ISOLATION AND CHARACTERIZATION OF A LYTIC BACTERIOPHAGE C1 SPECIFIC TO THE MARINE BACTERIUM *VIBRIO PARAHAEMOLYTICUS* IN QUANG NINH PROVINCE

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ABSTRACT

Acute hepatopancreatic necrosis disease caused by *Vibrio parahaemolyticus* is the main cause leading to economic losses to shrimp farming. In addition, the emergence of multiple drug-resistant *Vibrio* has underscored the urgent need for alternative strategies such as phage therapy. In the present study, 7 bacteriophages specific to *Vibrio* spp. were isolated from water and sludge samples collected at the shrimp farms in Quang Ninh province. Among them, phage C1 was highly effetive in lysing *Vibrio panurili* A1.2, *Vibrio alginolyticus* A2, *V. alginolyticus* D1, and especially *V. parahaemolyticus* H3. Using transmission electron microscopy, the phage C1 was observed to belongto the Podoviridae family with a head diameter of 52.73 nm and a very short tail. Phage C1 exhibited the highest titer (9.6±0.12 log PFU/mL) at an optimal MOI ratio of 0.0001. Assessement of lysis potential revealed that phage C1 was able to maintain strong lytic activity for 10 hours. Notably, C1 had a brief latent phase (<10 min) with a high burst size of 294 PFU/mL and resilience to a decent range of temperatures (20-50°C) and pHs (5-11). These findings further enhance our understanding of biological characteristics *Vibrio* phage from local shrimp farms and its potential as a biocontrol agent for vibriosis included by *Vibrio* spp.

PHÂN LẬP VÀ NGHIÊN CỨU ĐẶC ĐIỂM CỦA THỂ THỰC KHUẨN CÓ KHẢ NĂNG LY GIẢI VI KHUẨN BIỂN *VIBRIO PARAHAEMOLYTICUS* TẠI TỈNH QUẢNG NINH

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TÓM TẮT

Bệnh hoại tử gan tụy cấp do vi khuẩn *Vibrio parahaemolyticus* là nguyên nhân chính gây thiệt hại kinh tế tới ngành nuôi tôm. Hơn nữa, sự xuất hiện của *Vibrio* đa kháng thuốc đã nhấn mạnh tầm quan trọng của liệu pháp thể thực khuẩn như phương pháp thay thế nhằm kiểm soát dịch bệnh. Trong nghiên cứu này, 7 thể thực khuẩn đặc hiệu với *Vibrio* spp. được phân lập từ các mẫu nước và bùn thu tại các trại nuôi tôm ở tỉnh Quảng Ninh. Trong đó, thể thực khuẩn C1 có khả năng ly giải *Vibrio panurili* A1.2, *Vibrio alginolyticus* A2, *V. alginolyticus* D1 và đặc biệt là *V. parahaemolyticus* H3. Sử dụng kính hiển vi điện tử quét, thể thực khuẩn C1 có đặc điểm giống với họ Podoviridae dựa vào đường kính đầu 52,73 nm và đuôi rất ngắn. Phage C1 thể hiện hiệu giá cao nhất (9,6 ± 0,12 log PFU/mL) ở tỷ lệ MOI tối ưu là 0,0001. Thể thực khuẩn C1 có thể duy trì hoạt động ly giải mạnh trong 10 giờ. Đáng chú ý, C1 có pha tiềm ẩn ngắn (<10 phút) với hệ số nhân vi rút là 294 PFU/mL, bền với dải nhiệt độ (20-50°C) và dải pH (5-11). Những kết quả trên nâng cao sự hiểu biết của chúng ta về đặc tính sinh học của thể thực khuẩn bản địa phân lập từ các trang trại nuôi tôm và minh chứng tiềm năng kiểm soát sinh học của thể thực khuẩn đối với bệnh vibriosis.

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1. Introduction

Vibriosis, caused by *Vibrio* pathogens, stands as one of the most prevalent infections posing a serious threat to shrimp worldwide. *Vibrio* species play significant roles in the biogeochemical cycles of aquatic ecosystems due to their high abundance and versatility [1]. However, some *Vibrio* spp. such as as *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* have been reported to be pathogenic to shrimp [2]. Acute hepatopancreatic necrosis disease (AHPND) caused by *V. parahaemolyticus* is the most severe disease devastating the global *shrimp* industry. Additionally, the consumption of shrimp and its related products contaminated with *Vibrio* spp. has been reported to cause serious food poisoning [3]. The emergence of antibiotics, as a quick and effective therapeutic strategy to control the spread of bacteria, has concided with excessive use, leading to the dispersal of multiple resistant strains and the presence of antibiotic residues in the environment, especially in shrimp [4]. Hence, exploration of alternative strategies to control *Vibrio* contamination in shrimp farming is imperative to improve production, reduce antibiotic use, and ensure food safety.

