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Antagonism *Pseudomonas diminuta* on The Growth of *Vibrio harveyi* with Mix Culture Method

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Abstract

One of the obstacles often encountered in white shrimp farming is the presence of bacterial diseases caused by *Vibrio* group bacteria. One of the efforts to control vibriosis is by using antagonistic bacteria as biocontrol agents, one of which is using *Pseudomonas diminuta*. In this study, *P. diminuta* and *Vibrio harveyi* were co-cultured to determine the optimal density and effective incubation time of *P. diminuta* which could provide the highest inhibition of *V. harveyi* growth. This study used a completely randomized design with six treatments P0 (*P. diminuta* 10⁵ CFU/ml), P1 (*V. harveyi* 10⁶ CFU/ml), P2 (*P. diminuta* 10⁵ CFU/ml+*V. harveyi* 10⁶ CFU/ml), P3 (*P. diminuta* 10⁶ CFU/ml+*V. harveyi* 10⁶ CFU/ml), P4 (*P. diminuta* 10⁷ CFU/ml+*V. harveyi* 10⁶ CFU/ml), P5 (*P. diminuta* 10⁸ CFU/ml+*V. harveyi* 10⁶ CFU/ml). The results of this study showed that P2, P3, P4, and P5 had decreased growth of *V. harveyi* when compared to control P1 (*V. harveyi* 10⁶ CFU/ml). The decrease in the growth of *V. harveyi* occurred in the 8th to 48th hour. *V. harveyi* in P3 (2.72 × 10⁸ CFU/ml), P2 (2.80 × 10⁸ CFU/ml), P4 (2.96 × 10⁸ CFU/ml) and P5 (2.90 × 10⁸ CFU/ml) at the 48th hour of incubation was lower than P1 (3.15 × 10⁸ CFU/ml). Based on the results of the Duncan test showed that P2, P3, P4, and P5 were significantly different (p < 0.05) from P1. From this study, it was concluded that administration of *P. diminuta* at a density of 10⁶ CFU/ml was able to reduce the growth of *V. harveyi*. The greatest decrease in the growth of *V. harveyi* occurred at the 48th hour.

INTRODUCTION

White shrimp is a species of shrimp that is cultivated in Indonesia because it has a fairly high economic value. Vibriosis disease is an obstacle that must be faced by business actors engaged in fisheries, especially white shrimp (*Litopenaeus vannamei*) (Sari *et al.*, 2015). Vibriosis

disease that attacks shrimp is a bacterium from the *Vibrio* group (Ramesh *et al.*, 2014). Control of the common bacterial disease vibriosis is done by using antibiotics. This resulted in a negative impact on consumer health in the form of antibiotic residues and caused

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contamination of the aquatic environment (Haditomo *et al.*, 2016). *Pseudomonas* sp. produces extracellular enzymes and has antagonistic abilities against *V. harveyi*. The existence of antagonism properties of a bacterium against other bacteria so that technology was developed in the prevention of Vibriosis that attacks shrimp (Firdaus, 2013).

Pseudomonas sp. is a gram-negative bacteria capable of producing chitinase enzymes. The chitinase enzyme works as a catalyst in the process of breaking down chitin polymers in the body armor of fish or shrimp into simpler monomer units (Mangunwardoyo *et al.*, 2009). *Pseudomonas* sp. is a bacterium capable of producing several enzymes such as protease, amylase, lipase, and chitinase which can break down proteins, carbohydrates, fats, and chitin into simpler compounds so that they can be used as biocontrol agents in aquaculture (Suji *et al.*, 2014).

The use of bacteria as a biocontrol agent will be increasingly important from an aquaculture ecosystem perspective, reducing and even eliminating the use of antibiotics to create an environmentally friendly cultivation system and prepare an organic aquaculture system, the issue of which has been getting stronger lately (Isnansetyo, 2005). Research results from Gram *et al.* (1999), *P. diminuta* produce antibacterial compounds that can inhibit bacterial growth, the compounds produced include antibiotics, siderophores, and bacteriocins. Supported by research results Kamei and Isnansetyo (2003), *Pseudomonas* sp. AMSN was able to inhibit the growth of *V. alginolyticus* because it produced the compound 2,4 diacetylploroglucinol.

