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In vitro and in vivo study of acyl homoserine lactone degrading *Bacillus* against *Vibrio harveyi*

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Abstract

Vibriosis caused by *Vibrio harveyi* is still a bottleneck for the development of shrimp culture industry. As quorum sensing (QS) regulates the virulence of *V. harveyi*, interference of QS might offer opportunity for controling *V. harveyi* in shrimp culture. The aim of this study was to evaluate the ability of AHL-degrading *Bacillus* to interfere with the AHL production and the growth of *V. harveyi* in vitro and in vivo on *Penaeus monodon* larvae. Based on biochemical and molecular study, the AHL-degrading *Bacillus* was confirmed as *B. subtilis* STC. The results showed that AHL-degrading *Bacillus* affected ($P<0.05$) the Total Bacterial Count (TBC) and Total Vibrio Count (TVC) within 24 and 48 hours in in-vitro and in-vivo study. The higher the *B. subtilis* STC was added, the less concentration of viable *Vibrio* cells in the system. The AHL concentration in in-vitro study were significantly different ($P<0.05$) between treated group and control. Meanwhile, the AHL concentrations in *P. monodon* culture were found the same ($P>0.05$) in all treatments. Yet, there was a trend that the higher of *B. subtilis* STC given, the smaller number of AHL available in the system. The survival rates of *P. monodon* larvae were significantly different ($P<0.05$) among treatments. The use of AHL-degrading bacteria, *B. subtilis STC*, at the concentration of $1x10^6$ CFU.ml^{-1} improved the survival rate of *P. monodon* larvae up to 65.56% when infected by *V. harveyi*. Therefore, this AHL-degrading bacteria could be used as biocontrol agent against vibriosis in *P. monodon* larvae culture.

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Introduction

Quorum sensing (QS), the process of cell to cell communication enables bacteria to do many tasks they cannot accomplish as individuals, and QS allows bacteria to collectively control processes including biofilm formation and the secretion of virulence factors (Fuqua, Winans, & Greenberg, 1994). In *Vibrio harveyi,* QS is accomplished by three different circuits (Henke & Bassler, 2004). The first circuit consists of HAI-1 (*harveyi* autoinducer-1), an acylated homoserine lactone (AHL) which is synthesized by LuxM, and recognized by the receptor LuxN (Cao & Meighen, 1989). The second circuit uses AI-2 as the autoinducer, LuxS as synthase, and LuxPQ as the receptor complex (Chen *et al*., 2002). The third circuit is CAI-1 (cholera autoinducer-1withCqsA (cholera QS autoinducer) as the synthase and CqsS (cholera QS Sensor) as the receptor (Henke & Bassler, 2004). *In vitro* studies showed that the HAI-1 signal is the strongest compared to the other two QS signals (Waters & Bassler, 2006), but all circuits are needed (Henke & Bassler, 2004). As the AHL is the strongest signal, then there is a possibility that disruption of the *V. harveyi* QS can be conducted through inhibition of that signal.

Bacillus species were amongst the first bacteria reported to degrade AHL by producing lactonase enzymes, which inactivate AHLs by opening the lactone ring (Dong, Xu, Li, & Zhang, 2000; Lee *et al*., 2002). Those microorganisms may produce that enzyme as a defence strategy against their rivals (Natrah *et al*., 2011). They were able to utilize AHL and enzymatic degradation product as sole carbon and nitrogen sources (Leadbetter & Greenberg, 2000; Tinh *et al*., 2007). Therefore, they can block the QS system of their bacterial competitor to obtain a selective advantage. Those reasons then were supported by the facts that *Bacillus* strains were also used increasingly as probiotics in aquaculture (Decamp, Moriarty, & Lavens, 2008; Hong, Duc, & Cutting, 2005).

