Pathogenic Assay of Probiotic Bacteria Producing Proteolytic Enzymes as Bioremediation Bacteria Against Vannamei Shrimp Larvae (*Litopenaeus vannamei*)

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Abstract

Application of bacteria in bioremediation of shrimp culture ponds is one of the methods used to clean internal pollutants. This study aimed to evaluate the pathogenicity of extracellular proteolytic enzyme produced by the probiotic bacteria as bioremediation bacteria on vannamei shrimp larvae culture. There were five probiotic bacteria, which were successfully isolated from the sediments served as substrate in mangrove area. The isolated bacteria were coded in number as 13, 19, 30, 33, and 36. Pathogenic bacteria Vibrio harveyi was used as positive control. Pathogenic assay was carried out in two different bacterial concentrations, i.e. 10^8 and 10^6 cells.mL^{-1}. The results showed that the lowest survival rate (SR) of shrimp larvae in positive control V. harveyi was 53 and 65%. Whereas isolates with the highest SR value (100%) were obtained from bacteria coded as 13 and 30. Isolates no. 19, 33 and 36 had SR of more than 90%. Total plate count (TPC) data showed that the bacteria increased significantly at the end of the study with an average increase value of 24%. The smallest TPC value was shown by bacterial isolate no. 19, while the largest was obtained from the isolate no. 13. These results suggest that all probiotic bacteria were not pathogenic to the vannamei shrimp larvae.

Keywords: *aquaculture, shrimp, bioremediation, pathogenesis, vibrio.*

Introduction

Marine and brackish aquaculture produced large amounts of organic waste that are released into the surrounding environment. Most of the organic wastes come from the remaining of feed (uneaten food), and/or feces (Qian *et al.*, 2001; Holmer *et al.*, 2007). Organic waste further stimulates bacterial activity, which decline dissolved oxygen. Due to limited oxygen supply, the aerobic decomposition process shifts to the anaerobic decomposition.

Mugnier *et al.* (2007) reported that a synergistic interaction between the compounds of anaerobic metabolism especially ammonia with anaerobic environmental conditions impact the physiology of shrimp *Litopenaeus stylirostris*. Therefore, it is necessary to remove the accumulated organic materials in sediment ponds using bioremediation methods. Bioremediation process is based on the activity of facultative anaerobic microorganisms, such as bacteria that are active in the process of degradation in aerobic and anaerobic conditions.

In addition to bad environmental factors due to accumulation of organic wastes generated by cultivated organisms, problems encountered in the development of shrimp farming are factors of disease (Smith and Davey, 1993). Disease is primarily determined by the presence of pathogenic bacteria in the environment. Therefore, a strategy to control the presence of pathogenic bacteria is crucial and it is most effective through biocontrol.

Bioremediation and biocontrol are considered as an effective integrated strategy to maintain the sanitation of ponds. For that reason, the technological development of bioremediation and biocontrol is important to perform in order to improve the effectiveness and the efficiency of aquaculture activities in the future. Bioremediation and biocontrol agents are commonly known as probiotics.

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Probiotics are microbial cells culture product applied to clean the environment from contamination material (the decomposition of organic matter), to suppress the pathogenic population (mechanism antagonist) and to improve the digestibility of food (Moriarty, 1999). Therefore, probiotic becomes an alternative solution in an integrated disease control program (environmental aspect).

The application of probiotic, according to Kamiso (2004), is one of the disease control measures that gives promising results together with the shrimp fry screening activities, the usage of vaccines, immunostimulant and healthy cultivation patterns (good management practices). One of the probiotics is a consortium of bacteria antagonistic against pathogens in the pond ecosystem. This consortium has a mutual non-antagonistic and non pathogenic. Therefore this study was carried out to evaluate the pathogenicity of bacteria producing proteolytic enzymes isolated from sediments of mangrove as candidate of probiotics towards vannamei shrimp larvae *L. vannamei*.

Materials and Methods

Research was conducted by experimental Laboratory at MKHA laboratory, Center for Development of Brackish Water Aquaculture (BBPBAP) - Jepara regency. The research consisted of three phases: bacterial culture, preparation of shrimp and pathogenic assay. Culture of bacterial isolates encoded in numbers (no.) 13, 19, 30, 33 and 36 were grown on Zobell 2216 E broth media enriched with 2% of glucose and 0.05% of ammonium nitrate. Larvae of vannamei *L. vannamei* were acclimated with *Artemia* as live feed. Bacterial isolates no. 13, 19, 30, 33, 36 and the positive control bacteria *Vibrio harveyi* were evaluated for their pathogenic activity against the larvae of *L. vannamei*. Bacterial suspensions tested were prepared in two concentrations, *i.e.* 10^8 cells.mL^{-1} and 10^6 cells.mL^{-1}. This study used *autoclave, orbital shaker, centrifuge*, micropipette, Spectrophotometer UV / Vis (Beckman), colony Quebec, tank aquarium size of 70 x 60 x 30 cm, glass jar 3 L, tank heater, aeration systems, and Water quality Checker.

