QUORUM SENSING AND QUORUM QUENCHING IN SELECTED

BACTERIA ISOLATED FROM DISEASED TILAPIA FISH

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ABSTRACT

Living in a polymicrobial community, bacteria communicate within and among species for interaction. This cell-cell communication in bacteria is termed quorum sensing (QS). Among the QS signaling molecules, *N*-acyl homoserine lactone (AHL) is one of the well characterized QS signaling molecules produced and used by vast group of *Proteobacteria*. This QS signaling molecule often associated with coordinating certain group behavior of bacteria such as the bioluminescence, biofilm formation and regulation of virulence factors expression. On the other hand, other than production, some bacteria also degrade the QS signaling molecules and this is termed as quorum quenching (QQ). It is believed that the bacteria possessed QQ as a strategy to survive in the competitive environment against the QS bacteria.

In these studies, five bacteria are isolated from diseased tilapia fish namely *Bacillus* sp. strain W2.2, *Klebsiella oxytoca* strain W4.2, *Pseudomonas* sp. strain W3, *Pseudomonas aeruginosa* strain W3.1 and *Serratia marcescens* sp. strain W2.3. The QS signaling molecules produced by these bacteria are characterized by thin layer chromatography (TLC) and liquid chromatography mass spectrophotometry (LCMS). Results showed that *P. aeruginosa* strain W3.1 produces a wide range of *Pseudomonas* quinolone signaling (PQS), and AHLs including unusually long-chain *N*-(3-oxohexadecanoyl)-$_L$-homoserine lactone (OC16-HSL), but none of these compounds are detected from *Pseudomonas* sp. strain W3. In contrast, *Pseudomonas* sp. strain W3 degraded *N*-butanoyl-$_L$-homoserine lactone (C4-HSL) and *N*-hexanoyl-$_L$-homoserine lactone (C6-HSL). *N*-dodecanoyl-$_L$-homoserine lactone (C12-HSL) was detected from spent supernatant from *S. marcescens* strain W2.3 and this is the first report stating long chain AHL produced by *Serratia* sp.. Bioassay using skimmed milk agar has confirmed

that with the exception of *Klebsiella* sp. strain W4.2, all isolates showed proteolytic activity, while assays using sheep blood agar showed that *Bacillus* sp. strain W2.2 and *Pseudomonas* sp. strains W3 and *P. aeruginosa* strain W3.1 have hemolytic activity.

ABSTRAK

Bakteria berkomunikasi antara sama spesis dan juga kumpulan yang beza untuk kehidupan. Komunikasi antara sel-sel bakteria ini dipanggil kuorum pengesanan (QS). Antara molekul-molekul isyarat korum pengesanan, *N*-acyl homoserine lactone (AHL) merupakan isyarat korum pengesanan yang paling popular di mana ia dihasilkan dan digunakan oleh sekumpulan besar *Proteobacteria*. Molekul isyarat ini sering dikaitkan dengan menyelaraskan tingkah laku kumpulan bakteria tertentu seperti bioluminescence, pembentukan biofilm dan memiliki faktor kebisaan. Selain daripada hasil, sesetengah bakteria juga mengguna and memisahkan molekul isyarat kuorum pengesahan dan ini dipanggil sebagai kuorum perencatan. Adalah dipercayai bahawa keupayaan kuorum perencatan (QQ) bakteria adalah strategi untuk bertahan dan hidup di suasana yang kompetitif.

Dalam kajian ini, 5 bakteria diasingkan daripada ikan tilapia yang sakit iaitu *Bacillus* sp. W2.2, *Klebsiella* sp. W4.2, *Pseudomonas* sp. W3, *Pseudomonas* sp. W3.1 dan *Serratia* sp. W2.3. Isyarat molekul QS yang dihasilkan oleh bakteria ini dicirikan oleh kromatografi lapisan nipis (TLC) dan kromatografi cecair mass spektrofotometri (LCMS). Keputusan menunjukkan bahawa *Pseudomonas* sp. W3.1 menghasilkan pelbagai isyarat quinolone *Pseudomonas* (PQS), dan pelbagai jenis AHLs termasuk AHL dengan rantai panjang yang luar biasa, *N*-(3-oxohexadecanoyl)-L-homoserine lactone (3-Oxo-C16-HSL). Walaubagaimanapun, isyarat luar biasa ini tidak dapat dikesan daripada *Pseudomonas* sp. W3. Sebaliknya, *Pseudomonas* sp. W3 menguraikan *N*-butanoyl-L-homoserine lactone (C4-HSL) dan *N*-hexanoyl-L-homoserine lactone (C6-HSL). Selain daripada *Pseudomonas* sp. W3.1, *N*-dodecanoyl-L-homoserine lactone (C12-HSL) dikesan dari kultur media yang dibelanjai oleh *Serratia* sp. W2.3 dan ini

adalah laporan pertama menyatakan pengesanan AHL dengan rantai panjang yang luar

biasa dari *Serratia* sp.. Ujian bakteria dengan agar susu telah mengesahkan bahawa

kecuali *Klebsiella* sp. W4.2 semua bakteria menunjukkan aktiviti proteolitik, manakala

bagi agar darah biri-biri, *Bacillus* sp. W2.2 dan *Pseudomonas* sp. strain W3 dan W3.1

juga dipaparkan aktiviti hemolitik.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

chromatography. C12‑HSL is mark by as "**" while OC16‑HSL is marked as "*". *P. aeruginos* W3.1 produces of both C12‑HSL and OC16‑HSL which *S. marcescens* W2.3 produces C12-HSL.

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
$\times$	Times
$\times g$	Gravity
℃	Celsius
μg	Microgram
μl	Microlittre
μm	Micron
μM	Micromolar
AB medium	*Agrobacterium* medium
ACN	Acetonitrile
AGE	Agarose gel electrophoresis
AHL	*N*-acyl homoserine lactone
AHQ	2-alkyl-4(1H)-quinolones
AI-2	Autoinducer 2
AIP	Autoinducer peptide
C12-HSL	*N*-dodecanoyl-$_L$-homoserine lactone
C16-HSL	*N*-hexadecanoyl-$_L$-homoserine lactone
C4-HSL	*N*-butanoyl-$_L$-homoserine lactone
C6-HSL	*N*-hexanoyl-$_L$-homoserine lactone
C7-AQ	2-heptyl-4-hydroxyquinolone
C7-HSL	*N*-heptanoyl-$_L$-homoserine lactone
C7-*N*-Oxide	2-heptyl-4-hydroxyquinoline-*N*-oxide
C7-PQS	2-heptyl-3-hydroxy-4(1H)-quinolone
C8-HSL	*N*-octanoyl-$_L$-homoserine lactone

C9-AQ	2-nonyl-4-hydroxyquinolone
C9-N-Oxide	2-nonyl-4-hydroxyquinoline-N-oxide
C9-PQS	2-hydroxy-2-nonyl-4(1H)-quinolone
DKP	Diketopiperazines
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSF	Diffusible signal factor
EDTA	Ethylenediaminetetraacetic acid
HHQ	2-heptyl-4-hydroxyquinolone
hr	Hour
kb	Kilobase pair
L	Littre
LB medium	Luria-Bertani medium
LCMS	Liquid chromatography mass spectrometry
M	Molarity
m/z	Mass to charge ration
mA	Miliampere
min	Minute
mL	Milliliter
mM	Milimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MS	Mass spectrometry
NCBI	National center for biotechnology information
ng	Nanogram
nM	Nanomolar

OC12-HSL N-(3-oxo-dodecanoyl)-$_L$-homoserine lactone

OC14-HSL N-(3-oxo-tetradecanoyl)-$_L$-homoserine lactone

OC16-HSL N-(3-oxo-hexadecanoyl)-$_L$-homoserine lactone

OC6-HSL N-(3-oxo-hexanoyl)-$_L$-homoserine lactone

OC8-HSL N-(3-oxo-octanoyl)-$_L$-homoserine lactone

OD   Optical density

OHC16-HSL   3-hydroxyhexadecanoyl-$_L$-homoserine lactone

PAME   Palmitate methyl ester

PBS   Phosphate Buffer Saline

PCR   Polymerase chain reaction

PQS   *Pseudomonas* quinolone signal

psi   Pounds per square inch

QQ   Quorum quenching

QS   Quorum sensing

R_f Retardation factor

s   Second

SAM S-adenosyl-$_L$-methionine

SOC   Super optimum broth

TBE   Tris-Boric Acid Ethylenediaminetetraacetic acid

TLC   Thin layer chromatography

TOF   Time of flight

V   Voltage

v/v   Volume per volume

w/v   Weight per volume

X-gal   5-bromo-4-chloro-indolyl-galactopyranoside

CHAPTER 1.0

INTRODUCTION

1.1 Research Background

Bacteria cell-cell communication is termed as quorum sensing (QS). It is a cell density dependent system involving the signalling molecules production and exportation to the extracellular environment (Joint, 2006). As the cell density increases, the QS signalling molecules accumulate until a threshold the signalling molecule-receptor protein complex will then coordinate certain group behavior of the bacteria (Dong & Zhang, 2005; Waters & Bassler, 2005; Williams *et al.*, 2007). For example, the production of hemolysin and proteinases are the several phenotypes regulated by QS (De Kievit & Iglewski, 2000; Swift *et al.*, 1997).

QS is mediated by signalling molecules, such as *N*-acyl homoserine lactone (AHL), autoinducer 2 (AI-2), and diffusible signal factor (DSF). Among various QS signalling molecules, AHL which was produced by *Proteobacteria* is one of the most commonly studied signalling molecules (Whitehead *et al.*, 2006). The carbon-3 of the acyl side chain can be an unsubstitute acyl side chain, 3-hydroxy or 3-oxo group (Hong *et al.*, 2012). Other than AHL, *Pseudomonas* harbors a second QS system which is mediated by a different QS signalling molecule, *i.e. Pseudomonas* Quinolone Signal (PQS) (Kim *et al.*, 2010). The precursor of PQS, *i.e.* 2-heptyl-4-hydroxyquinolone (HHQ) works hand in hand with PQS, intertwined with the AHL-mediated QS system, forming a complex and versatile regulation mechanism, monitoring phenotypes such as biofilm formation, exoproteases secretion, virulence factor production as well as

resistance against antibiotics (Déziel *et al*., 2004; Juhas *et al*., 2005; Kim *et al*., 2010; McGrath *et al*., 2006; Wade *et al*., 2005).

In addition to QS, quorum quenching (QQ) activity which is the interference or inactivation of QS system of the isolated bacteria was studied (Dong & Zhang, 2005). According to Dong & Zhang, (2005), the degraded AHL was postulated to be utilized by some bacteria as the source of carbon and nitrogen. Czajkowski & Jafra, (2009) suggested interference of QS allows the QQ bacteria to gain competition advantage.

