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Zia ullah
Abbottabad University of Science and Technology
Ibrar Khan
abrar@aust.edu.pk

Abbottabad University of Science and Technology
Iqbal Ahmed Alvi
Hazara University,

Research Article

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Posted Date: October 27th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11113818/v1

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Additional Declarations: No competing interests reported.

Characterization and Multiplicity of Infection: *Xanthomonas* Oryzae Bacteriophages XOPW and XOP2 for Biofilm Control

Zia Ullah[1], Ibrar Khan[1*], Iqbal Ahmed Alvi[2*]

1. Department of Microbiology, Abbottabad University of Science & Technology, 22010 Havelian, Pakistan
2. Department of Microbiology, Hazara University, 21300 Mansehra, Pakistan

1. Zia Ullah: ziahamdard13@gmail.com
2. Ibrar Khan: abrar@aust.edu.pk
3. Iqbal Ahmed Alvi: iqbalalvi@hu.edu.pk
4. * Correspondence**:** abrar@aust.edu.pk (IK),: iqbalalvi@hu.edu.pk

Acknowledgments

We thank the Higher Education Commission of Pakistan (HEC) for the Important Research Grant Scheme under project no. [14565/KPK/NRPU/R&D/HEC/2022] and Abbottabad University of Science and Technology for funding this research project and providing technical support.

Author's contributions

Conceptualization, reviewing and editing, writing the original draft, formal analysis, investigations, funding acquisition: ZU, IK, IAA.

Funding: We wish to acknowledge the Higher Education Commission of Pakistan (HEC) for the Important Research Grant Scheme under project no: 14565/KPK/ NRPU/R&D/HEC/ 2022] and Abbottabad University of Science and Technology for funding this research project and technical support.

Declarations

Ethics Approval and Consent to Participate

All experimental procedures involving bacterial strains and phages were conducted in accordance with institutional biosafety guidelines. As the study did not involve human participants or animal subjects, ethical approval and informed consent were not required.

Consent for Publication

Not applicable.

Abstract

Xanthomonas oryzae is a bacterial pathogen that causes leaf blight disease (BLB), a devastating rice disease that is globally epidemic and causes significant crop losses of 50%. This bacterium shows resistance to antibiotics and chemicals because of the misuse of chemicals and antibiotics, so bacteriophages are used as an alternative treatment against *X. Oryzae.* Bacteriophages are considered biocontrol agents because they target specific bacterial strains and lyse bacteria rapidly, controlling infection. For the first time, the current study was designed to isolate and characterize the specific lytic bacteriophage against *X. oryzae* from Hazara Division, Shinkiari, KPK Province, Pakistan, the major cause of BLB in rice, as well as evaluate the efficiency of bacteriophages in terms of multiplicity of infection for controlling *X. Oryzae* infection. In this study, all isolated phages showed a very narrow host range; XOPW and XOP2 infect only *X. oryzae.* All phages showed excellent stability at pH 6–8 while phage treatment was for 1 hour. Heat treatment results showed that even a 1-hour exposure at 80 ºC has a significant effect on phage stability, and phages become completely inactivated. Phages tolerated 60 ºC heat treatment to varying degrees, resulting in a 2-3 log reduction in titer. Similarly, heat treatment at 50ºC had a minor effect on phage titer. Phages suspended in SM buffer stored at 4 ºC showed a minor reduction in titer even after three months of storage. According to the findings, isolated phages were causing bacterial lysis by forming transparent plaques. The MOI studies were conducted at MOI 0.001, 0.01, 0.1, 1, 10, and 100, and the spectrophotometric technique was used to record the net decrease in the bacterial population. Phages XOP2 and XOPW showed diverse levels of efficacy, with bacterial growth being significantly suppressed at various MOIs. The isolated bacteriophages were highly effective at reducing the growth of bacteria. At MOI 1, 10, and 100, a slow but moderate decline in bacterial growth over time was seen in comparison to other MOI levels. 1, 10, and 100, bacterial growth was comparatively unchanged until 20 hours, remaining nearly parallel to the control. The XOPW and XOP2 phage effectively reduced the optical density (OD) of a 4-day-old biofilm in a dose-dependent and time-dependent manner. At all-time points (6, 8, and 24 hours), the higher concentration of phage (MOI 1) consistently resulted in a lower OD compared to the lower concentration (MOI 0.1). The antibacterial activity of all phages was sustained, despite some partial resistance, indicating their potential for therapeutic use. In this pressing situation, employing phages as a therapeutic tool to combat antibiotic-resistant *X. oryzae* provides another approach.

Keywords: *Xanthomonas oryzae*, Bacteriophage, Bacterial leaf blight (BLB), Colony forming unit, Multiplicity of infection (MOI)

Introduction

Rice is a staple food crop consumed by about 3.5 billion people in the world. Twenty percent of the world's dietary energy comes from rice, compared to five and nineteen percent from wheat and maize, respectively (Alexandratos & Bruinsma, 2012). Plant pathogens are becoming a major issue for the stability of the economy and society. Globally, plant pathogen populations are growing. Plant pathogens include bacteria, fungi and viruses (Selin, De Kievit, Belmonte, & Fernando, 2016). One of the most dangerous diseases of rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. oryzae comparatively specific crop targets (Eichenlaub & Gartemann, 2011). *Xanthomonas Oryzae pv. Oryzae* a Gram-negative bacterium that is a member of family Xanthomonadaceae subclass Gammaproteobacteria (Nakayinga, Makumi, Tumuhaise, & Tinzaara, 2021), is primary cause of bacterial leaf blight (BLB), one of the most destructive bacterial disease. It is mainly found in temperate and tropical regions of Asia in rainfed paddy and lowland irrigated (Gumma, Nelson, Thenkabail, & Singh, 2011).

In order to control rice bacterial blight, bismerthiazol is the most widely used bactericide. Streptomycin is an aminoglycoside antibiotic that is used to prevent rice blight. However, abuse of these medicines leads to resistance, which worsens the disease and reduces yield (Xu *et al.,* 2010). Phage therapy, a different approach to managing phytopathogens through naturally occurring bacteriophages, has thus been reassessed in light of the quest for ecologically safe substitutes for chemical anti-bacterials (Kocharunchitt, Ross, & McNeil, 2009). Since bacteria naturally oppose phages, man has an ally in the fight against bacterial illnesses. Actually, the discovery of bacteriophages occurred in the latter half of the 1900s, and Ernest Hankin was the first to describe their bactericidal properties in 1896 (Sulakvelidze, 2004).

Bacteriophages are made up of a capsid (head) and/or tail (Akhverdyan *et al.,* 2011). Genetic material, such as DNA or RNA, is encapsulated and shielded by the capsid (Akhverdyan *et al.,* 2011). The size of bacteriophages ranges from 24 to 400 nm (Ivanovska *et al.,* 2004). Consisting of two or more distinct proteins, the capsid typically has a geometric shape. There are two types of bacteriophages: icosahedral (20 facets) and filamentous (some). Enzymes cannot utilize the genetic material when the capsid is present, which makes it vital (Orlova, 2012). With a population estimated to be at least 1030, bacteriophages are thought to be the most prominent biological entity on the planet. Phages have been isolated in a variety of

environmental conditions, including hot springs with acidic water. One of the main sources of bacteriophage resources is marine water. One of the main reservoirs is seawater (O'Flynn, Coffey, Fitzgerald, & Ross, 2006). Due to their diverse life cycles, bacteriophages can play a variety of roles in the biology of bacteria and archaea. A phage's lytic and lysogenic life cycles are its two main phases. Two typical phases are included in both life cycles: i) Phage adsorption ii) Genetic material leaking through (Salmond & Fineran, 2015). When compared to conventional antimicrobials, phages have many advantageous characteristics, including the capacity to replicate, high host-bacteria specificity, low environmental impact, ease of discovery, innocuousness to eukaryotes, high efficiency at low multiplicity of infection (MOI), low cost, straightforward preparation, and effectiveness against multidrug-resistant bacteria (MDR) and in preventing biofilm formation (Loc-Carrillo & Abedon, 2011).