Isolates rejuvenation

Pure culture media was stored and was rejuvenated in Zobell 2216 E agar. Bacterial colonies were scratched with quadrant method at 2216 E Zobell media agar, were incubated for 24 hours at 35°C and were left to agitate in 150 rpm.

Culture refreshment

Culture refreshment was performed by taking 5 mL of culture solutions from each isolates no. 13, 19, 30, 33 and 36. Later, they were put into 20 mL of Zobell media 2216 E broth enriched with 2% of glucose and 0.05% of ammonium nitrate. Bacterial suspension was homogenized and was incubated for 24 hours at 35°C in 150 rpm.

Culture scale up

The scaling up of the bacterial culture was done by using 500 mL of media on the 1000 mL of erlenmeyer. The culture media was Zobell 2216 E broth enriched with 2% of glucose and 0.05% of ammonium nitrate. A volume of 1% (v/v) of inoculants at OD 0.01 on A600 was added to the culture media. Further, it was incubated at 35 °C, 30 ppt, in rpm for 24 hours.

Bacteria turbidity measurements

The dissolved oxygen (DO) determination was done by methods of Center for Development of Brackish Water Aquaculture (BBPBAP) Jepara by minor modification. Bacterial solution of 500 mL was centrifuged at 3000 rpm for 10 minutes. Supernatants were discharged, while the pellets were washed and were dissolved with 100 mL of PBS solution followed by homogenization using vortex. Bacteria turbidity was read at 600 nm.

Shrimp preparation

The shrimp larvae vanamei at *post-larval* stage (PL) 20 was used in this study. The fry/larvae were acclimatized for 3 days in the 100 x 100 x 30 cm tank aquarium equipped with shelter as a place of refuge. In addition, the walls of the tank were covered by black plastic to avoid stress on the shrimps. A net was also added on top of the tank to prevent the shrimps jumping out of the tank. The larvae of *L. vannamei* were fed with *Artemia* concentrated at 3-5 ind.mL^{-1} four times a day. The feeding period was at 07:00, 11:00, 15:00, and 19:00 of local time. Management of water quality was conducted by syphon and the water was changed every morning by replacing 10% of total volume.

Tank preparation and pathogenicity assay media

Aquarium with the dimension of 70x60x30 cm and glass jar of 3 L were prepared by washing them detergent and were left to dry. After they were dry, they were disinfected by 100 ppm chlorine for 24 hours. Disinfected jars were filled with 2 L sterile seawater each, were put into the aquarium previously prepared with 6 L sterile seawater and a heater.

Larvae of *L. vannamei* were cultured in sterile seawater. Sterile seawater was prepared by adding chlorine 30 ppm to the non-sterile seawater followed by neutralization using Na-thiosulfate 15 ppm. Level of chlorine in sterilized seawater was regularly evaluated using Chlorine test.

Pathogenic assay of probiotics

Suspension of candidate probiotics was poured into the sterile media filled with larvae of *L. vannamei* to reach the final density of 10^6 and 10^8 CFU.mL^{-1}. Experiments were carried out in two replicates with the positive control of *V. harveyi* bacteria. Larvae of *L. vannamei* with the density 10 ind.mL^{-1} were kept in a jar loaded with 2 L of sterile seawater and were fed with *Artemia* (3-5 ind.mL^{-1}). Pathogenicity of probiotics through larval mortality was observed during 5 days of culture. Survival rate (SR) of shrimp larvae was determined and was compared with the positive control based on the following formula (Effendi, 2000):

Bacteria colonies calculation

The abundance of bacteria was determined using spread plate method. The sample was diluted in a series of dilution, such as 10^{-1}, 10^{-2}, 10^{-3}, and so on, then was poured into a Petri dish, was incubated for 24 hours, and was counted. Bacterial colonies calculation was carried out by counting the number of bacterial colonies using a simple colony counter between 30 to 300 colonies on calculating tool Quebec colony counter machine by Collins *et al.,* 2001.

Bacterial colonies were expressed in colony-forming units (CFU).mL^{-1} or as a viable count.mL^{-1}. If the colonies in Petri dishes were more than 300 colonies, then the calculation could use the quadrant division, for example, one-quarter or one-eighth division calculation area (McIntosh *et al.,* 2001).