As a follow-up effort, it is necessary to carry out co-culture between *P. diminuta* and *V. harveyi* to determine the inhibition of *P. diminuta* on the growth of *V. harveyi*, which is expected to reduce the use of antibiotics in tackling Vibriosis. The update of this research is to look for indigenous bacteria from intensive shrimp

ponds that have the ability to fight against the pathogenic bacteria *V. harveyi* which causes many failures in shrimp farming.

METHODOLOGY

Ethical Approval

This research does not use living organisms as test animals, so it does not require ethical clearance. This study used bacteria isolated from the sediments of intensive shrimp ponds.

Place and Time

This research was conducted at the Laboratory of Microbiology and Fish Disease Analysis, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya in January - September 2018.

Research Materials

The materials used were *P. diminuta* isolated, purified and selected from intensive shrimp pond sediments, *V. harveyi* culture obtained from the Situbondo Brackish Water Cultivation Center, isolated from infected shrimp, shrimp, Tryptic Soy Broth (TSB) (Merck 1.05459.0500, Germany), Tryptic Soy Agar (TSA) (Merck 105458, Germany), Thiosulfat Citrat Bile Sucrose (TCBS) (Merck 1.0263.0500, Germany), NaCl powder, distilled water, KCl (Merck, Germany), MgSO₄ (Merck, Germany), and PBS solution.

The equipment used was 1 ml syringe, hot plate (Thermo Scientific Cimarec-2), autoclave (HVE-50 Hirayama, Japan), test tube, petri dish, incubator (Memmert Digital Incubator IN-55, Germany), centrifuge (TD 4C, China), spectrophotometer UV-VIS (Human X-MA Series), and Vortex D-Lab mx-s.

Research Design

This study used an experimental method with a completely randomized design (CRD) using six treatments including; (P0) *P. diminuta* 10⁵ CFU/ml; (P1) *V. harveyi* 10⁶ CFU/ml; (P2) *Vibrio harveyi* 10⁶ CFU/ml + *P. diminuta* 10⁵ CFU/ml; (P3) *V. harveyi* 10⁶ CFU/ml + *P.*

diminuta 10⁶ CFU/ml; (P4) *V. harveyi* 10⁶ CFU/ml + *P. diminuta* 10⁷ CFU/ml; (P5) *V. harveyi* 10⁶ CFU/ml + *P. diminuta* 10⁸ CFU/ml with four replicates for each treatment.

Work Procedure

Media Preparation

Preparation of TSB media was carried out by weighing 30 g of TSB powder and 15 g of NaCl powder dissolved in one liter of distilled water. Furthermore, the media is heated with a hot plate until homogeneous. The media was sterilized by autoclaving at 121 °C for 15 minutes with a pressure of 1 atm. Then it is poured into a sterile test tube and wait until it cools down. Preparation of TSA media was carried out by weighing 40 g of TSA powder plus 15 g of NaCl powder in one liter of distilled water. Furthermore, the media is heated on the hot plate while stirring until homogeneous. Then the TSA media was sterilized by autoclaving at 121 °C for 15 minutes with a pressure of 1 atm. The media is poured into a sterile petri dish and wait until it cools down and becomes solid. TCBS media was prepared by weighing 88 g of TCBS, 8.40 g of NaCl powder, 0.75 g of KCl, MgSO₄, and 6.94 g of dissolved in one liter of sterile distilled water. Next, the media is heated with a hot plate until it is homogeneous, then pour the media into the petri dish, and wait until it cools and becomes solid.

V. harveyi and *P. diminuta* Cultures

V. harveyi isolate was inoculated into one loop of TCBS media using the streak method. Based on the research by Mailoa and Setha (2011) the specific medium for *V. harveyi* is TCBS. Whereas *P. diminuta* isolates were rejuvenated by inoculating them using the streak method on TSA media. Furthermore, the bacterial isolates were stored in an incubator at 37 °C for 24 hours. *V. harveyi* and *P. diminuta* which had been inoculated on each media were re-cultured on TSB media by taking one colony using one loop of needle loops and incubating for 24 hours at 37 °C. After

that, *V. harveyi* and *P. diminuta* which had been inoculated for 24 hours were harvested by centrifuging at 1500 rpm, after which the supernatant was removed and PBS solution was added according to the initial volume and vortexed to equalize with McFarland standard scale 0.5. Then the absorbance value was calculated using a spectrophotometer with a wavelength of 550 nm.

