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Selective Media for *In Vitro* Activity Evaluation of Bacterial Biocontrol Against Pathogenic Vibrio

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***In vitro* activity test is a critical evaluation to screen the potential biocontrol agent. We developed a selective medium for quantitative *in vitro* activity evaluation of bacterial biocontrol agents against pathogenic *Vibrio* in aquaculture. Sensitivity test of bacterial biocontrol and *Vibrio* spp. to nine antibiotics showed that oxytetracycline inhibited the growth of *Vibrio* spp., but did not inhibit the growth of the bacterial biocontrol. This selective inhibition activity of oxytetracycline suggested this antibiotic might be supplemented to establish a selective medium. The MIC of oxytetracycline against *V. harveyi*, *V. parahaemolyticus,* and *V. algosus* were 120, 250, and 120 µg/ml, respectively. These concentration did not inhibit the growth of bacterial biocontrols. Therefore, oxytetracycline was supplemented at 250 µg/ml in Zobell agar medium. This medium was used as a selective medium to enumerate the density of bacterial biocontrol. *In vitro* activity test of bacterial biocontrol (RLP1) against *V. parahaemolyticus* showed that strain RLP1 at density of 10^4 and 10^6 cells/ml was able to kill *V. parahaemolyticus* during 6 h incubation. At lower density, 10^2 cells/ml, this bacterial biocontrol agent was able to kill the pathogenic *Vibrio* during 12 h incubation. This study discovered a selective medium for the bacterial biocontrol and *Vibrio* spp. and provided the results of its application in the evaluation of *in vitro* activity of a bacterial biocontrol agent against *V. parahaemolyticus*. The results also revealed that strain RLP1 is a potential bacterial biocontrol against vibriosis in marine aquaculture.**

Key words: selective medium, biocontrol, *Vibrio*, *in vitro*

INTRODUCTION

Vibriosis caused by *Vibrio* spp. is a devastating disease in either marine or brackishwater aquaculture. The disease infects all marine and brackishwater animal including fish, crustaceans, molusks and mammals. The causative agents of the disease are *V. anguillarum*, *V. alginolyticus*, *V. ordalii*, *V. salmonicida*, *V. vulnivicus*, *V. damsela*, *V. charchariae*, *V. cholerae*, *V. harveyi, V. fluvialis*, *V. parahaemolyticus, V. metchnikovii,* and *V. furnisii* (Austin & Austin 1993; Kamiso *et al*. 2005). Conventional countermeasure methods using antibiotics and disinfectants have tremendous negative impacts such as the development of bacterial resistant, the change of microbial composition in ecosystem and residual antibiotics or disinfectants in aquaculture products (Esposito *et al*. 2007). Vaccination has also been developed to control vibriosis in aquaculture. As vibriosis is caused by multi-species of vibrio and each species have broad variation of immunogenic property, an effective vaccine for all causative agents of vibriosis is difficult to be found.

One of the alternatives to control vibriosis is the use of bacterial bicontrol agents. Several bacterial strains with anti-*Vibrio* activity are reported including *Flavimonas* BY-9 (Haryanti *et al*. 1997), *Thalassobacter utilis* (Gatesoupe 1999), strains GSB-95030 and GSB-95033 (Roza *et al*. 2001), *Alteromonas haloplanktis* (Verschuere *et al*. 2000), strains MR 53 and PK 446 (Muliani *et al*. 2004). Almost the above references did not reported quantitative *in vitro* activity of the strains against *Vibrio*. Evaluation of the quantitative *in vitro* activity is a crucial to know the potency of the biocontrol agents. Isnansetyo *et al*. (2009a) evaluated anti-vibrio activity of a bacterial biocontrol, and found that *Pseudoalteromonas* sp. S2V2 is a potential biocontrol agents of vibriosis. To evaluate the quantitative potency of bacterial biocontrol agents against *Vibrio* spp., we have developed a selective medium based on Zobell medium composition.

Recently we have isolated several bacterial biocontrol agents against *Vibrio* and evaluated the potency of the isolates against *Vibrio* spp. We also have evaluated their *in vitro* and *in vivo* activity. This paper provides our recent study results on the development of a selective medium and the use of the medium to evaluate the potency of bacterial biocontrol agent quantitatively.

