Streptomyces sp. as a Biocontrol of Vibriosis on Larvae of Macrobrachium rosenbergii (de Man) Prawns

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**ABSTRACT**

Vibriosis is a limiting factor on production of *M. rosenbergii* (de Man) prawn hachlings. An alternative control of vibriosis can be undertaken through a biological pathways by using *Streptomyces* bacteria. Ten isolates of *Streptomyces* spp. were isolated in this research from rhizosphere of mangrove of *Rhizophora* spp. Three isolates namely *Streptomyces sp.1*, *Streptomyces sp.3* and *Streptomyces sp.4* could inhibit the growth of *V. anguillarum* (in vitro). *Streptomyces sp.1* could inhibit the growth *V. anguillarum* best with diameter of inhibition of 21.03±1.42 mm. Filtrates of *Streptomyces sp.1* could also inhibit *V. anguillarum* (in vitro) with MIC value of 10%. Aplication of *Streptomyces sp.1* culture could give significant different impacts (*p<0.05*) on the percentage of survival (SR) of prawn larvae that had been infected by *V. anguillarum* compared to the control. Treatment of *Streptomyces sp.1* culture could also significantly (*p<0.05*) reduce the total population of Vibrio on the maintenance media compared to the control.

Key words: *Streptomyces* sp., *Vibrio anguillarum*, *Macrobrachium rosenbergii* (de Man) Prawns, Vibriosis and Biocontrol Agent.

**INTRODUCTION**

The activity of nursery of shrimp larvae must be steril from patogenic infections. Infection on hatchling besides causing high financial lost, it may be also potential to be of an entry point of patogenic bacteria to the shrimp ponds (Atmomarsono *et al.*, 2010; Patang, 2012).
**Vibrio** spp. act as the main pathogenic bacteria causing vibriosis on animals kept in aquaculture systems (Thiruvarangan *et al.*, 2014). Increase in the population of *Vibrio* on aquaculture ponds can disturb physiological activities of shrimps that in turn may cause increase in mortality of captivated animals (Velmurugan *et al.*, 2015). Some species that have been reported to cause vibriosis such as *V. harveyi*, *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholera*, *V. fischeri* and *V. splendidus* (Morris, 2003; Azizunnisa and Shreeramulu, 2013; Vaseeharan et al., 2013). Control of vibriosis by fishermen was generally by using antibiotics. Some antibiotics such as from the group of quinolones, flumequine and fluoroquinolone are frequently used to supresse the vibriosis infections on aquaculture systems (Cabello, 2006; Balcazar *et al.*, 2006). In practical, the use of the antibiotics may have negative impacts to the health as well as harmful to the environment. Applying antibiotics on aquaculture can increase the resistance of the pathogenic bacteria as well as causing bio-accumulation of their residue on the captivated animals (Isnansetyo, 2009). The concept of biological control agents as what Aftabudin *et al.* (2013) andIsnansetyo (2009) stressed out was potential to be developed in controlling bacterial aquatic infections. As Selvakumar *et al.* (2010) postulated, *Streptomyces* bacterium is one of the biological controlling agents which has a potential to be applied in aquaculture systems. *Streptomyces* is well known to have capability in antagonistic activities against other bacteria, including on the group of patogenic *Vibrio* (Selvakumar *et al.*, 2010; Velmurugan *et al.*, 2015). The genus of *Streptomyces* is a group of Actinobacteria which are commonly exist in marine ecosystems (Velmurugan *et al.*, 2015). Mohana and Radhakrishnan (2014) added that *Streptomyces* which is isolated from the beach area, especially from the mangrove areas, have high antagonistic activities on negative Gram bacteria. Hong *et al.* (2009) reported that mangrove ecosystems act as a potential habitat for Actinomycetes, the bacteria on which habitats have been limited explored. As the mangrove occupied intertidal zones, Wang *et al.* (2003) stated that this ecosystem act as an extremely productive areas, so they have a high potential to be used as a source of isolates for microorganisms producing bioactive compounds. Based on this assumption, the objective of this research was to reveal the potential of *Streptomyces* act as a biocontrol agent controlling vibriosis diseases on shrimp larvae.

