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POTENCY OF *VIBRIO* ISOLATES FOR BIOCONTROL OF VIBRIOSIS IN TIGER SHRIMP *(PENAEUS MONODON)* LARVAE

WIDANARNI[1,2]. A. SUWANTO[u],SUKENDA[2],and B. W. LAY[4]

'Department of Biology, Faculty of Science and Mathematics, Bogor Agricultural University, Bogor 16144, Indonesia [2] Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor 16680, Indonesia [3]SEAMEO-BIOTROP, Jl. Raya Tajur Km 6, Bogor 16720. Indonesia ^Faculty of Veterinary Science, Bogor Agricultural University, Bogor 16680, Indonesia

ABSTRACT

This study was carried out to obtain *Vibrio* isolates able to function as biocontrol of vibriosis in shrimp hatchery. Thirty one *Vibrio* isolates were isolated from tiger shrimp larvae and hatchery environments, i.e. Labuan, Pangandaran, and Lampung, Indonesia. Pathogenic *V. harveyi* MR5339 was obtained from Maros, South-Sulawesi and was made as a rifampicin resistant mutant (RF^R) to screen for those 31 *Vibrio* isolates in *in vitro* assays and to allow us to monitor their presence in shrimp larvae and larval rearing water. Almost all *Vibrio* isolates could inhibit the growth of pathogenic *V. harveyi* MR5339 RF^R. SKT-b isolate from *Skeletonema* was the most effective to inhibit the growth of *V. harveyi* MR5339 Rf* and significantly reduced larval mortality in pathogen challenge assays. These prospective biocontrol bacteria, at concentration of 10" CFU/ml, did not show pathogenicity to shrimp larvae. SKT-b was Gram negative, short rod-shape, exhibited yellow colonies on TCBS and swarming on SWC-agar media, motile, utilized glucose and sucrose but not lactose: produced extra-cellular protease and amylase, but did not produce chitmase. Partial sequencing of 16S-rRNA gene SKT-b showed SKT-b similarity to *Vibrio alginofyticus.*

Keywords: shrimp larvae / biocontrol bacteria / vibriosis.

INTRODUCTION

Tiger shrimp *(Penaeus monodori)* culture in Indonesia has become more intensive and extensive because of high demand and economic value of this export commodity. However, the shrimp culture industry is associated with multiple problems such as diseases and poor environmental quality, which became the main constraint to reach the target of production. Bacterial disease that attacked at hatchery stage is the most serious threat and often caused mass mortality in shrimp larvae which greatly influenced the sustainable supply of healthy fry. This disease is often caused by a luminous bacterium identified as *Vibrio harveyi* (Lavilla — Pitogo *et al.* 1990; Pedersen *et al.* 1998).

To prevent such mass mortalities, shrimp hatcheries routinely use antibiotics. However, widespread antibiotics applications could result in antibiotic resistant pathogens (Karunasagar *et al.* 1994; Tjahjadi *et al.* 1994; Tendencia and de la Pena

*** Corresponding author: asuwanto@indo.net. id**

2001). Not only can resistant bacteria proliferate after an antibiotic has killed off the other bacteria, but also they can transfer their resistant genes to other bacteria which have never been exposed to the antibiotic (Verschuere *et al.* 2000). Furthermore, this approach is not predictable since *V. harveyi* strains that attack shrimp larvae are genetically diverse (Suwanto *et al.* 1998). The development of vaccine was hampered due to the lack of immune response-memory or the degradation of the absorbed vaccines (Alabi *et al.* 1999).

An alternative method of controlling pathogenic bacterial strains in shrimp cultures could be supplementation of pure cultures of natural bacterial isolates (biocontrol) which might produce chemical substances inhibiting the growth of pathogens. The approach basically employs the activity of microorganism that could suppress or inhibit the growth of *V. harveyi* without causing bad impact on the equilibrium system in a particular microbial community. This has been an established practice in husbandry of terrestrial animals (Fuller 1992; Ohhira *et al.* 1996), and has only recently been applied to aquatic systems such as fish culture (Gildberg *et al.* 1995) '-nd crustacean culture (Riquelme *et al.* 1997; Rengpipat *et al.* 1998).

