ABSTRACT

A wide range of Proteobacteria are known to produce signalling molecules, such as *N*-acyl-homoserine lactones (AHLs) to regulate genes expression in a population-dependent manner, thereby enabling group behavior acted in unison. This mechanism of cell-to-cell communication is known as quorum sensing (QS). In a polymicrobial community, while some bacteria are communicating with neighboring cells, others are interrupting the communication, and the interruption of communication is known as quorum quenching (QQ). In this study, the production and degradation of AHLs by bacteria isolated from Ulu Slim Hot Spring were investigated. *Pseudomonas aeruginosa* and *Methylobacterium* sp. were found to synthesize AHL in this study. Significant QQ activity was discovered among *Bacillus megaterium*, *Gordonia* sp., *P. aeruginosa*, *Pseudomonas otitidis*, *Roseomonas* sp. and *Rhodotorula mucilaginosa* using triple quadrupole liquid chromatography mass spectrometry (LC/MS/MS-QQQ). Degradation of AHLs by *Gordonia* sp. and *Rhodotorula* (a fungus) was documented for the first time in this study. Selected bacterial isolates have been isolated and identified using both Microflex™ LT MALDI-TOF approach as well as 16S rRNA gene sequencing approach. The bacterial isolates include *Kocuria rhizophila* CN12, *Stenotrophomonas maltophila* F2, *Chelatococcus* sp. M3, *Providencia rettgeri* M22, *Dermacoccus nishinomiyaensis* M25, *Schineria* sp. M27, *Cronobacter sakazakii* M30, *Exiguobacterium aurantiacum* M31, *Brevibacterium casei* M40, *Proteus mirabilis* M48 and *Micrococcus luteus* M71.

ABSTRAK

Pelbagai anggota Proteobacteria telah dilaporkan menghasilkan molekul-molekul isyarat seperti *N*-asil homoserin lakton (AHL) untuk mengawal selia ekspresi gen bergantung kepada ketumpatan populasi. Pengawalan ekspresi gen membolehkan populasi bakteria menunjukkan tingkah laku berkumpulan dan bertindak secara serentak. Mekanisma komunikasi antara sel-sel bakteria dikenali sebagai pengesanan kuorum (QS). Di dalam sebuah komuniti bakteria, sementara sekelompok bakteria berkomunikasi dengan sel-sel bakteria yang berdekatan, terdapat sekumpulan bakteria yang mengganggu proses komunikasi tersebut. Gangguan komunikasi tersebut dikenali sebagai perencatan kuorum (QQ). Dalam kajian tersebut, bakteria-bakteria yang merembeskan AHL dan juga menguraikan AHL telah dikaji. Penguraian AHL telah ditemui di bakteria-bakteria *Bacillus megaterium*, *Gordonia* sp., *P. aeruginosa*, *P. otitidis*, *Roseomonas* sp. dan juga *Rhodotorula mucilaginosa*, dengan menggunakan kromatografi cecair selaras spektrometer jisim. Aktiviti penguraian AHL oleh *Gordonia* sp. dan *Rhodotorula* (sejenis kulat) adalah kali pertama dilaporkan. Terdapat juga beberapa bakteria yang telah diasingkan dan dikenali dengan menggunakan kaedah Microflex™ LT MALDI-TOF dan juga kaedah penjujukan DNA bagi gen 16S rRNA. Bakteria-bakteria tersebut adalah seperti *Kocuria rhizophila* CN12, *Stenotrophomonas maltophila* F2, *Chelatococcus* sp. M3, *Providencia rettgeri* M22, *Dermacoccus nishinomiyaensis* M25, *Schineria* sp. M27, *Cronobacter sakazakii* M30, *Exiguobacterium aurantiacum* M31, *Brevibacterium casei* M40, *Proteus mirabilis* M48 serta *Micrococcus luteus* M71.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
×	Times
× *g*	Gravity
°C	Celsius
µg	Microgram
µL	Microlittre
µm	Micron
µM	Micromolar
ACN	Acetonitrile
Acyl-ACP	Acetylated acyl carrier protein
A-factor	2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone
AGE	Agarose gel electrophoresis
AHL	*N*-acyl homoserine lactone
AI-2	Autoinducer 2
AI-3	Autoinducer-3
C12-HSL	*N*-dodecanoyl-$_L$-homoserine lactone
C4-HSL	*N*-butanoyl-$_L$-homoserine lactone
C6-HSL	*N*-hexanoyl-$_L$-homoserine lactone
C7-HSL	*N*-heptanoyl-$_L$-homoserine lactone
C8-HSL	*N*-octanoyl-$_L$-homoserine lactone
C10-HSL	*N*-decaanoyl-$_L$-homoserine lactone
DKP	Diketopiperazines
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
DPD	4,5-dihydroxy-2,3-pentandione
DSF	Diffusible signal factor
EDTA	Ethylenediaminetetraacetic acid
EPS	Exocapsular polysaccharide
HCCA	α- cyano-4-hydroxy-cinnamic acid
HHQ	2-heptyl-4-hydroxyquinolone
hr	Hour
HS Agar	Hot spring agar
HSL	Homoserine lactone
Kb	Kilobase pair
L	Littre
LB medium	Luria-Bertani medium
LCMS	Liquid chromatography mass spectrometry
LC/MS/MS-QQQ	Triple Quadrupole Liquid Chromatography Mass Spectrometry
LuxI	Autoinducer synthase
M	Molarity
MBL	Metallo-β-lactamase
m/z	Mass to charge ration
mA	Miliampere
MALDI-TOF	Matrix-assisted laser desorption/ionization with time of flights
MEGA	Molecular Evolutionary Genetic Analysis
Min	Minute
Ml	Milliliter
mM	Milimolar

MOPS	3-(*N*-morpholino) propanesulfonic acid
MS	Mass spectrometry
N	Normality
NCBI	National center for biotechnology information
ng	Nanogram
nM	Nanomolar
3-oxo-C12-HSL	*N*-(3-oxo-dodecanoyl)-$_L$-homoserine lactone
3-oxo-C16-HSL	*N*-(3-oxo-hexadecanoyl)-$_L$-homoserine lactone
3-oxo-C6-HSL	*N*-(3-oxo-hexanoyl)-$_L$-homoserine lactone
3-oxo-C8-HSL	*N*-(3-oxo-octanoyl)-$_L$-homoserine lactone
3-oxo-C10-HSL	*N*-(3-oxo-decanoyl)-$_L$-homoserine lactone
Ntn	Terminal nucleophile
OD	Optical density
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PQS	*Pseudomonas* quinolone signal
psi	Pounds per square inch
QQ	Quorum quenching
QS	Quorum sensing
rRNA	Ribosomal RNA
s	Second
SAM	*S*-adenosyl-$_L$-methionine
SOB	Super optimum broth
TBE	Tris-Boric Acid Ethylenediaminetetraacetic acid
TOF	Time of flight

V	Voltage
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-indolyl-galactopyranoside
3OH-PAME	3-hydroxypalmitic acid methyl ester

CHAPTER 1.0

INTRODUCTION

In nature, bacteria function less as individuals and more as groups that are able to inhabit multiple ecological niches. Bacterial cell-to-cell communication, which is also commonly known as quorum sensing (QS) allows scientists to investigate the link between bacterial population density and their behaviors, the bacterial ecology as well as regulation of mechanism of bacterial phenotypes (Fuqua *et al.*, 1994; Parsek and Greenberg, 2005). It is believed that bacteria employ QS to gain maximum competition advantages, hence a group of microorganisms confer quorum quenching (QQ) – the interference of QS, to counter measure the benefits of QS, by producing AHL-inactivating enzymes, such as AHL-lactonases, AHL-acylases and AHL-oxidoreductases (Dong and Zhang, 2005; Hong *et al.*, 2012)

In this study, bacteria were isolated from Ulu Slim Hot Spring located in Perak, Malaysia followed by characterization of AHL molecules produced by these isolates as well as investigation of their AHL inactivation activities. Hot spring was selected as the isolation source to investigate the ubiquity of QS and QQ activities among microorganisms inhabiting hot aquatic environment. In this study, two approaches of identification were conducted, *i.e.* Microflex^{TM} LT Matrix-Assisted Laser Desorption/ Ionization with Time of Flights (MALDI-TOF) Biotyper approach and 16S rRNA approach. The investigation of AHL synthesis among these isolates involved (1) detection using bacterial biosensor which designed to detect the presence of AHLs, and (2) characterization of AHL molecules produced by bacterial isolates using triple quadrupole liquid chromatography mass spectrometry (LC/MS/MS-QQQ). For the assessment of QQ activities, whole cell inactivation assay was performed using

bacterial biosensor approach as well as analytical chemical investigation approach, *i.e.* LC/MS/MS-QQQ.

OBJECTIVES

The main objectives of this research project include the following:

1. To isolate QS and QQ mesophilic bacteria from Ulu Slim Hot Spring，
2. To identify the isolated bacterial strains using 16S rRNA gene sequencing analysis as well as MicroFlex MALDI-TOF Biotyper,
3. To study AHL production from QS bacterial isolates, and
4. To investigate QQ activity against various AHL molecules on the isolated strains.

CHAPTER 2.0

LITERATURE REVIEW

2.1 Communication within the Microbial World

Bacteria were for a long time thought to exist as primitive, individual cells that sought to primarily find nutrients and multiply independently of other members of their species. This simplistic view was altered with the discovery of quorum sensing (QS).

The phrase "QS" was first coined by Fuqua *et al.* (1994) and it is generally used to describe the phenomenon whereby the accumulation of signaling molecules enables a single bacterium to sense the number of bacteria (cell density) and therefore the population as a whole can make a coordinated response (Fuqua *et al.*, 1994). In other words, the bacteria community able to perform certain bacterial behaviors efficiently when a sufficiently large population of bacteria (quorum) has achieved. Fuqua *et al.* described this minimal behavioral unit as a quorum of bacteria (Fuqua *et al.*, 1994).

The discovery of QS led to the realization that bacteria are capable to coordinate activity to facilitate their adaptation to changing environmental conditions, including the competitive environments, which was once believe to be restricted to multicellular organisms. Therefore, QS allows bacteria to behave as multicellular organisms, forming a high cell density behavior, and to reap the benefits that could be unattainable to them as individuals (Schauder & Bassler, 2001). The realization that bacteria can communicate, cooperate and alter their behavior, according to changes in their social environment has led to an explosion of research in the area of microbiology.

2.2 QS Signalling Molecules

For true communication to occur, two crucial conditions must be achieved: (1) one or several individuals must produce a signal that can be perceived by other individuals, and (2) the perceivers must alter their behavior in response to this signal (Keller and Surette, 2006). In the context of bacteria-to-bacteria communication, what is a QS signal? According to Diggle *et al.* (2006), there are four fundamental characteristics of QS signals - (1) the synthesis of QS signal occurs at a specific stage of growth or in response to specific environmental changes; (2) the recognition and the binding of QS signals which accumulate in the extracellular environment to the specific bacterial receptor; (3) the accumulation of the QS signal to a critical threshold concentration in order to trigger a concerted or coordinated response; (4) the cellular response extends beyond the physiological changes required to metabolize or detoxify the molecule (Diggle *et al.*, 2006; Winzer *et al.*, 2002).

Traditionally, QS molecules have been classified into two major categories - the Gram-negative's *N*-acyl homoserine lactones (AHLs) and the Gram-positive's oligopeptides (Lowery *et al.*, 2008). However, it is becoming increasingly evident that bacteria are not limited by these two classes of signals, as several other structurally and chemically distinct QS molecules have been identified in both Gram-positive and Gram-negative bacteria, such as the 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal) (PQS), 4-hydroxy-2-heptyl-quinoline (HHQ) (Pesci *et al.*, 1999), diketopiperazines (DKP), furanosyl borate diester (autoinducer-2) (AI-2), autoinducer-3 (AI-3), 2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone (A factor), *cis*-11-methyl-2-dodecenoic acid (diffusible signal factor) (DSF) (Barber *et al.*, 1997), 3-hydroxypalmitic acid methyl ester (3OH-PAME) (Flavier *et al.*, 1997), long-chain fatty acid derivatives (oligopeptide autoinducer) (Novick, 2003), bradyoxetin (Loh *et al.*,

2002) and 4,5-dihydroxy-2,3-pentandione (DPD) (Figure 2.1) (Atkinson & Williams, 2009; Williams *et al.*, 2007). The repertoire of chemical molecules that are associated with bacterial cell-cell signaling continues to grow.

Although diverse groups of QS signal molecules have been discovered, the most studied QS signaling molecules are the AHLs, which includes the *N*-acyl-$_L$-HSL, *N*-(3-oxoacyl)-$_L$-HSL and *N*-(3-hydroxyacyl)-$_L$-HSL, produced by the Gram-negative bacteria (Figure 2.1). The earliest characterised example is the luminescence regulated by QS molecule in the symbiotic marine bacterium *Vibrio fischeri* which produce 3-oxo-C6-HSL (Eberhard *et al.*, 1981; Schaefer *et al.*, 1996).

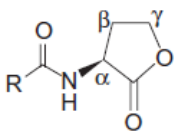
AHL molecules are synthesized by autoinducer synthase (LuxI) from, substrates *S*-adenosylmethionine (SAM) and acetylated acyl carrier protein (Acyl-ACP) – an intermediate of fatty acid biosynthesis.

All reported AHLs are characterized with an unsubstituted group at the β- and γ-position, and an *N*-acylated with a fatty acid acyl group at the α-position (Figure 2.1) (Chhabra *et al.*, 2005). Myriad structural variants of the basic AHL molecules have been discovered and they vary in length and degree of saturation of the acyl side chain as well as in the functional group located in C3 (Decho *et al.*, 2011). In most cases, the acyl chain has even number of carbons, ranged from 4 to 18 carbons (Chhabra *et al.*, 2005). Some bacteria such as *Rhodopseudomonas palustris, Bradyrhizobium* sp. and *Silicibacter pomeroyi*, are found to produce signaling molecule *p*-coumaroyl-HSL by using environmental *p*-coumaric acid rather than fatty acids from cellular pools to produce AHLs.

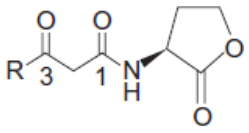
2.3 Bacterial Communication Using AHLs

Diverse Gram-negative bacterial cells communicate with each other by using diffusible AHLs to coordinate gene expression with cell population density. Accumulation of AHLs above a threshold concentration renders the population "quorate," and the appropriate target genes are activated, contributing to the change in bacterial behavior (Fuqua *et al.*, 1994).

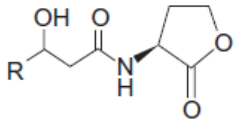
The production of AHLs in diverse range of Gram–negative bacteria has been reported together with their implications in regulating different cellular response and phenotypes. Examples of bacteria utilizing the AHLs as the signaling molecules were summarized in Table 2.1.



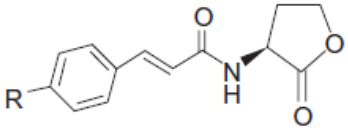
N-acyl-$_L$-HSL



N-(3-oxoacyl)-$_L$-HSL

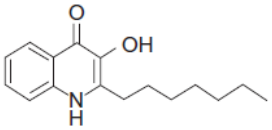


N-(3-hydroxyacyl)-$_L$-HSL

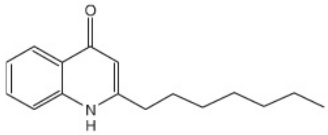


N-(ρ-coumaroyl)-HSL

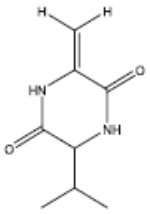
R = OH (ρC-HSL)



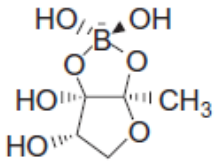
2-heptyl-3-hydroxy-4-quinolone (PQS)



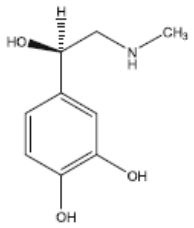
4-hydroxy-2-heptyl-quinoline (HHQ)



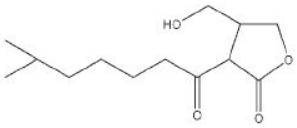
Diketopiperazines (DKP)



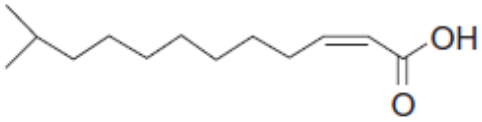
Autoinducer 2 (AI-2)



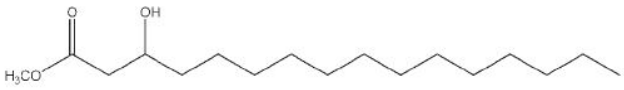
Autoinducer-3 (AI-3)



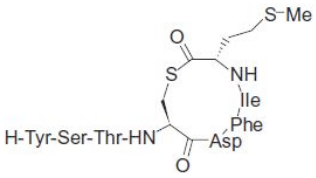
A-Factor



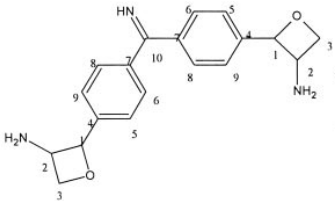
Diffusible Signal Factor (DSF)



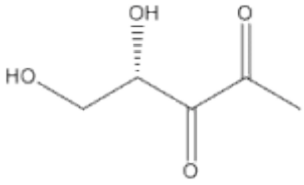
3-hydroxypalmitic acid methyl ester (3OH-PAME)



Group 1 *S. aureas* peptide thiolactone



Bradyoxetin



4,5-dihydroxy-2,3-pentandione (DPD)

Figure 2.1 Different classes of quorum sensing signalling molecules

Table 2.1 AHLs-producing bacteria and the QS-regulated behaviors

Organism	LuxI homolo-gue(s)	LuxR homolo-gue (s)	Major AHLs	Target genes and functions	Reference
Aeromonas hydrophila	AhyI	AhyR	C4-HSL	Serine protease and metalloprotease production, biofilm formation	Swift *et al.*, 1997
Aeromonas salmonicida	AsaI	AsaR	C4-HSL	*aspA* (extracellular exoprotease production)	Swift *et al.*, 1997
Agrobacterium tumefaciens	TraI	TraR	3-oxo-C8-HSL	*tra*, *trb* (Ti plasmid conjugation transfer)	Zhang *et al.*, 1993
Agrobacterium vitis	AvsI	AvsR	C16:1-HSL, 3-oxo-C16:1-HSL	Virulence	Hao and Burr, 2006
Acidovorax defluvii	n.d.	n.d.	3-hydroxy-C8-HSL	n.d.	d'Angelo-Picard *et al.*, 2005
Acidithiobacillus ferrooxidans	AfeI	AfeR	C14-HSL	Biofilm formation, response to iron	Rivas *et al.*, 2007
Acinetobacter baumannii	AbaI	AbaR	3-hydroxy-C12-HSL	*csu*-ecoded chaperone-usher pilus assembly system and the Bap protein for biofilm formation	Niu *et al.*, 2008
Burkholderia cenocepacia	CepI, CciI	CepR, CciR	C6-HSL, C8-HSL	Exoenzyme, biofilm formation, swarming motility, siderphore, virulence	Sokol *et al.*, 2003; Mallot *et al.*, 2005

Table 2.1 Continue

Burkholderia cepacia	CepI	CepR	C8-HSL	Protease & siderophore production	Lewenza *et al.*, 1999.
Burkholderia mallei	BmaI1, BmaI3	BmaR1, BmaR3, BmaR4, BmaR5	C8-HSL, 3-hydroxy-C8-HSL, C10-HSL	Virulence	Ulrich *et al.*, 2004b; Duerkop *et al.*, 2007
Burkholderia pseudomallei	PmlI1, PmlI2, PmlI3	PmlIR1, BpmR2, BpmR3	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL	Virulence, exoproteases	Valade *et al.*, 2004; Ulrich *et al.,* 2004a
Comamonas testosteroni	n.d.	TerR	n.d.	*sip48*-β-HSD gene, steroid degradation genes (Steroid catabolic pathway)	Pruneda-Paz *et al.*, 2004
Chromobacterium violaceum	CviI	CviR	C6-HSL	Violacein pigment, *hcnABC* operon, *cynS, cynT* operon (hydrogen cynida), phenazineantibiotics, *lasA* & *lasB* (exoproteeases) & chitinolytic enzymes production	McClean *et al.*, 1997
Edwardsiella tarda strain LTB-4	ExpI	ExpR	3-oxo-C6-HSL	Production of extracellular plant cell wall-degrading enzymes	Kõiv & Mäe, 2001

Table 2.1 Continue

Erwinia carotovora	CarI	CarR	3-oxo-C6-HSL	Biosynthesis of carbapenem antibiotic	Barnard *et al.*, 2007
Erwinia carotovora ssp. *Carotovora*	ExpI	ExpR	3-oxo-C6-HSL	*pecS* (regulator of pectinase synthesis)	Nasser *et al.*, 1998
Erwinia chrysanthemi	EagI	EagR	3-oxo-C6-HSL	n.d.	Swift *et al.*, 1993
Enterobacter agglomerans	GinI	GinR	C10-HSL, C12-HSL, C12:1-HSL	*ginA* (growth in ethanol-containing medium, acetic acid production)	Iida *et al.*, 2008
Massilia timonae	n.d.	n.d.	5-*cis*-3-oxo-C12-HSL; 5-*cis*-C12-HSL	n.d.	Krick *et al.*, 2007
Mesorhizobium sp.	MrlI1, MrlI2, MrlI3	n.d.	C12-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL	Symbiotic nodulation	Yang *et al.*, 2009
Mesorhizobium loti NZP2213	MsaI	MsaR	C6-HSL, C8-HSL	Extrapolysaccharide carbohydrate production	Penalver *et al.,* 2006
Methylobacterium extorquens AM1	MlaI	MlaR	C14:1-HSL, C14:2-HSL	Extrapolysaccharide carbohydrate production	Penalver *et al.,* 2006
	Lacks of homologs	n.d.	C6-HSL, C-oxo-C6-HSL, C8-HSL, C10-HSL	Emergence from lag phase	Burton *et al.*, 2005; Batchelor *et al.*, 1997

Table 2.1 Continue

Nitrosomonas europaea	OprI	OprR	3-oxo-C6-HSL	n.d.	Swift *et al.*, 1999
Obesumbacterium proteus	PagI	PagR	C4-HSL	*hrpL, hrpS* and *hrc* genes (gall formation in plant; IAA & cytokinins in regulation	Chalupowicz *et al.*, 2009
Pantoea agglomerans pv. *gypsophilae*	EanI	EanR	C6-HSL,3-oxo-C6-HSL	Biosynthesis of exocapsular polysaccharide (EPS), biofilm formation, and infection of onion leaves.	Morohoshi *et al.,* 2007
Pantoea ananatis	EsaI	EsaR	C6-HSL	Biosynthesis of stewartan and EPS	Koutsoudis *et al.,* 2006
Pantoea stewartii	n.d.	n.d.	C16-HSL	n.d.	Schaefer *et al.*, 2002
Paracoccus denitrificans	LasI	LasR	3-oxo-C12-HSL	*lasA, lasB, aprA, toxA*, exoprotease virulence factors and biofilm formation, RhlR expression	Latifi *et al.*, 1996; Pearson *et al.*, 1994; Pearson *et al.*, 1995; Davis *et al.*, 1998; Oinuma and Greenberg, 2011; Juhas *et al.*, 2004

Table 2.1 Continue

Pseudomonas aeruginosa	RhlI	RhlR	C4-HSL	*lasB*,*rhlAB*, (rhamnolipid), *rpoS*, type 4 pili, exoenzymes, lectins and pyocyanin, HCN	Latifi *et al.*, 1996; Pearson *et al.*, 1994; Pearson *et al.*, 1995; Davis *et al.*, 1998; Oinuma and Greenberg, 2011; Juhas *et al.*, 2004 Wood *et al.*, 1997; Zhang and Pierson, 2001
	n.d.	QscR, VqsR	-	n.d.	
	PhzI, CsaI	PhzR, CsaR	C6-HSL	*phz* – Phenazines antibiotic symnthesis, protease, colony morphology, aggregation	
Pseudomonas aurefaciens	PhzI, CsaI	PhzR, CsaR	C4-HSL, C6-HSL, 3-oxo-C6-HSL	Phenazine antibiotics (*phzA* & *phzB*), and antifungal activity	Maddula *et al.*, 2006
Pseudomonas chlororaphis	PhzI	PhzR	3-hydroxy-C6-HSL	Phenazines synthesis	Khan *et al.*, 2005
Pseudomonas fluorescens	PpuI	PpuR	3-oxo-C10-HSL, 3-oxo-C12-HSL	Biofilm formation	Dubern *et al.*, 2006
Pseudomonas putida	AhlI	AhlR	3-oxo-C6-HSL	Exo-polysaccharide, swimming motility, virulence	Quiñones *et al.*, 2005

