CHARACTERISATION OF QUORUM QUENCHING ACTINOBACTERIA ISOLATED FROM MANGROVE SWAMPS EXHIBITING LACTONASE ACTIVITY

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ABSTRACT

Sophisticated communication systems that link cell density and gene expression in many bacteria species aims to regulate a broad range of biological functions. Such cell-to-cell communication, termed quorum sensing (QS), depends on the production, diffusion, and recognition of small signal molecules. Quorum quenching (QQ), is a process that interrupt the key process of QS and could be potentially used to prevent microbial infections. Actinobacteria produce numerous secondary metabolites with diverse pharmacological activities. This study aims to isolate actinobacteria from mangrove swamps and to characterize their QQ enzymes as a potential remediation. A total of nine soil actinobacteria were isolated from mangrove swamps soil samples using selective agar. QQ activity was detected by screening of AHL degradation using *C. violaceum* CV026 biosensor assay and rapid resolution liquid chromatography (RRLC) analysis. All nine actinobacteria were able to degrade different QS molecules namely *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-*oxo*-*C6*-HSL) and *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-hydroxy-C6-HSL). It is found that these actinobacteria degrade AHL via enzymatic reaction through lactonase activity. For future work, next generation sequencing should be done to identify the QQ gene(s) involved.

ABSTRAK

Sistem komunikasi yang mengandungi rangkaian kepadatan sel dan gen dalam pelbagai spesis bakteria bertujuan untuk mengawal selia pelbagai fungsi biologi. Komunikasi sel-sel tersebut, yang digelar Sensing Korum (QS), bergantung kepada pengeluaran, penyebaran, dan pengiktirafan molekul isyarat kecil. Sebaliknya, Quenching Quorum (QQ), adalah satu proses yang mengganggu proses kunci QS dan berpotensi digunakan untuk mencegah jangkitan bakteria. Aktinobacteria memainkan kepentingan besar kerana kemampuan untuk menghasilkan entiti kimia baru dengan aktiviti farmakologi pelbagai. Marin aktinobacteria khususnya telah menghasilkan banyak novel metabolit sekunder. Kajian ini bertujuan untuk mengasingkan Aktinobacteria dari paya bakau dan untuk mencirikan potensi mereka untuk menghasilkan sebatian bioaktif yang boleh diterokai sebagai agen QQ. Sebanyak sembilan bakteria tanah telah diasingkan daripada kawasan paya bakau menggunakan agar selektif. Aktiviti QQ dikesan oleh siri bioassei dan kromatografi cecair resolusi pantas (RRLC) analisis. Pencilan dapat merendahkan pelbagai molekul QS iaitu *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*- (3-oxo-hexanoyl) -L-homoserine lactone (3-oxo-C6-HSL) dan *N*- (3-hydroxyhexanoyl) -L-homoserine lactone (3-hydroxy-C6-HSL). Ia didapati bahawa mengasingkan merendahkan AHL melalui tindak balas enzim melalui aktiviti lactonase. Untuk kerja-kerja masa depan, kajian menggunakan Next Generation Sequencing perlu dilakukan untuk mengenal pasti gen QQ yang terlibat.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
AHL	:	*N*-acyl homoserine lactone
ACP	:	Acyl carrier protein
α	:	Alpha
β	:	Beta
BLAST	:	Basic Local Alignment Search Tool
Bp	:	Basepair
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetracetic acid
et al.,	:	*et alia* (and others)
EtBr	:	Ethidium bromide
g	:	Gram
g	:	Gravity(relative centrifuge force)
h	:	Hour
HSL	:	Homoserine lactone
kb	:	Kilobase pair
□	:	Lambda
LB	:	Luria-Bertani
M	:	Molar
MOPS	:	3-(*N*-morpholino) propanesulfonic acid
min	:	Minute
mg	:	Miligram
ml	:	Mililitre

mM : Milimolar

OD : Optical density

% : Percentage

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

QS : Quorum Sensing

QQ : Quorum Quenching

rRNA : Ribosomal deoxyribonucleic acid

rpm : Revolutions per minute

RRLC : Rapid Resolution Liquid Chromatography

SAM : *S*-adenosylmethionine

Sp. : Species

TBE : Tris borate EDTA

UV : Ultra Violet

v/v : Volume/volume

w/v : Weight/volume

X-gal : 5-bromo-4-chlolo-3-indoyl-beta=D galacto-pyranoside

µg : Microgram

µl : Microlitre

CHAPTER 1.0

INTRODUCTION

Quorum sensing (QS) enables microorganisms to communicate by secreting signaling molecules namely autoinducers and contributes to the gene expression regulation in bacterial population density (Smith *et al*., 2003). Many Gram-negative bacteria use the *N*-acyl homoserine lactones (AHLs) as autoinducers (Miller *et al.*, 2001) in which AHLs consist of 4- to 18- carbon *N*-acyl side chain linked to a lactone ring (Pearson *et al*., 1999). AHLs were synthesized in an activity of LuxI synthase using substrates of *S*-adenosylmethionine and acylated acyl carrier protein (Swift *et al*., 1997). Integrity of AHLs is pH-dependent in which the lactone ring will hydrolyze under alkaline condition, resulting in homoserine structure with an opened lactone ring. Such process is reversible with a switch of pH to acidic condition (Byers *et al.,* 2002; Yates *et al.,* 2002). Gram-negative bacteria employ AHL as QS signals in their communication circuits to coordinate various physiological activities. These signals lead to activation survival processes in which include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller *et al*., 2001).

On the other hand, quorum quenching (QQ) is known as a process in which disrupts the QS signals by using several ways which includes enzymatic destruction of the signal molecules, development of antibodies or through QS signalling molecules blocking agents (Williams *et al.,* 2002; Bjarnsholt *et al.,* 2008; Lowery *et al.,* 2010). The first documented QQ enzyme was produced by a soil bacterium from the genus *Bacillus* in which was encoded by the *aiiA* gene. It was later characterized as an AHL-lactonase**.** Since then, these QQ systems have been found in various microorganisms to prevent the benefits of QS in an attempt to gain competitive advantage in polymicrobial environment (Lin *et al.,* 2003). Properties of QQ from species of genera *Bacillus* and

Pseudomonas have been well established (Chong *et al.,* 2012). Examples are the AiiA lactonase homologs of *Bacillus* and PvdQ and also QuiP of *Pseudomonas* (Dong *et al.,* 2005; Sio *et al.,* 2006). These reactions are produced enzymatically by lactonases such as AiiA, AttM, AiiB (Dong *et al*., 2000; Carlier *et al*., 2003) and AhlD (Medina *et al*., 2007). The disruption of QS signaling molecules is considered as a potential way of preventing and treating infections besides preventing plant diseases (Hosni *et al.,* 2011).

In view of this, we have isolated actinobacteria from the mangrove swamps which possess QQ properties. Actinobacteria are known as an important group producing half of all the known antibiotics that can be found from environmental sources (Naikpatil & Rathod, 2011) and has been described as pathogens antagonist. In this work, we identify whether these isolates exhibit QQ activity.

OBJECTIVES

The objective of this study: is to screen and characterize quorum quenching activity of Actinobacteria isolated from mangrove swamps.

1. To isolate and identify quorum quenching (QQ) actinobacteria from mangrove swamps using selective media.
2. To determine AHLs degradation activity and lactonase activity of actinobacteria with C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL using whole–cell AHLs inactivation assay and Rapid Resolution Liquid Chromatography (RRLC).

CHAPTER 2.0

LITERATURE REVIEW

2.1 Quorum Sensing

Quorum sensing (QS) described as regulation of gene expression in response to fluctuations in cell-population density. QS bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. Gram positive and Gram negative bacteria use QS communication circuits to regulate a diverse array of physiological activities (Miller & Bassler, 2001). These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. Gram negative bacteria use acylated homoserine lactones as autoinducers, and Gram positive bacteria use processed oligo-peptides to communicate (Novick & Geisinger, 2008**;** Ng & Bassler, 2009; Williams & Camara, 2009). Gram positive bacteria use peptides, namely autoinducing peptides (AIPs), as signaling molecules. When the extracellular concentration of the AIP is high, , it will binds to a cognate membrane-bound two-component histidine kinase receptor. Usually, binding activates the receptor's kinase activity, it auto-phosphorylates, and passes phosphate to a cognate cytoplasmic response regulator. The phosphorylated response regulator activates transcription of the genes in the QS regulator. In some cases of Gram positive bacteria QS, AIPs are transported back into the cell cytoplasm where they interact with transcription factors to modulate the transcription factor's activity and, in turn, modulate gene expression changes (Rutherford *et al.,* 2015)

2.1.1 *N*-Acyl Homoserine Lactones

Many Gram negative bacteria produce *N*-Acyl Homoserine Lactones (AHL) (Chen *et al.,* 2002; Fuqua *et al.,* 2001; Greenberg, 2000). AHL QS signals are highly conserved as they have the same homoserine lactone moiety unsubstituted in the β and γ-positions with an amide (*N*)-linked acyl side chain at the α-position (Chhabra *et al*., 2005; Cooley *et al*., 2008). The *N*-acylated side chain consisted of fatty acids in different chain length (ranging from 4-18 carbons), degree of saturation and the presence of substituent which could be either hyroxyl- or oxo- group at the C3 position (Swift *et al*., 1997). AHLs molecules are synthesized by the activity of LuxI synthase from substrates *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (Acyl-ACP), an intermediated of fatty acid biosynthesis.

The first describe of AHLs produce by QS bacteria in marine bioluminescence bacterium *Vibrio fischeri*. This bacterium produces QS signals which identified as 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6 AHL) (Eberhard *et al*., 1981; Schaefer *et al*., 1996). 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. Two important proteins, LuxR and LuxI play important roles in QS system. In example, *Agrobacterium tumefaciens* which is responsible for crown gall tumors in plants, used the plant opine hormones to interact with the bacterial protein OccR or AccR to regulate LuxR homologue TraR expression (Oger *et al*., 1998; Zhu & Winans, 1998).

Some Gram negative bacteria employ AHLs biosynthesis enzymes that related to the conventional LuxI-enzymes. It is include LuxM from *Vibrio harveyi* and AinS from *V. fischeri* and HdtS from *Pseudomonas fluoresces* that showed no significant homology with LuxI family members (Bassler *et al.,* 1993; Hanzelka *et al*., 1999; Laue *et al.,*

2000). This signals achieved with cytoplasmic membrane associated sensor kinase (Ryan & Dow, 2008).

Methylobacterium extorquens AM1 a member of pink-pigmented facultative methylotrophs produces several AHLs depending on carbon source (Penalver *et al.*, 2006). A novel AHL was discovered with double unsaturated carbon chain (*N*-(tetradecenoyl)) (C14; 2), detected by MS and proton NMR. These AHLs was synthesized by M1aI which also synthesis of C14:1-AHLs. This unsaturated AHL was originally described in *Rhizobium leguminosarum* (Lithgrow *et al.,* 2000). In addition, *M. extorquens* AM1 also produces C6-AHLs and C4-AHLs via MsaI (Penalver *et al.,* 2006). Examples of bacteria utilizing the AHL QS are summarized in Table 2.1

Table 2.1: Examples of AHL producing bacteria

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Aeromonas caviae Strain YL12	C4-AHLs C6-AHLs	N.D	N.D	Yan *et al.*, 2014
Aeromonas hydrophila	C4-AHLs, C6-AHLs	AhyI/R	Serine protease, metalloprotease production and biofilm formation	Swift *et al.*, 1997; Lynch *et al.*, 2002
Aeromonas salmonicida	C4-AHLs	Asal/R	Extracellular protease and metalloprotease	Swift *et al.,* 1997

Table 2.1, continued

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Agrobacterium Tumefaciens	3-oxo-C8-AHLs	TraI/R	Conjugation	Piper *et al.,* 1999
Burkholderia cepacia	C6-AHLs, C8-AHLs	CepI/R	Swarming motility, biofilm formation and siderosphere production	Lewenza *et al.,* 1999; Huber *et al.,* 2001; Tomlin *et al.,* 2004
B. cenocepacia	C6-AHLs, C8-AHLs	CepI/R	Biofilm formation, and siderosphere production	Sokol *et al.,* 2003
Chromobacterium violaceum	C10-AHLs	CviI/R	Biofilm formation, chitinase and violacein production	Stauff & Bassler, 2011
Erwinia carotovora subsp. *carotovora*	3-oxo-C6-AHLs	ExpI/R	Carbaenem antibiotic production. Exoenzymes	Bainton *et al*., 1992; Pirhonen *et al.,* 1993
Enterobacter asburiae	C4-AHLs	N.D	N.D	Yin *et al*., 2014

Table 2.1, continued

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Hafnia alvei	3-oxo-C6-AHLs 3-oxo-C8-AHLs	N.D	N.D	Tan *et al.,* 2014
Klebsiella pneumoniae Strain CSG20	C6-AHLs	N.D	N.D	Yun *et al*., 2013
Kluyvera sp.	3-oxo-C6 AHLs 3-oxo-C8 AHLs	N.D	N.D	Yunos *et al*., 2014
Pandoraea sp. RB-44	C8-AHLs	N.D	N.D	Robson *et al*., 2013
Pantoea stewartii	3-oxo-C6-AHLs	Esal/R	Adhesion and host cononization	Koutsoudis *et al*., 2006

Table 2.1, continued

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Pseudomonas aeruginosa	3-oxo-C12-AHLs	LasI/R and RhII/R	Exoenzymes, Biofilm formation, cell-cell spacing	Chapon *et al*., 1997; Passador *et al*., 1993; Glessner *et al.,* 1999
P. aureofaciens	C6-AHLs	PhzI/R	Phenazine antibiotic production	Pierson III *et al*., 1995
P. chlororaphis	C6-AHLs	Phzl/R	Phenazine-I-carboximide production	Chin *et al.,* 2001
P. fluorescens	3-hydroxy-C6-AHLs, 3-hydroxy-C14-AHLs	MpuI/R and HdtS	Phenazine antibiotic production	Shaw *et al*., 1997; Laue *et al.,* 2000
Pseudomonas putida	3-oxo-C10-AHLs, 3-oxo-C12-AHLs	Ppul/R	Maturation	Dubern *et al.,* 2006
Pseudomonas putida Strain T2-2	C8-AHLs C12-AHLs	N.D	Maturation	Jian *et al*., 2013

Table 2.1, continued

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Rhizobium leguminosarum	3-hydroxy-7-cis-C14-AHLs	RhiI/R	Nodulation, Bacteriocin, stationary phase survival	Gray, 1997; Rodelas *et al*., 1999; Thorne & Williams, 1999
Rhodobacter sphaeroides	7-cis-C14-AHLs	CerI/R	Community scape	Puskas *et al*., 2008
R. palustris	Pc-AHLs	Rpal/R	N.D	Schaefer *et al.,* 2008
Serratia liquefaciens	C4-AHLs	SwrI/R	Biofilm formation	Labbate *et al*., 2004
Vibrio anguillarum	3-hydroxy-C6-AHLs	VanM	Terminal hemorrhagic septicemia in fish	Milton *et al*., 2001
Vibrio sinaloensis strain T47	C4-AHLs	N.D	N.D	Tan *et al.,* 2013
V. fischeri	C8, 3-oxo-C6-AHLs	AinS and LuxI/R	Bioluminescence	Hanzelka *et al*., 1999

Table 2.1, continued

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
V. harveyi	3-hyroxy-C4-AHLs	LuxM	Phenazine antibiotic production	Bassler *et al*., 1993
Yersinia pseudotuberculosis	C6-AHLs, 3-oxo-C6-AHLs, C8-AHLs	Ypsl/R and Ytbl/R	Motility, Clumping	Atkinson *et al.,* 1999

Box 1. Structures of Some Quorum Sensing Signal Molecules

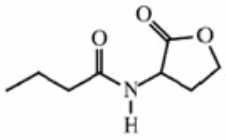
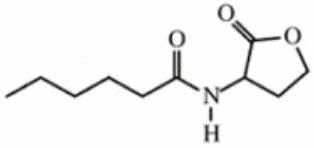
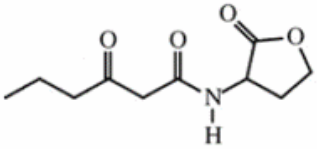
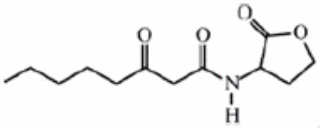
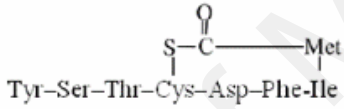
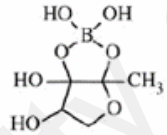
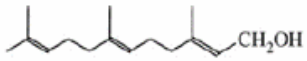
Signal	Structure	Organisms
C4-HSL (an AHL)		*Aeromonas hydrophila, Pseudomonas aeruginosa*
C6-HSL		*Erwinia carotovora, Pseudomonas aureofaciens, Yersinia enterocolitica*
3-Oxo-C6-HSL		*E. carotovora, Vibrio fischeri, Y. enterocolitica*
3-Oxo-C8-HSL		*Agrobacterium tumefaciens*
Autoinducing Peptide (AIP)-I		*Straphylococcus aureus* Group I strains
AI-2 (*S*-THMF-borate)		*Vibrio harveyi*
Competence and Sporulation Stimulating Factor (CSF)	Glu – Arg – Gly – Met – Thr	*Bacillus subtilis*
Farnesol		*Candida albicans*

Figure 2.1: Several chemical structures of *N*-acyl homoserine lactone molecules (Yannick *et al.,* 2013)

2.1.2 Other Signaling Molecules

There are other signaling molecules produce by Gram negative bacteria including 4-quinolones, namely 2-hepthyl-3-hydroxy-4-quinone (termed as *Pseudomonas quinolone* signal, PQS) and 4-hydroxy-2-heptyl-quinoline (HHQ) synthesized by *Pseudomonas aeruginosa* (Pesci *et al.,* 1999). The signaling of PQS is an incorporated with AHL QS pathway that is governed by Las and Rhl systems and act as upregulated gene in cystic fibrosis in lung infections (Collier *et al.,* 2002; Pesci *et al*., 1999). Precursor anthranilate were converted biosynthesis PQS into HHQ by pqsABCD gene products of pqsABCDE operon. HHQ is immediate precursor for PQS produced owned signaling molecules released before converted to PQS (Deziel *et al.,* 2004).

Xanthomonas campestris pv. campestris (Xcc), plant pathogen which synthesized virulence factor by diffusible signaling factor (DSF) was characterized as unsaturated fatty acid namely cis-11-methyl-2-dodecenoic acid (Barber *et al.,* 1997; Wang *et al*., 2004). Expanded of DSF-family signals by discovered of Xcc with a series of DSF structural analogues identified from *B. cenocepacia, Streptococcus mutans* and other Xanthomonads. DF (diffusible factor) which is second signal molecule was also found in Xcc and associated with the regulation of yellow pigment (xanthomonadins) and extracellular polysaccharides (EPS) production (Potlawsky & Chun, 1998).

Bradyrhizobium japonicum, a symbiotic bacterium from rhizosphere produced an iron mediated oxetane ring which contain bradyoxetin namely 2-{4-[[4-(3-aminooxetan2-yl)phenyl](amino)methyl]phenyl}oxetan-3-ylamine as signaling molecule that regulate genes for nodulation (Loh *et al.,* 2002).

Another bacterium, phytopathogen *Ralstonia solanacearum* were exerted different signaling pathway utilized 3-hydroxypalmitic acid methyl ester (3-OH PAME) for the regulation of virulence factor in causing wilting diseases of crops (Flavier *et al*., 1997).

Gram positive bacteria were different from Gram negative bacteria signals; they used post-translationally modified autoinducing peptide (AIP) molecules as intracellular communication (Novick, 2003). Secretion of peptide signals was achieved via dedicated ATP-binding cassette (ABC) exporter protein followed by recognition of the signals by membrane bound two-component sensor kinase proteins (Bassler, 1999). In examples, *Bacillus subtilis* secretion enables the bacterium to decide between competence for DNA uptake and sporulation through secretion of two extracellular peptides, ComX and CSF (Competence and Sporulation factor) (Ansaldi & Dubnau, 2004; Lazazzera & Grossman, 1998).

2.2 AHL Biosensor

AHL biosensors do not produce AHL molecules but contain a functional LuxR-family protein clone with a cognate target promoter. This target promoter regulates the transcription of a reporter gene that identified including bioluminescence, green fluorescent protein and production of pigment (Steindler & Venturi, 2007). The most explored AHL biosensor is Mini-Tn*5* mutant *C. violaceum* CV026, derived from *C. violaceum* ATCC 31532. It was developed after double mini-Tn*5* transposon mutagenesis on *cvil* gene responsible for C6-AHLs production while retaining the functionality of *cviR* gene that induces violacein production. CV026 detected short chain of AHLs which produce purple pigmentation upon exposure to exogenous AHLs ranging from C4-AHLs to C8-AHLs (McClean *et al.,* 1997).

Serratia sp. SP19 is another biosensor based on pigmentation production that relies on exogenous shot chain AHLs which produce bright red pigment, prodigiosin production. Triple mutations on *smal, pigX* and *pigZ* genes of C4-AHLs construct to produce *Serratia* sp. ATCC 39006. Mutation of *smal* causes the deficiency in synthesis C4-AHLs. *pigX* and *pigZ* genes are significant regulators for biosynthesisi of

prodiogisin and increase of prodiogison production from mutation of both genes. This mutation generated a non-AHL producing mutant which enhanced specificity towards exogenous AHL including C4-AHLs and 3-oxo-C4-AHLs (Poulter *et al.,* 2010).

Another detection of AHL by bioluminescence AHL biosensor involves emission of bioluminescence in presence of exogenous AHLs. Genetically engineered *E. coli* carrying AHL sensor plasmids namely pSB401, pSB406 and pSB1075 containing fusion of *luxRI'::luxCDABE, rhlRI'::luxCDABE* and *lasRI'::luxCDABE* which produce bioluminescence in presence of AHL molecules by activation of LuxR homologues (Winson *et al.,* 1998). These constructs enable identification of AHL producers through cross-streak and supernatant assays.

2.3 AHL QQ Enzymes

Many bacteria used QS regulation to gain maximal advantages of their population. On the other hand, natural QQ regulation was used to disarm the deleterious effect results from QS (Lin *et al*., 2003). These mechanisms play important roles in microbial and pathogen-host interactions (Dong *et al*., 2007). There are several groups of identified QQ enzymes in a range of living organism including bacteria and eukaryotes (Dong & Zhang, 2005). Inhibition quorum signals of AHL were achieved by destabilizing the LuxR family protein receptors for AHL signal molecules, blocking of AHL signal generators and degrading AHL signal molecules (De Kievit & Iglewski, 2000; Lin *et al.,* 2003; Uroz *et al*., 2003; Hong *et al.,* 2012). Three types of enzymes involved in degradation of AHL signal molecules which are AHL-lactonase (Dong *et al*., 2002), AHL-acylase (Lin *et al*., 2003) and AHL-oxidoreductase (Uroz *et al*., 2005). The first QQ enzyme encoded by the AiiA gene which later characterized as AHL-lactonase (Dong *et al*., 2001)

2.3.1 AHL Acylase

Acylase enzymes was hydrolyze amide bond of AHL to release fatty acid and AHLs (Sio *et al*., 2006). AHLs released as nitrogen source through mineralization of lactone ring whereas fatty acid released to utilize as an energy source (Wang & Leadbetter, 2005; Dong *et al*., 2007). *P. aeruginosa* PA01 exerted acylase activities which include PvdQ and QuiP. Other bacteria that produce acylase activities is *Ralstonia* strain XJ12B which significantly reduced swarming activity of *P. aeruginosa* PA01 as well as produce virulence factor of pyocyanin and elastase (Lin *et al.,* 2003).

Other bacterium that secreted AHL degrading enzyme found in *Streptomyces sp*. (Park *et al.,* 2005). The AhlM gene for AHL degradation from *Streptomyces* sp. strain M664 was cloned, expressed heterologously in *Streptomyces lividans.* AhlM hydrolyzed the amide bond of AHL and released homoserine lactone (Park *et al.,* 2005). AhlM exhibits a higher deacylation activity towards AHLs with long acyl chains rather than short acyl chain.

Soil bacterium, *Variovorax paradoxus*, has been reported to grow using different AHLs as the sole source of carbon, energy and nitrogen (Leadbetter & Greenberg, 2000). The degradation pathway starts with the cleavage of acyl side chain released homoserine lactone and carboxylic acid. This acid was used as carbon and energy source while the homoserine lactone used as nitrogen source. The presence of homoserine lactone in the AHL metabolic of *V. paradoxus*, suggested that the bacterium may produce and AHL-acylase and gene encoding for AHL-acylase remains to be cloned and characterized. Table 2.2 illustrated the various examples of AHL acylases found in various organisms.

Table 2.2: Examples of various acylases

Strain/ Source	Enzyme Abbreviation	AHL Degradation	References
P. aeruginosa PA01, *P. aeruginosa* PAI-A	PvdQ	AHLs with or without C3 substitutions and chain length from C11 to C14 but did not degrade short chain AHLs	Huang *et al.,* 2003; Sio *et al*., 2006
P. aeruginosa PA01	QuiP	AHLs with or without C3 substitutions and chain length from C7 to C14 but did not degrade C4-AHLs, C6-AHLs and 3-oxo-C6-AHLs	Huang *et al*., 2006
P. syringae B728a	HacA	C8-AHLs, C10-AHLs and C12-AHLs, but did not degrade C6-AHLs and 3-oxo-C6-AHLs	Shephere & Lindow, 2009

Table 2.2, continued

Strain/ Source	**Enzyme Abbreviation**	**AHL Degradation**	**References**
P. syringae B728a	HAcB	C6-AHLs, 3-oxo-C6-AHLs, C8-AHLs, C10-AHLs and C12-AHLs	Shephere & Lindow, 2009
Porcine (Kidney)	ACY1	C4-AHLs C10-AHLs	Xu *et al.,* 2004
R. erythropolis W2	QsdA	AHLs with or without C3 substitutions and chain length from C6 to C14 but less efficiency against AHLs without C3 substitution	Uroz *et al.,* 2005
Ralstonia sp. XJ12B	AiiD	3-oxo-C8-AHLs, 3-oxo-C10-AHLs and 3-oxo-C12-AHLs	Lin *et al.,* 2003
Streptomyces sp. M664	AhlM	C8-AHLs, C10-AHLs, 3-oxo-C12-AHLs, low activity towards 3-oxo-C6-AHLs, C6-AHLs and did nit degrade C4-AHLs	Park *et al*., 2005

V. paradoxus VAI-C	N.D	C4-AHLs, C6-AHLs, 3-oxo-C6-AHLs, C8-AHLs, C10-AHLs and C12-AHLs	Leadbetter & Greenberg, 2000

Legend: N.D: Not determined

2.3.2 AHL Lactonase

AHL-lactonase hydrolyses the homoserine lactone ring to produce acylhomoserine (Wang & Leadbetter, 2005). The lactonolysis involved cleavage of ester bond of homoserine lactone ring by attacking the lactone carbonyl. Then elimination process of an alcohol leaving group (Momb *et al*., 2006). Thus, the opening ring prevents binding of the molecules o the target transcriptional regulators (Dong *et al*., 2001). Lactonolysis were pH dependent in which relactonization of homoserine lactone ring were observed upon acidification of growth media to pH 2.0 (Yates *et al*., 2002).

The first documented QQ enzyme was produced by a soil bacterium from the genus *Bacillus sp.* 240BI in which was encoded by the *aiiA* gene, which was later characterized as an AHL-lactonase (Dong *et al.,* 2001). Homologues of aiiA were later discovered in the genus Bacillus including *Bacillus cereus, Bacillus thuringiensis* and *Bacillus thailandensis* (Dong *et al*., 2002; Ulrich, 2004). It was later found that the aiiA gene conserved ^{104}HXHXDH$^{109\sim}$H^{169} region that resembles the zinc binding motif of several enzymes in metallohydrolase superfamily (Dong & Zhang, 2005). Catalytic activities and proper of AHL lactonase requires a binuclear metal ion active site that harbors metal ions as chelation of metal ions from the enzyme were shown to greatly

reduce its catalytic rate (Thomas *et al*., 2005). An interaction between metal ions and substarte's leaving group during catalysis that facilitated the bond cleavage of lactone ring (Momb *et al*., 2006). Incorporation of metal ions in the enzyme including Zn^{2+}, Co^{2+}, Ca^{2+} and Mn^{2+} for catalysis has been reported in Table 2.3.

QQ enzymes activity was well documented in *Bacillus* species, *Agrobacterium tumefaciens* and *Klebsiella pneumoniae* (Huang *et al*., 2000; Park *et al*., 2003; Zhang *et al*., 2002). *A. tumefaciens* produce AHLs and regulates AHL-lactonase encoded by AttM. It has been reported that AttM expression is activated by starvation signal and (p)ppGpp stress alarmone (Zhang *et al.,* 2004).

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

As AHLs will undergo lactonolysis under alkaline conditions (Yates *et al.,* 2002) turnover of AHLs by alkaline lactonolysis was ruled out as no change in pH values was observed in the action mixtures after 24 hour. Biosensor CV026 is only applicable for detection of short chain AHLs and have well respond towards C6-AHLs (Chong *et al.,* 2012), the 3-hydroxy or 3-oxo substitutions and un-substituted homoserine lactones were tested to determine the broad range of actinobacteria QQ gene. The AHL lactonases from the MBL superfamily are known to have a signature zinc-binding motif HXHXDH ~ H. Crystallographic studies of AiiA, AiiB and Aid, have also revealed

many other amino acid residues that are functionally crucial. These amino acid residues play critical functions in the catalytic mechanism (Wah *et al.,* 2017).

Wah *et al.,* 2017 studied have been identified a number of functionally-important amino acid residues that were previously reported in the crystallographic studies of AiiA-type enzymes. A mutagenesis study of the active site of AiiA revealed that tyrosine (Y194) and aspartic acid (D108) residues are directly involved in the catalytic mechanism. Multiple alignment analysis of *aidP* and other AHL lactonases from the MBL superfamily revealed the presence of both amino acids (Y222 and D121) in *aidP*. The tyrosine residue provides stabilization for the substrate, while aspartic acid acts as a proton shuttle that tightens the active site, interacting with the hydroxyl leaving group of the product. Asp219 (D219) of *aidP*, which is homologous with D191 of AiiA and D213 in AiiB, is important in the formation of zinc bridging. G235, which is homologous with Gly207 of AiiA, was also detected in AidP; mutation of this amino acid residue which is located in the *N*-acyl binding region will cause a significant decrease in the activity of AiiA. Previously, *P. rifientoensis* has been reported to promote the growth of plants, even though a complete genome analysis of *P. rifientoensis* identified no gene sequence coding for *N*-acyl homoserine lactonase.

