

Isolation of bacteriophage from shrimp ponds in Soc Trang Province for inhibiting biofilm-forming *Micrococcus luteus*

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Abstract: The bacterial biofilm can support several pathogens to be resistant to antibiotics, fungicides, and parasiticides. Slime from shrimp ponds is the common habitat of some biofilm-forming bacteria and their natural enemies, especially bacteriophage. Therefore, this study aimed to isolate these biofilm-forming bacteria from the bottom slime of shrimp ponds and subsequently isolate several bacteriophage strains that were capable of infecting these bacteria. For the methods, the disadvantageous bacteria were collected from the samples and isolated through morphology, biochemical reactions, and molecular biological identification. The bacteriophage was simultaneously collected from the same slime samples and isolated through spot test and double agar overlay assay. Moreover, the bacterial biofilm detachment ability of the bacteriophage was evaluated through the optical density measurement of the bacterial biofilm with and without bacteriophage. As a result, the isolated biofilm-forming bacteria STM1 was both diplococci and tetrad, Gram-positive, non-motile, and yellow colony on the TSA medium. The catalase and oxidase activities of the STM1 were positive, and this strain did not ferment glucose and mannitol. Through morphological observations, biochemical reactions, and 16S rRNA gene analysis, STM1 was similar to *Micrococcus luteus* (PP495084.1) at 99.5 %. For the bacteriophage selection, four strains detaching bacterial biofilm were isolated at salinities between 5 and 20 ppt, wherein the phage pST9Δ2 strongly detached the STM1 biofilm (peaking at 82 % at 15ppt salinity).

Keywords: Bacteriophage, biofilm, *Micrococcus luteus*, Podoviridae, shrimp pond.

Classification numbers: 3.4.2, 3.7.2

1. INTRODUCTION

The genus *Micrococcus*, which are cocci, Gram-positive, are common in aquatic environments. In this genus, some species are probiotics while others become pathogens under stressful conditions [1]. Besides, there were several evidences that *Micrococcus* spp. was

implicated in the skin disease of some fish species. For instance, *Micrococcus luteus* causes scale loss, body ulcers, and hemorrhagic spots on the body of *Oreochromis niloticus* [2]. They also cause hyperpigmentation, skin lesions, and hemorrhagic manifestations in *Oncorhynchus mykiss* [3], and isolated *Micrococcus luteus* from *Cyprinus carpio* shows hemorrhagic lesions and body ulcers [4]. Although the pathogenicity of *Micrococcus* spp. in shrimp has not been reported yet, they can form biofilms [5–7] where various opportunistic bacterial pathogens can reside. They can adhere to the surface of the substrate to form microcolonies and quickly develop into indelible colonies. Since removing these plaques by physical methods is troublesome and capital-intensive, biological methods are in demand to control them effectively.

While other biological methods exploiting microorganisms cannot degrade the biofilm effectively, bacteriophage can penetrate and degrade biofilms by their lysine and depolymerase. Lysine exhibits amidase, lysozyme (muramidase), endopeptidase, or glucosaminidase activity [8], and it is an anti-biofilm agent at low concentrations [9]. The peptidoglycan hydrolases of lysine had antibacterial effects, which broke peptidoglycan bonds, degrading bacterial cell walls and shattering biofilm structures [10–12]. For depolymerase capability, they are divided into two groups based on mechanisms: hydrolases and lyases. Both groups focus on the degradation of polysaccharides, including capsule polysaccharides, lipopolysaccharides, O-polysaccharides, or exopolysaccharides generated in biofilms [13]. Therefore, this study aims to select potential bacteriophages that are inhibitory to the biofilm formation of *Micrococcus* spp. isolated from shrimp ponds.

2. MATERIALS AND METHODS

2.1. Samples collection

The bottom slime was collected at white-leg shrimp ponds in My Xuyen district, Soc Trang province, Viet Nam (1st pond: 9°27'42.4"N 105°56'11.4"E; 2nd pond: 9°27'20.4"N 105°55'52.2"E; 3rd pond: 9°32'01.5"N 105°58'09.3"E). In brief, three slime samples were collected from the bottom of each shrimp pond at 3 separate positions (the two ends and the middle), with 100 mL of each sample. Next, the three samples from the same shrimp pond were homogenized into a separate bottle. Then, samples were kept on ice and transported to the laboratory for subsequent isolation.

