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# Characteristics and Activity of Anti Quorum Sensing *Bacillus* spp. Isolated from *Penaeus vannamei* Shrimp Ponds

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**ABSTRACT**

**Certain strains of *V. parahaemolyticus* carry a gene that encodes a toxin that causes Acute hepatopancreatic necrosis disease (AHPND) in *P. vannamei*. AHPND attacks shrimp post larvae within 20-30 days after stocking causing up to 100% mortality. The expression of these virulent genes is controlled by the quorum sensing system. This system is inhibited by an anti-quorum sensing (AQS) mechanism. Several *Bacillus* strains have AQS mechanism by producing AHL-Lactonase enzyme. Therefore, this study aimed to obtain *Bacillus* spp. having AQS activity for controlling AHPND. The study was conducted from isolation and selection of *Bacillus* isolates, as well as determination of AQS activity. From 22 samples consisting of shrimp intestines, water and pond sediment samples, a total of 151 isolates of *Bacillus* spp. were isolated. The screening test for AQS activity obtained 11 isolates that showed AQS activity on *Cromobacterium violaceum*. Determination of violacein pigment in liquid cultures of *C. violaceum* showed the index value of the pigment formation was between 0.025-0.166 and 0.026-0.567 at 24-hour and between 48-hour incubations, respectively. The quantitative analysis of violacein production showed that there were six isolates of *Bacillus* could inhibit the pigment production more than 75%. The isolates were identified as *Bacillus cereus* (four isolates), *Bacillus thuringiensis* (one isolate), and *Bacillus velezensis* (one isolate), respectively. The molecular analysis had confirmed that the isolates have *aii*A genes encoding AHL-lactonase enzyme. These *Bacillus* isolates have potential application for controlling AHNPD disease.**

## 1. Introduction

Vannamei shrimp is an export commodity of fishery products in Indonesia. Disease attack is the main problem of shrimp production. One of the bacterial pathogens causing vibriosis is *Vibrio parahaemolyticus* (Longyant *et al.* 2008; Sarjito *et al.* 2018). Certain strains of *V. parahaemolyticus* carry a gene encoding a toxin that causes acute hepatopancreatic necrosis disease (AHPND in vannamei shrimp (Tran *et al.* 2013). AHPND was first reported in southern China in 2010 and subsequently in Vietnam, Thailand, and Malaysia (FAO 2013). This disease has been endemic in several countries in Asia such as China, Vietnam, Philippines, Thailand, Malaysia, and Mexico (Han *et al.* 2015; de la Peña *et al.* 2015; Soto-Rodriguez *et al.* 2015). AHPND attacks on shrimp post larvae within 20-30 days after stocking and causes up to 100% mortality (De Schryver *et al.* 2014). The Global Aquaculture Alliance (GAA 2013) estimates the loss of Asian shrimp aquaculture farms at one billion USD.

AHPND is caused by the binary gene pirAvp/pirBvp found on a plasmid of *V. parahaemolyticus* (Tran *et al.* 2013; Han *et al.* 2015; Lee *et al.* 2015). The expression of these genes is controlled by quorum sensing system. Quorum sensing (QS) is induced by a signal molecule called an autoinducer (AI) (Defoirdt *et al.* 2004). The signal molecule is an acyl homoserine lactones (AHL) molecule (Vinoj *et al.* 2014). The expression of this virulence factor gene can be inhibited by an anti-quorum sensing (AQS) mechanism. AQS can inhibit production of autoinducer enzymatic degradation (Natrah *et al.* 2014). A broad-spectrum autoinducer degrading enzyme is AHL lactonase enzyme encoded by the *aii*A gene. The AHL autoinducer degradation

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process by this enzyme does not depend on the variation of the acyl group (Defoirdt *et al.* 2004).

Several *Bacillus* strains such as *B. cereus, B. substilis,* and *B. thuringiensis* have *aii*A gene encoded AHL-Lactonase enzyme that can degrade the AHL autoinducer (Kalia 2013). These bacteria can be isolated from the pond environment, and the digestive tract of shrimp. And the *Bacillus* isolates was able to degrade autoinducers and inhibit QS in *V. parahemolyticus* (Defoirdt *et al.* 2008; Cao *et al.* 2012; Romero *et al.* 2014; Vinoj *et al.* 2014; Novita *et al.* 2015; Yuniarti *et al.* 2015; Torres *et al.* 2018). Therefore, *Bacillus* AQS isolates have potential application in shrimp ponds to control AHPND disease (Chu *et al.* 2010; Delshad *et al.* 2018).