**MATERIAL AND METHODES**

**Bacterial Strain and Media**

Strain of *V. anguillarum* causing vibriosis on prawn larvae of *M. rosenbergii* (*de Man*) was isolated from larvae ponds belong to Integrated Services Unit (UPT) of Nursery (Pembenihan), Office of Marine and Fisheries (Dinas Kelautan dan Perikanan), Bali Province (Bintari *et al.*, 2016). Strains of *Streptomyces* spp. were isolated from the soil of mangrove rhizosphere at the Mangrove Information Centre, Suwung Kawuh Village, Kuta District, Badung Regency, Bali, and isolation was carried out at Microbiology Laboratory, Biology Department, Faculty of Natural Sciences and Mathematics of Udayana University, Bali. *Vibrio anguillarum* was cultured on Trypic Soy Broth (TSB) (Merk™), *Trypic Soy Agar* (TSA) (Merk™) and *Alcaline Peptone Water* (10 g of pepton, 10 g of NaCl, 1000 ml of water, final pH or acidity of 8.5) media.
Streptomyces spp. was cultured on Yeast Extract Malt Agar (YEMA) media (0.5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.1 g of NaCl, 10 g of malt, 1 g of yeast extract, 15 g of agar, 600 ml of distilled water, 400 ml of sea water) and Yeast Extract Malt Broth / YEMB (media composition of 4 g (0.4%) of yeast extract, 10 g (1%) of malt, 0.4 g of glucose, 600 ml of distilled water, and 400 ml of sea water).

Isolating, identifying and characterisation of Streptomyces spp.
Isolation of Streptomyces spp. was undertaken through a serial dilution method. It was grown on YEMA media and incubated on 28°C for 5 days. The colony was characterised macroscopically, microscopically, undergone Gram stained tests and acidic resistency. Identification of the genus was undertaken as stated on the Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics (Lechevalier and Waksman, 1973) as well as on the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Antagonistic Activity of Streptomyces spp. against Vibrio anguillarum
Activity tests were carried out using dual culture methods (Bernal et al., 2015). In the test of activities, Streptomyces spp. was grown on YEMA culture for 5 days. Vibrio anguillarum was cultured on TSB for 24 hours (28±30°C) and standardized by Mc Farland 5%. The culture was grown on 200 µl TSA media through pour plate. When the media has already become solid, the culture of Streptomyces sp. (with the diameter of ± 5 cm) was placed in the centre of agar media. It was then incubated on 28°C for 24 hours. Investigation was undertaken by observing the existence of inhibition zones emerging on the media. The isolate of Streptomyces that have the highest inhibition capability (in mm), was utilized in the following tests.

Isolation of Streptomyces sp. Filtrate and Determination of Minimum Inhibitory Concentration (MIC)
The colony of Streptomyces sp. was grown on YEMA media for 5 days. The colony grown on 100 ml YEMB media was by taking 5 rounded colonies using a cork borer (diameter ± 5 mm), and then incubated on 80 rpm speed of shaker (Retnowati 2010; Kawuri, 2012). The culture was incubated for 22 days and then isolation of the filtrate was undertaken periodically by centrifugating the culture on 11,000 rpm speed for 15 minutes. Supernatant was then shievered by 0.45 µm filter papers (Charoensopharat et al., 2008). The filtrate activity tests was undertaken by applying diffusion well methods (Kawuri, 2012) for finding out the best incubation time of Streptomyces sp. isolates in producing metabolite compounds which was then followed by MIC tests. The filtrates for MIC tests were partitioned by N-butanol solution with the volume ratio of 1:1 (v/v). The water phase and N-butanol phase formed were evaporated by using evaporator on 40°C until getting concentrated filtrates. The MIC tests were undertaken on several concentrations of filtrates (v/v): 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10% as well as on 0% as control by applying diffusion well methods (Kawuri, 2012 ; Laidi et al., 2013).

The Potential of Streptomyces sp. as a biocontrol for Vibrosis on Prawn Larvae
Prawns larvae which was used on the M. rosenbergii (de Man) larvae test was in the IV stadium. Larvae was placed in the plastic bag with 500 ml brakish water (salinity of 10 ppt), as many as 50 individuals each. The larvae was aclimatised and given fodder of Artemia sp. as many as 4 naupli/larvae in the morning and in the afternoon. The experimental design applied was the complete randomized design with 5 levels of treatments of Streptomyces sp. cultures.
The isolate of *Streptomyces* sp. was cultured on YEMB media and incubated on 80 rpm speed shaker for 5 days. The culture was harvested and standardised on $10^5$ CFU/ml density. The suspension of the culture which was used in the treatment was 2.5 ml, 5 ml, 7.5 ml and 10 ml. Inoculation of the culture of the *Streptomyces* sp. was undertaken for 2 days respectively, each treatment undertaken once daily (Velmurugan et al., 2015), and on final treatment, change of 25% of maintenance water was undertaken. On the third days of treatments, *V. anguillarum* culture was inoculated as much as 10 ml. The isolate of *V. anguillarum* was cultured on APW media for 24 hours on ± 30°C. The bacteria were harvested through centrifugation on 5000 rpm for 5 minutes. The pellets gained were then suspended again on 10 ml of NaCl 0.9% (w/v) and standardized by McFarland 5% (Chau et al., 2011). Observation was made after 24 hours inoculation of the patogenic by observing the percentage of survival rates of larvae (%SR) and the abundance or the total of *Vibrio* bacteria on the water where it was kept. The value of %SR was calculated by using the Goddard (1996) formula:

$$SR = \frac{N_t}{N_0} \times 100\%,$$

where $SR = \text{level of survival rate of larvae (}); N_t= \text{number of larvae which is survive at the end of observation (individuals)}; N_0 = \text{Numbers of prawn larvae at the beginning of observation.}$

Data were analyzed through *Analysis of Variance* (ANOVA), through F test on the level of 5% and then followed by the Duncan (DMRT) test on the level of 5%.

**RESULT**

**Isolation of *Streptomyces* spp.**

Results of the isolation on rhizosphere of *Rhizophora* sp. showed that there were 10 isolates of *Streptomyces* with different macroscopic characteristics were successfully isolated. The general characteristic belongs to the isolates such as the colony were strongly attached to YEMA media, forming aerial hifa, Gram positive stained, cannot stand to acid, positive catalase, cained conidia with the size of 0.20-1.84 µm and having vegetative hifa with the diameter of 0.40-1.00 µm.

**Antagonistic activity test**

Antagonistic activity of the 10 isolates of *Streptomyces* was tested by utilizing dual culture methods. Results of the tests showed that 3 isolates namely *Streptomyces* sp.1, *Streptomyces* sp.3 and *Streptomyces* sp.4 (Figure 1) can inhibit the growth of *V. anguillarum* on the in vitro test (Table 1) which is indicated by the formation of inhibition zones around antagonistic bacteria (Figure 2). *Streptomyces* sp.1 has the best potential in inhibiting *V. anguillarum* by the diameter of inhibition of 21.03 ± 1.42 mm, which was then explorated for the next test.

**Filtrate activity test and determination of MIC**

Based on the result of the filtrate activity test, it was found that the *Streptomyces* sp.1 could produce metabolit compounds, the compound of which had antibacteria characteristics against *V. anguillarum*. *Streptomyces* sp.1 was active, starting producing metabolit compounds which had antibacteria characteristics on day 12th and the production was optimum on the day of 18th (Figure 3). The filtrate on the 18 day incubation periods was then undergone a purification process by extracting the filtrates by n-butanol solution for 24 hours. Results of purification processes showed that the filtrates on the n-butanol fase was capable in inhibiting the *V. anguillarum* in vitro by inhibition diameter of 20.4±0.31 mm.
Results of MIC tests showed that in this research, the concentration of the filtrates of 10% was the minimum concentration that was capable to inhibit the *V. anguillarum* by the inhibition capability of 12.5±0.13 (Table 2). Nevertheless the acquired value of MIC was still high, so, the reduction of filtrate concentration under 10% needed in order to get the maximum MIC value.

**The potential of *Streptomyces* sp1. as a biocontrol of vibriosis on prawn larvae**

Results of application tests showed that treatment of *Streptomyces* sp.1 culture brought impacts significantly (p<0.05) on increasing the percentage of the survival rate (%SR) of prawn larvae (Table 3.). Treatment of culture of *Streptomyces* sp.1 by volume of 5 ml (A2B1), 7.5 ml (A3B1) and 10 ml (A4B1) gave significantly different impacts (p<0.05) compared to the control (A0B1). Adding 10 ml culture of *Streptomyces* sp.1 on the media found to be the most effective on improving the percentage of survival rates of larvae. Application of *Streptomyces* sp.1 was also found to gave impacts on the decrease in the total population of Vibrio on the liquid component of the media (Table 3). Treatment of A4B1, A3B1 and A2B1 known to give significant different impacts (P<0.05) on the total Vibrio bacteria on the media compared to the control.

**DISCUSSION**

As many as 10 isolates of *Streptomyces* spp. were successfully isolated and characterized from rhizosphere of mangrove *Rhizophora* sp. Three isolates (Figure 1) namely *Streptomyces* sp.1, *Streptomyces* sp.3 and *Streptomyces* sp.4 had an antagonistic activity against *V. anguillarum* that caused vibriosis on the *M. rosenbergii* (de Man) larvae (Table 1, Figure 2). The rhizosphere of mangrove according to Usha et al. (2010) and Rao and Rao (2013) acted as a potential source of exploration of Actinomycetes. As it is located in intertidal areas, Wang et al. (2003) claimed that the mangrove areas were productive so they have a potential to be used as a source of microorganism isolates producing bioactive compounds.