2.2. Isolation of biofilm-forming bacteria

After transferring to the laboratory, the bottom slime was quickly vortexed left undisturbed under static condition. After 30 min, 10 mL of supernatant was transferred to 90 mL of the tryptone soya broth (TSB - Himedia - India) medium and incubated at 120 rpm at room temperature (27°C ± 3) for 24 h to increase the bacteria population. After the bacterial enrichment, 100 µL of 10⁵-diluted sample was spread on the tryptone soya agar (TSA – Himedia - India) medium, and incubated at room temperature for a day. Finally, individual colonies with different morphological characteristics on the TSA medium were selected (Figure 1) based on the morphological descriptions by Prisinda *et al.* [14].

2.3 Ability of biofilms formation

In detail, bacterial suspension (20 μ L) and 180 μ L the TSB medium were mixed into the well (microplate), and incubated at room temperature under the static conditions. After 48 h, the culture medium was removed from the wells, and the stagnated biofilm was stained with 200 μ L of aqueous 1 % crystal violet solution (Xilong, China, CAS 548-62-9) for 15 min. Then, the excess dye was removed from the well, and distilled water was gently pipetted several times to wash the biofilm until the washed water was transparent. The biofilm was subsequently dried at room temperature. Then, 200 μ L of 95 % v/v ethanol was added to solubilize the biofilm for measuring the OD at the wavelength of 600 nm. The biofilm-forming capacity was positively correlated to the measured OD value (the larger the measured value was, the higher the amount of biofilm was produced). Then, strains with strong biofilm-forming ability were selected and stored in 40 % v/v glycerol (final concentration) at -20 $^{\circ}$ C. They were observed for colonies, cells (morphology, motility, and Gram stain), and biochemical characteristics (oxidase, catalase, glucose, and mannitol fermentation).

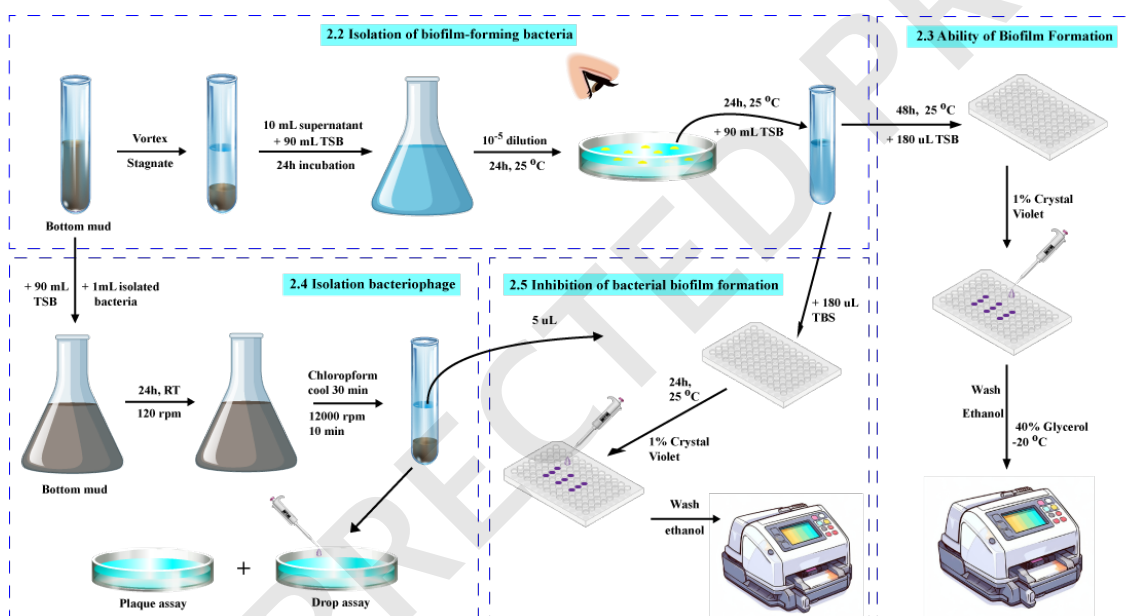


Figure 1. Illustrations of methods for isolating biofilm-forming bacteria, testing bacterial biofilm-forming ability, isolating bacteriophage, and testing their inhibition of bacterial biofilm formation.