Bacteriophages, composed of DNA or RNA enclosed in a protein coat, are prokaryotic viruses that infect and lyse host bacteria [3]. Virulent or lytic phages attach to host bacteria through specific receptors on the cell surface. Subsequently, the viral genetic materials are injected into the host, initiating cycles of replication until the phage-derived proteins are activated to lyse and kill bacterial cells [5]. Transmission electron microscopy (TEM) is still an effective method for the identification of phage. Various studies have proved that the use of phage could prevent vibriosis. Previous reports showed over 70% reduction in *V. parahaemolyticus* counts within one hour of phage application [6]. The efficacy of phages as biocontrol agents was also evident in a 40% reduction in shrimp mortality upon treatment with bacteriophage against *V. alginolyticus* [7].

In Vietnam, research investigating phages as biocontrol alternatives to antibiotics in shrimp farming has only gained momentum in recent years [8]. However, a common limitation of these studies is the lack of sufficient evidence and characterization of potential *Vibrio* phage, which may hinder the practical application of phage therapy in aquaculture. It is crucial to thoroughly evaluate their morphological characteristics and lytic specificity to host strain before utilizing phages for broad applications. The study aims were to isolate and characterize a *V. parahaemolyticus* phage isolated from shrimp farms collected in Quang Ninh province. These findings will offer valuable insights for future application of phages in managing *Virbio* infections in shrimp.

2. Methods

2.1. Bacteriophage isolation

Four bacterial strains *V. panurili* A1.2, *V. alginolyticus* A2, *V. alginolyticus* D1, and *V. parahaemolyticus* H3 were kindly provided by the VAST-Culture Collection of Microorganisms, Vietnam Academy of Science and Technology were used hosts for the isolation of bacteriophages. All bacterial strains were cultivated in Alkaline Saline Peptone Water (ASPW) medium (Peptone 10 g/L; NaCl 15 g/L; pH 6.2-7.0).

Bacteriophages were isolated from 18 water and 12 sludge samples collected in Quang Ninh province using the double agar layer method as described previously [9]. In brief, samples were centrifuged at 8000 rpm for 10 min to obtain supernatant which was then mixed with a respective volume of 2X ASPW medium. After 8 hours of incubation with shaking at 37°C, the mixture was centrifuged at 8000 rpm for 5 min and then incubated with *Vibrio* cultures under shaking conditions overnight. Afterwards, the mixture underwent centrifugation at 10000 rpm for 10 min at 4°C. The resulted lysis samples were later filtered through sterile 0.22 µm membrane filters and stored at 4°C. Lysis samples were then diluted with SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin, pH 7.0). The mixture was added to the exponential phase culture of *Vibrio* in 0.3% agar ASPW and then poured over pre-solidified 1.5% agar ASPW plates. The plates were

incubated overnight for plaque formation [9]. Single plaques were picked from the plate using a sterile pipette tip and the procedure was repeated at least four times for phage purification. Purified phage was stored in SM buffer at 4°C.

2.2. Transimission Electron Microscopy (TEM)

Bacteriophage sample was deposited on a copper grid for absorption in the dark for 30 min according to the previous protocol with a slight modification [10]. The sample was negatively stained using 1% phosphotungstic acid for 20 min and then dried for 30 min. The obtained phage samples were examined in a JEM-2100 transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

2.3. Determination of lytic activity against V. parahaemolyticus **H3**

Multiplicity of Infection (MOI) is the ratio of phage particles to potential *Vibrio* host cells, which was determined according to the method previously described [10]. Briefly, *V. parahaemolyticus* H3 at log phase (~10^8 CFU/mL) were mixed with phage suspension (~10^8 PFU/mL) to achieve an MOI of 0,001; 0,01; 0;1; 1; 10; 100. Non-infected *Vibrio* cells were used as controls. All samples were incubated at 37°C for 10 hours with shaking at 150 rpm and lysate samples were then centrifuged at 8000 rpm for 5 min at 4°C. The supernatants were filtered through a 0.22-μm membrane filter and phage titer and then assessed using the double-layer agar method. The MOI with the highest phage titer was considered the optimal MOI. The experiment was done in triplicate.