AiiA shows broad substrate specificity and a preference for substrates with long acyl chain AHLs; however, C6-HSL is mostly used as substrate in crystal studies of AiiA. Heterologous expression of AiiA in numerous pathogenic bacteria, including *P. aeruginosa, B. thailandensis* and *E. carotovora,* may reduce AHL accumulation and decrease their virulence expression, which indicates the potential use of AiiA as a strategy for antivirulence therapy. Furthermore, most of the AHL lactonases (both in the AiiA cluster and the AidC cluster) belonging to metallo-β-lactamase family are soil-derived. In this connection, identification of many marine bacteria with AHL-degrading activity, and one of these organisms, *Muricauda olearia* Th120, possesses a gene

encoding a novel AHL lactonase which represents a new cluster of AHL lactonase in the metallo-β-lactamase family (Tang & Zhang, 2014).

As QQ enzymes produced by bacteria have been shown to effectively prevent the plant pathogen, *Pectobacterium* sp., from causing soft root disease on plant tubers, a capability of *P. versutus* L10.15T to promote plant growth, and a potential for it to act as a biocontrol agent in horticulture. Using QQ as biocontrol strategy will minimize the selective pressure imposed on the targeted pathogens, and thus may reduce the development of resistance. The search for more effective QQ agents is important, as the resistance of bacteria to available antibiotics is increasing, and the development of alternative strategies against pathogens is critical. It has been recognized that polar and other extreme environments are potentially important sources of novel and industrially important enzymes. In this context, the discovery of psychrotolerant QQ bacteria with potential for use as biocontrol, remediation or growth promoting agents would be advantageous (Wah *et al.,* 2016).

Table 2.3: Examples of various AHL lactonase

Strain / Source	**Enzyme name**	**AHL Degradation**	**Metal Ion for activity**	**References**
Agrobacterium tumefaciens C58	AiiB, AttM	C6-AHLs, 3-oxo-C8-AHLs	N.D	Zhang *et al*., 2002; Haundecoeu et al., 2009
Arthrobacter sp. strain IBN110	AhlD	C4-AHLs, C6-AHLs, 3-oxo-C6-AHLs, C10-AHLs, and 3-	Zn^{2+}	Park *et al*., 2003

Table 2.3, continued

		oxo-C12-AHLs		
Bacillus sp. AI96	$AiiA_{A196}$	AHLs with or without C3 substitutions and chain length from C4 to C14 but less efficiency against 3-oxo-C6-AHLs, C12-AHLs and 3-hydroxy-C14-AHLs	Zn^{2+}	Cao *et al.,* 2012
Bacillus sp. 240BI	AiiA	3-oxo-C6-AHLs, 3-oxo-C8-AHLs and 3-oxo-C10-AHLs	Zn^{2+}	Dong *et al*., 2001
B. thuringiensis	AiiA homologues	3-oxo-C6-AHLs	Zn^{2+}	Lee *et al*., 2002
Geobacillus caldoxylosilyticus YS-8	N.D	C6-AHLs, 3-oxo-C6-AHLs	Co^{2+} , Mn^{2+} and Ni^{2+}	Seo *et al.,* 2011
Geobacillus kaustophilus HTA426	GKL	C6-AHLs, 3-oxo-C6-AHLs, C8-AHLs, C10-	Zn^{2+}	Chow *et al*., 2010

Table 2.3, continued

		AHLs and 3-oxo-C12-AHLs		
Klebsiella pneumoniae	AhlK	C6-AHLs and 3-oxo-C6-AHLs	N.D	Park *et al.,* 2003
Mycobacterium tuberculosis	PPH	C4-AHLs, 3-oxo-C8-AHLs, C10-AHLs	Zn^{2+}	Afriat *et al*., 2006
Mycobacterium subsp. *paratuberculosis* K-10	MCP	C4-AHLs, C6-AHLs, 3-oxo-C6-AHLs, 3-oxo-C8-AHLs	Mn^{2+}, Zn^{2+}	Chow *et al*., 2009
Ochrobactrum sp.	AidH	C4-AHLs, C6-AHLs, 3-oxo-C6-AHLs, 3-oxo-C8-AHLs, C10-AHLsand 3-hydroxy-AHLs	Mn^{2+}	Mei *et al.,* 2010
R. erythropolis W2	AhlA	AHLs with or without C3 substitutions and chain length from C6 to C14 but less efficiency against AHLs	Zn^{2+}	Uroz *et al.,* 2008

Table 2.3, continued

		without C3 substitution		
Sulfolobus sulfataricus P2	SsoPox	C8-AHLs, 3-oxo-C8-AHLs, 3-oxo-C10-AHLs, 3-oxo-C12-AHLs	Fe^{2+}	Elias *et al*., 2008
Human (airway epithelia)	PON	C6-AHLsand 3-oxo-C12-AHLs	Ca^{2+}	Greenberg *et al.*, 2004

2.3.3 AHL Oxidoreductase

AHL-oxidoreductases origin were identified in *R. erythropolis* reduces the keto-group of 3-oxo-AHLs to the corresponding 3-hydroxy derivative AHLs (Uroz *et al*., 2005). AHL Oxidoreductase does not destruct AHL structure which is different from AHL acylase and lactonase, but it causes signal disturbance of bacteria especially those that depend on oxo-AHL for regulation of QS-mediated genes (Chan *et al*., 2011). This AHL-oxidoreductase is inactive against hydroxylated and fully reduced AHLs (Uroz *et al*., 2005). This oxidoreductase activity was not specific to 3-oxo-AHLs but allowed the reduction of compounds such as *N*-(3-oxo-6-phynylhexanoyl) homoserine lactone which contain an aromatic acyl chain substituents and 3-oxododecanamide which lacks the homoserine lactone ring (Uroz *et al*., 2005).

Table 2.4: Examples of AHL-oxidoreductase

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

Legend : N.D : Not determined

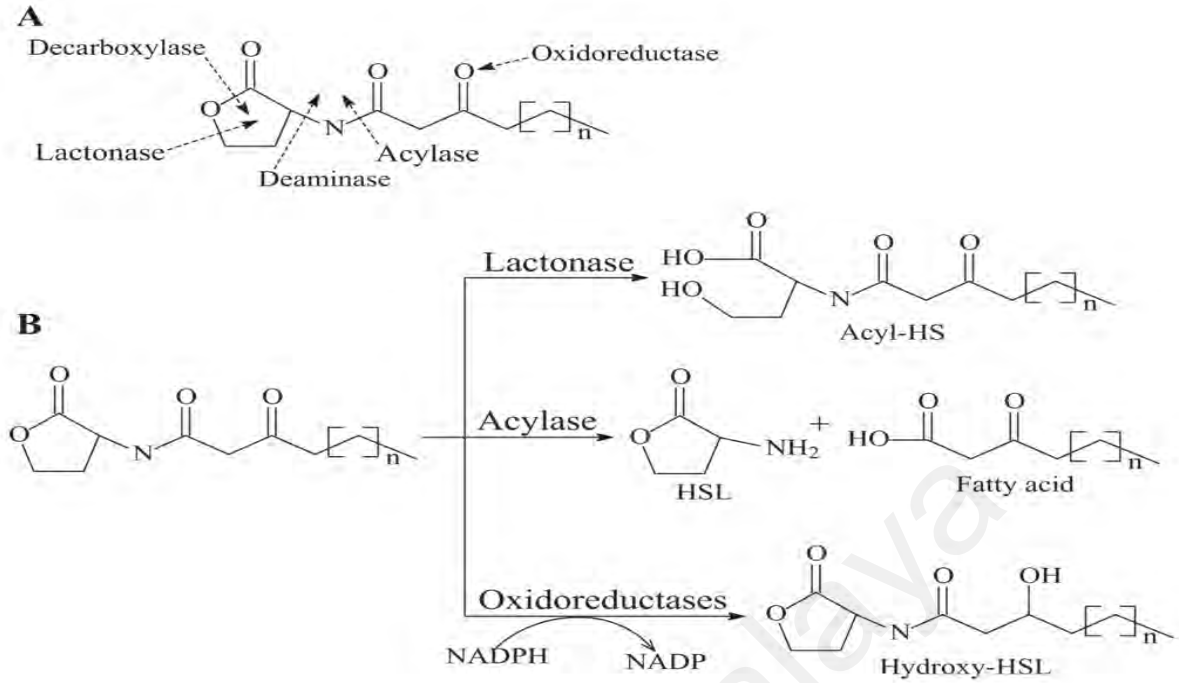


Figure 2.2: Structural modification of AHL molecules result from enzymatic reactions

2.4 Biotechnological Implication of QQ

Quorum quenching (QQ) refers to a process in which disrupts the QS signals by using several ways which includes enzymatic destruction of the signal molecules, development of antibodies or through QS signalling molecules blocking agents (Williams *et al.,* 2002; Bjarnsholt *et al.,* 2008; Lowery *et al.,* 2010). The disruption of QS signaling molecules is considered as a potential way of preventing and treating infections besides preventing plant diseases (Hosni *et al.,* 2011). Thus, both AHL-lactonase (AiiA) and amidase (AiiD, AhlM, AttM and PvdQ) could effectively apply to the control of AHL-mediated pathogenicity.

The discovery of various QQ enzymes and QS inhibitors may provide a new dimension on treatments for bacterial infections in both human and plants (Hentzer *et al.,* 2003). For example, *Rhodotorula mucilaginosa* isolated from Malaysian shoreline possesses QQ properties. *Rhodotorula* is known as saprophytic yeast present in the

environmental (Thournas *et al*., 2006) and has been described as a pathogen antagonist (Akhtyamova *et al.,* 2013).

Both *Rh*. *mucilaginosa* and *Pseudomonas* species produce a wide range of antimicrobials which help to confer a high competitive ability and to ensure dominance within their respective polymicrobial communities. Further studies of the biology of QQ are needed to fully characterize the eco-physiological consequences (Jonathan *et al*., 2013). Recently, QQ yeast *Trichosporon loubieri* has been reported to be isolated from tropical wetland waters and capable to grow on *N*-3-oxo-hexanoyl homoserine as carbon and nitrogen source for growth (Ghani *et al.,* 2014).

QQ enzymes have been studied in *P*. *aeruginosa* and *E. carotovora*, a plant pathogen, that they reduce the virulence to infect nematode *Caenorhabditis elegans* and tobacco plants (Dong & Zhang, 2005). The QQ enzyme was act as protection against virulence of *E. carotovora* to transgenic potato and tobacco plants (Dong *et al.,* 2000). Other used of QQ enzymes in *R. erythropolis* W2 can reduce pathogenicity of *Pectobacterium carotovorum* in potato tubers, indicated as potential biocontrol agent (Uroz *et al*., 2005).

In addition to this, the halogenated furanones produced by macroalgae *Delisea pulchra* can interfere with the expression of AHL regulated genes in *P. aeruginosa*, a pathogen responsible for chronic lung infections in patients with cystic fibrosis (Chun *et al.,* 2004; Hentzer *et al.,* 2003).

The impact of QQ enzymes may use in biocontrol of QS dependent pathogens although the prematurity of this approach is apparent in acceptability and biosafety issues. Furthermore, further analysis need to be done on assessing the stability, toxicity and side effects of theses enzymes in the context of pharmaceutical and agricultural applications (Dong & Zhang, 2005; Hong *et al*., 2012).

2.5 QQ in the Marine Environment: A Tremendous Resource to be developed

QQ may be a strategy used by microorganisms to gain benefit in a competitive environment. Also, it is believed that in the highly diverse marine ecosystem, microorganisms with capabilities of producing small QS inhibitors and QQ enzymes remain to be discovered. Romero *et al.,* propose that QQ is likely to be a common activity in marine bacteria because a high abundance of QQ bacteria was found among marine cultivable bacteria and a high frequency of QQ genes was discovered in marine metagenomes. Although only a few studies have been carried out to assay the AHL-degrading activity of marine bacteria, more than 30 species of QQ bacteria belonging to Alp haproteobacteria, Gammaproteobacteria Actinobacteria, Flavobacteriia and Firmicutes have been identified. Additionally, some QQ strains have revealed degradative activity only against long-chain AHLs. Because AHL lactonases normally present broad AHL inactivating activities while many acylases are specific to long-chain AHLs, lactonases more common for short chain AHL degradation. Therefore, many marine QQ bacteria may be still undiscovered, and the prevalence of QQ enzymes in marine bacteria may be higher than expected (Tang & Zhang, 2014).

Due to emergence of multidrug-resistant strains of bacteria and fungi, especially those causing nosocomial infections, there are an ever-increasing demand for novel antibiotics with broad antimicrobial spectra. Actinobacteria constituted a diverse group of microorganisms that are widely distributed over terrestrial, freshwater and marine habitats (Radhika *et al.,* 2011). Compared to terrestrial actinobacteria, very few studies have been conducted on marine actinobacteria. Marine ecosystem constitutes oceans, the deep sea and the sea floor, estuaries and lagoons, salt marsh and intertidal zones, coral reefs and mangrove swamps. Mangrove forests consist of woody trees and shrubs that plentifully thrive in the saline sediments of tropical and subtropical coastline. Due to the presence of high salinity, high temperature, extreme tides, high sedimentation and

high evaporation the muddy anaerobic mangrove sediment differs from the terrestrial one in terms of the microbial diversity (Giri *et al*., 2011).

The mangrove ecosystem remains largely unexplored, and therefore offers excellent opportunity for finding rare actinobacteria with unique properties, capable of producing many novel bioactive compounds such as antibiotics, enzymes and anti tumor agents. Rare actinobacteria are considered as the strains whose isolation frequency by conventional methods is much lower than that of commonly occurring actinobacteria strains (Naikpatil & Rathod, 2011). Among the actinobacteria, species of Streptomyces constitute second highest percentage after Nocardia in terms of distribution in the mangrove ecosystem (George *et al.,* 2012). They are prolific producers of secondary metabolites and about 80% of total antibiotics are produced from the species of Streptomyces (Sathiyaseelan & Stella, 2011). These habitats offer unique ecological niche supporting the occurrence and interactions between many rare microbial forms. Therefore, many studies have been done for determining the in vitro activity of actinobacteria isolated from a mangrove ecosystem and identification of a potent strain of actinobacteria effective against the bacterial and fungal pathogens of nosocomial origin. The high diversity and abundance of marine QQ bacteria may lead to the discovery of new QQ enzymes and AHL-degrading mechanisms. The findings indicate that marine microorganisms may be important resources for the discovery of new anti virulence strategies (Tang & Zhang, 2014).

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Bacterial Strains, Oligonucleotides

Table 3.1: Bacterial strains used in this study

Bacteria strain	Description	Source/ Reference
C. violaceum CV026	Mini-*Tn5* mutant derived from *C. violaceum* ATCC 31532 acts as AHL biosensor with formation of purple violacein pigment in presence of short chain exogenous AHL molecules	McClean *et al.,* 1997
B. cereus KM1S	Positive control for quorum quenching test based on strong degradation of AHLs	Dr Chan Kok Gan Department of Genetics University Malaya
E. coli Top 10	Negative control for quorum quenching tests as it does not possess degradation of AHLs.	Invitrogen

The growth *B. cereus* KM1S and *C. violaceum* CV026 were carried out at 28°C in LB media while *E. coli* Top 10 were cultured in LB media at 37°C. Incubation in broth was done with shaking at 220 rpm.

Table 3.2: Oligonucleotide used in this study

Primer	Sequence	Reference
16S rRNA forward primer 27F	5'-AGAGTTTGATCMTGGCTCAG-3'	Paola *et al*., 2002
16S rRNA reverse primer 515R	5'GTGCCAGCAGCCGCGGTAA-3'	Paola *et al*., 2002
16S rRNA reverse primer 1525R	5'AAGGAGGTGWTCCARCC-3'	Paola *et al*., 2002

3.2 Soil Sampling and Isolation of Bacteria

The Mangrove swamp sampling site (GPS: N 02°43.952 E 102°09.341) is located at Kuala Selangor, Malaysia. Sample was collected on April 2013 using 50-mL sterile polypropylene tubes. The pH of the soil sample collected was recorded as pH 8. Sample was immediately processed upon returning to the laboratory. To process the sample, the soil sample was serially diluted and spread onto different selective agar (actinobacteria isolation agar, ISP 4, ISP 2, yeast malt agar and starch casein agar) supplies with 80 µg/ml cycloheximide and 75 µg/ml nalidixic acid. Bacteria with observable different morphology were isolated after incubation for 14 days at 28 °C.

3.3 Chemical Reagents

All the chemical reagents used in this study are from analytical grade (or highest grade) purchased from Bio-Rad Laboratories Ltd., U.S.A.; Merck Germany; Promega

Ltd, U.S.A.; Sigma Chemical Corp., U.S.A.; Invitrogen Corp., U.S.A.; BDH Laboratory Supplies, England.; and Sigma-Aldrich, Switzerland

3.4 Equipments

Equipment that were used during this study included Eppendorf research micropipettes, Eppendorf mini spin centrifuge machine, Eppendorf thermomixer compact, Shimadzu UV1601 spectrophotometer, Olympus 1X71-22FL/PH research inverted microscope, Sartorius weighting balance, Hirayama autoclave machine, 2720 Thermal Cycles PCR machine, Cyberscan pH500 pH meter, UVP high performance ultraviolet transilluminator, Merck mili-Q water, Orbital shaker incubator, NBIOTEK. INC. shaking incubator, Perkin Elmer Cetus DNA thermal cycler, eyela oil bath, vortex mixer, Agilent 1200 Series Rapid Resolution Liquid Chromatography.

3.5 Commercial Kits

Table 3.3: Commercial Kits

Kits, Manufacturer	**Application**
i-TaqTM DNA polymerase kit iNtRON Biotechnology, Korea	PCR Amplification
QIAamp DNA Mini Kit Qiagen Pty. Ltd., Germany	Genomic DNA Extraction
QIAquick Gel Extraction Kit Qiagen Pty. Ltd., Germany	Purification of DNA from agarose gel
QIAquick PCR Purification Kit Qiagen Pty. Ltd., Germany	Purification of DNA from PCR

3.6 Growth Media and Buffer Solutions

3.6.1 Luria-Bertani (LB) Medium

Luria-Bertani broth preparation consisted of 1.0 % w/v trytone, 1.0 % w/v NaCl and 0.5 % w/v yeast extract and mixed in 500 ml of sterile distilled water and 500 ml sterile seawater (Sambrook & Maniatis, 1989). The solutions were then sterilised by autoclave. When cooled, antibiotic was added to the medium as indicated. For extraction of AHL molecules, the LB broth was supply with 50 mM of 3-(*N*-morpholino) propaneslfonic acid (MOPS) prior to inoculation of bacteria. MOPS maintained the media acidic that would prevent elevation of solution pH above the value of 7.0 as lactonolysis of AHL molecules will be induced at high pH (Yates *et al.*, 2002).

ISP Medium 1, ISP Medium 2 and ISP Medium 4 are used for characterizing *Streptomyces* and *Actinomycetes* species according to the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966).

3.6.2 ISP Medium 1

ISP medium 1 consists of 5.0 g Pancreatic Digest of Casein, 3.0 g Yeast extract and mixed in 250 ml water and 250 ml filtered sea water. The solutions were then sterilised by autoclave 121°C for 15 minutes.

3.6.3 ISP Medium 2

ISP medium 2 consist of 4.0 g Yeast extract , 10.0 g Malt extract,4.0 g Dextrose 4.0 g, 20.0 g agar and mixed in 250 ml water and 250 ml filteredsea water. The solutions were then sterilised by autoclave 121°C for 15 minutes.

3.6.4 ISP Medium 4

ISP medium 2 consist of 10.0 g Soluble Starch, 1.0 g Dipotassium Phosphate, 1.0 g Magnesium Sulfate USP, 1.0 g Sodium Chloride, 2.0 g Ammonium Sulfate,2.0 g Calcium Carbonate,1.0 mg Ferrous Sulfate, 1.0 mg Manganous Chloride, 1.0 g Zinc Sulfate 1.0 mg, and 20.0 g agar that mixed in 250 ml water and 250 ml filtered sea water. The solutions were then sterilised by autoclave at 121°C for 15 minutes.

3.6.5 Glycerol Yeast Extract Agar

Glycerol Yeast Extract Agar was prepared by mixing 0.5 mL Glycerol, 0.2 g Yeast extract, 0.01 g K_2HPO_4, 2.5 g Peptone, 1.5 g agar 1.5 g, that mixed in 250 ml distilled water and 250 ml filtered sea water. The solutions were then sterilised by autoclave 121°C for 15 minutes.

3.6.6 Phosphate Buffered Saline (PBS) 20mM, pH 6.5

PBS solution was prepared by mixing 0.23 g of NaH_2PO_4, 1.15 g of Na_2HPO_4 and 9.0 g NaCl in 1 L of distilled water. The pH of solution was then adjusted to the value of 6. The solutions were then sterilised by autoclave 121°C for 15 minutes.

3.6.7 5× Tris Borate EDTA (TBE) Buffer

5× x TBE stock solutions consisted of 54.0 g Tris base, 27.5 g boric acid and 3.72 g $Na_2EDTA.2H_20$ dissolved in 1 L of distilled water with pH adjusted to 8.0 before autoclave 121°C for 15 minutes.

3.6.8 Seawater

Seawater was collected at the same place with the soil mangrove swamps collected. Then, sea water was filtered using electric filter pump with 0.2 mm microfiber membrane filter for 2 hours. The pH of solution was then adjusted to the value of 8 and were then sterilised by autoclave 121°C for 15 minutes.

3.6.9 Yeast Malt Broth

Yeast Malt Broth consist of peptic digest of animal tissue, 5g, yeast extract, 3g, Malt extract, 3g, Dextrose, 10g dissolved in 250 ml distilled water and 250 ml filtered sea water. The solutions were then sterilised by autoclave 121°C for 15 minutes.

3.7 Stock Solutions

3.7.1 Synthetic *N*-Acyl Homoserne Lactones

Synthetic AHL molecules (C6-AHLs, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL) with concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL were obtained from Sigma-Aldrich® and Cayman Chemicals. AHLs were dissolved by using 100 % Acetonitrile (ACN) to the desired concentration.

3.8 DNA Ladder Marker

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

3.9 Sampling and Isolation of Environmental Bacteria

3.9.1 Mangrove Swamps Collection and Sampling Procedure

Sample was collected using 50 mL sterile polypropylene tubes from the soil mangrove swamps in Kuala Selangor (N 02°43.952 E 102°09.341), Malaysia. Sampling was performed on April 2013. The pH of the soil sample collected was recorded as pH 8. Samples were immediately processed upon returning to the laboratory to maintain the temperature and bacterial conditions.

3.9.2 Isolation of Bacterial Strains

Soil sample was serially diluted with Phosphate Buffered Saline (PBS) 20mM, pH 6.5 and spread onto different selective agar (ISP 4, ISP 2, yeast malt agar and starch casein agar) supplied with 80 µg/ml of cycloheximide and 75 µg/ml of nalidixic acid. Bacteria with observable different morphology (surface, color, elevation, sporulation and edge), were isolated after incubation for 14 days at 28 °C. Isolation procedure was preceding by selection of single bacteria colonies displaying distinctive morphology. Pure colony was obtained with several passages on yeast malt agar. Gram staining was then performed followed by observation under Olympus™ IX71 inverted research microscope (Olympus, Japan).

3.10 Molecular Identification of Bacterial Isolates

3.10.1 Genomic DNA Extraction and 16S rRNA Gene Amplification

Bacterial isolates were inoculated into yeast malt broth and incubated for 5 days, at 28°C in a shaking incubator at 220 rpm. The bacterial cells were harvested by centrifugation at 1200 *g* for 10 min. The cell pellets extracted using NucleoSpin Tissue (Qiagen) according to manufacturer's instruction. Total genomic DNA of the bacterial isolates was extracted using QIAamp DNA Mini Kit (Qiagen) according to

manufacturer instructions. PCR amplification of the 16S rRNA gene of nine isolated bacterial was carried out using the genomic DNA as template, primers 27F and 1525R were used for PCR of 16S rRNA gene. The expected size for amplified 16S rRNA genes is approximately 1.5 kb.

3.10.2 Polymerase Chain Reaction (PCR) Amplification

The target gene for identification of bacteria in this study was the 16S rRNA gene. 16S rRNA genes were PCR-amplified with the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and the reverse primer 1525R (5'AAGGAGGTGWTCCARCC-3') that the amplified fragments with expected size 1.5kb. The reagents from *i-Taq*™ DNA polymerase kit (INtron) were used for the PCR mixtures. The amount for each of the components used in the PCR mixtures is illustrated in Table 3.4. The PCR cycles for the amplification of 16S rRNA gene region consisted of initial denaturation 98°C, 2 min, denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension 72°C for 30 s and final stage 72°C at 10 min. Negative control was included for each PCR run by substituting cells with ultrapure water H_2O (MiliQ, Merck).

Table 3.4: PCR mixture for 16S rRNA gene amplification

PCR Component (Stock Concentration)	**Volume per sample (µl)**
ultrapure water H_2O	9.9
10 × Buffer containing 2 mM $MgCl_2$	1.5
dNTP (200 µM)	1.2
i-Taq™ DNA polymerase	0.2
27F Forward primer (10µM)	0.6

1525R Reverse primer (10µM)	0.6
Genomic DNA	1.0

3.10.3 Agarose Gel Electrophoresis

Electrophoretic examination of DNA samples were carried out using horizontal agarose gel at 1.0% (weight/volume) submerged in 1× TBE buffer. Agarose was melted in the microwave oven and EtBr was added to the final concentration of 0.5 µg/ml. The molten agarose was poured into gel cast for solidification. Agarose gel electrophoresis was start after load 3µg/ul of DNA samples and 0.2 µg of 1kb DNA ladder at 85 V for 45 minutes with 1.7 % (weight/volume) TBE. The gel was visualized on UVP ultraviolet transilluminator.

3.10.3.1 Purification and Sequencing

Gel Electrophoresis was carried out using Qiagen Extraction Kits protocol. 1.5 ml sterile centrifuge tube was prepared and label according to samples. 100 ul QG buffers were added and dissolved or 10 minutes at 50°C using thermomixer. After dissolved, sample was transfer into QIamp 2.0 ml tubes and spin at 1300 rpm for 1 minute. Then, 0.5 ml QG buffer were added and spin. After 1 minute's centrifugation, flow-through was discarding and 0.75 ml PE buffer were added. Samples were spin and transfer to new 1.5 ml centrifuge tubes. Then, 30 ul 0f EB buffer were added and incubate for 1 minutes and spin. Purified fragments were sending to 1st Base Lab Sdn Bhd for sequencing.

3.10.4 Sample preparation for MALDI-TOF MS and data analysis (Bruker Daltonics, Coventry, UK)

A fresh single bacterial colony was smeared onto a MSP 96 target polished steel BC plate, overlaid with formic acid (1.5 µL, 70%), and air-dried. Subsequently, the sample was overlaid with MALDI matrix (1 µL, 10 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) and air-dried again. The target plate was then subjected to MALDI-TOF MS analysis.

MS measurements were performed on a Microflex MALDI-TOF (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer (equipped with UV laser at wavelength 337 nm) equipped with the Bruker FlexControl software Version 3.3 (Build 108). The spectra were recorded in the linear positive ion mode and analyzed over a mass range of 2 to 20 kDa. The acceleration voltage was 20 kV. Each spot on the target plate was measured by the MBT-autoX.axe autoExecute method which enables the auto manipulation of the laser emission. Every measurement resulted from six series of 40 laser shots at different positions on the spotted product. The bacterial spectra were then analyzed in the Bruker MALDI Biotyper Real Time Classification (RTC) Version 3.1 (Build 65) software. The dendrogram was created by the standard MALDI Biotyper MSP creation method (Bruker Daltonics, Bremen, Germany), where distance values are relative and are always normalized to a maximum value of 1,000. Using the Biotyper software and taking a list of mass signals and their intensities into consideration, dendrograms were generated by similarity scoring of a set of mass spectra. Dendrograms shown had graphical distance values between species constructed from their reference spectra and a correlation function was used for calculating distance values. Biotyper 2.0 software (Bruker Daltonics) was used for the identification of bacterial isolates (Emami *et al*., 2012).

The matching of unknown spectra to the main spectra was evaluated based on dedicated score values. For this, peak information for the main spectrum was transformed to a maximum accessible score value. The results were reported as the best match to the Bruker database with the corresponding score value which was calculated on the final score according to which the identification results were evaluated as follows: if the logarithmic value of the final score was between 2.3 and 3, the isolate was identified at the level of species; for values between 2 and 2.3, the identification was secured at the level of genus and probable species identification; for values between 1.7 and 2, the identification at the level of genus was probable; and for values lower than 1.7, no reliable identification was made (Eigner *et al*, 2009).

3.10.5 Analysis of 16S rRNA sequence

3.10.5.1 16S rRNA gene sequence analysis

Genomic DNA of bacterial isolates was extracted using NucleoSpin Tissue and used for PCR as DNA template. 16S rRNA genes were PCR-amplified with the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and the reverse primer 1525R (5'AAGGAGGTGWTCCARCC-3'). The obtained gene sequencing was visualized using Applied Biosystem Sequence Scanner v1.0 followed by acquisition of good quality sequences via trimming.

3.10.5.2 Phylogenetic Analysis

Gene sequences were compared with GenBank database in National Center of Biotechnology Information using the BLASTN program followed by sequence alignment and phylogenetic analyses using the Molecular Evolutionary Genetic Analysis (MEGA) version 6.06 with parameter Neighbour-Joining algorithm and bootstrap 1,000 re-samplings. The model used was nucleotide Maximum Composite

Likelihood (MCL) to estimate the evolutionary distances between all sequences at one time. An appropriate outgroup namely a taxon which is distantly related but nevertheless sufficiently conserved of homologues to each of the in group taxa was selected to produce a rooted tree for phylogenetics analysis of the bacterial isolates (Tamura *et al*., 2011).

3.11 Detection of AHL Degradation Activities

Degradation of AHLs by the soil isolates was screened by using whole-cell AHLs inactivation assay used in conjunction with the biosensor *Chromobacterium violaceum* CV026. Residual AHLs was indicated by the purple pigmentation of CV026, and the decreased purple pigmentation indicated the degradation of AHLs. Degradation kinematic of AHLs will be measured using Rapid Resolution Liquid Chromatography (RRLC). Furthermore, AHLs degradation product will be identified using Liquid Chromatography Mass Spectrometry (LC-MS) (Agilent Inc., USA).