The term MOI (multiplicity of infection), which describes the quantity of virus delivered per cell during infection, explains the bacteriophages/bacteria ratio. Only fluid systems with a large number of host cells can use MOI. Phage-against-bacteria investigations, both in vitro and in vivo, often employ MOIs ranging from 0.01 to 100. To make sure there is enough phage in the media, the MOI is typically 100. But not every phage reproduces or survives in the same manner. Determining the phages' resistance and their replication and lytic cycles with respect to environmental conditions is crucial. Although phages have been extensively studied for medicinal purposes in both the US and the EU for decades, neither region presently has any phage products that have been approved by regulatory agencies or that are undergoing phase-III clinical trials for human use. Researchers from all over the world needed systemically designed controlled trials to demonstrate the safety and effectiveness of phages, although these trials revealed no negative effects on humans (Viertel, Ritter, & Horz, 2014).

Materials and methods

Bacterial culture

Reference strains of *X. oryzae* 3M (PQ577762, SUB14856888) and IB (PQ577759, SUB14856888) from the Department of Microbiology, Abbottabad University of Science and Technology were used in the current investigation. Samples of bacteria were streaked over fresh YDC agar medium and incubated overnight at 37°C. Gram staining and biochemical tests were used to confirm the reference strains. Before every experiment, fresh bacterial samples were subcultured and used for further processing. By regularly subculturing, the culture's viability and purity were preserved.

Isolation of bacteriophages from sewage

Sewage and rice field soil samples were collected from District Mansehra. In order to isolate bacteriophages, the samples were collected in a sterilized pot and transferred to the Microbiology Lab of Abbottabad University. The samples were centrifuged for 10 minutes at 15,000 rpm after being agitated for two minutes. For sample processing, 10 mL of sterile 5 X nutrient broth, 40 mL of soil and rice field water sample, and one microliter of an overnight culture of *X. oryzae* strains were added as an inoculant in a flask. The flask was placed in a shaking incubator (120 rpm) and incubated overnight at 37°C for the overnight. The flask's contents were centrifuged for ten minutes at 10,000 rpm after incubation. The clear supernatant was collected in a fresh, sterile Falcon tube and stored at 4°C. A spot test was used to detect whether bacteriophages were present in the filtrate or not (J. Liu, Chia, & Tan, 2021).

Detection of bacteriophages in the filtrate

After bacteriophage enrichment, the existence of an *X. oryzae* phage was detected by a spot test following: 100 μL of an overnight-grown *X. oryzae* culture is spread on a nutrient agar plate for the spot test. After applying 7 μL of the filtrate containing suspect bacteriophage, the plates were allowed to dry for nearly 10 minutes. The plates were then incubated for the overnight at 37°C. Next, the plates were examined to see if bacteriophages had produced a definite lysis zone. The presence of a particular bacteriophage is indicated by the presence of the clear zone, or plaque (J. Liu et al., 2021).

Purification of bacteriophages using a double layer agar assay

To isolate Xoo specific phages, surface-sterilized BLB leaves were cut into pieces, immersed in 1% (w/v) peptone water in tubes and shaken at 30 °C for 4 h. The peptone water suspension was filtered through a 0.22 μm filter. The filtrate was then mixed with an overnight Xoo strain cultured in LB liquid medium and fresh LB liquid medium in the ratio 2:1:10 and the mix ture was shaken at 180 rpm/min at 30 °C for 10 h. After centrifugation at 4000 g at 4 °C for 15 min, the supernatant was filtered through a 0.22 μm filter to remove bacterial cells. The phage filtrate (2 μl) was then spotted onto the top layer of a bacterial lawn and incubated at 30 °C. Bacterial lawns were prepared by mixing an overnight Xoo culture (4 × 107 CFU/ml) (1 ml) with molten (about 48 °C) soft (0.4% agar) LB medium (5 ml) and then overlaying on a solid LB medium. After the appearance of a clearing zone (plaque), individual plaques of different sizes and clarity were picked with sterile pipette tips and soaked in 1 ml of SM (Saline Magnesium) buffer (Ogunyemi et al., 2019) at 4 °C overnight. The phage suspension was filtered through

a 0.22 µm filter, serially 10-fold diluted, and spotted onto a bacterial lawn for plaque formation. Single-plaque isolation was done three times to obtain a pure phage that formed plaques of consistent size and clarity. The purified phages were stored in SM buffer (Ogunyemi et al., 2019).

Characterization of bacteriophages

Determination of host ranges of phages.

The host ranges of the phages were determined by examining their infection on the different strains of the Xoo bacteria in this study based on the soft agar overlay method described above. The phage stock was diluted to about 1×10^8 plaque-forming units (PFU)/ml and spotted (2 µl) onto the top layer of the bacterial lawn and incubated at 30 °C. The appearance of clearing zones indicated cell lysis of the host bacteria by the respective phage(M. Liu, Hu, Xia, He, & Jin, 2023).

Determination of thermal stability of bacteriophages

Determining the bacteriophages' temperature stability is crucial since it offers information on phage storage and transit. Over a broad range of incubation temperatures, Xanthomonas phages can remain viable. Phage *X. oryzae* can thrive in temperatures ranging from 20 to 50°C (Civerolo, 1970).

Determination of pH stability of bacteriophages

To ensure a phage lifespan, the pH, ionic strength, and storage medium must all be at their ideal levels for phage survival. With the help of HCl and NaOH, the medium's pH was changed. pH paper was used to measure the pH. Aliquots of known titers of bacteriophages were placed at pH 2–9 for an hour to assess the pH stability of the isolated bacteriophages. The double-layer agar overlay method determines bacteriophage titer following incubation (M. Liu et al., 2023).

Long term storage stability

Phage strains of Xanthomonas vary in what their storage temperature should be. At +4°C in nutrient broth for three month, but at +20°C, it decreases to 2×10^7 pfu/ml in the same amount of time (Gašić *et al.,* 2018)**.** Keeping Xanthomonas phages in nutrient broth at +4°C prolongs their shelf life. To maintain phage efficacy throughout storage and before biocontrol

treatments, the proper storage conditions for each kind of phage should be established (Gill & Hyman, 2010).

Bacterial growth reduction

In-vitro phage therapy is another term for phage-mediated bacterial growth suppression. In this experiment, bacteriophages' ability to inhibit bacterial growth were assessed. A previously defined technique was used to test the reduction in bacterial growth of the bacteriophages. Three flasks containing 50 mL of broth were added 8-hour-old culture of *X. oryzae* strains. The first flask served as a control, whereas the second flask contained bacteria and the third flask contained bacteriophage and bacteria. By measuring optical density at 600 nm (O.D 600), the growth reduction was ascertained. A graph was created and used to compare the decreased growth to the control. The experiment was completed independently and repeated three times (Du *et al.,* 2009).

