เฟจจากธรรมชาติที่สามารถกำจัด *Pseudomonas aeruginosa*
Potency of Natural Phage in *Pseudomonas aeruginosa* Lysis

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บทคัดย่อ

เฟจ หรือ แบคเทอริโอเฟจเป็นศัตรูในธรรมชาติของแบคทีเรีย เฟจสามารถดำรงชีวิตได้โดยอาศัยแบคทีเรียเพื่อเพิ่มจำนวน ปัจจุบันแบคทีเรียก่อโรคหลายชนิดเกิดภาวะดื้อยาปฏิชีวนะ ทำให้เฟจเป็นอีกทางเลือกหนึ่งที่สามารถนำมาใช้กำจัดแบคทีเรียที่ดื้อยาปฏิชีวนะ *Pseudomonas aeruginosa* เป็นเชื้อแบคทีเรียที่มีรายงานการดื้อยาเนื่องจากสามารถสร้างไบโอฟิล์มซึ่งมีส่วนช่วยเสริมให้มีการดื้อยาได้มากกว่าแบคทีเรียชนิดอื่น งานวิจัยนี้ศึกษาการคัดแยกเฟจจากธรรมชาติที่สามารถทำลาย *P. aeruginosa* จากการศึกษารูปร่างของเฟจด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบว่าเฟจ PAMFUP2 จัดอยู่ในแฟมมิลี *Myoviridae* มีวงจรชีวิตแบบ lytic สามารถทำลาย *P. aeruginosa* โดยทำให้เกิดวงใส (clear zone lysis) และมีความจำเพาะกับ *P. aeruginosa* TISTR 1287 สามารถทำลาย *P. aeruginosa* TISTR 357 แต่ไม่สามารถทำลายแบคทีเรียสายพันธุ์อื่นที่ใช้ในการทดสอบ โดยเฟจ PAPMFU2 น้อยกว่า 20% สามารถทำลาย *P. aeruginosa* TISTR 1287 เมื่อบ่มในสารละลายที่มีค่า pH เท่ากับ 3 ที่อุณหภูมิ 70°C โดยคงความสามารถในการเข้าทำลายเมื่อสารละลายมีค่า pH อยู่ในช่วง 3-12 การเติมเฟจเพียงครั้งเดียวสามารถยับยั้งการเจริญของแบคทีเรียได้นาน 7 ชั่วโมง อีกทั้งเฟจ PAPMFU2 ยังสามารถยับยั้งการเกิดไบโอฟิล์มของ *P. aeruginosa* TISTR 1287 ได้อย่างมีนัยสำคัญ ($P < 0.01$) จากคุณสมบัติข้างต้นจึงสามารถนำเฟจจากธรรมชาติ PAMFUP2 มาใช้ในการกำจัด *P. aeruginosa* ได้ในอนาคต

คำสำคัญ: เฟจ, ไบโอฟิล์ม, *Pseudomonas aeruginosa*

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ABSTRACT

A phage, also known as a bacteriophage, is a bacteria's natural predator. Phages can sustain themselves by relying on bacteria to increase their numbers. Many pathogenic bacteria are now resistant to antibiotics. Therefore, phages can be used as an alternative method to kill antibiotic-resistant bacteria. *Pseudomonas aeruginosa* is an antibiotic-resistant bacterium because it can produce biofilm, which contributes to more resistance than other bacterial species. In this study, a natural phage capable to destroy *P. aeruginosa* was isolated. It was found that the shape of the PAMFUP2 lytic phage under a transmission electron microscope belonged to the *Myoviridae* family. It had a lytic life cycle that was able to destroy *P. aeruginosa* by causing a clear zone lysis. The phage PAMFUP2 was specific to *P. aeruginosa* TISTR 1287 strain in a specificity test. It was also able to inhibit *P. aeruginosa* TISTR 357, but not the other bacterial strains. The phage PAPMFU2 less than 20% could destroy *P. aeruginosa* TISTR 1287 when incubated in a solution with a pH value of 3 and a temperature of at 70°C. The stability of destructive capacity was maintained when the pH solution was in the range of 3-12. Additionally, a single PAMFUP2 phage could inhibit bacteria growth for up to 7 hours. Phage PAPMFU2 significantly ($P < 0.01$) inhibited biofilm formation of *P. aeruginosa* TISTR 1287. Based on the above characteristics, natural phage PAMFUP2 could be used to destroy *P. aeruginosa* in the future.