MATERIALS AND METHODS

Bacterial Strains and Media. Bacterial biocontrol MIR2, MIR3, and RLP1, and pathogenic *Vibrio* spp. including *V. alginolyticus* (26 S), *V. parahaemolyticus* (29 S), *V. fluvialis* (24 SK), *V. algosus,* and *V. harveyi* were used in this study. All the bacterial strains were bacterial collection of the Fish Disease Laboratory, Department of Fisheries, Faculty of Agriculture, Gadjah Mada University,

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Indonesia. Media used in this study were Zobell 2216E medium and Thiosulphate Citrat Bile Salt Agar medium (TCBS Agar) (Oxoid, Hampshire, England).

Antibiotics Sensitivity Test of Bacterial Biocontrol and *Vibrio* spp. Antibiotics sensitivity was evaluated by the disk agar diffusion method on double layers agar of Zobell medium as previously described (Isnansetyo & Kamei 2005; Isnansetyo *et al*. 2009b). The test was conducted with enrofloxacine (PT. Bayer Indonesia), oxytetracycline (Zalmweg Raamsdonksveer-the Nentherland), streptomycin (PT. Duta Kaisar Pharmacy Solo-Indonesia), penicillin (PT. Duta Kaisar Pharmacy Solo-Indonesia), Kanamycin (PT. Meiji Indonesian Pharmaceutical Industries Surabaya-Indonesia), novobiocin (Oxoid LTD, Basingstoke, Hants- England), amoxicillin (Ceva Sante Animale Libourne Cedek-France), chloramphenicol (Basingstoke Hampshire-England), Sulfonamide (PT. Wonderindo Pharmatama Jakarta-Indonesia). The test was carried out at antibiotic concentrations of 5 and 10 µg/disk. Incubation was conducted at 30 °C for 24 h, and activity of the antibiotics was determined by measuring the inhibition zone. An antibiotic with selective and high activity was selected for further experiment.

Minimum Inhibitory Concentration of Antibiotic. The minimum inhibitory concentration of antibiotic was evaluated by agar dillution method (CLSI 2006) on Zobell agar medium supplemented with a selected antibiotic at various concentration. Bacterial strains were inoculated on the medium and incubated at 30 °C for 24 h.

Selective Medium. Selective medium based on Zobell agar medium supplemented with a selected antibiotic was established. By supplementing the selected antibiotic, the bacterial biocontrol agent were able to grow well on the medium, but the growths of pathogenic *Vibrio* spp. were inhibited. The medium was used to enumerate the bacterial biocontrol agent in the further experiment. Selectiveness of the medium was tested by streaking the bacteria on the selective medium and incubated at 30 °C for 24 h.

Antagonistic Test of Bacterial Biocontrol Against *V. parahaemolyticus.* The porpose of this experiment was to select a bacterial biocontrol with higest anti-*Vibrio* activity which will be used in the further experiment. This study used time course experiment (Isnansetyo & Kamei 2003). *V. parahaemolyticus* was co-cultured with MIR2, MIR3, or RLP1. Control treatment was culture of *V. parahaemolyticus* without any biocontrol. Each treatment was done in duplicates.

V. parahaemolyticus was inoculated in 15 ml of Zobell broth medium at final density of 10^6 cells/ml. Then bacterial biocontrol, strains MIR2, MIR3, or RLP1 immediately inoculated to the medium at the same initial density. The bacterial density was estimated by McFarland standard. After incubation at 30 °C for 24 h, bacterial density was enumerated by pour plate method on the selective medium for bacterial biocontrol and TCBS agar medium for *V. parahaemolyticus*. The plates were incubated at 30 °C for 48 h.

Antagonistic Test of a Selected Bacterial Biocontrol at Various Initial Densities Against *V. parahaemolyticus.* A selected bacterial biocontrol with the highest anti-*Vibrio* activity obtained from the previous experiment was used in this experiment. The purpose of this experiment was to know the effect of initial density of a biocontrol bacterium on its growth inhibitory activity against *V. parahaemolyticus*. *V. parahaemolyticus* at an initial density of 10^6 cells/ml was co-cultured with a bacterial biocontrol at an initial densities of 10^2, 10^4, or 10^6 cells/ml. The control treatment was culture of *V. parahaemolyticus* without any bacterial biocontrol.

Time course experiment (Isnansetyo & Kamei 2003) was used in this study. The culture and co-culture were conducted in 15 ml Zobell broth medium in Erlenmeyer. The medium was inoculated with *V. parahaemolyticus* at the initial density of 10^6 cells/ml and the bacterial biocontrol at various densities as described above. The culture were incubated at 30 °C for 48 h. During incubation, the growth of the bacterial biocontrol and *V. parahaemolyticus* were examined periodically by the pour plate method on the same agar medium as described in the previous experiment.

