

CHAPTER 1.0

INTRODUCTION

Quorum sensing (QS) describes the events of bacterial cell-to-cell communication through diffusible signals that bind to the receptor protein, resulting in regulation of gene expression in response to bacterial cell density (Fuqua *et al.*, 2001). Cell density dependent gene regulations in many proteobacteria employ intercellular signalling molecules namely *N*-acyl homoserine lactones (AHL). Many bacteria employed QS to gain maximal competition advantages, hence various organisms confer quorum quenching (QQ) to counter measure the benefits of QS (Lin *et al.*, 2003). Specifically, inhibition of AHL signalling could involve degradation of the molecules by acylase, lactonase and oxidoreductase activities (Dong *et al.*, 2002; Lin *et al.*, 2003; Uroz *et al.*, 2005; Hong *et al.*, 2012). This study involved the isolation of soil bacteria from Cameron Highlands followed by characterization of both production and degradation of AHL molecules among these isolates. Interest was drawn in selecting soil from Cameron Highlands as isolation source was due to its landscapes at elevated altitudes that offers diverse bacterial communities haboring the soils. For decades, transition of the terrains in Cameron Highlands into agricultural lands followed by proliferant use of pesticides and fertilizers would have affected the composition of native microbial communities in the soils (Ngan *et al.*, 2005; Widenfalk *et al.*, 2008). As such, isolation of these bacteria enables us to look into the ubiquity of QS and QQ bacteria inhabiting montane soils that are contaminated with human activities.

The objectives of this research project include the following:

1. To isolate QS and QQ bacteria from soil sample collected from Cameron Highlands.
2. To identify the isolated bacteria strains using 16S rDNA gene sequencing analysis.
3. To study AHL production from these bacteria isolates.
4. To determine the isolated bacteria QQ activity against various AHL molecules.

CHAPTER 2.0

LITERATURE REVIEW

2.1 Quorum Sensing

The generic term ‘quorum sensing’ (QS) describes the bacterial cell-to-cell communication mechanisms through diffusible signals that act to induce gene expression in response to bacterial cell density (González and Marketon, 2003; Williams *et al.*, 2007). Bacterial QS involves release, detect and respond to small signal molecules, in a cell density-dependent manner, thereby regulating the expression a set of target genes (Dong and Zhang, 2005). These signals, known as autoinducers (AI), are synthesized at specific stages of growth or in response to changes in the environment and induce a concerted response once a critical concentration has been reached (González and Marketon, 2003).

When a bacterial population reaches a threshold cell density, the concentration of the QS signal is sufficient to induce gene expression either directly by interacting with a transcriptional regulator or indirectly by activation of a signalling cascade (Fuqua *et al.*, 2001). Thus, the concentration of QS signal reflects the number of bacterial cells and the sensing of a threshold level of signal indicates that the population is “quorate”; that is, ready to make a collective decision (Cooley *et al.*, 2008). With the presence of specific kinase or response regulator, coordinated behavioral change in bacteria is initiated followed by the expression of QS dependent target genes. Secondary metabolite production, bioluminescence, competence, plasmid transfer, biofilm development and virulence factor production are known to be regulated QS in Gram

positive and Gram negative bacteria (Chen *et al.*, 2002; Hentzer *et al.*, 2003). Bacterial QS signal molecules are chemically diverse, ranging from peptides used by Gram positive bacteria, 4-Alkyl-4-quinolones (AHQs) commonly found in *Pseudomonas*, to acylated homoserine lactones in many proteobacteria (Fletcher *et al.*, 2007).

2.1.1 *N*-Acyl Homoserine Lactones

Several types of bacterial cell-cell communication signals have been identified in the past two decades which *N*-acylhomoserine lactone (AHL) being the most commonly found in Gram negative bacteria (Chen *et al.*, 2002). AHL QS signals are highly conserved as they have the same homoserine lactone moiety unsubstituted in the β - and γ -positions with an amide (*N*)-linked acyl side chain at the α -position (Cooley *et al.*, 2008). The *N*-acylated side chain consisted of fatty acids that vary in term of chain length (ranging from 4-18 carbons), degree of saturation, and the presence of substituent which could be either hydroxy- or oxo- group at the C3 position (Figure 2.1) (Swift *et al.*, 1997). AHL molecules are synthesized by LuxI synthase from substrates *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (Acyl-ACP), an intermediate of fatty acid biosynthesis.

The use of AHLs as QS signal was first described in marine bioluminescence bacterium *Vibrio fischeri* whereby light production is regulated by QS signals identified as 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6 HSL) (Eberhard *et al.*, 1981; Schaefer *et al.*, 1996). Subsequently the presence of AHLs in diverse range of Gram negative bacteria has been reported together with their implications in regulating different cellular responses and phenotypes. It has become clear that many bacteria

possess the QS mechanism with LuxI/LuxR signal-response circuit as the backbone however with additional complexity. For instance, in phytopathogen *Agrobacterium tumefaciens* which is responsible for crown gall tumours in plants, uses the plant opine hormones to interact with the bacterial protein OccR or AccR to regulate the expression of the LuxR homologue TraR (Oger *et al.*, 1998; Zhu and Winans, 1998). Besides, opportunistic pathogen *Pseudomonas aeruginosa* employs two pairs of LuxI/LuxR like systems namely LasI/R and RhlI/R that function in tandem to regulate expression of virulence factors and biofilm formation (Glessner *et al.*, 1999).

A new group of homoserine lactone termed as *p*-coumaroyl homoserine lactone (pC-HSL) has been discovered in photosynthetic bacterium *Rhodospseudomonas palustris* that was found to produce the compounds using *p*-coumaric acid rather than fatty acids from cellular pools. Synthesis of pC-HSL molecules were catalyzed by RpaI, a LuxI homolog in which the expression of RpaI was found to be enhanced by presence of *p*-coumaric acid (Schaefer *et al.*, 2008). To address the uniqueness of pC-HSL production by *R. palustris*, other bacteria including nitrogen fixing symbiont *Bradyrhizobium* sp. BTAil and coastal bacterioplankton *Silicibacter pomeroyi* DSS-3 were also capable to utilize *p*-coumarate for the synthesis of pC-HSL (Kalia and Purohit, 2011).

Some Gram negative bacteria employ HSL biosynthetic enzymes that are unrelated to the conventional LuxI-enzymes. Instances include LuxM from *Vibrio harveyi* and AinS from *Vibrio fischeri* and HdtS from *Pseudomonas fluorescens* that showed no significant homology with LuxI family members (Bassler *et al.*, 1993; Hanzelka *et al.*, 1999; Laue *et al.*, 2000). Subsequently, perception of the signals were

achieved with cytoplasmic membrane-associated sensor kinase (Ryan and Dow, 2008).

Examples of bacteria utilizing the AHL QS are summarized in Table 2.1.

Table 2.1 Examples of AHL producing bacteria

| Bacteria | AHLs | QS Systems | Target Genes and Phenotypes | References |
|---|----------------|-------------------|---|---|
| <i>Aeromonas hydrophila</i> | C4-HSL, C6-HSL | AhyI/R | Serine protease, metalloprotease production and biofilm formation | Swift <i>et al.</i> , 1997; Lynch <i>et al.</i> , 2002 |
| <i>Aeromonas salmonicida</i> | C4-HSL | AsaI/R | Extracellular protease | Swift <i>et al.</i> , 1997 |
| <i>A. tumefaciens</i> | 3-oxo-C8-HSL | TraI/R | Conjugation | Piper <i>et al.</i> , 1999 |
| <i>Burkholderia cepacia</i> | C6-HSL, C8-HSL | CepI/R | Swarming motility, biofilm formation and siderosphere production | Lewenza <i>et al.</i> , 1999; Huber <i>et al.</i> , 2001; Tomlin <i>et al.</i> , 2004 |
| <i>B. cenocepacia</i> | C6-HSL, C8-HSL | CepI/R | Biofilm formation and siderosphere production | Sokol <i>et al.</i> , 2003 |
| <i>Chromobacterium violaceum</i> | C10-HSL | CviI/R | Biofilm formation, chitinase and violacein production | Stauff and Bassler, 2011 |
| <i>Erwinia carotovora</i> subsp <i>carotovora</i> | 3-oxo-C6-HSL | ExpI/R | Carbapenem antibiotic production, exoenzymes | Bainton <i>et al.</i> , 1992; Pirhonen <i>et al.</i> , 1993 |
| <i>Pantoea stewartii</i> | 3-oxo-C6-HSL | EsaI/R | Adhesion and host colonization | Koutsoudis <i>et al.</i> , 2006 |
| <i>P. aeruginosa</i> | 3-oxo-C12-HSL | LasI/R and RhlI/R | Exoenzymes, biofilm formation, cell-cell spacing | Chapon-Herve <i>et al.</i> , 1997, Passador <i>et al.</i> , 1993, Glessner <i>et al.</i> , 1999 |

| | | | | |
|------------------------------------|-------------------------------------|-------------------|--|---|
| <i>P. aureofaciens</i> | C6-HSL | PhzI/R | Phenazine antibiotic production | Pierson III <i>et al.</i> , 1995 |
| <i>P. chlororaphis</i> | C6-HSL | PhzI/R | Phenazine-1-carboximide production | Chin-A-Woeng <i>et al.</i> , 2001 |
| <i>P. fluorescens</i> | 3-hydroxy-C6-HSL, 3-hydroxy-C14-HSL | MpuI/R and HdtS | Phenazine antibiotic production | Shaw <i>et al.</i> , 1997; Laue <i>et al.</i> , 2000 |
| <i>P. putida</i> | 3-oxo-C10-HSL, 3-oxo-C12-HSL | PpuI/R | Maturation | Dubern <i>et al.</i> , 2006 |
| <i>Rhizobium leguminosarum</i> | 3-hydroxy-7-cis-C14-HSL | RhiI/R | Nodulation, bacteriocin, stationary phase survival | Gray, 1997; Rodelas <i>et al.</i> , 1999; Thorne and Williams, 1999 |
| <i>Rhodobacter sphaeroides</i> | 7-cis-C14-HSL | CerI/R | Community escape | Puskas <i>et al.</i> , 1997 |
| <i>R. palustris</i> | pC-HSL | RpaI/R | N.D. | Schaefer <i>et al.</i> , 2008 |
| <i>Serratia liquefaciens</i> | C4-HSL | SwrI/R | Biofilm formation | Labbate <i>et al.</i> , 2004 |
| <i>Vibrio anguillarum</i> | 3-hydroxy-C6-HSL | VanM | Terminal hemorrhagic septicemia in fish | Milton <i>et al.</i> , 2001 |
| <i>V. fischeri</i> | C8-HSL, 3-oxo-C6-HSL | AinS and LuxI/R | Bioluminescence | Eberhard <i>et al.</i> , 1981; Hanzelka <i>et al.</i> , 1999 |
| <i>V. harveyi</i> | 3-hydroxy-C4-HSL | LuxM | Bioluminescence | Bassler <i>et al.</i> , 1993 |
| <i>Yersinia pseudotuberculosis</i> | C6-HSL, 3-oxo-C6-HSL and C8-HSL | YpsI/R and YtbI/R | Motility, clumping | Atkinson <i>et al.</i> , 1999 |

Legend: N.D. : Not Determined

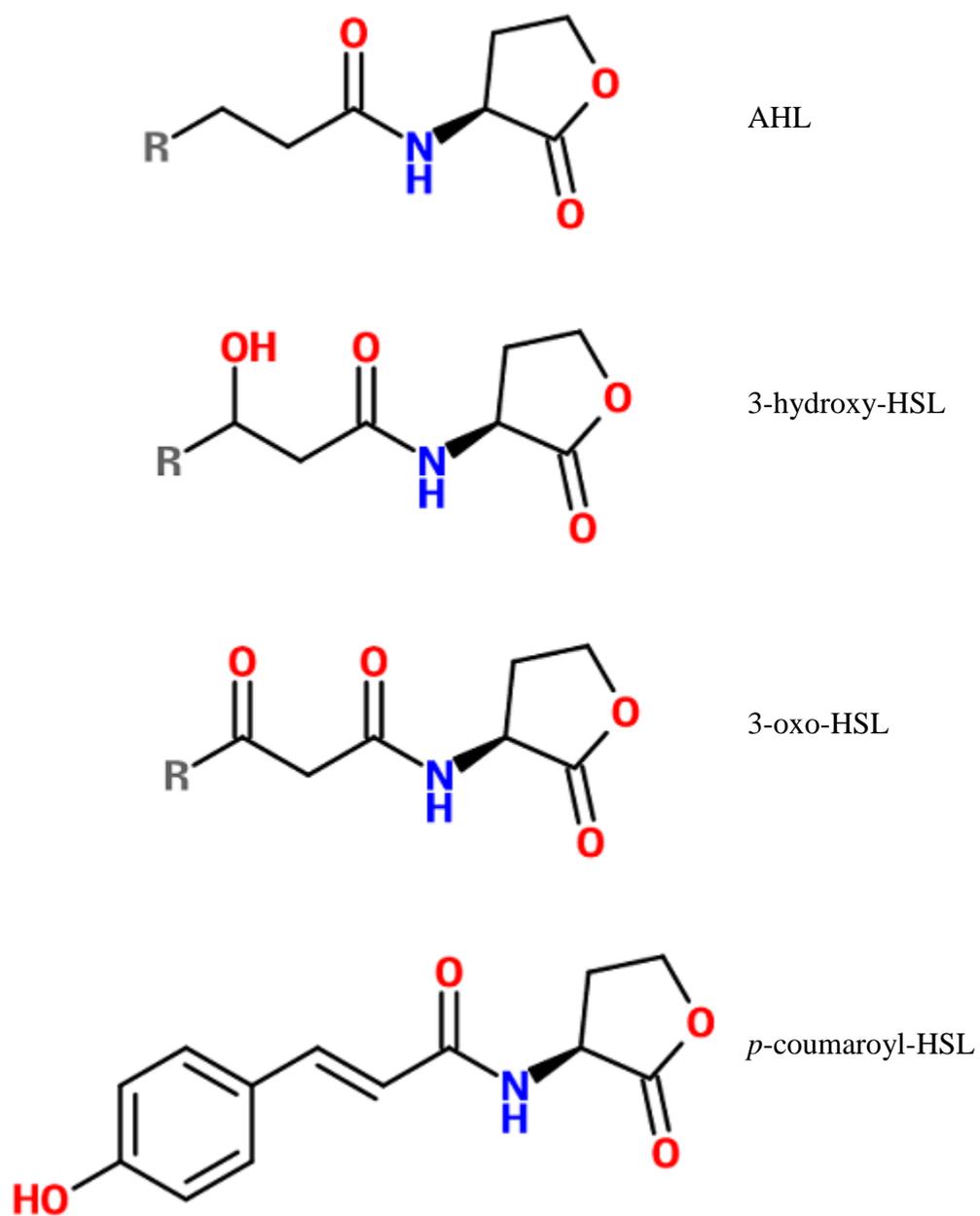


Figure 2.1 Several chemical structures of *N*-acyl homoserine lactone molecules and R corresponds to the carbon chain length ranging from C1 to C15.

2.1.2 Other Signalling Molecules

Aside from AHLs, the existence of other signalling molecules among Gram negative bacteria is apparent. Some of the well-studied intercellular signaling molecules are 4-quinolones, namely 2-heptyl-3-hydroxy-4-quinolone (termed as *Pseudomonas* quinolone signal, PQS) and 4-hydroxy-2-heptyl-quinoline (HHQ) synthesized by *Pseudomonas aeruginosa* (Pesci *et al.*, 1999). The signalling of PQS is incorporated in the AHL QS pathway that is governed by Las and Rhl systems and known to be upregulated in cystic fibrosis patients during lung infections (Pesci *et al.*, 1999; Collier *et al.*, 2002). Biosynthesis of PQS is initiated with anthranilate as precursor being converted into HHQ by *pqsABCD* gene products of *pqsABCDE* operon. HHQ as the immediate precursor for PQS was also believed to be a signalling molecule itself and the release from one cell would be taken up by another followed by conversion into PQS (Déziel *et al.*, 2004).

Plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) synthesizes virulence factor that is mediated by diffusible signalling factor (DSF) which was later characterized as unsaturated fatty acid namely *cis*-11-methyl-2-dodecenoic acid (Barber *et al.*, 1997; Wang *et al.*, 2004). The list of DSF-family signals has been expanding since its discovery in *Xcc* with a series of DSF structural analogues identified from *B. cenocepacia*, *Streptococcus mutans* and other Xanthomonads. A second signal molecule namely diffusible factor (DF) was also found in *Xcc* and was associated with regulation of yellow pigment (xanthomonadins) and extracellular polysaccharides (EPS) production (Potlawsky and Chun 1998). Mass spectrometry analysis later confirmed the structure of the DF as butyrolactones (Chun *et al.*, 1997). Next, *Bradyrhizobium*

japonicum, a symbiotic bacterium from rhizosphere which produces an iron mediated oxetane ring containing bradyoxetin namely 2-{4-[[4-(3-aminooxetan-2-yl)phenyl](imino)methyl]phenyl}oxetan-3-ylamine as signalling molecule that regulates genes for nodulation (Loh *et al.*, 2002). Another bacterium, phytopathogen *Ralstonia solanacearum* exerting a different signalling pathway utilizes 3-hydroxypalmitic acid methyl ester (3-OH PAME) for the regulation of virulence involved in causing wilting disease of crops (Flavier *et al.*, 1997).

Unlike the Gram negative signalling, Gram positive bacteria employ post-translationally modified autoinducing peptide (AIP) molecules as a mean of intercellular communication (Novick, 2003). Secretion of the peptide signals are achieved via a dedicated ATP-binding cassette (ABC) exporter protein followed by recognition of the signals by membrane bound two-component sensor kinase proteins (Bassler, 1999). In *Bacillus subtilis*, such secretory machinery enables the bacterium to decide between competence for DNA uptake and sporulation through secretion of two extracellular peptides, ComX and CSF (Competence and sporulation factor) (Ansaldi and Dubnau, 2004; Lazazzera and Grossman, 1998).

