QUORUM QUENCHING POTENTIAL OF ACTINOBACTERIA ISOLATED FROM SELECTED HABITATS IN PENINSULAR MALAYSIA

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

Quorum sensing (QS) is a cell-to-cell communication process that involves bacteria in a community regulating and coordinating various population density-dependent behaviours via signaling molecules commonly known as autoinducers. Currently, inhibition of QS is being targeted as a novel approach to curb bacterial pathogenicity. The discovery of anti-quorum sensing compounds has been crucial due to the tremendous increase of multidrug-resistant bacteria. In this study, Actinobacteria are targeted for their ability to attenuate quorum sensing as they are well known for their remarkable capabilities in producing a wide variety of secondary metabolites. A total of 207 actinobacterial isolates were screened for their ability to inhibit violacein synthesis in *Chromobacterium violaceum* CV026. Three actinobacterial isolates which are positive for violacein inhibition were then tested to determine their inhibitory activity against swarming and pyocyanin production by *Pseudomonas aeruginosa* PAO1. Based on the analysis of 16S rRNA gene sequences, these isolates belong to the genera *Micromonospora*, *Rhodococcus* and *Streptomyces*. Interestingly, this is the first report presenting anti-quorum sensing activity by a member of the genus *Micromonospora*. Pertaining to the findings of this study, members of the family Actinobacteria have a prospective ability in quenching QS signals.

ABSTRAK

‘Quorum sensing’ (QS) adalah satu proses komunikasi antara satu sel dengan satu sel yang lain dimana proses ini melibatkan komuniti bakteria dalam mengatur dan menyelaras reaksi bakteria yang bergantung kepada kepadatan bakteria melalui isyarat molekul yang dikenali sebagai ‘autoinducers’. Buat masa ini, pendekatan secara menghalang ‘quorum sensing’ adalah merupakan salah satu pendekatan baru untuk membendung dari bakteria yang berbahaya. Penemuan bahan anti-sensing kuorum adalah sangat penting kerana disebabkan peningkatan bakteria yang berupaya hidup walau dibasmi menggunakan pelbagai antibiotik. Dalam kajian ini, aktinobakteria telah digunakan kerana kemampuan mereka untuk melemahkan sensing korum disebabkan mereka terkenal dengan keupayaan yang luar biasa mereka dalam menghasilkan pelbagai jenis metabolit sekunder. Sebanyak 207 aktinobakteria telah disaring untuk mencari aktinobakteria yang berupaya menghalang sintesis violacein dalam *Chromobacterium violaceum* CV026. Sebanyak 3 tiga aktinobacterial yang positif untuk menghalang sintesis ‘violacein’ kemudiannya diuji untuk menentukan aktiviti yg melarang mereka terhadap baceteria yang boleh bergerak dan pengeluaran ‘pyocyanin’ dari *Pseudomonas aeruginosa* PAO1. Berdasarkan analisis urutan gen 16S rRNA, 3 aktinobakteria ini tergolong dalam genera *Micromonospora*, *Rhodococcus* dan *Streptomyces*. Menariknya, ini adalah laporan yang pertama melaporkan aktiviti anti-kuorum dari genus *Micromonospora* ini.Melalui hasil kajian ini, jelaslah bahawa Aktinobacteria mempunyai prospektif dalam keupayaan pelindapkejutan isyarat QS.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
×	:	times
µg	:	microgramme
µL	:	microlitre
3-oxo-C12-HSL	:	*N*-(3-oxo-dodecanoyl)-homoserine lactone
3-oxo-C6-HSL	:	*N*-(3-oxo-hexanoyl)-homoserine lactone
ACN	:	Acetonitrile
AHL	:	*N*-acyl homoserine lactones
AI-1	:	Autoinducer 1
AI-2	:	Autoinducer 2
AI-3	:	Autoinducer 3
bp	:	base pair
℃	:	degree Celsius
C10-HSL	:	*N*-decanoyl-homoserine lactone
C4-HSL	:	*N*-butanoyl-homoserine lactone
C6-HSL	:	*N*-hexanoyl-homoserine lactone
cm^2	:	square centimetre
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
g	:	gramme
g	:	gravitational force
G+C	:	guanine and cytosine
HCl	:	Hydrochloric acid

ISP2	:	International *Streptomyces* Project Medium 2
LB	:	Luria-Bertani
M	:	Molar
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	milligramme
$MgCl_2$	:	Magnesium chloride
mL	:	millilitre
NJ	:	Neighbor-Joining
nm	:	nanometre
OD	:	Optical density
PCR	:	Polymerase chain reaction
QQ	:	Quorum quenching
QS	:	Quorum sensing
rpm	:	revolutions per minute
rRNA	:	ribosomal ribonucleic acid
SB	:	Sodium borate
sdH_2O	:	sterile distilled water
w/v	:	weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The emergence of antibiotic resistance has increased the urge of intense drug discovery with novel antimicrobial mechanisms. One of such attempt to prevent the consequence of bacterial resistance is to disrupt the bacterial cell-to-cell communication, or more commonly known as quorum sensing (QS).

Quorum sensing is an essential microbial system that synchronizes and regulates cell concentration-dependent bacterial behavior. Some of the QS-controlled processes include regulation of virulent factors production, formation of biofilm and antibiotic synthesis. Like human being, bacteria too, have their respective languages of communication. Bacteria 'talk' to each other by secreting autoinducer molecules as a form of chemical messenger to exchange information among them. Autoinducers 1 (AI-1), produced by Gram negative bacteria, are one of the most extensively studied group of signaling molecules to date, whereas for Gram positive bacteria, QS is typically mediated by modified oligopeptides.

Attenuation of quorum sensing, also known as quorum quenching (QQ), is a system that disrupts the social communication of bacteria. Interruption of quorum sensing is currently being targeted to control the vicious pathogenicity of virulent bacterial strains. QQ is an excellent system in overcoming antibiotic resistance because it does not exert any bactericidal effects. Inhibition of quorum sensing can be achieved via the degradation of the signal molecules, disruption of signal generator and interference of receptor gene activation.

Thus far, numerous studies have been conducted in the effort of discovering compounds with QQ activity from various sources (Dong *et al*., 2002; Manefield *et al*., 1999; Martín-Rodríguez *et al*., 2014; Tan *et al*., 2013). Some examples of such sources include bacteria, fungi, marine algae and even higher plants.

In this project, the members of the Actinobacteria family were targeted to study their potential in inhibiting quorum sensing by using *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1 as the model organisms. The class Actinobacteria was selected due to the remarkable ability of its members to produce a wide range of biologically active metabolites. To the best of our knowledge, there are two studies that have reported the production of AHL acylase from *Streptomyces* spp. (Chankhamhaengdecha*et al*., 2013; Park*et al*., 2005), whereas multiple studies have described the production of AHL lactonase by members of the genus *Rhodococcus* and anti-QS secondary metabolites by *Streptomyces* spp. (Cirou *et al*., 2012; Fukumoto *et al*., 2016; Ooka *et al*., 2013; Park *et al*., 2006). Hence, we strongly believed that Actinobacteria isolated from the tropical ecosystems are able to produce a broad range of compounds with QQ properties.

The objectives of this study are:

a. To investigate the ability of isolated Actinobacteria to inhibit quorum sensing system of *C. violaceum* CV026.
b. To screen the crude extracts from selected actinobacterial isolates for their ability to inhibit QS-regulated virulence factors by *P. aeruginosa* PAO1.
c. To identify selected actinobacterial isolates with anti-quorum sensing activity based on their 16S rRNA gene sequences.

CHAPTER 2: LITERATURE REVIEW

2.1 Quorum sensing

Bacterial cell-to-cell communication, often termed as quorum sensing (QS), is a population density-dependent process that involves the exchange of information in a certain bacterial community through the accumulation and secretion of chemical molecules as which alters gene expression and thus, changes their behaviour accordingly (Waters and Bassler, 2005; West *et al*., 2012). Bacterial interaction via the signal-response mechanism which relies on concentration of cells shows these tiny creatures are able to carry out complex tasks very much like multicellular organisms. Besides being able to perform the standard physiological activities, quorum sensing enables bacteria to thrive in the competitive microbial communities (March and Bentley, 2004).

2.1.1 The many languages of bacteria

Having already acquired the ability to socially interact with each other, what more interesting is that these microorganisms use different languages to communicate with one another. In general, bacteria produce and secrete chemical molecules termed as autoinducers to transmit and receive signals (Hense *et al*., 2007). AI-1 molecules, commonly termed as *N*-acyl homoserine lactones (AHLs), are one of the first groups of bacteria-associated signals to be discovered and well-characterised, which are primarily utilized by Gram-negative bacteria, for instance, *P. aeruginosa* and *Vibrio fischeri* (Bassler, 1999; Smith *et al*., 2003).

AHLs are made up of a homoserine lactone core linked to an acyl side chain of various lengths and substitutions that determines the species specificity of these molecules (Jimenez *et al*., 2012). The acyl side chain varies in length consisting of 4 to 18 carbons, where AHLs with acyl moiety of less than 8 carbons are categorized as short chain AHLs and those with 8 and more are long chain AHLs (Scott *et al*., 2006). Generally, it is known that short chain AHLs diffuse freely through the cell membrane whereas long chain AHLs are transported out of the cells via active efflux system (Greenberg, 2000).

In addition to AI-1 molecules, Gram-negative bacteria are also known to produce AI-2 signalling compounds, typically by *Salmonella typhimurium* and *Vibrio harveyi*, A-signaling amino acids by *Myxococcus xanthus* (Visick and Fuqua, 2005), 3-hydroxypalmitic acid methyl ester by *Ralstonia solanacearum* (Flavier *et al*., 1997) and a compound with unknown structure, AI-3, utilised by enterohemorrhagic *Escherichia coli* (Scutera *et al*., 2014). On the other hand, oligopeptides autoinducers are synthesized by Gram-positive bacteria for their cell-to-cell interaction (Waters and Bassler, 2005). Unlike free diffusion of AI-1 through the plasma membrane, peptide signals by Gram-positive bacteria are lugged out of the cells through oligopeptides exporters.

2.1.2 Overview of bacterial communication mechanism

2.1.2.1 Gram-negative bacteria

The marine bacteria *Vibrio fischeri* and *V. harveyi* are some of the very first models used for studying the quorum sensing mechanism. The QS system in *V. fischeri* is known to be regulated mainly by the LuxIR-protein family (Bassler, 1999; Chugani

and Greenberg, 2010), where LuxI synthase directs the synthesis of acyl-homoserine autoinducer molecules and LuxR is a transcriptional activator protein that regulates the expression of the *luxCDABE* operon (Bassler, 1999; Engebrecht and Silverman, 1984). The *luxCDABE* genes encode the enzyme luciferase that catalyses the light-emission phenomenon in *V. fischeri*, known as bioluminescence.

On the other hand, *V. harveyi* consists of two distinct density-dependent luminescence system; one responds to the AI-1 signalling molecules and the other responds to the AI-2 molecules (Surette *et al*., 1999). The first signal-response system is coordinated by the expression of the *luxM* and *luxN* genes. LuxM protein, previously known as LuxLM (Henke and Bassler, 2004a) is required for the production of autoinducers, specifically 4-hydroxyl C4-HSL (Mok *et al*., 2003; Surette *et al*., 1999) whereas LuxN is closely similar to the LuxR-type autoinducer-binding protein. As for the second signal-response system in *V. harveyi*, *luxS* is necessary for the synthesis of AI-2 molecules, also commonly known as furanosyl borate diester which responses to the cognate regulator protein, LuxPQ (Henke and Bassler, 2004b; Mok *et al*., 2003). Autoinducers produced by both *V. fischeri* and *V. harveyi* are identical but these molecules cannot be used interchangeably (Bassler *et al*., 1994). The flowchart below summarises the mechanism of quorum sensing in general for *Vibrio* spp. (Figure 2.1).

A similar quorum sensing regulatory tools and mechanism is also observed in *P. aeruginosa*, a clinically-important opportunistic pathogen (Chugani and Greenberg, 2014; Schuster and Greenberg, 2006). In *P. aeruginosa*, the quorum sensing mechanism is employed to regulate the production of extracellular virulence factors such as exotoxin A, elastase, pyocyanin, rhamnolipids, superoxidase dismutase, lectins, alkaline protease

and factors that aid in the production of biofilms. This bacterium possesses two distinct quorum sensing systems, namely, LasI-LasR and RhlI-RhlR system which are responsible for the synthesis of these virulence determinants (Antunes *et al*., 2010).

In the LasI-LasR system, *lasI* encodes the signal synthase protein, which produces *N*-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL), and *lasR* is necessary for the synthesis of a LuxR-type protein that binds to the cognate autoinducer molecule, which in turn activates the expression of relevant virulence-associated genes. Similarly, for the *rhl* system, RhlI is required for the production of *N*-butanoyl-homoserine lactone (C4-HSL). These autoinducers then form complexes with the transcriptional activator, RhlR (Chugani and Greenberg, 2014; Fuqua, 2006; Smith and Iglewski, 2003) to promote the activity, inter alia, of *rhlAB* operon and *rhlC* gene which code for enzymes required for the biosynthesis of rhamnolipid (Medina *et al*., 2003). Both LasR/3-oxo-C12-HSL and RhlR/C4-HSL complexes also induces a positive feedback loop by activating the transcription of *lasI* and *rhlI*, respectively (Schuster and Greenberg, 2006). It is also known that the quorum sensing system in *P. aeruginosa* works hierarchically, whereby the binding of LasR to 3-oxo-C12-HSL activates the *rhl* system (Latifi *et al*., 1996; Medina *et al*., 2003).

Additionally, *P. aeruginosa* also consists of a gene that encodes for an orphan receptor protein, QscR (Lequette *et al*., 2006). This LasR-RhlR homolog does not possess any cognate autoinducer synthase gene but responds to 3-oxo-C12-HSL (Antunes *et al*., 2010; Fuqua, 2006) generated by the *las* system.

