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Highly Spesific *Bacillus cereus*-Phages Isolated from Hospital Wastewater in Banyumas Regency

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Abstract—*Bacillus* cereus is pathogenic bacteria that frequently contaminate food by producing entero and emetic toxins. B. cereus had shown resistance to various antibiotics. An alternative to control B. cereus is the use of bacteriophages. This study aims to determine whether bacteriophages isolated from hospital wastewater in Banyumas Regency is highly specific to B. cereus. The research was carried out descriptively through isolation, purification, enumeration, specificity, effectivity, and host-lysing rate test. B. cereus phages were isolated from hospital wastewater in Banyumas Regency with various titer, i.e. 108-109 PFU.ml-1. The PKRW-1, PKCL-1, PKSR-2, RSBMS-2, RSBMT-1, RSAJP-1, RSAJP-2, RSAJK-2, RSAJE-1, and RSAJE-3 phages have a great specificity and effectivity of infection to B. cereus. Total of 7 isolated phages have a fast host-lysing rate by the 2nd hour of incubation.

Key words—*Bacillus cereus, Bacteriophage, Hospital wastewater, Specific.*

INTRODUCTION

Foodborne disease is widespread and become a global health problem. Contamination of pathogenic bacteria often found in uncooked foods [1]. *Bacillus cereus* is a pathogen that is commonly found in foods, beside of *Salmonella* sp., *Staphylococcus aureus*, *E. coli*, and *Vibrio* sp. [2]. *B. cereus* contamination has been reported to cause a wide range of disease in some countries, such as Netherlands, Norway, United States, New Zealand, and Indonesia from 2006-2012, has even led to several outbreaks cases [3,4,5,6,7].

Food decontamination is not effective in controlling *B. cereus*. Decontamination limited to the physical-chemical methods, such as UV rays, gamma radiation, and high-temperature treatment. Meanwhile, *B. cereus* can form endospores as a form of defense against environmental stress, such as high temperatures and limited sources of nutrients. In addition, *B. cereus* is psychotropic bacteria, therefore, it can survive low temperatures storage [8].

Bacillus cereus is a Gram positive, rod-shaped, sometimes arranged in pairs or short chains, facultative anaerobe, motile, and form endospores [9]. The optimum growing temperature is at 37°C and a pH range of 4.3-9.3. *B. cereus* concentration found in food under normal circumstances is less than 10^2 cells/g of food. The infective dose of *B. cereus* range is 10^4-10^{11} cells/g of food [10].

B. cereus produce enterotoxin and emetic toxin, which can cause poisoning when included in the human digestive tract. Food poisoning is causing two distinct syndromes, the type of diarrhea and emetic. Diarrhea is characterized by abdominal cramps, vomiting, fever and sometimes vomiting. Emetic is characterized by nausea and vomiting [1]. *B. cereus* can also cause infection, such as bacteremia, septicemia, meningitis, pneumonia, central nervous system infections, endocarditis, pericarditis, and respiratory infections [12].

Antibiotics often used to treat the *B. cereus* infection. However, *B. cereus* has developed resistance to various antibiotics [13,14,15]. Therefore, some of those problems, we need an alternative to control food contamination and *B. cereus* infection, that is the use of bacteriophages.

Bacteriophage is obligate intracellular parasites of bacteria [16]. The specificity of bacteriophages to host is high enough, because it is specific to a particular species or bacterial strains. Bacteriophage specifically and effectively control the bacterial population by lysing the host cell [17]. Bacteriophage application is relatively more efficient, specific, and inexpensive, since bacteriophage capable of producing the bacterial peptide and cell wall hydrolitic enzyme, bacteriophage may be reproduced biologically, bacterial resistance to bacteriophage infection is relatively low [18], and has no effect on other cells, including human, animal, and plant cells [19].

Bacteriophage can be isolated from various samples, such as soil, waste, water, food, and bacteria-infected tissues [20]. Studies on the specific bacteriophage infecting *B. cereus* was still limited. This study aims to determine whether higly specific *B. cereus* phages can be isolated from hospital wastewater in Banyumas Regency.

