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Characterization of acyl-homoserine lactonase gene from *Brevibacillus brevis* strain B37

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Acyl-homoserine lactonase (EC 3.1.1.25) is a metallo-betalactamase, specifically hydrolyzed N-acyl-homoserine lactones (AHL) secreted by Gram-negative bacteria. AHL lactonase has been reported as a potential substitute for synthetic anti-bacterial, such as reduce the severity of plant diseases caused by *Xanthomonas campestris* pv. *campestris*, and *Pectobacterium catrotovorum*. The exploration of lactonase producing organisms has been widely reported. AHL-lactonase is produced by Bacillaceae bacteria such as *Bacillus thuringiensis*, *B. cereus*, and *B. antrachis*. AHL-lactonase produced by Bacillaceae bacteria was translated from *aiiA* gene. In our previous study, *aiiA* novel gene was detected in *Brevibacillus brevis* B37 but has not been characterized. This study aimed to clone *aiiA* gene isolated from *B. brevis* B37 by polymerase chain reaction (PCR) with a pair of degenerated primers, to reveal homology comparison with others *aiiA* genes and amino acids, to express *aiiA* gene in *Escherichia coli* BL21 (DE3), and also to assay quorum quencher ability. The *aiiA* gene was successfully isolated with 753 bp and 250 amino acids. The *aiiA* gene and the AiiA protein from *B. brevis* B37 had high similarity with *aii*A and AiiA from *B. thuringiensis* group. The deduced amino acid sequence contained conserved sequence region ^{103}SHLHFDH^{109} and ^{166}TPGHTPGH^{173} as characteristic of the metallo betalactamase family. Additionally, the *aii*A_{B37} gene was expressed in *E. coli* BL21 (DE3) and the expressed AiiA protein could attenuate the expression of violacein produce by *Chromobacterium violaceum* and decrease the expression of soft rot symptom caused by *Dickeya dadantii*.

Keywords: Acyl-homoserine lactone, *aiiA* gene, AiiA protein, quorum quenching, quorum sensing

Introduction

Quorum sensing (QS) is a communication mechanism between Gram-negative bacteria based on the population level. QS induced by the signal molecule N-acyl homoserine lactone (AHL) as an auto-inducer. AHL with high concentrations can induce or suppress the expression of certain genes[1]. AHL is used as a signal molecule to detect their population density. The mechanism of QS is bacteria secreting AHL as a signal molecule in the extracellular environment. When the population density reaches a threshold, the AHL binds the receptor protein and activates the expression of downstream-related genes[2].

QS system in many Gram-negative bacteria is involved in important biological functions, such as luminescence, antibiotic production, plasmid transfer, motility, biofilm formation, surface-attachment, antimicrobial agent resistance, virulence, and regulation of pathogenesis-related gene expression[3-4]. AHL has been known to play a role in the activation of plant pathogenic bacteria virulence factors i.e. *Pantoea stewartia* and the *Erwinia* group[5-8].

AHL can be degraded by AHL-lactonase and AHL-acylase[9]. AHL-lactonase is a metallo-betalactamase (metalloenzyme) that inactivates AHL. Metalloenzymes are enzymes that have iron cofactors. The mechanism of AHL degradation by the AHL-lactonase enzyme is by breaking the ester ring in the lactone ring bond of AHL[10-12]. AHL-lactonase can be used as an alternative to control pathogenic bacteria that is safer than the use of antibiotics because antibiotics can lead to the emergence of a pathogen generation that is more resistant to antibiotics[13]. Several studies have reported the effectiveness of AHL-lactonase in suppressing the virulence of pathogenic bacteria, including the ability to suppress virulence of *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*[14-17], *Pectobacteriumaeru carotovorum* causing soft rot

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disease[18], *Xanthomonas campestris* pv. *campestris* causing black rot disease[19].

In our previous study, AHL-lactonase has been detected in *Brevibacillus brevis* B37[20]. But, It's still uncharacterized. This study aimed to clone *aii*A gene isolated from *B. brevis* B37 by PCR with a pair of degenerated primers, to reveal homology comparison with others *aii*A genes and amino acids, to protein construction using Swiss-Model (https://swissmodel.expasy.org), to express *aii*A gene in *Escherichia coli* BL21 (DE3), and also to assay quorum quencher ability.