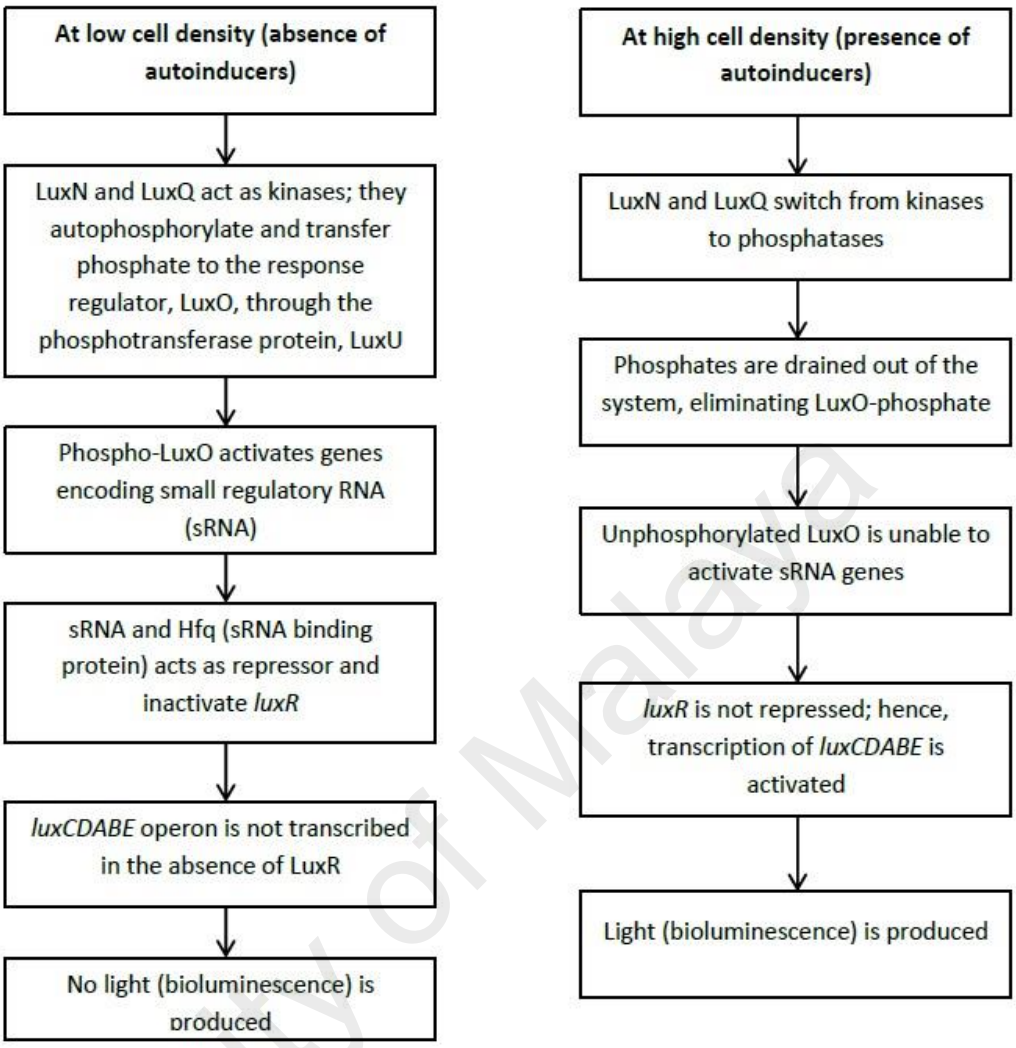

Figure 2.1: Quorum sensing mechanism in *Vibrio* spp. Adapted from: (Henke and Bassler, 2004b; Lenz *et al*., 2004; Lilley and Bassler, 2000).

2.1.2.2 Gram-positive bacteria

Unlike Gram-negative bacteria, Gram-positive bacteria utilise the peptide quorum sensing system to facilitate bacterial communication. These bacteria produce and secrete short modified oligopeptides typically known as autoinducing peptides (AIP) (Waters and Bassler, 2005; Yarwood and Schlievert, 2003). QS is often known to also play a vital role in population density-dependent processes in Gram-positive bacteria; pathogenesis of

Staphylococcus aureus, microcin production in *Lactobacillus sake* and conjugation in *Enterococcus faecalis*, to name a few (Bassler, 1999).

The opportunistic human pathogen, *Staphylococcus aureus*, which is known to be the etiologic agent of various diseases (Koenig *et al*., 2004; Kong *et al*., 2006), is often used as a model in studying the peptide-based signalling system in Gram-positive bacteria. This system is modulated by the staphylococcal accessory gene regulator (*agr*) locus that controls two different promoters, P2 and P3, which regulates the transcription of RNAII and RNAIII, respectively (Otto, 2004; Yarwood *et al*., 2004; Yarwood and Schlievert, 2003). The signalling molecule, AIP, in this case, is encoded by *agrD*. The *agr*-mediated quorum sensing system in *Staphylococcus aureus* (Figure 2.2) regulates the production of multiple virulence-related factors such as enterotoxins, cytolysins, hyaluronidases and staphylokinases (Novick, 2003).

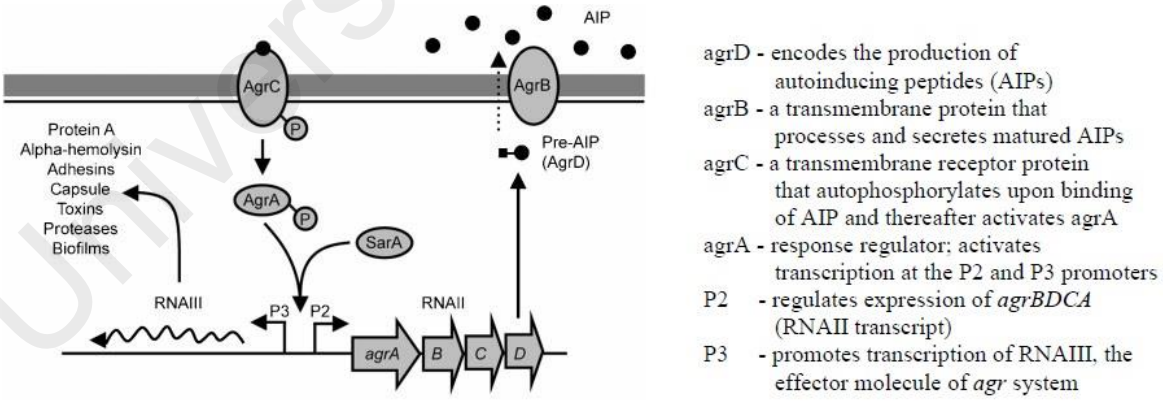

Figure 2.2: Quorum sensing mechanism in *S. aureus* (Antunes *et al*., 2010).

2.2 Quorum sensing to overcome antibiotic-resistant bacteria

In the recent years, the quest for novel drugs has been massive and so is the increase in antibiotic-resistant bacteria. The usage of antimicrobials that target and kill bacterial cell has led to the evolution of these resistant microorganisms. The fact that many of these bacteria rely on the quorum sensing system to regulate and produce virulence-associated factors has given rise to the idea of manufacturing drugs that interferes with their quorum sensing mechanism (Rampioni *et al*., 2014), rendering them avirulent. Interference of quorum sensing can be achieved via the degradation of the signalling molecules and the disruption of signal generator (LuxI, for instance) and signal receptor (LuxR, for instance) (Rasmussen and Givskov, 2006b).

2.2.1 Quorum sensing inhibitors and resistance towards quorum sensing

To date, several inhibitors with the ability to collapse a QS cascade have been discovered (Rasmussen and Givskov, 2006a). Enzymatic degradation of signalling molecules have been known to be one of the widely used approaches in quenching these QS signals. The two most common enzymes, known as AHL lactonase and AHL acylase can be found in a diverse range of prokaryotes. AHL lactonase, which cleaves the homoserine lactone ring of the AHL molecule, is usually produced by *Bacillus* spp., for instance, *Bacillus thuringiensis* (Dong *et al*., 2004), *Bacillus anthracis* (Ulrich, 2004) and *Bacillus cereus* (Reimmann *et al*., 2002), also including a handful of different species from the phyla Actinobacteria, Firmicutes, and Proteobacteria (Kalia, 2013). AHL acylase, on the other hand, hydrolyses the amide bond linking the acyl side chain and homoserine lactone moiety and is frequently produced by *Pseudomonas* spp. (Huang *et al*., 2003; Huang *et al*., 2006; Sio *et al*., 2006).

Besides using enzymatic approach to degrade the quorum sensing signals, a few studies have reported the usage of antagonistic molecules, generated by prokaryotes, that directly interferes and competes with the binding of signalling molecules to the receptor protein. Two phenyethylamides metabolites produced by a marine bacterium, *Halobacillus salinus*, were described as analogues that mimic the AHL signals and disrupt the signal-receptor binding in *Vibrio harveyi*, which in turn inhibits its bioluminescence production (Teasdale *et al*., 2009).

In addition to the two different approaches stated above, a third strategy, involving the interruption of signal generation, is another tactic employed to manipulate bacterial quorum sensing. A recent study reported that the major constituent of clove extract, known as eugenol, has the ability to inhibit the activation of both *las* and *pqs* systems, which plays a direct role in the pathogenicity of *P. aeruginosa* (Zhou *et al*., 2013).

As reported by many (Koh *et al*., 2013; Vasavi *et al*., 2013; Zhang and Dong, 2004), anti-virulence compounds that interferes with bacterial quorum sensing system are less likely to exert a selective pressure that leads to the evolution of drug-resistant strains, unlike regular antibiotics which work contrariwise. These anti-virulence compounds are able to combat bacterial infections mediated by quorum sensing systems without killing or inhibiting their growth.

However, this alternative strategy which is believed to overcome antibiotic resistance appears to have a few setbacks. According to the study conducted by Defoirdt *et al*. (2010), variation in the core genes of quorum sensing increases the probability of

rendering quorum sensing disruption insensitive, which, is reported to be one of the few factors contributing to resistance towards quorum sensing. This is highly due to the fact that natural selection occurs when there is variation on certain traits which equally happens to affect the fitness of individuals (bacteria).

Although multiple studies have demonstrated the ability of bacteria to develop resistance towards quorum sensing (García-Contreras *et al*., 2013; Kalia *et al*., 2014; Maeda *et al*., 2012), the risk of acquiring resistance is much lower than that of towards conventional antibiotics. Hence, to prevent a similar consequence, it is suggested that studies associated with discoveries of anti-virulence compounds should take into account additional experiments to confirm their quorum sensing inhibitory activities (Defoirdt *et al*., 2013). It has also been proposed that the search for potential quorum sensing inhibitors should be directed in a way that reduces the threat of resistance development, for instance, using inhibitory compounds with broad range activities, as suggested by Defoirdt and co-workers (Defoirdt *et al*., 2010).

2.3 Discovery of potential novel quorum quenchers from Actinobacteria

The phylum Actinobacteria is a group of Gram-positive bacteria with high G+C content in their DNA. These bacteria can be ubiquitously found in the terrestrial and marine ecosystems. Members of the class Actinobacteria are often regarded as important producers of various extracellular enzymes and bioactive secondary metabolites (Ballav *et al*., 2012; Gao and Gupta, 2005; Lam, 2006; Manivasagan *et al*., 2014; Subramani and Aalbersberg, 2012). Many studies have reported the production of valuable compounds by these biotechnologically important bacteria, for example, roseoflavin, an antibacterial compound produced by *Streptomyces davawensis* (Matsui *et al*., 1979);purpuromycin, an

antifungal compound by *Actinoplanes ianthinogenes* (Coronelli *et al*., 1974);diazepinomicin, an antibacterial, anticancer and anti-inflammatory compound by *Micromonospora* sp. (Charan *et al*., 2004) andabyssomicin, an antibacterial compound by *Verrucosispora* sp. (Riedlinger *et al*., 2004).

Actinobacteria are regarded as an inexhaustive source of active metabolites (Berdy, 2012; Ser, 2015), and hence, they have been targeted to study their potential in producing quorum quenching compounds. Numerous studies are being conducted to explore the quorum quenching potential of Actinobacteria. One such example is the inhibition of *fsr* quorum sensing system in *Enterococcus faecalis* by siamycin I, a peptide antibiotic produced by *Streptomyces* sp. Y33-1 (Nakayama *et al*., 2007). The fsr quorum sensing system regulates the production of virulence-associated proteases by this bacterium. According to another recent study conducted by Hassan *et al*. (2016), it was reported that extract of *Streptomyces coelicoflavus* strain S17 inhibited violacein production by *Chromobacterium violaceum*. Further studies concluded that the active component of the extract, 1*H*-pyrrole-2-carboxylic acid reduced production of virulence factors by *P. aeruginosa* PAO1 without affecting cell growth. Table 2.1 shows examples of compounds with quorum sensing inhibitory activity isolated from Actinobacteria.

Table 2.1: Examples of quorum sensing inhibitory compounds from Actinobacteria

Compound	Source (bacterial species)	Activity	References
Arthroamide	*Arthrobacter* sp.	Inhibits *agr*-mediated signalling in *S. aureus*	Igarashi *et al*., 2015
Siamycin I	*Streptomyces* sp. Y33-1	Inhibits virulence-related proteases in *E. faecalis* at submicromolar concentration	Nakayama *et al*., 2007
Maniwamycin C-F	*Streptomyces* sp. TOHO-M025	Inhibits quorum sensing activity of *C. violaceum* CV026	Fukumoto *et al*., 2016
Piericidin A1	*Streptomyces* sp. TOHO-Y209 and TOHO-O348	Inhibits quorum sensing activity of *C. violaceum* CV026	Ooka *et al*., 2013
Piericidin A and Glucopiericidin A	*Streptomyces xanthocidicus* KPP01532	Inhibits quorum sensing activity of *C. violaceum* CV026 and reduce virulence gene expression of *Erwinia carotovora* subsp. *atroseptica*	Kang*et al*., 2016
Cyclodepsipeptides WS9326A, WS9326B and cochinmicin II/III	Actinomycete strains DSW812 and GMKU369	Inhibits *agr* and *fsr* quorum sensing systems in *S. aureus* and *E. faecalis*, respectively	Desouky *et al*., 2015
SdLi crude extract	*Streptomyces* sp.	Inhibits QS-dependent virulence factors (urease and haemolysin) by *Proteus mirabilis*	Younis, Usup, & Ahmad, 2015

Although earlier studies focused only on the production of AHL-degrading enzymes by Actinobacteria (Chankhamhaengdecha *et al*., 2013; Park *et al*., 2006; Park *et al*., 2005), recent discoveries substantiate the fact that members of this family are able to produce active metabolites to quench quorum signals.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals, solutions and media preparation

All media were prepared accordingly and topped up with distilled water to 1000 mL, adjusted to pH 7 and autoclaved at 121℃ for 15 minutes.

