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Antimicrobial Activity of *Bacillus cereus* and *Bacillus thuringiensis* on Pathogenic *Vibrio harveyi* in *Litopenaeus vannamei*

Marissa Maya Masitoh[1], A. M. Hariati[2] and M. Fadjar[2]
[1]*Master Program on Faculty of Fishery and Marine Science, University of Brawijaya, Malang 65145, Indonesia*
[2]*Department of Aquaculture, University of Brawijaya, Malang 65145, Indonesia*

ABSTRACT: The bacterial strain of *Bacillus cereus* and *Bacillus thuringiensis* has been known to produce antimicrobial activity against pathogenic *Vibrio harveyi*. The effect of *B. cereus* and *B. thuringiensis* were tested by in vitro and in vivo. In vitro test was used to analyze antagonism characteristic of bacteria using the paper disc diffusion method. In vivo test was applied to evaluate antimicrobial activity of *B. cereus* and *B. thuringiensis* (10^5 CFU ml^{-1}) on survival rate and histopathology of *Litopenaeus vannamei* challenged with *V. harveyi*. The results showed that *B. thuringiensis* had a greater inhibitory activity of 18.60–35.97 mm. Both Bacillus bacteria treatment resulted in survival rate of 100%, compared with 75% in the treatment without Bacillus. It can be concluded that *B. cereus* and *B. thuringiensis* have potential applications for controlling pathogenic *V. harveyi* in shrimp aquaculture.
Key words: *Bacillus cereus*, *Bacillus thuringiensis*, *Inhibitory Activity*, *V. harveyi*.

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INTRODUCTION

Aquaculture is the world's fastest growing food production sector. However, disease outbreaks have caused serious economic losses in several countries. Vibrio species are among the most important bacterial pathogens of cultured shrimp. They are responsible for several diseases and mortalities up to 100% due to vibriosis have been reported [1]. Using antibiotics in potential negative consequences of using antibiotics in aquaculture for the prophylactic treatment of diseases are the development of drug resistant bacteria and reduced efficacy of antibiotic treatment for human and animal diseases [2].

In the search for more effective and environmentally friendly treatments, using bacteria like Bacillus provides a solution to these problems. Bacillus have antimicrobial (bacteriocin) which usually occurs in all growth phases and finishes at the end of logarithmic phase [3]. Luis-Villaseñor et al. [4] investigated that the effect of Bacillus showed trait inhibitory to Vibrio and ability to adhere and grow on intestinal mucus.

The purpose of this study was to investigate antimicrobial activity of *B. cereus* and *B. thuringiensis* against *Vibrio harveyi* under in vitro and in vivo conditions.

MATERIAL AND METHODS

Bacterial strains

A virulent strain of *V. harveyi*, was used as a pathogenic strain. Strain were taken from the stock culture collection of our laboratory. *B. cereus* and *B. thuringiensis*, obtained from isolated from the gastrointestinal of L. vannamei and cultured in duplicate in the general media (nutrient agar with 1.5% w/v NaCl) for 18-24 hours at a temperature of 30°C. Pure isolates were taken after subculture on Tryptic Soya Agar (TSA) was used as an antagonistic strain.

Antagonism assay

The methods of paper disc diffusion assays were used in this study. Both groups of the bacterial strains (the tested strains including the compared strain and the pathogenic strain) were briefly grown in tryptic soya broth (TSB), incubated at 32°C for 24 h. After 24 h, each sterilised paper disc was immersion with B. cereus and B. thuringiensis with different concentration (10^4, 10^5 and 10^6 CFU ml^{-1}). And then placed on the surface of an agar plate (TSA) which was previously inoculated with the indicator pathogen at a concentration of about 10^7 CFU ml^{-1}. The plate was then incubated at 32° C for 24 h and the inhibition zone around paper disc was recorded.

SDS-PAGE electrophoresis test for molecular weight

For detection of bacteriocin activity use polyacrylamide gels and stained with silver nitrate to determine the molecular weight of the separated protein. Low molecular mass standards ranging from 10 to 225 kDa were purchased.

