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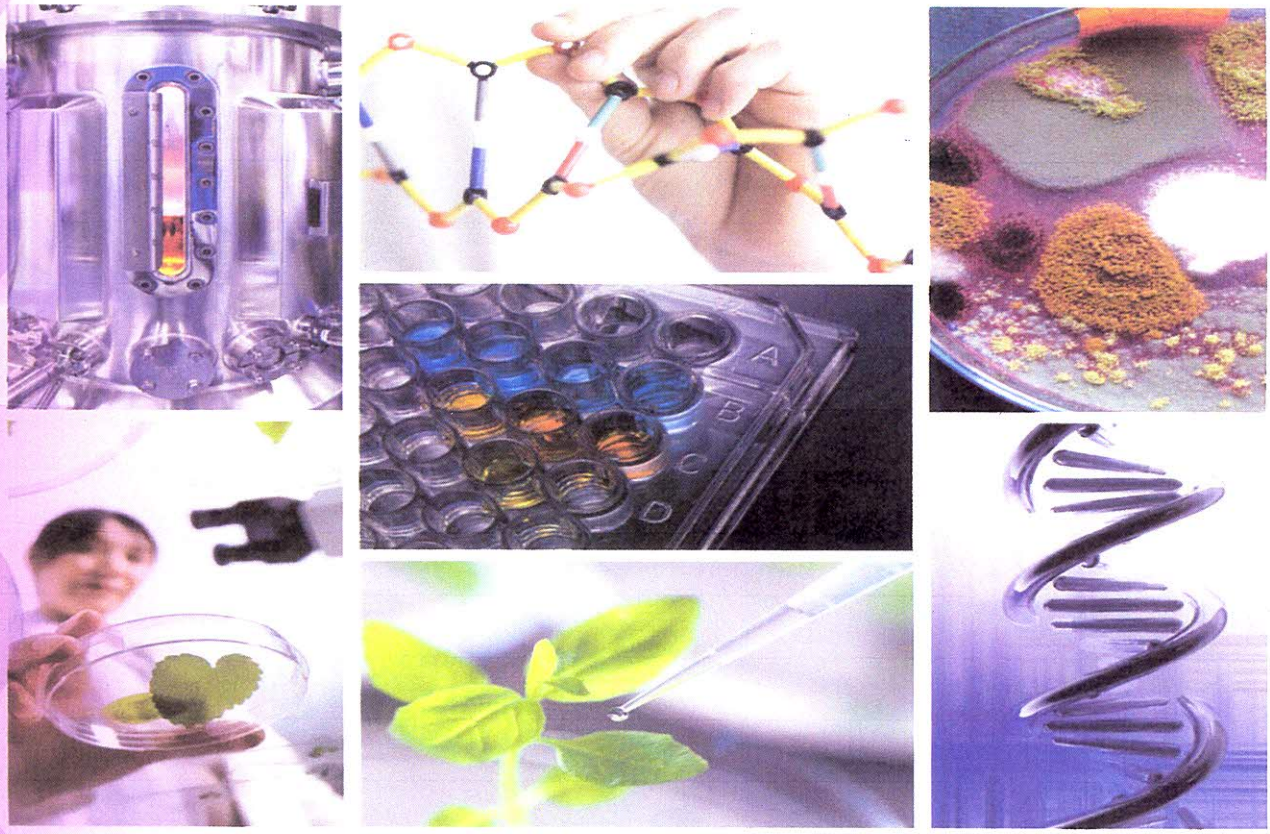
TSB 2013
Conference Proceedings

The 25th Annual Meeting of The Thai Society for Biotechnology and International Conference



Agro-Industrial Biotechnology for Global Sustainable Prosperity

Joint Symposia
BIOPRODUCT AND SYSTEMS BIOTECHNOLOGY
JATROPHA UPDATES 2013

Organized by

KASETSART UNIVERSITY
มหาวิทยาลัยเกษตรศาสตร์

THAI SOCIETY
FOR BIOTECHNOLOGY
สมาคมเทคโนโลยีชีวภาพแห่งประเทศไทย

GREETINGS

Message from the TSB President

On behalf of the organizing committee of the Thai Society for Biotechnology (TSB), it is my great pleasure to welcome you to "the 25th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2013)", which will be held during 16th - 19th October, 2013 at the Emerald Hotel, Bangkok, Thailand. This year the conference will be hosted by the Faculty of Agro-Industry, Kasetsart University under the theme of "Agro-Industrial Biotechnology for Global Sustainable Prosperity".

