Quorum quenching of Bacillus cereus INT1c against Pseudomonas syringae

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Abstract. Quorum Quenching is a process to prevent AHL signal accumulation resulting in the virulence factors and pathogenicity genes. The aims of this study were to determine the ability of the AHL (Acyl Hocerine Lactone)-lactonase produced by Bacillus cereus INT1c and its potential as a biocontrol agent against Pseudomonas syringae. The experiments were swarming motility inhibition of P. syringae and inhibition assay of P. syringae on snap beans, INT1c produced extracellular and intracellular AHL-lactonase which could inactivate quorum sensing process of C. violaceum. The crude enzymes of INT1c were precipitated optimally at 70% saturation of ammonium sulphate. The Bacillus could reduce rot symptom of snap beans caused by P. syringae and motility of the pathogen. Swarming motility distance of the pathogen on control plates were 12.25 mm while the pathogen was co-cultured with INT1c was 3.3 mm. The average length of rot symptoms on positive control was longer (6.4 cm) than the snap beans inoculated with INT1c (3.92 cm). The population of P. syringae on the snap beans was decreased. Due to nutrient competition between P. syringae and INT1c. These results indicated that INT1c was potentially developed as a biocontrol agent.

1. Introduction
Quorum Quenching (QQ) is an enzymatic process to cut off communication of bacteria, including their ability to cause disease. One of the principles to control bacterial pathogens with quorum sensing (QS) as the basis is to prevent the accumulation of AHL signal thus the genes involved in the virulence process could not be expressed. AHL signal accumulation can be prevented by degrading the signal molecule. The compounds or molecules that can prevent the QS process are called as anti-QS [1].

P. syringae is a phytopathogen which causes brown spot and soft rot disease on snap beans. Generally, prevention method of these diseases is still based on chemical (bactericidal) and antibiotic usage that will kill the phytopathogen bacteria. However, it turns out that the chemical and antibiotic usage have a negative impact, such as the pathogenic bacteria resistance widespread and causing a variety of environmental problems that adversely affect the health of animals and humans. The discovery about bacteria virulence factors regulated by quorum sensing system provides an alternative strategy to overcome the bacteria pathogenicity by inhibiting the quorum sensing system. P. syringae
virulence factors in the form of EPS (Exopolysaccharide), pectinase and motility are also regulated by quorum sensing mechanism with 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) as signal molecules.

AHL-Lactonase is an anti-QS enzyme which belongs to degrade enzyme. AHL-Lactonase inhibits the QS process by hydrolyzing the lactone ring in AHL molecule thus there is no expression of pathogenicity encoding genes. Aiiia gene is one of AHL-Lactonase encoding genes and was first discovered in Bacillus sp. [2]. In the previous study Bacillus cereus INT1c was isolated from agricultural land in East Nusa Tenggara and has been detected to have quorum quenching activity and AiiA AHL Lactonase gene [3].

Bacillus cereus INT1c was known to have anti-quorum sensing activity against Chromobacterium violaceum which is a biosensor bacterium producing violacein (purple pigment) encoded by Vio gene [4]. The activity of AHL-lactonase Bacillus in degrading C. violaceum AHL is indicated by QQ zone formed around the paper disc.

2. Methods
2.1. The growth curve of Bacillus cereus INT1c
The culture suspension was prepared by inoculating one loop bacterial culture into 50 mL LB medium and incubated in a shaker (130 rpm) until reaching OD=0.8. A total of 1% culture suspension was poured into 100 mL LB cultures and incubated in a shaker at room temperature. The cell turbidity measurement at 600 nm with two replications was done every 3 hours for 36 hours.

2.2. AHL-lactonase activity assay
Two loops of Bacillus cereus INT1c were inoculated into 50 mL Luria Broth (LB) medium, then incubated for 24 hours in a shaker at room temperature. The cultures then centrifuged for 10 minutes at 10000 rpm. A total of 80 mL supernatant then was dropped onto the sterile paper disc and placed on the surface of the semi-solid Luria Agar plate which has been inoculated with C. violaceum (1%). The plates then incubated at room temperature for 24 hours [5]. The formation of QQ zone around the paper disc was observed which showed AHL degradation activity by AHL-Lactonase enzyme was observed.

