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# Characteristics of anti-*Vibrio harveyi* compounds produced by *Bacillus* spp. isolated from shrimp ponds

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**Abstract.** *Rusmana I, Isramilda, Akhdiya A. 2021. Characteristics of anti-*Vibrio harveyi *compounds produced by* Bacillus *spp. isolated from shrimp ponds. Biodiversitas 22: 4872-4879.* Pathogenic *Vibrio* spp. such as *Vibrio vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. anguillarum*, and *V. harveyi* caused shrimp diseases. The application of bacterial probiotics can control the growth of pathogenic *Vibrio* in shrimp. *Bacillus* spp. can produce antimicrobial compounds that inhibit the growth of pathogenic *Vibrio* spp. Isolation of *Bacillus* from several pond water samples, pond sediment, and shrimp intestines was successfully collected 175 isolates of *Bacillus* spp. Three isolates, i.e., Ltw54, Lts36, dan Lts40 had a high inhibitory index and stability in inhibiting the growth of *Vibrio harveyi.* Verification assay showed that *Bacillus* sp. Lts40 had the highest antimicrobial activity. The competition assay showed that *Bacillus* sp Lts40 isolates inhibited the growth of *V. harveyi* up to 81,8%. The antimicrobial compound produced by *Bacillus* sp. Lts40 was stable at the pH range of 3-11 and remained stable after heating at 100ºC for 20 minutes. The purification results using the chromatographic filtration technique showed that the protein fraction with a molecular weight of 47,38 kDa effectively inhibited *V. harveyi* growth. *Bacillus* sp. Lts40 isolate has potential application as a probiotic agent in shrimp ponds to control the growth of *V. harveyi* that causes luminous vibriosis diseases and the antimicrobial substance is potentially to be developed and produced as an anti-*V. harveyi* product that can be applied in a shrimp hatchery.

**Keywords:** Antimicrobial substances, *Bacillus*, shrimp pathogen, *Vibrio harveyi*

## INTRODUCTION

Some pathogenic *Vibrio* spp. are leading to various 'vibriosis' diseases. These diseases have a significant negative impact on shrimp production (de Souza Valente C and Wa 2021). In shrimp larval rearing, one of the primary disease problems is the zoea syndrome. This disease could result in larval mortality of up to 90 %. Pathogenic *Vibrio* spp. such as *Vibrio vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. anguillarum* dan *V. harveyi* are the main factors causing the diseases. The most important pathogenic *Vibrio* in shrimp cultures is *V. harveyi*. This bacterium is the main cause of bacterial infection in shrimp that can occur starting from the larval level. Bacterial attack at the larval stage can cause mass mortality (Roza 1993). *Vibrio harveyi* (Johnson & Shunk 1936) Baumann et al. (1981) is the dominant group of vibrios from the shrimp pond sediment (40%) and shrimp gut (40%). Pathological symptoms of the diseases were indicated by loss of digestive capacity and nutrient absorption which result in delayed shrimp molting and subsequently causing the death of the larvae with cumulative mortality up to 100% at stage two of zoea (Kumar et al. 2017).

The use of probiotic bacteria is an alternative way to control diseases caused by pathogenic bacteria in shrimp ponds (de Souza Valente and Wa 2021). Probiotics are live microbes that exert beneficial effects on the host by altering the microbial community, improving nutritional value, enhancing the host defense mechanism to disease, and improving environmental quality (Verschuere et al. 2000). Some probiotic bacteria could produce bactericidal or bacteriostatic compounds that inhibit the growth of pathogenic bacteria. Shrimp probiotics commonly used are *Bacillus* that can produce antimicrobial compounds. *Bacillus* can produce antimicrobial polypeptides such as bacteriocins that can inhibit the growth of other bacteria (Irina et al. 2001). *Bacillus* is present in the sediments and digestive tract of shrimp (Verschuere et al. 2000). Application of *Bacillus* sp. as a probiotic in shrimp culture improved resistance performance of Pacific white shrimp (*Litopenaeus vannamei*) against *V. harveyi*. Probiotic applications significantly improved shrimp growth parameters such as weight gain, average daily growth, feed conversion ratio, and survival rate than those of the control against *V. harveyi* (Harpeni et al. 2018). However, information on the characteristic of the antimicrobial compounds produced by the indigenous *Bacillus* spp. isolated from Indonesian shrimp ponds are still limited. Therefore, this paper describes the characteristics of antimicrobial activity against *V. harveyi* and molecular weight of the compounds produced by the indigenous *Bacillus* spp. isolated from several areas of Indonesian shrimp ponds and their activity stability to pH and temperature treatments.