Not only the sharing of the ongoing discovery towards the biotechnology, but also two pronounced awards to be given at the TSB 2013 namely the Taguchi prize and the Ajinomoto lecture. Those awards are given to the outstanding young and senior biotechnologists who have done innovative and intensive research on biotechnology. Interestingly, TSB 2013 will have 5 eminent keynote speakers to share their experience for the sustainable prosperity of agro-industrial biotechnology and 18 invited speakers from Australia, China, Germany, India, Japan, United Kingdom, United State of America and Thailand, as well as the presentations in 8 parallel sessions. Moreover, there will be 2 joint symposiums; "Bioproduct and Systems Biotechnology" and "Jatropha Updates 2013". Definitely, this conference will offer the great opportunity for all the participants to obtain the advancement of biotechnology at the same time to strengthen or expand the existing networks to new international members.

To enhance the collaboration in Asia and international, TSB 2013 offers the special rates for the members of the AFOB (Asian Federation of Biotechnology), KSBB (Korean Society for Biotechnology and Bioengineering), SBJ (Society for Biotechnology, Japan) and BEST (Biochemical Engineering Society of Taiwan). I hope that TSB 2013 will be a good drive for the Asia and International biotechnology collaboration and this event will be the part of the success of world biotechnology uniting in the future.

Sincerely yours,

Associate Professor Penjit Srinophakun

President

Thai Society for Biotechnology (TSB)

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TABLE OF CONTENT

O-08-004

Exploration of Newly Isolated Bacteriophage from Beef Tripe and Chicken Intestine as Biosanitizing Agent for Controlling Biofilm Formation

Efendi Oulan Gustav Hakim Nata Buana, Edwin Yulian Ellyastono, Sari Kurniawati and Agustin Krisna Wardani*

ABSTRACT

Biofilm formation has long been studied as a form of bacterial adaptation for surviving in kinds of harsh condition and considered as one of the hygiene problems especially for food industry. It is highly resistant toward antimicrobial agent thus alternate solution such as bacteriophages have been suggested. Previous study showed that bacteriophage isolated from sewage is effective for eliminating biofilm. However, the very exploration is still poorly investigated and further explorations/ isolations from different sources are still needed. Three bacteriophages have been isolated from beef tripe and chicken intestine—namely φBS6, φBS8, and φUA7. Those three phages have been applied for inhibiting *Pseudomonas fluorescens*, *Escherichia coli*, and *Bacillus subtilis* which are biofilm forming bacteria. Out of the three, *B. subtilis* growth was successfully inhibited by adding 1.12×10^{7} PFU.mL^{-1} of φBS6, 1.00×10^{4} PFU.mL^{-1} of φBS8, and 1.00×10^{4} PFU.mL^{-1} of φUA7. However, the three phages were not able to inhibit the growth of *P. fluorescens* and *E. coli*. Phage infection (φBS6) has been done for observing the effectiveness of *B. subtilis* biofilm inhibition.

Key words: isolation, bacteriophage, bio-sanitizing, biofilm, beef tripe, and chicken intestine

INTRODUCTION

Biofilm is one of the adaptation forms of bacteria which compromise of some steps, including surface attachment, colonization, and envelopment into a matrix. Most of bacteria are found in biofilm forms in order to preserve their existences. Biofilm formation has become a major problem and given many negative impacts as they are naturally more resistant toward antimicrobial substances (Simões *et al.*, 2008). Biofilm resistance are said to be 100 – 1000 times stronger than their planktonic counterparts (Sillankorva, 2008).

