BIODIVERSITAS
Volume 21, Number 7, July 2020
Pages: 2871-2877

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210701

Isolation and selection of *Bacillus cereus* specific phages from hospital wastewater

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Manuscript received: 27 April 2020. Revision accepted: 1 June 2020.

Abstract. *Rovik A, 'Aziz S, Pramono H. 2020. Isolation and selection of* Bacillus cereus *specific phages from hospital wastewater. Biodiversitas 21: 2871-2877. Bacillus cereus* (*B. cereus*) is a pathogenic bacterium that frequently contaminates food by producing entero and emetic toxins. *B. cereus* has shown resistance to various antibiotics, especially β-lactam antibiotics. An alternative to control *B. cereus* contamination is the use of bacteriophages. This study aimed to isolate and screen *B. cereus* specific phages from hospital wastewater in Banyumas District. The research was conducted descriptively through isolation, purification, titer determination, host ranges, and adsorption rate determination. A total of 29 isolates of *B. cereus*-phages were isolated from hospital wastewater in Banyumas with various titers, ranged from 0.14-3.76 x 10^7 PFU.mL^{-1}. Isolated phages could be grouped into two, narrow host range (14 phages) and broad activity spectra (15 phages) that infect both Gram-positive and negative bacteria i.e. *B. subtilis, B. fragilis, B. licheniformis, Citrobacter freundii, Klebsiella pneumoniae, Salmonella thypi,* and *Escherichia coli*. The infections had a latency period of 60-120 minutes. The decrease of culture absorbance value ranged from 0.06 to 0.41, while the control tended to increase by 0.39. The largest decreases were showed by phage isolates RSBMS-2 and RSBMT-1 with 0.41 and 0.37, respectively.

Keywords: *Bacillus cereus,* Bacteriophage, Banyumas, hospital wastewater, specific

INTRODUCTION

Foodborne disease is widespread and has become a global health problem. Several foodborne pathogens are commonly contaminating cooked and uncooked foods, such as *Bacillus cereus* (*B. cereus*), *Salmonella* spp., *Staphylococcus aureus*, *E. coli*, and *Vibrio* spp. (Whong et al. 2006; Ziane et al. 2014). *B. cereus* contamination was reported to cause a wide range of diseases in some countries from 2006-2018, such as the Netherlands, Norway, United States, New Zealand, France, and Indonesia, that even led to several outbreak cases (Scallan et al. 2011; Sumarno et al. 2011; Lim et al. 2012; OzFoodNet 2012; EFSA 2013; Glasset et al. 2016; Lentz et al. 2018).

Food decontamination is not fully effective in eliminating *B. cereus* since it can only reduce the microbial contamination level in or on foods. Food decontamination is limited to the physical-chemical methods, such as the application of ultraviolet (UV) rays, gamma radiation, and high-temperature treatment. Physical-chemical treatments may cause further undesirable effects e.g. potential changes of food sensory qualities and residues remaining in the food (EFSA 2009). To minimize these risks, the intensity of the treatments has to be limited, which implies lowering the effectiveness. Meanwhile, *B. cereus* can produce endospores as a defense against environmental stress, such as high temperatures, limited sources of nutrients, and low-temperature storage (El-Arabi et al. 2013).

Bacillus cereus is Gram-positive, rod-shaped, which are sometimes arranged in pairs or short chains, facultatively anaerobic, motile, and form endospores (Pelczar and Chan 1986). They are found in food under normal circumstances with less than 10^2 cells/g. Their higher presence in food e.g. 10^4-10^{11} cells/g may be infective to humans (Lake et al. 2004) since *B. cereus* produces entero and emetic toxins which cause poisoning. When *B. cereus* entering the human digestive tract, their toxins cause two distinct syndromes: diarrhea and emetic types (Logan and Rodrigez-Diaz 2006). This opportunistic bacterium can also cause infections, such as bacteremia, meningitis, pneumonia, pericarditis, endocarditis, central nervous system, and respiratory infections (Schoeni and Wong 2005).

Some studies reported that *B. cereus* has developed resistance to various antibiotics, especially β-lactam antibiotics. Several strains are also resistant to ampicillin, oxacillin, penicillin, amoxicillin, rifampin, rifamycin, cefepime, erythromycin, tetracycline, and fluoroquinolones (Schlegelova et al. 2003; Logan and Rodrigez-Diaz 2006 Mutalib and Abdullah 2006; Fenselau et al. 2008; Bottone 2010; Kim et al. 2015; Savic et al. 2016; Owusu-Kwarteng et al. 2017; Sood et al. 2017; Park et al. 2018; Yu et al. 2020). Those problems arise the interest in bacteriophage application as an alternative way to control the *B. cereus* contamination.