2.4. One-step growth curve

The one-step growth curve of phage was determined as previously described with slight modifications [11]. Phage suspension was mixed with 500 µL of *V. parahaemolyticus* H3 culture (~10^8 CFU/mL) at optimal MOI and incubated at 37°C for 15 min for phage absorption. The mixture was then centrifuged at 10,000 rpm for 30 seconds at room temperature to remove free phage particles. The pellet was resuspended in 10 mL ASPW. A 100 µL sample was taken every 20 min for 1.5 hours to determine phage titer by plaques counting. The experiment was performed in triplicate. Phage burst size was calculated based on the growth curve using the formula:

$$Burst\ size\ = \frac{Phage\ average\ at\ plateau\ phase}{Number\ of\ infecting\ phage}$$

In which, the number of infecting phage is the initial total phages minus free phage.

2.5. Effects of temperature and pH on bacteriophage stability

Thermal and pH stability of phages was assessed according to the method previously described [10]. For thermal stability, phage suspension of 10^8 PFU/mL was incubated on ASPW for 1 hour at different temperatures (20°C, 35°C, 40°C, 50°C, 60°C, 70°C, 80°C). For pH stability, ASPW medium was prepared at pH 3-11. A volume of 1 mL phage suspension was mixed with 9 mL of ASPW at respective pH to achieve 10^8 PFU/mL phage and then incubated at 35°C for 1 hour. The titer of phages was determined using the double layer-agar method.

3. Results and Discussion

3.1. Screening of Vibrio lytic bacteriophage

Bacteriophages specific to 4 *Vibrio* host strains were isolated from water and sludge samples collected in Quang Ninh province. Transparent zones on the host bacterial lawn agar, called phage plaques, indicate the presence of *Vibrio* lytic phages. Purification of phages was carried out after repeated sub-cultures until separated plaques appeared uniform in shape and size. A total of 7 presumtive phages were obtained from pure subcultures using three successive single-plaque isolations (Table 1). Three phages B1, B3 and B4 displayed infectivity towards single bacterial

strain such as *V. alginolyticus* D1 or *V. paraheamolyticus* H3. In contrast, phage C1 had the most potent lytic activity against all bacterial strains, followed by phage C2. Since phage C1 displayed significant lytic activities against all tested *Vibrio* strains with visible transparent zones, C1 was selected for further characterization.

Table 1. *Screening for lytic bacteriophage on different Vibrio strains*

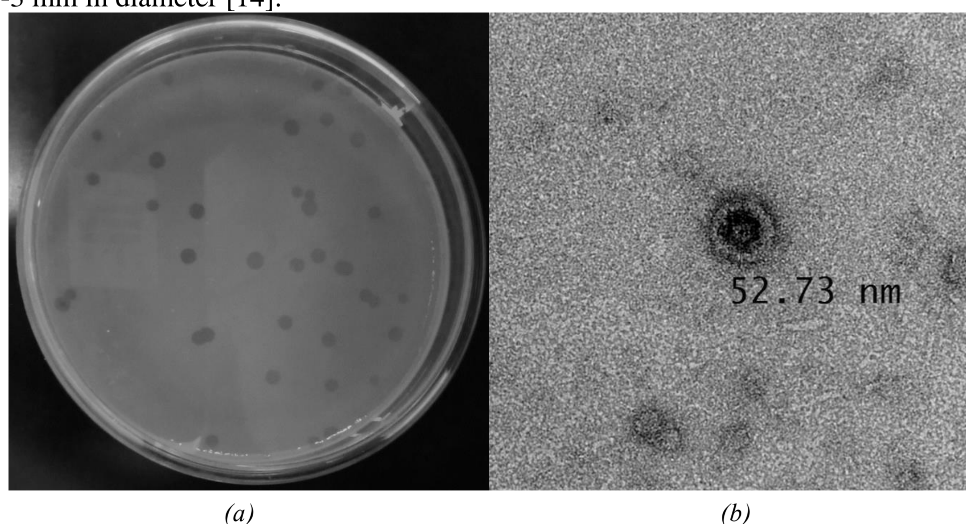
Host / Phage	*Vibrio panurili* **A1.2**	*V. alginolyticus* **A2**	*V. alginolyticus* **D1**	*V. paraheamolyticus* **H3**
C1	++	++	++	++
C2	+	+	+	+
B1	-	-	+	-
B2	-	-	+	+
B3	-	-	-	+
B4	-	-	-	+
Q4	-	-	+	+

Note: ++, clear plaques; +, plaques with heavy turbidity; -, no plaques formed.