## MATERIALS AND METHODS

### Screening of bacterial isolates producing antimicrobial compounds

Three isolates of *Bacillus* spp. (Lts36, Lts40, and Ltw54 isolates) and *V. harveyi* from the collection of Microbiology Laboratory, Department of Biology, IPB University) were re-cultured in a seawater complete (SWC) agar medium. One liter SWC medium consists of 5 g yeast extract, 5 g peptone, 3 g beef extract, 600 mL seawater, and 400 mL distilled water. Inoculated SWC were incubated at room temperature (±26ºC) for two days. The antimicrobial activity of *Bacillus* spp. was assayed against *V. harveyi* using a double layer method. A total of 50 µL of the *V. harveyi* culture with the cell's density of $10^8$ CFU/mL was suspended in 50 mL of a melted semi-solid SWC medium. As much as 10 mL of the culture was poured on a solid surface SWC medium plate.

Furthermore, the bacterial isolates of *Bacillus* spp. were inoculated triplicates onto the plates and then incubated for 24 hours at room temperature (±26ºC). The bacterial isolates that produce antimicrobial compounds form the inhibition zone around the bacterial colony. Calculation of inhibition index using the formula as follows:

$$\text{Inhibition index} = \frac{(\text{diameter of inhibition zone} - \text{diameter of bacterial colony})}{\text{Diameter of bacterial colony}}$$

### Growth inhibition assay in mixed cultures of *Bacillus* spp. isolates against *Vibrio harveyi*

Growth inhibition assay was carried out using mixed cultures in a flask on the SWC liquid medium. The co-culture used different ratios of the selected *Bacillus* sp. and *V. harveyi* inoculum. The inoculum ratios of the *V. harveyi* and the selected *Bacillus* sp were 1:1, 1:2, 1:4, and 1:10, respectively. Five flasks containing 50 mL of the SWC liquid medium were inoculated with 100 µL of the *V. harveyi* culture with a cell density of $10^8$ CFU/mL. Then, *Bacillus* spp. were inoculated at the ratio of 1:1, 1:2, 1:4, and 1:10, respectively. Growth control of *V. harveyi* used the same medium without the addition of selected *Bacillus* sp. inoculum. Incubation was conducted on a shaker incubator for 24 hours at room temperature (±26ºC). The number of cell density were calculated triplicates using a plate count method on SWC agar. The percentage of growth inhibition of *V. harveyi* was calculated using a formula as follows:

$$\text{Percentage og growth inhibition} = \frac{(\text{control cell density} - \text{treatment cell density})}{\text{control cell density}} 100\%$$

### Determination of optimum production of antimicrobial compounds during bacterial growth

Bacillus sp isolates that had the highest inhibition activity against *V. harveyi* the A total of 2 mL of the selected *Bacillus* sp. culture with the cell density of $10^8$ CFU/mL was inoculated into 200 mL of SWC liquid medium and incubated on a shaker for 120 hours at room temperature (±26ºC). Five mL of cultures were taken every six hours for the inhibitory activity assay. The culture of *Bacillus* sp. was centrifuged at the speed of 4500 G for 15 minutes. Assays of antimicrobial activity against *V. harveyi* were conducted using a cell-free supernatant by the disk diffusion method (Lisboa et al. 2006).

### Precipitation of antimicrobial compounds using ammonium sulfate

The selected *Bacillus* isolate was cultured in the SWC liquid medium and incubated for the optimum time. The cultures were then centrifuged at 4500 g for 15 minutes at 4ºC. The obtained supernatant was precipitated by salting out method using different concentrations of ammonium sulfate. A total of 500 mL was precipitated by adding ammonium sulfate gradually ranging from 00-10% of initial concentration until the final concentration of 70-80% at 4ºC using a magnetic stirrer at slow speed. The precipitated proteins were separated from the liquid by centrifugation at 4500 g for 15 minutes at 4ºC. The precipitate was dissolved using 0.1 M phosphate buffer pH 7. Suspensions were assayed for their inhibitory activity against *V. harveyi*. The optimum ammonium sulfate for precipitation is the concentration that has the highest inhibitory activity. The inhibitory activity was determined using the disk diffusion method (Lisboa et al. 2006). Protein concentrations were measured using the Bradford method (Bradford 1976). The protein standard for protein measurement was Bovine Serum Albumin (A3294, Sigma-Aldrich).

