Isolation of lytic bacteriophages and their potential to control \textit{Cronobacter spp.} - opportunistic food-borne pathogens

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Abstract. Bacteriophage could be an effective and safe method to decrease the presence of pathogens in food. In this study, lytic phages were isolated and examined for their efficacy to control pathogens. It was isolated using the "plaque assay" method with the indicator bacteria, \textit{Cronobacter spp.} and the lytic ability of isolated phage was confirmed using turbidity test. The isolated φKA5 from chicken skin samples was able to inhibit the growth of bacterial host (\textit{Cronobacter spp.}) specifically \textit{Cronobacter malonaticus} strain 05CHPL53. Infection of \textit{Cronobacter malonaticus} using φKA5 for 360 minutes showed the significant reduction of \textit{Cronobacter} growth. Based on morphological classification with the ICTV system, the φKA5 can be classified into the \textit{Myoviridae} family with a contractile tail. These data suggested that isolated phage from raw chicken skin are potential agents for controlling \textit{Cronobacter spp.}

1. Introduction

Billions of people in the world are at risk of unsafe food. Several of them die annually due to the consumption of contaminated food [1]. One of the pathogenic bacteria which infect food is the \textit{Cronobacter spp}. \textit{Cronobacter spp.} is a pathogenic bacterium which may cause neonatal meningitis, septicemia, and necrotic enterocolitis to infants with a 40-80% mortality rate. The transmission routes are contamination in Powdered Infant Formulas (PIF). Five hundred forty-four (544) cases of \textit{Cronobacter spp.} infection were identified from 2003 to 2009 among people, especially children below five years old, in the six states of the United States [2]. The most recent report occurred in October 2016, in which 156 cases of Foodborne Acute Gastroenteritis (AGE) attacked high school students due to \textit{Cronobacter sakazakii} [3]. In addition, other pathogenic bacteria, which also cause foodborne diseases in 2016-2017 were \textit{Salmonella}, fifty-five cases of \textit{Salmonella bovismorificans} happened in the Netherlands [4]. The control of pathogenic bacteria using antimicrobial agents such as antibiotics has experienced many obstacles, for example, their uncontrolled use leads to resistance [5] and Multi-Drug Resistance, (MDR) [6] which cause ineffective treatment of pathogenic bacteria by several antibiotics. Resistance to antibiotics has been widely spread to several pathogens such as \textit{Staphylococcus aureus, Escherichia coli, Campylobacter jejuni, and Pseudomonas aeruginosa} [7].

Bacteriophage is a virus which naturally attacks bacteria. Virulent phage may cause rapid lysis and death in the host bacterial cells [8]. Bacteriophages work precisely and efficiently against target bacteria. They are not pathogenic in humans, and their life is only as long as the life of the targeted bacteria [9]. In 2006, the FDA (US Food and Drug Administration) stated the safety of bacteriophage used in food, and it goes into GRAS (Generally Recognized as Safe) [7]. This study was conducted to
find lytic bacteriophage isolates and to know their potential to be a biocontrol of pathogenic bacteria *Cronobacter* spp, which cause foodborne illness.

2. Materials and Method

2.1. Samples

Soil samples (from Dinoyo), liquid waste of slaughterhouses and cow dung (from Gadang slaughterhouse), cow intestines and chicken intestines (from traditional market of Blimbing), and chicken skin (from traditional market of Merjosari, Malang) were used to isolate the bacteriophage and bacterial host.

2.2. Isolation and characterisation of bacterial host strains

The bacterial hosts were isolated by diluting the samples. The last three dilutions were poured on plates containing *Tryptic Soy Agar* (TSA) using the *spread plate* method. The plate containing the culture was incubated at 37°C for 24 hours. Colonies appearing on the plates were purified by three consecutive single colony passages. Susceptible strains were stocked in TSB (*Tryptic Soy Broth*) with 15% glycerol (v/v) at -20°C. Isolated bacterial strains were used as hosts for the detection of lytic bacteriophages from the same samples. Bacterial host strain was simply identified by Gram staining and KOH test. Bacterial hosts’ genomic DNA extraction, PCR-mediated amplification of 16S rDNA gene using universal bacterial primer sets and purification of PCR products was carried out to determine the type and characteristics of host.