Bacteriophages are obligate intracellular parasites of bacteria (Calendar 2004). They offer many advantages as bio-control agents of pathogenic bacteria: (i) high

specificity infection to a particular species or bacterial strain; (ii) self-replication and self-limiting which implies to their continuous multiplication activities as long as there is still a bacterial host; (iii) bacterial resistance to phage infection is low; (iv) low toxicity, since they consist mostly of proteins and nucleic acids; (v) they are easy to isolate and propagate; (vi) they can generally withstand food processing stresses; (vii) they have proved to have a prolonged shelf-life; and (viii) they have no effect on other cells, including human, animal, and plant cells (Snyder and Champness 2003; Sulakvelide and Kutter 2005; Parisien et al. 2007).

Phages can be found wherever bacteria are present. Therefore, they can be found in some sample sources, such as soil, sewage, food, bacteria-infected tissues, waste, and water (Popova et al. 2012). Hospital wastewater is coming from any source or activities, such as hospitals and medical centers, cleaning services, laboratories, and others. Therefore, organic materials, antibiotics, disinfectants, and pathogenic bacteria are suggested to be the major constituents (Pauwels and Verstraete 2006; Al-Gheethi et al. 2018). Those wastewaters contribute to the high rates of antibiotic-resistant bacteria, both Gram-positive and negative bacteria (Moges et al. 2014). Studies on the specific bacteriophages infecting *B. cereus* are still limited. Thus, isolation and characterization of new phages are essential for discovering effective bio-control agents against *B. cereus*. This study aimed to isolate and screen *B. cereus* specific phages from hospital wastewater in Banyumas District, Indonesia.

MATERIALS AND METHODS

Sampling

The wastewater was collected from six hospitals in Banyumas District, Central Java, Indonesia, i.e.: Margono Soekarjo Hospital, RSUD Banyumas Hospital, RSUD Ajibarang Hospital, Puskesmas Cilongok I, Puskesmas Rawalo, and Puskesmas Soekaraja I. The sample was taken by purposive random sampling from the installation system of wastewater treatments. A total of 30 mL of wastewater was collected using a sterile container from each study site, then directly transported to the laboratory.

Solution and medium preparation

Phage buffer solution. A 0.8 g NaCl, 0.02 g KCl, 0.144 g $Na_2HPO_4.2H_2O$, and 0.024 g KH_2PO_4 were diluted with sterile aquadest and adjusted to pH 7.4 (for 100 mL final volume). Next, the solution was sterilized by autoclaving.

NaCl-polyethylene glycol solution. An 8 g Polyethylene glycol 8000 (Sigma) and 11.7 g NaCl were added with a 75 mL phage buffer solution, then adjusted to pH 7.5. Next, the solution was sterilized by autoclaving.

Luria Bertani medium. A 5 g Yeast extract, 5 g NaCl, 10 g Tryptone, 1 g L-Tryptophan, and 15 g agar were diluted with sterile aquadest and adjusted to pH 7.5 (for 1000 mL final volume). Next, the medium was sterilized by autoclaving. Note: the overlay/top medium was using a 5 g agar.

Total phage isolation

Wastewater was filtered with a 0.45 µm membrane filter (Sartorius). The filtrate (F1) was supplemented with a phage buffer solution and incubated in a shaker incubator S1-600 at room temperature, then centrifuged at 8,000x*g* for 20 minutes (Thermo Centrifuge). Next, the supernatant was filtered with a 0.45 µm membrane filter (Sartorius). The filtrate (F2) was precipitated overnight at 4°C with NaCl-polyethylene glycol precipitation, then centrifuged at 10,000x*g* at room temperature (Thermo Centrifuge) for 20 minutes. The supernatant was discarded, the pellet was re-suspended with a phage buffer solution and stored as crude stock at 4°C.

Isolation of *Bacillus cereus* specific phage

A total of 5 µL of phage crude stock were mixed with 100 µL of 8 hours-cultured *B. cereus*, 10 µL $MgSO_4$ (10 mM), and 10 µL $CaCl_2$ (10 mM). The mixture was incubated at 37°C (Memmert) for 15 minutes, then mixed with Luria Bertani medium and over-lay-cultured on the solid growth medium. The medium was incubated at 37°C (Memmert) for 24 hours, then the formed plaque was observed.