Table 2.1 Continue

Pseudomonas syringae	RaiI, CinI	RaiR, CinR	3OH-C14:1-HSL, 3OH-(slc)-HSL etc.	Growth and symbiotic nitrogen fixation (root nodulation)	Daniels *et al.*, 2002; Rosemeyer *et al.*, 1998
Rhizobium etli	CinI, RhiI, RaiI	CinR, RhiR, RaiR, TraR, BisR, TriR	3-hydroxy-7-*cis*-C14-HSL, 7-*cis*-C14-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL	*rhiABC* (rhizosphere genes), root nodulation/ symbiosis, bacteriocin, plasmid transfer, growth inhibition, stationary phase survival/ adaption, quorum sensing regulatory cascade	Wisniewski-Dyé *et al.*, 2002; Rodelas *et al.*, 1999; Danino *et al.*, 2003; Wilkinson *et al.*, 2002
Rhizobium leguminosarum bv *viciae*	SolI	SolR	C6-HSL, C8-HSL	n.d.	Flavier *et al.*, 1997.
Ralstonia solanacearum	GtaI	n.d.	C14-HSL, C16-HSL	Gene transfer agent production	Schaefer *et al.*, 2002
Rhodobacter capsulatus	CerI	CerR	C14:1-HSL	Cellular aggregation, community escape	Puskas *et al.,* 1997
Rhodobacter sphaeroides	n.d.	n.d.	C6-HSL, 3-oxo-C6-HSL	n.d.	Gram *et al.*, 2002
Roseobacter sp.	SmaI	SmaR	C4-HSL, C6-HSL	Production of carbapenem, prodigiosin, pectate lyase and cellulose	Thomson *et al.*, 2000

Table 2.1 Continue

Serratia sp ATCC 39006	SwrI	SwrR	C4-HSL, C6-HSL	Swarming motility; production of serrawattin, protease and S-layer protein; biofilm formation; butanediol fermentation	Eberl *et al.*, 1996a; Eberl *et al.*, 1996b
Serratia liquefaciens	SwrI	SwrR	C4-HSL, C6-HSL	Swarming motility; production of serrawattin, protease and S-layer protein; biofilm formation; butanediol fermentation	Eberl *et al.*, 1996a; Eberl *et al.*, 1996b
Serratia marcescens					
Strain CH-1	SpnI	SpnR	C6-HSL, 3-oxo-C6-HSL, C7-HSL, C8-HSL	Sliding motility; production of biosurfactant, prodigiosin and nuclease	Horng *et al.*, 2002
Strain SS-1	SmaI	SmaR	C4-HSL, C6-HSL	Swarming motility; heamolytic activity; production of caseinase and chitinase; biofilm formation	Coulthurst *et al.*, 2006
Strain 12	SmaI	SmaR	C4-HSL, C6-HSL	Swarming motility; heamolytic activity; production of caseinase and chitinase; biofilm formation	Coulthurst *et al.*, 2006

Table 2.1 Continue

Serratia plymythica					
	SplI	SplR	3-hydroxy-C6-HSL, 3-hydroxy-C8-HSL	n.d.	Ovadis *et al.*, 2004
Strain IC1270	SplI	SplR	C4-HSL, C6-HSL, 3-oxo-C6-HSL	Production of nuclease, protease, chitinase and antibacterial compounds, butanediol fermentation	Van Houdt *et al.*, 2007
Strain RVH1	SprI	SprR	C6-HSL, 3-oxo.C6-HSL	Production of lipase, protease, chitinase	Christensen *et al.*, 2003
Serratia proteamaculans B5a	SinI	SinR, ExpR, TraR	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL	Exopolysaccharide Production, symbiotic nodulation	Marketon *et al.*, 2003
Sinorhizobium meliloti	n.d.	n.d.	3-hydroxy-C8-HSL	n.d.	d'Angelo-Picard *et al.*, 2005

Table 2.1 Continue

Sphingomonas agrestis	n.d.	n.d.	3-hydroxy-C8-HSL	n.d.	d'Angelo-Picard *et al.*, 2005
Variovorax paradoxus	LuxI	LuxR	3-oxo-C6-HSL	*LuxICDABE* (bioluminescence)	Engebretcht *et al.*, 1983
Vibrio fischeri	VanI, VanM, VanN	VanR	3-oxo-C10-HSL, C6-HSL, 3-hydroxy-C6-HSL	n.d.	Milton *et al.*, 1997; Milton *et al.*, 2001
Vibrio anguillarum	n.d.	n.d.	3-hydroxy-C4-HSL	Virulence	Dunphy *et al.*, 1997
Xenorhabdus nematophilus	YenI	YenR, YenR2	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	*fleB* (flagellin structural gene) expression, Swimming and swarming motility	Atkinson *et al.*, 2006a
Yersinia enterocolitica	YpeI	YpeR	3-oxo-C6-HSL, 3-oxo-C8-HSL	n.d.	Kirwan *et al.*, 2006
Yersinia pestis	YpsI, YtbI	YpsR, YtbR	3-oxo-C6-HSL, C6-HSL, C8-HSL	Motility, clumping	Atkinson *et al.*, 1999; Atkinson *et al.*, 2008

Table 2.1 Continue

Yersinia pseudotuberculosis	YukI	YukR	36-HSL, 3-oxo-C6-HSL, C8-HSL	n.d.	Atkinson *et al.*, 1999

Legend: n.d.: not determine

Table 2.1 Continue

2.4 QS and Evolution

Understanding altruistic behaviors of organisms as well as microorganisms is one of the challenging tasks to evolutionary biologists as natural selection appears to favour selfish and uncooperative individuals (Hamilton 1963; Hamilton 1964). Cooperation has been documented in the animal kingdom back in mid 1900's (Kropotkin, 1902; Skutch, 1935). However, the social behavior in microorganisms has only been investigated since 1990 (Shimkets, 1990).

According to several literatures, QS had been evolutionary selected for because it optimizes growth and survival at the population level (West *et al.*, 2006). This has been challenged, as there is a drawback of this form of microbial communication and cooperation, as such cooperation is vulnerable to invasion by cheaters who do not cooperate but gain the benefit from other cooperation (Diggle *et al.*, 2007). Similar condition does occur in the field of economics and human morality where it is termed as "the tragedy of the commons' (Hardin, 1968).

Hamilton's kin selection theory provides an explanation for cooperation or communication between relatives in which by helping a close relative to reproduce, this enable the passing of its own genes to the next generation, albeit indirectly (Hamilton 1963; Hamilton 1964). According to the kin selection theory, altruistic cooperation is

favoured when $rb\text{-}c>0$, where c is the fitness cost to the altruist; b is the fitness cost to the beneficiary; and r is their genetic relatedness. This predicts that individuals should be more likely to cooperate when social partners are more closely related.

According to Keller and Surette, the nature of interactions through QS signalling molecules is not just solely cooperative communication. It involves other more dynamic interactions such as signalling molecules modification. Besides, these signalling molecules might play an important role in intraspecies as well as interspecies conflicts. When investigating the mechanism of bacterial responses to signalling molecules produces by other bacteria or the host, several aspects need to be taken into account, *i.e.* (1) the nature of the chemical interaction, (2) the molecular basis of the signalling molecules (speed of evolution, quantity of substances produces, specificity of the chemical), and (3) chemical moiety of the signalling molecules (Keller and Surette, 2006).

It is important to study microbiology from the perspective of ecology and evolution in order to understand QS as well as to develop bacteria as model organisms in ecology-evolution.

2.5 Interference of QS

QQ is the mechanism which interferes the bacterial cell-to-cell communication or QS. Bacteria are ubiquitous, present in all niches, but the resources in almost all the niches are limited. Thus, in the condition where the bacterial population of a niche competes for limited resources, the ability to interference or disrupt QS provides the bacterial species an advantage over other that relies on QS (Dong and Zhang, 2005; Waters & Bassler, 2005).

The interference of QS can be achieved in several ways, such as (1) inhibition of AHL biosynthesis by inhibiting the enzymes involved in the biosynthesis of acyl chain (acyl-acyl carrier protein) (ACP) and *S*-adenosylmethionine synthase, (2) destruction of QS signalling molecules via enzymatic reactions, and (3) inhibition of LuxR homolog protein (QS receptors) from binding to the signalling molecules (Dong and Zhang, 2005; Hong *et al.*, 2012).

According to Dong and Zhang, there are four possible ways which the AHL molecules can be degraded or inactivated, namely lactonase, decarboxylase, acylase and deaminase (Dong and Zhang, 2005) (Figure 2.2). Of these four enzymes, only two have been discovered, which are lactonase, which hydrolyzes the ester bond of the lactone ring, and acylase, which cleaves the peptide (amide) bond of the lactone ring. According to Hong *et al.*, there are three types of enzymes which have been reported to degrade or inactivate the AHLs molecules, which are AHL-lactonase, AHL-acylase and AHL-oxidoreductase (Hong *et al.*, 2012) (Figure 2.3).

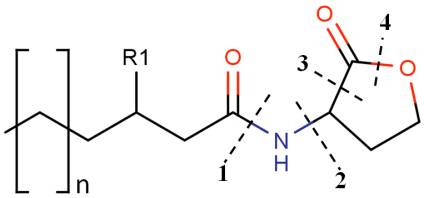


Figure 2.2 Four possible ways of enzymatic degradation of AHL molecule. Broken lines mark the position of possible cleavages by the following enzymes: 1. acylase; 2. deaminase; 3. decarboxylase; 4. lactonase.

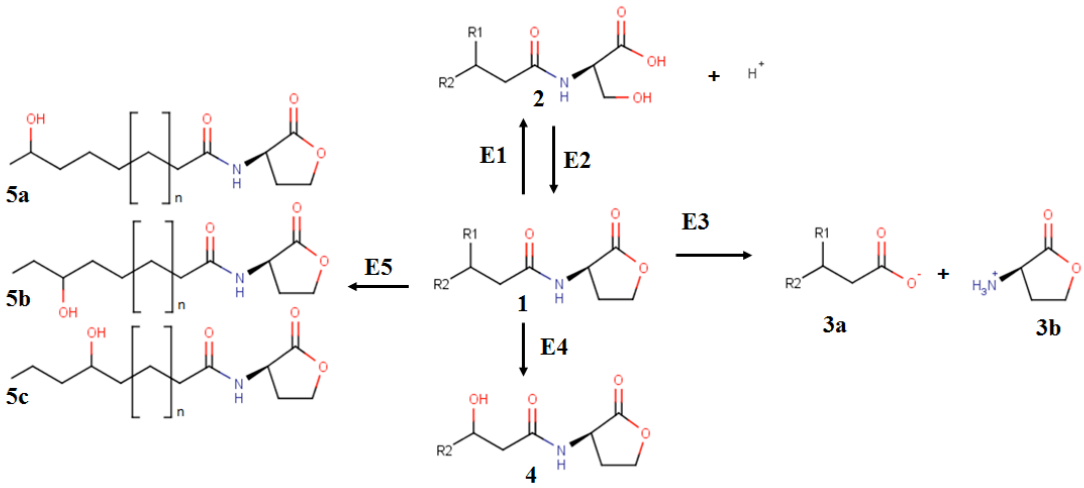


Figure 2.3 Structural modification of AHL molecules via quorum quenching enzymatic reactions. AHL-lactonase **(E1)** cleave ester bond of lactone of the AHL **(1)**, yielding *N*-acyl homoserine **(2)**. Relactonization **(E2)** of homeserine lactone via acidification causes the formation of functional AHL molecule. Acylase **(E3)** cleaves the amide bond of AHL, releasing fatty acid **(3a)** and homoserine lactone **(3b)**. Oxidoreductase from *Rhodococcus erythropolis* W2 and *Burkholderia* sp. GG4 **(E4)** inactivate AHL by substituting the oxo group at C3 position with hydroxyl group **(4)**. CYP102A1 from *Bacillus megaterium* **(E5)** reduces the acyl side chain of AHL at ω-1 **(5a)**, ω-2 **(5b)** and ω-3 **(5c)** position. R1 corresponds to the side chain of the C3 position, *i.e.* 3-oxo-, 3-hydroxy- and 3-unsubstituted. R2 corresponds to the acyl side chain of AHL. n corresponds to the alkyl group.

2.5.1 AHL-Lactonases

The hydrolysis homoserine lactone (HSL) of the AHL molecule, producing acyl homoserine is known as lactonolysis (Yates *et al.*, 2002). This process involves cleavage of the ester bond of the HSL ring by attacking the lactone's carbonyl followed by the elimination of an alcohol leaving group (Momb *et al.*, 2006; Momb *et al.*, 2010).

Therefore, the opening of the ring prevents the binding of the molecules to the LuxR homologs. However, the lactonolysis was found to be pH-dependent in which relactonization of the HSL ring were observed upon acidification of growth media or buffer to pH 2.0 (Yates *et al.*, 2002).

The first documentation of AHL-lactonase was AiiA, which was purified from *Bacillus* sp. strain 240B1 (Dong *et al.*, 2000). It has a 250-residue-long amino acid sequence and a conserved sequence motif of ^{104}HXHXDH^{109}~H^{169}, which is similar to the zinc binding motif of several metallohydrolases of metallo-β-lactamase (MBL) superfamily of protein (Dong *et al.*, 2000).The crystal structure of AiiA from *Bacillus thuringiensis* strain BTK shows the presence of two Zn^{2+} ions at the active centre of the enzyme (Kim *et al.*, 2005). According to Thomas *et al.* (2005) and Momb *et al.* (2008), the metal ions are essential for the cleavage of the ester bond on the lactone ring and the proper folding of the enzyme. Molecular and biochemical characterization of various reported AHL-lactonases has been reported as illustrated in Table 2.2.

Table 2.2 Characterization of various microbial AHL-lactonases

Strain or source	Name of enzyme	Protein Family	AHL degradation	Metal ion required	References
Agrobacterium tumefaciens c58	AttM	MBL superfamily	C6-HSL, 3-oxo-C8-HSL	Zn^{2+}	Zhang *et al.*, 2002

Arthrobacter sp. IBN110	AhlD	MBL superfamily	3-oxo-C6-HSL, C4-HSL, C6-HSL, C8-HSL, C10-HSL	Zn^{2+}	Park *et al.*, 2003
Bacillus sp. 240B1	AiiA	MBL superfamily	3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL	Zn^{2+}	Dong *et al.*, 2000
Geobacillus kaustophilus HTA426	GKL	Amidohydrolase superfamily	C6-HSL, C8-HSL, C10-HSL, 3-oxo-C8-HSL, 3-oxo-C12-HSL	Zn^{2+}	Chow *et al.*, 2010
Microbacterium testaceum StLB037	AiiM	α/β hydrolase fold family	3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL	None	Wang *et al.*, 2010
Mycobacterium avium subsp. *paratuberculosis* K-10	MCP	Amidohydrolase superfamily	C7-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL. C12-HSL	Mn^{2+}	Chow *et al.*, 2009

Table 2.2 Continue

Mycobacterium tuberculosis	PPH	Amidohydrolase superfamily	C4-HSL, C10-HSL, 3-oxo-C8-HSL	Mn^{2+}	Afriat *et al.*, 2006
Ochrobactrum sp. T63	AidH	α/β hydrolase fold family	C4-hsl, c6-hsl, 3-oxo-C6-HSL, 3-oxo-C8-HSL, C10-HSL	Mn^{2+}	Mei *et al.*, 2010
Rhodococcus erythropolis W2	QsdA (also known as AhlA)	PTE superfamily	AHL with or without substitution on carbon 3 and with an acyl chain ranging from 6 to 14 carbons	Zn^{2+}	Uroz *et al.*, 2008
Solibacillus silvestris StLB046	AhlS	MBL superfamily	C6-HSL, 3-oxo-C6-HSL, C10-HSL, 3-oxo-C10-HSL	Zn^{2+}	Morohoshi *et al.*, 2012
Sulfolobus solfataricus P2	SsoPox	Amidohydrolase superfamily	C8-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL	Co^{2+} & Fe^{3+}	Merone *et al.*, 2005; Elias *et al.*, 2008

Legend: MBL: Metallo-β-lactamase; PTE: Phosphotriesterase

Table 2.2 Continue

2.5.2 AHL-Acylases

AHL-acylases degrade the amide bond of a wide variety of AHL molecules yielding HSL and fatty acids (Leadbetter and Greenberg *et al.*, 2000). The first documentation of AHL-acylase was from *Variovorax paradoxus* VAI-C (Leadbetter and Greenberg, 2000). 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).

The second reported AHL-acylase is the AiiD from *Ralstonia* sp. XJ12B, which degrades and grows equally rapidly with short- and long-chain AHLs as sole source of carbon and nitrogen (Lin *et al.*, 2003). Shortly after the discovery of AiiD, another AHL-acylase, *i.e.* PvdQ (PA2385), from *P. aeruginosa* PA01 was reported by Huang *et al.* (2003). It is believed that the PvdQ involves in the regulation of the self-produced 3-oxo-C12-HSL. Another AHL-acylase, *i.e.* QuiP (PA1032), which is also from *P. aeruginosa* PA01was discovered in 2006 by Huang *et al.* (2006). QuiP has preference to degrade AHLs with long-chain acyl side chain (more than 6 carbons) and it is constitutively expressed during growth. The expression of QuiP, PvdQ and AHL synthases are believe to regulate the production of AHLs in order to communicate and to ensure that cell-to-cell communication is not disrupted (Huang *et al.*, 2006). Table 2.3 illustrated the various examples of AHL-acylases discovered in various microorganisms.

Table 2.3 Examples for various documented AHL-acylases

Strain or source	Name of enzyme	Protein family	AHL degradation	References
Anabaena sp. PCC7120	AiiC	n.d.	AHLs with or without substitution on carbon 3 and with an acyl side chain ranging from 4 to 14 carbons	Romero *et al.*, 2008
Comamonas sp. D1	n.d.	n.d.	AHLs with or without substitution on carbon 3 and with an acyl side chain ranging from 4 to 16 carbons	Uroz *et al.*, 2007
Pseudomonas aeruginosa PA01	PvdQ	Ntn-hydrolase	AHLs with or without substitution on carbon 3 and with an acyl side chain ranging from 10 to 14 carbons	Huang *et al.*, 2003; Sio *et al.*, 2006
Pseudomonas aeruginosa PA01	QuiP	Ntn-hydrolase	AHLs with or without substitution on carbon 3 and with an acyl side chain ranging from 7 to 14 carbons	Huang *et al.*, 2006

Pseudomonas syringae B728a	HacA	Ntn-hydrolase	C8-HSL, C10-HSL, C12-HSL	Shepherd *et al.*, 2009
Pseudomonas syringae B728a	HacB	Ntn-hydrolase	AHLs with or without substitution on carbon 3 and with an acyl side chain ranging from C6 to C12	Shepherd *et al.*, 2009
Ralstonia sp. XJ12B	AiiD	Ntn-hydrolase	3-oxo-C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL (less activity against 3-oxo-C6-HSL)	Lin *et al.*, 2003
Ralstonia solanacearum GMI1000	Aac	n.d.	C7-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL	Chen *et al.*, 2009
Rhodococcus erythropolis W2	n.d.	n.d.	3-oxo-C10-HSL	Uroz *et al.*, 2005
Shewanella sp. MIB015	Aac	n.d.	C8-HSL, C10-HSL, C12-HSL	Morohoshi *et al.*, 2008
Streptomyces sp. M664	AhlM	Ntn-hydrolase	C8-HSL, C10-HSL, 3-oxo-C12-HSL	Park *et al.*, 2005

Table 2.3 Continue

Variovorax paradoxus VAI-C	n.d.	n.d.	C4-HSL, C6-HSL, 3-oxo-C6-HSL C8-HSL, C10-HSL, C12-HSL, C14-HSL	Leadbetter and Greenberg, 2000

Legend: n.d.: not determine; Ntn: N-terminal nucleophile

Table 2.3 Continue

2.5.3 AHL-Oxidoreductases

AHL-oxidoreductase was first reported in 2005 by Uroz *et al.*, in which the *Rhodococcus erythropolis* W2 reduces the keto group of 3-oxo-AHLs to the corresponding 3-hydroxy derivatives (Uroz *et al.*, 2005). Unlike AHL-lactonases and AHL-acylases, no destruction of the AHL structure is observed but in turn signal disturbance of bacteria that depend on oxo-AHLs for regulation of QS-mediated gene expression was resulted (Chan *et al.*, 2011).

Unlike *R. erythropolis* W2 which first modifies the AHLs through an oxidoreductase activity followed by degradation of the modified AHLs, namely hydroxyl-AHLs, through an amidolytic activity, *Burkholderia* sp. GG4 does not degrade the modified AHL. While other bacteria may still able to use the modified AHL as QS molecules, the producer of this oxo-AHL will be deprived of its cognate QS oxo-AHL to bind to its LuxR receptor (Chan *et al.*, 2011). This phenomenon is known as "signalling confusion" which does not involve destruction of the AHL structures (Hong *et al.*, 2012). Table 2.4 illustrated the examples of AHL-oxidoreductases.

Table 2.4 Examples of documented AHL-oxidoreductases

Strain	Taxonomy (class)	AHL degradation	Reference
Bacillus megaterium CYP102A1	Firmicutes	Oxidizes C12-HSL to C20-HSL to corresponding ω-1, ω-2 and/or ω-3 hydroxylated AHLs	Chowdhary *et al.*, 2007
Burkholderia sp. GG4	Proteobacteria	Reduces 3-oxo-AHLs to corresponding 3-hydroxy derivatives	Chan *et al.*, 2011
Rhodococcus erythropolis W2	Actinobacteria	Converts C8-HSL to C14-HSL to corresponding 3-hydroxy derivatives	Uroz *et al.*, 2005

2.6 More Than Just Interference of Communication

QQ enzymes have been thought solely play an important role in interfering QS in order to ensure success in competition for the limited natural resources (Czakowski and Jafra, 2009). This has been proved by Park *et al.* in which *aiiA*-defective *B. thuringiensis* has a relatively lower survival rate, competency and adaptability compared to the wild type (Park *et al.*, 2008).

Besides playing an important role in microbial competition, the QQ enzyme is believed to be part of the microbial carbon and nitrogen metabolism mechanism where the QQ enzymes break the AHL molecules apart followed by downstream degradation by other enzymes. The products of these series of enzymatic degradation are carbon and

nitrogen compounds which required for growth. This postulation was derived from several experiments where several QQ bacterial strains, such as *V. paradoxus*, *Arthrobacter* sp. and *N. kongjuensis*, able to growth on medium with AHLs as the sole source of carbon and nitrogen (Leadbetter and Greenberg, 2000; Park *et al.*, 2003; Yoon *et al.*, 2006).

According to Haudecoeur and Faure (2009), two lactonases, *i.e.* AttM and AiiB, *in A. tumefaciens* involved in the regulation of QS-mediated gene expression such as conjugation, transfer of Ti plasmid and production of virulence factor. Similarly, two acylases in *P. aeruginosa*, *i.e.* PvdQ and QuiP, may also play an important role in regulated QS-regulated activities, such as production of virulence factor, biosynthesis and maturation of pyoverdine siderophore, regulation of AHLs production and iron homeostasis (Lehoux *et al.*, 2000; Huang *et al.*, 2003; Jimenez *et al.*, 2010).

The accumulation of 3-oxo-AHLs will lead to the formation of tetramic acid derivatives, which is a bactericidal agent against Gram-positive bacteria as well as a primordial siderophore that chelate diverse metal cations. The inactivation of AHL molecules via lactonases of *Bacillus* sp. will prevent the formation of tetramic acid derivatives, playing an important role in controlling the toxicity effects of AHLs (Kaufmann *et al.*, 2005).

According to Rashid *et al.* (2011), *Chryseobacterium* which isolated from plant might play a crucial role in providing protection to its host as the secreted QQ enzyme produced interferes the bacterial communication. Thus, this bacterial species helps in preventing the plant pathogen from invading the host.

In a nutshell, several roles of QQ enzymes have been proposed, from interference of QS to metabolism of AHL as the source of carbon and nitrogen, from regulation of

QS-mediated gene expression to detoxification, and symbiotic interaction with the host. A huge number of QQ enzymes where their role remain a mystery. Hence, further investigation is needed to gain insight into the role of QQ enzymes.

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains, Plasmid and Oligonucleotides

Bacterial strains used in this study were illustrated in Table 3.1. Table 3.2 and 3.3 showed the plasmid as well as primers used in this study, respectively.