Passage of *V. harveyi* on White Shrimp

The *in vivo* test on shrimp aims to vicious *V. harveyi* bacteria. *V. harveyi* which had been inoculated on TSB media for 24 hours was harvested by centrifuging, after which the supernatant was removed and added to PBS solution according to the initial volume and vortexed to calculate the absorbance value using a spectrophotometer with a wavelength of 550 nm. Next, the density of *V. harveyi* 10⁶ and 10⁸ CFU/ml was determined to be injected into white shrimp as much as 0.1 ml/head using a 1 ml syringe intramuscularly in the third abdominal segment (Chau *et al.*, 2011) previously the shrimp were stunned using ice cubes at 15 °C. The shrimp that had been injected with *V. harveyi* were put into the rearing tank by observing every six hours for two days. When the shrimps move abnormally, the shrimps are taken to be inoculated from the hepatopancreas, gills and intestines. Furthermore, the bacteria from the shrimp organs were inoculated using the streak method on TSA media for 24 hours, if bacterial colonies grew, then one colony was taken and re-cultured on TCBS media to determine whether the bacteria growing was *V. harveyi*.

Determination of Lethal Dose (LD₅₀) of *V. harveyi*

Determination of Lethal Dose (LD₅₀) aims to determine the dose of *V. harveyi* that can kill 50% of the total shrimp population infected with *V. harveyi*. *V. harveyi* which had been inoculated on TSB media for 24 hours was harvested by

centrifuging, after which the supernatant was discarded and washed with PBS solution twice, then PBS was added according to the initial volume and vortexed to then calculate the absorbance value using a spectrophotometer with a wavelength of 550 nm. Next, the density of *V. harveyi* 10⁸, 10⁷, 10⁶, and 10⁵ CFU/ml was determined for the LD50 test. *V. harveyi* was injected intramuscularly into the white shrimp as much as 0.1 ml/head in the third abdominal segment, previously the white shrimp were stunned first using ice cubes at a temperature of 20 °C. In the LD₅₀ test, four treatments and three replications were used, each consisting of 10 shrimps.

Mix Culture of *P. diminuta* with *V. harveyi*

The bacterial growth test in this study used the co-culture method. This method is carried out by growing *P. diminuta* and *V. harveyi* on the same medium, namely TSB media. *P. diminuta* with different bacterial densities of 10⁸, 10⁷, 10⁶, and 10⁵ CFU/ml were each put into culture tubes, and *V. harveyi* at an LD50 dose of 10⁶ CFU/ml into each culture tube containing *P. diminuta* (P2, P3, P4, P5) and then incubated for 24 hours. After that, co-culture was carried out by taking 1 ml of each bacterial culture to grow on TSB media using two bacterial controls, namely *V. harveyi* control using the resulting LD50 dose of 10⁶ CFU/ml (P1) and *P. diminuta* control with a density 10⁵ CFU/ml (P0) which according to Widanarni *et al.* (2003) inoculated probiotic candidate bacteria to be used using a density of 10⁵ CFU/ml. In addition, at a density of 10⁵ CFU/ml, it is still within the safe limit in water (Hardiyani *et al.*, 2016). After that, it was incubated at 37 °C for 48 hours.

Calculation of Bacterial Density

Calculation of the number of bacteria in this study was carried out by counting bacterial colonies using the Total Plate Count (TPC) method. Calculations were made with multilevel dilution, namely at 10⁻¹ to 10⁻⁸ dilutions. Bacterial dilution aims to count the cultured bacteria provided that the colony count is 30-300 (Waluyo, 2007). The incubation results were centrifuged for 15 minutes at 1500 rpm, then discarded the supernatant and added PBS according to the initial volume. Dilution was carried out by taking 1 ml of *P. diminuta* and *V. harveyi* cultures in culture media and adding 9 ml of PBS to a test tube, then vortex until homogeneous.

From dilutions 10⁻⁷ and 10⁻⁸, 0.1 ml was taken each inoculated on TSA media for counting *P. diminuta*, and another 0.1 ml was taken and inoculated on TCBS media for counting *V. harveyi* using the spread plate method. Furthermore, the number of bacteria was counted during the incubation period at 8, 16, 24, 32, 40, and 48 hours (Szala *et al.*, 2012). The formula for calculating bacteria using the TPC method according to Waluyo (2007) is as follows:

$$\text{Bacteria Count (CFU/ml)} = \Sigma \text{bacteria/dish} \times \frac{1}{\text{sample volume}} \times \frac{1}{\text{dilution factor}}$$

Data Analysis

Data from this study were analyzed using Analysis of Variance (ANOVA). If the results obtained have the effect of giving treatment to the results, then a post hoc test is carried out using Duncan's multiple range test.