Purification of *Bacillus cereus* phage

Every single plaque was picked up by using an inoculum needle, then inserted into the micro-centrifuge tube containing 5 µL phage buffer solution, then mixed with 100 µL of 8 hours-cultured *B. cereus* and incubated at 37°C for 24 hours by shaking at 40 rpm (S1-600 Shaker Incubator). The culture was filtered through a 0.45 µm membrane filter (Sartorius). A 5 µL phage suspension (F3) was taken and mixed with 100 µL of 8 hours-cultured *B. cereus*, 10 µL $MgSO_4$ (10 mM), and 10 µL $CaCl_2$ (10 mM). The mixture was incubated at 37°C (Memmert) for 15 minutes, then mixed with Luria Bertani medium and over-lay-cultured on the solid growth medium. The medium was incubated at 37°C (Memmert) for 24 hours, then the formed plaque was observed. This step was repeated 3 times to ensure the phage was pure, by observing the phage lytic zone (plaque). The filtrate (F4) from the last purification stage was precipitated with NaCl-polyethylene glycol precipitation and stored as pure stock phage at -80°C (Thermo Scientific, USA).

Phage titer determination

A total of 10 µL of pure stock phage was diluted with 90 µL of phage buffer solution, then diluted up to 10^6 by using sterile aquadest. A 10 µL suspension from two final dilutions was taken. Each suspension was inoculated on 100 µL of 8 hours-cultured *B. cereus* that was supplemented with 10 µL $MgSO_4$ (10 mM) and 10 µL $CaCl_2$ (10 mM). The mixture was incubated at 37°C (Memmert) for 15 minutes, then mixed with Luria Bertani medium and over-lay-cultured on the solid growth medium. The medium was incubated at 37°C (Memmert) for 24 hours. The formed plaque was calculated as the number of Plaque Forming Units per milliliter ($PFU.mL^{-1}$).

Phage host ranges

Phage host ranges were evaluated by using a liquid test. The first round test used an 8 hours-cultured *B. cereus*, *Escherichia coli*, *Enterobacter aerogenes*, *Shigella* sp., *Vibrio* sp., *Salmonella typhi*, *Citrobacter freundii*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. A further test was conducted by infecting the phage to bacterial host among Bacillus genera, i.e. *Bacillus cereus*, *Bacillus subtilis*, *Bacillus fragilis*, and *Bacillus circulans*. First, 10 µL of pure stock phage (~10^6 PFU.mL^{-1}) was infected into 7 mL of 8 hours-cultured bacterial hosts, then incubated at 37°C for 6 hours by shaking at 60 rpm (S1-600 Shaker Incubator). Culture absorbance values were measured using a UV-Vis spectrophotometer at λ600 nm (Thermo Scientific). The control was the liquid culture of bacteria without phage infection.

Adsorption rate determination

The narrow host ranges phages were further tested for their adsorption rates. A 100 µL pure stock phage (~10^6 PFU.mL^{-1}) was infected into 50 mL of 8 hours-cultured *B. cereus* (MOI of 0.01), then incubated at 37°C by shaking at 60 rpm (S1-600 Shaker Incubator). The control was a liquid culture of bacteria without a phage infection. Culture absorbance values were measured using a UV-Vis spectrophotometer at λ600 nm (Thermo Scientific) at 0, 0.5, 1, 2, 4, 6, 8, 10, and 12 hours of incubation.

RESULTS AND DISCUSSION

Bacteriophages with highly specific and effective infections have the potential to be applied in some fields, such as bio-control of food contamination (Guenther et al. 2009; Iswadi 2010; Leverentz et al. 2003) and phage therapy (Kutter et al. 2010; Marza et al. 2006). In this research, bacteriophage isolation was done from hospital wastewater which was expected to contain the bacterium host. Hospital wastewater is coming from any source or activities, such as hospitals and medical centers, cleaning services, laboratories, and others. Therefore, organic materials, antibiotics, disinfectants, and pathogenic bacteria are suggested to be the major constituents (Pauwels and Verstraete 2006; Al-Gheethi et al. 2018). Those wastewaters contribute to the high rates of antibiotic-resistant bacteria, both Gram-positive and negative bacteria (Periasamy and Sundaram 2013; Moges et al. 2014). According to Weinbauer (2004), bacteriophages can be isolated from aquatic environments, including sewage. The aquatic environment contains organic and inorganic materials that support bacterial growth.