Key words: Phage, Biofilm, *Pseudomonas aeruginosa*

INTRODUCTION

The gram-negative bacterium *Pseudomonas aeruginosa* is commonly found in plants, soil, and water (Wu and Li, 2015). In hospitals, *P. aeruginosa* antibiotic resistance is recognized as a serious problem (Livermore, 2002) because it adapts to its surroundings (Hirsch and Tam, 2010). Consequently, using antibiotics to treat infections is problematic. Discovering an effective method to treat *P. aeruginosa* infection is critical for hospital care. *P. aeruginosa* multidrug resistance (MDR) is caused by a combination of mechanisms, including lactamases, aminoglycoside-modifying enzymes, target site modifications, and multi-drug efflux pumps (Santajit *et al*., 2011). Antibiotic classes that cause MDR include aminoglycosides, antipseudomonal penicillin, cephalosporins, carbapenems, and fluoroquinolones (Hirsch and Tam, 2010).

Bacteriophage or phage is a virus that infects bacterial cells (Ofir and Sorek, 2018). It can be found in almost any environment (Casto *et al*., 2016). Bacteriophages were first discovered in 1915 by William Twort (Clokie *et al*., 2011). In 1917, Felix d'Herelle realized that bacteriophage is specific and have potentiality to lyse bacterial cells (Clokie *et al*., 2011). The lytic cycle of a bacteriophage begins with infection when the phage attaches to the host's surface and inserts phage nucleic acid into the host cell. The phage genes will then be expressed, replicated, and packed to form phage particles in bacterial cells. At the end of the lytic cycle, the bacteria cell is lysed, and a new phage is released (Ofir and Sorek, 2018). It has been reported that MDR bacteria can be killed by phages (Lindberg, 1973). Phages have numerous advantages, including the ability to infect only specific bacterial cells (Loc-Carrillo and Abedon, 2011). Phage numbers are self-controlling as they can multiply when the target host bacterial strains are present and increase their numbers only at the infection site until the target bacteria are eliminated (Dufour *et al*., 2015). Therefore, bacteriophage therapy is a treatment option that has the potential to be a viable solution for antibiotic resistance (Rohde *et al*., 2018).

Phage has the potential to be a natural alternative method for lowering the number of *P. aeruginosa* infections in food (Hemalata *et al*., 2020). In 2013, Hungaro *et al.* reported

that phage has been shown to be more effective to reduce *Salmonella enterica* infection in chicken skin than chemical agents. They suggested that phage could be used as an alternative treatment agent instead of traditional synthetic preservatives in the food industry. According to Reyneke *et al.* (2020), they reported that bacteriophage isolated from rainwater can be used as a biocontrol as it can destroy and effectively regulate the reproduction of *P. aeruginosa* infection. Forti *et al.* (2018) successfully recovered 23 lytic phages from sewage samples on inhibition of 40 strains of *P. aeruginosa* from cystic fibrosis patients hosts. Similarly, Adnan *et al.* (2020) successfully isolated phage MA1 from Pakistan water resources. In this work, the basic features of the *P. aeruginosa* TISTR 1287 lytic phage isolated from natural sources were examined.

MATERIALS AND METHODS

1. Bacteriophage isolation

P. aeruginosa TISTR 1287 was obtained from Thailand Institute of Scientific and Technology Research (TISTR). It was grown on Luria Broth (LB) media (Himedia ®, India) for 16-20 hours at 37°C. It was used to employ as a host strain for phage isolation from soil sample collected from reforestation area at Mae Fah Luang University, Thailand (DMS Latitude: 20°02'81.0"N and DMS Longitude: 99°53'46.0"E). A total 50 grams of soil sample was suspended and properly mixed in 50 ml of water. The sample was centrifuged at 4500 rpm for 5 minutes at 4°C. The supernatant of phage was collected and filtered with a pore size of 0.22 µm membrane filter (Nest®, Korea). An 8.6 ml of supernatant was added to 100 µl of the host strain's overnight cell culture and 1 ml of 10x LB media (Himedia ®, India). The supernatant was shaken at 180-200 rpm for 24 hours at 37°C. Supernatant was centrifuged at 4500 rpm for 5 minutes at 4°C and filtered through a 0.22 µm membrane filter before being kept at 4°C. The cell lysis zone of isolated phage was assessed using a spot test.