Table 3.1 Bacterial strains used in this study

Bacterial strain	Description	Source/ Reference
Bacillus cereus	AHL-lactonase producing bacteria which inactivate AHL molecules by hydrolyzing the lactone ring, serving as positive control in AHL inactivation assay.	Dr Chan Kok Gan, Department of Genetics and Molecular Biology, Malaya University.
Chromobacterium violaceum strain CV026	Double mini-Tn*5* mutant derived from *Chromobacterium* violaceum ATCC 31532, Hg^R, *cviI*::Tn*5* *xylE*, Kan^R, Sm^R, violacein-negative, white mutant, defective in the production of AHL. It serves as AHL biosensor with the formation of purple violacein pigment in the presence of exogenous short chain AHLs.	McClean *et al.*, 1997

Erwinia carotovora strain Attn	AHL-producing mutant that serve as the positive control in quorum sensing assay.	Dr Chan Kok Gan, Department of Genetics and Molecular Biology, University of Malaya
Erwinia carotovora strain A20	Mutant strain which defective for AHL production	Dr Chan Kok Gan, Department of Genetics and Molecular Biology, University of Malaya
Escherichia coli strain DH5α	F^-, φ80dlacZΔM15, Δ(*lac*ZYA-*arg*F) U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_κ^-, m_κ^+), *pho*A, *sup*E44, λ^-, *thi*1, *gyr*A96, *rel*A1, nalidixic acid resistant. It serves as negative control in AHL inactivation assay as it does not possess AHL-degrading ability.	Sambrook *et al.*, 1989

Table 3.1 Continue

Bacterial strains *E. carotovora*, *C. violaceum* and *B. cereus* were cultivated in Luria-Bertani (LB) medium at 28°C. Bacterial strain *E. coli* DH5α was cultured in LB medium at 37°C.

Table 3.2 Plasmid used in this study

Plasmid	**Description**	**Source/ Reference**
pGEM®-T Easy Vector	F1 ori, Amp^R, SP6 and T7 promoters, used as cloning vector	Promega

Table 3.3 Oligonucleotide used in this study

Primer	**Description**	**Length (-mer)/ Reference**
16S rRNA forward primer 27F	5' – AGA GTT TGA TCM TGG CTC AG – 3'	20, Ott *et al.*, 2004
16S rRNA reverse primer 1525R	5' – AAG GAG GTG WTC CAR CC – 3'	17, Dewhirst *et al.*, 1999
16S rRNA forward primer 515F	5' GTG CCA GCA GCC GCG GTA A – 3'	19, Kotilainen *et al.*, 1998
SP6 Promoter	5' – ATT TAG GTG ACA CTA TAG – 3'	18, Universal primer
T7 Promoter	5' – TAA TAC GAC TCA CTA TAG GG – 3'	20, Universal primer

Legend: M = A + C; W = A + T; R = A + G

3.1.2 Chemical Reagents

All chemical reagents used in this study were of highest grade, purchased form Merck, Germany; Promega Ltd., USA; Sigma Chemical Crop., USA; BD Difco™ Laboratories, USA; BDH Ltd., England, Illumina, USA; Qiagen Pty. Ltd., Germany; iNtRON Biotechnology Inc., Korea; Cayman Chemical Company, USA.

3.1.3 Commercial Kits

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

Table 3.4 Commercial kits used in the study

Commercial kits (manufacturer)	Application
MasterPure™ Gram Positive DNA Purification Kit and MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies, USA)	Extraction of genomic DNA
i-Taq™ DNA polymerase kit (iNtRON Biotechnology Inc., Korea)	PCR amplification
QIAquick Gel Extraction Kit (Qiagen Pty. Ltd., Germany)	Purification of amplified DNA from agarose gel
pGEM®T Easy Vector System (Promega, USA)	Cloning of targeted gene and transform into host
QIAquick Spin Miniprep Kit (Qiagen Pty. Ltd., Germany)	Plasmid DNA extraction

3.1.4 Growth Media and Buffer Solutions

All media were sterilized by autoclaving at 121°C, 15 psi for 20 min, unless stated otherwise. Heat-labile solutions were sterilized by membrane filtration with syringe filter at pore size of 0.22 μm.

3.1.4.1 Luria-Bertani (LB) Medium

LB broth was prepared according to Sambrook *et al.* (1989), consisting of 1.0% w/v trypton, 1.0% w/v sodium chloride (NaCl) and 0.5% w/v yeast extract in 1.0 L of distilled water. LB agar was prepared by adding 1.5% w/v Bacto^{TM} Agar (BD) to the LB broth. For extraction of AHL molecules, bacterial isolates were grown in LB broth buffered with 50 mM of 3-(*N*-morpholino) propanesulfonic acid (MOPS) to pH 6.5 in order to maintain the acidity of the bacterial culture. Elevation of pH to above pH 7.0 will trigger lactonolysis of AHL molecules (Yates *et al.*, 2002). All ingredients were dissolved in distilled water and autoclaved.

3.1.4.2 Super Optimal Broth (S.O.B) Medium

S.O.B. medium was prepared as described in Sambrook *et al.* (1989), consisting of 2.0% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl and 2.5 mM of potassium chloride (KCl) in 1.0 L of distilled water. After autoclave sterilization, addition of filter-sterilized magnesium sulfate ($MgSO_4$) to the final concentration of 20 mM (Sambrook *et al.*, 1989).

3.1.4.3 S.O.C. Medium

S.O.C. medium was prepared by addition of filter-sterilized D-glucose into S.O.B. to the final concentration of 10 mM (Sambrook *et al.*, 1989).

3.1.4.4 Hot Spring Water Growth Medium (HS Agar)

In order to capture and cultivate greater diversity of bacteria that inhabit in the hot spring, a new growth medium was prepared. It consists of 0.1% w/v glucose and 0.1% w/v casamino acid in 1.0 L of filtered hot spring water. All ingredients were dissolved in hot spring water and autoclaved.

3.1.4.5 1 × Phosphate Buffer Saline (PBS)

PBS solution consisted of 154 mM NaCl, 2 mM monosodium phosphate (NaH_2PO_4), 8.5 mM disodium phosphate (Na_2HPO_4) and 2.7 mM KCl in 1.0 L of distilled water. The solution pH was adjusted to pH 6.5 before autoclaving.

3.1.4.6 5 × Tris Borate EDTA (TBE) Buffer

The 5 × TBE solution consisted of 445.76 mM Tris base, 444.77 mM boric acid (H_3BO_3) and 10 mM $Na_2EDTA{\cdot}2H_2O$ in 1.0 L of distilled water with pH adjusted to 8.0 prior to autoclaving. To prepare 1 × TBE solution, the 5 × TBE solution was diluted five times with distilled water.

3.1.5 Synthetic *N*-Acyl Homoserine Lactone

Synthetic AHL were purchased from Sigma-Aldrich® (USA) and Cayman Chemical Company (USA). AHLs were dissolved with acetonitrile (ACN) to targeted concentration and kept in −20°C.

3.1.6 Ampicillin Solution

Ampicillin was dissolved in sterile distilled water, yielding final concentration of 100 mg/ml. The antibiotic solution was filter-sterilized and kept in microcentrifuge tubes followed by storage at −20°C.

3.1.7 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-gal) Solution

X-gal powder was dissolved in dimethylformamide (DMF), yielding final concentration of 20 mg/ml. The solution was filter-sterilized and stored at −20°C.

3.1.8 DNA Size Reference Marker/ DNA Ladder

The GeneRuler^{TM} 1 kb DNA ladder (Fermentas Int. Inc., Canada) was used in this study.

3.2 Methods

3.2.1 Sampling

Water from Ulu Slim Hot Spring was collected on October 2011. The GPS position of the sampling site was at N 3°53’55’’, E 101°29’52’’. A total of 600 mL of hot spring water was collected using autoclaved scotch bottles from three points of the hot spring as replicates and transported back to the laboratory within 3 hours. The pH value and the temperature of the hot spring water were measured.

3.2.2 Isolation of Environmental Bacteria

A ten-fold serial dilution was performed on the collected hot spring water, using 1 × PBS as the diluent. Next, a total of 100 µL of diluted hot spring water was spread onto LB agar as well as HS agar, followed by incubation at 37°C for 5 consecutive days. Subsequently, isolation procedure was performed by selection of single bacterial colonies displaying distinctive morphologies. The bacterial isolates were streaked on until pure colonies were obtained. Pure bacterial isolates were proceeded to Gram staining and examination under the Olympus 1X71-22FL/PH microscope. Gram type of bacteria were determined and micrographs were captured using Olympus Cell^D Imaging System.

3.2.3 Bacterial Identification using Microflex^{TM} LT

The identities of the bacterial isolates were determined via direct smear method on a Bruker Microflex LT with the biotyping software. Bacterial colonies of fresh overnight culture were applied on polished steel MSP 96 target polished steel BC plate

(Bruker Daltonics). The smeared bacterial isolates were overlaid with 1 µL of α- cyano-4-hydroxy-cinnamic acid (HCCA) matrix. The suspensions were air dried at room temperature prior to analysis using Microflex LT and MALDI-TOF BioTyper (version 3.1) software package (Bruker Daltonik GmbH). The results of analysis were considered valid whenever the acquired score were above 2.00. When the acquired score was less than 2.00, the sample was proceeded to protein extraction method, where bacterial colony was suspended in 300 µL of distilled water, 900 µL of absolute ethanol was added, and the components were mixed well. The cell suspension was centrifuged at 12,500 × *g* for 2 min. The supernatant was discarded and the cell pellet was suspended in 80 µL of 70% formic acid. Subsequently, 80 µL of ACN was added and the suspension was centrifuged at 12,500 × *g* for 2 min. Finally, 1 µL of supernatant was spotted onto MSP 96 target plate and air dried at room temperature before it was overlaid with 1 µL of HCCA matrix followed by analysis (Eigner *et al.*, 2008).

3.2.4 Detection of *N*-Acyl Homoserine Lactone (AHL) Production

3.2.4.1 Detection of AHL Production using Biosensors

Bacteria were screen for the presence of short chain AHLs using cross streaking approach with the biosensor *C. violeceum* strain CV026. *E. carotovora* strain Attn served as positive control while *E. carotovora* strain A20 served as negative control (Section 3.1.1). The production of purple pigment by CV026 after one day incubation at 28 °C indicates the production of short chain AHL by the bacteria (Chan *et al.*, 2011).

3.2.4.2 AHL Extraction

Bacterial isolates with positive results are Section 3.2.4.1 was cultured in 100 mL of LB broth, supplemented with 50 mM of MOPS and incubated in a shaking incubator with 200 rpm agitation at 37 °C for 16 to 18 h. The bacterial culture was centrifuged at maximum speed, collecting only the cell-free supernatant for AHL extraction. The cell-free supernatant was extracted twice with equal volume of acidified ethyl acetate (containing 0.1 % v/v glacial acetic acid). The crude extract was concentrated to dryness and stored in -20 °C (Chan *et al.*, 2011).

3.2.4.3 Triple Quadrupole Liquid Chromatography Mass Spectrometry (LC/MS/MS-QQQ) Analysis

The crude extract from Section 3.2.4.2 was resuspended in 500 µl of ACN and centrifuge at maximum speed for 10 min. A total of 100 µl of the resuspended crude extract was placed into the insert.

Synthetic AHLs, *e.g. N*-butanoyl-$_L$-homoserine lactone (C4-HSL), *N*-hexanoyl-$_L$-homoserine lactone (C6-HSL), *N*-(3-oxo-hexanoyl)-$_L$-homoserine lactone (3-oxo-C6-HSL), *N*-heptanoyl-$_L$-homoserine lactone (C7-HSL), *N*-octanoyl-$_L$-homoserine lactone (C8-HSL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL), *N*-decanoyl-$_L$-homoserine lactone (C10-HSL), *N*-(3-oxo-decanoyl)-$_L$-homoserine lactone (3-oxo-C10-HSL), *N*-dodecanoyl-$_L$-homoserine lactone (C12-HSL), *N*-(3-oxo-dodecanoyl)-$_L$-homoserine lactone (3-oxo-C12-HSL) and *N*-hexadecanoyl-$_L$-homoserine lactone (3-oxo-C16-HSLs), were used as standard. The stock solutions for these synthetic AHLs (1 g/L) were dissolved in ACN and stored at -20 °C (Wong *et al.*, 2013).

The chemical separation system applied was Agilent 1290 Infinity UHPLC System, coupled with Agilent ZORBAX Rapid Resolution High Definition SB-C18 Threaded Column (2.1 mm × 50 mm, 1.8 μm particle size). The flow rate was adjusted to 0.5 mL/min at 37°C, and the injection volume was 1 μL. The mobile phase used in this experiment was water with 0.1 % v/v formic acid and acetronitrile. The gradient profile applied was as follows (time; water with formic acid: acetronitrile): 0 min; 80:20. 7 min: 50:50, 12 min; 20:80, and 14 min; 80:20. The MS detection from UHPLC separated compounds was performed using Agilent 6490 Triple Quadrupole LC/ MS System. The probe capillary voltage was set at 3 kV, flow of sheath gas was set at 11 mL/h with the nebulizer pressure of 20 psi. The desolvation temperature was set at 200 °C.

The experiment was conducted using precursor-ion scanning mode. Positive ion mode was applied with Q3 set to monitor for *m/z* value of 102 and Q1 was adjusted to scan a mass range from *m/z* 80 to *m/z* 400. The Agilent MassHunter – Qualitative Analysis (Version B.05.00) was used for the MS data analysis. The analysis was based on precursor ion, product ion, retention index, and the comparison of electron ionization (EI) of mass spectrum of the synthetic AHLs. Coupling with the presence if targeted precursor ion and the chemical fingerprinting, the molecular mass of *m/z* 102 confers the presence of homoserine lactone of AHL molecules.

3.2.5 Detection of AHL Inactivation Bioactivities

3.2.5.1 Whole Cell AHL Inactivation Assay

Detection of AHL degradation exhibited by the bacterial isolates was performed using biosensor *C. violeceum* strain CV026. *E. coli* DH5α and 1 × PBS solution were

used as the negative controls while the *B. cereus* served as the positive control of this experiment.

Overnight culture of bacterial isolates were adjusted to $OD_{600\ nm}$ of 1 using sterile LB broth. A total of 10 mL of the adjusted bacterial culture was centrifuged at 10,625 × *g* for 10 min. The cell pellet was resuspended in 1 mL of 1 × PBS (pH 6.5) followed by centrifugation and removal of supernatant. The cell pellet was again resuspended with 300 µL of 1 × PBS (pH 6.5) forming the resting cell needed for the experiment.

Appropriate amount of targeted AHL was dispensed into microcentrifuge tubes and allowed to evaporate prior to addition of 300 µL resting cells, reconstitute the AHL to the final concentration of 0.2 µg/µL. The mixture of AHL and bacterial cell suspension was then incubated at 37 °C, except *B. cereus* which was incubated at 28 °C with shaking at 220 rpm. Aliquot of 100 µL of cell suspension was withdrawn at 0 h, 24 h and 48 h followed by heat inactivation at 95 °C for 5 min. After the second aliquot at 24^{th} h, equal volume of 0.2 N HCl was added into the cell suspension followed by incubation and heat inactivation at the 48^{th} h.

A total of 300 mL of LB agar was seeded with 100 mL of overnight culture of *C. violeceum* strain CVO26 and allowed to solidify at room temperature. Aliquot of 10 µL from the 0^{th} h and 24^{th} h AHL-cell suspension mixture, and 20 µL from the 48^{th} h AHL-cell suspension mixture, were progressively dispensed onto paper discs placed on the solidified LB agar with *C. violeceum* strain CV026, followed by overnight incubation. The presence of the purple colour zone indicates the presence of AHL molecule, and *vice versa*. The presence of purple zone at the 0^{th} h but absence in the 24^{th} h indicates the degradation of AHL. The absence of purple zone in the 24^{th} h but presence of purple zone in the 48^{th} h indicates the mechanism of AHL degradation is due to AHL-lactonase.

The purple colour zone at the 48th h was due to relactonization of *N*-acyl homoserine (Yates *et al.*, 2002)

3.2.5.2 Investigation of QQ activities using LC/MS/MS-QQQ

Sample preparation for LC/MS/MS-QQQ required whole cell inactivation assay as describe in Section 3.2.5.1, with the exception of substituting the heat inactivation protocol with the addition of two times volume of ethyl acetate. The cell suspension was extracted with ethyl acetate twice. The crude extract was evaporated to dryness. LB agar seeded with *C. violeceum* strain CV026 was not needed in this experiment. The crude extract was resuspended with 100 µL acetronitrile and centrifuge for 10 min at maximum speed. A total of 90 µL of crude extract was placed into the insert prior to mass spectrometry analysis. Synthetic AHL was used as the standard.

The chemical separation system applied was Agilent 1290 Infinity UHPLC System, coupled with Agilent Poroshell 120 EC-C18 column (4.6 mm × 100 mm, 2.7 µm) with elution procedure consisted of an isocratic profile of acetronitrile/ water with 0.1 % formic acid (35:65, v/v) for short chain AHLs and acetronitrile/ water with 0.1 % formic acid (65:35, v/v) for long chain AHLs. A constant flow rate of 0.5 mL/min was applied At 37 °C. The parameter setting of MS was similar to Section 3.2.4.3 and the analysis was performed using Agilent MassHunter–Qualitative Analysis (Version B.05.00) (Chong *et al.*, 2012).

3.2.6 Molecular Identification of Bacterial Isolates

3.2.6.1 Genomic DNA Extraction

Bacterial isolates were inoculated into 10 mL of LB broth and incubated in a shaking incubator with 200 rpm agitation at 37 °C. The bacterial cells were harvested by centrifugation at 8,421 × *g* for 10 min. The cell pellet was proceeded for DNA extraction using MasterPure™ DNA Purification Kit for Gram-negative bacterial isolates and MasterPur MasterPure™ Gram Positive DNA Purification Kit (for Gram-positive bacterial isolates) (Epicentre® Biotechnologies, USA) for Gram-positive bacteria and MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies, USA) for Gram-negative bacteria.

3.2.6.2 Polymerase Chain Reaction (PCR) Amplification

The 16S rRNA gene of bacterial isolates was selected for the purpose of bacterial identification. Amplification of the gene involved forward primer 27F and 1525R as the reverse primer, yielding the amplicon of approximately 1.5 kb in length. The reagents from *i-Taq*™ DNA polymerase kit (iNtRON Biotechnology Inc., Korea) were used for the setup of the PCR mixtures. The amounts for each components used in the PCR mixture were illustrated in Table 3.5. The PCR thermal profile for the 16S rRNA gene amplification was as follow: initial denaturation at 94 °C for 5 min, followed by 30 cycles of amplification process consist of denaturation at 94 °C for 30 s, annealing at 58 °C to 63 °C for 30 s, extension at 72 °C for 90 s, and lastly the final extension protocol of 72 °C for 5 min. A negative control was included for each gene amplification experiment by substituting the bacterial genomic DNA with autoclaved ultrapure water (Merck Millipore, Germany) (Chong *et al.*, 2012).

Table 3.5 PCR setup for 16S rRNA gene amplification

PCR Component	Volume per sample (µL)
Ultrapure water	9.9
10 × buffer containing 2 mM $MgCl_2$	1.5
dNTP (200 µM)	1.2
Taq polymerase (5 U/µL)	0.2
27F forward primer (10 µM)	0.6
1525R reverse primer (10 µM)	0.6
Genomic DNA (~1 ng)	1.0
Total volume	15.0

3.2.6.3 Agarose Gel Electrophoresis (AGE)

AGE was performed using horizaontal 1.0 % w/v agarose gel, submerged in 1 × TBE buffer. The gel was pre-stained with 0.5 mg/mL of ethidium bromide (EtBr). The AGE was carried out at 80 V, 400 mA and 40 min. The stained agarose gel was visualized on UVP ultraviolet transilluminator. The desired DNA band(s) was determined by comparing the size of DNA band to 1 kb DNA ladder (Fermentas).

3.2.6.4 Gene Cloning, Transformation and Gene Sequencing

The desired band on the agarose gel was excised and purified using QIAquick Gel Extraction Kit (Qiagen Pty. Ltd., Germany) and proceeded to gene cloning using pGEM®T Easy Vector System (Promega, USA). Experiments were conducted according to the manufacturer's protocol. The ligation product was transformed into the competent cell via heat-shock protocol. The chemically competent cells were prepared according to Sambrook *et al.*, 1989. The transformed cells were incubated at 37 °C for 1 h with shaking at 150 rpm. A total of 100 µL of the transformed cell culture was spread onto LB agar supplemented with 100 µg/mL of X-gal followed by the incubated at 37 °C for 18 h.

Selection of successful transformants carrying the recombinant plasminds containing the desired 16S rRNA amplicons involved the blue/ white selection. Only the white colonies were selected and followed by PCR which used the bacterial colony as the template of genomic DNA. PCR was performed as stated in Section 3.2.5.2 with modification. The primers used were T7 forward primer and SP6 reverse primer. The initial denaturation at 94 °C was extended to 10 min. The thermal profile of primer annealing phase was 58 °C for 30 s. For each PCR reaction, 10.9 µL of ultrapure water was used.

Colony which shown positive results for 16S rRNA gene cloning was cultured in LB broth supplemented with 100 µg/mL of amplicillin. The culture was incubated in a shaking incubator with 200 rpm agitation at 37 °C for 16 to 18 h followed by plasmid extraction using QIAquick Spin Miniprep Kit (Qiagen Pty. Ltd., Germany). The extracted plasmid was stored at -20 °C.

Sequencing analysis of extracted recombinant plasmid was outsourced to 1st Base (Malaysia) with 16S rRNA 515F forward primer, M13F forward primer and M13R reverse primer as the sequencing primers. The gene sequences were then visualized using Applied Biosystem Sequence Scanner (v1.0), The sequences were then compared with those in the GenBank databases using BLASTN programme through the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1990). The identities of the bacterial isolates were evaluated based on the nearest identity to those deposited in the databases.

Phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis (MEGA) (version 5.2), in which the neighbor joining algorithm with bootstrap value of 1000 and maximum composite likelihood method was applied (Tamura *et al.*, 2011).

CHAPTER 4.0

RESULTS

4.1 Bacteria Identification using Microflex™ LT Biotyper

A total of 18 strains of bacteria have been isolated from the mesophilic region of the Ulu Slim Hot Spring. Only one bacterial isolate, *i.e.* strain F1, grow one the HS agar, while the rest were isolated from LB agar. All the isolated bacteria were proceeded for Microflex™ LT MALDI-TOF bacterial identification. The results were shown in Table 4.1.

Table 4.1 Bacterial identity determined using Microflex™ LT and 16S rRNA gene

Bacterial Isolate	Bacterial identity based on Microflex™	Score in Microflex™ LT
CN12	*Kocuria rhizophila*	2.111
CN14	*Rhodotorula mucilaginosa*	1.889
F1	*Methylobacterium rhodesianum*	2.159
F2	*Stenotrophomonas maltophila*	1.923
M3	n.d.	n.d.
M7	*Pseudomonas aeruginosa*	2.425
M18	*Pseudomonas otitidis*	2.457
M22	*Providencia rettgeri*	2.025
M25	*Dermacoccus nishinomiyaensis*	1.687

M27	n.d.	n.d.
M30	*Cronobacter sakazakii*	2.133
M31	*Exiguobacterium aurantiacum*	1.977
M40	*Brevibacterium casei*	2.381
M45	*Gordonia aichiensis*	1.763
M48	*Proteus mirabilis*	2.463
M52	*Bacillus megaterium*	2.325
M66	*Roseomonas mucosa*	2.004
M71	*Micrococcus luteus*	2.383

Legend: n.d. = not determined

Table 4.1 Continue

4.2 Molecular Identification of Isolates

The phylogenetic tree of the 16S rRNA gene sequences were constructed using MEGA (version 5.2). The algorithm used was neighbour-joining method with bootstrap value of 1,000. An 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. Phylogenetic analysis supporting the identification of each isolates were shown in Figure 4.1 to Figure 4.10.