Widanarni and Suwanto (2000) reported the presence of several *Vibrio* isolates associated with the shrimp from the egg stadia to post larva, as well as their rearing environment. Based on their physiological and genetical characters, the isolates were distinguishable from *V. harveyi* that has been proved to be pathogenic on shrimp larva. Therefore, further studies should be conducted to evaluate its potency as biocontrol in shrimp hatchery. Similar finding was also reported by Riquelme *et al.* (1997) in which *Vibrio* isolates associated with scallop larvae were proved to be a potential probiotic in scallop culture in Chile.

The purpose of this research was to obtain new bacterial isolates as improved biocontrol agents which can be applied to solve the problem of bacterial disease in shrimp culture, and to study their ability to inhibit the growth of *V. harveyi.*

MATERIALS AND METHODS

Isolation of *Vibrio* candidates for biocontrol

Vibrio isolates for biocontrol were isolated from tiger shrimp larvae and hatchery environments, i.e. Labuan, Pangandaran, and Lampung, Indonesia. Samples were taken from eggs, larvae (nauplius, zoea, and mysis) and post-larvae of tiger shrimp, natural shrimp feed (i.e. *Artemia* and *Skeletonemd),* seawater, and rearing water of each stage of shrimp larvae. All samples were spread on thiosulphate citrate bile salt agar (TCBS, Oxoid). The culture was incubated at room temperature (28-31)°C, for 24 hours. Subsequently, different morphological types of colonies were randomly selected for further study. Pathogenic *V. harveyi* MR5339 was obtained from BALITDITA (Research Institute for Coastal Aquaculture) Maros, South-Sulawesi.

Sensitivity test of *Vibrio* to rifampicin

Purified isolates of *Vibrio* candidates for biocontrol and pathogenic *V. harveyi* MR5339 were grown in seawater complete agar (SWC-agar) (5 g bactopeptone, 1 g yeast extract, 3 ml glycerol, 15 g agar, 750 ml seawater, and 250 ml distilled water) supplemented with rifampicin (Rf) 50 u.g/ml. After overnight incubation at room temperature (28-31)°C, the bacteria were scored for their antibiotic sensitivity by streaking on the appropriate antibiotic-containing media.

Rifampicin-resistant mutant of *V. harveyi*

One milliliter of a 24 hour culture of *V. harveyi* MR5339 was centrifuged at 5000 rpm for 1 minute. Following the removal of the supernatant, the bacterial pellet was resuspended in 100 (il of sterile seawater. The suspension was then spread onto SWC-agar containing Rf (50 u.g/ml). Colonies of luminescent bacteria grown on SWC-agar + Rf were restreaked onto new media to obtain isolated *V. harveyi* MR5339 resistant to rifampicin.

Determination by *in vitro* test of biocontrol *Vibrio*

The inhibitory effects of each isolate candidate for biocontrol were tested against *V. harveyi* MR5339 Rf*, known to be a pathogen of shrimp larvae. Tested isolate candidates for biocontrol was added to 10 ml of SWC-broth in test tubes at 10^6 cells/ml. Onto the same test tubes were also added overnight liquid culture of *V. harveyi* MR5339 Rf* at 10^2cell/ml. All test tubes were incubated overnight at room temperature. Appropriate dilutions will be chosen for seeding the mixed culture on SWC-agar supplemented with Rf (50 |ag/ml). If the number of colony forming unit (CfU) of *V. harveyi* MR5339 Rf* from the control tubes (i.e. tubes inoculated only with *V. harveyi* MR5339 Rf^1*) is larger than the number of *V. harveyi* of the mixed cultures (i.e. *V. harveyi* MR5339 Rf* which is mixed with the candidate biocontrol isolate), this could identify potential candidates of biocontrol isolates that inhibit the growth of the *V. harveyi* MR5339 Rf* pathogen.