2.3. Isolation of bacteriophage

Bacteriophages were isolated through a *plaque assay* method [10]. *Plaque assays* is one of the most accurate methods for the direct quantification of infectious virons and antiviral substances in cell culture monolayers under solid or semisolid overlay media. Approximately 1 g of sample was suspended in 9 ml TSB (*Tryptic Soy Broth*) in a sterile 15 ml centrifuge tube and mixed thoroughly on a shaker for 1 hour at room temperature. The sample was thereafter centrifuged at 10,000 r.p.m. for 15 minutes then the supernatant further filtered through a 0.20 μm pore size syringe filter (Millipore corp, Billerica, MA). The supernatant (5 ml) was added to equal amount of TSB (*Tryptic Soy Broth*) and inoculated with an early log-phase (0.1 ml) host culture. After overnight enrichment at 30°C with gentle shaking, the culture was centrifuged at 10,000 r.p.m. for 15 minutes. The supernatant obtained from the final enrichment step was filter sterilized through a 0.20 μm pore size syringe filter and checked for the presence of phages by the soft agar overlay method. The soft agar was prepared by adding 100 μl phage lysate to 300 μl of an overnight culture of indicator strain and mixed with 3 ml of liquid soft agar at 45°C. The mixture of soft agar was mixed thoroughly then spread over the hard agar and left until it has solidified and formed a double layer. The double layers were incubated at 37°C for 24 hours and checked for the presence of plaques.

2.4. Lytic ability analysis of bacteriophages

In order to determine the lytic ability of bacteriophage, two tubes containing 7 ml of TSB were prepared and 100 μl of 20-hour host culture were added in each tube. One tube was used for control (without phage lysate addition), while another test tube was added with 100 μl CaCl₂ 0.3M and 100 μl phage lysate. The test tubes were incubated at 37°C and observed every 30 minutes until clearing was observed when host cell infected by bacteriophages.

2.5. Host range analysis of bacteriophages

To evaluate the lytic spectrum of the obtained bacteriophages, some pathogenic bacterial strains were used. Double layer agar plates with different bacterial strains were prepared. One hundred microliters (100 μl) of pathogenic bacteria was added into two tubes containing 40 ml TSB media. As much as 300 μl CaCl₂ 0.3 M and 200 μl phage lysate was added. The test tubes were incubated at 37°C and
observed every 30 minutes for approximately 6 hours. Observed inhibition of cell growth as marked by clearing where the lysate was added.

3. Results and Discussion
3.1. Isolation of bacterial host strains and bacteriophages
Table 1 shows the results of host and bacteriophage isolation from several samples. The isolation by plaque assay indicated the plaque formation from slaughterhouse waste (L12, L24, L33, and L37), cow intestine (US8), and chicken skin (KA5 and KA6).

| Sample                  | Number of Host | Host Reference No. | Plaque Formation | Number of Plaque | Plaque Reference No. |
|-------------------------|----------------|-------------------|------------------|------------------|----------------------|
| Slaughter house liquid  | 30             | L                 | +                | 4                | L12; L24; L33; L37   |
| Soil                    | 5              | T                 | -                | -                | -                    |
| Cow dung                | 5              | KS                | -                | -                | -                    |
| Chicken Intestine       | 5              | UA                | -                | -                | -                    |
| Cow intestine           | 5              | US                | +                | 1                | US8                  |
| Chicken skin            | 5              | KA                | +                | 2                | KA5; KA6             |

Notes: L (Liquid waste); T (Soil); KS (Cow Dung); UA (Chicken Intestine); US (Cow intestine); KA (Chicken skin)

Some samples such as slaughterhouse waste (L12, L24, L33, and L37) and cow intestines (US8) did not show any lytic bacteriophage activity because they did not form plaques (data are not presented). However, lytic bacteriophage was found in chicken skin samples (KA5 and KA6) were incubated at 37°C for 24 hours as shown in Figure 1. Clear plaque was found in KA5, a clear area which looks quite contrast to the yellowish white field of the host culture. The obvious appearance of plaque in KA5 indicates that the obtained bacteriophage isolates were lytic bacteriophages. Whilst, in KA6 the plaque was cloudy, which shows lysogenic phage. Thus, the φKA5 isolate was chosen for the next stage of research.

![A](imageA.png)  ![B](imageB.png)

Figure 1. Plaque formation on (A) KA5 and (B) KA6 during the incubation at 37°C for 24 h
The appearance of plaque in the double layer shows the presence of phages. The clear plaque shows lytic phages while the cloudy one shows lysogenic phages [11].