3.11.1 Whole-Cell AHL Inactivation Assay

AHL inactivation assay was conducted to screen bacterial isolates for their AHL-degradation activity (Chan *et al.*, 2011). Overnight grown bacterial cells were harvested by 1300 rpm centrifugation for 3 minutes at room temperature, cell pellets were washed twice and resuspended in PBS buffer (100 mM, pH 6.5). Selected concentration of synthetic AHLs (C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL, Sigma-Aldrich, St. Louis, MO, USA) were dispensed into sterile micro-centrifuge tubes and dried by evaporation for 3 hours in the fume hood. AHLs were dispensed into micro-centrifuge tubes and left dried in the fume hood. Cells suspension (in PBS buffer) was added to rehydrate the AHL to the final concentration of 0.5 μM. Mixtures were then incubated

at 28°C with shaker at 220 rpm for 0 h, 24 h and 48 h. Aliquots of AHLs were with drawn at 0 h, 24 h and 48 h interval. Residual AHLs were detected using *C. violaceum* CV026. For AHL inactivation assay, reaction mixtures were stopped by heat inactivation at 95°C for 5 min and 10 µL of reaction mixture was spotted onto sterile paper disc placed on *C. violaceum* CV026 lawn and incubated overnight. Reduction of purple pigments after 24 h and 48 h of incubation indicates AHL degradation. PBS buffer and *B. cereus* served as negative and positive controls, respectively. Degradation of short chain AHLs are evident by loss of purple pigmentation shown by *C. violaceum* CV026 and the results will be recorded (Chan *et al*., 2009).

3.11.2 Screening of AHL Degradation using *C. violaceum* CV026 Biosensor

Cell suspension (10 µL) was dispensed onto disc placed on the CV026 overlay followed by overnight incubation. Reduction of violet color zone size indicates significant degradation of AHL over the incubation period. The negative controls for the experiment involved incubation of AHL with washed *E. coli* TOP 10 and PBS solution whereas *B. cereus* was employed as positive control.

3.11.3 AHL Extraction

Bacteria were cultured in LB broth buffered to pH 6.5 with 50 mM of MOPS (100 mM, pH 6.5) and incubated overnight with shaking at 28°C. The spent culture supernatant was extracted twice by addition of an equal volume of ethyl acetate followed by vortex mixing for 1 min. The organic solvent was dried in fume hood. The dried extracts were resuspended in 1 mL of ethyl acetate and dried again in fume hood. Finally, 200 µL of acetonitrile (HPLC grade) was added and vortexed for 3 min to dissolve the extracted AHLs. The mixture was left overnight at room temperature and

centrifuged at 12,000 rpm for 10 min to remove any insoluble residue. Aliquots (75 μL) of the extract were withdrawn from the top layer and placed in sample vials for mass spectrometry analysis (Yates *et al*., 2002).

3.11.4 Identification of AHL Lactonase Activity with CV026 overlay

Overnight cultures of QQ cells were harvested by centrifugation for 1300 rpm for 3 minutes. Cell pellets were washed twice and re-suspended in phosphate buffered saline (PBS) (100 mM, pH 6.5). Selected known concentrations of synthetic AHLs (C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL, Sigma-Aldrich, St. Louis, MO, USA) were dispensed into sterile micro-centrifuge tubes and dried by evaporation. Bacteria cell suspensions were then added to rehydrate the AHLs to final concentrations of 0.5 μM. The mixtures were then incubated at 28 °C with shaking (220 rpm) for 0 h and 24 h. All reactions were stopped by heat inactivation at 95 °C. For the detection of AHL degradation, 10 μL of reaction mixture was spotted onto sterile paper discs placed on a *Chromobacterium violaceum* CV026 lawn and incubated overnight at 28 °C. AHL inactivation assays involved incubation of *E. coli* TOP10 and PBS buffer as negative controls. Re-lactonisation with acidification using hydrochloric acid (HCl, 0.2 M) was performed as reported. The purple pigments with the addition of hydrochloric acid (HCl) suggested re-lactonisation of the digested AHL indicated that both the isolates produced lactonase in the presence of 3-oxo-C6-HSL (Yates *et al*., 2002).

3.11.5 Verification of QQ Activities through RRLC Analysis

Sample preparation for RRLC analysis was performed similar to the whole-cell AHLs inactivation assay described. AHLs were extracted twice using ethyl acetate, followed by drying in the fume hood and resuspend in 100 μL of 100% acetonitrile for

RRLC analysis. AHLs degradation was analyzed using Agilent Technologies 1200 series RRLC system (Agilent Inc., USA). Briefly, AHLs samples were separated using Agilent Poroshell120 EC-C18 column (4.6 mm × 100mm, packed with 2.7 μm particle size) with the elution procedure consisting of an isocratic profile of acetonitrile/water (35:65, v/v). Flow rate of 0.7 mL/min was applied and AHL detection was carried out at 210 nm (Chong *et al.,* 2012). Known amounts of synthetic AHLs (0.2, 0.4, 0.6, 0.8 and 1.0 μg/μL) were included as standards. AHL incubated with *E. coli* TOP10 cells and PBS served as a negative controls. RRLC will confirm the degradations of AHLs by reduction of mili-absorbance unit (mAU).

3.11.6 Identification of AHL Lactonase Activity with RRLC Analysis

Overnight cultures of QQ cells were harvested by centrifugation. Cell pellets were washed twice and re-suspended in phosphate buffered saline (PBS) (100 mM, pH 6.5). Selected known concentrations of synthetic AHLs (C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL, Sigma-Aldrich, St. Louis, MO, USA) were dispensed into sterile micro-centrifuge tubes and dried by evaporation. Cell suspensions were then added to rehydrate the AHLs to final concentrations of 0.5 μM. The mixtures were then incubated at 28 °C with shaking (220 rpm) for 0 h and 24 h. All reactions were stopped by heat inactivation at 95 °C. For the detection of AHL degradation, 10 μL of reaction mixture was spotted onto sterile paper discs placed on a *Chromobacterium violaceum* CV026 lawn and incubated overnight at 28 °C. AHL inactivation assays involved incubation of *E. coli* TOP10 and PBS buffer as negative controls. Re-lactonisation with acidification using hydrochloric acid (HCl, 0.2 M) was performed as reported (Chong *et al.,* 2012). Flow rate of 0.7mL/min and the spectrum will be monitored at 210nm. Agilent Chemstation software (version B.04.01) was used for data collection and analysis.

3.11.7 Verification of QQ Activities via Lactonase through RRLC Analysis

Procedure was followed as RRLC analysis protocol (Chong *et al.,* 2012). Sample preparation for RRLC analysis was performed similar to the whole-cell AHLs inactivation assay described. AHLs were extracted twice using ethyl acetate, followed by drying in the fume hood before resuspended in 100 µL of acetonitrile for RRLC analysis. AHLs degradation was analyzed using Agilent Technologies 1200 series RRLC system. Briefly, AHLs samples were separated using Agilent Poroshell120 EC-C18 column (4.6 mm × 100mm, packed with 2.7 µm particle size) with the elution procedure consisting of an isocratic profile of acetonitrile/water (35:65, v/v). Flow rate of 0.7 mL/min was applied and AHL detection was carried out at 210 nm (Chong *et al.,* 2012). Known amounts of synthetic AHLs (0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL) were included as standards. AHL incubated with *E. coli* TOP10 cells and PBS served as a negative controls. RRLC will confirm the degradation of AHLs by reduction of mili-absorbance unit (mAU).

CHAPTER 4.0

RESULTS

4.1 Soil Sampling and Isolation of Bacteria

The Mangrove swamp sampling site (GPS: N 02°43.952 E 102°09.341) is located at Kuala Selangor, Malaysia. Sample was collected on April 2013 using 50-mL sterile polypropylene tube. The pH of the soil sample collected was recorded as pH 8. Sample was immediately processed upon returning to the laboratory. To process the sample, the soil sample was serially diluted and spread onto different selective media (ISP 4, ISP 2, yeast malt agar and starch casein agar) supplied with 80 µg/ml cycloheximide and 75 µg/ml nalidixic acid. Bacteria with observable different morphology were isolated after incubation for 14 days at 28 °C. Pure colony was obtained with several passages on yeast malt agar. Nine bacteria with different morphologies were isolated and selected for further experiments. Table 4.1 illustrated a summary of appearance and morphology of bacterial colonies obtained from agar plates.

4.2 Gram Staining

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

Table 4.1: Morphology of bacterial colonies

Isolate	Abbreviation	Gram Staining	Surface	Elevation	Sporulation	Edge	Colour
1	NP1	+	Contoured	Pulvinate	Endospore	Entire	Gray-brown
2	NP2	+	Contoured	Pulvinate	Endospore	Entire	Brown
3	NP3	+	Contoured	Pulvinate	Endospore	Serrated	Yellowish green
4	NP4	+	Contoured	Pulvinate	Endospore	Serrated	Dark brown
5	NP5	+	Contoured	Pulvinate	Endospore	Serrated	White yellow
6	NP6	+	Contoured	Pulvinate	Endospore	Entire	Greenish orange
7	NP7	+	Contoured	Pulvinate	Endospore	Entire	Orange brown
8	NP8	+	Contoured	Pulvinate	Endospore	Entire	Grey white
9	NP9	+	Contoured	Pulvinate	Endospore	Entire	Grey

4.3 Molecular Identification and Phylogenetic Analyses of Bacteria Isolates

The obtained nucleotide sequences were compared with those deposited in the GenBank database using the BLASTn followed by sequence alignment. Table 4.2 illustrated the nearest identities for the nine strains. The 16S rRNA sequences showed 99% similarity to the known bacteria and 0.00 E-value. Bacterial isolates of NP3 and NP4 were clustered into genus Actinomycetes, NP1, NP2, NP6, NP7, NP8 and NP9 were clustered into the genus *Streptomyces,* and NP5 were clustered into genus *Kitasatospora* with 99% homology.

Table 4.2 : Identification of Microbial Strains using NCBI BLASTN alignment search analysis

Isolate name	Closest match	Similarity (%)	Sequence Length (bp)	Accession no. of closest match
NP1	*Streptomyces costaricanus strain* NBRC 100773	99%	1480 bp	NR_041414
NP2	*Streptomyces atratus* strain NRRL B-16927	99%	1487 bp	NR_043490
NP3	*Actinoalloteichus cyanogriseus* strain NRRL B-2194	99%	1481 bp	NR_044137
NP4	*Actinobacterium* J95	99%	1440 bp	KP216753
NP5	*Kitasatospora papulosa* strain JCM 7250	99%	1484 bp	NR_126320
NP6	*Streptomyces luridus* strain NBRC 12793	99%	1439 bp	NR_112280

Table 4.2, continued

Isolate Name	Closest match	Similarity (%)	Sequence Length (bp)	Accession no. of closest match
NP7	*Streptomyces albofaciens* strain NBRC 12833	99%	1432 bp	NR_112296
NP8	*Streptomyces kanamyceticus* strain NRRL B-2535 16S	99%	1502 bp	NR_043822
NP9	*Streptomyces prasinopilosus* strain R12-4	99%	1394 bp	KT751524

4.4 Phylogenetic Analysis

The phylogenetic tree of 16S rRNA gene sequences were constructed using Molecular Evolutionary Genetic Analyss (MEGA) version 6.06. Construction of phylogenetic tree was based on neighbor-joining method with Bootstrap test with the value of 1000 replicates. Phylogenetic analyses supporting the identification of each isolates were shown in Figure 4.1 to Figure 4.9.

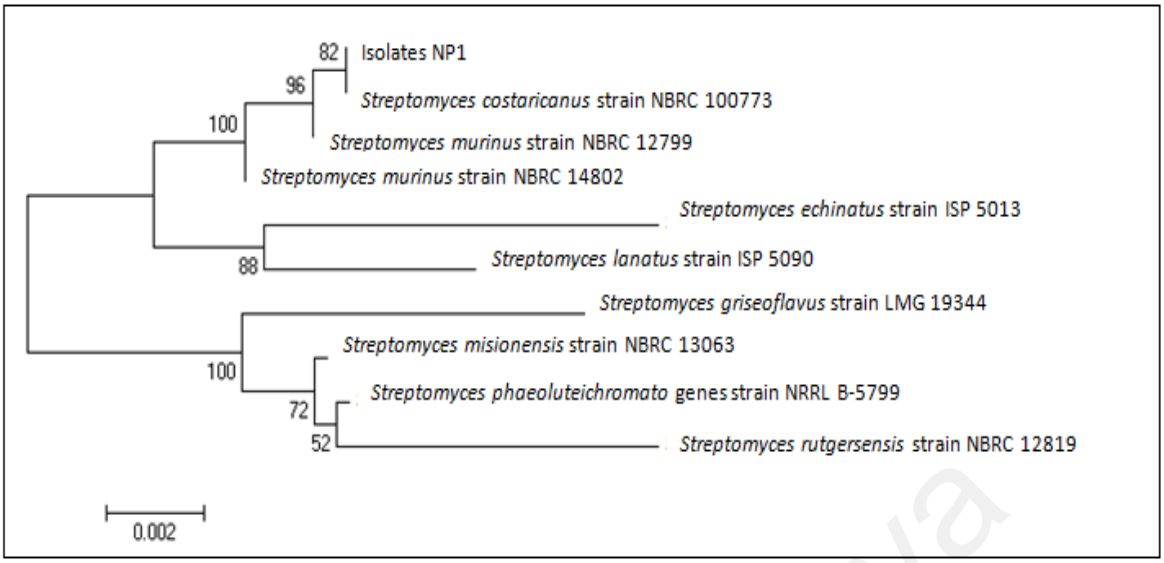


Figure 4.1: 16S rRNA gene based phylogenetic analysis of *Streptomyces contaricanus* NP1

Legends: bar shows 0.002 nucleotide changes. Numerical values at the nodes are bootstrap values.

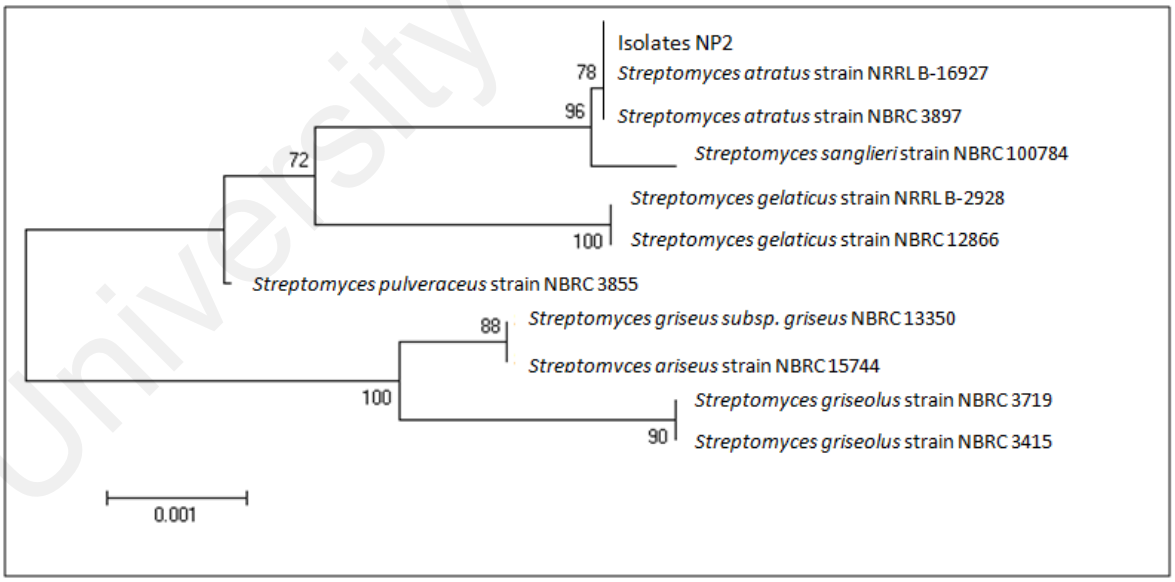


Figure 4.2: 16S rRNA gene based phylogenetic analysis of *Streptomyces atratus* NP2

Legends: bar shows 0.001 nucleotide changes. Numerical values at the nodes are bootstrap values.

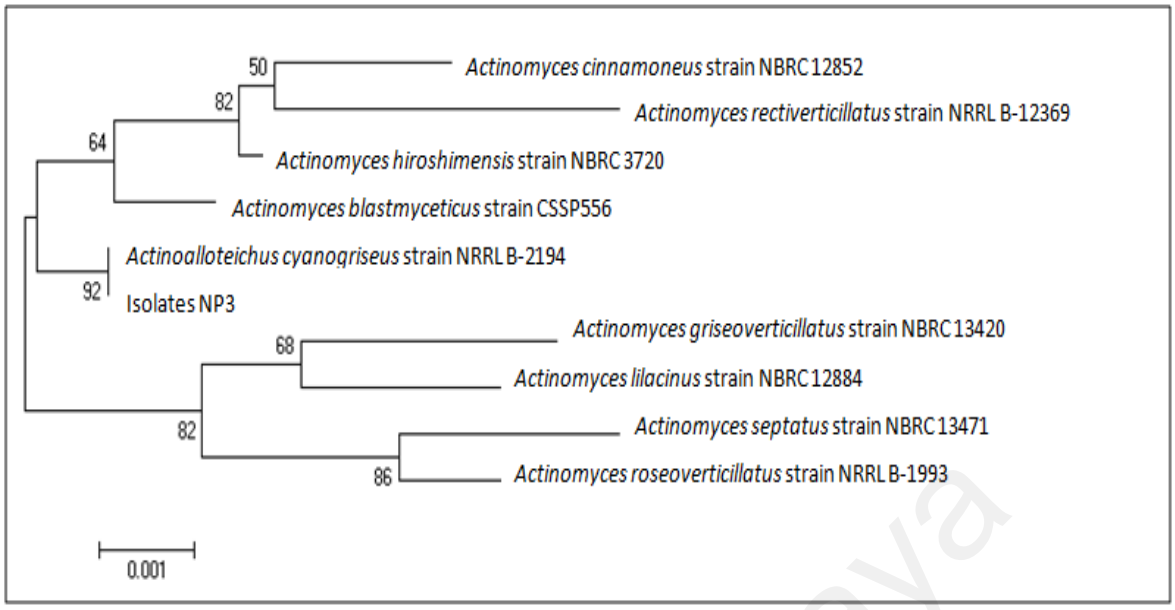


Figure 4.3: 16S rRNA gene based phylogenetic analysis of *Actinoalloteichus cyanogriseus* NP3

Legends: bar shows 0.001 nucleotide changes. Numerical values at the nodes are bootstrap values.

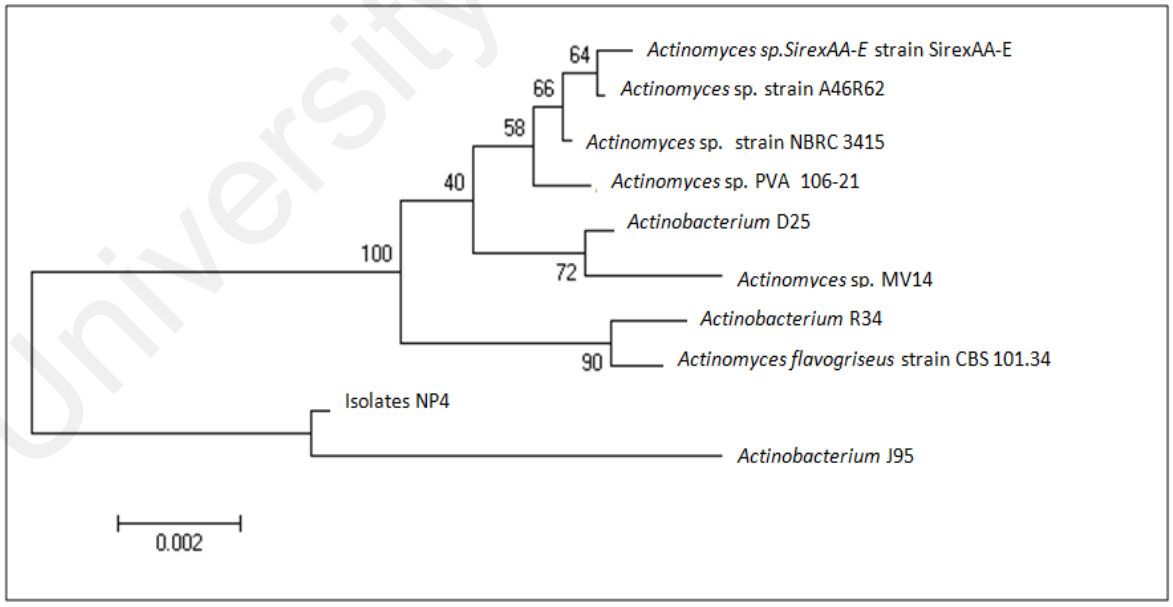


Figure 4.4: 16S rRNA gene based phylogenetic analysis of *Actinobacterium* NP4

Legends: bar shows 0.002 nucleotide changes. Numerical values at the nodes are bootstrap values.

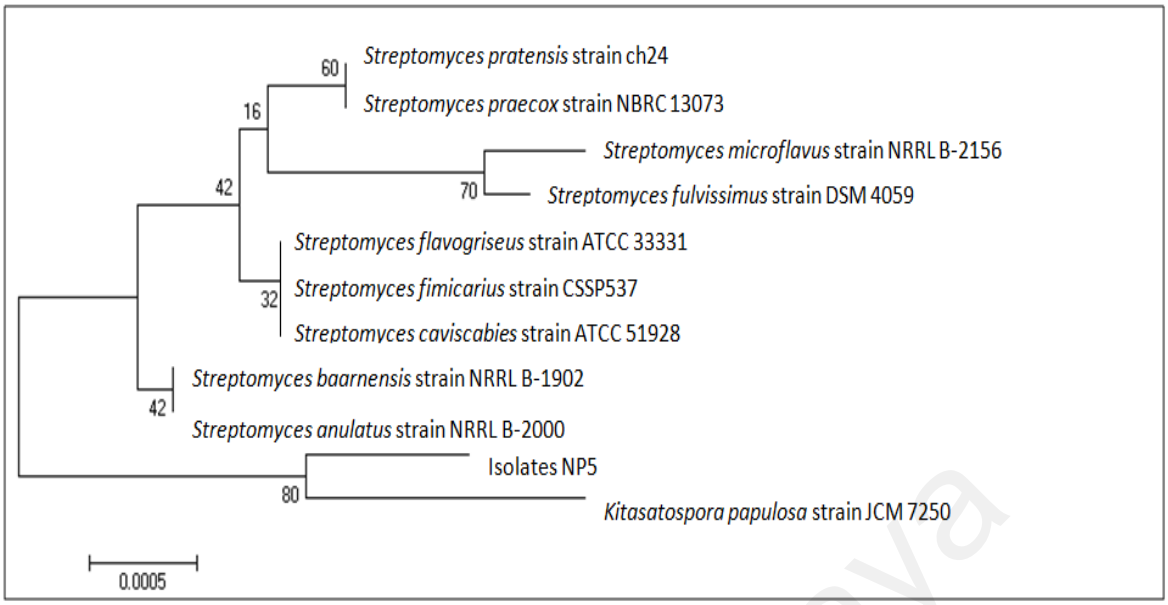


Figure 4.5: 16S rRNA gene based phylogenetic analysis of *Kitasatospora papulosa* NP5

Legends: bar shows 0.0005 nucleotide changes. Numerical values at the nodes are bootstrap values.

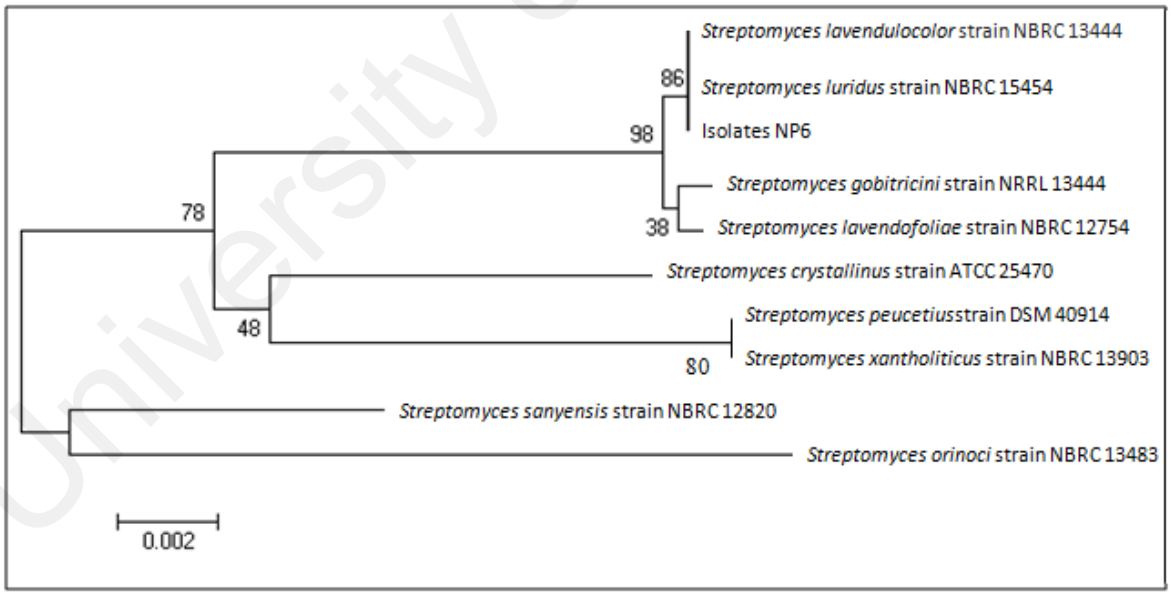


Figure 4.6: 16S rRNA gene based phylogenetic analysis of *Streptomyces luridus* NP6

Legends: bar shows 0.002 nucleotide changes. Numerical values at the nodes are bootstrap values.

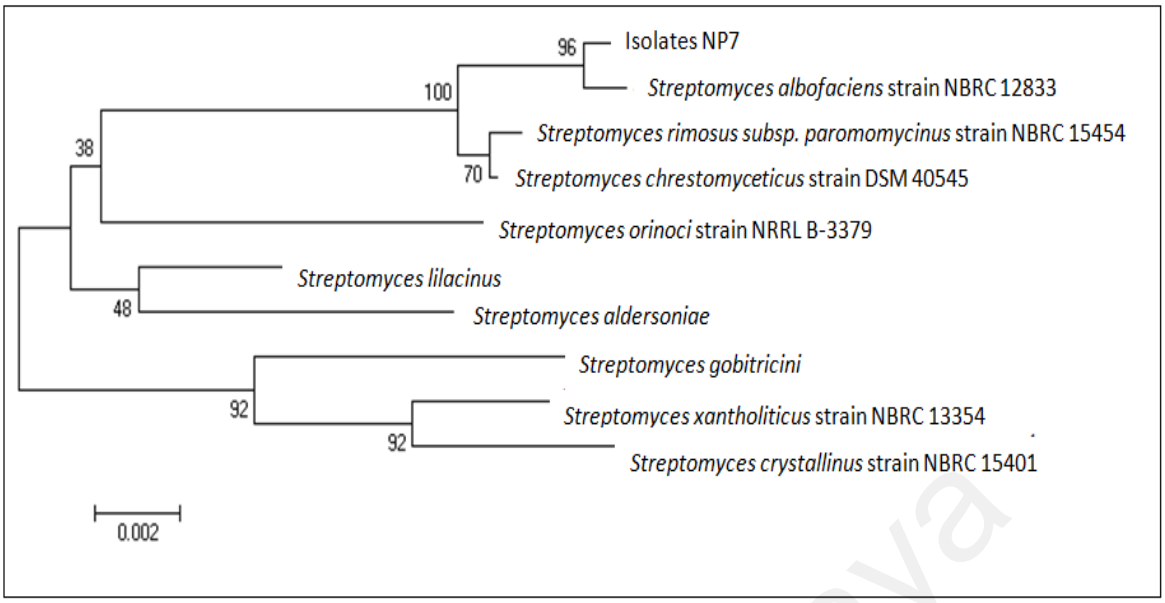


Figure 4.7: 16S rRNA gene based phylogenetic analysis of *Strepomyces albofaciens* NP7

Legends: bar shows 0.002 nucleotide changes. Numerical values at the nodes are bootstrap values.

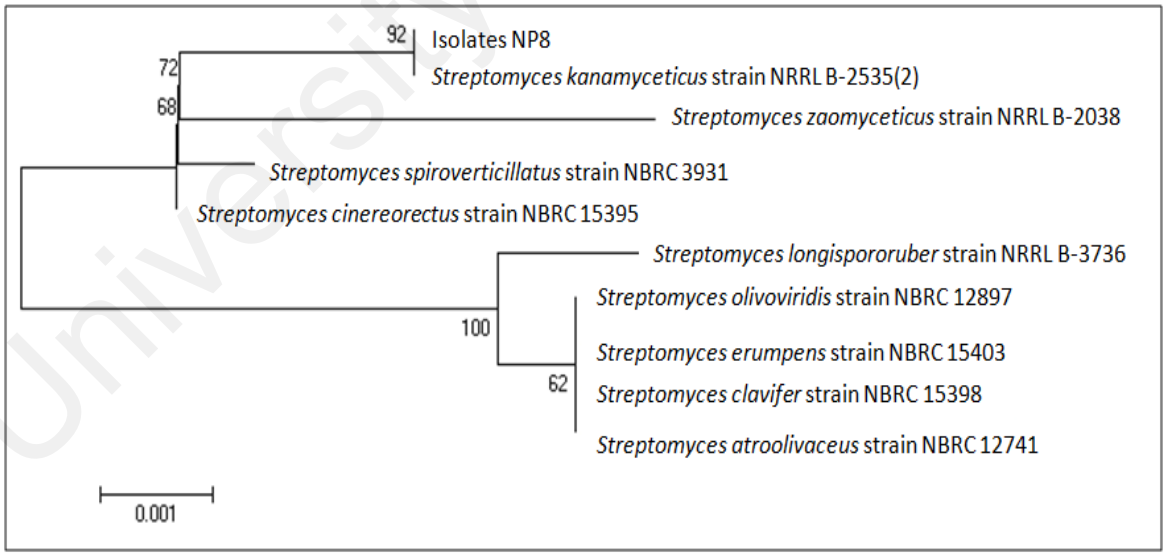


Figure 4.8: 16S rRNA gene based phylogenetic analysis of *Streptomyces kenamyceticus* NP8

Legends: bar shows 0.001 nucleotide changes. Numerical values at the nodes are bootstrap values.