### Dialysis

The dialysis process was carried out to wash out ammonium sulfate salt from the precipitated protein. The dialysis membrane preparation was performed by boiling method for 10 minutes in a solution of 2% sodium carbonate and 0005% EDTA. Then, the carbonate solution was washed by boiling distilled water for 10 minutes. The dialysis membrane was stored in a buffer solution at 4ºC. One end of the membrane was tied, and the precipitated protein was inserted. The salting-out processes were conducted using 0,01 M phosphate buffer pH 7 with 100 times the total volume of the sample in the membrane. The phosphate buffer was replaced every two hours and incubated at 4ºC overnight. The dialysis results were assayed for their inhibitory activity against *V. harveyi* using the disk diffusion method (Lisboa et al. 2006), and the protein content was measured using the Bradford method (Bradford 1976).

### Purification using a gel filtration column of chromatography

The protein purification was performed using a Hi Prep 16/60 Sephacryl S-200 high-resolution column chromatography connected to a Sweden AKTA purifier system. Sephacryl S-200 matrix was slowly inserted into the chromatographic column length of 60 cm with a 1 cm diameter. The dialysis processes were applied to the column, and the elution was carried out using 0.1 M phosphate buffer (pH 7) solution with a flow rate of 0.5 mL/min. The elution process was observed by the detector at the wavelength of 280 nm and 254 nm. Every 3 mL of

eluent fraction was automatically collected in a tube. The collected fractions were grouped based on the retention time in the column. These fractions were determined their inhibitory activity against *V. harveyi* using the disk diffusion method (Lisboa et al. 2006). The Sephacril S-200 column was also used to determine the molecular weight of the protein fractions by creating a standard curve of the eluents volume versus the log of protein standard molecular weight.

**Effect of pH on the stability of antimicrobial compounds**

The selected fractions from the dialysis were treated with solutions of pH 3, 5, 7, 9, and 11. The acid solution was 1 M HCl, while the alkaline solution was 1 M NaOH. The pH treatments were conducted at room temperature (±26ºC) for two hours. The inhibitory activity assay was conducted against *V. harveyi* using the disk diffusion method (Joshi et al. 1976).

**Effect of temperature on the stability of the antimicrobial compound**

The selected fractions from the dialysis were heated at 50ºC and 100ºC for 10 and 20 minutes. The inhibitory activity was conducted against *V. harveyi* using the disk diffusion method (Joshi et al. 1976).

## RESULTS AND DISCUSSION

**Inhibitory activity of selected *Bacillus* sp. isolates**

The inhibitory activity of the selected *Bacillus* sp. against *V. harveyi* showed that all selected *Bacillus* sp. could inhibit the growth of *V. harveyi* (Table 1). The inhibitory activity of the three selected *Bacillus* sp was categorized as high inhibition against *V. harveyi.* The highest inhibition index against *V. Harvey i* was performed by the *Bacillus* sp Lts40, with an inhibitory index of 7,0.

The mixed culture assay in a liquid media showed that the *Bacillus* sp. Lts40 isolate could inhibit the growth of *V. harveyi* (Figure 1) in all inoculum ratios. The highest *V. harveyi* population was at the 1:10 ratio. The approximate cell density of *Bacillus* sp. Lts40 at the 1:10 ratio was 61,5 x $10^{10}$ CFU/mL. Meanwhile, the cell density of *V. harveyi* was 6,8 x $10^{10}$ CFU/mL. Growth inhibitory activity of *Bacillus* sp Lts40 at inoculum ratio of 1:1 against *V. harveyi* was 81.8 %. The highest growth inhibition of *V. harveyi* by *Bacillus* sp Lts40 (88.7%) was obtained at the inoculum ratio of 1:10.

**Optimum production of antimicrobial compounds during the growth of *Bacillus* Lts 40**

The bacterial growth of *Bacillus* sp. Lts40 in SWC medium showed that the end of the log phase was achieved at 18 hours of incubation. The inhibitory activity was started at 6 hours up to the end of incubation. However, the highest inhibitory activity was obtained at 72 hours of incubation (Figure 2).

**Characteristics of antimicrobial compounds of *Bacillus* sp. Lts40 isolate**

The results of the protein precipitation using ammonium sulfate ($(NH_4)_2SO_4$) showed that both supernatant and precipitated protein showed inhibitory activity against *V. harveyi* (Figure 3). The concentrations of protein precipitates were relatively not significantly different in different ammonium sulfate concentrations. However, the highest inhibitory activity of the precipitates in the treatment of 60-70% $(NH_4)_2SO_4$. However, the highest specific inhibitory activity was obtained from the precipitate resulted from 30-40% $(NH_4)_2SO_4$ treatment on the precipitates before or after dialysis (Table 2). The dialysis process could increase the specific inhibition activity up to 2.4 times.