Plaque assay (Dong *et al.*, 2018) was used to evaluate phage titer in test tubes by combining 5 ml of soft agar pre-incubated at 50 °C, phage filtrate, and bacterial culture (OD = 1.0 at 600 nm.). The phage-bacteria combination was placed on LB agar plates and allowed to dry for a while. The phage was purified by choosing a single clear plaque after 16 hours of incubation period at 37°C.

2. Phage amplification, purification, and stabilization

Phage was multiplied and precipitated with 2.5M NaCl and 20% PEG8000 (Bio Basic, Canada). A single phage plaque was combined with 4.8 mL of LB media containing 100 µl of overnight host culture. Then, it was incubated for 24 hours at 37°C with shaking at 180-200 rpm. Supernatant was centrifuged at 4500 rpm for 5 minutes at 4°C and filtered through a 0.22 µm Syringe Filter (Nest®, Korea). The single plaque was picked and amplified for 3 rounds. Then, phage supernatant was mixed with 1 ml of 2.5 M NaCl in 20% (w/v) PEG8000 and chilled on ice for 30-60 minutes before being centrifuged at 9000 rpm for 30 minutes at 4°C. The supernatant was removed and centrifuged at 9000 rpm for 5 minutes at 4°C. All remaining supernatant was discarded. A 200 µl of 1X Phosphate Buffered Saline (PBS) was used to resuspend the precipitated phage. The spot test technique was used to identify plaque forming units in the purified stock, and then the appropriate dilutions were plated. The stock was stored at -20°C in 20% glycerol.

The stability of the phage PAMFUP2 at different temperatures and pH levels was studied. A phage solution at 10^6 PFU/mL was incubated at 4, 20, 30, 40, 50, 60, 70, 80 and 100°C for 1 hour to determine their thermal stability. Using the double-layer overlay approach (Dong *et al.*, 2018), the phage titers were then calculated. The ratio of phage titers at different temperatures to those held at 4°C was used to calculate the relative titer. Three separated experiments were carried out. The stability of the phage PAMFUP2 at various pH levels was investigated.

A phage solution (10^6 PFU/mL) was incubated with Tryptic soy broth (TSB) media (Himedia ®, India) with pH 1.0 to 14.0. Using the double-layer overlay approach, the number of phages was counted after incubation. The relative titer was calculated by comparing phage titers at various pH to TSB medium without changing the pH. Three separated experiments were carried out in the study.

3. Transmission Electron Microscope (TEM) study

The phage suspension was applied to the formal carbon film surface with 200 mesh copper grids. In a negative staining procedure, phage was stained with 2% (w/v) uranyl acetate on a carbon covered grid. The grids were examined using a Hitachi transmission electron microscope (Model HT7700, Japan) and operated at 80 kV. The morphology of phages PAMFUP2 was observed using Transmission Electron Microscopy. The sizes of each phage's 10 viral particles were measured and averaged.

4. Analysis of the Host Range

Pure culture of 14 bacterial strains used to evaluated for phage PAMFUP2 specificity. There were five strains of *P. aeruginosa* (TISTR 1287, DMST 37186, TISTR 357, TISTR 781 and TISTR 1101), three strains of *Escherichia coli* (TG1, TISTR 527 and TISTR 780), two strains of *Bacillus cereus* (ATCC 11778 and TISTR 687), two strains of *Salmonella typhimurium* (TISTR 1470 and TISTR 2519), *Enterobacter aerogenes* TISTR 1540 and *Staphylococcus aureus* TISTR 746. Briefly, 1 µl of 10^6 PFU/mL phage suspensions was spotted onto bacterial lawns and incubated overnight at 37°C. The spot morphology was evaluated and classified as "+", a clear lysis zone; "T", a turbid lysis zone; and "-" no lysis. Three biological replicates were subjected to host range analysis utilizing the spot testing method.

5. Killing assay of bacteriophage

To evaluate lytic kinetics (Guo *et al*., 2019), phages with variable multiplicity of infection (MOI) 0.1, 1 and 10 were cultured with mid-log-phase of host strain *P. aeruginosa* TISTR 1287 culture (OD600 = 0.5) on 96-well culture plates at 37°C and 180 rpm. As a control assay, 100 µl of LB broth and 100 µl of *P. aeruginosa* TISTR 1287 (OD600 = 0.5) were employed in a 96 well culture plate. After incubation, the kinetic data were acquired by utilizing a microplate reader (SPECTROstar® Nano, U.S.A) to track the change in absorbance at 600 nm for 7.5 hours at 30-minute intervals.