Food industries have faced and given additional attention for biofilm formation as one of the hygiene problem for long period until nowadays. In order to lower its formation, industries have applied chemical sanitizer—mostly oxidant substances (chlorine, H_2O_2) and surfactant. However, those chemical sanitizers have some limitations and drawbacks such as rising the resistance of bacteria inside biofilm, ineffective for biofilm controlling (Simões *et al.*, 2008), and great chance for cross-contaminating food products. Given the situation as stated, it is a necessity for exploring alternate solution to control biofilm. Biological entity such as bacterial virus or commonly known as bacteriophage has been suggested as bio-sanitizing agent (Sillankorva, 2008).

Bacteriophage is virus which specifically infects and uses bacteria metabolism system to replicate themselves. Bacteriophages have very effective bactericidal activity and several advantages over other antimicrobial agents. Most notably, phages replicate at the expense of infectious bacteria, are available in abundance where they are most required, and so far, no serious or irreversible side effects of phage therapy have been described (Sulakvelidze and Kutter, 2005) in Gutiérrez *et al.*, 2010).

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Previous study by Sillankorva (2008) in Portugal showed that bacteriophage isolated from sewage were effective for controlling *Pseudomonas fluorescens* biofilm. However, the study of bacteriophage and biofilm are still limited in number, thus this study was aimed for exploring bacteriophage from different sources for strengthening bacteriophage potency as bio-sanitizing agent. Indonesia have rich and varied foodstuff which can be used as another source for bacteriophage isolation. Livestock innards such as beef tripe and chicken intestine are high in bacteria count and expected high in bacteriophage count (Klieve and Swain, 1993 in Joerger, 2003). The isolated bacteriophage would later be used for controlling some pathogens or spoilage bacteria and its biofilm.

MATERIALS AND METHODS

Materials and methods are explained bellow with each steps of the method are repeated two or three times, depend on the situation.

Bacterial strains and growth

Three different bacteria which form biofilm were used in this study as infection target (*Pseudomonas fluorescens*, *Escherichia coli*, *Bacillus subtilis*). The bacteria were supplied by Microbiology Laboratory, Faculty of Agricultural Technology, Brawijaya University and stored at -20°C in 40% glycerol as stock culture.

Culture media and chemical materials

Two different media for the bacteria were used for cultivation and biofilm formation; Trypticase Soy Broth and Agar (TSB and TSA) for *P. fluorescens*, Luria Bertani for *E. coli* and *B. subtilis*. For the purpose of host bacteria and phage isolation, Nutrient Broth and Agar (NA and NB) were used instead. Phosphate Buffer Saline (PBS) pH 7 was used for washing the biofilm and $CaCl_2$ 0.3 M was used as cofactor for phage infection. Gram-identification of the host bacteria used 3% KOH instead.

Host bacteria isolation

Two samples were used for the host bacteria isolation; beef tripe and chicken intestine. Each sample was weighed ±5 gram and put inside a stomacher bag with 45 mL sterile distilled water. Samples homogenization was carried on high speed for 120 minutes or more until the samples turned into aliquot. The dilution was carried on for at least five fold and platted onto NA. Plate was incubated at 37°C for 20 hours. Single colonies were then transferred into NA slant as culture stock.

Bacteriophage isolation

Double layer assay was used for bacteriophage isolation by mixing an overnight host culture inside NB with filtrated aliquot (through 0.22 µm filter membrane) and $CaCl_2$ 0.3 M into soft NA thoroughly. The mixture then poured onto hard NA and let it set. The double layer then incubated at 37°C for 24 hours and observed for plaque forming.

Phage lysate stock and titering

Samples aliquot with formed plaque were used for phage amplification by briefly adding 2.5% host bacteria (20 hours of age) with 2.5% samples aliquot and $CaCl_2$ 0.3 M into Nutrient Broth. Once the turbidity has decreased, the mixture was filtered for collecting high titer bacteriophage (lysate). The lysate was then counted its phage by double layer method and measured in $PFU.mL^{-1}$.