**Table 1.** Inhibitory activity of the three selected *Bacillus* sp. isolated from shrimp ponds against *Vibrio harveyi* using a double-layer method assay (average ± standard error; n=3).

| *Bacillus* sp. isolate | Disk diameter (mm) | Diameter of inhibition zone (mm) | Inhibition index |
|---|---|---|---|
| Lts40 | 2.5 ± 0 | 20.0 ± 0.6 | 7.0 ± 0.2 |
| Lts36 | 2.5 ± 0 | 11.5 ± 0.3 | 3.6 ± 0.1 |
| Ltw54 | 2.5 ± 0 | 13.0 ± 0.3 | 4.2 ± 0.1 |

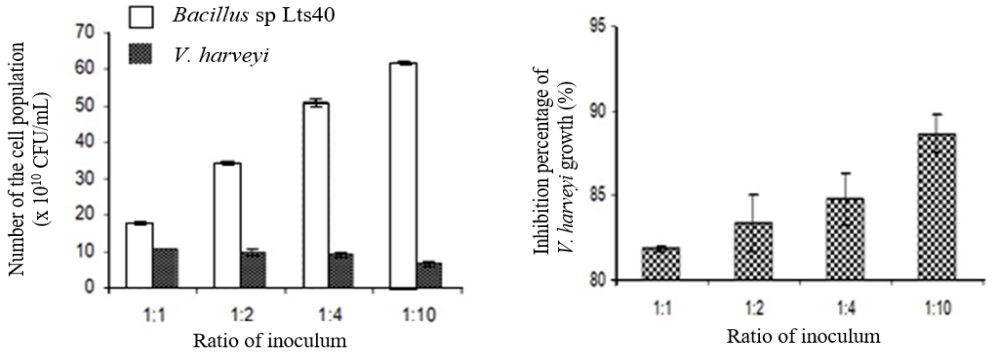


**Figure 1.** Inhibitory activity of *Bacillus* sp. Lts40 against *Vibrio harveyi*, (A) Number of cells population of *Bacillus* sp Lts40 and *V. harveyi* in the mixed culture with different ratio of inoculum, (B) The percentage of growth inhibition of *V. harveyi* by *Bacillus* sp Lts40. Bars indicate standard error (n=3).

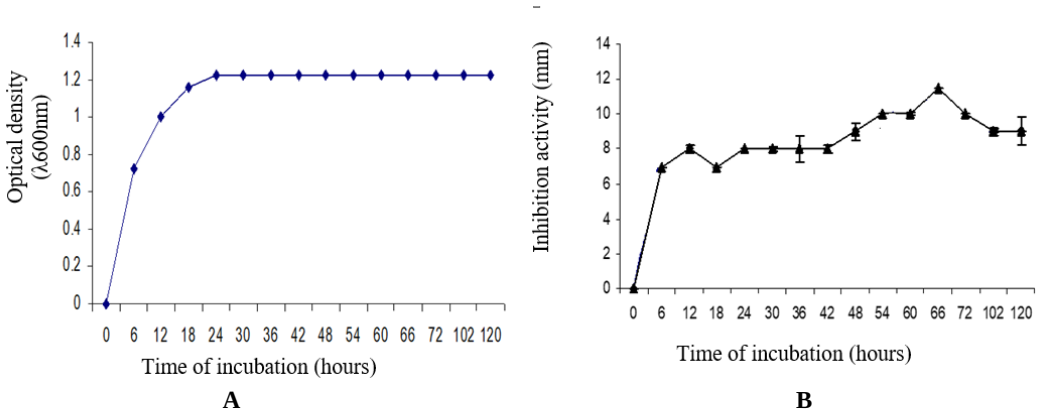


**Figure 2.** The optimum production of antimicrobial compounds during the growth of *Bacillus* sp. Lts40. (A) the growth curve of *Bacillus* sp. Lts40 isolate in seawater complete medium, and (B) Inhibitory activity of supernatant of the culture medium of *Bacillus* sp Lts40. The inhibitory activity was determined using a disk diffusion method. Bars indicate standard error (n=3).

