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Xanthomonas sp. L019 INHIBITION BY BACTERIOPHAGE ISOLATED FROM WILD RICE (*Oryza rufipogon*)

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ABSTRACT

Bacteriophages are widely distributed in ecosystems, facilitating isolation. The study reported 16 phage strains capable of infecting *Xanthomonas* sp. L019. The host spectrum survey showed that phage strain B22 infected all 8 host bacterial strains; phage strains 13 and SR1 infected 6 bacterial strains, including *Xanthomonas* sp. (L19, 12280), *Erwinia carotovora* (TCDT3), and *Ralstonia* spp. (OT6, OT4, OT1); phage strains 21, PCR1, and TCDT7 infected 5 bacterial strains, including *Xanthomonas* sp. (L19, L20, 12280), *E. carotovora* (TCDT3), and *Ralstonia* sp. (OT6); and phage strains 2B2 and SR10 infected 4 bacterial strains, including *Xanthomonas* sp. (L19, 12280), *E. carotovora* (TCDT3), and *Ralstonia* sp. (OT6). The study of the lysis pattern of phage strains 13, 21, 3B and B22 showed the formation of clear lysis patterns, typical of lytic phage strains. The turbidimetry assay showed significant phage strain lysis, as indicated by reduced OD values. Investigation of the effect of phage on bacterial density showed that phages reduced it by approximately 1 log CFU/mL. The biofilm degradation test demonstrated significant control by all 4 phage strains against *Xanthomonas* sp. L019. Phage 13 exhibited the most optimal lytic ability across all 5 time points, reducing bacterial density to 5.88 log CFU/mL and biofilm OD to 0.24, compared to the control value of 0.6. These results highlight the potential of phages as alternative therapeutics, affirming their effectiveness in controlling pathogenic bacteria and contributing to green, sustainable agriculture.

Keywords: Biofilm, controlling pathogenic, sustainable agriculture, Vietnam.

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INTRODUCTION

Xanthomonas sp. is a Gram-negative bacterium that causes various severe diseases, such as leaf spot, leaf blight, and black spot, in economically important monocot and dicot crops worldwide. Notably, diseases caused by *Xanthomonas* sp. pose a significant threat to rice-growing regions. The genus *Oryza* holds agricultural importance, and the *Oryza rufipogon* species complex (ORSC), the wild progenitor of cultivated rice, is a valuable genetic resource for resistance to biotic and abiotic stresses, suitable for fundamental research (Büttner & Bonas, 2010). Due to its rapid spread, difficulty in treatment, and limited control, *Xanthomonas* sp. is a serious problem for agriculture worldwide. In addition, chemical control of some of these diseases causes negative impacts on health and the environment (Marin et al., 2019).

There is an urgent need for an alternative method to combat bacterial diseases in crops, and phage therapy is a promising emerging method (Goodridge, 2010). Bacteriophage therapy is attracting the attention of researchers as bacterial resistance to pesticides becomes widespread, with the potential for biological control against crop diseases. Phage therapy has shown positive results and can improve existing treatments in agriculture. The success of phage therapy depends on identifying effective phages that inhibit plant pathogenic bacteria to enhance sustainable agriculture worldwide (Villalpando-Aguilar et al., 2023). Tam et al. (2024) showed that bacteriophage BT56, applied at all densities, reduced the disease rate. Treatment with bacteriophage BT56 at 10^7 PFU/mL and 10^8 PFU/mL reduced disease rate and severity equally, and more effectively than treatment with 10^6 PFU/mL, 16 days post-infection, in controlling bacterial wilt caused by *Ralstonia solanacearum* on chrysanthemum under greenhouse conditions. Huy et al. (2016) studied the use of bacteriophages in controlling rice grain rot caused by *Burkholderia glumae*. Under greenhouse conditions, all six phage strains effectively reduced grain rot when a phage suspension at 10^8 PFU/mL density was sprayed two hours before artificial infection. Studies using three individual phages and a mixture of three phages demonstrated the potential of phages as a biocontrol strategy to control Welsh onion leaf blight caused by *Xanthomonas axonopodis* pv. *allii* (Nga et al., 2021). Bacteriophages showed similar efficacy to $CuSO_4$ in reducing disease incidence and significantly reducing the number of *Xanthomonas arboricola* pv. *juglandis* bacteria causing walnut leaf blight (Retamales et al., 2022). In addition, phages were combined with stimulants and chemical pesticides against leaf blight on green onions caused by *Xanthomonas* sp. (Vui et al., 2019).