2.2 AHL Biosensors

Identification of bacterial QS systems via AHL production has been hasten with the development of bacterial biosensors that are able to detect the presence of AHL compounds. These biosensors do not produce AHL molecules but contain a functional LuxR-family protein cloned together with a cognate target promoter which positively regulates the transcription of a reporter gene that displays phenotypes such as

bioluminescence, green fluorescent protein and pigment production (Steindler and Venturi, 2007).

C. violaceum CV026, a Mini-Tn5 mutant derived from *C. violaceum* ATCC 31532 is one of the widely exploited AHL biosensor. It was developed after mini-Tn5 transposon mutagenesis on *cviI* gene responsible for C6-HSL production while retaining the functionality of *cviR* gene that induces violacein production. Such defect results in white colony mutant that will only produce purple pigmentation upon exposure to exogenous AHLs ranging from C4-HSL to C8-HSL (McClellan *et al.*, 1997). Therefore CV026 can be used as a simple biosensor for detection of short chained AHLs.

Another pigmentation based biosensor is the *Serratia* sp. SP19 that relies on exogenous short chained AHLs for the observation of bright red pigment, prodigiosin production. Construction of this biosensor was performed via triple mutation on *smal*, *pigX* and *pigZ* genes of C4-HSL producing *Serratia* sp. ATCC 39006. Mutation of *smal* causes the deficiency in C4-HSL synthesis. The product of *pigX* and *pigZ* genes are significant regulators for the biosynthesis of prodigiosin and mutation of both genes showed increment of prodigiosin production. As such the resulting mutation has generated a non AHL producing mutant with enhanced sensitivity towards exogenous short chained AHLs especially C4-HSL and 3-oxo-C4-HSL (Poulter *et al.*, 2010).

Another mode of AHL detection involves emission of bioluminescence in the presence of exogenous AHLs. Genetically engineered *E. coli* carrying AHL sensor plasmids namely pSB401, pSB406 and pSB1075 containing fusion of *luxRI'::luxCDABE*, *rhlRI'::luxCDABE* and *lasRI'::luxCDABE* respectively were able to

exert bioluminescence in presence of AHL molecules that activates the LuxR homologues (Winson *et al.*, 1998).

2.3 QQ Ceasing the Chatter

QS regulation has been employed by many bacteria to gain maximal advantages from the competitive surroundings as well as exploitation of other bacteria, fungus, plants and animals (Dong and Zhang, 2005). As such, naturally occurring and well evolved QQ mechanisms were found from various organisms to disarm the deleterious effect resulted from QS of other competitors (Lin *et al.*, 2003). These mechanisms that play important roles in microbe–microbe and pathogen–host interactions are widely conserved in prokaryotes and eukaryotes organisms (Dong *et al.*, 2007). Several groups of QQ enzymes and inhibitors have been identified in a range of living organisms, including bacteria and eukaryotes (Dong and Zhang, 2005).

2.3.1 AHL QQ Enzymes

Inhibition of AHL quorum signals could be achieved by i) destabilizing the LuxR family protein receptors for AHL signal molecules, ii) blocking AHL signal generators and iii) by degrading AHL signal compounds (De Kievit and Iglewski, 2000; Lin *et al.*, 2003; Uroz *et al.*, 2003; Hong *et al.*, 2012). For the degradation of AHL signal molecules, three types of enzymes including AHL-lactonase (Dong *et al.*, 2002), AHL-acylase (Lin *et al.*, 2003) and AHL-oxidoreductase (Uroz *et al.*, 2005), conferring distinctive AHL degradation pathways from each another has been documented. The

structural modifications of AHL molecules resulted from these enzymes are illustrated in Figure 2.2.

2.3.1.1 AHL Acylase

In general, acylase enzymes hydrolyze the connecting amide bond of AHL to release fatty acid and homoserine lactone (HSL) (Sio *et al.*, 2006). The fatty acid released is utilized as an energy source whereas the HSL released can be exploited as nitrogen source through mineralization of the lactone ring (Wang and Leadbetter, 2005; Dong *et al.*, 2007). A classic example of bacteria exerting acylase activities would be *P. aeruginosa* PA01 where two AHL acylase homologues namely PvdQ and QuiP, was found in the bacterium. Further studies found that the implication for the bacterium expressing the enzymes was to decrease the accumulation of the native 3-oxo-C12 HSL signalling molecules (Huang *et al.*, 2003; Huang *et al.*, 2006). Another instance of AHL acylase was from *Ralstonia* strain XJ12B in which the isolation and the expression of the gene in *P. aeruginosa* PA01 had significantly reduced the swarming ability as well as production of virulence factor pyocyanin and elastase (Lin *et al.*, 2003). Beside prokaryotes, acylase 1 isolated from porcine kidney had also been described to deacylate C4-HSL and C10-HSL (Xu *et al.*, 2003). Table 2.2 illustrated the various examples of AHL acylases found in various organisms.

Table 2.2 Examples of various AHL acylases.

| Strain or Source | Enzyme Abbreviation | AHL Degradation | References |
|---|---------------------|---|--|
| <i>Pseudomonas</i> PAI-A, <i>P. aeruginosa</i> PA01 | PvdQ | AHLs with or without C3 substitutions and chain length from C11 to C14 but did not degrade short chain AHLs | Huang <i>et al.</i> , 2003, Sio <i>et al.</i> , 2006 |
| <i>P. aeruginosa</i> PA01 | QuiP | AHLs with or without C3 substitutions and chain length from C7 to C14 but did not degrade C4-HSL, C6-HSL and 3-oxo-C6-HSL | Huang <i>et al.</i> , 2006 |
| <i>P. syringae</i> B728a | HacA | C8-HSL, C10-HSL and C12-HSL C14 but did not degrade C6-HSL and 3-oxo-C6-HSL | Shepherd and Lindow, 2009 |
| <i>P. syringae</i> B728a | HacB | C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL and C12-HSL | Shepherd and Lindow, 2009 |
| Porcine (Kidney) | ACY1 | C4-HSL and C10-HSL | Xu <i>et al.</i> , 2003 |
| <i>R. erythropolis</i> W2 | QsdA | AHLs with C3 substitutions and chain length from C6 to C14 but less efficiency against AHLs without C3 substitution | Uroz <i>et al.</i> , 2005 |
| <i>Ralstonia</i> sp. XJ12B | AiiD | 3-oxo-C8-HSL, 3-oxo-C10-HSL and 3-oxo-C12-HSL | Lin <i>et al.</i> , 2003 |
| <i>Streptomyces</i> sp. M664 | AhIM | C8-HSL, C10-HSL, 3-oxo-C12-HSL, low activity towards 3-oxo-C6-HSL, C6-HSL and no activity towards C4-HSL | Park <i>et al.</i> , 2005 |

| | | | |
|---------------------------|-----|---|--------------------------------|
| <i>V. paradoxus</i> VAI-C | N.D | C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL and C12-HSL | Leadbetter and Greenberg, 2000 |
|---------------------------|-----|---|--------------------------------|

Legend: N.D: Not Determined

2.3.1.2 AHL Lactonase

On the other hand, AHL-lactonases hydrolyses the homoserine lactone ring producing acylhomoserine (Wang and Leadbetter, 2005). The lactonolysis involves cleavage of the ester bond of the homoserine lactone ring by attacking the lactone's carbonyl followed by elimination of an alcohol leaving group (Momb *et al.*, 2006). Thus the opening of the ring prevents binding of the molecules to the target transcriptional regulators (Dong *et al.*, 2001). However the lactonolysis were found to be pH dependent in which relactonization of the homoserine lactone ring were observed upon acidification of growth media to pH 2.0 (Yates *et al.*, 2002).

The first documentation of AHL-lactonase was from *Bacillus* sp. 240BI which was later termed as AiiA (Dong *et al.*, 2000). Homologues of AiiA were later discovered in the genus of *Bacillus* including *Bacillus cereus*, *thuringiensis* and *thailandensis* (Dong *et al.*, 2002; Ulrich, 2004). It was later found that the AiiA contains a conserved $^{104}\text{HXHXDH}^{109}\sim\text{H}^{169}$ region that resembles the zinc binding motif of several enzymes in metallohydrolase superfamily (Dong and Zhang, 2005). The amino acid residues His-106, Asp-108, His-109 and His-169 are conserved in metallohydrolases (Dong *et al.*, 2000).

Catalytic activities and proper folding of AHL lactonase requires a binuclear metal ion active site that harbors metal ions as chelation of metal ions from the enzyme were shown to greatly reduce its catalytic rate (Thomas *et al.*, 2005). In addition, Momb

et al., 2006 proposed an interaction between the metal ions and substrate's leaving group during catalysis that facilitated the bond cleavage of lactone ring. Incorporation of metal ions in the enzyme including Zn²⁺, Co²⁺, Ca²⁺ and Mn²⁺ for catalysis has been reported as illustrated in Table 2.3.

In addition, human paraoxonases encoded by the *PON* genes exhibit lactonase activities against C6-HSL and oxo-C12-HSL with varying inactivation rate depending on cell types and tissues likely to be exposed to pathogens were found to have highest inactivation of the AHL signals (Chun *et al.*, 2004; Greenberg *et al.*, 2004). Furthermore, the activities of human paraoxonases have been demonstrated over 30 different non AHL type lactones by exhibiting a range of other physiologically important hydrolytic activities, including drug metabolism and detoxification of nerve agents (Dong and Zhang, 2005).

Table 2.3 Examples of various AHL lactonases

| Strain or Source | Enzyme Name | AHL Degradation | Metal Ion for Activity | References |
|---------------------------------------|-------------|---|------------------------|--|
| <i>Agrobacterium tumefaciens</i> C58 | AiiB, AttM | C6-HSL and 3-oxo-C8-HSL | N.D | Zhang <i>et al.</i> , 2002; Haudecoeur <i>et al.</i> , 2009 |
| <i>Arthrobacter</i> sp. strain IBN110 | AhID | C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL and 3-oxo-C12-HSL | Zn ²⁺ | Park <i>et al.</i> , 2003 |

| | | | | |
|--|----------------------|--|--|-----------------------------|
| <i>Bacillus</i> sp. AI96 | AiiA _{AI96} | AHLs with or without C3 substitution and chain length from C4 to C14 but less efficiency against 3-oxo-C6-HSL, C12-HSL and 3-hydroxy-C14-HSL | Zn ²⁺ | Cao <i>et al.</i> , 2012 |
| <i>Bacillus</i> sp. 240BI | AiiA | 3-oxo-C6-HSL, 3-oxo-C8-HSL and 3-oxo-C10-HSL | Zn ²⁺ | Dong <i>et al.</i> , 2001 |
| <i>B. thuringiensis</i> | AiiA homologues | 3-oxo-C6-HSL | Zn ²⁺ | Lee <i>et al.</i> , 2002 |
| <i>Geobacillus caldxylosilyticus</i> YS-8 | N.D | C6-HSL and 3-oxo-C6-HSL | CO ²⁺ , Mn ²⁺ and Ni ²⁺ | Seo <i>et al.</i> , 2011 |
| <i>Geobacillus kaustophilus</i> HTA426 | GKL | C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL and 3-oxo-C12-HSL | Zn ²⁺ | Chow <i>et al.</i> , 2010 |
| <i>Klebsiella pneumoniae</i> | AhlK | C6-HSL and 3-oxo-C6-HSL | N.D | Park <i>et al.</i> , 2003 |
| <i>Mycobacterium tuberculosis</i> | PPH | C4-HSL, 3-oxo-C8-HSL and C10-HSL | Zn ²⁺ | Afriat <i>et al.</i> , 2006 |
| <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10 | MCP | C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL | Mn ²⁺ , Zn ²⁺ | Chow <i>et al.</i> , 2009 |

| | | | | |
|---|--------|---|------------------|---|
| <i>Ochrobactrum</i> sp. | AidH | C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3- oxo-C8-HSL, C10- HSL and 3- hydroxy-AHLs | Mn ²⁺ | Mei <i>et al.</i> , 2010 |
| <i>R. erythropolis</i> W2 | AhlA | AHLs with C3 substitutions and chain length from C6 to C14 but less efficiency against AHLs without C3 substitution | Zn ²⁺ | Uroz <i>et al.</i> , 2008 |
| <i>Sulfolobus</i> <i>sulfataricus</i> P2 | SsoPox | C8-HSL, 3-oxo- C8-HSL, 3-oxo- C10-HSL and 3- oxo-C12-HSL | Fe ²⁺ | Elias <i>et al.</i> , 2008 |
| Human (airway epithelia) | PON | C6-HSL and 3- oxo-C12-HSL | Ca ²⁺ | Chun <i>et al.</i> , 2004; Greenberg <i>et</i> <i>al.</i> , 2004 |

Legend: N.D: Not Determined

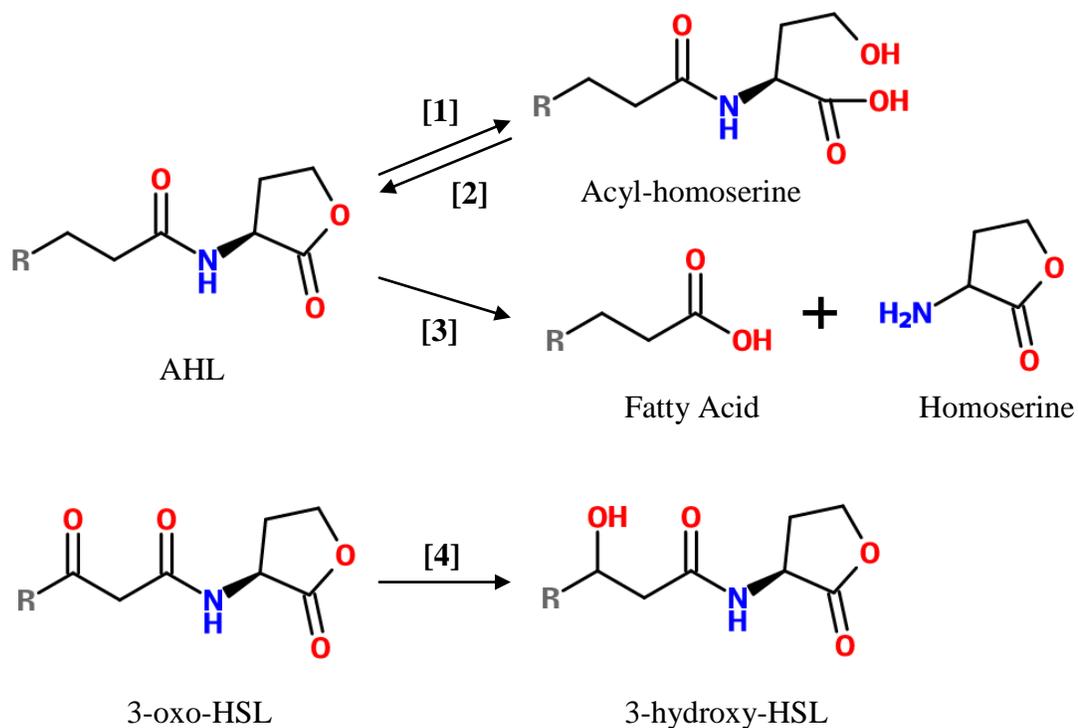
2.3.1.3 AHL Oxidoreductase

The third enzyme, AHL-oxidoreductases which was originally identified in *R. erythropolis* reduces the keto-group of 3-oxo-AHLs to the corresponding 3-hydroxy derivative AHLs (Uroz *et al.*, 2005). Unlike the acylases and lactonases, no destruction of the AHL structure is observed but in turn signal disturbance of bacteria that depend on oxo-AHL for regulation of QS-mediated genes were resulted (Chan *et al.*, 2011). However, this AHL-oxidoreductase is inactive against hydroxylated and fully reduced AHLs (Uroz *et al.*, 2005). Examples of reported AHL oxidoreductases were illustrated in Table 2.4.

Table 2.4 Examples of AHL oxidoreductases

| Strain or Source | Enzyme Abbreviation | AHL Degradation | References |
|-------------------------------------|---------------------|--|--------------------------------|
| <i>Bacillus megaterium</i> CYP102A1 | N.D | Oxidation of AHLs to hydroxylated AHLs at ω -1, ω -2 and ω -3 carbons of acyl chain | Chowdhary <i>et al.</i> , 2007 |
| <i>Burkholderia</i> sp. GG4 | N.D | Reduction of 3-oxo-C4-HSL, 3-oxo-C6-HSL and 3-oxo-C8-HSL to corresponding 3-hydroxy compounds | Chan <i>et al.</i> , 2011 |
| <i>Rhodococcus erythropolis</i> W2 | N.D | Reduction of AHLs ranging from 3-oxo-C8-HSL, to 3-oxo-C14-HSL to corresponding 3-hydroxy compounds | Uroz <i>et al.</i> , 2005 |

Legend: N.D: Not Determined



Legend:

[1]: Lactonase activity via cleavage of ester bond of lactone ring

[2]: Relactonization

[3]: Acylase activity via cleavage of amide bond between acyl group and lactone ring

[4]: Oxidoreductase activity via substitution of oxo group at C3 position into hydroxy group

Figure 2.2 Structural modification of AHL molecules resulted from enzymatic reactions

2.4 Biotechnological Implications of QQ

Given the importance of AHL QS systems in bacterial virulence towards eukaryotic hosts, the control of bacterial infections could be strategized by interfering the QS signalling of pathogens. Concentrations of AHL is essential in regulating virulence gene expression, thus degradation or disruption of the signal would clearly reduce the pathogenicity of the infections (Dong *et al.*, 2000). With the discovery of various QQ enzymes and QS inhibitors, such approach were made feasible as treatments for bacterial infections in both humans and plants (Hentzer and Givskov, 2003). Instances include the ability of halogenated furanones produced by macroalgae *Delisea pulchra* to interfere with the expression of AHL regulated genes in *P. aeruginosa*, a pathogen responsible for chronic lung infections in patients with cystic fibrosis (Chun *et al.*, 2004; Hentzer *et al.*, 2003). Expression of QQ enzymes in *P. aeruginosa* and plant pathogen, *E. carotovora* reduces their virulence to infect nematode *Caenorhabditis elegans* and tobacco plants, respectively (Dong and Zhang, 2005). Protection against the virulence of *E. carotovora* were also achieved by transgenic potato and tobacco plants expressing AHL degrading enzymes (Dong *et al.*, 2000).