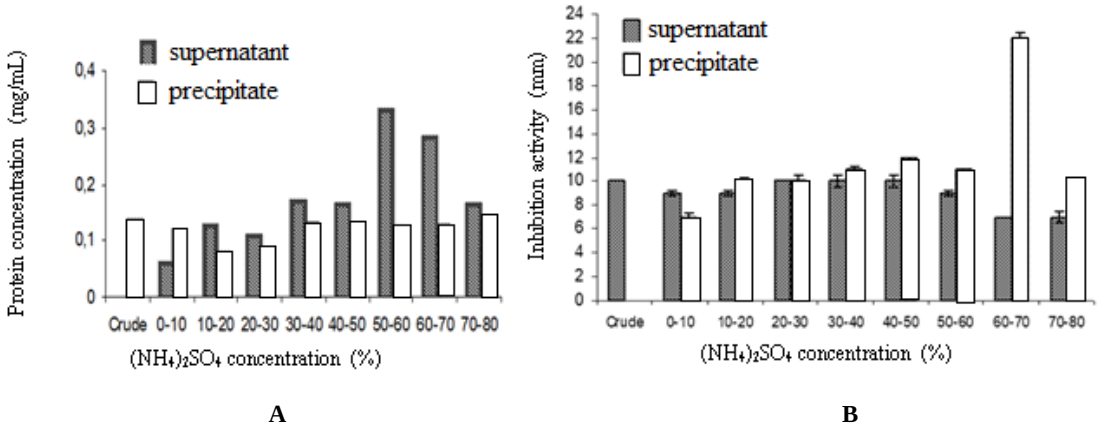


**Figure 3.** Inhibitory activity of antimicrobial compounds of *Bacillus* sp. Lts40 against *Vibrio harveyi* after protein precipitation using ammonium sulfate ($(NH_4)_2SO_4$). A. Protein concentration in the supernatant and precipitate after precipitation using different concentrations of ($(NH_4)_2SO_4$). B. Inhibitory activity of the supernatant and precipitate after precipitation using different concentrations of ($(NH_4)_2SO_4$). The inhibitory activity was determined using a disk diffusion method. Bars indicate standard error (n=3).

Purification of *Bacillus* sp. Lts40 antimicrobial substance using a Hi Prep 16/60 Sephacryl S-200 high-resolution column with a Sweden AKTA purifier system resulted in 15 peak protein fractions (Figure 4). All eluent fractions showed antimicrobial activity against *V. harveyi*. However, the highest antimicrobial activity against *V. harveyi* (30 mm) was no 21 fraction (Figure 5). Concentration of the protein fraction no 21 was 0.0167 mg/mL. Based on the standard protein retention time, the molecular weight of this protein fraction is 47.38 kDa.

### Stability of antimicrobial compounds from *Bacillus* sp. Lts40 at different pH and temperature

The inhibitory activity of antimicrobial compounds from *Bacillus* sp Lts40 at different pH (pH 3-11) remained stable (Figure 6) and not significantly different among pH treatments. However, the optimum inhibitory activity against *V. harveyi* was obtained at pH 5 with a diameter of inhibition was 22 mm. Furthermore, the stability of inhibitory activity of antimicrobial compounds from *Bacillus* sp. Lts40 was determined at different temperatures and the length of exposure. The results showed that the antimicrobial compounds remained stable after being heated at 50ºC and 100ºC for 10 and 20 minutes (Table 3).

## Discussion

Three selected *Bacillus* sp. isolates have antimicrobial activity against *V. harveyi*. The *Bacillus* sp. Lts40 performed the highest inhibitory activity against *V. harveyi*. *Bacillus* sp. can produce antimicrobial compounds such as bacteriocin and polypeptide antibiotics. *Bacillus subtilis* can produce an antimicrobial substance such as Cerein (Oscariz and Pisabarro 2000). Factors that can affect the inhibitory activity of antimicrobial compounds are their functional groups, the resistance of the bacterial targets, the concentration of the active substances, and the density of the target bacteria (Rachmaniar 1997). Moreover, other factors influencing antimicrobial activity are 1) type, number, and growth phase of bacteria, 2) concentration of antimicrobial compounds, 3) temperature and length of contact time, and 4) physic-chemical properties of substrates such as pH, water content, and surface tension (Frazier and Wasthoff 1981).