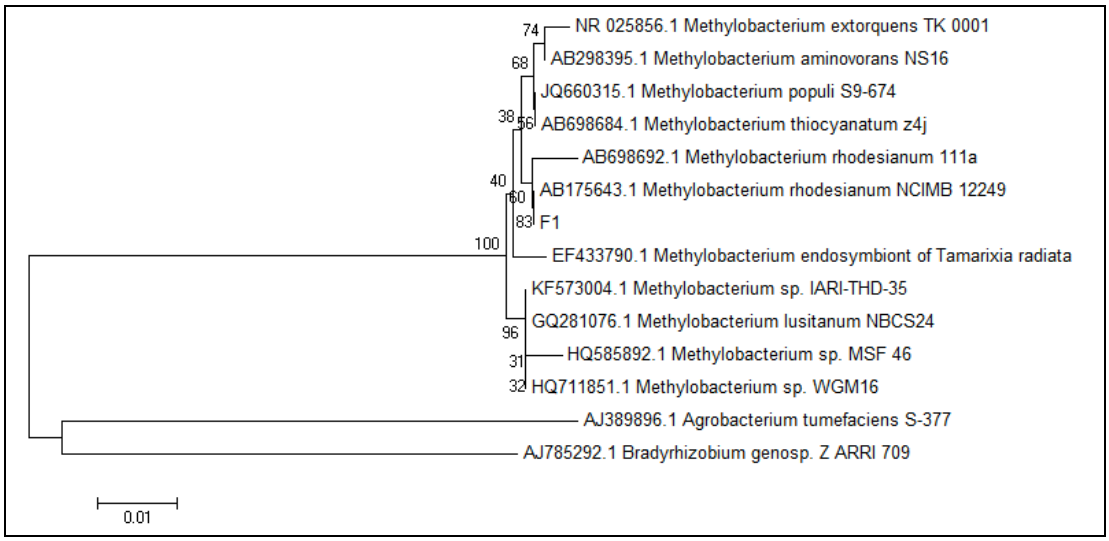


Figure 4.1 16S rRNA gene based phylogenetic analysis of *Methylobacterium rhodesianum* F1

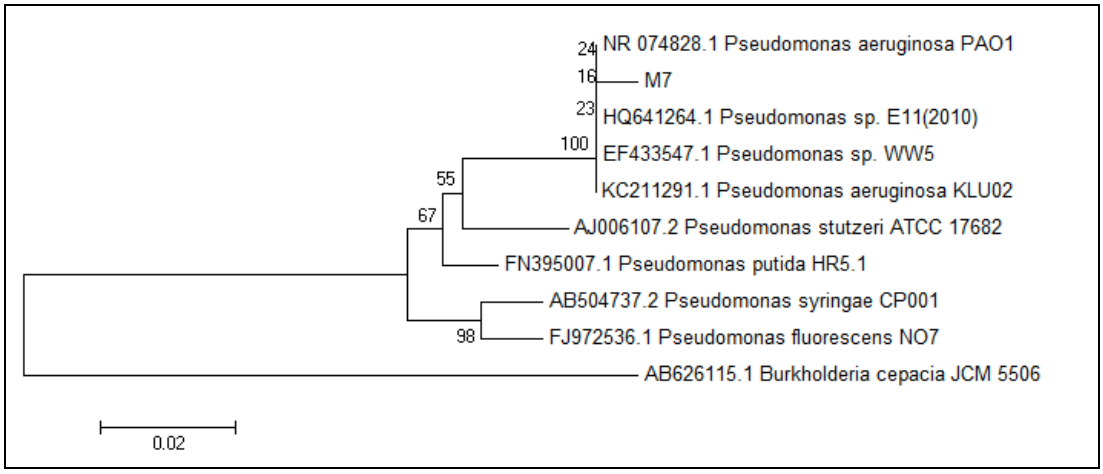


Figure 4.2 16S rRNA gene based phylogenetic analysis of *Pseudomonas aeruginosa* M7

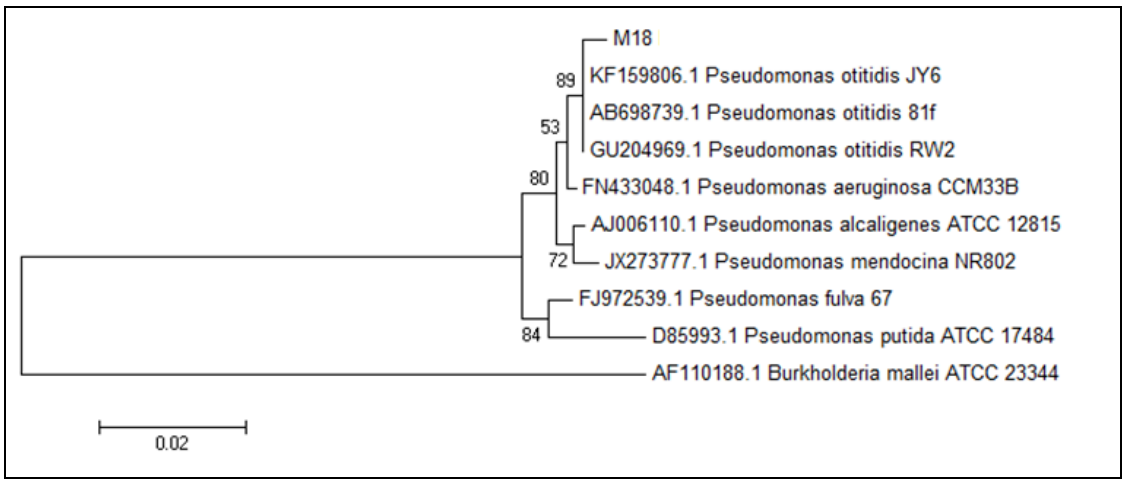


Figure 4.3 16S rRNA gene based phylogenetic analysis of *Pseudomonas otitidis* M18

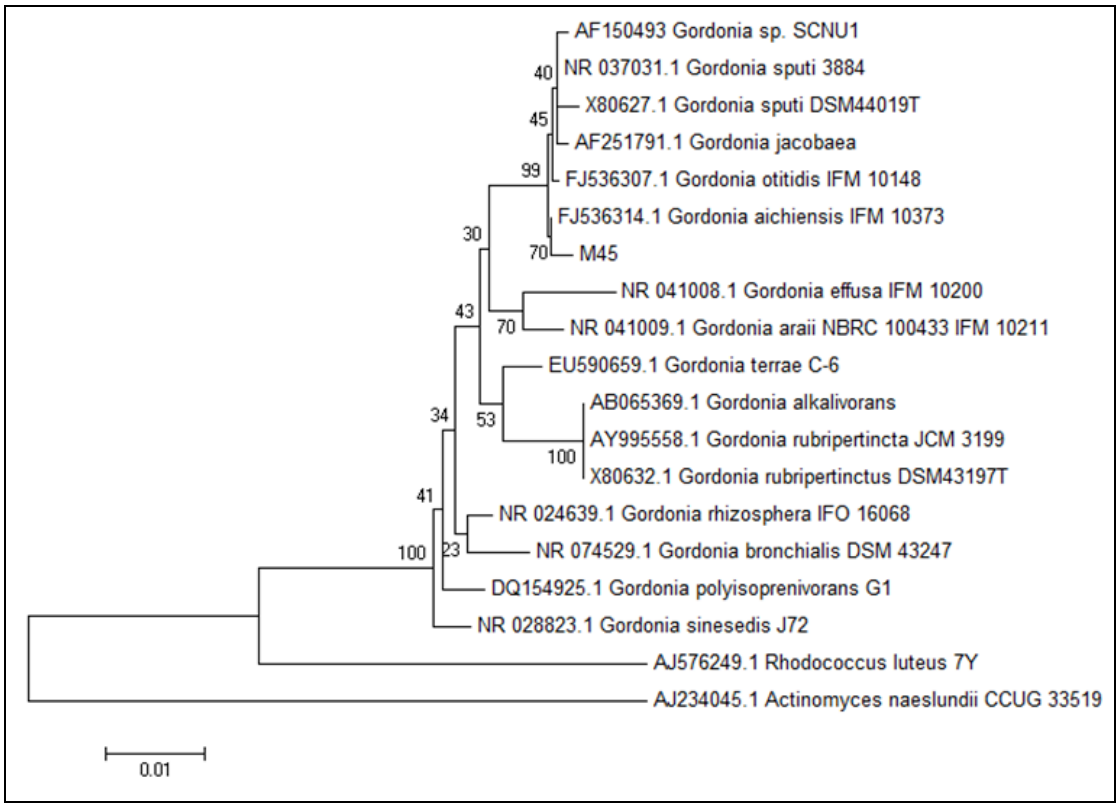


Figure 4.4 16S rRNA gene based phylogenetic analysis of *Gordonia* sp. M45

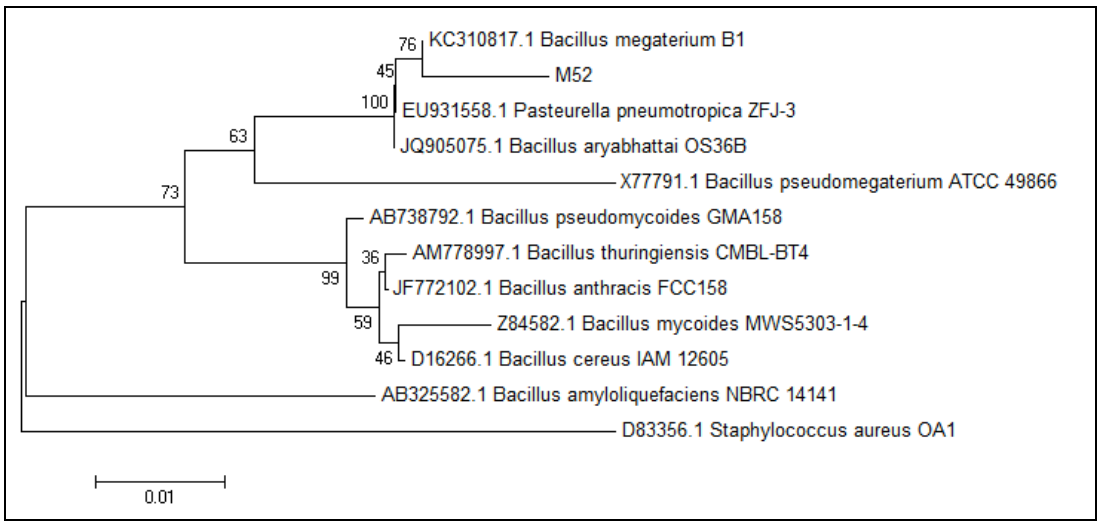


Figure 4.5 16S rRNA gene based phylogenetic analysis of *Bacillus megaterium* M52

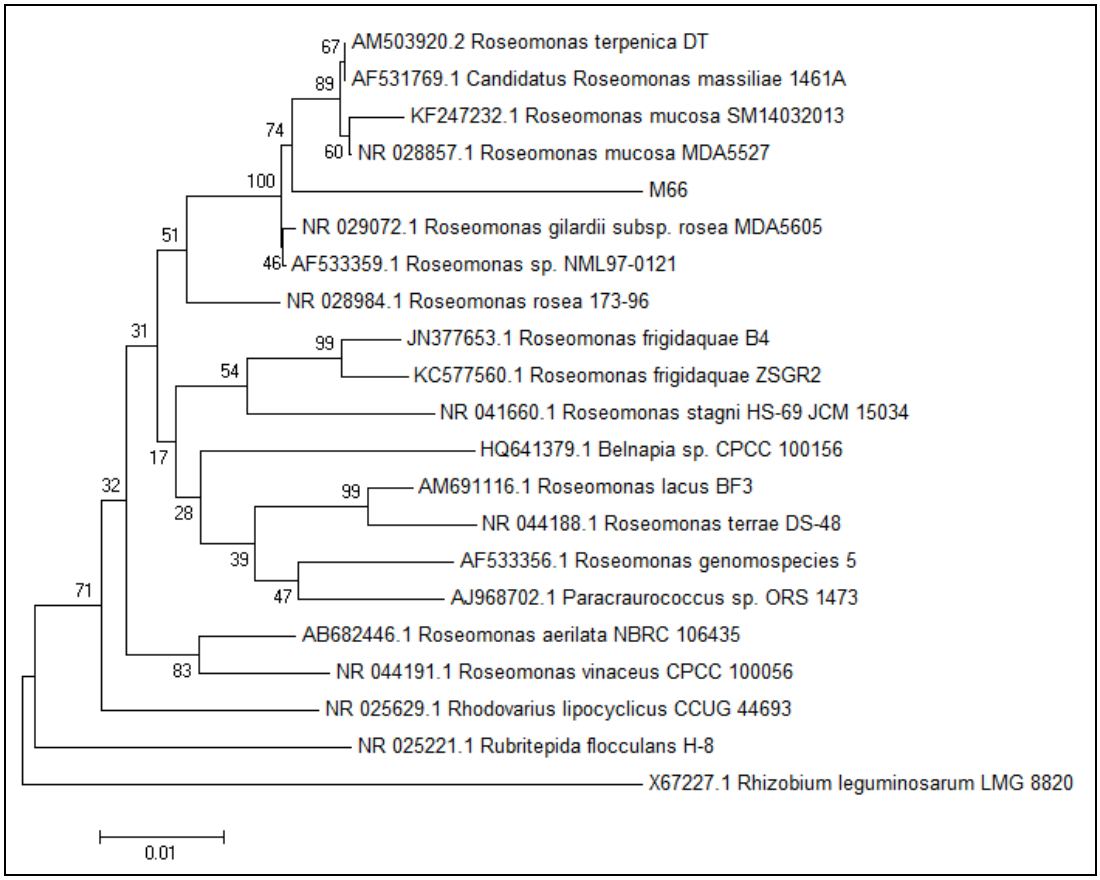


Figure 4.6 16S rRNA gene based phylogenetic analysis of *Roseomonas* sp. M66

There are several isolates with relatively lower score or unidentified using Microflex™ LT, such as strain F2, M3, M27 and M31. Therefore, 16S rRNA gene amplification and sequencing was performed in order to determine the identities of the isolates.

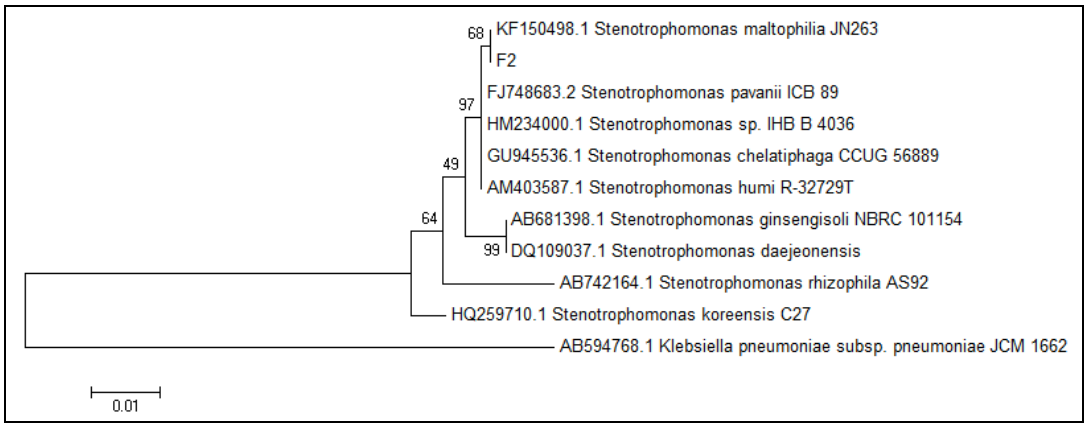


Figure 4.7 16S rRNA gene based phylogenetic analysis of *Stenotrophomonas maltophila* F2.

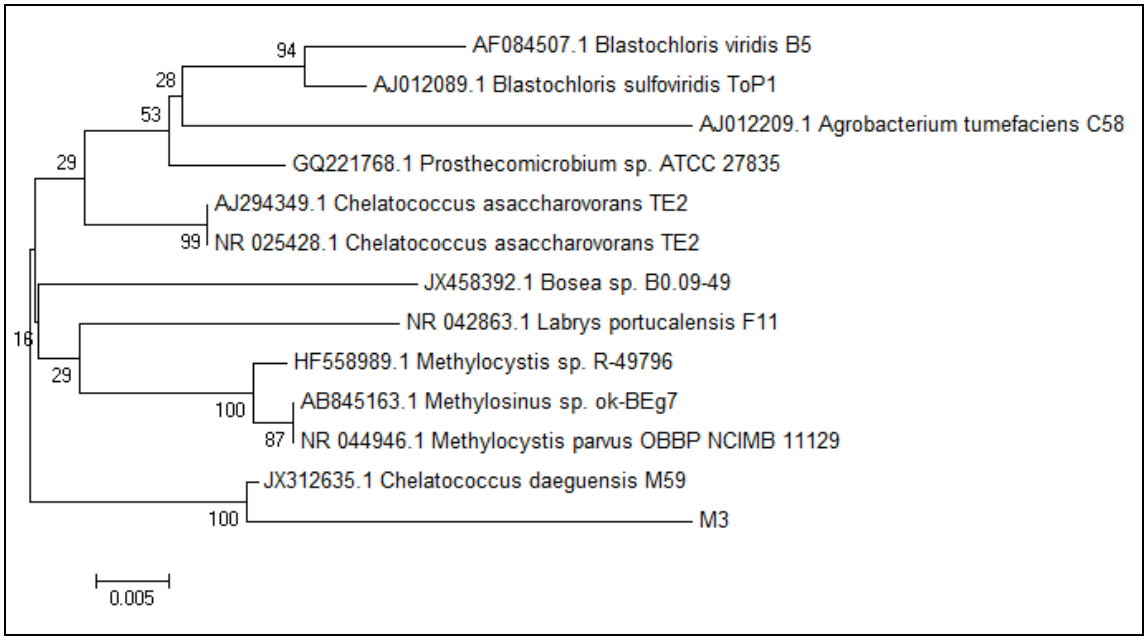


Figure 4.8 16S rRNA gene based phylogenetic analysis of *Chelatococcus daeguensis* M3. The identity of this bacterial strain was unable to be determined using Microflex™ LT. 16S rRNA gene of this strain has reveal it relativity to *Chelatococcus daeguensis*.

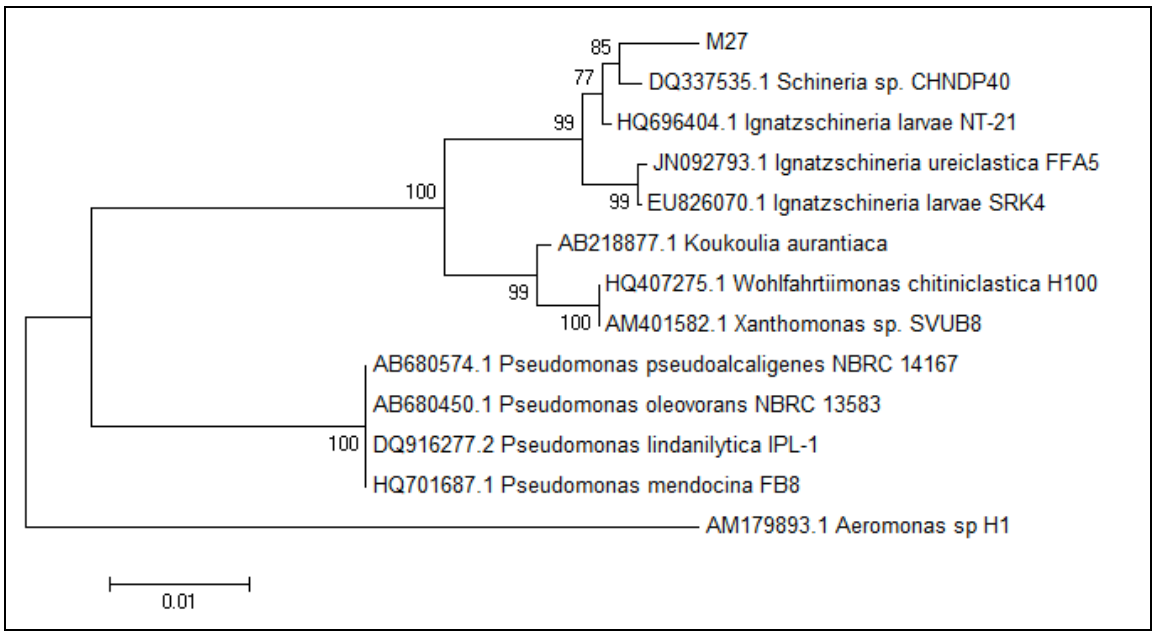


Figure 4.9 16S rRNA gene based phylogenetic analysis of *Schineria* sp. M27. The identity of this bacterial strain was unable to be determined using Microflex™ LT. 16S rRNA gene of this strain has reveal it relativity to *Schineria* sp.

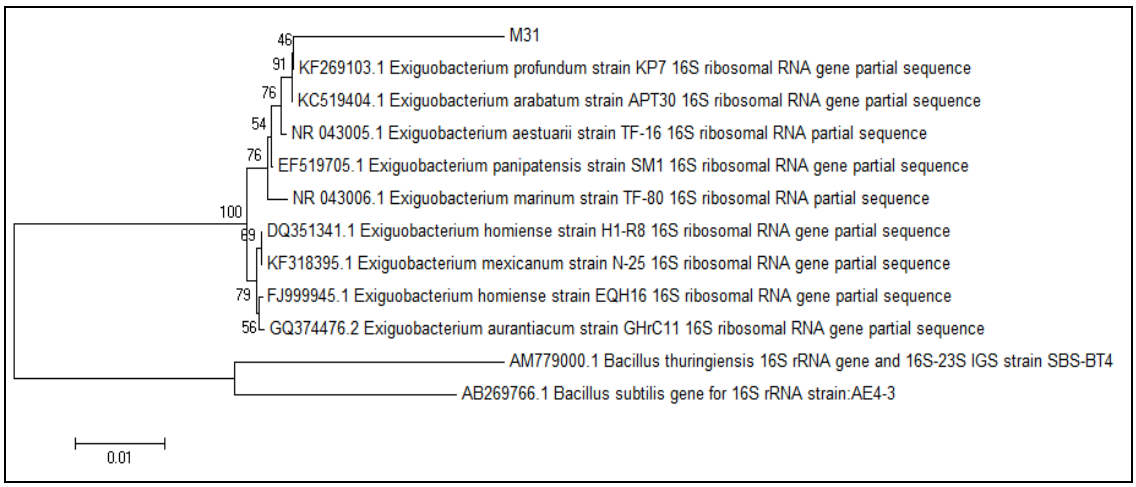
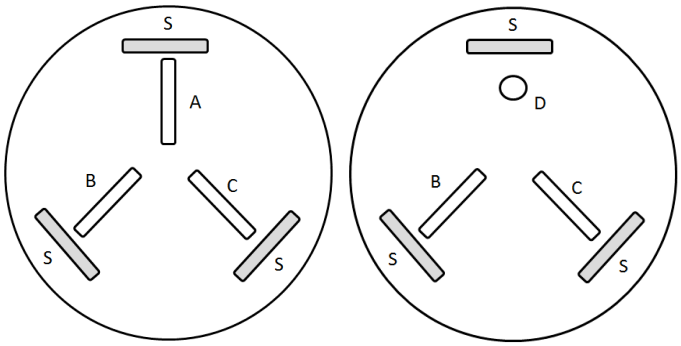


Figure 4.10 16S rRNA gene based phylogenetic analysis of *Exigiobacterium* sp. M31.

4.3 Detection of AHL Production

4.3.1 Detection of AHL Production using Biosensor

Production of purple colour pigment, *i.e.* violacein, of the biosensor *C. violaceum* strain CV026 was induced by the presence of exogenous short chain AHLs (McClean *et al.*, 1997). *E. carotovora* Attn which carries the functional *carI* gene responsible for the production of OC6-HSL served as the positive control for this assay. The *carI* defective mutant *E. carotovora* A20 served as the negative control. Among all the tested bacterial isolated, only *P. aeruginosa* strain M7 induced the purple pigment formation of CV026 (Figure 4.12).



(I) (II)

Figure 4.11 Layout of the CV cross streak assay. S represents the biosensor *C. violaceum* strain CV026. A represents the bacterial isolates from this study. B and C represent positive control (*E. carotovora* strain Attn and *E. corotovora* strain A20, respectively). As bacterial isolate F1, *i.e. Methylobacterium mucilagonosa* was not cultivated on LB agar, therefore, the bacterial isolate was cultivated in 100 mL of HS broth followed by AHL extraction and finally resuspended in 100 µL of ACN. The circle D indicates the AHL crude extract of bacterial isolate. Similarly, bacterial isolate M48, *i.e. Proteus mirabilis*, swarm extensively, making cross streak with *C. violaceum* CV026 was unable to be performed. Therefore, similar with *Methylobacterium rhodesianum* strain F1, M48 was cultivated in 100 mL of LB broth followed by AHL extraction and finally resuspended in 100 µL of ACN. The circle indicated the AHL crude extract of bacterial isolate.