The integration of QQ compounds and enzymes may offer a rational strategy in biocontrol of QS dependent pathogens although the prematurity of this approach is apparent in terms of its general acceptability and biosafety issues. In fact, much work remain to be done on assessing the stability, delivery, toxicity and possible side effects of these enzymes in the context of pharmaceutical and agricultural applications (Dong and Zhang, 2005; Hong *et al.*, 2012).

CHAPTER 3.0
MATERIALS AND METHODS

3.1 Bacteria Strains, Plasmid and Oligonucleotides

Table 3.1 Bacterial strains used in this study

| Bacterial strain | Description | Source/Reference |
|---------------------------|--|---|
| <i>C. violaceum</i> CV026 | Mini-Tn5 mutant derived from <i>C. violaceum</i> ATCC 31532 acts as biosensor with formation of purple violacein pigment in presence of short chain exogenous AHL molecules. | McClellan <i>et al.</i> , 1997 |
| <i>E. carotovora</i> Attn | Positive control for quorum sensing tests due to its capability of producing AHL molecules to be detected by <i>C. violaceum</i> CV026. | Dr. Chan Kok Gan, Department of Genetics, University of Malaya |
| <i>E. carotovora</i> A20 | Negative control for AHL production as it does not produce AHL molecules. | Dr. Chan Kok Gan, Department of Genetics, University of Malaya |

| | | |
|-----------------------|---|---|
| <i>B. cereus</i> | Positive control for quorum quenching tests due to its strong quorum quenching activity. | Dr. Chan Kok Gan, Department of Genetics, University of Malaya |
| <i>E. coli</i> Top 10 | Negative control for quorum quenching tests as it does not possess quorum quenching ability. It was also used as host for TA cloning. | Invitrogen |

The growth of *E. carotovora* and *B. cereus* KM1S were carried out at 28 °C in Luria Bertani (LB) media for 18-20 hours while *E. coli* were cultured in LB media at 37 °C. Incubation in broth was done with shaking at 220 rpm.

Table 3.2 Plasmid used in this study

| Plasmid | Description | Source/Reference |
|----------------------------------|---|-------------------------|
| pGEM [®] -T Easy Vector | F1 ori, Amp ^R , used as cloning vector | Promega |

Table 3.3 Oligonucleotide used in this study

| Primer | Sequence | Length (-mer), Reference |
|----------------------------------|-------------------------------------|--------------------------------------|
| 16S rDNA forward primer 27F | 5'-AGA GTT TGA TCM TGG CTC AG-3' | 20, Ott <i>et al.</i> , 2004 |
| 16S rDNA reverse primer 1525F | 5'-AAG GAG GTG WTC CAR CC-3' | 17, Dewhirst <i>et al.</i> , 1999 |
| M13F forward primer | 5'GTA AAA CGA CGG CCA GT-3' | 17, Universal Primer |
| M13R reverse primer | 5'-CAG GAA ACA GCT ATG ACC-3' | 18, Universal Primer |
| T7 | 5'-TAA TAC GAC TCA CTA TAG GG-3' | 20, Universal Primer |
| SP6 | 5'-TTC TAT AGT GTC ACC TAA AT-3' | 19, Universal Primer |

3.2 Chemical Reagents

All the chemical reagents used in this study is of analytical grade (or highest grade) purchased from Bio-Rad Laboratories Ltd., U.S.A.; Merck, Germany; Promega Ltd, U.S.A.; Thermo Fisher Scientific, U.S.A.; Sigma Chemical Corp., U.S.A.; Invitrogen Corp., U.S.A.; BDH Laboratory Supplies, England; and Ajax Pacific Specialty Chemicals Limited ABN., Australia.

3.3 Equipments

Equipments and instruments that were used during the course of this study included Eppendorf Research micropipettes, Eppendorf mini spin centrifuge machine, Eppendorf thermomixer compact, Shimadzu UV1601 spectrophotometer, Olympus 1X71-22FL/PH research inverted microscope, Sartorius weighting balance, Hirayama autoclave machine, 2720 Thermal Cycles PCR machine, Cyberscan pH500 pH meter, UVP high performance ultraviolet transilluminator, Exelo water distiller, Merck mili-Q water synthesizer, Orbital shaker incubator, N-BIOTEK. INC. shaking incubator, Perkin Elmer Cetus DNA thermal cycler, Eyela oil bath, Vortex mixer, Agilent 1200 series Rapid Resolution Liquid Chromatography and Agilent 6500 Q-TOF LC/MS system.

3.4 Commercial Kits

The commercial kits used in the study are described in Table 3.4.

Table 3.4 Commercial kits.

| Kit, Manufacturer | Application |
|---|--------------------------------------|
| <i>i-Taq</i> TM DNA polymerase kit, iNtRON Biotechnology, Korea. | PCR amplification |
| QIAamp DNA Mini Kit, Qiagen Pty. Ltd., Germany | Genomic DNA extraction |
| QIAquick Gel Extraction Kit , Qiagen Pty. Ltd., Germany | Purification of DNA from agarose gel |
| QIAquick PCR purification Kit , Qiagen Pty. Ltd., Germany | Purification of DNA from PCR |
| QIAquick Spin Miniprep Kit, , Qiagen Pty. Ltd., Germany | Plasmid DNA extraction |

3.5 Growth Media and Buffer Solutions

Unless otherwise stated, preparation of the growth media and solutions stated in this study required sterilization by autoclaving at 121 °C, 15 psi for 20 mins. Sterilization of heat sensitive solutions was done via filter sterilization with syringe filter (Sartorius, Germany) at pore size of 0.22 µm.

3.5.1 Luria-Bertani (LB) Medium

Preparation of Luria-Bertani broth consisted of 1.0 % w/v trytone, 1.0 % w/v NaCl and 0.5 % w/v yeast extract in 1 L of distilled water. To solidify agar, Bacto agar was added to broth medium (1.5 % w/v) to prepare LB agar (Sambrook *et al.*, 1989). The solutions were then autoclave sterilised. When cooled, antibiotic was added to the medium as indicated.

For extraction of AHL molecules, the LB broth was supplemented with 50 mM of 3-(N-morpholino) propanesulfonic acid (MOPS) (Merck, Germany) prior to inoculation of bacteria. MOPS maintained the media acidic that would prevent elevation of solution pH above the value of 7.0 as lactonolysis of AHL molecules will be induced at high pH (Yates *et al.*, 2002).

3.5.2 S.O.C Medium

SOC medium was prepared as described in Sambrook *et al.*, (1989) consisting of 2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl and 2.5 mM KCl in 100 ml distilled water. After autoclave sterilisation, addition of filter sterilised MgSO₄ and glucose solutions to the final concentration of 10 mM and 20 mM, respectively, was added aseptically to the solution.

3.5.3 Phosphate Buffered Saline (PBS)

PBS solution was prepared by mixing 0.23 g of NaH₂PO₄, 1.15 g of Na₂HPO₄ and 9.0 g of NaCl in 1 L of distilled H₂O. The pH of solution was then adjusted to the value of 6.5 prior to autoclave sterilization.

3.5.4 5× Tris Borate EDTA (TBE) Buffer

Briefly, 5× TBE stock solutions consisted of 54.0 g Tris base, 27.5 g boric acid and 3.72 g Na₂EDTA•2H₂O dissolved in 1 L of distilled water with pH adjusted to 8.0 before autoclaved.

3.6 Stock Solutions

3.6.1 Synthetic N-Acyl Homoserine Lactones

Synthetic AHL molecules were obtained from Sigma-Aldrich[®] and Cayman Chemicals. AHLs were dissolved by using acetonitrile (ACN) to the desired concentration.

3.6.2 Ampicillin Stock Solution

Ampicillin (Sigma Chemical Corp., U.S.A) stock solution was prepared by dissolving in sterile distilled water resulting in the final concentration of 100 mg/ml. The antibiotic stock was filter sterilized into new sterile microcentrifuge tubes followed by storage of the aliquots at -20 °C until further usage.

3.6.3 5-bromo-4-chloro-3-indoyl-beta-D-galacto-pyranoside (X-gal) Stock Solution

X-gal is a synthetic sugar analogous to lactose, was used for blue/white colony screening during transformation step in this study. The stock solution at concentration of 20 mg/ml was prepared by dissolving the X-gal powder with dimethylformamide (DMF) and filter sterilized with syringe filter (Sartorius, Germany) at pore size of 0.22 µm before storage at -20 °C.

3.7 DNA Ladder Marker

DNA ladder markers used in this study were GeneRuler™ 100 bp DNA Ladder and GeneRuler™ 1kb DNA ladder purchased from Fermentas International Inc., Canada.

3.8 Sampling and Isolation of Environmental Bacteria

3.8.1 Soil Collection and Sampling Procedure

Sampling of the soil was done in a compound several meters away from a tea farm in Cameron Highland, Malaysia. The GPS position for the site was at N04°32'707' E101°25'275', at an elevation of 1,206 m above sea level. Collection time of the soil was at 1145 a.m. at ambient temperature of approximately 28 °C. The soil was sampled at the subsurface level (to the depth of 5 cm) and placed into a sterile 50-ml centrifuge tube. Large particles such as stones and roots was removed. Suspension of the soil was prepared with 1 g of the soil dispersed in 5 ml PBS (pH 6.5) via vortex agitation.

3.8.2 Isolation of Bacterial Strains

Ten fold serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5}) were performed for the suspended soil sample by using sterile 1× PBS (pH 6.5) into sterile microcentrifuge tubes. Small portion of each dilution was transferred and plated on LB agar plates followed by incubation at 28°C for 10 consecutive days. Isolation procedure was proceeded by selection of single bacterial colonies displaying distinctive morphologies. Each of the identified single colonies is streaked onto LB agar plates until pure cultures were obtained. Gram staining was then performed followed by observation under OlympusTM IX71 inverted research microscope and the micrographs were captured using Olympus Cell[^]D imaging system.

3.9 Molecular Identification of Bacteria Isolates

3.9.1 Genomic DNA Extraction

Bacterial isolates were inoculated into LB broth and incubated overnight in a shaking incubator at 200 rpm. The bacteria cells were harvested by centrifugation at 8421 *g* for 10 min. The cell pellets were subjected to genomic DNA extraction using QIAamp DNA Mini Kit (Qiagen) with accordance of the manufacturer's instructions.

3.9.2 Polymerase Chain Reaction (PCR) Amplification

The target gene for identification of bacteria in this study was the 16S rDNA gene. Amplification of the gene involved forward primer 27F and 1525R as reverse primer that would yield the amplified fragments with expected size of 1.5kb. The reagents from *i-Taq*TM DNA polymerase kit (Intron Biotechnology, Korea) were used for setup of the PCR mixtures. The amounts for each of the components used in the PCR mixtures were illustrated in Table 3.5.

The PCR cycles for the amplification of 16S rDNA gene region consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 1 min 30 s and final extension step for 5 min.. A negative control was included for each PCR run by substituting bacterial cells with ultrapure H₂O (MiliQ, Merck).

Table 3.5 PCR mixture for 16S rDNA amplification.

| PCR Component (Stock Concentration) | Volume per sample (μl) |
|--|--|
| Ultrapure H ₂ O | 9.9 |
| 10 \times Buffer containing 2 mM MgCl ₂ | 1.5 |
| dNTP (200 μ M) | 1.2 |
| <i>Taq</i> polymerase (5 U/ μ l) | 0.2 |
| 27F Forward primer (10 μ M) | 0.6 |
| 1525R Reverse primer (10 μ M) | 0.6 |
| Genomic DNA | 1.0 |
| Total volume | 15.0 |

3.9.3 Agarose Gel Electrophoresis

Electrophoretic examination of DNA samples were carried out using horizontal agarose gel at 1.0 % w/v submerged in 1 \times TBE buffer. Melting of the agarose mixture was done in the microwave oven followed by supplementation of EtBr to final concentration of 0.5 μ g/ml. Upon cooling, the molten agarose was then poured into a gel cast for solidification.

Agarose gel electrophoresis was carried out after loading 3 μ l of DNAs samples and 0.2 μ g of 1 kb DNA ladder (Vivantis, Malaysia) and electrophoresis was carried out at 80 V until the loading dye front approached about 1.0 cm from the edge of the gel. The gel was visualized on UVP ultraviolet transilluminator (Bio-Rad Laboratories, U.S.A).

3.9.4 Cloning, Transformation and Sequencing

3.9.4.1 Ligation of Purified PCR Products

The purified PCR products were ligated into pGEM[®]-T Easy Vector (Promega Ltd. U.S.A.) according to the manufacturer's protocol. The ligation mixtures were then incubated overnight at 4°C. Table 3.6 illustrated the amount for each components used in the ligation.

Table 3.6 Setup for ligation of purified PCR products.

| Component | Standard 1× reaction (μl) |
|----------------------------------|---------------------------|
| 2× Rapid ligation buffer | 2.5 |
| pGEM [®] -T Easy Vector | 0.5 |
| Purified PCR product | 1.5 |
| T4 DNA ligase | 0.5 |
| Total volume | 5.0 |

3.9.4.2 Preparation of Chemical Competent Cells

A single colony of *E. coli* Top 10 grown on LB agar plate culture was inoculated in 10ml of LB broth followed by overnight incubation at 37°C in shaking incubator at 220 rpm. An amount of 2 ml overnight culture was withdrawn and transferred into fresh 100 ml of LB broth. The culture was then incubated in 37°C with shaking at 220 rpm until OD_{600nm} reaches approximately 0.5 and the conical flask containing the culture was placed on ice for approximately 20 min. This was followed by 50 ml of the culture being transferred into a chilled 50-ml polypropylene tube.

The cells were harvested by centrifuging at 1663 *g* at 4°C for 10 min. The supernatant was discarded and the cells were collected gently in 10 ml of chilled 1.0 M of CaCl₂. The cells were then harvested again by centrifuging at 1663 *g* at 4°C for 10 min. Supernatant was discarded and the cells were resuspended gently in 1 ml of 0.1 M of CaCl₂. The cells were kept in 4°C for 16 h as to increase the competency of the cells. Subsequently the cells were mixed with glycerol solution (80% v/v) and dispensed into sterile 1.5-ml microcentrifuge tube in 50 µl aliquots before storage at -80°C.

3.9.4.3 Transformation using Heat Shock Method

An amount of 2 µl ligation product was added into 50 µl of thawed competent cells. The tube was then chilled on ice for 20 min followed by heat shock at 42°C for 35 s. The tubes were then returned to ice for 2 min before adding 450 µl of warm S.O.C medium. The transformed cells were then incubated at 37°C for 1 h with shaking at 150 rpm. Finally, 100 µl of the cell suspension were spread on LB agar supplemented with 100 µg/ml of ampicillin and 20 µg/ml of X-gal. The plates were then incubated at 37°C for 18 h.

3.9.4.4 Selection of Recombinants and Confirmation by Colony PCR

Selection of transformants carrying recombinant plasmids containing the desired 16S rDNA amplicons involved blue/white colony screening. The pGEM[®]-T Easy Vector used in the study contained cloning region within the coding sequence of β -galactosidase and the insertion of the sequences into the cloning region would disrupt the reading frame of β -galactosidase resulting in non-functionality of the enzyme. Hence, the presence of X-gal allowed the bacterial colonies containing plasmid with insert to be distinguished from those containing non-recombinant plasmid. Appearance of blue colony indicated breakdown of X-gal due to functional β -galactosidase activities as the reading frame encoding the enzyme was not disrupted by the insertion of sequence in the plasmid. In contrary, transformants carrying plasmid with insert were identified as white colonies.

Subsequently, verification of successful cloning and transformation was performed through colony PCR. The T7 and SP6 forward and reverse primers were used to amplify the internal fragment of the insert DNA. A single colony was picked from agar plate and diluted in 50 μ l of ultrapure H₂O. The composition of PCR mixture was shown in Table 3.7. The colony PCR conditions were 1 cycle of initial denaturation at 94°C for 10 min followed by 25 cycles of denaturation at 94°C for 30 s, primers annealing at 58°C for 30 s, DNA extension at 72°C for 90 s and 1 cycle of final extension at 72°C for 7 min. A negative control was included for each PCR run by substituting bacterial cells with ultrapure H₂O. Agarose gel eletrophoresis that showed amplicon with size of about 1.7 kb confirmed the actuality of the selected colony as a transformant carrying plasmid inserted with the 16S rDNA sequences.

Table 3.7 PCR mixture for transformant screening.

| PCR Component (Stock Concentration) | Volume per sample (µl) |
|--|-------------------------------|
| Ultrapure H ₂ O | 9.9 |
| 10× Buffer containing 2 mM MgCl ₂ | 1.5 |
| dNTP (200 µM) | 1.2 |
| <i>Taq</i> polymerase (5 U/µl) | 0.2 |
| T7 Forward primer (10 µM) | 0.6 |
| SP6 Reverse primer (10 µM) | 0.6 |
| Genomic DNA | 1.0 |
| Total volume | 15.0 |

3.9.4.5 Plasmid Extraction

A single colony of the transformant was inoculated into LB broth supplemented with 100 µg/ml of Ampicillin. The culture was then incubated overnight at 37°C with shaking before subjected to plasmid extraction. Commercial kit QIAprep Spin Mini Kit (Qiagen, Germany) was used to extract the plasmid and was performed according to manufacturer's instruction. The obtained pure plasmid DNA was stored at -20°C.