The collected wastewater came from Margono Soekarjo Hospital (RSMG), RSUD Banyumas (RSBMS and RSBMT), RSUD Ajibarang (RSAJK, RSAJP, and RSAJE), Puskesmas Cilongok I (PKCL), Puskesmas Rawalo (PKRW), and Puskesmas Soekaraja I (PKSR). Isolating lytic phages is essential for discovering the bio-control agents. The samples were added to the upper layer of a double-layer agar plate. Every single plaque from the lawn was harvested. Lytic phages begin lysing the host cells in a matter of minutes or hours after infection, producing hundreds to thousands of new phages (Calender 2004). Lytic phage infection produces clear plaques on a lawn of bacterial host, because of the destruction of the cells. The phages (plaques) purification step was repeated 3 times to remove the contaminant phages and keep the consistency of the formed plaque (Figure 1). However, the plaque was not formed by every phage filtrate. Hyman and Abedon (2010) stated that bacteriophage may be unable to form plaques on a solid bacterial growth medium, but still be able to infect host cells in liquid bacterial culture.

The collected samples have various bacteriophage titers (Figure 2). The isolated phages were enriched by infecting them to 8 hours-cultured *B. cereus* for 24 hours to produce quantifiable titer. The highest titer was originating from RSAJP, PKRW, and RSAJE, i.e. 2.05-3.76 x 10^7 PFU.mL^{-1}. Bacteriophage titers in most water systems range from 10^4-10^8 PFU.mL^{-1} (Weinbauer 2004). This titer value could indicate the time of phage generation which is controlled by the lysis time. The longer lysis time resulted in a larger burst size (Wang 2006). The phage titer in wastewater samples is influenced by some factors, such as environmental factors and the presence of host bacteria. The presence of organic compounds has an important role in phage replication because organic compounds greatly affect the activity of the host bacterium (Ackerman 2007). Meanwhile, several physical and chemical factors can reduce the bacteria and inactivate the phage (Al-Gheethi et al. 2018).

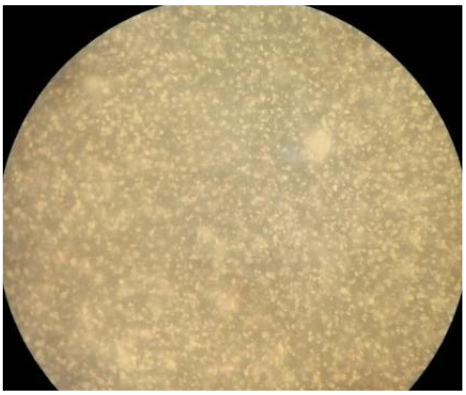

Figure 1. Formed plaques on *Bacillus cereus* growth medium

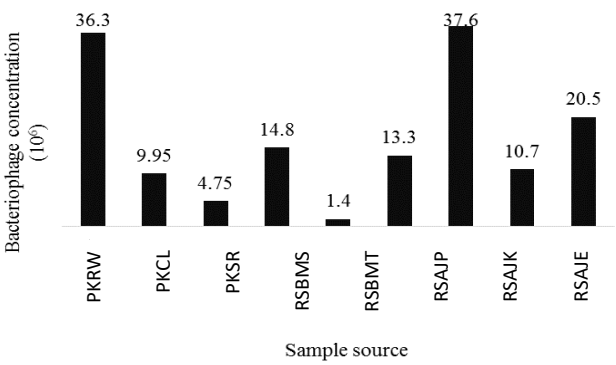

Figure 2. The titer of *Bacillus cereus* phage isolated from hospital wastewater

Table 1. Phage host ranges

Phage isolate	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	+								

Note: (+) the culture absorbance values were decreased in 3 repetitions

Table 2. Phage infection specificity among *Bacillus* genera

Phage isolate	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	+			+

Note: (+) the culture absorbance values were decreased in 3 repetitions

The phage host range was determined by using a liquid culture infection. A total of 10 µL of purified phage stock (~10^6 PFU.mL^{-1}) was infected into 7 mL of 8 hours-cultured bacteria. The titer of phage and host bacteria are important factors in phage replication. At low levels of host bacteria, the phage infection may not occur. At least 10^4 CFU.mL^{-1} bacteria are needed to get a successful phage replication (Goyal et al. 1987). The research used bacteria that commonly found in aquatic environments, both Gram-positive and negative, which are *Salmonella typhi, Enterobacter aerogenes, Shigella* sp., *Klebsiella pneumoniae, Citrobacter freundii, Escherichia coli, Staphylococcus aureus,* and *Vibrio* sp. Most of these bacteria have also been reported to be resistant to the commonly used antibiotics and as such have led to the outbreak of several diseases and infections (Al-Gheethi et al. 2018).

