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Influencing Factors on Up-stream Process of Edwardsiella ictaluri Bacteriophage Preparation

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Catfish filet (*Pangasianodon hypophthalmus* (Sauvage) is an important export product of East Asian countries. Aquaculture of these fish has often been threatened by a serious disease, bacillary necrosis of pangasius (BNP) that is caused by *Edwarsiella ictaluri* bacteria. Bacteriophage therapy is considered an alternative reagent to antibiotics for controlling the disease. In this study, a batch process was used to prepare vB_EiA_PVN09 bacteriophage product toward its application in aquaculture of catfish in Vietnam. The influencing factors in the upstream process investigated here included medium type, pH, divalent cations, infection time point, MOI value, aeration, and temperature. The stability of phage product (raw lysate) during storage was also assessed. The results showed that *E. ictaluri* medium (EIM) broth was the best for formation of phage progenies (PFU mL^{-1}) but did not indicate phage product stability during storage. Tryptic Soy Broth (TSB) broth was selected to investigate the influencing factors. It was found that the most sensitive factor was medium pH, which, when lower than 6.0, would completely suppress the process. The second sensitive factor was phage infection time point. Adding phage later than 4 h reduced 1 – 2.5 log PFU mL^{-1}. Mg^{2+} or Ca^{2+} could enhanced phage yields by 1.5 - or 3-fold. MOI (0.01 - 1.0) and temperature (24 - 30 °C) did not cause significant difference in the yield while aeration (0 - 150 rpm) had slightly effects. The preparation made under conditions of pH 6.0 containing 0.6 mM Ca^{2+}, 150 rpm, at 28 °C, phage infection point at 3 h with MOI = 0.1 and further incubation for 5 h was able to produce 9.72 ± 0.40 log PFU mL^{-1} (n=5) phage progenies. Phage product had a half-life of about 30 d at room temperature. Our study has pre-established an upstream process for *E. ictaluri* bacteriophage preparation in a 40 mL batch with verified factors.

1. Introduction

The striped catfish (*Pangasianodon hypophthalmus*) is the most important aquaculture fish species in Vietnam because of the high demand for catfish fillet products for export. Tripped catfish farming takes place mainly in the Mekong Delta area in Vietnam. During the rainy season, when temperatures drop below 28 °C, a serious disease called bacillary necrosis of pangasius (BNP) caused by *Edwardsiella ictaluri* bacteria (Dung et al., 2008) may be developed in this fish species. BNP is diagnosed in Vietnam striped catfish showing symptoms of light-coloured nodules in internal organs such as the kidney, liver, and spleen (Ferguson et al., 2001). The fatality rate of BNP disease reported in catfish fingerlings/juveniles and adults was 90-100 % and 30-50 %, threatening striped catfish production (Vietnam Journal of Seafood., 2020). The use of antibiotics to cure and prevent the disease were very common in Vietnam before 2020, also posing risks to consumers and the environment.
Recently, phage therapy has been considered as an alternative solution to antibiotics in aquaculture because antibiotics react non-specifically to both malicious and beneficial bacteria and induce the antibiotic resistance in bacterial pathogens, while bacteriophages combat these pathogens more specifically and naturally (Kowalska et al., 2020). Phage products used in therapy so far have mainly been liquid (Żaczek et al., 2020). Phage products for fish have only been deployed in research environments, with no commercial use as of yet (Kowalska et al., 2020). Batch mode is always the first choice for production for research because it is simple, cheap and

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can produce high phage concentrations (Tanir et al., 2021). The phage titer produced in batch reactors varies depending on phage type, its host, and processing techniques (Kowalska et al., 2020). The phage candidate for production must be lytic, not lysogenic, so that multiple phage cycles will be yielded with a host clearance from environment (Tanir et al., 2021). The upstream processing to produce a lytic phage product is generally formed in four consecutive steps: first, host bacterium is grown until it reaches a desired physical state for phage infection; second, phage is added for infection, depending on multiplicity of infection (MOI); third is post-infection incubation for multiple phage cycles; fourth is the removal of uninfected host cells to obtain the raw lysate containing active phage progenies (Tanir et al., 2021).