2.4. Isolation of bacteriophage

The slime collected from the shrimp pond (10 mL) was added with 1 mL biofilm-forming bacteria (selected from the above experiment) before injected into 90 mL of the TSB medium. The mixture was then incubated on a shaker (120 rpm) at room temperature for 24 h to increase the phage population. Then, the culture solution was mixed with 1 % v/v chloroform solution, cooled for 30 minutes to kill bacterial cells, and centrifuged at 12,000 rpm for 10 minutes to collect the supernatant, which might contain phage. The spot method described by [15] and the double agar overlay assay described by [16] were used to determine the presence of phage and select phages, respectively. In brief, soft TSA medium (0.5 % agar) at a volume of 5 mL was initially overlay inoculated with 100 μ L host culture ($OD_{550} \sim 0.5$) before poured over the sterilized TSA medium (1.5 % agar) [15]. The ten-fold serial dilutions of the lysates of each

phage were spotted (10 µL) onto the overlay inoculated with the original host, dried, and incubated at 27-30 °C overnight. The bacteriophage forming visible plaques from the drop spots was subsequently selected for the double agar overlay assay.

Turning to the plaque assay Ács *et al.* [16], the bottom layer was prepared with sterilized TSA medium (1.5 % agar). The top agar layer contained soft TSA medium (0.5 % agar), which was initially mixed with the biofilm-forming bacteria and the selected bacteriophage before poured onto the bottom layer. After 24h of incubation at room temperature, the bacteriophage forming visible plaques on the agar plates was chosen for evaluating the inhibition of bacterial biofilm formation.

2.5. Inhibition of bacterial biofilm formation

The bacterial suspension (20 µL), 180 µL of TSB medium, and 5 µL phage were mixed into the well (microplate) and incubated at room temperature under static conditions. After 24 h, the culture medium was removed from the wells, and 200 µL of aqueous 1 % crystal violet solution was introduced for 15 min. Then, the excess dye solution was removed, and distilled water was gently pipetted to wash until the washed water became transparent. After naturally dried at room temperature, the wells were added 200 µL of 95 % ethanol to solubilize the biofilm before measuring the OD at a wavelength of 600 nm. The inhibition capacity is inversely proportional to the measured OD value. The lower the OD value was measured, the higher the inhibition capacity of the phage was. The inhibition capability of biofilm formation was suggested to evaluate as the following formulation:

$$IC (\%) = \left(\frac{OD_c - OD_s}{OD_c} \right) \times 100 \quad (1)$$

in which: IC: inhibition capability; OD_c: Optical density of control without phage under 600nm wavelength; OD_s: Optical density of sample treated with phage under 600nm wavelength.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of biofilm-forming bacteria

Three bacterial strains, namely STM1, STM2 and STM3, were successfully isolated from shrimp ponds in My Xuyen district, Soc Trang province. Figure 2A shows the grayscale image of the microplate after staining and their average optical densities measured at the wavelength of 600 nm (Figure 2B). Obviously, the wells containing STM1 were darker, and the average OD value was significantly the highest (1.18). Therefore, the bacteria STM1 was the strongest strain in biofilm formation.

The colony characteristics and cell morphology were compared with the results of Breed *et al.* [17]. They were small, yellowish, glistening, raised on agar medium, as shown in Figure 2C. The cell morphologies observed under the microscope with 1000 magnification were spheres, 1.0 - 1.2 µm in diameter, occurring in diplococci and tetrad, non-motile, and Gram-positive (Figure 2D).

According to the biochemical characteristics illustrated in Table 1, *M. luteus* showed morphological characteristics and biochemical activities similar to some published reports [2, 17]. Although the Bergey's Manual [17] described that *M. luteus* was negative for catalase and oxidase, Saleh *et al.* [2] reported that *M. luteus* could be positive for those.