The antagonistic ability of *Streptomyces* on pathogenic bacteria in aquaculture has been frequently reported. Some species of *Streptomyces* such as *Streptomyces* strains VM-8, VM-15, VM21 (Velmurugan et al., 2015) and the *Streptomyces* strain A1 (Chau et al., 2011) which were isolated from the sediment of ponds was revealed to be having potentials as the biocontrol against vibriosis of shrimps. Some species of *Streptomyces* such as *S. flocculus*, *S. pancagri* (Bernal et al., 2015), *S. xantholithicus*, *S. aureofasiciplic*, *S. vastus*, *S. galbus*, *S. rimosus* (Sahu et al., 2007) and *S. fradiae* (Aftabuddin et al., 2013) were also known to have antagonistic activity against the patogenic Vibrio on the aquaculture.

The genus of *Streptomyces* has been known broadly to be able to produce metabolite compounds such as antibiotics (Remya and Vijayakumar, 2008), enzymes and melanines (Manivasagan et al., 2013) as well as siderophores (You et al., 2005) which were having function in antagonistic activities. Results of exploration on *Streptomyces* sp.1 on this research showed that this isolate can actively producing secondary metabolite compounds on water culture. This was shown by the capability of inhibition of the filtrate produced by the *Streptomyces* sp.1 (on the in vitro test) against *V. anguillarum*. On this research it was revealed that the optimum production of metabolite compound by *Streptomyces* sp.1 occurred on the day 18<sup>th</sup> of the incubation periods (Figure 3), with MIC value of 10% (Table 2). The filtrate of *Streptomyces* has a high potential in producing antibacterial compounds.
The filtrate of the *S. violaceusniger* strain HAL64 was reported to contain high quantities of casinostatines, the compounds of which was known to be effective in inhibiting Gram positive and negative bacteria (El-Naggar, 2007). The filtrates of *S. longwoodensis* and *S. viridiviolaceus* were also known to contain vast spectrum of antibiotics against patogenic bacteria and fungy (Remya and Vijayakumar, 2008).

### Table 1. Inhibition capability of the isolate of Streptomyces on the *V. anguillarum*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolates of the Streptomyces</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Streptomyces</em> sp.1</td>
<td>21.03±1.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td><em>Streptomyces</em> sp.2</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.</td>
<td><em>Streptomyces</em> sp.3</td>
<td>9.57±0.75&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td><em>Streptomyces</em> sp.4</td>
<td>8±0.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.</td>
<td><em>Streptomyces</em> sp.5</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.</td>
<td><em>Streptomyces</em> sp.6</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.</td>
<td><em>Streptomyces</em> sp.7</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8.</td>
<td><em>Streptomyces</em> sp.8</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>9.</td>
<td><em>Streptomyces</em> sp.9</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.</td>
<td><em>Streptomyces</em> sp.10</td>
<td>0±0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>11.</td>
<td>Chloramfenicol positive control</td>
<td>31.43±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes:
1). Values on the table ± standard deviation (average of 3 replications).
2). Different letters on the same columns indicated significantly different results (P<0.05) based on Duncan tests.

### Table 2. Results of MIC test of filtrates of Streptomyces sp.1 against the *V. anguillarum*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Filtrate consentation</th>
<th>Diameter of inhibition power (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100%</td>
<td>20.4±0.31a</td>
</tr>
<tr>
<td>2.</td>
<td>90%</td>
<td>17.8±0.05ab</td>
</tr>
<tr>
<td>3.</td>
<td>80%</td>
<td>17.6±0.05ab</td>
</tr>
<tr>
<td>4.</td>
<td>70%</td>
<td>17.4±0.12ab</td>
</tr>
<tr>
<td>5.</td>
<td>60%</td>
<td>16.4±0.15abc</td>
</tr>
<tr>
<td>6.</td>
<td>50%</td>
<td>15.8±0.14bc</td>
</tr>
<tr>
<td>7.</td>
<td>40%</td>
<td>15.8±0.15bc</td>
</tr>
<tr>
<td>8.</td>
<td>30%</td>
<td>14.9±0.07bc</td>
</tr>
<tr>
<td>9.</td>
<td>20%</td>
<td>12.5±0.13c</td>
</tr>
<tr>
<td>10.</td>
<td>10%</td>
<td>12.5±0.13c</td>
</tr>
<tr>
<td>11.</td>
<td>Control (n-butanol)</td>
<td>0.00±0.00d</td>
</tr>
</tbody>
</table>