Materials and Methods

Bacterial Strain and Chemicals

Brevibacillus brevis B37 obtained from Laboratory of Plant Bacteriology, Institut Pertanian Bogor, Indonesia was used as a genome source. *E. coli* DH5α was used as a host cell in the cloning process, and *E. coli* BL21 (DE3) was used as a host cell in gene expression. Two kinds of plasmid were used in this study, namely pTZ57R/T plasmid (InsTAclone Thermo Scientific) and pET-28a plasmid (Novagen). pTZ57R/T plasmid was used as a vector cloning and pET-28a was used as vector expression. The genome of *B. brevis* B37 was extracted using gene jet DNA extraction kit (Thermo Scientific). Restriction endonuclease (*Bam*HI and *Sal*I), T4 DNA ligase, DNA ladder 1 Kb, agarose, and isopropyl ß-D-1-thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS), acrylamide, and TEMED were purchased from Thermo Scientific. All the chemicals used in this research were analytical grade.

Multiplication of AHL-lactonase Gene

The genome of *B. brevis* B37 was extracted using gene jet DNA extraction kit (Thermo Scientific). The AHL-lactonase gene was isolated using a pair of degenerative primers *aii*A-F1 5'-CGC GGA TCC ATG ACA GTA AAR AAR CTT TAT TTC-3' and *aii*A-R1, 5'-AGC GGT CGA CTC ACT ATA TAT AYT CMG GGA ACT C-3' (The underlined nucleotides are the *Bam*HI and *Sal*I enzyme restriction sites). PCR reaction consisted of 12.5 µL PCR ready mix DreamTaq 2X (Thermo Scientific), 1 µL 20 pmol of each primer, 1 µL template DNA, and water-free nuclease up to total volume 25 µL. The PCR was conducted as follows: pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. Both *aii*A and pET-28a were restricted by *Bam*HI and *Sal*I endonuclease and were purified. The *aii*A_{B37} gene was ligated into pTZ57R/T plasmid using T4 DNA ligase. After ligation, pTZ57R/T::*aii*A_{B37} was transformed into *E. coli* DH5α for multiplication.

Sub-cloning AHL-lactonase Gene into *E. coli* BL21 (DE3) using pET-28a Plasmid

The preparation of the *aii*A gene fragment was initiated by isolation of the plasmid pTZ57R/T:: *aii*A_{B37}. The *aii*A_{B37} gene fragment was taken by cutting the pTZ57R/T:: *aii*A_{B37} obtained by double digestion method using *Bam*HI and *Sal*I enzymes according to the protocol digestion (Thermo Scientific). The restriction fragment was electrophoresed and was purified with high yield gel/PCR fragment extraction kit. The results of gene purification are ready to be used for ligation. The ligation composition included 1 µL of plasmid pET-28a, 1 µL of *aii*A_{B37} gene fragment, 1 µL of 10X ligation buffer, 6.67 µL of nuclease-free water, and 0.34 µL of T4 DNA ligase. Ligation was carried out at 4ºC overnight. The result of ligation between the pET-28a plasmid and the *aii*A gene fragment is called pET-28a::*aii*A_{B37}. The pET-28a was transformed into *E. coli* BL21 (DE3) competent cell and incubated at 37ºC overnight. The success of sub-cloning was determined by selecting it on media containing the antibiotic kanamycin and also was detected of *aii*A gene by PCR.

Expression of Recombinant Protein

E. coli BL21 (DE3) which had been transformed with pET-28a::*aii*A_{B37} was grown on LB agar containing 25 µg mL^{-1} of kanamycin and incubated at 37°C overnight. A single colony was taken and inoculated on 3 ml of Luria Bertani (LB) containing kanamycin 25 µg mL^{-1}. It was incubated in a water bath incubator at a speed of 100 rpm at 37°C for one night. Furthermore, 150 µL of the culture was inoculated at 15 ml of LB containing 25 µg mL^{-1} of kanamycin and incubated at 37°C at 150 rpm. Every 30 minutes, the OD_{600} is measured to reach 0.5. The culture was divided into two parts, the culture without being induced and the culture that was induced by IPTG. The culture was supplemented with IPTG with a final concentration of 0.75 mM. The incubation was continued for 12 hours at 37°C at 100 rpm. Cells were harvested by taking 1.5 mL into a microtube and centrifuged at 6000 rpm for 5 minutes at 4°C. The cell pellets were stored in a freezer at -70°C until the protein was analyzed.