3.9.4.6 Sequencing Analysis

Sequencing analysis was carried out with the purified recombinant plasmids by outsourcing to 1st Base (Malaysia) with forward primer M13F and reverse primer M13R were employed as sequencing primers to obtain the partial sequences of the 16S rDNA gene. The obtained gene sequences were then visualised using Applied Biosystem Sequence Scanner v1.0 followed by acquisition of good quality sequences via trimming. The sequences were then compared with those in the GenBank databases using the BLASTN program through the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov>). The genus and species of bacterial isolates were then evaluated based on the nearest identity to those deposited in the databases.

3.9.4.7 Phylogenetic Analysis

The 16S rDNA sequences were first saved as FASTA file type prior to any phylogenetic analysis. Phylogenetic analysis was done using the Molecular Evolutionary genetic Analysis (MEGA) version 5.0 downloadable from <http://www.megasoftware.net> (Tamura *et al.*, 2011). Phylogenetic tree was constructed using neighbor-joining method with Bootstrap test with the value of 1000. The model used was nucleotide maximum composite likelihood (MCL) to estimate the evolutionary distances between all sequences at one time. An appropriate outgroup namely a taxon which is distantly related but nevertheless sufficiently conserved of homologues to each of the ingroup taxa was selected to produce a rooted tree.

3.10 Detection of Acyl Homoserine Lactone Production

3.10.1 CV026 Cross Streak

Preliminary screening for AHL production among the isolates required cross streaking the sample with biosensor CV026. A loopful of bacterial isolates was streaked down the center of an LB agar plate. After flaming, a loopful of biosensor CV026 was cross streaked at 90 degree angle perpendicular to the original streak of the isolates. The cross streaks were to be close together as to allow diffusion of exogenous AHLs from the isolates streak lines to CV026. Observation of violacein production depicted by formation of purple pigments in CV026 streak lines would indicate the secretion of short chained AHLs from the isolate.

3.10.2 AHL Extraction

Selected bacteria were cultured in LB broth supplemented with 50 mM of MOPS and incubated for 18-20 h at 28 °C with shaking at 200 rpm. An equal volume of acidified ethyl acetate (containing 0.1 % v/v glacial acetic acid) was added to the overnight culture followed by vortex agitation for 1 minute. The upper layer of the mixture was transferred into a new tube and the procedure was repeated twice. The tube was then dried in fume hood and the dried extracted contents were resuspended with 1 ml of ethyl acetate. The mixture was then transferred into a new 1.5-ml centrifuge tube for further drying. An amount of 200 µl HPLC grade acetonitrile was then added and vortex for 3 min. The mixture was left overnight at room temperature and was centrifuged at full speed for 10 min. An amount of 75 µl of the mixture was withdrawn

from the top and was placed in insert or sample vial before sending for mass spectrometry analysis.

3.10.3 Mass Spectrometry Analysis

Mass spectrometry analysis of AHL molecules was carried out by Dr. Choo Yeun-Mun from Chemistry Department, University Malaya. Agilent RRLC 1200 system was employed as the LC delivery system with the use of Agilent ZORBAX Rapid Resolution HT column (2.1 mm × 100 mm, 1.8 μm particle size). Analysis was carried out using flow rate of 0.3 ml/min at 60°C and the injection volume was 20 μl. Mobile phases A and B were 0.1% v/v formic acid in water and 0.1% v/v formic acid in acetonitrile, respectively. The gradient profiles were as follows (time: mobile phase A: mobile phase B): 0 min: 60:40, 5 min: 20:80, 7 and 10 min: 5:95, and 11 and 13 min: 60:40. The high-resolution electrospray ionization mass spectrometry (ESI-MS) were performed with the Agilent 6500 Q-TOF LC/MS system. The MS experiment was performed in the ESI-positive mode. The probe capillary voltage was set at 3 kV, desolvation temperature at 350°C, sheath gas at 11 ml/h, and nebulizer pressure at 50 psi (Wong *et al.*, 2012).

3.11 Detection of AHL Degradation Activities

3.11.1 Whole Cell AHL Inactivation Assay

Detection and quantification of AHL degradations exhibited by the bacterial isolates in the study was performed through bioassay with the use of biosensor CV026 as well as Rapid Resolution Liquid Chromatography (RRLC). Sample preparation for both methods required the whole cell inactivation assay (Chan *et.al*, 2011).

Overnight culture of bacteria isolates were harvested by centrifugation at 10,625 g for 5 minutes. The cell pellets were then washed once with 500 μ l and twice with 300 μ l with PBS (pH 6.5) followed by resuspension at 300 μ l. Appropriate amount of selected AHLs were dispensed into microcentrifuge tubes and allowed to evaporate prior to addition of the washed cell suspensions. Subsequently, the cells suspension was added to reconstitute the AHL to the final concentration of 0.2 μ g/ μ l . The mixtures were then incubated at 28°C with shaking at 220 rpm. Aliquots of 25 μ l cell suspension was withdrawn at 0 h, 3 h and 24 h of incubation followed by heat inactivation at 95°C for 5 min with Thermomixer (Eppendorf, Germany).

3.11.2 Screening of AHL Degradation using CV026 Biosensor

Cell suspension (10 μ l) was progressively dispensed onto discs placed on a CV026 overlay followed by overnight incubation. Reduction in violet colour zone size would indicate significant degradation of the AHL over the incubation period. The negative controls for the experiment involved incubation of AHL with washed *E. coli* Top 10 cells and PBS solution whereas *B. cereus* was employed as positive control.

3.11.3 Verification of QQ Activities through RRLC Analysis

Sample preparation for RRLC analysis required whole cell inactivation assay as described in Section 3.11.1 with the exception of final concentration of AHL to be 0.05 μ g/ μ l after rehydration with cell suspension. Extraction of the residual AHL was done with ethyl acetate followed by evaporation of the extract to dryness. Extracted AHL was

then resuspended in acetonitrile before loading into RRLC. The Agilent Technologies 1200 series RRLC system equipped with a vacuum degasser, a binary pump SL, an autosampler, and a diode-array detector (DAD) was used to analyze AHLs. The samples were separated in a Agilent Poroshell 120 EC-C18 (4.6 mm × 100mm, 2.7 μm) column with elution procedure consisted of an isocratic profile of acetonitrile/water (35:65, v/v) for short chained AHL and acetonitrile/water (65:35, v/v) for long chained AHL. A constant flow rate of 0.7 ml/min was applied and AHL detection was carried out at 210 nm with exception of *p*-coumaroyl homoserine lactone (pC-HSL) which was at 306 nm (Schaefer *et al.*, 2008). Known amounts of synthetic AHLs were also loaded as standard and AHL incubated with *E. coli* Top 10 or PBS were used as negative controls.

CHAPTER 4.0

RESULTS

4.1 Soil Sampling and Isolation of Bacteria

Sampling of the soil was done in May of 2010 in an area near to a tea plantation in Cameron Highland, Malaysia. The GPS position for the site was at N04°32,707' E101°25,275' at elevation of 1,206 m above sea level. The pH of the soil sample was pH 5.22. The sampled montane soil was resuspended with PBS and the ten-fold serially diluted mixtures are inoculated on LB agar plates followed by incubation at 28°C with accordance to the ambient temperature when the soil was sampled. Nine bacteria displaying different morphologies were isolated and selected for further experiments. Table 4.1 illustrated a summary of the appearance and morphology of bacterial colonies obtained from LB agar plates.

4.2 Gram Staining

The pure cultures of the nine isolated bacteria were stained according to the standard Gram staining procedure. Microscopic observations based on the shape and size of each of the bacteria was done under the light microscope at 1000 × magnification size (oil emersion). Table 4.1 showed the Gram staining results of the isolates.

Table 4.1 Morphology of bacterial colonies.

| Isolate | Abbreviation | Gram Reaction | Surface | Elevation | Edge | Colour |
|----------------|---------------------|----------------------|----------------|------------------|-------------|---------------|
| 1 | BT1 | + | Contoured | Flat | Serrated | White |
| 2 | BT2 | + | Contoured | Flat | Serrated | Pale Yellow |
| 3 | BT3 | - | Smooth | Pulvinate | Entire | Pale Yellow |
| 4 | BT4 | + | Contoured | Flat | Filamentous | Pale Yellow |
| 5 | BT5 | - | Smooth | Pulvinate | Entire | Pale Yellow |
| 6 | BT6 | + | Smooth | Convex | Entire | Pale Yellow |
| 7 | BT7 | - | Smooth | Pulvinate | Entire | Pale Yellow |
| 8 | BT8 | - | Smooth | Pulvinate | Entire | Pale Yellow |
| 9 | BT9 | - | Smooth | Pulvinate | Entire | Pale Yellow |

4.3 Genomic DNA Extraction and 16S rDNA Gene Amplification

Total genomic DNA of the bacterial isolates were extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. PCR amplification of the 16S rDNA gene of each isolates was carried out using the genomic DNA as template, primers 27F and 1525R for amplification. The expected size for the amplified 16S rDNA genes are of approximately 1.5 kb. The amplicons were then gel purified, ligated into pGEM[®]-T Easy cloning vector followed by tranformation into chemically competent *E. coli* Top 10. Screening of clones with correct insert were accomplished with blue/white colony selection and colony PCR. Sequencing analysis was performed using vector specific universal primers M13F and M13R for the acquisition of nucleotide sequences.

4.4 Molecular Identification and Phylogenetic Analysis of Bacterial Isolates

The obtained nucleotide sequences were compared with GenBank databases using the BLASTN followed by sequence alignment. Table 4.2 illustrated the nearest identities for the nine strains. The 16S rDNA sequences revealed that isolates BT3, BT5 and BT7-9 shared 99% similarity to that of *Pseudomonas frederiksbergensis* whereas the sequence for BT6 has been 99% similar to *Arthrobacter* sp. On the other hand, isolates BT1, BT2, and BT4 were clustered into the genus *Bacillus* with 99% homology with BT4 being specifically identified as *B. cereus*.

Table 4.2 Details of BLASTN alignment search analysis.

| Sample Name | Identity | Accession Number of Subject | E-Value | Similarity with Known Bacteria |
|--------------------|------------------------------|------------------------------------|----------------|---------------------------------------|
| BT1 | <i>Bacillus</i> sp. | AY853168 | 0.0 | 943/944 (99%) |
| BT2 | <i>Bacillus</i> sp. | GU905015 | 0.0 | 918/919 (99%) |
| BT3 | <i>P. frederiksbergensis</i> | HQ242750 | 0.0 | 1003/1006 (99%) |
| BT4 | <i>B. cereus</i> | AJ539175 | 0.0 | 849/851 (99%) |
| BT5 | <i>P. frederiksbergensis</i> | HQ242750 | 0.0 | 945/947 (99%) |
| BT6 | <i>Arthrobacter</i> sp. | JF772504 | 0.0 | 666/667 (99%) |
| BT7 | <i>P. frederiksbergensis</i> | HQ242750 | 0.0 | 766/768 (99%) |
| BT8 | <i>P. frederiksbergensis</i> | HQ242750 | 0.0 | 896/901 (99%) |
| BT9 | <i>P. frederiksbergensis</i> | HQ242750 | 0.0 | 683/685 (99%) |

4.5 Phylogenetic Analysis

The phylogenetic tree of the 16S rDNA gene sequences were constructed using Molecular Evolutionary Genetic Analysis (MEGA) version 5. Construction of phylogenetic tree was based on neighbor-joining method with Bootstrap test with the value of 1000 replicates. The numbers between each clades indicate the percentage of the 1000 bootstrap replications that the members of the certain clade always belongs that clade. Appropriate outgroup sharing a distantly related taxon that is yet sufficiently conserved to each of the ingroup taxa was assigned to produce a rooted tree. Unrooted trees with absence of an outgroup will not display the direction of evolution for each of the considered taxa. Phylogenetic analysis supporting the identification of each isolates were shown in Figure 4.1 to Figure 4.9.

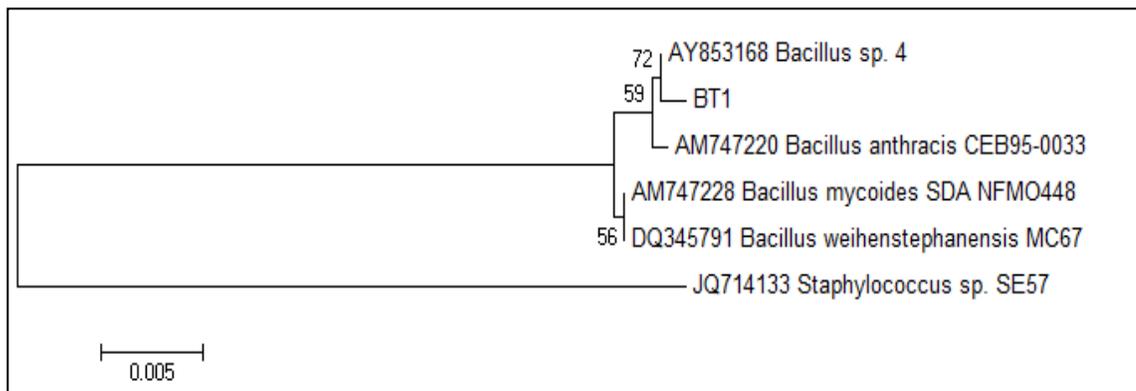


Figure 4.1 16S rDNA gene based phylogenetic analysis of *Bacillus* sp. BT1.

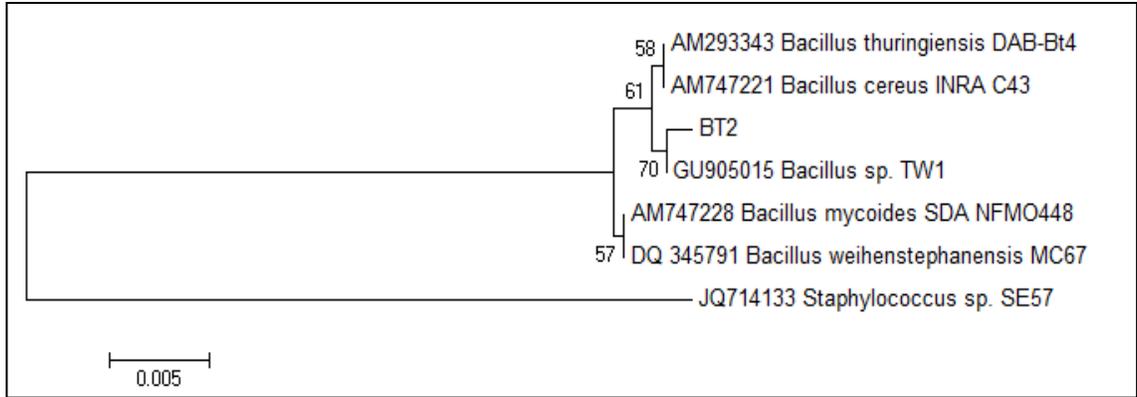


Figure 4.2 16S rDNA gene based phylogenetic analysis of *Bacillus* sp. BT2.

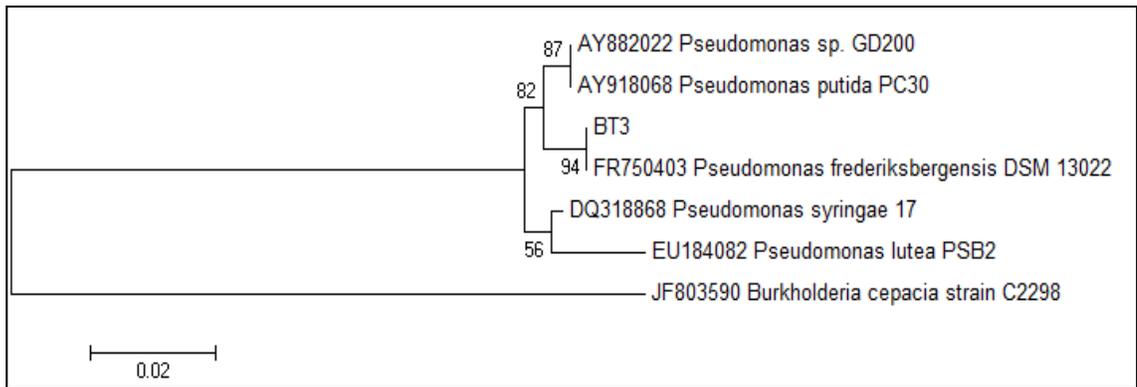


Figure 4.3 16S rDNA gene based phylogenetic analysis of *P. frederiksbergensis* BT3.

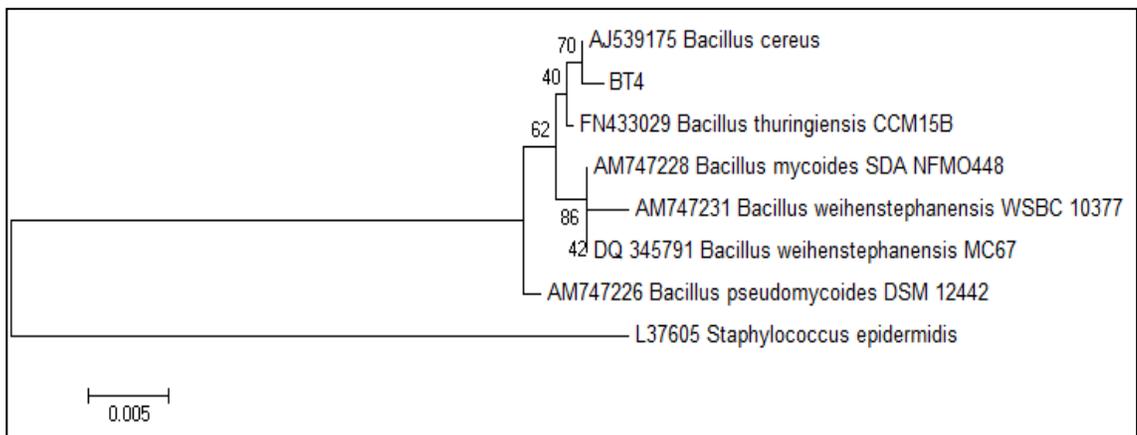


Figure 4.4 16S rDNA gene based phylogenetic analysis of *B. cereus* BT4.