Several bacteriophages specific to *E. ictaluri* were studied as phages ΦeiDWF and ΦeiAU (Walakira et al., 2008), PEi21 (Yasuike et al., 2014), MK7 (Hoang et al., 2018), phages G1, G7, G8, G9 (Hoang and Pham, 2021), phages PVN06 and PVN09 (Nguyen et al., 2021), but no phage product based on them has been developed to control the disease in fish (Schulz et al., 2022). In this study, bacteriophage vB_EiA_PVN09, a safe phage for aquaculture based on its verified genome (Nguyen et al., 2021) was produced toward its application in fish. The host used in the production was *E. ictaluri* strain E1, which has been studied previously (Hoang et al., 2018) for isolation and characterization of vB_EiA_PVN09 (Nguyen et al., 2021). Technical parameters in the first three consecutive steps of the process described above consisting of medium type, pH, divalent cations, infection time point, MOI value, aeration and temperature were studied. For convenience to use in a tropical area like Mekong Delta, the lifetime of end-product during storage at room temperature was also investigated.

2. Materials and methods

2.1 Materials

The bacterial host strain *Edwardsiella ictaluri* E1 (Hoang et al., 2018) and its bacteriophage vB_EiA_PVN09 (Nguyen et al., 2021) were isolated and characterized prior to this study. Bacteria and phage were in stocks kept at -70 °C. Tryptic Soy Broth (TSB, Himedia, India), Brain Heart Infusion (BHI, Merck, Germany) and *E. ictaluri* Medium (EIM, Alphachem, Vietnam) media and chemicals were high quality grades.

2.2 Selection of broth medium and effect of MOI values on the phage production

Lytic phage cycles use the biosynthesis of the host to make new phage DNAs and proteins for assembly. This process therefore depends directly on the host's development factors such as nutrition (medium composition), environmental condition (pH, oxygen, temperature, ions) and number of phages per host cell (MOI). TSB, BHI and EIM are three broths routinely used for isolation and growth of the host *E. ictaluri* bacteria, and so were used here to evaluate phage production efficiency. Experiments were carried out in glass flasks each containing 40 mL of either TSB, BHI or EIM broth. The preparation process was started by adding 5 % volume of the overnight-cultured bacterial E1 inoculum (adjusted to OD_{600nm} of 0.5 (ChromTech, USA) and incubated with 150 rpm shaking at 30 ± 2 °C (Jisico, Korea). After a 3-h incubation, with the host bacterial population at about 10^7 CFU mL^{-1}, phage infection was conducted by adding stock bacteriophage vB_EiA_PVN09 to reach a MOI of 0.01, 0.1 or 1.0. Phage formation time course was followed within 5 h post-infection. At each hour interval, the mixture was withdrawn and centrifuged at 10,000 rpm, 5 min at 4 °C (5415R, Germany). Phage titer in the supernatant was quantified by a plaque assay method using the host bacterium as a lawn (Hoang et al., 2018). In order to test the lifetime of the product during storage, phage lysate was filtered through a 0.45 µm membrane and then divided in two samples, one kept at room temperature (30 ± 2 °C) and one in a refrigerator (4 ± 2 °C). Phage titer in samples was counted (PFU mL^{-1}) weekly after the date of manufacture using a plaque assay method (Hoang et al., 2018).

2.3 Phage infection point

In addition to nutrition, environmental conditions and MOI, physiological state of the host population at lag-phase, log-phase or stationary phase may also be critical for phage infection and amplification. The phage must be added into the host being cultured in order to start the phage cycles. The time at which to add the phage was the target of investigation. The experiment was performed in a 40 mL TSB medium and incubation condition as described above. Two variants were used: (a) *without phage infection* to assess the host population, and also (b) *phage infection* to vary the phage-infection times. In (a) variant, the host cells were enumerated (CFU mL^{-1}) using TSA plates (Dung, 2010) every hour of incubation. In (b) variant, a volume (0.01 % v/v) of phage stock (10^9 PFU mL^{-1}) was added into the host growing culture at 0, 1, 2, 3, 4, 5, 6 or 7 h, and further incubated for 5 h. Following an hour interval, phage lysates were obtained in the manner described above and phages were counted (PFU mL^{-1}) according to a previously published method (Hoang et al., 2018). The actual MOI value was calculated using the bacterial cell number (CFU mL^{-1}) counted at each host growth interval (0 – 7 h) and the added phage titer (PFU mL^{-1}).

2.4 Effects of medium pH, aeration, divalent cations, and temperature in the phage production

Because pH, oxygen, temperature and ions are the main environmental factors that influence bacterial growth, they were considered to be critical to phage cycles. Phage production was performed in a glass flask containing 40 mL TSB medium. Medium pH, aeration, divalent cations (Mg^{2+}/Ca^{2+}) concentration, and processing temperature were investigated in the experiments, with the conditions shown in Table 1. The time course of phage formation was followed at an hour interval within 5 h post-infection. Phage lysates were obtained at each hour interval by mean described above and phages were counted (PFU mL^{-1}) by plaque assay (Hoang et al., 2018).