1.2 Objectives

In this study, bacteria were suspected to be the causative agent of an outbreak in year 2009. It killed more than 50 % of the tilapia fish stock of Malaysia. The diseased tilapia fish were delivered to our laboratory in 2009 from a local fish farm in Terengganu, Malaysia. Five bacteria were isolated and identified from the diseased tilapia fish. QS and QQ activities were performed on these potential fish pathogens.

OBJECTIVES

The four objectives in this study are as follow:

1. to identify the pathogens from the diseased tilapia fish，

2. to examine the production of virulence factors namely proteinase and hemolysin from these isolates,

3. to characterize the AHL produced by isolates, and

4. to study QQ activity of from the isolates.

CHAPTER 2.0

LITERATURE REVIEW

2.1 QS

The discovery of bacteria communication signals has shift the perspective on bacteria from an independent living unicellular organism to a complex community where bacteria communication occurs among each other (Atkinson & Williams, 2009; Waters & Bassler, 2005). These interactions happen both within the same species and cross species (Waters & Bassler, 2005). This cell-cell communication was termed as quorum sensing (QS) (Fuqua *et al*., 1994).

QS involve four steps where the bacteria synthesize the signalling molecules and release them into the extracellular environment (Williams, 2007). As the population density increases, the concentration of the signalling molecules in the extracellular environment increases as well. When the concentration of the signalling molecules reaches its threshold level, it will diffuse into the bacteria cells, binds with the receptor protein. This signalling molecule- receptor protein will interact with the target promoter, subsequently up-regulate or down-regulate certain phenotypes (Atkinson & Williams, 2009; Bassler, 2002; Dong & Zhang, 2005; Jayaraman & Wood, 2008; Williams, 2007).

The first reported QS-regulated bioactivity was the bioluminescence of *Vibrio fischeri* which inhabited in the light organ of the Hawaiian bobtail squid (*Eupryma scolopes*) (Callahan & Dunlap, 2000; Defoirdt *et al*., 2007; Fuqua & Greenberg, 2002). A minute amount of *V. fischeri* is harbored in the light organ of the squid during day

time. The bacteria population gradually increases after hours of incubation as well as the signalling molecules that trigger the luciferase expression when the signalling molecules reach its threshold concentration. This bioluminescence was needed by the squid to counterilluminate its shadow and avoid predation in the night (Callahan & Dunlap, 2000; Defoirdt *et al*., 2007; Dunlap, 1999; Waters & Bassler, 2005). The luminescence was "turned off" by the squid by pumping out large amount of bacteria from its light organ, hence the bacteria has insufficient signalling molecules to trigger the production of luciferase (Dunlap, 1999; Waters & Bassler, 2005).

Besides bioluminescence, QS regulates a vast array of phenotypes, such as the production of chitinolytic enzymes, exoproteases as well as natural antibiotic violacein by *Chrombacterium violaceum* (Chernin *et al*., 1998; McClean *et al*., 1997). QS too regulates several phenotypes which are life-threatening such as biofilm formation, production of virulence factors and swarming activity, in several notorious pathogens, such as *Pseudomonas aeruginosa* (Cvitkovitch *et al*., 2003; De Kievit *et al*., 2001), *Serratia marcescens* (Rice *et al*., 2005), *Serratia liquefaciens* (Labbate *et al*., 2004), *Burkholderia cepacia* (Huber *et al*., 2001), *Burkholderia cenocepacia* (O'Grady *et al*., 2012), *Vibrio cholera* (Hammer & Bassler, 2003), and *Streptococcus mutans* (Cvitkovitch *et al*., 2003). More attention was given to the studies of bacteria QS when it was learn that the expression of virulence factor for some of the bacteria are reported to be QS regulated, for instance *V. cholera* (Hammer & Bassler, 2003), *B. cenocepacia* (O'Grady *et al*., 2012), *Pectobacter* (Põllumaa *et al*., 2012) and *P. aeruginosa*.

There are various types of QS signalling molecules for example the AHL, 2-alkyl-4(1H)-quinolones (AHQs), autoinducer peptide (AIP), DSF, palmitate methyl ester (PAME), diketopiperazines (DKP) (Dong & Zhang, 2005; Kim *et al*., 2010; Lyon & Novick, 2004; Uroz *et al*., 2008; Williams *et al*., 2007; Yin *et al*., 2012). Of all the mentioned signalling molecules, AHL has been widely studied by most research institutions. AHL is basically a group of signalling molecules which produced by most Gram-negative bacteria, specifically *Proteobacteria* (Leadbetter & Greenberg, 2000; Uroz *et al*., 2008). Several reports showed that single genus of bacteria may employ more than one type of QS system for example, *Pseudomonas*, *Burkholderia*, and *Alteromonas* have been reported to produce AHL as well as AHQ as QS signalling molecules (Fletcher *et al*, 2007; McGrath *et al*., 2006).

In the AHL-mediated QS system, the AHL synthase encoded by *luxI* gene is responsible for the production for AHL whereas the *luxR* gene which encodes for AHL receptor play a crucial role in regulating QS-regulated phenotypes as well as facilitating a positive autoinductive loop in driving the production of AHL (Atkinson & Williams, 2009). The AHL was made up of a lactone ring and acylated side chain (Williams *et al*., 2007) where the lactone ring of AHL was synthesized by AHL synthase from *S*-adenosyl-L-methionine (SAM) and its acyl chains was originated from lipid metabolism, carried by various acyl-carrier proteins (Reading & Sperandio, 2005). This acyl side chain normally range from 4 to 18 carbons where the longer acyl side chain stand a chance to have unsaturated bond in the middle of the acyl chain (Fuqua & Greenberg, 2002). The third carbon (C3) of the acyl side chain could be fully reduced, fully oxidized carbonyl or carry a hydroxyl group (Hong *et al*., 2012). Some other groups or molecules such as cystein, biotin and fluorescence might be incorporated within the

AHL molecules as well and this has an effect on the binding affinity of modified-AHL to its native AHL receptor (Fuqua & Greenberg, 2002).

2.1.1 QS of *Pseudomonas aeruginosa*

P. aeruginosa listed as one of the top three human opportunistic pathogen was consistently found infecting immunocompromised host (Stover *et al*., 2000). It was recorded as the most common Gram-negative bacteria responsible for nosocomial infection (Van Delden & Iglewski, 1998). The resulting infection could cause morbidity and mortality of the host yet the emergence of multidrug resistance strain makes treatment more challenging (Erickson *et al*., 2002; Livermore, 2002). Besides human, *P. aeruginosa* was recovered from sick tilapia fish in Eygpt and its close species *Pseudomonas angulliseptica* was reported to cause more than 95 % mortality of the infected fish (Eissa *et al*., 2010).

QS of *P. aeruginosa* has been studied extensively and was reported to be regulating its virulence factor production, biofilm formation, swarming ability and pyocyanin production (Dubern & Diggle, 2008; Köhler *et al*., 2000; Pesci *et al*., 1997). Its genome carries at least two complete QS systems (Pesci *et al*., 1997). Las and Rhl systems are responsible for AHL regulatory circuits (Zhu *et al*., 2004).

In brief, *lasI* gene is responsible for autoinducer *N*-(3-oxododecanoyl)-$_L$-homoserine actone (OC12-HSL) synthesis in Las system (Smith *et al*., 2002). The OC12-HSL binds to its transcription factor LasR-like family protein

and the complex activates the expression of several virulence genes such as the *lasB* gene that code for the production of elastase, *toxA* for exotoxin A, *aprA* for alkaline protease and *xcpP* and *xcpR* for secretory pathway of *P. aeruginosa* (Passador *et al.*, 1993; Pesci *et al.*, 1997; Storey *et al.*, 1998; Van Delden & Iglewski, 1998). On top of virulence gene, LasR/OC12-HSL complex activates the expression of *rhlR* genes of another QS system of *P. aeruginosa*, the Rhl system (Juhas *et al.*, 2004; Van Delden & Iglewski, 1998). *rhlI* synthesize *N*-butanoyl-$_L$-homoserine lactone (C4-HSL) which binds with the *rhlR* cognate transcriptional factor activating the expression of genes that encode for the synthesis of rhamnosyltransferase, elastase, alkaline protease, and cyanide (Pearson *et al.*, 1997; Smith *et al.*, 2002; Van Delden & Iglewski, 1998). In addition to C4-HSL and OC12-HSL, *P. aeruginosa* was reported to produce *N*-(3-oxotetradecanoyl)-$_L$-homoserine lactone (OC14-HSL), however the mechanism and function of this autoinducer was undetermined (Wong *et al.*, 2012).

The third well known QS system of *P. aeruginosa* was termed as PQS system. The active autoinducer involved in this system are 2-heptyl-3-hydroxy-4(1H)-quinolone, commonly known as PQS and its immediate precursor, 2-heptyl-4-hydroxyquinolone (HHQ) (Fletcher *et al.*, 2007; Häussler & Becker, 2008). PQS belongs to the 4-quinolone chemical family and its synthesis depends on *pqs*ABCDE operon which is also responsible for the synthesis of various type of hydroxyquinolone (Fletcher *et al.*, 2007; Gallagher *et al.*, 2002; Wilder *et al.*, 2011). PQS or HHQ binds to the receptor protein, PqsR with high affinity, enhancing the binding of PqsR to *pqsA* promoter, self-regulating the synthesis of PQS compound (Déziel *et al.*, 2004; Fletcher *et al.*, 2007; Wade *et al.*, 2005). This system works together with the AHL regulated QS system, mainly the Rhl system, co-ordinating more than 100 genes expression including the

pyocynin production, secondary metabolites production and biofilm development (Aendekerk *et al.*, 2005; D éziel *et al.*, 2004; Dubern & Diggle, 2008; McGrath *et al.*, 2006; Wade *et al.*, 2005; Williams & C ámara, 2009).

2.1.2 QS of *Serratia marcescens*

S. marcescens belongs to *Enterobacteriaceae* family often associated with food spoilage and causal agent of various diseases (Van Houdt *et al.*, 2007; Wang *et al.*, 2012). It usually occurs as bloody red colonies due to the production of prodigiosin (Thomson *et al.*, 2002; Williams, 1973). The production of prodigiosin is regulated by the QS system of *S. marcescens* (Thomson *et al.*, 2002). Studies on *S. marcescens* strain SS-1, and strain MG1 (previously known as *S. liquefaciens* MG1) reviewed two AHL based QS which are the Spn system and Swr system (Horng *et al.*, 2002; Labbate *et al.*, 2004; Lindum *et al.*, 1998; Van Houdt *et al.*, 2007; Van Houdt *et al.*, 2006; Wei & Lai, 2006; Williams, 1973).

