Macrothink Institute™

Aquatic Science and Technology
ISSN 2168-9148
2017, Vol. 5, No. 2

The Effectiveness of Candidate Probiotic Bacteria to Control Vibriosis Disease

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Received: February 21, 2017 Accepted: June 20, 2017 Published: July 24, 2017

doi:10.5296/ast.v5i2.11594 URL: http://dx.doi.org/10.5296/ast.v5i2.11594

Abstract

The study aims to isolate, characterize, and examine probiotic bacteria's inhibitory ability against *Vibrio harveyi* bacteria, both *in-vitro* and *in vivo*. Methods used in the study consist of 1) An Isolation of Candidate Probiotic Bacteria, 2) An Antagonistic Test of Candidate Probiotic Bacteria *in vitro*, 3) An Identification of Bacteria, 4) A Pathogenicity Test of Candidate Probiotic Bacteria, 5) An Antagonistic Test of Candidate Probiotic Bacteria against *V. harveyi* in vivo. According to the isolation of candidate probiotic bacteria, there are 18 isolated candidate probiotic. After being tested for its inhibitory ability in vitro, there are 8 isolates with zone of inhibition as follows: isolate MM 7 from intestine (22 mm), isolate MM 6 from intestine (12 mm), isolate MM 10 from sea water (10 mm), isolate MM 5 from intestine (9 mm), isolate MM 4 from intestine (8 mm), isolate MM 3 from intestine (7 mm), isolate MM 2.2 from intestine (7 mm), isolate MM 2.1 from intestine (7 mm). Eight genera of the candidate probiotic bacteria is derived from Portunid crab, they are *Staphylococcus, Streptococcus, bacillus, vibrio, Alcaligenes, Lactobacillus, micrococcus.* Before proceeding the *V. harveyi* bacterial challenge test in vivo, three potential isolates consisting of MM6, MM7 and MM10 as the probiotic bacteria are pathogenicity-tested against *V. harveyi*. The survival rate of Portunid crab on pathogenicity test using MM6, MM7 and MM10 generates 91.11-100%, while the control generates 100% survival rate. Variance analysis result through post-hoc Tukey's Honest Significant Difference (HSD) test at 95% confidence interval indicates that isolate MM7 and MM10 are significantly able to increase hatchling Portunid crab's survival rate.

Keywords: Probiotic, Isolation, *In vitro*, Identification, *Portunus pelagicus*, *In vivo*

1. Introduction

Both mangrove crab and Portunid crab are promising export commodity. Nowadays, Portunid crab is one of fishery commodity and has an important economic value. Indonesian export for this commodity increases every year to meet global demand, such as US, Europe, Australia and some parts in Asia. Based on the data made available by the Department of Maritime Affairs and Fisheries, the demand for crab and Portunid crab from sea food restaurateur across US reaches 450 ton monthly. This demand has not been met due to catch limits for natural preservation and minimum fish farming production.

Portunid crab, *Portunus pelagicus*, is generally known as Blue Swimming Crab or Flower Crab. High export demand leads to exploitation of Portunid crab. Alternative fish farming is required to keep Portunid crab consumption continued and well-circulated. However, the constraints faced by farmer are low crab's survival rate and unavailability of effective hatchery technology.

One of deadly disease that attacks crab in hatchery and fishpond is vibriosis, caused by bacteria from a group called *Vibrio*. Vibriosis disease found by fish farmer is also called as *udang menyala* or *kunang-kunang*. Report says that *V. harveyi* bacteria is quite pathogenic that leads to mass mortality of crab larvae. At population density of 10^3 cfu/ml, this bacterium may cause mortality more than 50% (Boer et al., 1993; Roza, 1997).

Medical treatment using antibiotics and chemotherapy agents becomes the prominent treatment in aquaculture industry. However, the use of antibiotics may arouse resistant pathogenic microorganism and stack residue that remains in fish's body and environment (FAO/WHO/OIE 2006).

By the time hatchery production is carried out, biological control is highly anticipated. The control may utilize beneficial bacteria to cut down harmful microorganism population or bacteria with toxic-decomposition ability. It will be more beneficial if there is a bacteria that is able to accelerate crab larvae's growth rate and survival rate and (Maeda and Nogami, 1989; Maeda and Liae, 1992). The use of probiotic bacteria isolated from various source as biocontrol against *V. harveyi* has been made in many reports. The bacteria used as probiotic may be isolated from healthy shrimp larvae (Widanarni and Suwanto, 2000), sea water (Chytanya et al., 2002), sea sediment (Muliani, 2002), sponge (Sasanti, 2004), intestine and water used in freshwater fish farming (Mulyati, 2010).