Disruption of AHL signals had been proven to be an effective strategy in combating disease outbreaks caused by several organism such as *Erwinia carotovora* (Pan *et al*., 2008), *Erwinia amylovora* (Dong, Gusti, Zhang, Xu, & Zhang, 2002; Molina *et al*., 2003), *Burkholderia cepacia* complex (Wopperer *et al*., 2006), and *Pseudomonas aeruginosa* (Sio *et al*., 2006). In aquaculture, some studies also showed that AHL-degrading bacteria approach increased the survival rate of several aquatic organism against pathogens such as larvae turbot, *Scopthalmus maximus* (Tinh, Yen, Dierckens, Sorgeloos, & Bossier, 2008) and giant freshwater prawn, *Macrobrachium rosenbergii* (Nhan *et al*., 2010) against *V. harveyi*, and zebrafish against *Aeromonas hydrophila* (Cao, He, Zhou, & Zhang, 2012). However, the study on the use of AHL-degrading *Bacillus* against *V. harveyi* has never been done in shrimp (*Penaeus monodon*). The aim of this study was to evaluate the ability of AHL-degrading *Bacillus* to interfere with the AHL production and the growth of *V. harveyi* in vitro and in vivo using *Penaeus monodon* larvae.

Material and methods

Bacterial strains and culture media

Bacillus STC used in this study was isolated from the digestive tract of shrimp cultured in East Java Indonesia. In the previous study, *Bacillus* STC was identified as a AHL-degrading bacteria, meanwhile *V. harveyi* BB 120 (ATCC BAA-116) was identified as a AHL-producing bacteria. Both bacteria were cultured in TSB (with 2% of NaCl) at 30°C (120 rpm) for 24 hours. *Agrobacterium tumefaciens* NTL4 (pZLR4, ATCC ®BAA-2240™) was used as AHL bioreporter. This strain produces the blue pigment in the presence of exogenous AHLs (Piper, Bodman, & Farrand, 1993). This bioreporter was grown in a buffered (pH 6.5) normal Luria-Bertani (LB) medium containing 25 μg ml^{-1} gentamycin for 24 hours and measured to an optical density of 0.5 at 600 nm.

Strain identification

The identity of AHL-degrading *Bacillus* STC was confirmed biochemically based on the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). This isolate was also confirmed molecularly by sequencing a 1500-bp 16SrRNA gene using a primer

pair of F1 5'-AAG GAG GTG ATC CAG CC-3' and R1 : 5'-GAG TTT GAT CCT GGG TCA G-3'. The PCR program conducted were 95°C for 1 min, 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1.5 min and a final extension period of 72°C for 1 min. The 16SrRNA sequencing of the obtained PCR products was carried using ABI Prism 310. That sequence was been compared with the gene Bank database using the program BLASTN. Phylogenetic tree was generated using MEGA 4.0 program (Tamura *et al*., 2007).

In-Vitro study

Co-culture assay of AHL-degrading *Bacillus* STC and *V. harveyi* BB 120 were conducted based on the method of Gram *et al*. (1999). In separately, *Bacillus* and *V. harveyi* BB120 were cultured in TSB (with 2% NaCl) at 30°C on a shaker at 120 rpm for 24 hours. In 100 ml TSB, *V. harveyi* BB 120 was inoculated with initial bacterial count of 10^6 CFU. ml^{-1}. The *Bacillus* STC was added in those flasks at the initial count of 0, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ CFU ml^{-1}. Those co-culture flasks were incubated at 30°C on shaker (120 rpm) for 24 hours. By using standart spread plate method, *Bacillus* STC and *V. harveyi* BB 120 were enumerated at 24 and 48 hours on nutrient agar and TCBS agar, respectively.

AHL Concentration

The AHL concentration in co-culture study was measured using *Agrobacterium tumefaciens* NTL4 (pZLR4, ATCC ® BAA-2240™). One (1) ml of co-culture was centrifugated at 7000 g (10 minutes). Five (5) µl supernatant was loaded on the bioassay plate at the one end of bioassay plate (Zhang *et al*., 2007). The bioreporter were spotted at the gradually futher distance from the samples. The bioassay plate was incubated at the 28°C for 24 hours. The relative amount of AHL was measured based on the standard curve of $y = 5.479 \ln(x) - 6.679$ ($r^2=0.988$), in which x is diffusion distance (cm) and y is the concentration of AHL (µM).

