PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF YELLOW-GREEN FLUORESCENT SIDEROPHORES PRODUCED BY *PSEUDOMONAS OTITIDIS* B1

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PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF YELLOW-GREEN FLUORESCENT SIDEROPHORES PRODUCED BY *Pseudomonas otitidis* B1

By

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ABSTRACT

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Pseudomonas otitidis is associated with otic infection in human ears. It is closely related to *Pseudomonas aeruginosa* and it shares the common features of the *Pseudomonas* genus. As iron is an essential element needed by most microorganisms to survive, microorganisms synthesize iron-scavenging agents known as siderophores to sequester iron from the environment. In this study, *Pseudomonas otitidis* B1, isolated from an ex-mining lake in Kampar, was used for the production of yellow-green fluorescent siderophores in simple succinate medium (SSM). After 24-hour incubation, the supernatant of the bacterial culture was subjected to spectrophotometric analysis and the chrome azurol sulphonate (CAS) agar diffusion assay for detection of siderophores. Siderophore was then extracted and purified in a more stable form as ferric-siderophore complex. After running through ion-exchange chromatography, the siderophore underwent gel filtration chromatography after iron removal for final purification. Upon

purification, the antimicrobial and growth-promoting properties of iron-free siderophore were tested against several Gram-postive and Gram-negative bacteria. The crude siderophores of *P. otitidis* B1 gave a characteristic peak at 404 nm upon UV-VIS spectrophotometry analysis. After purification, the characteristic peak of the siderophore was conserved. No growth inhibition activity against all Gram-positive bacteria and Gram-negative bacteria were displayed by the partially purified iron-free siderophore. However, it was found to promote the growth of *Staphylococcus aureus* ATCC 25933 where a zone of denser growth was detected around the disc filled with the partially purified sample. Further investigations on the structural and biological properties of this siderophore are necessary especially its growth-promoting properties on bacteria should be further investigated.

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Last but not least, I want to thank my friends and family for their moral support and assistance in any aspect during completion of this project. Thank you all.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Yeoh Chew Chie

APPROVAL SHEET

This project report entitled "**PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF YELLOW-GREEN FLUORESCENT SIDEROPHORES PRODUCED BY *Pseudomonas otitidis* B1**" was prepared by YEOH CHEW CHIE and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **YEOH CHEW CHIE** (ID No: **10ADB05402**) has completed this final year project entitled "PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF YELLOW-GREEN FLUORESCENT SIDEROPHORES PRODUCED BY *Pseudomonas otitidis* B1" under the supervision of Dr. Kho Chiew Ling (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(YEOH CHEW CHIE)

TABLE OF CONTENTS

		Page
ABSTRACT		**ii**
ACKNOWLEDGEMENT		**iv**
DECLARATION		**v**
APPROVAL SHEET		**vi**
PERMISSION SHEET		**vii**
TABLE OF CONTENTS		**viii**
LIST OF TABLES		**xii**
LIST OF FIGURES		**xiii**
LIST OF ABBREVIATIONS		**xv**
CHAPTER		
1	INTRODUCTION	1
2	LITERATURE REVIEW	5
	2.1 *Pseudomonas*	5
	2.1.1 *Pseudomonas otitidis*	7
	2.2 Importance of Iron to Life	8
	2.2.1 Iron Uptake System of Gram-negative Bacteria	9
	2.3 Siderophores	10
	2.3.1 Types of Siderophores	12
	2.3.2 Siderophores of *Pseudomonas* spp.	15
	2.3.3 Pyoverdine	16

2.4 Detection and Characterization of Siderophores 17
2.5 Applications of Siderophores 18
2.5.1 Environmental Applications 19
2.5.2 Agriculture Applications 19
2.5.3 Medical Applications 21

3 MATERIALS AND METHODS 24
3.1 Experimental Design 24
3.2 Bacterial Strain 25
3.3 Apparatus and Equipment 25
3.4 Chemicals and Media 25
3.5 Preparation of *Pseudomonas otitidis* B1 Culture 26
3.5.1 Revival of *P. otitidis* B1 26
3.5.2 Preparation of Overnight Culture of *P. otitidis* B1 26
3.5.3 Long-term Storage of *P. otitidis* B1 26
3.6 Detection of Siderophores 27
3.6.1 Spectrophotometric Analysis 27
3.6.2 Chrome Azurol Sulphonate Agar Diffusion Assay 27
3.7 Scale-up Production of Siderophores 29
3.8 Purification of Siderophores 29
3.8.1 Ferration of Siderophores 29
3.8.2 Ammonium Sulfate Precipitation 30
3.8.3 Phenol-chloroform Extraction 30
3.8.4 Ion-exchange Chromatography 31
3.9 Purification of Iron-free Siderophores 32
3.9.1 Preparation of Iron-free Siderophores 32

3.9.2 Gel Filtration Chromatography 32
3.9.3 Detection of Iron-free Siderophores 33
3.10 Antimicrobial Properties of Siderophores 34
3.10.1 Antimicrobial Test of Siderophores 34

4 RESULT 36
4.1 Production of Siderophores 36
4.2 Detection of Siderophores 37
4.2.1 Spectrophotometric Analysis 37
4.2.2 Chrome Azurol Sulphonate (CAS) Agar Diffusion Assay 38
4.3 Purification of Siderophores 39
4.3.1 Ion-exchange Chromatography 41
4.3.2 Preparation of Iron-free Siderophores 43
4.3.3 Gel Filtration Chromatography 43
4.3.4 Detection of Iron-free Siderophores 45
4.4 Antimicrobial Properties of Siderophores 46

5 DISCUSSION 49
5.1 Production of Siderophores 49
5.2 Detection of Siderophores 50
5.2.1 Spectrophotometric Analysis 50
5.2.2 Chrome Azurol Sulphonate (CAS) Agar Diffusion Assay 51
5.3 Purification of Siderophores 52
5.3.1 Ammonium Sulfate Precipitation 53
5.3.2 Phenol-chloroform Extraction 54
5.3.3 Ion-exchange Chromatography 55

5.3.4 Preparation of Iron-free Siderophores 57

5.3.5 Gel Filtration Chromatography 58

5.3.6 Detection of Iron-free Siderophores 59

5.4 Antimicrobial and Growth-promoting Activities of Siderophores 59

5.5 Future Prospects 61

6 CONCLUSIONS 63

REFERENCES 65

APPENDICES 77

LIST OF TABLES

Table		Page
2.1	Examples of siderophores produced by fluorescent pseudomonads and non-fluorescent pseudomonads.	15
A	List of apparatus and equipment with their respective manufacturers.	76
B	List of chemicals, reagents and pre-mixed media with their respective manufacturers.	77

LIST OF FIGURES

Figure		Page
2.1	The five *Pseudomonas* groups arranged based on rRNA homology.	6
2.2	Neighbor-joining tree analysis for some of the *Pseudomonas* species.	7
2.3	Iron uptake systems in Gram-negative bacteria.	10
2.4	Functional groups in different types of siderophores.	13
2.5	Pyoverdine structure of *Pseudomonas fluorescens* (ATCC 13525 strain).	17
2.6	Application of siderophores in medicinal industry.	21
3.1	Overview of the experimental design of this project.	24
4.1	Production of yellow-green fluorescent pigment.	36
4.2	Absorption spectrum of crude siderophores.	37
4.3	CAS agar diffusion assay.	38
4.4	Difference in color of the 24-hour culture before and after addition of $FeCl_3$.	39
4.5	Absorption spectra of crude and ferrated siderohpores.	40
4.6	Phenol-chloroform extraction of ferrated siderophores.	41
4.7	Spectra of eluents in ion-exchange chromatography.	42

4.8	Comparison of color difference of the sample before and after iron removal.	43
4.9	Spectra of eluents in gel filtration chromatography.	44
4.10	The color of the sample after gel filtration chromatography.	45
4.11	CAS agar diffusion assay for detection of iron-free siderophores.	46
4.12	Effect of partially purified siderophore on the growth of Gram-postive bacteria.	47
4.13	Effect of partially purified siderophore on the growth of Gram-negative bacteria.	48
D	Ammonium sulfate precipitation table.	80

LIST OF ABBREVIATIONS

°C	degree Celsius
°	degree
%	percentage
× *g*	multiplies of Earth's gravitational acceleration
$(NH_4)_2SO_4$	ammonium sulphate
µl	microliter
µm	micrometer
16S	16 Svedberg
ABC	ATP-binding cassette
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CAS	chrome azurol sulphonate
CCl_4	carbon tetrachloride
cm	centimeter
cm^3	cubic centimeter
CM Sephadex	carboxymethyl Sephadex
CO_2	carbon dioxide
DFO	Desferrioxamine B
DFX	Desferal
DNA	deoxyribonucleic acid

DZR	dexrazoxane
ESI-MS	Electrospray ion-mass spectrometry
Fe	iron (Ferrum)
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
$FeCl_3$	ferric chloride
$FeCl_3.6H_2O$	ferric chloride hexahydrate
Fhu	ferric hydroxamate-binding proteins
g	gram
HCl	hydrochloric acid
HDTMA	hexadecyltrimethylammonium
HPLC	high performance liquid chromatography
L	litre
LB	Luria-Bertani
M	molar
mg	milligram
MH	Müeller Hinton
ml	millilitre
mm	millimeter
mM	millimolar
MS	mass spectrometry

MW	molecular weight
NaCl	sodium chloride
nm	nanometer
NMR	nuclear magnetic resonance
PBP	periplasmic binding protein
PCR	polymerase chain reaction
PDTC	pyridine-2,6-dithiocarboxylic acid
PGPR	plant growth-promoting rhizobacteria
PIPES	1,4-piperazinediethanesulphonic acid
Pvd	pyoverdine
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
sp. nov.	species nova
spp.	species (plural)
SSM	simple succinate medium
TLC	thin layer chromatography
UV-VIS	ultraviolet-visible
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Pseudomonas species are very common in natural microbial community (Liu, et al., 2009). The genus *Pseudomonas* was created by Migula in 1894 (Liu, et al., 2009), and to date, there are 207 species that have been described and published (LPSN, 2013). Almost all species share the basic features. According to Palleroni (2005), they are Gram negative, straight rods bacteria with one or several polar flagella. The members of *Pseudomonas* are non-spore forming, and also, they undergo aerobic chemoorganotrophic metabolism (Meldrum, 1999). The ability of some *Pseudomonas* spp. to produce pigments is an important tool for species differentiation. Some *Pseudomonas* spp. are capable of producing the blue pigment, pyocyanin; some produce carotenoid pigments; and some produce yellow-green fluorescent pigments (Palleroni, 2005; Young, 1947).

Iron is needed by most of the living cells (Bhattacharya, 2010). Iron exits in two forms as ferric ions (Fe^{3+}) and ferrous ions (Fe^{2+}). Most of the irons on the earth present as the oxidized form ferric ions and is not readily available (Moody, 1986). Although iron is abundant in the earth, its bioavailability is relatively low as ferric and ferrous ions hydrolyze to polymeric hydroxides at neutral pH under aerobic condition (Moody, 1986). To overcome the problem of iron limitation,

microorganisms developed a system for high-affinity uptake of iron mediated by low molecular weight chelators known as siderophores (Visca, et al., 1992).

Siderophore is a low molecular weight, ferric-specific chelating agent that is produced under low iron condition. It is important in scavenging iron from the surroundings and in making minerals which is essential to microorganisms (Neilands, 1995). Crichton and Charloteaux-Wauters (1987) reported that probably all aerobic and facultative anaerobic microorganisms are capable of producing siderophores.

Siderophore-mediated iron assimilation systems involve ferric-binding siderophores and the cognate membrane receptors (Neilands, 1984). The synthesis of siderophore is tightly regulated by iron (Neilands, 1976). Siderophores are generally classified as hydroxamates or phenolate-catecholates (Crichton and Charloteaux-Wauters, 1987). A third class is extended as carboxylate siderophores for siderophores that contain neither of the mentioned classes (Nagoba and Vedpathak, 2011). Bacteria are able to produce both hydroxamates and phenolate-catecholates types while fungi can only produce hydroxamates type. Some plants use hydroxamate siderophores in exploiting iron (Barash, 1990). Some *Pseudomonas* species may even produce siderophores of both the hydroxamate and catechol groups (Barash, 1990).