In the study by Horng *et al.*, (2002), *spnR* of *S. marcescens* SS-1 acts contradict to majority of the *luxR* in the QS system, as it negatively regulates the sliding activity, pigment and nuclease production of *S. marcescens* SS-1. This depressed nuclease gene by *spnR* can be restore by N-(3-oxohexanoyl)-$_L$-homoserine lactone (OC6-HSL) synthesize by *spnI*. Aside from OC6-HSL, AHL synthase, *spnI* also synthesize N-hexanoyl-$_L$-homoserine lactone (C6-HSL), N-heptanoyl-$_L$-homoserine lactone (C7-HSL) and N-octanoyl-$_L$-homoserine lactone (C8-HSL) (Horng *et al.*, 2002; Van Houdt *et al.*, 2007; Wei & Lai, 2006; Wei *et al.*, 2006). Further studies on *S. marcescens* strain SS-1 shows that its *spnR* falls within the Tn*3* family transposon

making it a mobile QS system that might transfer within different host (Wei *et al*., 2006).

On the other hand, the main element involve in Swr system are the AHL synthase, *swrI*, the translational regulators *swrR* and the signalling molecules, C4‑HSL and C6‑HSL (Labbate *et al*., 2004; Van Houdt *et al*., 2007; Wei & Lai, 2006). The Swr system positively regulates the production of biosurfactant with C4‑HSL which facilitates the motility of *Serratia* (Labbate *et al*., 2004; Lindum *et al*., 1998). In addition to the biosurfactant production, Swr too coordinates the biofilm maturation, butanediol fermentation and extracellular lipase (Labbate *et al*., 2004; Lindum *et al*., 1998; Rice *et al*., 2005; Van Houdt *et al*., 2006; Wei & Lai, 2006).

2.2 Detection of AHLs with Biosensors

Certain QS bacteria have been mutated and genetically modified to act as biosensors for rapid screening for the presence of QS signalling molecules (Farrand *et al*., 2002; Llamas *et al*., 2004; McClean *et al*., 1997). Most of the QS biosensors are constructed by either modification of the bacterial QS gene or by the insertion of plasmid reporter vector into the bacteria (Steindler & Venturi, 2007; Winson *et al*., 2006). The presence and detection of QS signalling molecules was shown by possessing certain characteristic such as pigment formation and bioluminescence (McClean *et al*., 1997; Winson *et al*., 2006).

2.3 QQ

The interruption of QS activity was termed as QQ (Dong & Zhang, 2005). The interruption can be enzymatically degradation of the QS signalling compound or by the introduction of the antagonist that mimics the QS autoinducer which blocks the autoinducer synthase or receptor protein (Adonizio *et al*., 2006; Czajkowski & Jafra, 2009; Dong & Zhang, 2005; Hong *et al*., 2012; Uroz *et al*., 2008). The QS antagonist can be natural compound which are extracted and purified from plants (Chong *et al*., 2011; Krishnan *et al*., 2012; Tan *et al*., 2012).

The enzymatic degradation of the AHL can be performed by 4 categories of enzymes, namely the lactonase and decarboxylase that target and break the lactone ring, while the acylase and deaminase target and cleave the acyl side chain (Dong & Zhang, 2005). To date only lactonase and acylase were found with an addition enzyme that disturb but not disrupt the AHL based QS system (Chan *et al*., 2011; Dong & Zhang, 2005; Hong *et al*., 2012). These enzymes were frequently reported to be produced by bacteria such as *Bacillus cereus* (lactonase), *P. aeruginosa* (acylase) and *Burkholderia* (oxidoreductase) (Chan *et al*., 2010; Chan *et al*., 2011; Dong *et al*., 2002; Sio *et al*., 2006). Studies suggest that bacteria degrades AHLs in the environment for several reason including to compete for limited nutrient source, recycling the energy source from AHL, and regulating its QS system (Czajkowski & Jafra, 2009; Haudecoeur *et al*., 2009; Leadbetter & Greenberg, 2000; Park *et al*., 2008; Park *et al*., 2003).

2.4 Tilapia Fish

Tilapia Fish is the common name for approximately 70 species of fishes in *Cichlidae* family (Gonzales & Brown, 2006). It is cultivated in both tropical and subtropical countries and is supplied as food fish in countries around the world (Lee *et al.*, 2005). In year 2010, tilapia is reported as one of the major aquaculture production that makes up to 7.6 % of the world-wide freshwater fish production (Bostock *et al.*, 2010). The major tilapia fish suppliers in the world include China, Eygpt, Philippines, Indonesia, Thailand, Brazil, Bangladesh, Vietnam, Taiwan and Malaysia (Sayed, 2006; Young & Muir, 2002).

The high growth rates, short generation time, ability of adapt to wide range of environment, resistant to stress and diseases, feeding on low trophic levels and artificial feeds has make it a popular aquaculture candidate in developing countries (Sayed, 2006). According to several studies, tilapia fish able to tolerate a wide range of water salinity where certain species can grow and reproduce normally in water with more than 50 % of water salinity making it a good candidate for fish farmers, especially in countries with limited freshwater source (El-Sayed, 2006). On the other hand, its lower protein requirement with the credit of the high demand from the market resulting the extensive cultivation of tilapia by fish farmers (El-Sayed, 2006; Fitzsimmons, 2000).

Malaysia being one of the major suppliers of farmed tilapia fish has increased the cultivation volume of tilapia dramatically within 10 years. According to several reports, the production of farmed tilapia fish in Malaysia increased by 15 fold from 1345 million tonnes to 26872 tonnes from year 1992 to 2002 (Sayed, 2006). In year 2010,

Malaysia was estimated to produce 120000 million tonnes of tilapia fish (Josupeit, 2005). Most of this fish was sold to the domestic market of Malaysia, while a small portion from the total harvested product was export as high value products, such as fish fillets, to foreign market (Bostock *et al*., 2010; Fitzsimmons, 2000; Josupeit, 2005). With the increasing demand for tilapia fish globally, tilapia fish has become an important aquaculture product for Malaysia.

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Materials

Chemicals used in this work were purchased from Sigma, USA; Merck, Germany; Amresco, USA; BDH Ltd. UK; and BD DifcoTM Laboratories, USA. Solvents used in this work were supplied by Fisher Scientific, UK. While AHL used were purchased from Cayman, USA. Buffers and culture mediums were sterilized by autoclaving at 121 °C, 15 psi for 15 min before use, while the heat sensitive material including inorganic supplement, antibiotics, glucose, skimmed milk and X-gal were filtered sterilize using syringe filter (Sartorius, Germany) with pore size of 0.22 μm. The pH of each buffer and medium were adjusted with 1 M of HCl and 1 M of NaOH.

3.1.1 Buffer

3.1.1.1 Phosphate Buffer Saline (PBS)

PBS was prepared with 0.0023 % (w/v) NaH_2PO_4, 0.0115 % (w/v) Na_2HPO_4, and 5.4 % (w/v) tris base in distilled water. The pH of the buffer was adjusted to 6.5 prior to autoclave.

3.1.1.2 Tris-Boric Acid Ethylenediaminetetraacetic acid (TBE)

TBE was prepared by mixing 0.37 % (w/v) ethylenediaminetetraacetic acid (EDTA), 2.75 % (w/v) of boric acid and tris base 5.40 % (w/v) in 1 L of distilled water

making it $5 \times$ TBE. The pH of TBE was adjusted to 6.5 before autoclaving. Five times dilution on $5 \times$ TBE was performed in order to prepare $1 \times$ TBE.

3.1.2 Culture Medium

3.1.2.1 Luria-Bertani (LB) Medium

Bacteria were maintained on LB medium which contain 1.0 % (w/v) NaCl, 1.0 % (w/v) peptone and 0.5 % (w/v) yeast extract. LB agar was prepared by solidifying LB medium with 1.5 % (w/v) of bacto agar. If AHL extraction is needed, 50 mM of 3-(N-morpholino) propanesulfonic acid (MOPS) was added into the medium.

3.1.2.2 *Agrobacterium* (AB) Medium

AB medium contain 0.3 % (w/v) K_2HPO_4, 0.1 % (w/v) NaH_2PO_4, 0.1 % (w/v) NH_4Cl, 0.03 % (w/v) $MgSO_4.7H_2O$, 0.015 % (w/v) KCl, 0.0005 % (w/v) $CaCl_2$ and 0.00025 % (w/v) $FeSO_4.7H_2O$. In order to culture *A. tumefaciens* NTLZ pZLR4 0.5 % (w/v) glucose and 150 μg/ml gentamycin was added into AB medium (Yin *et al*., 2012). AB agar was prepared by solidifying AB medium with 1.5 % (w/v) bacto agar.

3.1.2.3 Super Optimum Broth (SOC)

SOC was prepared by with 2.0 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) $MgCl_2$, 0.05 % (w/v) NaCl and 20 mM glucose. The broth was adjusted to pH 7.0 with NaOH pellet.

3.1.2.4 Skimmed Milk Agar

Skimmed milk agar for proteolytic assay was made of 0.05 % (w/v) yeast extract, 0.1 % (w/v) tryptone, 1.0 % (w/v) NaCl, 1.5 % (v/v) skim milk and solidified with 1.5 % (w/v) bacto agar.

3.1.3 Reaction Mix

3.1.3.1 Polymerase Chain Reaction (PCR) Master Mix for Gene Amplification

Each PCR reaction mix contain $1 \times$ PCR buffer, 800 μM dNTP mix, 20 mM $MgCl_2$, 10 nM forward primer, 10 nM reverse primer, 1 unit taq DNA polymerase, 1 ng genomic DNA and top up each reaction to 15 μl with distilled water.

3.1.3.2 Ligation Mix

Ligation mix for each sample contain $1 \times$ rapid ligation buffer 5 ng/μl pGEM®-T easy vector (Promega, USA), 0.3 unit/μl T4 DNA ligase and 1 μg purified PCR product.

3.1.4 Biosensors and Strains

Table 3.1 : Biosensors, Positive Control, Negative Control and Host for Transformation Used in This Project

Sample	Description	Reference
Chromobacterium violaceum 026	A double mini Tn*5* mutant derived from *C. violaceum* ATCC 31532. It is a violacein-	(McClean *et al*., 1997)

(CV026)	negative, white mutant defective in the production of the AHLs. Production of purple colour pigment, *i.e.* violacein, is inducible by the addition of exogeneous short chain AHLs, range from C4-HSL to C8-HSL.	
Escherichia coli DH5α	Host that carries no plasmid that yield high quality and concentration of inserted plasmid.	(Taylor *et al.*, 1993)
Bacillus cereus	Serve as positive control in the AHL inactivation assay. It is capable of degrading AHL by targeting the lactone bond and amide linkage, via AHL lactonase, encoded by *aiiA* homologues	(Chan *et al.*, 2010)
Erwinia carotovora GS101	Restrictionless, modificationless derivatives that produces OC6‑HSL for the regulation of its production of carbapenem.	(McGowan *et al.*, 1995)
Erwinia carotovora PNP22	Mutants derive from GS101 with defective *carI* gene which code for the production of AHL synthase. It serve as the negative control for the cross streak due to its inability to synthesize OC6‑HSL.	(McGowan *et al.*, 1995)
Agrobacterium tumefaciens NTL4 (pZLR4)	Biosensor that based on the *traI/R* to detect a broad range of AHLs	(Farrand *et al.*, 2002)

3.1.5 Primers

Table 3.2: Primer Used to Amplify16S rRNA, and to Screen for the Presence of Insert in pGEM®-T.