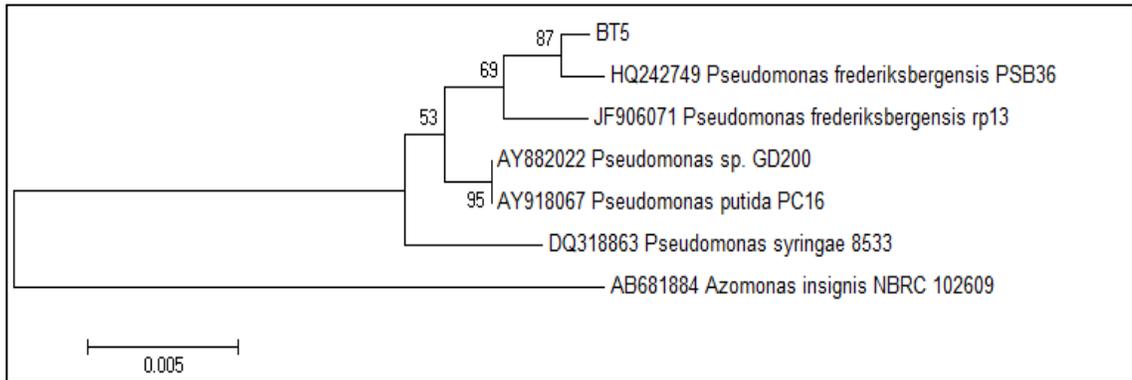


Figure 4.5 16S rDNA gene based phylogenetic analysis of *P. frederiksbergensis* BT5.

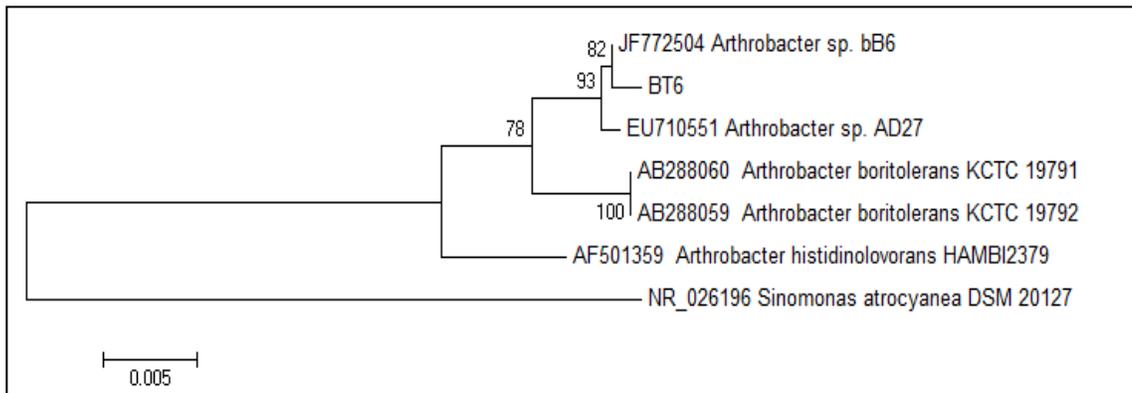


Figure 4.6 16S rDNA gene based phylogenetic analysis of *Arthrobacter* sp. BT6.

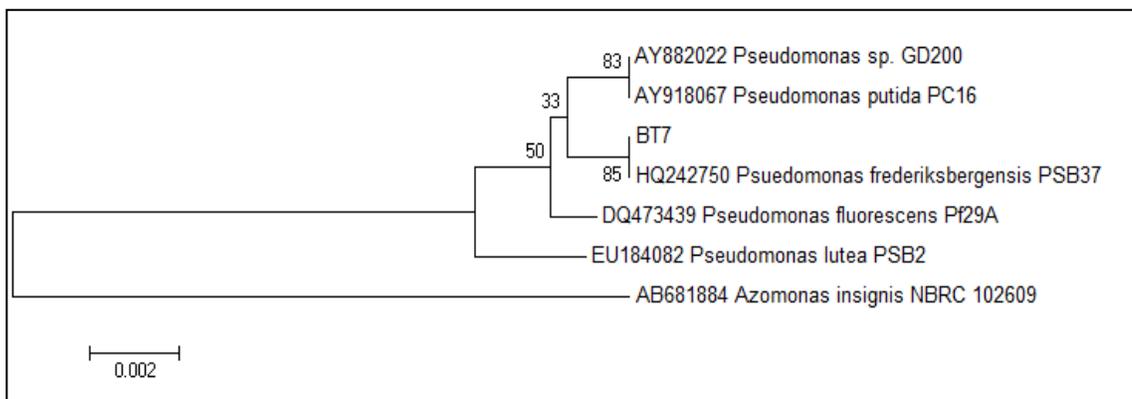


Figure 4.7 16S rDNA gene based phylogenetic analysis of *P. frederiksbergensis* BT7.

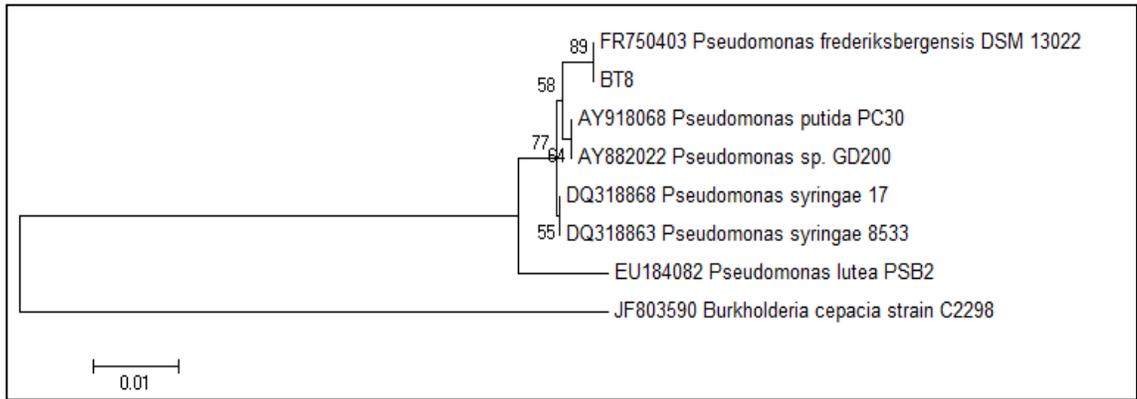


Figure 4.8 16S rDNA gene based phylogenetic analysis of *P. frederiksbergensis* BT8

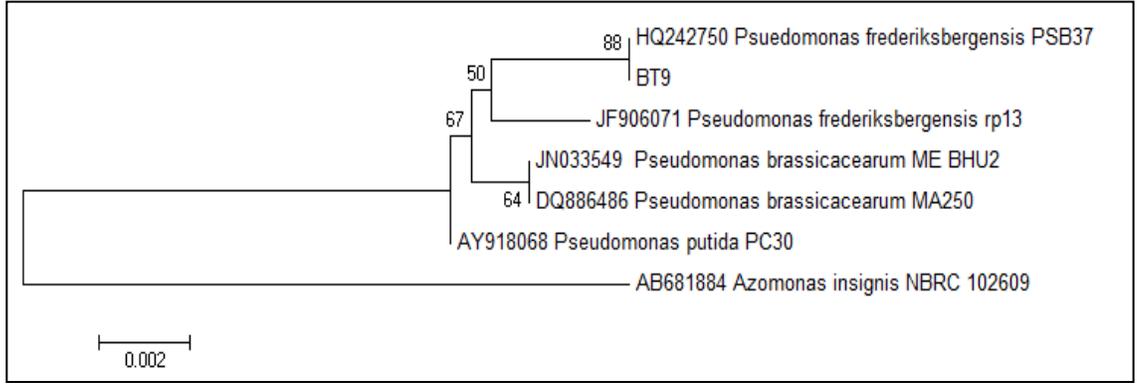


Figure 4.9 16S rDNA gene based phylogenetic analysis of *P. frederiksbergensis* BT9.

Legend:

— : Horizontal bars at the bottom of each tree represent evolutionary distances as changes per nucleotide. Numbers next to the branch nodes represent gene accession numbers.

4.6 Nucleotide Sequence Depositions

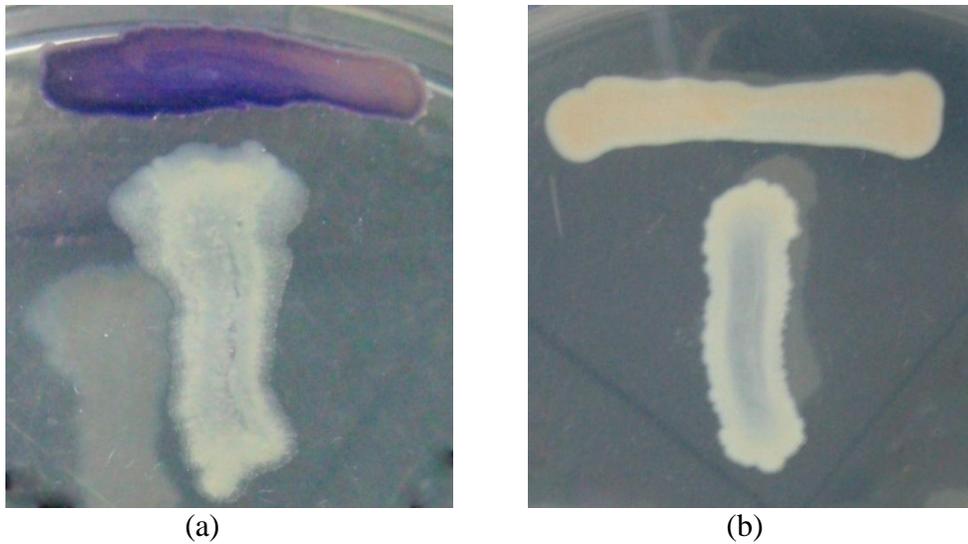
The 16S rDNA sequences were deposited into GenBank through NCBI BankIt submission tool. The acquired GenBank accession numbers for each of the deposited sequence are shown in Table 4.3.

Table 4.3 Assigned GenBank accession numbers for the identified isolates.

| Strain | Definition | GenBank Accession Number |
|--------|---|--------------------------|
| BT1 | <i>Bacillus</i> sp. strain BT1 | JN695052 |
| BT2 | <i>Bacillus</i> sp. strain BT2 | JN695053 |
| BT3 | <i>P. frederiksbergensis</i> strain BT3 | JN695055 |
| BT4 | <i>B. cereus</i> strain BT4 | JN695054 |
| BT5 | <i>P. frederiksbergensis</i> strain BT5 | JN695056 |
| BT6 | <i>Arthrobacter</i> sp. strain BT6 | JQ014617 |
| BT7 | <i>P. frederiksbergensis</i> strain BT7 | JN695057 |
| BT8 | <i>P. frederiksbergensis</i> strain BT8 | JN695058 |
| BT9 | <i>P. frederiksbergensis</i> strain BT9 | JN695059 |

4.7 CV026 Cross Streak

Preliminary screening for quorum sensing activities among the bacterial isolates was performed through cross streak with *C. violaceum* CV026 biosensor. Observation of purple pigment formation on the biosensor streak line would indicate the production of exogenous short chained AHL molecules from the tested isolates. Observation of purple pigmentation of the biosensor was illustrated on Figure 4.10 (a) whereas no AHL production was shown as in Figure 4.10 (b). Table 4.4 indicated the results from the CV026 cross streak. No violacein production was triggered upon cross streaking with isolates *Bacillus* sp. BT1 and BT2, *B. cereus* BT4 and *Arthrobacter* sp. BT6 as these isolates belong to the Gram positive bacteria whereby no AHL production was expected. Hence these isolates were not included in subsequent experiments that involved AHL extraction and AHL detection via liquid chromatography mass spectrometry (LCMS).



Legend:

- (a): CV026 cross streak with *P. frederiksbergensis* strain BT3
- (b): CV026 cross streak with *Bacillus* sp. strain BT1

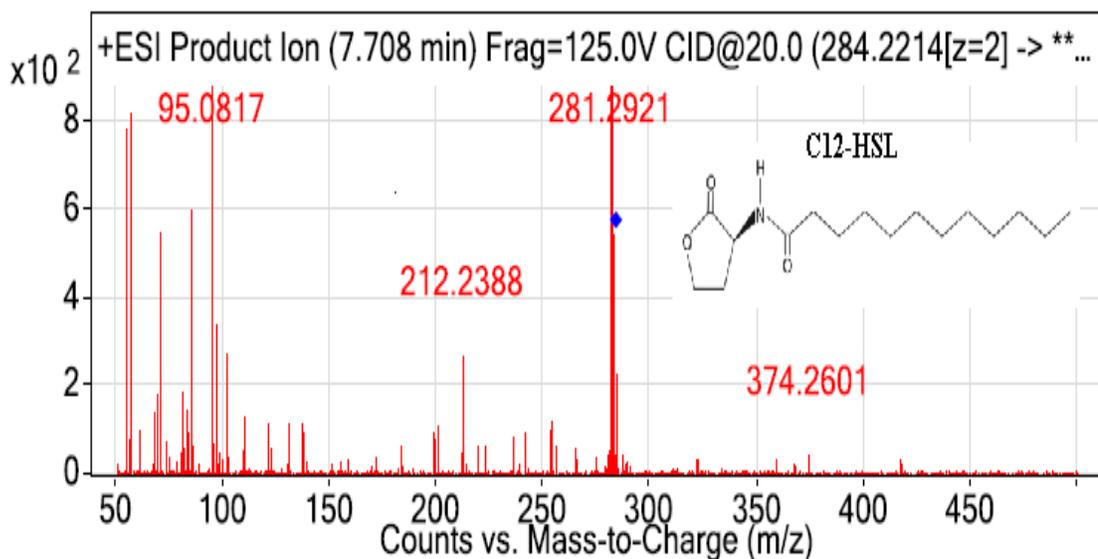
Figure 4.10 Representative result of detection of violacein production using CV026 cross streak.

Table 4.4 Results from the CV026 cross streak on the isolates

| Isolate | Violacein production |
|---|----------------------|
| <i>Bacillus</i> sp. strain BT1 | - |
| <i>Bacillus</i> sp. strain BT2 | - |
| <i>P. frederiksbergensis</i> strain BT3 | + |
| <i>B. cereus</i> strain BT4 | - |
| <i>P. frederiksbergensis</i> strain BT5 | + |
| <i>Arthrobacter</i> sp. strain BT6 | - |
| <i>P. frederiksbergensis</i> strain BT7 | + |
| <i>P. frederiksbergensis</i> strain BT8 | + |
| <i>P. frederiksbergensis</i> strain BT9 | + |
| <i>E. carotovora</i> Attn | + |
| <i>E. carotovora</i> A20 | - |

4.8 LCMS Analysis of AHL Extracts

Overnight cultures of QS isolates were subjected to AHL extraction as described in Chapter 3.10.2. The extracts were then analyzed with Agilent 6500 Q-TOF LCMS system and Agilent MassHunter software was used for the MS data analysis. The presence of *N*-dodecanoyl-L-homoserine lactone (C12-HSL) (m/z value of 284.2214) as depicted in Figure 4.11 was confirmed in the extract of isolate BT9 from the mass spectrometry results. No AHL molecules were detected from extracts of other isolates although these isolates were known to trigger violacein production from biosensor CV026 indicating the occurrence of short chain AHL production from these isolates.



Legend: ◆ Identification of C12-HSL at m/z value of 284.2214

Figure 4.11 ESI-MS spectrum of C12-HSL (m/z 284.2214, 7.708 min)

4.9 QQ Activities of Montane Soil Bacterial Isolates

4.9.1 CV026 Biosensor Overlay

The assessments of QQ activities among the bacterial isolates was carried out using CV026 biosensor overlay and RRLC analysis as described in Section 3.11.2 and Section 3.11.3, respectively after the whole cell inactivation assay. All the isolates were initially screened for QQ activity with CV026 overlay by incubating *N*-hexanoyl-L-homoserine lactone (C6-HSL) with cell suspension of each isolates for 0 and 24 h. Results from the CV026 overlay was depicted in Figure 4.12 and Table 4.5 where isolates *Bacillus* sp. BT1 and BT2, *B. cereus* BT4, *Arthrobacter* sp. BT6, and *P. frederiksbergensis* BT8 and BT9 had shown significant degradation of C6-HSL leaving lesser or undetectable amounts of the AHLs by the biosensor.