Bacterial growth curve

Each strain of bacteria has a unique growth pattern. A bacterial growth curve for the *X. oryzae* strains was done by using the spectrophotometer density technique. Through the use of OD_{600} and subsequent culture on culturing media, a link between OD and CFU was determined. Two flasks with 50 mL of LB broth were taken. A culture of bacteria was added to one flask and incubated for two hours at 37°C. A spectrophotometer was then used to quantify the optical density of bacteria at 600 nm. The number of colony-forming units (CFU) must be properly estimated to determine MOI**.** A connection between the optical density at 600nm wave length (OD_{600}) and the bacterial count was determined every one hour. The culture was then serially diluted in nutrient broth and spread (100 μL) on nutrient agar plates. Aliquots of culture based on the spreading technique were plated on nutrient agar plates concurrently. After that, the plates were incubated at 37°C for the entire night. The bacterial colonies were then counted using a formula (Schneider, Tsoungui Obama, Kamanga, Kayanula, & Adil Mahmoud Yousif, 2022).

$$CFU/mL = \frac{numbers\ of\ colonies \times dilution\ factor}{volume}$$

Determination of MOI for bacterial growth reduction

Multiplicity of infection is defined as the ratio of phage to bacteria. Reduction tests are the most crucial tool for determining whether or not bacteria are growing (S. T. J. B. a. D. T. M. f. t. P. Abedon & Disease, 2018). A known titer of fresh *X. oryzae* cultured overnight was utilized

in this experiment. This experiment evaluates the capacity of bacteriophages to suppress bacterial growth at different MOI.

96-well microtiter plate assay

Using a serial dilution technique, all bacteriophages were kept in 96-well plates at different multiplicities of infection (MOI), specifically 0.001, 0.01, 0.1, 1, 10, 100. To calculate the MOI, dilutions of bacteriophage were prepared. For this, six sterile Eppendorf tubes were used. In the first Eppendorf tube, 900 uL of broth was mixed with 100 uL of bacteriophage. In the remaining dilutions (2–6), 100 uL of bacteriophage was added to 900 uL of broth. Subsequently, in a 96-well microtiter plate assay, 200uL broth in each well of row 1 was poured. In row 2, 100 uL of broth and 100 uL of bacteria were poured. In rows 3, 4, 5,6,7,8, 100 uL of bacteria and 100 uL of bacteriophage were poured. Then, in rows 9, 10, 11, 12, 100 uL of broth and 100 uL of bacteria were added. Incubation of the 96-well microtiter plate was done for 2 hours at 37°C. After incubation, ELISA was used to monitor it (Benala *et al.,* 2021).

Biofilm formation kinetics by host bacterium

A culture of Xoo at OD600 = 0.8 was mixed into liquid LB medium (1:50), and 150 µl aliquots were added into wells of a 96-well plate (Corning-Costar Corp., Corning, NY, USA), which was then incubated at 30 °C for 12 h. Liquid LB medium was used as a blank. After incubation, the Xoo cultures in wells were discarded, and the wells were washed twice with double-distilled water and air-dried. 150 µl of each phage stock at the optimal MOI, or SM buffer without phages as a control, was then added into the wells and incubated at 30 °C for 12 h. The cultures were then discarded, and the wells were washed twice with double-distilled water and air-dried. The bacterial cells attached to the wells were stained with 150 µl of 1% (w/v) crystal violet for 30 min at room temperature and then were washed twice with double distilled water and air-dried. The dye was dissolved by adding 150 µl of 33% acetic acid, and its intensity was measured at 570 nm with a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, USA) (Ogunyemi et al., 2019).

Results

Bacterial growth on media

The isolated *X. Oryzae* was a Gram negative rod-shaped, non-sporing, motile bacterium. YDC agar is one of the various culture media on which *X. oryzae* grow. On YDC agar medium colonies of *X. Oryzae* show yellow smooth yellow color.

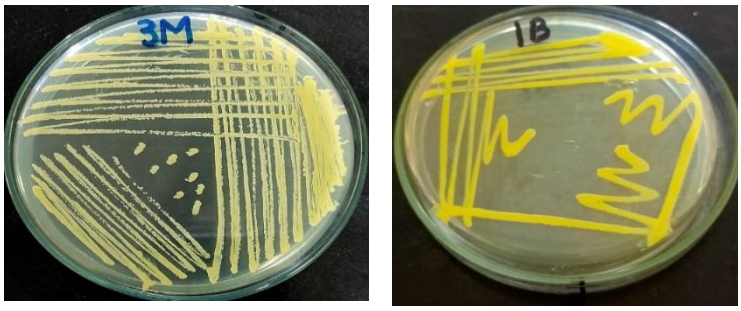


Figure 1: Characterization of bacterial isolates morphologically

Spot test for identification of *Xanthomonas Oryzae-specific* bacteriophages

In 2 out of 4 sewage samples, the presence of bacteriophages specific to *X. oryzae was* detected, which showed lytic activity (Figure 2). The bacteriophage obtained from a rice field (soil sample) in district Mansehra was named XOP2, while the bacteriophage obtained from a sewage sample of a rice field was named XOPW. However, the bacteriophages obtained from other samples, 2 of the 4 water and soil samples, had *X. Oryzae* infectious bacteriophage, according to spot testing, while the other samples had no lytic zone.

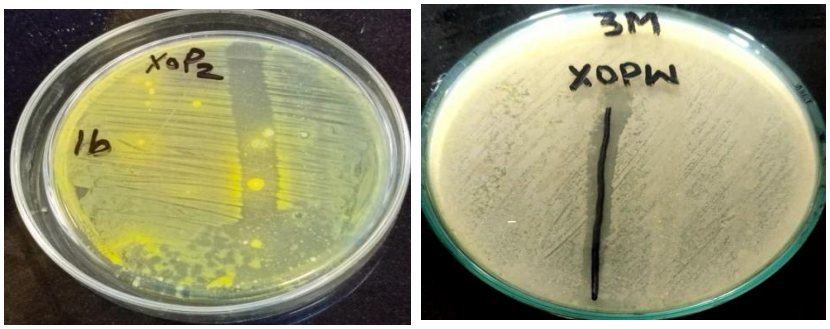
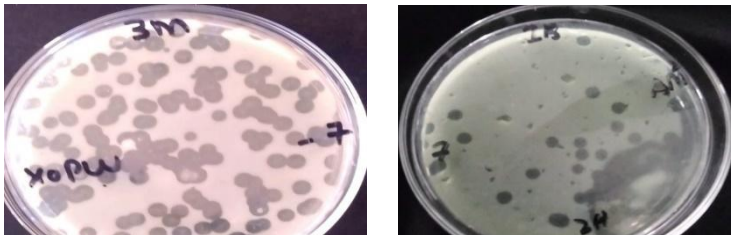


Figure 2: Detection of bacteriophages XOPW and XOP2 through spot test

Quantify bacteriophage activity through double layer assay

In contrast to *X. Oryzae*, the isolated phage generated a transparent, circular plaque on the double-layer agar plate. The plaque is made up of two circles, the inner circle being completely transparent and the outer circle encircling it. Bacteriophages generate a depolymerase enzyme when there is a hazy layer like this one around the plaque. The halo that surrounds the plaque shows that the phage is producing soluble enzymes, such as depolymerase, that allow the bacterial host cell to be released from its capsule. (Figure3) shows that XOPW was isolated against 3M (A) and XOP2 was isolated against IB (B).