Controlling of AHPND disease caused by *V. parahaemolyticus* in vanamei shrimp culture in Indonesia by utilizing the AQS mechanism from *Bacillus* spp. still has not been done much. Therefore, this study was conducted to obtain *Bacillus* spp. AQS Isolates. The selected isolates that characterized showing AQS activity can be further developed as biocontrol agents of AHPND disease in shrimp aquacultures.

## 2. Materials and Methods

### 2.1. Isolation of *Bacillus* spp.

Samples such as shrimp intestine, water and sediment shrimp ponds were collected from several shrimp ponds in Karawang (West Java), Tanggerang (Banten) and Lampung (Lampung), Indonesia. One gram of the samples was serially diluted in 9 ml of 0.85% NaCl solution. The suspension treated by heat shocking at 80°C for 5 minutes, then the appropriate dilutions were spread on 50% SWC agar medium. Incubation was done aerobically at room temperature for 24 to 48 hours. Bacterial colonies grown in the agar plates were then purified to obtain a pure culture of the isolates.

### 2.2. Selection of *Bacillus* Isolates Having AQS Activity

Screening of pure culture of isolated *Bacillus* that had AQS activity was carried out by detecting anti quorum sensing activity using the disc diffusion method. The purified bacterial isolates were grown in Luria-Bertani broth (LB) medium on a shaker for 18 hours until the Optical Density (OD)600 nm reached 0.8-1.0 (equivalent to 1 x $10^{-9}$ CFU/mL). The culture was then centrifuged at 6 00 x g for 10 minutes. The supernatant was filtered using a 0.22 um syringe filter. A total of 100 ul of supernatant was dripped onto a sterile paper disk (6 mm) on the surface of Luria-Bertani agar (LA) medium containing 1% *C. violaceum* (v/v). Incubated was done at 28°C for 24 hours. Sterile LB was used as a negative control. AQS activity of the isolates was indicated by the presence of a no purple zone around the paper disk. This zone was formed due to interference with QS *C. violaceum* by enzymes possessed by the test bacterial supernatant, so that the production of the purple pigment violaceum was inhibited (McLean *et al.* 1997). The diameter of the clear zone was measured from the outer edge of the paper disk to the edge of the colorless zone. The AHL degradation index is calculated by the following formula:

$$\text{AQS activity index} = \frac{\text{Non-purple zone diameter}}{\text{Paper disk diameter}}$$

### 2.3. Determination of Inhibition Violacein Production by AQS Activity

Determination of violacein Production Inhibition by AQS activity was conducted based on the method reported by Chaudari *et al.* (2014). As much as 300 ul of AQS bacterial supernatant was added to 30 ml of LB medium which was inoculated with 1% *C. violaceum* (v/v). The cultures were incubated on a shaker incubator at room temperature for 24 hours and 48 hours. The violacein pigment was extracted from the cultures by centrifugation at 10,000 rpm for 15 minutes. And the pellets were washed with 1.5 ml DMSO and then centrifuged again at 10000 rpm for 15 minutes. The pure culture of *C. violaceum* without addition of supernatant of the culture *Bacillus* isolates was used as a control. Violacein dissolved in DMSO was measured their absorbance at 585 nm, while bacterial cells were measured at 600 nm. The violacein unit index was measured by calculating the ratio of the absorbance at 585 nm and 600 nm (Chaudari *et al.* 2014). And the inhibition percentage of violacein was calculated form percentage ratio of violacein unit index of the treatment and the control.

### 2.4. Identification of Bacterial Isolates Based on 16S rRNA Gene

The selected bacterial isolates were identified based on the PCR product sequences of 16S rDNA gene. PCR amplification used primers of 63F and 1387R (Marchesi *et al.* 1998). The temperatures set up of the PCR machine were at 95°C for 5 minutes for pre-denaturation, 95°C for 1 minute for denaturation, 55°C for 1 minute for annealing at, 72°C for 1.5 minutes for elongation, and 72°C for 10 minutes for post elongation. The PCR was run for 30 cycles. The PCR products were electrophoresed on 1% agarose, then purified for sequencing. The sequence results of

the PCR products were analyzed using the Basic Local Alignment Search Tool-Nucleotide (BLAST-N) in the the National Center for Biotechnology Information (NCBI) Gene Bank website [www.ncbi.nlm.nih.gov]. Phylogenetic analysis was done using the maximum likelihood method with 1,000x bootstraps of the MEGA X software.