According to these matters, there should be a search of biocontrol agents with more potential characters to cope with Vibriosis disease in Portunid crab.

2. Research Methods

2.1 The Isolation of Candidate Probiotic Bacteria

Probiotic bacteria is isolated from the intestine (digestive organ) of Portunid crab (*P. Pelagicus*). The sample from digestive organ (intestine) is weighed out 1g, ground by mortar, then 9 ml physiological solution (NaCl 0.85%) is added. After stirring up the intestine sample and physiological solution, 0.1 ml of this mixture is poured into 0.9 ml physiological solution in eppendorf to get 10^{-1} concentrated solution or 10 times dilution. Such dilution is continuously repeated to get 10^{-7} and 10^{-8} concentrated solution. As much as 0.1 ml from respective concentration is spread equally on Sea Water Complete (SWC-agar) media, and is incubated at room temperature (28-31^{o}C) for 24-48 hours long.

Bacteria from fishpond is isolated, here are the steps: 1 ml water sample is poured into eppendorf using sterile micropipette from which 0.1 ml of the sample is added into 0.9 ml physiological solution in eppendorf. After stirring up the water sample and physiological solution, 0.1 ml of this mixture is poured into 0.9 ml physiological solution in eppendorf to get 10^{-1} concentrated solution or 10 times dilution. Such dilution is continuously repeated to get 10^{-3} and 10^{-4} concentrated solution. As much as 0.1 ml from respective concentration is spread equally on SWC-agar media, and is incubated at room temperature (28-31^{o}C) for 24-48 hours long. Sprout out colony is identified morphologically based on shape, color, edge, elevation, and size of colony (Austin, 1993; Hadioetomo, 1993).

2.2 The Antagonistic Test of Candidate Probiotic Bacteria In Vitro

Isolate *V. harveyi* and probiotic bacteria at the age of 24 hours are diluted in order to have same bacteria density at 10^{6} CFU/ml. *V. harveyi* is spread on SWC-agar media at 0.1 ml and a disc paper of 6 mm in diameter–with 0.05 ml probiotic bacteria suspension drip on it– are laid upon agar media. After being incubated for 24 hours at room temperature, the widest zone of inhibition is calculated in diameter. The isolate generating clear zone of inhibition indicates an ability to hamper *V. harveyi* growth. The bacterium with the widest zone of inhibition gets promoted to be used for the further test.

2.3 The Identification of Bacteria

The act of characterizing includes visual morphological observation on the colony visually, shape and gram stain color. Kinds of test taken are catalase test, oxidase test, oxidative-fermentative (OF) test and motility test, as well as its reaction on enzyme and sugar. The identification applies Bergey's Manual of Determinative (Holt et al., 1994).

2.4 The Pathogenicity Test of Candidate Probiotic Bacteria

Before the crab and *V. harveyi* getting tested, pathogenicity of the selected bacteria should be tested on crab Portunid crab. One lup from each isolate is grown up in SWC-liquid media in apart. The culture is put into shaker water bath at 28-29^{o}C. It is then centrifuged at speed of 3,000 rpm for 24 hours. The formed cell pellet is resuspended in physiological solution and poured into crab larvae's hatchery media to meet 10^{6} CFU/ml concentrated solution. The crab larvae is kept in a jar containing 2 liter sterile sea water where the crab density 10 crabs/liter. The crabs are fed up with *Artemia* 3-5 individual/ml. Pathogenicity of candidate probiotic bacteria is observed based on larvae mortality for 7 days cultivation. In the end of the experiment, crab larvae's survival rate is calculated and compared to the control, i.e. a treatment without candidate probiotic bacteria addition. Location of experimental unit after randomisation.

2.5 The Antagonistic Test of Candidate Probiotic Bacteria against V. harveyi In Vivo

The most potential and non-pathogenic isolate of candidate probiotic bacteria has its inhibitory ability tested against *V. harveyi* in Portunid crab. The isolate of candidate probiotic bacteria is inoculated into crab larvae's hatchery container to meet concentration 10^{6} CFU/ml for one day. After being co-cultivated together with 15 Portunid crab larvae (Megalopa) per container for 6 hours, *V. harveyi* is inoculated into crab hatchery container to meet concentration 10^{6} CFU/ml.