Notes:
1). Values on the table ± standard deviation (average of 3 replications).
2). Different letters on the same columns indicated significantly different results (P<0.05) based on Duncan tests.
The culture of *Streptomyces* sp.1 which was applied on maintenance containers was known to be capable of increasing the percentage of survival rates of larvae and decreasing the total of Vibrio on the maintenance water (Table 3). Adding 10 ml of the culture of *Streptomyces* sp.1 on the maintenance media was known to be the most effective on increasing the percentage of survival rates of larvae as much as 26.8 ± 6.09 %, and decreasing the total of Vibrio from the origin population, from $1 \times 10^7$ CFU/ml into $1.7 \times 10^3$ CFU/ml. Most of the species of Vibrio were opportunistic patogenic bacteria on the aquaculture. According to Kimura et al. (1998), when the total Vibrio on the culture system increased, it then would be correlated with the incrementation of infections. Based on this, the application of *Streptomyces* on the culture system will be excellent because it will be able to reduce the total population of the Vibrio.

### Table 3. The percentage of Survival Rates (SR) of larvae of *Macrobrachium rosenbergii* (*in vivo* tests).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacteria Culture of <em>Streptomyces</em> spp. 1 (A)</th>
<th>Bacteria Culture of <em>V. anguillarum</em> (B)</th>
<th>% SR</th>
<th>Total of Vibrio at the Beginning (CFU/ml)</th>
<th>Total of Vibrio at the End (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0B1</td>
<td>0 ml</td>
<td>10 ml</td>
<td>9.6 ± 1.67b</td>
<td>$10^7$</td>
<td>$5.28 \times 10^3$ab</td>
</tr>
<tr>
<td>A1B1</td>
<td>2.5 ml</td>
<td>10 ml</td>
<td>18 ± 6.75ab</td>
<td>$10^7$</td>
<td>$4.62 \times 10^3$ab</td>
</tr>
<tr>
<td>A2B1</td>
<td>5 ml</td>
<td>10 ml</td>
<td>23.2 ± 11.09a</td>
<td>$10^7$</td>
<td>$2.12 \times 10^3$bc</td>
</tr>
<tr>
<td>A3B1</td>
<td>7.5 ml</td>
<td>10 ml</td>
<td>25.2 ± 4.14a</td>
<td>$10^7$</td>
<td>$2.1 \times 10^3$bc</td>
</tr>
<tr>
<td>A4B1</td>
<td>10 ml</td>
<td>10 ml</td>
<td>26.8 ± 6.09a</td>
<td>$10^7$</td>
<td>$1.7 \times 10^3$c</td>
</tr>
</tbody>
</table>

Notes:
1). Values on table ± standard deviation (average of 5 replications).
2). Different letters on the same columns indicated significantly different results (P<0.05) based on Duncan tests.

![Figure 1. The colony of *Streptomyces* sp.1 (A), *Streptomyces* sp.3 (B) and *Streptomyces* sp.4 (C) on the YEMA media.](image-url)
This result also supported a research by Aftabuddin et al. (2013), the research of which found that the application of *Streptomyces* sp. on the larvae ponds would be effective in reducing the population of the Vibrio. On that research it was known that the *Streptomyces fradiae* which was applied on the maintenance ponds of larvae of *P. monodon* can reduce the population of the Vibrio significantly compared to the control. *Streptomyces fradiae* was known to be able to secret an antibiotic as a mechanism of inhibiting the pathogenic Vibrio. The ability of the antagonistic microbes in reducing the population of the Vibrio spp. on the maintenance media according to Banaerjee et al. (2007) can also caused by the production of vibriostatic compounds and because of niche competitions between the Vibrio bacteria and the antagonistic bacteria. The ability of the Streptomyces in controlling the vibriosis infections on shrimps has also been reported by Velmurugan et al. (2015). On his research, the culture of *Streptomyces VM-15*, *Streptomyces VM-21*, and *Streptomyces VM-8* were found to be effective in reducing vibriosis infections on post larvae of *P. monodon*.
Das et al. (2010) were also reported that the application of the culture of Streptomyces sp. on the concentration of 1% (v/v) was known can reduce vibriosis infection by V. harveyi and V. proteolyticus on adult Artemia sp. (age 15 days).

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