Inhibition of pathogenic *V. Harveyi,* in vivo

L. vannamei were introduced into 5 treatment (each in tripled) filled with filtered seawater at salinity of 20 ppt. The set A was inoculated with *B. cereus* at the concentrations of 10^5 CFU ml^{-1} in rearing medium to facilitate attachment or colonization on the larvae for 24 hours. After one day was then exposed to test pathogen (V. harveyi) at 10^7 CFU ml^{-1} for 2 hour. The set B was inoculated with *B. thuringienis* at the concentrations of 10^5 CFU ml^{-1}. After one day was then exposed to test pathogen (*V. harveyi*) at 10^7 CFU ml^{-1} for 2 h. The set C was inoculated with *B. cereus* and *B. thuringiensis* (combined treatment) at the concentrations of 10^5 CFU ml^{-1}. After one day was then exposed to test pathogen (*V. harveyi*) at 10^7 CFU ml^{-1} for 2 h. The set K(n) received no bacterial inoculums and served as control. The set K(-) was inoculated with pathogen alone at 10^7 CFU ml^{-1} in rearing medium to serve as negative control. The number of CFU ml^{-1} in overnight culture of baceria were standardized from OD measurements at 600 nm. Mortality in each set was recorded for twelve days and no water exchange was done during that period.

Statistical Analysis

Results were presented as mean ± mean standard deviation of three replicates. Statistical analysis was performed using SPSS 20.0. To determine the effect of treatment on the response of each parameter used ANOVA analysis followed by Duncan's multiple range test. Levels of significance are expressed as $P < 0.05$.

RESULTS AND DISCUSSION

Antagonism assay

Antimicrobial activity of *B. cereus* and *B. thuringiensis* against *V. harveyi* were performed by paper disc with different concentrations. The diameters of the inhibitory zones of *B. thuringiensis* was 18.60–35.97 mm (Table 1). We suggested that *B. thuringiensis* showed a greater inhibitory activity than others. The antagonistic effect of these isolates on the growth of indicator pathogen could be determined by the appearance of clear inhibition zones around the paper disc. Bacillus species could produce a large number of antimicrobials [3].

Table 1. Antagonistic activity of *B. cereus* and *B. thuringiensis* strains against *V. harveyi*

Bacteria	Inhibition zones (mm)		
	10^4	10^5	10^6
B. cereus	16.47±0.67	22.80±1.14	31.70±1.96
B. thuringiensis	18.60±1.06	25.13±1.07	35.97±1.58
B. cereus + *B. thuringiensis*	13.79±0.81	20.81±1.47	25.46±0.64

SDS-PAGE electrophoresis test for molecular weight

Direct detection of the bacteriocin was performed by SDS-PAGE. Following electrophoresis, several contaminating proteins were detected in sample. The band had an apparent molecular mass of about 10-225 kDa.

Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by Abriouel et al. [5]. The results (Fig. 1) showed that *B. cereus* had molecular weights of 15.53 kDa and 25.21 kDa while *B. thuringiensis* had molecular weights of 12.19 and 23.25 kDa which there are antimicrobial polypeptides of intermediate size.

Inhibition of pathogenic *V. harveyi* in vivo

Survival rate: The results revealed that *B. cereus* and *B. thuringiensis* lead to reduce shrimp mortality under in vivo conditions. Both Bacillus treatments resulted 100 % survival rate of shrimp, while survival rate of 75% was obtained in not treated shrimp (Figure 2). Bacillus significantly increased survival rate and some digestive enzyme activities of shrimp larvae [6].

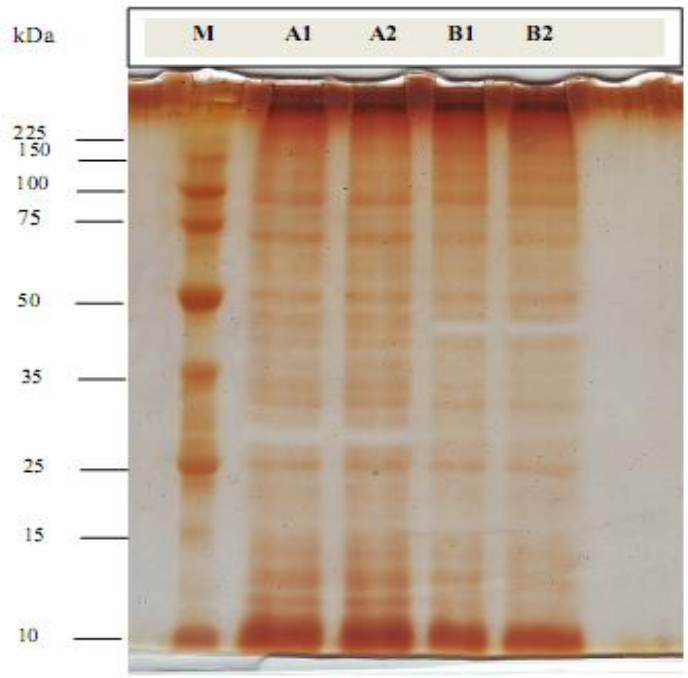

Figure 1. SDS-PAGE analysis protein profile. Line (M): Silver nitrate-stained gel, low range protein standard; line (A1 and A2) protein profile *B. cereus*; line (B1 and B2) protein profile *B. thuringiensis*.