6. Biofilm prevention assay

The ability of phage to inhibit biofilm formation was tested by mixing phage suspensions of 10^8 PFU/well with 150 µl aliquots of diluted *P. aeruginosa* 1287 culture in flat-bottomed polystyrene microtiter plate. The biofilms prevention test was performed with minimal modifications (Guo *et al*., 2019). Briefly, an overnight culture of *P. aeruginosa* 1287 was diluted 1:100 with fresh TSB medium and incubated at 37°C for 4, 8, and 24 hours. TSB without phage was used as a control. The formation of biofilms was then assessed using crystal violet-stained biomass. For crystal violet (CV) staining, the plates were rinsed three times with 0.9 % NaCl solution. The biofilms were dyed with 220 µl of 0.1 % CV solution for 10 minutes. They were washed three times with 0.9 % NaCl solution to remove the excessive CV, and dried in the air. To dissolve the bound CV, 220 µl of 30% acetic acid was applied. The eluted stain was transferred to a new microtiter plate and the absorbance at 590 nm was measured. Three separate experiments were carried out. The T-test was employed to differentiate between significant differences.

RESULTS AND DISCUSSION

1. Plaque of bacteriophages with *P. aeruginosa* TISTR 1287 as a host

The plaque characteristics of bacteriophages isolated from soil sample are shown in Figure 1. Single plaques had a round shape with a diameter of 3.51 ± 0.77 mm. (n = 17). Spot testing revealed a distinct cell lysis zone on the plaque, indicating the presence of an isolation phage specific to *P. aeruginosa* TISTR 1287 as the host. The

number of amplified phage using enrichment method increased the phage yield to 7.00×10^3 PFU/mL (data not shown). After 3 rounds of amplification and purification, isolated phage was named PAMFUP2. Bacteriophage can be found in almost any environment. A number of lytic phages against clinical *P. aeruginosa* ATCC PAO strain were isolated from sewage at Ilam University of Medical Sciences (Azizian *et al*., 2015). Phee *et al.* (2013) identified 80 phages from *P. aeruginosa* environmental and clinical sources. There are 2 phages named JBD4 and JBD44a isolated from *P. aeruginosa* ATCC15524 and *P. aeruginosa* ENV110BP, respectively. According to our findings, there is a reforestation area in which lytic phages could be isolated. The phage PAMFUP2 was successfully isolated from soil samples collected from reforestation area at Mae Fah Luang University's in Thailand using the enrichment method. This is the first time a *P. aeruginosa* TISTR 1287 lytic phage isolated from an uncontaminated environment has been reported.

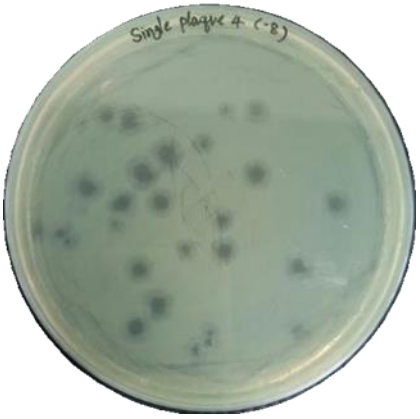

Figure 1 Single clear plaques of isolated phage PAMFUP2 after amplification.

2. Stability of phage PAMFUP2 under various temperature and pH

Thermal stability study shows that phage PAMFUP2 could survive at various temperatures (Figure 2). Phage PAMFUP2 showed high stability at 4 to 60°C temperatures. A few phage PAMFUP2 particles could survive at 70°C. However, no survival phage was observed when phages were incubated at 80 and 100°C temperatures. The phage PAMFUP2 has the same temperature tolerance range as other members of the same family. It can survive at temperatures ranging from 4 to 60°C, with fewer than 20% surviving at 70°C. In comparison, phage vB PaeM SCUT-S1 has a greater proportion of survivors at 70°C (Guo *et al*., 2019).

Infectivity of the phage PAMFUP2 maintained at pH 3.0 to 12.0 is shown in Figure 3. The phage PAMFUP2 had a poor infectivity when it was incubated at pH 3.0. Only about a quarter of the phages survive. From pH 5.0 to 11.0, it is fairly stable. There is a relative survival of more than 70%. There were no plaques found at pH 1.0, 2.0, 13.0 and 14.0, indicating that they were completely inactive. Phage PAMFUP2 is acid sensitive when compared to phage MA1 (Adnan *et al*., 2020), which can live at pH 3.0. This phage PAMFUP2 is basic and able to withstand pH = 12.0, whereas other phages, such as vB PaeM SCUT-S1 and vB PaeM SCUT-S2, demonstrated lower resistance to basic solution. They can withstand a maximum pH of 11.0 (Guo *et al*., 2019).