Name	Sequence	Reference
27F	AGA GTT TGA TCM TGG CTC AG	(Chong *et al.*, 2012)
1525R	AAG GAG GTG WTC CAR CC	(Chong *et al.*, 2012)
515F	GTG CCA GCM GCC GCG GTA A	(Budsberg *et al*, 2003)
1174F	GAG GAA GGT GGG GAT GAC GT	(Geerlings *et al.*, 2001)
SP6	ATT TAG GTG ACA CTA TAG	(Burrell *et al.*, 1998)
T7	TAA TAC GAC TCA CTA TAG GG	(Burrell *et al.*, 1998)

3.2 Methods

3.2.1 Isolation of Potential Fish Pathogen

Three diseased tilapia fish were transported on ice to the laboratory from the fish pond of a local fish farm in 2009. Fish surface was sterilized with 70 % (v/v) ethanol, followed by swabbing the gills and inner muscle of the fishes with sterile cotton swab as there was no significant lesion at the fish surface. The cotton swab was immersed in 1.0 ml of 1 × PBS and diluted hundred fold before spreading 100 µl of each sample on LB agar followed by incubation in 28 ℃. The bacterial isolates were maintained at 28 ℃.

3.2.2 Gram Stain

A drop of bacteria culture was smeared and heat fixed onto glass slide. Sample was stained with crystal violet followed by washing the stain under the running tap

water for 30 s. Next, iodine was applied onto the slide for 1 min as the mordant to trap crystal violet in cell wall of Gram-positive bacteria, preventing the stain from being removed during destaining process with acetone. Lastly, the sample was counterstained with safranin. The stained samples were viewed under microscope (Olympus, Japan), at 100 × magnification with the aid of emersion oil.

3.2.3 Genomic DNA Extraction

Bacterial genomic DNA was extracted by using QIAamp DNA Mini kit (Qiagen, USA) as described by manufacture in the user manual. Genomic DNA was eluted with 100 μl of elution buffer and kept in -20 ℃.

3.2.4 Agarose Gel Electrophoresis (AGE)

AGE was performed with 1.0 % (w/v) agarose gel, submerged with Tris Borate EDTA (TBE) buffer at 80 V, 400 mA and 40 min. The gel was pre-stained with 0.5 mg/ml of ethidium bromide (EtBr). The stained agarose gel was viewed under the UV illuminator (UVP, USA) and the desired DNA band(s) was determined by comparing the size of DNA band to 1 kb DNA ladder.

3.2.5 Bacterial Identification

The identities of the isolates were identified by their 16S rRNA nucleotides sequences. Genomic DNA of the bacteria was extracted as described in Section 3.2.3. The 16S rRNA was amplified with primer pair 27F forward primer and 1525R reverse

primer (Chong *et al*., 2012). PCR was performed at the following condition where it was programmed to be 1 cycle of denaturation at 94 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 63 ℃ for 30 s and extension at 72 ℃ for 90 s, with 1 cycle of final extension at 72 ℃ for 7 min. The PCR products of 16S rRNA amplification were checked and purified from the gel via AGE as described at Section 3.2.4.

3.2.6 PCR Product Purification

After performing AGE as described in Section 3.2.4, the desired PCR product was purified from agarose gel with QIAquick PCR Purification Kit (Qiagen, USA) as per the manufacturer's instruction. Purified DNA was eluted with 50 μl of sterile distilled water and kept at -20 ℃.

3.2.7 Competent Cell

Escherichia coli DH5α chemically competent cell was prepared according to Russell Sambrook *et al*., (2001). First, 100 ml of sterile LB broth was seeded with 1 ml of overnight *E. coli* DH5α culture and was incubated at 37 °C shaking incubator until the OD_{600} reaches 0.5. The culture was placed on ice for 30 min to slow down the cell activity and later it was pelleted at 8000 × *g* for 10 min. The cell pellet was washed twice with 10 ml of 1 M calcium chloride ($CaCl_2$). Finally, the cell pellet was resuspended with 1 ml of 100 mM $CaCl_2$ gently followed by incubation in 4 ℃ for 16 hr in order to increase the competency before adding 16 % (v/v) glycerol and stored at -80 ℃.

3.2.8 Ligation and Transformation

The purified PCR product was inserted into pGEM®-T (Promega, USA) as descript in the manufacture protocol. This mixture was incubated overnight at 4 ℃ without shaking before transforming into the host *i.e E. coli* DH5α competent cell.

Prior to transformation, competent cells were thawed on ice before used. It was added with 2 µl of the ligation product and the tube was tapped gently to mix the reaction. The mixture was placed on ice for 10 min followed by heat shock transformation at 42 ℃ for 40 s. Next, the cell was immediately transferred to ice for 2 min.

For recovery, 500 µl of SOC was added to the transformant and it was immediately incubated at 37 ℃ for 90 min before spreading on the LB agar plates added with 100 µg/ml ampicillin and 20 µg/ml X-gal.

White colonies resulted from the disruption of *lacZ* gene during insertion will be selected for the screening. The screening of transformant was performed by PCR using primers SP6 and T7 (Burrell *et al*., 1998). The PCR condition was set at 1 cycle of denaturation at 94 ℃ for 5 min, followed by 25 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s and extension at 72 ℃ for 90 s, with 1 cycle of final extension at 72 ℃ for 7 min. The PCR product was subjected to AGE as described at Section 3.2.4.

3.2.9 Plasmid Extraction

Transformant was cultured in LB broth supplemented with 100 μg/ml ampicillin at 37 ℃ for 18 hr prior to plasmid extraction. Plasmid extraction was performed as described in the user manual of QIAprep Spin Miniprep kit (Qiagen, USA). Plasmid was eluted with 50 μl of elution buffer.

The extracted plasmid was sent to First Base Laboratories for Sanger sequencing. The 16S rRNA sequence was sequenced using primers SP6 and T7 and two internal primers namely 515F and 1174F (Budsberg *et al*., 2003; Burrell *et al*., 1998).

3.2.10 Phylogenetic Analysis

DNA fragments were aligned with MEGA (version 5) with Cluster W algorithm (Tamura *et al*., 2011). The aligned 16S rRNA sequence was compared against National Center for Biotechnology Information (NCBI) 16S ribosomal RNA gene sequences database via BLASTN, whereas the rest of the aligned sequences were compared with the NCBI-nt database. Next, the phylogenetic trees were constructed with MEGA (version 5) based on maximum-likelihood (Tamura-Nei model) analysis with bootstrap value of 1000, expressed as percentages of 1000 replicates which are shown at branch (Tamura *et al*., 2007; Tamura *et al*., 2011). The gene nucleotide sequences were then deposited into GenBank.

3.2.11 Screening for Hemolytic Activity

Hemolytic activity of bacteria isolated from diseased tilapia fish was screened with 5.0 % (v/v) sheep blood agar (BD, USA). The presence of hemolytic activity was detected with the presence of halo-zone on the agar.

3.2.12 Screening for Proteinase Activity

Bacteria isolated from diseased tilapia fish was screened for proteinase activity using skimmed milk agar (containing 0.05 % (w/v) yeast extract, 0.1 % (w/v) tryptone, 1.0 % (w/v) NaCl and 1.5 % (v/v) skimmed milk, solidified with 1.5 % (w/v) bacto agar. The presence of proteinase activity was shown with the presence of halo zone on the agar.

3.2.13 Screening of QS activity

Bacteria were screen for the presence of short chain AHLs using cross streaking approach with the biosensor CV026. *E. carotovora* GS101 served as positive control while *E. carotovora* PNP22 served as negative control. Purple pigment production by CV026 after one day incubation at 28 ℃ indicates the production of short chain AHL by the bacteria.

3.2.14 AHL Extraction

A loop full of bacteria was cultured overnight in 5.0 ml of LB broth buffered with 50 mM MOPS. A total of 1.0 ml of the bacterial culture was sub-cultured into 200 ml of

fresh LB-MOPS and incubated for 18 hr. The spent supernatant was extracted three times with equal volume of acidified ethyl acetate (0.1 % (v/v) acetate acid). The extract was air dried in fume hood and kept in -20 ℃ (Wong *et al*., 2012).

3.2.15 Thin Layer Chromatography (TLC)

The extracted AHL as described in Section 3.2.14 was resuspended in 100 μl of acetonitrile (ACN). Characterization of long chain AHLs was performed by using normal phase TLC plate, 20 cm × 20 cm, Silica gel 60, F_{254}, (Merck, Germany). The TLC plate was buffered by 5 % (v/v) KH_2PO_4 and was dried at 90 ℃ for an hour. After drying, 10 μl of the AHL extracts were spotted on the normal phase TLC plate and 0.05 μg of synthetic *N*-dodecanoyl-$_L$-homoserine lactone (C12-HSL) and 0.05 μg of *N*-(3-oxohexadecanoyl)-$_L$-homoserine lactone (OC16-HSL) were used as standard for this assay. It was chromatographed with 95 % (v/v) dichloromethane: 5 % (v/v) methanol. Two days before developing the TLC plate, *A. tumefaciens* NTL4 pZLR4 was cultured in AB medium at 28 ℃. The developed TLC plate was air dried before overlaying the biosensor seeded AB agar supplied with 0.5 % (w/v) glucose and 150 μg/ml gentamycin on the TLC plate (Chang *et al*., 2012; Yin *et al*., 2012).

3.2.16 Liquid Chromatography Mass Spectrometry/Mass Spectrometry (LCMS/MS)

Further characterization of the QS signalling molecules produced by the bacteria was carried out by LCMS. Before the LCMS analysis, AHLs extracted from 2 ml of spent supernatant were dissolved in 150 μl of ACN. Next, 100 μl of the dissolved extract was sent for LCMS analysis using Agilent RRLC 1200 system for separation

and Agilent 6500 Q-TOF MS/MS (Agilent, Germany) for high resolution mass spectrometry. The column used for chemical analysis was Agilent ZORBAK Rapid Resolution HT column (Agilent, Germany). The analysis was performed according to the reported method by Ortori *et al*., (2011). In brief, the gradient elution was performed with water and ACN constituted 0.1 % (v/v) formic acid as mobile phase. The analysis started with an isocratic elution of 10 % (v/v) of ACN, followed by a linear gradient elution which increases the 10 % of ACN to 50 % over 0.5 min. Next, a second gradient elution was performed in which the mobile phase was increase from 50 % (v/v) ACN to 99 % (v/v) over 4 min. Lastly, an isocratic elution was performed with 99 % of ACN over 1.5 min. After each round of analysis, the column was re-equilibrated for 2.9 min before the next injection. This analysis was conducted at 45 ℃ with the flow rate of 0.4 ml/min.