### 2.5. Detection of *aii*A Gene Encoding AHL Lactonase of the *Bacterial* Isolates

The selected bacterial isolates were grown on SWC media for 18 hours. The bacterial cells pellet was harvested by centrifugation at a speed of 6,000 xg for 10 minutes. The genomic DNA of the isolates was extracted using the Mericon DNA Bacteria Plus Kit (Qiagen). The presence of the *aii*A genes was determined using primers pair of *aii*AF and *aii*AR (Dong *et al.* 2002). The amplification of the genes was set up at 94°C for 10 minutes for pre-denaturing, 94°C for 30 seconds for denaturation, 52°C for 30 seconds for annealing, 72°C for 1 minute for elongation at, and 72°C for 5 min for post elongation. The PCR was run for 30 cycles. The PCR product was purified using the QIA-quick PCR Purification Kit (Qiagen). Then the sequences of the PCR products were analyzed using the Basic Local Alignment Search Tool. -Nucleotide translation protein (BLAST-X) of the NCBI Gene Bank website. The phylogenetic tree of the amino acid sequences was constructed using the maximum likelihood method with 1,000x bootstraps of the MEGA X software.

## 3. Results

Isolation of *Bacillus* spp. bacteria was carried out from 22 samples consisting of samples of shrimp intestines, water and pond sediments of white shrimp (*Penaeus vannamei*) from several locations in Karawang (West Java), Tangerang (Banten), and Lampung (Lampung), Indonesia. A total of 151 isolates of *Bacillus* spp. Were successfully isolated from bacteria isolated from different sources (shrimp intestine, pond water and pond sediment). The results of the screening for AQS activity showed that 11 Bacillus isolates performed AQS activity on *C. viloaceum* consisting of 3 isolates from Karawang samples, 2 isolates from Tangerang samples, and 6 isolates from Lampung samples (Table 1). AQS activity was indicated by the presence of a non-purple colony zone.

The results of the screening of ASQ activity using disk diffusion method to select isolates that had inhibitory activity on the production of violacein pigment by *C. violaceum* indicate that the culture supernatant of 11 *Bacillus* spp. isolates can inhibit the formation of violacein pigment indicated by the presence of a non-purple zone around paper discs (Table 2 and Figure 1). The non-purple zone index values the isolates were from 0.33 to 2.83. The non-purple zone around the paper discs indicates the presence of AQS activity which inhibits the QS mechanism in *C. violaceum*. The three highest values of violacein pigment inhibition indexes were shown by the supernatants of LpgA01, LpgS03, and LpgS01 isolate (Table 2).

Quantitative determination of pigment production inhibition in liquid cultures of *C. violaceum* with the addition of culture supernatant of *Bacillus* spp. showed that the index value of violacein pigment formation was between 0.025-0.166 and 0.026-0.567 at 24-and 48-hour incubations (Table 3). Some culture supernatants of the isolates also inhibited the growth

Table 1. Isolation results of *Bacillus* spp. which has anti-quorum sensing (AQS) activity isolated from samples of shrimp intestines, water, and pond sediments in several locations of shrimp ponds

| Pond location | Sample | | Number of Isolated *Bacillus* | Number isolate with Positive AQS | Isolate code |
|---|---|---|---|---|---|
| | Type | Number | | | |
| Karawang | Shrimp intestine | 4 | 20 | 2 | KwgU01; KwgU02 |
| | Pond water | 1 | 8 | - | |
| | Pond sediment | 2 | 16 | 1 | KwgS01 |
| Tanggerang | Shrimp intestine | 2 | 16 | 2 | TggU01; TggU02 |
| | Pond water | 1 | 8 | - | |
| | Pond sediment | 3 | 18 | - | |
| Lampung | Shrimp intestine | 3 | 24 | 2 | LpgU01; LpgU02 |
| | Pond water | 2 | 19 | 1 | LpgA01 |
| | Pond sediment | 4 | 22 | 3 | LpgS01; LpgS02; LpgS03 |
| Total | | 22 | 151 | 11 | |

Table 2. Diameter of the non-purple zone of *C. violaceum* by addition culture supentants of *Bacillus* spp. isolated from samples of shrimp intestines, pond water and pond sediments in several locations of shrimp ponds

| Isolate code | Diameter of Non-purple zone (mm) | Indexes of non-purple zone |
|---|---|---|
| LpgS01 | 16 | 1.67 |
| LpgS02 | 10 | 0.67 |
| LpgS03 | 18 | 2.00 |
| LpgU01 | 14 | 1.33 |
| LpgU02 | 8 | 0.33 |
| LpgA01 | 23 | 2.83 |
| TggU01 | 9 | 0.50 |
| TggU02 | 8 | 0.33 |
| KwgU02 | 13 | 1.17 |
| KwgS01 | 9 | 0.50 |
| KwgU01 | 12 | 1.00 |
| Control (-) | 0 | 0.00 |

of *C. violaceum*, as seen from the absorption value at 600 nm which was lower than the absorption value in the negative control (Table 3). And to get the best isolate that has the AQS mechanism, the percentage inhibition of violacein pigment production per absorption unit index 600 nm which is equivalent to per unit cell was calculated (Table 4). The results of the analysis showed that there were six *Bacillus* isolates could inhibit pigment production per index unit with the inhibition percentage more than 75%, namely LpgS01, LpgS02, LpgS03, LpgU01, LpgU02, and LpgA01 isolates.