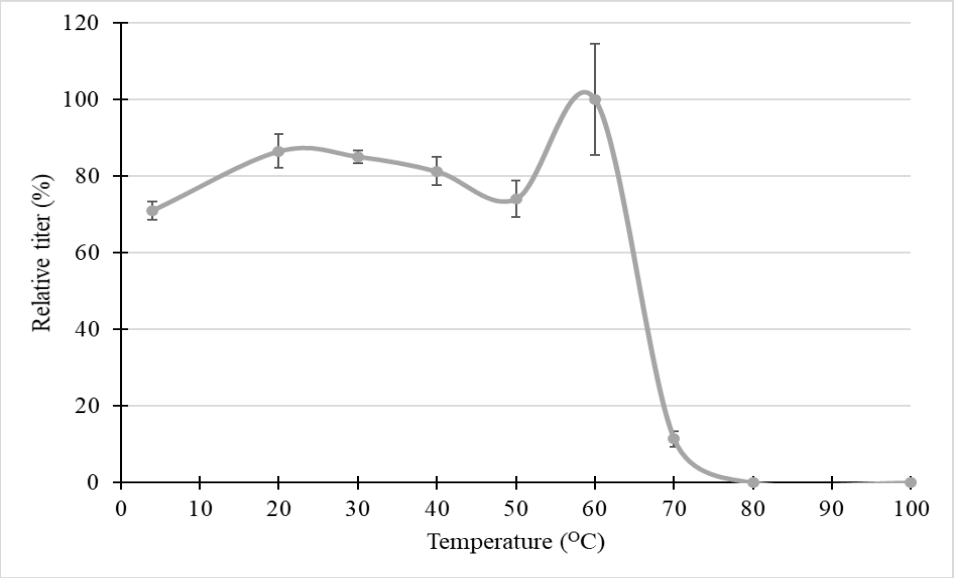

Figure 2 The relative number of infectious PAMFUP2 phage particles after incubating at 4, 20, 30, 40, 50, 60, 70, 80, 100°C temperatures for 1 hour.

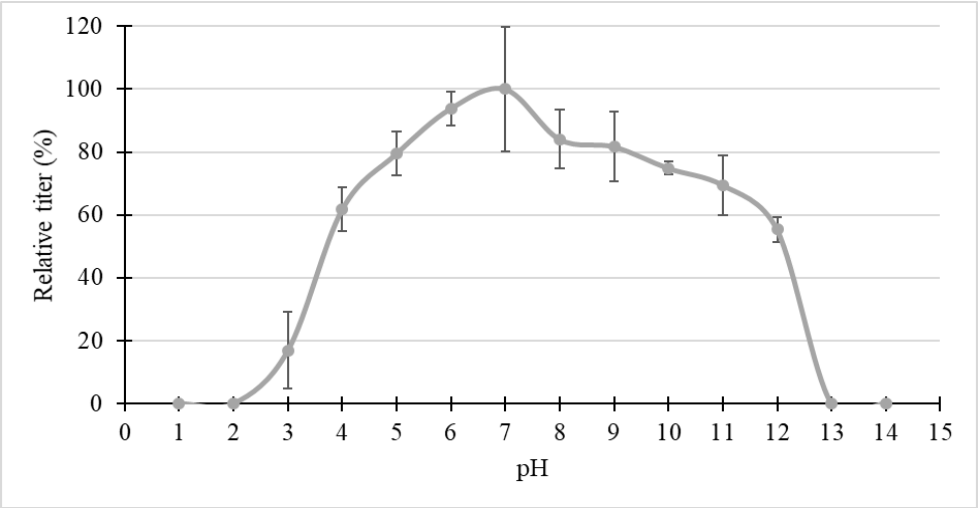

Figure 3 The relative number of infectious PAMFUP2 phage particles after incubating at various pH values (1.0 to 14.0) for 1 hour.