For these reasons, in this study, we aimed to investigate the ability of bacteriophages to control *Xanthomonas* sp. and develop biological therapies for plant disease treatments. First, we isolated bacteriophage strains from rice rhizosphere soil samples and diseased rice samples, then evaluated the ability of the bacteriophage strains to control *Xanthomonas* sp. L019 using turbidity measurement and spread plate counting. In addition, we examined the ability of the bacteriophage strains to degrade *Xanthomonas* sp. L019 biofilms. The findings in this study will help explore the potential of bacteriophages as an alternative therapy in the treatment of plant pathogenic bacteria, contributing to the development of safe and environmentally friendly biological therapies.

MATERIALS AND METHODS

Bacteria cultures and preparations

The bacterial strains used in this study were obtained from the Advanced Virology Laboratory, Institute of Food and Biotechnology, Can Tho University. They included *Xanthomonas* sp. L20, *Xanthomonas* sp. L19, *Xanthomonas oryzae* VTCC 12280, *Erwinia carotovora* sp. carotovora TCDT3, *Ralstonia* sp. OT6, *Ralstonia* sp. OT4, *Ralstonia* sp. OT2, and *Ralstonia* sp. OT1. The bacterial strains were cultured in Nutrient Broth (NB, Himedia, India) and Nutrient Agar

(NA, Himedia, India) at 37 ^{o}C with shaking at 120 rpm for 24 hours.

The study used 6 bacteriophage strains provided by the Advanced Virology Laboratory, Institute of Food and Biotechnology. The strains were SR1, SR10, TCDT7, PCR1, BCM, and 3B.

Isolation and survey of bacteriophage plaque morphology

Bacteriophages were isolated from rice rhizosphere soil and rice samples in Can Tho city using the double-layer agar method (Kropinski et al., 2009a). Water, mud, and rice leaf samples were placed in the Nutrient medium and incubated at room temperature for 24 hours to enrich the original sample for bacteriophages. Then, the bacterial suspension was mixed with 1% chloroform, kept cold for 30 minutes, after which the solution was centrifuged at 12,000 rpm for 10 minutes at 4 ^{o}C to collect the clear supernatant as a crude bacteriophage solution (Wommack et al., 2009).

The plaque morphology was determined using the double-layer agar method (Kropinski et al., 2009b). To do this, 100 µL of phage suspension at an appropriate dilution was mixed with 100 µL of bacterial suspension in a 1.5 mL Eppendorf tube and incubated at room temperature for 15 minutes. The suspension mixture was then transferred to 0.5% NA agar medium, spread on a plate containing 1.5% NA agar medium. The plates were incubated, and the plaque morphology was observed after 24 hours.

Determination of host range

The host range of bacteriophages was determined by the double-layer agar surface drop method using the previously mentioned bacterial strains. Briefly, 3 µL of each bacteriophage strain suspension was dropped onto NA plates containing different bacterial strains. After overnight incubation, lysis spots were observed and classified as clear, turbid, or non-lysed, based on their clarity against the turbid bacterial background (Luzon-Hidalgo et al., 2021). The experiment was repeated three times to ensure reliability.