A **B**

Figure 3: Bacteriophage activity (A) XOPW for 3M strain and (B) XOP2 for IB strain

Propagation of isolated phages

Clear, translucent plaques show that the isolated phages exhibit lytic activity, which suggests that they could be used as therapeutic agents. Plates exhibiting clear plaques through the double-layer method were selected for propagation of bacteriophages. Phages were then incubated in nutrient broth as shown in Figure 4. To determine the phage titer.

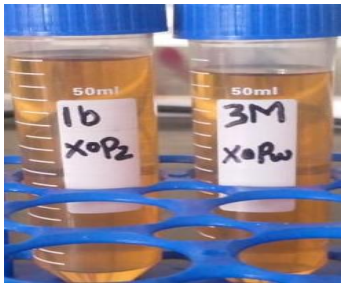


Figure 4: Phage XOP2 and XOPW propagation in liquid media

Bacteriophages that were isolated had high titer

The 3M and IB phage titers were found to be 9×10^9 pfu /ml (XOPW) and 3×10^8 pfu /ml (XOP2), respectively, after a day of phage propagation in a liquid medium (Figure 4). Our isolated phages' titers are high enough to imply that bacterial infections could be treated with a minimal amount of phage lysate (Figure 5).

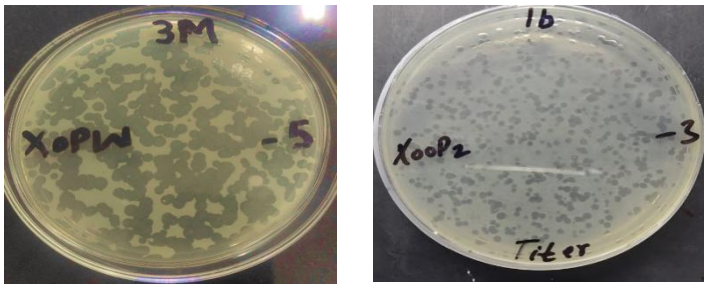


Figure 5: Titer of purified bacteriophages (XOPW, XOP2)

Characterization of Bacteriophages

Spectrum of activity analysis

It was discovered that the phages XOP2 and XOPW were very strain-specific against isolates of *X. oryzae;* bacteriophages were able to infect and generate lytic zones; however, no infectivity was seen for other genera. XOP2 bacteriophage exhibited no infectivity against other examined taxa, but they were able to infect and generate lytic zones against isolates of *X. oryzae.* Based on the results of the host range specificity test, the XOPW was able to infect *X. oryzae* but no other genera of bacteria (Table 2).

Table 1: Host range spectrum of bacteriophage against various bacterial cultures

Bacterial culture	Spot test XOP2	Spot test XOPW	Source
Xanthomonas oryzae IAM	_	_	Sewage sample
Xanthomonas oryzae IB	+	_	Rice field water sample
Xanthomonas oryzae 3B	_	_	Sewage sample
Xanthomonas oryzae 1M	_	_	Sewage sample
Xanthomonas oryzae 3M	_	+	Rice soil sample
Xanthomonas oryzae 5M	_	_	Rice field soil sample
Xanthomonas oryzae 6M	_	_	Sewage sample
Xanthomonas oryzae ZH	_	_	Sewage sample
Xanthomonas translucence LS2	_	_	Sewage sample
Xanthomonas translucence G2	_	_	Sewage sample

Xanthomonas translucence G3	_	_	Sewage sample
Neisseria meningitides	_	_	Sewage sample

Thermal stability analysis

The XOPW titer was unaffected at 4ºC however, 2 log, 1 log, 2 log, and 3 log reductions in titer were observed at 25ºC, 37 ºC, 50 ºC and 60 ºC respectivel. Heat treatment at 37ºC, 4ºC for up to 1 hour did not affect the titer of XOP2 phage. Minor reduction in titer was observed for phage XOP2 at 25ºC and a 3 log reduction occurred at 50 ºC and a 4 log decrease at 60 ºC for up to 1-hour heat treatment. At 80 ºC all phages became completely inactivated. No significant differences were observed for 1hour treatment for any phage at any temperature.

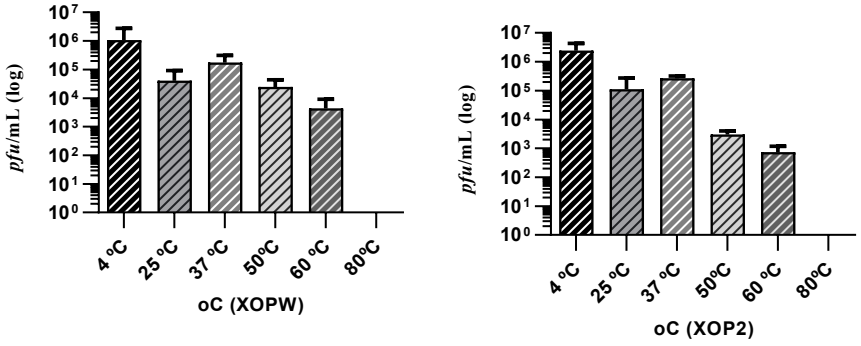


Figure 6: Thermal stability of XOP2 and XOPW phage at different temperature

pH stability of bacteriophages

Phage treatment at pH 2-9 for 1 hour showed slight change in titer. XOPW treatment for 1 hour at pH 3, 4, 5, 6, 8 resulted in 6-log, 4-log, 3-log, 1-log, 5-log, and 6-log reduction in titer, respectively. The best activity was observed at pH 5- 7, and no activity was observed at pH 2. The same results were observed for XOP2 after 1-hour treatment at these pH values (Figure7).

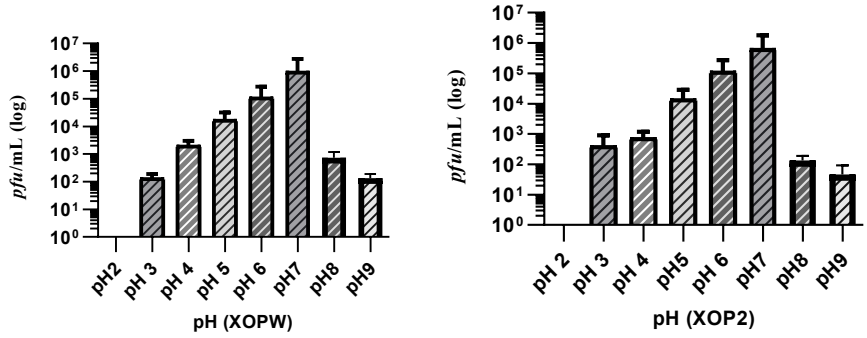


Figure 7: Effect of various pH on the viability of XOPW and XOP2 phage

Storage stability

The Xanthomonas phages were active by showing clear plaques after being stored at room temperature for one month. After a month of storage, the phage XOP2 titer was shown to be stable. The best storage temperature for XOP2 was recorded at 4°C, and a decrease of 1 log was recorded at 25°C and 37°C, respectively (Table 2). The best storage temperature for XO PW was recorded at 4°C, and at 25°C and 37°C, a drop of 1 log was seen.