In-Vivo Study

Group of 30 (thirty) *P. monodon* PL-20 was maintained in 3 litre glass container and reared until PL-30. *Bacillus* STC was pre-cultured in TSB on a 120 rpm shaker at 30°C for 24 hours. The bacterial culture was added to water at initial concentration of *Bacillus* count of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ CFU.ml^{-1} for every 5 days. As a control, a group of *P. monodon* was reared without *Bacillus* STC treatment. All treatments were conducted triplicate to ensure the feasibility. Challenge test with *V. harveyi* BB 120 was conducted after 10 days in which the *P. monodon* reached the size of PL-30. The infection was carried out by added the *V. harveyi* BB 120 at the initial concentration of $1x10^6$ CFU.ml^{-1} to every glass container. Three (3) ml water was drawn from each container once in 5 days since the adding of *V. harveyi BB 120* to monitor the Total Bacterial Count (TBC) and Total Vibrio Count (TVC). The AHL concentrations in the shrimp culture system were also measured based on the above described method. The survival rate of *P. monodon* was calculated at the day 6 after challenge test. During the culture, *P. monodon* larvae were fed with regular feed at the level of 10% of body weight.

Statistical Analysis

One-way analysis of variance (ANOVA) was carried out to compare the control and treatments at a significance level of $P<0.05$. Duncan's Multiple Range Test was used to determine significant differences between treatments (SPSS, version 13.0).

Result and discussion

Strain identity

The *Bacillus* STC isolate was checked biochemically (data not shown) to confirm the identity. There were positive on the *B. subtilis* identification keys: gram positive rod, endospora-forming bacteria, anerobic facultative, positive strach hidrolysis, positive VP dan positive citrate. Therefore, based on biochemical results, the *Bacillus* isolate was identified as *B. subtilis* STC. Molecularly, *B. subtilis* STC was closely related to seven other Bacillus in the gene Bank such as *B. subtilis* strain B7, *B. cereus* ULT 15 and *B. thuringiensis* strain JMH48. The phylogenetic analysis of *B. subtilis* STC and other closely related *Bacillus* strain in the gene bank was depicted in Fig. 1.

In-Vitro Assay

Co-culture assay allowed one to evaluate the interaction between two organisms' trough antagonism process and competition in an environment with limited nutrients. In the present study, we intended to figure out the interaction process between *V. harveyi* BB 120 as a shrimp pathogen and *B. subtilis* STC as a bio-control candidate. Total bacterial count (TBC) dan Total *Vibrio count (TVC)* in the co-culture system within 24 and 48 hours were documented in Table 1.

Table 1. TBC and TVC in the co-culture system *B. subtilis* STC and *V. harveyi* BB 120.

B. subtilis STC (CFU mL-1)	TBC (log CFU mL-1)		TVC (log CFU mL-1)	
	24h	48h	24h	48h
Control	9.76± 0.06 ^{e}	7.20± 0.16 ^{a}	8.73± 0.08 ^{a}	7.19± 0.08^{a}
1x10^{4}	7.03 ± 0.02 ^{a}	7.34 ± 0.03^{ab}	6.55 ± 0.04 ^{b}	5.29± 0.04^{b}
1x 10^{5}	7.53 ± 0.05 ^{b}	7.46 ± 0.01^{b}	5.96 ± 0.04 ^{c}	4.80± 0.04^{c}
1x 10^{6}	9.40 ± 0.06 ^{c}	7.34 ± 0.04^{ab}	5.74 ± 0.06 ^{d}	3.93± 0.06^{d}
1x 10^{7}	9.52 ± 0.05 ^{d}	7.41 ± 0.05^{b}	5.69 ± 0.04 ^{d}	3.92± 0.04^{d}

Mean ± S.E. in the same column having the same letter are not significantly different at P<0.05.

There was a significant effect (P<0.05) of the *B. subtilis* STC on the TBC after 24 and 48 hours. There was an increase of TBC with increasing concentrations of *B. subtilis* STC within 24 hours. However, the TBC of control (*V. harveyi* BB 120 monoculture) increased significantly compared to other treatments. This was possible as with only one type of bacteria, there would be no competition for nutrients and space. Thus, the *V. harveyi* BB 120 could grow optimally. After 48 hours, the TBC value did not indicate a particular trend. Furthermore, there was a significant difference (P<0.05) recorded for TBC in the co-culture system among treatments. Yet, the TBC in all treatments ranged in value of 10^{7} CFU.ml^{-1}. This phenomenon showed that after 48 hours cultured bacteria would not grow as there was a limit of nutrients available.

