ISOLATION, CHARACTERIZATION, AND APPLICATION OF BACTERIOPHAGES AGAINST SEVERAL FOOD SPOILAGE BACTERIA: *Bacillus subtilis*, *Bacillus cereus*, AND *Shewanella Putrefaciens*Felicia Roseline Sujonoputri¹, Diana Elizabeth Waturangi^{1*}, Yogiara¹

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ABSTRACT

Introduction: Bacteriophages can be the natural alternative method to prevent food spoilage caused by bacteria. This research was conducted to isolate bacteriophages from a soil sample, retail food, and wastewater from fish and then the bacteriophages will be characterized for their activity against several food spoilage bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, and *Shewanella putrefaciens* and will be further investigated for application as food preservation.

Experimental: *B. cereus* (Atma Jaya culture collection), *B. subtilis* ATCC 6633, and *S. putrefaciens* ATCC 8071 were used for bacteriophage isolation. Food samples (rice, pasta, tofu, tempeh), soil samples (black soil and laterite), and wastewater sample of freshwater fish and seawater fish were used in this research. The overall process consists of eight steps: inoculum preparation, sample collection, bacteriophage isolation, purification and enrichment, titer determination, host range determination, the efficiency of plating (EOP), and bacteriophage application.

Results: A total of four bacteriophages were isolated with *B. cereus*, *B. subtilis*, and *S. putrefaciens* as host bacteria. Bacteriophage titers observed around 10^9 PFU mL⁻¹. Bacteriophages that isolated with *B. cereus* and *B. subtilis* as host bacteria tend to have high EOP with the same species bacteria. All the *Bacillus* phages (S1-BC, S2-BC, and S1-BS) can reduce the *Bacillus* species bacteria concentration for more than 90%.

Conclusion: Refers to their activity, the isolated bacteriophages in this study might have a great prospect to be used as food biocontrol and also can be further tested to make a phage cocktail.

Keywords: Agar overlay assay, Bacteriophages, *Bacillus cereus*, *Bacillus subtilis*, Food biocontrol, *Shewanella putrefaciens*

INTRODUCTION

Food spoilage is a metabolic process that causes changes in sensory characteristics, such as off-odor, textural changes, and visible growth of the colony in food. This spoilage is mediated by microbes that use food as their carbon and energy source. Some microbes are commonly found in many spoiled foods such as *Bacillus cereus*, *Bacillus subtilis*, and *Shewanella putrefaciens* (Rawat, 2015).

B. cereus has been recognized as a cause of food-borne disease and also known as a food spoilage agent. The proteolytic and lipolytic properties from these bacteria can affect the sensory characteristics from food for example sweet coagulation of milk and cream, ropy pastries (Jessberger et al., 2020). The prevalence of *B. subtilis* in food products related to food spoilage has received little attention and only limited information is available. However, several studies have showed that *B. subtilis* can produce enterotoxins and emetic toxins resulting in food contamination (Krasowska et al., 2015). *S. putrefaciens* can cause the spoilage in both fresh and packed fish leads to off-odors even at the low cell numbers of bacteria. *S. putrefaciens* can produce trimethylamine (TMA), hydrogen sulphide (H₂S), methyl mercaptan (CH₃SH), dimethyl sulphide ((CH₃)₂S), and other compounds resulting in intensive off-odors (Yang et al., 2019).

Food preservation is usually done by physical or chemical method. However, these methods have been known to reduce the sensory or organoleptic properties of the food products. Preservation using an excessive amount of antibiotic can increase the antibiotic resistance capability in bacteria which give a negative impact on human antibacterial treatment (Thapar and Garcha, 2017).

Bacteriophages as the natural alternative method to prevent food spoilage is very promising to be used. Several publications reported that bacteriophages have gained great attention as food preservation because they are viruses that can targeting bacteria specifically to disrupt bacterial metabolism and can cause bacteria to lyse without disrupting human, animal, or plant cells (Yang et al., 2019). It can be isolated from a wide range of foods, such as rice, fish products, meat products, dairy products, fermented foods, and from soil (Oh et al., 2017; Yang et al., 2019). For food preservation, bacteriophage must be lytic type and non-transducing as the minimum requirements for ensuring safety (FDA, 2006). Bacteriophage can be formed into phage cocktail which can be used to prevent bacteriophage-resistant bacteria mutants (Mirzaei and Nilsson, 2015).

This research aimed to isolate and characterize lytic bacteriophages for *B. cereus*, *B. subtilis*, and *S. putrefaciens* from a soil sample, retail food, and wastewater from fish and analyze their capability as food biocontrol.