Protein Expression Analysis Using SDS-PAGE

SDS-PAGE Preparation

There are two types of the gel used, namely: stacking gel and separating gel[21]. The materials used were solution A (14.6 g acrylamide, 0.4 g bis-acrylamide, ddH_2O up to 50 ml), buffer B (1.5 M Tris-HCl pH 8.8, 0.4% SDS), buffer C (0.5 M Tris-HCl pH 6.8, 0.4% SDS), solution D (10% ammonium persulfate), and TEMED. All materials are mixed and printed on the SDS-PAGE print. The composition of SDS-PAGE gel-making materials occurs in Table 1.

SDS-PAGE running

The bacterial protein stored with 300 µL buffer loading sample (1 M Tris, 2 g SDS, 17.4% glycerol, 10 mg bromophenol blue, ddH_2O up to 45 mL, 5 mL 2-mercaptoethanol). The sample was vortexed for 15 minutes. The samples were heated at 95^oC for 10 minutes. A total of 50 µL of samples were taken and put on an SDS-PAGE gel. The SDS-PAGE electrophoresis was carried out at 150 volts for 4 hours in a running buffer (15 g Tris-base, 72 g glycine, 5 g SDS, ddH_2O to 1 L).

Gel Staining

The gel was put in a staining container then add 100 mL of distilled water. The mix of gel was heated with high-level heat for 1 min and agitated for 4 min at room temperature. This step was repeated three times by replacing the distilled water. Furthermore, 20 mL of PAGE-blue staining (Fermentas) was added to the agar and was incubated for 30 sec at a high temperature. The agitated gel was allowed to stain for 60 min. Furthermore, rinsing was carried out by adding 100 mL of water to agar and incubating for 5 min. The rinsing process was repeated 3 to 5 times until the protein bands were visible.

Qualitative Assay of AHL-lactonase (Quorum Quenching Activity)

E. coli BL21 (DE3) recombinant was tested against *C. violaceum* to determine the expression of *aii*A gene. Tests were carried out with recombinant supernatant and *E. coli* BL21 (DE3) pellets. The supernatant was prepared to utilize recombinant *E. coli* BL21 centrifuged at a speed of 10000 rpm for 5 minutes then the supernatant was filtered using 0.2 µm Millipore (Minisart, Sartorius Stediem Biotech, Germany). The method used in anti-QS testing is the disc diffusion assay method with a double layer culture plate technique. The method is the same as the method for anti-QS testing in the screening section for AHL-lactonase-producing bacteria following Song *et al* [22] method.

Table 1 — Composition of SDS-PAGE agar.

Materials	Separating gel 12.5%	Stacking gel
ddH_2O	12 mL	7.2 mL
Solution A	15 mL	1.8 mL
Buffer B	9 mL	-
Buffer C	-	3 mL
Solution D	170 µL	45 µL
TEMED	20 µL	20 µL

Bioinformatics Analysis

The results of nucleotide tracing were compared with sequences available in the NCBI GenBank database data with basic local alignment search tools (BLAST) software[16]. The analysis of the amino acid conserved areas of the *aii*A gene obtained was carried out using MEGA X software. The signal peptide prediction was analyzed by signalP-5.0 software (http://www.cbs.dtu.dk/services/SignalP/). The construction of the three-dimensional protein structure of the AHL-lactonase enzyme was carried out using Swiss-Model software (http:// swissmodel.expasy.org/). Estimates of the size of the protein molecular weights, pI values of the amino acid, and simulation of the expression of AHL-lactonase from the *aii*A gene sequence were simulated using the ExPaSy software (http://web.expasy. org/compute_pi/)[23].