2.3. Protein measurement
The protein content was determined by Micro Assay technique using a spectrophotometer. The measurement used Commasie Brilliant Blue G-250 as a dye and Bovine Serum Albumin (BSA) solution as a protein standard. The mixture absorbance then was measured using spectrophotometer at 595 nm [6].

2.4. Crude extract enzyme preparation
Bacillus cereus INT1c was grown overnight in 5 ml Luria Bertani (LB) broth medium at 28°C in a rotary incubator at 130 rpm. Furthermore, the cell biomass was collected by centrifugation at 10000 x g in 4°C for 5 minutes then washed with Phosphate Buffer Saline (PBS) pH 7.4. The mixture then was centrifuged again at 10000 g in 4°C. Thereafter, the pellet was resuspended with cold PBS and sonicated in an ice bath. The cell lysate was centrifuged at 12,500 g in 4°C for 15 minutes and the supernatant was as the crude enzyme [7, 8].

2.5. Precipitation of AHL-lactonase
The crude enzyme was precipitated with ammonium sulphate at saturation range 40-80%. Ammonium sulphate was added into supernatant while stirred for 1 hour in an ice bath. Crude extract enzyme was stored overnight at 4°C and then centrifuged at 10000 rpm in 4°C for 15 min. The whole protein precipitate then was dialyzed using a dialysis bag (Sigma D0405). The dialysis process was performed for 12 hours in 0.01 M phosphate buffer pH 7 with 100× sample volume.
2.6. *P. syringae* swarming bioassay
The swarming bioassay was done by inoculating *P. syringae* into LB medium and incubated for 24 hours. A total of 50 mL *P. syringae* culture then dropped onto sterile paper disc placed on the LA semisolid plate medium (0.6% agar) which contain Bacillus *cereus* INT1c. The plates then were incubated at room temperature. The swarming movement was observed and measured after 96 hours incubation [9].

2.7. Quorum sensing inhibition of *P. syringae* in snap beans (*P. vulgaris*) by Bacillus *cereus* INT1c
Snap beans were placed in a plastic mica with sterile distilled water-moistened cotton inside then sprayed with 3 mL *Bacillus cereus* INT1c (10^6-10^9 CFU/mL). After the surface of beans dried, each treatment unit then was incubated for 24 hours. Each bean then was pierced by needles 4 times and dripped with 10 mL *P. syringae* culture (10^6-10^9 CFU/mL). The experimental units used are beans, and three pieces of beans then taken as a sample. The positive control is *P. syringae* culture, while the negative control was *Bacillus cereus* INT1c. The inoculated beans then incubated in a plastic mica for 2-3 days. Disease symptoms were quantified by measuring the average length of symptoms after 72 hours incubation.

3. Result and discussion
3.1. The growth curve of Bacillus *cereus* INT1c
*Bacillus cereus* INT1c was able to grow well in LB medium at room temperature. The growth of *Bacillus cereus* INT1c had lag phase at 0-1 hour after incubation, the exponential phase occurred from 3-4 hours after incubation. The growth tends to be stable up to 24 hours of incubation (figure 1). *Bacillus cereus* INT1c was able to adapt well to grow in a new medium. The AHL-lactonase were produced in exponential phase.

![Figure 1. The growth curve of Bacillus cereus INT1c in LB medium.](image)

Microorganisms which have quorum quenching activity have been widely identified in both Gram negative and Gram positive bacteria [10]. In this study, a group of bacteria from *Bacillus* which have quorum quenching activity were isolated from agricultural land. AHL degrading bacteria isolated from soil rhizosphere and tobacco plants generally belong to *Bacillus* group [11]. *Bacillus cereus* INT1c produced extracellular AHL-lactonase, an enzyme which bound to the outer membrane of a cell. Tang et al. (2015) also reported that AHL-lactonase from *Olearia muricauda* is an extracellular enzyme [12].

3.2. AHL-lactonase activity assay
The AHL degradation activity of *Bacillus cereus* INT1c characterized by the presence of quorum quenching zone around the paper disc on *C. violaceum*, meanwhile the control showed no quorum quenching zone around the paper disc (figure 2).
Figure 2. Degradation AHL of *C. violaceum* by AHL-lactonase of *Bacillus cereus* INT1c (A) LB medium as control (B) extracellular AHL-lactonase.