The experiment is repeated three times, including the control (positive control: being inoculated with *V. harveyi*, and negative control: neither with *V. harveyi* nor candidate probiotic bacteria). Observation is carried out for 10 days, and in the end of experiment, the

survival rate of crab larvae is calculated. In addition, the bacteria population from both crab larvae and water in hatchery media every three day. The experiment feeds *Artemia* to crab larvae at 3-5 individual/ml. It is carried out triplicate (three identical repetitions). Location of experimental unit after randomisation.

2.6 Data Collection Technique

The data analysis for inhibition method in vitro and bacteria identification is presented descriptively. Meanwhile, bacteria population with pathogenicity method and inhibition method in vivo, where survival rate is calculated, is analyzed statistically on SPSS 10.0 for Windows (Steel and Torrie, 1989). If any influence on the treatment occurs, the analysis is followed by Duncan's test.

3. Research Findings and Discussion

3.1 The Isolation of Candidate Probiotic Bacteria

There are 18 isolates of candidate probiotic bacteria; these are isolated from crab's intestine (digestion), fishpond water and sea water where every bacteria colony shows different appearance. Thirteen of 18 bacteria isolates are isolated from vanname shrimp's intestine, 4 other isolates are from fishpond and one isolate is from sea water. All of these 18 isolates have their inhibitory ability tested against *V. harveyi.*

The isolated intestine and fishpond water affirm the suggestion of Irianto (2003) that the selection of probion for aquaculture could be subtracted from various sources: fish's intestine or other aquatic creatures, from soil, mud, and water.

3.2 The Antagonistic Test of Candidate Probiotic Bacteria In Vitro

There are 18 bacteria isolates made from intestine, fishpond water and sea water. They have their antagonistic tested in vitro against *V. harveyi* by Kirby-Bauer method (Lay, 1994). This selection makes 8 bacteria isolates that are able to generate the widest zone of inhibition against the growth of *V. harveyi*. Bacteria isolate with the widest zone of inhibition is the isolate MM 7 made from intestine (22 mm), isolate MM 6 from intestine (12 mm), isolate MM 10 from sea water (10 mm), isolate MM 5 from intestine (9 mm), isolate MM 4 from intestine (8 mm), isolate MM 3 from intestine (7 mm), isolate MM 2.2 from intestine (7 mm), isolate MM 2.1 from intestine (7 mm).

Transparent zone surrounding the colony or *V. harveyi* growth area indicates that the antimicrobial substance is able to inhibit *V. harveyi* growth. Sensitivity of *V. harveyi* against antimicrobial substance is represented by the width of transparent area surrounding *V. harveyi* growth zone. This transparent zone indicates *V. harveyi* sensitivity against antimicrobial substance, in addition to antimicrobial substance's diffusion rate in medium. According to Lay (1994), this rate of diffusion shall be taken into account in determining antimicrobial substance's effectiveness.

3.3 The Biochemical Identification of Bacteria

Identification means pragmatic classification that takes a deeper emphasizing on specific characters, a character that distinguishes a species from other species or a character possessed by all members in their group (Cowan, 1974). Specific character derived from the isolated bacteria in this study is found by taking physiological or biochemical test. According to Bergey's Manual of Determinative (Holt et al., 1994), these are bacteria genus found in the study: *Staphylococcus, Streptococcus, bacillus, vibrio, Alcaligenes, Lactobacillus,*

micrococcus.

The product of MM 2.1 isolate characterization falls under genus *Staphylococcus*; it indicates that MM 2.1 is gram-positive, coccus, non-motile, catalase-positive, and oxidase-negative bacteria. The product of MM 2.2 isolate characterization falls under genus *Streptococcus*; it indicates that MM 2.2 is gram-positive, coccus, motile, catalase-positive, and oxidase-negative bacteria. The product of MM 3 isolate characterization falls under genus *Bacillus*; it indicates that MM 3 is gram-positive, bacillus, motile, catalase-positive, and oxidase-positive bacteria. The product of MM 4 and MM 10 isolate characterization falls under genus *Vibrio*; it indicates that MM 4 and MM 10 are gram-negative, bacillus, motile, catalase-positive, and oxidase-positive bacteria. The product of MM 5 isolate characterization falls under genus *Alcoligenes*; it indicates that MM 5 is gram-negative, coccus, motile, catalase-positive, and oxidase-positive bacteria. The product of MM 6 isolate characterization falls under genus *Lactobacillus*; it indicates that MM 6 is gram-negative, coccus, motile, catalase-positive, and oxidase-positive bacteria. The product of MM 7 isolate characterization falls under genus *Micrococcus* ; it indicates that MM 7 is gram-positive, coccus, motile, catalase-positive, and oxidase-positive bacteria.