It is worthy noting that *V. paraheamolyticus* H3 strain was the most sensitive host (Table 1). Despite being the same species, *V. alginolyticus* A2 was quite resistant to isolated phages as compared to *V. alginolyticus* D1. *V. paraheamolyticus* were often reported to be related to infections, especially AHPND, in shrimps [8]. Similar to the present study, six phages from sediments and seafood in Korea were capable of lysis multiple *V. paraheamolyticus* hosts, of which the most efficient phage Vpp2 had lytic properties on 3 different *Vibrio* species [12]. In addition, a bacteriophage VVP1 from a shrimp grow-out pond was able to infect both *V. parahaemolyticus* and *V. alginolyticus* [13]. A broad host-specific bacteriophage seemed to be an attractive feature, especially in applications for preventing *Vibrio* spp. in shrimp farms.

3.2. Morphological analysis of bacteriophage C1

Plaques of C1 phage exhibited high transparency and round shape, with diameters of approximately 1-3 mm after 10 h incubation (Figure 2A). Halo around the plaques was also observed for C1 phage, suggesting depolymerization enzymes activity on bacterial cell envelops that potentially contribute to phage-derived enzymes approach for phage therapy. This result was similar to that of lytic phage R16F against *V. parahaemolyticus* 1.1997^{T}, with clear plaques of about 2-3 mm in diameter [14].



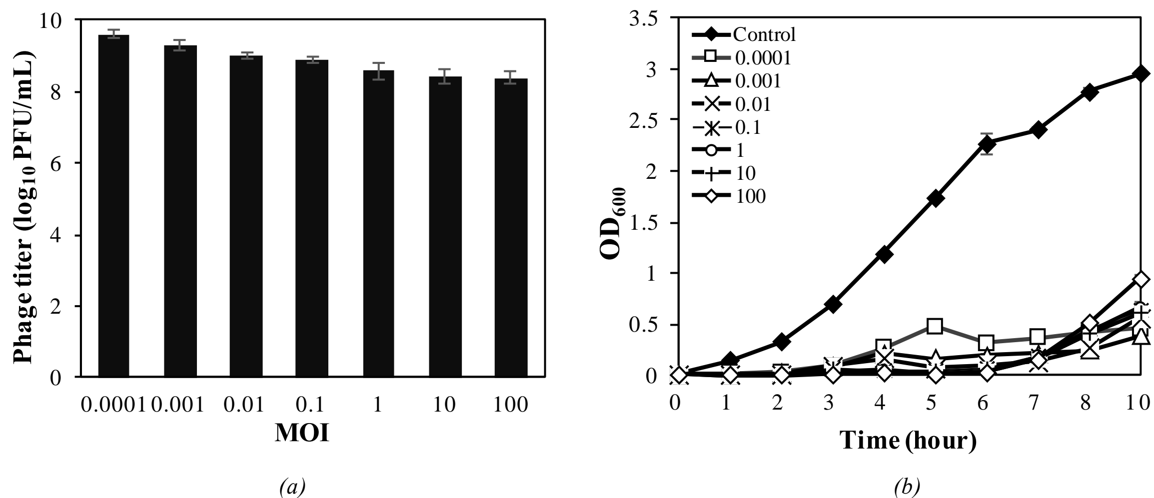
(a) *(b)*

Figure 2. *Plague morphology (a) and transmission electron photographs of bacteriophage C1 at a magnification of 1000X (b).*

TEM revealed that C1 consisted of a head diameter of approximately 52.73 nm resembling an isometric shape, and a very short tail (Figure 2B). In agreement with our findings, a Podoviridae phage vB_VpaP_DE10 also possessed an icosahedral shape with a diameter of 52.4 □ 2.5nm [15]. These indicated that C1 could be a putative member of the Podoviridae family under the Caudovirales order according to classification guidelines by the International Committee on Taxonomy of Viruses (ICTV) 2020 [16]. Of note, a large number of studies identified Vibrio lytic phages belonging to Siphoviridae family [4], [8]. In Vietnam, only V. parahaemolyticus lytic phage isolated from Can Tho province was reported to belong to Demerecviridae [8]. Hence, the current study was the first identification of Podoviridae phage from a shrimp farm in Vietnam.