Results and Discussion

Sequence Analysis of The AHL-Lactonase Gene from *B. brevis* B37 (*aii*A_{B37})

Since the infection mechanism of *Dickeya dadantii* was observed that was mediated by AHLs, we speculated that AHL-lactonase might be one of the biocontrol mechanisms of *B. brevis* B37 on *D. dadantii*[20]. AHL-lactonase gene from *B. brevis* B37 was obtained by PCR using a pair of degenerative primer *aii*A-F1 and *aii*A-R1 was successfully amplified (Fig. 1). The nucleotide sequence analysis result consisted of 753 bases encoding 250 amino acids (Fig. 2). The simulation of the translation of the *aii*A gene inserted in pET-28a was analyzed to confirm that the gene reads as a codon. The simulation results show that the four genes *aii*A inserted in pET-28a can be read according to the original codon and there is no translation shift. The *aii*A gene is translated from the start codon that belongs to pET-28a and several bases including

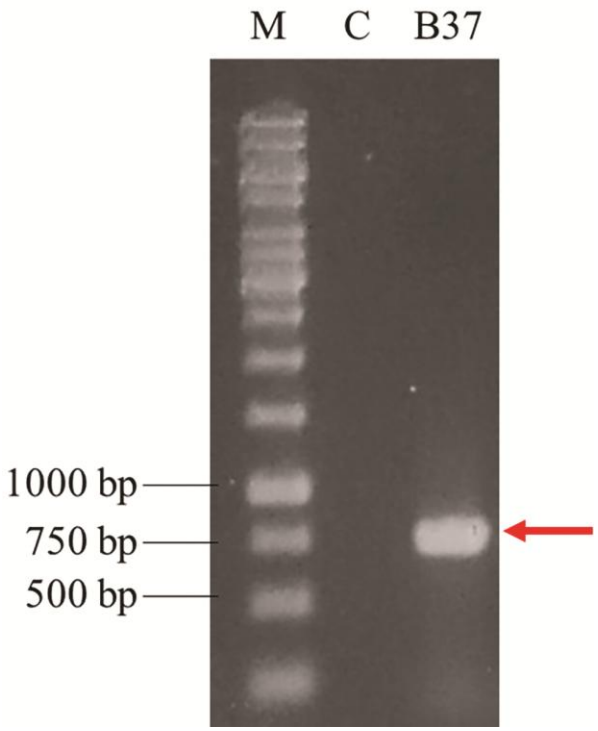

Fig. 1 — Isolation of the AHL-lactonase gene from *B. brevis* B37 using degenerative primer *aii*A-F1 and *aii*A-R1. M-Marker 1 Kb (Thermo Scientific), C-negative control (*E. coli* DH5α), B37-*B. brevis* B37.

fragments that encoding 6X His. The addition of some of the translated amino acids can affect the size of the protein inserted genes. One example is the simulation of the *aii*A B37 gene translation and there is no difference in expression for the *aii*A gene from other isolates (i.e. *Bacillus* sp. GG3, *Bacillus* sp. GG6, and *B. thuringiensis* BT2). The difference in nucleotides was marked in the nucleotides underlined in the *aii*A gene and did not affect protein expression (Fig. 2).

Protein Analysis of AHL-lactonase from *B. brevis* B37

The result of the protein analysis using MEGA X software showed that AiiA$_{B37}$ protein conserved domain which contains 3 major parts, namely GloB (Thr-78 through Asp-236), metallo-betalactamase super family (Leu-33-Thr-195), and metal-dependent hydrolases of the betalactamase super family II (Asn-22 through Val-161). This indicates that the *aii*A$_{B37}$ gene is characterized by the metallo-betalactamase group (Dong *et al*. 2000)[10]. Besides, *aii*A$_{B37}$ genes have a conserved section, namely the amino acids ^{103}SHLHFDH^{109} and ^{166}TPGHSPGH^{173} (Fig. 3). This is like the character of *aii*A genes from other Bacillaceae[24].

Signal analysis using Signal P-5.0 software showed that the *aii*A$_{B37}$ gene did not find any peptide signal regions. This is the same as the report from Pan *et al*. (2008) who performed a peptide signal analysis of the *aii*A gene sequence from *B. subtilis* BS1. But, the lactonase from other species such as lactonase from *Acinetobacter lactucae* had peptides signal cleavage site between position 47 and 48: VNA-AQ. Probability: 0.8567 (Accession number WP_057105863). The peptides signal with a length of 5-30 amino acids present in the N-terminal of protein synthesis products to determine protein translocation[25-26]. This bioinformatic analysis is important to determine the nucleotide and amino acid characters of a gene, protein domain, and protein structure[27].