METHOD

a. Sampling

Water samples were taken from hospital wastewater in Banyumas Regency. Samples were taken by purposive random sampling technique. Wastewater sample (30 ml) was collected using a sterile bottle.

b. Isolation of total bacteriophages [21] with modifications

Water sample was filtered with 0.45 µm membrane filter. The filtrate (F1) supplemented with phage buffer solution and incubate in a shaker incubator at room temperature, then centrifuged at 8000 g for 20 min. Supernatant was filtered with 0.45 µm membrane filter. The filtrate (F2) precipitated with polyethylene glycol 8000 (8% w/v)-1 M NaCl overnight at 4°C, then centrifuged at 10000 g for 20 min at room temperature. The supernatant was discarded, the natant was resuspended with phage buffer solution and stored as crude stock at 4°C.

c. Isolation of bacteriophage infecting *B. cereus* [21] with modifications

A total of 5 µL crude stock was mixed with 100 µL liquid *B. cereus* culture, 10 µL $MgSO_4$, and 10 µL $CaCl_2$. The mixture was incubated for 15 minutes at 37°C. Then, mixed with Luria Bertani medium and stocked in a sterile petri dish. Media was incubated for 24 hours at 37°C, then the plaque was observed.

d. Purification of bacteriophage infecting *B. cereus* [21]

Every single plaque inserted into the micro centrifuge tube containing 5 µL phage buffer solution, then mixed with 100 µL liquid *B. cereus* culture and incubated for 24 hours at 37°C by shaking at 40 rpm. Culture filtered through 0.45 µm membrane filter. A total of 5 µL phage suspension (F3) was taken and mixed with 100 µL liquid *B. cereus* culture, 10 µL $MgSO_4$, and 10 µL $CaCl_2$. The mixture was incubated for 15 minutes at 37°C. Then, mixed with Luria Bertani medium and stocked in a sterile petri dish. Media was incubated for 24 hours at 37°C, then the plaque was observed. These step was done 3 times to ensure the phage is pure. The activity observed with the phage lytic zones formed (plaque). The filtrate from the last purification stage precipitated with polyethylene glycol 8000 (8% w/v) -1 M NaCl and stored as phage pure stock at -80°C.

e. Calculation of bacteriophage concentration [21] with modification

A total of 10 µL of phage pure stock was diluted with 90 µL of phage buffer solution, then diluted to 10^6. A total of 10 µL suspension from two final dillution were taken. Each suspension was inoculated on 100 µL liquid *B. cereus* culture that supplemented with 10 µL $MgSO_4$ and 10 µL $CaCl_2$. The cultures were incubated for 15 min at 37°C. The mixture was incubated for 15 minutes at 37°C. Then, mixed with Luria Bertani medium and stocked in a sterile petri dish. Media was incubated for 24 hours at 37°C. Formed plaque was calculated as the number of Plaque Forming Units per milliliter (PFU.ml^{-1}).

f. Specificity test of bacteriophage infection to *B. cereus* by spectrophotometry at λ600 nm [22] with modifications

Bacteria used were *B. cereus, Escherichia coli, Enterobacter aerogenes, Shigella* sp., *Vibrio* sp., *Salmonella typhi, Citrobacter freundii, Staphylococcus aureus*, and *Klebsiella pneumoniae*. A total of 10 µL phage pure stock was infected in 7 mL liquid bacteria culture, then incubated for 6 hours at 37°C by shaking at 60 rpm. Culture absorbance values were measured using a UV-Vis spectrophotometer at λ600 nm. Liquid culture of bacteria without phage infection was the control.

g. Effectivity test of bacteriophage infection to *B. cereus* by spectrophotometry at λ600 nm [22] with modifications

Effectivity test conducted by means such as specificity test using *Bacillus cereus* ATCC 11778, *Bacillus subtilis, Bacillus fragilis, Bacillus circulans,* and *Bacillus cereus* non ATCC 11778.

h. Lytic rate test of bacteriophage infection to *B. cereus* by spectrophotometry at λ600 nm [22] with modifications

A total of 100 µL phage pure stock was infected in 50 mL liquid *B. cereus* ATCC 11778 culture, then incubated in incubator shaker with 60 rpm at 37°C. Liquid culture of bacteria without phage infection was the control. Culture absorbance values were measured using a UV-Vis spectrophotometer at λ600 nm within 0, 0.5, 1, 2, 4, 6, 8, 10, and 12 hours.

RESULTS AND DISCUSSION

Sampling and isolation of total bacteriophages

Wastewater samples that have been taken are Margono (RSMG), Banyumas (RSBMS and RSBMT), Ajibarang (RSAJK, RSAJP, and RSAJE) Hospital, Public Health Center Cilongok I (PKCL), Rawalo (PKRW), Soekaraja I (PKSR). Bacteriophage isolation carried out from the environment which is expected to contain the bacterium host. According to [23], bacteriophages can be isolated from aquatic environments, including sewage. Meanwhile, bacteria in freshwater environments may be concentrated on the surface of solids than in water bodies.