The host ranges data could divide the isolated phages into two groups (Table 1). The phages with narrow host range (14 phage isolates) and broad activity spectra (15 phage isolates) that infected more than single host bacteria, both Gram-positive and negative. Lee (2008) found that *B. cereus* phage has a narrow host range or high infection specificity since it did not infect untargeted bacteria. Phages have a high infection specificity towards species and even strains of certain bacteria, but some phages can infect different genus of bacteria (Logan and Rodrigez-Diaz 2006). Some studies have isolated phages with broad activity spectra from sewage, wastewater, and soil (Khan et al. 2002). However, it has been suggested that repeated phage purification using single host strain may increase infection specificity which further narrowing the phage host-range (Jensen et al. 1998).

Phages contact with the bacterial host by passive diffusion, while the adsorption and entry processes are mediated by specific receptors (Bielke et al. 2007). These receptor sites are located on different parts of bacteria, such as proteins, carbohydrates, lipopolysaccharides, pili, flagella, capsular, and others. Some of them are present all the time, while other sites present only in the logarithmic growth of bacteria (Goyal et al. 1987; Hyman and Abedon 2010). Phage interaction to receptors of bacterial host determines the infection specificity and bacterial host ranges. For example, phage specific to smooth-type lipopolysaccharide (LPS) display an extremely narrow host range specificity while phage recognizing rough-type LPS shows a broader host range. The structure of peptidoglycan and teichoic acid are similar between *S. aureus* and genus Bacillus, therefore phage can infect both *S. aureus* and *B. subtilis* (Rakhuba et al. 2010).

Some isolated phages were unable to lyse the indicator bacteria, i.e. *E. aerogenes, Shigella* sp., *S. typhi, S. aureus,* and *Vibrio* sp. showed that the tested strains may resistant to the infection of *B. cereus* specific phages (Table 1). Phage-resistance mechanisms encoded by bacteria serve to limit the phage host range. Bacteria have developed their resistance to phage infection through some mechanisms, such as the receptor's loss, genome uptake, and phage adsorption blocks (Hyman and Abedon 2010; Moller et al. 2019). For example, *E. coli* with tsx gene mutation displayed resistance to bacteriophage T6 infection (Rakhuba et al. 2010). Stk2 was found to be activated by a phage protein and caused cell death in *S. aureus* (Moller et al. 2019).

The phages with narrow host range were further assessed for the infection specificity among Bacillus genera. Four phage isolates could infect *B. fragilis, B. subtilis,* and *B. circulans* other than *B. cereus* (Table 2). Thorne and Holt (1974) found that *B. cereus* phage CP-51 can also infect *B. cereus* (6464, 9239, and T) and even *B. anthracis*. However, the phage could not infect *B. subtilis* and *B. licheniformis.* Some phages were reported to have infection specificity toward different strains and species (Holmfeldt et al. 2007; McLaughlin et al. 2006).

Adsorption rate determination was done by infecting 50 µL of phage (~10^6 PFU.mL^{-1}) into a 50 mL bacterial culture of 8 hours-cultured *B. cereus* (MOI of 0.01). The host-lysing rate test showed that most of the phages were able to lyse the host indicated by the decrease of culture absorbance value (Figure 3). The rate of adsorption may vary depending on the concentration of phage and bacterial host (Moller et al. 2019). The absorption rate is affected by some non-specific physical-chemical factors, such as temperature, acidity, presence of certain substances and ions on medium, and physiological condition of host bacteria (Rakhuba et al. 2010). Some independent studies have shown that the presence of calcium and magnesium ions plays an important role in phage adsorption (Marks and Sharp 2000; Thorne and Holt 1974). For example, with 10 mM supplementation of calcium and magnesium, the majority of PBC1 phage particles were found to be attached to the bacterial host after 15 minutes of incubation (Kong and Ryu 2015). Therefore, it is essential to supplement the bacterial growth medium with calcium and magnesium.