The AHL degradation activity is characterized by the presence of quorum quenching zone around the paper disc on media inoculated with *C. violaceum*. *C. violaceum* is a Gram-negative bacterium producing purple pigment (violacein) through quorum sensing mechanism which uses AHL signals in the form of N-hexanoyl homoserine lactone (HHL) [13]. AHL-lactonase works by hydrolyzing the AHL lactone ring and has a varied substrate [14]. The results of AHL hydrolysis process are homoserine lactone and free fatty acids which can be metabolized and used by bacteria as sources of carbon, nitrogen, and energy [15].

3.3. Precipitation of AHL-lactonase

The result of precipitation *Bacillus cereus* INT1c crude extract using ammonium sulphate showed that ammonium sulphate was able to precipitate protein in all concentrations. The highest precipitate protein content was obtained using 70% of ammonium sulphate (salting out) (figure 3). All saturation of ammonium sulfate carried out to concentrate the extracellular *Bacillus. cereus INT1c* protein cause loss of AHL-lactonase activity (figure 4A).

The AHL-lactonase precipitate of *Bacillus* sp. NTT3a were further tested to determine whether the AHL is extracellular or intracellular enzyme. The precipitate showed that AHL-lactonase was an extracellular enzyme (figure 4B). It was proved the higher quorum quenching index of precipitated than that of supernatant.

![Figure 3.](image)

Figure 3. The effect of ammonium sulphate saturation against AHL-lactonase precipitate of *Bacillus cereus* INT1c (■ : pellet, □ : supernatant).
The AHL-lactonase enzyme of *Bacillus cereus* INT1c was precipitated using ammonium sulphate. The enzymes precipitation aimed to increase the activity by reducing the content of other compounds from crude extract enzyme. The enzymes from *Bacillus cereus* INT1c salted out at 70% saturation of ammonium sulphate. The salting out occurs as a result of competition between ions of ammonium salts and the enzyme molecules that interact with water molecules. A high level of ions, causes the protein molecules interact with each other to form a precipitate [16].

The absence of quorum quenching zone by an enzyme from 40%-80% saturation of ammonium sulphate (figure 4A) was caused by the proteins denaturation process. Protein denaturation is defined as a change or modification of secondary, tertiary and quaternary structures in protein molecules without breaking the covalent bonds. When the protein was denaturated, it will lose its biological activity [16]. Protein will lose its original conformation if pH, salt concentration, temperature, chemical materials or other aspects in the environment changed. In enzyme precipitation process, ammonium sulphate salt can break the hydrogen bonds by breaking the hydrophobic bonds and increasing the solubility of hydrophobic proteins in water. That process causes the protein loses its solubility to aggregate, thus the enzyme will lose its ability to degrade AHL.

3.4. *P. syringae* swarming bioassay

The result after 96 hours showed that *P. syringae* inoculated on medium had swarming distance around 3.05 centimeters while *P. syringae* and *Bacillus cereus* INT1c had swarming distance around 1.2 centimeters (figure 5). This difference due to inhibition process of *P. syringae* quorum sensing by *Bacillus cereus* INT1c (figure 6).
Figure 5. The swarming ability of *P. syringae* in LA semisolid medium

![Swarming ability of P. syringae in LA semisolid medium](image)

**Figure 6.** The morphology of the swarming ability of *P. syringae* in LA semisolid medium

(A) *P. syringae* (B) *P. syringae* and *Bacillus cereus* INT1c.

The result of in-vitro assay during 96 hours of incubation showed that in medium inoculated with *P. syringae* had a movement about 3.05 centimeters from the paper discs, meanwhile the *P. syringae* and *Bacillus* sp. NTT3a had a narrower movement about 1.2 centimeters (figure 5). The results occurred due to quorum sensing of *P. syringae* was inhibited by *Bacillus cereus* INT1c. Quinones et al. (2005) reported that the motility of *P. syringae* regulated by quorum sensing system [9]. *P. syringae* move by producing extracellular proteins such as mucus consisting of polysaccharides and biosurfactant which are virulence factor resulting in the symbiotic and pathogenic interaction between *P. syringae* and plants [17].