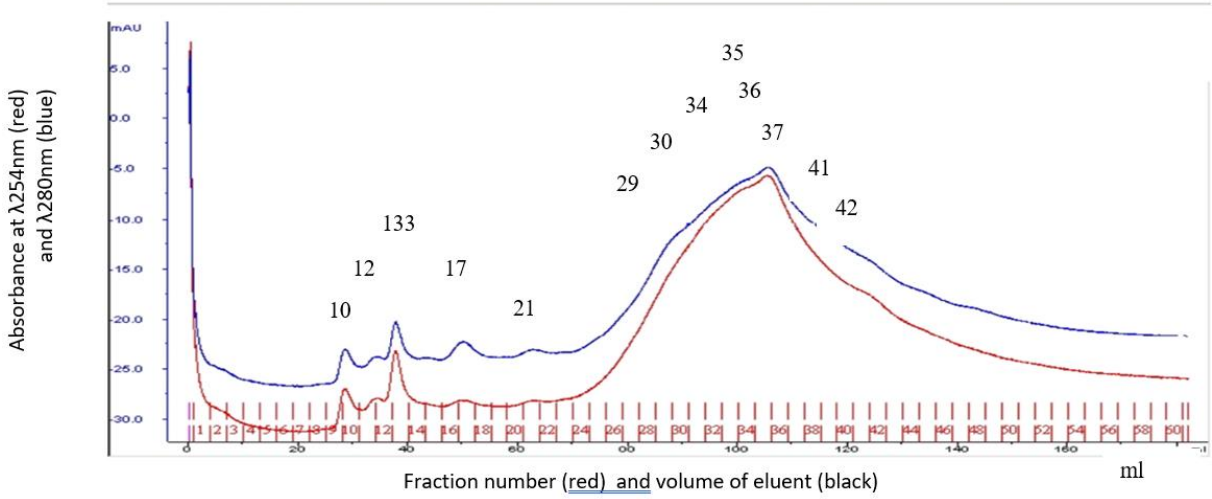


**Figure 4.** The absorbance of protein fractions from *Bacillus* sp. Lts40 at the wavelength of 254 nm (red) and 280 nm (blue). The number in the graph were the number of fractions that analyzed for antibacterial activity. The number in the graph were the number of fractions that were analyzed for antibacterial activity against *Vibrio harveyi*. The purification was conducted using a Hi Prep 16/60 Sephacryl S-200 High Resolution, with a Sweden AKTA purifier system.

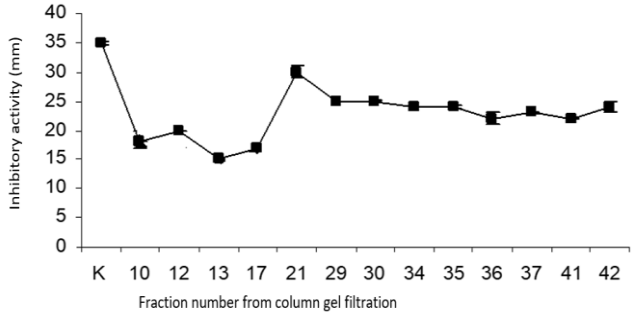


**Figure 5.** The inhibitory activity of the 15 selected fractions from *Bacillus* sp. Lts40 against *V. harveyi*. The purification was conducted using a Hi Prep 16/60 Sephacryl S-200 high resolution, with a Sweden AKTA purifier system. The inhibitory activity was determined using a disk diffusion method. Bars indicate standard error (n=3) and K is control of supernatant.

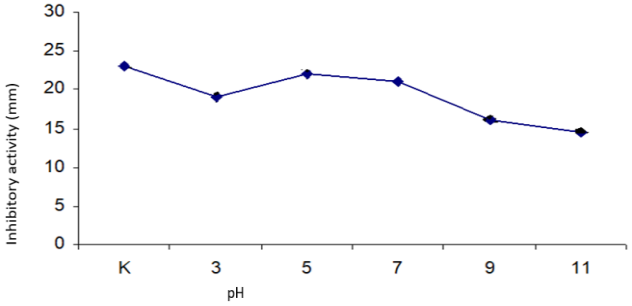


**Figure 6.** Inhibitory activity of the antimicrobial compounds from *Bacillus* sp. Lts40 at the pH range of 3-11 for two hours at room temperature (±26ºC). The inhibitory activity was determined using a disk diffusion method. K indicates control of supernatant.

