Isolation and anti-\textit{Escherichia coli} biofilm activity of lytic bacteriophages isolated from water environment in vitro

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Abstract. Bacterial biofilms have emerged from contamination and infection problems in the medical, industrial and in the food and beverage processing. Bacteriophages are potential as biocontrol agents against bacterial biofilms as they specifically prevent or destroy the bacterial biofilm formation. The use of bacteriophages is safe from humans and animals as they only infect specific bacteria in specific pathways and have non-toxic properties to humans and animals. The objective of this study was to isolate and to know anti-\textit{Escherichia coli} biofilm activity of lytic bacteriophages from the water environment in vitro. Seven \textit{Escherichia coli} bacteriophages were isolated from different water sources. These bacteriophages \textit{E. coli} were analyzed to know its potential for prevention, inhibition or degradation against \textit{E. coli} biofilm in vitro. Data were analyzed using Minitab response surface. The results reveal that all seven \textit{E. coli} bacteriophages showed that they were potential to prevent, inhibit and degrade \textit{E. coli} biofilm in vitro. However, only \textit{E. coli} IV phage has the highest activity to prevent \textit{E. coli} biofilm formation by 40\% using filtrate dilution $10^{-3}$-$10^{-5}$ incubated for 10-28 hours. The highest biofilm inhibition activity was shown by \textit{E. coli} I phage at 40\% using filtrate dilution $10^{-2}$ and $10^{-4}$ incubated for 48-60 hours. Meanwhile, the highest biofilm degradation activity, 100\%, was achieved by \textit{E. coli} III phage and \textit{E. coli} IV phage using filtrate dilution $10^{-3}$-$10^{-5}$ and $10^{-2}$-$10^{-5}$ incubated for 10-15 hours and 10-31 hours, respectively. Further study is needed to reveal the bacteriophages identity and to optimize the number of bacteriophages particles which can be used efficiently and effectively to control \textit{E. coli} biofilm.

1. Introduction
Biofilm is a microbial community that consists of a pool of single or many populations that is attached to abiotic or biotic surfaces by extracellular polymeric substances [1]. The cells that form biofilms have different properties compared to planktonic cells, such as resistance increase to antimicrobial agents [2]. Recently, studies reported that the planktonic-biofilm transition is a complex and highly regulated process [3]. Biofilm formation is commonly considered as the cause of the antimicrobial agents fails and an assessed 65-80\% of all bacterial infections in humans are supposed to be associated with biofilms [4], but no available specific antimicrobials target to biofilms. Biofilm cells are more resistant between 10 and
1,000 times to conventional antibiotics compared to planktonic cells [5]. Some researchers have taken genetic and molecular approaches to study bacterial biofilms and genes that are important for the initial interaction of the cell surface, biofilm maturation and the return of biofilm microorganisms to the form of planktonic growth. This suggests that biofilm formation serves as a new model system for the study of microbial development [3].

*Escherichia coli* is useful for some scientists as a model to study the initiation of biofilm formation that depends on nutrients, motility and mannose [6] and as a model for studies of surface colonization. *E. coli* forms a biofilm as an attempt to self-defend. Physiologically, biofilms can increase the cost of stress, as an immunological defense, and as a protector of drugs as well as antibiotics [7].

Biofilms is a common problem in the food industry, the milk processing industry, fish, poultry, meat and ready-to-eat foods because they can resist to antimicrobial agents and cleansing [8] and drinking water systems [9]. Similarly with the rise of numerous drug-resistant bacteria, the use of bacteriophages has increased new interest as promising anti-microbial agents [10]. Lytic bacteriophages can deliver effective and natural interventions to reduce pathogenic bacteria in fresh commodities or new products [11].

The bacteriophage is a virus that infects specifically only to target bacteria and cannot infect humans, animals, or plants. Phage therapy has been used for about a century as an antimicrobial agent [12]. They are an intracellular obligate parasite that can replicate in bacterial cells by using several or all host cell as biosynthetic machines [13]. *E. coli* phages are a promising bio control agent against *E. coli* infection [10]. Several studies associated to bacteriophages have been accomplished. Carter *et al* [14] reported that bacteriophage reduced bacterial levels in contaminated beef by 94% and in lettuce by 87% after five minutes of contact time. Moreover, control of spoilage bacterial phage in cooled raw meat can result in a significant extension in storage period [15]. Phage cocktails also reduce biofilm formation and prevent the appearance of phage-resistant mutants that occur with a single phage.