RESULTS

Sensitivity of *Vibrio* spp. and bacterial biocontrol to nine antibiotics was tested by the disk agar diffusion. The results showed that the bacterial strains had broad variation of sensitivity to antibiotics tested. The variation was indicated by the different of inhibitory zone among antibiotics and bacterial strains. All bacterial strains tested were sensitive to amoxicillin, sulfonamide, kanamycin and streptomycin at 10 µg/disk, but several bacterial strains were resistant to the antibiotics at 5 µg/disk (Table 1). Two bacterial strains, V3 and V5 were resistant to penicillin and enrofloxacine either at 5 or 10 µg/disk. At the same concentration, chloramphenicol inhibited all bacterial strains except MIR2 and MIR3. Two bacterial biocontrol, MIR3 and RLP1, and two pathogenic Vibrio, V3 and V5 were sensitive to novobiocin. All bacterial biocontrol were resistant to oxytetracycline either at 5 or 10 g/disk. However, two strains of pathogenic *Vibrio*, V2 and V4 were sensitive to the same antibiotic at 10 g/disk. As oxytetracycline did not inhibit the growth of all bacterial biocontrols, we chose the antibiotic for the further experiment.

Minimum Inhibitory Concentration (MIC) test of oxytetracycline at 10 to 500 µg/ml indicated that the MIC of oxytetracycline varied among the bacterial strains tested. This antibiotic inhibited pathogenic *Vibrio* except *V. alginolyticus* and *V. fluvialis* at 250 µg/ml without inhibiting any bacterial biocontrol. Bacterial biocontrols, MIR2 was inhibited by this antibiotic at 300 µg/ml. Furthermore, two other bacterial biocontrols, MIR3 and RLP1 were not inhibited by this antibiotic even at 500 µg/ml. Briefly, MICs of bacterial biocontrols, MIR2, MIR3, and RPL1 were 300, >500 and >500 µg/ml, respectively. In

Table 1. Inhibition zone of antibiotics against bacterial biocontrol and patogenic *Vibrio* spp.

Antibiotics concentrations	Bacterial biocontrol			*Vibrio* spp.*				
	MIR2 (mm)	MIR3 (mm)	RLP1 (mm)	Vh (mm)	Vp (mm)	Valgi (mm)	Valgo (mm)	Vf (mm)
5 µg/disk								
Amoxicillin	15.5	18.4	16.2	15.1	24.8	9.8	20.8	28.0
Sulfonamide (Sulfadiazine sodium, sulfadimidine, and sulfamerazine)	27.8	28.6	28.2	24.5	13.1	33.0	12.4	10.0
Enrofloxacine	11.7	13.1	11.2	9.7	9.6	0	12.4	0
Kanamycin	0	0	13.5	19.2	21.7	19.2	19.3	25.8
Streptomycin	12.6	8.1	10.0	21.7	25.3	21.2	22.3	25.4
Oxytetracycline	0	0	0	0	0	0	10.4	0
Chloramphenicol	0	0	8.5	24.8	26.7	26.9	22.6	27.5
Novobiocin	12.0	0	0	11.3	11.6	0	12.5	0
10 µg/disk								
Amoxicillin	19.6	22.7	18.9	19.6	26.7	14.8	29.8	28.3
Sulfonamide (Sulfadiazine sodium, sulfadimidine, and sulfamerazine)	29.3	31.8	29.2	27.6	21.4	35.4	28.5	28.3
Enrofloxacine	15.1	16.0	19.2	13.3	10.0	0	12.8	0
Kanamycin	16.2	14.0	13.3	32.6	22.7	26.8	22.0	38.8
Streptomycin	16.5	15.1	22.6	25.6	26.6	22.0	23.7	26.0
Oxytetracycline	0	0	0	0	13.9	0	14.5	0
Cloramphenicol	0	0	9.1	26.2	26.9	27.4	22.8	27.8
Novobiocin	18.0	0	0	18.0	16.0	0	20.3	0
Penicillin**	13.8	16.6	21.9	15.8	13.5	0	16.2	0

*Vh: *V. harveyi*, Vp: *V. parahaemolyticus* (29S), Valgi: *V. alginolyticus* (26S), Valgo: *V. algosus*, Vf: *V. fluvialis* (24SK); **: 60 IU/disk.

addition, MICs of pathogenic *Vibrio*, *V. harveyi*, *V. parahaemolyticus* (29S), *V. alginolyticus* (26S), *V. algosus* and *V. fluvialis* were 120, 250, 300, 120, and 300 µg/ml, respectively. This results indicated that oxytetracycline at 250 µg/ml inhibited selectively three pathogenic *Vibrio*, *V. harveyi*, *V. parahaemolyticus* (29S), and *V. algosus* without inhibiting any bacterial biocontrol.