RESULTS AND DISCUSSION

Calculation of the density of *P. diminuta* and *V. harveyi* co-cultured on TSB media with different *P. diminuta* densities using the TPC method which can be seen in Figure 1 and presented in Table 1.

Table 1. The average density of *P. diminuta* (P0) and *V. harveyi* at 0 to 48 hours.

Treatment	Hour (CFU/ml)						
	0	8	16	24	32	40	48
P0 <i>P. diminuta</i>	10 ⁵	4.94×10 ^{6c}	5.49×10 ^{6c}	6.89×10 ^{6c}	6.53×10 ^{8c}	5.24×10 ^{8d}	3.21×10 ^{8c}
P1 <i>V. harveyi</i>	10 ⁶	4.75×10 ^{6c}	5.30×10 ^{6c}	6.69×10 ^{6c}	6.45×10 ^{8c}	5.05×10 ^{8cd}	3.15×10 ^{8bc}
P2 <i>V. harveyi</i>	10 ⁶	3.27×10 ^{6b}	3.82×10 ^{6b}	5.11×10 ^{6b}	5.06×10 ^{8ab}	4.72×10 ^{8ab}	2.80×10 ^{8a}
P3 <i>V. harveyi</i>	10 ⁶	2.62×10 ^{6a}	3.17×10 ^{6a}	4.48×10 ^{6a}	4.96×10 ^{8a}	4.67×10 ^{8a}	2.72×10 ^{8a}
P4 <i>V. harveyi</i>	10 ⁶	3.48×10 ^{6b}	4.03×10 ^{6b}	5.18×10 ^{6b}	5.28×10 ^{8b}	4.9×10 ^{8bc}	2.96×10 ^{8ab}
P5 <i>V. harveyi</i>	10 ⁶	3.34×10 ^{6b}	3.90×10 ^{6b}	5.30×10 ^{6b}	5.15×10 ^{8ab}	4.85×10 ^{8abc}	2.90×10 ^{8ab}

Notations indicated by different superscript letters in the same column show significant differences (p<0.05).

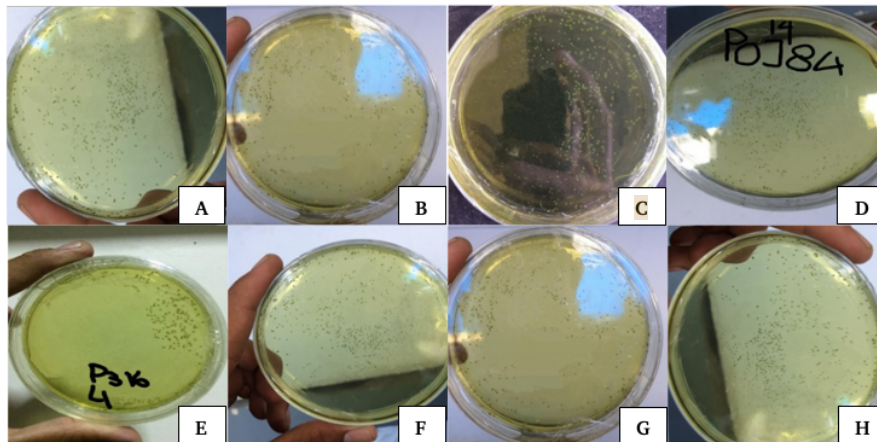


Figure 1. The Total Plate Count (TPC) method. A: *V. harveyi* 10⁶CFU/ml (control) 8th hour, B: *V. harveyi* 10⁶CFU/ml (control) 16th hour, C: *V. harveyi* 10⁶CFU/ml (control) 24th hour, D: *V. harveyi* 10⁶CFU/ml (control) 32nd hour, E: *P. diminuta* 10⁶CFU/ml+*V. harveyi* 10⁶CFU/ml 8th hour F: *P. diminuta* 10⁶CFU/ml+*V. harveyi* 10⁶CFU/ml 16th hour, G: *P. diminuta* 10⁶CFU/ml+*V. harveyi* 10⁶CFU/ml 24th hour, H: *P. diminuta* 10⁶CFU/ml+*V. harveyi* 10⁶CFU/ml 32nd hour.