Table 2. AHL concentration in co-culture system of *B. subtilis* STC and *V. harveyi* BB 120.

B. subtilis STC (CFU.ml^{-1})	AHL (µM)	
	24 h	48 h
Control	3.19 E-02 ± 0.02^{a}	8.25 E00 ± 0.00^{a}
1x10^{4}	1.72 E-02 ± 0.01^{a}	1.31 E-03 ± 0.00^{b}
1x 10^{5}	1.31 E-03 ± 0.00^{b}	3.49 E-05 ± 0.00^{c}
1x 10^{6}	7.96 E-05 ± 0.00^{c}	2.09 E-07 ± 0.00^{d}
1x 10^{7}	2.09 E-07 ± 0.00^{d}	4.20 E-09 ± 0.00^{e}

Mean ± S.E. in the same column having the same letter are not significantly different at P<0.05.

In addition to TBC, the Total Vibrio Count (TVC) from co-culture system was enumerated by culturing them in TCBSA plate. There was a significant differences (P<0.05) of the TVC after 24 hours. The TVC of control (V. *harveyi* BB 120 monoculture) increased by an average of 8.73 (log CFU ml^{-1}) within 24 hours. On the other hand, with the increasing concentration of *B. subtilis* STC, the TVC value decreased significantly. *B. subtilis* STC with initial concentration of 10^{4}-10^{7} CFU ml^{-1} were able to reduce the TVC by 24.91 to 34.80% after the first 24 hours and 26.39 to 45.46% for the next 24 hours. Purivirojkul *et al.* (2006) found that *B. pumilus* NW01, NW02 *B. sphaericus* and *B. subtilis* NW03

reduced the population of *V. harveyi* up to 39.10; 43.62 and 34.46%. The results of co-culture showed that *B. subtilis* STC was potential to be developed as a biocontrol agent. Competition for nutrients or energy available would determine the composition of microbial populations which live in the same ecosystem. Microbial ecosystem in the aquatic environment is usually dominated by heterotrophic bacteria which compete for organic substrate either for carbon or energy source (Sihag and Sharma, 2012).

Table 3. TBC and TVC in the shrimp culture system with the administration of *B. subtilis* STC.

B. subtilis STC (CFU mL^{-1})	TBC (log CFU mL-1)			TVC (log CFU mL-1)		
	Day 6	Day 11	Day 16	Day 6	Day 11	Day 16
Control	2.09± 0.10 ^{a}	6.32± 0.13 ^{c}	6.22± 0.09 ^{a}	0.00± 0.00^{a}	6.13± 0.06 ^{a}	5.16± 0.11 ^{a}
1x10^{4}	5.18 ± 0.20 ^{b}	5.13 ± 0.03 ^{a}	5.34 ± 0.03 ^{b}	0.00± 0.00^{a}	3.33 ± 0.01 ^{b}	2.76 ± 0.02 ^{b}
1x10^{5}	5.11 ± 0.06 ^{b}	5.57 ± 0.02 ^{b}	5.46 ± 0.01 ^{c}	0.00± 0.00^{a}	3.24 ± 0.11 ^{b}	2.69 ± 0.05 ^{b}
1x10^{6}	6.06 ± 0.05 ^{c}	6.48 ± 0.04 ^{c}	5.34 ± 0.04 ^{d}	0.00± 0.00^{a}	2.51 ± 0.07 ^{c}	2.41 ± 0.07 ^{c}
1x10^{7}	7.01 ± 0.03 ^{d}	7.02 ± 0.05 ^{d}	6.41 ± 0.05 ^{d}	0.00± 0.00^{a}	2.34 ± 0.12 ^{d}	2.49 ± 0.05 ^{c}