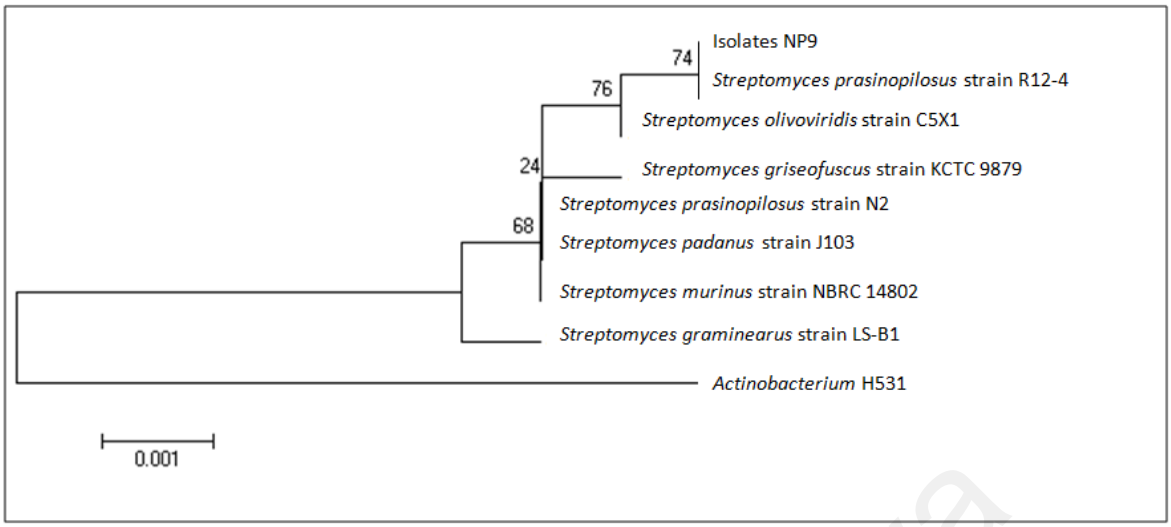


Figure 4.9: 16S rRNA gene based phylogenetic analysis of *Streptomyces prasinopilosus* NP9

Legends: bar shows 0.001 nucleotide changes. Numerical values at the nodes are bootstrap values.

4.5 Quorum Quenching (QQ) Activities of Bacterial Isolates

4.5. 1 Whole Cell AHL Inactivation Assay

Table 4.3: Summarization of degradation of AHLs inactivation assay of nine isolates.

AHLs / Isolate	**C6-HSL**	**3-oxo-*C6*-HSL**	**3-hydroxy-C6-HSL**
B. cereus (positive control)	Degraded	Degraded	Degraded
E. coli Top10 (negative control)	Not degraded	Not degraded	Not degraded
PBS buffer (negative control)	Not degraded	Not degraded	Not degraded
Isolate NP1	Slow degraded after 48h	Slow degraded after 48h	Slow degraded after 48h
Isolate NP2	Slow degraded after 48h	Slow degraded after 48h	Slow degraded after 48h
Isolate NP3	Fast degraded after 24h	Fast degraded after 24h	Fast degraded after 24h
Isolate NP4	Fast degraded after 24h	degraded after 24h	Fast degraded after 24h

	Table 4.3, continued		
AHLs / Isolate	**C6-HSL**	**3-oxo-*C6*-HSL**	**3-hydroxy-C6-HSL**
Isolate NP5	Slow degraded after 48h	Slow degraded after 48h	Fast degraded after 24h
Isolate NP6	Fast degraded after 24h	Slow degraded after 48h	Fast degraded after 24h
Isolate NP7	Slow degraded after 48h	Slow degraded after 48h	Slow degraded after 48h
Isolate NP8	Slow degraded after 48h	Slow degraded after 48h	Slow degraded after 48h
Isolate NP9	Slow degraded after 48h	Slow degraded after 48h	Slow degraded after 48h

4.5.2 Screening of AHL Degradation using CV026 Biosensor

Detection of QQ activity using CV026 overlay

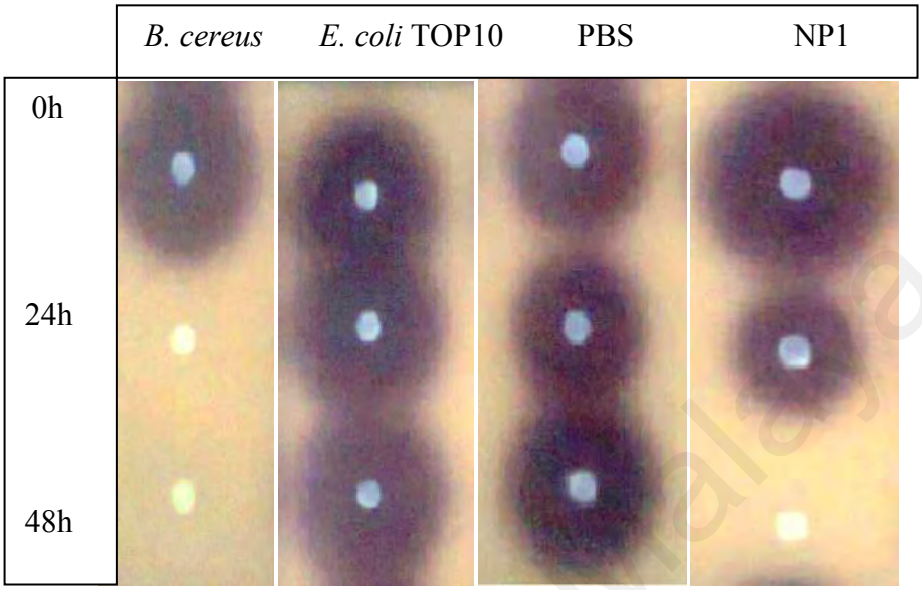


Figure 4.10: Detection of Quorum Quenching activity using CV026 overlay. Bacterial suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with C6-AHLs for 0 h, 24 h and 48 h (Figure 4.10**)**. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h and 48h of incubation. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls. Results in Figure 4.10 shows that isolates NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9 degrade the C6-AHLs.

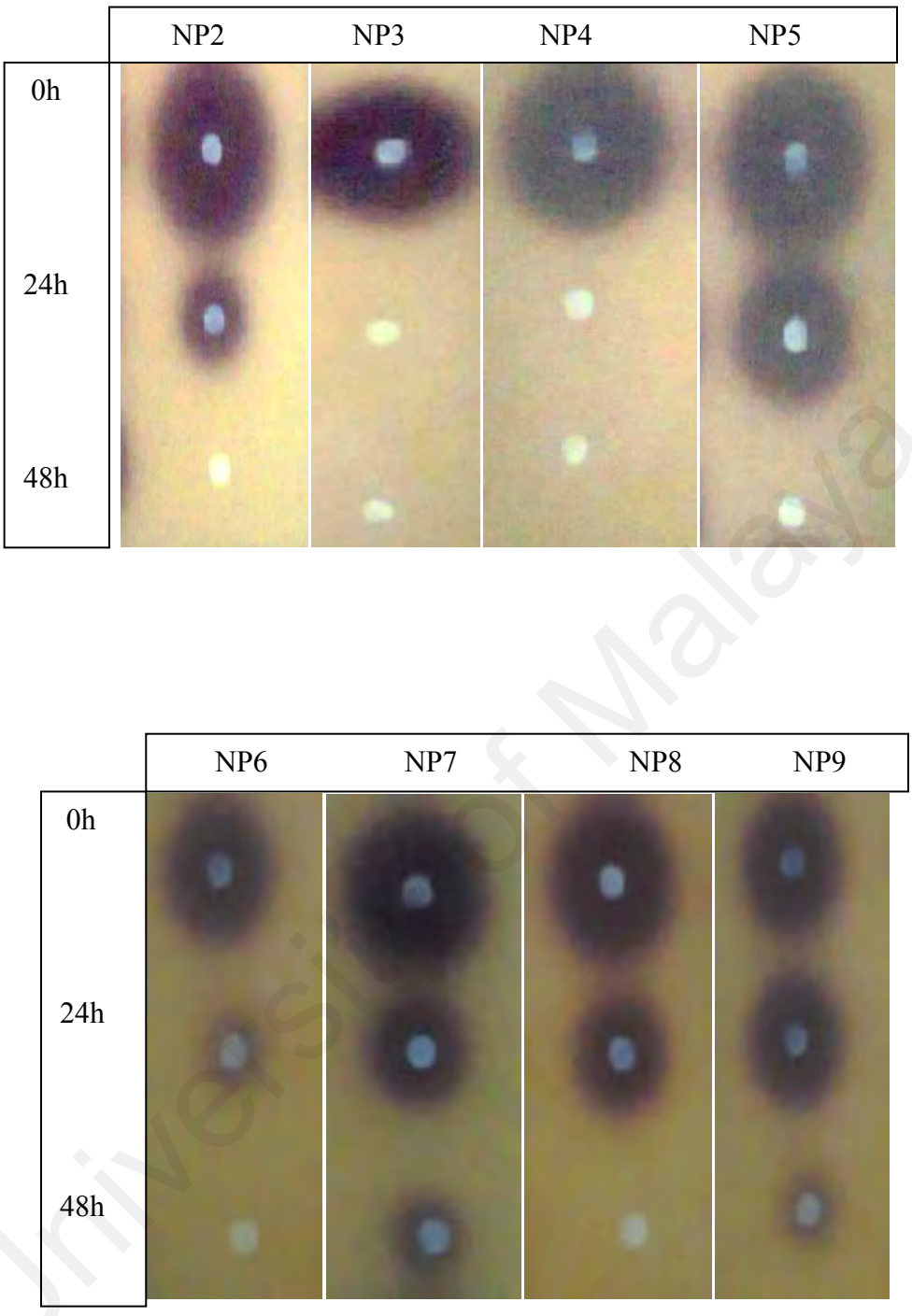


Figure 4.10, continued

Disappearance or reduced purple pigment shows AHL degradation. *B. cereus* served as the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls.

To detect QQ against with 3-oxo-C6-AHLs, bacterial isolates (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-oxo-C6-HSL for 0 h, 24 h and 48 h. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h and 48 h of incubation (Figure 4.11). Among the isolates, NP1, NP2, NP3, NP4, NP5, NP6, NP7 and NP8 showed degradation of 3-oxo-C6-HSL.

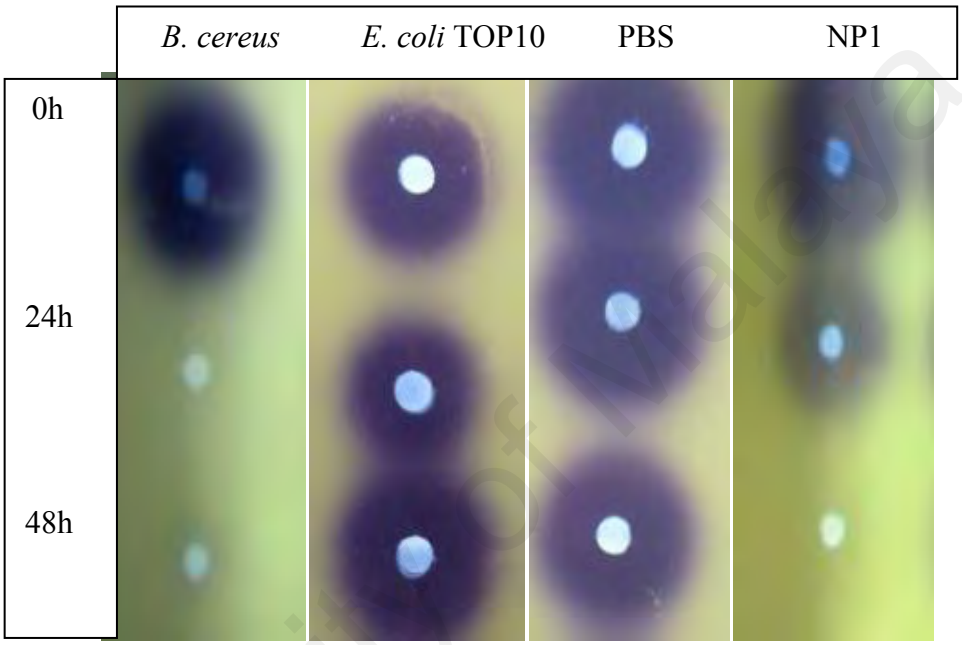
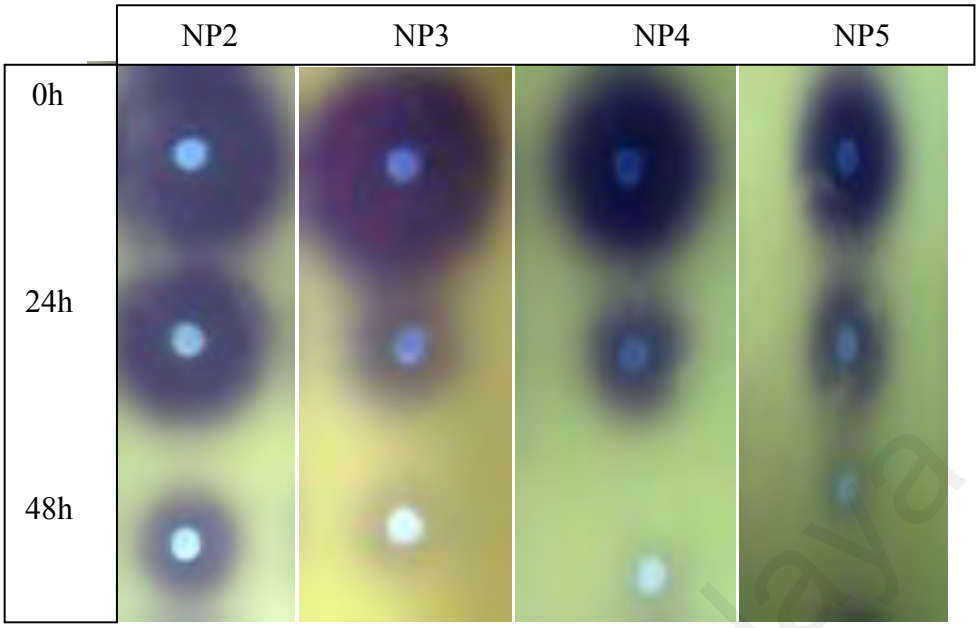


Figure 4.11: Detection of QQ activity using CV026 overlay. Bacterial suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-oxo-C6-HSL for 0 h, 24 h and 48 h as indicated on the left of figure. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h and 48 h of incubation. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls.



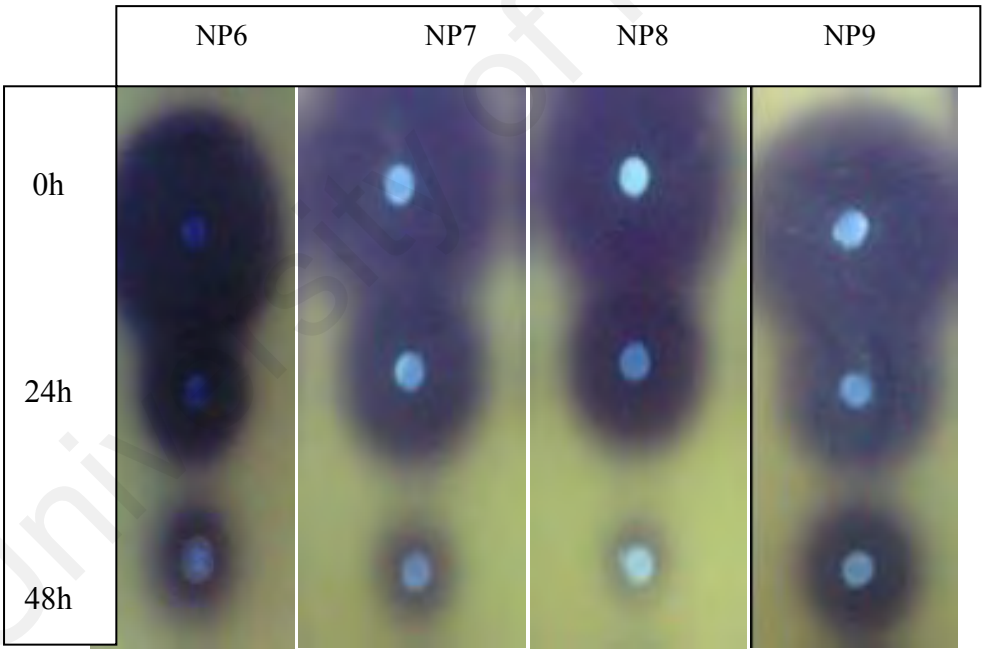


Figure 4.11, continued

To detect QQ against with 3-hyroxy-C6-HSL, bacterial isolates (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-hyroxy-C6-HSL for 0 h, 24 h and 48 h. Among the isolates, NP1, NP2, NP3, NP4, NP5, NP6 and NP9 showed degradation of 3- hyroxy-C6-HSL (Figure 4.12).

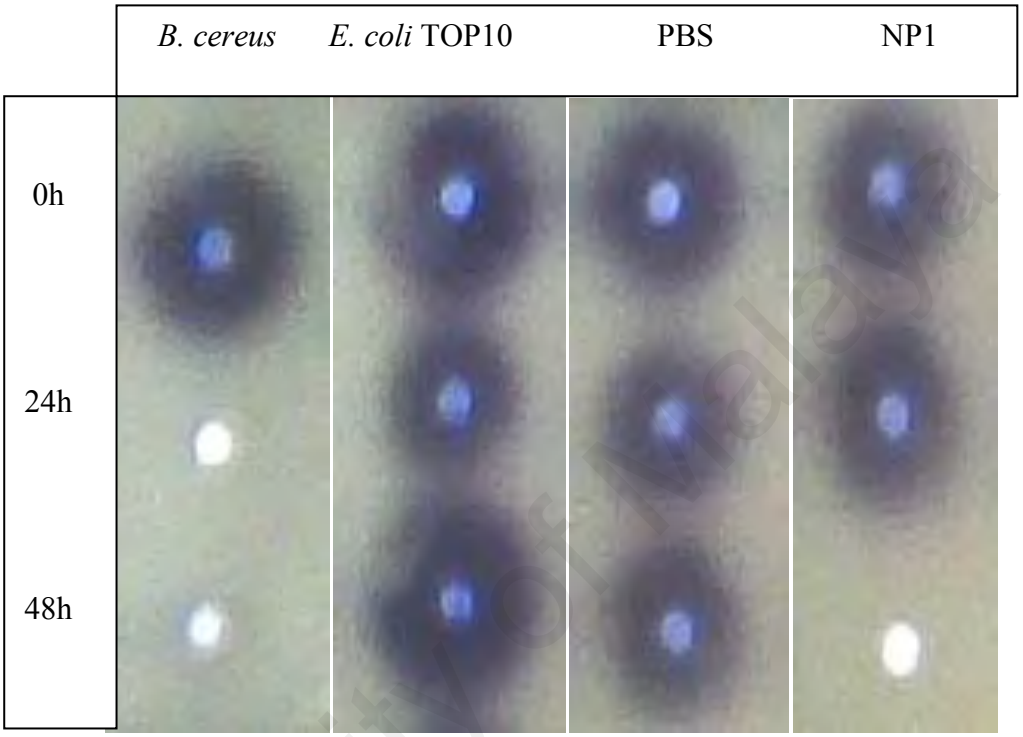


Figure 4.12: Detection of QQ activity using CV026 overlay. Bacterial suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-hyroxy-C6-AHLs for 0 h, 24 h and 48 h as indicated on the left of figure. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h and 48 h of incubation. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls.

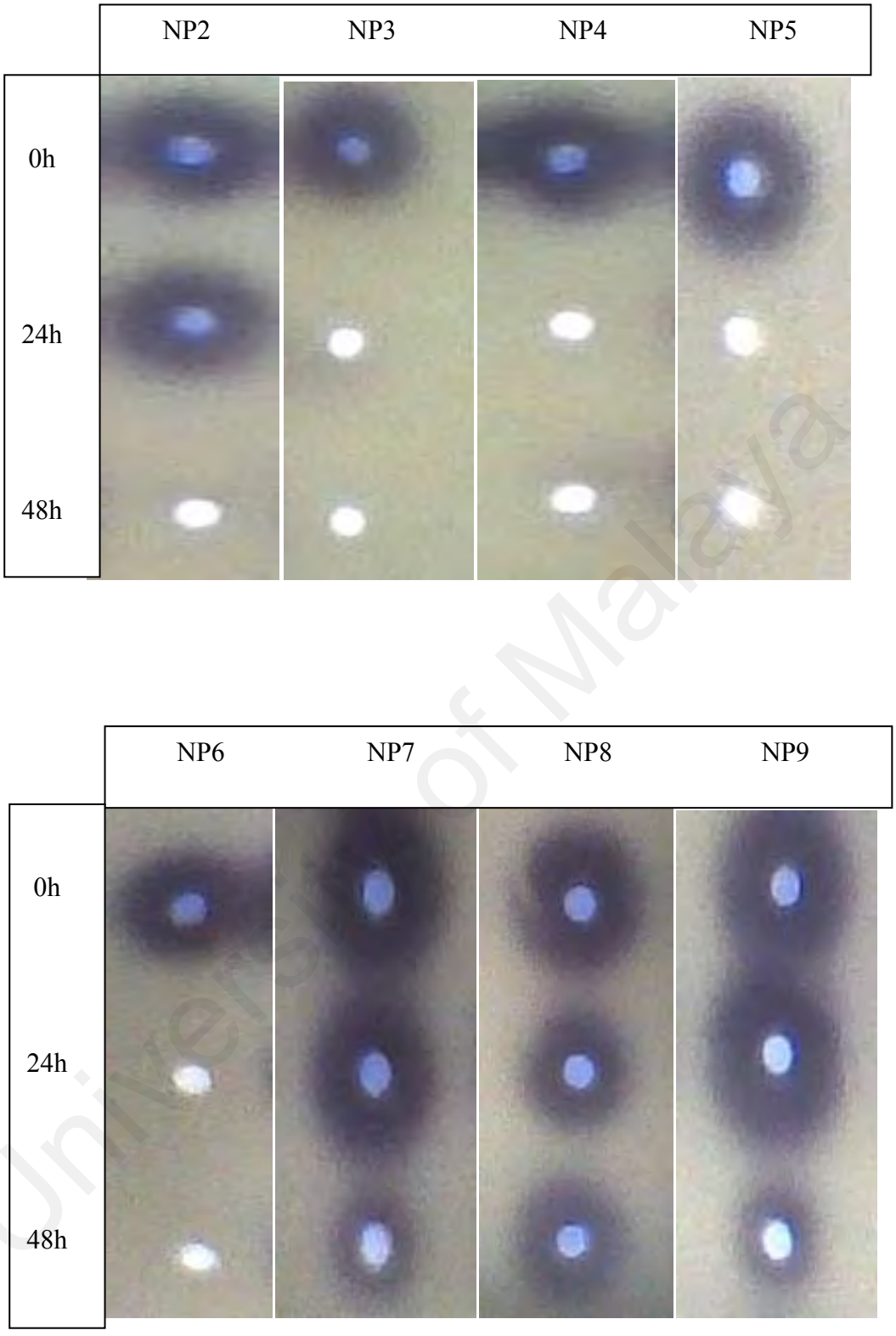
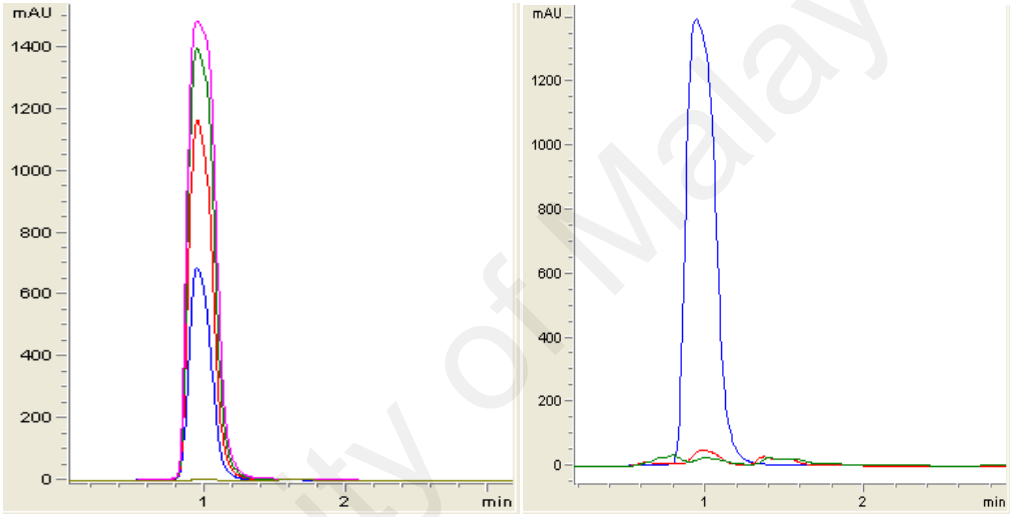


Figure 4.12, continued

4.5.3 Verification of QQ Activities through Rapid Resolution Liquid Chromatography (RRLC) Analysis

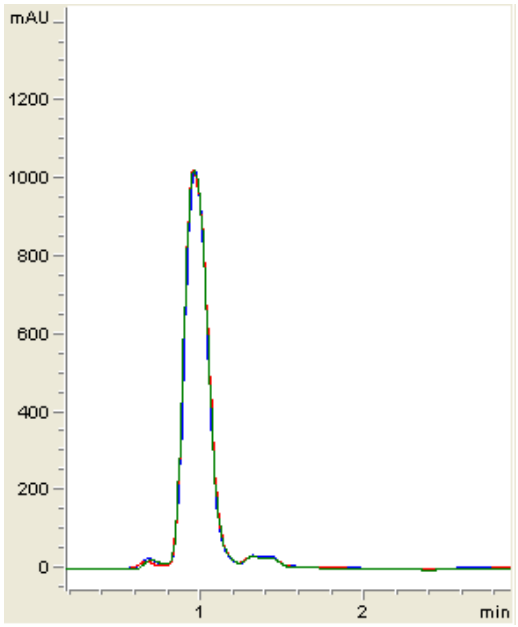
RRLC was used to monitor the degradation of AHLs by the isolates over the period of 48 hr (Figure 4.13) (a – m). Synthetic AHL was used as standards for comparison purpose. It appears that degradation of C6-HSL was observed by all of the isolates (Figure 4.10). Similarly, degradation of 3-oxo-C6-HSL (Figure 4.11), 3-hydroxy -C6-HSL (Figure 4.12) were observed by all of the isolates.



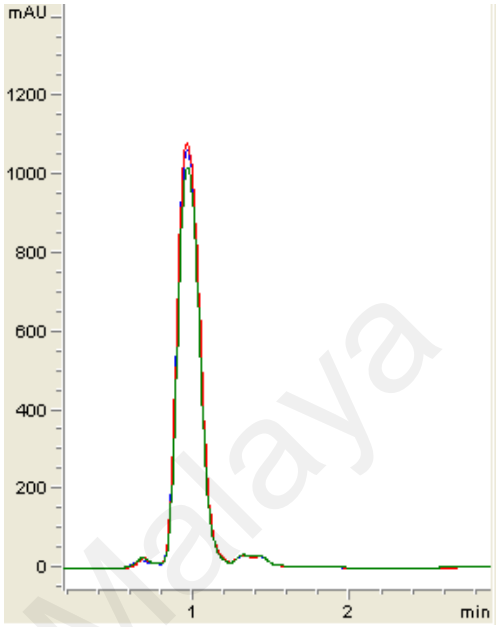
(a) **(b)**

Figure 4.13**:** RRLC analysis of C6-AHLs degradation. Residual C6-HSL (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), both after degradation 24 h (red) and 48 h (green) was monitored at 210 nm. Degradation of C6-HSL is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram. (**a**) Synthetic C6-AHLs was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* aspositive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)**

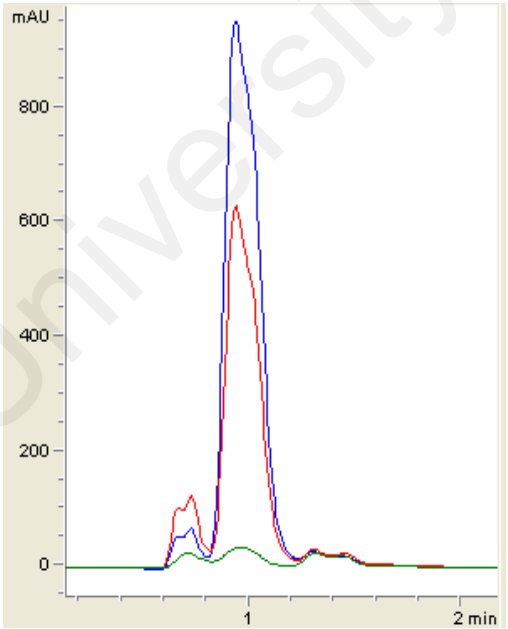
NP9, respectively. Degradation of C6-HSL was observed by all of the samples isolates.