Target bacteria inhibition range

The range of bacteriophage inhibition towards target bacteria's growth was observed by adding 2.5% of 20 hours target bacteria into each respective media. The culture were incubated at 37°C for 30 minutes and followed by adding 2.5% of bacteriophage lysate and $CaCL_2$ 0.3 M. The mixtures were incubated 37°C and have its turbidity observed every 30minutes until any differences in turbidity with control.

Biofilm formation

Biofilms were formed under static condition for 72 hours on stainless steel slide (SS slide) 1x1 cm as described by Cerca *et al.* (2004) with some alterations. Briefly, sterile SS slides were put into three microplates (6 cm in diameter) an added with 10mL each respective media with 0.25% glucose. The 20 hours age culture were then added as much as 50µL and incubated at 37°C. Every 12 hours the media was changed with new one until 72 hours of age.

Biofilm infection

After the biofilms were formed on SS slide, the biofilms were washed with PBS (pH 7) and put into new microplates. Infection was carried by adding 3 mL media, 3 mL phage lysate, and 3 mL $CaCl_2$ 0.3M, while the control was not given any lysate. The infected mixture were then incubated for 4 hours and 6 hours, followed with analysis for its bacteriophage (from the broth) and bacteria count (from SS slide).

RESULTS

Host bacteria and bacteriophage isolation

Ten isolates from each samples were observed and taken as stock culture for further bacteriophage isolation. From 10 isolates of beef tripe and 10 isolates of chicken intestine, only three isolate which form plaque—namely BS6 and BS8 from beef tripe; UA7 from chicken intestine. The plaque formation can be seen on Figure 1 which shown the plaque from host bacteria BS6.

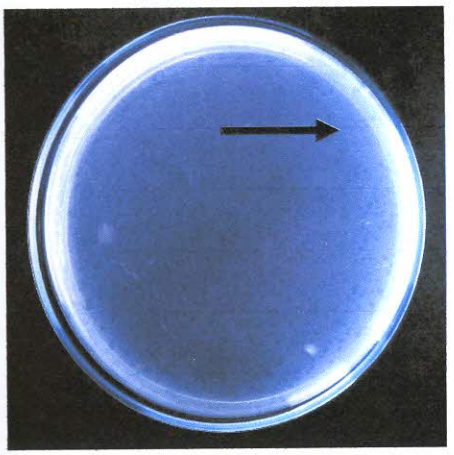

Figure 1 Plaque forming in beef tripe sample (BS6 host bacteria) that showed bacteriophage presence. Plaques are formed via an outward diffusion of phage virion that is fed by bacterial infection (Clokie and Kropinski, 2009). → *Arrow indicating plaque formation*

The plaque formation is indicating that bacteriophage is present in the samples. However, bacteriophage is specific for some bacteria, thus only several host bacteria isolates from the samples can be infected and shown the plaque

formation. From those plaques, the number of bacteriophage can be doubled by infecting the host bacteria with one loop of plaque or adding the filtrate of aliquot samples. In this study, aliquot samples were preferred for doubling the bacteriophage number.

Bacteriophage lytic activity

Even though plaque has been formed for BS6, BS8, and UA7, it does not guarantee that the bacteriophage present on the plaque is virulent. To prove the lytic activity and the virulence of the bacteriophage, turbidity test was used as first step. By comparing the turbidity of infected and uninfected host bacteria, lytic activity of bacteriophage can be observed. The differences of turbidity from infected and uninfected host bacteria are shown in Figure 2.

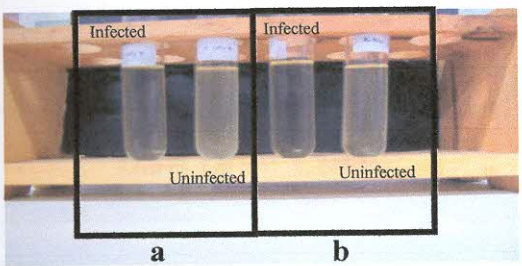

Figure 2 Turbidity comparison of infected and uninfected (a) UA7 and (b) BS8 host bacteria. The infected tube has the host bacteria lysed by the bacteriophage, thus the turbidity decreased. The uninfected tube only has the host bacteria grown, thus the turbidity increased.