Water quality

Measurement of several water quality parameters including DO, temperature, salinity and pH were based on *in-situ* analysis using water quality checker. The water quality of larvae shrimp culture media was observed every day during the experiment.

Data analysis

Survival Rate (SR) of *L. vannamei* larvae was analyzed using the one way and two way analysis of variance (ANOVA) followed by post-hoc Tukey test (p = 0.05) for a significant difference. As for the TPC, the data were analyzed using one way and three way ANOVA followed by post-hoc Tukey test (*P*=0.05) for a significant difference. The data of water quality were analyzed by means of descriptive tabulation based on minimal and maximal interval

Results and Discussion

Pathogenicity assay was performed in this study was meant to evaluate whether the isolated probionts used in this study were pathogenic to the larvae of *L. vannamei.* It is important to consider the pathogenicity of probionts prior to application since it becomes the primary requirements of probiotics (Gomez-Gil & Roque, 1998).

Figure 1 showed that isolates probiotics no. 13 and 30 at 10^6 and 10^8 CFU.mL^{-1} had the same SR (survival rate) value, which was 100%. On the other hand, isolates no. 19 and 36 at 10^6 CFU.mL^{-1} had the same SR value, 95%, while the value of SR at 10^8 CFU.mL^{-1} was 90%. Isolate 33 at concentration 10^6 and 10^8 had SR value by 93% and 90%. The SR of larvae *L. vannamei* introduced with pathogenic bacteria *V. harveyi* at 10^6 and 10^8 CFU.mL^{-1} were quite low with 65% and 53%, respectively. Based on the SR data above, the isolates probiotics encoded 13, 19, 30, 33 and 36 were not pathogenic because the SR reached more than 90%. Amin & Hendrajat (2008) reported that the application of probiotics in shrimp vannamei live media generates SR 92-94%, implied that the use of probiotics in shrimp farming can provide a significant effect to the survival rate of the shrimps.

According to Irianto (2003), *Bacillus sp* used as probiotic bacteria did not affect the survival rate of shrimp larvae. Verschuere *et al*. (2000) stated that the addition of probiotic bacteria to the media could contribute to the maintenance of shrimp larvae in the digestive system as well as biocontrol. In the positive control, larvae of *L. vannamei* introduced to *V. harveyi,* the survival rate dropped to nearly 50%. It indicates that the pathogenicity of *V. harveyi* managed to decrease the population of *L. vannamei* larvae to 50%. This result is in accordance with that of Lavilla-Pitogo e*t al.* (1998) implying that high mortality in shrimp culture often occurs during the post-larvae, juvenile and young shrimp. Normally, the mortality of shrimp happens 48 h after the infection caused by *V. harveyi*. *V. harveyi* is an opportunistic pathogen, thus, the pathogenicity of this bacteria increases if the amount of bacteria is high and the immunity of the host weakens (Wood, 1974).

Results of two-way ANOVA analysis of SR data showed that difference treatment towards the isolates could influence significantly the SR values

(P<0.05). SR of positive control was the lowest and was significantly different (P<0.05) with the bacterial isolates of candidate probiotic. Isolates no. 33, 36 and 19 had SR values that were not significantly different (P<0.05). But these isolates differed significantly (P<0.05) against isolates no. 13 and 30. Isolates no. 13 and 30 had values of SR that were not significantly different (P<0.05). It seems that different concentrations of bacterial suspension affected significantly the SR values (P<0.05). Bacterial suspension of 10^8 cells.mL^{-1} had smaller significantly different of SR values (P<0.05) to the treatment of concentration of 10^6 cells.mL^{-1}.

Table 1. One Way Anova of SR Value based on the Pathogenicity Assay of Probiotics in Different Concentration.

Treatments	Bacteria Concentrations	
	10^6	10^8
Isolate 13	100 ± 0,0 ^{b}	100 ± 0,0 ^{c}
Isolate 19	95 ± 7,1 ^{b}	90 ± 3,5 ^{b}
Isolate 30	100 ± 3,5 ^{b}	100 ± 0,0 ^{c}
Isolate 33	93 ± 0,0 ^{b}	90 ± 0,0 ^{b}
Isolate 36	95 ± 0,0 ^{b}	90 ± 0,0 ^{b}
V harveyi	65 ± 0,0 ^{a}	53 ± 0,0 ^{a}

Description: Data are mean ± standard deviation; Different italic letters on the back of data on the same column are significantly different (P<0.05); the concentration of bacteria in units of CFU.mL^{-1}.