Fluorescent pseudomonads mainly synthesize pyoverdine siderophore, a yellow-green, water-soluble pigment in scavenging iron from the environment and transporting iron into the cell (Meyer, 2000). Proven by the research of Visca, et al. (1992) research, pyoverdine synthesis and expression of its associated receptor proteins are affected only by ferrous ions, Fe^{3+}. Meanwhile, pyochelin is another common type of siderophores synthesized by fluorescent pseudomonads. In contrast, pyochelin is poorly water-soluble and has relatively low affinity for Fe^{3+} but with higher affinity of binding to other transition metals such as copper(II), cobalt(II), nickel(II), and molybdenum(VI) ions (Visca, et al., 1992).

Siderophores are useful in various applications and functions. Firstly, siderophores can be used as drug delivery agents in medical application which is known as the "Trojan Horse" strategy. Antimicrobial agents conjugate with siderophores to form Sideromycins and utilize the iron transport system of siderophores to defeat the bacteria (Nagoba and Vedpathak, 2011). Siderophores are also clinically useful in treating iron overload disease and cancer (Kalinowski and Richardson, 2005). Besides, plant growth-promoting rhizobacteria (PGPR) produce extracellular siderophores to deprive native microflora by complexing with environmental iron (Kloepper, et al., 1980).

In this project, the subject of this study was *Pseudomonas otitidis* B1, which is a recently recognized *Pseudomonas* species associated with otic infections in human (Thaller, et al., 2011). The ability of the bacteria to produce yellow-green fluorescent siderophores (unpublished data) was first studied. This was followed by siderophore purification and the determination of its antimicrobial properties. The five main objectives of this study were:

i. To produce siderophores of *P. otitidis* B1 in simple succinate medium (SSM).
ii. To detect the siderophores produced by spectrophotometric assay and chrome azurol sulphonate (CAS) agar diffusion assay.
iii. To extract the ferrated siderophores produced through ammonium sulphate precipitation, phenol-chloroform extraction and ion-exchange chromatography.
iv. To prepare the iron-free siderophores and to partially purify the iron-free siderophores by gel filtration chromatography.
v. To characterize the antimicrobial properties of the siderophores.

CHAPTER 2

LITERATURE REVIEW

2.1 *Pseudomonas*

Pseudomonas species are very common and have been isolated from human, plants and various habitats including soil, water and air (Clark, et al., 2006). Since Migula created the genus *Pseudomonas* in 1894 (Liu, et al., 2009) until now, there are 207 species have been described and assigned with valid published names (LPSN, 2013). *Pseudomonas* is Gram negative bacteria and the members share similar morphological features (Palleroni, 2005). They are straight rod bacteria with one or several polar flagella. Besides, the members of *Pseudomonas* are non-spore forming and they undergo aerobic chemoorganotrophic metabolism (Meldrum, 1999). Most of the *Pseudomonas* species are mesophilic however, some, such as *Pseudomonas fluorescen*s, are psychrophilic as they can grow at 4°C (Palleroni, 2005). The ability of pigmentation plays an important role in diagnosis of some species. Some *Pseudomonas* are capable of producing phenazine pigments; some produce carotenoid pigments; and some produce yellow-green fluorescent pigments (Palleroni, 2005; Young, 1947).

According to Palleroni, et al. (1973), *Pseudomonas* species are classified into five RNA homology groups using rRNA-DNA hybridization approach. Based on their

research, the five groups (as shown in Figure 2.1) are distantly related to one another phylogenetically.

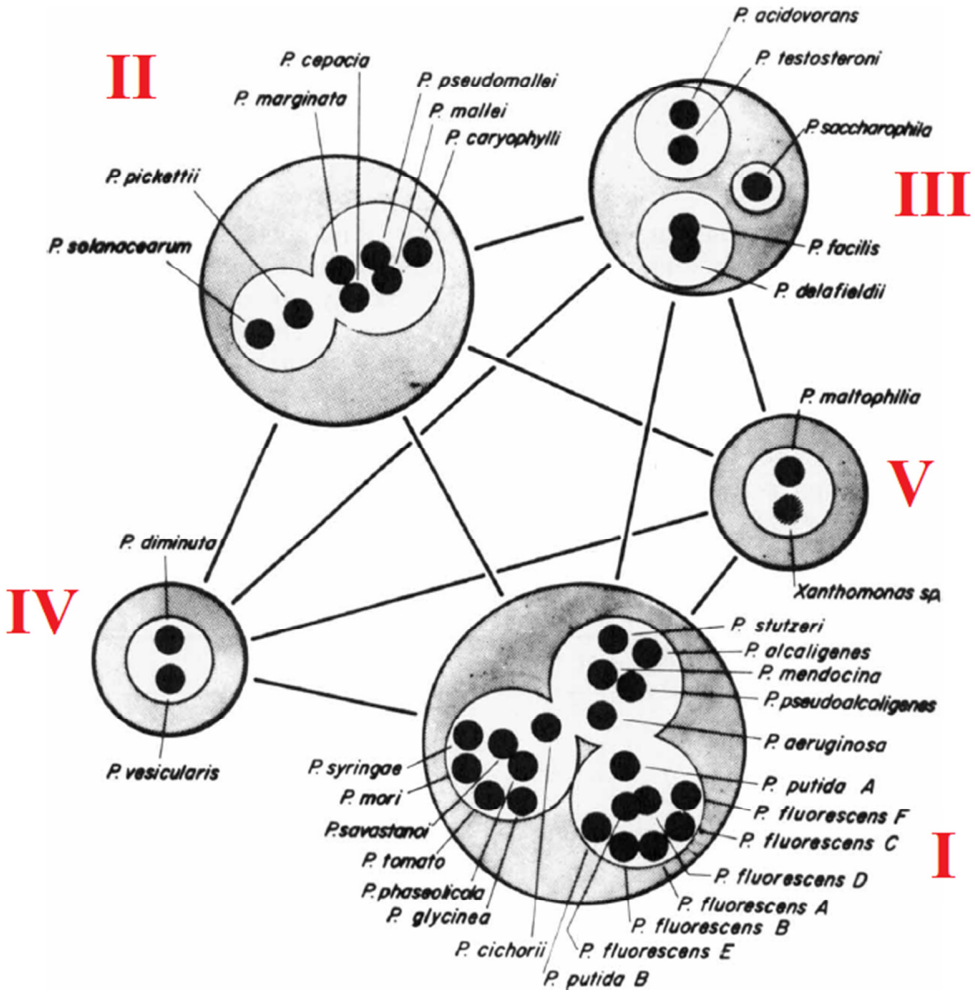

Figure 2.1: The five *Pseudomonas* groups arranged based on rRNA homology. The shaded circles represent rRNA homology while the white circles represent DNA homology of *Pseudomonas* species (Palleroni, et al., 1973).

2.1.1 *Pseudomonas otitidis*

P. otitidis was recently identified by isolation from humans' ears with otic infections such as acute otitis externa, acute otitis media and chronic suppurative otitis media (Thaller, et al., 2011). *P. otitidis* also shares the basic properties of the genus *Pseudomonas* as the Gram reaction is negative and it is a motile rod shaped bacteria (Clark, et al., 2006). As in Figure 2.2, Clark, et al. (2006) showed that *P. otitidis* sp. nov. is closely related to *Pseudomonas aeruginosa* where their 16S rRNA gene sequences are 98.6% similar.

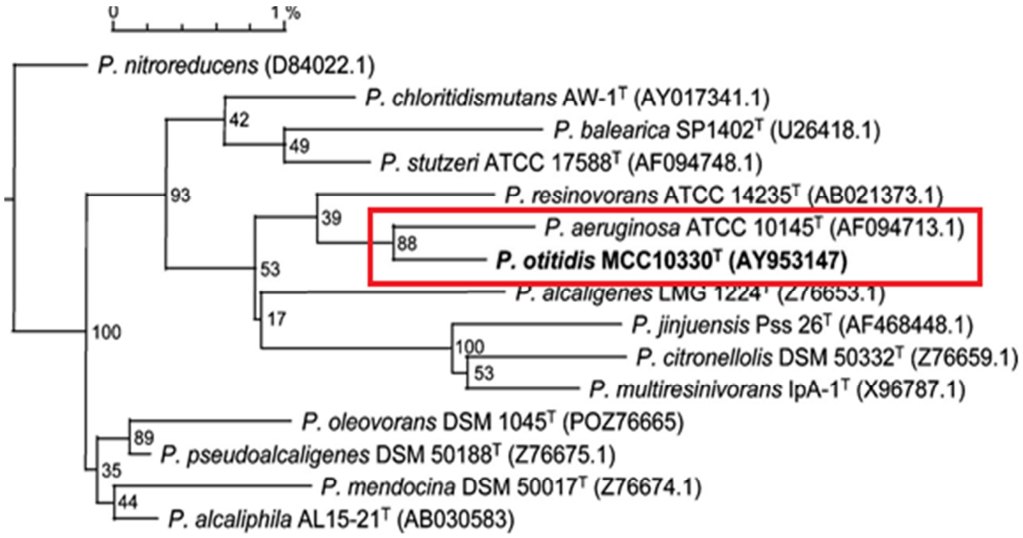

Figure 2.2: Neighbor-joining tree analysis for some of the *Pseudomonas* species. *P. otitidis* and *P. aeruginosa* grouped under the same clade based on 16S rRNA gene sequence analysis (Clark, et al., 2006).

2.2 Importance of Iron to Life

Iron is an essential element that is needed by all organisms to survive except certain strains of lactobacilli (Heli, Mirtorabi and Karimian, 2011). Iron is involved in many biochemical and life supporting activities such as respiration, nitrogen fixation, DNA and chlorophyll biosynthesis and some enzymatic system (Barash, 1990). Although iron is abundant in the earth, its bioavailability is relatively low (Moody, 1986). Iron exist in two form, depending on the oxidation state of the ions, as ferrous ions, Fe^{2+} or ferric ions, Fe^{3+}(Moody, 1986). Ferrous ions are unstable and are likely to undergo Fenton reaction under aerobic condition where ferric ions and reactive oxygen species are produced. Ferric ions are then accumulated to form insoluble polymer hydroxides (Krewulak and Vogel, 2008).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

Due to this problem, through evolution, microorganisms had developed a high-affinity iron uptake system which consists of iron chelators, siderophores and the corresponding membrane receptors (Visca, et al., 1992).

2.2.1 Iron Uptake System of Gram-negative Bacteria

Pseudomonas species are Gram-negative bacteria (Palleroni, 2005). Iron acquisition can be done by transferrin, lactoferrin, siderophore, or heme (Krewulak and Vogel, 2008). Gram-negative bacteria are protected by a permeable outer membrane. In the mechanism of iron uptake by all the mentioned pathways, outer membrane receptor, ATP-binding cassette (ABC) transporter and periplasmic binding protein (PBP) are the basic components required (Krewulak and Vogel, 2008). The energy required for transportation of iron across the membrane can be driven by the proton motive force created by the TonB system which consists of TonB, ExbB and ExbD (Krewulak and Vogel, 2008). Some bacteria such as *Escherichia coli* and *Vibrio cholerae* acquire iron from heme (Occhino, et al., 1998) while others such as *Listeria monocytogenes* are able to acquire iron by reducing iron bound to transferrin (Deneer and Boychuk, 1993). Besides, some bacteria such as *P. aeruginosa* produce iron-chelating siderophores to bind iron under low iron condition (Neilands, 1981). Figure 2.3 shows transferrin, siderophore and heme iron uptake system in Gram-negative bacteria.