3.2.17 Resting Cell Preparation

A loop of bacteria was cultured overnight at 28 ℃ except *E. coli* DH5α was cultured at 37 ℃. The absorbance OD_{600} of the overnight culture was adjusted to OD_{600} = 1.0 before pelleting the cell at 5000 × *g*. The bacteria cells were washed three times with 200 μl of PBS. Lastly, it was resuspended in 100 μl of PBS.

3.2.18 Whole Cell Inactivation Assay

Synthetic C4-HSL and C6-HSL with the final concentration of 0.5 μg/μl was air dried in a 1.5 ml tube. Next, 100 μl of resting cell was added to the tube followed by incubation of 0 hr and 24 hr at 28 ℃. Heat inactivation was carried out at 95 ℃ for 3

min. Lastly, 10 µl of each sample was spotted on agar seeded with CV026 and was incubated at 28 ℃ for purple pigment formation.

CHAPTER 4.0

RESULTS

4.1 Bacteria Identification

Five bacteria were isolated from diseased tilapia fish using LB Agar. Strain W2.3 produced red pigment and possessed swarming characteristic; strain W3.1 produced green pigment and possessed swimming characteristic; strain W3 appeared as white round colony; strain W2.2 appeared to be white colony with uneven shape and edge, with flat rough surface while strain W4.2 appeared to be smooth, raised round colony on LB agar plates. Gram stain profile showed that strain W2.3, W3.1, W3 and W4.2 to be Gram-negative bacteria while strain W2.2 is a Gram-positive bacterium.

The % of similarity of the isolates' 16S rRNA genes nucleotides with the 16S rRNA available in the database and the phylogenetic analysis (refer Figure 4.1) showed that strain W2.3 was assigned to *S. marcescens*, strain W3.1 was assigned to *P. aeruginosa*, strain W3 was assigned to *Pseudomonas* sp., strain W2.2 was assigned to *Bacillus* sp. and strain W4.2 was assigned to *Klebsiella oxytoca*. The 16S rRNA sequence of strain W2.3, W3.1, W3, W2.2, and W4.2 can be obtained from NCBI with the accession number of JF317350.1, JF317349.1, JF487789.1, JF487790.1 and JF317350.1.
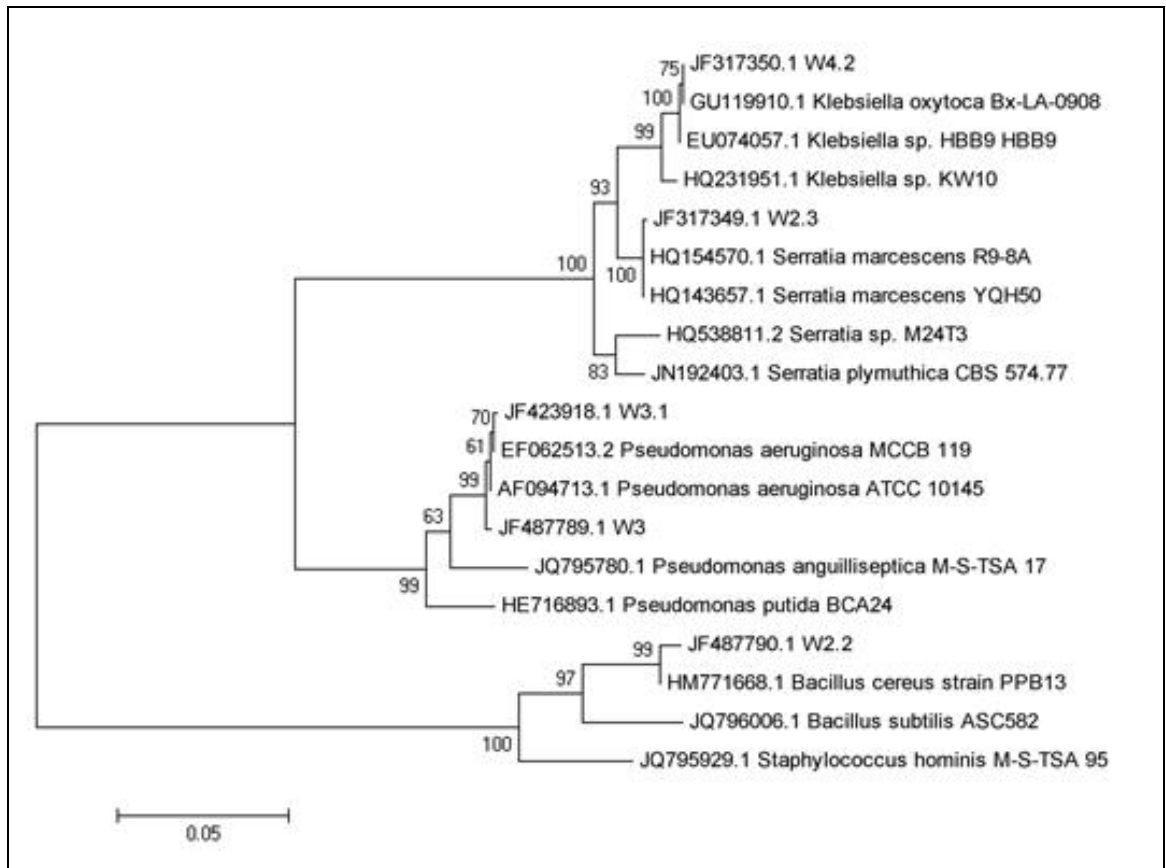
Figure 4.1 Phylogenetic analysis of 16s rRNA sequences of strain W2.3: *Serratia marcescens*, W3.1: *Pseudomonas aeruginosa*, W3: *Pseudomonas* sp., W2.2: *Bacillus* sp. and W4.2: *Klebsiella oxytoca*. The evolutionary distance is 0.05 changes per nucleotide position.

4.2 Screening for Virulence Factors Expression

Hemolysin and protease are important virulence factors that enable the bacteria to infect its host. Thus, hemolysin production was screened using 5.0 % (v/v) sheep blood agar. Similarly, proteinase activity was screened using 1.5 % (v/v) of skimmed milk agar. The production of hemolysin and exoprotease productions were determined by the presence of clear zone around the bacteria colony after one day of incubation.

Based on Figure 4.2 (A), *Bacillus* sp. strain W2.2, *Pseudomonas* sp. strain W3 and *P. aeruginosa* strain W3.1 showed the formation of a halo zone around its colony,

28

indicating the presence of hemolytic activity. Meanwhile almost all strains isolated from diseased tilapia fish, except *K. oxytoca* strain W4.2 produces proteinase. This can be observed from the formation of clear zone around the colony (Figure 4.2(B)).

4.3 Screening for Short Chain AHL Production by CV026

Production of purple colour pigment, *i.e.* violacein, was induced by the presence of exogenous short chain AHLs (McClean *et al*., 1997). *E. carotovora* GS101 which carries *carI* gene that responsible for the production of OC6-HSL was used as the positive control while the *carI* defect mutant *E. carotovora* PNP 22 served as negative control (McGowan *et al*., 1995). Among the tested isolates, only *P. aeruginosa* strain W3.1 triggered the purple pigment formation of CV026 (Figure 4.3).
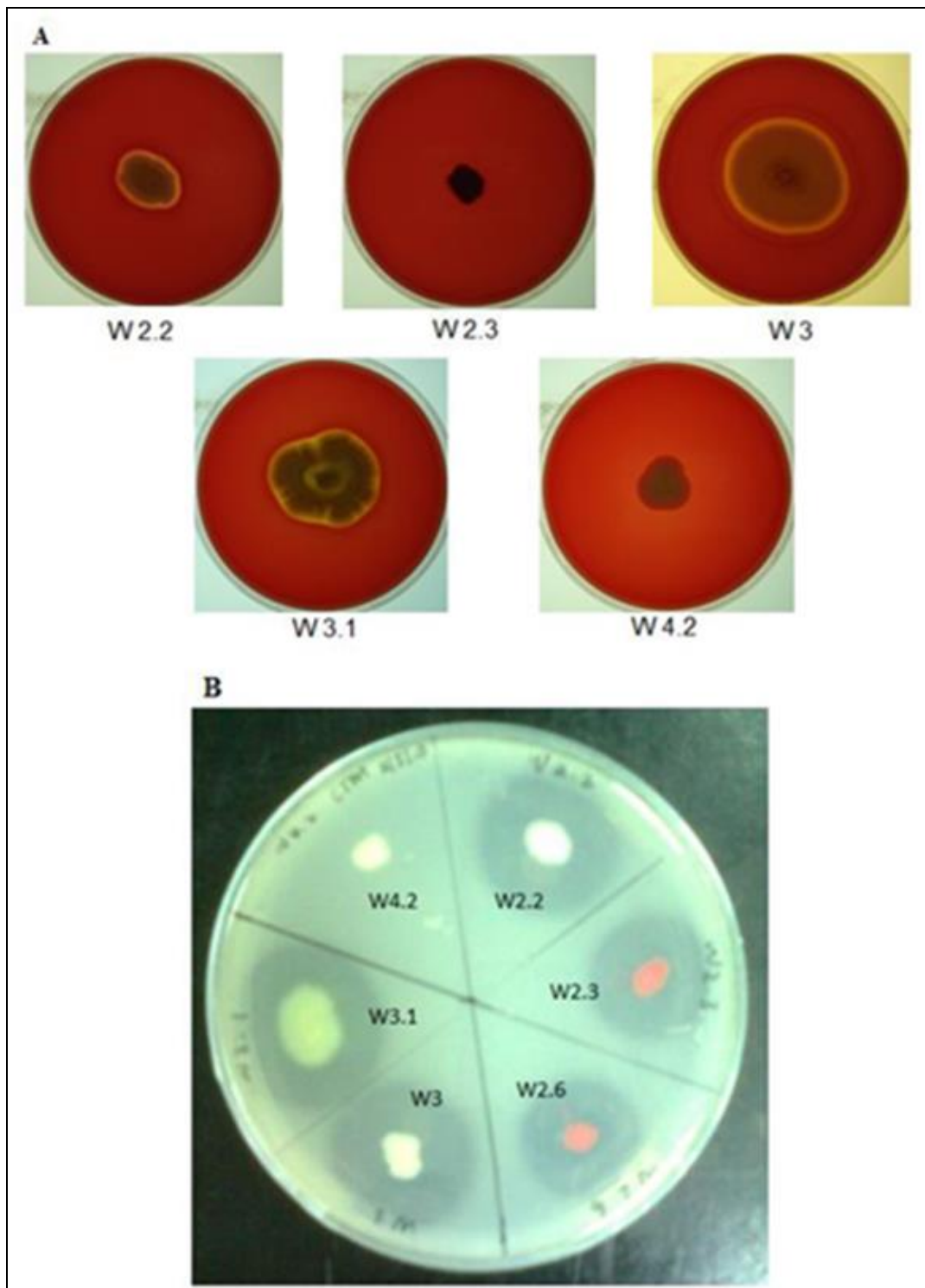
Figure 4.2 Screening of virulence factor, *i.e.* hemolysin, with (A) 5.0 % (v/v) sheep blood agar for hemolytic activity. *Bacillus* sp. strain W2.2, *Pseudomonas* sp. strain W3 and *P. aeruginosa* strain W3.1 produced hemolysin and caused lysis of sheep blood. (B) 1.5 % (v/v) skimmed milk agar for proteolytic activity. *Bacillus* sp. strain W2.2, *Pseudomonas* sp. strain W3, *P. aeruginosa* strain W3.1 and *S. marcescens* strain W2.3 produced protease and degraded casein.
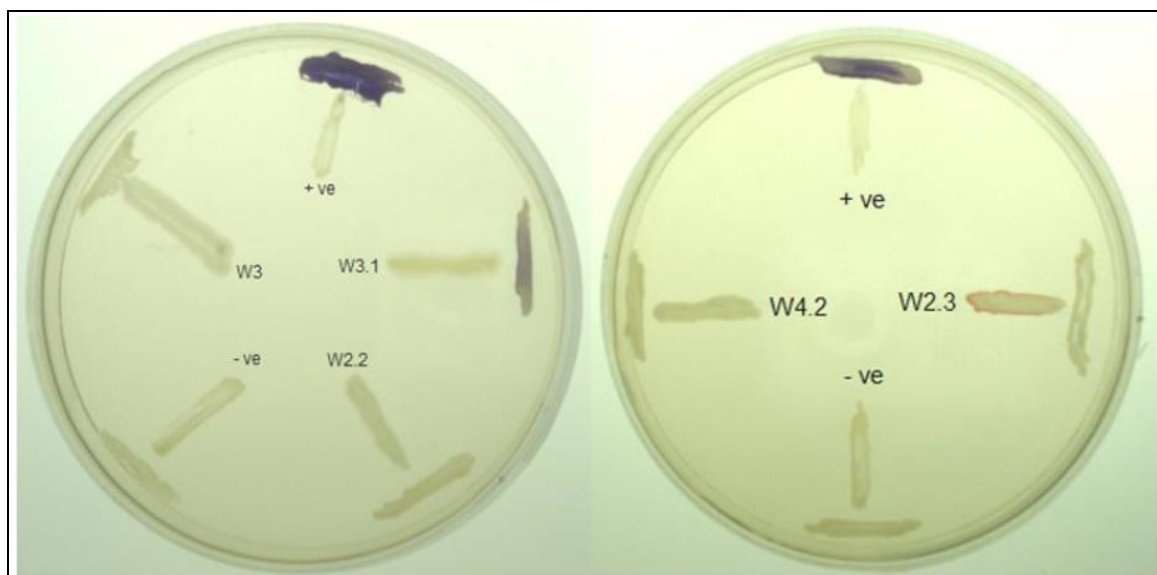
Figure 4.3 Screening of short chain AHL production with CV026. The AHL produced by the bacteria will induced the purple pigment formation of CV026. *E. carotovora* GS 101 is the positive control whereas *E. carotovora* PNP22. Only *P. aeruginosa* strain W3.1 induced the purple pigment formation.