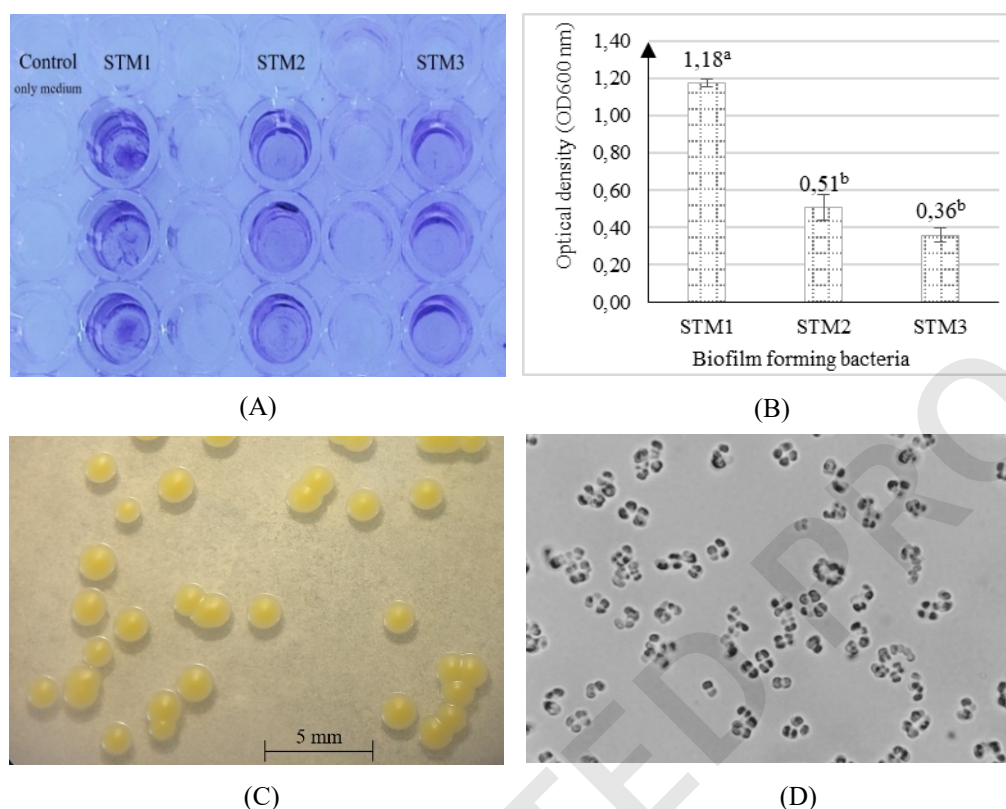


Figure 2. (A) Grayscale image of a microplate containing 3 isolated biofilm-forming bacteria after staining, (B) the average optical densities measured from these stained bacterial biofilms, (C) Morphology of *Micrococcus luteus* STM1 on TSA medium after 24h incubation, and (D) their cell morphology under the microscope at 100X magnification. In the chart, means followed by the same letter are not significantly different at the 5 % level using Tukey's test.

Table 1. Morphological and biochemical characteristics of *Micrococcus luteus* in this study compared with references.

Characteristics	STM1 in this study	<i>M. luteus</i> [18]	<i>M. luteus</i> [19]
Pigment	Yellow	Yellow	Yellow
Colony morphology	Circular, convex, entire	Small, spherical, smooth	Circular, convex, entire
Cell morphology	Tetra head coccus	Tetra head coccus	Tetra head coccus
Motility	Non-motile	Non-motile	Non-motile
Gram	Positive	Positive	-
Catalase	Positive	Positive	-
Oxidase	Positive	Positive	Negative
Glucose fermentation	Negative	Negative	Positive
Mannitol fermentation	Negative	Negative	Positive

Moreover, a comparison of 16S rRNA gene sequences showed that STM1 had 99.50 % similarity in sequence and biochemical characteristics (Figure 3) with *Micrococcus luteus* (the PP495084.1 sequence was registered in NCBI). Based on the BLAST output, thirteen 16S rRNA sequences, including 12 strains from *Micrococcus* genus and the outgroup *Renibacterium*

salmoninarum were collected for phylogeny construction by MEGA X [18]. There was a total of 790 positions in the final dataset. The phylogenetic tree was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The percentage of trees in which the associated taxa clustered together is shown below the branches. The strain STM1 16S rRNA sequence showed 99.5 % identity to *Micrococcus luteus*. From the phylogenetic tree, this strain was clustered with *Micrococcus luteus* strains due to the low value of genetic distance. The low intra-genetic diversity of *Micrococcus* species was also illustrated by Bala *et al.* [19] in the microbiota from wastewater. The *Micrococcus* genus is well-known as an opportunistic pathogen that attacks livestock when their immune system is weak. Besides the pathogenicity of *Micrococcus* spp., it can also create biofilms [20], which is a potential accommodation for other opportunistic pathogens.