Results of one-way ANOVA from the SR data as presented in Table 1 indicated that the different concentrations of bacteria isolated from the sediment of mangrove significantly influenced SR value (P<0.05). Bacterial suspension of 10^6 CFU.mL^{-1} showed to influence the survival rate significantly (P<0.05). Positive control had the lowest SR value and significantly different (P<0.05) from the other 5 bacterial isolates candidate as probiotic. However, the SR values of isolates between no. 13, 19, 30, 33, and 36 did not show a significant different (P<0.05).

Different type of bacterial isolates at concentrations of 10^8 CFU.mL^{-1} influenced significantly the SR values (P<0.05). Positive control had the lowest SR value and showed significant different (P<0.05) with the other 5 bacterial isolate. Isolates no. 19, 33 and 36 had values of SR, which were not significantly different to each other (P<0.05). In addition, isolates no. 13 and 30 had values of SR which were not significantly different (P<0.05).However, a significantly different (P<0.05) was shown by isolates no. 13 and 30.

Figure 2 showed that generally the bacteria had an increasing density in the end of the research. Isolates no13 concentrated at 10^8 and 10^6 CFU.mL^{-1} increased in the density of bacteria, by 55% and 88%. The density of isolates no. 19, 30, 33 and 36 at 10^8 and 10^6 CFU.mL^{-1} were 4 and 22%, 42 and 69%, 23 and 27%, 21 and 43%, respectively. Meanwhile, the density of *V. harveyi* was 1 and 33%.

The results of three way ANOVA analysis of TPC log data presented indicated that isolates no. 19 had the lowest TPC log value, while no. 13 had the highest TPC log value. *V. harvayi,* and isolate of the probiotic no. 19, 33, 36, 30 and 13 had great TPC log value and showed a significant differenceone to another (P<0.05). Furthermore, different bacterial suspension (10^6 and 10^8 CFU.mL^{-1}) affected the TPC significantly (P<0.05), in which the TPC of bacterial suspension at 10^8 CFU.mL^{-1} was higher then the 10^6 CFU.mL^{-1}. Based on the observation time, the initial log TPC was significantly

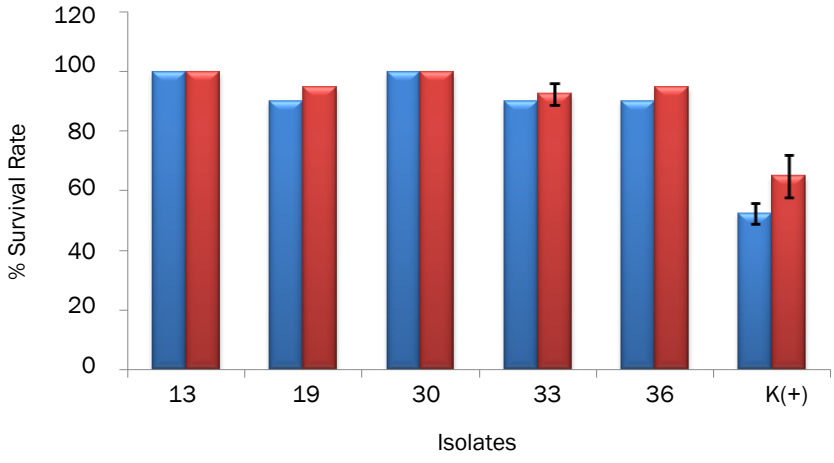

Figure 1. Percentage of survival rates in pathogenic assay from difference probiotics
Note. = 10^8, = 10^6