The results of the alignment of 16S rRNA gene sequences using BLAST-N showed that all selected isolates were had similarity 98-100% with genus *Bacillus* (Table 5). There were four isolates (LpgS03, LpgU01, LpgU02, and LpgA01 isolates) had similarity

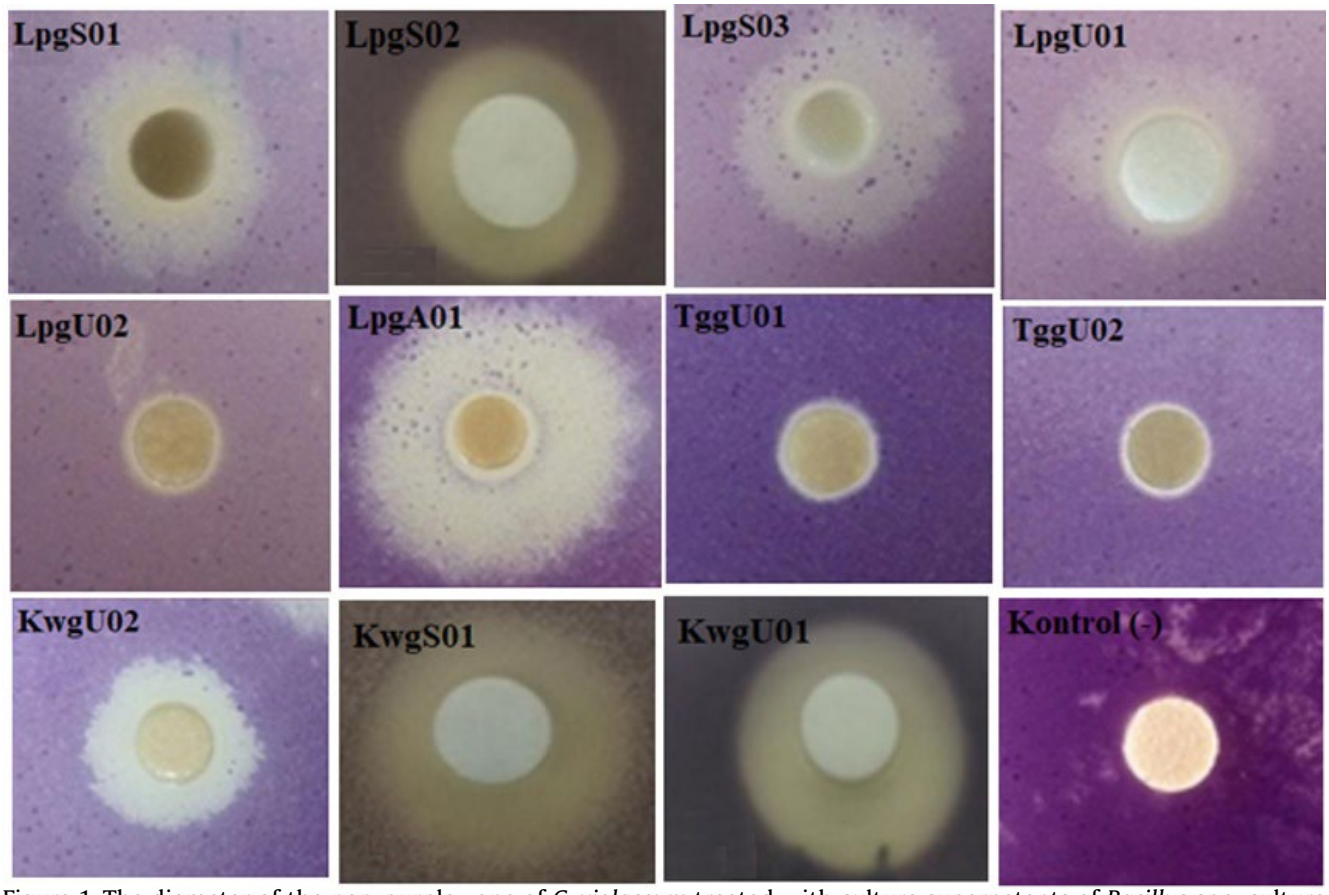


Figure 1. The diameter of the non-purple zone of *C. violaceum* treated with culture supernatants of *Bacillus* spp. culture supernatant. isolated from samples of shrimp intestines, pond water and pond sediments in several locations of shrimp ponds