Pathogenicity assay of biocontrol bacteria to shrimp larvae

Shrimp larvae were incubated with the isolates which showed positive growth inhibitory activity against *V. harveyi* MR5339 Rf*. Bacterial isolates as candidate of biocontrol agent were resuspended in sterile seawater. Shrimp larvae were cultured in 2-liter shrimp rearing tank (20 individuals per tank). Bacterial suspension was added into the tank at the final concentration 10^6 CFU/ml. After incubation at room temperature, survival rate of larvae at 5 days was recorded.

Pathogen challenge test

This test was carried out to study the efficacy of *Vibrio* candidate biocontrol isolates towards pathogenic *V. harveyi* in shrimp larvae. The best three biocontrol isolates, based on *in vitro* test, were selected for pathogen-challenged assay. Suspension of each isolate was placed in the shrimp rearing tank for 6 hours before shrimp larvae were introduced into the tank. After cocultivation of isolates with shrimp larvae for 6 hours, *V. harveyi* MR5339 Rf* was introduced into the culture tank. This experiment was performed in three replications. Tanks treated with only *V, harveyi* MR5339 Rf* or without bacteria were employed as controls. Total *Vibrio* population (counted on TCBS media), *V. harveyi* MR5339 Rf* (counted on TCBS media + Rf) in the rearing media, larval mortality monitored daily for 5 days and the survival rate of shrimp larvae were determined as described previously (Hala 2002).

Microbiological and physiological properties

Cell shape, motility, and Gram staining were evaluated using an Olympus BH2-RFC microscope. A number of physiological characteristics were analyzed using Microbact kit test (Medved Science Pry. Ltd. Australia) and production of extra-cellular protease, chitinase and amylase monitored on SWC-agar supplemented with skim milk, colloidal chitin and starch were tested, respectively. The presence of extra-cellular hydrolytic enzymes could be used to provide further information on the pathogenicity or virulence of the isolates.

Amplification and sequencing of 16S-rRNA gene

Since many strains of marine bacteria share similar physiological and morphological characteristics, identification of bacteria strictly based on such parameters is not reliable. Therefore, it is essential to perform DNA sequencing of the 16S-rRNA gene in order to provide reliable additional information for bacterial identification.

Modified phenol-chloroform-isoamylalcohol treatment and ethanol precipitation were used to extract the genomic DNA (Sambrook *et ul.* 1989). The 16S-rRNA genes were amplified by PCR using specific primers of 63f and 1387r from genomic DNA provided by Ready-To-GO PCR Beads (Pharmacia-Biotech, Uppsala, Sweden). These primers were successful to work with a broad range of environmental samples (Marches! *et al.* 1998).

A part of 16S-rRNA gene was sequenced to infer the closest related organism from Ribosomal Database Project (RDP) maintained in the University of Illinois, Urbana-Champaign. The sequencing reactions were done by using the Big Dye Ready Reaction Dye Deoxy Terminator kit, purify with ethanol-sodium acetate precipitation. The reactions were run on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Cetus, Norwalk, Conn).

RESULTS AND DISCUSSION

Isolation of *Vibrio* candidates for biocontrol

Thirty one *Vibrio* isolates were isolated from tiger shrimp larvae and hatchery environments, i. e. Labuan, Pangandaran, and Lampung (Table 1). Of these isolates, 25 isolates were found to be associated with eggs, larvae, post-larvae, and the rearing water of each stage; 1 isolate was obtained from seawater reservoir: 4 isolates were obtained from natural feed (i.e. 2 isolates from *Artemia* and 2 isolates from *Skeletonema).* All of the isolates were non-luminous yellow colonies on TCBS and swarming on SWC-agar media.