Bacteriophages are increasingly being applied and considered for a variety of practical applications, ranging from food decontamination and human therapy. This study was aimed at selecting and enriching specific *E. coli* phages from various water source environment. Moreover, their anti-biofilm activity in preventing, inhibiting and degrading *E. coli* biofilms growth in vitro was assessed.

2. Materials and Methods

2.1. Sampling

Water sampling locations were well and river water in Ambon and Ternate areas, Maluku Islands, waters in Sumbawa area, West Nusa Tenggara, drinking water depots in Sentul, West Java and areas around Bogor Botanical Gardens, West Java, Indonesia.

2.2. Preparation of *E. coli* culture

*E. coli* cultures were originated from drinking water depots in Sentul, West Java and waters in Sumbawa, West Nusa Tenggara. Previously, *E. coli* that was purified by the quadrant method and incubated for 24 hours at 37°C to obtain a single colony that used as a test bacteria. Pure bacteria was planted in 50 ml LB broth 1X, and incubated overnight at 37°C in a shaker water bath.

2.3. Isolation of bacteriophage

Bacteriophage isolation was carried out after amplification procedure. Bacteriophage amplification was carried out with 40 ml of sample inserted into 5 ml LB broth 10x and 5 ml of overnight culture of *E. coli*, then incubated at 37°C for 24 hours. Ten ml of bacteriophages filtrate was pipetted for amplification and centrifuged at 2000 rpm for 5 minutes. The supernatant was filtered with 0.22 µm filter membrane (filtrate
1). *E. coli* phage amplification was carried out by taking as much as 4 ml of filtrate I then added with 1 ml of *E. coli* culture and 5 ml of LB broth 10X, incubated at 37 °C and shaker for 24 hours.

### 2.4. Storage of bacteriophages

The plaques seen in the scrubbing dish were then inserted into the Eppendorf tube which contained PBS solution and centrifuged at 3000 rpm for 10 minutes. The supernatant was taken and added with chloroform as much as 1 drop per 1 ml PBS then stored at 4°C.

### 2.5. Biofilm-forming bacteria formation and growth test

The test of the formation and growth of *E. coli* bacterial biofilms was carried out to find out how much time *E. coli* bacteria needed to form biofilms. *E. coli* biofilm growth test was carried out using the Microtiter Plate Biofilm Assay method. A total of 100 µL of *E. coli* suspension (OD 0.5) and 200 µL of liquid LB media were inserted into the microplate well, then incubated at 37°C for 24 hours, 48 hours and 72 hours. After incubation, measurement of OD (Optical Density) suspension was performed using a microplate reader. Measurements are carried out periodically according to the incubation period. After incubation, the microplate was washed 3 times by running water, dried afterward and added 200% of 1% crystal violet solution for 15 minutes. Subsequently, the microplate was rinsed again and dried, then added 200% ethanol of 200 µL and then left for 15 minutes. *E. coli* biofilm was measured based on optical density (OD) by using iMarkBiorad Microplate Reader at a wavelength of 595.5 nm. Biorem A and Biorem B which were enzyme compounds were used as a positive control solution.

### 2.6 Anti-biofilm activity test in vitro

#### 2.6.1. Activity to prevent biofilm growth

Biofilm formation was tested in-vitro using the 96 wells microtiter plate flat-bottom method of polystyrene. Tests were carried out on 7 bacteriophages with the various dilution level of 103, 105 and 107, with varying contact times. In this test, the negative control used was a microplate well containing bacterial suspension and LB media without the addition of bacteriophage, and the positive controls used were bio rem A and B. A total of 200 µL of bacteriophage was first included in each well, except for the well of the negative control and positive control and blank. After incubating for 60 minutes, the bacteriophage in the microplate well is removed and bacterial suspension and liquid LB media were added 100 µL each on microplate well samples and negative controls, then incubated for 24 hours, 48 hours and 72 hours at 37°C.

After that, the microplate was washed by using running water three times, then added 200 µL of crystal violet 0.02% to each microplate well and incubated at room temperature for 15 minutes. The microplate was washed again by using running water three times. A 200 µL of 96% ethanol was added to each microplate well and subsequently incubated at room temperature for 15 minutes. Each microplate well was read for OD by using the iMark-Biorad Microplate Reader tool at a wavelength of 595.5 nm.