Once the difference in turbidity is clear, second step was taken by making the lyse curve of each host bacteria by each bacteriophage. This step was used to measure how long the host bacteria growth can be subdued and determine the right interval to infect the host bacteria after its inoculation. The lyse curves of each host bacteria are shown in Figure 3.

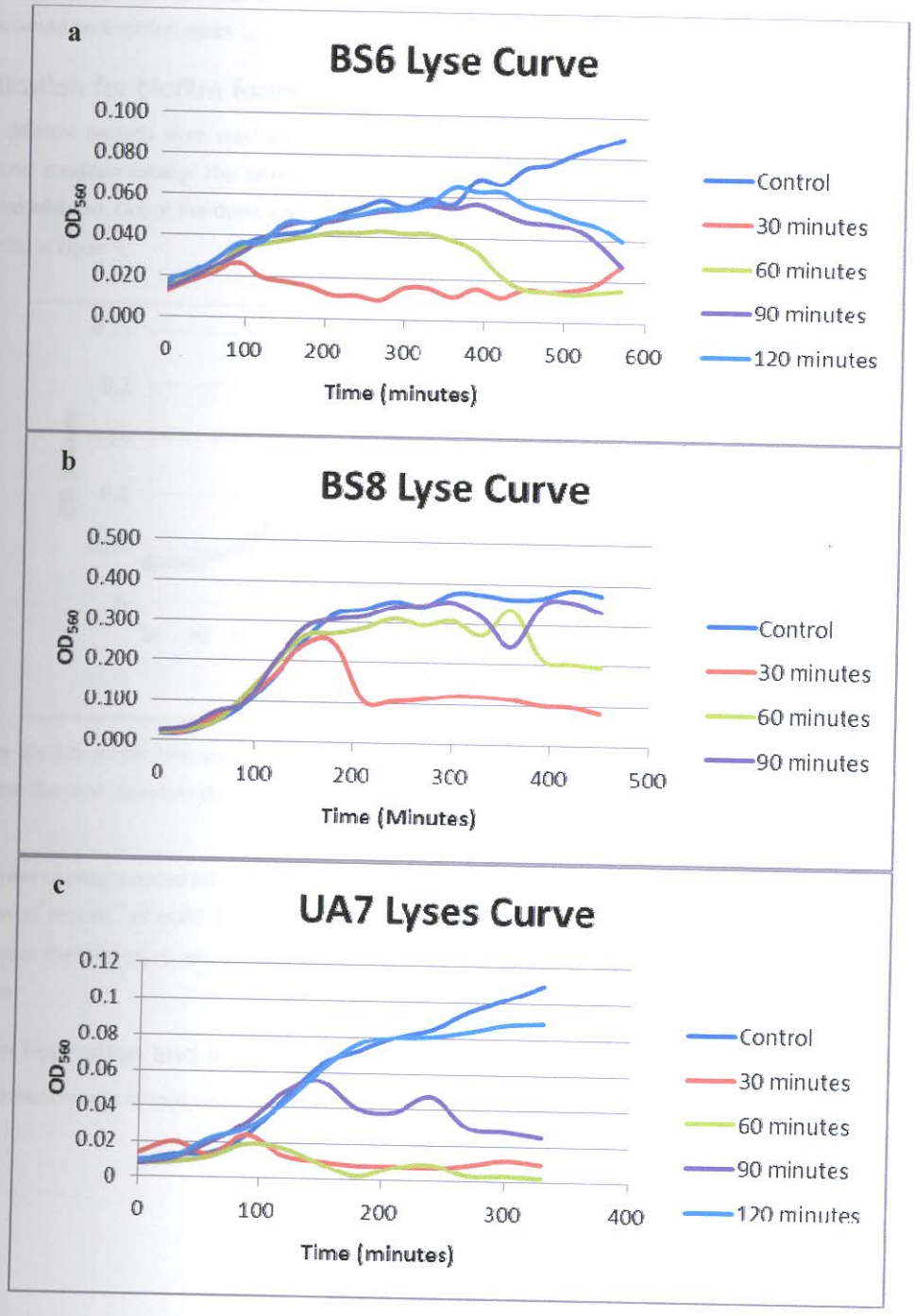

Figure 3 Lyse curve of (a) BS6, (b) BS8, and (c) UA7 host bacteria.