Based on the appearance and characteristics of wastewater samples, the estimated number of bacteria in the waste is quite a lot. This estimation is based on the environmental conditions (sewage) that support for bacterial growth, so that the phage has enough host. The aquatic environment containing organic and inorganic materials [9]. The presence of organic compounds has an important role to phage replication, because organic compounds greatly affect the activity of the host bacterium.

The amount of phage in wastewater samples is influenced by some factors, such as environmental factors and the presence of bacterial host. Several physical and chemical factors can inactivate the phage [24]. The detergents content in wastewater influence the presence of phage. The chlorine content in the detergent efficiently kill the bacteria. These things may have an impact on the reduction of total phage in the wastewater.

The phage stock is stored at 4°C to maintain the phage stability. Temperature is an important factor for phage resistance. According [1], phage storage temperature is the most decisive factor of phage activity, such as adhesion, penetration, and phage multiplication.

The observation of plaque at this stage indicates a number of various data. The highest phage amount was originating from RSAJK, RSAJE, and RSBMT, i.e. 10-12 plaques (Table 1). Number of isolated phage is highly dependent on the presence of the target bacteria in wastewater, *Bacillus cereus*.

Table 1. Bacteriophage Isolation

Sample code	Plaque amount
PKRW	3
PKCL	5
PKSR	2
RSMG	7
RSBMS	9
RSBMT	10
RSAJP	8
RSAJK	12
RSAJE	12

Plaque was purified 3 times with the same working step to purify and keep the consistency of plaque (Figure 1). The results showed that an increasing amount of plaque formed from each sample with a range between 5-58 plaques in every dish (data not shown). Generally, there is an increase of plaque formed on each stage of purification.

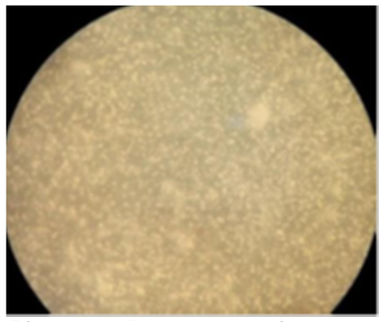

Figure 1. Plaques on B. cereus growth

Concentration amount of phage isolate was very various depent on the amount of phage in sample (Figure 2). However, plaque was not formed from every filtrate. According to [11], bacteriophages may be unable to form plaques on solid bacterial growth medium, but still able to infect host cells in liquid bacterial culture.

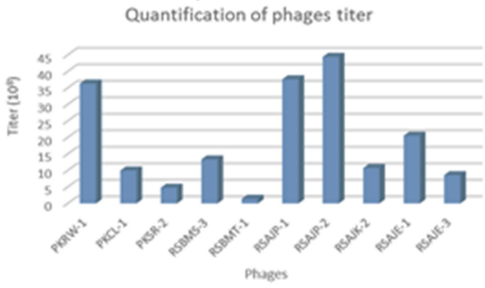

Figure 2. Quantification of phage titer

Absorbance value measurement results show that all phages can infect *B. cereus*, seen from a decrease in the absorbance values of culture. There are several phage that has a low level of specificity. However, all phages infect no more than 33% of total testing bacterium (Table 2). This is demonstrated by the ability of phage infects bacteria other than *B. cereus*.

Phage isolates were only able to infect *B. cereus* showed a high degree of infection specificity. A total of 14 from 29 phages isolate were highly spesific (Table 2). Phage has a high infection specificity towards species and even strains of certain bacteria, but some phages can

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infect more than one species of bacteria. There are several types of phage that can infect more than one species, and even genus of bacteria [14]. This relates to the phage ability to recognize receptors on the host cell.