Luria-Bertani (LB) medium

Yeast extract	5 g
Tryptone	10 g
NaCl	10 g
Agar	12 g

GSM medium [modified from (Park *et al.*, 2005)]

Glucose	20 g
Starch	10 g
Soybean meal	25 g
Beef extract	1 g
Yeast extract	4 g
K_2HPO_4	0.25 g
NaCl	2 g
$CaCO_3$	2 g
Sodium propionate	4 g
Agar	15 g

Swarm agar (Norizan *et al*., 2013)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	8 g

International Streptomyces Project Medium 2 (ISP2) (Shirling and Gottlieb, 1966)

Yeast extract	4 g
Malt extract	10 g
Glucose	4 g
Agar	15 g

3.2 Bacterial strains and growth requirements

3.2.1 Biosensors

Chromobacterium violaceum is a Gram-negative bacterium that gives rise to purple-pigmented colonies, due to the production of violacein, a purple, water-insoluble pigment (Morohoshi *et al*., 2008). The QS-controlled production of violacein by *C. violaceum* is mediated by the autoinducer *N*-hexanoyl-homoserine lactone (C6-HSL) (Blosser and Gray, 2000).

C. violaceum CV026, on the other hand, is obtained through a mini-Tn*5* transposon mutagenesis of the wild type strain ATCC 315232 (McClean *et al*., 1997) and lacks the ability to readily produce violacein, unless supplied with exogenous AHLs (C6-HSL). Pertaining to that, *C. violaceum* CV026 is often used as biosensors for the study of short chain (C4 to C6) AHL-mediated QS interruption (Morohoshi *et al*., 2010). Alternatively, long chain AHLs, with side chains typically ranging from C10 to C14 in length, obstructs violacein production.

P. aeruginosa is widely known for its pathogenicity and innate resistance towards antimicrobials. These multidrug-resistant bacteria are often associated with clinical infections such as hospital-acquired pneumonia, bacteremia, skin infections, urinary tract infections and infections in immunocompromised patients (Driscoll *et al*., 2007; Hentzer *et al*., 2003; Sader *et al*., 2015).

In this study, strains *C. violaceum* CV026 and *P. aeruginosa* PAO1 were used as biosensors to investigate the QS-inhibitory properties of actinobacterial isolates. These strains were regularly cultured in Luria-Bertani (LB) medium. The cultures were incubated overnight for 16 hours with shaking (180 rpm) at 28℃ for *C. violaceum* CV026 and at 37℃ for *P. aeruginosa* PAO1 when needed.

3.2.2 Actinobacterial isolates and culture conditions

A total of 207 Actinobacteria isolated from the terrestrial, marine and mangrove ecosystem in various locations around Malaysia (Appendix A) were obtained from the culture collection of Microbial Resource Laboratory, University of Malaya, Kuala Lumpur to be used in this study. All isolates were grown on GSM medium at 28℃ for 12 days before conducting the respective anti-quorum sensing-associated bioassays.

3.3 Screening for anti-quorum sensing activity

Prior to conducting the agar diffusion assay, bioassay plates were prepared to detect the inhibition of violacein synthesis (Park *et al*., 2005). LB agar plates were overlaid with 4.5 mL of soft LB agar (0.8% agar) seeded with 0.5 mL overnight culture of *C. violaceum* CV026 at an OD_{600} of 1.5 and *N*-hexanoyl-L-homoserine lactone (C6-HSL) at a final concentration of 4×10^{-5} mg mL^{-1}. A sterile cork borer was used to remove agar plugs of Actinobacteria culture grown on GSM medium and these plugs were placed

accordingly on the bioassay plates after complete solidification. The long chain AHLs, *N*-decanoyl-L-homoserine lactone (C10-HSL) was suspended in acetonitrile (ACN) and used as positive control. The plates were incubated at 28℃ for 16 hours and observed for the formation of translucent halo zone against a background of purple pigmentation, which indicates anti-QS activity.

3.4 Production and preparation of extracts

Actinobacteria isolates with violacein inhibitory activity were grown in larger scale for compound extraction, with slight modifications from the original protocol by (Pazhanimurugan *et al*., 2012). Selected isolates were cultivated on at least 20 plates of GSM medium each, for 12 days at 28℃. The mycelial mass, along with the agar, was then cut into small pieces (approximately 1 cm^2) and transferred to a conical flask containing 500 mL of ethyl acetate, followed by hexane (Chenia, 2013), for the extraction of non-polar compounds. The agar pieces were soaked for 24 hours before being filtered out using Macherey-Nagel Filter Paper MN615 (Macherey-Nagel, Germany). Both extracts were evaporated under reduced pressure using a rotary evaporator (Eyela, Japan), weighed and reconstituted in dimethyl sulfoxide (DMSO) to obtain a final concentration of 10 mg mL^{-1}. All extracts were stored in -20℃ until further usage.

3.5 Growth inhibitory activity of actinobacterial isolates against *C. violaceum* CV026 and *P. aeruginosa* PAO1

To rule out any possibilities of antibacterial activity against the biosensors, a 96-well microtiter plate-based growth curve study was conducted based on previous work (Priya *et al*., 2013) with slight modification. Overnight cultures of biosensors were prepared in LB medium and diluted accordingly to an optical density of 0.1 at 600 nm and aliquoted to the wells of the microtiter plate, followed by addition of actinobacterial

extracts to obtain final concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. The plates were incubated for 24 hours at 28℃ and 37℃ for *C. violaceum* CV026 and *P. aeruginosa* PAO1, respectively, with absorbance reading measured at 600 nm every 30 minutes using Infinite M200 Pro microplate reader (TECAN, Switzerland).

3.6 Violacein quantification assay

A microtiter plate-based assay was conducted for the quantification of violacein production (Tan *et al*., 2012). Overnight suspension of *C. violaceum* CV026, with an optical density of 1.5 at 600 nm, was supplemented with 3.75 µg mL^{-1} of C6-HSL, prior to aliquoting it accordingly into the wells of a 96-well plate, in triplicates. Crude extracts of the isolates were also added to the wells at final concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}, respectively. DMSO was used as the negative control. The 96-well plate was incubated at 28℃ in a shaking incubator. After 24 hours, the plate was placed in an oven and left to dry at 60℃. Upon drying, 100 µL of DMSO was added into each well to solubilise the violacein and the optical density of the solution was read at 590 nm using a microplate reader, with pure DMSO as the blank. Absorbance reading lower than that of control (DMSO) indicates inhibition of violacein production.

3.7 Anti-virulence assays using *P. aeruginosa* PAO1

3.7.1 Inhibition of swarming motility

P. aeruginosa PAO1 swarming assay was carried out as previously described (Norizan *et al*., 2013) with slight alterations. Swarm agar was prepared accordingly and 5 mL of the agar was mixed with the actinobacterial crude extracts and poured onto plates which already contains pre-warmed swarm agar. Appropriate volumes of extracts were added into the swarm agar to obtain final concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. After two hours (or until the agar has solidified), 2 µL of *P. aeruginosa*

PAO1 overnight culture was inoculated in the middle of each agar plates before incubating them at 37℃. The extent (or inhibition) of swarming was observed after an incubation period of 16 hours.

3.7.2 Inhibition of pyocyanin production

Extraction of pyocyanin was performed according to a previously described protocol (Priya *et al*., 2013). To begin with, 5 mL of *P. aeruginosa* PAO1 cultures (OD_{600} = 0.1) were prepared in sterile universal bottles. The crude extracts were added accordingly to the cultures in each bottle to achieve final concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}, respectively. DMSO was used as the negative control. The cultures were incubated at 37℃. After 16 hours, the bacterial suspensions were centrifuged (6 000 *g*, 28℃) for 5 minutes to obtain cell-free supernatant using SIGMA 1-14 centrifuge machine (Sartorius, Germany). Three millilitres of chloroform was then added to the supernatant of *P. aeruginosa* PAO1 cultures. After a brisk mixing, the organic layer formed was transferred to a new tube, followed by addition of 1 mL of 0.2 M hydrochloric acid (HCl) to the tube. Upon centrifugation (4 700 *g*, 28℃) for 10 minutes, the absorbance of the top layer was measured at 520 nm. A lower absorbance reading (compared to that of control) indicates that pyocyanin production is inhibited.

3.8 Molecular identification of actinobacterial isolates

3.8.1 Extraction of genomic DNA

For DNA extraction purposes, actinobacterial isolates were cultured and grown at 28℃ for 7 days on ISP2 medium. Genomic DNA extraction was performed using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany). To begin with, appropriate amount of mycelium (of each isolate) is scraped off and placed in a microcentrifuge tube containing 180 µL of Buffer T1. After the addition of 20 µL of lysozyme (50 mg mL^{-1}),

the tube was incubated at 37℃ for 60 minutes. Twenty-five microliters of Proteinase K was then added to the same tube and the sample was incubated again for at least 1 to 3 hours (overnight if necessary) at 56℃. Upon incubation, the sample was lysed by adding Buffer B3, vortexed and incubated at 70℃ for about 10 minutes. Following that, the suspension was centrifuged (11 000 g, 5 minutes) and about 200 µL of molecular-grade ethanol was added to the supernatant to adjust the DNA binding conditions.

Upon DNA binding to the silica membrane of NucleoSpin® Tissue Column, washing steps were carried out using Buffer BW and Buffer B5. The genomic DNA was then eluted using pre-warmed Buffer BE and its concentration was determined using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

Gel electrophoresis was also performed to check the integrity of extracted DNA using a 0.8% (w/v) agarose gel and 1× SB buffer, at 100V for 30 minutes. The gel was then stained using GelRed™ Nucleic Acid Gel Stain (Biotium, USA) before being viewed in UV Transilluminator MUV21 (Major Science, USA). The genomic DNA was stored at -20℃ until further usage.

3.8.2 Polymerase chain reaction (PCR) and 16S ribosomal RNA (rRNA) gene sequences analysis

The amplification of 16S rRNA gene was carried out using Swift™ Maxi thermal cycler (ESCO, Singapore) based on the formulation in Table 3.1. The universal primers used in this study were 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane *et al*., 1985), with the following PCR conditions: initial denaturation at 95℃ for 2 minutes, 35 cycles of amplification (denaturation at 95℃ for 30 seconds, annealing at 55℃ for 30 seconds, extension at 72℃ for 1 minute and 30 seconds) and lastly, a final extension at 72℃ for 10 minutes.

Table 3.1: Concentration and final volume of components used for the amplification of 16S rRNA gene

Components	Final concentration	Final volume (µL)
5× Green GoTaq® Flexi Buffer (Promega, USA)	1×	10.0
GoTaq® Flexi DNA Polymerase (Promega, USA)	1.5 U	0.15
dNTPs	0.2 mM	1.0
Magnesium chloride ($MgCl_2$)	1.5 mM	3.0
Forward primer (27f)	0.2 µM	1.0
Reverse primer (1492r)	0.2 µM	1.0
Sterile distilled water (sdH_2O)	-	31.85
DNA template	≈ 50 ng	2.0
Total	-	**50**

Gel electrophoresis was then conducted to ensure the quality of the amplified product using 1% (w/v) agarose gel and 1×SB buffer (20× SB buffer was prepared by adding 450 mL distilled water to 10 mL of 10N NaOH and pH was adjusted to 8.5 by gradually adding boric acid. The solution is then topped up with 500 mL distilled water, filter sterilised and diluted to 1× working concentration (Brody and Kern, 2004), at 100V for 20 minutes, followed by the gel staining and viewing steps. The PCR product obtained was also subjected to purification using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) before being outsourced to First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. The sequencing services are performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, USA) via the Applied Biosystems Genetic Analyzer (Thermo Fisher Scientific, USA). Sequences analysis and phylogenetic affiliation of the isolates were computed via alignment with the EzTaxon-e database (Chun *et al*., 2007) using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.1 (Tamura *et al*., 2011).

3.9 Statistical analysis

All assays were performed in triplicates and all graphs were plotted with standard deviation represented by error bars. Significance of data obtained were analyzed via paired t-tests using GraphPad Prism 6 software, where p values less than 0.05 ($p < 0.05$) are considered significant.

CHAPTER 4: RESULTS

4.1 Anti-quorum sensing activity of actinobacterial isolates

4.1.1 Qualitative analysis of violacein inhibition

A total of 207 actinobacterial isolates (Appendix A) were screened for their inhibitory properties against synthesis of violacein using *C. violaceum* CV026 supplemented with C6-HSL. Out of 207, 25 isolates exhibited positive activity by forming a white-turbid zone against the purple-pigmented background in the agar diffusion assay.

Of the 25 isolates that showed violacein inhibitory activity (Table 4.1), three morphologically distinct isolates, namely H16, H54 and TAV14 (Figure 4.1), were selected for further analysis. Figure 4.2 shows the inhibition of violacein production (formation of white-turbid zone) by isolate H16, H54 and TAV14. These three isolates were grown in larger scale and subjected to subsequent assays.