Table 3. Inhibitory activity of violacein accumulation of *C. violaceum* by culture supernatants of *Bacillus* spp. isolates. The control of violacein accumulation was a single culture of *C. violaceum* without the culture supernatant of *Bacillus* spp. isolates

| Isolate code | Violacein absorbance (λ 585 nm) after incubation | | Cells absorbance (λ 660 nm) after incubation | | Cells absorbance (λ 660 nm) after incubation | |
|---|---|---|---|---|---|---|
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| LpgS01 | 0.072 | 0.149 | 0.963 | 1.480 | 0.075 | 0.100 |
| LpgS02 | 0.076 | 0.142 | 0.962 | 0.792 | 0.079 | 0.179 |
| LpgS03 | 0.073 | 0.156 | 1.594 | 2.029 | 0.077 | 0.107 |
| LpgU01 | 0.028 | 0.041 | 0.951 | 1.454 | 0.025 | 0.026 |
| LpgU02 | 0.106 | 0.268 | 1.105 | 1.557 | 0.133 | 0.286 |
| LpgA01 | 0.122 | 0.346 | 0.793 | 1.003 | 0.156 | 0.335 |
| TggU01 | 0.133 | 0.631 | 0.780 | 1.034 | 0.166 | 0.567 |
| TggU02 | 0.133 | 0.478 | 0.799 | 1.112 | 0.136 | 0.333 |
| KwgU02 | 0.062 | 0.137 | 0.983 | 1.434 | 0.069 | 0.116 |
| KwgS01 | 0.162 | 0.581 | 0.890 | 1.183 | 0.146 | 0.339 |
| KwgU01 | 0.086 | 0.136 | 0.989 | 0.982 | 0.087 | 0.138 |
| Control (-) | 0.624 | 1.673 | 1.56 | 2.041 | 0.400 | 0.820 |

Table 4. Percentage of inhibition of violacein pigment formation in *C. violaceum* per unit index treated with culture supernatant of *Bacillus* spp. isolates

| Isolate code | Inhibition percentage of violacein formation per unit index (%) on incubation: | |
|---|---|---|
| | 24 h | 48 h |
| LpgU01 | 93.8 | 96.8 |
| LpgS01 | 81.3 | 87.8 |
| LpgS03 | 80.8 | 87.0 |
| KwgU02 | 82.8 | 85.9 |
| KwgU01 | 78.3 | 83.2 |
| LpgS02 | 80.3 | 78.2 |
| LpgU02 | 66.8 | 65.1 |
| TggU02 | 66.0 | 59.4 |
| LpgA01 | 61.0 | 59.1 |
| KwgS01 | 63.5 | 58.7 |
| TggU01 | 585 | 30.9 |

Table 5. The bacterial species similarity of the selected isolates base on 16S rDNA genes analysed using the Basic Local Alignment Search Tool-Nucleotide (BLAST-N) in the the National Center for Biotechnology Information (NCBI) Gene Bank website [www.ncbi.nlm.nih.gov]

| Isolate code | Bacterial species similarity | Nucletide leght ratio of similar/ alignment (bp) | Percent identity (%) | E-value | Request Search ID (RID) |
|---|---|---|---|---|---|
| LpgS01 | *Bacillus thuringiensis* BRAW PT | 1213/1227 | 98.86 | 0 | MUD5EZJW013 |
| LpgS02 | *Bacillus velezensis* HSB1 | 998/999 | 99.90 | 0 | MUE81J8H013 |
| LpgS03 | *Bacillus cereus* B10 | 1254/1254 | 100 | 0 | MUF7K4ZT016 |
| LpgU01 | *Bacillus cereus* ATCC 14579 | 1225/1248 | 98.16 | 0 | MUG0P6TR013 |
| LpgU02 | *Bacillus cereus* B10 | 1244/1266 | 98.26 | 0 | MUGMCKXB016 |
| LpgA01 | *Bacillus cereus* EM6 | 1255/1258 | 99.76 | 0 | MUH5HGTE013 |
| TggU01 | *Bacillus thuringiensis* BRAW PT | 1252/1259 | 99.44 | 0 | MUHJKGU1016 |
| TggU02 | *Bacillus* sp. hb10 | 1247/1256 | 99.28 | 0 | MUJ0J6V5016 |
| KwgU02 | *Bacillus subtilis* H1 | 1253/1264 | 99.13 | 0 | MUJAV3TB013 |
| KwgS01 | *Bacillus subtilis* TW3 | 1191/1222 | 97.46 | 0 | MUJRSN21013 |
| KwgU01 | *Bacillus coagulans* BSCB 2 | 1329/1332 | 99.77 | 0 | MUK8GD3J013 |