Lytic activity of phage

Investigation of bacteriophage lytic ability by the turbidimetric method

The lytic ability of bacteriophages was determined by OD measurement. 100 µL of bacterial culture was mixed with an equal volume of phage lysate (~1x10^{6} PFU/mL) (Alves et al., 2014). The mixture was incubated at room temperature. Turbidity (OD_{600}) was measured at 0, 2, 4, 6, and 24 hours. The experiment was repeated 3 times to ensure the reliability of the study.

Investigation of bacteriophage lytic ability by the spread counting method

The lytic ability of bacteriophages was performed by the spread counting method as described by Van et al. (2022) with some modifications. The bacterial suspension was grown in NB medium for 24 hours. 100 µL of bacteriophage suspension was added to 100 µL of bacterial suspension (10^{-6} dilution) in a 1.5 mL Eppendorf tube and mixed well. After 30 minutes of incubation, the entire mixture was spread on NA agar (the control was a bacterial suspension without bacteriophage addition). After 24 hours, the number of colonies formed on the agar plate was observed and recorded.

Biofilm inhibition assay

The biofilm removal ability of bacteriophages was assessed according to the method of (Gao et al., 2024; Korzeniowski et al., 2022) with some modifications. The bacterial suspension was grown in NB medium for 24 hours. 200 µL of the bacterial suspension was taken and placed in a 96-well plate, and incubated for 24 hours. After 24 hours, the wells of the 96-well plate were washed 3 times with 0.9% NaCl. 200 µL of bacteriophages was added to the plate and incubated for 4 hours (the control was the well without bacteriophages). After incubation, the solution was removed, and the wells were

washed 3 times with 0.9% NaCl 3 times. 200 µL of 0.1% crystal violet dye solution was added to the 96-well plate and left for 20 minutes. Subsequently, the dye solution was removed, and the wells were rinsed until the washing solution was colorless. Then, 200 µL of 30% acetic acid was added for 10 minutes to dissolve the biofilm, and the absorbance was determined using a plate reader at 600 nm.

Data analysis

Data were acquired and statistically processed using analysis of variance (ANOVA) to examine significant differences between treatments using Fisher's Least Significant Difference (LSD) and Tukey's Honestly Significant Difference (HSD) tests performed in the software MINITAB ver.16. Data were calculated and presented as mean ± standard deviation and graphed using Microsoft Excel 2016.

RESULTS AND DISCUSSION

Isolation and survey of bacteriophage plaque morphology

Ten bacteriophage strains capable of infecting *Xanthomonas* sp. L019 were isolated from rice rhizosphere soil and rice samples (Fig. 1) namely: 21, 33, 3B3, B22, 2B2, L03, L12, L20, and L24. Lytic spots appeared on the opaque bacterial background at 4 hours, with diameters ranging from 8 mm to 12 mm at the inoculation site. These spots remained clear for 24 hours, which is typical of lytic bacteriophages that kill host bacteria upon infection and multiplication, creating a clear, transparent area (Jurczak-Kurek et al., 2016). The morphology and size of the lytic spots created by bacteriophage strains differ due to variations in the infection method, as well as the time and growth rate of the bacteriophage (Ngoc et al., 2021). Because they formed clear, stable lytic spots faster than other strains (after only 3 hours), four phage strains (3B, B22, 21, and 13) were selected for further investigation of their morphological characteristics, bacterial lytic ability, and biofilm-lysing ability.