Each bacteriophage has its own optimal lyse time. From each lyse curve, it was known that φBS6 could lyse the host even after 120 minutes after its inoculation, φBS8 gave the best growth inhibition at 30 minutes after its host

inoculation, and φUA7 could lyse the host at 90 minutes after its inoculation. This period points are useful if the phages would be amplified again.

Application for biofilm forming bacteria

Three different bacteria were used for phages application; *P. fluorescens*, *E. coli*, and *B. subtilis* which was in its planktonic condition initially. The assumption was the inhibition of biofilm could be occurred if the planktonic cells could be inhibited. Out of the three, only *B. subtilis* could be inhibited by the addition of three phages. The graph is presented in Figure 4.

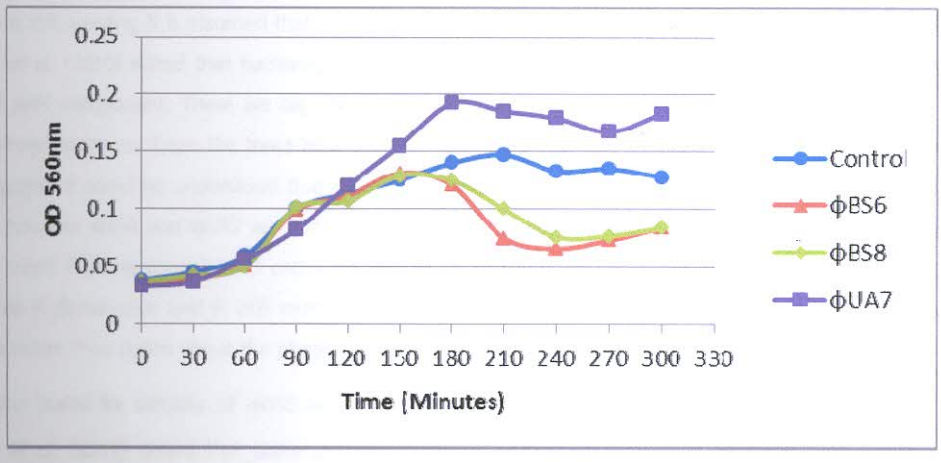

Figure 4 *Bacillus subtilis* lyse curve by φBS6, φBS8, and φUA7 addition, 30 minutes after the bacteria inoculation. φBS6 gave the most significant decrease of the bacteria.

The number of phages added into *B. subtilis* for the curve are 1.12×10^{7} PFU.mL^{-1} of φBS6, 1.00×10^{4} PFU.mL^{-1} of φBS8, and 1.00×10^{4} PFU.mL^{-1} of φUA7. By referencing to the curve, it was known that φBS6 addition with 1.12×10^{7} PFU.mL^{-1} phages gave the most significant decrease out of three phages, thus φBS6 will be used for further work for *B. subtilis* inhibition.

Biofilm Formation and Infection

B. subtilis biofilm were formed under static condition and shown in Figure 5.

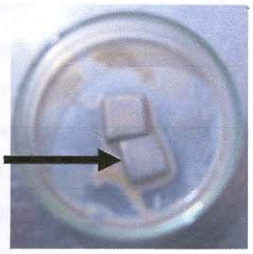

Figure 5 *B. subtilis* biofilm formed under static condition with media renewal every 12 hours. → *Arrow indicating biofilm formation.*

The biofilm was then infected by adding φBS6 and incubated for 4 and 6 hours, followed by bacteriophage and bacterial count. Results showed that uninfected biofilm has 1.50×10^{6} CFU.mL^{-1} bacterial count while the 4 hours infected biofilm has 9.50×10^{5} CFU.mL^{-1} and 6 hours infected biofilm has 4.90×10^{5} CFU.mL^{-1} bacterial count, which was 1 log cycle lower compared to the uninfected biofilm. For the bacteriophage count, the 6 hours infected biofilm has 1.73×10^{7} PFU.mL^{-1} count whereas the 4 hours infected biofilm has 9.70×10^{6} PFU.mL^{-1}.