3.3. Determination of optimal multiple of infection

Given that optimal MOI is the value at which a maximal phage yield is achieved [9], the optimal MOI of phage C1 was determined using *V. parahaemolyticus* H3 as the host (Figure 3A). At MOI of 0.0001, the highest bacteriophage titer at 9.6 ± 0.12 log PFU/mL was observed after 10-hour incubation at 37°C, indicating that 0.0001 was the optimal MOI. In agreement with our results, Yu *et al.* reported that the optimal MOI for *V. owensii* phages P7A and P8D was 0.0001. Meanwhile, the optimal MOI of three *Podoviridae* phages Vp22, Vp02 and Vp33 was 0.001, 0.1 and 1, achieving phage titers of 9.52, 10.02, and 8.34 log PFU/mL, respectively [10]. This discrepancy indicates varying MOI could be observed for different phages. Moreover, a lower MOI of C1 implies that fewer phages are required to infect the same number of bacteria, rendering it a favorable option to minimize application costs in phage therapy development.



(a) *(b)*

Figure 3. *Determination of optimal MOI (a) and lytic activity (b) of C1 against V. paraheamolyticus H3 at different MOI for 10 h.*

In vitro lysis activity of phage C1 against *V. paraheamolyticus H3* was evaluated using seven MOI ratios (Figure 3B). For the control, the OD_{600} value of *V. parahaemolyticus* H3 kept increasing and reached approximately OD_{600} of 3.0 after 10 h. Meanwhile, all phage-treated samples exhibited a significant reduction of OD_{600} during 10 h. This result indicates a highly effective lytic activity of phage C1 on the host *V. parahaemolyticus* H3 regardless of the MOI ratios. The vibriophage vB-VpP_MGD2 of the *Podoviridae* family, demonstrated a complete inhibition of *V. parahaemolyticus* strains only at a high MOI of 100, while lower MOIs only sustained the effect for about 4 hours [17]. Although there was a slight increase in OD_{600} for all C1 treated samples at around 7 hours, bacterial growth remained suppressed throughout the 10-hour infection. This rebounding effect could imply the emergence of phage-resistant progeny possibly due to unexplored mutations in the host bacteria or selective pressure on phage infectivity. Bacterial resistance is often eliminated by using phage cocktails.

3.4. Bacteriophage one-step growth curve

One-step growth curve was constructed to further characterize the life cycle of bacteriophage C1 on host *V. parahaemolyticus* H3. As depicted in Figure 4, the life cycle of phage C1 includes a short latent phase of less than 10 min, a burst phase lasting about 40 min with a burst size of 294 PFU per infected cell, followed by a plateau phase (Figure 4). This demonstrated phage C1 ability to produce a large number of progeny in a short time while effectively infecting the host. In agreement with our findings, *Podoviridae* phages, including vB_VpaP_DE10, vB_VpP_DE17, and vB_VpP_MGD2, were reported with a short latent phase from 5-25 min and burst period from 25-40 min [15], [18]. C1 had a stronger lysis capacity at 294 PFU/infected cells, as compared to vB_VpaP_DE10 (244 PFU/infected cells). In addition, GHSM17 phage was considered to have an excellent lytic effect on *V. parahaemolyticus* with a longer latency period (20 min) and rise phase (100 min) and a higher burst size of 316 PFU/cells [17]. Taken together, phage C1 possesses attractive characteristics for being a phage therapy candidate.

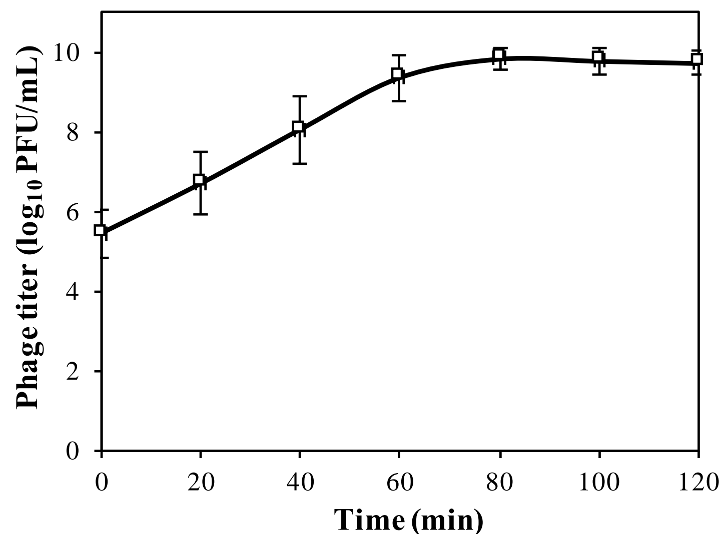


Figure 4. *One-step growth curve of bacteriophage C1 on host V. parahaemolyticus H3*