Table 2: Storage stability of *Xanthomonas Oryzae* bacteriophages

	Titer Before Storage	**Titer After Storage**		
		4°C	25°C	37°C
XOP2	8×10^{8} *pfu*/Ml	6×10^{9}	5×10^{8}	4×10^{8}
XOPW	7×10^{9} *pfu*/Ml	6×10^{8}	5×10^{7}	4×10^{8}

Growth Reduction Assay

After 28 hours of observation, the antibacterial activity of XOP2 and XOPW showed an excellent bacterial reduction ability by suppressing and reducing the original bacterial count. Because these phages are extremely uncommon and no resistant mutants have emerged after XOP2 infection, the phage may connect to several different bacterial receptors. Phages by themselves have not been shown to have such an effective bacterial reduction potential. XOP2 may be a viable candidate for phage therapy based on suppression of bacterial growth. Through

the bacterial growth reduction experiment, XOP2 and XOPW were evaluated. For the next 24 hours, the OD600 was recorded every two hours using a spectrophotometer to monitor the efficacy of both phages in inhibiting bacterial growth compared to the control (Figures 8 and 9).

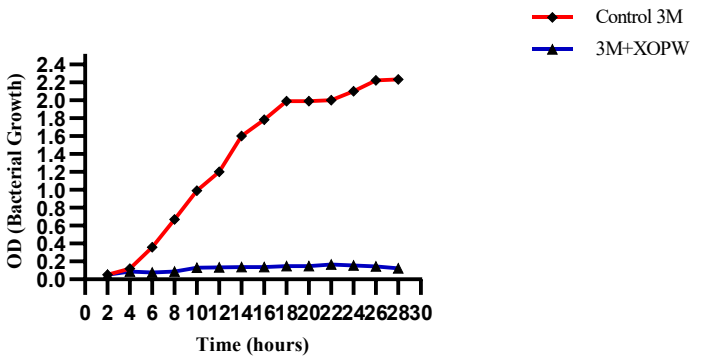


Figure8. Bacterial growth reduction potential of XOPW bacteriophage

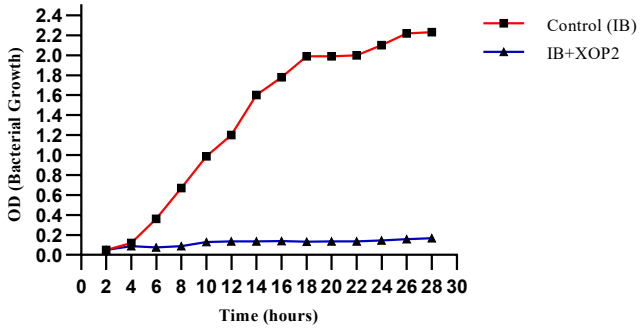


Figure 9. Bacterial growth reduction potential of XOPR bacteriophage

Determination of bacterial growth curve of 3M and IB strain

The correlation between CFUs/mL and O.D of 3M was plotted on the graph, and a curve was obtained. The graph showed a strong positive correlation between OD600 and CFU/mL. As the bacterial population increases (higher CFU/mL), the OD600 also rises. The upward trend of the red line indicated that as the number of bacteria increases (higher CFU/mL), the turbidity of the culture also increases (higher OD600) (Figure 10). 3M was selected for experiments because it was isolated from a rice field. *X. Oryzae* is mostly associated with rice leaf disease; therefore, we have planned to use *X. Oryzae* in rice field infections.

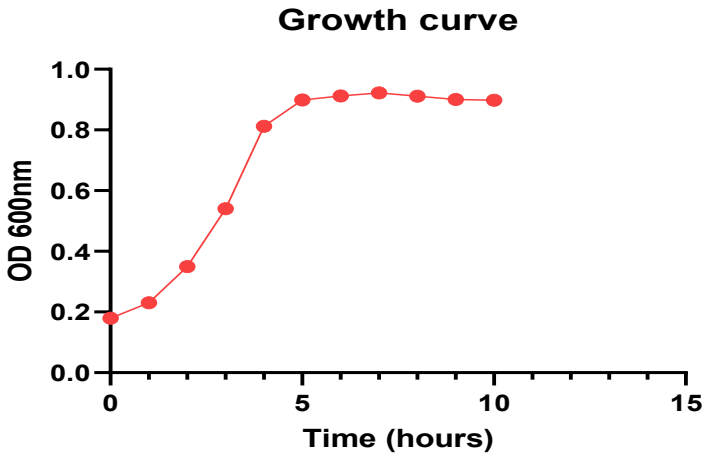


Figure 10: Represents the correlation between OD_{600} and CFU/mL of 3M strain

The correlation between CFUs/mL and O.D of IB was plotted on the graph, and a curve was obtained (Figure 11). The graph demonstrated a strong positive correlation between OD_{600} and CFU/mL. As the number of bacteria increases (higher CFU/mL), the turbidity of the culture also increases (higher OD600), as shown by the red line's upward trend. Due to its isolation from the rice field, IB was chosen for the experiments. Since rice leaf disease is primarily linked to *X. Oryzae*, we intend to use this pathogen to combat infections in the rice field.

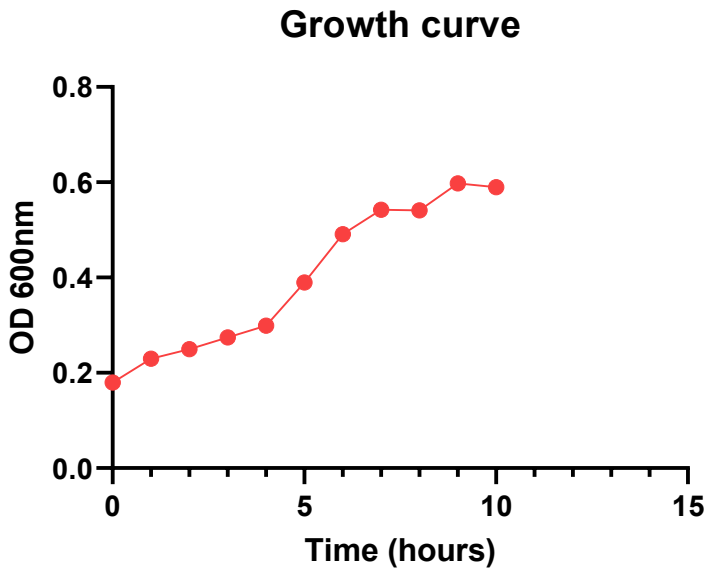


Figure 11: Represent correlation between OD_{600} and CFU/mL of IB strain

Effect of phage XOPW on bacterial growth reduction at different MOI

The effectiveness of phage as a therapeutic agent was evaluated in vitro using the bacterial growth reduction test. The isolated bacteriophages were highly effective at reducing the growth of bacteria. The effectiveness of phage XOPW in inhibiting bacterial growth was evaluated by comparing optical density (OD) data over different multiplicities of infection (MOI) and time intervals. The control group (3M) had much higher OD values for bacterial growth than the phage-treated groups, demonstrating the virus's strong bactericidal effect. At MOI = 0.001, a slow but moderate decline in bacterial growth over time was seen in comparison to other MOI levels. Following 24 hours, the OD was still greater than other phage treatments but lower than the control (3M). A slight decrease in bacterial growth was observed at MOI 0.01 up to 16 hours, but by 24 hours, this decline was identical to that observed at MOI 1. At MOI 0.1, a decrease in growth was observed until 16 hours, after which it began to increase until 24 hours and reached the MOI 1 reduction. At MOI 1, bacterial growth initially decreased until 8 hours, and then bacterial growth became static. At MOI 10, it decreased until 16 hours and then began to increase until 24 hours. Although bacterial growth was significantly lower than the control. At MOI 100, the highest bacterial growth reduction was observed compared to the control and all other MOIs, as shown in Figure 12. a. The graph clearly shows the lowest OD values at MOI 100 throughout the 24 hours, indicating the strongest inhibition of bacterial growth.