3. Morphology of phage

According to electron microscopic imaging in Figure 4, phages PAMFUP2 particle exhibited icosahedral heads and contractile tails, indicating that it belonged to the order *Caudovirales* and the *Myoviridae* family. Phage PAMFUP2 particle consisted of 57.75 ± 3.66 nm in width and 60.51 ± 2.74 nm in height of icosahedral head with a contractile tail of 124.00 ± 5.50 nm in length and 17.64 ±1.60 nm in width, respectively. Our report resembles that of Guo *et al.* (2019) using *P. aeruginosa* PA01 as a host, and 12 virulent phages were recovered from a water sample in Guangzhou, China. There are only 2 different phages named vB_PaeM_SCUT-S1 and vB_PaeM_SCUT-S2. These phage structure revealed from TEM photograph with icosahedral head with contractile tail. They are belong to *Myoviridae* family (Guo *et al*., 2019).

Olszak *et al.* (2015) reported 28 found phages using 18 strains of *P. aeruginosa* as hosts and there are 2 strongest lytic activities named PA5OCT and KT28. Phage PA5OCT morphological features studied revealed that

it belongs to order *Caudovirales* and the *Myoviridae* family. In addition, Forti *et al.* (2018) reported all 23 isolated phages belong to the order *Caudovirales* that can lyse *P. aeruginosa*. Adnan *et al.* (2020) isolated phage MA-1 from wastewater using *P. aeruginosa* 2949 as a host. Phage MA-1 structure belongs to *Myoviridae* family. This lytic phage produced clear plaques against susceptible bacteria having well defined boundaries. These plaques were ranging in diameter from 1.5 to 3.0 mm. The size of the clear cell lysis plaques generated by phage PAMFUP2 is comparable to that of MA-1. The phage PAMFUP2's morphology was found to be very distinct from that of other isolated phages. Phage PAMFUP2 has a smaller icosahedral head and a shorter tail size than phages PA5OCT (Olszak *et al*., 2015), E215, E217 (Forti *et al*., 2018), MA-1(Adnan *et al*., 2020), vB PaeM SCUT-S1 and vB PaeM SCUT-S2 (Guo *et al*., 2019), which are all members of the *Myoviridae* family and Order *Caudovirales*.

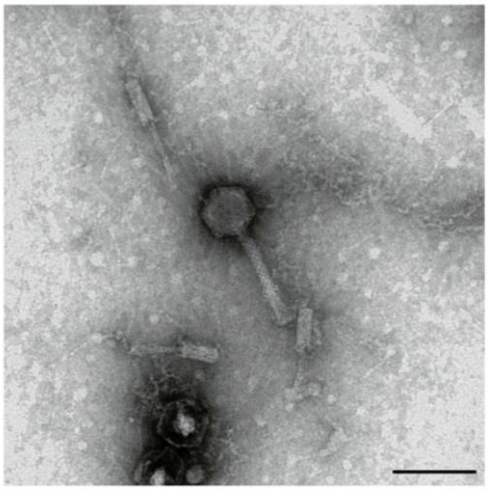

Figure 4 Image of negative stained phage PAMFUP2 particle using 2% (w/v) uranyl acetate. Phage particle was visualized under a Transmission Electron Microscopy operating at a voltage of 80 kV (scale bar = 100 nm).

4. The specificity of phage PAMFUP2

Phage PAMFUP2 only caused selective lysis on *P. aeruginosa* TISTR 1287 and *P. aeruginosa* TISTR 357 (Table 1). However, it could marginally lyse two strains of *P. aeruginosa* TISTR 37186 and *P. aeruginosa* TISTR 1101, whereas there was no activity observed against the other strains used in this study. According to these data, phage PAMFUP2 had a high-specific phage based on its host range. It has been reported that the other specificity of phages vB PaeM SCUT-S1 and vB PaeM SCUT-S2 showed selective lysis on *P. aeruginosa* strains ATCC 15442, ATCC 27853, PAO1, PALWL1.001, PALWL1.002 and PALWL1.003. Both phages did not display a distinct lysis zone with *Stenotrophomonas maltophilia* ATCC 51331 (Guo *et al*., 2019). Bacteriophage vB PaeM LS1 could infect all *P. aeruginosa* strains 0212, 0205, 1-1, 5-1-1, 5-2-1, DLB1 and DLG isolated from mink and *P. aeruginosa* strains PA01, DY-1, DY-2, DY-5, DY-6, DY-9, DY-10, DY-11, DY-12, DY-13, DY-14 and DY-15 isolated from human (Yuan *et al*., 2019). Adnan *et al*. (2020) reported that the MA-1 phage has a moderate *P. aeruginosa* host range (6/20), which makes it comparable to this result.