In the present study, *B. subtilis* STC, which previously identified as AHL-degrading bacteria, were expected to break down the AHL produced by *V. harveyi* BB 120. Therefore, the concentrations of AHL in the co-culture system were evaluated (Table 2). There were significantly differences ($P<0.05$) of AHL concentration within treatments. The higher the concentration of *B. subtilis* STC, the lower the concentration of existing AHL in the system. *B. subtilis* STC was able to eliminate the AHL signal constantly on the surrounding environment as an attempt to maintain its population. After the nutrients in the system began to decrease, the *B. subtilis* STC would utilize AHL produced by *V. harveyi* BB 120. This can be a prove that B. *subtilis* STC produced an AHL degrading enzyme which degrade the AHL produced by *V. harveyi* BB 120. Some organisms such as *Variovoraxparadoxus*, *Bacillus thuringiensis*, *Acinetobacter* sp. were able to use the AHL as a source of carbon and nitrogen (Leadbetter and Greenberg, 2000; Hu *et al.*, 2003; Kang *et al.*, 2004). These bacteria used the AHL degradation mechanisms as an effort to take advantage of its competitors. Several possible mechanisms in the AHL degradation process were as part of a defense mechanism against producing antibiotics bacteria in the adjacent environment (González and Keshavan, 2006), as a source of carbon, nitrogen and energy (Leadbetter and Greenberg, 2000), and biocontrol (Rasmussen and Givskov, 2006; Dong and Zhang, 2005). Furthermore, those mechanisms would help the AHL-degrading bacteria for dominating its environment (Dong *et al.*, 2001; Park *et al.*, 2003).

Table 4. AHL concentration in shrimp culture system with the administration of *B. subtilis* STC.

B. subtilis STC (CFU mL^{-1})	Concentration of AHL (µM)		
	Day 6	Day 11	Day 16
Control	0.00± 0.00^{a}	0.01± 0.00^{a}	4.38 E-04± 0.00^{a}
1x10^{4}	0.00± 0.00^{a}	2.23 E-11± 0.00^{a}	0.00± 0.00^{a}
1x10^{5}	0.00± 0.00^{a}	1.11 E-11± 0.00^{a}	0.00± 0.00^{a}
1x10^{6}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
1x10^{7}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}

The AHL concentration was affected by the density of *V. harveyi* as the AHL producer. In this study, it was found that the higher of viable *Vibrio* cells, the higher of the AHL concentration in the co-culture system. That fact was in accordance with the statement of Miller and Bassler (2001) who discovered that the bacteria used the QS system to determine the density of the population. In addition, González & Keshavan

(2006) stated that the AHL concentration in the environment is an indicator of the total population of the AHL-producing bacteria.

In Vivo Study

Several studies have shown that the application of bacteria as a bio-control in vitro has not necessarily given the same effect when it had applied in vivo. This in vivo study was carried out to give a confirmation that *B. subtilis* STC able to degrade AHL produced by *V. harveyi BB 120* in the shrimp culture system. Furthermore, *B. subtilis* STC was able to act as biocontrol of vibriosis in shrimp culture. The addition of the *B. subtilis* STC in the culture system was considered effective as shrimp can directly contact with the bacteria. The addition of microorganisms in the culture media is a means of biocontrol in fish or shrimp farming systems. The microorganisms can be digested and give the probiotics effects on the host animal (Verschuere *et al.*, 1999). After administration of *B. subtilis* STC, the TBC and *TVC* in shrimp culture system were counted (Table 3).