MATERIAL AND METHODS

The overall process in this research consists of eight steps including: bacterial strains preparation, sample collection, bacteriophage isolation, purification and enrichment, titer determination, host range determination, efficiency of plating (EOP), and bacteriophage application. In this research, mostly all the steps were done using the agar overlay assay method (Adams, 1959).

Inoculum Preparation

B. cereus from Atma Jaya culture collections, *B. subtilis* ATCC 6633, and *S. putrefaciens* ATCC 8071 were used in this study. Bacterial cultures were inoculated onto Luria agar (LA) and incubated at 37°C and 28°C (for *S. putrefaciens*) overnight.

Sample Collection

Different types of food samples (rice, pasta, tofu, tempeh), black soil (S1), laterite soil (S2), and wastewater samples of freshwater fish (SF) and seawater fish (SW) were collected in Jakarta, Indonesia.

Bacteriophage Isolation for *B. cereus* and *B. subtilis*

The bacterial host strain was grown at 37°C overnight at 120 rpm. Six grams of sample and 300 µL of bacteria culture were added into 30 mL of Luria broth (LB) and then incubated at 37°C overnight at 150 rpm. The medium was centrifuged at 6300 x g for 15 minutes and filtered using a 0.2 µm syringe filter. The filtrate was centrifuged again at 6300 x g for 10 minutes to make sure the filtrate was clean and tested for the presence of bacteriophages using the agar overlay assay (Adams, 1959; Oh et al., 2017).

Bacteriophage Isolation for *S. putrefaciens*

The bacterial host strain was grown to the mid-log phase at 28°C overnight at 120 rpm. Then, 30 mL of each wastewater sample was centrifuged at 6300 x g for 10 minutes and then filtered using a 0.45 µm syringe filter. Subsequently, 5 mL of filtrate was mixed with 100 µL of host bacteria and added into 5 mL of 2X LB then incubated at 28°C overnight. The culture was centrifuged at 6300 x g for 10 minutes and filtered using a 0.2 µm syringe filter. After that, 10 µL of the filtrate was mixed with 100 µL of host bacteria and then mixed it in 3 mL of soft agar and poured onto LA plate according to the agar overlay assay (Adams, 1959).

Bacteriophage Purification and Enrichment

Phages were purified by removing a single plaque using a sterile tip onto the micropipette. The plaque was transferred into 500 µL of SM buffer (50 mM Tris-hydrochloride (Tris-HCl) [pH 7.5], 0.1 M NaCl, 8 mM magnesium sulphate heptahydrate (MgSO₄·7H₂O), and 0.01% (w/v) gelatine) and then vortexed. The plaque was then diluted with a 10-fold serial dilution (100 µL of phages: 900 µL of SM buffer). After that, 400 µL of bacteria culture was mixed with 100 µL of the respective phage dilutions. The phage-host mixture was incubated at 37°C and 28°C (for *S. putrefaciens*) to ensure phage adsorption to the host bacteria. Phages were plated according to the agar overlay assay (Adams, 1959; Gencay et al., 2016).

Bacteriophage Titer Determination

A series of 10-fold dilutions with SM buffer were made of bacteriophage lysate solution up to 10⁻⁸. 100 µL of diluted bacteriophage solution was mixed with 100 µL of mid-log phase bacteria culture in 3 mL of soft agar. The soft agar mixture was then poured onto the agar and incubated at 37°C and 28°C (for *S. putrefaciens*) overnight. The number of visible plaques was counted between 30 to 300 plaques and calculated as plaque-forming unit (PFU) mL⁻¹ (Gencay et al., 2016).

Host Range Determination

Two *Escherichia coli* pathotypes (EHEC and EPEC), *Vibrio cholerae*, *B. cereus*, and *B. subtilis* were tested for susceptibility towards isolated bacteriophages. Briefly, 100 µL of bacteriophage solution was mixed with 100 µL of tested bacteria in 3 mL of soft agar. The mixture was then poured on top of agar and incubated at 37°C and 28°C (for *S. putrefaciens*) overnight (Sufa et al., 2018).

Efficiency of Plating (EOP)

Bacteriophages that can lyse the tested bacteria in the host range determination method were continued to the EOP. The EOP was calculated by dividing the average PFU of target bacteria by the average PFU of host bacteria (Mirzaei and Nilsson, 2015).

Bacteriophage Application

Rice and pasta sample were sterilized for 15 minutes at 121°C. Tested bacteria (*B. cereus*, *B. subtilis*, *V. cholerae*) were inoculated 10⁴ colony-forming unit (CFU) cm⁻² onto the surface of the food surface followed by phage application at concentration 10⁹ PFU mL⁻¹ (S1-BC, S2-BC, and S1-BS). Samples were incubated at 25°C overnight. For the control, the same volume of SM buffer was used instead of phage (Hyman, 2019). Samples were suspended with 9 mL of SM buffer. A 10-fold serial dilution was made then spread onto the LA plate and incubated at 37°C overnight. The appropriate dilutions giving number colony between 30 to 300 colonies were expressed as CFU mL⁻¹ (Shin et al., 2011).