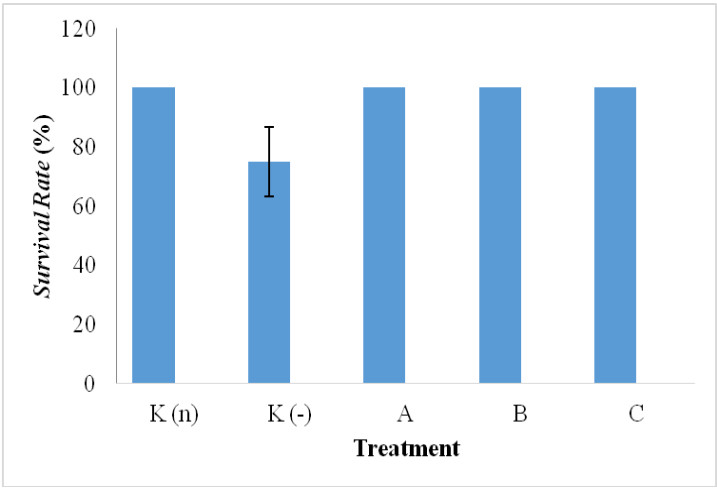

Figure 2. Survival rate recorded during twelve days

Total Haemocyte Counts (THC) : The total haemocyte counts showed significant differences among the treatments. The average of total haemocyte was 5.93 x 10^5 sel mm^{-3} – 89.17 x 10^5 sel mm^{-3} (Table 2). THC of crustaceans plays an important role in regulating the physiological functions including hardening of exoskeleton, wound repair, carbohydrate metabolism, transport and storage of protein and amino acid, haemolymph coagulation and the confinement of invasive organisms by clot formation, phagocytosis, and encapsulation [7].

Table 2. Total haemocyte counts among different treatments

Treatment	THC (x 10^5 sel mm^{-3})
K (n)	12.75 ± 0.75^{d}
K (-)	15.73 ± 0.75^{a}
A	37.3 ± 1.53^{b}
B	89.17 ± 3.75^{a}
C	5.93 ± 0.12^{c}

Fig K(n) : not infected with *V. harveyi* bacteria; Fig K(-): Infected with 10^7 CFU/ml *V. harveyi* bacteria; Fig A: Adding *B. cereus* and infected *V. harveyi*;Fig B: Adding *B. thuringiensis* and infected *V. harveyi*; Fig C: Adding *B. cereus* and *B. thuringiensis* than infected *V. harveyi*.

THC in crustaceans rapidly drops following the injection of foreign material. *B. cereus* and *B. thuringiensis* in *L. vannamei* can be increased significantly after the injection of *V. harveyi*. This study demonstrated promising

results for immune response stimulation in *L. vannamei*. Moreover, Rengpipat et al. [8] explained that *Bacillus* S11 surface antigens, or their metabolites might act as immunogens for shrimp immune defense. *Bacillus* S11 cell wall peptidoglycan might elicit an immune function in shrimps.

Histopathology of Hepatopancreas

The hepatopancreas of control K(n) maintained a good shape and colour. The hepatopancreas of infected larvae were grey and showing vacuolization (Figure 3). The hepatopancreas or digestive gland is a sensitive indicator of diverse physiological states, such as metabolic level, ecdysis phase, nutritional status, disease, etc., so that its use as a general indicator of the physiological condition of the shrimp has been proposed [9].

Figure 3 detected the sites of bacterial presence in the tissue, and the changes caused by the pathogens could be evaluated. Obviously, death will occur when a sufficiently large number of hepatopancreatic cells are damaged by the pathogen, rendering the organ non functional. Robertson et al. [10] explained that the hepatopancreas of chronically infected larvae were grey and aciated, with balls of necrotic tissue. Shrimp with infected by virus or bacteria, pathology of hepatopancreas showing severe necrosis, loss of structure, atrophy of tubule epithelial cells, vacuolation and rounding and sloughing of cells into the lumen [11].