**Table 2.** Antimicrobial activity of protein precipitates from culture media of *Bacillus* sp Lts40 from the best ammonium sulfate precipitation before and after purification using membrane dialysis (average ± standard error; n=3).

| Concentration of ammonium sulfate (%) | Before dialysis | | | After dialysis | | |
|---|---|---|---|---|---|---|
| | Protein concentration (mg/mL) | Antimicrobial activity (mm) | Specific activity (mm/mg/mL) | Protein concentration (mg/mL) | Antimicrobial activity (mm) | Specific activity (mm/mg/mL) |
| 30-40 | 0.0192 ± 0.0005 | 19 ± 1.5 | 989.58 ± 59.21 | 0.0077 ± 0.0006 | 18 ± 1.2 | 2,337.66 ± 347.72 |
| 60-70 | 0.1653 ± 0.0043 | 35 ± 2.5 | 211.74 ± 10.00 | 0.1104 ± 0.0075 | 30 ± 1.7 | 271.74 ± 27.85 |

**Table 3.** Inhibitory activity of antimicrobial compounds from *Bacillus* sp. Lts40 after temperature treatments (average ± standard error; n=3)

| Temperature treatments (ºC) | Length of temperature treatments (minutes) | Disk diameter (mm) | Inhibition zone (mm) | Inhibition index |
|---|---|---|---|---|
| 50 | 10 | 2.5 ± 0.0 | 20 ± 1.0 | 8.0 ± 0.4 |
| | 20 | 2.5 ± 0.0 | 19 ± 1.0 | 7.6 ± 0.4 |
| 100 | 10 | 2.5 ± 0.0 | 16 ± 0.6 | 6.4 ± 0.2 |
| | 20 | 2.5 ± 0.0 | 15 ± 1.0 | 6.0 ± 0.4 |

*Bacillus* sp. Lts40 isolate produced extracellular antimicrobial compounds produced during bacterial growth, either in the log phase or stationary phase, and the optimum production was in 72 hours of incubation. Therefore, its culture supernatant showed inhibitory activity against *V. harveyi*. Antimicrobial compounds produced by *Bacillus* sp. Lts40 isolate is a protein. And the protein is produced during bacterial growth. So, this indicates that the antimicrobial compound of *Bacillus* sp. Lts40 isolate is a bacteriocin. Bacteriocins are proteins or peptides generally produced at the exponential growth phase and synthesized in the ribosome (Ray and Daeschel 1981). The inhibitory activity seemed to decrease after 72 hours of incubation, it may be indicating the reduction of digestive enzymes, reabsorption of antimicrobe (Dajani and Wannamaker 1969), or releasing proteases from cell autolysis that degrade the antimicrobial compounds (Jo et al. 1996).

Precipitation of antimicrobial compounds indicated that the treatment of 60-70% ammonium sulfate was the best concentration in precipitating antimicrobial compounds from *Bacillu*s sp. Lts40. However, precipitation of antimicrobial compounds using 30-40% ammonium sulfate resulted in the best specific antimicrobial activity. Dialysis processes that wash out ammonium sulfate affected the inhibitory activity of antimicrobial compounds. Therefore, dialysis processes could increase the specific inhibitory activity of the antimicrobial compounds up to 2.4 times.

The highest antimicrobial activity of purified antimicrobial substance of *Bacillus* sp. Lts40 is a protein that has molecular weight of 47.38 kDa. This result supports the indication that the antimicrobial substance of *Bacillus* sp. Lts40 is a bacteriocin. Bacteriocins are proteinaceous antimicrobial agents that are classified into three classes. Class I bacteriocins are known as antibiotics which have a molecular weight of less than five kDa. Class II bacteriocins have a molecular weight of less than ten kDa, and Class III bacteriocins consist of proteins with a molecular weight of more than 30 kDa (Parada et al. 2007; Mokoena 2017). Based on its molecular weight, the antimicrobial compound from *Bacillus* sp. Lts40 is presumably a class III bacteriocin. Several *Bacillus* spp produce bacteriocins with different ranges of molecular weight. Molecular weight of bacteriocins produced by *Bacillus coagulans* is 3-4 kDa (Marrec 1998), and bacteriocins from *Bacillus polyfermenticus* is 14.3 kDa (Lee et al. 2001). Other characteristics of bacteriocins produced by other bacteria are presented in Table 4.

The antimicrobial substance from *Bacillus* sp. Lts40 was stable in a pH range of 3-11. Inhibitory activity of *Bacillus* sp. Lts40 against *V. harveyi* was stable in acidic and alkaline conditions. The stability of antimicrobial compounds in a wide pH range due to their amino acid compositions contains cysteine. It will form disulfide bonds and have more than one active site. If there is a change in pH, it will change the degree of ionization of the ionic clusters of amino acids of the proteins. Increasing the inhibitory activity due to the ionization of the ionic group on the active site leads to a more stable conformation (Denniston et al. 2001).