Subsequently, we tested the selective medium based on Zobell agar medium supplemented with oxytetracycline at 250 µg/ml, and the medium was inoculated with all bacterial strains. Result showed that all bacterial biocontrol grew well on the medium, but tree pathogenic *Vibrio*, *V. harveyi*, *V. parahaemolyticus* (29S), and *V. algosus* was not able to grow on the medium (Figure 1). This result indicated that the medium selectively inhibited these three pathogenic *Vibrio*. All pathogenic *Vibrio* were able to grow well on TCBS agar. In contrast, there was no any bacterial biocontrol grew on the medium.

Antagonistic test in co-culture of each bacterial biocontrol and *V. parahaemolyticus* showed that all the bacterial biocontrol were able to inhibit the growth of this pathogenic *Vibrio* (Figure 2). At the initial density of 10^6 cells/ml, MIR2 strain killed *V. parahaemolyticus* completely after 12 h incubation. Higher activities were exhibited by strains MIR3 and RLP1 which were able to kill this pathogenic *Vibrio* after 6 h incubation. In contrast, density of *V. parahaemolyticus* in culture without any bacterial biocontrol increased sharply after 6 h incubation and reached 9 x 10^8 cells/ml after 48 h incubation. All bacterial biocontrols grew well in the co-culture. These three bacterial biocontrols reached the highest bacterial density, 3.3 x 10^8, 5.0 x 10^8, and 1.2 x 10^9 cells/ml at 24 h incubation for MIR2, MIR3, and RLP1, respectively. The densities were relatively constant after 24 h incubation. The results indicated that among the three bacterial

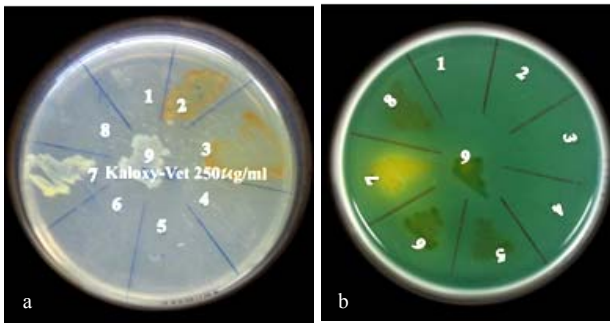

Figure 1. Growth of bacterial biocontrols and pathogenic Vibrios on selective Zobell agar medium (a) and TCBSA medium (b). 1: MIR2, 2: MIR3, 3: RLP1, 4: uninoculated, 5: *V. harveyi*, 6: *V. parahaemolyticus* (29S), 7: *V. alginolyticus* (26S), 8: *V. algosus*, 9: *V. fluvialis* (24SK).

biocontrol, RLP1 exhibited highest growth rate. Therefore in the further experiment we evaluated the effective bacterial cell density of RLP1 to eradicate *V. parahaemolyticus*.

An experiment with various initial bacterial densities indicated that RLP1 was effectively kill *V. parahaemolyticus* even at low initial density (Figure 3). At the initial density of 10^2 cells/ml, this strain was able to eradicate *V. parahaemolyticus* within 12 h incubation. At higher initial density, RLP1 killed completely this pathogenic *Vibrio* readily within 6 h incubation. In this experiment, RLP1 showed the same growth pattern as its growth pattern in Figure 2. This strain had high growth rate even at low initial density. The density was relatively constant after 12 h incubation for initial bacterial densities of 10^4 and 10^6 cells/ml. However, the bacterial density became relatively constant after 24 h incubation when RLP1 was inoculated at an initial bacterial density of 10^2 cells/ml.

