Isolation of *Bacillus* strains inhibition disease resistance of Acute Hepatopancreatic Necrosis Disease on the Shrimp in Can Gio, Vietnam

L T Phung¹, T T M Phuong², N K Phung³ and M Nicolas⁴

¹ Ho Chi Minh City University of Natural resources and Environment (HCMUNRE), Vietnam
² Ho Chi Minh City University of Science (HCMUS) - Vietnam National University, Ho Chi Minh City, Vietnam
³ Department of Science and Technology, Ho Chi Minh City, Vietnam
⁴ Université Côte d’Azur. Parc. Valrose, 06103 Nice Cedex 2, France

*ltphung@hcmunre.edu.vn

Abstract. Recently, the outbreak and severe damage to the shrimp farming industry is Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND). The selection of *Bacillus* strains inhibition disease resistance of the acute hepatopancreatic necrosis disease on the shrimp is a research filed that is much attention due to *Bacillus subtilis* is a group of beneficial bacteria that presents in the majority of biological products for aquaculture, especially for shrimp. Competitiveness of *Bacillus subtilis* towards the harmful bacteria firstly is the number of bacterial cells. Beside, *Bacillus* has capacity to create a large amount of extracellular enzymes aid digestion, antibiotics or the inhibitor has the characteristic for resistance to the strains of *Vibrio Parahaemolyticus*. In this work, the inhibition ability of the *Bacillus* strains for the AHPND on the brackish water shrimp is studied. In which, the bacteria is isolated and filtered from shrimp farm in Can Gio province that contain the characteristic of antagonizing *Vibrio parahaemolyticus*, which cause AHPND on shrimp in laboratory conditions. The results show that *Bacillus subtilis* produced 2,5 – Diketopiperazines capable to inhibit the growth of pandemic strain of *Vibrio parahaemolyticus* in Landy media for highest activity was 200 AU/mL after 24 h of culture. This research is create the possibility of using probiotics to control the pantogenic clones *Vibrio parahaemolyticus* that cause AHPND for brackish water shrimp farming in Can Gio province as well as other areas in Vietnam.

1. Introduction

In recent years, one of the many shrimp deaths has been known as *Acute Hepatopancreatic Necrosis Disease* – AHPND, also known as *Early Mortality Syndrome* - EMS. The agent is determined to be due to *Vibrio parahemolyticus*, AHPND strains causing serious damage to Vietnam's shrimp industry. So far, there is almost no specific treatment for this disease, most of the strains of *Vibrio parahaemolyticus* are completely resistant to oxytetracyclin, an antibiotic that is mainly mixed into periodic shrimp feed [1]. The use of antibiotics to treat diseases is not effective, and also affects the environment, growth and quality of shrimp [2]. Many domestic and foreign studies have shown that the use of *Bacillus* to inhibit some species of pathogenic *Vibrio spp.* has been effective because they
are capable of developing and creating spores in the stomach and digestive system, secrete antimicrobial compounds, regulates the immune system and secretes quorum sensing enzymes [3]. Studies of author groups such as Hong and etc., 2005; Dong et al., 2001; Mujeeb and partner., 2015 found inhibitors in Bacillus bacteria isolated from the environment in giant freshwater shrimp ponds (Macrobrachium rosenbergii) to combat pathogenic bacteria such as Aeromonas hydrophila, Vibrio parahaemolyticus, Vibrio vulnificus and bacteria Escherichia Coli [4] [5]. These research results show that Bacillus bacteria are capable of developing and creating spores in the stomach and digestive system, secrete antimicrobial compounds, regulating the system immunity and secretion of quorum sensing enzymes [6]. So some of the current products are bacteria that make spores in which Bacillus strains are most used [7].

In our country, biological products used in aquaculture are imported at high prices and are not really suitable for the conditions of Vietnam [8]. In particular, no research has been done to produce biological products against shrimp AHPND. Therefore, it is necessary to study the selection of Vibrio parahaemolyticus resistant strains that cause AHPND bacteria on shrimp to produce biological products for AHPND prevention and treatment. In this study, we evaluated the antagonistic properties of Bacillus strains isolated and selected from the shrimp pond environment in Can Gio with Vibrio parahaemolyticus causing AHPND syndrome on shrimp. This contributes to the diversity of strains and anti-AHPND properties in probiotics products [9].