Table 1. Codes and sources of *Vibrio* isolates

No.	Code	Source	Location
1.	SW	Seawater	Labuan
2.	N-a	Nauplius	Labuan
3.	NT	Nauplmstank water	Labuan
4.	Z-a	Zoea	Labuan
5.	PL„-a	Post-larvae 6	Labuan
6.	PL„-b	Post-larvae 6	Labuan
7.	PU-c	Post-larvae 6	Labuan
8.	PL„-d	Post-larvae 6	Labuan
9.	PLn-a	Post-larvae 1 1	Labuan
10.	PL,,-b	Post-larvae 1 1	Labuan
11.	PLnT-a	Post-larvae 1 1 tank water	Labuan
12.	PLnT-b	Post-larvae 1 1 tank water	Labuan
13.	SKT-a	*Skeletonema*	Labuan
14.	SKT-b	*Skeletonema*	Labuan
15.	PL_2	Post-larvae 2	Pangandaran
16,	PL_7T-a	Post-larvae 2 tank water	Pangandaran
17,	PL_2T-b	Post-larvae 2 tank water	Pangandaran
18.	PL,	Post-larvae 3	Pangandaran
19.	PL,T	Post-larvae 3 tank water	Pangandaran
20.	PL„	Post-larvae 6	Pangandaran
21.	PL„T-a	Post-larvae 6 tank water	Pangandaran
22.	PL„T-b	Post-larvae 6 tank water	Pangandaran
23.	PL_7	Post-larvae 7	Pangandaran
24.	PL_7T-a	Post-larvae 7 tank water	Pangandaran
25.	PL_7T-b	Post-larvae 7 tank water	Pangandaran
26.	AT-a	*Artemia*	Pangandaran
27.	AT-b	*Anemia*	Pangandaran
28.	E	Egg	Lampung
29.	N-b	Nauplius	Lampung
30.	N-c	Nauplius	Lampung
31.	Z-b	Zoea	Lampung
32.	*MR5W) (V.harveyi)*	Infected shrimp larvae	Maros-SULSEL

Pathogenic *V. harveyi* MR5339 was obtained from BALITDITA (Research Institute for Coastal Aquaculture) Maros, South-Sulawesi and was isolated from infected shrimp larvae. This isolate formed green colonies on TCBS and became luminous on TCBS or SWC-agar media. *V. harveyi* MR5339 was further employed for both *in vitro* and pathogen challenge assay to screen for potential biocontrol bacteria.

Sensitivity test of *Vibrio* to rifampicin

Sensitivity testing *of Vibrio* isolates including *V. harveyi* MR5339 showed that all of the isolates were sensitive to rifampicin. Therefore, a rifampicin resistant mutant of *V. harveyi* MR5339 was employed in the assay for biocontrol *in vitro* and pathogen challenge to screen for potential biocontrol bacteria. Tjahjadi *et al.* (1994) reported that almost all of the bacteria that were isolated from seawater and hatchery-rearing water at Kalianget, East Java, including *V. harveyi*, were resistant to various antibiotics except rifampicin. Rifampicin, a bactericidal antibiotic, is active against gram-positive and some gram-negative bacteria. This drug interferes with transcription processes in bacteria and thus far was never used in shrimp hatchery.

In vitro assay **for biocontrol activity**

The inhibitory effects of each *Vibrio* isolate candidate for biocontrol were tested against *V. harveyi* MR5339 *Rf* (Figure 1). Three out of 31 isolates tested i.e. SKT-b, PL_2T-a and N-c were isolated from *Skeletonema*, post-larvae 2 rearing water and nauplii showed the best result. After 24 hours of incubations, only very few colonies (10^2-10^3 cells/ml) of *V. harveyi* MR5339 Rf* were grown on culture media inoculated with *V. harveyi* MR5339 Rf* at concentration of 10^2 cells/ml and *Vibrio* candidates for biocontrol at concentration of 10^6 cells/ml. The number of colonies in the control experiments (inoculated only with *V. harveyi* MR5339 Rf*) could reach $5x10^8$ cells/ml. The other tested isolates could not significantly inhibit the growth of MR5339 *Rf*, Therefore, only SKT-b, PL_2T-a, and N-c were further studied in the pathogenicity challenge experiments.

Before performing challenge test with *V. harveyi* MR5339 RF^R in shrimp larvae, some potential isolates candidate for biocontrol were analysed for their pathogenicity to shrimp larva. After 5 days of exposure with biocontrol isolates at $1Q^6$ CFU/ml, it had confirmed that SKT-b, PL_2T-a and N-c isolates were not pathogenic to shrimp larvae. These facts were indicated by relatively similar values of survival rate to those of control group (Table 2).