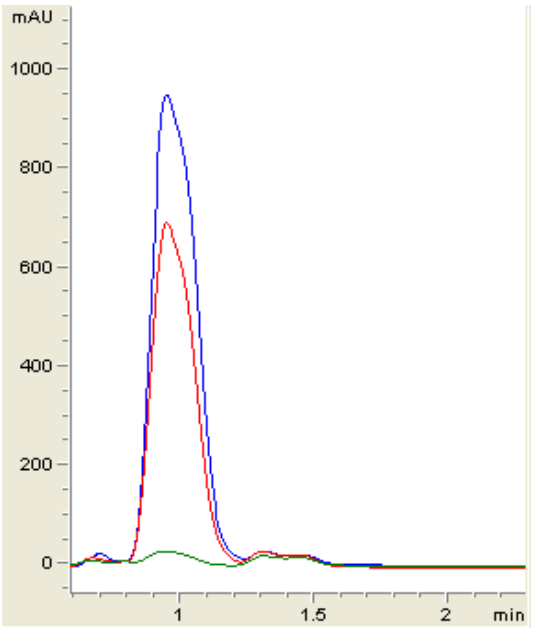
(c)



(d)

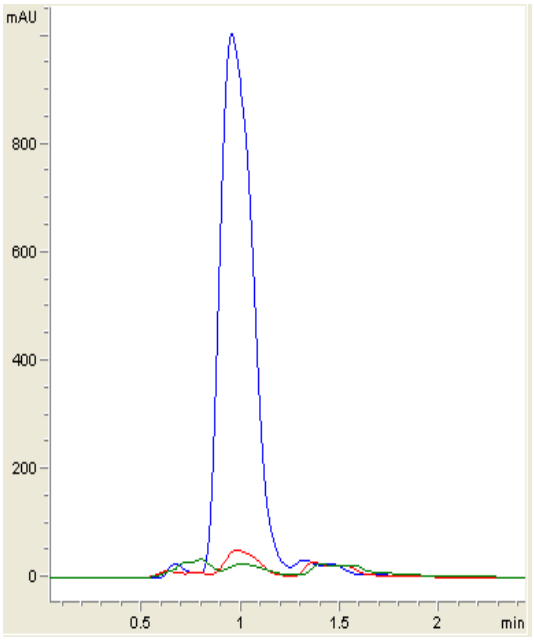


(e)

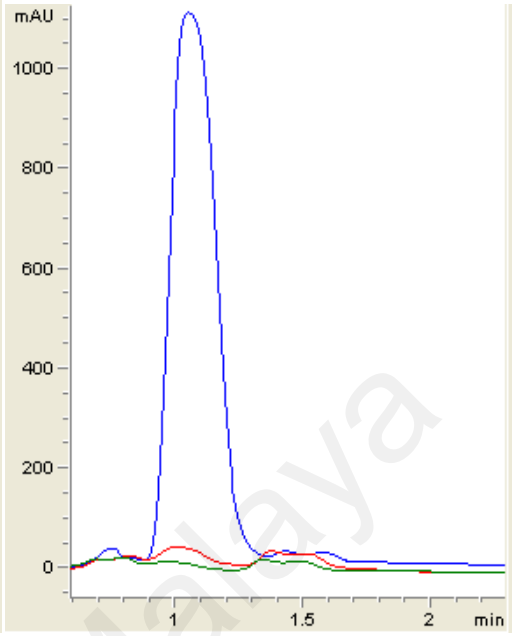


(f)

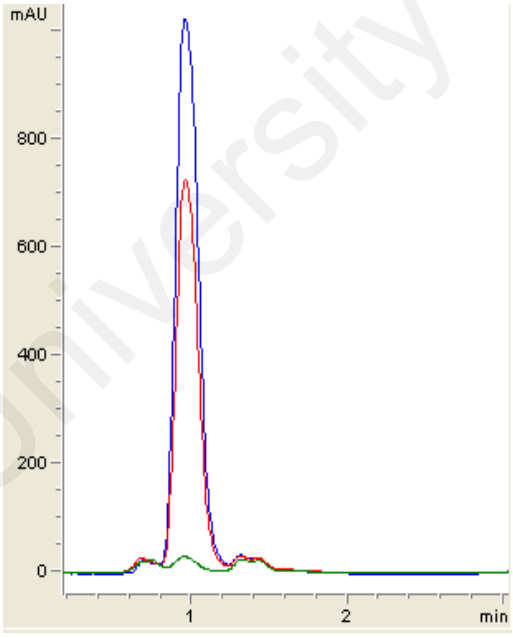
Figure 4.13, continued



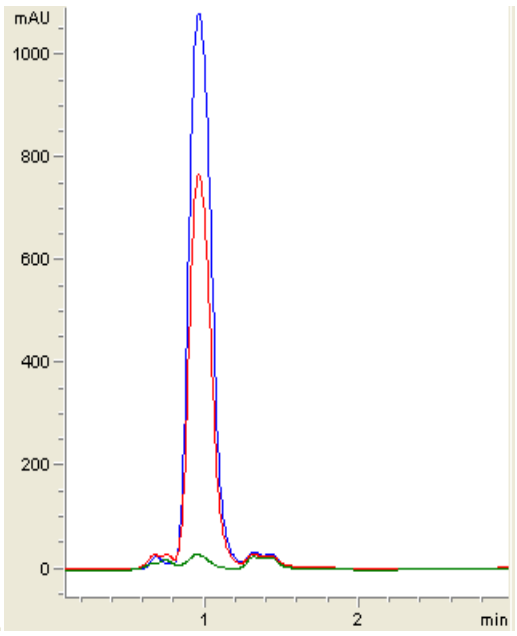
(g)



(h)

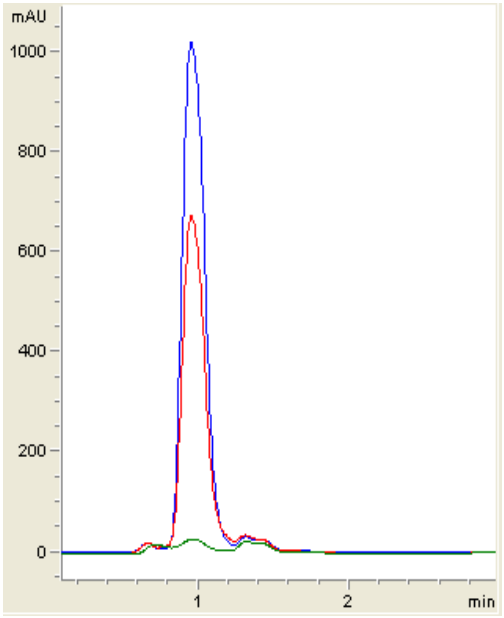


(i)



(j)

Figure 4.13, continued



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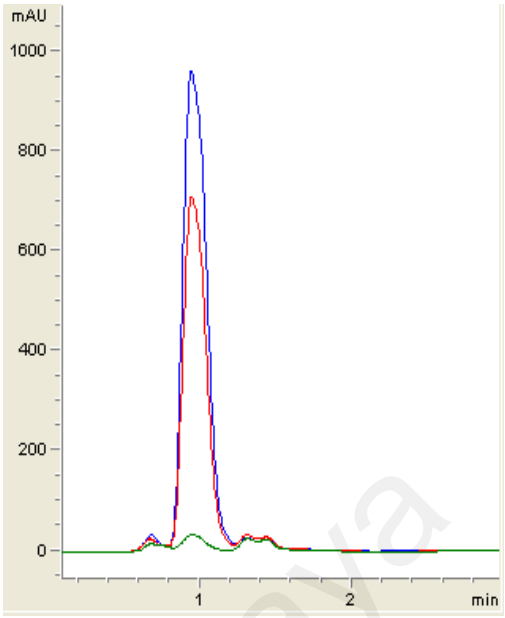
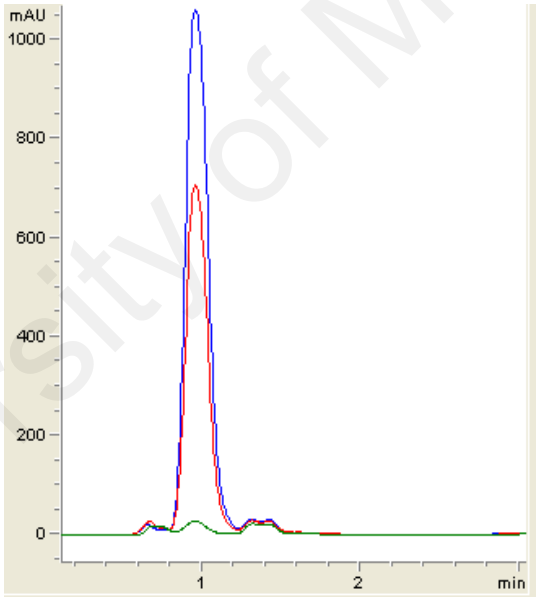


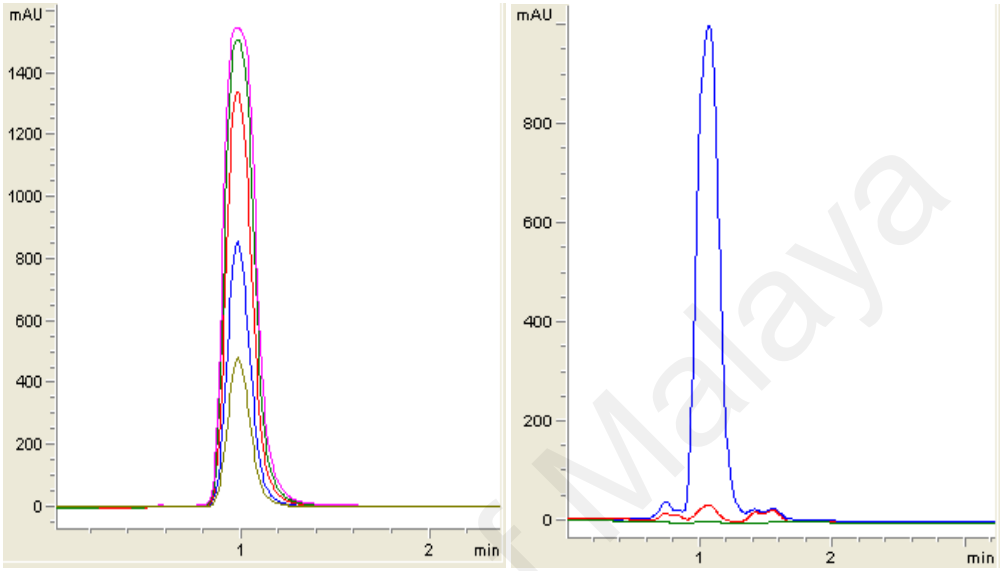
(l)



(m)

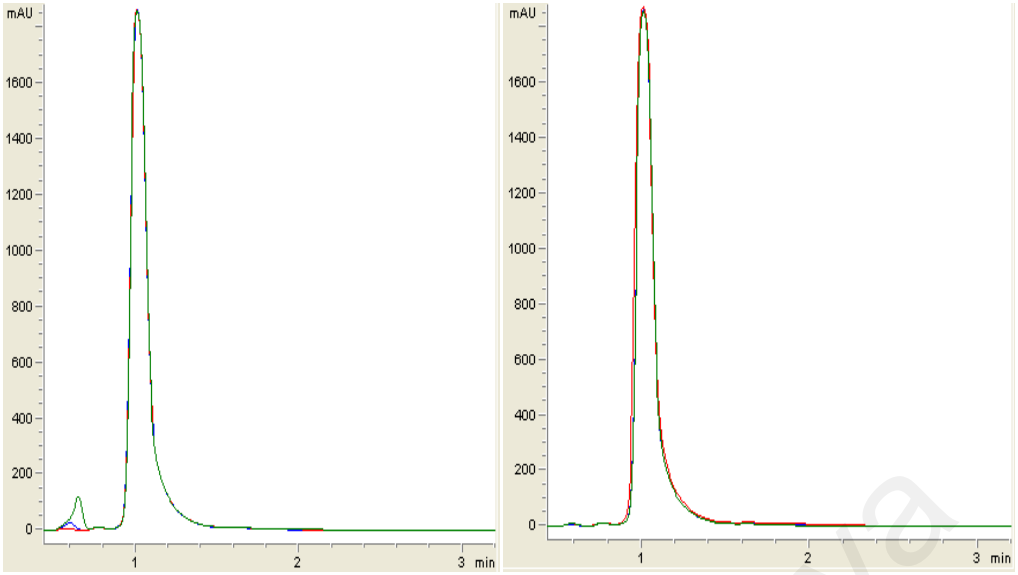
Figure 4.13, continued

RRLC was used to monitor the degradation of AHLs by the isolates over the period of 48 hr (Figure 4.14) (a – m). Synthetic AHL was used as standards for comparison purpose. It appears that degradation of 3-oxo-C6-HSL was observed by all of the isolates

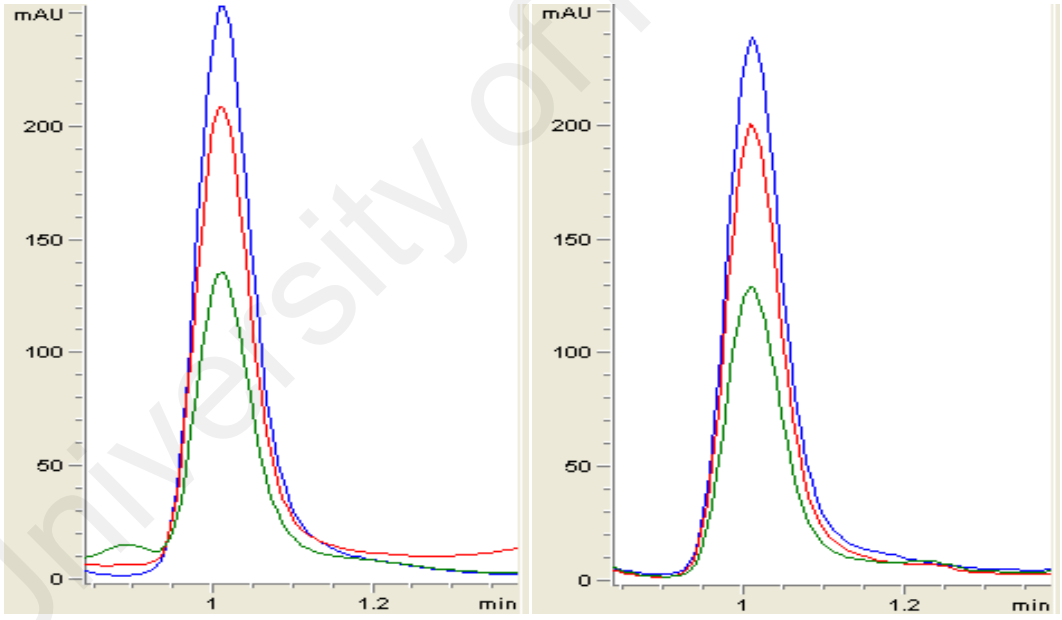


(a) **(b)**

Figure 4.14: RRLC analysis of 3-oxo-C6-HSL degradation. Residual 3-oxo-C6-HSL (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), both after degradation 24 h (red) and 48 h (green) was monitored at 210 nm. Degradation of 3-oxo-C6-AHLs is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram (**a**) Synthetic 3-oxo-C6-AHLs was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* as positive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)** NP9 respectively.

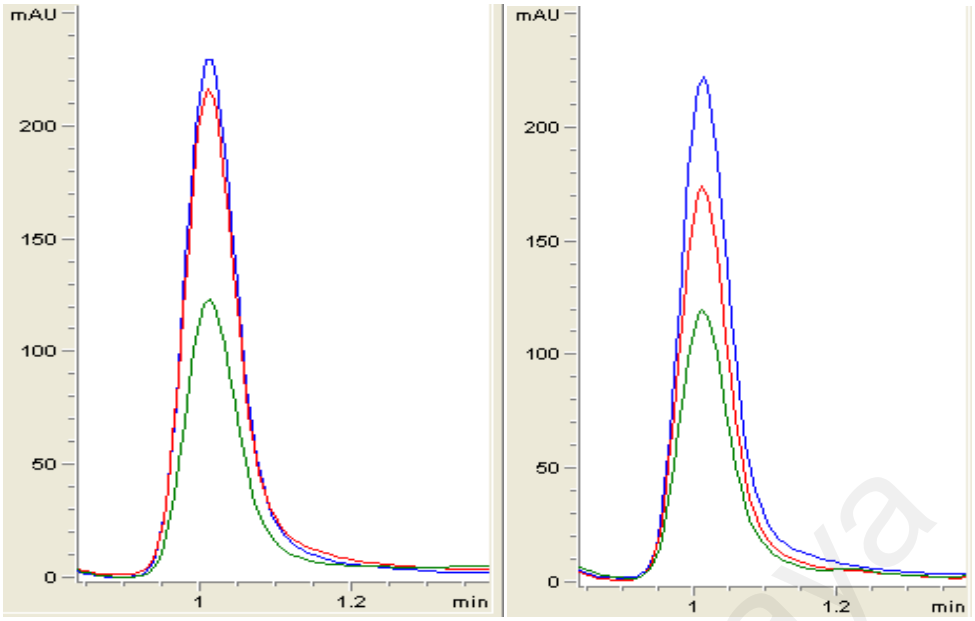


(c) **(d)**

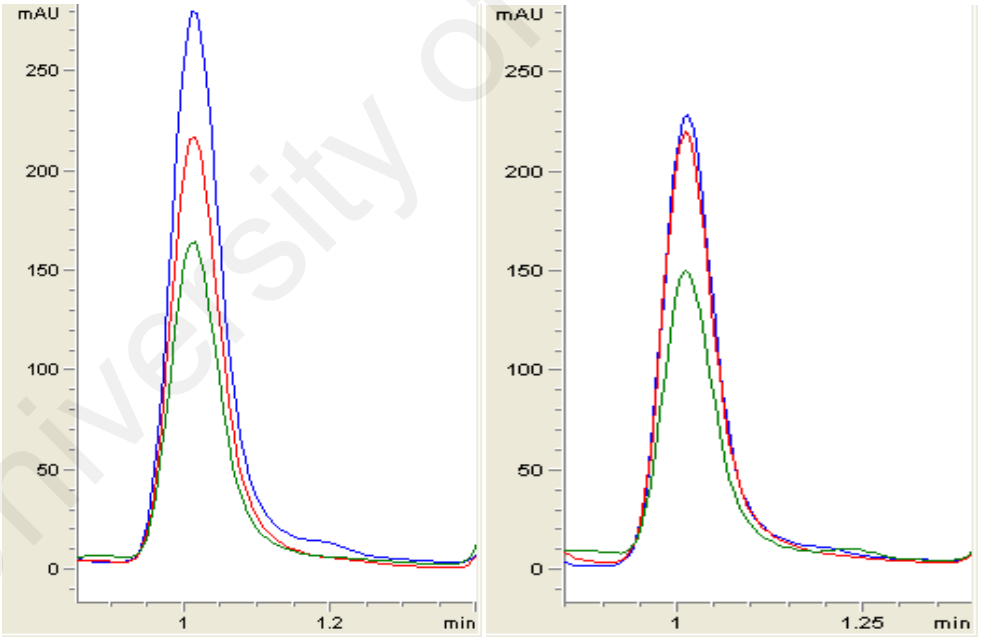


(e) **(f)**

Figure 4.14, continued

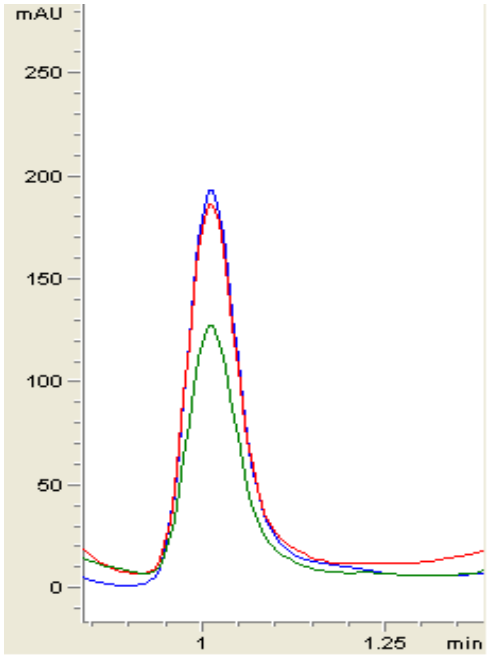


(g) **(h)**

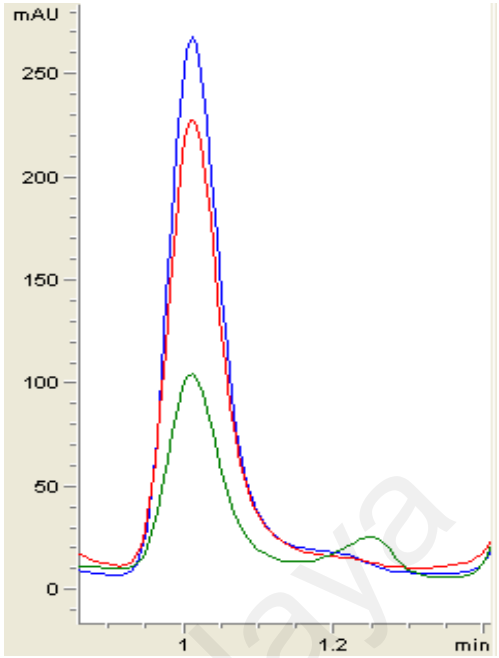


(i) **(j)**

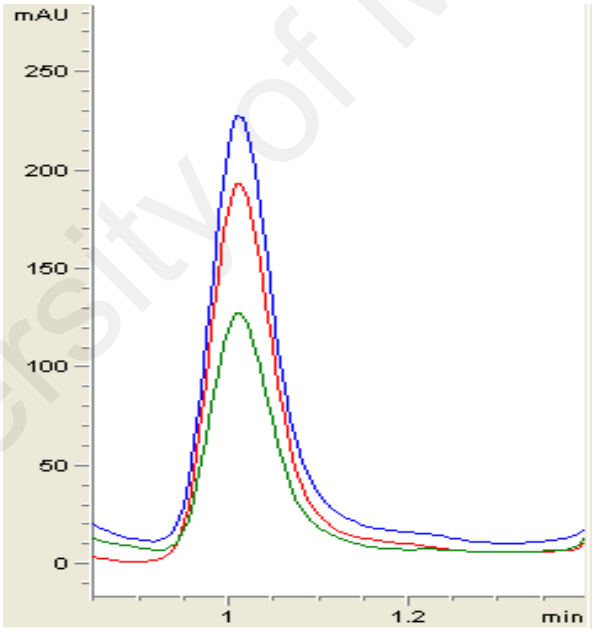
Figure 4.14, continued



(k)



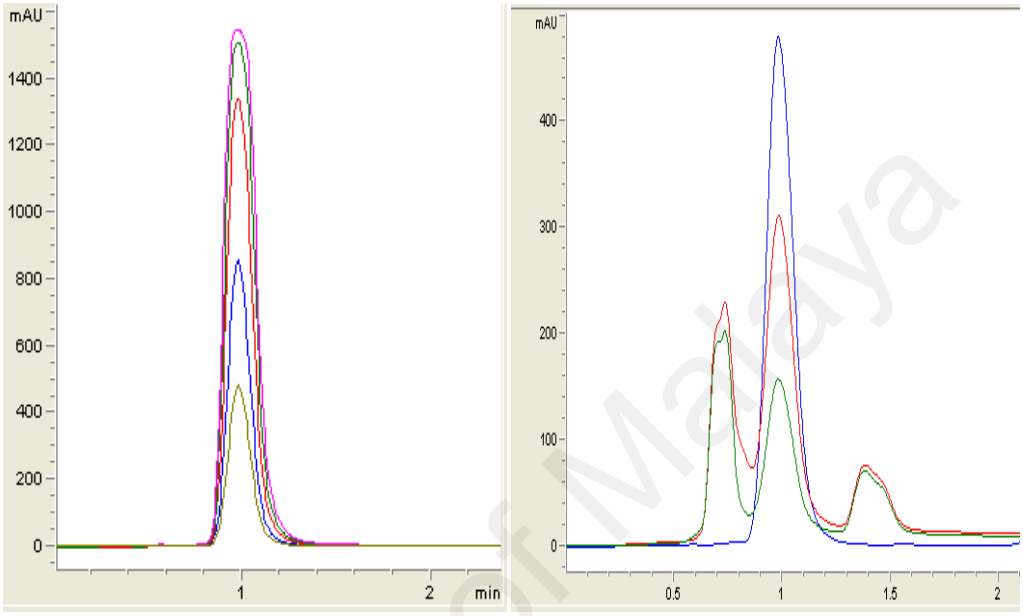
(l)



(m)

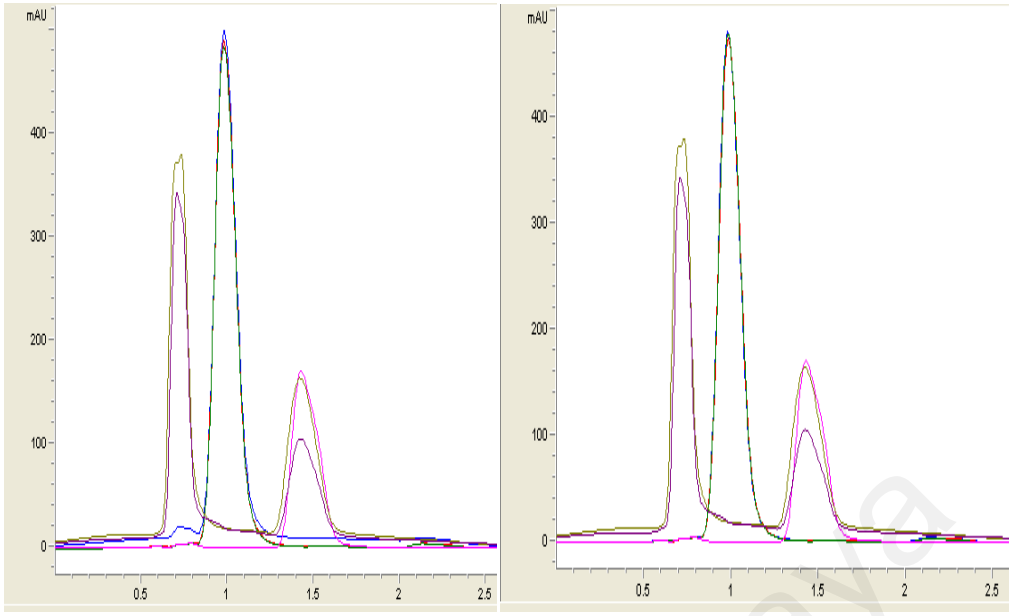
Figure 4.14, continued

RRLC was used to monitor the degradation of AHLs by the isolates over the period of 48 hr (Figure 4.15) (a – m). Synthetic AHL was used as standards for comparison purpose. It appears that degradation of 3-hydroxy-C6-HSL was observed by all of the isolates.

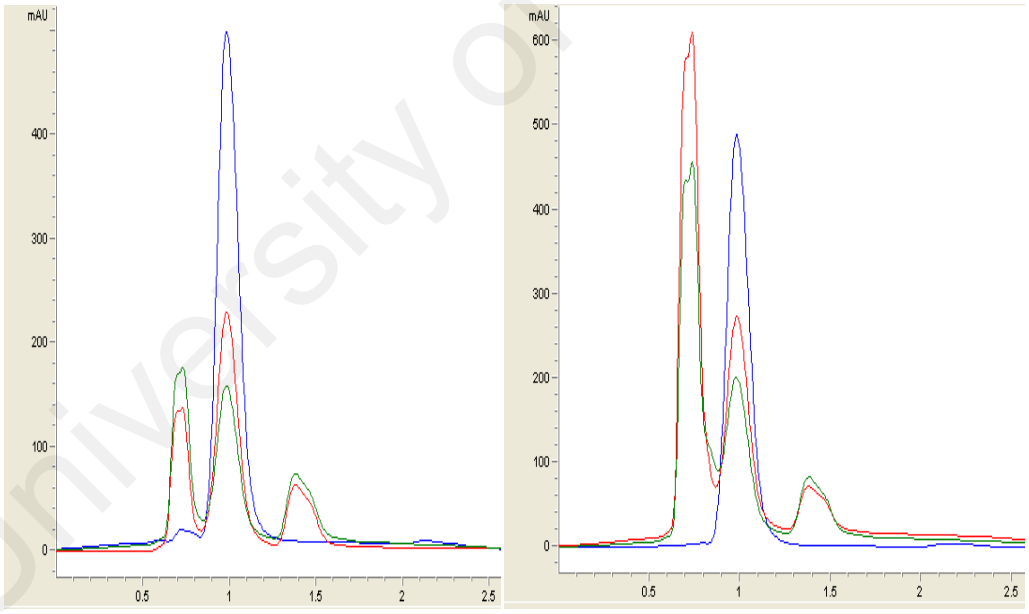


(a) **(b)**

Figure 4.15: RRLC analysis of 3-hydroxy-C6-HSL degradation. Residual 3-hydroxy-C6-HSL (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), both after degradation 24 h (red) and 48 h (green) was monitored at 210 nm. Degradation of 3- hydroxy-C6-AHLs is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram (**a**) Synthetic 3- hydroxy-C6-AHLs was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* as positive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)** NP9, respectively.