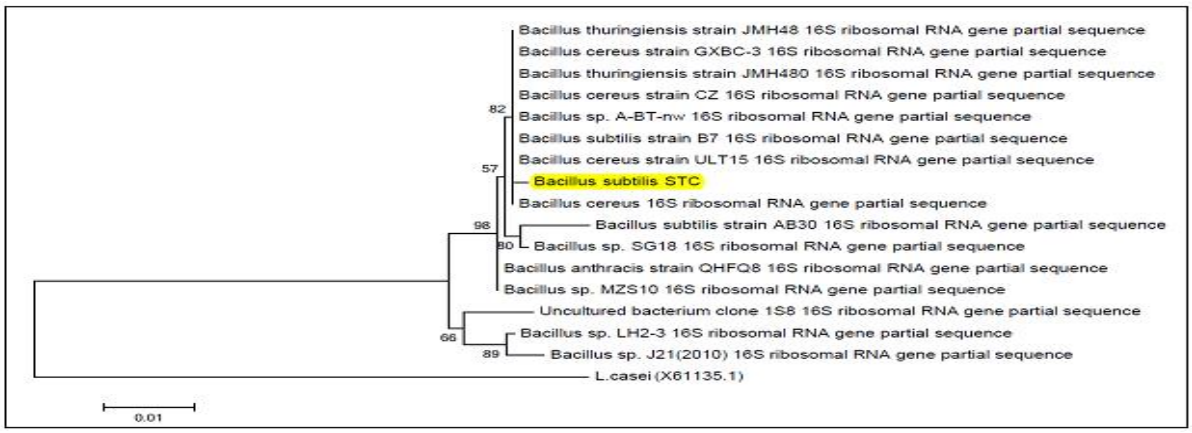

Fig. 1. A maximum likelihood tree shows the phylogenetic position of *B. subtilis* STC with other closely related *Bacillus* in the gene bank. *Lactobacillus casei* was set as an out-group. The evolutionary distance was 0.01 changes per nucleotide position. Numbers indicated bootstrap level (%) determined from 1000 resample data. Total final nucleotide examined was 1.304 bp.

Bacteria usually present abundantly in the shrimp pond environment. They play an important role in geochemical cycles and the health status of aquatic animals which live in it. In marine waters, if the concentration of bacteria present was more than 10^6 cells ml^{-1}, then the protozoa would grow quickly by eating those bacteria (Maeda *et al.*, 1997). Consequently, the concentration of bacteria would be still in the range of 10^6 cells ml^{-1}. In this study, however, the total bacterial counts among treatments were found to exceed the range of 10^6 CFU.ml^{-1}. Rengpipat *et al.* (1998) also found that after supplementation of probiotic bacteria for 100 days the total bacterial count in the culture and the tiger prawn shrimp digestive system were 10^{7-8} CFU and 10^{10} CFU.ml^{-1}, respectively. In general, the total number of bacteria found in the in-vivo test was lower than that in in-vitro test.

The addition of *B. subtilis* STC with various concentrations and the challenge test using *V. harveyi* BB 120 on day 10 had an effect on the composition, concentration and dominance of the bacterial population in the shrimp culture system. There were significant differences ($P<0.05$) recorded for the TBC and TVC in the shrimp culture system among treatments. Excluding control (no *B. subtilis* STC), the TBC increased as the increase of *B. subtilis* STC given in the shrimp culture. For TVC, the higher the *B. subtilis* STC was added, the less concentration of viable *Vibrio* cells in the system. These in vivo results then gave a confirmation on the in vitro study previously conducted.

As *B. subtilis* STC are AHL-degrading bacteria, the AHL concentrations in the shrimp culture systems were analyzed (Table 4) after administration of those

in the shrimp culture. It was found that there were no significantly differences (P>0.05) for AHL concentration between treatments and control group. However, there was a trend that the higher of *B. subtilis* STC given, the smaller number of AHL available in the system. There were still AHL's on the day 11 and 16 in all treatments, yet those could not be detected by bioreporter *A. tumefaciens* used in this study. It seems that the AHL concentration could be detected if the *V. harveyi* reached the population of 10^3 CFU. ml^{-1}. The same thing was experienced by Buch *et al.* (2003) who only could detect the AHL extracted from liver, kidney and muscle of salmon in vitro and in vivo when the concentration of AHL-producing bacteria (*V. anguillarum*) reached the number of 10^7 cells.ml^{-1}. They used *A. tumefaciens* (pZLR4) and *Chromobacterium violaceum* (CV026) as the AHL bioreporters. To clarify the phenomena, it is necessary to evaluate the AHL production in aquaculture systems by using other methods.

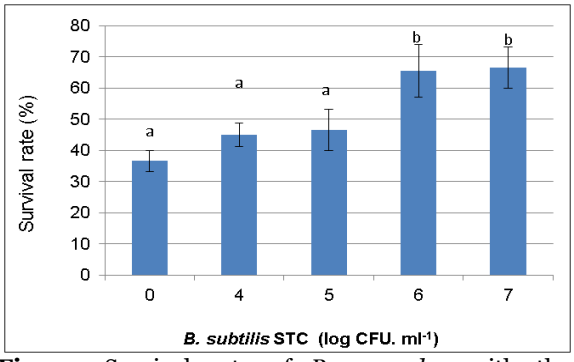

Fig. 2. Survival rate of *P. monodon* with the treatments of various concentrations of *B. subtilis* STC.