Table 1 Host range analysis of bacteriophage PAMFUP2 against different *P. aeruginosa* strains and other bacterial strain

Bacterial species	Strains	Specificity of Phage PAMFUP2
P. aeruginosa	TISTR 1287	+
	DMST 37186	T
	TISTR 357	+
	TISTR 781	-
	TISTR 1101	T
Escherichia coli	TG1	-
	TISTR 527	-
	TISTR 780	-
Bacillus cereus	ATCC 11778	-
	TISTR 687	-
Enterobacter aerogenes	TISTR 1540	-
Staphylococcus aureus	TISTR 746	-
Salmonella typhimurium	TISTR 1470	-
	TISTR 2519	-

Remark: "+" a clear lysis zone; "T" a turbid lysis zone; "-" no lysis.

5. Lysis kinetics

The growth curves of *P. aeruginosa* TISTR 1287 infected with various MOI phages PAMFUP2 are shown in Figure 5. The result showed that phage PAMFUP2 could control the number of *P. aeruginosa* TISTR 1287 in 60 minutes and continued to limit the growth of *P. aeruginosa* TISTR 1287 up to 450 minutes. Only single time phage addition could control *P. aeruginosa* TISTR 1287 up to 7 hours. The phage PAMFUP2 takes shorter time to suppress and reduce the number of bacterial host *P. aeruginosa* TISTR 1287 as compared to the phage vB PaeM SCUT-S1 and vB PaeM SCUT-S2 (Guo *et al*., 2019). They reported that these 2 phages inhibited bacterial host *P. aeruginosa* PA01 growth after 3.5 hours of incubation. Therefore, the host cell lysis becomes inefficient after 12 hours of culture.

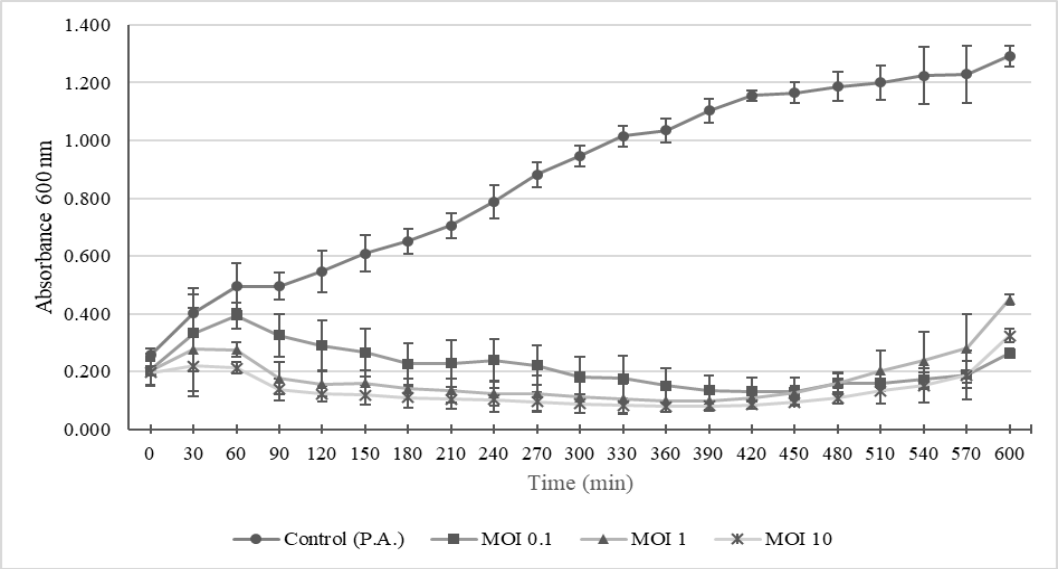

Figure 5 Lysis kinetic of phage PAMFUP2 against the growth of *P. aeruginosa* TISTR 1287. Bacterial growth was monitored at different multiplicity of infection (MOI) of 0.1, 1 and 10 for 10 hours. (P.A. is *P. aeruginosa* TISTR 1287)

6. Biofilm inhibition assay

Figure 6 shows the ability of phage PAMFUP2 to inhibit biofilm formation of *P. aeruginosa* TISTR 1287. Phage PAMFUP2 demonstrated significant limited biofilm formation at 8 ($P < 0.01$) and 24 hours ($P < 0.001$) incubation, whereas biofilm biomass was not significant at 4 hours incubation. Phage PAMFUP2 not only inhibited *P. aeruginosa* TISTR 1287 growth (Figure 5), but also inhibited biofilm formation. These finding results were similar to those of phages MA-1 (Adnan *et al*., 2020), vB PAeM LS1 (Yuan *et al*., 2019), JBD4, and JBD44a (Phee *et al*., 2013). In our results, the lysis kinetics discovered that the phage PAMFUP2 could kill *P. aeruginosa* TISTR 1287 for up to 7 hours without adding new phage particles (Figure 5). The phage PAMFUP2 had effectively suppressed planktonic cell growth for 7 hours of treatment and exhibited good results in preventing biofilm development (Figure 6 and 7).