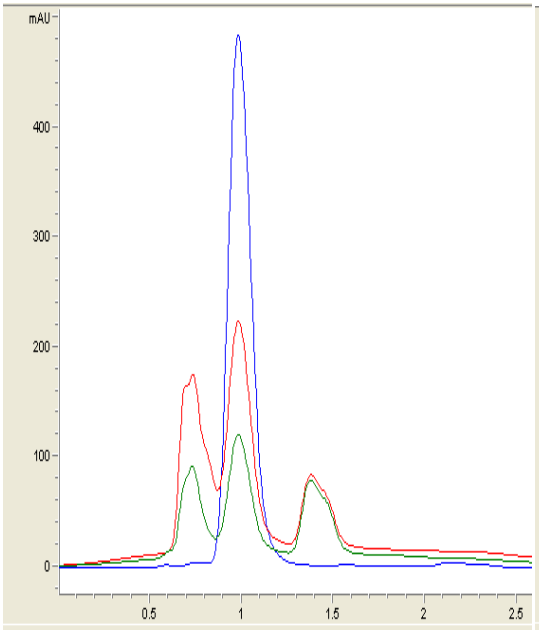


(c) **(d)**

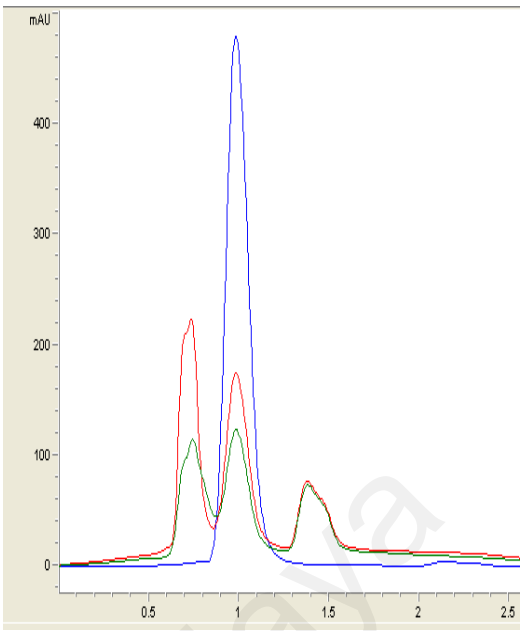


(e) **(f)**

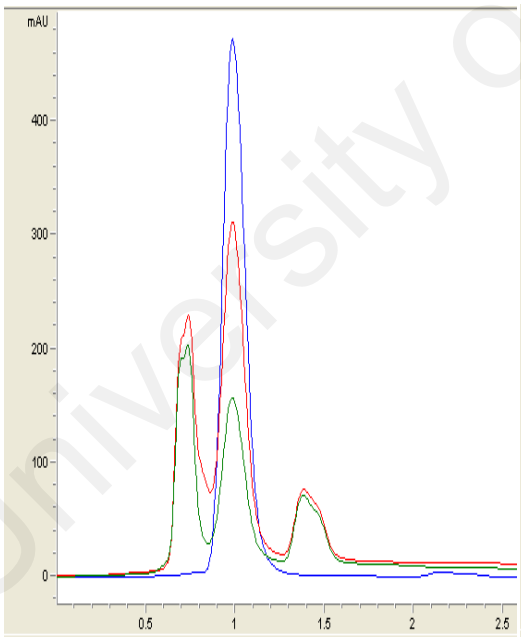
Figure 4.15, continued



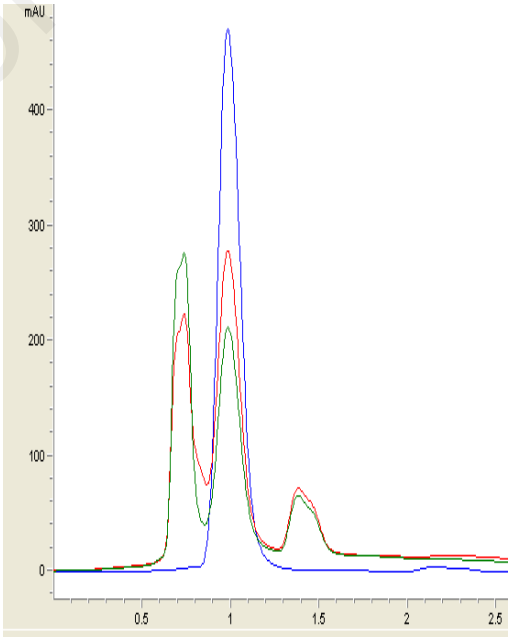
(g)



(h)

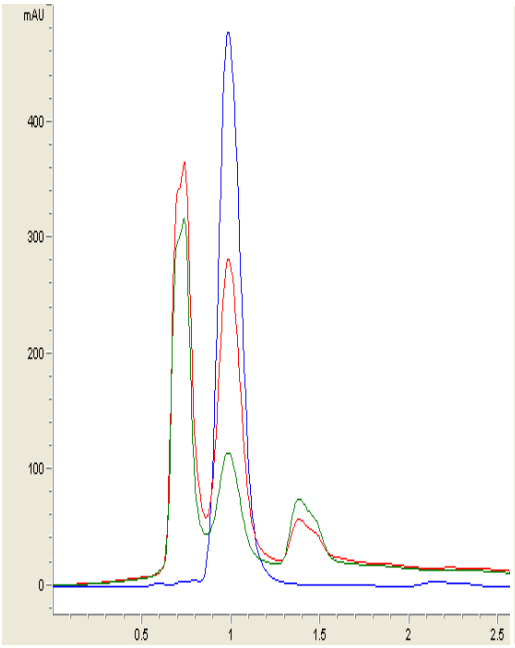


(i)



(j)

Figure 4.15, continued



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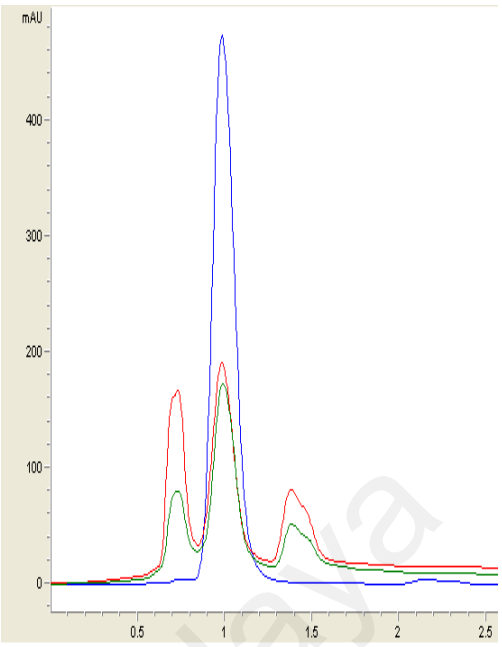

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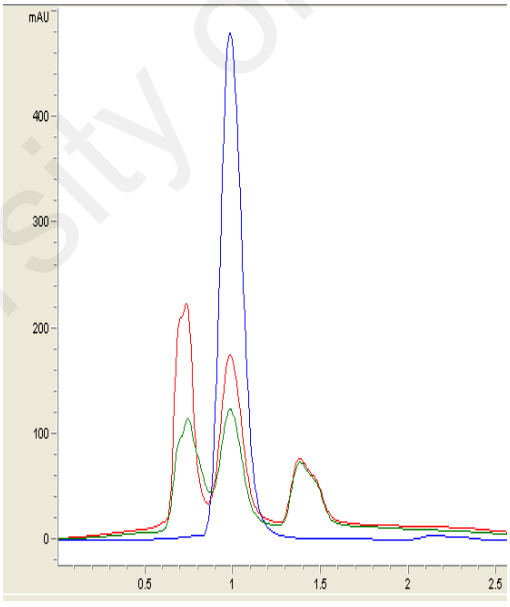


(m)

Figure 4.15, continued

4.5.4 Identification of AHL Lactonase Activity

Table 4.4: Summary of lactonase activity of nine isolates.

AHLs / Isolate	C6-HSL	3-oxo-*C6*-HSL	3-hydroxy-C6-HSL
B. cereus (positive control)	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation
E. coli Top10 (negative control)	No lactone ring form after degradation	No lactone ring form after degradation	No lactone ring form after degradation
PBS buffer (negative control)	No lactone ring form after degradation	No lactone ring form after degradation	No lactone ring form after degradation
Isolate NP1	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation
Isolate NP2	Slow formation of lactone ring after degradation	Lactone ring form after degradation	Slow formation of lactone ring after degradation
Isolate NP3	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation
Isolate NP4	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation

Table 4.4, continued

AHLs / Isolate	C6-HSL	3-oxo-C6-HSL	3-hydroxy-C6-HSL
Isolate NP5	Slow formation of lactone ring after degradation	Lactone ring form after degradation	Lactone ring form after degradation
Isolate NP6	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation
Isolate NP7	Lactone ring form after degradation	Lactone ring form after degradation	Slow formation of lactone ring after degradation
Isolate NP8	Lactone ring form after degradation	Slow formation of lactone ring after degradation	Lactone ring form after degradation
Isolate NP9	Lactone ring form after degradation	Lactone ring form after degradation	Lactone ring form after degradation

In order to identify AHL degradation caused by lactonase enzyme, bioassay was performed and data was shown in Figure 4.16. Positive QQ activity can be seen as the reduction of purple pigments after 24 h of incubation. The purple pigments with the addition of hydrochloric acid (HCl) suggested re-lactonisation of the digested AHL indicated that both the isolates produced lactonase in the presence of 3-C6-HSL (Figure 4.16). Isolates NP1, NP2, NP3, NP4, NP5, NP6, NP7, and NP9 appears to degrade AHL via lactonase activity. When tested with 3-oxo-C6-HSL (Figure 4.17) and 3-hydroxy-C6-HSL (Figure 4.18), similar results were obtained suggesting a broad activity of the lactonase in these isolates.

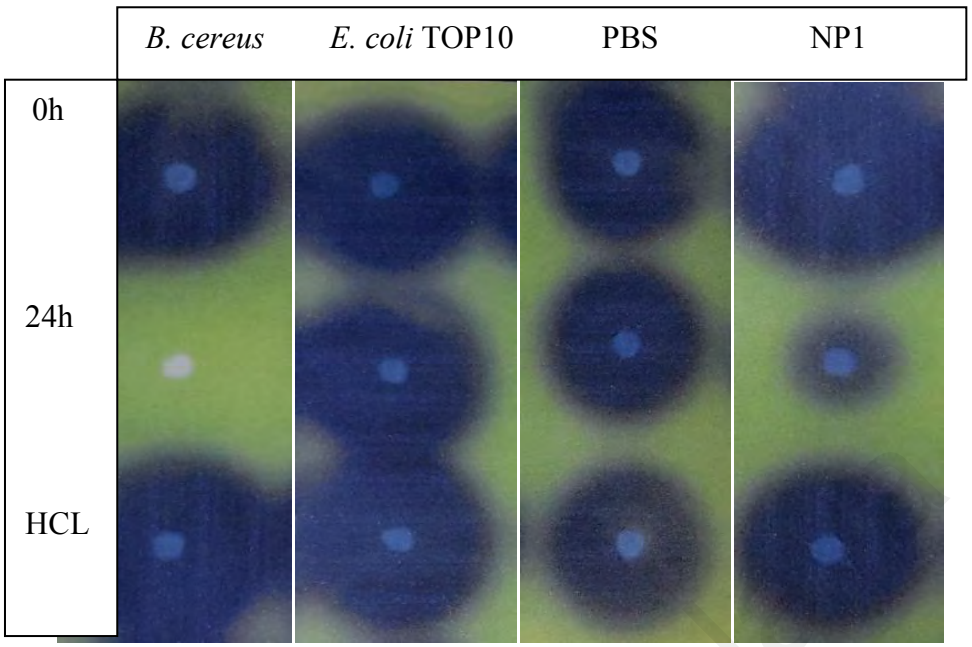
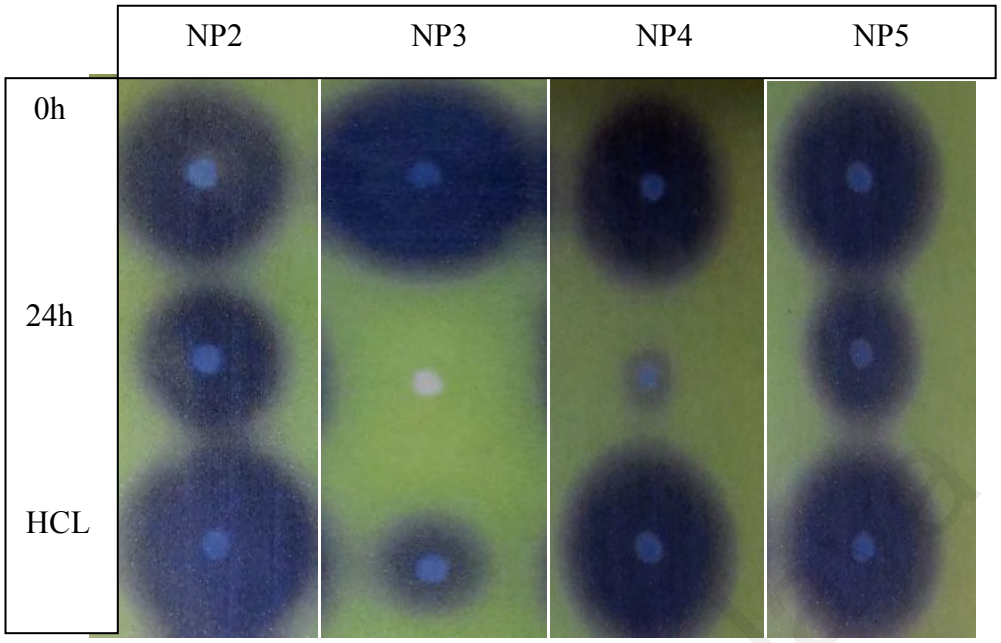


Figure 4.16: Detection of lactonase activity using CV026 overlay. Bacteria suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-C6-HSL for 0 h and 24 h as indicated on the left of figure. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls for this test. The purple pigments with the addition of hydrochloric acid (HCl) suggested re-lactonisation of the digested AHL indicated that both the isolates produced lactonase in the presence of 3-oxo-C6-HSL.



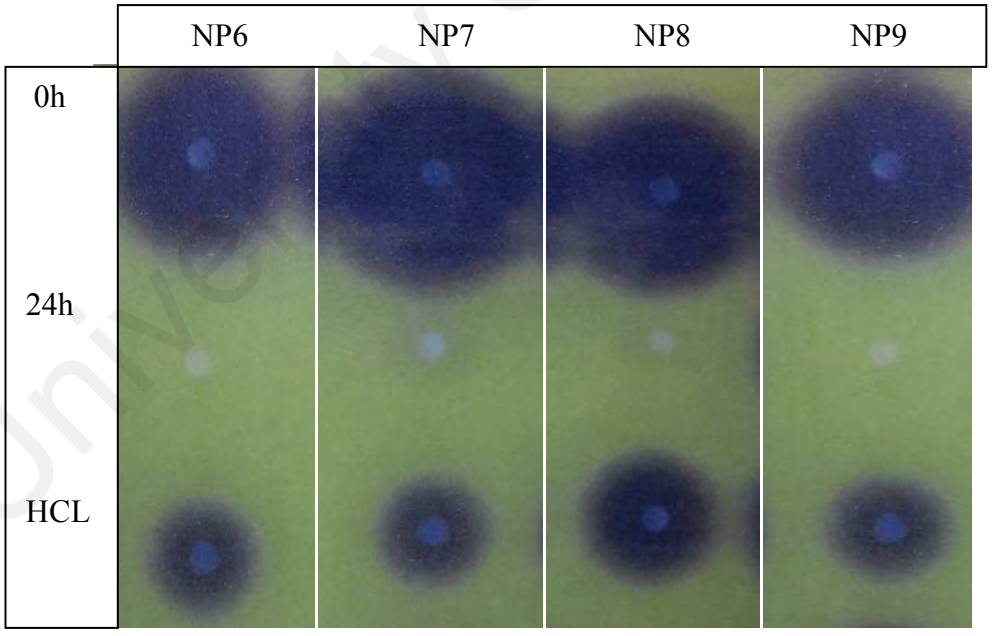


Figure 4.16, continued

Lactonase activity of isolates tested with 3-oxo-C6-HSL using CV026 overlay.

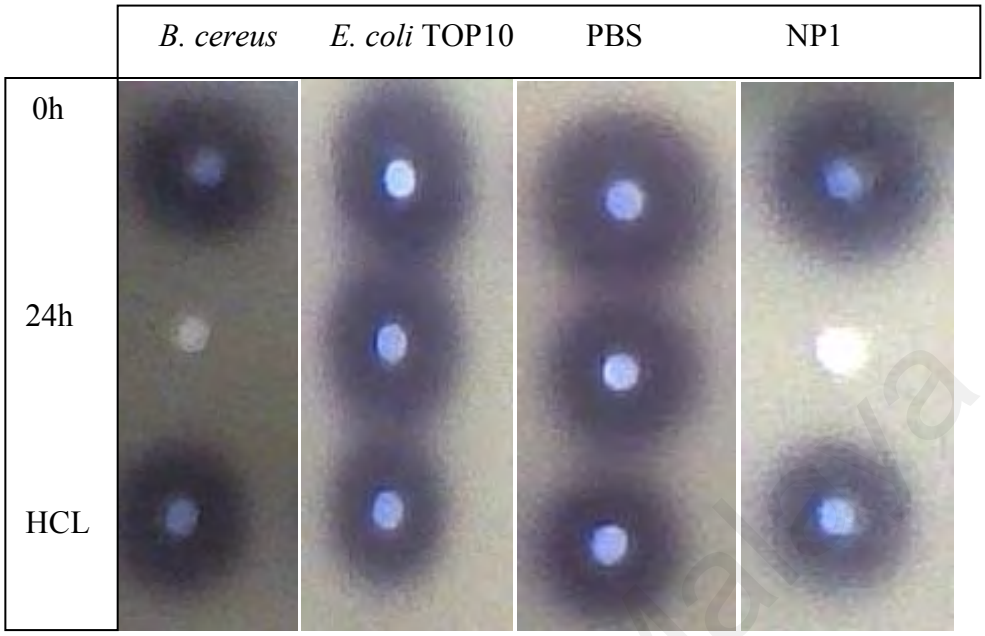
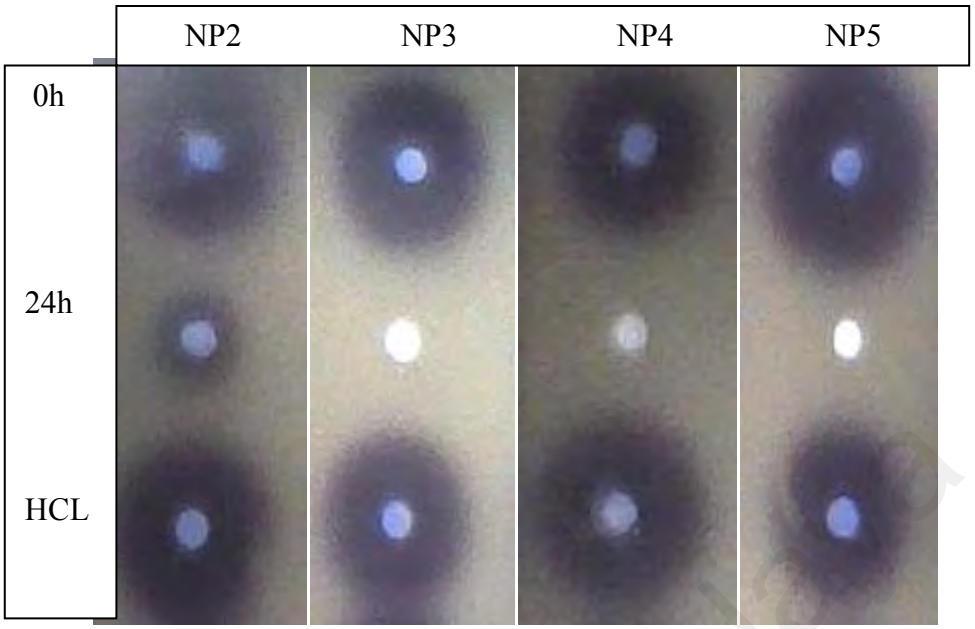


Figure 4.17: Detection of lactonase activity using CV026 overlay. Bacteria suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-oxo-C6-HSL for 0 h and 24 h as indicated on the left of figure. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h of incubation. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls. The purple pigments with the addition of hydrochloric acid (HCl) suggested re-lactonisation of the digested AHL indicated that both the isolates produced lactonase in the presence of 3-oxo-C6-HSL.



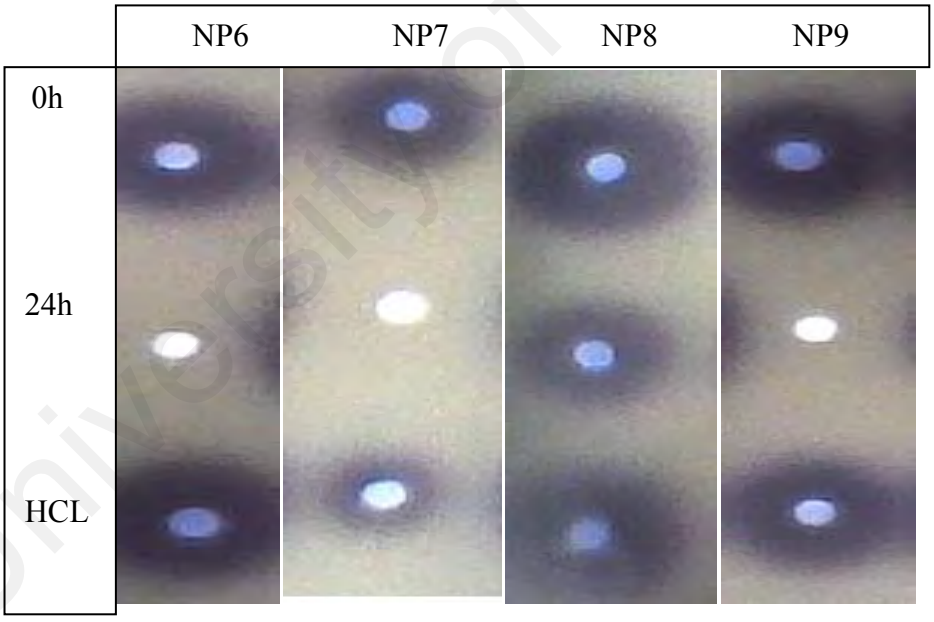


Figure 4.17, continued

Lactonase activity of isolates tested with 3-hydroxy-C6-HSL using CV026 overlay.

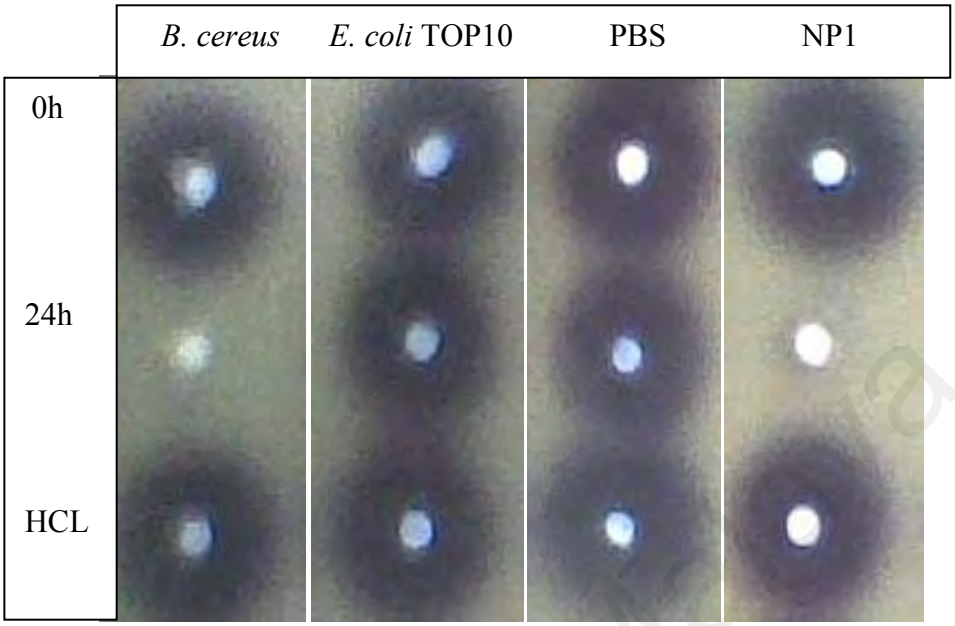
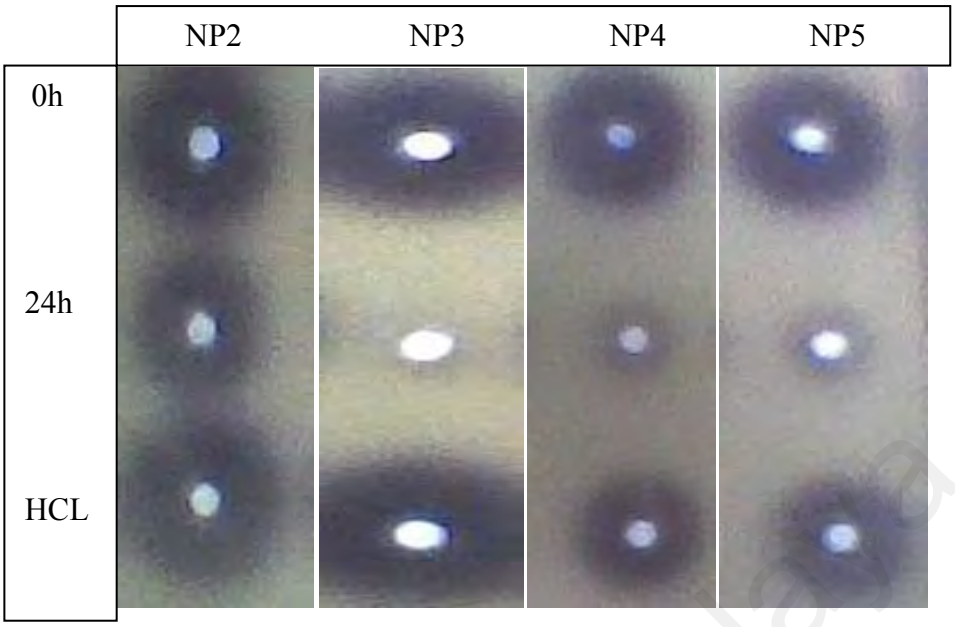


Figure 4.18: Detection of lactonase activity using CV026 overlay. Bacteria suspensions (NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, and NP9) were incubated with 3-hydroxy-C6-HSL for 0 h and 24 h as indicated on the left of figure. Positive QQ activity can be seen as the abolishment of purple pigments after 24 h of incubation. *B. cereus* represents the positive control while *E. coli* TOP10 and PBS buffer acted as negative controls. The purple pigments with the addition of hydrochloric acid (HCl) suggested re-lactonisation of the digested AHL indicated that both the isolates produced lactonase in the presence of 3-hydroxy-C6-HSL.



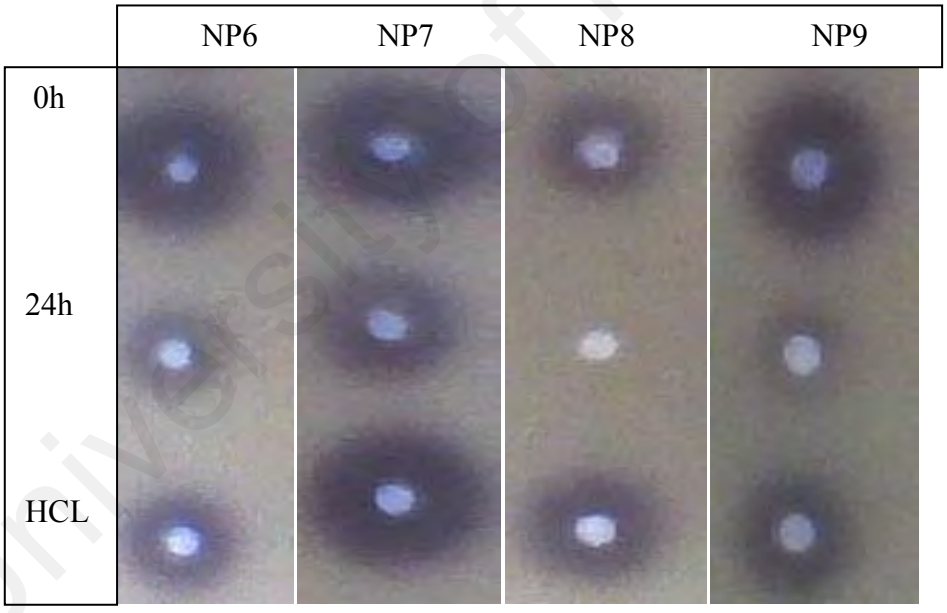
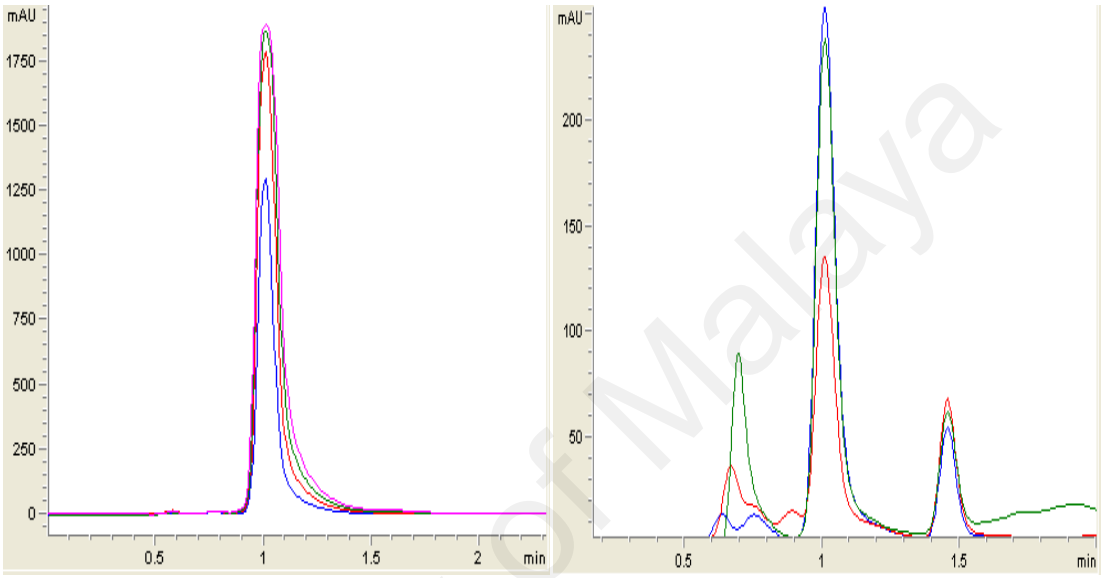


Figure 4.18, continued

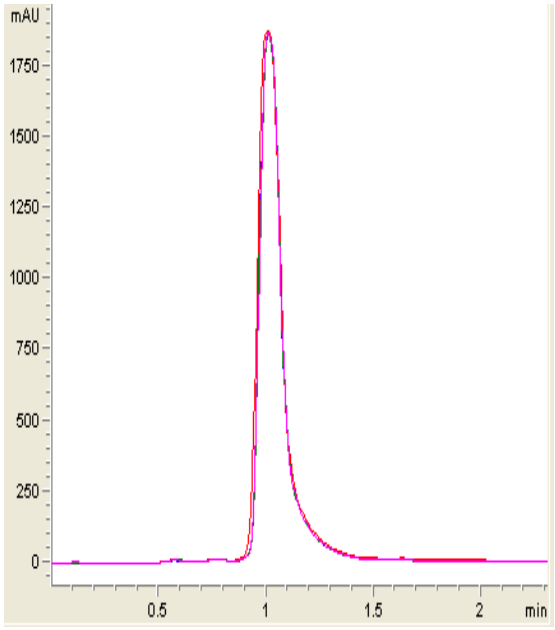
4.5.5 Verification of QQ Activities via lactonase through RRLC Analysis

RRLC was used to verify the lactonase activity by the isolates over the period of 48 hr and after HCI added (Figure 4.19). Synthetic AHL (C6-HSL) was used as standards to see the elution time for comparison purpose.

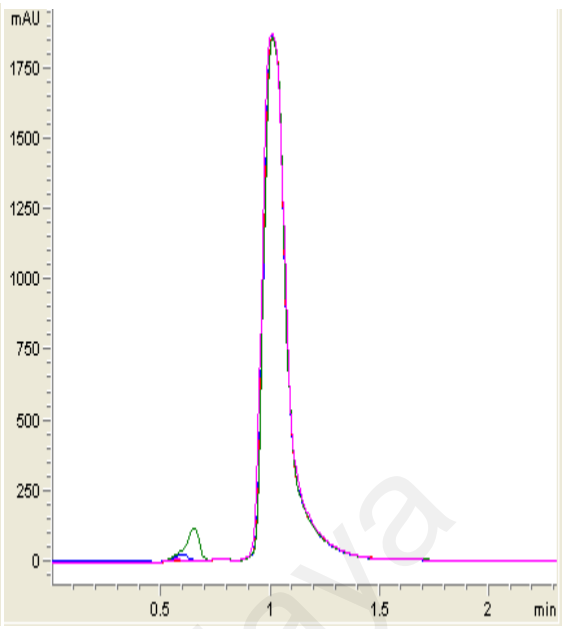


(a) **(b)**

Figure 4.19: Verification of QQ Activities via lactonase through RRLC analysis of C6-HSL degradation. Residual C6-HSL (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), after degradation 24 h (red) and after HCl added (green) was monitored at 210 nm. Degradation of C6-HSL is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram (**a**) Synthetic C6-HSL was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* as positive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)** NP9, respectively.