with *Bacillus cereus*, two isolates (KwgU02 and KwgS01 isolates) had similarity with Bacillus subtilis, two isolates (LpgS01 and TggU01 isolates) had similarity with *Bacillus thuringiensis*, and one isolate had similarity with *Bacillus coagulan* (KwgU01 isolate) and *Bacillus velezensis* (LpgS02 isolate), respectively. However, based on the phylogenetics tree, the bacterial isolates were clustered in two groups. The LPGS02 and KWGU02 isolates were separated in different cluster that that of the other isolates (Figure 2).

Molecular analysis using a PCR machine showed that the eleven selected isolates had the *aii*A gene encoding AHL lactonase enzymes. The electrophoresis gel agarose of the all-isolate's PCR products showed DNA bands size of 800 bp (Figure 3). The BLAST-X analysis of the PCR products sequences confirmed that the all sequences had similarity 97-100% with N-acyl homoserine lactonase of *Bacillus* group (Table 6). The analysis showed that almost all isolates had *aii*A genes that similar to that of *Bacillus cereus*. Additionally, the *aii*A gene of LpgS01 was similar to *Bacillus thuringiensis* serovar aizawai. The BLAST-X results also showed that KwgU02 isolate had the lowest similarity value (97%) to the *Bacillus cereus* AiiA sequences, while the other isolates had similarity between 99-100%.

## 4. Discussion

The isolation results showed that the obtained isolates that had AQS activity were from shrimp intestine samples, namely 6 isolates and followed by sediment samples as many as 4 isolates. Meanwhile, only one isolate of *Bacillus* spp. with AQS activity was isolated form pond water samples. The AQS activity was determine by the non-purple zone on an agar plate of *C. violaceum* cultures. The non-purple zone was formed due to the presence of AQS activity which could inhibit *C. violaceum* in producing violacein pigment. The use of this bacterium as an AQS activity test is relatively easy to observe because the contras of purple color violacein pigment. And the violacein formation in *C. violaceum* is controlled by the quorum sensing (QS) mechanism. The inhibition of violacein formation is an indication of a failure induction of genes expression that responsible for the pigment production due to AQS activity (Hoshino 2011). Therefore, *C. violaceum* bacterium is widely used as a test bacterium to screen bacteria that have AQS activity (Chu *et al.* 2010; Romero *et al.* 2011; Novita *et al.* 2015; Oh *et al.* 2017). Production violacein pigment production in *C. violaceum* is influenced by AHL concentration as an autoinducer to induce gene targets expression involved in production of violacein pigment. Romero *et al.* (2011) reported that the percentage of bacteria that had AQS activity obtained from sediment samples was more than that of pond water samples. While Defoirdt *et al.* (2011) and Vinoj *et al.* (2014) reported that *Bacillus* spp. isolated from the intestine samples of *P. vannamei* was able to AHL.

The supernatants from the eleven selected *Bacillus* isolates were suspected to synthesize enzymes that can degrade AHL so that the degraded AHL compounds cannot form complexes with regulatory proteins that can induce the expression of genes that responsible for the formation of violacein pigment. There were six *Bacillus* isolates could inhibit pigment production per index unit with the inhibition percentage more than 75%, namely LpgS01, LpgS02, LpgS03, LpgU01, LpgU02, and LpgA01 isolates. The enzyme will inactivate of the expression of the vioABCD operon (Stauff and Bassler 2011). AHL compounds regulate the expression of the genes related to the production of violacein pigments (Blosser and Gray 2000). The mutant of *C. violaceum* CV0blu in a gene that responsible for synthesizing AHL, cannot produce AHL so that it cannot produce the pigment violacein. However, with addition of AHL compounds extracted from *V. fisheri* and wild strains of *C. violaceum,* the mutant strain of *C. violaceum* CV0blu was able to re-synthesize the violacein pigment (Blosser and Gray 2000). The decrease in violacein levels together with the decrease in the absorbance value at 600 nm indicated that the isolates also produced antimicrobial compounds, in addition to producing AHL-degrading enzymes. Antimicrobial compounds such as antibiotics can inhibit the growth of bacteria (Nagorska *et al.* 2007). In contrast to the activity of antimicrobial compounds, the AQS mechanism does not inhibit the growth or kill bacterial cells, but only inhibits the synthesis of the violacein pigment.