3.4 The Pathogenicity Test of Candidate Probiotic Bacteria

Pathogenicity test result of several isolates is provided in Figure 1. The presented figure illustrates that all tested candidate probiotic bacteria are pathogen-free. This finding is detected from the value of survival rate that there are no much difference between all treatments and the control. The survival rate of hatchling Portunid crab at the pathogenicity test of isolate MM 6 generates 91% survival rate, isolate MM 7 generates 100% survival rate, isolate MM 10 generates 95% survival rate, while the control generates 100% survival rate. One of the most prominent criteria in selecting candidate probiotic bacteria is that the organism has to be nonpathogenic to the host.

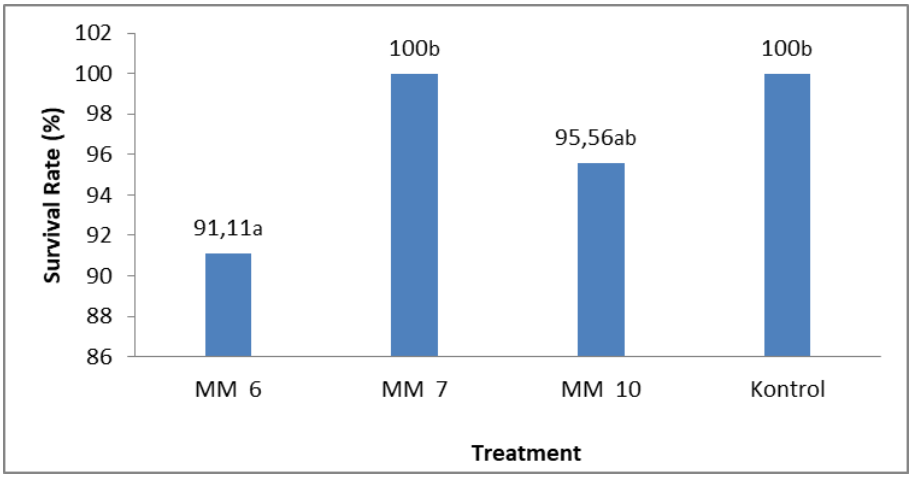

Figure 1. Portunid crab survival rate at pathogenicity test

This research finding indicates that all isolates and their respective concentration 10^6 cell/ml which had been infected through soaking method do not induce infection and disease in the hatchling Portunid crab with 100% survival rate. Same finding has been preceded by Aly et al. (200). They found that *Bacillus subtilis* and *Lactobacillus acidophilus* which were injected to *intraperitoneal* (IP) is not dangerous and does not cause mortality to Tilapia nilotica

(*Oreochromis niloticus*) for 15 days observation. Muliani (2002) also suggested that the bio-controlled soaking of candidate bacteria which was isolated from sea sediment (BL 542) and sea water (BL546 and BL548) in Balam Lompo Island, South Sulawesi at density 10^5, 10^6, 10^7, and 10^8 CFU/ml for 24 hours will not make giant tiger prawn pathogenic.

Verschuere et al. (2000) suggested that probiotic as a living microbial agent is beneficial for the host. It means that probiotic bacteria may not be pathogenic to its host, both in normal and stress state. In brief, the three isolates remains safe and are able to depress the growth of pathogenic bacteria.

3.5 The Inhibition of Candidate Probiotic Bacteria against V. harveyi In Vivo

The two most potential isolate made from candidate probiotic bacteria isolates by in vitro test is isolate MM 7 and MM10 as they had their effectiveness tested to inhibit *V. harveyi*'s attack to the hatchling Portunid crab. Observation is carried out to find out larvae's survival rate and bacteria population in hatchery water.

Variance analysis result through post-hoc Tukey's Honest Significant Difference (HSD) test at 95% confidence interval indicates that isolate MM7 and MM10 are significantly able to increase hatchling Portunid crab's survival rate. It is said so because statistically, crab's survival rate is far different from positive control (Figure 2). The isolate seems inhibit *V. harveyi* growth that it is able to increase Portunid crab's survival rate. It is found from Portunid crab's survival rate at the positive control (inoculated by *V. harveyi*). At this positive control, the survival rate is lower than the one added by probiotic isolate; 40% vs 75-85%. According to Verschuere et al. (2000), probiotic bacteria is capable to guarantee crab's feed improvement and nutrition intake, and to develop host's response sensitivity against the disease.