(c)



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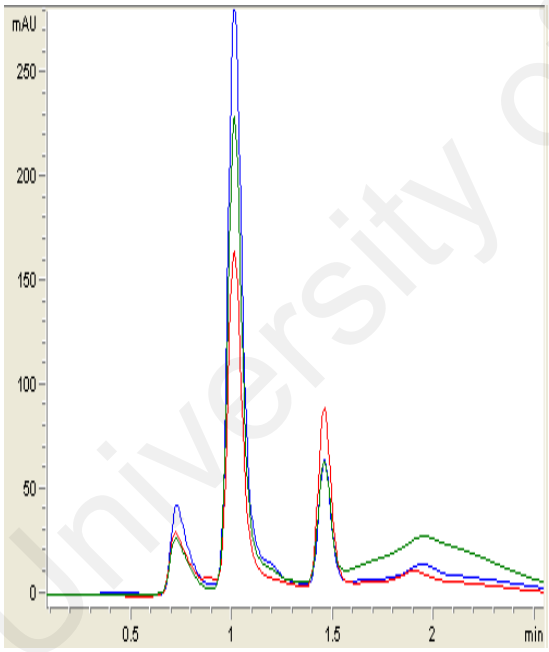

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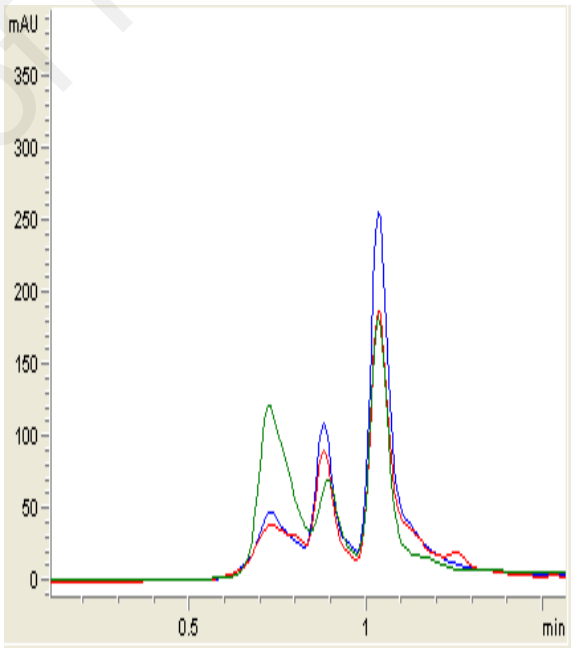
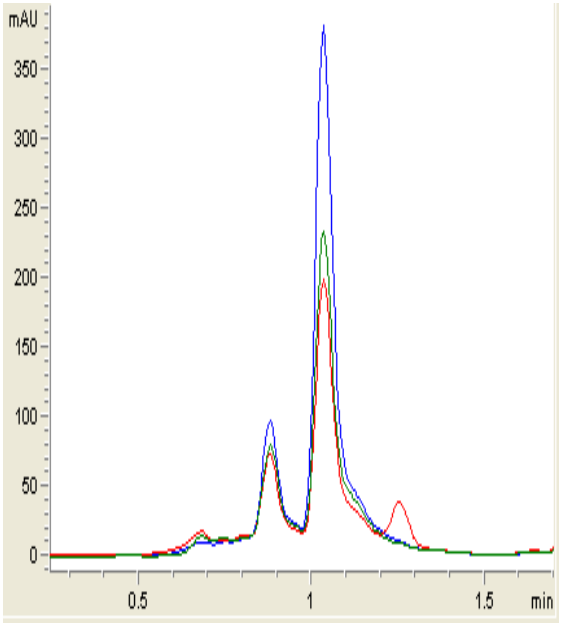
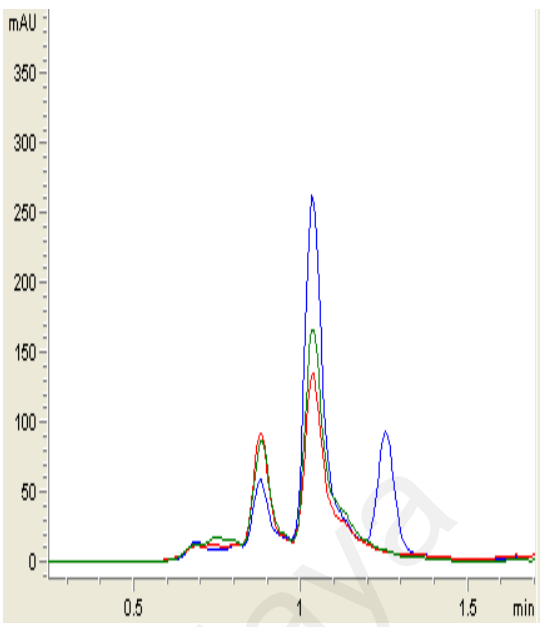


(f)

Figure 4.19, continued



(g)



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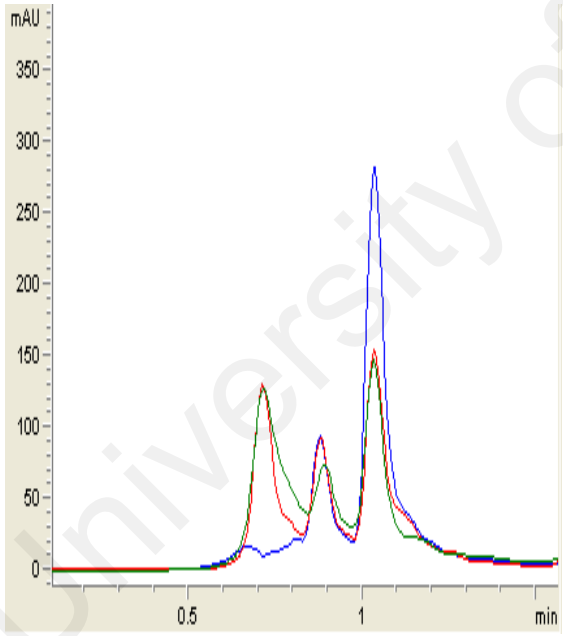

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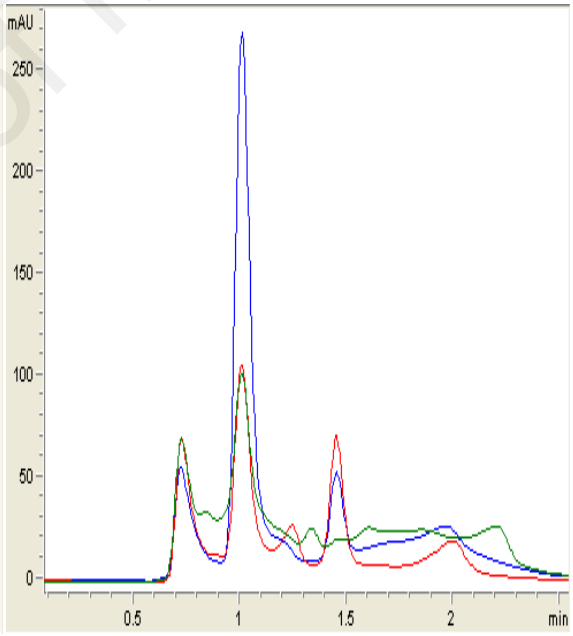
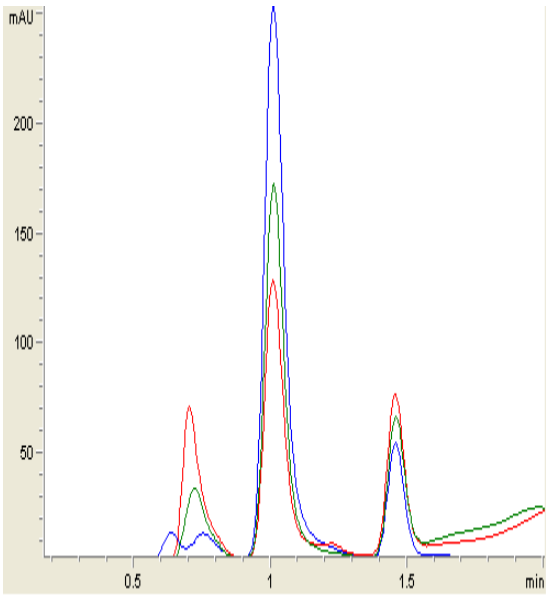


(j)

Figure 4.19, continued



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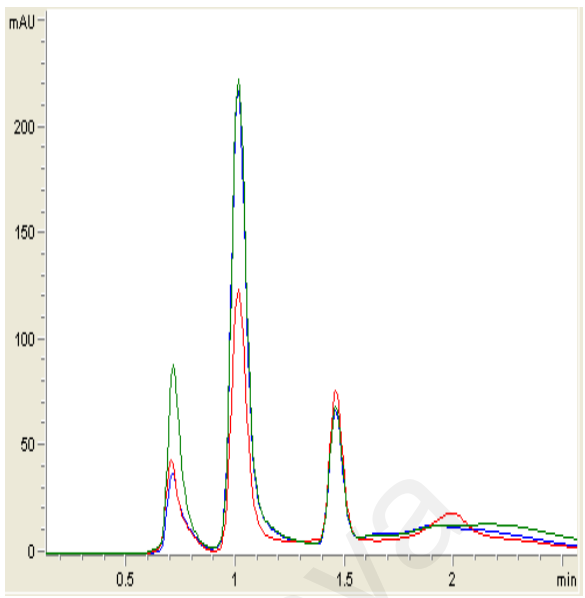
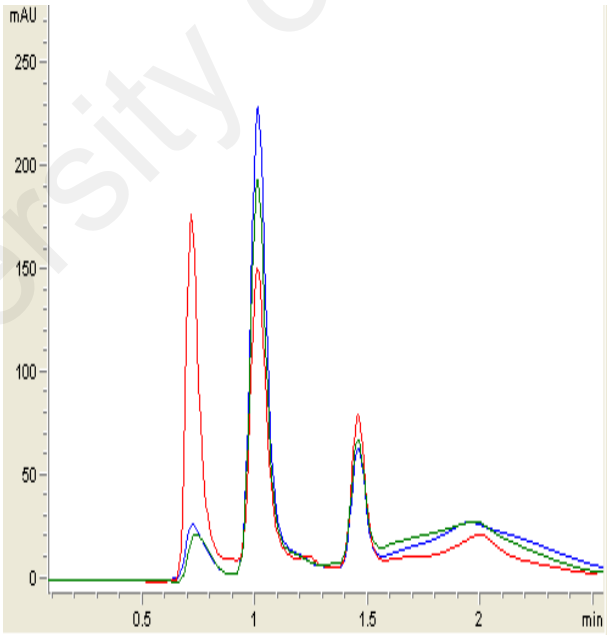


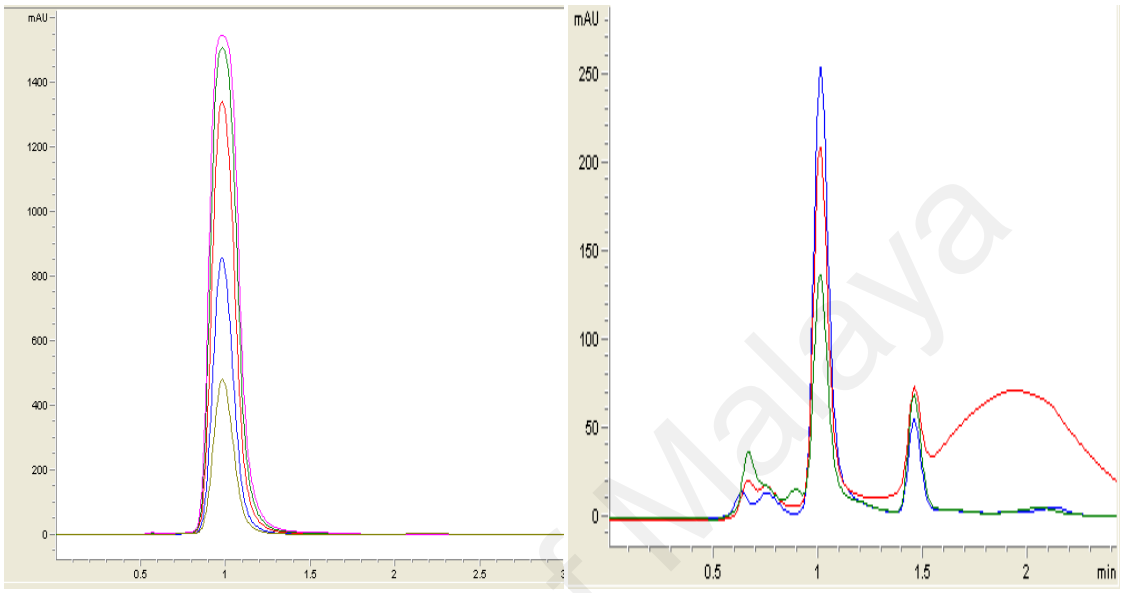
(l)



(m)

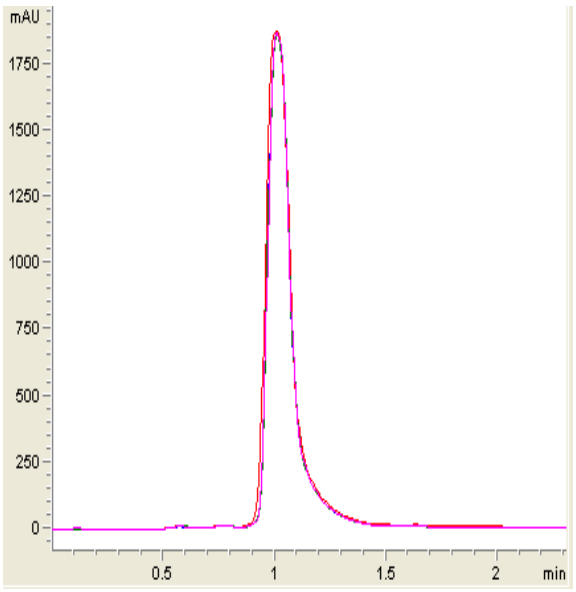
Figure 4.19, continued

RRLC was used to verify the lactonase activity by the isolates over the period of 48 hr and after HCI added (Figure 4.20). Synthetic AHL (3-oxo-C6-HSL) was used as standards to see the elution time for comparison purpose.



(a) **(b)**

Figure 4.20: Verification of QQ Activities via lactonase through RRLC analysis of 3-oxo-C6-HSL degradation. Residual 3-oxo-C6-HSL (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), after degradation 24 h (green) and after HCl added (red) was monitored at 210 nm. Degradation of 3-oxo-C6-HSL is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram (**a**) Synthetic 3-oxo-C6-HSL was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* as positive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)** NP9, respectively



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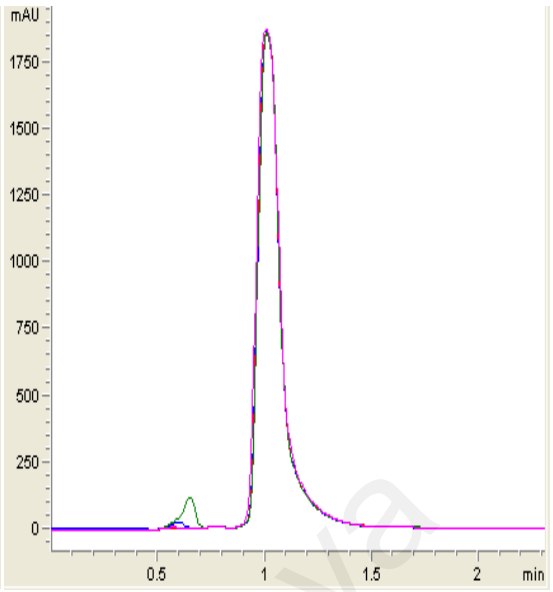

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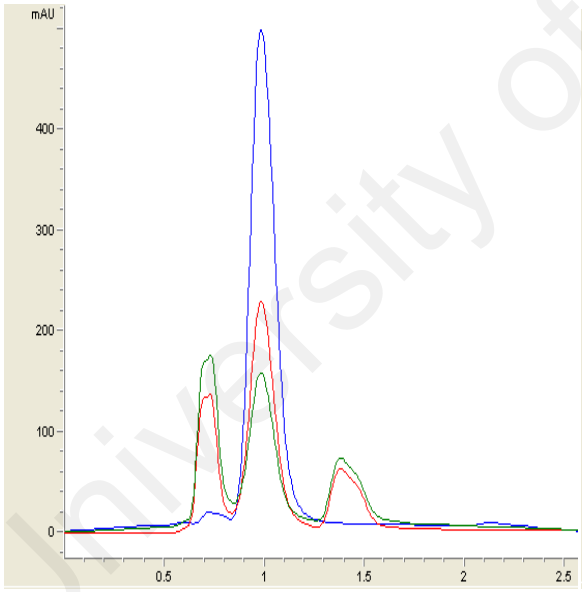

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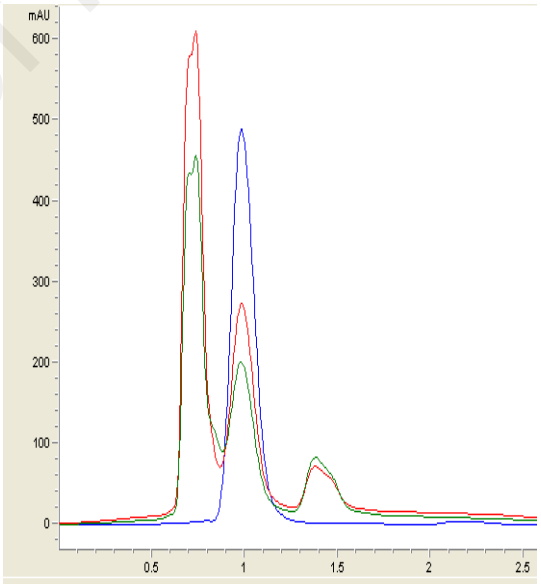
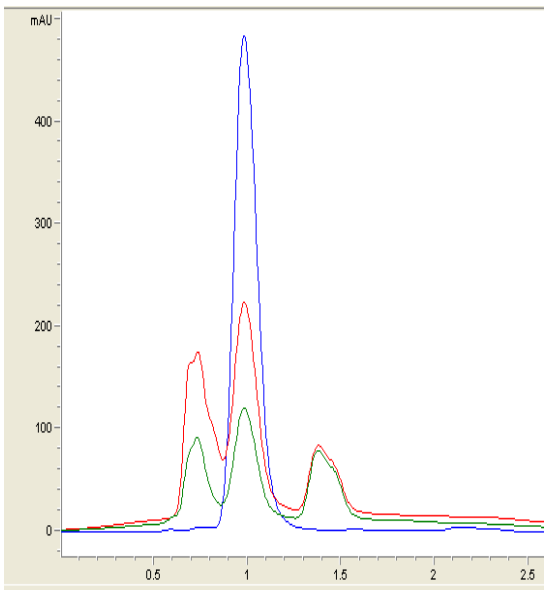
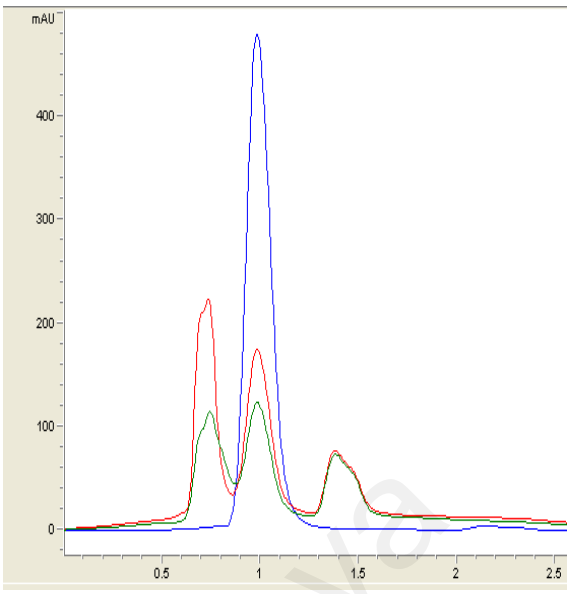


(f)

Figure 4.20, continued



(g)



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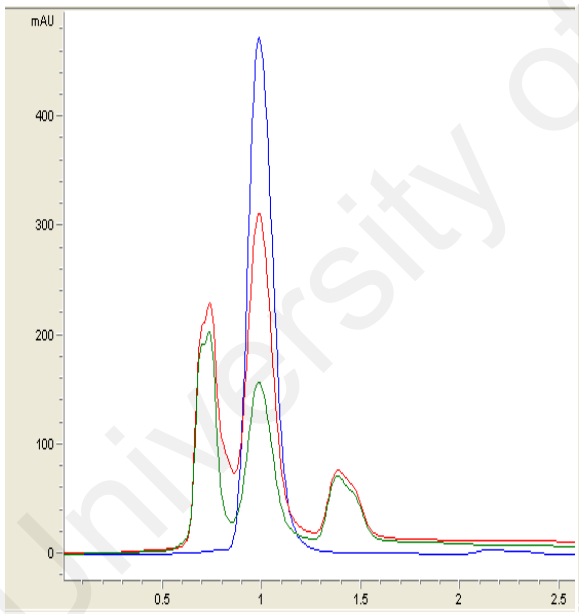
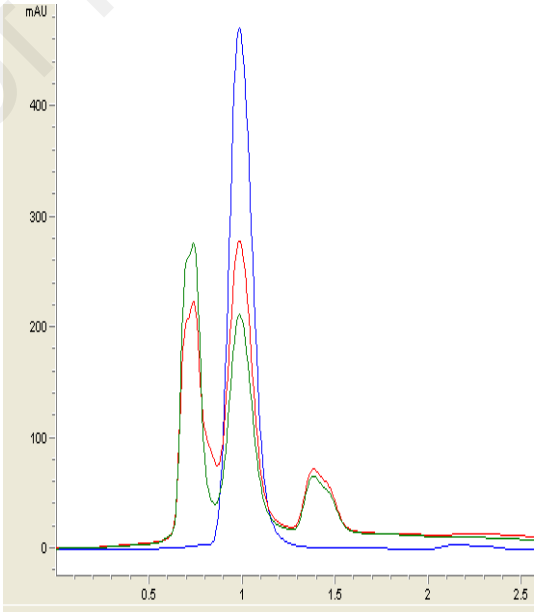
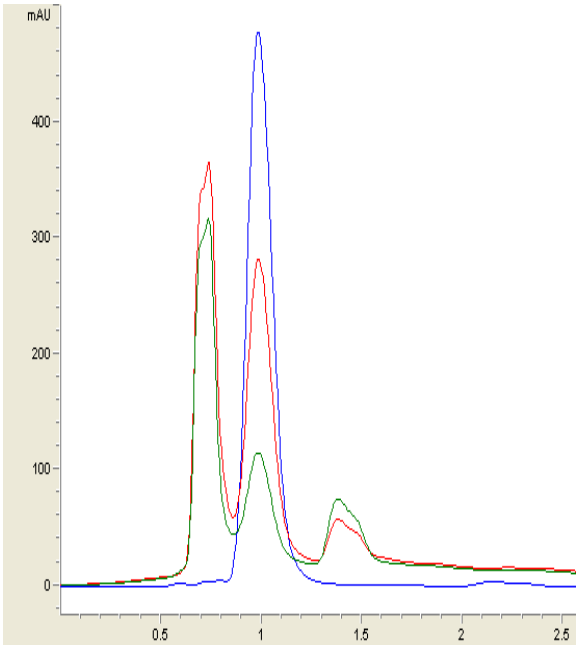


(i)



(j)

Figure 4.20, continued



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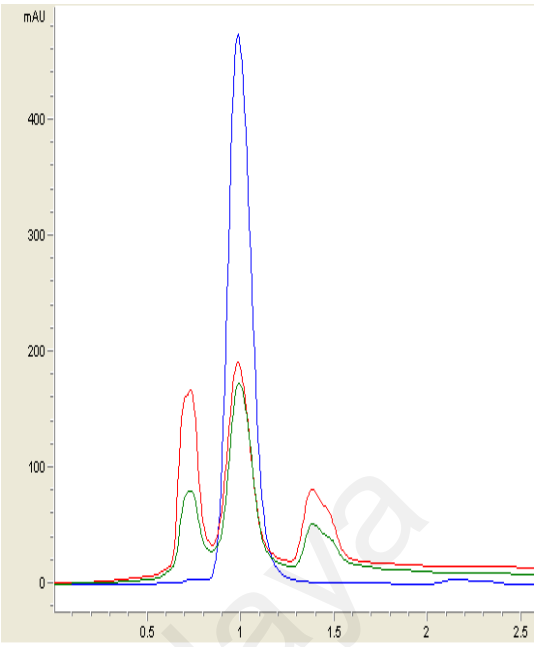
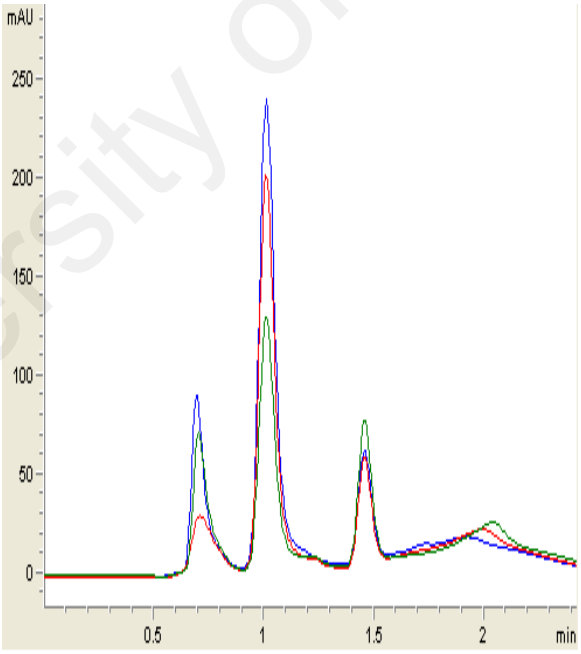


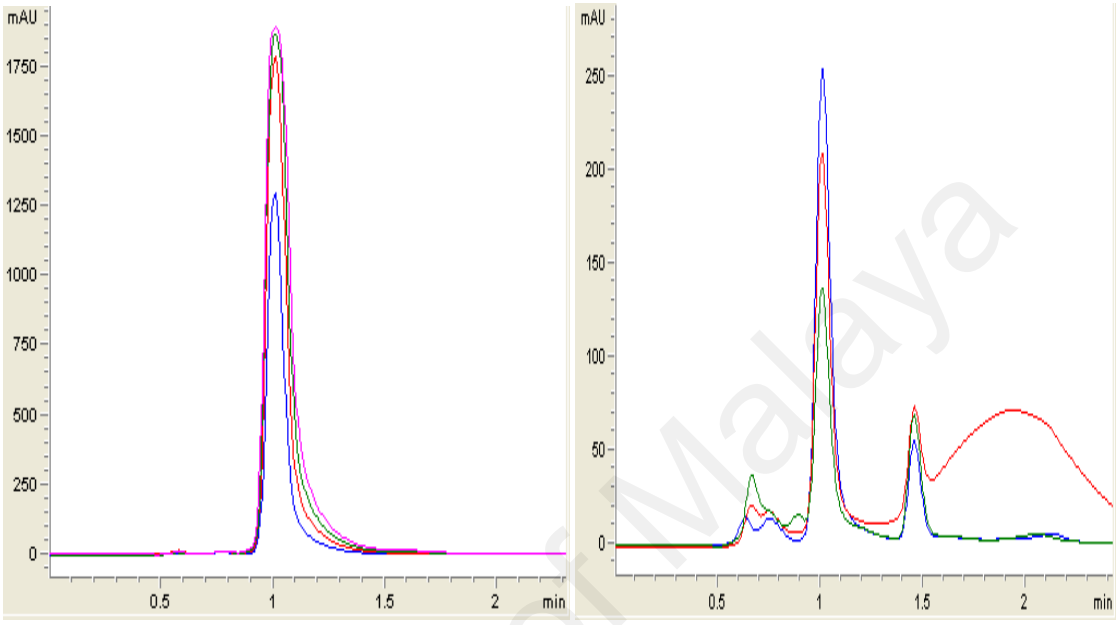
(l)



(m)

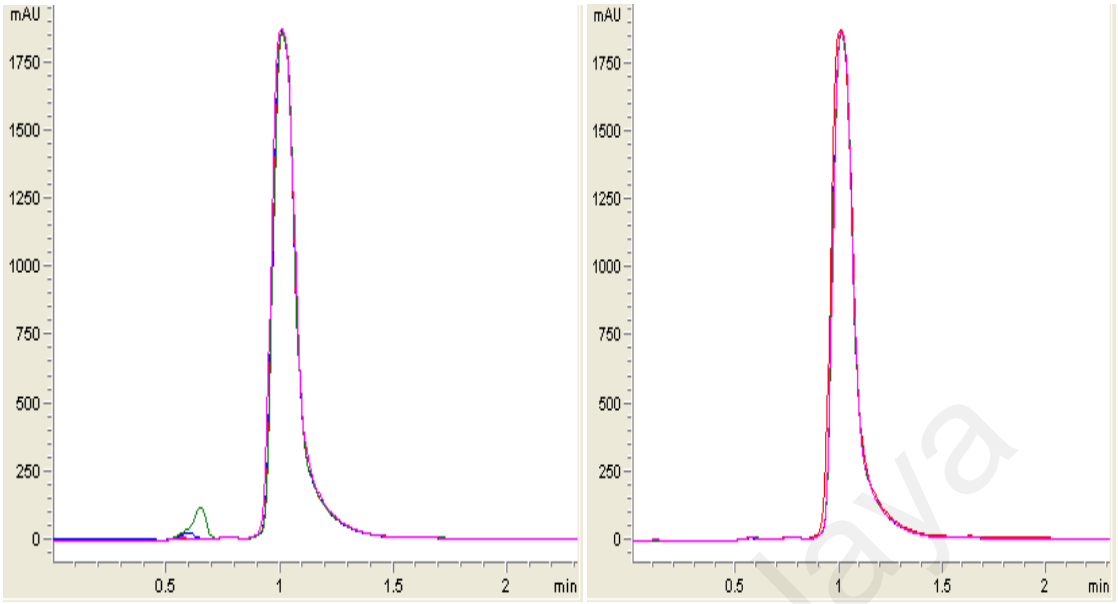
Figure 4.20, continued

RRLC was used to verify the lactonase activity by the isolates over the period of 48 hr and after HCI added (Figure 4.21). Synthetic AHL (3-hydroxy-C6-HSL) was used as standards to see the elution time for comparison purpose.