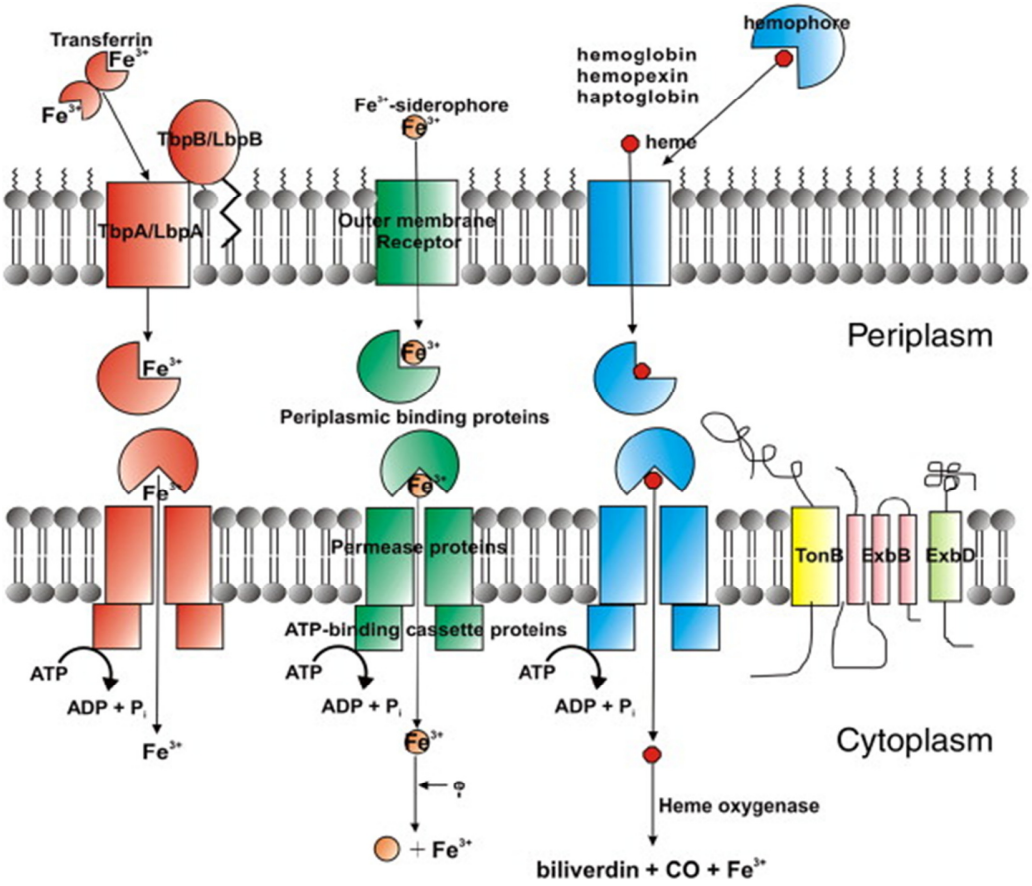

Figure 2.3: Iron uptake systems in Gram-negative bacteria. For each pathway of iron uptake, the basic components are outer membrane receptor, PBP and ABC transporter. TonB system establishes ion gradients to create energy for iron transport (Krewulak and Vogel, 2008).

2.3 Siderophores

Siderophores are low molecular weight (about 500 to 1000 Daltons) secondary metabolites, ferric-specific chelating agents that are produced under iron-limiting condition (Meldrum, 1999). It is important in scavenging iron from surrounding

and making iron readily available to microorganisms (Neilands, 1995). Siderophores have high affinity towards ferric ions (affinity constant of approximately 10^{30}) (Meldrum, 1999). It is also reported that siderophores are capable of binding other metals such as molybdenum, cadmium, calcium, nickel, lead and etc (Bhattacharya, 2010).

Many researches had proven that siderophores produced by various bacteria imposed virulence to many animals and plants. The *in vivo* growth of infectious bacteria in host is mainly due to the ability of their iron scavengers to compete with the host for iron (West and Buckling, 2002). According to Meyer, et al. (1996), *P. aeruginosa* produces pyoverdins which can cause fatal infection in patients with cystic fibrosis. It was also reported that *Staphylococcus aureus* produce hydroxamate-type siderophores that may cause several skins and wounds infections by invading and destructing tissues (Dale, et al., 2004).

Fungi are also capable of synthesizing various types of iron chelators to aid in iron uptake. Ferricrocin of *Aspergillus fumigatus* is a siderophore involved in iron distribution within and between cellular (Wallner, 2009). Chen, Lin and Chung (2013) found that *Alternaria alternate* excretes coprogen that induces virulence to citrus. Fusarinines, ferrichromes and rhizoferrins are some other examples of siderophores produced by fungi (Hollinsworth and Martin, 2009).

2.3.1 Types of Siderophores

To date, almost 500 types of siderophores produced by different microorganisms have been recognized (Krewulak and Vogel, 2008). Siderophores are generally classified as hydroxamates and phenolate-catecholates (Crichton and Charloteaux-Wauters, 1987). A third class of siderophores known as carboxylate has been extended for siderophores that contained neither of the two classes. The classification of siderophores is based on the functional group moieties of the compound to bind and coordinate metal ions (Nagoba and Vedpathak, 2011).

Hydroxamates are typically produced by both bacteria and fungi (Barash, 1990). Hydroxamates contain N-hydroxyornithine moieties that contribute to the hydroxamic acid groups (Figure 2.4a) (Crichton and Charloteaux-Wauters, 1987). Most of the hydroxamate siderophores consist of three hydroxamate groups which form the hexadentate octahedral when complex with a Fe^{3+} (Ali and Vidhale, 2013). Ferrichrome, as shown in Figure 2.4b, is an example of hydroxamate siderophore produced by many types of fungi such as *Aspergillus* sp. and *Trichophyton* sp. (Varma and Chincholkar, 2007).

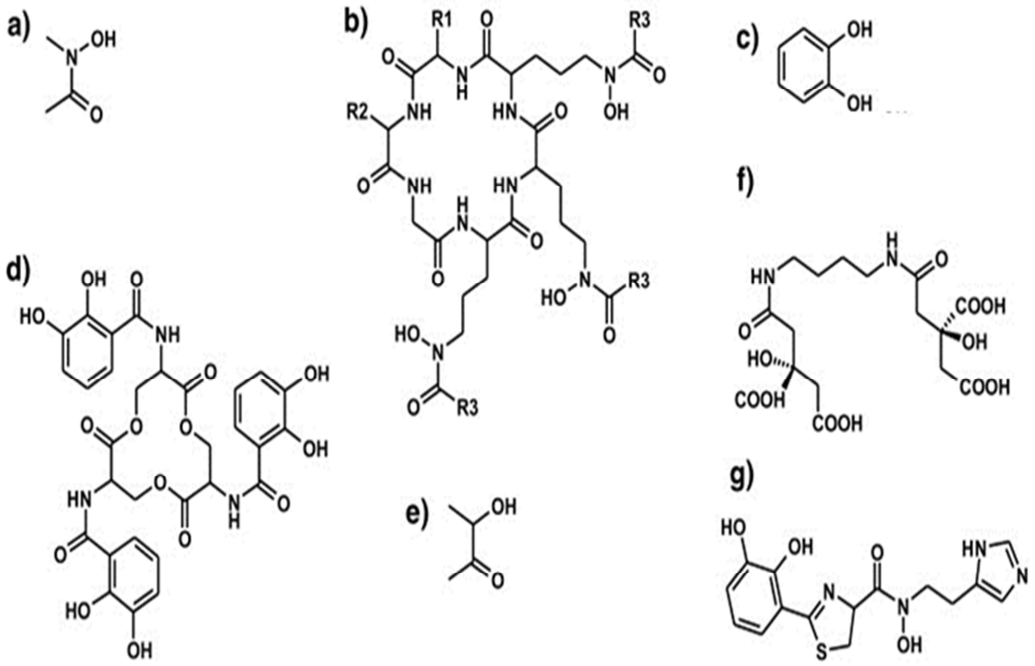

Figure 2.4: Functional groups in different types of siderophores. a) Hydroxamic acid, functional group for hydroxamate siderophores. b) Ferrichrome produced by many types of fungi. c) Catechol, functional group for catocholate siderophores. d) Enterobactin produced by *S. typhimurium*. e) α-Hydrocarboxylic acid, functional group for caboxylate siderophores. f) Rhizoferrin produced by the phylum Zygomycota. g) Anguibactin, catechol-hyrdoxamate mixed siderophore produced by *Vibrio anguillarum* (Krewulak and Vogel, 2008).

Phenolate-catecholates are usually produced by some bacteria only but not produced by fungi (Varma and Chincholkar, 2007). Similar to hydroxamate, hexadentate octahedral complex is formed when catecholate groups (Figure 2.4c) bind to a Fe^{3+} (Ali and Vidhale, 2013). *Salmonella typhimurium* produces enterobactin (Raymond, Dertz and Kim, 2003) which consists of three catecholate

groups as shown in Figure 2.4d. Enterochelin is another name of enterobactin which was used to describe *E. coli* catecholate siderophore (Raymond, Dertz and Kim, 2003).

Certain bacteria such as *Rhizobium* and *Staphylococcus* as well as Mucorales fungi produce carboxylate siderophores (Ali and Vidhale, 2013; Varma and Chincholkar, 2007). Carboxylates utilizes carboxyl and hydroxyl groups contributed by hydrocarboxylic acid (Figure 2.4e) to coordinate iron (Ali and Vidhale, 2013). Rhizoferrin as shown in Figure 2.4f which is observed among the phylum Zygomycota contains two citric acids connected by a diaminobutane (Varma and Chincholkar, 2007).

According to Barash (1990), there are some siderophores that contains both catechol and hydroxamate groups. For example, anguibactin, as shown in Figure 2.4g, produced by *Vibrio anguillarum* consisted of catechol group and secondary amide function which corresponded to the hydroxamate group (Actis, et al., 1986). *Pseudomonas* spp. are also capable of producing catechol-hydroxamate mixed siderophores such as pseudobactin and pyoverdin (Barash, 1990).

2.3.2 Siderophores of *Pseudomonas* spp.

Over the years, many researches on the siderophores produced by various species of *Pseudomonas* have been conducted. Based on the characteristics of the siderophores produced by *Pseudomonas*, this genus can be categorized as fluorescent pseudomonads and non-fluorescent pseudomonads (Meyer and Abdallah, 1980). According to Bultreys, et al. (2001), fluorescent pseudomonads are characterized by the ability to produce fluorescent siderophores. In contrast, non-fluorescent pseudomonads produce colorless siderophores which are also able to bind strongly to Fe^{3+} (Meyer and Abdallah, 1980). Some examples of siderophores produced by both types of *Pseudomonas* are illustrated in Table 2.1.

Table 2.1: Examples of siderophores produced by fluorescent pseudomonads and non-fluorescent pseudomonads.

Species	Siderophores	References
Fluorescent pseudomonads		
P. fluorescens	Pyoverdine	Meyer and Abdallah (1978)
P. aeruginosa PAO1	Pyochelin	Visca, et al. (1992)
P. putida B10	Pseudobactin	Buyer, Sikora and Kratzke (1990)
Non-fluorescent pseudomonads		
P. stutzeri ATCC 17488	Desferriferrioxamine E	Meyer and Abdallah (1980)
P. corrugata	Corrugatin	Meyer, et al. (2002)

2.3.3 Pyoverdine

Pyoverdine is the main siderophore synthesized by many fluorescent pseudomonads which gives the characteristic yellow-green fluorescent color (Meyer, 2000). This water soluble pigment is believed to contribute to the pathogenic and saprophytic effects on plants and animals (Cody and Gross, 1987). Although the mechanism of iron sequester from animal hosts are not known, pyoverdine of *P. aeruginosa* acquires iron from host proteins such as transferrin and lactoferrin (Meyer, et al., 1996). Gross (1985) suggested that the plant phytotoxin produced by *Pseudomonas syringae* is an iron-binding pyoverdine siderophore.

Pyoverdine is a catechol-hydroxamate siderophore which consists of three main parts in the structure, i) quinoline chromophore, ii) peptide chain and iii) acid side chain (Figure 2.5) (Fuchs, et al., 2001). Pyoverdines share a common feature in which the fluorescent chromophores are made up of 2,3-diamino-6,7-dihydroxyquinoline where the catecholate group resides (Visca, et al., 1992). Dicarboxylic acid side chain is bounded to the chromophore at N-terminal (Fuchs, et al., 2001). N-hydroxyornithines of the octapeptide contribute the hydroxamic acid groups which complex iron in action with the catecolate group of chromophore (Cody and Gross, 1987). It is reported by Fuchs, et al. (2001) that the pyoverdines of *Pseudomonas* strains are differed by the peptides which are found to consist of six to twelve amino acids present in a linear or cyclic form.

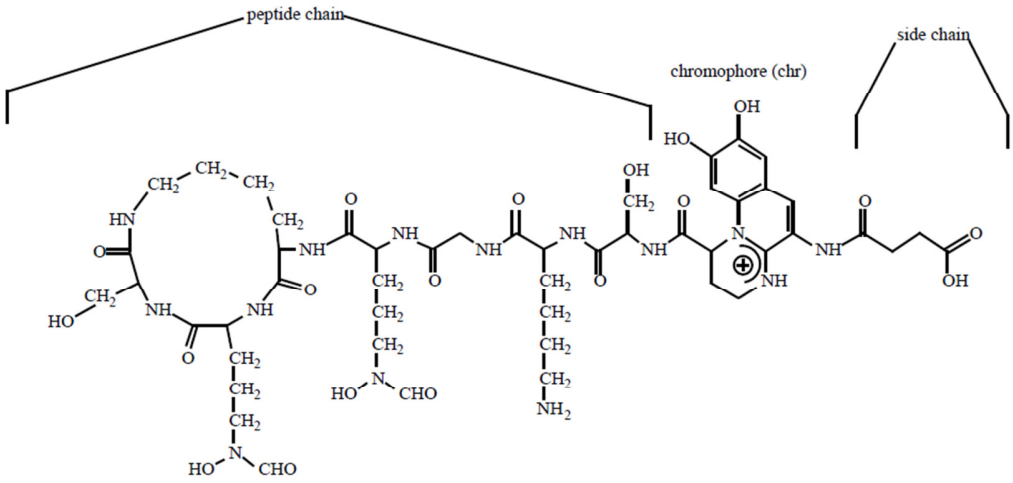

Figure 2.5: Pyoverdine structure of *Pseudomonas fluorescens* (ATCC 13525). Pyoverdine is made up of three parts; chromophore, peptide and side chain (Fuchs, et al., 2001).