3.5. Temperature and pH stability of bacteriophage

The ability to withstand environmental physical and chemical factors, such as temperature and acidity, is critical in determining an effective candidate for industrial applications [12]. Phage C1 could survive temperature below 60°C and remained stable from 20- 50°C with titers of around 8 log PFU/mL (Figure 5A). However, after being exposed to 70°C, C1 titer decreased noticeably to only about 5 log PFU/mL which was then completely diminished at 80°C. Sensitivity to different pH levels is illustrated in Figure 5B with high activity of C1 sustained from pH 5-11 (~ 8.0 log PFU/mL). Meanwhile, C1 phage titer was significantly affected at pH 4.0 (7.1 ± 0,106 log PFU/mL) and no infectivity was observed at pH 3.0. Likewise, increasing the pH level to 12 witnessed complete inactivation of C1.

In a previous study, vB_VpP_DE17 was assessed at a wider temperature range and displayed stability from 4-50°C as well as a consistent pH of 5-10 [18]. Some phage members of the *Siphoviridae* family, for example, two phages BA3 and CA8, were more sensitive to temperature and pH changes with optimal ranges from 20-40°C and pH 5-7 [19]. These results indicate that different phage strains tolerate different thermal and acidity conditions. The ability of C1 to resist environmental stressors indicates C1 could be a useful phage for applications against pathogenic *Vibrio* spp.

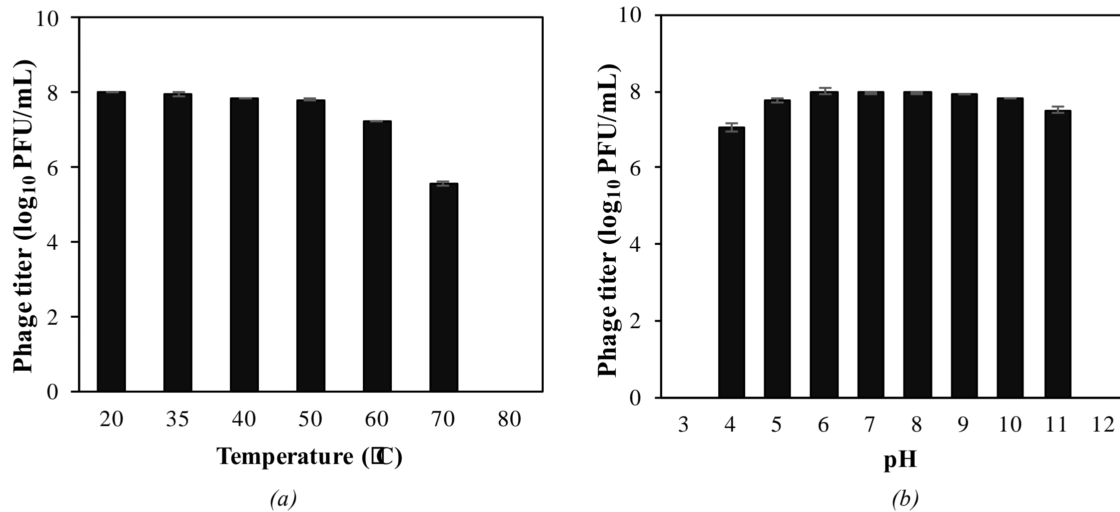


Figure 5. *Effects of different temperature (a) and pH (b) on the stability of bacteriophage C1*

4. Conclusion

In this study, we provided for the first time the isolation of a *Podoviridae* vibriophage C1 from shrimp farms in Quang Ninh province. Phage C1 displayed a broad host range against 4 tested *Vibrio* strains. Using *V. parahaemolyticus* H3 as a host, the optimal MOI for bacteriophage propagation was determined to be 0.0001 and lytic potential was sustained for 10 h. Importantly, C1 exhibited a short latent period, a big burst size and the ability to tolerate an adequate range of temperature and pH levels, making it a desirable candidate for phage therapy. Future exploration of the C1 phage genome, including analysis of lysogenic and virulent genes, could provide additional insights into its safety and suitability as an alternative biocontrol agent for treating shrimp diseases caused by *Vibrio.*

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