Anova testing of the average density of *P. diminuta* (P0) and *V. harveyi* was carried out at each observation time, namely the 8th, 16th, 24th, 32nd, 40th, and 48th hours. The test results showed significantly different (p < 0.05). This can be seen in Table 1. The results of the co-culture test of *P. diminuta* and *V. harveyi* in the P1 control treatment (*V. harveyi*) at 32nd hours could reach 6.45×10⁸CFU/ml. When compared

in the P2, P3, P4, and P5 treatments cultured with *P. diminuta*, it showed that *P. diminuta* could reduce the density of *V. harveyi* while P1 4.75×10⁶CFU/ml at 8th hours. At the 16th hour, the P2, P3, P4, and P5 treatments were significantly different from P1.

Based on the Duncan test at P2, P4, and P5 there was no significant difference until the 48th hour. At the 8th to 32nd hours of treatment P2, P3, P4, and P5 the density

of *V. harveyi* increased, but the mean test results at 32nd hours showed that P3 was significantly different from P1 and P4,

but P3 was not significantly different from P2 and P5. At 40th hours *V. harveyi* began to decline.

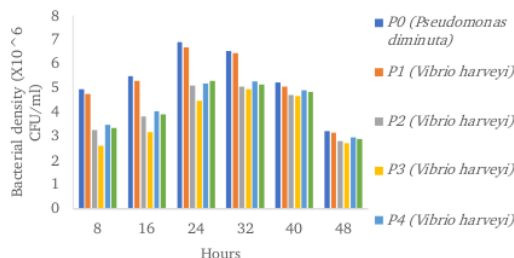


Figure 2. Density graph of *V. harveyi* and *P. diminuta* (P0).

The addition of *P. diminuta* caused a decrease in the density of *V. harveyi*. This is shown in Figure 2. that the density of *V. harveyi* in the P2, P3, P4, and P5 treatments was lower than the P1 control (*V. harveyi*) from the 8th to 48th hour. In Figure 2 it can be seen that the development of *V. harveyi* at 8th to 48th hours developed normally according to the growth phases of bacteria in general. In treatment P1, the optimum point for the exponential phase of *V. harveyi* was 6.45×10^8 CFU/ml, which occurred at 32nd hours. The optimum point for the exponential phase of *P. diminuta* with the highest density was found in treatment P0 (*P. diminuta*) which was 6.53×10^8 CFU/ml while the optimum point for the exponential phase for *V. harveyi* with the lowest density was found in P3 which was 4.96×10^8 CFU/ml (Figure 2).

One of the antagonistic tests can be carried out using the co-culture method containing more than one type of microorganism. Co-cultures can be carried out on all groups of microorganisms and all types of bacteria consisting of a mixture of fungal and bacterial organisms, mold and yeast, or other combinations where the components are sufficiently related (National Research Council, 1992). The results of the study showed that in monocultures *P. diminuta* (P0) and *V. harveyi* experienced a normal growth phase. *V. harveyi* co-culture with the addition of *P. diminuta* at different

densities of 10^5 , 10^6 , 10^7 and 10^8 CFU/ml showed a decrease in *V. harveyi* density at the 8th to 48th hour compared to the P1 treatment (*V. harveyi* without *P. diminuta*) (Table 1). This shows that the addition of *P. diminuta* can reduce the density of *V. harveyi*. Semangun (2006) suggests that antagonistic mechanisms in microbes can occur in three ways, namely direct parasitization due to the presence of toxic secondary metabolites, and competition in terms of space and nutritional needs.

The results of co-culture at the 16th hour of treatment P4 (added *P. diminuta* 10^7 CFU/ml) obtained a density of *V. harveyi* 4.03×10^6 CFU/ml and P5 (added *P. diminuta* 10^8 CFU/ml) obtained a density of *V. harveyi* of 3.90×10^6 CFU/ml. In the P4 and P5 treatments, the density of *V. harveyi* was still higher compared to the P3 treatment. This is presumably due to the limitations of *P. diminuta* in treatments P4 and P5 in reducing the density of *V. harveyi*. This conjecture is based on the statement of Verschueren *et al.* (2000) that the use of probiotic bacteria in high concentrations does not guarantee better protection against the host.