#### 2.6.2. Biofilm growth inhibition activities

Tests were carried out on bacteriophages with various concentrations of 10³, 10⁵ and 10⁷. In this test bacteriophage (50µL) was added to each microplate well and simultaneously added with bacterial suspension and LB media (150µL). Negative control used is a microplate well containing bacterial suspension and liquid LB media without the addition of bacteriophage. In the inhibitory test, no positive control was used as a comparison. Then the microplate was incubated for 3 days (24 hours, 48 hours, and 72 hours) at 37°C. After the incubation period, the microplate is washed according to the treatment of the inhibitory activity above. Next, Optical Density (OD) is measured.
2.6.3. Biofilm degradation activity. This test was carried out for the prevention and inhibition biofilm growth. Negative control used was a microplate well containing bacterial suspension and liquid LB media without the addition of bacteriophage. After formation of biofilm at incubation period certain, the host bacteria were removed by washing three times by using running water for one hour. After that, the wells were added with 200 µL of bacteriophage culture and incubated for 24 hours at 37ºC. Each microplate was added with 200 µL of 1% crystal violet to each well and incubated at room temperature for 15 minutes. The microplate was washed according to the prevention and inhibition treatments. Optical density was measured for each treatment.

2.7. Data analysis
The quantitative data was in the absorbance value which as analyzed by averaging the percentage of OD in each biofilm. The data were analyzed using the Mini Tab17 (Response surface) software. The end result was a contour plot image.

3. Results and Discussion

3.1. Isolation of samples and biofilm formation
After isolation of *E. coli* from various water samples, 18 *E. coli* isolates was collected. *E. coli* code 23 was the best growth isolates. This isolate was originated from water sources in the Maluku Islands. This isolate was selected for bacteriophage assay in this study because this *E. coli* isolate 23 was a typical *E. coli* to fit with the requirement of this study based on the density of the biomass. The average value of optical density *E. coli* 23 was 0.329 after incubation in a shaker incubator at 37ºC for 4 hours. This OD value of *E. coli* growth was linear with the time of incubation or in the other word, the longer the incubation time, the greater the OD biofilm value. This showed that *E. coli* 23 isolate had stable biofilm growth.

Figure 1 shows that the average of the optical density of *E. coli* biofilm is 0.207 and the best time needed to form a biofilm is three days at 37ºC. *E. coli* is a common bacterium in the digestive tract of humans and animals. In some digestive tracts *E. coli* is harmless, but many are pathogenic, namely those that infect the digestive tract and outside the digestive tract, for example, *E. coli* which infects the urinary tract [16]. *E. coli* includes bacteria that can form biofilms. According to [7] the physiology of biofilms of these bacteria can increase tolerance to stress and for immunological defense.

![Figure 1](image-url)  
*Figure 1. E. coli* biofilm formation and growth through microtiter biofilm assay plate method.*


3.2. Isolation of bacteriophages

The results from bacteriophage isolation from various water sources in Indonesia showed that seven bacteriophage filtrates were isolated namely bacteriophage *E. coli* I (EC Rth Bogor), bacteriophage *E. coli* II (DAM Filtrate 2, Sentul), bacteriophage *E. coli* III (DAM 2 F2, Sentul), *E. coli* IV bacteriophage (filtrate 2, Bogor Botanical Garden), *E. coli* V bacteriophage (2G Sumbawa), *E. coli* bacteriophage VI (1M, Sumbawa) and *E. coli* VII bacteriophage (2M, Sumbawa). Confirmed results the presence of bacteriophages on *E. coli* grown on solid media is the formation of lysis on the distribution of host bacteria that look like plaque. Bitton [17] states that infection by bacteriophages in host bacteria results in the formation of plaques with size and morphology that vary depending on the type of bacteriophage. The occurrence of lysis can be produced by concentrating phage to a certain point. Worley-Morse *et al* [18] stated that lysis activity depends on bacteriophage concentration. It has been reported that lysis of bacterial hosts is the last event in the cycle of lytic bacteriophage infection [19]. Plaques scattered throughout the media surface was then stored at 4°C [20]. Figure 2 below shows the lysis zone in *E. coli* bacterial isolates.