Figure 4.1: Actinobacterial isolates on ISP2 agar after 14 days incubation period (from left: isolate H16, isolate H54 and isolate TAV14)

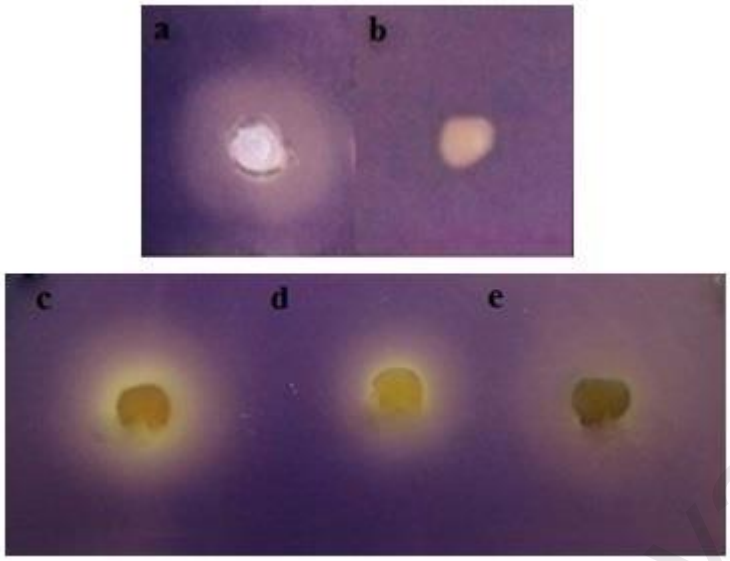

Figure 4.2: Inhibition of violacein production: (**a**) C10-HSL (positive control), (**b**) Uninoculated agar plug (negative control), (**c**) Isolate H16, (**d**) Isolate H54 and (**e**) Isolate TAV14

Table 4.1: Degree of violacein inhibition by actinobacterial isolates

Isolate	Inhibition of violacein production	Isolate	Inhibition of violacein production
2OPSa	+	H54	++
RC1	++	H84	++
RA4	+	H125	++
RA6	+	PE37	+
RA8	+	SE30	+
HT1	+	J10	+
TPS1	+	J18	+
TAV4	+	J27	+
TAV7	+	J28	+
TAV8	+	J44	+
TAV14	+	J45	+
TAV18	+	TPS3	+
H16	++		

(++: strong inhibition; +: mild inhibition)

4.1.2 Quantification of violacein reduction

The three selected isolates with positive activity were cultivated onto 20 GSM agar plates each. Ethyl acetate and hexane were used as the extraction solvent in this

experiment to extract the potential anti-QS compounds. Table 4.2 shows the pigment colours of crude extracts of the three isolates.

To quantify the reduction of violacein production, extracts of ethyl acetate and hexane were tested at three different concentrations (0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}) for their inhibitory properties.

Table 4.2: Colour of pigments of ethyl acetate and hexane extracts of isolates H16, H54 and TAV14 as determined according to ISCC-NBS (Kelly, 1965).

Isolate	**Pigmentation of extracts**	
	Ethyl acetate	**Hexane**
H16	Deep orange yellow	Light greenish yellow
H54	Vivid reddish orange	Vivid orange
TAV14	Vivid orange yellow	Brilliant greenish yellow

Based on the violacein quantification assay, generally, both ethyl acetate extracts (Figure 4.3) and hexane extracts (Figure 4.4) of all three isolates H16, H54 and TAV14 were observed to have significantly ($p < 0.05$) reduced violacein production when compared to negative control, with exception to ethyl acetate extract of isolate H54. However, for isolate TAV14, the reduction was observed to have occurred in a concentration-independent manner. Inhibition of violacein production was notably greater when treated with hexane extracts compared to those of ethyl acetate.

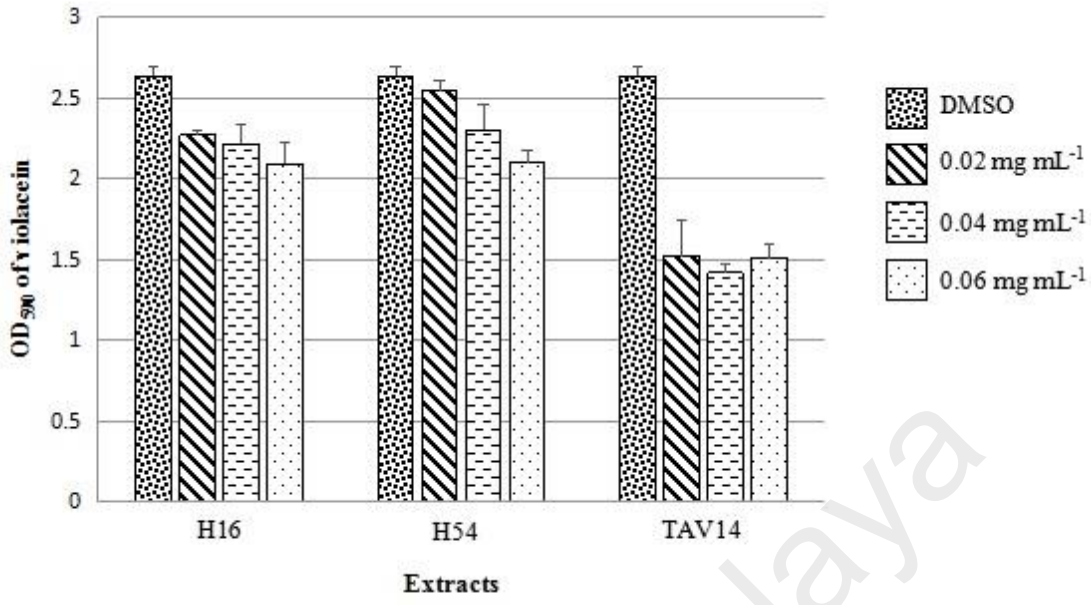

Figure 4.3: Inhibition of violacein production of *C. violaceum* CV026 by ethyl acetate extracts of isolate H16, isolate H54 and isolate TAV14 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. *****DMSO-control.

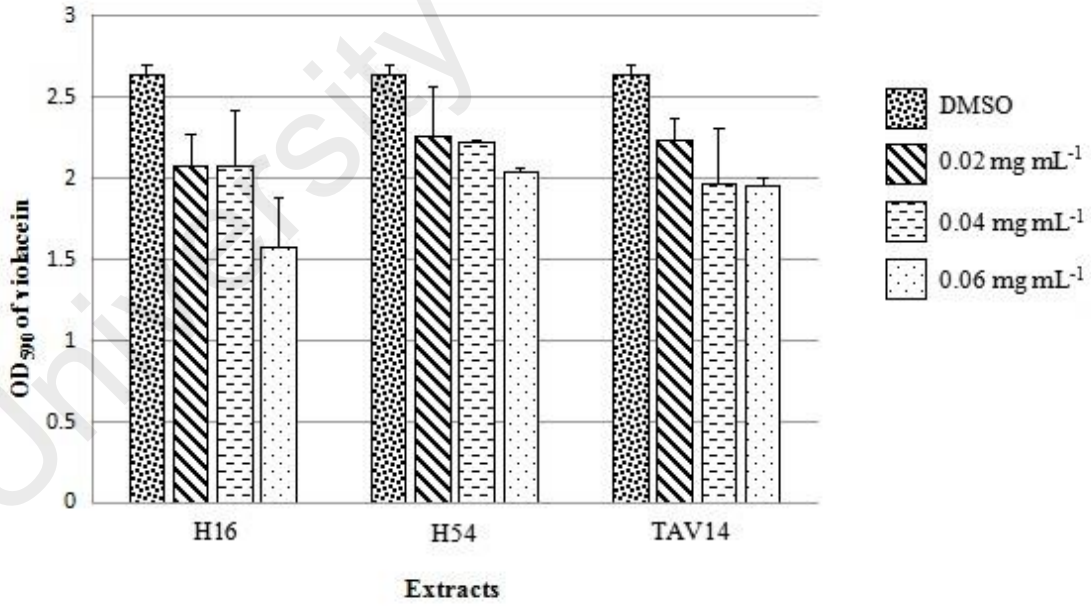

Figure 4.4: Inhibition of violacein production of *C. violaceum* CV026 by hexane extracts of isolate H16, isolate H54 and isolate TAV14 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. *****DMSO-control.

4.2 Inhibition of virulence factors production by *P. aeruginosa* PAO1

4.2.1 Swarm-inhibitory activity by *P. aeruginosa* PAO1

In this study, ethyl acetate and hexane extracts of the selected three Actinobacteria were qualitatively assessed for their ability to inhibit swarming activity of *P. aeruginosa* PAO1. Maximum inhibition was observed when PAO1 was treated with ethyl acetate extract of isolate H16 (Figure 4.5) whereas hexane extract of the same isolate (Figure 4.6) only showed mild inhibition at a concentration of 0.06 mg mL^{-1}.

On the other hand, for isolate H54, only the ethyl acetate extract at a concentration of 0.06 mg mL^{-1} was able to greatly decrease the swarming ability of *P. aeruginosa* PAO1 (Figure 4.7), whereas, a mild reduction was noted when the biosensor was treated with hexane extract of the same isolate (Figure 4.8).

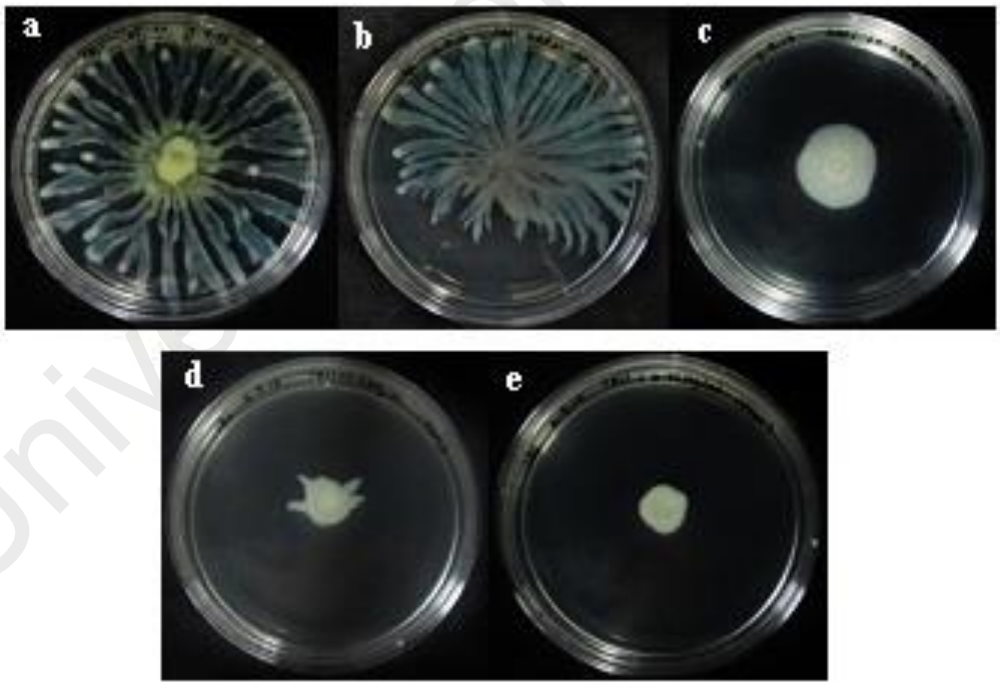

Figure 4.5: Inhibition of *P. aeruginosa* PAO1 swarming by ethyl acetate extract of isolate H16 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. (**a**) *P. aeruginosa* PAO1 (control-no extract added); (**b**) DMSO only; (**c**) 0.02 mg mL^{-1}; (**d**) 0.04 mg mL^{-1} and (**e**) 0.06 mg mL^{-1}.

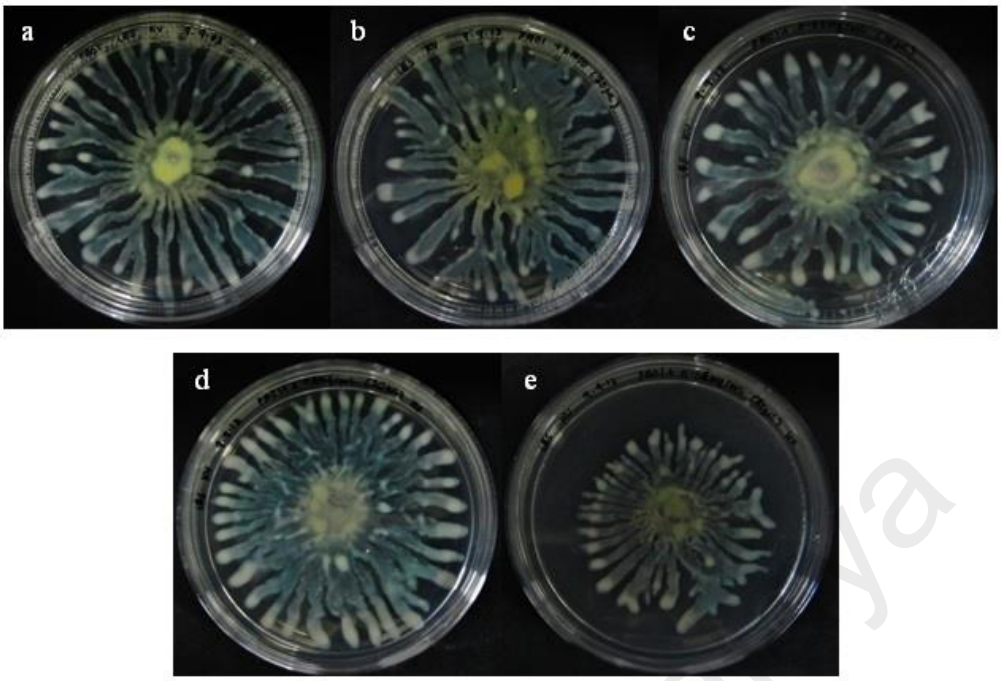

Figure 4.6: Inhibition of *P. aeruginosa* PAO1 swarming by hexane extract of isolate H16 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. (**a**) *P. aeruginosa* PAO1 (control-no extract added); (**b**) DMSO only; (**c**) 0.02 mg mL^{-1}; (**d**) 0.04 mg mL^{-1} and (**e**) 0.06 mg mL^{-1}.