2.4 Detection and Characterization of Siderophores

Several methods were developed to detect and characterize siderophores. Highly specific test for hydroxamate siderophores can be carried out using Csàky test to measure the concentration of the nitrite end product (Cody and Gross, 1987). Different known concentrations of hydroxylamine hydrochloride are usually used to construct a standard curve for the quantification of the end products (Visca, et al., 1992). Another method for hydroxamate detection which is known as Neilands assay was described by Emery and Neilands (1960) where the periodic oxidation of hydroxamic acid results in intense absorption at 264 nm. Arnow assay is commonly used for the detection of phenolate-catecholate siderophores where nitrous acid and excess sodium chloride are added to sample supernatant to

yield an orange red color compound (Bhattacharya, 2010). However, these specific tests mentioned are not able to detect the occurrence of carboxylate siderophores due to the absence of specific functional groups. Hence, chrome azurol sulfonate assays are more often used to detect the presence of siderophores (Neilands, 1995). It is a colorimetric assay in which the removal of iron from iron-CAS-hexadecyltrimethylammonium bromide (HDTMA) complex changes the color from blue to orange (Neilands, 1995). The structure of siderophores can be determined by the combination of mass spectrometry and nuclear magnetic resonance (NMR) as the paramagnetism of ferric ion causes direct NMR analysis to be impossible (Neilands, 1995).

2.5 Applications of Siderophores

Despite the pathogenicity in causing diseases in plants and animals, the ability of siderophores to chelate iron drives various applications in the biotechnology field. Researches regarding the uses of siderophores are intense in recent years and siderophores have been proven to contribute to environmental, agriculture and even medicinal fields (Ali and Vidhale, 2013).

2.5.1 Environmental Applications

Since the industrial revolution, actinide compounds are found to be contaminating soils and groundwater which threatens the environmental condition (Ali and Vidhale, 2013). According to John, et al. (2001), plutonium (IV) ions are chemically similar to Fe^{3+} which enables siderophores to complex with Pu(IV) in the contaminating environment. The research of John, et al. (2001) showed that the mobility and solubility of Pu can be affected by the compatibility of microorganisms to the Pu-siderophores complexes and hence contributes to the bioremediation of actinide contamination in the environment.

2.5.2 Agriculture Applications

Siderophores are found to be involved in promoting plant growth and suppressing soil-borne diseases in the agriculture field (Barash, 1990; Ali and Vidhale, 2013). Researches against the plant growth promotion of corn, sugar beet, tea and etc are revealed to be related to the ability of rhizobacteria to produce siderophores (Moon, et al., 2008; Chakraborty, Chakarborty and Basnet, 2006; Molina, et al., 2005). Seed inoculated with *Pseudomonas fluorescens-putida* groups are able to promote the growth of plants and increase the crop yield (Kloepper, et al., 1980).

2.5.2.1 Plant Growth-promoting Rhizobacteria (PGPR)

Many soil bacteria are plant growth-promoting rhizobacteria which promote the growth of plants. These rhizobacteria grow in close proximity with plants produce siderophores to deprive iron from phytopathogens in the rhizophere area of the plants, causing the inaccessibility of iron to the phytopathogens (Barash, 1990; Das, Kumar and Kumar, 2013). The ability of PGPR in suppressing the growth of deleterious microorganisms is termed as biopesticides (Vessey, 2003).

Reported by Moon, et al. (2008), *P. fluorescens* SBW25 encodes genes related in iron uptake mechanisms which show similarities to siderophores receptors genes and siderophores biosynthetic enzyme genes. These characteristics of *P. fluorescens* SBW25 are important for sugar beet colonization as the *Pseudomonas* are able to control against the growth of the phytopathogen, *Pythium ultimum (*Moon, et al., 2008). Another example is discovered by Chakraborty, Chakarborty and Basnet (2006) where the inoculation of *Bacillus magaterium*, which is capable of synthesizing siderophores, to the tea rhizosphere is able to decrease the population of the plant pathogen, *Fomes lamaoensis*.

2.5.3 Medical Applications

Siderophores and their derivatives contribute to a wide range of applications in the healthcare industry. Figure 2.6 summarizes the major medical applications of different types of siderophores.

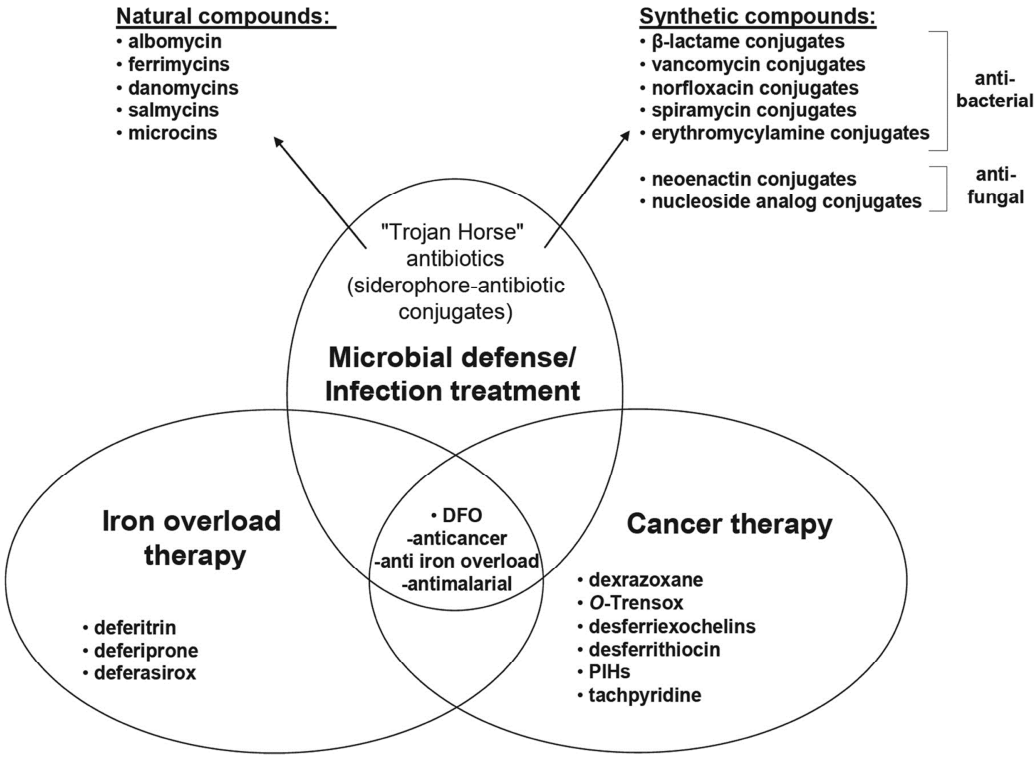

Figure 2.6: Applications of siderophores in the medicinal industry. Therapeutic potentials of various siderophores and the derivatives in treating iron overload diseases, cancer, malaria, infections and etc (Miethke and Marahiel, 2007).

2.5.3.1 Drug Delivery - Trojan Horse Strategy

Siderophores which are able to selectively introduce antibiotics by utilizing the iron-siderophore uptake system of the antibiotic-resistant bacteria are known as sideromycins (Miethke and Marahiel, 2007). Sideromycins are made up of two parts; the siderophore part that forms complex with iron and later recognized by the Fe-siderophore complex receptor on the bacteria cell; and the antibiotic part that enacts antibiotic function to the pathogenic bacteria (Nagoba and Vedpathak, 2011).

Natural and synthetic sideromycins have been discovered and synthesized and are being used to achieve drug delivery into pathogens. Experiment done by Pramanik, et al. (2007) showed that the natural sideromycin, albomycin, reduced Enterobacteriaceae, *Staphylococcus aureus* and *Streptococcus pneumoniae* in mice infected with these bacteria. Synthetic sideromycins are also developed to counteract pathogens that are harmful to human. Human infection by *P. aeruginosa* may contribute to cystic fibrosis (Smith, et al., 2012). Kinzel and Budzikiewics (1999) developed a pyoverdine-cephalexin conjugate that utilizes the iron uptake mechanism to deliver cephalexin into *P. aeruginosa*.

2.5.3.2 Iron Overload Therapy

Periodic blood transfusions of β-thalassemia patients often lead to iron overload diseases such as hemochromatosis and hemosiderosis (Nagoba and Vedpathak, 2011). Such conditions are unfavorable and require iron removal especially from the liver. Deferoxamine, deferiprone and deferasirox are the iron-chelating agents that have been used in treating iron overload diseases (Makis, et al., 2013). Among all, deferasirox and deferiprone, which can be taken orally, are the more popular way of treatment as compared to the deferoxamine injection (Makis, et al., 2013).

2.5.3.3 Anti-malaria and Cancer Therapy

Iron chelation by siderophores is shown to be able in treating malaria, a sickness caused by *Plasmodium falciparum* (Nagoba and Vedpathak, 2011). *In vivo* or *in vitro* treatments are made available by the iron-chelating Desferrioxamine B (DFO) which is produced by *Streptomyces pilosus* (Nagoba and Vedpathak, 2011). The deprivation of iron by DFO from the parasite's surrounding causes the parasite to die (Miethke and Marahiel, 2007). As for cancer therapy, the clinical trial carried out by Swain, et al. (1997) showed that dexrazoxane (DZR) protects breast cancer patients from heart failure due to overdose of doxorubicin.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

The overview of the experimental design of this project is summarized in Figure 3.1.

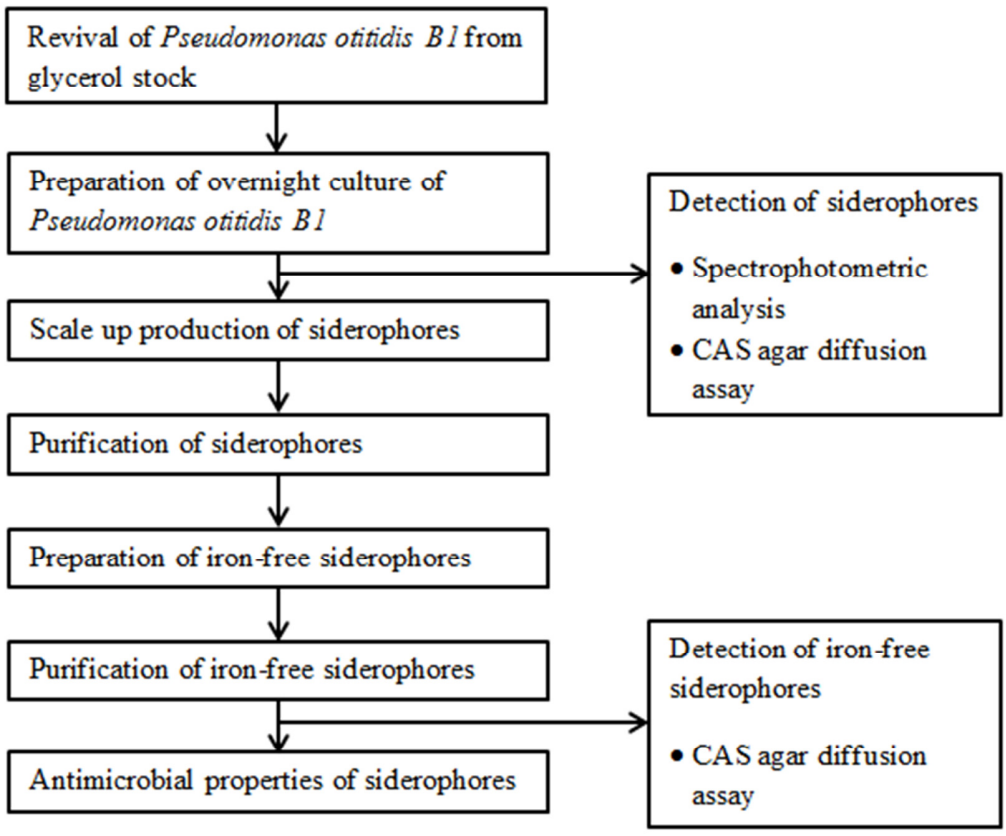

Figure 3.1: Overview of the experimental design of this project.