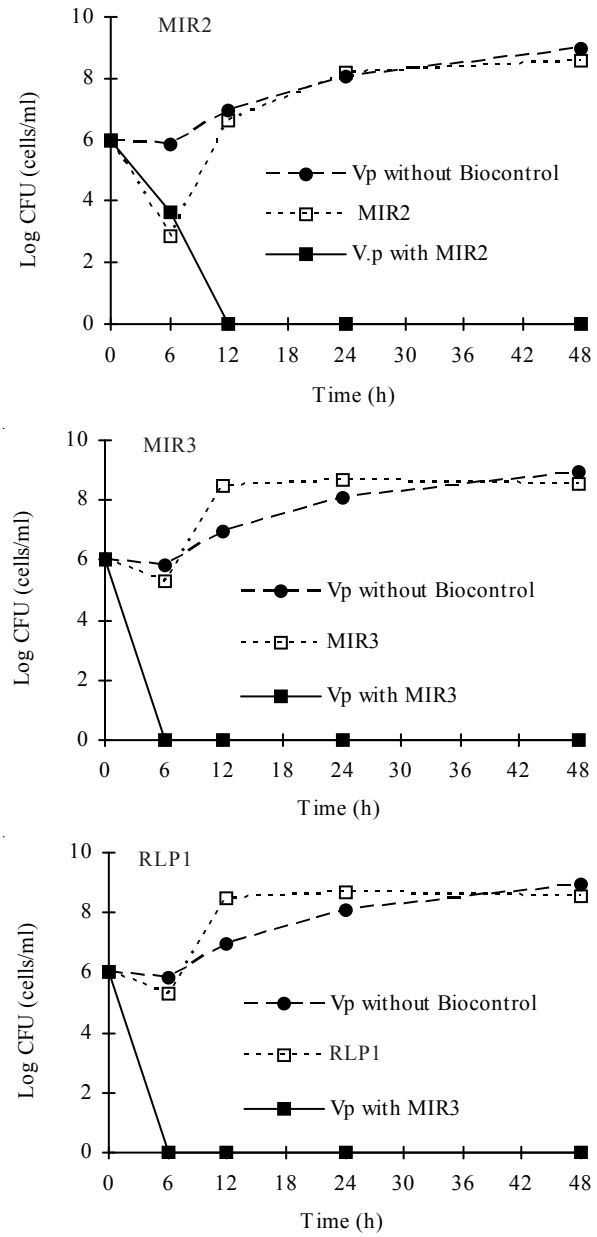

Figure 2. Anti-*Vibrio* activities of bacterial biocontrol, MIR2, MIR3, and RLP1 in co-culture with *V. parahaemolyticus* in Zobell broth medium. Vp without biocontrol, density of *V. parahaemolyticus* in the culture without any biocontrol; MIR2, density of MIR2 in the co-culture with *V. parahaemolyticus*; Vp with MIR2, density of *V. parahaemolyticus* in the co-culture with MIR2; MIR3, density of MIR3 in the co-culture with *V. parahaemolyticus*; Vp with MIR3, density of *V. parahaemolyticus* in the co-culture with MIR3; RLP1, density of RLP1 in the co-culture with *V. parahaemolyticus*; Vp with RLP1, density of *V. parahaemolyticus* in the co-culture with RLP1.