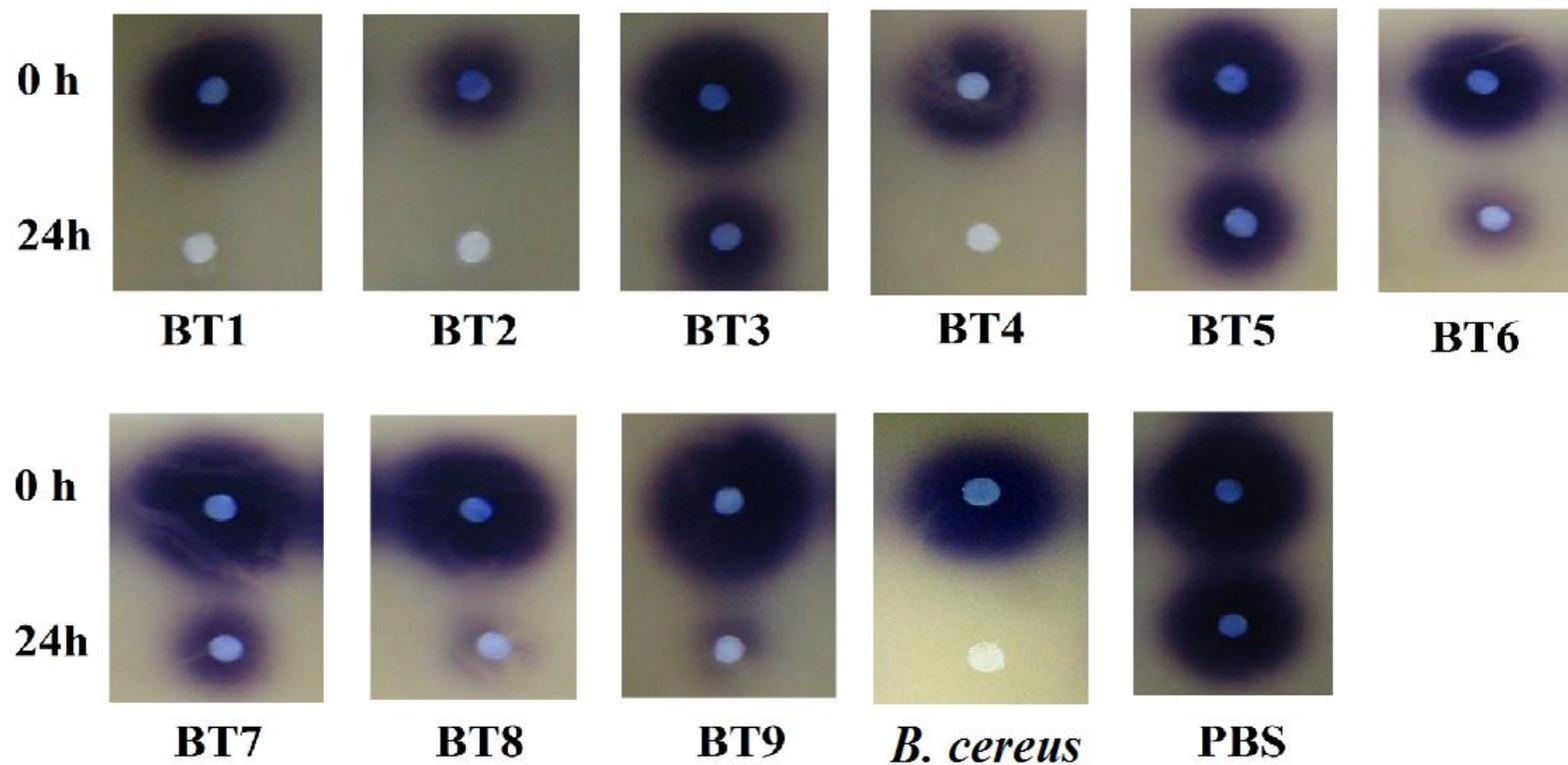


Figure 4.12 Detection of QQ activities using CV026 overlay. Substrate C6-HSL (0.2 $\mu\text{g}/\mu\text{l}$) was incubated with bacterial cell suspensions for 0 h and 24 h. Positive result in QQ activity was depicted by the reduction or abolishment of purple pigments after 24 h of incubation. The positive and negative controls were *B. cereus* and PBS, respectively.

Table 4.5 **Detection of C6-HSL degradation activities using CV026 overlay**

| Isolate | C6-HSL degradation |
|---|---------------------------|
| <i>Bacillus</i> sp. strain BT1 | ++ |
| <i>Bacillus</i> sp. strain BT2 | ++ |
| <i>P. frederiksbergensis</i> strain BT3 | - |
| <i>B. cereus</i> strain BT4 | ++ |
| <i>P. frederiksbergensis</i> strain BT5 | - |
| <i>Arthrobacter</i> sp. strain BT6 | ++ |
| <i>P. frederiksbergensis</i> strain BT7 | + |
| <i>P. frederiksbergensis</i> strain BT8 | ++ |
| <i>P. frederiksbergensis</i> strain BT9 | ++ |
| <i>B. cereus</i> (Positive control) | ++ |
| PBS (Negative control) | - |

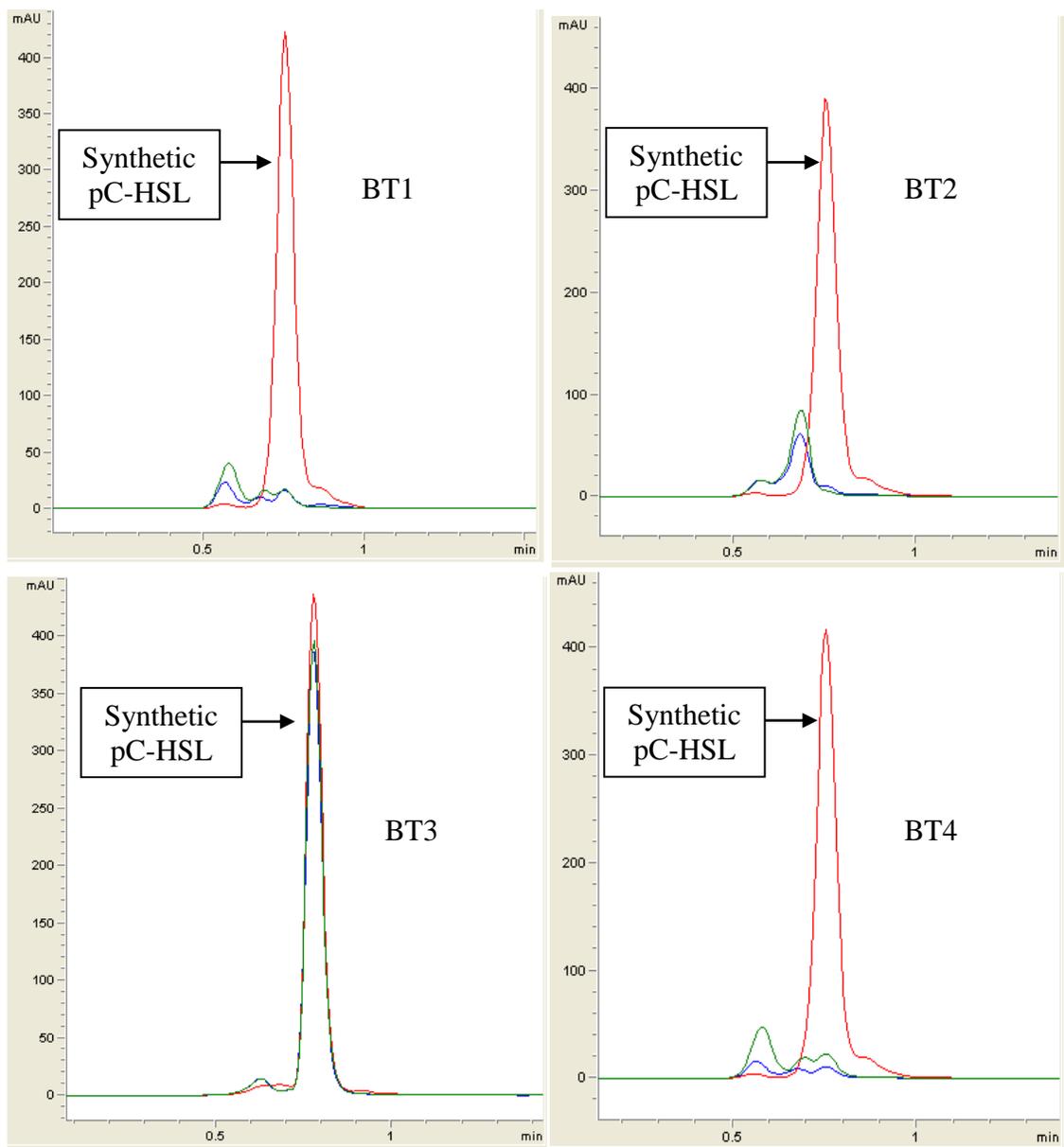
Legend:

- ++ : Strong quenching activity
- + : Modest quenching activity
- : No activity

4.9.2 RRLC Analysis of Various AHLs Degradation

RRLC analysis was performed after the incubation of the cell suspensions for 0, 3 and 24 h with *p*-coumaroyl homoserine lactone (pC-HSL) as substrate for degradation. Degradation of pC-HSL (elution time of 0.75 ± 0.01 min) was monitored at detection wavelength of 306 nm. The reduction of milli-absorbance unit (mAU) in the chromatogram corresponds to degradation of the substrate AHLs. Significant degradation of pC-HSL was observed for *Bacillus* sp. isolates BT1 and BT2, *B. cereus* BT4, *Arthrobacter* sp. BT6, and *P. frederiksbergensis* BT8 and BT9 with no degradation being demonstrated from *P. frederiksbergensis* isolates BT3 and BT5 followed by modest degradation from *P. frederiksbergensis* isolate BT7. *B. cereus* and PBS was employed as positive and negative control respectively for the RRLC analysis. Figure 4.13 illustrated the observed chromatograms of both positive and negative results for pC-HSL degradation.

Furthermore, isolates *Bacillus* sp. isolates BT1 and BT2, *B. cereus* BT4, *Arthrobacter* sp. BT6, and *P. frederiksbergensis* BT8 and BT9 was also tested with substrates *N*-decanoyl-L-homoserine lactone (C10-HSL), *N*-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) representing long chain and oxo group substituted AHLs. Detection of both AHLs were carried out at elution time of 0.95 ± 0.02 min and at 210 nm detection wavelength. Degradation of the AHLs was depicted by the reduction of milli-absorbance unit (mAU) in the chromatogram as shown in Figure 4.14 and Figure 4.15. Significant degradation of both tested AHLs were observed for all the tested isolates. *B. cereus* and PBS was also served as positive and negative control, respectively for the analysis. Results from the RRLC analysis was shown in Table 4.6.



Legend:

- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

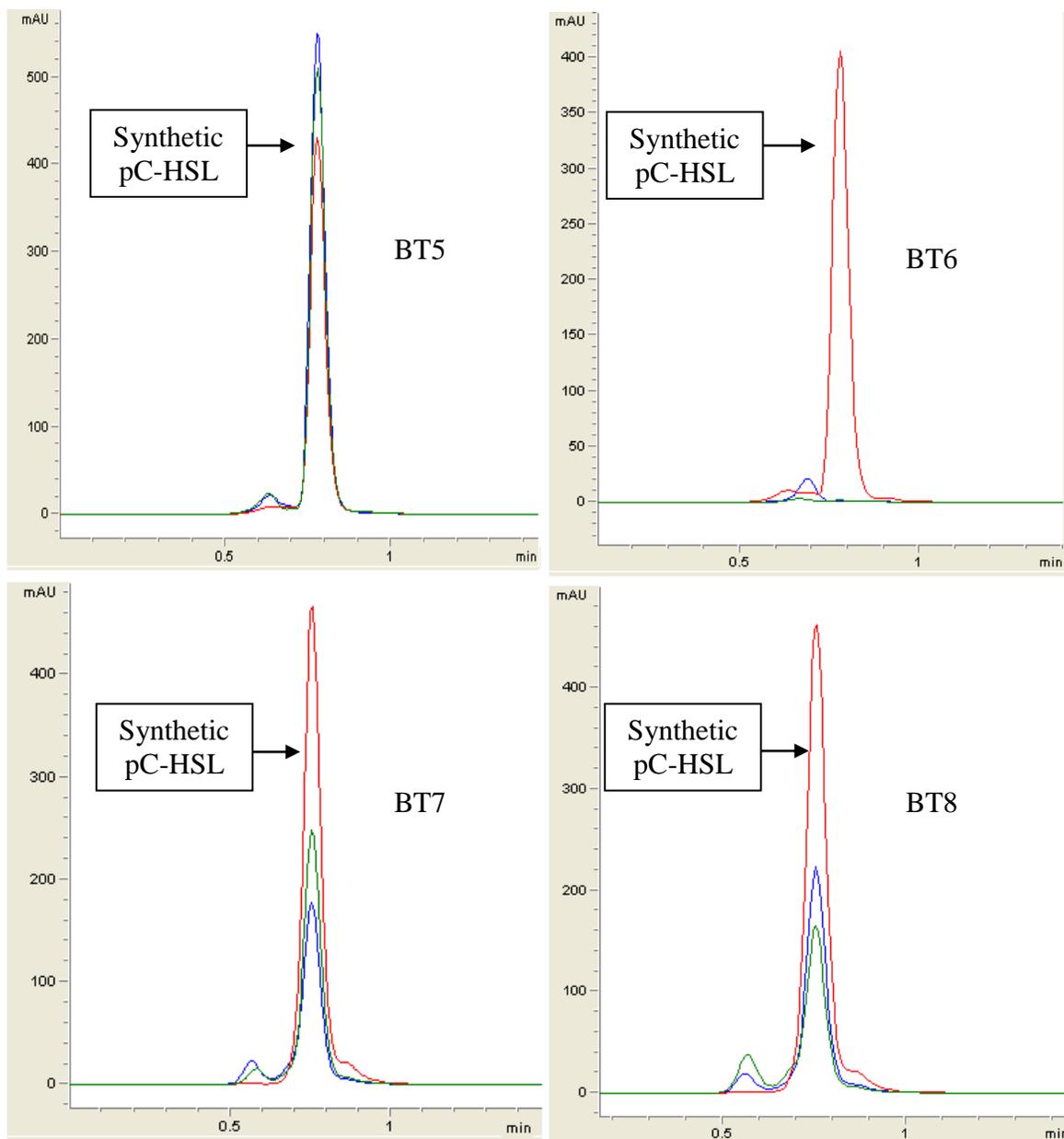
BT1: *Bacillus* sp. strain BT1

BT2: *Bacillus* sp. strain BT2

BT3: *P. frederiksbergensis* strain BT3

BT4: *Bacillus cereus* strain BT4

Figure 4.13 RRLC analysis of pC-HSL degradation.



Legend:

- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

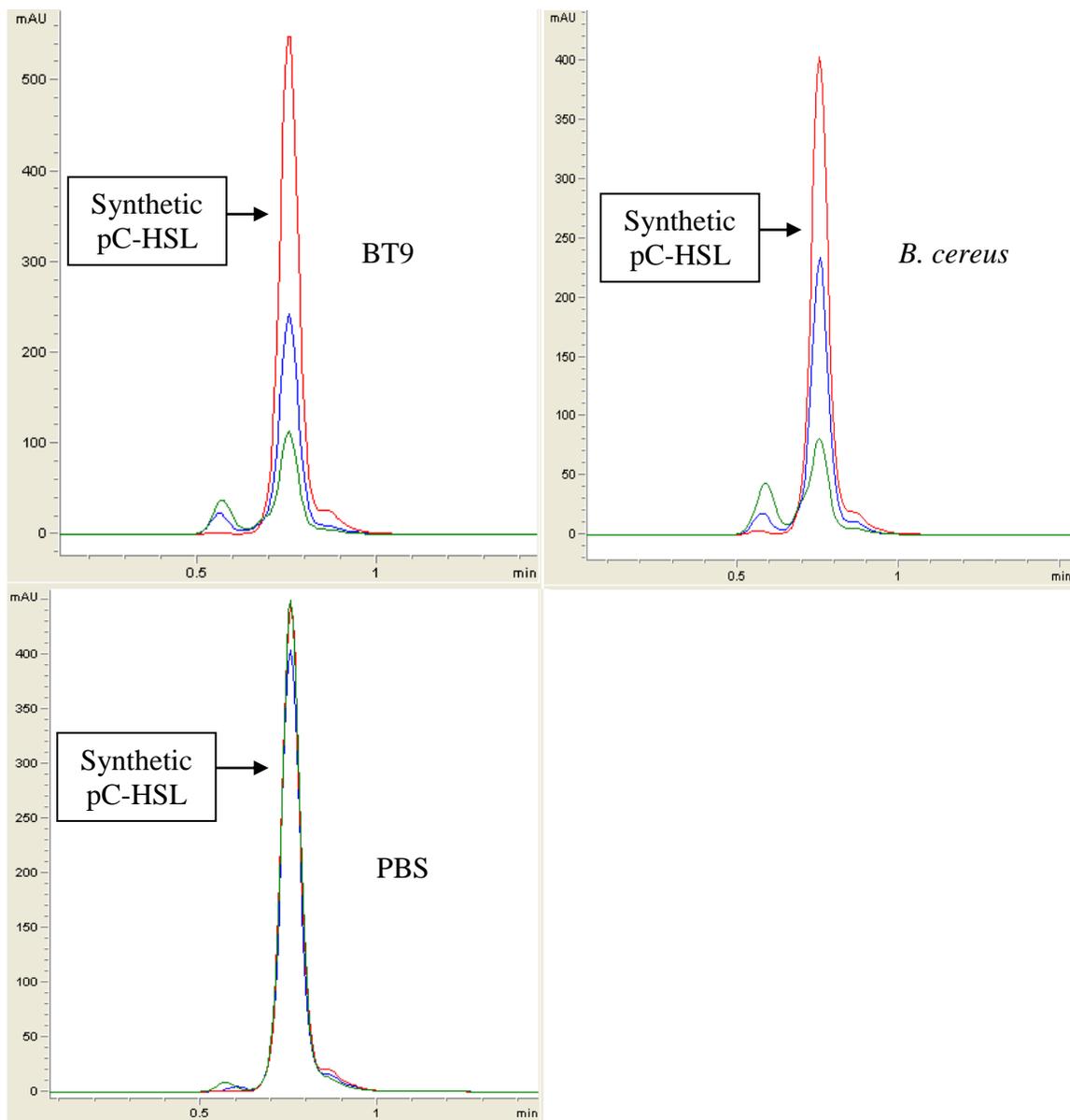
BT5: *P. frederiksbergensis* strain BT5

BT6: *Arthrobacter* sp. strain BT6

BT7: *P. frederiksbergensis* strain BT7

BT8: *P. frederiksbergensis* strain BT8

Figure 4.13 RRLC analysis of pC-HSL degradation (Continued).