DNA sequence of *M. luteus* STM1:

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GAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTC
TCTGGGCTGTAACGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGT
AGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCATTCCACGTTTCCGCGCCGCAGCTA
ACGCATTAAGTGCCCCGCTGGGGAGTACGGCCGCAAGGCTAAACTCAAAGGAATTGACGGGG
GCCCCGACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTT
GACATGTTCTCGATCGCCGTGGAGATACGTTTCCCTTTGGGGCGGGTTCACAGGTGGTGCATG
GTTGTCGTGAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCA
TGTTGCCAGCACGTCATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGA
GGACGACGTCAAATCATCATGCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACA
ATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCT
GCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATAC
GTTCCCGGGCCTTGACACACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCGAAGCCGG
TGGCCTAACCTTGTGGGGG
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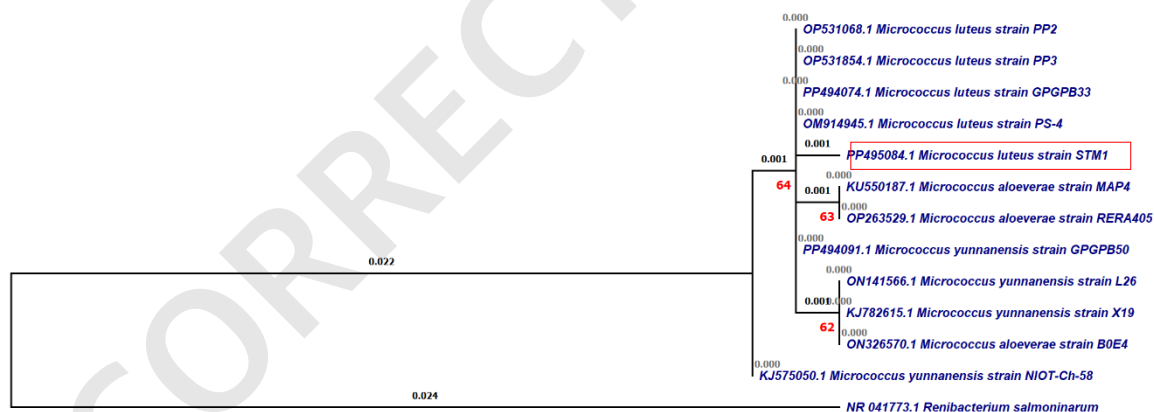


Figure 3. DNA sequence and phylogenetic tree of *M. luteus* STM1 based on 16S rRNA gene sequences.

3.2. Bacteriophage isolation

From the slime of a shrimp pond, four phages, which can degrade the biofilm of *Micrococcus luteus* STM1, were isolated. Figure 4 shows the results for evaluating the inhibition capacity in STM1 by the drop method and the plaque method.