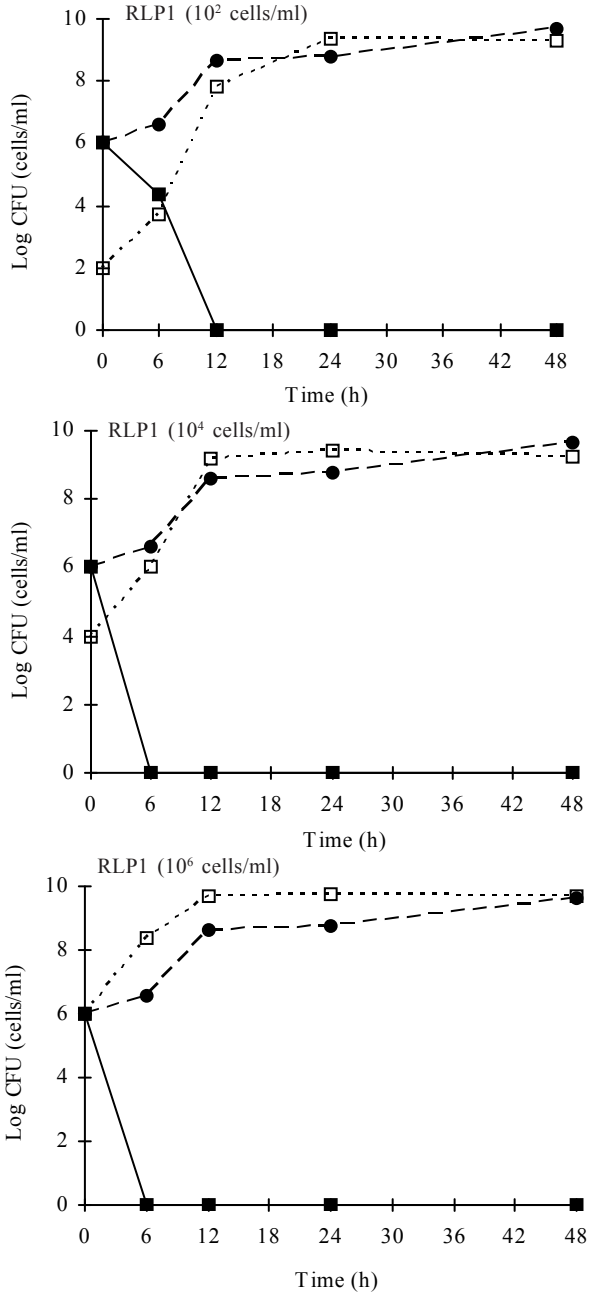

Figure 3. Anti-*Vibrio* activities of a bacterial biocontrol, RLP1 at various initial cell densities in co-culture with *V. parahaemolyticus* in Zobell broth medium. Vp without biocontrol, density of *V. parahaemolyticus* in the culture without RLP1; RLP1, density of RLP1 in the co-culture with *V. parahaemolyticus*; Vp with RLP1, density of *V. parahaemolyticus* in the co-culture with RLP1. •: Vp without biocontrol, □: RLP1, ■: Vp with RLP1.

DISCUSSION

One of alternatives to control vibriosis in aquaculture is the use of bicontrol agents besides the application of vaccination. These two countermeasure methods recently have become the focus of research as their environmental friendly properties and low side effect. *In vitro* activity test of biocontrol agent candidates is a crucial step in the development of biocontrol agent. We have developed a method for the evaluation of quantitative *in vitro* activity of bacterial biocontrol agents based on the co-culture in broth medium with pathogenic *Vibrio* spp. The *in vitro* activity is conventionally evaluated qualitatively or semi-quantitatively on agar medium. To establish the method, we firstly evaluated the sensitivity of bacterial biocontrol and pathogenic *Vibrio* spp. to antibiotics. Secondly, we investigated MIC of selected antibiotic, and supplemented the antibiotic on Zobell agar medium to establish a selective medium. Thirdly, this medium has been used to

enumerate the growth of the bacterial biocontrol co-cultured with a pathogenic *Vibrio* in a broth medium.

Sensitivity test indicated that bacterial biocontrols used in this study are resistant to oxytetracycline either at 5 and 10 µg/disk. MIR2 and MIR3 are also resistant to chloramphenicol, but RLP1 is sensitive to this antibiotic. MIC test indicated that the MICs of oxytetracycline to the bacterial biocontrols are 300 µg/ml or higher, but MICs of *Vibrio* spp. are 300 µg/ml or lower. Therefore, oxytetracycline has been chosen to establish a selective medium although some pathogenic *Vibrio* spp. are resistant to this antibiotic. As all the bacterial biocontrol are resistant to oxytetracycline at 250 µg/ml, but *V. harveyi*, *V. parahaemplyticus,* and *V. algosus* are sensitive at this concentration, we supplemented Zobell agar medium with oxytetracycline at 250 µg/ml to established a selective medium.

Isnansetyo *et al*. (2009a) have reported that a bacterial biocontrol, *Pseudoalteromonas* sp. S2V2 resistant to oxytetracycline at 10 µg/disk. Oxytetracycline is a broad spectrum antibiotic which is traditionally used to control both Gram negative and Gram positive bacteria in aquaculture (Saeed 1995). However, the sensitivities of bacteria to the antibiotic vary in different strain and species of bacteria. Zafran *et al*. (1997) have reported that oxytetracycline inhibits the growth of *Vibrio* sp. at concentration more than 15 µg/ml. This antibiotic has much higher MICs to an oxytetracycline-resistant strain of *V. harveyi* (Nakayama *et al*. 2006; Isnansetyo *et al*. 2009b). The above references indicate the variation sensitivity of bacteria to oxytetracycline and the resistant development of bacteria. Moreover, the high incidence of resistance to oxytetracycline was found in *Vibrio* (Nonaka *et al*. 2002; Hameed *et al*. 2003; Kim *et al*. 2003; Nonaka *et al*. 2007).