Pathogen challenge test

Three of the most potential *Vibrio* isolates i.e. SKT-b, PL_2T-a and N-c for biocontrol based on *in vitro* test, were assayed for their activities in inhibiting colonization of *V. harveyi* MR5339 Rf* in shrimp larvae. Observations were carried

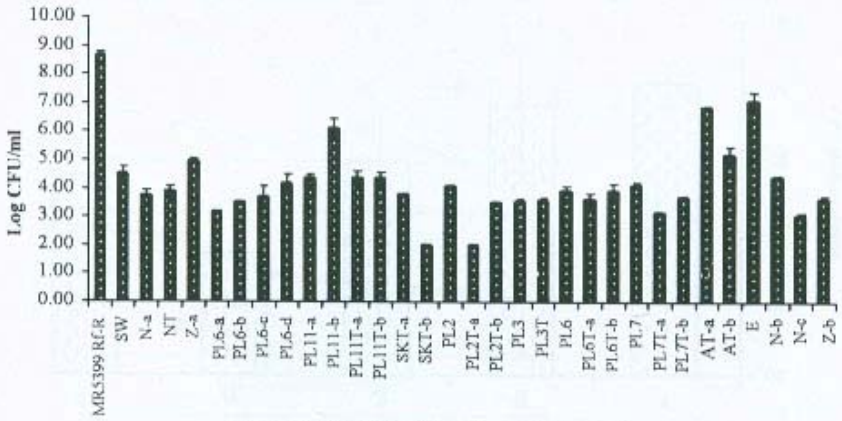


Figure 1. *In vitro* assay for the inhibition of *V. harveyi* MR5339 Rf^R by biocontrol bacteria

Table 2. Pathogenicity effect of biocontrol bacteria to shrimp larvae

Code	Survival rate (%)
SKT-b	98.3 ± 2.9^a
PL_2T-a	96.7 ± 2.9^a
N-c	96.7 ± 2.9^a
Control (without bacteria)	91.7 ± 2.9^a

Note : Values followed by the same letters are not significantly different at 5% confidence level.

out on larval survival rates and total population of *Vibrio* and *V. harveyi* MR5339 Rr* both in the rearing water and in the dead larvae.

Results showed that each of the three biocontrol candidates significantly ($P<0.05$) could increase survival rates of shrimp larvae reared in seawater inoculated with 10^3 cells/ml of MR5339 Rf". Larval survival rates in each of biocontrol treatment, i.e. SKT-b, PL_2T-a and N-c were 93%, 90% and 82%, respectively. On the other hand, treatment with MR5339 Rf* inoculation without biocontrol isolate showed only 68% survival rate of shrimp larvae (Figure 2). The isolates presumably increased survival rates of shrimp larvae as survival rates of control group (without addition of biocontrol or *V. harveyi* MR5339 Rf^R isolates) were lower than treatments with biocontrol isolates. However, observation on shrimp larvae fitness after inoculation is required to determine the physiological condition of larvae.