Table 1: Experimental conditions for investigation of the influence factors

Factors	pH	Aeration	Temperature	Divalent cations
Infection time at 3 h	x	x	x	x
MOI of 0.1	x	x	x	x
TSB medium pH 6.0	4.0, 5.0, 6.0, 7.0, 8.0, 9.0	x	x	x
150 rpm sharking	x	0 rpm, 50 rpm, 150 rpm	x	x
Temperature of 30 ± 2 °C	x	x	24, 26, 28, 30 °C	x
Divalent cations	-	-	-	0, 0.2, 0.4, 0.6, 0.8, 1.0 mM

2.5 Statistical analysis

One way ANOVA (SPSS 22.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to assess the significance between the phage yields in each parameter group. A value of $p < 0.05$ was considered of statistically significance.

3. Results

3.1 Type of medium and effective MOI

The medium affects not only the growth kinetics and physiology of the bacteria but also the phage adsorption and phage yields (João et al., 2021). Differences in medium composition caused differences in phage yields as shown in Figure 1. Highest progenies were around 8 log PFU mL^{-1}, 9 log PFU mL^{-1} and 10 log PFU mL^{-1}, obtained in TSB, BHI and EIM, respectively. Rate of progeny formation was fastest in BHI broth, which reached the maximum within 2 h (Figure 2b), while the rate in EIM medium was slowest reaching the maximum at 5 h (Figure 1c). BHI broth composition is based on serum conditions that support growth of the pathogenic bacteria in animal blood. Given that the *E. ictaluri* E1 bacterium was isolated from catfish liver and kidney (Hoang et al., 2018), serum condition may be suitable to accelerate phage-host interaction. EIM is a selective medium that usually contains agents, bile, etc. to suppress the development of the enteric bacteria, so it may also suppress the acceleration of phage formation as well. TSB broth is the simplest composition and used for general microbiological purposes. Many heterotrophic bacteria, whether of environmental or animal origins, can grow in TSB. The time of 3 h to get maximal yield in TSB (Figure 1a) indicated that, without any selective agent, such

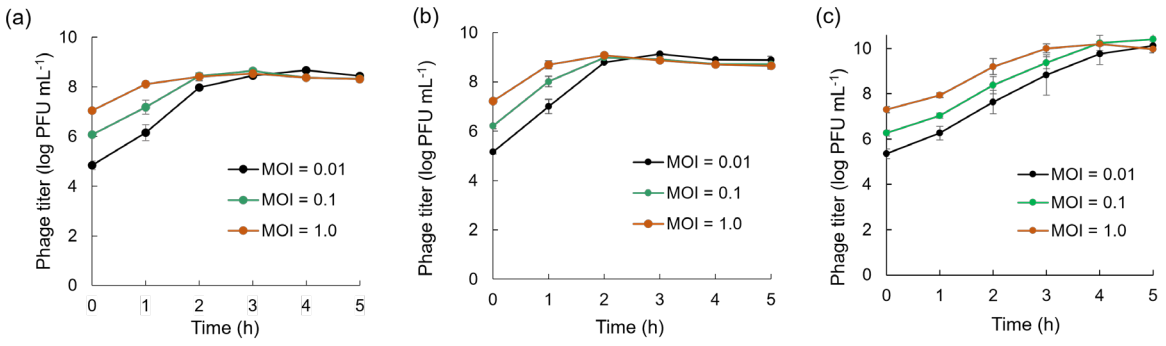

Figure 1: Effect of broth type medium and MOI value on phage production yield (PFU mL^{-1}). (a) TSB; (b) BHI and (c) EIM.

as bile, it would be better for phage cycles. In TSB, the effect of the MOI value was not in the maximum, but in the acceleration rate. Higher MOI in a range of 0.01 - 1.0 had a faster rate of acceleration. Three MOI values produced similar yields (p value of 0.229, 0.348, and 0.376 calculated for TSB, BHI and EIM variant).