3.2 Bacterial Strain

The bacteria strain used throughout this project is *Pseudomonas otitidis* strain B1, isolated from an ex-tin mining lake in Kampar, Perak (Chin, 2010) which was identified through 16S rRNA gene sequencing and gene analysis (unpublished data). The bacterium was designated as POB1 and was maintained in glycerol stock.

3.3 Apparatus and Equipment

All apparatus and equipment used in this project and their respective manufacturers are listed in Appendix A. All autoclavable glasswares were autoclaved at 121°C for 15 minutes unless otherwise stated.

3.4 Chemicals and Media

All chemicals, reagents and pre-mixed media used in this project and their respective manufacturers are listed in Appendix B. All heat-stable media and solutions were autoclaved at 121°C for 15 minutes unless otherwise stated. The components of all agar, media, buffer and solutions used in this project are attached in Appendix C.

3.5 Preparation of *Pseudomonas otitidis* B1 Culture

3.5.1 Revival of *P. otitidis* B1

P. otitidis B1 was revived from glycerol stock by streaking on Luria-Bertani or LB agar. The agar plate was incubated overnight at 37°C. The plate was stored at 4°C until further use.

3.5.2 Preparation of Overnight Culture of *P. otitidis* B1

Single colony from the culture plate was transferred into a sterile universal bottle containing 5 ml of LB broth. The culture was incubated overnight at 37°C with agitation at 200 rpm. The culture was stored at 4°C until further use.

3.5.3 Long-term Storage of *P. otitidis* B1

Glycerol stock was prepared for long-term storage. Freshly prepared overnight culture was used to mix with 60% (v/v) glycerol solution to make up a final concentration of 20% (v/v) glycerol solution in a 1.5-ml cryovial. The cryovial was then stored at -80°C.

3.6 Detection of Siderophores

Spectrophotometric analysis and chrome azurol sulphonate (CAS) agar diffusion assay were used for siderophore detection at this stage.

3.6.1 Spectrophotometric Analysis

About 5 ml of the bacterial culture grown in SSM was transferred into a 50 ml centrifuge tube and was centrifuged at 9,000 rpm for 12 minutes. The supernatant was then undergone spectrophotometric analysis using a double-beam UV-VIS spectrophotometer (PerkinElmer Inc., USA, MA). The absorbance of the supernatant was scanned from 300 nm to 800 nm where the wavelength with the highest absorbance was recorded.

3.6.2 Chrome Azurol Sulphonate Agar Diffusion Assay

The CAS agar diffusion assay used in this study was a slight modified version of the original protocol from Shin, et al. (2001). Prior to carrying out this assay, 1% (v/v) of *P. otitidis* B1 was cultured in 50 ml of SSM in a 250-ml conical flask, incubated at 30°C for 24 hours with agitation at 200 rpm. This culture was grown for 72 hours. About 1 ml of the culture was harvested into a 1.5-ml microfuge tube at 24-hour intervals. The microfuge tube was centrifuged and the supernatant was kept at 4°C until use.

3.6.2.1 Preparation of CAS Agar

Prior to CAS agar preparation, iron (III) solution was prepared by mixing 1 mM $FeCl_3.6H_2O$ in 10 mM HCl. Then, 6.05 g of PIPES (1, 4 – piperazinediethanesulphonic acid) was dissolved in 180 ml of distilled water with the pH adjusted to pH 6.8 followed by adding 2 g of agarose before autoclaving. In another conical flask, 30 mg of CAS was dissolved in 25 ml of distilled water. The orange mixture was then added with 5 ml of the iron (III) solution prepared previously which turned the solution purple. While swirling, the purple solution was slowly poured into a pre-mixed solution of 36.45 mg HDTMA (hexadecyltrimethylammonium) dissolved in 20 ml of distilled water where it turned into dark blue color after mixing. After that, 20 ml of the dark blue solution was added to the autoclaved agarose-containing-solution and mixed thoroughly. The mixture was then poured into sterile petri dishes. Five holes were then punched onto the solidified CAS agar with a cork borer.

3.6.2.2 Incubation of CAS Agar

Of the five holes on the CAS agar, one of the holes was filled with 35 µl of 2.5 mM of Desferal which acted as a positive control. Another hole was filled with 35 µl of SSM which acted as a negative control. The remaining three holes were loaded with 35 µl of culture supernatant each collected at 24^{th} hour, 48^{th} hour and 72^{th} hour, respectively. The agar plate was incubated in an upright position at

37°C for 16 hours. The resultant diameters of the orange halo formed surrounding the holes were then measured and recorded.

3.7 Scale-up Production of Siderophores

A total of 3 L of cultures were to obtain and the cultivations of the cultures were divided into six separated batches, with 500 ml for each batch. In each of the 250 ml conical flask, 1% (v/v) of *P. otitidis* B1 was grown in 50 ml of SSM. The cultures were grown at 30°C for 24 hours with agitation at 200 rpm.

3.8 Purification of Siderophores

In this stage, siderophores were purified as iron-siderophore complexes.

3.8.1 Ferration of Siderophores

The addition of ferric ions to siderophores was done batch by batch (500 ml of culture per batch). In each batch, after 24 hours of incubation, the 500 ml of culture was pooled together into a 1 L conical flask. Then, 2 ml of 1 M $FeCl_3$ stock was added to the 500 ml of culture and the mixture was adjusted to pH 7. The mixture was then stirred for 20 minutes. After that, the mixture was subjected to 10,000 × *g* centrifugation for 20 minutes to remove the cells and other

precipitate. The resultant supernatant was concentrated 50-fold under reduced pressure with a rotary evaporator (Büchi, Switzerland) at 40°C.

3.8.2 Ammonium Sulphate Precipitation

Contaminating proteins in the concentrated supernatant were precipitated by 20%, 50% and finally 100% saturation of ammonium sulphate. Appropriate amount of ammonium sulphate was added according to the proportion as suggested in Appendix D depending on the volume of concentrated supernatant obtained previously. The mixture of supernatant and ammonium sulphate was stirred thoroughly for 10 minutes and was centrifuged at 9,000 rpm for 15 minutes. The pellet was discarded and the resultant supernatant was proceeded with a higher saturation of 50% and then 100%.

3.8.3 Phenol-chloroform Extraction

The extraction process was conducted in a fume hood. The volume of the supernatant from the previous step was measured. Then, an equal volume of phenol-chloroform solution [1:1, (w/v)] was used to extract the supernatant in a separatory funnel. The mixture was shaken vigorously. The aqueous layer was then discarded. The organic layer was used to repeat the above steps for two to three times. After that, the organic layer was mixed and shaken vigorously with three volumes of diethyl ether and half volume of distilled water. The resultant

aqueous layer was kept aside while the organic layer was re-extracted three times by adding half volume of water until the top layer's brown color became lighter. The top layer was then discarded. The aqueous layer that was kept aside previously was mixed and shaken with two volumes of diethyl ether. The resultant organic layer was discarded. The aqueous layer was freeze-dried into powder form. The powder form sample was weighted and stored in a vial at room temperature.

3.8.4 Ion-exchange Chromatography

CM Sephadex C-25 beads of about 15 g were pre-soaked overnight in 50 mM pyridine-acetate buffer [1%, (w/v)] where the buffer was adjusted to pH 5. The beads were packed in a burette as elution column by using the same buffer. The powdered sample obtained previously was dissolved in distilled water to get a concentration of 200 mg/ml. The dissolved sample was added to the column. The column was eluted with the same buffer and the eluents were collected in 1.5-ml microcentrifuge tubes. Next, all the collected microcentrifuge tubes were undergone spectrophotometric analysis where the absorbance of the samples was scanned over the range of 300 to 800 nm. Through the analysis from the graph obtained, those sample tubes without any matching peak at about 400 nm to 410 nm were discarded. The remaining sample tubes that contained iron-siderophore complexes were pooled together and freeze-dried. The powdered form sample was weighted and stored in a vial at room temperature.

3.9 Purification of Iron-free Siderophores

At this stage, siderophores were purified as iron-free siderophores by removing the iron prior to purification.

3.9.1 Preparation of Iron-free Siderophores

The powdered sample obtained previously was dissolved in distilled water [1%, (w/v)]. Aqueous acetic acid (10%, v/v) was used to adjust the pH of the mixture to pH 4. After that, three volumes of 5% (w/v) 8-hydroxyquinoline/chloroform were added to the mixture before it was shaken vigorously in a separatory funnel. The pH of the aqueous top layer was re-adjusted to pH 4 and was re-extracted with three volumes of 5% (w/v) 8-hydroxyquinoline/chloroform for another four times. The aqueous layer obtained was washed with chloroform to remove the excess 8-hydroxyquinoline until the aqueous layer became clear.

3.9.2 Gel Filtration Chromatography

Sephadex G-25 beads of about 15 g were pre-soaked overnight in distilled water. The beads were packed in a burette as elution column by using distilled water. The powdered sample obtained previously was dissolved in distilled water to get a concentration of 200 mg/ml. The column was eluted with the distilled water after the dissolved sample was added to the column. The eluents were collected in 1.5-ml microcentrifuge tubes. Next, all the collected microcentrifuge tubes were

undergone spectrophotometric analysis where the absorbance of the samples was scanned over the range of 300 to 800 nm. Through the analysis from the graph obtained, those sample tubes without any matching peak at about 400 nm to 410 nm were discarded. The remaining sample tubes were pooled together and freeze-dried. The powdered form sample was weighted and stored in a vial at room temperature.

3.9.3 Detection of Iron-free Siderophores

In order to verify the presence of iron-free siderophores after gel filtration chromatography, CAS agar diffusion assay was carried out. The partially purified sample obtained after gel filtration chromatography was dissolved in sterile distilled water to obtain a concentration of 0.2 mg/µl. A fresh *P. otitidis* B1 culture supernatant was also collected for CAS agar diffusion test.

One of the holes on the CAS agar plate was filled with 35 µl of the 24-hour culture supernatant. Another hole was filled with 35 µl of the 0.2 mg/µl partially purified sample. Two of the remaining holes were filled with 35 µl of SSM and distilled water, respectively, as negative controls. The last hole was filled with 35 µl of 2.5 mM of Desferal which acted as the positive control. The CAS agar plate was incubated in an upright position at 37°C for 16 hours. The resultant diameters of the orange halo formed surrounding the holes were then measured and recorded.

3.10 Antimicrobial Properties of Siderophores

In this study, the antimicrobial properties of siderophores were tested against eight strains of bacteria, namely *Enterobacter aerogenes* ATCC 13408, *Escherichia coli* ATCC 25922, *Proteus vulgari*, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermis* ATCC 12228 and *Bacillus spizizenii* ATCC 6633. These test bacteria were grown on LB agar at 37°C for 16 hours a day before the antimicrobial test.

3.10.1 Antimicrobial Test of Siderophores

A 0.5 McFarland solution was prepared as standard. The absorbance of the solution was measured at 600 nm to make sure that the reading fall within the range of 0.008 to 0.100. About 50 ml of 0.85% NaCl (saline solution) was prepared and autoclaved. Then, about 3 to 4 ml of the saline solution was poured into eight sterile universal bottles respectively.

From each of the test bacterial culture plate, appropriate amount of single colonies were selected and transferred into the universal bottle. The universal bottle was shaken briskly to ensure all the colonies were dissolved in the saline solution. The mixture was then compared optically to the 0.5 McFarland standard solution. Additional colonies were added to the universal bottle if the turbidity of the test

bacterial species did not reach the turbidity of the standard. A sterile swab was dipped into the universal bottle and tapped lightly onto the side wall of the universal bottle. Then, the swab was swabbed evenly on the Müeller-Hinton (MH) agar plate three times; with each time, the agar plate was rotated by 60°. Next, two sterile blank discs were placed onto the swabbed agar plate using a sterile forceps.

The partially purified sample obtained after gel filtration chromatography was dissolved in sterile distilled water to obtain a concentration of 0.05 mg/µl. The sample solution was filtered through 0.45-µm filter membrane to remove impurities. Then, 13 µl (0.65 mg) of the filtered sample solution was pipetted onto one of the blank disc on the MH agar plate. Another blank disc was filled with 13 µl of distilled water as negative control. All the agar plates were incubated at 37°C for three days. Antimicrobial activities were observed at 24-hour intervals for three days.