DISCUSSION

Out of the three bacteria, only *B. subtilis* that can be inhibited by the phages while the other two (*P. fluorescens* and *E. coli*) was still growing. It is assumed that the receptor of three phages are not suitable for *P. fluorescens* and *E. coli*. Rakhuba *et al.* (2010) stated that bacteriophage has several receptors for its attachment and it depends as well at host cell wall component. There are big differences in Gram positive and negative host receptors, thus narrowing bacteriophage spectrum. From the three host bacteria, BS6 is the only Gram positive bacteria while the other two are Gram negative. It could be understood that φBS6 gave the best inhibition on *B. subtilis* since both of them are Gram positive, however φBS8 and φUA7 are still able infecting *B. subtilis*. On the other hand, φBS8 and φUA7 could not properly infect *P. fluorescens* and *E. coli* even though all of them are Gram negative. Given the situation, it is well stated that *P. fluorescens* and *E. coli* infection condition (time of phage addition and infection period) are still not optimum rather than stated about the phages receptors.

Assumption made for inability of φBS8 and φUA7 to infect *P. fluorescens* and *E. coli* are the attachment type. Jakutyte *et al.* (2011) stated that there are two types of phage attachment in bacterial surface—reversible and irreversible attachment. In the reversible attachment, there is a possibility that the phage could be dissociated from the bacteria while the opposite happened in irreversible attachment. It is expected that the two phages (φBS8 and φUA7) bind irreversibly onto the two bacteria (*P. fluorescens* and *E. coli*) which resulted in improper bacteria inhibition. This assumption could be used as fundamental background for further study of φBS8 and φUA7 infection behavior.

The biofilm formation of *B. subtilis* in this study used static condition, even not fully representing the real biofilm condition in industry. Infection with φBS6 into *B. subtilis* biofilm resulted in bacterial count decrease. The infected biofilm has its bacterial count decreased by 1 log cycle compared to the uninfected biofilm. The bacteriophage count showed that by adding 1.12×10^{7} PFU.mL^{-1} of φBS6 resulted in decreasing count of 4 hours infected biofilm (9.70×10^{6} PFU.mL^{-1}) followed by rapid increase in 6 hours infected biofilm (1.73×10^{7} PFU.mL^{-1}). The initial phage decrease is assumed as phages attachment phase or latent phase. Calsina *et al.* (2011) stated that the latent periods (in general as the period when infected individuals are not infectious yet) are typically assumed to be either exponentially distributed or fixed, which can be understood as the stage where phages attach its receptor into the host cell wall and inject its genetic material with no or less lyse occurred, thus number of phage count decreased in the beginning. After the latent phase, it is followed by the period of lyse occurring in which the phage count was increased rapidly. The increasing of phage count, marks the burst phase of phages which correlated to bacteria lyse/ death.

The interval and how long the time needed for changing from latent to burst phase depend on each phage. Referencing the results, it is still too early to state that 4 hours and 6 hours give the optimum interval/ period for phage infection in biofilm. Optimizations of infection are still needed for the best *B. subtilis* biofilm inhibition by φBS6 with more than 1 log cycle.

CONCLUSION

There are three phages that have been isolated from beef tripe and chicken intestine—namely φBS6, φBS8, and φUA7. From the host of each phage, BS6 is the only Gram positive bacteria while the other two are Gram negative bacteria. φBS6, φBS8, and φUA7 can inhibit the growth of *B. subtilis* biofilm even though the decreasing of the bacteria only for 1 log cycle. There are still infection condition improvement and optimization that have to be done in future study.

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