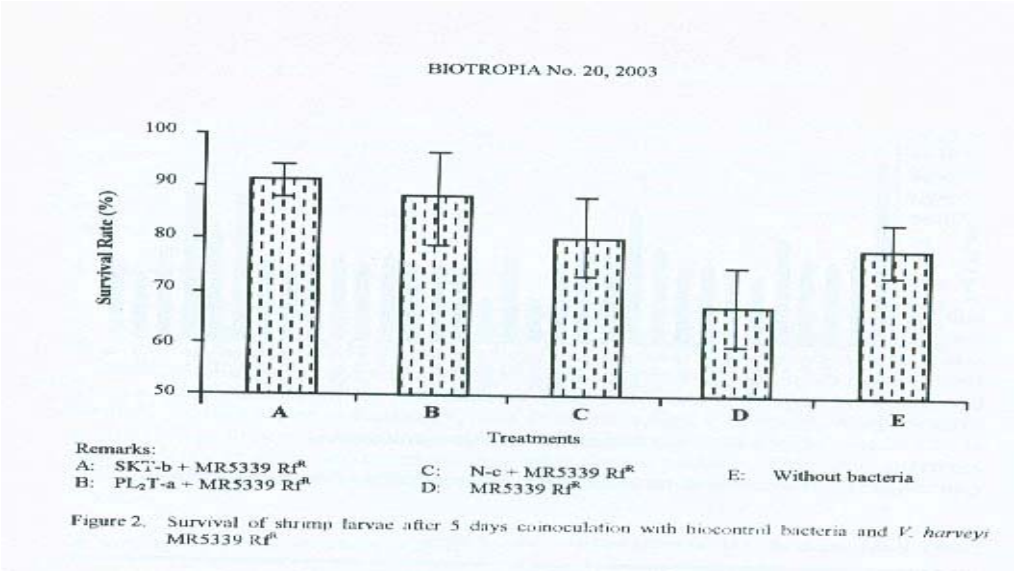


Figure 2. Survival of shrimp larvae after 5 days coinoculation with biocontrol bacteria and *V. harveyi* MR5339 Rf^R

Rengpipat *et al.* (1998) reported the use *of Bacillus* Strain S11 as a probiotic administered in enriched *Artemia* to larvae of the black tiger shrimp *(P. monodon).* At two weeks, *P. monodon* survival was significantly different between the control groups (85%) and the treated groups (89%). When challenged with a pathogenic *V. harveyi* strain D331, the shrimp treated with probiotics showed a higher survival (13%) when compared to the control group (4%). Haryanti *et el.* (2000) also reported that inoculation with BY-9 strain at a dose of 10^6 CFU/ml to shrimp larvae, resulted to a survival rate of 59.3%, while for the control only 14.7%.

The increased survival rate might be due to the growth inhibition of *V. harveyi* MR5339 Rf* on shrimp larvae by biocontrol bacteria. Although no significant difference was observed in the number of *V. harveyi* MR5339 Rf* either in culture media (Figure 3) or dead larvae (Figure 4), high mortality in shrimp larvae for treatment without biocontrol isolates indicated the effect of the inhibition. However, the way how the mechanisms occurred need to be studied further. According to Verschuere *et al.* (2000), action mechanism of probiotic bacteria or biocontrol could be divided into several ways as follows: (1) production of inhibitory compounds, (2) competition for chemicals or available energy, (3) competition for adhesion sites, (4) enhancement of the immune response, (5) improvement of water quality, (6) interaction with phytoplankton.

To get an insight about action mechanism of probiotic bacteria, especially on their competition for adhesion sites, biocontrol bacteria should be tagged with molecular marker so that the existence of bacteria in shrimp larvae could be