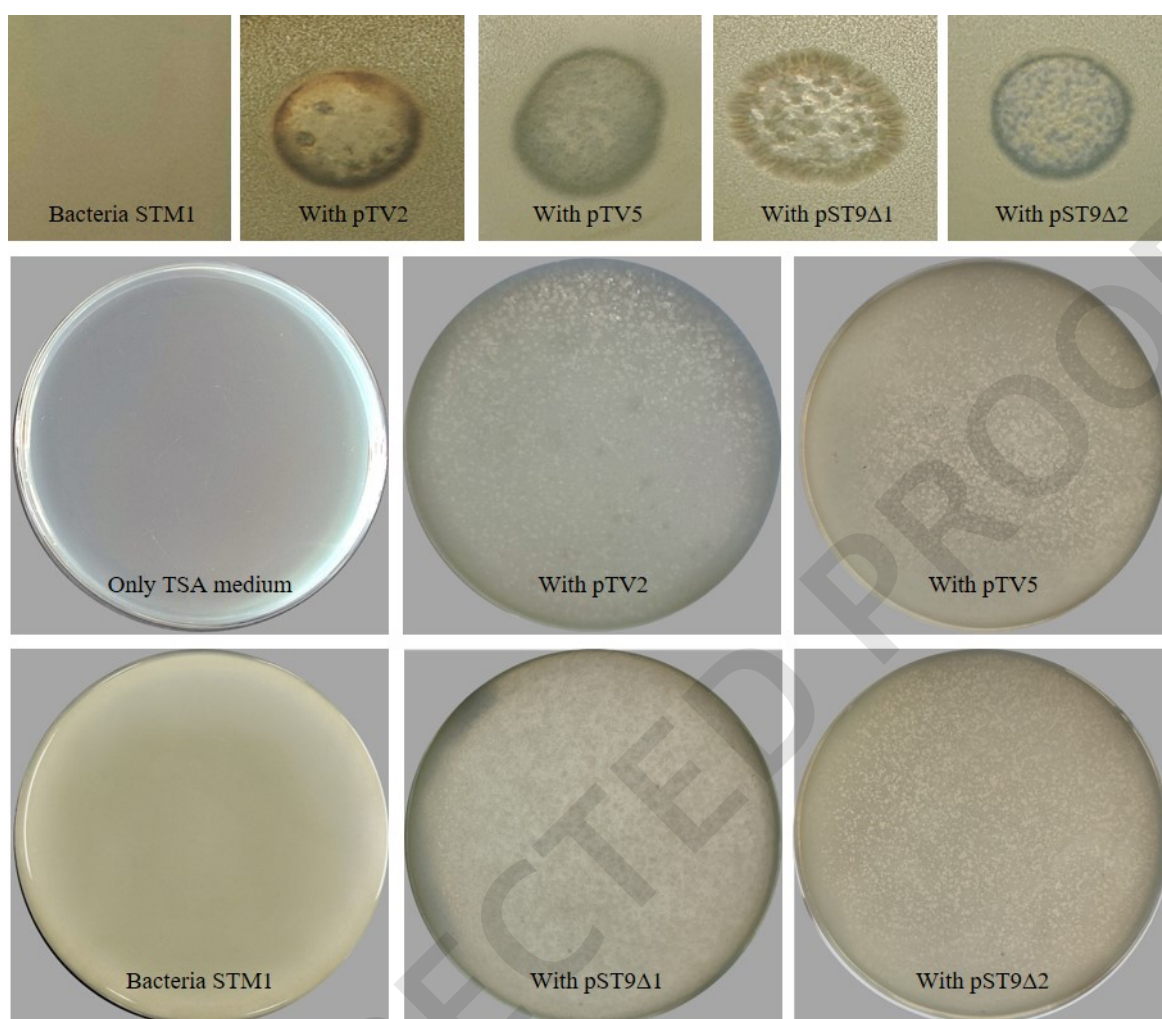


Figure 4. The inhibition ability of isolated phage strain to *Micrococcus luteus* STM1 by drop method and plaque assay.

For the plaque method, the infectivity of the four phages was shown by the morphology of the plaque, which was small and similar to the plaque of phage in *Micrococcus luteus* isolated from dairy cattle feces [5]. Since the plaque morphology of the phage pTV2 and pST9Δ1 was not clear, these two phage strains had little effect on the bacterial density of STM1. Phage pTV5 and pST9Δ2 showed clearer cell lysis ability than pTV2 and pST9Δ1, as the plaque was clearer and brighter. This inhibitory to control bacteria has been determined to be related to the enzyme lysine [12]. In brief, lysine disrupts peptidoglycan-specific bonds and has a strong bactericidal effect against susceptible bacteria.

3.3. Bacteriophages inhibit biofilm formation

STM1 bacteria can grow and form biofilms at salinities varied between 5 and 20 ppt, which was the optimized salinity range for whiteleg shrimp culturing. Table 2 shows the average OD values measured from stained STM1 biofilm after treated with 4 isolated phage strains. The more effectively the phage inhibited the growth of STM1 as well as their biofilm

formation, the lower the OD value was measured. Overall, all 4 phage strains could inhibit the biofilm formation of STM1 when the salinity is greater than or equal to 10 ppt. Moreover, the biofilm inhibition of four phage strains hit a peak at 15 ppt salinity before simultaneously declining at the salinity of 20 ppt.

Table 2. The OD value of *Micrococcus luteus* STM1 biofilm after being treated with 4 isolated bacteriophage strains.