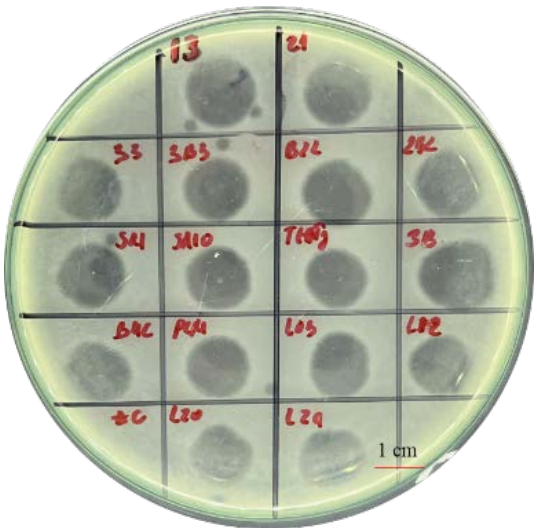

Figure 1. Infectivity ability of bacteriophage strains on *Xanthomonas* sp. L019. *Note:* The red symbols depicted in the image identify 16 phage clones, with the following designations: 13, 21, 33, 3B3, B22, 2B2, SR1, SR10, TCDT7, PCR1, 3B, BMC, L03, L12, L20, and L24

Morphological examination of the lytic spots showed that the four phage strains (3B, B22, 21, and 13) all produced lytic spots with a clear center and an outer halo on the *Xanthomonas* sp. L019 background, typical of lytic phages. The presence of halos, a transparent area around the lytic spots, indicates lysis of the host cell membrane (Fig. 2). This result is similar to that of phage vB_Pae575P-4, which produced haloed lysates and could lyse *Psendomonas aeruginosa* strains isolated from patients with cirrhosis (Jurczak-Kurek et al., 2016), and vB-EcoS-95, which formed transparent patches (diameter 2.5 ± 0.5 mm) with halos on *Escherichia* (Topka et al., 2019). The phages produced lysates ranging from 2 mm to 6.2 mm in diameter. Phage 13 produced the largest (approximately 6.2 mm), with a small center and a large halo, while phage 21 produced the smallest (2 mm) with a larger center and a halo. The remaining two phage strains, phage 3B and B22, showed quite similar morphological results. However, phage 3B had a larger diameter and clearer morphological appearance than phage B22.

Lysate diameter on the bacterial background was affected by incubation time, which influences lysate size, lysis efficiency, and phage concentration. Longer incubation times resulted in larger diameter lysates, and shorter times in smaller lysates. Conversely, phages with very short incubation times can infect other host cells sooner but have lower replication rates due to reduced burst size. Essentially, phages with the maximum growth rate (fitness) have an intermediate (optimal) incubation time (Shao & Wang, 2008).

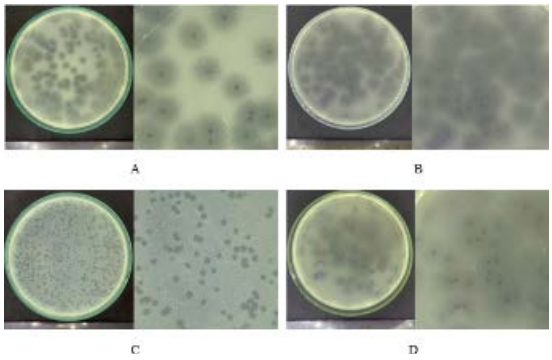

Figure 2. Plaque morphology of bacteriophage strains on host *Xanthomonas* sp. L019. (A) Phage 13. (B) Phage 3B. (C) Phage B22. (D) Phage 21

Host specificity

The host range of 16 phage strains was investigated by the double-layer agar drop method with eight host bacterial strains provided by the laboratory (Table 1). The results of Table 1 showed that phage strain B22 was capable of infecting all 8 bacterial strains. Phage strains 13 and SR1 were capable of infecting 6 bacterial strains, phage strains 21, PCR1 and $TCDT_7$ were capable of infecting 5 bacterial strains, and phage strains 2B2 and SR10 were capable of infecting 4 bacterial strains. The remaining phage strains had a limited ability to infect or were specific to *Xanthomonas* sp. L019. The isolated phage strains generally had a broad host range, exhibiting varying levels of infection (large or small, clear or opaque lysis spots), while some had specific hosts. According to (Dicks & Vermeulen, 2024), some phages could infect several strains within the same species or infect strains from many different bacterial genera. These phages were called multihost phages. Conversely, some phages had a narrow host range, limited to a single bacterial species or specific strains within a species (Abhilash et al., 2008).