Measurement of violacein pigment production by *C. violaceum* can be calculated based on the violacein units formed during the bacterial growth phase (Chaudhari *et al.* 2018). The violacein pigment is synthesized from tryptophan substrate by enzymes resulting from the expression of the vioABCD operon (Stauff and Bassler 2011). Violacein pigment production is positively regulated by AHL (Devescovi *et al.* 2011; Stauff and Bassler 2011). And differences in pigmentation violacein levels of *C. violaceum* produced in culture can be quantified photometrically (Chaudhari *et al.* 2018). AQS activity of


Figure 2. The phylogenetics tree of the 16S rDNA genes of the selected bacterial isolates isolated from shrimp ponds that showing anti-qourum sensing activity. The tree was construted using the maximum likelihood method with 1,000x bootstraps of the MEGA X software

the six cultures supernatant *Bacillus* spp. isolates was shown that the *Bacillus* spp. isolates AHL degrading enzymes, possibly AHL-lactonase enzyme. These six *Bacillus* spp. isolates have potential to be develop as a bacterial control to inhibit shrimp AHNPD diseases.

The BLAST-N analysis of 16S rDNA gene sequences indicated that all selected isolates were *Bacillus* spp. The six potential isolates that had high AQS were *Bacillus cereus* (LpgS03, LpgU01, LpgU02, and LpgA01 isolates), *Bacillus thuringiensis* (LpgS01 isolate), and *Bacillus velezensis* (LpgS02 isolate), respectively. Hagstrom *et al.* (2002) stated that isolates that had 16S rRNA sequence similarities more than 97% could represent the same species, while sequence similarities between 93-97% could represent the identity of bacteria at the genus level but different species. These isolates were confirmed to have the *aii*A genes. The *aii*A gene is one of the genes encoding AHL lactonase which was detected in the *Bacillus* spp. group (Chen *et al.* 2013). AHL lactonase is also encoded