According to Leadbetter and Greenberg (2000), AHL's were not available in the environment for quite long time. They stated that if the AHL accumulated in the environment over a period of time then its function as a QS signal would be lost. AHL molecules rapidly diffused into bacterial cells and subsequently in-activation process occured (Medina-Martínez *et al.*, 2007). In addition, AHL instability can be caused by environmental conditions as well as the activity of the enzyme itself. AHL is a type of molecule that is unstable at high pH, especially AHL with short acyl chains (Yates *et al.*, 2002). Some evidences showed that the higher organisms had AHL-degrading enzyme as well (Chun *et al.*, 2004; Yang *et al.*, 2005). However, Yang *et al.*, (2005) found that fish and chicken had no AHL inactivation ability and no report for crustacea. Therefore, the possibility of shrimp ability to degrade AHL needs a confirmation in a future study. There were AHL-producing and AHL-degrading bacteria in ecosystem (Hu *et al.*, 2003; Yang *et al.*, 2005). Furthermore, those two types of bacteria have different strategies to take an advantage over the other and then create an ecological equilibrium. Even, some bacteria such as *A. tumefaciens* had an ability to produce and degrade AHL (Hu *et al.*, 2003).

Survival Rate

In the present research, AHL degrading bacteria, *B. subtilis* STC, was supplemented to the culture media with the intention that it could control the pathogenistic bacteria *V. harveyi* BB 120. The effect of *B. subtilis* STC addition on the survival rate of *P. monodon* larvae can be seen in Fig. 2. In general, the survival rate of *P. monodon* larvae in this study was quite high, ranged from 30-73% after challenge test with *V. harveyi* BB 120 at the day six. Lavilla-Pitogo *et al.* (1990) found a mass mortality in the larval and juvenile of *P. monodon* after bathing with *V. harveyi* and *V. Splendidus* in 48 hours. The high survival rate might due to the good environment of the culture system which then it would increase the immune system of shrimp larvae. The survival rates of P. monodon larvae were significatly different (P<0.05) among treatments. In this study, survival of *P. monodon* after challenge test found to increase with the administration of *B. subtilis* STC on the water compared to controls. Furthermore, the Duncan Post Hoc test revealed that the concentration of *B. subtilis* STC which effectively improved the survival rate of *P. monodon* was equal to $1x10^6$ CFU.ml^{-1}. Based on the observation during research, it was found that shrimp larvae mortality occurred not only because of a *V. harveyi* BB 120 infection but also due to cannibalism. Deaths due to the infection were characterized by the presence of clinical symptoms of red discoloration. According to Aguirre-Guzmán *et al.* (2004), the signs of vibriosis are lethargy, tissue necrosis, slow growth,

slow metamorphosis, body malformations, bioluminescence and melanization. In addition, shrimp attacked by vibrio becomes weak, slow swimming, loss of appetite, red discoloration on pleopod and abdominal.

Based on the in vivo test, it can be seen that the application of the AHL-degrading bacteria *B. subtilis* STC on the culture media gave a beneficial effect on survival of *P. monodon* larvae. Similar results were obtained by Tinh *et al.* (2008) who added a mixed culture of AHL-degrading bacteria on turbot (*Scophthalmus maximus*) larval rearing system and obtained an increase oflarvae survivalrate. Further study using the same mixed culture of those bacteria found an increase in survival rate of *Macrobranchium* larvae (Nhan *et al.*, 2010).That mixed culture of bacteria was isolated from the digestive system of fish and shrimp which then it enriched with AHL. In shrimp seed production, improvement of survival and quality of larvae is very important in order to improve cost efficiency. As many antibiotics misuse in aquaculture, it is no longer effective of using it against vibriosis. Application of AHL-degrading bacteria could lead to more sustainable aquaculture production by replacing the antibiotics to control diseases.

Conclusion

AHL-degrading *B. subtilis* STC could reduce the population of *V.harveyi* and its AHL production in vitro and in vivo. Furthermore, the use of AHL-degrading *B. subtilis STC* at the concentration of $1x10^6$ CFU.ml^{-1} improved the survival rate of *P. monodon* larvae up to 65.56% when infected by *V. harveyi*.

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