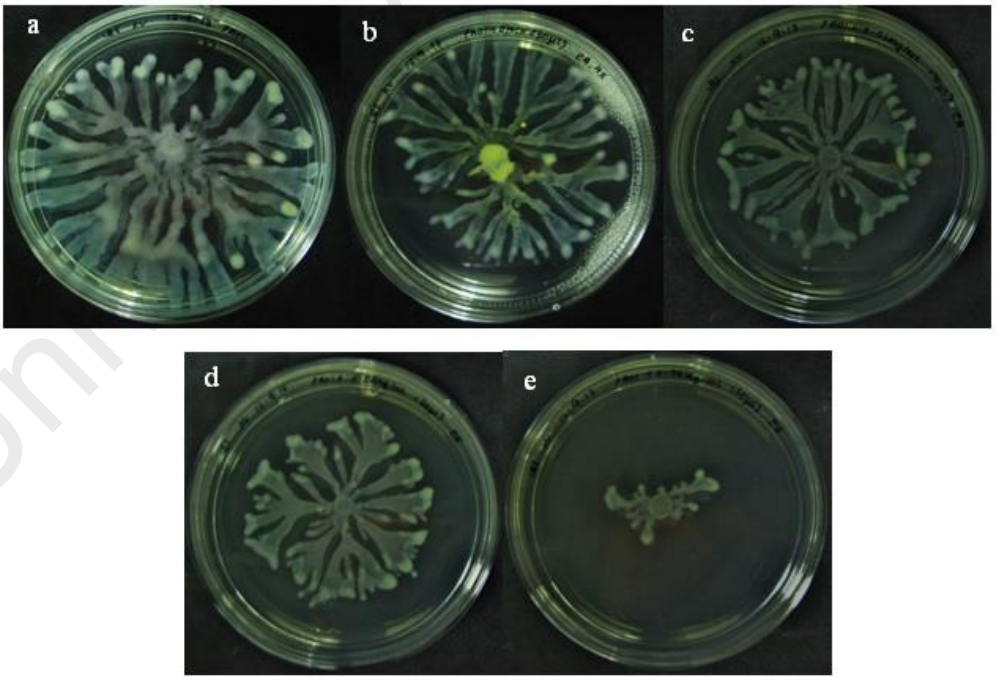

Figure 4.7: Inhibition of *P. aeruginosa* PAO1 swarming by ethyl acetate extract of isolate H54 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. (**a**) *P. aeruginosa* PAO1 (control-no extract added); (**b**) DMSO only; (**c**) 0.02 mg mL^{-1}; (**d**) 0.04 mg mL^{-1} and (**e**) 0.06 mg mL^{-1}.

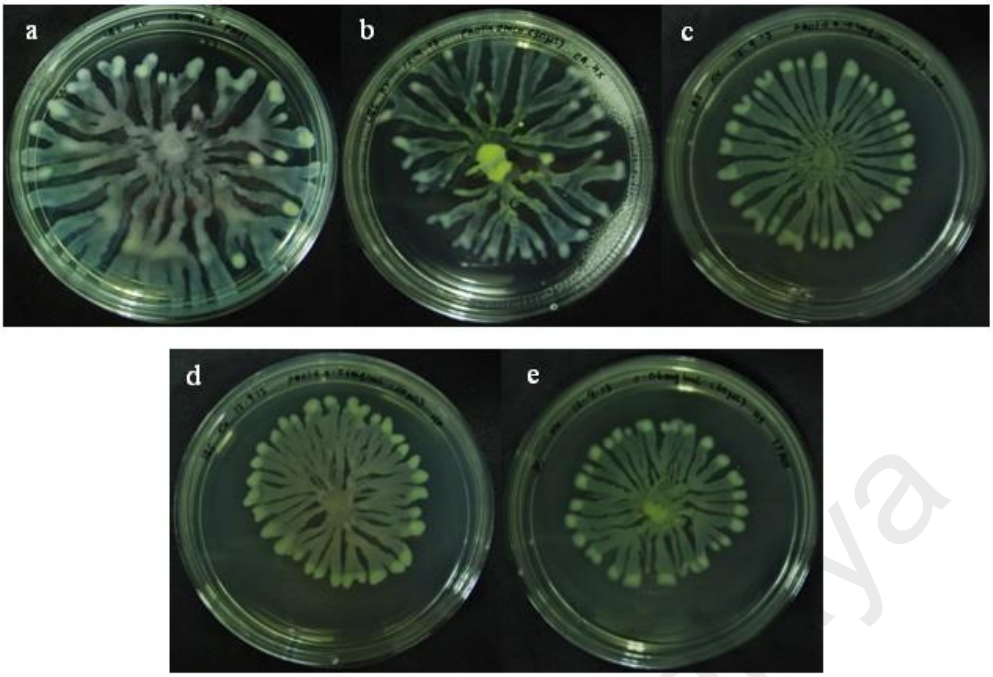

Figure 4.8: Inhibition of *P. aeruginosa* PAO1 swarming by hexane extract of isolate H54 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. (**a**) *P. aeruginosa* PAO1 (control-no extract added); (**b**) DMSO only; (**c**) 0.02 mg mL^{-1}; (**d**) 0.04 mg mL^{-1} and (**e**) 0.06 mg mL^{-1}.

As for isolate TAV14, only the hexane extract of this isolate (Figure 4.9) completely inhibited swarming of PAO1 at all three concentrations. However, none of the ethyl acetate extract of isolate TAV14 affected the swarming capacity of PAO1 (Appendix C). Table 4.3 summarises the swarm-inhibitory activity of ethyl acetate and hexane extracts of all three isolates - H16, H54 and TAV14.

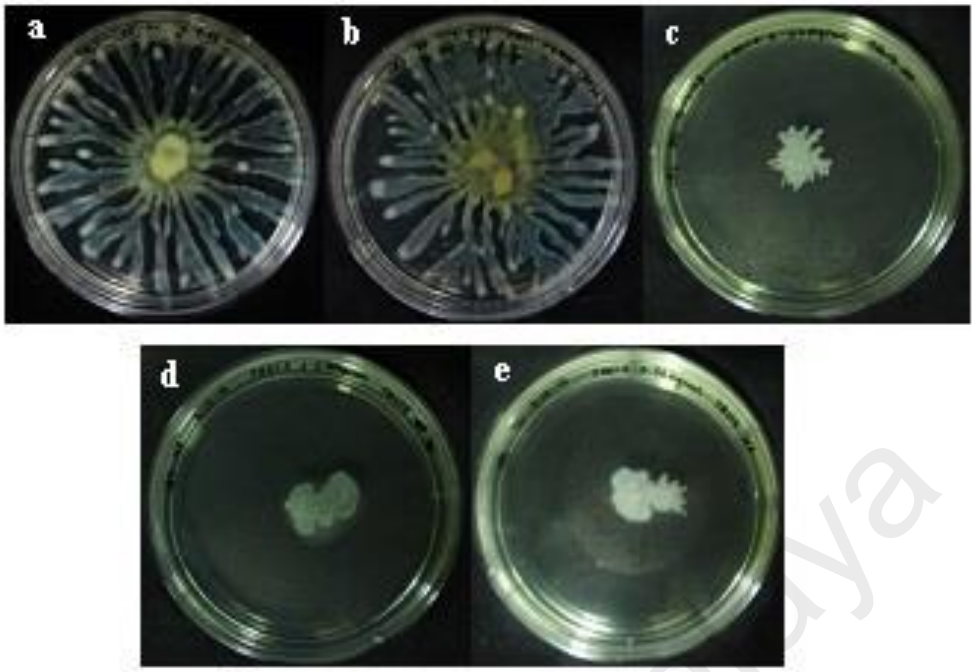

Figure 4.9: Inhibition of *P. aeruginosa* PAO1 swarming by hexane extract of isolate TAV14 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. (**a**) *P. aeruginosa* PAO1 (control-no extract added); (**b**) DMSO only; (**c**) 0.02 mg mL^{-1}; (**d**) 0.04 mg mL^{-1} and (**e**) 0.06 mg mL^{-1}.

Table 4.3: Inhibition of swarming by ethyl acetate and hexane extract of isolates H16, H54 and TAV14.

Isolate / **Concentration of extracts (mg mL^{-1})**		**H16**	**H54**	**TAV14**
Ethyl acetate	0.02	++	+	-
	0.04	++	+	+
	0.06	++	++	-
Hexane	0.02	-	+	++
	0.04	-	+	++
	0.06	+	+	++

(++ : complete inhibition; + : mild inhibition; - : no inhibition)

4.2.2 Attenuation of pyocyanin production

As demonstrated in this study, only ethyl acetate extract of isolate H16 (Figure 4.10), hexane extract of isolate H54 (Figure 4.11) and ethyl acetate of isolate TAV14

(Figure 4.12) was able to show observable decrement in pyocyanin production in *P. aeruginosa* PAO1. The decrements, however, are not significant.

The production of pyocyanin also decreased when subjected to treatment with hexane extract of isolate TAV14 at concentrations of 0.04 mg mL^{-1} and 0.06 mg mL^{-1}, but not 0.02 mg mL^{-1} (Appendix D). Otherwise, none of the other extracts (Appendix D) showed any reduction in synthesis of pyocyanin by PAO1. However, interestingly, an uncommon effect was observed when *P. aeruginosa* PAO1 was treated with ethyl acetate extract of isolate H54 (Appendix D) where the production of pyocyanin appeared to have increased in a concentration-dependent manner.

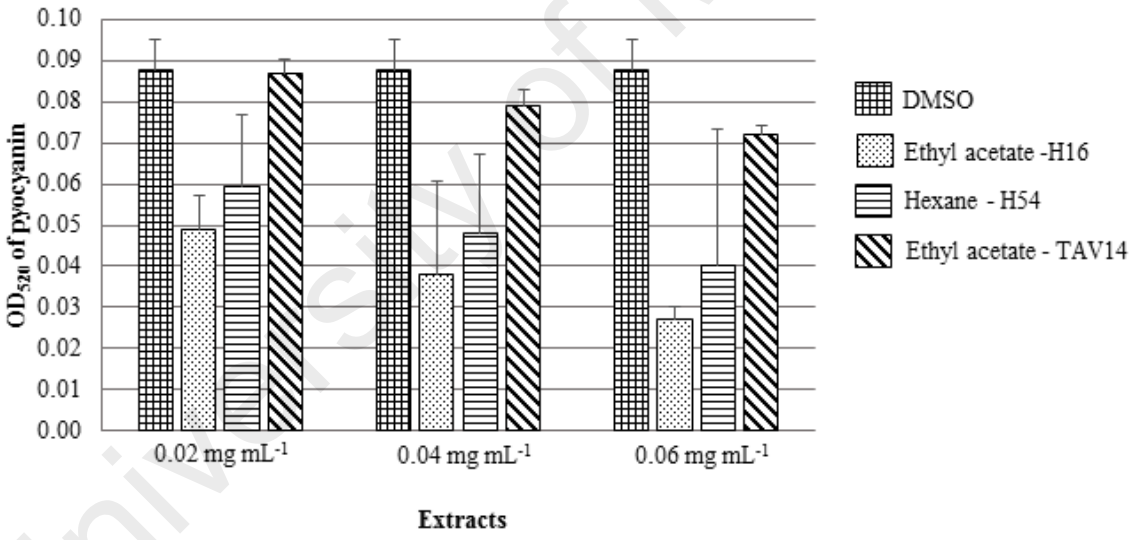

Figure 4.10: Inhibition of pyocyanin production of *P. aeruginosa* PAO1 by ethyl acetate extract of isolate H16, hexane extract of isolate H54 and ethyl acetate extract of isolate TAV14 at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} and 0.06 mg mL^{-1}. *DMSO–control.

4.3 Growth curve study of *C. violaceum* CV026 and *P. aeruginosa* PAO1

To eliminate the possibility of bactericidal effect on the biosensors - *C. violaceum* CV026 and *P. aeruginosa* PAO1, ethyl acetate and hexane extracts of isolates H16, H54 and TAV14 were subjected to antibacterial screening against the biosensors. Growth curves of biosensors treated with extracts at concentrations of 0.02 mg mL^{-1}, 0.04 mg mL^{-1} (Appendix B) and 0.06 mg mL^{-1} (Figure 4.13-4.16) were plotted based on data obtained at 30 minutes intervals over a 24 hour incubation period. All graphs plotted showed no observable growth inhibitory activity when compared alongside untreated controls.

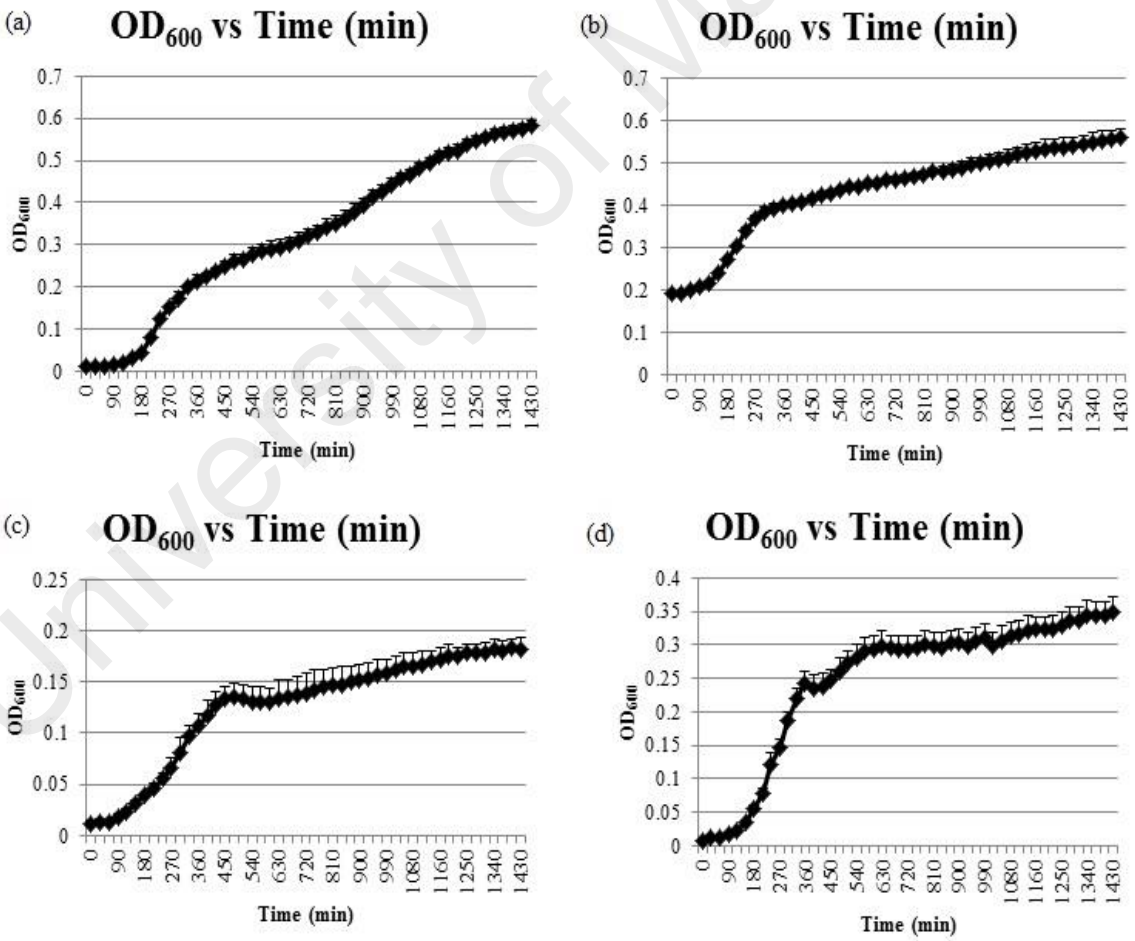

Figure 4.11: Growth curve of *C. violaceum* CV026. **(a)** CV026 only, **(b)** CV026 and DMSO, **(c)** CV026 and ethyl acetate extract of isolate H16 (0.06 mg mL^{-1}) and **(d)** CV026 and hexane extract of isolate H16 (0.06 mg mL^{-1}).