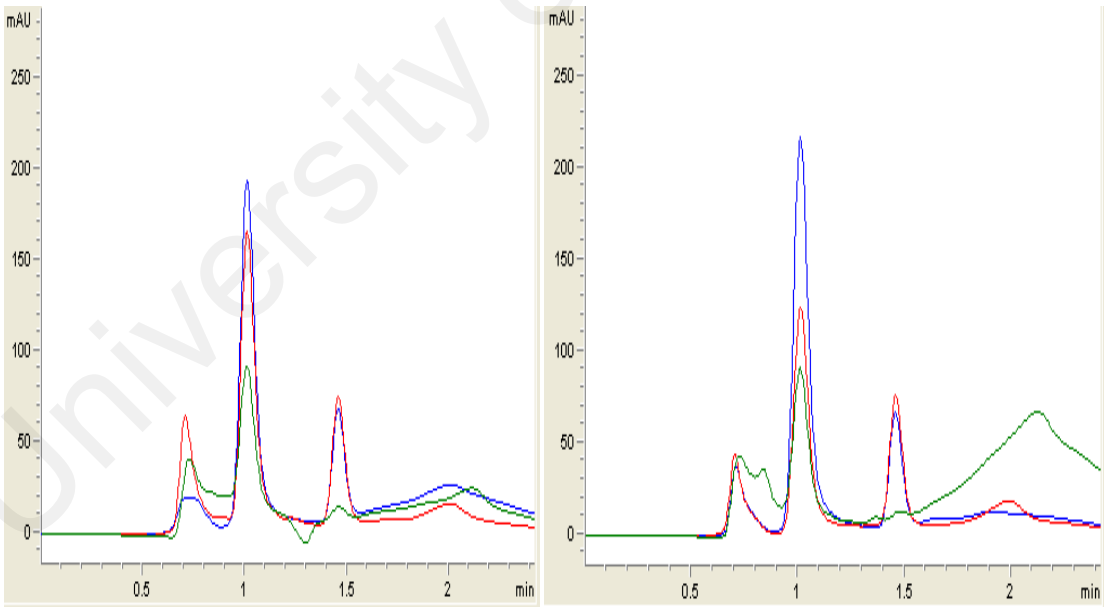


(a) **(b)**

Figure 4.21: Verification of QQ activities via lactonase through RRLC analysis of 3-hydroxy-C6-HSL degradation. Residual 3-hydroxy-C6-AHLs (with elution time of 1.00 min ± 1.2 s), before degradation for 0 h (blue), after degradation 24 h (green) and after HCl added (red) was monitored at 210 nm. Degradation of 3-hydroxy-C6-HSL is depicted by the reduction of milli-absorbance units (mAU) in the chromatogram (**a**) Synthetic 3-hydroxy-C6-HSL was used at 0.2, 0.4, 0.6, 0.8 and 1.0 µg/µL as standards (corresponding to peaks with ascending height); (**b**) *B. cereus* as positive control; (**c**) both *E. coli* TOP10; and (**d**) PBS buffer acted as the negative controls; (**e**) NP1; (**f**) NP2; **(g)** NP3; **(h)** NP4; **(i)** NP5; **(j)** NP6; **(k)** NP7; **(l)** NP8; and **(m)** NP9 respectively

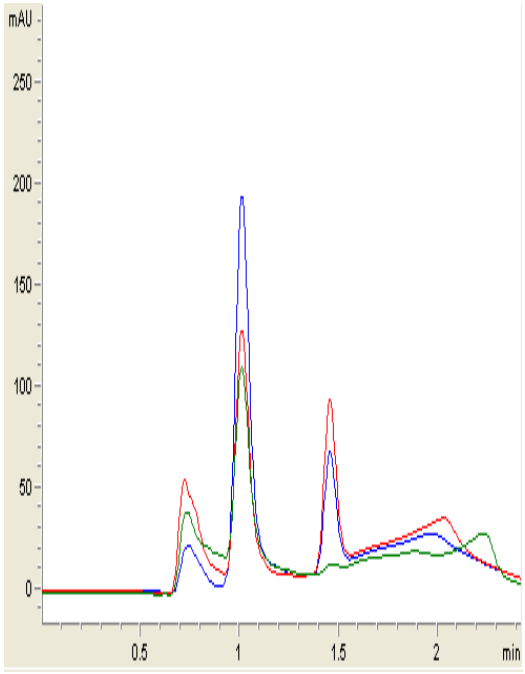


(c) **(d)**



(e) **(f)**

Figure 4.21, continued



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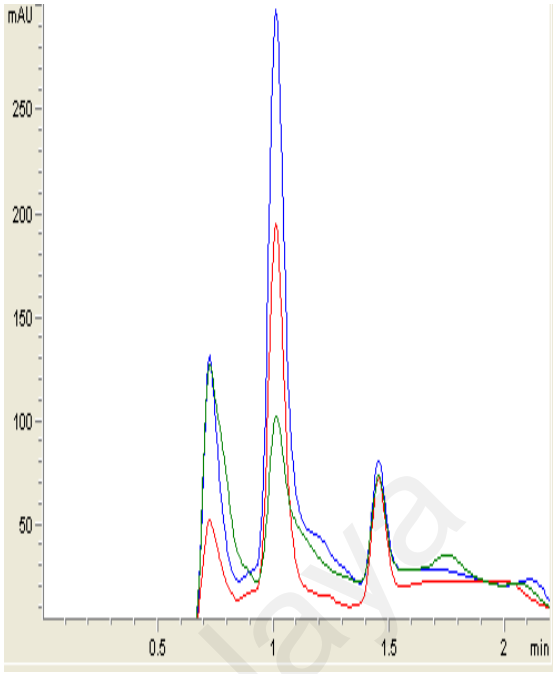

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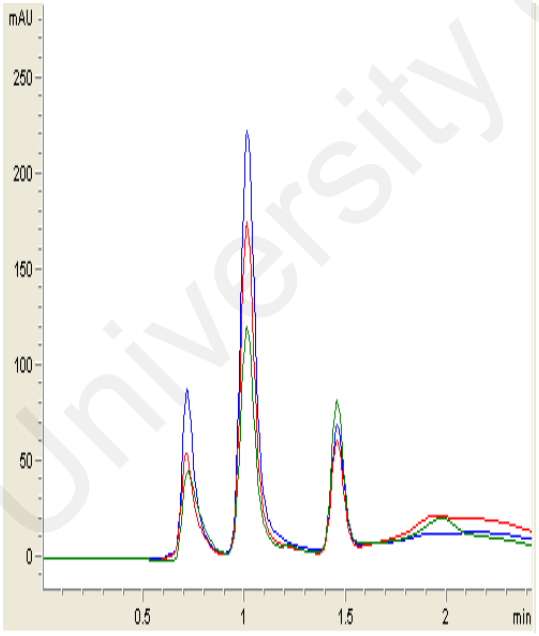

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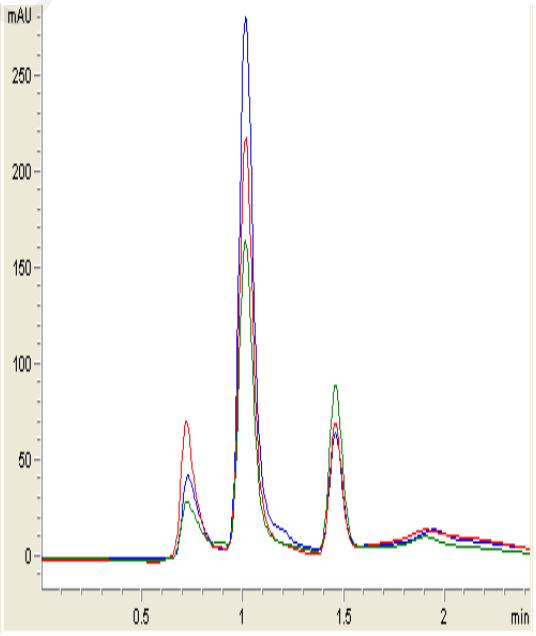
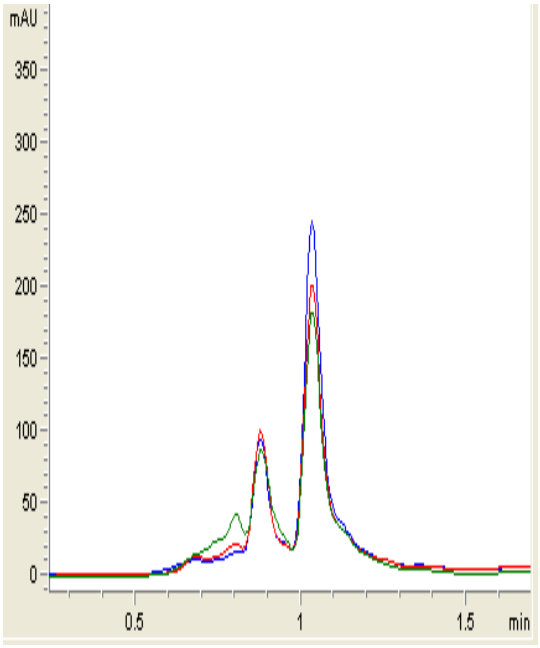


(j)

Figure 4.21, continued



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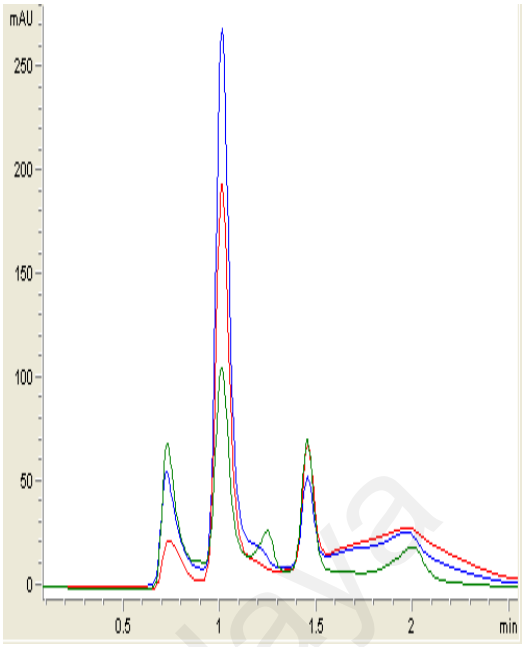

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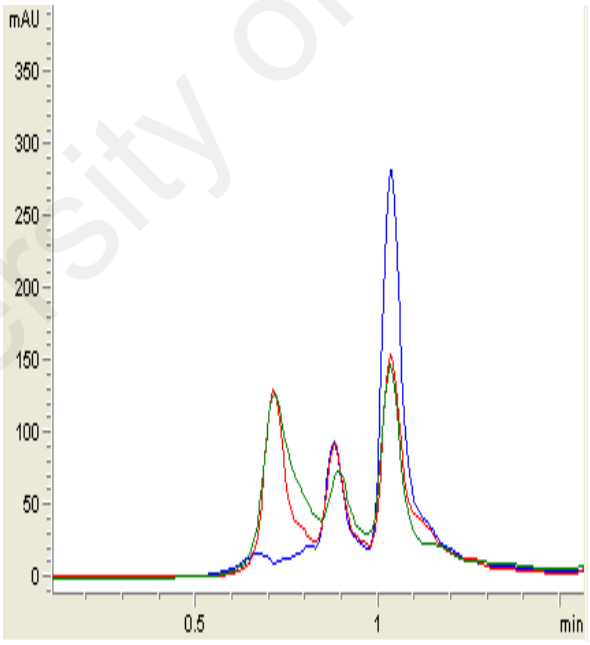

(m)

Figure 4.21, continued

CHAPTER 5.0

DISCUSSION

5.1 Isolation and Identification of Bacterial Strains

Mangrove swamps from Malaysian Nature Park area were selected as isolation source of this study with the aim to isolate QQ actinobacteria. Mangrove swamps are unique woody plant communities that are highly productive ecosystems that contain high populations of micromonosporae and novel actinobacteria (Hong *et. al*., 2009). Thus, selective medium ISP Medium 1, ISP Medium 2 and ISP Medium 4 were used for characterizing *Streptomyces* species according to the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). Bacterial culture broth was extracted with ethyl acetate. MOPS-buffered media (pH 6.5) was used to prevent lactonolysis of AHLs (Yates *et al.,* 2002). Moreover, pH values were checked at the time of sampling and compared to the pH values of non-inoculated control medium. No change of pH was observed during growth of bacterial isolating. PBS and *E. coli* TOP10 served as negative controls did not show any AHL degradation activity. This indicated AHLs was degraded by the activity of bacteria cultivated.

In the molecular work, to eliminate any risk of contamination from extraneous DNA, the PCR reaction mixture including polymerase, without the template and primers was treated with DNAse followed by heat inactivation of the DNase. When the DNase-treated PCR was used with sterile distilled water (negative control), no PCR products was observed. This indicated the DNase treatment removed all contamination. Using this approach, molecular identification was done to identify the soil isolates by amplifying their 16S rRNA genes. Using this approach, nine bacteria isolated all shared >99% similarity to actinobacteria based on BLASTn search and the identities were

further supported by phylogenetics analysis conducted with MEGA version 6.06. Bootstrap analyses for 1000 resamplings were performed to provide confidence estimates for tree analysis. The BLAST search showed that NP1, NP2, NP6, NP7, NP8, NP9 were closely to *Streptomyces* sp., while NP3 and NP4 closely to actinobacteria and NP5 closely to *Kitasatospora* sp.

5.2 Detection of Quorum Quenching (QQ) activity

AHL degradation activity among the isolates was first assessed with whole-cell inactivation assay using C6-HSL as substrate followed by detection of residual AHL with CV026 as biosensor. Then, these isolates were tested whether they can degrade 3-oxo-C6-HSL and 3-hydroxy-C6-HSL using RRLC analysis. All this nine actinobacteria showed AHL degradation, which post QQ activity. Thus, this nine actinobacteria exhibited QQ activity by degrading C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL.

Subsequently, these isolates showed significant C6-HSL degradation, and further tested for 3-oxo-C6-HSL and 3-hydroxy-C6-HSL degradation where these AHLs represent 3-oxo and 3-hydroxyl group substituted AHLs. Significant degradation of the tested AHLs was observed for all nine tested isolates.

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

Isolates from samples NP2, NP3, NP4 and NP6 in a Streptomyces group showed strong QQ activity after 24h incubation with C6-AHLs while isolates NP1, NP5, NP7, NP8 and NP9 shown degradation of AHLs after 48h incubation using CV026 overlay

test. Isolates NP1, NP3 and NP4 showed strong QQ activity after 24h incubation with 3-oxo-C6-AHLs while isolates NP2, NP5, NP6, NP7, NP8 and NP9 showed degradation of AHLs after 48h incubation using CV026 overlay test. Isolates NP3, NP4, NP5 and NP6 shown strong QQ activity after 24h incubation with 3-hydroxy-C6-AHLs while samples NP1, NP2, NP6, NP7, NP8 and NP9 shown degradation of purple pigmentation after 48h incubation using CV026 overlay test. Taken these data together, it shows that these isolates show broad QQ activity against various types of AHLs.

Further confirmation degradation of C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL were shown using RRLC as repicted by the reduction of milli-absorbance units (mAU) in the chromatogram. Isolates NP3 and NP4 from *Streptomyces* genus shown the most rapid degradation of C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL. Both NP3 and NP4 have been reported to produce bioactive compound for bioremediation (Wong *et al.,* 2013). This is first documented both of these species exhibit QQ activity. Further studies of the QQ properties are needed to fully characterize the biological roles.

In the last decades, QQ bacteria activity was widely explored. *Bacillus* sp. is AHLs degrading bacterium isolated from soil (Dong *et al*., 2000). Since then, a range of QQ bacteria including members of α, β and γ-proteobacteria have been identified from soil, plant rhizosphere and biofilm samples as well as from laboratory bacterial culture collections (Dong *et al*., 2002; Lee *et al*., 2002; Huang *et al*., 2003; Lin *et al*., 2003; Sio *et al*., 2006). AHLs degrading bacteria such *as Klebsiella. Pseudomonas, Ralstonia, Variovorax, Comamonas* and *Agrobacterium* spp. have been identified (Leadbetter and Greenberg, 2000; Lee *et al.,* 2002; Huang *et al.,* 2003; Lin *et al*., 2003; Uroz *et al*., 2003). Gram-negative AHLs-degrading bacteria belonging to the *Sphingomonadaceae, Bradyrhizobiaceae* and *Rhizobiaceae* families have been isolated from tobacco rhizosphere.

In this work *actinobacteria* have been found in mangrove swamps which are rich in nutrient and minerals. Actinobacteria are a group of organisms that morphologically resemble fungi and physiologically resemble bacteria and production of antibiotics is their most important contribution to mankind with the group producing half of all the known antibiotics (Yang *et al.,* 2005). Application of antibiotics in plant disease control has been expanding rapidly and hundreds of antibiotics are already commercially available for plant disease (Sun *et al.,* 2005). Actinobacteria species produce a wide range of antimicrobials which help to confer a high competitive ability and to ensure dominance within their respective microbial communities (Wong *et al.,* 2013). It may be that QQ activity also plays a key role in enabling actinobacteria and other microbial weed species to dominate their respective habitats.

Recently, the ability to degrade AHL has been identified in unrelated genera of soil bacteria, including proteobacteria (Hewitt *et al.,* 2000), low GC Gram positive bacteria (Park *et al.,* 2003), and high GC Gram positive bacteria (Chan *et al.,* 2011). However, no extracellular AHL degrading enzyme has yet been reported. We speculate that the QQ activity regulate using either lactonase or acylase enzyme. However, further analysis of its QQ mechanism should be conducted to confirm this speculation.

Isolate NP1 which closely to *Streptomyces contaricanus* based on BLAST results, was previously shown to have anti-nematodal and antibiotic properties (Esnard *et al.,* 1995). Isolate NP2 which closely to *Streptomyces atratus* sp. on BLAST resulted, was reported produces atramycin A, hydrazidomycins A, hydrazidomycins B, hydrazidomycins C, rufomycins A and rufomycins B (Maria *et al*., 2013). Isolate NP3, *Actinoalloteichus cyanogriseus* shows phylogenetic analysis of *'Actinoalloteichus cyanogriseus'* based on its 16S rRNA sequence confirmed that the organism belongs to the family *Pseudonocardiaceae*. It contains glutamic acid, alanine and meso-diaminopimelic acid as cell wall amino acids, and menaquinone 9 (H4) (Tamura *et al*.,

2000). Actinobacteria, especially *Streptomyces* sp., are recognized as the producers of many bioactive metabolites that are useful to humans in medicine, such as antibacterials (Mahajan, 2012), antifungals (Gupte *et al.,* 2002), antivirals, antithrombotics, immunomodifiers, anti-tumor drugs and enzyme inhibitors; and in agriculture, including insecticides, herbicides, fungicides and growth promoting substances for plants and animals (Bressan, 2003 & Atta, 2009) Actinobacteria derived antibiotics that are important in medicine include aminoglycosides, anthracyclines, chloramphenicol, macrolide, tetracyclines which produces by *Actinobacterium* sp. related to isolate NP4. Isolate NP5, *Kitasatospora papulosa*, is under *Streptomyces* group which produce secondary metabolites as antibacterial and anticancer effect was established. *Streptomyces luridus* NP6, is a bacteria produce luridin as secondary metabolite (Waksman, 1961). Isolate NP7 closely to *Streptomyces albofaciens*, produces oxytetracycline Terramycin (Thirumalachar & Bhatt, 1960; Pemodet *et al.*, 1993). *Streptomyces kanamyceticus* NP8 is a species from which the antibiotic kanamycin is isolated (Garrod *et al.,* 1981). Isolate NP9, *Streptomyces prasinopilosus*, produced prasinomycin compound (Ettlinger *et al*., 1958).

All these actinobacteria were reported with capability produce bioactive metabolites but not reported as QQ degraded bacteria. Thus, heterologous expression of AiiA gene from lactonases enzymes in actinobacteria may reduce AHL accumulation and decrease their virulence expression, which indicates the potential use of AiiA as a strategy for antivirulence therapy. Thus, we believe that the actinobacteria have potential as biocontrol agents which would delay food spoilage while novel AHL inactivating enzymes may have utility as therapeutic agents. Further work includes whole genome sequencing of atinobacteria in order to identify the QQ gene of these strains (Ghani *et al.,* 2014).

5.3 AHLs lactonase activity

In this work, it is confirmed that isolates NP1, NP2, NP3, NP5, NP6, NP7 and NP9 degraded AHL via lactonase activity. The degradation of AHLs could be due to enzymatic degradation of AHL signals. Thus far, two major types of AHL–degrading enzymes have been extensively studied namely lactonase and acylase (Hong *et al*., 2012). Lactonases degrade AHL by hydrolyzing the lactone bond whereas acylases cleaves the amide linkage between the lactone ring and the acyl side chain (Dong & Zhang, 2005).

The opened ring of the AHL resulted from lactonase activity can be relactonized in acidic condition. The first reported cloning of AHL lactonase was *aiiA* from *Bacillus* sp. 240B1 (Dong *et al*., 2000). Subsequently, *aiiB* and *attM* from *Agrobacterium* sp., *ahlD* from *Arthrobacter* sp., *and qsdA* from *Rhodococcus erythropolis* strain W2, *aiiM* from *Microbacterium testaceum* StLB037 and *aidH* from *Oclrobacterium* sp. have been reported (Huang *et al*., 2012).

QQ activity of *Bacillus* is caused by presence of lactonase activity. One of the well characterized lactonase homologs is AiiA homologs from soil bacterium *Bacillus* sp. containing zinc binding motif HXHXDH (Dong *et al.,* 2001; Dong *et al.,* 2002). Furthermore, Park *et al.,* 2003 reported an AHL lactonase isolated from *Arthrobacter* termed as AhlD, also reveals the presence of HXHXDH motif in the deduced amino acid sequence. Phylogeneric analyses reflecting the sequence variation of various AHL degrading lactonases shows low sequence homology between AiiA and AhlD enzymes despite both sharing the highly conserved motif that is essential for activity (Dong & Zhang, 2005).

Pseudomonas is another well documented QQ bacterium. *Pseudomonas* produce QQ acylase enzyme that cleaves the amide linkage between fatty acid Cain and homoserine lactone moiety. The reported *Pseudomonas* AHL acylases include QuiP (PA1032) and

PvdQ (PA2385) from *P. aeruginosa* followed by HacA (Psyr_1971) and HacB (Psyr_4858) from *P. syringe* strain B728A (Huang *et al.,* 2003; Huang *et al.,* 2006; Shepherd & Lindow, 2009).

5.4 Potential roles of QQ

This work show that QQ actinobacteria are present in this mangrove swamps habitat. This implies the relevance of active QQ microorganism responsible for the turnover of QS compounds in mangrove swamps environment albeit the precise ecological role of the QQ mechanism remains unknown. Degradation of AHL molecules may implicate the utilization of AHLs as sources of energy and growth.

The potential for microbiological of these actinobacteria QQ properties is important since AHL-mediated signaling molecules mechanisms are widespread and highly conserved in many pathogenic bacteria, these isolates can be explored for novel anti-infective therapies (Williams *et al.*, 2007; Finch *et al.,* 1998). Also, QQ bacteria sharing the same habitat with QS bacteria could gain a competitive advantage by degrading AHL signal molecules. Enzymes that degrade AHLs might have commercial value as biocontrol against QS pathogens (Bauer & Robinson, 2002).

To the best of my knowledge, this finding may be important for the development of novel antimicrobial strategy. Thus, isolated strains of actinobacteria have potential as biocontrol agents which would control QS-mediated virulence determinants by exploring the AHL inactivating enzymes in this study. Three differences type of AHL used to see the ability of this actinobacteria produce broad range of AHL activities. Furthermore it is may have futher medical benefit if future investigation and gene applications may done.

5.5 Limitation of study and future work

Marine actinobacteria have been the focus on research over the past decade for new drugs discovery due to its unique adaptation in the harsh sea environment. It is believed that marine actinobacteria could produce compounds that are rare and unique compared to the terrestrial actinobacteria. Despite its potential, marine actinobacteria are critically difficult to culture in laboratory because these actinobacteria lives in extreme environment in the sea with high salt concentration, high pressure, low temperature, and constant pH changes of seawater in its natural environment. Hence, in this study, optimum condition to culture marine actinobacteria was achieved by culturing the marine actinobacteria from marine sponges on different culture condition such as different types of isolation media, pH, sea salt concentration, temperature, and incubation time. The harsh conditions in the ocean such as high salt concentration, high pressure, anaerobic condition, and low temperature have made it difficult to manipulate and maintain in the laboratory.

The pH 8 adjusts is much similar to the pH of natural seawater. A suitable pH range is crucially important for bacterial growth since bacteria are really sensitive to the hydrogen ion concentration of their environment. The unsuitable pH condition may affect the large bioproteins (i.e., enzymes) within the metabolism of actinobacteria, and this will halt the metabolism in the cells or production of bioactive compounds. Temperature can be a limiting factor of the bacterial growth because it can affect the chemical and biochemical processes in the cell. Every bacterium phylotype has its own minimal, maximal, and optimal temperatures that allow its growth and each bacterium can grow in a certain range of temperature. However, the most favourable growth only can be achieved at the optimal temperature (Pomeroy & Wiebe, 2001).

All these nine actinobacteria growth in 50% seawater. The need of seawater for the growth of isolates is critically important especially during the isolation of marine

bacteria from its natural sources. The isolation media should resemble its natural environment as closely as possible in order to the marine actinobacteria to grow on the isolation plates. The usage of isolation media prepared by using seawater is one of the ways to provide a suitable growth condition for marine bacteria. There is a difference between marine and terrestrial bacteria in term of salt requirement because the growth of marine bacteria is more preferable in media with seawater (McLeod, 1965).

The influence of incubation time on the growth of marine actinobacteria was up to 14 days. Actinobacteria colony started to appear on yeast malt agar only after 3 days of incubation times at 28 °C. Longer incubation time is needed for marine actinobacteria because marine actinobacteria are really slow growing in nature (Lam, 2006) and that may contribute to their metabolites diversity.

Study done by Vasavada *et al.*, 2006 showed that the used of media, pH, salinity and carbon and nitrogen affect the growth and antibiotic production by actinobacteria. The search for novel metabolites producer requires a large number of isolates. According to Oskay *et al.,* 2004, actinobacteria diversity might be influence by the diversity of plants species grown on that particular soil. It was also noted that different plants produce different chemical metabolite which might useful for the microbes around it. In order to survive in a threatens environment the actinobacteria need to adapt to the environment (Jeffrey, 2008).

Future studies should include the study of another QQ enzyme namely acylase which can be confirm by targeting the release of acyl side chain reacted chemically with 5-dimethylamino-1-napthalensesulphonyl chloride (DANSYL chloride) (Lin *et al.,* 2003). Confirmation of lactonases enzymes should be done using LC-MS-MS. Also, cloning of the QQ genes among these isolates will allow enzymatic reaction studies. In addition to this, whole genome sequencing could be performed to understand the whole gene regulation and physiological effect of these QQ isolates. Novel actinobacteria gene

might be benefit for future biocontrol or bioremediation agents. Mechanism interaction of production secondary metabolites of actinobacteria and the relation of Quorum Quenching activity may have future medical benefit, should be study in details.

CHAPTER 6.0

CONCLUSION

Identification of quorum quenching (QQ) Actinobacteria from mangrove swamps using selective media (ISP media 1, ISP media 2, and ISP media 4, Yeast malt agar) has successfully growth nine difference actinobacteria which has QQ activity. These nine actinobacteria was proceed with identification of broad range of AHLs degradation activity using C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL by whole–cell AHLs inactivation assay and Rapid Resolution Liquid Chromatography (RRLC). Identification of lactonase was shows formation of purple pigmentation after 48h incubations for all isolates. The ability of this nine actinobacteria producing lactonase anzyme might lead to novel lactonase gene in future investigation.

QQ bacteria isolation from mangrove swamps has yielded nine actinobacteria isolates, one *Kitasatospora* sp. and six *Streptomyces* spp. isolates. Assessment of QQ activity against various AHLs showed that nine isolates significantly degraded C6-HSL, 3-oxo-C6-HSL and 3-hydroxy-C6-HSL. Further investigation is required to confirm the QQ genes of these isolates. The study showed actinobacteria isolated from mangrove samples has QQ activities.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

PUBLICATIONS

Norshazliza A. G.; Joanita S.; Zahidah I.; Xin-Yue C.; Wai-Fong Y.; and Kok-Gan C. *Rhodotorula mucilaginosa*, a Quorum Quenching Yeast Exhibiting Lactonase Activity Isolated from a Tropical Shoreline. *Sensors* **2014**, *14*, 6463-6473

Norshazliza A. G.; Siti Nur Maisarah N.; Xin Yue Chan.; Wai-Fong Y.; and Kok-Gan C. *Labrenzia sp. BM1*: A broad range Quorum Quenching bacterium degrading various *N*-acyl homoserine lactones. *Sensors* **2014**,14, 11760-11769

POSTER PRESENTATION

Quorum Quenching activity bacteria isolated from Malaysia Shoreline, Monash University, Kuala Lumpur, 2014.

APPENDIX

SEQUENCE OF CLOSEST MATCH

Isolates NP 1

ACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTT
CGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTG
CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCATCT
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CTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCC
GGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC
AGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCTCGG
GGCTTAACCCCGAGTCTGCAGTCGATACGGGCTAGCTAGAGTGTGGTAGGG
GAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGG
TGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGC
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TCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGG
CTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTG
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CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGA
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Isolates NP2

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CGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTT
CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGT
CCCGCATGGGACGGGGTTGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
CTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCC
GGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC
AGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGCTTGTTGCGTCGGTTGTGAAAGCCCGG
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Isolates NP3

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CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACCTGCC
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GACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGT

Isolates NP4

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CGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCT
ACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
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Isolates NP5

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Isolates NP6

GCTTAACACATGCAAGTCGAACGATGAACCCGCTTCGGTGGGGGATTAGTG
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CCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCG
GTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTG
GGACGAAGTCGT

Isolates NP7

CATGCAAGTCGAACGATGAACCCCTTCGGGGGGGATTAGTGGCGAACGGGT
GAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACG
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GCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCC
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Isolates NP8

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Isolates NP9

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GGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGA
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GGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCACGTCGATTGTGAAGCTCGGGGCTTAACCCCGAGTCTGCAGTC
GATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGC
GGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCT
GGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
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GTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACAC
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