants in the emulsion that have antibacterial activity. Based on this, the addition of bacteriophages proved to be able to increase the inhibition of double emulsions.

There are 2 emulgators used, namely cremophor RH 40 and Span 80. Cremophor RH 40 is nontoxic so it does not cause antibacterial activity. While Span 80 belongs to the class of fatty acid partial esters also known as sorbitan monooleat with the molecular formula C24H44O6 (Rowe *et al*., 2006). Fatty acids that have more than 10 carbon atoms (C) can cause protoplasm in bacteria to lyse, resulting in a bactericidal effect on bacteria (Agustini *et al*., 2017).

In general, SM buffer bacteriophages have broader inhibition than emulsion formulas with bacteriophages. It is due to the presence of layers by an internal oil phase and an external water phase, which slows the release rate of active ingredients for a certain period (Pachuau *et al*., 2009). In addition, hydrocolloids of xanthan gum also reduce the mobility of active ingredients in the emulsion (Devi *et al*., 2019). It means that bacteriophages of the emulsion formula need more prolonged incubation than in the SM buffer.

The Cremophor RH 40 and Span 80 factors affect the inhibitory response with no significant. At the same time, the interaction factor of the two emulsions had a significant effect (p-value = 0.0019) to inhibitory response. The higher composition of span 80 gave a positive response indicated by the bright color (red) and more significant inhibition on E. coli culture. However, a combination of span 80 and cremophor RH 40 at upper concentrations was a negative response marked in blue. Figure 6 shows that the formula with the largest inhibitory diameter is the FB formula with a low Cremophor RH 40 composition (1%) and a high Span 80 (4.5%).

Determination of the Optimum Formula. The optimum formula for bacteriophage ϕPT1b W/O/W emulsions determined by evaluating four procedures with variations in the number of span 80 and cremophor RH 40. The multiple emulsions result in the best formulas response to viscosity, droplets size, and E. coli inhibition (Table 5).

The formula with no bacteriophage has inhibition against E. coli (Table 4 and Fig. 6). Each formula with bacteriophages (F1, FA. FB, and FAB) has significant differences (sig. <0.05) compared with emulsion without bacteriophages (N1, NA, NB, and NAB). These results indicated that bacteriophage addition can increase the inhibition of W/O/W emulsions. The inhibition zone on non-bacteriophages emulsion is supposed to be the emulator's antibacterial activity. Two emulsifiers used in this study were Cremophor RH 40 and Span 80. Cremophor RH 40 is a common material in oral cleaning products that is nontoxic to bacteria (Florence and Whitehill, 1985). Span 80 belongs to the partial esters of the group of fatty acids, also known as sorbitan monooleate, with the formula Mole C24H44O6 (Müller *et al*., 2017; Rowe *et al*., 2006). Fatty acids with more than 10 carbon atoms (C) can cause protoplasm in bacterial lysis resulting in a bactericidal effect on bacteria (Desbois and Smith, 2010; Agustini *et al*., 2017).

CONCLUSIONS

Cremophor RH 40, span 80, and their interactions increased the viscosity response and droplets size. The span 80 only enhanced the inhibitory response to E. coli. The optimum formula for bacteriophages ϕPT1b W/O/W emulsions was FB with a composition of 1% Cremophor RH 40 and 4.5% span 80. The FB formula fulfilled 90.5% of the criteria to obtain the optimum formula. These results are indications that bacteriophages-based fruit washing can potentially be used as a preventive tool of foodborne disease spread from E. coli-contaminated fruit. However, further studies are required to evaluate several conditions such as stability of those bacteriophages under various stress conditions and different temperatures for extended periods of time.

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CONFLICT OF INTEREST

There is no conflict of interest.

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