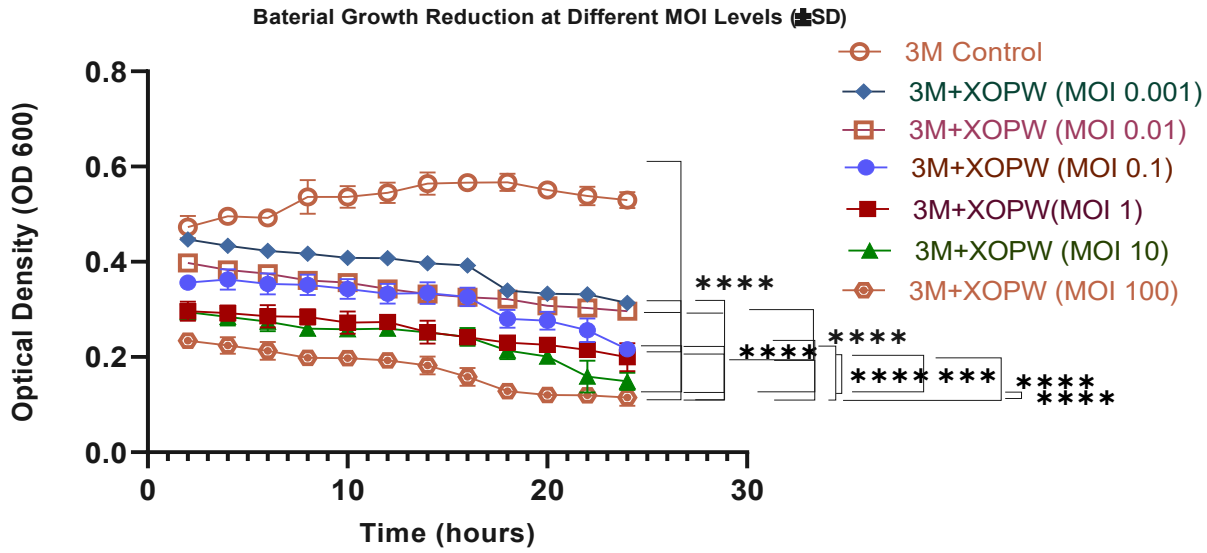


Figure 12: Effect of bacteriophage XOPW on corresponding bacteria 3M reduction at different MOI

Effect of phage XOP2 on bacterial growth reduction at different MOI

The bacterial growth reduction test was used to assess phage XOP2 efficacy as a therapeutic agent in vitro. The corresponding control (IB) was contrasted with the phage-mediated bacterial reduction assay. At MOI 0.001, bacterial growth was comparatively unchanged until 16 hours, remaining nearly parallel to the control. After 16 hours, the bacterial growth increased until 24 hours, though the OD reached was still slightly reduced compared to the control at 24 hours. Bacterial growth showed a slight decline at MOI 0.01 until 16 hours. The OD was at a level comparable to MOI 1 at 24 hours. This implies that the phage had a slightly higher initial impact at this MOI than at MOI 0.001. Up to 16 hours, there was a slight decrease in bacterial growth at MOI 0.01. At MOI 0.1, a decline in growth was noticed until 16 hours; however, it started to rise until 24 hours and became equal to the reduction of MOI=1. The OD was comparable to MOI 1 at 24 hours. This indicates that the phage's initial impact at this MOI was slightly higher than 0.001. Bacterial growth initially decreased until 8 hours at MOI 1. Eight hours later, there was no further noticeable rise in OD, and the bacterial growth remained static. The bacterial growth decreased until 16 hours at MOI 10. The OD at 24 hours was still significantly lower than the control. In comparison to the control and all other MOIs over the course of the 24-hour period, the bacterial growth was considerably lower at MOI 100, as shown in Figure 13. Accordingly, MOI 100 appears to be the most successful MOI for limiting bacterial growth.

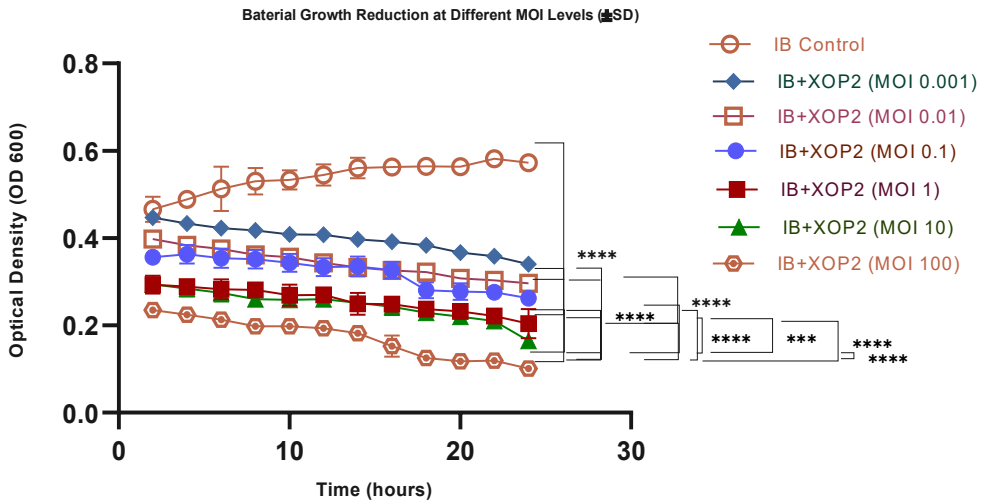


Figure 13: Effect of bacteriophage XOP2 on corresponding bacteria IB reduction at different MOI

3M and IB bacteria established mature biofilm at 96 hours' post-inoculation

The maturation stages of biofilm were determined by CV staining methods and the viable cell count method. At 24 h of incubation, 3M established biofilm, resulting in 3.0×10^3 CFUs/mL. Maximum CFUs/mL was produced at 96 hours post-inoculation, which resulted in 3.3×10^8 CFUs/mL, and IB bacteria established biofilm result in 24 h was 4.0×10^2 CFU/mL, and after 96 hours of incubation, maximum biofilm was produced at 4.3×10^9. Similar results were observed for the CV method, where the highest biomass was observed at 96 h with an OD_{595} value of 3.54. One and three-log reductions in the bacterial count were found on the 5th and 6th day of biofilm growth, respectively (Fig. 14).