Table 1. Host range of the 16 phages against eight bacterial strains

No.	Phage code	Sources	L019	12280	TCDT3	L020	OT6	OT4	OT2	OT1
1	13	Isolate	+++	++	-	+	++	-	++	+
2	21	Isolate	+++	++	++	+	++	-	-	-
3	33	Isolate	+++	-	-	-	-	-	-	+
4	3B3	Isolate	+++	-	-	-	-	-	-	-
5	B22	Isolate	+++	+	++	+	++	++	++	+
6	2B2	Isolate	+++	-	-	+	-	++	-	+
7	SR1	laboratory	+++	+	++	-	+	++	-	+
8	SR10	laboratory	+++	+	++	-	++	-	-	-
9	$TCDT_7$	laboratory	+++	+	++	+	++	-	-	-
10	PCR1	laboratory	+++	+	++	-	++	-	++	-
11	3B	laboratory	+++	-	-	+	++	-	-	-
12	BMC	laboratory	+++	-	-	-	-	-	-	-
13	L03	Isolate	+++	-	++	+	-	-	-	-
14	L12	Isolate	+++	-	-	-	-	-	-	-
15	L20	Isolate	+++	-	-	-	-	-	-	-
16	L24	Isolate	+++	-	-	-	-	-	-	-

Note: +++ main host; ++ clear; + turbid; - non-lysed.

Lytic activity of phage

Investigation of bacteriophage lytic ability by the turbidimetric method

The impact of bacteriophages on bacterial growth was monitored on 96-well plates by measuring the turbidity of the treatments at an optical density of 600 nm. This method evaluated the interaction of bacteriophages and bacteria at 0, 2, 4, 6, 8, and 24 hours (Fig. 3). For the four treatments with bacteriophages 3B, B22, 21, and 13, the OD value decreased at all time points. These results were statistically significant ($P < 0.05$). Bacteriophage 13 had the highest lytic ability, with the most significant reduction in OD observed at 6 and 8 hours. The remaining bacteriophages also showed significant lytic activity. A study of phage Sasha showed initial lytic activity against *Salmonella anatum*, followed by regrowth of both phage-resistant and phage-insensitive bacterial populations after 9 hours (Xie et al., 2018). When bacteria and phages were grown in the same well, host density initially increased, peaked during phage-mediated host cell lysis, and then decreased. The growth of lytic phages would necessarily reduce the density of bacteria. The turbidity measurements indicate that these phage strains, particularly phage strain 13, can significantly reduce bacterial density. A study of the growth curve of *Enterococcus faecalis* 271 bacteria showed that bacterial growth was rapidly inhibited by phage vB_EfaS-271 (Topka-Bielecka et al., 2021).

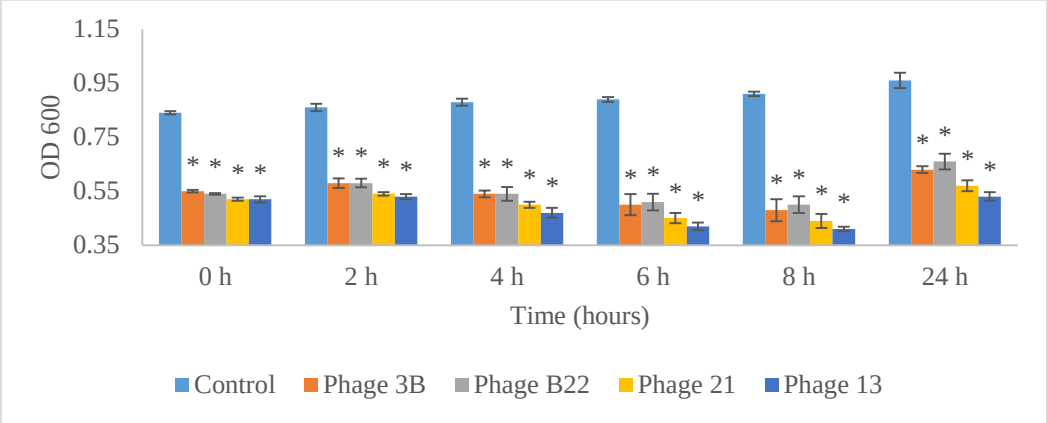

Figure 3. Lytic activity of four bacteriophage strains against *Xanthomonas* sp. L019 at five time points