4.4 Thin Layer Chromatography (TLC)

4.4.1 TLC Analysis of AHLs Extracts of the Isolates from Diseased Tilapia Fish

The chromatographed TLC was overlaid with AB agar lawn, seeded with biosensor *A. tumefaciens* NTL4 pZLR4 as described in Section 3.2.15. Different types of AHLs presence were separated according to their polarity. The cleavage of chromophor of X -gal occurred when *A. tumefaciens* NTL4 pZLR4 was activated by the presence of AHLs. Hence, blue pigment formation was observed (Adonizio *et al*., 2006). The retardation factor (R_f) were calculated based on the formula:

$$R_f = \frac{\text{Migration Distance of substance}}{\text{Migration Distance of solvent front}}$$

From the TLC (Figure 4.4), the R_f value of synthetic C12 -HSL is 0.82 and 0.57 for synthetic OC16 -HSL. By comparing the chromatograms of AHL extracted from

strain W3.1 suggesting the production both OC12‑HSL and OC16‑HSL, but the R_f value cannot be calculated as the blue spots for different types of AHLs were merge. Technique with higher sensitivity and stronger separation power was required to fully characterize the AHLs produced by this strain. Strain W3.1 was suspected as OC12‑HSL producer instead of C12‑HSL as the blue spot in the chromatogram carries a "comet" tail that resembled the characteristic of an AHL with 3-oxo substituted site chain. The R_f value of the AHL produced by *S. marcescens* strain W2.3 is 0.80. By comparing the migration distance between the sample and standard it was detected to be C12‑HSL.
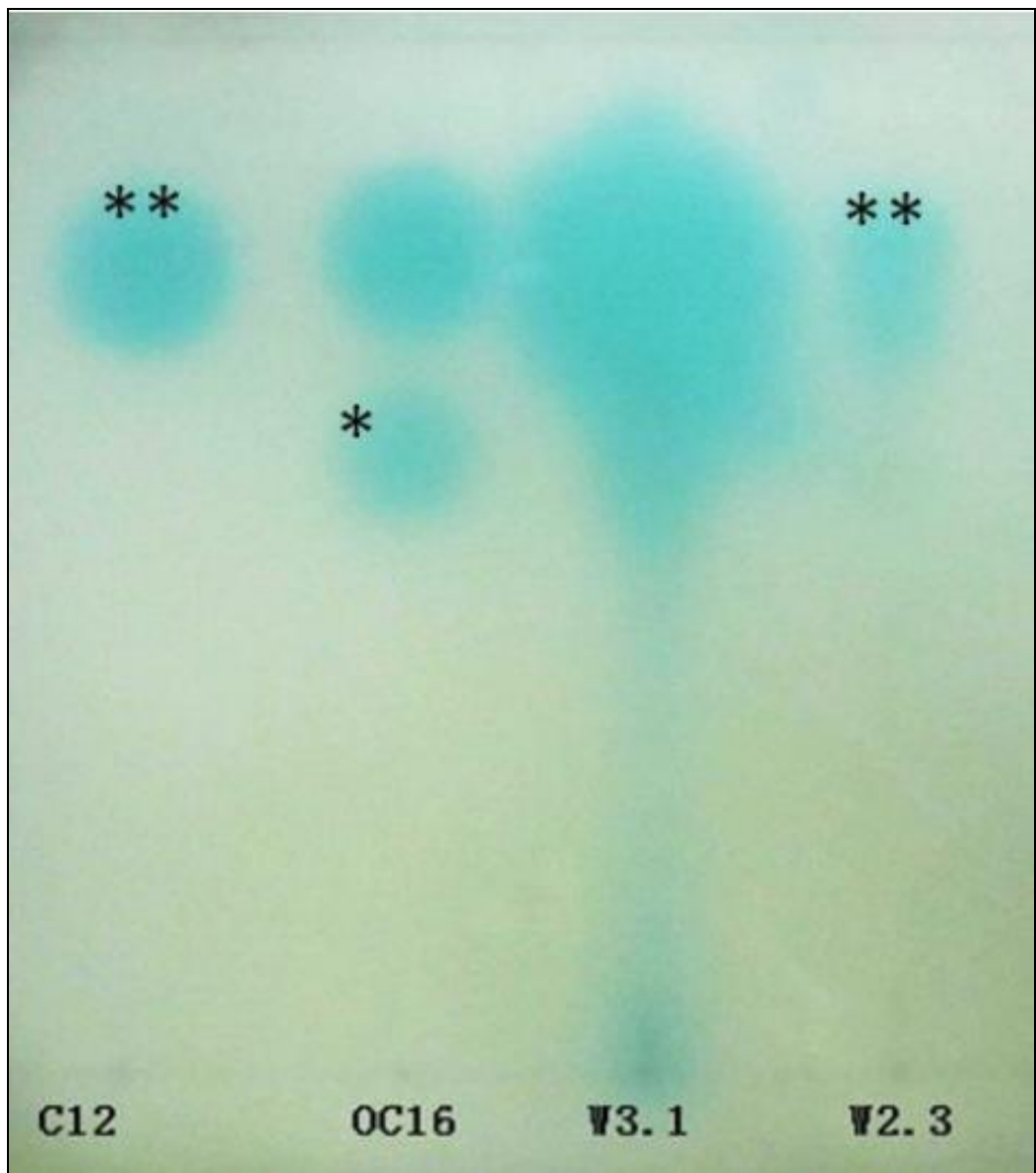
Figure 4.4 Characterization of long chain AHL produced by *P. aeruginosa* strain W3.1 and *S. marcescens* strain W2.3 by *A. tumefaciens* NTL4 pZLR4 with thin layer chromatography. C12-HSL is mark by as "**" while OC16-HSL is marked as "*".*P. aeruginos* strain W3.1 produces of both C12‑HSL and OC16‑HSL while *S. marcescens* strain W2.3 produces C12-HSL.

4.5 LCMS/MS

AHL extracted as described in Section 3.2.14 were subjected to LCMS/MS analysis in order to compensate the sensitivity of biosensor and also the low separation resolution of TLC. The presence of different of QS signalling molecules were

confirmed by the detection of its precursor ion at certain retention time with the aid of reference to the standard of each molecule.