In the 16th hour of P3 treatment with the addition of 10^6 CFU/ml *P. diminuta* showed better results than P2 (with the addition of 10^5 CFU/ml *P. diminuta*) in inhibiting the growth of *V. harveyi*. In treatment P3 the density of *V. harveyi* reached 3.17×10^6 CFU/ml while in P2 the density of *V. harveyi* reached 3.82×10^6

CFU/ml. This shows that *P. diminuta* with a density of 10^6 CFU/ml is optimum in reducing *V. harveyi*. The antagonism of *P. diminuta* towards *V. harveyi* in reducing the density of *V. harveyi* occurred from the 8th to the 48th hour in the P3 treatment. Supported by the statement of Verschuere *et al.* (2000) that the ability of bacteria to inhibit the growth of pathogenic bacteria is caused by several factors, namely the production of metabolites such as bacteriocins, siderophores, proteases, hydrogen peroxide or influencing the pH of the media by producing certain acidic compounds.

The process of inhibition of *V. harveyi* by *P. diminuta* peaked at the 48th hour at P3 with a density of *V. harveyi* 2.72×10^8 CFU/ml, this density was lower than that of the control (P1), which was 3.15×10^8 CFU/ml. This is presumably due to the presence of an inhibitory compound produced by *P. diminuta*, namely bacteriocins. Inhibition by bacteriocin compounds through inhibiting the formation of cell walls and the formation of nucleic acids in pathogenic bacterial cells by damaging cell membranes, causing lysis or bacteriolysis (Martínez-Cuesta *et al.*, 2000).

In addition to the bacteriocins that inhibit the growth of *V. harveyi*, there are other inhibitory compounds produced by *Pseudomonas*, namely siderophores that will compete in the utilization of Fe ions, because siderophores are specific proteins that bind Fe ions with a low molecular weight that can dissolve precipitated Fe so that it can be used for the growth of microorganisms. According to Garrity (2005) *P. diminuta* is able to produce siderophores in the form of pyoverdine, pseudobactin, pyochelin, and salicylic acid.

Another factor that caused the decrease in *V. harveyi* was due to the presence of an antibiotic compound produced by *P. diminuta*, namely 2,4 diacetylploroglucinol. This compound functions as an antibiotic whose role is almost the same as the bacteriocin

compound, namely inhibiting the formation of cell walls, inhibiting the formation of nucleic acids, and forming pores in the target cell membrane (Williams *et al.*, 1996; Chotiah, 2013). In addition, the antibiotic compound 2,4 diacetylploroglucinol causes lysis and displays antiviral activity against DNA and RNA viruses with envelopes (Brazelton *et al.*, 2008).

The results of this study showed that *P. diminuta* could reduce *V. harveyi* in each co-culture treatment at 8th to 48th hours compared to control P1. At the 48th hour, the lowest number of *V. harveyi* was found in P3 which was 2.72×10^8 CFU/ml compared to the other treatments. From the results of the Duncan test, that co-culture with the addition of 10^6 CFU/ml *P. diminuta* at P3 gave significantly different results from the other treatments (P2, P4, and P5). This shows that *P. diminuta* 10^6 CFU/ml can provide the highest inhibition against *V. harveyi* compared to *P. diminuta* 10^5 , 10^7 , and 10^8 CFU/ml. The high inhibition of *V. harveyi* in the P3 treatment was due to the density of *P. diminuta* in the P3 treatment 10^6 CFU/ml, which was able to work optimally in inhibiting the growth of *V. harveyi*. Muliani *et al.* (2010) stated that the density of probiotic candidate bacteria applied to the shrimp rearing water was 10^6 CFU/ml optimum for inhibiting the growth of *V. harveyi* which was characterized by a decrease in the density of *V. harveyi*. This is also supported by the results of Firdaus (2013) which stated that *Pseudomonas* sp. with a density of 10^6 CFU/ml can provide the highest inhibition of the growth of *V. parahaemolyticus*.

CONCLUSION

From the research results it can be concluded that *P. diminuta* can inhibit the growth of *V. harveyi*. *P. diminuta* at a density of 10^6 CFU/ml provided the highest inhibition of *V. harveyi* growth. The effective incubation time of *P. diminuta* which could provide the highest inhibition of *V. harveyi* growth occurred at the 48th hour.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest among the authors.

AUTHOR CONTRIBUTION

The contribution of each author is as follows; Ahmad Syahrul Mubarak collecting and analyzing data, Woro Hastuti Satyantini participated in conception, experimental design, drafting, and manuscript preparation, Sri Subekti drafted the manuscript and revision.

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