Investigation of bacteriophage lytic ability by the spread counting method

To clarify the impact of phage strains on the density and colony phenotype of *Xanthomonas* sp. L019, a study on the lytic ability of phage strains by the spread counting method, was conducted. The results are shown in Figures 4 and 5. Statistical analysis confirmed that all four phage strains were able to lyse *Xanthomonas* sp. L019, with phage 13 demonstrating the highest lytic activity, consistent with results from the turbidimetric assay. Compared to the control, phage 13 treatment reduced bacterial density from 7.72 log CFU/mL to 5.88 log CFU/mL. Similarly, bacterial density decreased with phages 21, B22, and 3B, to 6.37, 6.57, and 6.74 log CFU/mL, respectively. Several other studies have reported similar reductions in bacterial density. For example, bacteriophages reduced *Vibrio parahaemolyticus* by 4 log CFU/mL in 16 hours (Dechamma et al., 2022) and completely inhibited 107 CFU/mL of *Klebsiella pneumoniae* 0915 in 6 hours (Peng et al., 2023). These studies confirm the strong control ability of bacteriophages against pathogenic bacteria.

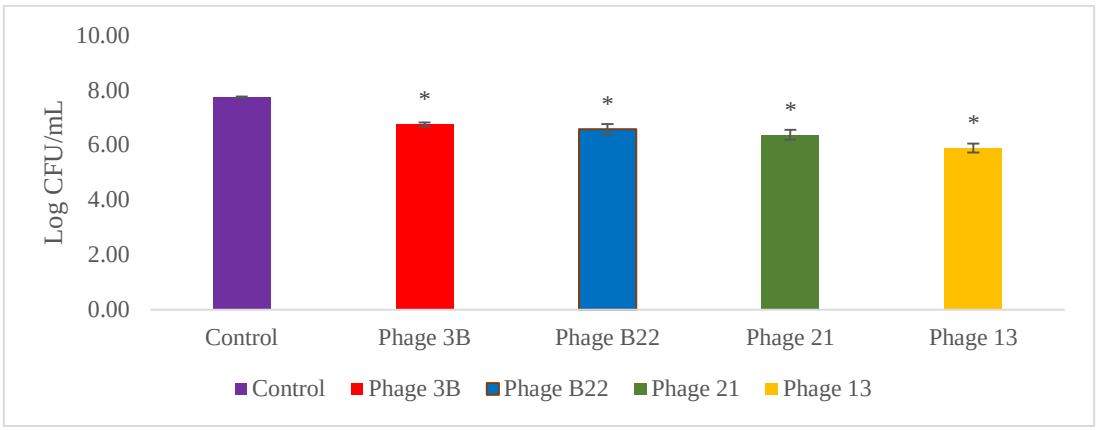

Figure 4. Lytic activity of four bacteriophage strains in reducing *Xanthomonas* sp. L019 density