3.5. Quorum sensing inhibition of *P. syringae* in snap beans (*P. vulgaris*) by *Bacillus cereus* INT1c

The result after 72 hours of incubation showed that negative control (beans inoculated by *Bacillus cereus* INT1c) had no rot symptoms. The positive control had a visible rot symptom, and beans inoculated with *Bacillus cereus* INT1c showed lighter rot symptoms than the positive control (figure 8). It occured due to the inhibition process of *P. syringae* quorum sensing by *Bacillus cereus* INT1c. The average length of rot symptoms on positive control was 6.4 centimeters meanwhile on the beans inoculated by *Bacillus cereus* INT1c was 4.55 centimeters (figure 7). *Bacillus cereus* INT1c was able to inhibit the rotting process caused by *P. Syringae* in beans with percentages up to 68.01% and (table...
1. The *P. syringae* cells number in the QS inhibition assay was calculated using Total Plate Count (TPC) method. The result showed that *P. syringae* cells number in all treatments did not significantly different ($10^9$ CFU/mL). The result proved that this process is an anti QS process, not an antibiosis process (table 2).

**Figure 7.** The effect of *Bacillus cereus* INT1c against snap bean rotting process by *P. Syringae*.

**Figure 8.** Quorum quenching bioassay of *P. syringae* by *Bacillus cereus* INT1c, snap beans were inoculated by (B) *Bacillus cereus* INT1c and *P. syringae*, (C) *P. syringae* (E) *Bacillus cereus* INT1c (F) LB medium.

**Table 1.** The percentage of rotting inhibition in snap bean by *Bacillus cereus* INT1c.

| Treatments    | Snap bean length average (cm) | Lession length average (cm) | Inhibition percentage (%) |
|---------------|--------------------------------|-----------------------------|----------------------------|
| PSG           | 16.37                          | 6.60                        | **59.67**                  |
| PSG + INT1c   | 16.96                          | 5.43                        | **68.01**                  |
Table 2. *P. syringae* cells number based on Total Plate Count (TPC) method.

| Treatments      | Cells Number ± SD (CFU's/mL) |
|-----------------|------------------------------|
| PSG             | $6 \times 10^8 \pm 4.24 \times 10^7$ |
| PSG + INT1c     | $5.7 \times 10^8 \pm 4.24 \times 10^7$ |

The result of quorum sensing inhibition of *P. syringae* in snap beans (*Phaseolus vulgaris*) showed sligther rot symptoms on snap beans inoculated with *Bacillus cereus* INT1c than the other inoculated with *P. syringae*. *P. syringae* as phytopathogen bacteria able to utilize pectin by hydrolyzing the glycosidic bond in pectate polymer and causes soft rot disease in snap beans. Extracellular pectinase production of *P. syringae* is controlled through quorum sensing mechanism that involves compounds which act as signaling molecules. The AHL-lactonase from *Bacillus cereus* INT1c was able to inhibit pectinase production which is one of *P. syringae* virulence factors. Estimation of the bacterial population number was done by the total plate count (TPC) method to prove the quorum sensing inhibition process in *P. syringae*. The result showed that the inhibition is an anti-quorum sensing process not antibiotic process, because AHL-lactonase from *Bacillus cereus* INT1c prevents only the accumulation of AHL signal resulting the pathogenicity encoding genes expression failure without killing *P. syringae* cells. The rot symptoms inhibition percentage proved that *Bacillus cereus* INT1c was potential to be developed as a biocontrol agent based on the inhibition of pathogenic bacteria. This control mechanism is more environmentally friendly than antibiosis mechanism, because it does not cause the pressure on phytopathogen to develop more new resistant strains against antibiosis compounds.

4. Conclusion

*Bacillus cereus* INT1c produced extracellular and intracellular AHL-Lactonase. Quorum quenching activity of *Bacillus cereus* INT1c could inhibit *P. syringae* in LA medium along with decreasing rot symptoms in beans. The quorum quenching ability of *Bacillus cereus* INT1c can be developed as a biocontrol for plant diseases caused by *P. syringae*.

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