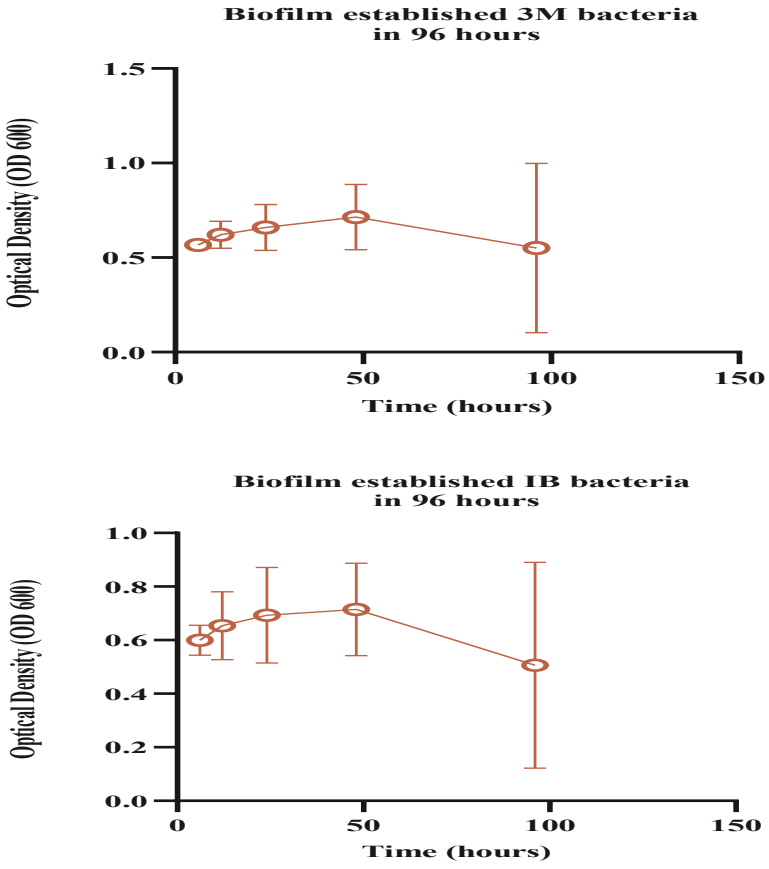


Figure 14. 3M and IB established biofilm in 96 hours

XOPW and XOP2 significantly removed mature biofilm

The XOPW phage effectively reduced the optical density (OD) of a 4-day-old biofilm in a dose-dependent and time-dependent manner. At all-time points (6, 8, and 24 hours), the higher concentration of phage (MOI 1) consistently resulted in a lower OD compared to the lower concentration (MOI 0.1). While the Control group showed a steady increase in biomass over 24 hours (rising from an OD of ~5.85 to ~7.785), the phage-treated groups maintained significantly lower OD levels. The most pronounced reduction occurred at the 24-hour mark, where the MOI 1 treatment prevented the biomass accumulation seen in the control. The 6,8 and 24-hours treatment was more effective, removing 44.4%, 55.6%, 62.4%,28.6%, 44.4%, and 41.1% of biofilm at MOI-1 and 0.1, respectively, and the difference between both MOIs and between 6-hour and 24-hour was statistically significant (p-value <0.0087). The disruptive efficacy of the XOP2 Phage against *Xanthomonas Oryzae* (IB), 24-hour-old mature biofilms, was evaluated across multiple time intervals (6, 8, and 24 hours) and infection doses MOI 0.1and MOI. Treatment with XOP2 resulted in a vigorous, dose-dependent clearance of biofilm biomass at all tested time points. At a lower concentration MOI 0.1, XOP2 demonstrated substantial biofilm reduction, peaking at 32.41% after 8 hours of exposure. Elevating the treatment concentration to a higher dose MOI-1.0 significantly accelerated biomass disruption, yielding a 37.57% reduction within the first 6 hours and culminating in a maximum eradication rate of 56.31% by the 24-hour interval. These findings demonstrate that high-dose XOP2 exposure provides both rapid and sustained destabilization of established biofilm matrices over an extended period. The difference between MOIs and between 6 hours and 24-hours was statistically significant (p-value < 0.0211).

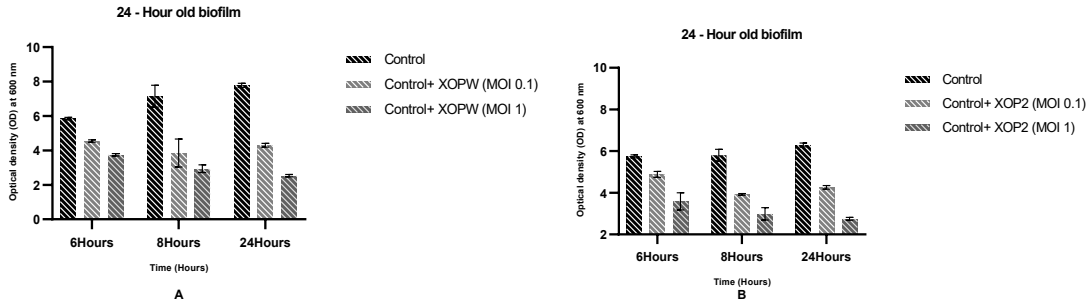


Figure 15: Biofilm removal (CFU/mL reduction) following XOPW and XOP2 treatment for 6, 12, and 24 h against 1–4-day-old *Xanthomonas Oryze* (3M and IB) biofilms at MOI -0.1 and MOI-1.

Table 3: The efficacy of XOPW and XOP2 bacteriophages against 3M and IB bacterial biofilms is detailed in the table below, summarizing recent in vitro

Treatment Group	Time (h)	Control (OD_{600})	MOI=0.1 (OD_{600})	MOI=0.1 Reduction (%)	MOI=1 (OD_{600})	MOI=1 Reduction (%)
3M+XOPW	6	5.85	4.55	22.88%	3.750	36.44%
	8	7.15	3.85	42.46%	2.94	56.12%
	24	7.78	4.30	45.3%	2.53	68.68%
IB+XOP2	6	5.75	4.88	15.13%	3.59	37.57%
	8	5.80	3.92	32.41%	2.99	48.45%
	24	6.29	4.26	32.33%	2.75	56.31%

Discussion

One of the oldest known diseases, *Xanthomonas oryzae* causes bacterial blight (BLB) that affects rice crops, causing yield losses of over 50% (Jabeen *et al.,* 2012). The most used bactericide for treating rice bacterial blight is bismerthiazol (Svircev, Roach, & Castle, 2018). Abuse of these medications, however, causes resistance, which exacerbates the illness and lowers yield (Xu *et al.,* 2010). Therefore, phage therapy an alternate method for controlling phytopathogens (Svircev *et al*., 2018). To fully maximize the use of phages as a biocontrol technique, however, we still need to get a better understanding of both phage ecology and the intricate phage–host interactions in diverse plant settings (Goodridge, 2004).

The *Xanthomonas oryzae* strains 3M and IB (PQ577762, PQ577759) taken from the Department of Microbiology, Abbottabad University of Science and Technology were used in the current study. Samples of bacteria were streaked over fresh YDC agar and cultured overnight at 37°C. On YDC agar, *X. oryzae* produces smooth yellow colonies. For the isolation of bacteriophages against pathogenic bacteria, the different samples of rice field soil water were the main source of bacteriophage isolation in the current study. The bacteriophages were isolated from Sewage water and rice field soil samples from District Manshera. Four samples were tested, and two of them were positive for bacteriophages against the Xanthomonas strain 3M and IB. A similar study was conducted by Kauffman *et al.,* (1973) he took five soil samples and 2 water samples from 15 sites and tested for the presence of phages specific for *X. oryzae.* Spot testing discovered that the samples contained bacteriophages. In the previous study, Rehman *et al.,* (2014) isolated phage NRO8 from sewage effluent, which was found effective against *X. oryzae* strains.