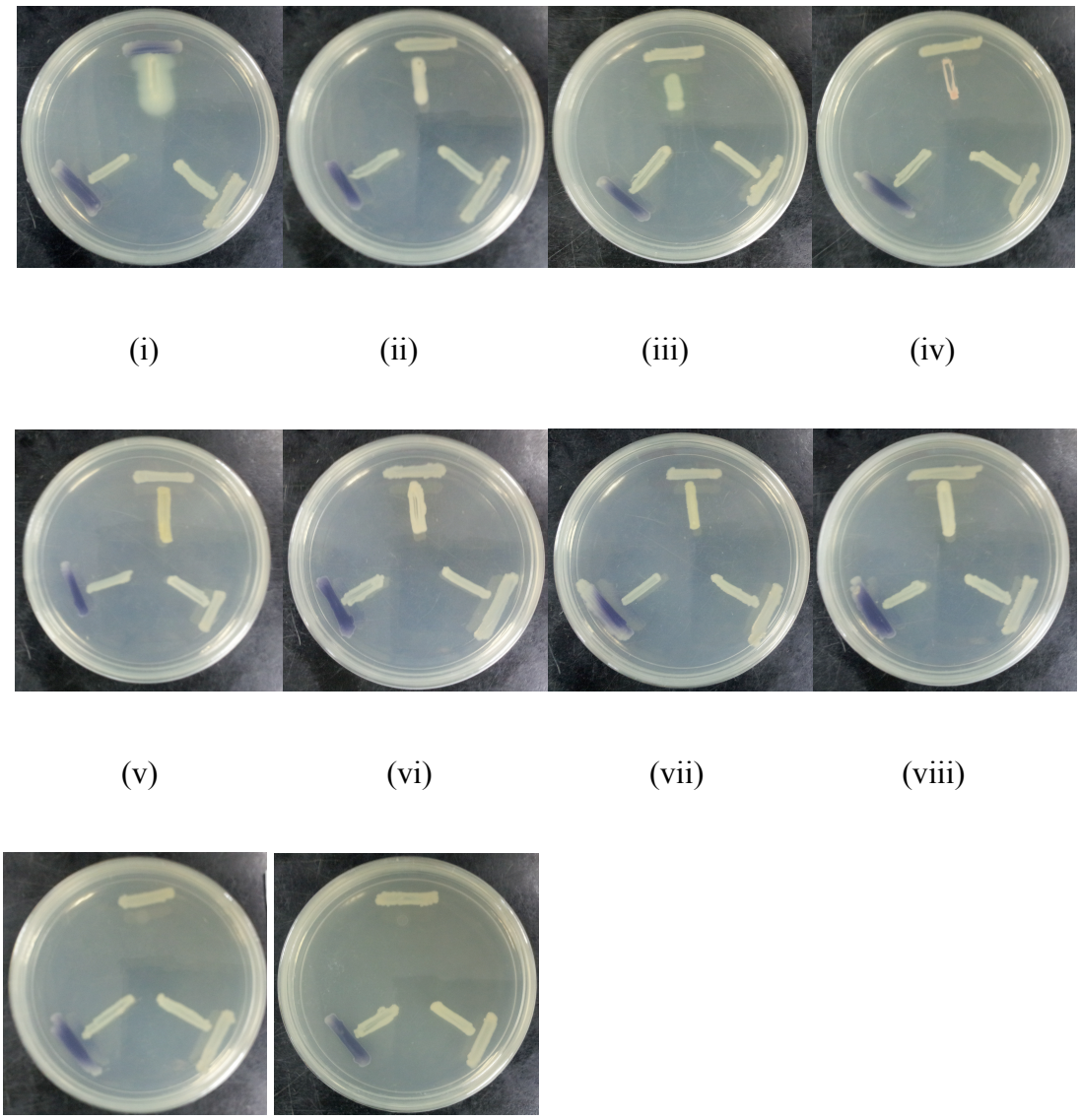


Figure 4.12 Results of the CV026 cross streak. All bacterial isolates exhibited no short chain AHL production expect *P. aeruginosa* strain M7. (i) *P. aeruginosa* strain M7, (ii) unidentified strain M3, (iii) *P. otitidis* strain M18, (iv) *Roseomonas mucosa* strain M66, (v) *Cronobacter sakazakii* strain M30, (vi) unidentified strain M27, (vii) *Stenotrophomonas maltophila* strain F2, (viii) *Providencia rettgeri* strain M22, (ix) *Proteus mirabilis* strain M48 and (x) *Methylobacterium rhodesianum* strain F1.

4.3.2 Detection of Synthetic AHL Molecules using LC/MS/MS-QQQ

AHL crude extracts of all Gram-negative bacterial isolates, as described in Section 3.2.4.2, were subjected to LC/MS/MS-QQQ analysis in order to compensate the sensitivity of biosensor. Besides, the biosensor *C. violaceum* strain CV026 can only detect short chain AHL molecules but not the long chain AHL molecules. The presence of different of QS signalling molecules were confirmed by the detection of product ion, *m/z* 102 that indicate the presence of lactone ring, the fragmentation of the precursor as well as the precursor ion at a targeted retention time with the aid of reference to the standard, *i.e.* synthetic AHL molecules.

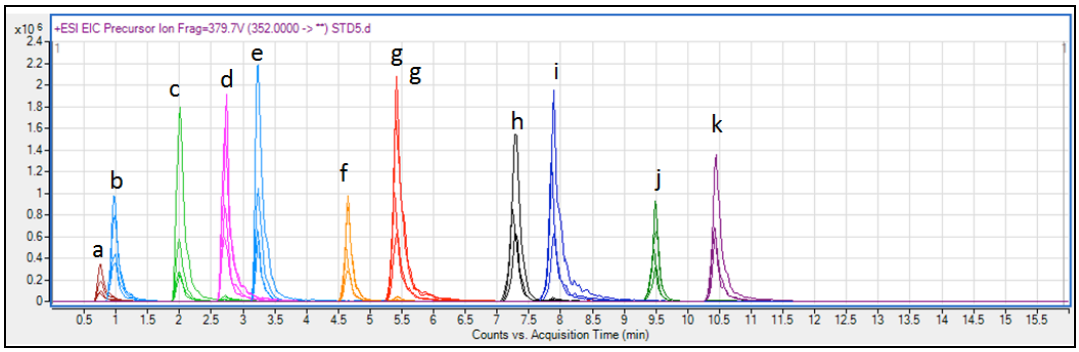


Figure 4.13 Chromatogram of various synthetic AHL molecules. (a) C4-HSL, (b) 3-oxo-C6-HSL, (c) C6-HSL, (d) 3-oxo-C8-HSL, (e) C7-HSL, (f) C8-HSL, (g) 3-oxo-C10-HSL, (h) C10-HSL, (i) 3-oxo-C12-HSL, (j) C12-HSL and (k) 3-oxo-C16-HSL.

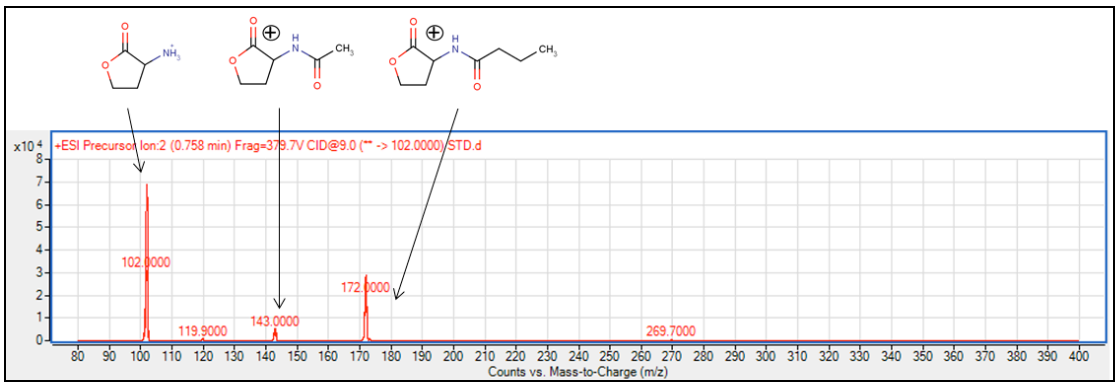


Figure 4.14 ESI-MS Spectrum of C4-HSL. The retention time of C4-HSL is 0.758 min. The precursor ion is *m/z* 172 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

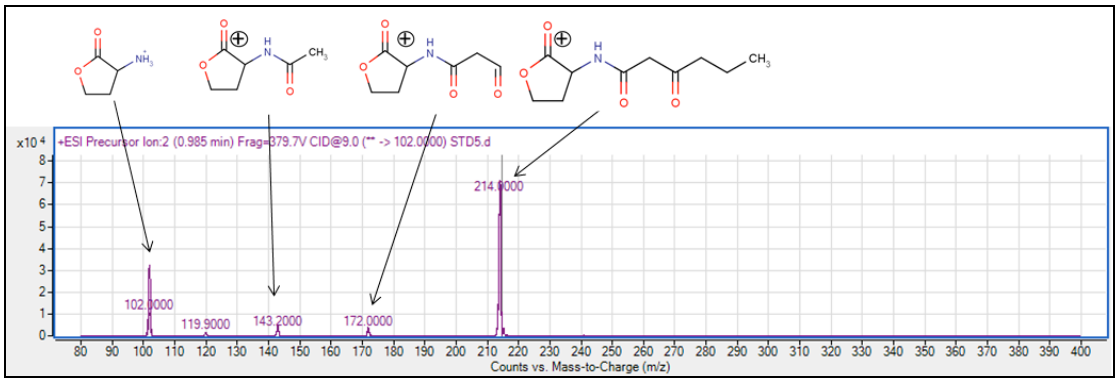


Figure 4.15 ESI-MS Spectrum of 3-oxo-C6-HSL. The retention time of 3-oxo-C6-HSL is 0.985 min. The precursor ion is *m/z* 214 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143 and *m/z* 172, with the structures shown above.

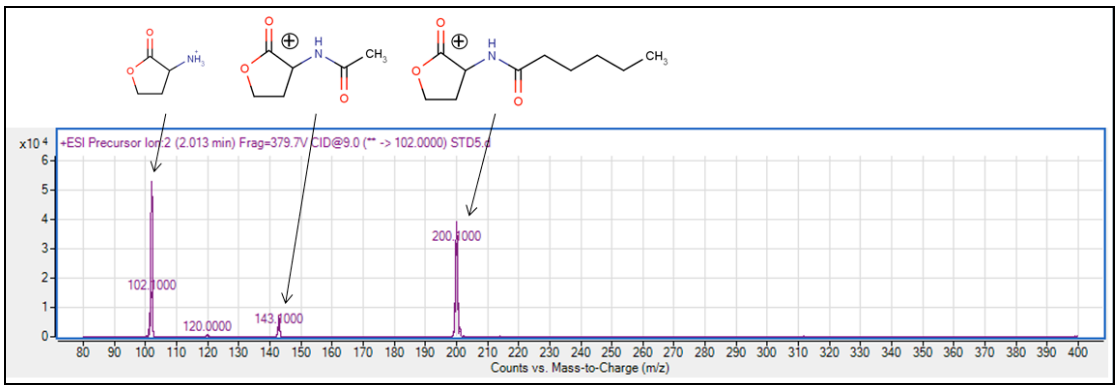


Figure 4.16 ESI-MS Spectrum of C6-HSL. The retention time of C6-HSL is 2.013 min. The precursor ion is *m/z* 200 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

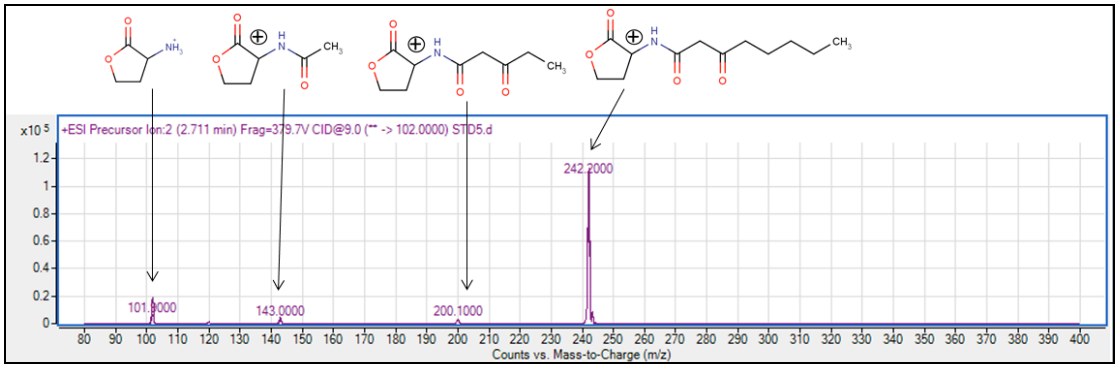


Figure 4.17 ESI-MS Spectrum of 3-oxo-C8-HSL. The retention time of 3-oxo-C8-HSL is 2.711 min. The precursor ion is *m/z* 242 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143 and *m/z* 200, with the structures shown above.

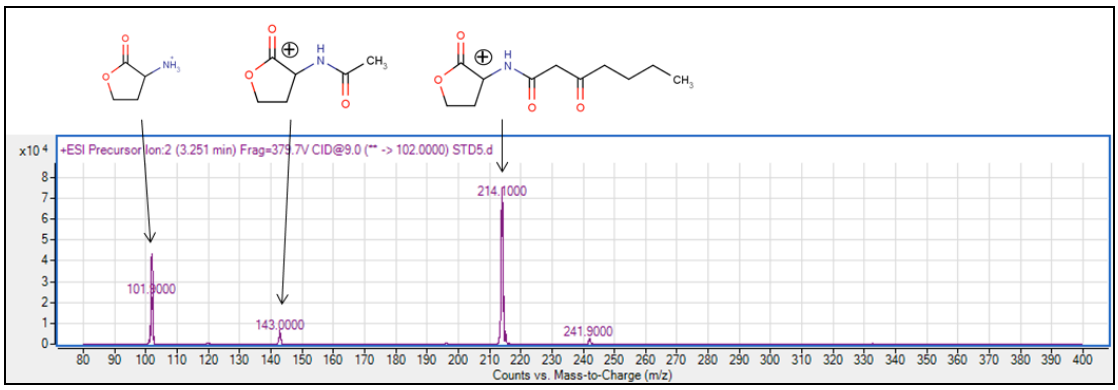


Figure 4.18 ESI-MS Spectrum of C7-HSL. The retention time of C7-HSL is 3.251 min. The precursor ion is *m/z* 214 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

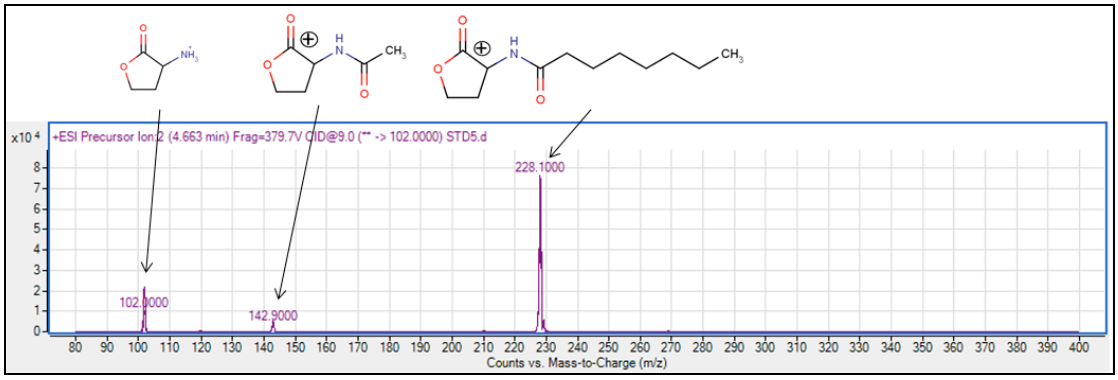


Figure 4.19 ESI-MS Spectrum of C8-HSL. The retention time of C8-HSL is 4.663 min. The precursor ion is *m/z* 228 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

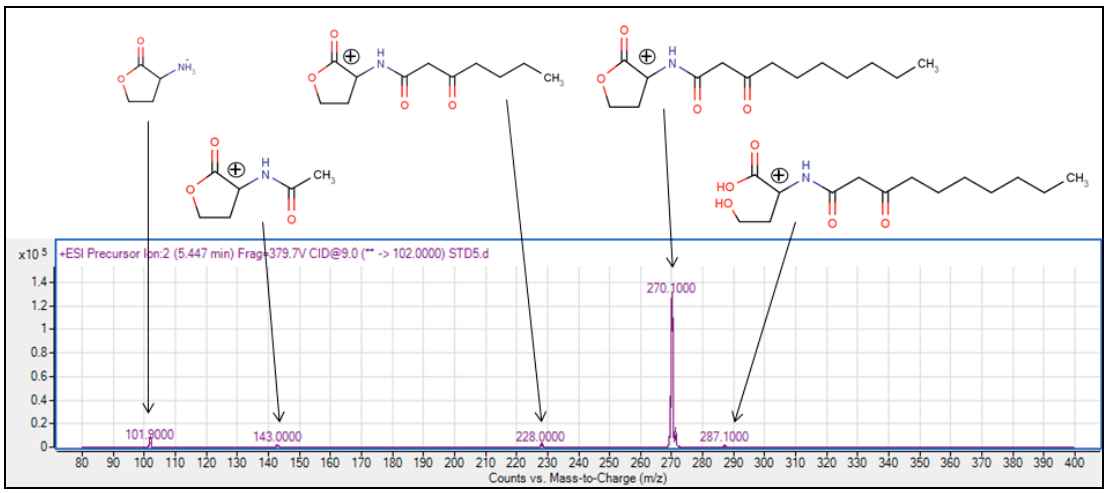


Figure 4.20 ESI-MS Spectrum of 3-oxo-C10-HSL. The retention time of 3-oxo-C10-HSL is 5.447 min. The precursor ion is *m/z* 270 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143 and *m/z* 228, with the structures shown above. A small amount of open-ring structure of 3-oxo-C10-HSL (*m/z* 287) was found.

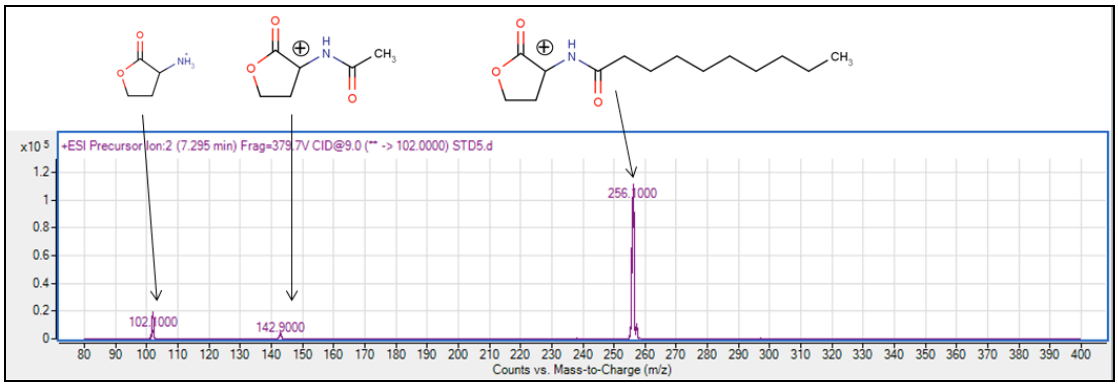


Figure 4.21 ESI-MS Spectrum of C10-HSL. The retention time of C10-HSL is 7.295 min. The precursor ion is *m/z* 256 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

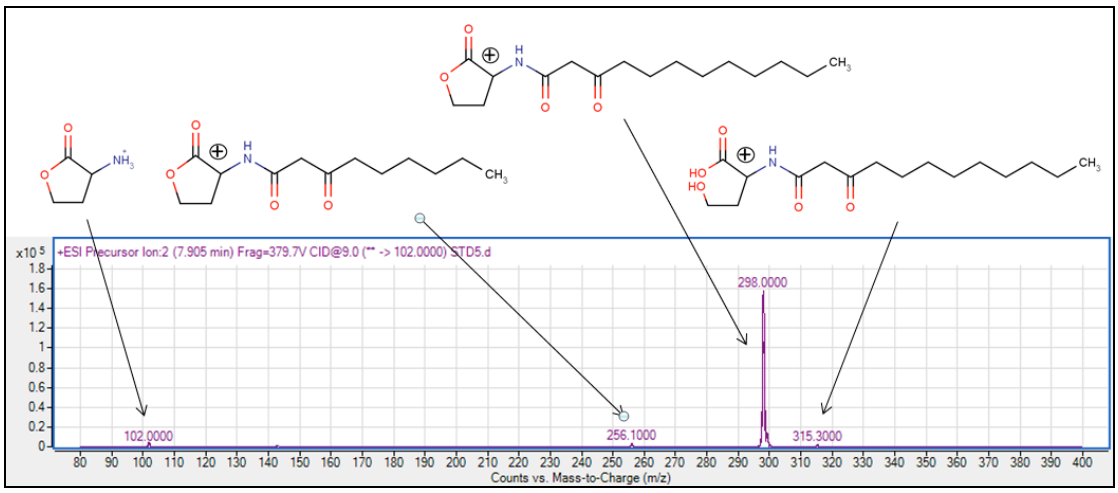


Figure 4.22 ESI-MS Spectrum of 3-oxo-C12-HSL. The retention time of 3-oxo-C12-HSL is 7.905 min. The precursor ion is *m/z* 298 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 256, with the structures shown above. A small amount of open-ring structure of 3-oxo-C12-HSL (*m/z* 315) was found.

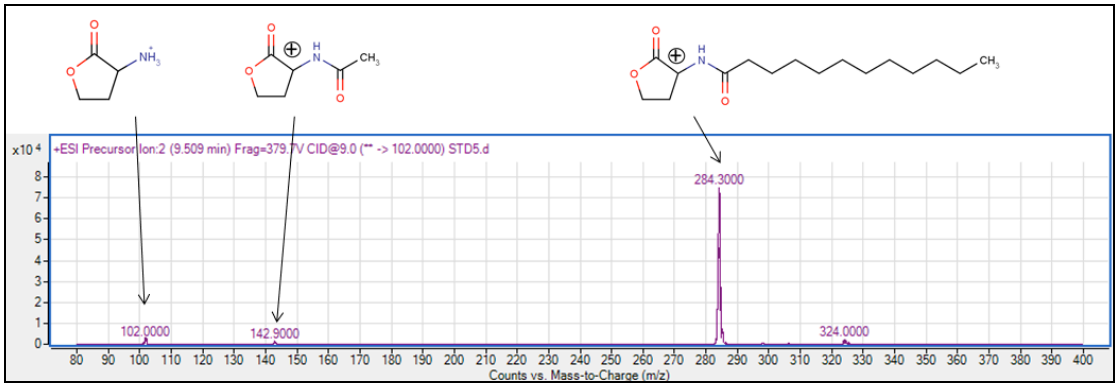


Figure 4.23 ESI-MS Spectrum of C12-HSL. The retention time of C12-HSL is 9.509 min. The precursor ion is *m/z* 284 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 143, with the structures shown above.

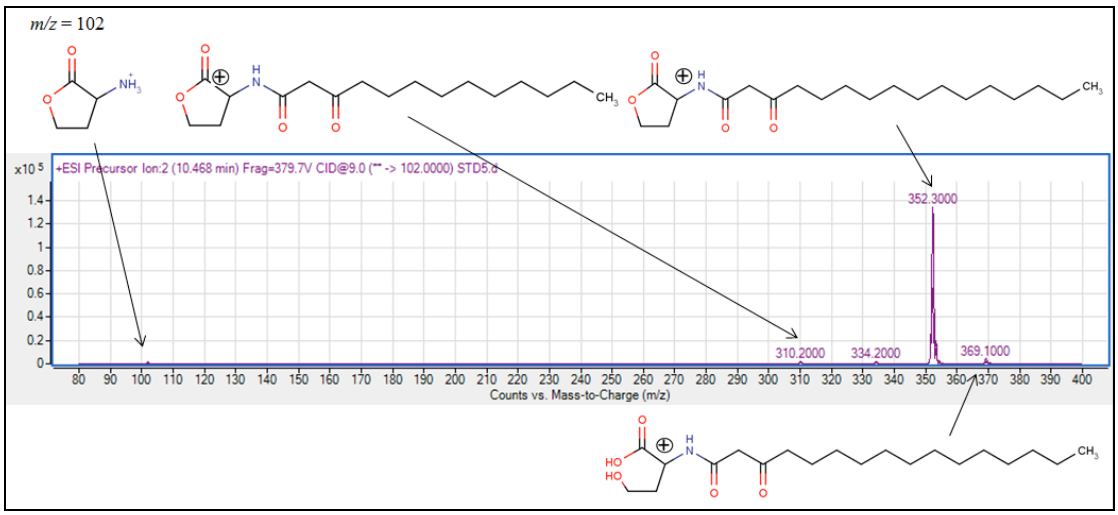


Figure 4.24 ESI-MS Spectrum of 3-oxo-C16-HSL. The retention time of 3-oxo-C16-HSL is 10.468 min. The precursor ion is *m/z* 352 with the product ion of *m/z* 102, indicating the presence of homoserine lactone, as well as *m/z* 310, with the structures shown above. A small amount of open-ring structure of 3-oxo-C16-HSL (*m/z* 369) was found.

4.3.3 Detection of AHL Molecules from Crude Extracts using LC/MS/MS-QQQ

The AHL produced by *Pseudomonas aeruginosa* strain M7 and *Methylobacterium rhodesianum strain* F1 were illustrated in Figure 4.25 and 4.26, respectively.