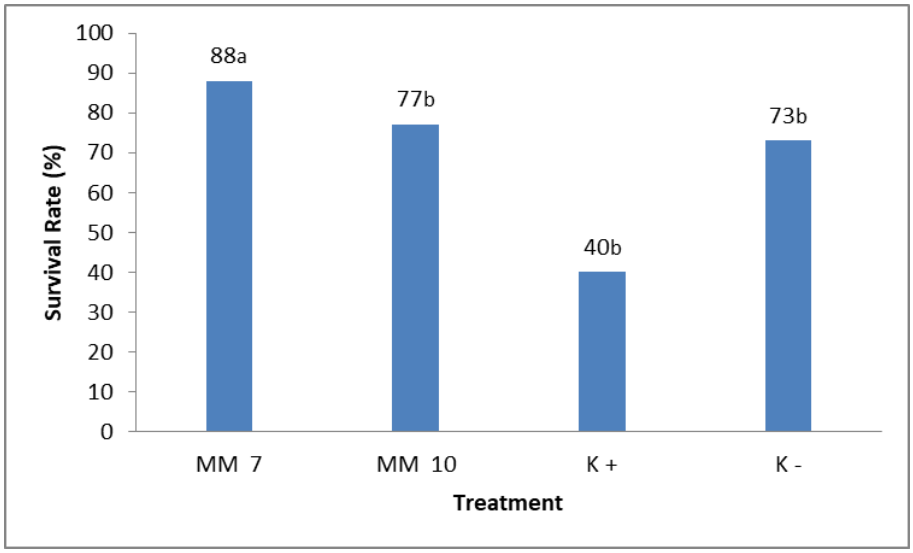

Figure 2. Survival rate *in vivo*

Rengpipat et al. (1998) has found that *Bacillus* S11, as the probiotic bacteria, was effective by treating the bacteria with probiotic for 100 days. It was then challenge-tested against *V. harveyi* for 10 days, and it made out that the giant tiger prawn's survival rate reached 100%, compared to 26% at the control. Haryanti et al. (2000) reported that the addition of strain BY-9 at concentration 10^6 CFU/ml into the giant tiger prawn's hatchery container made 59.3% survival

rate, while it was only 14.7% at the control. Riquelme et al. (1997) reported that the addition of strain C30 and SV1 into the shell (*Argopecien pururatus*) larvae's hatchery container was significantly raising the larvae's survival rate, even though the strain did not do the same after being challenge-tested against *V. Anguiilarum*. Isolate C30 is known because it can hamper pathogenic *Vibrio anguillarum* in vitro, while there is no inhibition using SV1. In 2006, Agustina reported that a feeding treatment plus probiotic bacteria KL4 made the survival rate at 84.45%, followed by a feeding treatment plus probiotic bacteria BBI at 80.00%, while the control only had survival rate at 55.56%.

The survival rate of hatchling crab increases when *V. harveyi* growth is inhibited by probiotic bacteria. It is illustrated in Figure 3 that the giving of candidate probiotic bacteria into isolate MM7 and MM10 makes *V. harveyi* no longer visible in the third day of bacterial inoculation. At the positive-control treatment, since the very first day to the end of the observation day, *Vibrio* population remains alive. At the negative-control treatment (without bacteria placement), the bacterium remains alive. It occurs because both the hatchling crab and hatchery water have bacteria from the nature.