Based on the amino acid sequence that has been obtained from *aii*A$_{B37}$ gene sequence showed that the protein structure of AHL-lactonase can be predicted. The construction results show that the protein construction results of the *aii*A$_{B37}$ gene have a protein structure that is very similar to the N-acyl homoserine lactone hydrolase protein (Accession number 2a7m.1.A) with an identity of 93.6%. The results of the AHL-lactonase protein construction had shown in Figure 4. The analysis results also show that the AHL-lactonase structure has a binding site to the Zn atom. The structure of the comparator AHL-lactonases, namely from *B. subtilis*, and *B. thuringiensis* also had a protein structure similar to that of AiiA from the analyzed isolates. This character is also owned by AHL-lactonase from *B. weihenstephanensis* (Sakr *et al*. 2013)[23]. Estimation of AiiA protein molecular weight from the *aii*A$_{B37}$ gene sequence has a pI value / molecular weight around 5.23 / 28096.30 Da.

The expression results showed that the *aii*A$_{B37}$ gene sequence was successfully expressed on *E. coli* BL21 (DE3) under the T7 promoter. The results of protein analysis using SDS-PAGE showed the size of the AHL-lactonase protein was around ~32 kDa. This site consists of a 3.8 kDa carrier protein present in plasmid pET-28a and protein AHL-lactonase ~28 kDa (Fig. 5).

Qualitative Analysis of AHL-Lactonase

AHL-lactonase activity is also called quorum quenching. Based on the assay of recombinant *E. coli* BL21 (DE3) that is inserted by *aii*A$_{B37}$ gene showed inhibition of expression violacein of *C. violaceum* (around 34%) and inhibit the virulence of *D. dadantii*