***Pseudomonas aeruginosa* strain M7**

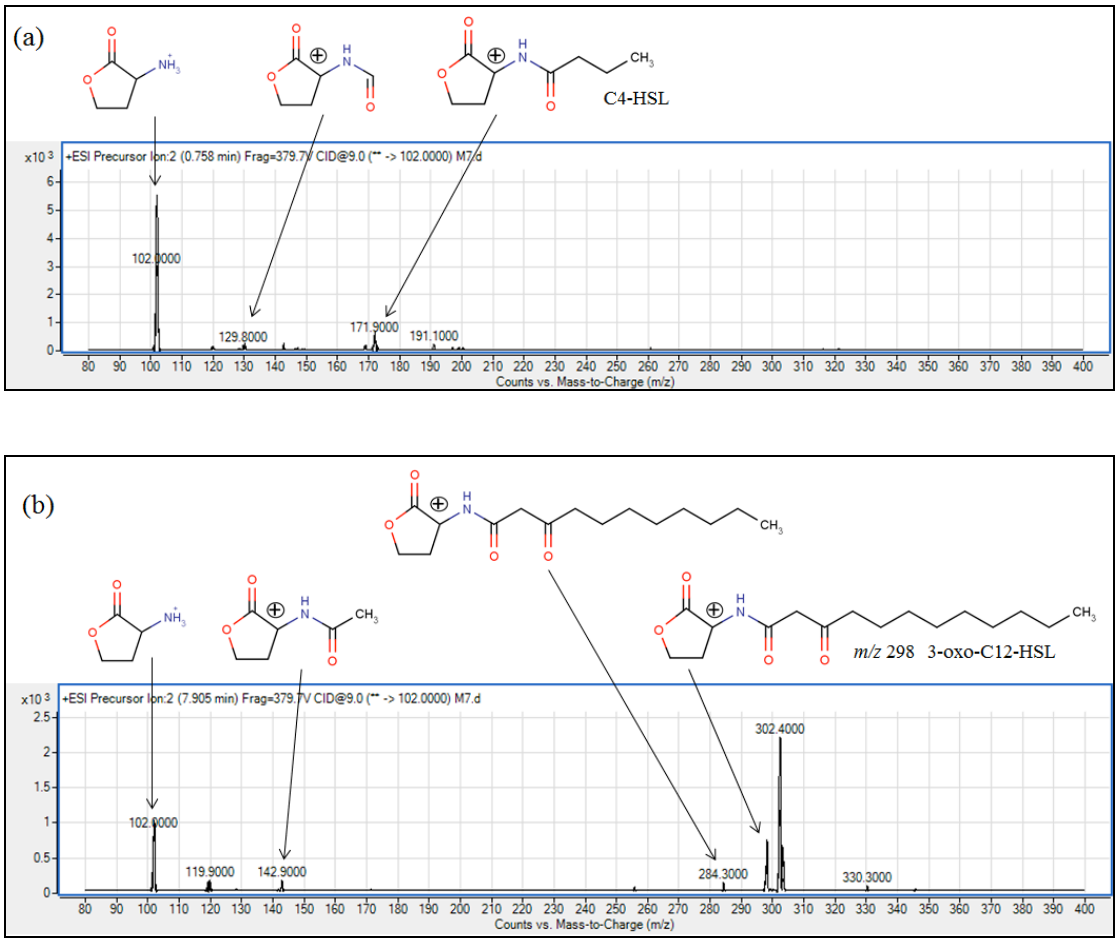


Figure 4.25 ESI-MS Spectrum of AHL crude extract from *Pseudomonas aeruginosa* strain M7. The ESI-MS spectrum indicates the presence of (a) C4-HSL (*m/z* 171.9000, 0.758 min) and (b) 3-oxo-C12-HSL (*m/z* 298, 7.905 min).

***Methylobacterium rhodesianum* strain F1**

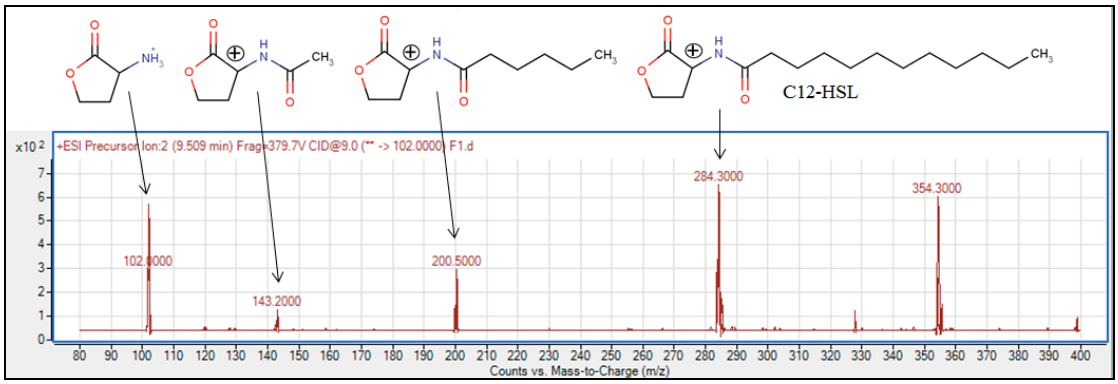


Figure 4.26 ESI-MS Spectrum of AHL crude extract from *Methylobacterium rhodesianum* strain F1. The ESI-MS spectrum indicates the presence of C12-HSL (*m/z* 284.3000, 9.509 min).

4.4 Investigation of Bacterial Quorum Quenching Activities

4.4.1 Screening for QQ Bacteria via Whole Cell Inactivation Assay

The assessments of QQ activities was performed using biosensor *C. violaceum* strain CV026 as described in Section 3.2.5.1. All the bacterial isolates were initially screened for QQ activities by incubating *N*-hexanoyl-$_L$-homoserine lactone (C6-HSL) with cell suspension of each bacterial isolates. Results from the whole cell inactivation assay were depicted in Figure 4.27 where sample *R. mucilaginosa* strain CN14, *P. aeruginosa* strain M7, *P. otitidis* strain M18, *G. aichiensis* strain M45, *B.* megaterium strain M52 and *R. mucosa* strain M66 exhibit QQ phenotype. *B. cereus* served as the positive control, while *E. coli* strain DH5α as well as 1 × PBS served as negative controls in this assay.

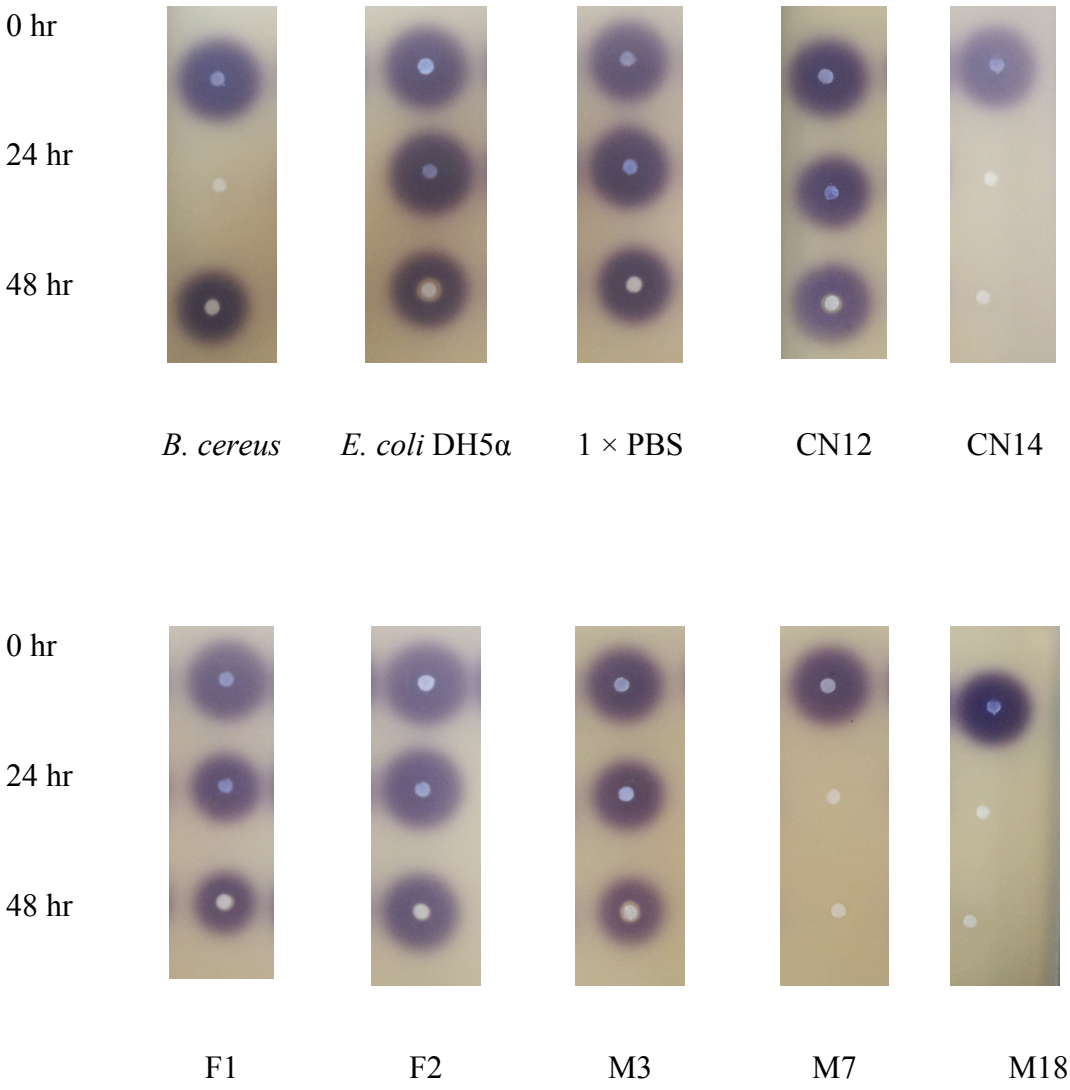
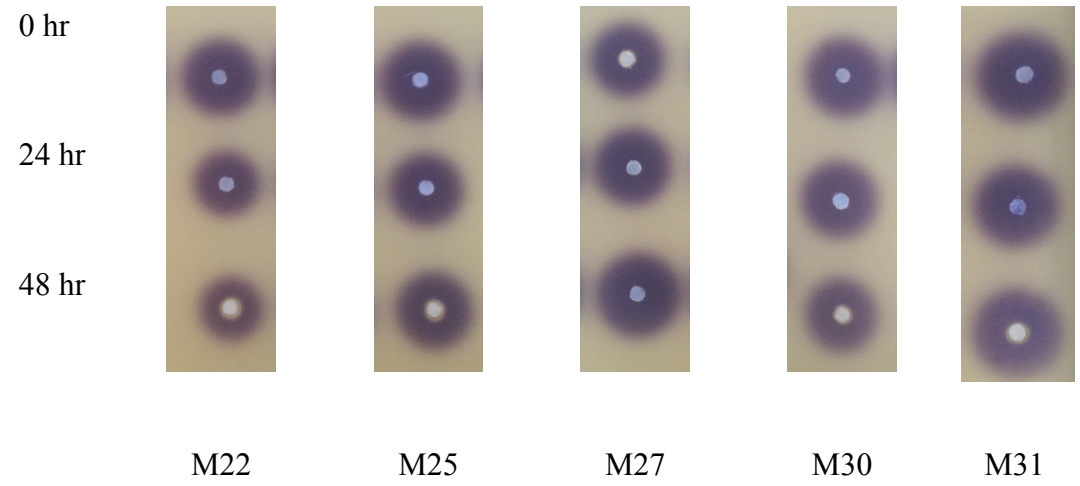
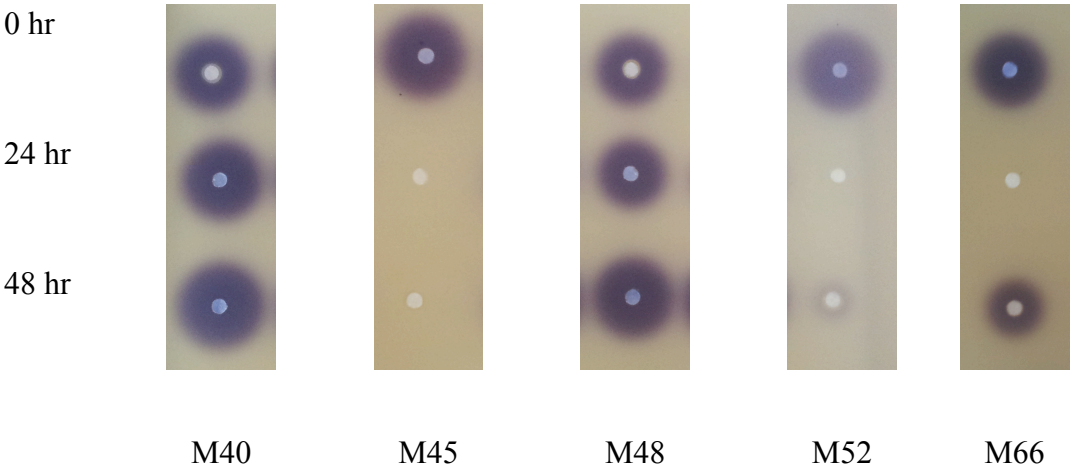


Figure 4.27 Screening for QQ bacteria using whole cell inactivation assay. The 48 hr represents relactonization protocol which aim to investigate the mechanism of QQ involved on the tested isolates, *i.e. B. cereus*, *E. coli* DH5α, *Kocuria rhizophila* CN12, *Rhodotorula mucilaginosa* CN14, *Methylobacterium rhodesianum* F1, *Stenotrophomonas maltophila* F2, strain M3, *P. aeruginosa* M7, *P. otitidis* M18, *Providencia rettgeri* M22, *Dermacoccus nishinomiyaensis*M25, strain M27, *Cronobacter sakazakii* M30, *Exiguobacterium aurantiacum* M31, *Brevibacterium casei* M40, *Gordonia aichiensis* M45, *Proteus mirabilis* M48, *Bacillus megaterium* M52, *Roseomonas mucosa* M66, *Micrococcus luteus* M71 and 1 × PBS.





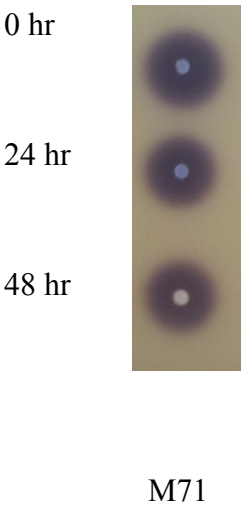


Figure 4.27 Continue

According to Figure 4.27, the QQ mechanism of isolates can be divided into two main categories, *i.e.* AHL-lactonase and non-AHL-lactonase. Only *R. mucosa* strain M66 produced AHL-lactonase which proved by the relactonization of inactivated AHL. The other isolates such as *R. mucilaginosa* strain CN14, *P. aeruginosa* strain M7, *P. otitidis* strain M18 and *G. aichiensis* strain M45 inactivated AHL molecules via non-lactonase based approach. There is a slight degree of AHL reformation at the 48 hr of strain *B. megaterium* strain M52. Therefore, one may deduce the possibility of pipetting error, or other form of enzymatic reactions.

4.4.2 Investigation of QQ Activities using LC/MS/MS-QQQ

Isolates with positive results at the Section 4.4.1 were proceeded to AHL inactivation assay using LC/MS/MS-QQQ, tested with various AHLs, *e.g.* C4-HSL, C6-HSL, 3-oxo-C8-HSL and 3-oxo-C12-HSL.

Inactivation of C4-HSL

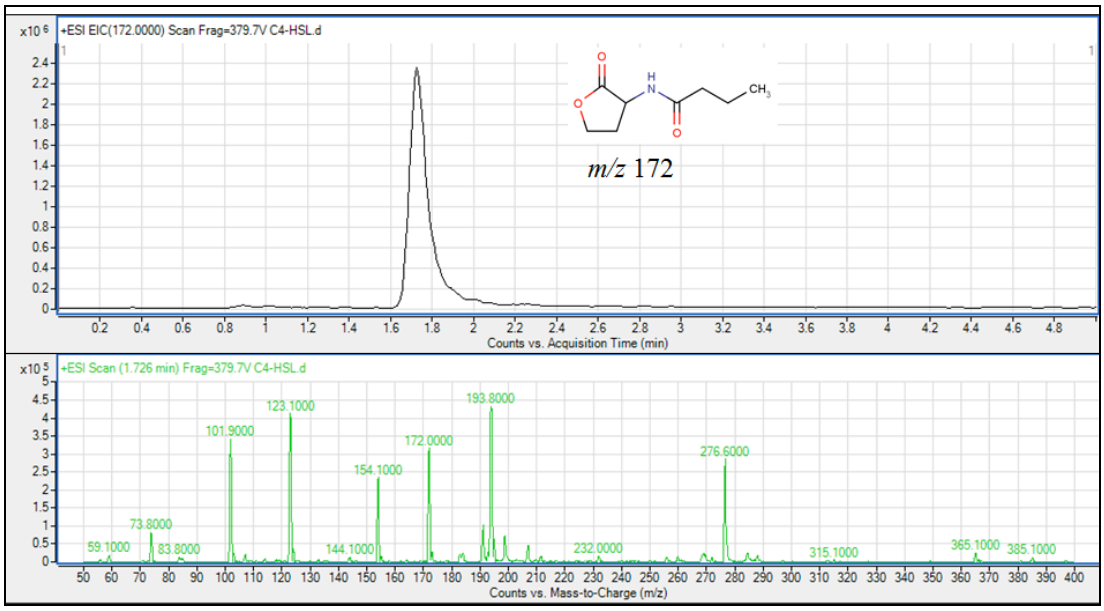


Figure 4.28 Chromatogram and ESI-MS spectrum of C4-HSL (*m/z* 172.0000, 1.726 min)

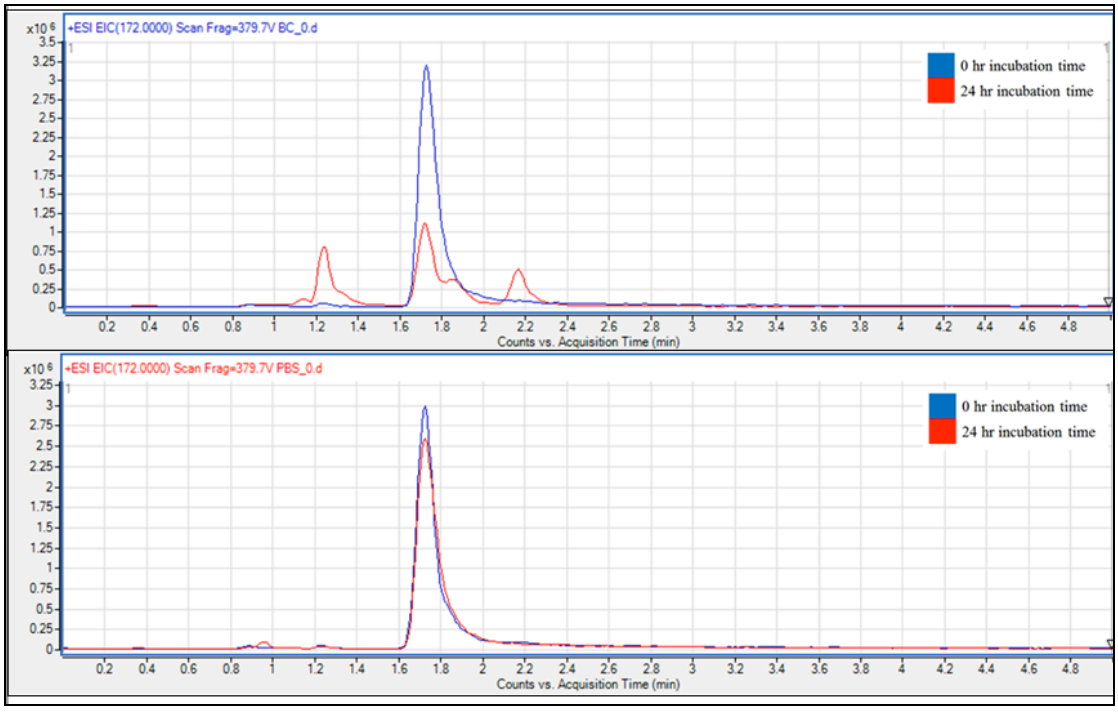


Figure 4.29 Chromatogram of C4-HSL treated with (top) *B. cereus* and (bottom) 1 × PBS, serving as positive control and negative control, respectively.

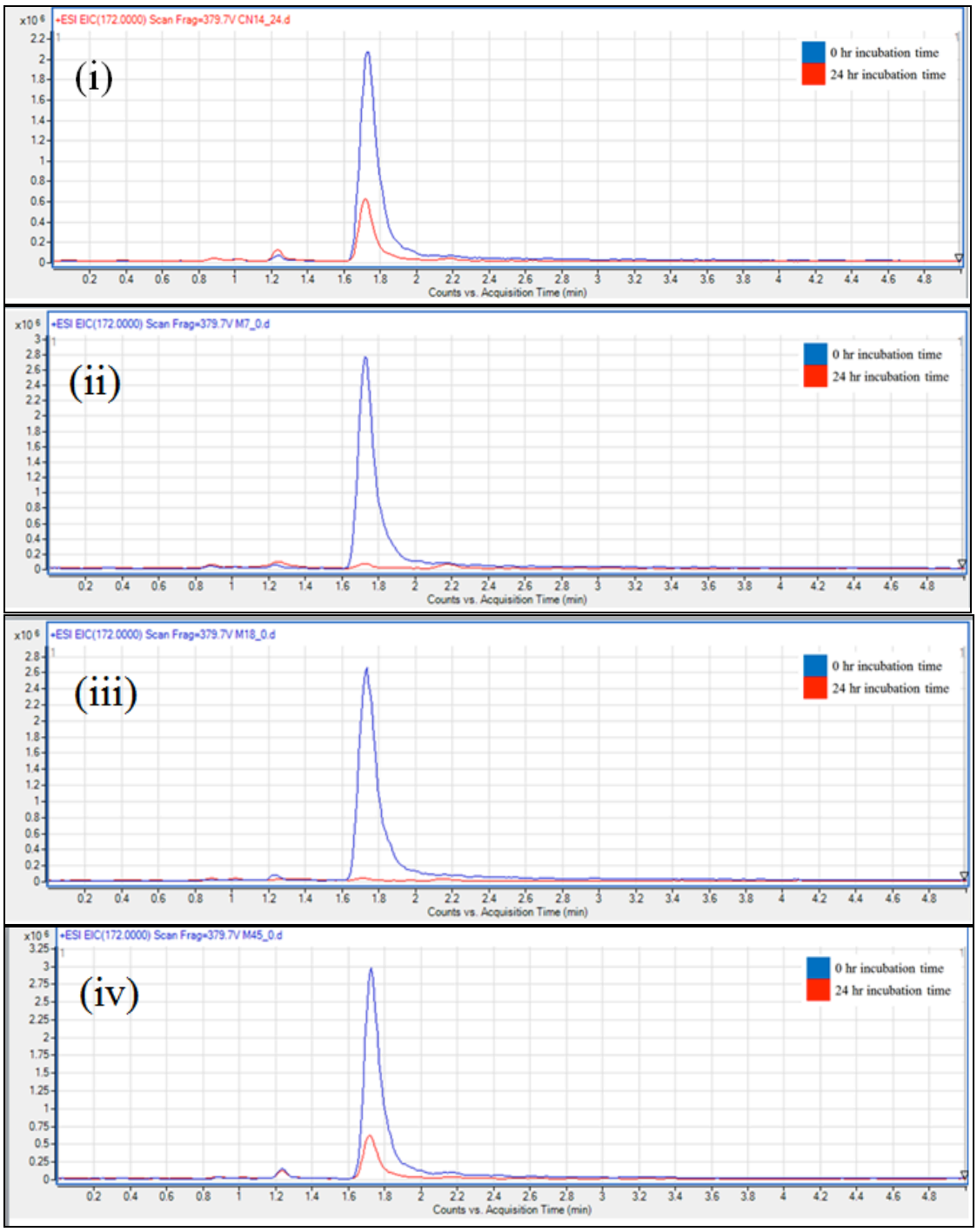


Figure 4.30 Chromatogram of C4-HSL treated with different isolates. (i) *R. mucilaginosa* strain CN14, (ii) *P. aeruginosa* strain M7, (iii) *P. otitidis* strain M18 and (iv) *G. aichiensis* strain M45, (v) *B. megaterium* strain M52 and (vi) *R. mucosa* strain M66.