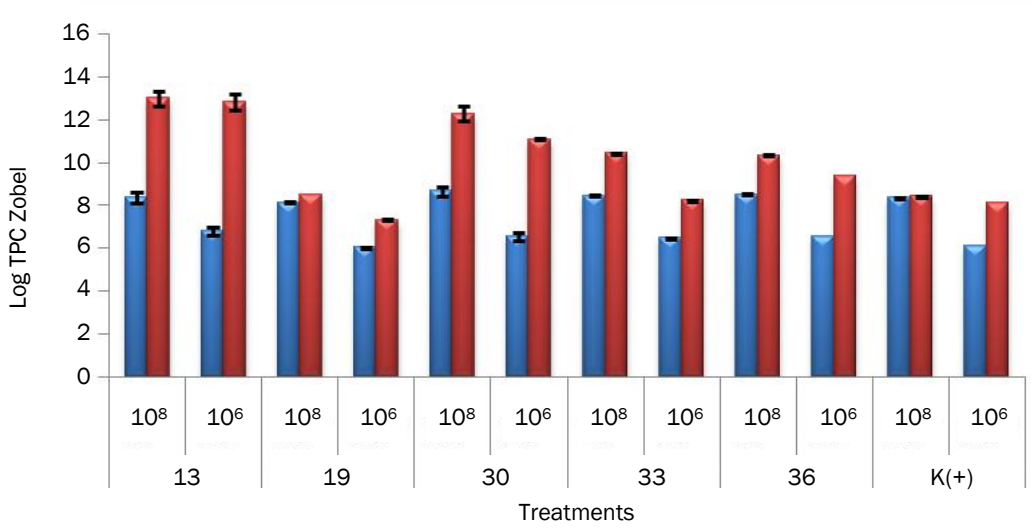

Figure 2. Log TPC At Pathogenicity Assay from Different Isolates of Probiotic
Note. = initial, = end

Table 2. One Way Anova of TPC Log Value In Pathogenicity Assay of Different Probiotics Isolates.

Treatments	Bacteria Concentrations	
	10^6	10^8
Isolate 13	12,851 ± 0,031 ^{e}	13,005 ± 0,347 ^{c}
Isolate 19	7,352 ± 0,023 ^{a}	8,518 ± 0,023 ^{a}
Isolate 30	11,117 ± 0,023 ^{d}	12,297 ± 0,029 ^{c}
Isolate 33	8,243 ± 0,026 ^{b}	10,454 ± 0,029 ^{b}
Isolate 36	9,390 ± 0,020 ^{c}	10,352 ± 0,024 ^{b}
V harveyi	8,146 ± 0,363 ^{b}	8,431 ± 0,367 ^{a}

Description: Data are mean ± standard deviation; Different italic letters on the back of data on the same column are significantly different (*P*<0.05); the concentration of bacteria in units of CFU.m^{-1}.

lower (*P*<0.05) than the final log TPC, *i.e.* after incubation.

Table 2 displayed the significant difference of bacteria concentrations based on the TPC log in probiotics isolates after incubation (p<0.05). Bacterial suspension at 10^6 CFU/ mL showed that isolates no. 19 had the lowest TPC log value and lower significantly different (p<0.05) against isolates 13, 30 and 36. Isolate no. 33 and *V. harveyi* was not significantly different (p<0.05). Isolates 36 and 30 were significantly lower to each other (p<0.05).

Treatment of concentration 10^6 CFU.mL^{-1} indicated that *V. harveyi* had the lowest TPC log value and did not differ significantly (*P*<0.05) against isolates no. 19. Isolate no. 36 and 33 were not significantly different (p <0.05). Isolates no. 13 and 30 were also not significantly different (*P*<0.05).

Water quality of the media had an important role in supporting the shrimp life. The pathogenicity. DO, temperature, salinity and pH were the parameters observed during this study. Results of these observations are presented in Table 3. The water quality of shrimp culture media had enormous influence on the growth, SR, molting frequency, as well as the population of pathogenic bacteria. DO, temperature, salinity and pH of the media used to culture the shrimp larvae ranged from 5.40 to 6.28 ppm, 27.50 to 32.50°C, 25.00 to 35.00‰ and 7.00 to 8.46, respectively.

The optimal conditions for shrimp cultivation as mentioned by Anonymous (2003), are DO> 4 mg.L^{-1} (tolerance of at least 0.8 mg.L^{-1}), temperature of 28-31°C (tolerance to 16-36° C), salinity 10-35 ppt (tolerance to 50 ppt), and pH of 7.00 to 8.70. In this study, the water quality of the media used was still in the favorable range for the shrimps. Wedemeyer (1977) stated that a favorable environmental condition during the shrimp cultivation activity would increase resistance to disease; while poor environmental conditions leading to stress and might provoke the susceptibility of shrimps to disease.

Table 3. Parameter Water Quality In Situ

Parameters	Data range
DO	5,40 - 6,28 ppm
Temperature	27,50 - 32,50 °C
Salinity	25,00 - 35,00 ‰
pH	7,00 - 8,46

Conclusions

Positive control showed lower survival rate compared to the other probiotics isolate, with SR value at 53 and 65%. Whereas isolates with the highest values of SR were isolates no. 13 and 30 with 100% of SR. Isolates no.19, 33 and 36 had value of SR inferior than 90%. TPC log data indicated that the bacteria had increased significantly in the end of the research with an average value of 24%. The smallest TPC log value in end of the research was isolates no.19, whereas the largest was isolates no.13. The results showed that probiotic bacteria no. 13, 19, 30, 33 and 36 were not pathogenic to the larvae of *L. vannamei*.

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