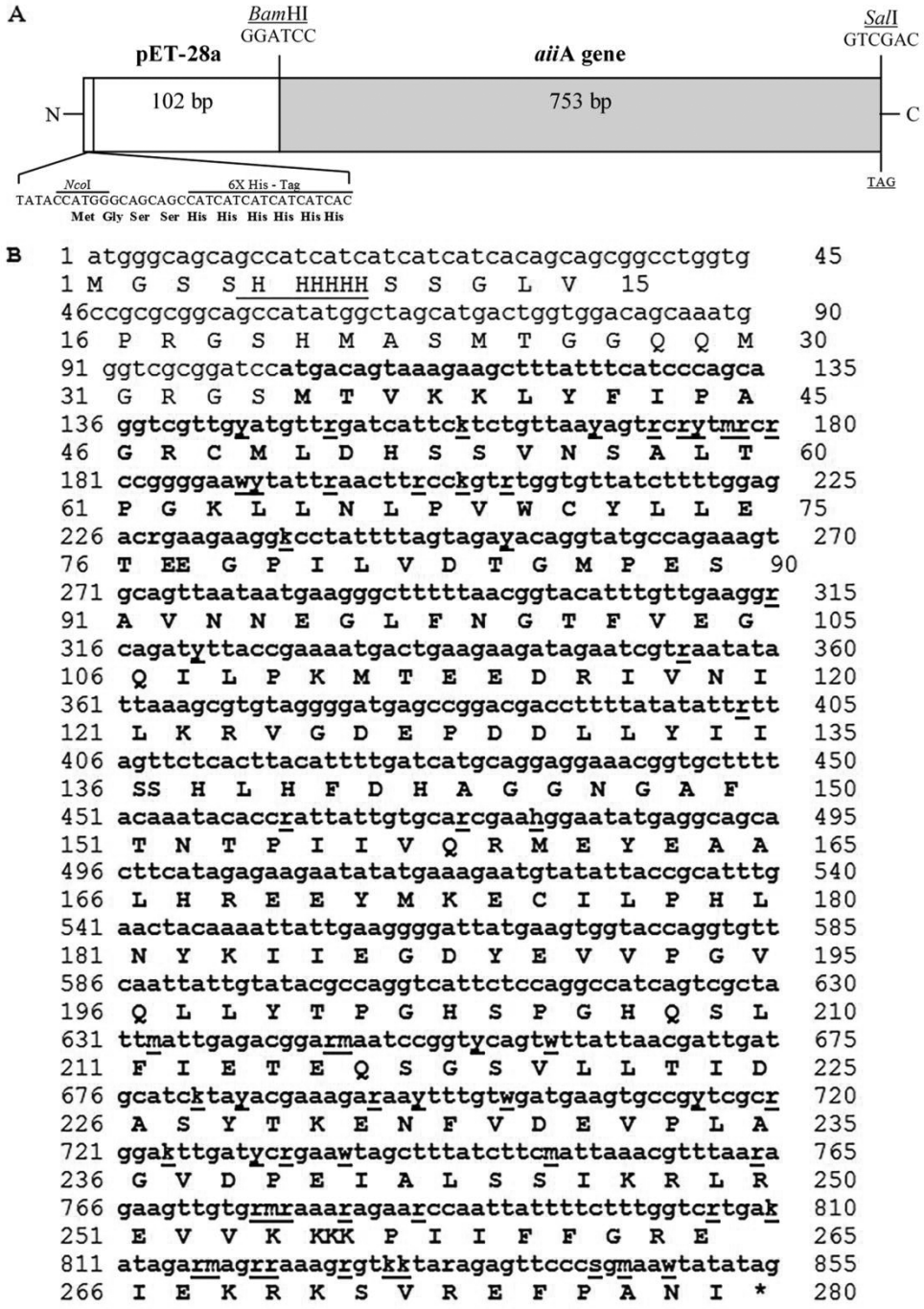

Fig. 2 — The fusion illustration of pET-28a protein with the *aii*A gene expressed from the start codon (ATG) in pET-28a to the stop codon in the *aii*A gene (TAG) (A) and the results of the analysis of the pET-28a :: aiiAB37 (B) sequence). 6X His-Tag (underline), nucleotides and amino acids (bold).

causing the soft rot symptoms on potatoes (around 57%) (Fig. 6). The effectiveness of AHL-lactonase in inhibiting virulence factors can inhibit the development of soft rot for up to 48 hours, but it is not known how long the AHL-lactonase enzyme remains active.

Discussion

Quorum sensing (QS) is communication between cells to improve or suppress gene expression as a response to fluctuations in cell population density. QS in bacteria produce and release chemical signaling molecules called autoinducer that increases with

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AiiA Bb_B37     101 ISSHLHFDHAGGNGA-60 aa-EVVPGVQLLYTPGHSPGHQS 175
AiiA Bs_BS1     101 ISSHLHFDHAGGNGA-60 aa-EVVPGVQLLYTPGHSPGHQS 175
AiiA Bt_B2      101 ISSHLHFDHAGGNGA-60 aa-EVVPGVQLLYTPGHSPGHQS 175
AiiA Bt_HD11    101 ISSHLHFDHAGGNGA-60 aa-EVVPGVQLLYTPGHSPGHQS 175
AiiA BT_BT2     101 ISSHLHFDHAGGNGA-60 aa-EVVPGVQLLYTPGHSPGHQS 175
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Fig. 3 — Alignment of the two conserved regions AiiA$_{B37}$ with those of other AiiA proteins. Sequences are compared to *B. subtilis* BS1 AiiA (Accession number DQ000640); *B. thuringiensis* B2 AiiA (Accession number AAL98718); *B. thuringiensis* HD11 AiiA (Accession number AAM92126); *B. thuringiensis* BT2 AiiA (Accession number LC055758.1).

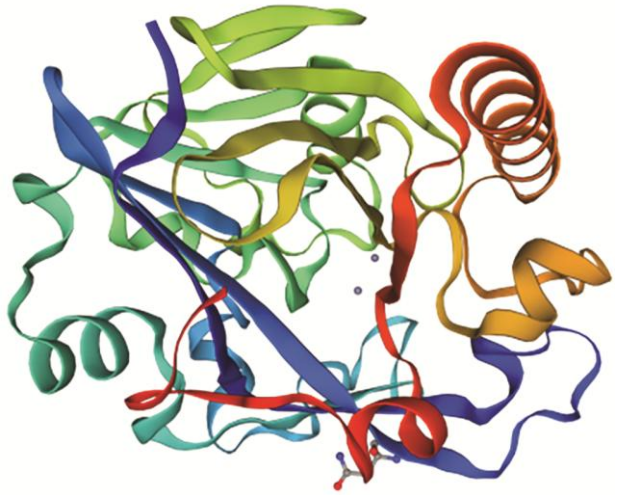

Fig. 4 — Tertiery protein structure prediction model of AHL-lactonase enzyme was constructed by Swiss-Model. The two arrows show the suggested metal ligands for dinuclear zinc binding.