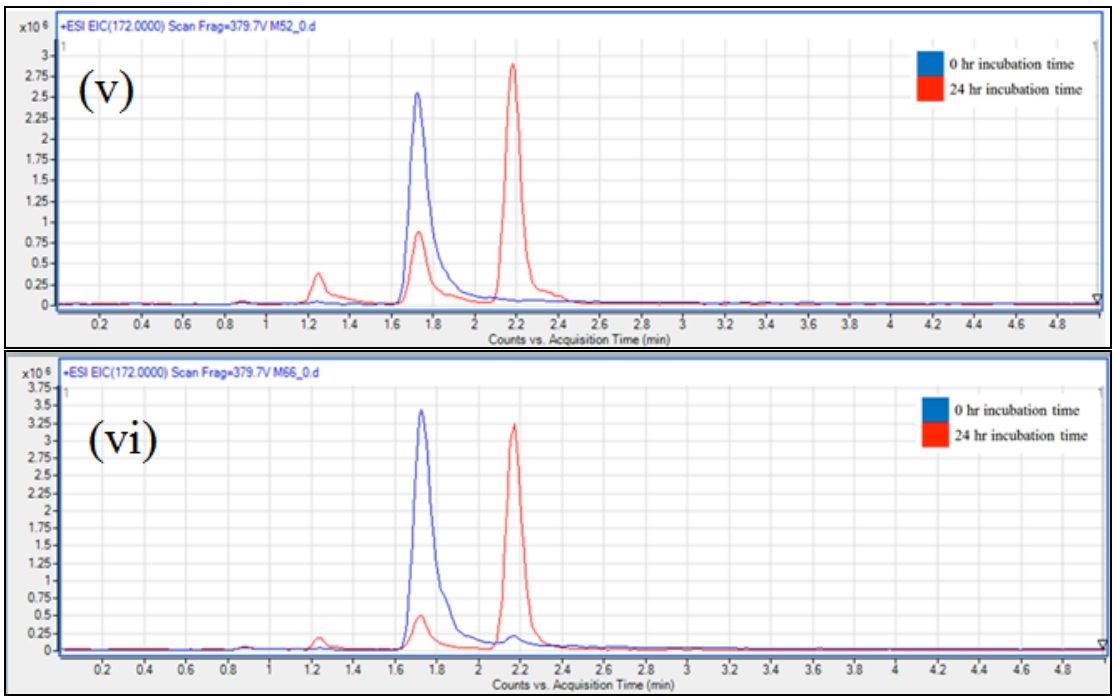


Figure 4.30 Continue

Inactivation of C6-HSL

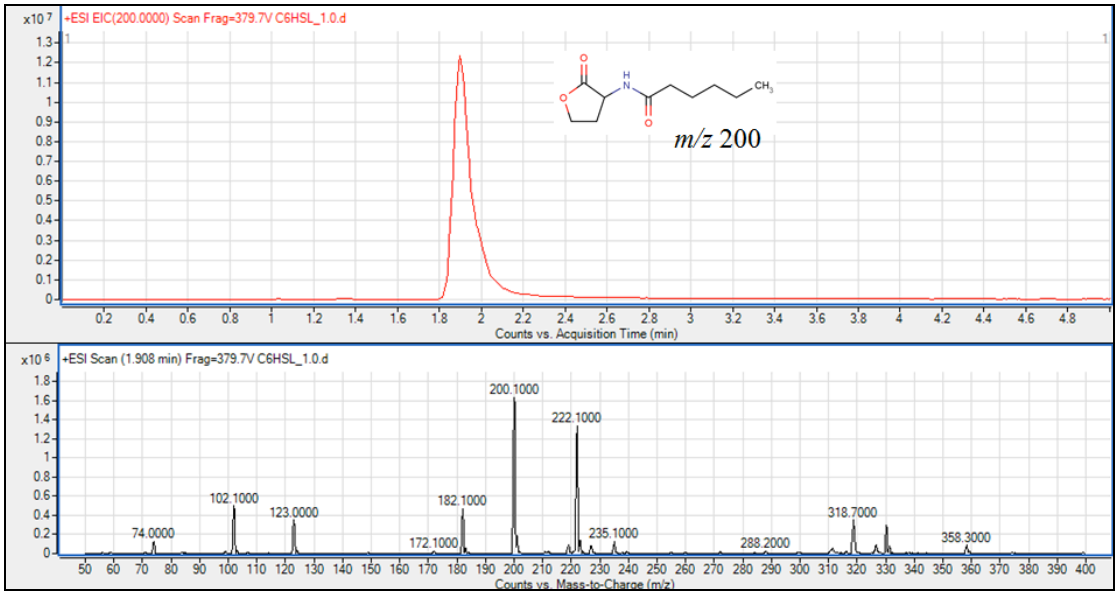


Figure 4.31 Chromatogram and ESI-MS spectrum of C6-HSL (*m/z* 200.0000, 1.908 min)

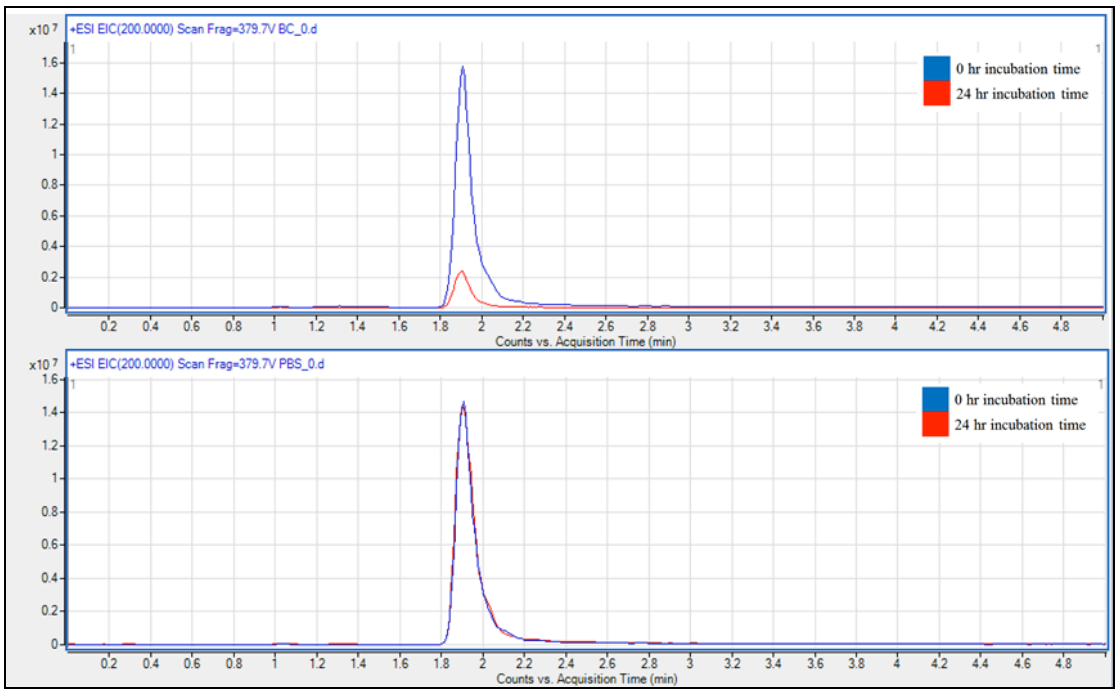


Figure 4.32 Chromatogram of C6-HSL treated with (top) *B. cereus* and (bottom) 1 × PBS, serving as positive control and negative control, respectively.

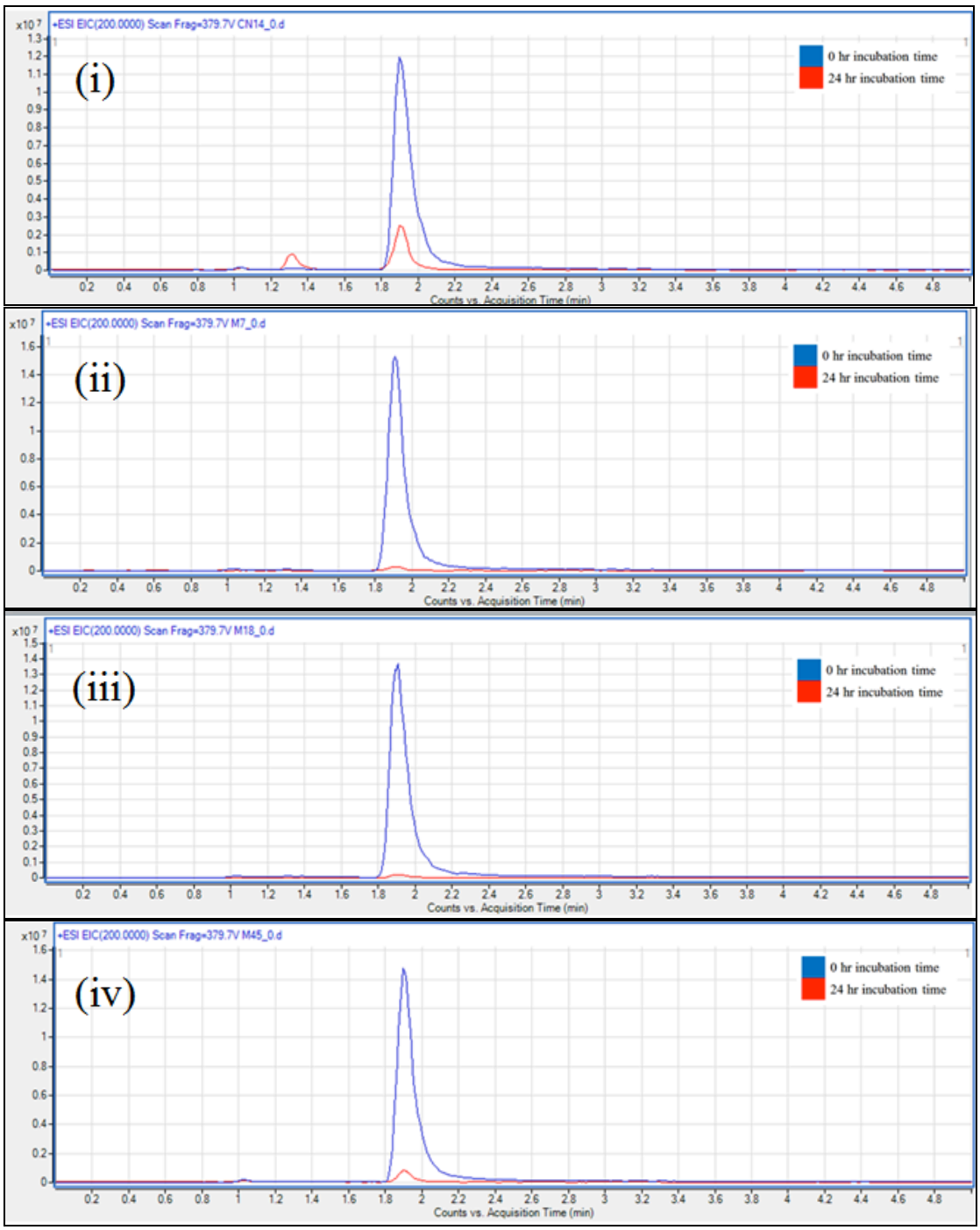


Figure 4.33 Chromatogram of C6-HSL treated with different isolates. (i) *R. mucilaginosa* strain CN14, (ii) *P. aeruginosa* strain M7, (iii) *P. otitidis* strain M18 and (iv) *G. aichiensis* strain M45, (v) *B. megaterium* strain M52 and (vi) *R. mucosa* strain M66.

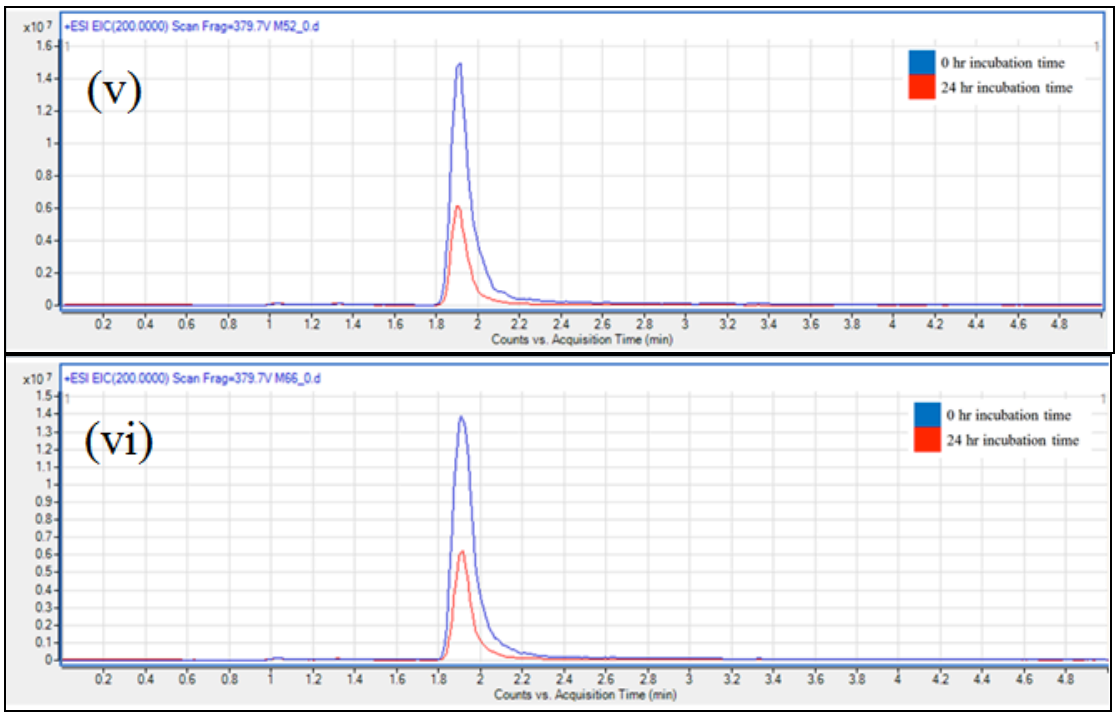


Figure 4.33 Continue

Inactivation of 3-oxo-C8-HSL

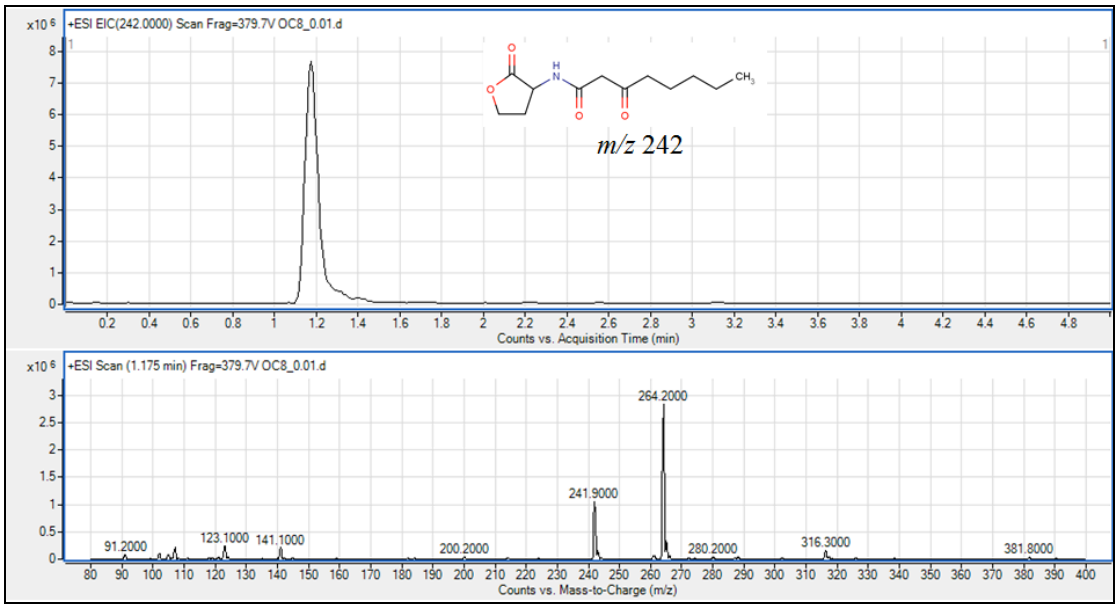


Figure 4.34 Chromatogram and ESI-MS spectrum of 3-oxo-C8-HSL (*m/z* 241.0000, 1.175 min)

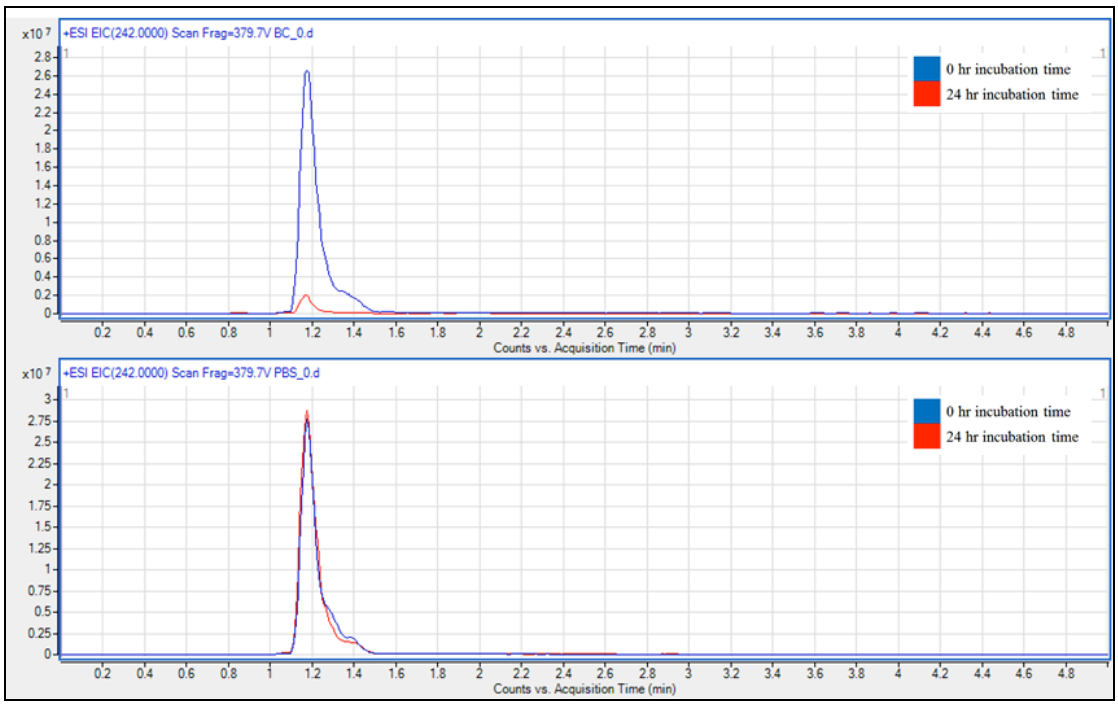


Figure 4.35 Chromatogram of 3-oxo-C8-HSL treated with (top) *B. cereus* and (bottom) 1 × PBS, serving as positive control and negative control, respectively.

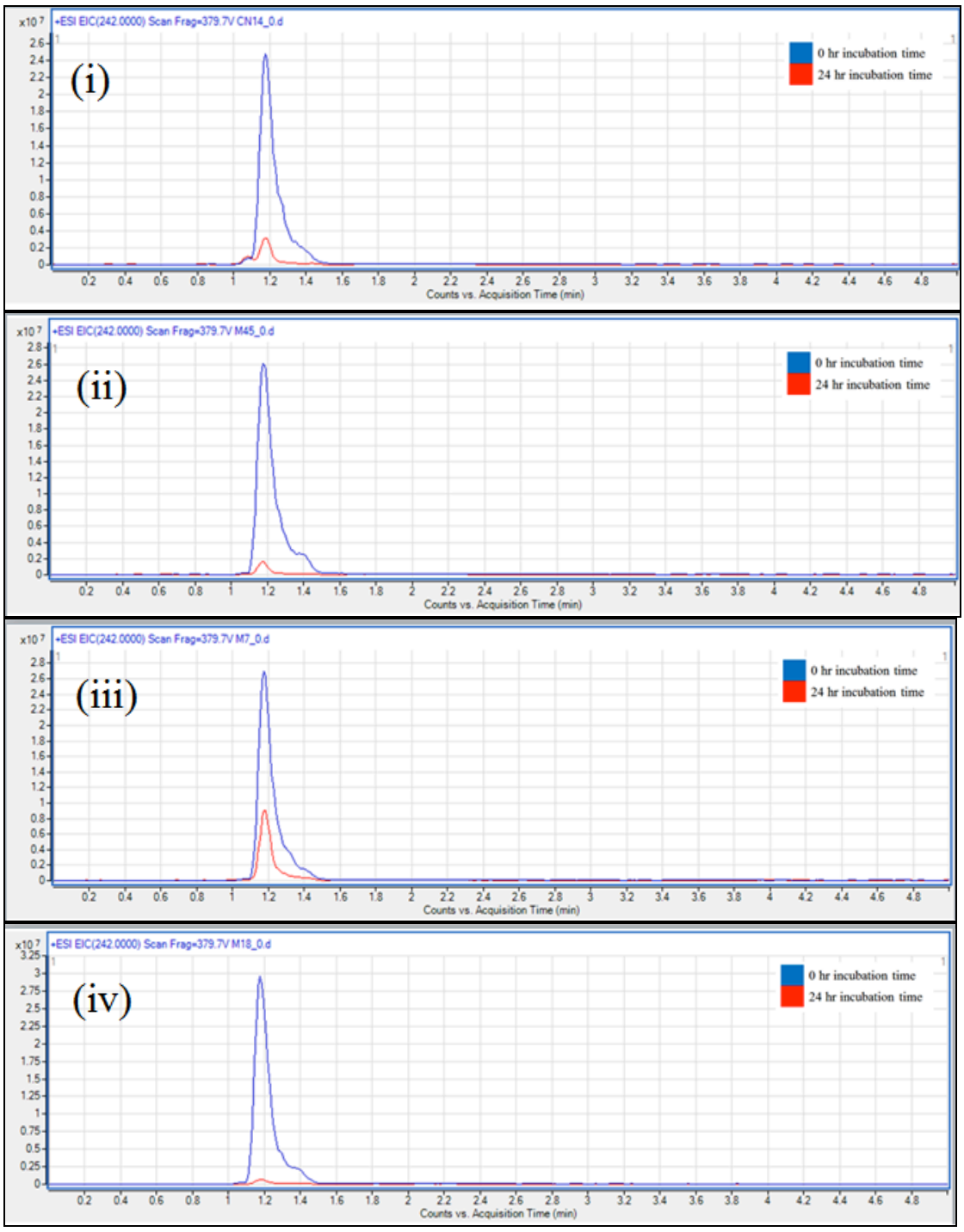


Figure 4.36 Chromatogram of 3-oxo-C8-HSL treated with different isolates. (i) *R. mucilaginosa* strain CN14, (ii) *P. aeruginosa* strain M7, (iii) *P. otitidis* strain M18 and (iv) *G. aichiensis* strain M45, (v) *B. megaterium* strain M52 and (vi) *R. mucosa* strain M66.