Treatment	Salinity			
	5ppt	10ppt	15ppt	20ppt
Control	3.37 ^a ± 0.29	3.78 ^a ± 0.32	3.55 ^a ± 0.70	3.18 ^a ± 0.91
pTV2	2.61 ^{ab} ± 0.82	1.62 ^{bc} ± 0.25	1.12 ^c ± 0.37	1.72 ^b ± 0.37
pTV5	2.89 ^{ab} ± 1.01	1.98 ^{bc} ± 0.63	1.04 ^c ± 0.21	1.71 ^b ± 0.57
pST9Δ1	3.88 ^a ± 0.19	2.26 ^b ± 0.63	2.68 ^b ± 0.33	2.27 ^{ab} ± 0.49
pST9Δ2	1.64 ^b ± 0.85	1.32 ^c ± 0.40	0.63 ^c ± 0.09	1.55 ^b ± 0.41

Note: Each treatment was replicated three times. Means ± SD followed by the same letter is not significantly different at the 5% level in the same column using Tukey's test.

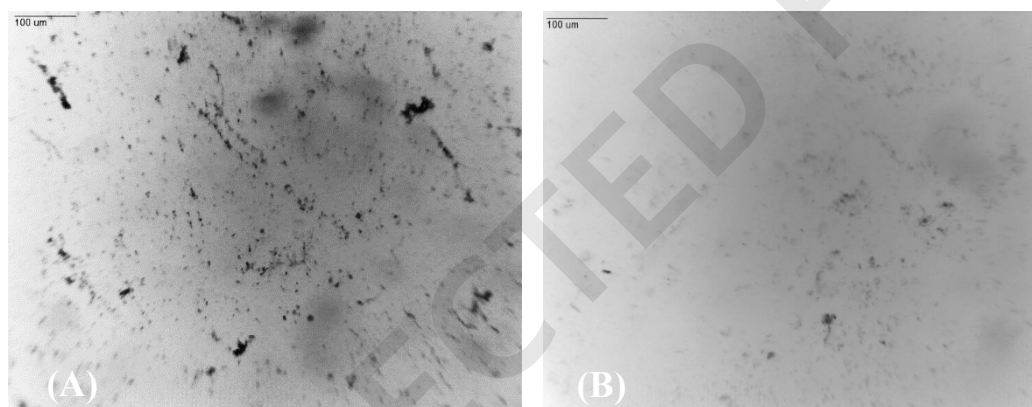


Figure 5. Optical microscopic images of STM1 biofilms at 15 ppt salinity at 1000 magnification (A) Control, (B) treated with pST9Δ2 phage.

Obviously, pST9Δ2 was a potential phage that showed the highest inhibition ability at all saline levels. In detail, at the salinity of 5 ppt, phage pST9Δ2 inhibited up to 51 % of bacterial biofilm, whereas the other strains did not show inhibitory ability (Table 2). At a salinity of 10 ppt, the proportion of biofilm inhibited by the phage pST9Δ2 was the highest (65 %). The ability of biofilm inhibition in pST9Δ2 was shown most effectively at 15 ppt salinity when it inhibited 82 % of the biofilm production (as shown in Figure 5). Although the proportion of biofilm inhibited by the phage pST9Δ2 decreased at the salinity of 20 ppt (51 %), it was still a more effective phage strain for inhibiting STM1 biofilm.

4. CONCLUSION

This study isolated 3 strains of biofilm-forming bacteria from the bottom slime of shrimp ponds, wherein *Micrococcus luteus* STM1 showed the highest capacity of biofilm formation. In addition, 4 bacteriophage strains that inhibit *Micrococcus luteus* STM1 biofilm formation were also isolated from the bottom slime of shrimp ponds. Among them, the bacteriophage pST9Δ2

showed the potential to eliminate up to 82 % of biofilm formation compared with the control treatment. However, the morphology and the identification of bacteriophage have not been studied yet. Therefore, the upcoming studies on pST9Δ2 should consider studying the morphological and genomic characteristics for its family identification.

CRedit authorship contribution statement. Tran Huu Hau: Methodology, Investigation, Formal analysis. Cao Nguyen Thuy Quynh: Investigation. Thach Thi Hoang Oanh: Investigation. Nguyen Pham Anh Thi: Methodology, Supervision. Truong Thi Bich Van: Methodology, Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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