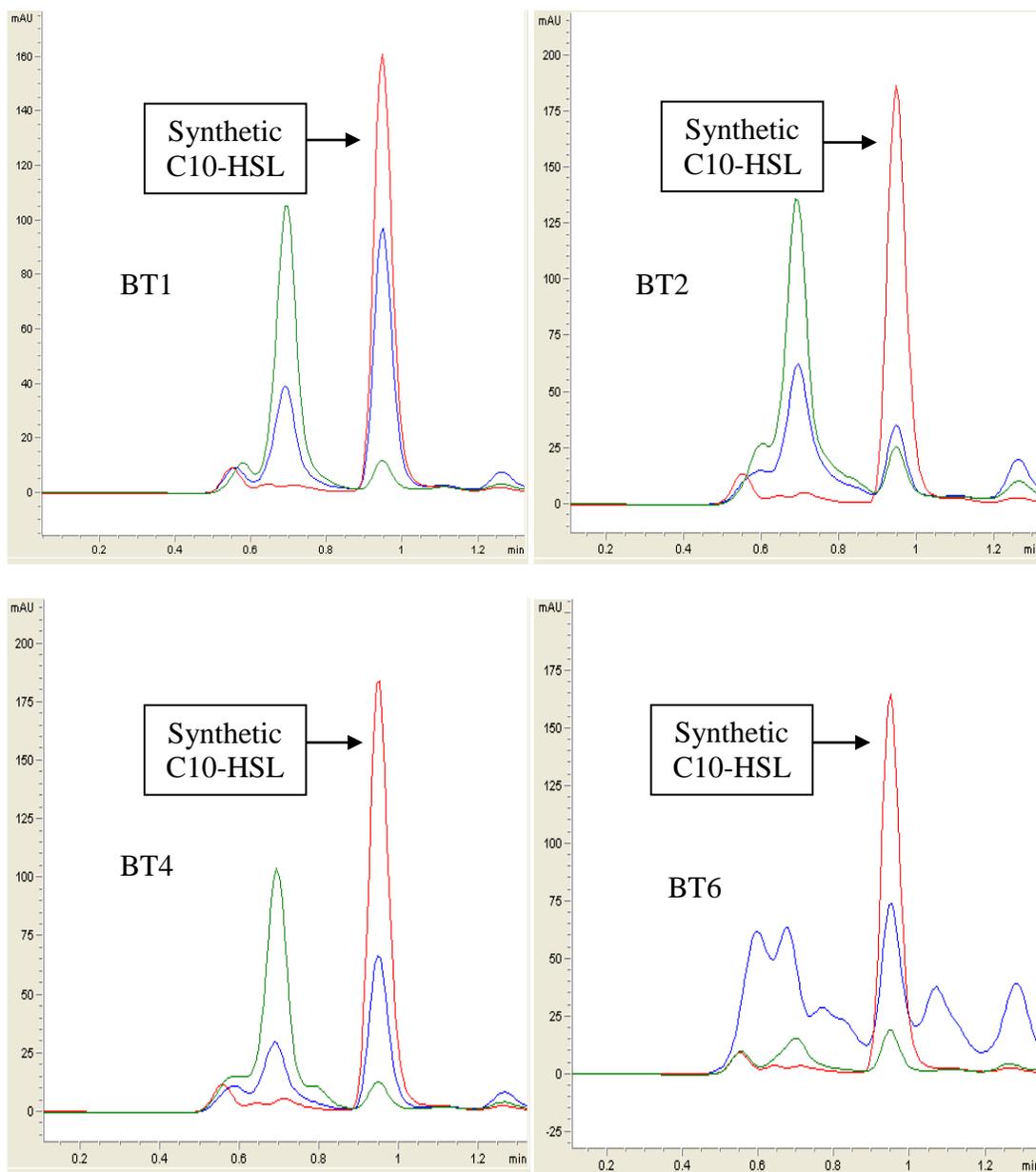
Legend:

- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

BT9: *P. frederiksbergensis* strain BT9

PBS: Phosphate buffered saline

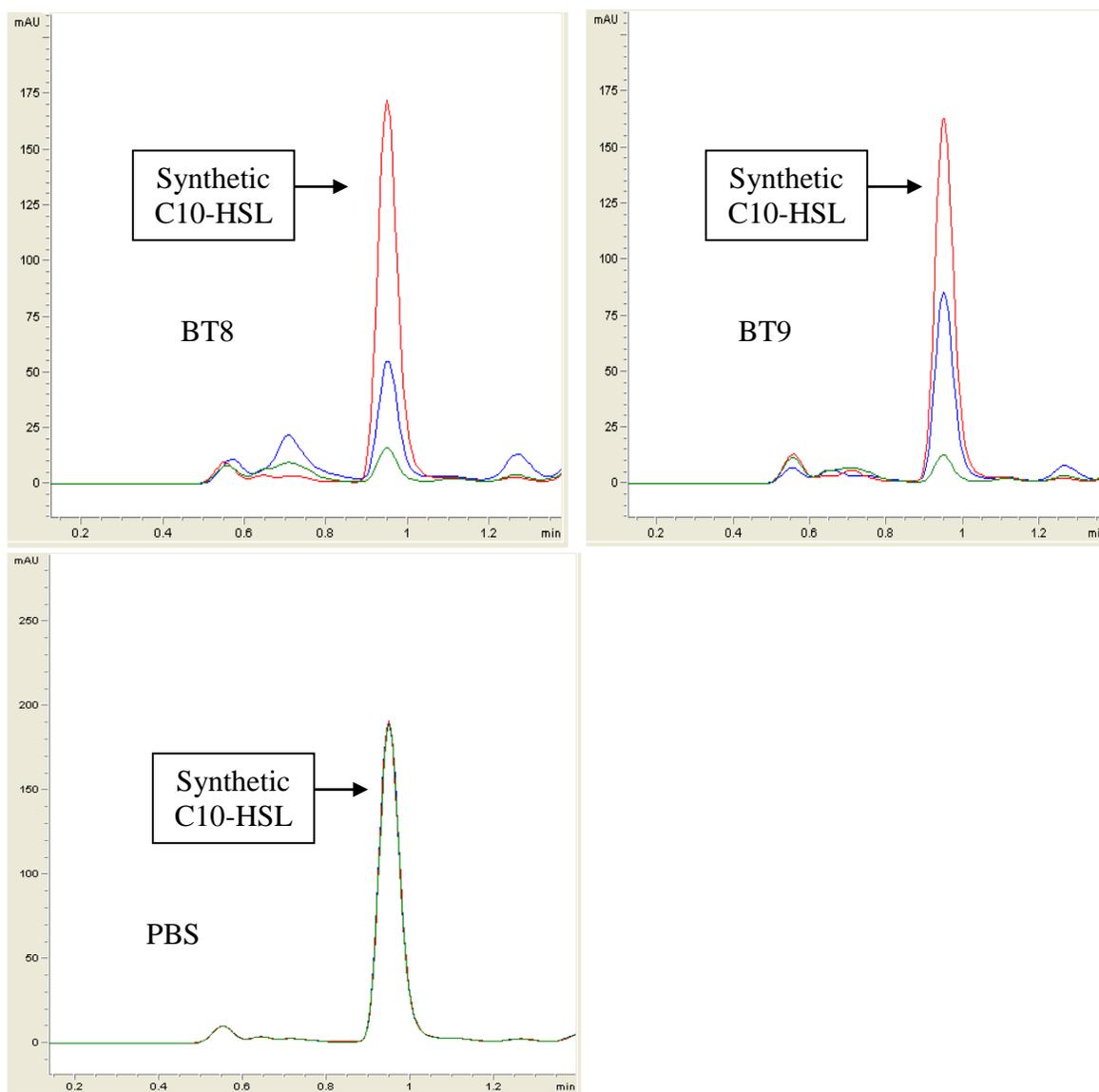
Figure 4.13 RRLC analysis of pC-HSL degradation (Continued).



Legend:

- 0 h incubation time
 - 3 h incubation time
 - 24 h incubation time
- BT1: *Bacillus* sp. strain BT1
 BT2: *Bacillus* sp. strain BT2
 BT4: *Bacillus cereus* strain BT4
 BT6: *Arthrobacter* sp. strain BT6

Figure 4.14 RRLC analysis of C10-HSL degradation.



Legend:

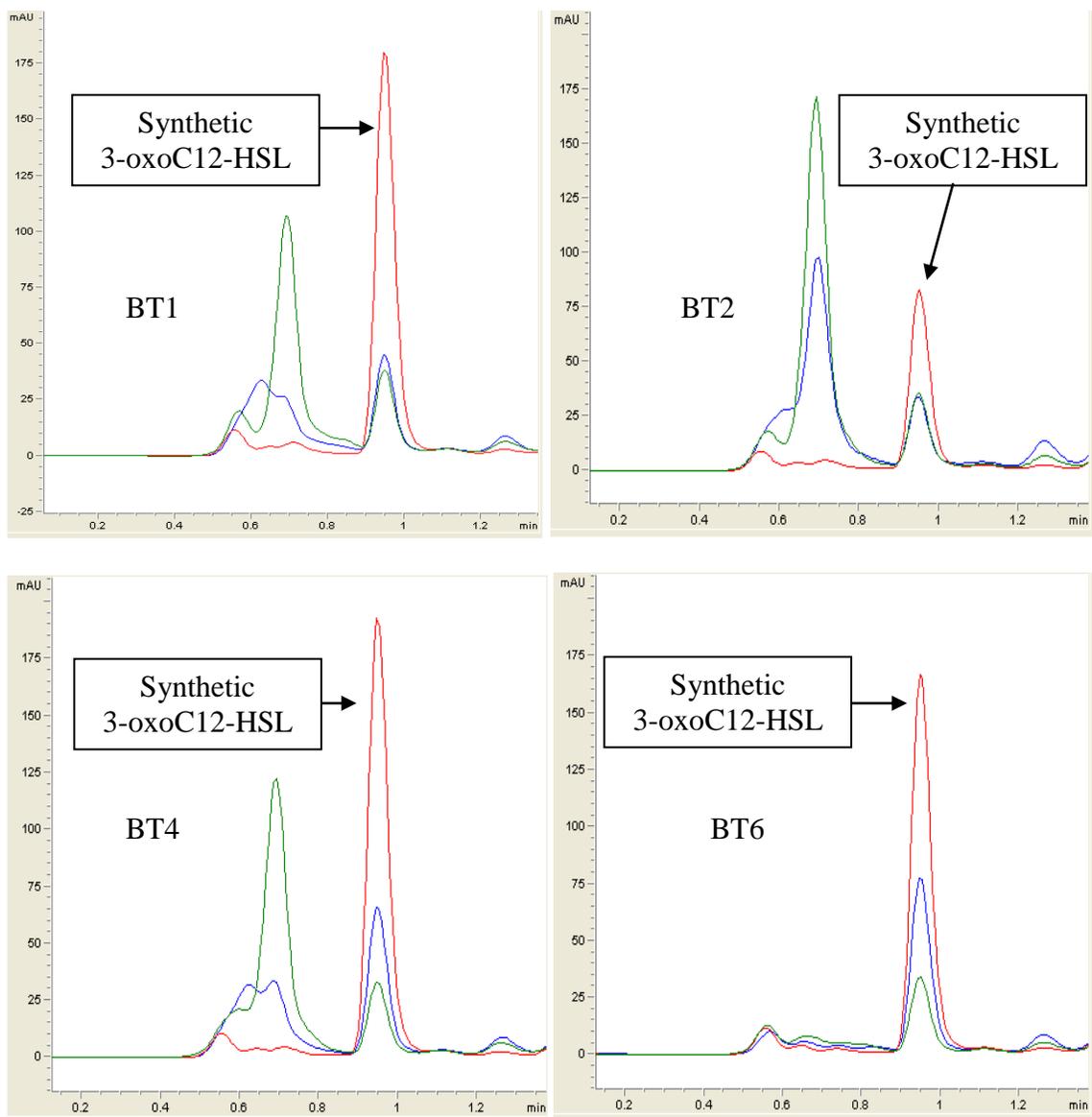
- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

BT8: *P. frederiksbergensis* strain BT8

BT9: *P. frederiksbergensis* strain BT9

PBS: Phosphate buffered saline

Figure 4.14 RRLC analysis of C10-HSL degradation (Continued).



Legend:

- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

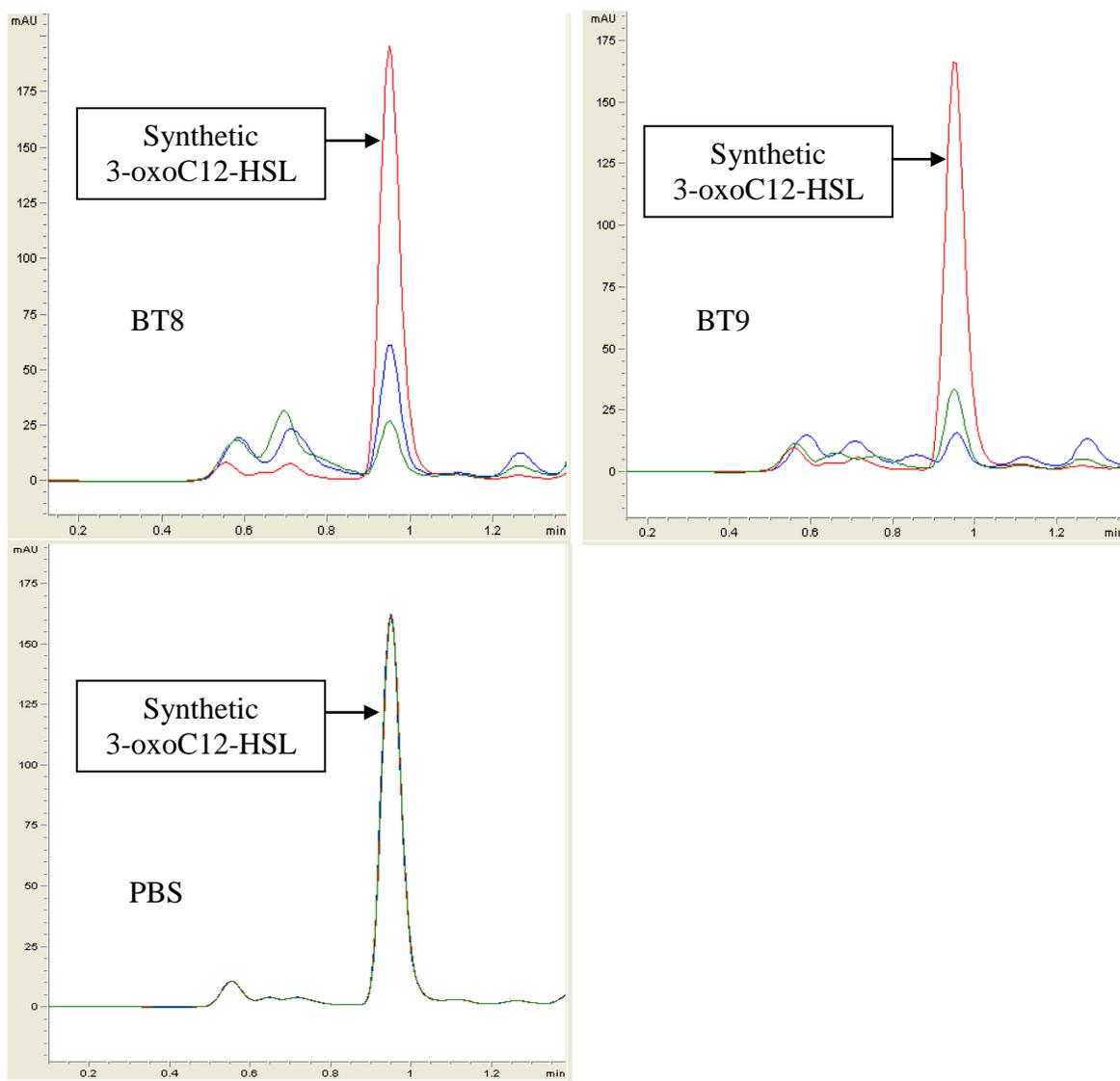
BT1: *Bacillus* sp. strain BT1

BT2: *Bacillus* sp. strain BT2

BT4: *Bacillus cereus* strain BT4

BT6: *Arthrobacter* sp. strain BT6

Figure 4.15 RRLC analysis of 3-oxo-C12-HSL.



Legend:

- 0 h incubation time
- 3 h incubation time
- 24 h incubation time

BT8: *P. frederiksbergensis* strain BT8

BT9: *P. frederiksbergensis* strain BT9

PBS: Phosphate buffered saline

Figure 4.15 RRLC analysis of 3-oxo-C12-HSL (Continued).