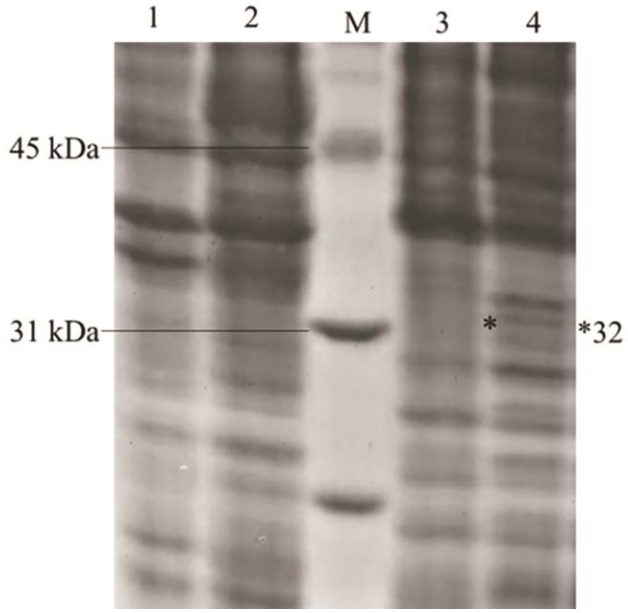

Fig. 5 — SDS-PAGE analysis AiiA produced in *E. coli* BL21(DE3). M-Marker, 1-*E. coli* BL21, 2-*E. coli* BL21+pET28a, 3-uninduced *E. coli* BL21+pET-28a::*aii*A$_{B37}$, 4-*E. coli* BL21+pET-28a::*aii*A$_{B37}$ induced by 0.75 µL IPTG.

increasing cell population density[28]. Several studies have reported that there is a threshold minimum limit of autoinducer concentration that can trigger the expression of certain genes and signal delivery mechanism, and target genes controlled by the QS system are different for each species[29]. The QS system using AHL as an autoinducer has been studied of many pathogenic bacteria, including those in the genus *Agrobacterium, Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia, Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahnella, Ralstonia, Rhodobacter, Rhizobium, Serratia, Vibrio, Xenorhabdus*, and *Yersinia*. Among the pathogenic bacteria, many studies of QS systems are LuxR-LuxI systems homology and its molecular signal similarity is N-acyl homoserine lactone (AHL) and was first studied in *Vibrio fischeri*[30], and other plant pathogenic bacteria i.e. *A. tumefaciens*[31], *Erwinia carotovora*[32], and *E. chrysanthemi*[33].

Based on the previous report, QS had an important role in the pathogenicity of pathogenic bacteria. Furthermore, a recent study is to explore the QS signal degradative enzyme, such as AHL-lactonase and AHL-acylase (Kalia, 2014)[9]. AHL-lactonase is produced by *B. cereus, B. thuringiensis, B. mycoides, B. fusiformis, B. sphaericus, B. weihenstephanensis, B. subtilis*, and other bacteria[34-37]. This study report that the gene coding for AHL-lactonase from *B. brevis* B37 is *aii*A gene. Similarity, sequence analysis showed that *aii*A$_{B37}$ gene is similar to *aii*A gene from *B. thuringiensis.* Lee *et al*.[38] reported that *B. thuringiensis* subsp. *morrisoni* had strong AHL-degrading activity.