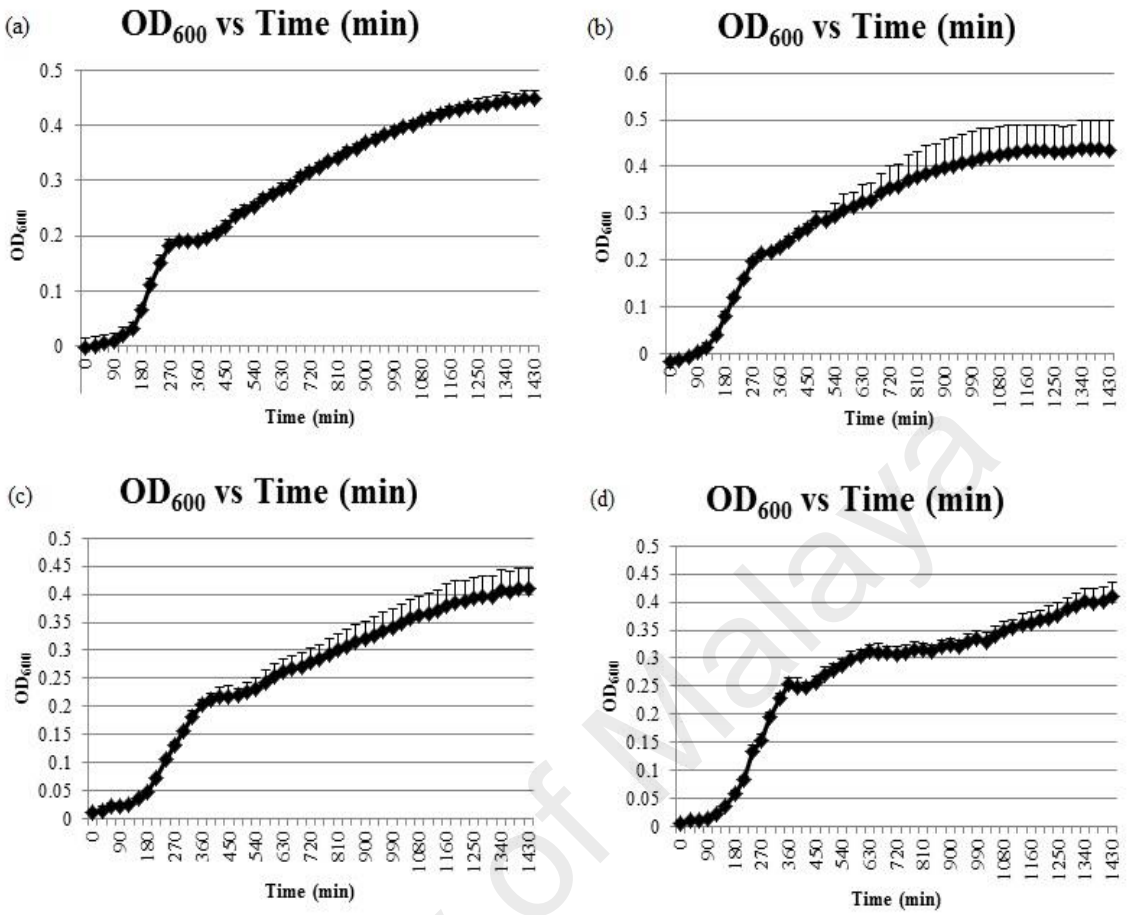

Figure 4.12: Growth curve of *C. violaceum* CV026 (continued). **(a)** CV026 and ethyl acetate extract of isolate H54 (0.06 mg mL^{-1}), **(b)** CV026 and hexane extract of isolate H54 (0.06 mg mL^{-1}), **(c)** CV026 and ethyl acetate extract of isolate TAV14 (0.06 mg mL^{-1}) and **(d)** CV026 and hexane extract of isolate TAV14 (0.06 mg mL^{-1}).

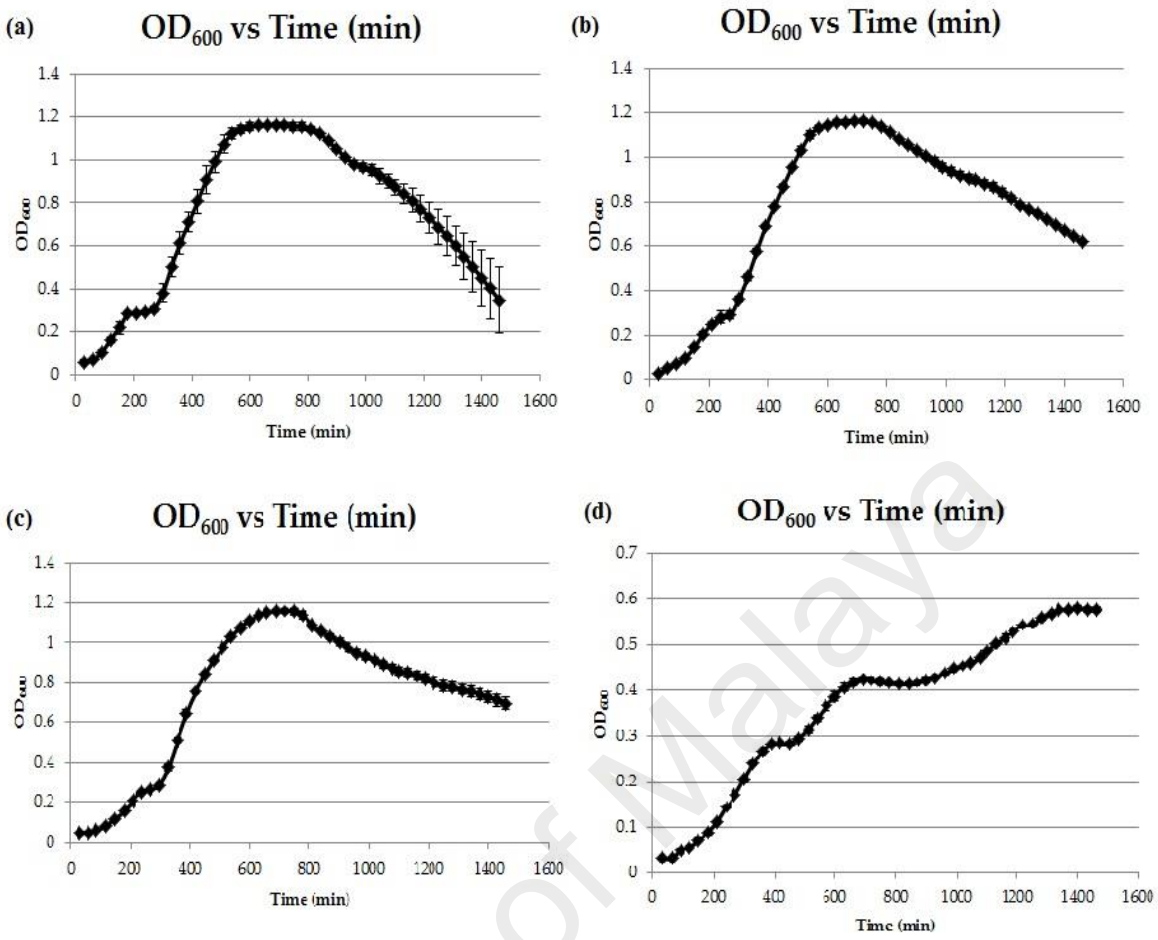

Figure 4.13: Growth curve of *P. aeruginosa* PAO1. **(a)** PAO1 only, **(b)** PAO1 and DMSO, **(c)** PAO1 and ethyl acetate extract of isolate H16 (0.06 mg mL^{-1}) and **(d)** PAO1 and hexane extract of isolate H16 (0.06 mg mL^{-1}).

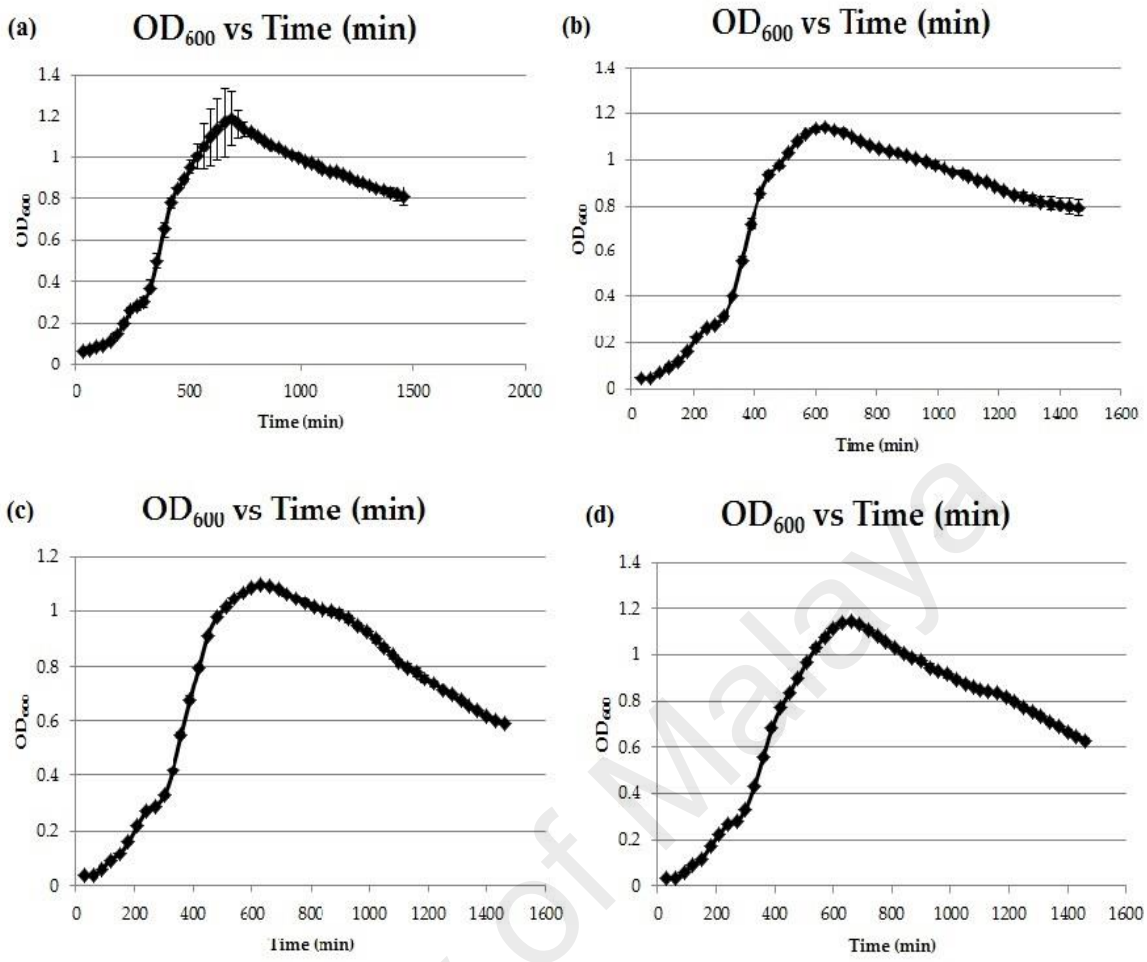

Figure 4.14: Growth curve of *P. aeruginosa* PAO1 (continued). **(a)** PAO1 and ethyl acetate extract of isolate H54 (0.06 mg mL^{-1}), **(b)** PAO1 and hexane extract of isolate H54 (0.06 mg mL^{-1}), **(c)** PAO1 and ethyl acetate extract of isolate TAV14 (0.06 mg mL^{-1}) and **(d)** PAO1 and hexane extract of isolate TAV14 (0.06 mg mL^{-1}).

4.4 Identification of actinobacterial isolates H16, H54 and TAV14

All three actinobacterial isolates were identified based on the analyses of partial 16S rRNA gene sequences (>1300 bp). A similarity search against the EzTaxon database (http://www.ezbiocloud.net/eztaxon; Kim *et al*., 2012a) revealed that the isolates belonged to three different genera in the class Actinobacteria, namely *Streptomyces*, *Rhodococcus* and *Micromonospora*. Isolate H16 is found to be closely related to *Streptomyces chiangmaiensis* TA4-1^T with a similarity of 99.43%, isolate H54 is 100%

similar to *Rhodococcus ruber* DSM 43338^T and isolate TAV14 on the other hand is 100% identical to *Micromonospora aurantiaca* ATCC 27029^T (Table 4.4).

Phylogenetic trees were also constructed for all three isolates based on 16S rRNA gene sequences of closely related species within the same respective genera (Figure 4.17-4.19). The Neighbor-Joining (NJ) method (Saitou and Nei, 1987) was used to infer the evolutionary relationship between species.

Interestingly, to date, this is the first report on anti-QS activity of a member from the genus *Micromonospora*. The partial 16S rRNA gene sequences of isolates H16, H54 and TAV14 (Appendix E) were deposited in the GenBank database under the accession numbers KJ810557, KJ810558 and KJ810559, respectively.

Table 4.4: Identity of isolates with anti-quorum sensing activity based on their 16S rRNA gene sequences.