Table 4.6 RRLC analysis for various AHL degradations.

| Isolate | pC-HSL degradation | C10-HSL degradation | 3-oxo-C12-HSL degradation |
|---|---------------------------|----------------------------|----------------------------------|
| <i>Bacillus</i> sp. strain BT1 | + | + | + |
| <i>Bacillus</i> sp. strain BT2 | + | + | + |
| <i>P. frederiksbergensis</i> strain BT3 | - | N.D | N.D |
| <i>B. cereus</i> strain BT4 | + | + | + |
| <i>P. frederiksbergensis</i> strain BT5 | - | N.D | N.D |
| <i>Arthrobacter</i> sp. strain BT6 | + | + | + |
| <i>P. frederiksbergensis</i> strain BT7 | + | N.D | N.D |
| <i>P. frederiksbergensis</i> strain BT8 | + | + | + |
| <i>P. frederiksbergensis</i> strain BT9 | + | + | + |
| <i>B. cereus</i> (Positive control) | + | + | + |
| PBS (Negative control) | - | - | - |

Legend:

+: Significant degradation was observed

- : No significant degradation was observed

N.D: Not Determined

CHAPTER 5.0

DISCUSSION

5.1. Isolation and Identification of Bacterial Strains

Soil from Malaysian montane area was selected as isolation source in this study as to assess the QS and QQ abilities of bacteria inhabiting soils at elevated altitudes and landscapes. To our knowledge, no work has been reported on QS and QQ in tropical montane soil. Subsequent isolation procedure had resulted in nine bacteria isolates where all isolates shared 99% similarity to *Arthrobacter* sp., *Bacillus* and *P. frederiksbergensis* based on BLAST search and the identities were further supported by phylogenetics analysis.

Three bacilli were identified in the study with isolates *Bacillus* sp. BT1, BT2 and isolate BT4 specifically as *Bacillus cereus*. *Bacillus* as one of the widely characterized bacterial genera is a member of phylum Firmicutes that can be obligate aerobes or facultative anaerobes (Kunst *et al.*, 1997). Naturally, in response to environmental or nutritional stresses, the cells of *Bacillus* produce highly resistant dormant endospores in which the dispersal of the spores arguably reflects the robust presence of this bacteria in diverse environments (Earl *et al.*, 2008).

One of the isolates namely BT6 was identified as *Arthrobacter* sp. that is known for its prevalence in common soils and some extreme environments (Mongodin *et al.*, 2006). Interestingly, KG medium has never been able to enrich *Arthrobacter* from tropical soil (Chan *et al.*, 2010). The genus of *Arthrobacter* was described to be obligate aerobes from the Actinobacteria phylum which was distinguished from the genus of *Corynebacterium* due to several morphological dissimilarities (Jones and Keddie, 1992).

The study has also interestingly identified five *P. frederikbergensis* isolates namely BT3, BT5, BT7, BT8 and BT9. The *Pseudomonas* genus belonging to the phylum Proteobacteria is known to exhibit various metabolic activities and consequently colonises a wide range of niches (Euzéby, 1997; Spiers *et al.*, 2000). The *Pseudomonas* species identified in this study was first nomenclated by Andersen *et al.*, 2000 after isolation of this phenanthrene-degrading bacteria from coal gasification site in Frederiksberg, Copenhagen, Denmark. Some of the descriptions of this species by Andersen *et al.* (2000), include smooth and pale yellowish colonies, does not produce fluorescence under UV emission, and shows no growth at 37 °C and presence of D-xylose. Morphological and biochemical properties of the isolated *P. frederikbergensis* in this study were shown to fit the descriptions mentioned in the article. The match of phenotypic characteristics coupled with phylogenetic analysis has further infer the presence of this particular *Pseudomonas* species in highland soil.

Despite the ubiquity in soil environment, members of *Pseudomonas*, *Bacillus* and *Arthrobacter* genera are also well known for their resistance against chemicals (Chen and Tomasek, 1991; Setlow, 2006; Schweizer *et al.*, 1999). This may be the adaptive behaviour of these isolates found in tea plantation where pesticides and inorganic fertilizers are widely used. For instance, members of *Bacillus* have been known to resistant against diphenyl ether herbicides and decompose a series of phenylamide and urea herbicides (Lee *et al.*, 2000; Engelhardt *et al.*, 1973; Wallnöfer, 1969). *Arthrobacter aurescens* was found to metabolize herbicide atrazine into cyanuric acid regulated by *trzN*, *atzB* and *atzC* genes (Sajjaphan *et al.*, 2004). *Pseudomonas* strains have also demonstrated significant capacity in degrading substrates consisting of aromatic and halogenated derivatives as well as recalcitrant organic residues (Spiers *et al.*, 2000). Thus, selective environment created by

the altered soil biodiversity would have favored the growth of certain microorganisms that includes *Pseudomonas*, *Bacillus* and *Arthrobacter* in this study.

5.2. AHL Production of *P. frederiksborgensis* Isolates

Preliminary screening for AHL production among the isolates in this study was performed with CV026 cross streak. All *Pseudomonas* isolates triggered the violacein production of biosensor CV026 suggesting production of short chain AHL.

For precise identification of AHLs produced, spent supernatants of QS positive bacterial isolates were extracted with acidified ethyl acetate and analyzed with Agilent 6500 Q-TOF LCMS system. Prior to the extraction, bacteria were cultured in LB broth buffered with 50 mM of MOPS to pH 5.5 to avoid spontaneous AHL degradation induced by pH changes during incubation (Chan *et al.*, 2009). Extraction was then carried out during early stationary phase or at least late exponential phase which is after 18 to 20 hours of incubation with addition of acidified ethyl acetate. Acidification of ethyl acetate with acetic acid was to minimize the amount of lactone hydrolysis during extraction and storage (Gould *et al.*, 2006).

Mass spectrometry analysis of the supernatant of *P. frederiksborgensis* isolate BT9 revealed a peak at m/z value of 284.2214 indicating the molecular ion (M+H) of C12-HSL (Niu *et al.*, 2008). As such production of C12-HSL by *P. frederiksborgensis* isolate BT9 was deduced. However, MS analysis did not reveal any AHL production from other isolates although these isolates have triggered CV026 violacein production. One possible explanation for such discrepancy would be the degradation of AHL by the *Pseudomonas* isolates during the incubation period. Since the culture pH was well maintained with MOPS, the likelihood of abiotic alkaline hydrolysis of the produced AHL would be

minimal. It is speculated that the AHLs have been enzymatically degraded by the *Pseudomonas* isolates at a certain time point during the incubation. This is supported by studies showing the fluctuation of AHL concentration at different growth phase of bacteria is associated with expression of AHL degrading enzymes (AHLase) that are growth phase dependent. For instance, concentration of 3-oxo-C12-HSL in *P. aeruginosa* PAO1 was heavily reduced by AHL acylase when the growth entered early stationary phase (Huang *et al.*, 2003). Elevated expression of AHL lactonase coupled with sharp decline of 3-oxo-C8-HSL level in *A. tumefaciens* was also observed during stationary phase (Zhang *et al.*, 2002). As such, during the harvest time of 18-20 h, the expression of AHLase in *Pseudomonas* isolates in this experiment would have resulted in degradation of AHLs into amounts that fell below the detection limit of the LCMS instrument even though detection of synthetic AHL standards was feasible in this experiment. Besides, for the explanation of positive results in CV026 cross streak, the diffusion of AHLs in solid agar in contrast with liquid media, is not affected by the existing AHLase in the isolate itself. This can be verified with CV026 cross streak with co-culture of QS positive *E. carotovora* Attn and *Bacillus* sp. that expresses AHLase (Appendix 3). Violacein production was still evident in CV026 indicating the diffusion of AHL produced by *E. carotovora* Attn was not influenced by the presence of AHLase producing *Bacillus* sp.

5.3. Degradation of AHLs

AHL degradation activity among the isolates was first assessed with whole cell inactivation assay using C6-HSL as substrate followed by CV026 overlay. These isolates were then tested against pC-HSL as substrate followed by detection using RRLC analysis. As a result, *Bacillus* sp. isolates BT1 and BT2, *B. cereus* BT4, *Arthrobacter* sp. BT6, and *P. frederiksbergensis* isolates BT8 and BT9 had showed significant degradation of both C6-HSL and pC-HSL. On the other hand, degradation of both AHLs was not observed in *Pseudomonas* isolates BT3 and BT5. This would imply that both isolates do not exhibit QQ phenotypes or possess substrate specific AHLase that do not target the tested AHLs.

Subsequently, isolates demonstrating significant C6-HSL and pC-HSL degradation were then tested for C10-HSL and 3-oxo-C12-HSL that represent long chain and oxo group substituted AHL substrates, respectively. Significant degradation of the tested AHLs was observed for all six tested isolates as shown in Table 4.6.

Results from whole-cell AHL inactivation assay and RRLC analyses on degradation of various AHL substrates indicated strong QQ activity with broad substrate specificity among the isolates of the genera of *Pseudomonas*, *Bacillus* and *Arthrobacter*. Such data illustrated that these soil QQ isolates exhibited broad AHL inactivation activity regardless of the AHL molecules *N*-acyl side chain length and degree of saturation at C3 position.

The QQ properties of these isolates have been well documented. For instance, QQ of *Bacillus* has been known to confer lactonase activity in hydrolyzing the lactone ring of the AHL. One of the well characterized lactonase homologs would be the AiiA homologs from soil bacterium *Bacillus* sp. containing zinc binding motif HXHXDH. (Dong *et al.*, 2001, Dong *et al.*, 2002). Besides, in 2003, Park *et al.* reported an AHL lactonase isolated

from *Arthrobacter* termed as AhlD, also revealed the presence of HXHXDH motif in the deduced amino acid sequence. Phylogenetic analyses reflecting the sequence variation of various AHL degrading lactonases revealed low sequence homology between AiiA and AhlD enzymes despite both sharing the highly conserved motif that is essential for activity (Dong and Zhang 2005). On the other hand *Pseudomonas* has been identified to produce QQ acylase enzymes that target the amide linkage between fatty acid chain and homoserine lactone moiety. The reported AHL acylase found to be expressed in *Pseudomonas* include QuiP (PA1032) and PvdQ (PA2385) from *P. aeruginosa* followed by HacA (P syr_1971) and HacB (P syr_4858) from *P. syringae* strain B728A (Huang *et al.*, 2003; Huang *et al.*, 2006; Shepherd and Lindow, 2009).

5.4 Degradation of Aroyl-HSL

Aroyl-HSL is nomenclated based on the presence of aromatic side chain whereby in this experiment the *p*-coumaroyl HSL contains moiety derived from aromatic acid namely *p*-coumarate (Cooley *et al.*, 2008). In addition to a recent work by Momb *et al.*, 2010 that described the QQ activity of AiiA homolog from *B. thuringiensis* against aroyl HSL, *Arthrobacter* and *Pseudomonas* isolates in this study were also found to inactivate pC-HSL. Such observation may offer an addition to the collection of aroyl HSL degraders. However the mechanism of pC-HSL inactivation by these isolates remains unknown. Nonetheless in addition to degradation of other AHLs in this study, recognition of AHL substrates by the enzyme active sites appeared not to be influenced by the length or substitution of the AHL side chain.

5.5 AHL Signalling Turnover in Soil Environment

The diverse distribution of QQ bacteria in this particular highland soil environment is evident with these QQ isolates from three distinct phyla exert two different AHL degradations. This implies the prevalence of active QQ microorganisms responsible for the turnover of QS compounds in the environment though the precise ecological role for the QQ mechanism remains unknown. However, it is proposed that environmental competition has led to the expression of AHL degrading enzymes for the aim of insulating the signalling activities of AHL producing Gram negative bacteria (Park *et al.*, 2006; Lee *et al.*, 2002). Degradation of AHL molecules may also implicate the utilization of AHLs as source of carbon and nitrogen. Leadbetter and Greenberg, 2000 demonstrated the use of homoserine lactones (HSL) as nitrogen source by *V. paradoxus* based on observation of cleavage and mineralization of the HSL ring. Studies conducted by Yang *et al.*, 2006 also described the utilization of HSL as sole carbon source by soil *Arthrobacter* and *Burkholderia*. Mineralization of the ¹⁴C- radiolabeled AHL were found to be recovered as ¹⁴CO₂ thus implicating the biological degradation of signalling molecules as a mean of preventing accumulation of these signalling molecules in the environment (Wang and Leadbetter, 2005; Yang *et al.*, 2006)

5.6 Future Work

Future studies would involve verification of QQ properties of isolates exhibiting strong QQ activities such as *Bacillus* sp. BT1 and BT2, *B. cereus* BT4, *Arthrobacter* sp. BT6, and *P. frederiksbergensis* BT8 and BT9. One of the approach to determine which QQ mechanism exerted by the isolation is by monitoring the AHL degradation product. As AHL acylases cleave the amide bond of AHL producing homoserine lactone (HSL), this degradation product could be detected via dansylation process. Release of HSL could be reacted chemically with 5-dimethylamino-1-naphthalenesulphonyl chloride (DANSYL chloride) which will hence increase the hydrophobicity of HSL allowing more resolved separation during chromatography (Lin *et al.*, 2003). On the other hand, AHL lactonases cleave the lactone ring of AHL that results in ring opening of the compound. However, the corresponding *N*-acylhomoserine could be converted back to AHL by acidification process that involve incubation at acidic pHs lower than pH 2.0 (Yates *et al.*, 2002). Alternatively, molecular work directed to clone the QQ genes among these isolates that include PCR amplification using specific primers followed by cloning and sequencing would also be required in the future. The interest to understand the global gene regulation and physiological effect of QS in *P. frederiksbergensis*, whole genome sequencing of these isolates is currently being performed . Whole genome sequencing of *P. frederiksbergensis* is being performed based on the interest to understand the global gene regulation and physiological effect of both QS and QQ in this strain.

CHAPTER 6.0

CONCLUSION

Bacterial isolation from Malaysian tropical montane soil has yielded five *Pseudomonas* isolates, three *Bacillus* isolates and one *Arthrobacter* isolate based on 16S rDNA identification. LCMS analysis has identified the production of long chain AHL namely C12-HSL in spent supernatant of *P. frederiksbergensis* isolate BT9 via identification of a peak at m/z value of 284.2214. Assessment of QQ activity against various AHLs showed that six isolates belonging to *Pseudomonas*, *Bacillus* and *Arthrobacter* significantly degraded C6-HSL and pC-HSL. Further degradations of C10-HSL and 3-oxo-C12-HSL by these six isolates demonstrated strong QQ activity with broad AHL substrate specificity. Besides, C12-HSL producing *P. frederiksbergensis* isolate BT9 is showed the strongest AHL degradation activity indicative of the coexistence of QS and QQ system in the organism. Nonetheless further investigations are required to confirm QS-mediated genes of *P. frederiksbergensis* isolate BT9, which perhaps include QQ genes. Approaches in verifying the QQ properties among isolates exhibiting strong QQ activities were also essential.

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Appendix 1

16S rDNA Sequences

>*Bacillus* sp. BT1

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CCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGG
CAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA
CACGAGCTGACGACGACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAG
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TGAGTTTCAGCCTTGC GGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTAACT
TCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCG
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TCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTAC
GCATTTACCGCTACACATGGAATTCCAATTCCTCTTCTGCACTCAAGTCTCC
CAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATC

> *Bacillus* sp. BT2

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>*Pseudomonas frederiksbergensis* BT3

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>*Bacillus cereus* BT4

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> *Pseudomonas frederiksbergensis* BT5

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> *Arthrobacter* sp. BT6

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> *Pseudomonas frederiksbergensis* BT7

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> *Pseudomonas frederiksbergensis* BT8

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> *Pseudomonas frederiksbergensis* BT9

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Appendix 2

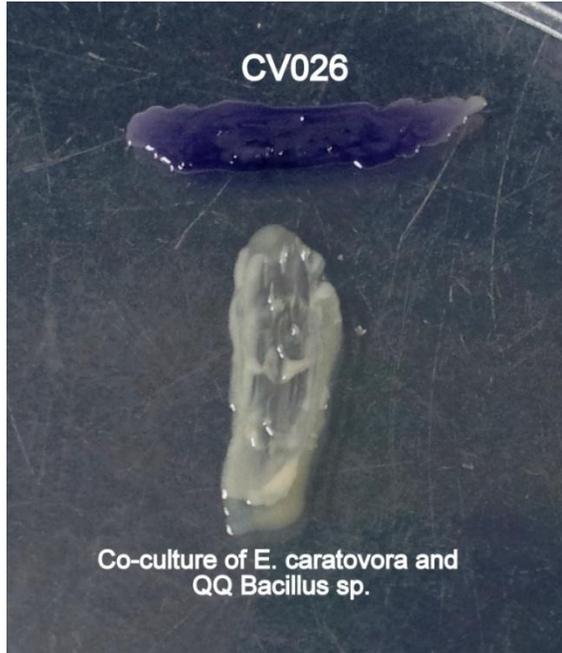


Image 1

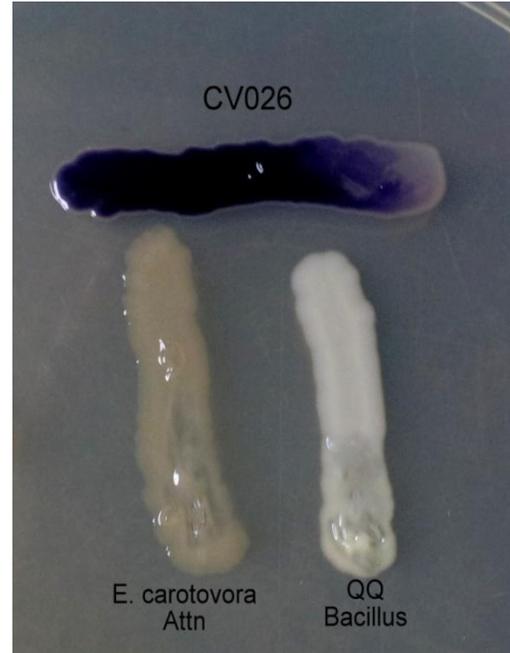


Image 2

CV026 cross streak experiment were performed with the QS positive *E. carotovora* Attn and *Bacillus* sp. that expresses AHLase. *E. carotovora* Attn and *Bacillus* sp. were streaked concurrently with CV026 in an agar plate (Image1) and co-culture of both strains was streaked as one line as in another agar plate (Image 2). Violacein production was observed in both plates with the presence of quorum quenching bacteria (*Bacillus* sp.) thus indicating the diffusion of AHL molecules in solid agar was not affected by presence of AHLase in the bacterial colonies.