![Figure 2. Bacteriophage plate.](image)

3.3. Optimization of the prevention activity of escherichia coli biofilm growth

The data analyzed at this stage was the density of *E. coli* biofilm at 595 nm wavelength. The optimization results of the activity test for the prevention of *E. coli* biofilm growth by *E. coli* bacteriophages are as follows:

Figure 3 shows the optimization of the test for the prevention of biofilm growth by *E. coli* bacteriophage. The results was obtained in the form of a Contour plot. Green zone shows bacteriophage can reduce biofilm growth. The more concentrated green, the higher the ability of bacteriophages to reduce biofilm growth, conversely if the blue color is more concentrated the bacteriophage will be lower in its ability to reduce biofilm growth and even in some cases cause the increase of biofilms. Figure 3 shows that *E. coli* bacteriophages I, III, IV and V can prevent biofilm growth, on average the prevention of biofilm growth occurs at $10^3$ dilution level, while bacteriophages II, VI and VII, cannot prevent biofilm growth, because the percentage of prevention ability of biofilm growth is 0% even the biofilm growth increases to more than 10%. The mechanism of prevention of biofilms formation is the bacteria which was attacked by bacteriophages was not able to produce extracellular polymeric substance...
(EPS) which it was needed for formation biofilms. Bacteria produces EPS to enable it to attach the surface and form biofilm.

Based on the contour plot, the best in preventing biofilm growth was *E. coli* IV phages originating from water sources in Bogor Botanical Gardens, with prevention activities of 40% at $10^3$-$10^5$ dilution level and time of incubation ranged from 10 to 28 hours. The difference among *E. coli* phages was caused by the number of phages of the different filtrates. In this test, the number of phages in the IV phage is more likely than other phages. The contact time of *E. coli* phage to prevent biofilm formation started after ten hour, but 30 hour for other phages.

**Figure 3.** Contour plot between contact times versus dilution factor concentration bacteriophage I-III (line 1), bacteriophage IV-VI (line 2) and bacteriophage VII (line 3).

Bacteriophage can penetrate bacterial biofilms. When it reaches the bacteria it will replicate to produce new phages that can cause the death of the bacteria. In addition to this preventative treatment in this study, bacteriophages have been previously inoculated before bacterial inoculation which it caused bacterial biofilm formation was prevented. Sharma *et al* [21] showed that the process of biofilm formation took several steps, namely (i) bacterial planktonic cells will move from liquid to the surface of solid objects, the cell attachment process is still temporary; (ii) early development of biofilm structures (irreversible); (iii)
maturation of biofilms and emitting chemical signals as a means of communication between bacterial cells; (iv) more and more biofilms formed and form a three-dimensional structure containing veiled cells in several groups that are connected to each other, then cell dispersion from the biofilm to return to the planktonic state. Furthermore, [22] said that the increasing number of phage titers and reduced resistance in biofilms showed that phages could replicate in susceptible cells in biofilms.

3.4. Optimization of the inhibition activity of escherichia coli biofilm growth

The next activity tested was the growth inhibition of \textit{E. coli} biofilm. In this activity, the negative control was \textit{E. coli} bacteria without the addition of \textit{E. coli} bacteriophage, whereas the positive control enzyme compounds were Biorem A and Biorem 10 which functioned as cleaning agents. In the inhibitory activity, bacterial phages and suspensions are added simultaneously, in contrast to prevention and degradation activities. The optimization results of \textit{E. coli} biofilm growth inhibition activity test by bacteriophage \textit{E. coli} was obtained as showed below.

![Contour plot between contact times versus dilution factor concentration bacteriophage I-III (line 1), bacteriophage IV-VI (line 2), and bacteriophage VII (line 3).](image-url)
3.5. Optimization of escherichia coli biofilm growth degradation activity

These results showed *E. coli* bacteriophages was able to degrade biofilm growth (Figure 5). The optimization results of the activity degradation test by *E. coli* phage against *E. coli* biofilm growth was showed in Figure 5. The result showed *E. coli* I, II, III and IV bacteriophages was able to inhibit biofilm growth. The best *E. coli* bacteriophage which it was able to inhibit biofilm growth is *E. coli* phage I. This phage at $10^2$ to $10^4$ dilution level with 48-60 hours incubation time an inhibit biofilm growth by 40%. However, *E. coli* II, III and IV bacteriophages at $10^2$ dilution with starting after 10 hours were able only by 20%. However *E. coli* phages V, VI, and VII was not able to inhibit biofilm growth with the percentage inhibitory ability is -40%, -20%, and -50%, respectively. In the contrary, at treatment by *E. coli* phages V, VI, and VII, biofilm growth increased by 40%, 20%, and 50% respectively. No inhibition capability of *E. coli* phage V, VI, and VII might be caused by communication occurred among the bacterial cells in biofilm where its formation took place that allowed bacteria to proliferate and censor the resulting infection.