CHAPTER 4

RESULTS

4.1 Production of Siderophores

Upon inoculation of *Pseudomonas otitidis* B1 in simple succinate medium (SSM), the culture medium was transparent (Figure 4.1a). After 24 hours of incubation at 30°C with an agitation speed of 200 rpm, the culture medium turned in yellow-green fluorescent in color as shown in Figure 4.1b.

a) b)

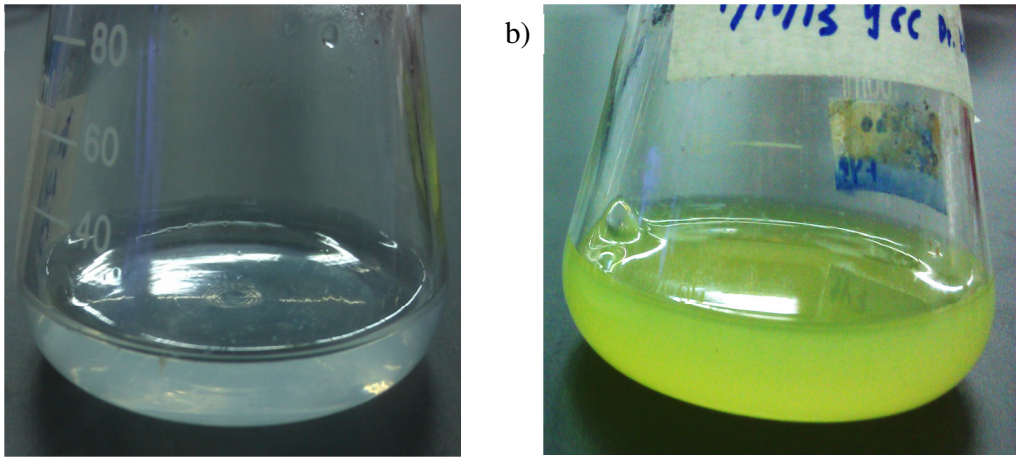

Figure 4.1: Production of yellow-green fluorescent pigment. a) At 0 hour, SSM with *P. otitidis* B1 was transparent. b) After 24 hours of incubation at 30°C with agitation at 200 rpm, the culture medium turned in yellow-green fluorescent in color.

4.2 Detection of Siderophores

Spectrophotometric analysis and chrome azurol sulphonate (CAS) agar diffusion assay were carried out for siderophore detection.

4.2.1 Spectrophotometric Analysis

The supernatant obtained from a 24-hour culture was scanned through a double-beam UV-VIS spectrophotometer. The crude siderophores formed a peak at about 404 nm with shoulders at about 330 nm and 445 nm (Figure 4.2).

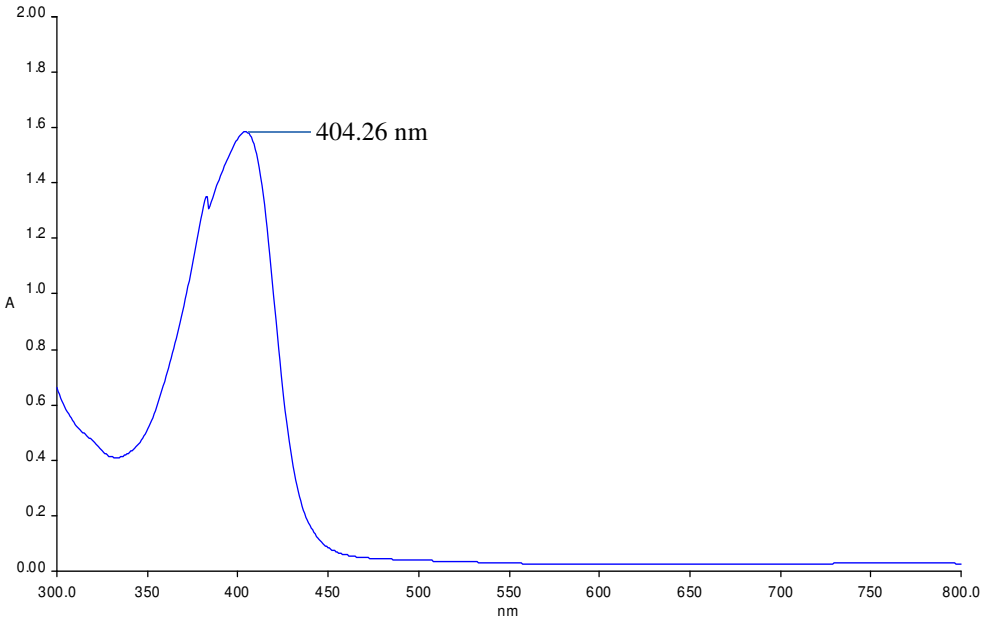

Figure 4.2: Absorption spectrum of crude siderophores. The crude yellow-green pigment gave rise to a peak at about 404 nm.

4.2.2 Chrome Azurol Sulphonate Agar Diffusion Assay

In Figure 4.3, after 16 hours incubation of CAS agar, an obvious and large orange halo was observed around the well filled with Desferal (2.5 mM) which acted as the positive control. With 0.5 cm wide of the well itself, the diameter of the positive control well was 1.4 cm. The negative control was represented by SSM and there was no orange halo formation around the well filled with SSM. The well filled with 24-hour culture supernatant showed a slightly larger orange halo, with 0.7 cm diameter, around the well than the 48-hour and 72-hour culture supernatants. Both the 48-hour and 72-hour culture supernatants formed smaller orange halos, with only 0.6 cm in diameters.

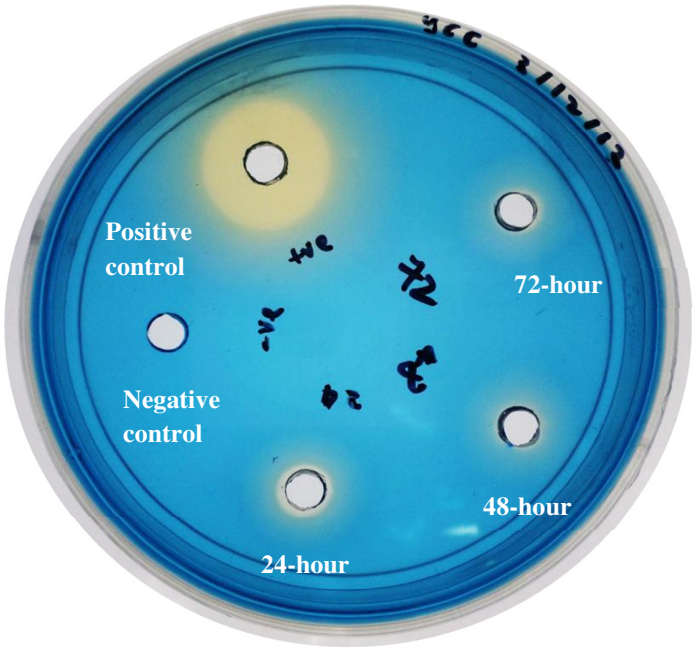

Figure 4.3: CAS agar diffusion assay. Orange halos were observed around the wells filled with Desferal (2.5 mM) which acted as positive control as well as the 24-hour, 48-hour and 72-hour culture supernatants.

4.3 Purification of Siderophores

The addition of ferric chloride, $FeCl_3$ (1 M), caused the 24-hour, yellow-green fluorescent colored culture to turn to brown color. The difference in color of the culture before and after addition of $FeCl_3$ is as shown in Figure 4.4.

a) b)

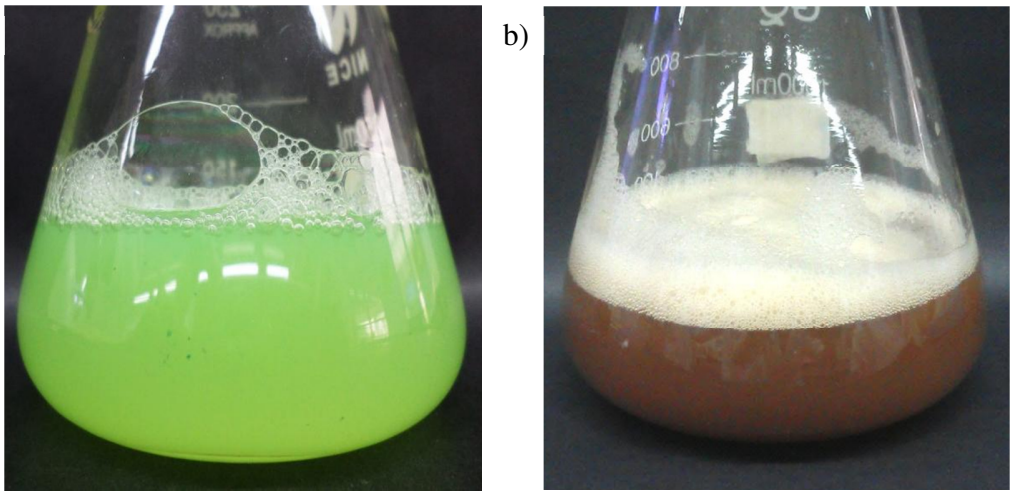

Figure 4.4: Difference in color of the 24-hour culture before and after addition of $FeCl_3$. a) Before addition of $FeCl_3$, the culture appeared yellow-green fluorescent in color. b) After addition of $FeCl_3$, the culture turned brownish in color.

The supernatant of the ferrated culture was then scanned through a double-beam UV-VIS spectrophotometer. In Figure 4.5, the ferrated siderophores showed a peak at about 399 nm with shoulders at about 345 nm and 430 nm.

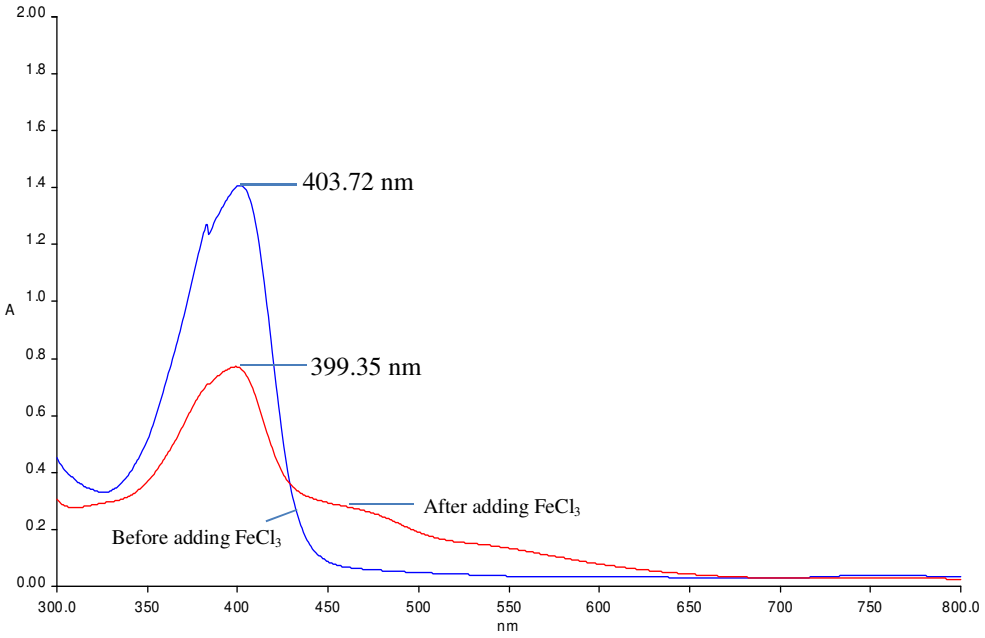

Figure 4.5: Absorption spectra of crude and ferrate siderohpores. The peak of ferrated siderophores was at 399 nm as compared to the crude culture peak at 404 nm.

After that, the supernatant of the ferrated siderophores was subjected to ammonium sulphate precipitation and phenol-chloroform extraction to remove impurities. The treatment of phenol-chloroform extraction resulted in purer ferrated siderophores which can be seen where the diethyl ether layer in the last extraction step appeared in transparent color as shown in Figure 4.6. The bottom aqueous layer of the extraction was then undergone freeze-drying to obtained powdered form of sample.