3.2 Effect of medium type on the product lifetime

The phage lysates produced in TSB, BHI and EIM broth had lifetime differences of up to 100 d in storage under 4 °C and room temperature, as shown in Figure 2. Phage activity in three broths was retained well during storage at 4 °C, but not at room temperature. The phage number lost about 1.0 log PFU mL^{-1} in TSB condition (Figure 2a) and 2.0 log PFU mL^{-1} in BHI and EIM (Figure 2b and 2c) after 100 d at room temperature. EIM was suitable for higher phage yields (Figure 1c) but did not provide a good lifetime duration of the product. TSB, the main input material for the process, is commercially sold and commonly found at a low price, so it is good for reducing production cost. All components of TSB, including soy, yeast extract and salt, are also safe and nutritious for fish. TSB was chosen as the target of investigation regarding technical parameters of the production process. Estimated half-life of the raw lysate (free of host cells) produced in TSB stored under room temperature (26 ± 4 °C) was about 30 d (Figure 2a), retaining more than 10^7 PFU mL^{-1} after three months.

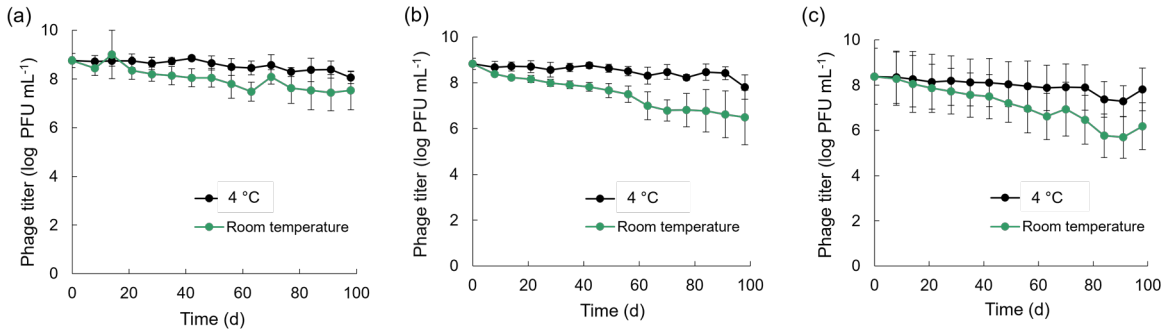

Figure 2: Effect of medium type to time life of the products during storage. (a) TSB; (b) BHI and (c) EIM, n = 3.

3.3 Time for infection in TSB medium

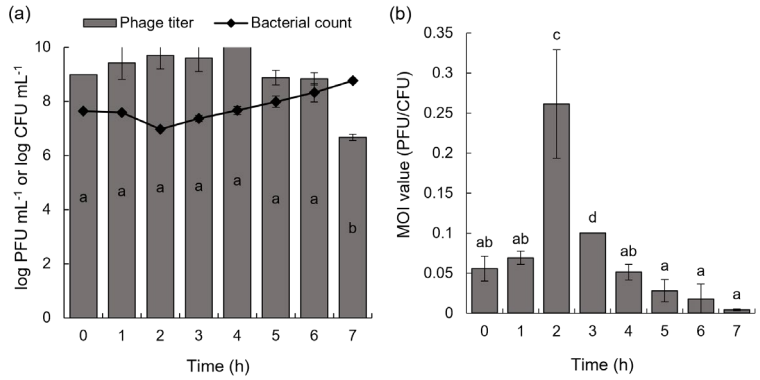

Figure 3: (a) Bacterial growth curve and phage yields at different infecting times and (b) the corresponding MOI values, n = 4

One critical question pertaining to the whole process is which physical state of host cells would be best for phage infection and amplification. In many cases of bacteria at development state, the early or midden log-phase was normally the point for infection. Our results showed that lag-phage, not log-phase, was the best for phage yield (Figure 3a). Before 4 h, the bacterial cells were still at adaptation step (Figure 3a, CFU mL^{-1} curve), with phage yield slowly increasing and then highest at 4 h (Figure 3a, columns). Interestingly, at 4-h culture, the host cells entering early-log phase with cells increased (CFU mL^{-1}) while infection at 5–7 h caused phage yield to drop 1–2.5 log PFU mL^{-1}. These reductions could not have been due to MOI (Figure 3b) because all MOI values were in a range 0.1 - 1.0 investigated and had similar yields (Figure 1a). The explanation for infection time point in *E. ictaluri* species falling at lag-phage rather than in log-phage also seen in the other bacteria (Wang, 2006), is not known. One reason might be the specific physical characteristics of pathogenic *E. ictaluri* strains, which have

been found in fish organs (Pirarat et al., 2016) and observed as able to survive inside macrophages of catfish (Booth et al., 2009).