Isolate	Closest match	Strain	Accession No.	Pairwise similarity (%)	Query Length (bp)
H16	*Streptomyces chiangmaiensis*	TA4-1^T	AB562507	99.43	1409
H54	*Rhodococcus ruber*	DSM 43338^T	X80625	100.00	1352
TAV14	*Micromonospora aurantiaca*	ATCC 27029^T	CP002162	100.00	1337

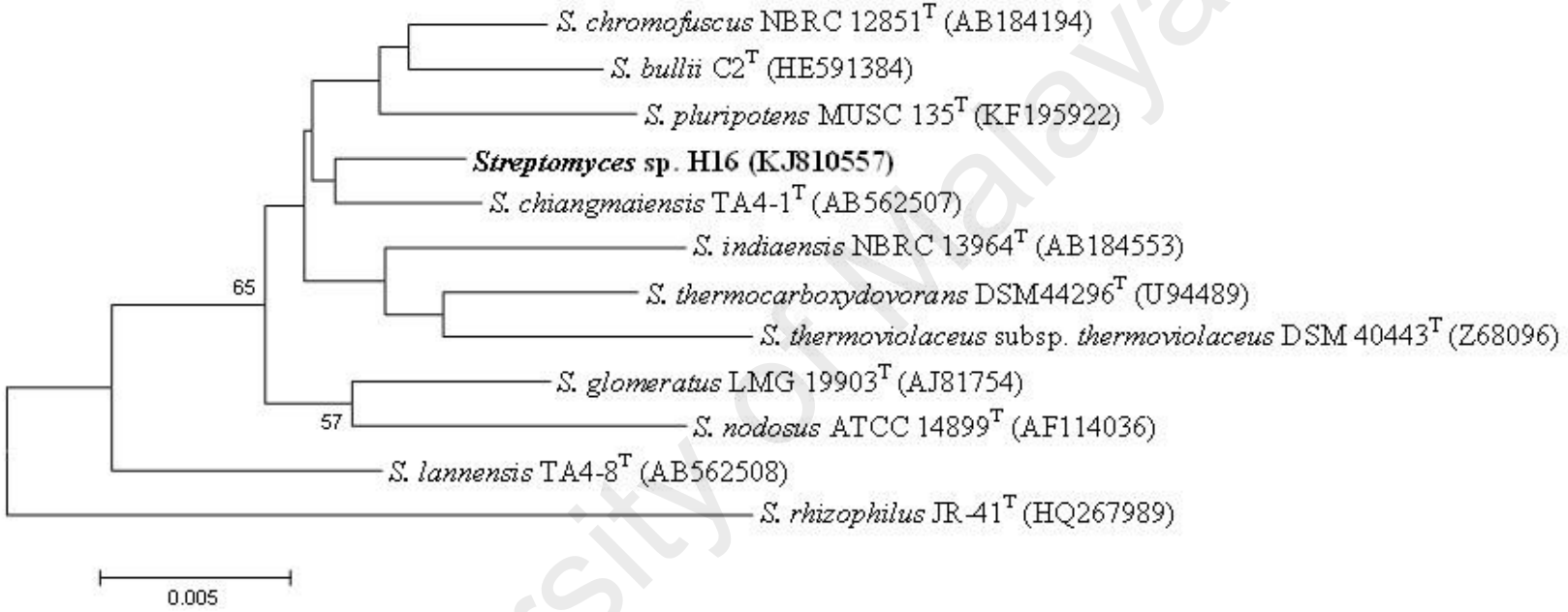

Figure 4.15: Neighbor-Joining (NJ) phylogenetic tree based on almost complete 16S rRNA gene sequences of *Streptomyces* sp. H16 and related type species. Number at nodes represents bootstrap percentages (1000 resamplings).

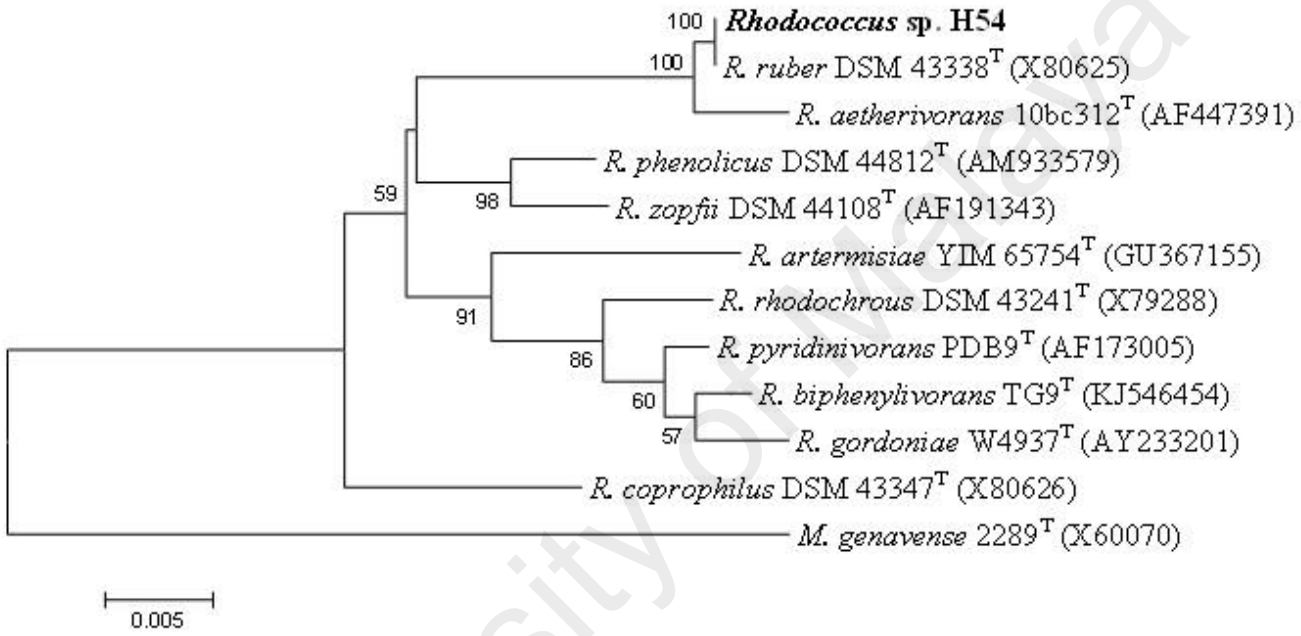

Figure 4.16: Neighbor-Joining (NJ) phylogenetic tree based on almost complete 16S rRNA gene sequences of *Rhodococcus* sp. H54 and related type species. Number at nodes represents bootstrap percentages (1000 resamplings).

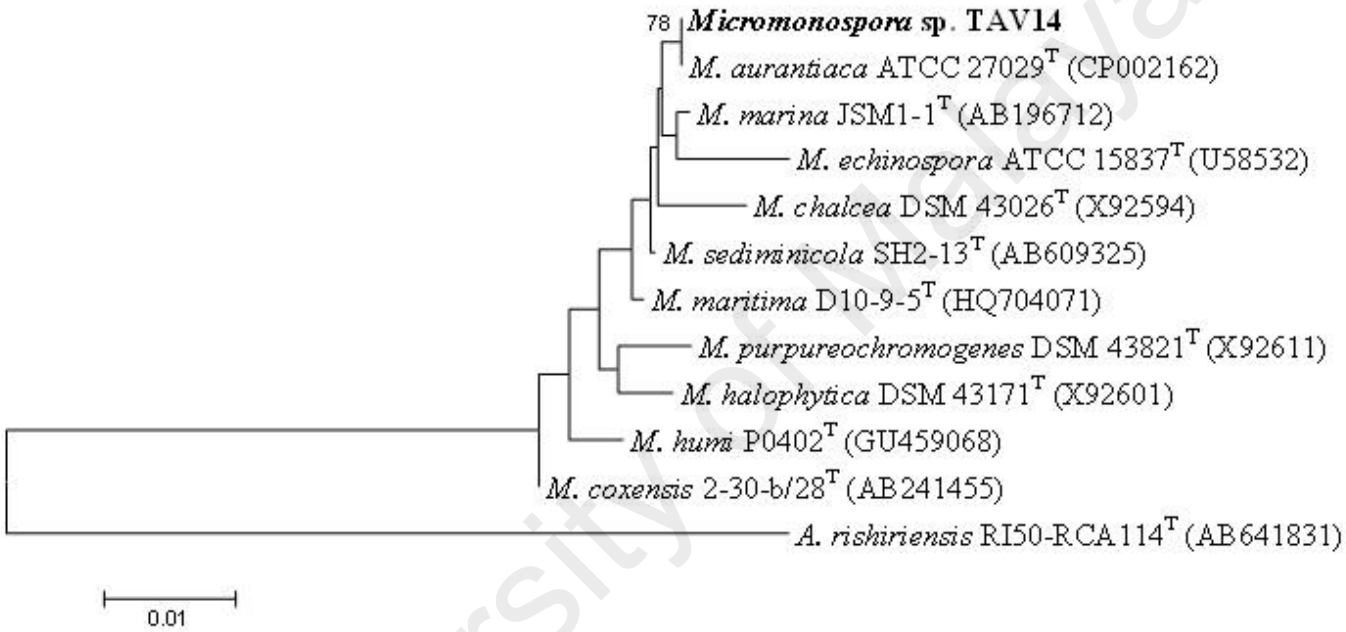

Figure 4.17: Neighbor-Joining (NJ) phylogenetic tree based on almost complete 16S rRNA gene sequences of *Micromonospora* sp. TAV14 and related type species. Number at nodes represents bootstrap percentages (1000 resamplings).

CHAPTER 5: DISCUSSION

5.1. Quorum quenching activities of Actinobacteria

Being the most biotechnologically active group of prokaryotes, the class Actinobacteria has always been of major scientific interest particularly in drug discovery. This distinct clade of Gram-positive bacteria is commonly distributed in both terrestrial and marine environments. They can also be found in areas with extreme conditions, such as hot springs, deep-sea floor and arid deserts, which is believed to have contributed to their genetic and metabolic diversity (Claverías *et al*., 2015; Manivasagan *et al*., 2013; Mohammadipanah and Wink, 2015). As such, the diverse genera under the class Actinobacteria have been reported to be the major producers of a wide range of secondary metabolites and extracellular enzymes.

In this study, actinobacterial isolates collected from various locations including the terrestrial environments, marine (coastal area and sea sponge) and high altitude regions were screened through to determine their ability to produce compounds with anti-quorum sensing potential.

In the present work, 207 actinobacterial isolates were selected to determine their ability to disrupt bacterial QS system of *C. violaceum* CV026. Based on the results obtained, 25 out of 207 isolates were able to inhibit production of violacein in the preliminary agar diffusion bioassay. Three morphologically distinct isolates, with strong inhibitory activity, namely *Streptomyces* sp. H16, *Rhodococcus* sp. H54 and *Micromonospora* sp. TAV14 were selected for further analyses.

The active anti-QS component(s) of the three isolates were extracted using ethyl acetate and hexane, as reported by previous studies (Abed *et al*., 2013; Ooka *et al*., 2013). These solvents are commonly used for the extraction of secondary metabolites from Actinobacteria (Sharma *et al*., 2011; Valan *et al*., 2012), as well as other microorganisms (Supaphon *et al*., 2013; Kumar *et al*., 2013; Darabpour *et al*., 2012). In addition, extracts of ethyl acetate and hexane have been reported to have potent biological activities (Supaphon *et al*., 2013; Vijayakumar *et al*., 2012). The compound extraction method employed in this study favored the recovery of non-polar secondary metabolites.

Before conducting the assays, the concentrations of crude ethyl acetate and hexane extracts were standardised to 0.02 mg mL^{-1}, 0.04 mg $mL^{-1}$and 0.06 mg mL^{-1}. At concentrations higher than 0.06 mg mL^{-1}, the extracts were found to inhibit growth of both biosensors; *P. aeruginosa* PAO1 and *C. violaceum* CV026 (data not shown). Similarly, previous studies have shown that compounds tested at subinhibitory (lower) concentrations inhibited quorum sensing activity but were lethal otherwise (Husain *et al*., 2016; Garske *et al*., 2004; Skindersoe *et al*., 2008). Table 5.1 shows a brief summary of the QS-inhibitory activity of ethyl acetate and hexane extracts of all three isolates used in the present study.

Table 5.1: A summary of inhibition of QS-regulated activities by extracts of *Streptomyces* sp. H16, *Rhodococcus* sp. H54 and *Micromonospora* sp. TAV14

Extracts of isolates \ **QS-mediated activities**		**Inhibition of violacein production by CV026**	**Inhibition of swarming motility by PAO1**	**Inhibition of pyocyanin production by PAO1**
Streptomyces sp. H16	Ethyl acetate	✔	✔	✔
	Hexane	✔	✔	✘
Rhodococcus sp. H54	Ethyl acetate	✔	✔	✘
	Hexane	✔	✔	✔
Micromonospora sp. TAV14	Ethyl acetate	✔	✘	✔
	Hexane	✔	✔	✘

(✔: presence of activity; ✘: absence of activity)

5.1.1 Inhibition of violacein production by CV026

Violacein is a purple-pigmented natural indolocarbazole compound produced by some Gram-negative bacteria including *Janthinobacterium lividum*, *Pseudoalteromonas tunicata*, *Duganella* sp. and mainly *C. violaceum* (Hoshino, 2011; Fang *et al*., 2015; Ryan and Drennan, 2009). The biosynthetic pathway of violacein is encoded by the gene cluster *vioABCDE* and initiated by the precursor molecule L-tryptophan (Sánchez *et al*., 2006; Ryan and Drennan, 2009).

In this current study, the mutant strain, *C. violaceum* CV026 was used as a bioindicator to quantify the reduction in violacein production. Generally, the findings obtained from violacein quantification assay showed that even at a low concentration of 0.02 mg mL^{-1}, both ethyl acetate and hexane extracts of all three isolates,*Streptomyces* sp. H16, *Rhodococcus* sp. H54 and *Micromonospora* sp. TAV14 inhibited violacein formation, highlighting the high potency of anti-QS compounds present in the extracts.It is postulated that the crude extracts may have interfered with the regulation and/or expression of the gene cluster involved in biosynthesis of violacein (Morohoshi *et al*.,

2010) or exerted a direct antagonistic effect on the AHL molecules (Hong *et al*., 2012), which, in this case, are the short chain AHLs C6-HSL.

It was observed that crude extracts of hexane of two isolates (*Streptomyces* sp. H16 and *Rhodococcus* sp. H54) exhibited stronger violacein inhibitory activity than those of ethyl acetate, which is comparable to a similar study conducted by Chenia (2013), where hexane extracts of *Kigelia africana* (common name: Sausage tree) fruit showed the best violacein inhibitory activity compared to extracts of ethyl acetate and other solvents. In another study performed by Salini *et al*. (2015), hexane extract of *Hyptis suaveolens* (common name: Pignut) also showed greater inhibitory effect than extracts of ethyl acetate. Active compounds with violacein-inhibiting properties, are however, often recovered from extracts of ethyl acetate (Fukumoto *et al*., 2016; Ooka *et al*., 2013; Kang *et al*., 2016; Teasdale *et al*., 2009).