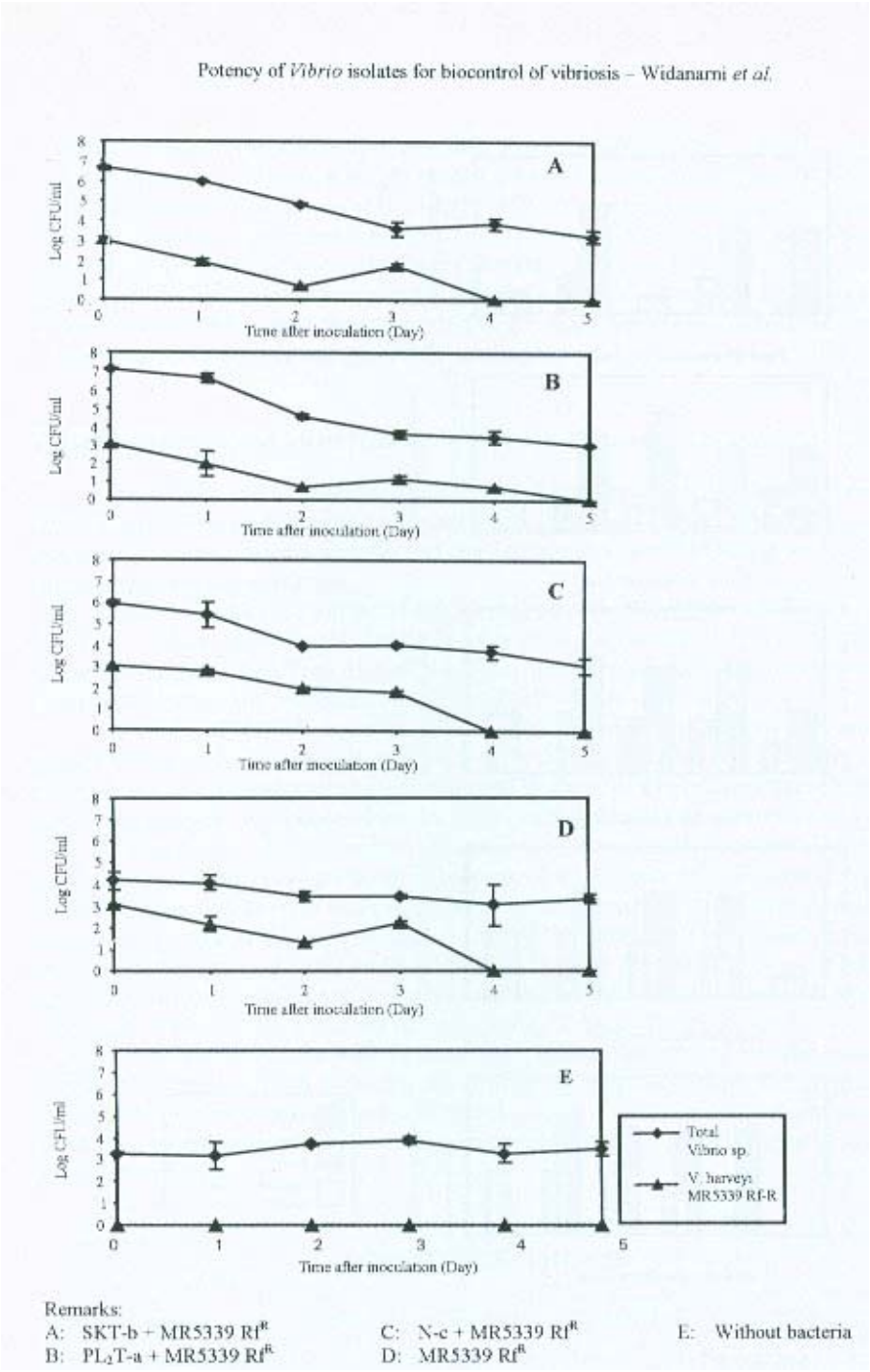
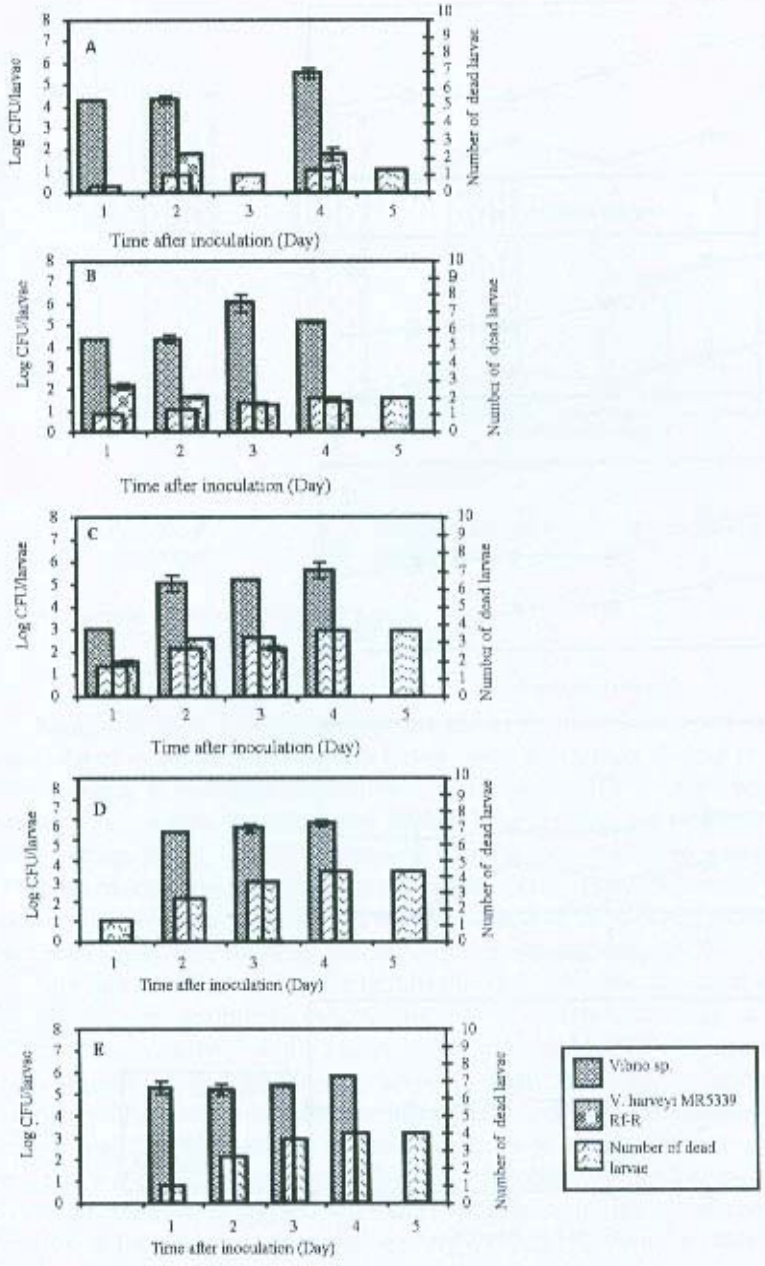