3.4 Effect of medium pH, aeration, divalent cations, and temperature in TSB medium

Effect of medium pH is shown in Figure 4a. It is clear that the phage yields strongly depend on pH. Lower pH than 6.0 could completely suppress the phage production process altogether. This suppression at low pH might reflect the pH sensitivity of progenies' activity (Nguyen et al., 2021) or the pH inhibition of phage multiple phage cycles. In a large pH range, 6.0 – 9.0, phage production had a similar rate and maximum (Figure 4a), signifying an advantage for processing.

Aeration, or shaking level, influenced the process shown in Figure 4b as higher dissolved oxygen (150 rpm) induced a faster rate of progeny formation, while no shaking (0 rpm) caused slower that. The host *E. ictaluri* is facultative, so it is not surprising that high oxygen accelerated progeny formation. The process with 150 rpm produced 5-6 fold higer maximal yield than that in the other conditions. At low oxygen (0 rpm), the yield could reach almost the same maximum as in 50 rpm conditions ($p = 0.001$). It should be emphasized that phage vB_EiA_PVN09 was isolated from fish body (Nguyen et al., 2021), therefore, oxygen needed at a low concentration for the entire phage cycles observed in this study might also hold some significance regarding phage-host interaction in-vivo.

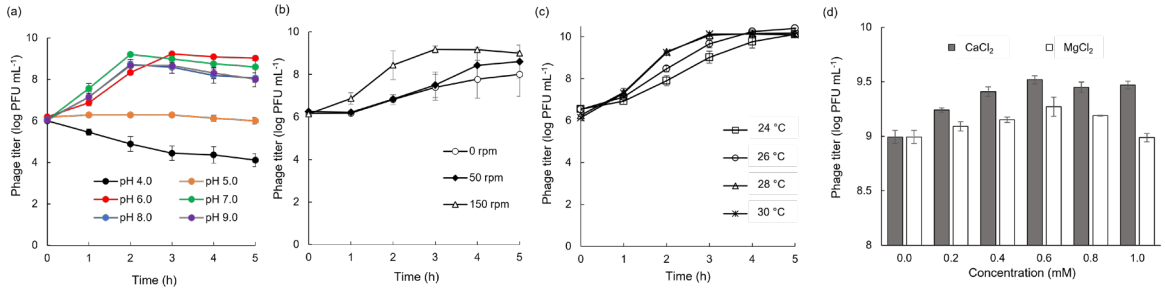

Figure 4: Influence factors. (a) Medium pH; (b) Aeration; (c) Temperature and (d) Divalent cations.

Temperature effecting to phage yield is shown in Figure 4c. The yield was not significant difference between 24 – 30 °C, $p = 0.11$. Phage- host interaction was not significantly sensitive within this temperature range although it was not that when water dropped lower than 28 °C it is easier for fish to catch a disease (Dung et al., 2008). Phage – bacteria interaction may not relate to such critical temperature point for pathogenicity in-vivo.

Divalent cation like Ca^{2+} or Mg^{2+} could enhance permeability of the bacterial cell envelope (Clifton et al., 2015), and thus may influence bacteriophage infection and amplification. As shown in Figure 4d, 0.6 mM concentration of Mg^{2+} or Ca^{2+} slightly increased phage yield of a maximum of about 1.5 - and 3-fold. At a concentration higher than 0.6 mM, Mg^{2+} suppressed the process, while Ca^{2+} did not. Ca^{2+} also enhanced phage-host activity in bacteria such as *E. coli* (Chen et al., 2016), *Lactobacillus* sp. (Ma et al., 2018), even to the extent required for bacteriophage to kill *Bacillus cereus* (Bandara et al., 2012).

4. Conclusions

This work is the first about condition for maximal mutiple cycles of an *E. ictaluri* bacteriophage, in a particularly case of vB_EiA_PVN09 phage and its host *E. ictaluri* E1. The factors affecting to the phage yield significantly were medium broth type, phage infection point, medium pH, and Mg^{2+}/Ca^{2+} concentration. These factors are important for phage - host interations as observed here in-vitro and might also hold meaning for this phage combating its host in-vivo. The process verified with the following procedure: (1) process was begun with about 10^7 – 10^8 CFU mL^{-1} fresh bacteria in medium TSB broth with 0.6 mM Ca^{2+}, pH 6.0, and incubation at 150 rpm at 28 °C; (2) phage with MOI of 0.1 was added at 3-h host culture; (3) post-infection incubation for 5 h. Through this pre-established process, a phage product of 9.72 ± 0.40 log PFU mL^{-1} (n=5) was obtained. Phage product had a half-life of about 30 d at room temperature, which is convenient for agricultural uses in South Vietnam. More research is needed for scaling up the preparation volume and formulating the end-product to increase the phage lysate lifetime.

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