Using the double-layer method, the filtrate was inoculated in triplicate onto PSA double-layer plates supplemented with the bacterial host in semi-solid (0.7% agar) and solid (1.5% agar) PS medium. Following that, the plates were incubated for 24 h at 37°C until plaques formed. This procedure was used for bacteriophage isolation. Four samples of total XOPW and XOP2 clearly show plaques against 3M and IB. A similar study was conducted by Jain *et al.,* (2017), which showed that out of five samples, three samples gave a clear plaque against *Xanthomonas oryzae* strains. Following a day of phage propagation in liquid culture, the 3M and IB phage titers were found to be 9×10^9 pfu /mL and 3×10^8 pfu /mL. A similar study was conducted by Abedon (2011), which shows phage titer in this range for *Xanthomonas oryzae* phage NRO8 was 4.1×10^8*pfu*/mL.

Host specificity of Xoo phages was determined by spot test XOPW and XOP2 have been found to infect all strains of *X. oryzae*. Furthermore, XOPW and XOP2 were unable to infect other strains of *Xanthomonas translucens* and *Salmonella gallinaruin*. A similar study was conducted by (S. T. Abedon, 2011) which shows Xoo-sp1 phage infects strains of *X. oryzae*. Phage particles become inactive due to structural element damage caused by temperature, pH, and UV exposure, among other environmental conditions. For XOP2, phage activity was best recorded at 4ºC, 25ºC, 37ºC, and 50 ºC. For XOPW phage activity was best recorded at 4 ºC to 50 ºC. The pH range of 3 to 9 was determined to be optimal for phage XOP2. Phage was discovered to function best at a pH of 5 to 8. A similar study was conducted by Gill and Abedon (2003), which shows that Xanthomonas phage NR08 is active at temperatures ranging from 4 to 60 degrees Celsius, Xanthomonas phage NR08 is active at pH values ranging from 4 to 9. The Xanthomonas bacteriophages XOP2 and XOPW can be kept for up to one month at room temperature by producing translucent, clear plaques that demonstrate the bacteriophage's lytic activity. The best storage temperature for XOPW and XOP3 was recorded at 4°C, 25°C, and 37°C. A similar study was conducted by Abedon (2011), which shows a decrease of 2 and 3 log was recorded for Xanthomonas phage NR08 at 4, 25, and 37 degrees centigrade.

After 28 hours of observation, the antibacterial activity of XOP2 and XOPW showed an excellent ability to reduce bacteria by both blocking and reducing the original bacterial inoculum. XOP2 and XOPW were evaluated using the test for bacterial growth decrease. Using a spectrophotometer to record the OD600 every two hours for the next twenty-four hours, the capacity of both phages to inhibit bacterial growth was observed and compared to the growth of the control. XOPW prevented bacterial growth for 28 hours, and XOP2 was prevent for 26 hours; after 26 hours, slightly resistance show bacteria to phage. It was previously observed that phages with the capacity to decrease *X. oryzae* growth might stop the bacterial growth for up to 16 hours (Jamal, Hussain, Das, & Andleeb, 2015)The bacterial growth curve was determined, and the strong positive correlation that was found is shown in the graph with the general rule that more turbidity results from increased bacterial growth. The MOI was quickly calculated by estimating the *Xanthomonas Oryzae* (3M and IB) concentrations using this standard curve. (Cuppers, Smelt, & biotechnology, 1993) described an observed linear relationship between the log of the initial inoculum size and the time taken for the incubating culture to reach a specified turbidimetric detection level due to a 10^6 CFU /ml culture.

The changes in bacterial growth over time highlight the dynamics of phage-bacteria interactions and the importance of MOI in achieving effective treatment. Results showed that

the optimal MOI for phages to inhibit growth of *Xanthomonas Oryzae* was 100. However, MOIs 0.01 and 1 showed similar reductions in bacterial growth at 24 hours. MOIs 0.001, 0.01, 0.1, 1, 10, and 100 can be used to treat bacterial infections, while their effectiveness varies. A similar study was conducted by Liu *et al.* 2021); the optimum MOI of phages NF-1 and NF-3 was ascertained by counting the average pfu at four distinct MOIs. The outcome demonstrated that the ideal MOI for NF-1 and NF-3 isolates is 0.1. The emergence of phage-resistant bacteria caused a minor reduction in OD towards the end of 24 hours at certain MOIs (0.001, 1, 0.1), but it was significantly less than the control. Phages that can inhibit the growth of *Xanthomonas Oryzae* can halt bacterial growth for as long as 16 hours. (Wang *et al.,* 2019) shown that the bacteriophage vB_SauS_SA2 causes the number of bacteria to decrease every hour, and after eight hours, the bacteria develop phage resistance. Both XOPW and XOP2 phages demonstrate potent, dose- and time-dependent antibiofilm efficacy against established, mature *Xanthomonas oryzae* matrices. At a high multiplicity of infection (MOI 1.0), both phages significantly outpace their lower-dose counterparts (MOI 0.1), preventing the steady biomass accumulation observed in the untreated controls. This accelerated biomass disruption is particularly evident in high-dose XOP2 treatment, which achieves a substantial 54.4% reduction within the first 6 hours. Ultimately, both phages reach peak eradication at the 24-hour interval, with XOPW removing 62.4% and XOP2 clearing 61.7% of the mature biofilm. The statistically significant differences recorded between the tested MOIs and across time points underscore the critical interplay of viral load and exposure kinetics in matrix destabilization. Mechanistically, this rapid initial degradation suggests that the phages may possess matrix-degrading depolymerase enzymes capable of penetrating the dense extracellular polymeric substance (EPS) layer. However, the plateauing efficacy observed between 8 and 24 hours at lower MOIs may point to the emergence of transient phenotypic resistance or metabolic dormancy within inner biofilm layers. Future studies should focus on evaluating these phages in combination therapies or exploring their genomic profiles for encoded endolysins to optimize biocontrol strategies. In conclusion, these findings present XOPW and XOP2 as highly effective, fast-acting biological agents for mitigating resilient *X. oryzae* infestations in agricultural systems. Previous studies have shown that phages can penetrate the inner layers of biofilms by degrading components of the exopolymeric biofilm matrix and thus can be used to remove or control infectious biofilms (Ogunyemi et al., 2019).

Conclusion

XOPW and XOP2 are novel phages against the 3M and IB strains of *Xanthomonas oryzae*. It has an excellent growth reduction ability against mature bacteria. Although it has a narrow host range, it has shown high infectivity against specific Xanthomonas strains. XOPW and XOP2 have shown stability and activity under varying physiological conditions. The findings of the study showed that the

phage is an appropriate substitute to be used for the elimination of life-threatening bacteria. This study resulted in *Xanthomonas Oryzae.* A specific phage, which is a valued finding associated with microbiology and other fields, can be applied safely for therapeutic purposes after *in vivo* studies.

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