Other in vitro tests reported that virulent phage vB PAeM LS1 which was isolated from local hospital sewage had significantly reduced biofilm formation of lung-infected *P. aeruginosa* DGL strain after 8 hours of treatment (Yuan *et al*., 2019). Moreover, phages JBD4 and JBD44a showed in vitro biofilm inhibition on *P. aeruginosa* strain UCBPP-PA14 (PA14) after 24 and 96 hours of treatment (Phee *et al*., 2013). Adnan *et al.* (2020) also reported that of phage MA-1 could inhibit *P. aeruginosa* 2949 growth and biofilm formation at 24, 48, and 74 hours. Similar to these finding, phage PAMFUP2 was able to inhibit biofilm formation after incubation of *P. aeruginosa* TISTR 1287 and phage PAMFUP2 for 24 hours (Figure 6). All of these characteristics suggest that phage PAMFUP2 could be an alternative treatment for eliminating *P. aeruginosa* TISTR 1287.

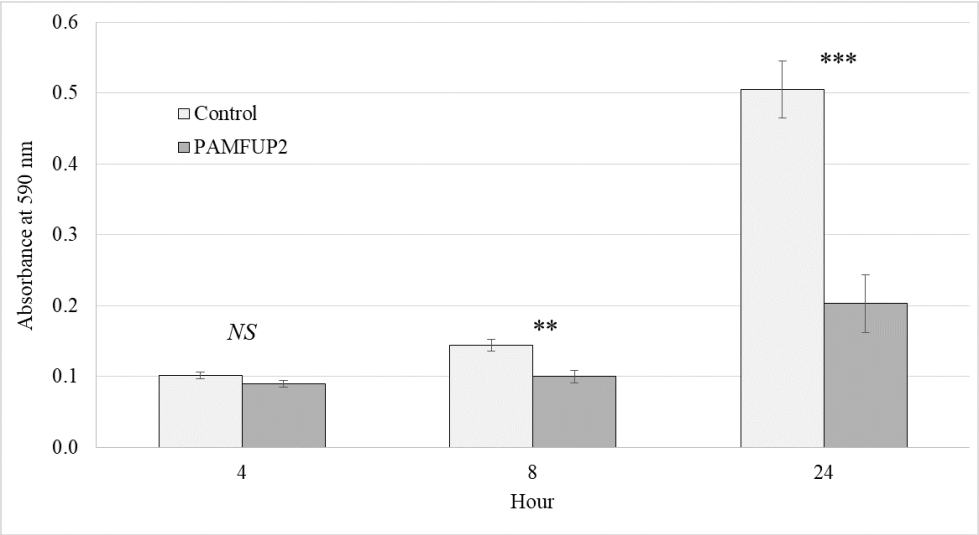

Figure 6 Effect of phage treatment on biofilms formation over 24 hours. Crystal violet staining is used to assess biomass. Lighter bars indicated *P. aeruginosa* TISTR 1287 (control) and dark bars showed co-culture of *P. aeruginosa* TISTR 1287 with 10^9 PFU/mL phage PAMFUP2. (NS = Non-significant difference, ** = Significant difference ($P < 0.01$), and *** = Significant difference ($P < 0.001$).

Figure 7 As a surrogate for biofilm growth, the absorbance (optical density) of dissolved crystal violet was measured at 590 nm. *P. aeruginosa* TISTR 1287 was compared to *P. aeruginosa* TISTR 1287 incubated with phage PAMFUP2 for 24 hours.

CONCLUSION

Phage PAMFUP2 had ability to inhibit biofilm formation of *P. aeruginosa* TISTR 1287 growth in vitro. The infectivity property could be maintained at pH 3 to 12 and 4 to 70 ^oC temperatures.

SUGGESTIONS

The lysis kinetic will be prolonged for a longer period. The phage cocktail should be investigated further to broaden the scope of phage treatment applications. In the hereafter, the capacity of the phage to kill additional clinical strains of *P. aeruginosa* will be studied. Furthermore, the genome of a phage that has been isolated will be studied.

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