E. coli BL21 (DE3) recombinant with *aii*AB37 gene had the potential to inhibit the violacein production of *C. violaceum* and inhibit the virulence of *D. dadantii* was higher than its expression in the wild type. Not only in terms of virulence, but QS in *Erwinia* also controls some other virulence determinants and secondary metabolites[39]. The potential for degradation of the QS signal in

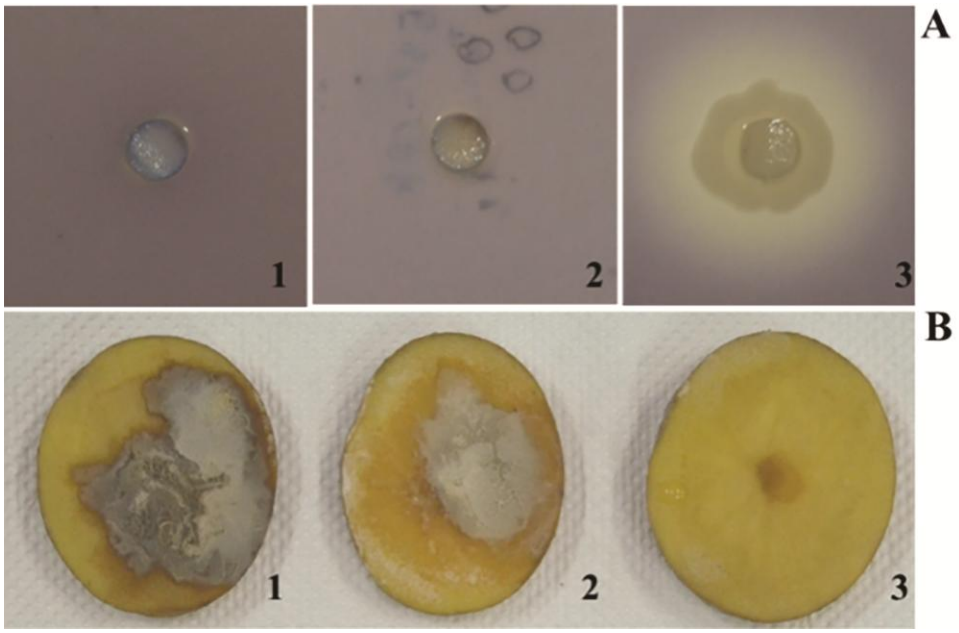

Fig. 6 — Quorum quenching ability of *E. coli* BL21(DE3) recombinant *aii*A genes from *B. brevis* B37. A-assayed on *C. violaceum;* B-assayed on *D. dadantii*, 1-control, 2-uninduced with IPTG, 3-induced IPTG.

bacteriology is important to study for several reasons. AHL is known to be stable in acidic conditions but unstable in alkaline conditions[40].

In this research, the assay of quorum quenching (QQ) ability was evaluated under laboratory conditions. The QQ ability showed by decreasing soft rot disease on *Phalaenopsis* orchid. Other research showed that *B. athina* HN-8 significantly reduces the severity of black rot disease caused by *X. campestris* pv. *campestris*[41]. The biocontrol efficiency of QQ bacteria may not be exhibited ideally in-plant test[42-43]. AHL lactonase was produced by *B. brevis* B37 as an option to control pathogenic bacteria.

In the future, AHL-lactonase had multiple benefits to be developed such as an effective biocontrol agent, and considered for commercial development for management strategy[44]. The efficiency under field conditions was previously reported. In China, *Lactobacillus plantarum* strain BY is used for biocontrol of softrot disease caused by *Pectobacterium carotovorum*[45]. AHL-lactonase can control both plant pathogenic bacteria and others. It is a useful enzyme and related gene for the biocontrol of infectious diseases caused by bacterial pathogens[46].

Conclusions

In this study, the AHL-lactonase from *B. brevis* B37 was successfully determined. Both gene and amino acid of AHL-lactonase from *B. brevis* B37 had similarity with AHL-lactonase from *aii*A gene of *B. thuringiensis.* The gene *aii*A_{B37} successfully cloned and expressed in *E. coli.* The gene *aii*A B37 had 753 base pairs, 325 amino acids, not find any peptide signal regions, and had conserve regions[103] SHLHFDH[109] and [166]TPGHSPGH[173]. AHL-lactonase protein from *B. brevis* B37 had 28 kDa. The expressed protein potentially inhibits the quorum sensing of *C. violaceum* and *D. dadantii.* In the future, this gene potentially used as a quorum sensing inhibitor to control pathogenic bacteria.

Acknowledgments

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