![Contour Plot](image)

**Figure 5.** Contour plot between contact times versus dilution factor concentration bacteriophage I-III (line 1), bacteriophage IV-VI (line 2), and bacteriophage VII (line 3).
According to [23], bacterial cells in biofilms has capability to make quorum sensing enabling the bacteria to communicate with each other through chemical signals. When quorum sensing is active, the auto inducer is secreted by bacterial cells to the extracellular environment. The bacteria can increase the formation and maturation of the biofilm, when high density is reached. Moreover, [24] described that those bacteria are protected by an exopolymere matrix that binds to macromolecules and inhibits their diffusion into biofilms. Whereas bacteriophages are able to infect and proliferate in bacterial cells that grow as biofilms. According to [18], bacteriophage concentrations and environmental factors such as the temperature are essential for lysis actions and effect on many aspects of the biological system. Theoretically, inoculum containing bacterial cell concentrations less than $10^8-10^9$ CFU / ml will produce more effective MOI (Multiple of Infection), so that it can deliver a more significant reduction bacterial colonization and biofilm formation [25].

The result showed that all *E. coli* phages were able to degrade biofilm growth. The best bacteriophage that was able to reduce the biofilm growth was *E. coli* III phage and *E. coli* phage IV. The *E. coli* phages III activity at $10^{-3}$ to $10^{-5}$ dilution with a time of 10-15 hours was able to reduce the growth of the best biofilm by $> 100\%$. Furthermore $10^{-2}$ to $10^{-5}$ dilution with an incubation time of 10-37 hours can reduce biofilm growth by 80-120%. While *E. coli* IV phage showed better degradation of biofilm growth at $10^{-2}$ to $10^{-5}$ dilution and 80-100% after 10-37 hours. At $10^{-2}$ to $10^{-5}$ dilution and 10-50 hours incubation time, the phage IV was able to reduce biofilm growth by 40 -60%. This results indicated continuing infection by new phages. Continuous infection by new phages was produced continuously was able to infect new bacterial cells as long as they were able to support their replication [25].

Furthermore, *E. coli* bacteriophages I, II, V, VI, and VII were also able to degrade biofilm growth by 80% (*E. coli* I), 75% (*E. coli* VI and VII bacteriophages) and 50% (*E. coli* II and V bacteriophage). Brussow and Kutter [26] stated that most bacteriophages could not effectively infect bacteria in the stationary phase. According to [27], there were factors that determine phage therapy such as isolation, analysis, and identification of phage species besides purification levels for various applications and products preparation for phage therapy. Moreover, [28] revealed that host specifications of bacteriophages was determined by bacteriophages introduction as the "lock and key" theory of enzymes, that is receptors from host bacteria that have been identified by proteins of bacteriophages.

Bacteriophages can be isolated from various water sources and can then be used for phage therapy against infectious diseases. Infectious diseases caused by gram-negative bacteria, such as *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Vibrio vulnificus*, and *Salmonella spp.* and gram-positive bacteria such as *Enterococcus faecium* and *Staphylococcus aureus* [29].

### 4. Conclusion

The results showed that the seven *E. coli* phage isolated from various sources had ability to prevent, inhibit, and degrade biofilm growth. *E. coli* IV phage was the best phage in preventing biofilm growth with the 40% activity at $10^{-3}$-$10^{-5}$ dilution and incubated for 10-28 hours, whereas for biofilm inhibition shown by bacteriophage *E. coli* I 40% at $10^{-2}$ and $10^{-4}$ dilutions with an incubation period of 48-60 hours. The seven bacteriophages (I-VII) were able to degrade biofilm growth. The results of optimization of degradation activity reached 100% was showed by bacteriophages III and IV at dilutions $10^{-1}$-$10^{-5}$ and $10^{-2}$-$10^{-5}$ with incubation times of 10-15 hours and 10-31 hours respectively.

This research showed the various effectively and efficiency of prevention, inhibition and reduction of *E.coli* biofilm by variety of *E.coli* phages those were collected from water environment. In fact, for some reason such as a quorum sensing state acquired by *E.coli* made the viral activity was reduced or vanish.
5. References

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