2. Materials and Methods

2.1 Materials and sample preparation

Luria Bertani culture medium (LB) was prepared by 10 g Trypton, 5 g Yeast Extract, 10 g NaCl, 1 liter distilled water. Tryptone Soy Agar medium (TSA) was prepared by 22.5 g Trypton, 1 g Pepsoy, 26 g Agar, 1 liter distilled water at pH = 7.3. Tryptone Soy Broth medium (TSB) was prepared by 22.5 g Trypton, 1 g Pepsoy, 25 g NaCl, 1 liter distilled water at pH = 7.3. Landy medium was prepared by 20 g Glucose, 1 g High yeast, 5 g L-sodium glutamate, 0.5 g MgSO4, 0.5 g KCl, 1 g KH2PO4, 0.15 mg FeSO4, 0.5 mg MnSO4, 0.16 mg CuSO4, 1 liter distilled water at pH = 7.0 - 7.6. M9 medium was prepared by 1 g/L Casamino acid, 1 g/L Na2HPO4, 2 g/L KH2PO4, 1 g/L NH4Cl, 4 g/L NaCl, distilled 960 mL of water, 10 mL of MgSO4 (24g/L); 10 mL of CaCl2 (1.5 g/l); 1 mL of vitamin B1 (10 g/L); 100 mL of glucose (200 g/L) adjust mild acidic medium at pH = 4.

2.2. Isolation of pantogenic bacteria Vibrio parahaemolyticus causing AHPND disease on farmer shrimp in Can Gio province

The pantogenic clones Vibrio parahaemolyticus that cause AHPND was isolated [10] from diseased shrimp collected in Can Gio province. A total of 4 samples of specimens characterized by AHPND were collected, (each sample collected 5-10 diseased shrimps). Samples were inoculated with Chrome agar (selective medium for Vibrio sp.) at 28 °C and tested after 24 h. Select specific colonies for Vibrio parahaemolyticus (purple colonies) then purify by implanting beards on Chrome agar medium until only one type of colony is observed on the plate. Purple colonies were selected for storage for analysis. The bacterial strains V. parahaemolitcicus were stored at -80 °C in nutrient broth (NB, Oxoid) containing 15% glycerol and 1% (w/v) NaCl.

2.3. Extraction of 2.5-Diketopiperazines from Bacillus subtilis

The bacteria Bacillus in this work were isolated from sediments in shrimp ponds in Can Gio province. Bacillus are grown in M9 medium [11]. Incubate bacteria Bacillus subtilis at 20°C, stirring for 96h (4 days) to achieve a initial content of 1 x 107 CFU/mL. Then the biomass obtained was added ethyl acetate at a concentration of 150 mL/liter of biomass, then organic phase was separated after 20 min of settling with Na2SO4 solution. The solution obtained was filtered through filter paper, dried at 45°C and placed in a desiccator for 24 h, then stored at -20 °C for experiments [12].
2.4 Test of resistance according to the method of perpendicular straight line. 
Investigation of antagonistic ability of strains *Bacillus* on TSA Agar [12]. Steps has the following improvements: transplanting *Bacillus* bacteria along a straight line on agar plates, incubated at 37 °C for 24 h, conducting *Vibrio parahemolyticus* in the horizontal lines perpendicular to the bacterial path, continue to incubate at 37 °C for 24 h. Antimicrobial activity was determined by measuring the antibacterial zone distance in mm units.

2.5 Evaluation of antagonistic properties of selected *Bacillus subtilis* with *Vibrio parahemiliticus*. 
We proceeded to spread 50 µl solution of *Vibrio parahemolyticus* (10⁵CFU/mL) on TSA medium to allow the medium to dry for 15 min. Subsequently, the bacteria *Bacillus subtilis* examined (single or mixed) were drilled into wells with a volume of 50µl 24-48 h of incubation at 28-30 °C in concentration ranges 10³, 10⁵, 10⁷ and 10⁹ CFU mL. The experiment was done in triplicate, after 24 h the antibacterial was defined by ring diameter (D).

Antimicrobial activity (AU/mL) by dilution method twice in a row. Determination of the highest dilution shows the sterile ring and the determination of antimicrobial activity (AU/mL) of fermentation: AU/mL = Df x (1/V); were AU isactive unit; Df is the highest dilution has inhibit ring. To increase the concentration of bacteria to be examined in suitable environment: Prepare a petri dish with 15ml of TSA agar medium. Covering 100 µl of *Vibrio paraheamolyticus* to be examined on agar plates; perforated holes with 5mm diameter on the agar surface; 40 µl of 2.5-DKP inoculant were diluted twice in succession into wells; Incubate at 30ºC, observe after 24 hours. Observe the formation of the sterile ring and the highest dilution, determine the antibacterial activity (AU/mL).

3. Results and Discussion

3.1. Isolation and screening of *Vibrio parahaemolyticus* 
Results of isolating and screening *Vibrio parahaemolyticus* strain was showed that 4 samples were appeared *Vibrio parahaemolyticus* (in 10 samples collected). Image of colonies *Vibrio* sp. growing on selective medium presented in Figure 1: Purple lilac colonies of *Vibrio parahaemolyticus* moss green to turquoise belonging to *Vibrio vulnificus* and *Vibrio cholerae* and colorless (creamy color) belonging to *Vibrio alginolyticus*. *Vibrio spp*. strains are confirmed via biochemical reactions, *Vibrio parahaemolyticus* reaction to oxidase, catalase, has the ability to ferment sugars.