3.2. Spectrum inhibition of bacteriophages to pathogenic bacteria
The phage lysates were infected to several target pathogenic bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Listeria monocytogenes* with the sample KA5. The result show that phages from φKA5 could not lyse all the target of pathogenic bacteria (data are not presented) except KA5 isolates (Figure 2).

![Figure 2. Lytic ability of φKA5 during incubation at 37°C](image)

Figure 2 shows that host KA5 was successfully lysed by φKA5 and the growth of KA5 was significantly decreased. In the 60 minutes, the host growth appears to decrease, which is indicated by the decrease of the OD value. In 360-minute incubation, the inhibition rate of growth of the target 1000x pathogen bacteria is lower than the treatment without bacteriophage infection. Bacteriophages are specifically related to their host cell, and they usually only infect a single bacterial species, or even specific strains in a particular species [12]. Monovalent phages can only attack one type of bacterial species [13]. The lysis ability of φKA5 can be used to inhibit and reduce the growth of KA5 host bacteria.

3.3. DNA sequencing of KA5 host bacteria
The lysis ability of φKA5 may inhibit and reduce the growth of KA5 host bacteria. However, the exact type of bacterium isolate in KA5 is not found. Therefore, the molecular identification of bacteria is also conducted to determine the specific type of host cell and phylogeny. The result shows that the sequence has a 97% similarity (data are not presented). The following table shows the results of molecular identification of the host bacteria in KA5.

| Host Bacterial Parameter | Unit | Result | Method                              |
|--------------------------|------|--------|-------------------------------------|
| Ribosomal 16S-rRNA       | -    | Cronobacter malonaticus strain 05CHPL53 | PCR (Polymerase Chain Reaction) Sequencing |

Table 2. Identification of host bacterial (KA5)

Notes: PCR-16S-rRNA sequence shows that the sample is identified as *Cronobacter malonaticus* strain 05CHPL53 with 97% similarity rate.
Table 2 shows that the bacterial isolate in KA5 is Cronobacter malonicicus strain 05CHPL53. Cronobacter spp is a pathogen currently classified as an opportunistic pathogen usually found in foods with low humidity, including that in infant formula milk [14]. It is one of the pathogenic bacteria which causes foodborne illness [15]. The success of φKA5 in lysing the host bacterial cell of KA5, one of the subspecies of Cronobacter spp. (Cronobacter malonicicus strain 05CHPL53) can be made as a biocontrol of the pathogen.

3.4. The morphological characterisation of bacteriophages
The morphological characterisation of bacteriophage aims to determine the classification of bacteriophages. They are classified based on ICTV rules in 13 different families. Figure 3 shows the visual appearance of bacteriophages using TEM.

![Figure 3. Transmission electron microscopy of Cronobacter phage (φKA5).](image)

The morphology in the bacteriophage isolates is in the head and tail. Bacteriophages with head and tail morphology are in the order of Caudovirales with three families from, Myoviridae, Siphoviridae, and Podoviridae. The family division is based on the differences in tail structure, Myoviridae with contractile tails, Siphoviridae with long non-contractile tails and Podoviridae with short non-contractile tails [16]. Table 3 shows the morphological classification of KA5 bacteriophages according to ICTV.

| Bacteriophage | Morphological Identification | Characteristics Based on ICTV |
|---------------|------------------------------|-----------------------------|
| φKA5          | Head and Tail                | Unwrapped Contractile Tail  |

| φKA5          | Unwrapped Contractile Tail  |

The morphological features in TEM visualization and when compared with the literature shows that φKA5 is probably a bacteriophage from the Myoviridae family. Myovirus is generally lytic, and it often has a broader host range than the other bacteriophages with tail although it infects different bacterial species [16]. However, to obtain more accurate classification, gene sequencing on φKA5 should be carried out.

4. Conclusions
One lytic bacteriophage specific to Cronobacter malonicicus was successfully isolated from the chicken skin samples (φKA5). However, φKA5 cannot infect several targeted bacteria such as Escherichia coli, Salmonella typhimurium, Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Shigella flexneri, Pseudomonas aeruginosa, Streptococcus pyogenes, and Listeria monocytogenes. Thus, the lytic bacteriophage of φKA5 can be potentially used as a biocontrol of pathogenic bacteria Cronobacter malonicicus.
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