The culture absorbance values decreased after 1-2 hours of phage infections (Figure 4). In other words, the infections had a latency period of 60-120 minutes. The decrease of culture absorbance values ranged from 0.06 to 0.41, while the control tended to increase by 0.39. The largest decreases in values were showed by phage isolates, RSBMS-2 and RSBMT-1, 0.41 and 0.37, respectively, while the lowest was isolate PKCL-1 with 0.06. The absorbance value of bacterial culture infected by phage isolate PKRW-1 tended to increase by 0.14. Lytic phages will be replicated immediately after entering the host bacteria. During the first 30 minutes after infection, tens to hundreds of new phages will be produced and released (Davis et al. 1990). Lee (2008) found that *B. cereus* lytic phages FWLBc1 and FWLBc2 are capable of producing 322 and 300 new phages from the infected cells and have a latency period of 106 and 102 minutes at 37°C, respectively.

In conclusion, a total of 29 isolates of *B. cereus* phages were isolated from hospital wastewater in Banyumas District. A total of 14 phages were narrow host range, while 15 phages were broad activity spectra that infect both Gram-positive and negative bacteria i.e. *B. subtilis, B. fragilis, B. licheniformis, Citrobacter freundii, Klebsiella pneumoniae, Salmonella thypi,* and *Escherichia coli.* Phage isolates RSBMS-2 and RSBMT-1 had the largest decrease of bacterial culture absorbance values, 0.41 and 0.37, respectively, with the latency period of 60-120 minutes.

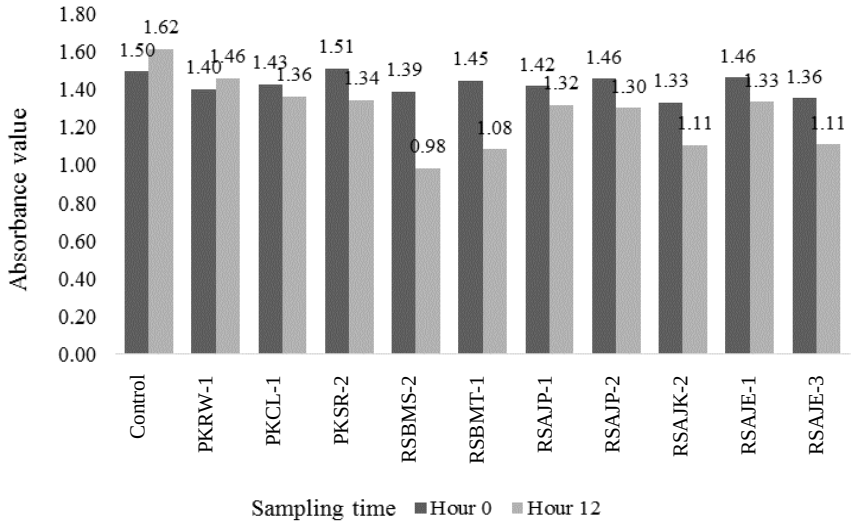

Figure 3. The absorbance value of bacterial culture after 12 hours incubation

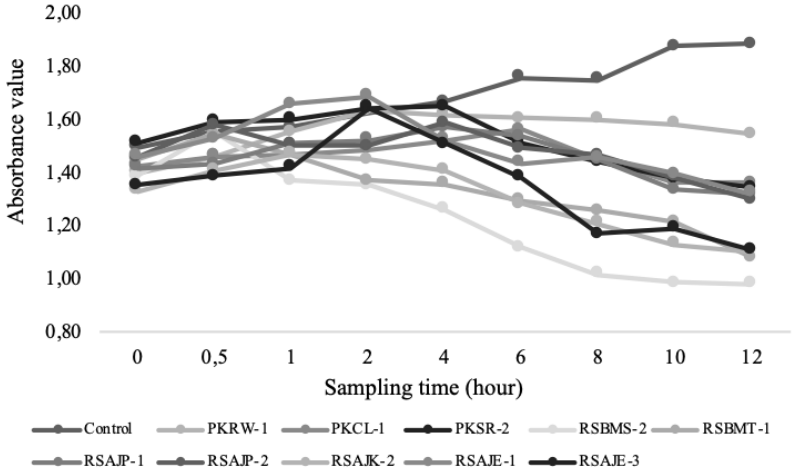

Figure 4. Adsorption rate determination of *Bacillus cereus* specific phages

ACKNOWLEDGEMENTS

The researchers extend their thanks to the Laboratory of Microbiology, Faculty of Biology, Universitas Jenderal Soedirman, Banyumas, Indonesia for funding support.

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