Table 2. Specificity test

Phages isolate	Bacterial Host								
	B. cereus	*C. freundii*	*Shigella* sp.	*K. pneumoniae*	*E. aerogenes*	*S. typhi*	*Vibrio* sp.	*E. coli*	*S. aureus*
PKRW-1	+								
PKRW-2	+							+	
PKRW-3	+							+	
PKCL-1	+								
PKCL-2	+	+							
PKCL-3	+								
PKSR-1	+			+					
PKSR-2	+								
RSMG-1	+							+	
RSMG-2	+			+					
RSMG-3	+	+							
RSBMS-1	+	+							
RSBMS-2	+								
RSBMS-3	+								
RSBMT-1	+								
RSAJP-1	+								
RSAJP-2	+								
RSAJP-3	+			+					
RSAJP-4	+			+					
RSAJK-1	+								
RSAJK-2	+								
RSAJK-3	+			+					
RSAJK-4	+			+					
RSAJE-1	+								
RSAJE-2	+	+							
RSAJE-3	+								
RSAJE-4	+	+						+	
RSAJE-5	+	+						+	
RSAJE-6	+								

The effectivity test results showed that some phages capable of infecting bacteria other than *B. cereus*, that are *B. fragilis, B. subtilis,* and *B. circulans* (Table 3). Those show the level of specificity and effectivity of phage infection to the host. According to [26], *B. cereus* phage CP-51 can infect several other strains, that are *B. cereus* (6464, 9239, and T) even in *B. anthracis*. However, the phage can not infect *B. subtilis* and *B. licheniformis.*

Lytic phage lysing their host bacteria in a matter of minutes or hours after infection. The infection produces hundreds to thousands of new phages [16]. The lytic rate test showed that most of phages was able to decrease the absorbance value after 1 hour infection (Figure 4 and 5). According to [27], *Bacillus cereus* lytic phage FWLBc1 and FWLBc2 are capable of producing 322 and 300 new phages from any infected cells, and have a latency period of 106 and 102 minutes at 37°C.

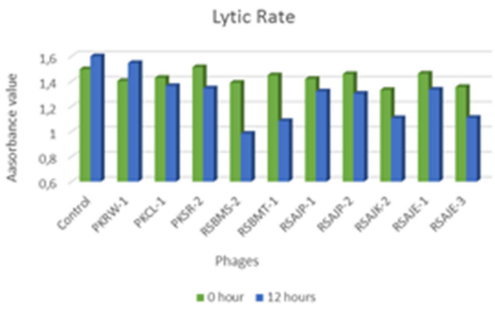

Figure 3. Lytic activity of B. cereus phages

Table 3. Effectivity Test

Phages isolate	Bacterial Host			
	B. cereus	*B. subtilis*	*B. fragilis*	*B. circulans*
PKRW-1	+			
PKCL-1	+			
PKCL-3	+	+		
PKSR-2	+			
RSBMS-2	+			
RSBMS-3	+		+	+
RSBMT-1	+			
RSAJP-1	+			
RSAJP-2	+			
RSAJK-1	+			+
RSAJK-2	+			
RSAJE-1	+			
RSAJE-3	+			
RSAJE-6	+			+

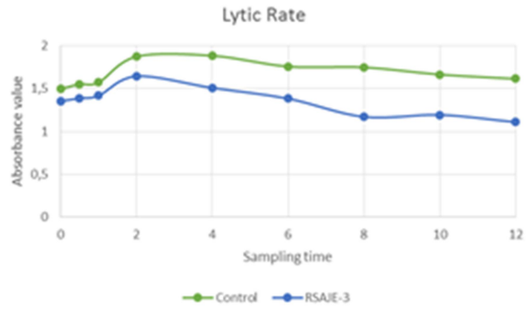

Figure 4. Lytic rate of phages RSAJE-3

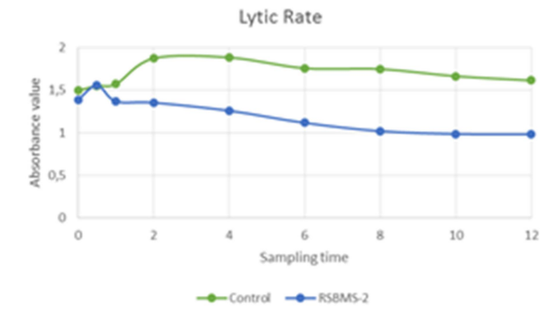

Figure 5. Lytic rate of phages RSBMS-2

Phages with great effectivity and specificity of infection has the potential to be applied in various fields. Some have applied as biocontrol for contamination of food [28,29,30,31]. In addition, phage was also applied in the clinical field as a treatment of various diseases [32,33,34].

CONCLUSION

Highly specific and effective *Bacillus cereus* phages isolated from hospital wastewater in Banyumas Regency were PKRW-1, PKCL-1, PKSR-2, RSBMS-2, RSBMT-1, RSAJP-1, RSAJP-2, RSAJK-2, RSAJE-1, and RSAJE-3.

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