![Figure 1](image1.png)

**Figure 1.** Image results of colonies of *Vibrio parahaemolyticus* (a), (b), (c) when isolated and (d) after grown on selection medium

3.2. Production of 2,5 – Diketopiperazines 
*Bacillus subtilis* bacteria isolated from Can Gio shrimp pond belong to aerobic microorganism group. This study was determined the conditions and environmental components of *Bacillus subtilis* strain to
create antibacterial active ingredients for *Vibrio parahemoliticus* resistance causing AHPND in cultured shrimp. A Landy medium is a suitable culture medium for *Bacillus subtilis* strain, create field cultures containing high antimicrobial activity, specific environmental conditions: pH 7.0; shaking speed 150 rounds/min at a temperature of 37 °C and appropriate temperature is 37 °C cultured for bacterial breeding; 0.5% salt activity was expressed at 200 AU/mL, and after 24 h the activity was highest at 400 AU/mL. From the beginning of 48 h, the antimicrobial activity gradually decreased. These results show that some of the characteristics of these strains differ from those published in previous studies, such as the best *Bacillus marisflavi* [13] strain in the range of 2-5% NaCl, and *Bacillus vietnamensis* is at 1% NaCl [14]. This difference may be due to the isolation of *Bacillus subtilis* strains, which are geographically and climately different. The result of antibacterial activity of *Bacillus* strain according to the perpendicular straight line method are presented in Figure 2.

![Figure 2](image-url)

(a) *Bacillus* sp Bac 4  
(b) *Bacillus* sp Bac 6  
(c) *Bacillus* sp Bac 7  
(d) *Bacillus* sp Bac 10

**Figure 2.** Observing growth inhibition of *Bacillus subtilis* strains that are resistant to *Vibrio parahemoliticus*.

The *Bacillus subtilis* strains with the highest activity of inhibiting *Vibrio Parahaemolyticus* growth inhibition were tested. After 72 h of culture on agar medium at a temperature of 30 °C, the diameter of the inhibition ring appears around the site of *Bacillus subtilis* for 4 microorganisms tested on B4, B6, B7, B10, respectively as shown in Figure 3 and Table 1:
The antagonistic results of Bacillus subtilis strains for Vibrio parahaemolyticus shows that Bacillus subtilis B4 and Bacillus subtilis B6 were able to inhibit the growth of Vibrio parahaemolyticus larger than Bacillus subtilis B7 and Bacillus subtilis B10 (The sterile rings with B4 and B6 were clearer and larger than those seen with B7 and B10). The antagonistic capacity is measured by the antibacterial ring diameter, evaluating the antagonistic capacity according to Sumathi and Reetha (2012) [16]. The data obtained from the experimental of Antagonistic activity of Bacillus subtilis for Vibrio parahaemolyticus strains showed that the expressed more strongly after 48h and 72h of culture. Because of growth inhibitors for Vibrio parahaemolyticus strains produced by Bacillus subtilis, possible in later growth phases of bacterial growth cycle. All of our experiments were performed on disk agar media. To apply highly effective in the environment of shrimp culture, we checked the antagonistic ability of Bacillus subtilis with Vibrio parahaemolyticus on liquid medium.

Due to the limited time, we only tested the antagonistic ability of two strains of Bacillus sp showing the best antagonistic activity against 4 tested microorganisms, with one pathogenic Vibrio parahaemolyticus in fluid medium. The number of Vibrio parahaemolyticus cells grows in the liquid medium before and after the addition of Bacillus subtilis.
4. Conclusions
The results showed that *Bacillus subtilis* was isolated from natural environment in Cangio province, capable of antagonizing *Vibrio parahaemolyticus* causing AHPND in shrimp. Our results suggest that bacteria *Bacillus subtilis* that isolated from Can Gio Province can produce 2.5-Diketopiperazines that have the capacity to inhibit growth of the pathogen *Vibrio parahemolyticus*. Besides, these *Bacillus subtilis* have growth characteristics suitable to shrimp culture conditions in our country such as pH tolerance (5-9), salinity tolerance (0-0.5%), suitable temperature for culture process, and antimicrobial resistance is 37°C. This is of great significance in the process of creating probiotics from *Bacillus subtilis* with strong antibacterial properties for mass production, to contribute to the control of acute hepatopancreatic necrosis of shrimp improve shrimp farming environment conditions, reduce economic risks for human.

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