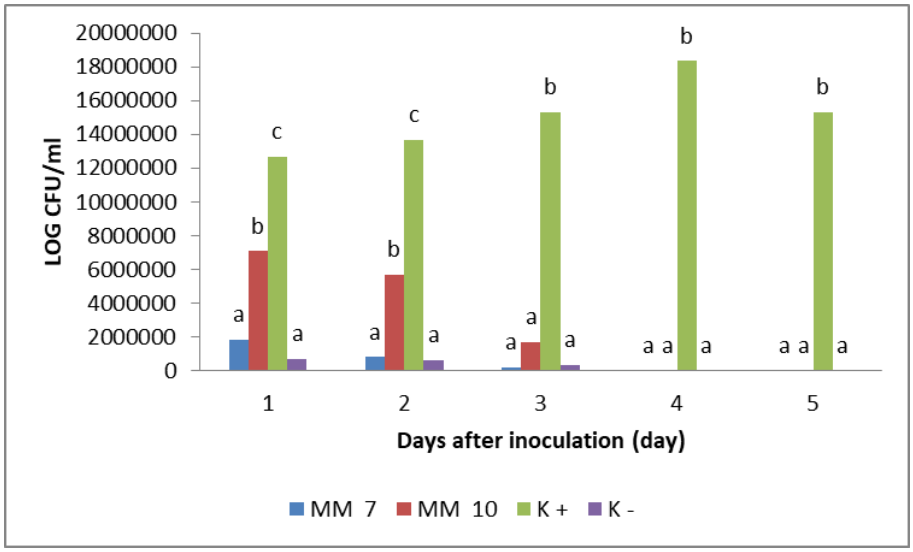

Figure 3. *V. harveyi* population in hatchery water container

Low *V. harveyi* population in hatchery water after probiotic addition is promoted by probiotic bacteria. This bacterium releases antibacterial compound or other compound to inhibit pathogenic bacteria, such as antibiotic, bacteriocins, siderospore, lysozyme, protease, and organic acid (Sugita et al., 1998). Nakamura et al. (1999) also suggests that bacteria playing a role as biocontrol agent will release vibriostatic or vibrisidal compound. The release of such compound creates niche competition between V. harveyi and biocontrol agent. The ability possessed by microbe to inhibit pathogenic microbe indicates a quality to maintain its micro flora balance in fish's digestive system. This ability relates to microbe's quality to release antimicrobial compound, a peptide synthesized in rybosom (Aslamyah, 2006). Normal flora in intestine has an important function to suppress pathogenic bacteria and virus, stimulate local and systemic resistivity, and alter metabolic activity of the intestine's microbe. Moreover, probiotic microbe may also suppress pathogenic microbe because of receptor competition, rise of mucous production in the intestine, and competition of nutrition (Salminen and Wright,

1993). This research finding confirms an opinion that the addition of bacterial isolate will reduce mortality due to *V. harveyi* attack.

It is possible that the decrease of bacterial population during Portunid crab treatment might be pre-colonized by other type of bacteria. Other bacterial colonization might be occurred too, as it happened in the study by Rengpipat et al. (1998). The experiment was given to shrimp larvae, and it was pre-colonized by other bacteria, in addition to the inoculated *Bacillus* S11. Decreasing *V. harveyi* population in the hatchery container may also be stimulated by the effectiveness of Portunid crab's immune system. It proves that the addition of probiotic bacterial isolate is more effective to improve crab's immune system to fight against *V. harveyi*, compared to the positive-control. This finding affirms the former research taken by Nikoskelainen et al. (2003) whose potential probiotic bacterium was *Lactobacillus rhamnosus*. They found that this bacterium was capable of improving rainbow trout's immune system. Irianto and Austin (2002) made the same conclusion; the two used probiotic to control furunculosis in rainbow trout fish. Gomez-Gil et al. (2000) suggested that probiotic is an agent with capability to prevent damage on the host's body which is caused by pathogen, generally from competition. Meanwhile, most of these probiotic bacteria generate a substance with an ability to inhibit growth or attachment of harmful microorganism.

Rengpipat et al. (1998, 2000) suggested that the increase of giant tiger prawn's survival rate in the treatment with the addition of *Bacillus* S11 probiotic bacteria occurs because the probiotic bacteria compete exclusively in shrimp's digestive system. In fact, the giving of probiotic at earlier stage of larvae will promote more optimum result since larvae's digestive system and its environment was pre-dominated by favorable bacteria (Rengpipat et al., 1998).

4. Conclusion

Two probiotic bacterial isolates, MM7 isolated from intestine and MM10 isolated from sea water, have potential inhibitory ability against *V. harveyi* growth in Portunid crab's body.

These two isolates are able to improve Portunid crab's survival rate which were pre-infected by *V. harveyi*. Thus, these isolates may be utilized as probiotic.

Acknowledgement

We would like to express our gratitude for the Director of Pangkep State Polytechnic for Agriculture and the chief of Research & Community Service Unit who have made facilities available for us. This study was funded by the Multi-year Research Program (competitive research grants of DP2M DIKTI, year 2012 and 2013). Our gratitude is also for our team, Irfani Baga, Suryati and to all technicians Seniati, Nurhayati, Basse Risfayati, and Ma'mur who have helped us to organize this study.

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