Figure 3. Number of colonies of *Vibrio* sp. And *V. harveyt* MR5339 Rĩ in larva rearing thanks



Remarks:

A: SKT-b + MR5339 Rf^R	C: N-c + MR5339 Rf^R	E: Without bacteria
B: PL_3T-a + MR5339 Rf^R	D: MR5339 Rf^R	

Figure 4. Number of dead larvae, *Vibrio* sp., and *V. harveyi* MR5339 Rf^R in pathogen challenge test

detected. One of the molecular markers extensively used for studying bacterial activity in the e nvironment is *gf'p* (green fluorescent protein) gene isolated from a jellyfish *(Aequorea victoria)* (Manning 1997). If expressed, the gene will produce green fluorescence GFP protein under UV light, so that the bacteria could be easily observed. Moreover, as molecular gene marker, GFP could provide several advantages such as no requirement for exogenous substrate or energy source for their visualization. GFP assays were reported to be sensitive, stable, non toxic, and did not disturb cell function and growth (Josenhans *et al.* 1998; Ling *et al.* 2000).

Characterization and identification of biocontrol bacteria

SKT-b was Gram negative, short rod-shape, produced yellow colonies on TCBS, and exhibited swarming activity on SWC-agar. This isolate was motile, could utilize glucose and sucrose but not lactose; produced protease and amylase, but did not produce chitinase.

Partial sequencing (500 bp of the 5'-end) of 16S-rRNA gene of SKT-b showed that the isolate showed similarity to *Vibrio alginolyticus* (83% of similarity). Complete sequencing of the 16S-rRNA gene, however, should give more definitive information about the taxonomic position of this isolate.

Austin *et al.* (1995) reported that *V. alginolyticus* was effective in reducing diseases caused by *V. anguillarum* and *V. ordalii,* however, other strains of this bacterium has been associated with vibriosis in shrimp. Therefore, characterization and identification of biocontrol strain were crucial steps to be carried out to assess the pathogenicity of biocontrol bacteria to the shrimp larvae. Moreover, characterization and identification of biocontrol strain were also important for mass production, quality control and patenting to protect commercial interest (Gomez-Gil *et al.* 2000). According to Riquelme *et al.* (1997), *Vibrio* might be more promising to be developed as probiotics when compared to other probiotic species for hatchery applications, since vibrios are commonly associated with larvae in culture, and auto-inhibition could limit the growth of pathogenic *Vibrio.*

In conclusion, *Vibrio* isolates from tiger shrimp larvae and hatchery environments have the ability in reducing shrimp larvae mortality in pathogen challenge assays. The isolates are potential to be developed as a biocontrol agent and therefore as an alternative to chemical treatment in preventing luminous bacterial diseases in shrimp hatcheries.

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