4.5.1 Characterization of AHL Molecules Produces by the Isolates from Disease Tilapia Fish using LCMS/MS

Only *P. aeruginosa* strain W3.1 produced short chain AHL while both *P. aeruginosa* strain W3.1 and *S. marcescens* strain W2.3 produces long chain AHLs. The mass-to-charge ration (*m/z*) of the precursor ion received from the chemist confirmed the occurrence of C4-HSL (*m/z*: 172), OC12-HSL (*m/z*: 298) and OC16-HSL (*m/z*: 354.26) (Figure 4.5 (A)) from strain W3.1. The precursor ion of C12-HSL (*m/z*: 288.22) was detected from the AHL extract confirmed the production of C12-HSL by *S. marcescens* strain W2.3 (Figure 4.5 (B)). In addition to AHLs, *P. aeruginosa* strain W3.1 also produces 2-heptyl-3-hydroxy-4(1H)-quinolone (C7-PQS) (*m/z*: 260), 2-heptyl-4-hydroxyquinolone (C7-AQ) (*m/z*: 244), 2-hydroxy-2-nonyl-4(1H)-quinolone (C9-PQS) (*m/z*: 288), 2-nonyl-4-hydroxyquinolone (C9-AQ) (*m/z*: 272), 2-heptyl-4-hydroxyquinoline *N*-oxide (C7-*N*-Oxide) (*m/z*: 260), and 2-nonyl-4-hydroxyquinoline *N*-oxide (C9-*N*-Oxide) (*m/z*: 288).
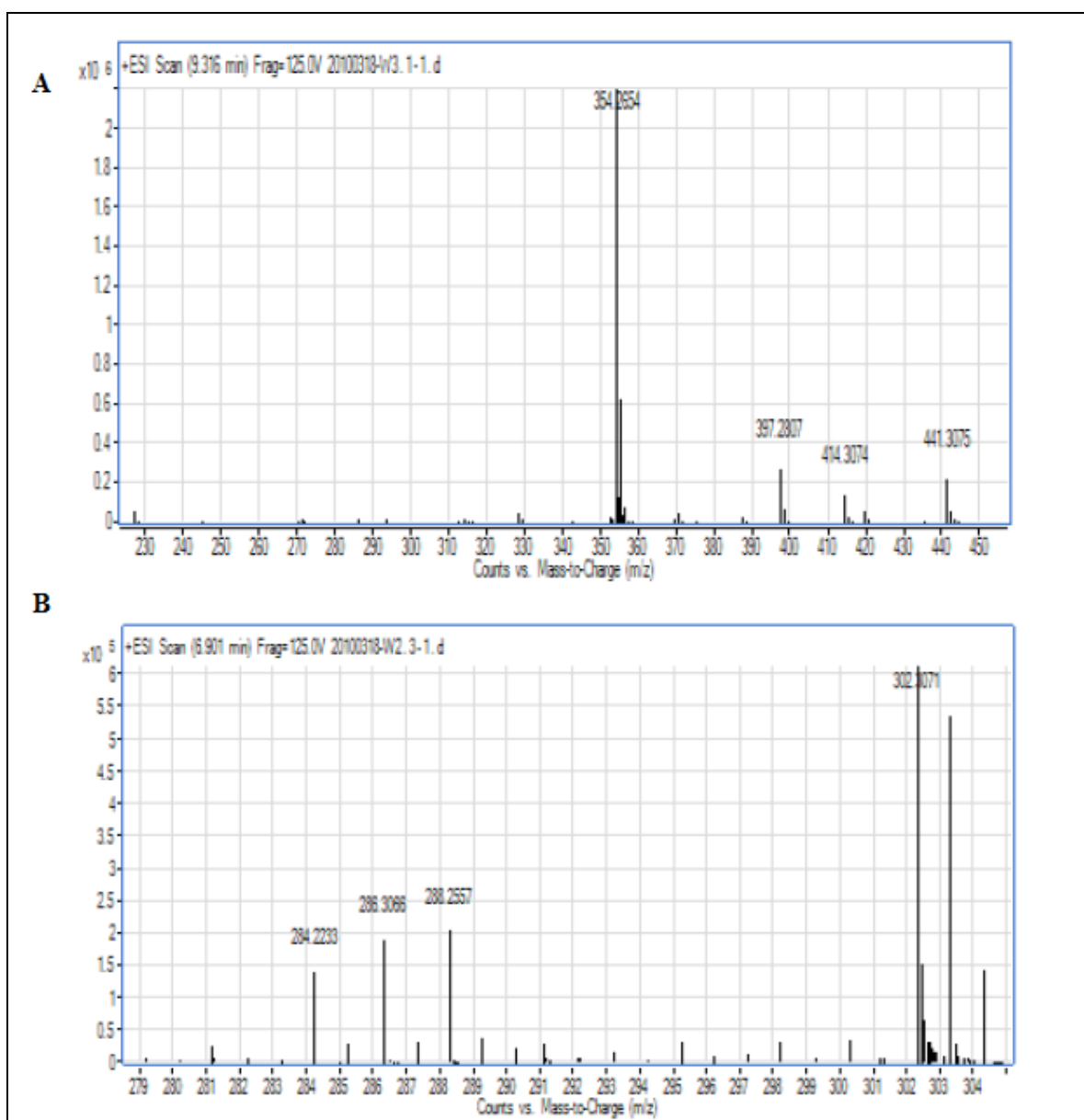
Figure 4.5 LCMS spectrum (A) *P. aeruginosa* strain W3.1 which was detected to produce OC16-HSL (*m/z* 354.26) and (B) *S. marcescens* strain W2.3 which was detected to produce C12-HSL (*m/z* 284.2233).

In summary, the AHL molecules producer isolated from diseased tilapia are as listed in Table 3. AHL molecules producers were *P. aeruginosa* strain W3.1 and *S. marcescens* strain W2.3. *P. aeruginosa* strain W3.1 produced both short and long chain AHLs, namely the C4-HSL, OC12-HSL and OC16-HSL. On top of AHLs, *P. aeruginosa* W3.1 also produced C7-PQS, C7-AQ, C9-PQS, C9-AQ, C7-*N*-Oxide, and C9-*N*-Oxide. *S. marcescens* strain W2.3 only produced C12-HSL.

35

Table 4.1: Summary of QS Molecules Produced by Isolates from Diseased Tilapia Fish

Strains	Bacteria Identity Based on 16S rRNA Gene	QS Signalling Molecules Detected by LCMS (*m/z* ration)
W3.1	*P. aeruginosa*	C4-HSL (*m/z*:172) OC12-HSL (*m/z*:298) OC16-HSL (*m/z*: 354.26) C7-PQS (*m/z*: 260) C9-PQS (*m/z*:288) C7-AQ (*m/z*:244) C9-AQ (*m/z*: 272) C7-*N*-Oxide (*m/z*: 260) C9-*N*-Oxide (*m/z*: 288)
W2.3	*S. marcescens*	C12-HSL (*m/z* :284.22)
W2.2	*Bacillus* sp.	Not Detectable
W3	*P. aeruginosa*	Not Detectable
W4.2	*K. oxytoca*	Not Detectable

4.6 Screening for QQ Activity

The degradation of the AHL was determined by the decrease in pigmentation or loss of pigment after 24 hr of incubation. *B. cereus* which was reported to be an AHL degrader served as the positive control for this QQ assay (Chan *et al*., 2010). The negative controls used in this assay involved the suspension buffer namely PBS buffer and *E. coli* DH5α cells (Taylor *et al*., 1993).

S. marcescens strain W2.3, *Bacillus* sp. strain W2.2, *K. oxytoca* strain W4.2, and *P. aeruginosa* strain W3.1 did not show any degradation on C4‑HSL (Figure 4.6(A)) and C6‑HSL (Figure 4.6 (B)) as no pigment loss was observed. Only *Pseudomonas* sp. strain W3 was able to degrade C4‑HSL as shown in Figure 4.6 (A) where there was a significant loss of pigment at 24 hr. In addition to C4‑HSL, C6‑HSL was also observed to be degraded by *Pseudomonas* sp. strain W3 as the purple pigmentation was reduced after 24 hr incubation.
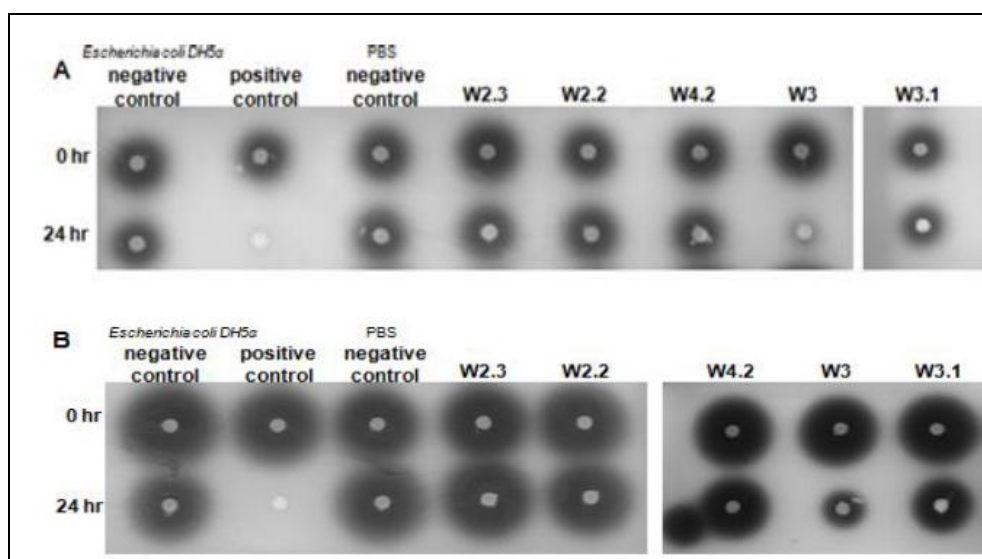
Figure 4.6 Residual of (A) C4‑HSL and (B) C6‑HSL was incubated with the bacteria for 0 and 24 hr. The AHL degradation was determined by either the decrease of pigment size or loss of pigment for the residual of 24 hr incubation.

CHAPTER 5.0

DISCUSSION

5.1 Bacteria Isolated from Diseased Tilapia Fish

The increasing demand of tilapia fish in domestic market of Malaysia has made it an important aquaculture product to Malaysia (Josupeit, 2005). In year 2009, the endemic disease has caused a hard hit to the fishery and aquaculture industry in Terrenganu, Malaysia. The aquaculture authority suspected the tilapia fishes are infected by bacteria which led them to seek for our aid for the search of causal agent. The 16S rRNA phylogenetic analysis identify the bacteria isolated from the fish tissue belongs to *Pseudomonas* sp., *S. marcescens*, *Klebsiella* sp. and *Bacillus* sp.. Since most of these bacteria are opportunistic pathogen to eukaryotes, thus, they might be the causal agent for this endemic disease.

Pseudomonas sp. was frequently isolated from freshwater fish or from the fish pond and could be the potential pathogen to the tilapia fish (Allen *et al*., 1983; Apun *et al*., 1999; Eissa *et al*., 2010). *Pseudomonas* sp. has been reported infecting freshwater fish such as, catfish (Meyer & Collar, 1964). A test on the effects of *Pseudomonas* sp. infection on tilapia fish has been carried out by Eissa *et al*, (2010) with different species of *Pseudomonas*. According to this study, *Pseudomonas* infection causes death of tilapia fish in which infection of tilapia fish by *P. angulliseptica* was the most lethal among the tested *Pseudomonas* species (Eissa *et al*., 2010).

S. marcescens is a normal microflora in various environment including soil and freshwater (Hejazi & Falkiner, 1997). It is well known as a human opportunistic pathogen which often associated with nosocomial infection (Hejazi & Falkiner, 1997; Kurz *et al*., 2003; Patterson *et al*., 2002). In addition to human, certain strain of *Serratia* has evolved into highly pathogenic strains attacking both freshwater and marine fishes as well as invertebrate (Aydin *et al*., 2001; Baya *et al*., 1992). However, to date, not much information regarding the *S. marcescens* infecting of freshwater fish are available.