When Zobell agar medium supplemented with oxytetracycline at 250 µg/ml, the growths inhibition of *V. harveyi*, *V. parahaemolyticus,* and *V. algosus* are indicated by no bacterial growth on this medium (Figure 1). However, this medium is not able to inhibit the growths of *V. fluvialis* 24SK and *V. alginolyticus* 26S. As there is no growth inhibition of bacterial biocontrols, MIR2, MIR3, and RLP1 this medium may be used to enumerate the bacterial density of these biocontrol bacteria in co-culture with *V. harveyi*, *V. parahaemolyticus,* and *V. algosus*. These results suggest that this medium is useful for *in vitro* activity evaluation of the bacterial biocontrol against *V. harveyi*, *V. parahaemolyticus,* and *V. algosus*, but the medium can not be used in the same evaluation against *V. fluvialis* 24SK and *V. alginolyticus* 26S. We suggest to supplementing Zobell medium with chloramphenicol for *in vitro* activity test of the bacterial biocontrol against these two pathogenic *Vibrio*. The bacterial density of all pathogenic *Vibrio* used in this study can be estimated by using TCBSA medium as there no growth of the bacterial biocontrols on this medium.

The above two selective media then be used to investigate the growth of MIR2, MIR3, and RLP1 co-cultured with *V. parahaemolyticus* to determine the potency of the three bacterial biocontrol. Results reveal that MIR2, MIR3, and RLP1 are potential biocontrol agents against *V. parahaemolyticus*. RLP1 and MIR3 seems having higher activity than MIR2. The former two biocontrol bacteria killed completely *V. parahaemolyticus* within 6 h incubation, but MIR2 need longer time to kill the pathogenic *Vibrio* (Figure 2). In the further experiment, we chose RLP1 as this bacterium grows faster than MIR3 in co-culture with *V. parahaemolyticus*. The growth in the beginning inoculation of bacterial biocontrol is crucial condition. The density of RLP1 increases immediately after inoculation in co-culture than other two bacterial biocontrol tested.

In this study we have evaluated quantitatively the *in vitro* activity of the biocontrol agents by time course experiment. It is the first report on the evaluation of the *in vitro* antagonistic activity using this method. Long and Azam (2001) have used Burkholder agar diffusion assay to investigate antagonistic activity of marine pelagic bacteria. The similar method has been used by Brinkhoff *et al*. (2004) to evaluate the antagonistic activity of *Roseobacter gallaeciensis* BS107. A semi quantitative test using well diffusion assay have been used to evaluate inhibitory activity of marine antagonistic bacterium belonging to the *Roseobacter* Clade against *V. anguillarum* (Bruhn *et al*. 2005). Nikoskelainen *et al*. (2001) have used co-culture method to evaluate growth inhibition activity of probiotics against fish pathogens, *A. salmonicida* SN1 and *V. anguillarum* 1-284. However, these authors did not find the growth inhibition effect in serial time course.

A time course experiment to evaluate the effectiveness of a biocontrol bacterium, RLP1 at various initial densities reveals that RLP1 is able to eradicate *V. parahaemolyticus* even at low inoculation density (Figure 3). When this bacterium biocontrol is inoculated at the initial density of 10^4 and 10^6 cells/ml, this bacterium is able to kill *V. parahaemolyticus* within 6 h incubation. This result is consistence with the result obtained from the previous experiment (Figure 2). When this biocontrol bacterium is inoculated at a lower initial density of 10^2 cells/ml, the longer killing time is needed. This bacterium completely kills *V. parahaemolyticus* within 12 h. The high antagonistic activities of RLP1 with short killing time at low initial density suggest that RLP1 is a potential biocontrol bacterium against vibriosis in aquaculture.

In summary, this study establishes a quantitative method for evaluation of *in vitro* activity of bacterial bioconrol agent. This paper also provides the first report on the evaluation of quantitative *in vitro* activity of biocontrol agent by establishing selective media with antibiotic supplementation and applying the time course experiment. These methods may be applied for various biocontrol and pathogenic bacteria by determining appropriate antibiotics and their concentration.

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