Notably, in the present study, ethyl acetate extract of *Micromonospor*a sp. TAV14 possessed the best violacein inhibitory activity, with 41% reduction at the lowest tested concentration (0.02 mg mL^{-1}), whereas only 0.7% to 19% of reduction was observed for other extracts at the same concentration. This finding was of particular interest since there are no reports on any anti-QS activities by members of this genus.

Recent studies also demonstrated the ability of natural products isolated from *Streptomyces* sp., i.e., Piericidin A1 (Ooka *et al*., 2013) and Maniwamycin C-F (Fukumoto *et al*., 2016) in inhibiting violacein production by *C. violaceum* CV026. Maniwamycin is one of many other active azoxy compounds produced by streptomycetes. In addition to quorum sensing inhibitory properties, members of the genus *Streptomyces* are also known to produce azoxy-containing natural compounds with antifungal activity,

for example, Elaiomycins D-F from *Streptomyces* sp. strain HKI0708 (Ding *et al*., 2012) and antibacterial activity, for example, Valanimycins from *Streptomyces viridifaciens* MG456-hF10 (Garg *et al*., 2008; Garg and Parry, 2010; Nakayama *et al*., 1989).

C. violaceum CV026 is often conveniently used as a biosensor for the study of violacein production, however, the disadvantage is that this biosensor has a detection limit of only responding to short chain AHLs (Anbazhagan *et al*., 2012). Hence, it is suggested that further experiments using AHLs of different lengths should be carried out prior to concluding the quorum sensing inhibitory potential of a test sample.

5.1.2 Inhibition of swarming motility

The intrinsic and rapid acquisition of antibiotic resistance by *P. aeruginosa* has always been a major challenge in the discovery of anti-infective drugs (Khosravi and Mohammadian, 2016; Tommasi *et al*., 2015; Yayan *et al*., 2015). To aid in an effective colonisation of its host, *P. aeruginosa* expresses multiple virulence-related phenotypic characteristics (McMorran *et al*., 2003; Yang *et al*., 2015), where swarming motility is one example of such phenotype. Swarming is generally described as the act of rapid and organised bacterial movement on semisolid surface driven by their flagellar bundles (Kazemian *et al*., 2015; Kearns, 2010; Overhage *et al*., 2008). This QS-controlled bacterial motility is often regulated by rhamnolipid, a class of biosurfactant molecules encoded by *rhl* gene that promotes swarming via reduction of surface tension (Köhler *et al*., 2000; O'May and Tufenkji, 2011; Oura *et al*., 2015; Rasamiravaka *et al*., 2015).

Being extensively studied for its virulence factors, *P. aeruginosa* PAO1 was used as the model organism for the present study. The results obtained from the assay conducted showed that all crude extracts of *Streptomyces* sp. H16, *Rhodococcus* sp. H54

and *Micromonospora* sp. TAV14, with exception to ethyl acetate extract of *Micromonospora* sp. TAV14, exhibited partial to complete inhibition of swarming activity of PAO1. Extracts of *Streptomyces* sp. H16 (ethyl acetate) and *Micromonospora* sp. TAV14 (hexane) showed the most potent inhibitory effect. As swarming motility of *P. aeruginosa* is primarily controlled by the *rhl* gene (Köhler *et al*., 2000), findings from this study suggest that the crude extracts may contain active molecules capable of interfering with the *rhl*-regulated biosynthesis of rhamnolipid. It is also envisaged that the extracts may have a potential to inhibit biofilm development, taking into account the fact that swarming motility is strongly correlated to the early stages of biofilm formation (Rasamiravaka *et al*., 2015).

A similar study conducted by Naik *et al*. (2013) showed that the active methanol extracts of two out of 72 actinomycete isolates (*Streptomyces* sp. NIO 10068 and *Streptomyces* sp. NIO 10058), containing cinnamic acid as the main compound, inhibited swarming by *P. aeruginosa* ATCC 27853. On the contrary, however, findings obtained from the experiment conducted by Kim *et al*. (2012b) revealed that culture supernatants of *Streptomyces* sp. BFI 230 and *Kribbella* sp. BFI 1562 increased the swarming motility of *P. aeruginosa* PAO1.

Similar to inhibition of violacein, hexane extracts of all isolates consistently inhibited swarming motility of *P. aeruginosa* PAO1, whereas ethyl acetate extracts of only *Streptomyces* sp. H16 and *Rhodococcus* sp. H54 inhibited swarming of PAO1. Several related studies reported that extracts of chloroform (Tan *et al*., 2012), ethanol (Datta *et al*., 2016) and methanol (Sarabhai *et al*., 2013) could also inhibit swarming motility of PAO1.

To date, there have been very few reports on swarming-inhibitory activity by prokaryotes, let alone members of the Actinobacteria group. Most of the studies carried out are focused on anti-swarming activities of plant extracts, essentially those with medicinal properties (Datta *et al*., 2016; Husain *et al*., 2015; Kalia *et al*., 2015; Kazemian *et al*., 2015; Rahman *et al*., 2015; Vasavi *et al*., 2016).

5.1.3 Attenuation of pyocyanin production

One of the most important and commonly studied virulence determinants in *P. aeruginosa* PAO1 is pyocyanin (1-hydroxy-5-methyl-phenazine) (Morkunas *et al*., 2012; Smith *et al*., 2003). This water-soluble blue-green phenazine pigment, often termed as 'blue pus', is an essential factor that induces chronic colonization of *P. aeruginosa* in airways of patients with cystic fibrosis (Carlsson *et al*., 2011). Pyocyanin is also known to exhibit high antimicrobial activity, conferring a selective advantage to *P. aeruginosa* against other bacteria (El-Fouly *et al*., 2015). Similar to the biosynthesis of other ominous exoproducts of *P. aeruginosa* PAO1, production of pyocyanin is strictly coordinated by an autoinducer-dependent regulatory system (Bratu *et al*., 2006). The derivatives of AHLs, namely C4-HSL, C6-HSL and 3-oxo-C6-HSL (*N*-3-oxohexanoyl-homoserine lactone) are the chemical messengers that play key roles in the hierarchical biosynthesis of this compound (Chong *et al*., 2011; O'Loughlin *et al*., 2013).

To initiate the synthesis of pyocyanin, the *las* system first triggers the activation of *rhlR* gene, followed by the binding of its cognate autoinducer molecules (production of which directed by *rhlI*) to the regulatory protein, RhlR (Balasubramanian *et al*., 2013; Pesci *et al*., 1997), which in turn induces the expression *phzABCDEFG* operons (Cao *et al*., 2001; Dekimpe and Déziel, 2009; O'Loughlin *et al*., 2013; Whiteley *et al*., 2000). Also, as reported by Brint and co-worker (Brint and Ohman, 1995), mutation in *rhl* system

showed extensive reduction in the synthesis of pyocyanin, which highlights the prominence of RhlI/RhlR complex in QS-modulated networks.

In the present study, ethyl acetate extracts of *Streptomyces* sp. H16 and *Micromonospora* sp. TAV14 and hexane extracts of *Rhodococcus* sp. H54 and *Micromonospora* sp. TAV14 (at 0.04 mg mL^{-1} and 0.06 mg mL^{-1}) were observed to have reduced pyocyanin production by *P. aeruginosa* PAO1. This finding is on par with a previous study performed by Naik *et al*. (2013) which reported the inhibition of pyocyanin by extracts of three *Streptomyces* spp., which contains cinnamic acid as the active constituent. In another study, it was also found that one of the major compounds purified from the ethyl acetate extract of *Streptomyces coelicoflavus* strain S17 and identified as 1*H*-pyrrole-2-carboxylic acid, a heterocyclic pyrrole derivative, decreased the production of virulence factors by *P. aeruginosa* PAO1, including pyocyanin (Hassan *et al*., 2016). These findings suggest that the crude extracts in the present study, particularly extracts of *Streptomyces* sp. H16, may contain carboxylic acid-based compound(s) with anti-QS activity against PAO1.

Besides that, the ethyl acetate extract of *Streptomyces* sp. H16 consistently inhibited both pyocyanin production and swarming motility of PAO1. Since both these virulence attributes are modulated by *rhl* system, it is suggested that the extract may contain *rhl* inhibitor (Rasamiravaka *et al*., 2015). Similarly, the study conducted by Babić *et al*. (2010) showed that an aminoglycoside antibiotic known as tobramycin produced by *Streptomyces tenebrarius* also reduced production of pyocyanin and inhibited swarming of PAO1 at a subinhibitory concentration.

Contrariwise, it was noted that ethyl acetate of *Rhodococcus* sp. H54 increased pyocyanin production in a dose-dependent manner. The study conducted by Kim *et al*. (2012) also presented a similar result where the culture supernatant of a *Streptomyces* sp. increased pyocyanin formation by PAO1. In another study performed by Ren *et al*. (2005), it was reported that a natural furanone the marine red alga, *Delisea pulchra*, increased the production of pyoverdine, another virulence factor produced by *P. aeruginosa* (Adonizio *et al*., 2008; Peek *et al*., 2012). This indicates that it is apparently not uncommon to have compounds with opposing effects.

Unlike the findings obtained from violacein and swarming inhibition assays, in this case, the degree of pyocyanin inhibition by both ethyl acetate and hexane extracts was comparably similar. Additionally, extracts of ethanol (Datta *et al*., 2016), methanol and dichloromethane (Okusa *et al*., 2014) were also found to actively inhibit pyocyanin production. These findings suggest that solvents with varying polarities can be used to extract a broad range of potential bioactive compounds.

This study also highlights the QS-inhibitory activities of the ethyl acetate and hexane extracts of *Micromonospora* sp. TAV14 as this is the first report on inhibition of violacein synthesis, swarming motility and pyocyanin production by a member of this genus. It is, therefore, essential to further explore the quorum quenching potential of different genera of the class Actinobacteria.

Besides inhibiting QS-controlled mechanisms in *C. violaceum* CV026 and *P. aeruginosa* PAO1, members of the class Actinobacteria are also known to produce compounds that are active against QS-mediated virulence in other bacteria. In a study conducted by (Kang *et al*., 2016), it was shown that the culture extract of *Streptomyces*

xanthocidicus KPP01532 consists of glucopiericidin A and piericidin A, which are the active compounds reported to have suppressed virulence-associated genes of *E. carotovora* subsp. *atroseptica* (*Eca*), an important plant pathogen. Similarly, Nakayama *et al*. (2007) also reported that at a sub-lethal concentration, the peptide antibiotic siamycin I produced by *Streptomyces* sp. Y33-1 suppressed the virulence-related genes of *E. faecalis*, namely *fsrBDC* and *gelE-sprE*, which are responsible for the synthesis of gelatinase and serine protease in this bacterium. Other peptide-related natural compounds produced by Actinobacteria include cochinmicin by *Microbispora* sp. ATCC 55140 (Desouky *et al*., 2015; Zink *et al*., 1992) and arthroamide by *Arthrobacter* sp. (Igarashi *et al*., 2015), two cyclodepsipeptide compounds that inhibit quorum sensing signaling in Gram-positive bacteria, predominantly the *agr* system in *S. aureus*.

5.2 Future work

Due to time and budget constraints, the bioactive component(s) were not isolated and purified from the extracts. Thus, future work should include purification of the ethyl acetate and hexane crude extracts via column chromatography and high-performance liquid chromatographic (HPLC) analysis to isolate active compound(s), followed by structure elucidation of isolated compound(s) through mass spectrometric (MS) and nuclear magnetic resonance (NMR) spectroscopic analysis. The pure compound(s) should then be further screened for their ability to attenuate QS-mediated virulence in important pathogens to determine the range of biological activity of the compound(s).

It would also be interesting to study the mode of action of the purified compound(s) as well, as this could lead towards application of the compound(s) as anti-pathogenic drugs.

CHAPTER 6: CONCLUSIONS

The present study demonstrates the ability of members of the class Actinobacteria in producing anti-QS compounds. A total of 207 actinobacterial isolates were screened to determine their ability in inhibiting QS-regulated activities in *C. violaceum* CV026. Twenty-five isolates exhibited inhibitory activity towards violacein production by *C. violaceum* CV026 where three isolates, H16, H54 and TAV14 were selected for further analysis. Based on 16S rRNA gene sequences analyses, these isolates were identified as *Streptomyces* sp. H16, *Rhodococcus* sp. H54 and *Micromonospora* sp. TAV14, respectively. Generally, crude ethyl acetate and hexane extracts of all three isolates inhibited swarming motility of *P. aeruginosa* PAO1, with exception to ethyl acetate extract of *Micromonospora* sp. TAV14. The extracts were also observed to have reduced pyocyanin production by PAO1, except for hexane extract of *Streptomyces* sp. H16. On the contrary, ethyl acetate of *Rhodococcus* sp. H54 increased pyocyanin formation.

Actinobacteria in this study showed promising QQ properties which may be a rich source of QQ compounds that could be useful to attenuate QS-mediated virulence determinants. Therefore, additional work should be carried out to isolate and purify the active QQ compounds from these isolates which can be applied as potent therapeutic and anti-infective agents.

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LIST OF PAPERS PRESENTED

1. **Poster presentation - International Congress of Malaysian Society for Microbiology (ICMSM) 2013, Langkawi Lagoon Resort, Kedah, 12-15 December 2015**

Title of poster: Actinomycetes as Potential Producers of Quorum Sensing Inhibitors

Authors: Kavimalar Devaraj, Geok Yuan Annie Tan, Kok-Gan Chan.

Category: General Microbiology

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*Best Poster Presentation Award, Category: General Microbiology

2. **Poster presentation - International Congress of Malaysian Society for Microbiology (ICMSM) 2015, Bayview Beach Resort, Penang, 7-10 December 2015**

Title of poster: Characterisation of the Novel *Streptomyces* sp. PT12 Isolated from a Marine Sponge

Authors: Kavimalar Devaraj, Geok Yuan Annie Tan, Kok-Gan Chan.

Category: General Microbiology

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