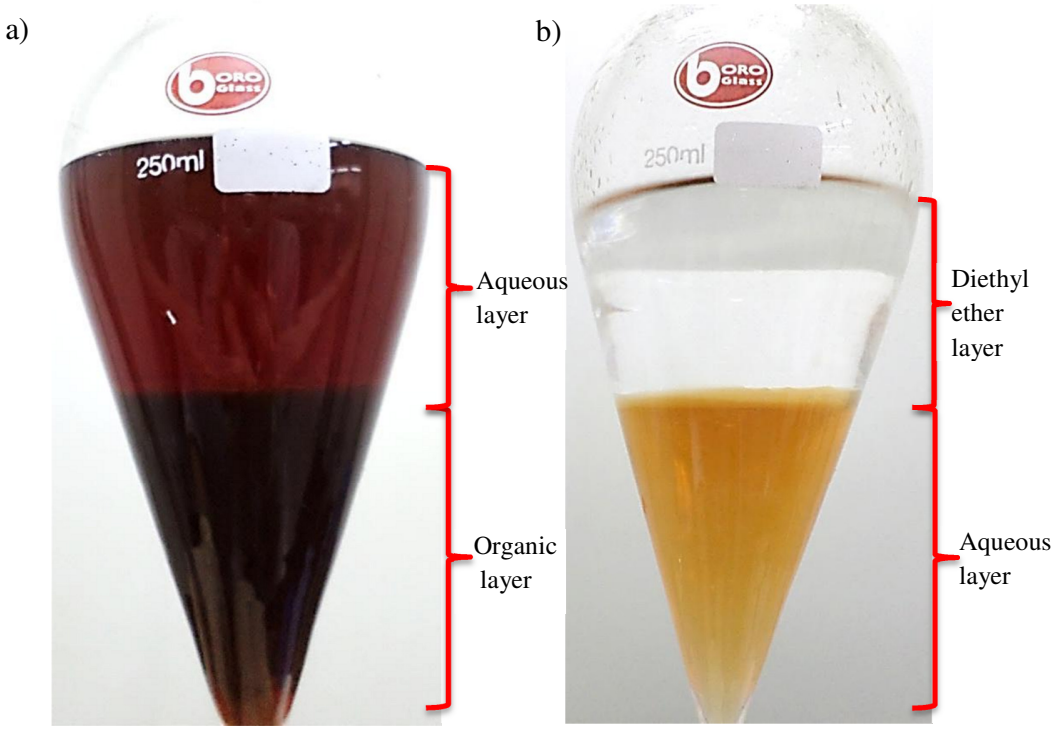

Figure 4.6: Phenol-chloroform extraction of ferrated siderophores. a) Extraction with phenol-chloroform solution [1:1, (w/v)], aqueous layer was discarded. b) Extraction with diethyl ether to remove phenol from ferrated siderophores.

4.3.1 Ion-exchange Chromatography

The powdered sample was dissolved in distilled water to a concentration of 200 mg/ml and was subjected to ion-exchange chromatography. The eluents were collected in 1.5-ml microcentrifuge tubes. Under UV-VIS spectrophotometric analysis, eluents which showed a peak at about 400 nm (eluents 2 to 19) were pooled together. In the meantime, other eluents (eluent 1 and eluent 20 to 45) with

gave peaks other than 400 nm were discarded as impurities were present. The example of spectra of desired eluents was shown in Figure 4.7. The eluents that were pooled together were then undergone freeze-drying to obtain powdered form of sample.

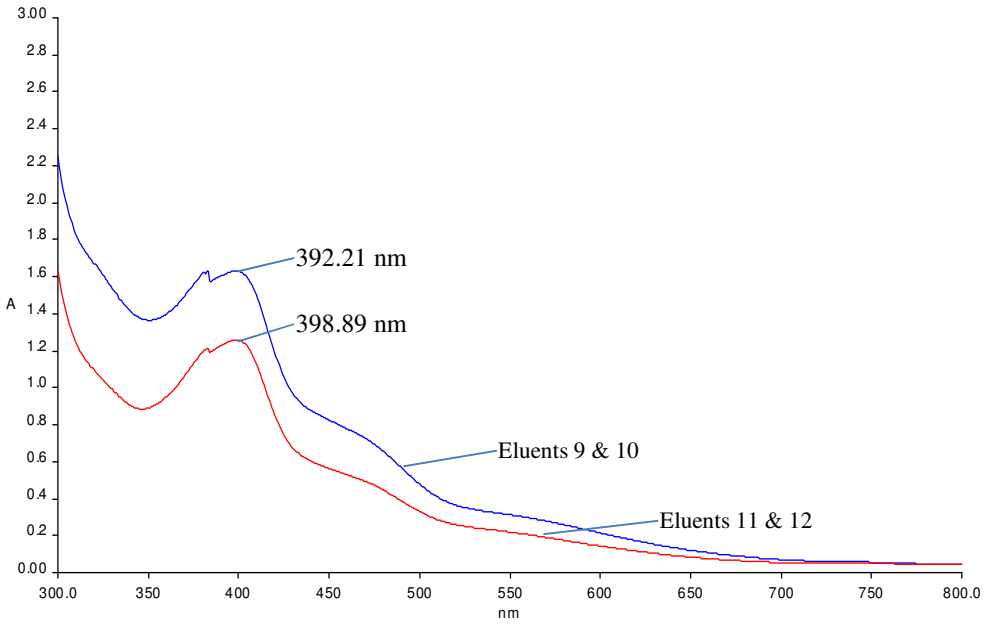

Figure 4.7: Spectra of eluents in ion-exchange chromatography. Eluents 9 and 10 showed a peak at about 392 nm while eluents 11 and 12 gave rise to a peak at about 399 nm. Both of the spectra showed strongest absorption at wavelength similar to that of the ferrated siderophores which is about 399 nm.

4.3.2 Preparation of Iron-free Siderophores

The powdered sample was dissolved in distilled water [1%, (w/v)] and was subjected to iron removal using 8-hydroxyquinoline/chloroform extraction. After removal of iron, the sample appeared to be lighter in color as compared to the sample before iron removal. The comparison of the color difference is as shown in Figure 4.8.

a) b)

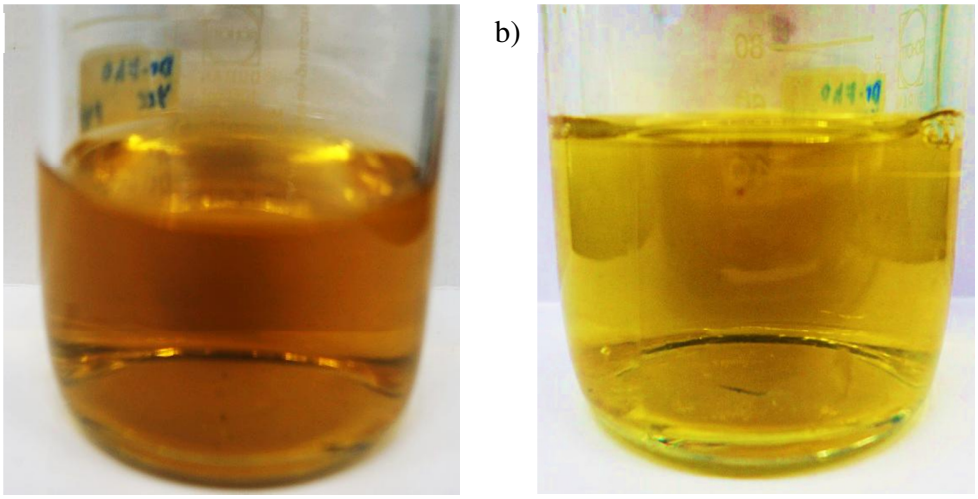

Figure 4.8: Comparison of color difference of the sample before and after iron removal. a) Before iron removal, the sample was in darker brown color. b) After iron removal, the sample appeared to be lighter in color.

4.3.3 Gel Filtration Chromatography

After iron removal, the sample was subjected to gel filtration chromatography. The eluents were collected in 1.5-ml microcentrifuge tubes. Under UV-VIS spectrophotometric analysis, eluents which showed a peak at about 404 nm

(eluents 9 to 17) were pooled together. In the meantime, other eluents (eluents 1 to 8 and eluents 18 to 22) which gave peaks other than 404 nm were eliminated. The example of spectra of desired eluents was shown in Figure 4.9. The eluents that were pooled together was more yellowish in color as shown in Figure 4.10. The sample was then undergone freeze-drying to obtained powdered form of sample.

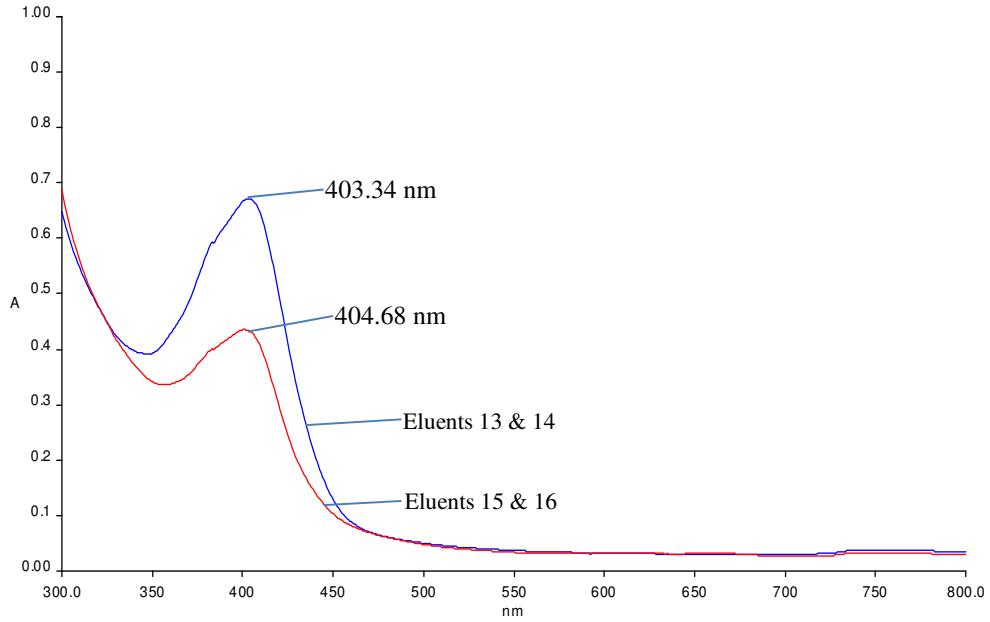

Figure 4.9: Spectra of eluents in gel filtration chromatography. Eluents 13 and 14 showed a peak at about 403 nm while eluents 15 and 16 gave rise to a peak at about 404 nm. Both of the spectra showed strongest absorption at wavelength similar to that of the crude siderophores which is about 404 nm.

Figure 4.10: The color of the sample after gel filtration chromatography. The pooled eluents appeared to be more yellowish in color than before undergoing gel filtration chromatography.

4.3.4 Detection of Iron-free Siderophores

CAS agar diffusion assay was carried out to detect as well as to compare the siderophores obtained from crude sample and partially purified sample after gel filtration chromatography. In Figure 4.11, as usual, after 16-hour incubation of CAS agar, an obvious and large orange halo (1.3 cm in diameter) was observed around the well filled with Desferal (2.5 mM) which acted as the positive control. The negative controls were represented by SSM and distilled water and there were no orange halo formation around both of the wells. Meanwhile, the well filled with partially purified sample showed a larger orange halo, with 0.9 cm in diameter, around the well than the crude culture supernatant which formed only 0.7 cm diameter orange halo.

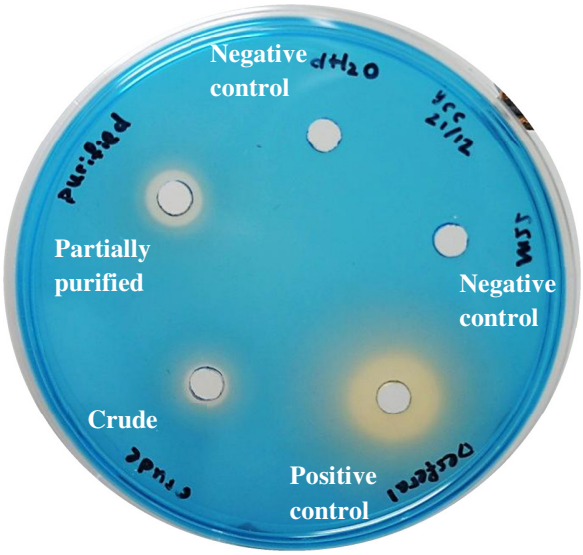

Figure 4.11: CAS agar diffusion assay for detection of iron-free siderophores. Orange halos were observed around the wells filled with positive control, the Desferal (2.5 mM), crude culture supernatant and partially purified sample. The orange halo of partially purified sample was larger than that for the crude sample.