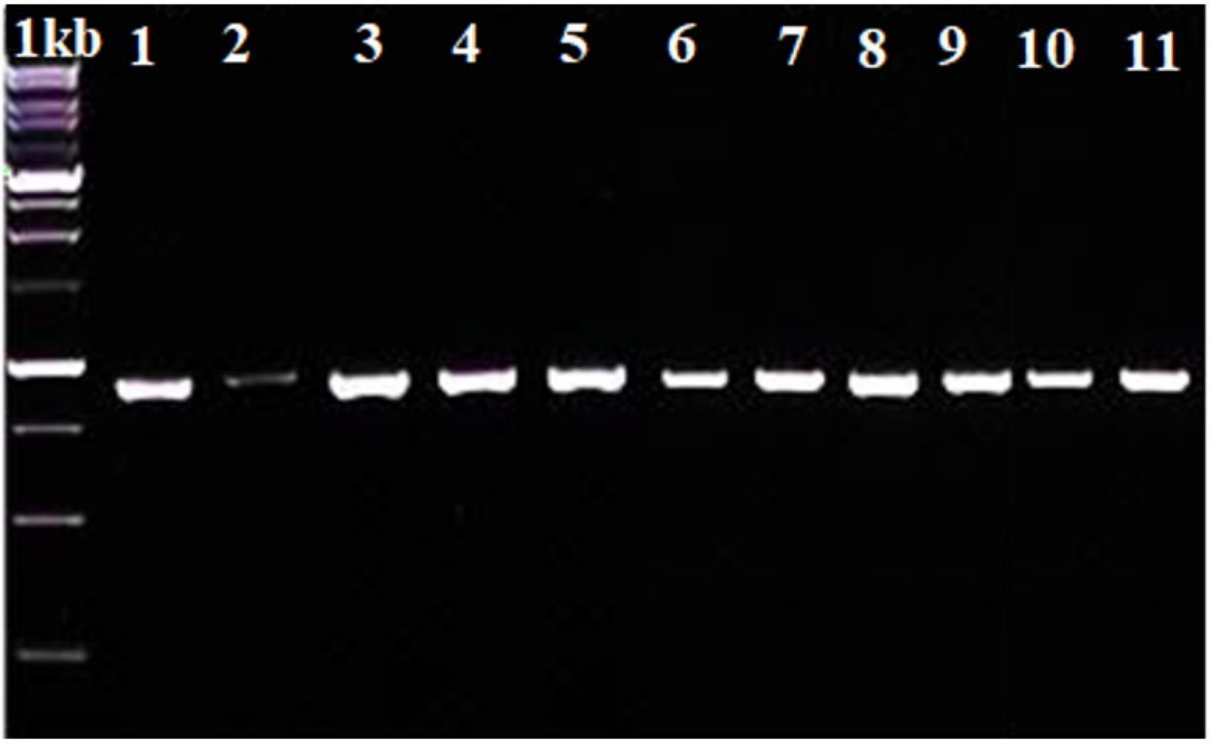


Figure 3. The amplicons 800 bp of *aii*A genes of the eleven selected isolates using specific primers of *aii*AF and *aii*AR visualised on 0.8% agarose. Simbols and number indicate as follow; M = Marker 1 kb, 1 = LpgS01 isolate, 2 = LpgS02 isolate, 3 = LpgS03 isolate, 4 = LpgU01 isolate, 5 = LpgU02 isolate, 6 = LpgA01 isolate, 7 = TggU01 isolate, 8 = TggU02 isolate, 9 = KwgS02 isolate, 10 = KwgS01 isolate, and 11 = KwgU01 isolate, respectively

Table 6. The Basic Local Alignment Search Tool-Nucleotide translation protein (BLAST-X) of the *aii*A gene amplicon sequences of the eleven selected isolates isolated from shimp ponds

| Isolate code | Bacterial species similarity | Maximun Score (bits) | Amino acid ratio of similar/ alignment | Percent identity (%) | E-value | Request Search ID (RID) |
|---|---|---|---|---|---|---|
| LpgS01 | AHL lactonase [Bacillus *thuringiensis* serovar aizawai] | 506 | 246/247 | 99.19 | 0 | MVH8EFST013 |
| LpgS02 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 507 | 246/247 | 99.6 | 0 | MVHYY4VS016 |
| LpgS03 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 100.0 | 0 | MVJDBYTJ013 |
| LpgU01 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 99.6 | 0 | MVJX904C016 |
| LpgU02 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 99.6 | 0 | MVK5W0P3013 |
| LpgA01 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 99.6 | 0 | MVKFXY1T013 |
| TggU01 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 99.6 | 0 | MVKNUZKA013 |
| TggU02 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 506 | 246/247 | 99.6 | 0 | MVM118FH016 |
| KwgU02 | N-acyl homoserine lactonase family protein [*Bacillus cereus*] | 494 | 240/247 | 97.17 | 6e-176 | MVM6TPH5016 |
| KwgS01 | N-acyl homoserine lactone lactonase [*Bacillus cereus*] | 490 | 238/240 | 99.17 | 9e-175 | MVMNFNXY013 |
| KwgU01 | N-acyl homoserine lactonase family protein [*Bacillus*] | 451 | 219/220 | 99.55 | 1e-159 | MVNJ1X2F016 |

by the attM genes (*Agrobacterium tumefaciens*), *ahl*D genes (*Arthrobacter* sp.), *qsd*A genes (*Rhodococcus erythropolis*), *Ahl*K genes (*Klebsiella pneumoniae*) and *aii*M genes (*Microbacterium testaceum*) (Chen *et al.* 2013).

The amino acid sequences of the AHL lactonase the isolates usually have a conserved motif, namely 107HxHxDH112. This motif is a characteristic of the metal-β-lactamase superfamily enzymes (Dong *et al.* 2002; Chen *et al.* 2013; Rusmana *et al.* 2017). The enzyme is composed of $Zn^{2+}$ ions bound to histidine and aspartate ligands (Rusmana *et al.* 2017). This motif was found in AiiA, AttM, AhlD proteins but not in AiiM (Wang *et al.* 2010). The AiiA proteins belong to the metallo-hydrolase-like metallod-β-lactamase superfamily. Metallo hydrolase is a hydrolytic enzyme that performs various biological functions, one of which is AHL lactonase which acts as a catalyst for hydrolysis of ester bonds at the opening of the AHL lactone ring so that the structure changes to acyl homoserine (Chen *et al.* 2013).

In summary, this study successfully characterized six isolates of *Bacillus* spp. isolated from shrimp ponds that had high anti-quorum sensing activity to inhibit the pigment production in *C. violaceum.* The selected isolates had inhibition AQS activity up to 75%. The isolates were identified as *Bacillus cereus* (four isolates), *Bacillus thuringiensis* (one isolate), and *Bacillus velezensis* (one isolate), respectively. The molecular analysis had confirmed that the isolates have *aii*A genes encoding AHL-lactonase enzyme. These *Bacillus* isolates have potential application for controlling AHNPD disease by anti-quorum sensing mechanisms.

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