RESULTS AND DISCUSSION

RESULTS

Bacteriophage Isolation

Three bacteriophages could be isolated from two types of soil, which is black soil and laterite soil (S1-BC, S2-BC, and S1-BS). One bacteriophage can be isolated from the wastewater of seawater fish (SW-SP). All positive results were classified as lytic bacteriophages due to the clear zone plaques on top of agar.

Bacteriophage Titer Determination

Bacteriophage titers observed around 10⁹ PFU mL⁻¹. The highest titer of bacteriophage was from S2-BC isolate with a titer of 1.84 ± 0.17 x 10⁹ PFU mL⁻¹ (Tab 1).

Table 1 Titer determination of isolated bacteriophages

Bacteriophage Isolate	Titer (PFU mL ⁻¹)
S1-BC	1.51 ± 0.13 x 10 ⁹
S2-BC	1.84 ± 0.17 x 10 ⁹
S1-BS	1.33 ± 0.10 x 10 ⁹
SW-SP	1.78 ± 0.06 x 10 ⁹

S1= Soil 1 (Black soil); S2= Soil 2 (Laterite soil); SW= Seawater

BC= *B. cereus*; BS= *B. subtilis*; SP= *S. putrefaciens*

Host Range Determination and EOP

Bacteriophages isolated with *B. cereus* as host bacteria could lyse *B. subtilis*, and vice versa. All the bacteriophages couldn't lyse different species of bacteria such as EHEC, EPEC, and *V. cholerae*. The S1-BC, S2-BC, and S1-BS bacteriophage showed high infection effectivity with EOP more than 0.5. The plating on the original strain of isolation resulted in EOP = 1.0 (Tab 2).

Table 2 Bacteriophages efficiency of plating (EOP)

Bacteriophage Isolate	Efficiency of Plating				
	BC	BS	EHEC	EPEC	VC
S1-BC	1.0	2.14	-	-	-
S2-BC	1.0	1.89	-	-	-
S1-BS	0.93	1.0	-	-	-
SW-SP	-	-	-	-	-

Bacteriophage Application

Pasta and rice were artificially contaminated with host bacteria to calculate bacteria reduction if samples were added with bacteriophages. S1-BC, S2-BC, and S1-BS bacteriophages significantly reduced bacterial concentration in samples by more than 90%. However, no significant reduction was observed when samples with S1-BC, S2-BC, and S1-BS bacteriophages were tested against *V. cholerae*. (Tab 3).

DISCUSSION

In this study, phages that were isolated from soil samples can invade *B. cereus* and *B. subtilis*; also, bacteriophage that was isolated from wastewater of seawater fish can invade *S. putrefaciens* as their host bacteria. These isolated phages could be classified as lytic type phages based on the clear zone plaques that appeared on top of the agar (Sufa et al., 2018). However, no phages could be obtained from food samples (tofu, tempeh, pasta, rice) and also wastewater of freshwater fish. This might be due to the food samples used in this study are processed foods so the processing method can affect the presence of host bacteria and due to different environment and interaction of biotic and abiotic components (Oh et al., 2017; Thung et al., 2017).

Bacteriophage titers were observed around 10⁹ PFU mL⁻¹. Other studies also showed a similar result with bacteriophage titers observed >10⁸ PFU mL⁻¹ which can be used for further analysis of the bacteriophage (Shin et al., 2011). Bacteriophage titer determination is needed to prevent higher bacteria concentration than the phage concentration for further analysis such as bacteriophage application (Klasse, 2015).

Phages that were isolated in this study are specific so they couldn't attack other bacteria with different species. A host range that is limited to a single species is desirable because it prevents the phage from killing other species of bacteria and leaving the rest of the host's microbiome intact (Hyman, 2019). Each bacteriophage has different tail fibers with receptor binding protein (RBP) that can bind to specific receptor on the host bacteria surface. Each host bacteria cell wall also has different components and additional constraints (Hyman, 2019).

The EOP method was done to determine which phage was the most effective in killing other bacteria. In this study, all the phages tested for EOP (S1-BC, S2-BC, and S1-BS) were indicated to be highly effective with EOP ≥ 0.5. EOP between any phage pair may vary with different host bacteria, it can be affected by the host resistance system and the absorption of phage into host cells (Hyman, 2019). High results of EOP bacteriophage might be useful for further research to make a bacteriophage cocktail (Mirzaei and Nilsson, 2015).