Bacillus sp. and *Klebsiella* sp. were naturally occurs in freshwater and were found in the intestine of freshwater fish (Apun *et al*., 1999; Austin, 2002; Kunst *et al*., 1997). Amylase producing *Bacillus* which aids in starch degradation has been isolated from the intestine of freshwater fish (Apun *et al*., 1999; Sugita *et al*., 1997). This is important for freshwater fish such as carps and tilapia where their foods are high in starch content (Sugita *et al*., 1997). However, there is also a report stating *Bacillus* could serve as the causal agent of freshwater fish septicemia condition in fish farm which leads to the death of the fishes (Oladosu *et al*., 1994).

Unexpectedly, *Streptococcus* which is the common freshwater fish disease causal agent was not found from the death infected fish. *Streptococcus* has causes several outbreaks to the tilapia fish farm around the world possess high morbidity and mortality rate of the fish (Sun *et al*., 2007; Weinstein *et al*., 1997). *Streptococcus*-infected tilapia fish turns lethargic, swims erratically, suffer dorsal rigidity, and eventually die if left without treatment (Weinstein *et al*., 1997). This infection is one of the most serious threats to tilapia industry (Pretto-Giordano *et al*., 2009). The effect of infected fish

towards public health gained more concern when the disease was passed from fish to human who had physical contact with the fish (Sun *et al*., 2007; Yagoub, 2009).

5.2 Virulence Factors and AHLs Detection

All isolates were screened for exoprotease and hemolysin production. Titball *et al*., (1985), founds the relations between caseinase and protease whereby the inhibition of caseinase from fish pathogen, *Aeromonas salmonicida* contributes to the suppression of hemolysin production. Proteolytic enzyme production is directly correlated with *Pseudomonas* pathogenicity as the concentration of the protease presence affects its invasiveness (Pavlovskis & Wretlind, 1979). In this assays, *S. marcescens* strain W2.3 showed exoprotease production while *P. aeruginosa* strain W3.1, *Pseudomonas* sp. strain W3, *Bacillus* sp. strain W2.2 were able to produce both exoprotease and hemolysin. The production of hemolysin and exoproteases suggested that these bacteria acquire nutrients from its host cells (Chan, 2009).

Since QS plays an important role in regulating the expression of virulence genes in certain bacteria, all isolates were subjected to the screening for the AHL and virulence factor production. In the studies, with the exception of *Bacillus* strain W2.2, all isolates are taxonomically classified in to the phylum of *Proteobacteria*. The major QS signalling molecules produced and utilized by *Proteobacteria* are AHLs (Case *et al*., 2008), therefore this study focused on the AHL characterization.

As *Pseudomonas* sp., *S.marcescens* and *Klebsiella* sp. are mainly short chain AHLs producer, a rapid detection of short chain AHL was hence conducted using biosensor CV026 (Van Delden & Iglewski, 1998; Van Houdt *et al*., 2007; Wilder *et al*., 2011). Only *P. aeruginosa* strain W3.1 triggered the purple pigment formation of CV026, indicating the production of short AHL. On the other hand, long chain AHL was detected from the spent supernatant of *P. aeruginosa* strain W3.1 via TLC with the aid of biosensor *A. tumefaciens* NTL4 pZLR. However TLC failed is not an accurate method to characterize the long chain AHL presence in the spent supernatant of *P. aeruginosa* strain W3.1.

Similar to the previously reported finding, C4-HSL and OC12-HSL were found in the spent supernatant extract of *P. aeruginosa* strain W3.1, as confirmed by LCMS/MS (Diggle *et al*., 2003; Zhu *et al*., 2004). These AHLs were shown to regulate the production of virulence factors, including hemolysin and proteases (elastase and alkaline protease) of *P. aeruginosa* (Zhu *et al*., 2002). Aside from C4-HSL and OC12-HSL, OC16-HSL was also detected from the spent supernatant of *P. aeruginosa* strain W3.1. This is the first report of *P. aeruginosa* producing OC16-HSL. To my knowledge, this is the AHL with the longest side chain synthesized by *P. aeruginosa*, but further studies are required in order to further understand the function of OC16-HSL produced by this *P. aeruginosa*.

Although AHL is the most studied QS signalling molecules, the function and production of long chain AHLs such as *N*-hexadecanoyl-L-homoserine lactone (C16-HSL) are poorly understood. Most of the long chain AHLs was identified in

α ‑Proteobacteria. For instance, *Rhodobacter capsulates* and *Paracoccus denitrificans* produces C16 ‑HSL, *Acidithiobacillus ferroxidans* produces 3 ‑hydroxy ‑hexadecanoyl ‑ᴌ‑ homoserine lactone (OHC16 ‑HSL) and *Sinorhizobium meliloti* produces OC16 ‑HSL and C16 ‑HSL (Farah *et al*., 2005; Marketon *et al*., 2002; Rivas *et al*., 2005; Schaefer *et al*, 2002). In *R. capsulates*, C16 ‑HSL is responsible in activating the production of gene transfer between *Rhodobacter* and its host (Schaefer *et al*., 2002). Meanwhile, QS of *Sinorhizobium meliloti* plays a role in controlling its symbiosis, and its long chain AHL might involve in nodulation efficiency *i.e.* the pink nodule formation (Marketon *et al*., 2002).

In addition to AHLs, PQS, HHQ and its *N* ‑oxide derivative were detected from spent supernatant of *P. aeruginosa* strain W3.1. These three compounds are inter ‑related where HHQ is the intermediate of PQS while *N* ‑oxide is synthesed via the similar AHQ synthesis pathway for HHQ synthesis (D éziel *et al*., 2004; Machan *et al*., 1992). Both PQS and HHQ are QS signaling molecules that regulate secondary metabolites production, virulence factor expression, and extracellular enzyme production (Dubern & Diggle, 2008; Gallagher *et al*., 2002; Pesci *et al*., 1999). On the other hand, the *N* ‑oxides PQS produced by *P. aeruginosa* is not classified as QS signalling molecule. It is an antibacterial compound against Gram positive bacteria. According MaChan *et al*, (1992), the production of PQS *N* ‑oxide by *P. aeruginosa* inhibits the growth of *Staphylococcus aureus* and this result explains the absences of *S. aureus* in the sputum of cystic fibrosis patient.

Production of virulence factors namely swarming ability, biofilm formation, protease and nuclease production of *Serratia* are regulated by its AHL-based QS system (Morohoshi *et al*., 2007; Wei & Lai, 2006; Wei *et al*., 2006; Williams, 1973). However, instead of synthesizing C_4 to C_8 HSL as previous reported, TLC and LCMS/MS results showed that *S. marcescens* W2.3 produced C12-HSL (Horng *et al*., 2002; Thomson *et al*., 2002; Van Houdt *et al*., 2007). The utilization of C12-HSL in regulating the virulence factor expression has been reported in *P. aeruginosa* (Erickson *et al*., 2002). However, more work is required to investigate the relationship between the C12-HSL produce by *S. marcescens* strain W2.3 and its regulation on pathogenicity.

5.3 QQ Activity

Bacteria degrade AHLs in the environment in order to acquire carbon and nitrogen for survival (Chan *et al*., 2010; Hong *et al*., 2012). Besides, it also serves as advantage towards survival in the competitive environment (Park *et al*., 2008). Since members of *Bacillus*, *Pseudomonas*, and *Klebsiella* genera have been reported to be degrade AHLs, hence in this study, C4-HSL and C6-HSL were used as the substrate of AHL degradation assay.

P. aeruginosa PAO1, a human pathogen harbors both QS and QQ genes (Huang *et al*., 2006; Latifi *et al*., 1995; Sio *et al*., 2006). It utilizes acylase as QQ enzyme selectively cleaving the acyl side chain of certain AHL (Sio *et al*., 2006). In this study, QQ activity was found in *Pseudomonas* sp. strain W3 but not *P. aeruginosa* strain W3.1. The absence of QQ activity in *P. aeruginosa* strain W3.1 may be due to the substrates used in the study. Previous studies showed that acylase of *P*. aeruginosa degrades

C7‑HSL and AHL with longer acyl side chain (Hong *et al*., 2012; Huang *et al*., 2006; Sio *et al*., 2006). In this study, C4‑HSL and C6‑HSL were used as the substrates for degradation, Hence, the selectivity of the QQ enzyme might be explained in this study.

Pseudomonas sp. strain W3 did not produce AHLs but it degraded both C4‑HSL and C6‑HSL. It is speculated that this strain is a social cheater where it used the AHL produced by other bacteria to co‑ordinates its gene expression, thus, saving the cost of producing QS signalling molecules (Wilder *et al*., 2011). Despite *Pseudomonas* strain W3 and *P*. *aeruginosa* W3.1 are closely related, the differences of QS and QQ activities exerted by both strains has suggested distinctive gene regulation in these two closely related species.

Bacillus sp. degrades AHLs via the production of lactonase enzyme, interrupting the QS by hydrolysing the lactone ring of AHL (Dong & Zhang, 2005; Dong *et al*., 2002). *B*. *cereus* known to quench OC6‑HSL and *N*‑(3‑oxo‑octanoyl)‑ʟ‑homoserine lactone (OC8‑HSL) was used as the positive control for this study (Chan *et al*., 2010). Contradictory to most of the reports, *Bacillus* sp. strain W2.2 did not shown to degrade both C4‑HSL and C6‑HSL. This also applies to *K*. *oxytoca* strain W4.2 which does not possesses any QQ activity on the tested AHLs, this finding is distinguishable from the *Klebsiella* sp. isolated from ginger's rhizosphere that was reported to degrade various AHL via lactonase activity (Chan *et al*., 2011).

CHAPTER 6.0

CONCLUSION

As a summary, five bacteria were isolated from this study, namely *P. aeruginosa* strain W3.1, *Pseudomonas* sp. strain W3, *S. marcescens* strain W2.3, *Bacillus* sp. strain W2.2 and *K. oxytoca* strain W4.2. Except *K. oxytoca* strain W4.2, all isolates produced either one or both virulence factors tested namely proteinase and hemolysin. This suggests that they could be the causal agent of the infectious disease.

Aside from C4‑HSL, OC12‑HSL and PQS, an unexpected long chain AHL, namely OC16‑HSL was detected from *P. aeruginosa* strain W3.1. Contradictory to the reported findings, an unusual long chain AHL *i.e.* C12‑HSL also detected from spent supernatant of *S. marcescens*. The detection of an unexpectedly long chain AHL from these well-studied bacteria *i.e.* *P. aeruginosa* and *S. marcescens* suggested that bacteria possess unknown QS mechanism. Therefore, more studies are required in order to further characterize and understand the mechanism of these unusual long chainAHL in these bacteria. Besides QS bacteria, *Pseudomonas* sp. strain W3 also degraded both C4‑HSL and C6‑HSL.

For future work, it is suggested that whole genome sequencing can be used to study the QS and QQ genes and the genes regulated by these systems.

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