Biofilm inhibition assay

The biofilm of *Xanthomonas* sp. L019 was formed in a 96-well plate for 24 hours and then treated with bacteriophages for 4 hours (Fig. 5). Based on OD values, bacteriophages 13 and 21 significantly reduced the *Xanthomonas* sp. L019 biofilm, with OD values of 0.24 and 0.27, respectively, compared to the initial biofilm formation value of 0.6. Bacteriophage 13 showed the optimal efficiency in removing biofilm compared to other bacteriophage strains. Bacteriophage strains B22 and 3B also reduced biofilm, with OD values of 0.3 and 0.36, respectively. The removal of bacterial populations in treated biofilms showed that bacteriophages were able to destroy biofilms by degrading EPS and reducing the number of viable cells (Goodarzi et al., 2021). Many studies have reported the biofilm-degrading ability of bacteriophages, such as bacteriophage ParuNE1, removing 60% of *Pseudomonas aeruginosa* CLI 22 biofilm (Enwuru et al., 2021), bacteriophage HZY2308 degrading 84.91% of bacterial biofilm after 24 hours of treatment (Wang et al., 2024), and bacteriophage mixtures degrading *Salmonella typhimurium* and *Salmonella enteritidis* biofilms by 44% and 63%, respectively, after treatment (Islam et al., 2019).

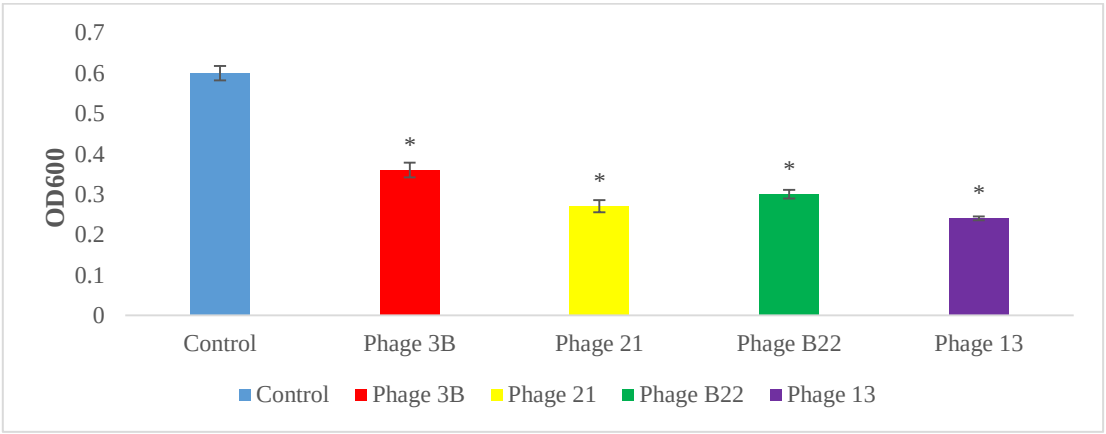

Figure 5. Effect of four strains of bacteriophage on inhibition of biofilm in a 96-well microplate after 24 h

CONCLUSION

Sixteen bacteriophage strains capable of infecting *Xanthomonas* sp. L019 were successfully isolated. Notably, one bacteriophage strain exhibited a broad host range, infecting 8/8 bacterial strains tested. Twelve bacteriophage strains demonstrated the ability to lyse several bacterial strains belonging to *Xanthomonas* sp., *E. carotovora* sp., and *Ralstonia* spp., displaying 4 distinct plaque morphology types: clear, clear with an opaque outer halo, opaque, and opaque with a clear outer halo. Among these, 4 bacteriophage strains, namely ϕ3B, ϕB22, ϕ21, and ϕ13, effectively lysed and controlled *Xanthomonas* sp. L019 populations, leading to a significant reduction in bacterial density. Furthermore, the ϕ3B, ϕB22, ϕ21, and ϕ13 bacteriophage strains inhibited biofilm formation and disrupted existing biofilms, with a reduction of up to 60%. Particularly, strain ϕ13 belonging to the class *Caudoviricetes* exhibited superior efficacy compared to other bacteriophage strains, indicating promising potential for biocontrol of bacterial plant diseases, an economically viable and environmentally friendly approach. These results suggest that ϕ3B, ϕB22, ϕ21, and ϕ13 can be considered as potential candidates for replacing chemical pesticides in biocontrol applications. However, further studies are necessary to evaluate the efficacy and consistency of field control, bacteriophage population dynamics, optimal application timing, influencing factors, and long-term ecotoxicological risks before field implementation.

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