Based on the results, all the *Bacillus* phages (S1-BC, S2-BC, S1-BS) reduced *B. cereus* and *B. subtilis* efficiently. The higher concentration of phages has a correlation with the efficiency of the bacteriophage application process to lyse targeted bacteria (Guenther et al., 2009). However, different results were found when the *Bacillus* phages were applied against *V. cholerae*. The reason why *V. cholerae* was added in this method because it is known that *V. cholerae* also can survive on cooked rice and pasta for up to 5 days and can multiply rapidly at ambient temperature (Maheshwari et al., 2011; Tang et al., 2013). This result was related to the host range determination result where phages can't lyse other species of bacteria. The reduction of bacteria can be affected by the food texture and matrix to absorb the phages' suspension and the distribution of phages in food samples (Fister et al., 2016). Phages' growth also depends on their ability to diffuse and contact with the host bacteria. The effectivity will lower due to

limited diffusion and contact with the host bacteria. This is also influenced by differences in receptors between phages and host bacteria (Hyman, 2019). This research discovers the effectiveness of bacteriophage against food spoilage bacteria and based on the results of this research, bacteriophages can be applied further and be beneficial for improving the quality of the products in the food industry. This research will help the researcher to uncover the critical areas of preventing food spoilage process that many researchers were not able to explore. Thus, a new theory on these phage application and phage cocktail combination may be arrived at. This research was only carried out until bacteriophage application with *Bacillus* phages, thereby authors recommend that further research needs to be conducted to determine the food application of *S. putrefaciens* phages, the minimum inhibitory multiplicity of infection, and also the morphology of isolated phages.

Table 3 Bacteriophages application on pasta and rice

Bacteriophage Isolate	Food Sample	Tested Bacteria	Control (CFU mL ⁻¹)	Bacteriophage treatment (CFU mL ⁻¹)	Bacteria reduction (log)	Bacteria reduction (%)
S1-BC	Pasta	<i>B. cereus</i>	3.60 x 10 ⁸	3.60 x 10 ⁵	3.00	99.90
		<i>B. subtilis</i>	4.50 x 10 ⁸	3.80 x 10 ⁵	2.98	99.89
		<i>V. cholerae</i>	3.10 x 10 ⁸	2.01 x 10 ⁸	0.19	35.16
	Rice	<i>B. cereus</i>	4.00 x 10 ⁸	2.10 x 10 ⁵	3.28	99.95
		<i>B. subtilis</i>	4.70 x 10 ⁸	3.80 x 10 ⁵	3.09	99.92
		<i>V. cholerae</i>	4.20 x 10 ⁸	2.35 x 10 ⁸	0.25	44.05
S2-BC	Pasta	<i>B. cereus</i>	3.60 x 10 ⁸	3.20 x 10 ⁵	3.05	99.91
		<i>B. subtilis</i>	4.50 x 10 ⁸	2.30 x 10 ⁵	3.19	99.94
		<i>V. cholerae</i>	3.10 x 10 ⁸	1.93 x 10 ⁸	0.20	37.74
	Rice	<i>B. cereus</i>	4.00 x 10 ⁸	3.50 x 10 ⁵	3.06	99.91
		<i>B. subtilis</i>	4.70 x 10 ⁸	2.40 x 10 ⁵	3.29	99.95
		<i>V. cholerae</i>	4.20 x 10 ⁸	2.71 x 10 ⁸	0.19	42.34
S1-BS	Pasta	<i>B. subtilis</i>	4.50 x 10 ⁸	2.00 x 10 ⁵	3.35	99.96
		<i>B. cereus</i>	3.60 x 10 ⁸	3.10 x 10 ⁵	3.06	99.91
		<i>V. cholerae</i>	3.10 x 10 ⁸	1.91 x 10 ⁸	0.21	38.39
	Rice	<i>B. subtilis</i>	4.70 x 10 ⁸	2.50 x 10 ⁵	3.27	99.94
		<i>B. cereus</i>	4.00 x 10 ⁸	3.30 x 10 ⁵	3.08	99.92
		<i>V. cholerae</i>	4.20 x 10 ⁸	2.87 x 10 ⁸	0.17	31.67

CONCLUSION

Four type of lytic phages were successfully isolated from black soil, laterite soil, and wastewater of seawater fish with *B. cereus*, *B. subtilis*, and *S. putrefaciens* as the host bacteria. All of the isolated phages showed high titer concentrations for more than 1 x 10⁹ PFU mL⁻¹. Phages isolated with *B. cereus* and *B. subtilis* as host bacteria tend to have high EOP and also can reduce the bacteria concentration effectively. All the isolated phages might have a great prospect to be used as food biocontrol and can be further tested to make phage cocktail.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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