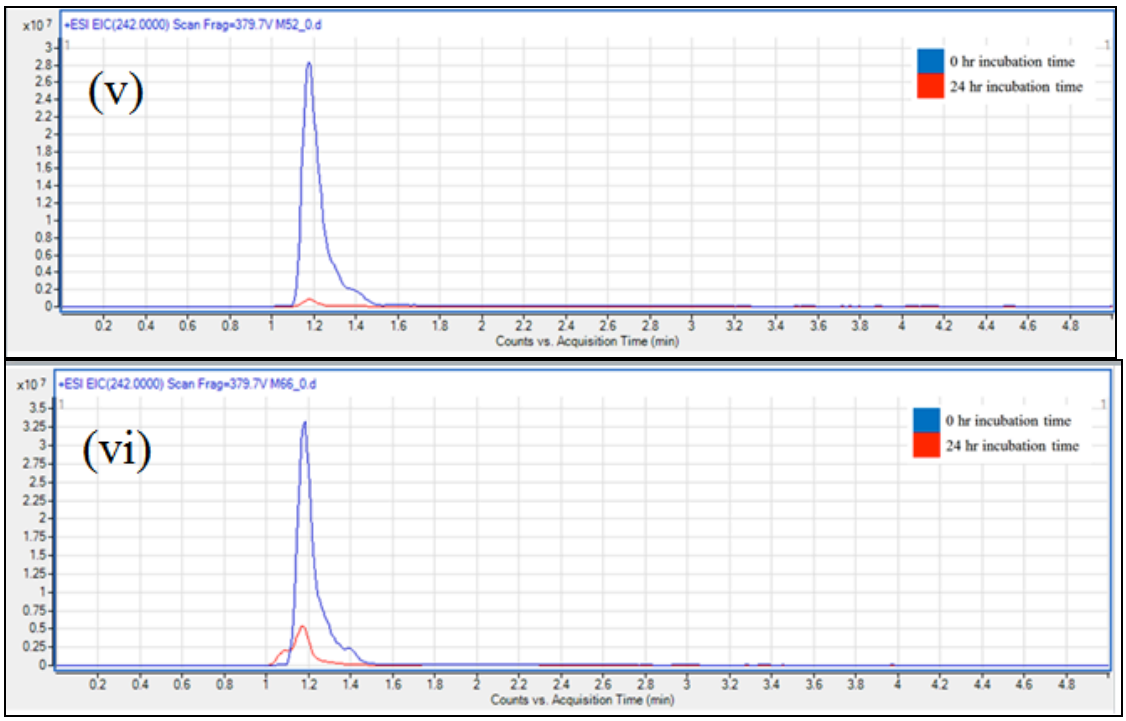


Figure 4.36 Continue

Inactivation of 3-oxo-C12-HSL

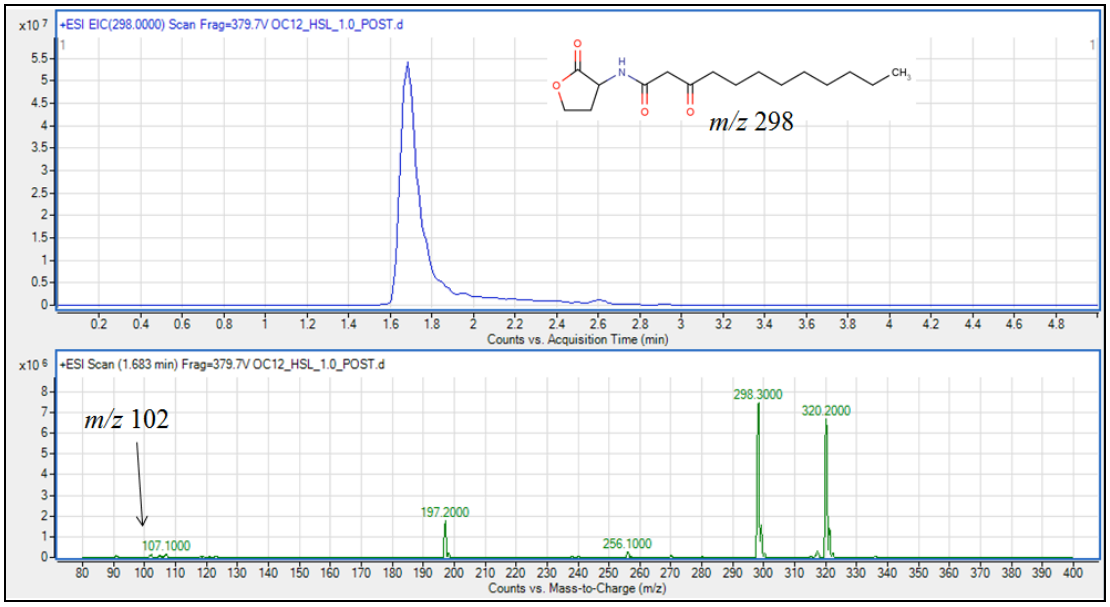


Figure 4.37 Chromatogram and ESI-MS spectrum of 3-oxo-C12-HSL (*m/z* 298.0000, 1.683 min)

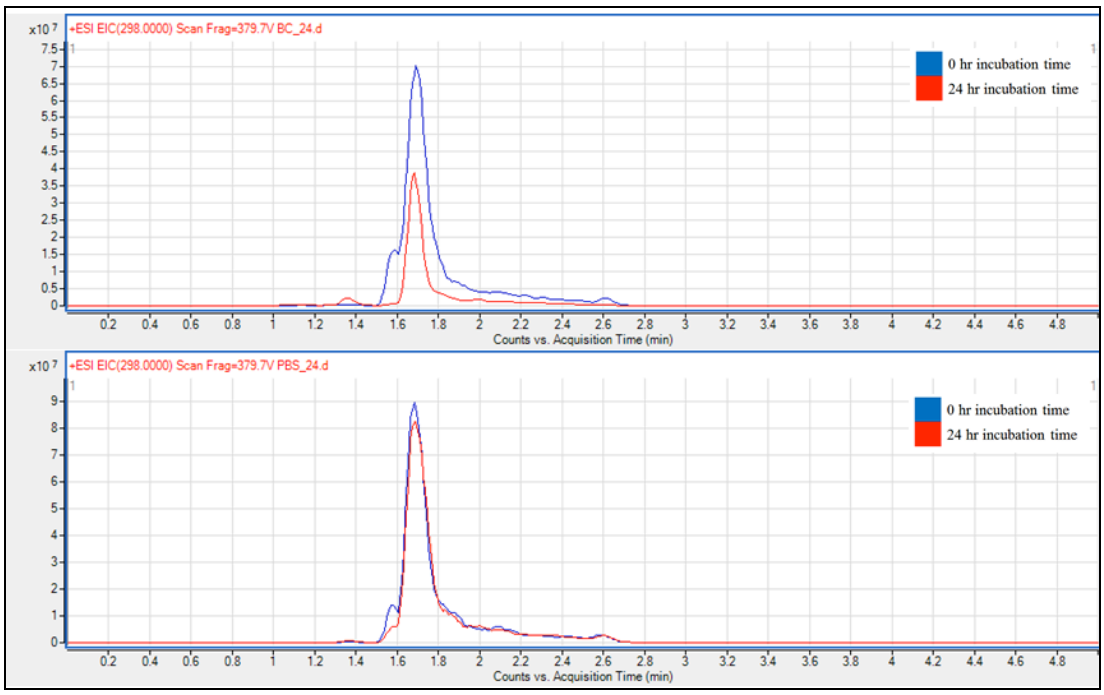


Figure 4.38 Chromatogram of 3-oxo-C12-HSL treated with (top) *B. cereus* and (bottom) 1 × PBS, serving as positive control and negative control, respectively.

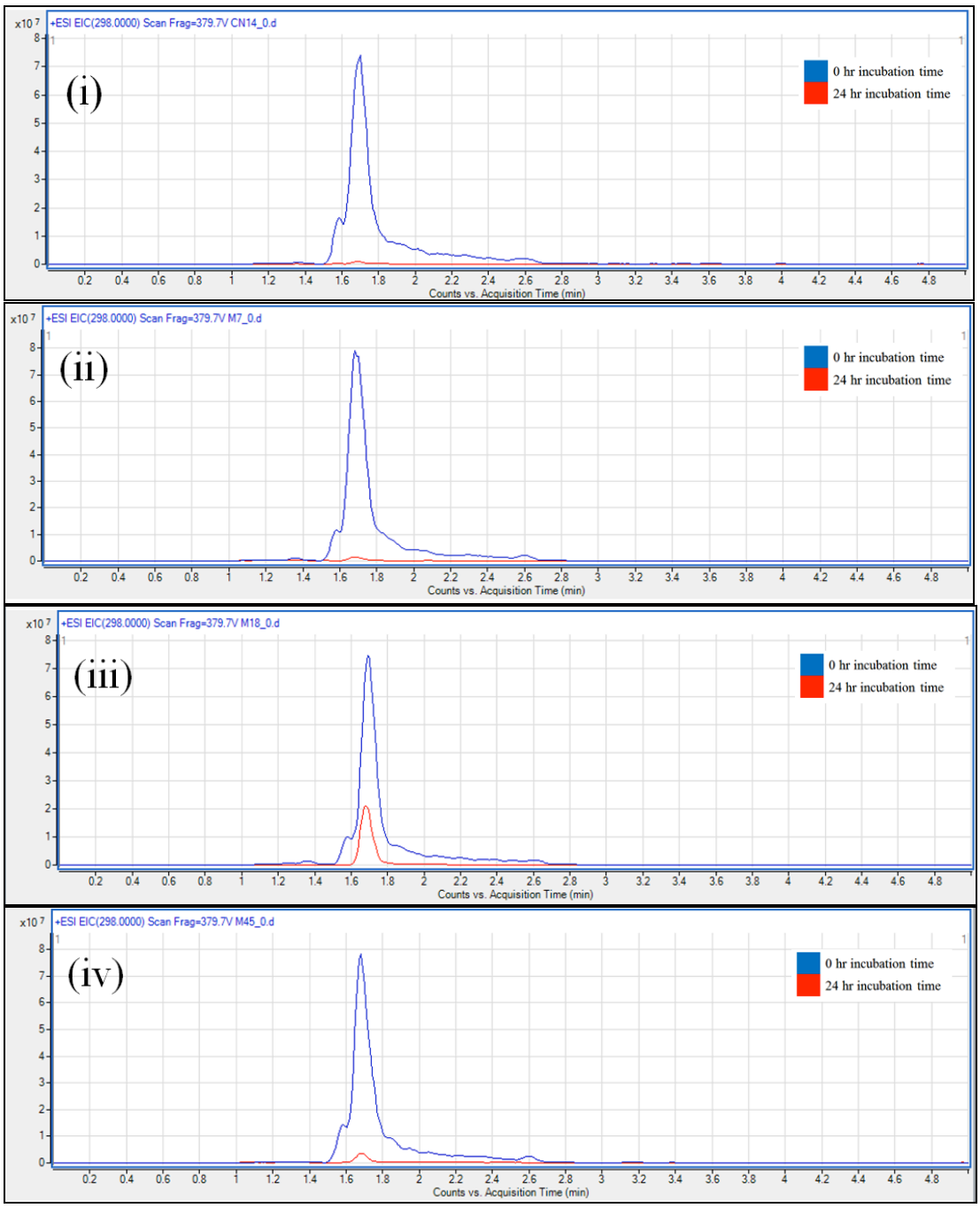


Figure 4.39 Chromatogram of 3-oxo-C12-HSL treated with different isolates. (i) *R. mucilaginosa* strain CN14, (ii) *P. aeruginosa* strain M7, (iii) *P. otitidis* strain M18 and (iv) *G. aichiensis* strain M45, (v) *B. megaterium* strain M52 and (vi) *R. mucosa* strain M66.

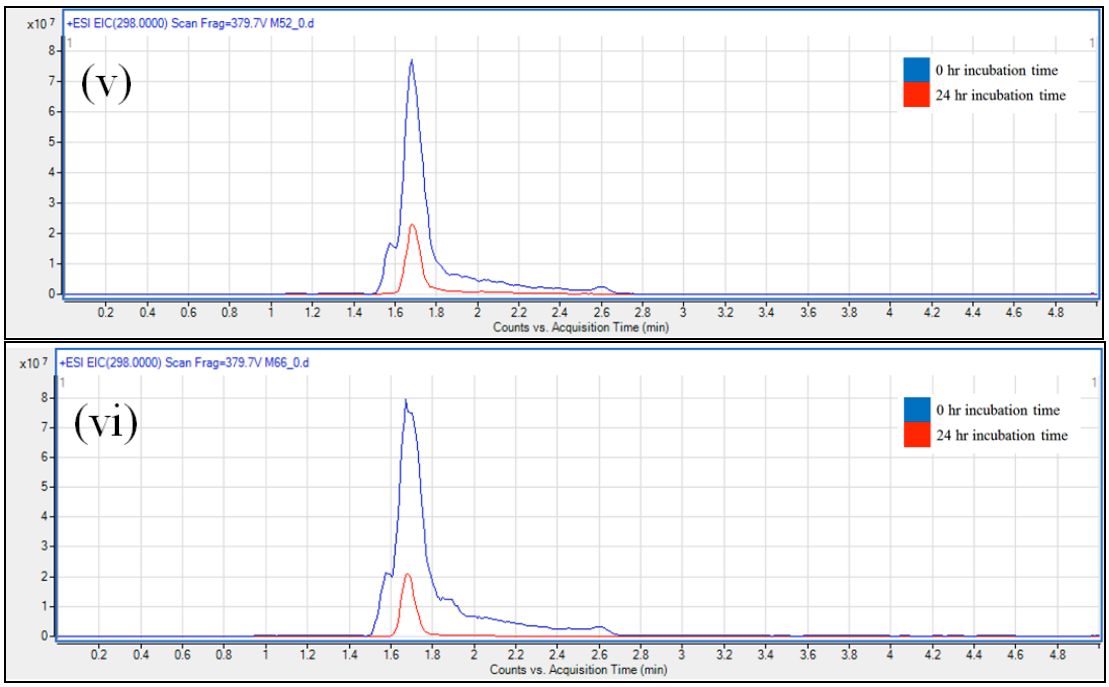


Figure 4.39 Continue

CHAPTER 5.0

DISCUSSION

5.1 Isolation and Identification of Isolates from Ulu Slim Hot Spring

Water from Ulu Slim Hot Spring was selected as isolation source in this study for the investigation of QS and QQ activities among the bacteria inhabiting in a relatively warm area, *i.e.* 35 to 38 °C. The hottest temperature of the hot spring can achieve up to 101 °C. As the boiling underground water flow out from the source and flow towards the peripheral of the hot spring, the temperature decreased. The water sample collected was from the peripheral area with the temperature of 38 °C.

Several isolates which were found in this study have been reported by other researchers to be isolated from hot spring or from hot and humid environments. For example, *Rhodotorula* (*R. mucilaginosa* CN14) has been isolated from Kusatsu Hot Spring, Japan (Nguyen *et al.*, 2001), *Chelatococcus* (*C. daeguensis* M3) has been isolated from a hot spring in Egypt (Ibrahim *et al.*, 2010), *P. aeruginosa* (*P. aeruginosa* M7 and *P. otitidis* M18) has been isolated from a hot spring in Hokkaido, Japan (Hasanuzzaman *et al.*, 2004), *Exiguobacterium* (*Exiguobacterium aurantiacum* M31) has been isolated from Yellowstone National Park, USA (Vishnivetskaya *et al.*, 2011), various *Bacillus* species (*Bacillus megaterium* M52) have been discovered from an Indonesian hot spring (Baker *et al.*, 2001).

5.2 AHL Production of *Pseudomonas* and *Methylobacterium*

Preliminary screening of AHL production among the isolates in this study was performed using biosensor *C. violaceum* strain CV026. Violacein production in this biosensor is inducible by AHLs evaluated with *N*-acyl side chains range from C4 to C8,

with varying degree of sensitivity. However, this biosensor was unable to detect any of the 3-hydroxyl-derivatives and with reduced sensitivity to most of the 3-oxo-derivatives (McClean *et al.*, 1997). Therefore, LC/MS/MS-QQQ was used in order to detect the presence of AHL and to characterized the secreted AHL.

In this study, only *P. aeruginosa* M7 induced the violacein production of biosensor suggesting the biosynthesis of short chain AHL molecules. The mass spectrometry analysis of the supernatant of *P. aeruginosa* M7 revealed a peak at *m/z* 171.9000 and ~298.0000, indicating the molecular ion (M+H) of C4-HSL and 3-oxo-C12-HSL, respectively. *P. aeruginosa* has been reported to produce C4-HSL (Pearson *et al.*, 1995) and 3-oxo-C12-HSL (Pearson *et al.*, 1994). According to Pearson *et al.* (1994), 3-oxo-C12-HSL, which synthesized by LasI, activates genes that encode exoprotease virulence factors, such as *lasA, lasB, aprA, toxA* etc., and lead to biofilm formation. C4-HSL is synthesized by RhlI, activates *lasB, rhlAB* (for rhamnolipid production) and *rpoS* (Pearson *et al.*, 1995).

The another pseudomonad, *i.e. P. otitidis* M18, failed to induce violacein production of the biosensor, suggesting two possibilities, (i) no AHL signalling molecule produced by this bacterial isolates, or (ii) only long chain AHL molecules was produced by this bacterial isolates. The LC/MS/MS-QQQ results (not shown) did not reveal any sign of AHL production.

In this study, *Methylobacterium rhodesianum* F1 failed to induce violacein production. The LC/MS/MS-QQQ results indicated the presence of a long chain AHL species, *i.e.* C12-HSL (*m/z* 284.3000). *Methylobacterium*, specifically *M. extorquens*, has been reported to possess two functional LuxI homologs, namely MsaI which responsible for the biosynthesis of C6-HSL and C8-HSL, as well as MlaI which responsible for the production of C14:1-HSL and C14:2-HSL, which are organized in

hierarchical manner, with MsaI activity required for full expression of *mlaI* (Penalver *et al.*, 2006). In 2007, Poonguzhali *et al.* investigated the AHL production of several species of *Methylobacterium*, *i.e. Methylobacterium* sp. CBMB120, *Methylobacterium* sp. CBMB130, *M. suomiense*, *M. extorquens*, *M. hispanicum* and *M. rhodinum*. These *Methylobacterium* species do produced various kinds of AHL, suggesting the production of AHL is widespread in *Methylobacterium*. The result of this study is likely to be the first report for the production of C12-HSL by *M. rhodesianum* F1.

5.3 Inactivation of AHL Molecules

5.3.1 AHL-degrading Fungus – *Rhodotorula mucilaginosa*

In this study, a fungal strain CN14, was isolated from the hot spring. Microflex™ LT MALDI-TOF Biotyper showed that this isolate belonged to *Rhodotorula mucilaginosa*, which is a member of Basidiomycota of the Urediniomycetes class.

R. mucilaginosa can be readily isolated from various environments such as olive mill wastewater (Jarboui *et al.*, 2013), agricultural soil (Abdul Salam *et al.*, 2013), human (Martini *et al.*, 2013), fresh water and marine (Fell *et al.*, 2011). They are capable of degrading various aromatic compounds, organophosphate compounds and xenobiotic compounds (Krastanov *et al.*, 2013, Bempelou *et al.*, 2013, Romero *et al.*, 2002). The degradation of AHL by *R. mucilaginosa* CN14 expands the list of diverse metabolic traits exhibited by members of this genus. This strain of fungi able to degrade a variety of AHLs, such as C4-HSL. C6-HSL, 3-oxo-C8-HSL as well as 3-oxo-C12-HSL. The relactonization assay by incubating the heat-killed cell suspension with equal volume of 0.2 N of HCl. The experiment proved that the QQ enzyme produced by this fungal strain is not AHL-lactonase.

It has become apparent that similar to bacteria, fungus also utilized QS mechanism to regulate population–level behaviors such as pathogenesis (Hogan, 2006). Various fungal signalling molecules have been identified, such as farnesol and tyrosol in *Candida albicans* (Hornby *et al.*, 2001; Chen *et al.*, 2004). In this study, no production of AHL was reported in this fungal isolate.

The discovery of AHL-inactivating activity in *R. mucilaginosa* CN14 raised the question about the reason of such catabolic activity in eukaryotic microorganism, in the hot spring environment. Some bacteria are known to produce chitin-degrading enzyme, *i.e.* chitinase, which regulated by QS (Chernin *et al.*, 1998). There is a scientific literature reported the presence and the extraction of extracellular chitinases from *P. aeruginosa* (Wang & Chang, 1997). In this study, *P. aeruginosa* M7 and *R. mucilaginosa* CN14 were isolated from the same sampling source. The inactivation of AHL could be a strategy developed by the fungi to interfere the QS system of the Gram-negative bacteria, and disrupt the deleterious bacterial functions, especially chitinases which degrade the chitin of the cell wall of fungus, and thereby giving the fungus survival advantage in the competitive habitats. Recently, an AHL-lactonase producing microscopic yeast, *i.e. Trichosporon loubieri* WW1C, was discovered (Wong *et al.*, 2013), suggesting the catabolic diversity of fungus in degrading AHL molecules.

5.3.2 QQ *Pseudomonas*

P. aeruginosa is a common bacterium found in diverse environmental condition due to its metabolic versatility. It is ubiquitous in the soil and water ecosystems, including the freshwater ecosystems. *P. aeruginosa* is well known for its ability in metabolizing a diverse chemical compounds ranging from organophosphate insecticides

to aromatic compound (Hsu *et al.*, 1979; Deziel *et al.*, 1996). In this study, *P. aeruginosa* and *P. otitidis* were able to degrade C4-HSL, C6-HSL, 3-oxo-C8-HSL and 3-oxo-C12-HSL, and the QQ enzymes from both microorganisms were not AHL-lactonases. This can be explained by the expression of two QQ-enzymes encoding genes, *i.e. pvdQ* and *quiP*, which are both AHL-acylases (Huang *et al.*,2003; Huang *et al.*,2006; Sio *et al.*,2006). As suggested by Huang *et al.* (2006), that these two AHL-acylases play crucial roles in regulating the production of AHL molecules in order for the bacteria to communicate and to ensure that cell-to-cell communication is not disrupted.

5.3.3 AHL-Lactonase of *Roseomonas*

Roseomonas is commonly found in soil, freshwater, lake sediment as well as playing a nasty role as a human pathogen causing severe bacteremia (Chen *et al.*, 2012; Furuhata *et al.*, 2008; Jiang *et al.*, 2006; Rihs *et al.*, 1993). In 2012, this bacteria has been reported to produce QQ enzyme and the genome of this bacteria has been sequenced (Chen *et al.*, 2012). This study further explains the mechanism of AHL-degradation by *Roseomonas* sp. M66, which is via AHL-lactonase approach. The can be proven by the relactonization assay shown in Figure 4.27 in which the addition of 0.2 N of HCl reform the functional AHL and thus inducing the violacein formation of the biosensor.

5.3.4 QQ *Gordonia* sp.

The actinomycete genus *Gordonia* has attracted much interest due to its ability to degrade xenobiotic compounds, environmental pollutants and various toxic compounds. Besides, *Gordonia* spp. also produce a variety of useful compounds, such as carotenoids, biosurfactant, gordonan (an acidic, cell aggregation-inducing polysaccharide) and gordonin (a form of glycosylated peptidolipids) (Arenskotter *et al.*, 2004). *Gordonia* species have been isolated from various environments, such as mangrove rhizosphere, hydrocarbon-contaminated soil, wastewater treatment bioreactors and on diseased human (Takeuchi *et al.*, 1998; Kummer *et al.*, 1999; Kim *et al.*, 2003; Verma *et al.*, 2006). This study has expanded the list of diverse metabolic traits exhibited by members of this genus.

5.3.5 Inactivation of AHL by *Bacillus megaterium*

Various members of *Bacillus* have been reported to possess the AHL-lactonase encoding gene, *i.e. aiiA*, which cleaves the ester bond of the lactone ring of AHL (Dong *et al.*, 2000). *B. megaterium* has been reported to inactivate AHL by oxidoreductase, in which the CYP102A1 oxidises the acyl chain of AHL at ω-1, ω-2 and ω-3 positions, independent of the presence or absence of the 3-oxo-group at C3 and occurs whether the lactone form is oxidesed or the hydroxyl-acid form is oxidised then recyclized (Chowdhary *et al.*, 2007).

In this study, upon additional of equal volume of 0.2 N HCl, a very minute amount of functional AHL has reformed, indicated by the formation of purple pigmented zone. The AHL inactivation might be contributed by the AHL-lactonase, forming a dysfunctional acyl homoserine, or by AHL-oxidoreductase, forming a slightly

edited signalling molecule, which fail to induce violacein production by the biosensor CV026.

5.4 Future Work

One of the major finding of this study is the identification of a QQ fungal strain, *i.e. R. mucilaginosa* CN14. Many intriguing questions arise from this finding: What is the mechanism of AHL-degradation of *R. mucilaginosa*? Little is known regarding eukaryotic quorum quenching as compared to quorum quenching in bacteria.

Similarly, the mechanism of QQ in *Gordonia* is still not known. Therefore, further study on the mechanism of QQ and the genes encoding for the QQ enzyme should be performed.

One of the approaches to determine which QQ mechanism is by investigating the AHL degradation products. AHL-acylases cleave the amide bond of AHL, forming HSL and fatty acid. HSL is readily to react with 5-(dimethyl amino)-naphthalene-1-sulfonyl chloride (DANSYL-chloride), forming dansylated homoserine lactone which has an increased hydrophobicity, thus allowing more resolved separation during chromatography (Lin *et al.*, 2003; Uroz *et al.*, 2008). In order to investigate the AHL-oxidoreductases, a more in depth and comprehensive analytical chemical analysis is required.

Whole genome sequencing of these bacterial isolates is currently undergoing. This is to study the genes present in the genome as well as to understand the global gene regulation and physiological effects of QS based on the organization of the genes.

CHAPTER 6.0

CONCLUSION

As a summary, a total of eighteen microorganisms have been isolated from Ulu Slim Hot Spring, at the lower temperature region, *i.e.* 35 – 38 °C. The isolated strains are *Kocuria rhizophila* CN12, *Rhodotorula mucilaginosa* CN14, *Methylobacterium rhodesianum* F1, *Stenotrophomonas maltophila* F2, *Chelatococcus* sp. M3, *Pseudomonas aeruginosa* M7, *Pseudomonas otitidis* M18, *Providencia rettgeri* M22, *Dermacoccus nishinomiyaensis* M25, *Schineria* sp. M27, *Cronobacter sakazakii* M30, *Exiguobacterium aurantiacum* M31, *Brevibacterium casei* M40, *Gordonia* sp. M45, *Proteus mirabilis* M48, *Bacillus megaterium* M52, *Roseomonas* sp. M66, *Micrococcus luteus* M71, and their identities were determined via MicroflexTM LT MALDI-TOF Biotyper as well as 16S rRNA sequencing. LC/MS/MS-QQQ analysis has identified the production of C4-HSL and 3-oxo-C12-HSL in spent supernatant of *P. aeruginosa* M7, as well as C12-HSL in spent supernatant of *M. rhodesianum* F1. This study also demonstrated the degradation of AHL by basidiomycetes yeast, *i.e. R. mucilaginosa* CN14, as well as five bacterial isolates, namely *P. aeruginosa* M7, *P. otitidis* M18, *Gordonia* sp. M45, *B. megaterium* M52 and *Roseomonas* sp. M66. Of these six isolates, only *Roseomonas* sp. M66 produced AHL-lactonase, while the rest produce other enzymes to inactivate or degrade AHL molecules. Further investigation to identify the AHL synthase genes, genes regulated by QS as well as QQ-enzymes encoding genes from these microorganisms are recommended. This can be achieved via whole genome sequencing. Besides, more in-depth studies on the QQ mechanisms of the QQ bacteria are suggested.

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