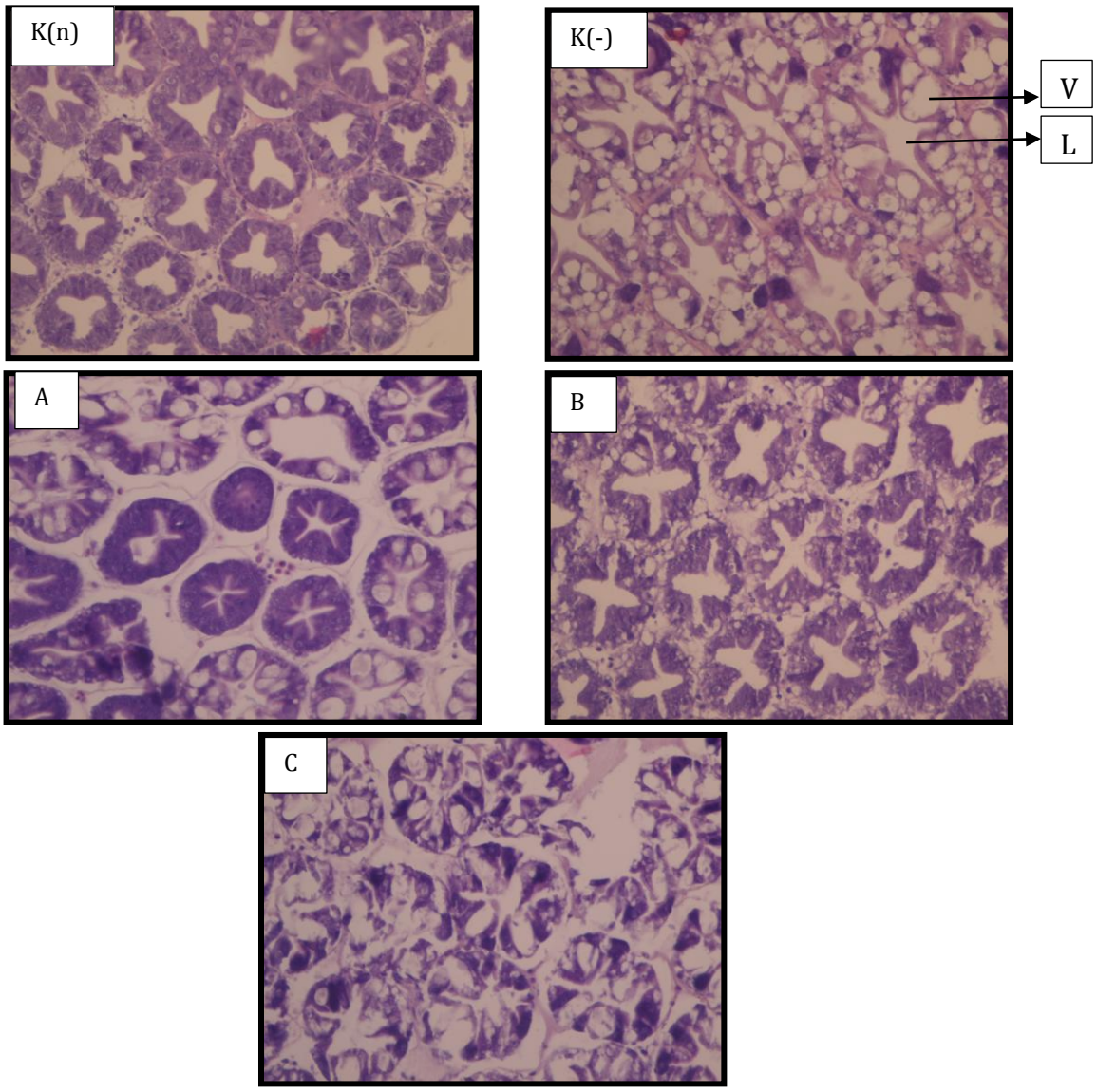

Figure 3. Histopathology of *L. vannamei* hepatopancreas (arrows indicated vacuolation)

Fig K (n): Hepatopancreas of control shrimps group (not exposed to *V. harveyi* bacteria); Fig K (-): Infected hepatopancreas of shrimps exposed to 10^7 CFU/ml *V. harveyi* bacteria. There are many vacuolation of cells (black arrow destruction of hepatopancreas tissue); Fig A: Adding *B. cereus* and infected *V. harveyi*. Some of tissues are healthy (stars/L) also vacuolation is observed; Fig B: Adding *B. thuringiensis* and infected *V. harveyi*. Some of tissues are healthy (stars/L) also vacuolation is observed but low than Fig A; Fig C: Adding *B. cereus* and *B. thuringiensis* and infected *V. harveyi*. There are many vacuolation of cells (black arrow), cells are finally destroyed. L (lumen) and V (vacuolation).

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Competing interests

The authors declare that they have no competing interests.

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