**Table 4.** The characteristics of bacteriocin from several bacteria

| Bacterial isolates | Molecular weight (kDa) | Stability | Purification method | References |
|---|---|---|---|---|
| *Brevibacterium linens* | 95 | Stable at 45°C 60 min, no activity at 60 °C | Gel filtration, SDS-PAGE | Kato et al. (1991) |
| *Lactobacillus brevis* | 10-30 | Stable at a broad range of temperatures and pH | Ion exchange | Benoit et al. (1991) |
| *Pediococcus acidilactici* | 3,6 | Stable at 100°C 40 min, Stable at pH 2-11 | Ion exchange, hydrophobic chromatography, HPLC, SDS-PAGE | Cintas et al. (1995) |
| *Bacillus coagulans* I4 | 3-4 | Stable at 60°C 90 min, pH 4-8 | SDS-PAGE | Marrec (1998) |
| *Lactobacillus acidophilus* 30SC | 3,5 | Stable at 121°C 20 min, stable at pH 3-10 | hydrophobic chromatography, SDS-PAGE | Oh et al. (2000) |
| *Bacillus polyfermenticus* | 14,3 | No activity at >70°C, stable at pH 2-9 | SDS-PAGE | Lee et al. (2001) |
| *Bacillus licheniformis* | 2 | Stable at 100°C, Stable at broad range of pH | Gel filtration, SDS-PAGE | Martirani et al. (2001) |
| *Bacillus cereus* | 1-8 | Stable at 75°C 15, stable at pH 3-10 | SDS-PAGE | Torkar and Matijasic (2003) |
| *Lactobacillus palntarum* F1 & *Lactobacillus brevis* OG1 | 1-10 | Stable at 121°C 10 min, stable at pH 2-6 | Ultrafiltration | Chythanya et al. (2002) |
| *Streptococcus thermophilus* ACA-DC 0001 | 30 | Stable 50°C 10 min, Stable at pH 2-10 | Gel filtration, ion exchange, and SDS-PAGE | Anastasios and George (2003) |
| *Bacillus amyloliquefaciens* | 5 | Stable at 100°C 60 min, stable at pH 2-8 | SDS-PAGE | Denniston et al. (2001) |
| *Bacillus* sp., Lts40 | 47.38 | Stable at 50-100°C 20 min, stable at pH 3-11 | Gel filtration | *This study* |

The antimicrobial compound of *Bacillus* sp. Lts40 is resistant to heat treatment up to a temperature of 100ºC for 20 minutes. This stability is possibly due to the amino acid composition of antimicrobial compounds from *Bacillus* sp. Lts40 naturally supports stability in complex circumstances. The disulfide bonds probably make this antimicrobial substance more heat stable. In general, some bacteriocins are resistant to heat and acidic conditions (Bhunia et al. 1988; Ahn and Stiles 1990; Kone and Fung 1992).

This study clearly shows that the *Bacillus* sp. Lts40 isolate has potential application as a probiotic agent in shrimp ponds to control the growth of *V. harveyi* that causes luminous vibriosis diseases. This bacterial isolate can inhibit the growth of *V. harveyi* up to 88.7%. Moreover, the antimicrobial substance produced by the isolate is a protein that is stable after heat shock treatments up to 100ºC. So, this substance is potentially to be developed and produced as an anti-*V. harveyi* product that can be applied in a shrimp hatchery.

In conclusion, inhibitory activity of three selected *Bacillus* sp. against *V. harveyi* was categorized as high. The highest inhibition index was performed by *Bacillus* sp Lts40 isolate. The highest growth inhibition of *V. harveyi* (88.7%) was performed by *Bacillus* sp. Lts40. The optimum production of antimicrobial compounds was obtained at 72 hours of incubation. Antimicrobial precipitation by 30-40% $(NH_4)_2SO_4$ had the highest specific inhibition activity, and dialysis processes could increase its specific inhibition activity up to 2.4 times. A gel filtration chromatography resulted in 15 peaks of protein fractions that have antimicrobial activity against *V. harveyi*. The highest inhibitory activity was protein fraction no 21 with a molecular weight of 47.38 kDa. The antimicrobial compound from *Bacillus* sp Lts40 remains stable at a pH range of 3-11 and a temperature of 100ºC for 20 minutes.

## ACKNOWLEDGEMENTS

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