4.4 Antimicrobial Properties of Siderophores

The powder sample after gel filtration chromatography was dissolved in sterile distilled water. Negative control (-ve) for every test bacterial species was represented by sterile distilled water. The partially purified sample was labeled as "sid." on each test bacterial MH agar plate. The amount of partially purified sample added onto each of the blank disc was 0.65 mg.

After 48 hours of incubation, as in Figure 4.12, there was no antimicrobial activity observed for Gram-positive bacteria tested. Nevertheless, growth promoting activity was observed on *Staphylococcus aureus* ATCC 25933 whereby the

density of the cell surrounding the disc filled with the partially purified siderophore sample was higher.

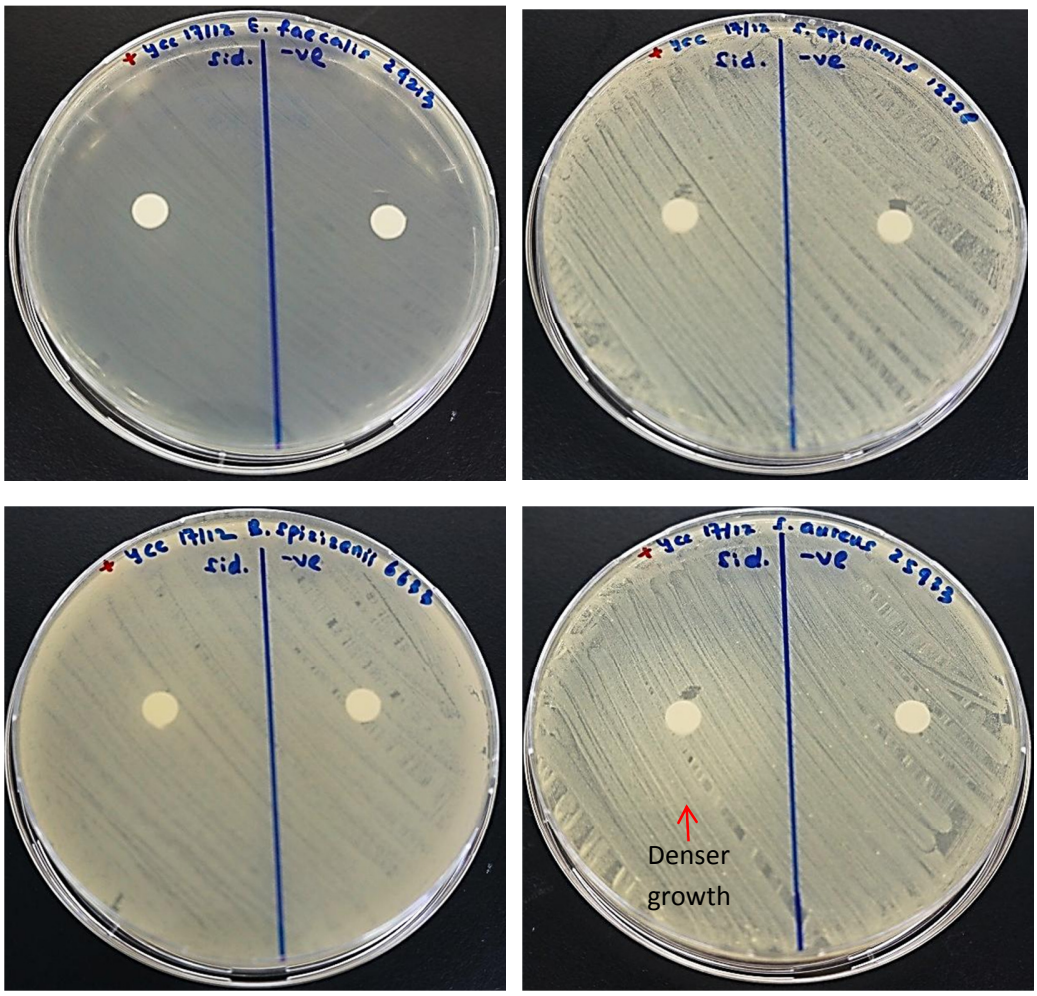

Figure 4.12: Effect of partially purified siderophore on the growth of Gram-postive bacteria. There was no antimicrobial activity observed for all the bacterial species tested (*E. faecalis* ATCC 29212, *S. epidermidis* ATCC 12228, *B. spizizenii* ATCC 6633, *S. aureus* ATCC 25923). However, only *S. aureus* ATCC 25923 showed growth promoting activity with a denser growth around the disc filled with partially purified siderophore.

On the other hand, for Gram-negative bacteria as shown in Figure 4.13, no antimicrobial activity or growth promoting activity was observed for all the bacteria tested.

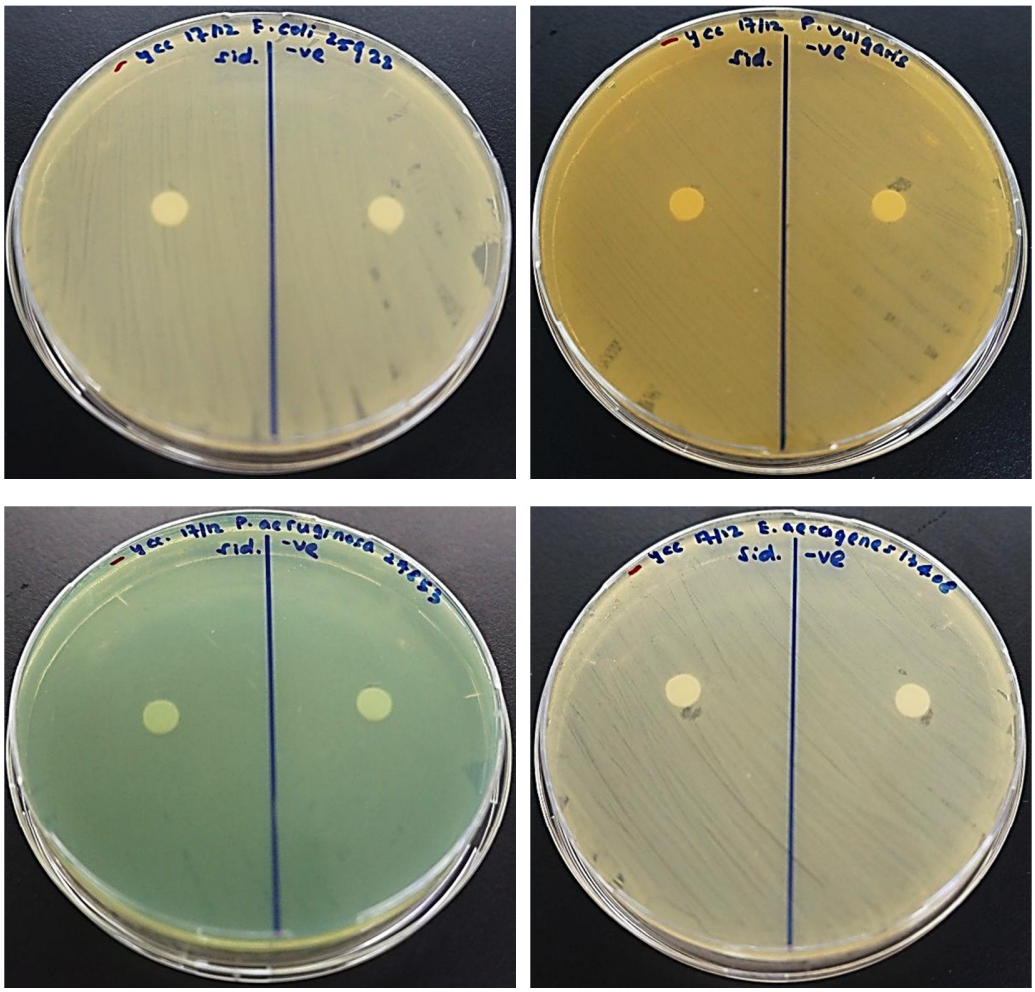

Figure 4.13: Effect of partially purified siderophore on the growth of Gram-negative bacteria. There was no antimicrobial activity and growth promoting activity observed for all the bacterial species tested (*E. coli* ATCC 25922, *P. vulgaris*, *P. aeruginosa* ATCC 27853 and *E. aerogenes* ATCC 13408).

CHAPTER 5

DISCUSSION

5.1 Production of Siderophores

Under iron-limiting condition, most microorganisms are capable of producing siderophores to scavenge iron from the surroundings (Visca, et al., 1992; Neilands, 1995). Based on this distinct characteristic, *Pseudomonas otitidis* B1 was subjected to iron-limited growth condition in simple succinate medium (SSM) in order to study its ability to produce the yellow-green fluorescent siderophores.

According to Clark, et al. (2006), *P. otitidis* is a non-fluorescent *Pseudomonas*. However, the research of Mehri, et al. (2011) showed that *P. otitidis* produced fluorescent pigment on King's B medium. The capability of *P. otitidis* to produce yellow-green fluorescent pigment was further confirmed in this study when the incubation of *P. otitidis* B1 in transparent SSM turned the medium to yellow-green fluorescent color after 24 hours.

The iron-deficient SSM was selected as the medium for cultivation of *P. otitidis*. Sreedevi, Preethi and Kumari (2014) found that siderophore production was at the highest when *Pseudomonas* isolates were cultured in succinate medium compared to in King's B medium, glycerol medium and asparagine medium. According to

Tailor and Joshi (2011), succinic acid is the best carbon source that can enhance siderophore production. Besides, no iron was introduced into the culture medium as the increasing iron concentration in the medium adverse the siderophore production (Rachid and Ahmed, 2005). Sreedevi, Preethi and Kumari (2014) also found out that the use of ammonium as nitrogen source yielded the highest siderophore level. The growth condition was further optimized by pH of the medium. Although Cody and Gross (1987) reported that the stability of iron-pyoverdine complex was higher at a more alkaline pH (pH 10), most of the bacteria still grow better under the physiological pH (pH 7) (Tailor and Joshi, 2011). Hence, the condition was compromised by using pH 7 instead of towards the alkaline condition. Tailor and Joshi (2011) also found that maximum siderophore production was achieved after 24 hours of incubation and at temperature of about 28°C to 30°C. Maximum production of siderophore normally happens in early stationary phase (Wensing, et al., 2010).

5.2 Detection of Siderophores

5.2.1 Spectrophotometric Analysis

Based on Meyer and Abdallah (1978) research, the free fluorescent pyoverdine of *P. fluorescens* formed a peak at 402 nm at pH 7 and the peak shifted to 410 nm at pH 10. The pH of the medium increased to pH 9 after 40 hours of incubation of *P. fluorescens* (Meyer and Abdallah, 1978). In this study, the fluorescent pigment produced by *P. otitidis* B1 gave rise to a maximum peak at 404 nm after 24 hours

of incubation. This indicates that 24 hours did not change much of the medium pH and the peak obtained at 404 nm was close to the wavelength of 402 nm as proposed by Meyer and Abdallah (1978). The similarities of the yellow-green color and the peak's wavalength of the pigment produced by *P. otitidis* B1 and the pigment mentioned by Meyer and Abdallah (1978) indicated the presence of the pyoverdine siderophore. Absorption of violet light (about 400 nm to 420 nm) at visible region corresponds to yellow-green color when observed (Kotz, 2005). This clearly indicates that the yellow-green fluorescence of the culture supernatant gave rise to the peak at 404 nm which is within the range of violet wavelength.

5.2.2 Chrome Azurol Sulphonate Agar Diffusion Assay

The CAS agar diffusion assay was based on the principle of iron removal by strong iron chelators which change the color of the CAS agar to facilitate easy analysis. According to Schwyn and Neilands (1987), the blue dye of the agar was due to the complex of chrome azurol S, ferric ion and hexadecyltrimethylammonium bromide (HDTMA). The addition of strong iron chelator, in this case, siderophores, removes ferric ion from the blue color dye complex and causes the agar color changes from blue to orange (Louden, Haarmann and Lynne, 2011). One important component in the CAS agar diffusion assay is the 1, 4 – piperazinediethanesulphonic acid (PIPES) which acts as a buffer to maintain the pH of the agar (Schwyn and Neilands, 1987). The pH

of the agar exceeding pH 6.8 changes the blue dye to green color (Louden, Haarmann and Lynne, 2011). Therefore, the pH was carefully adjusted to pH 6.8 to avoid the change of the agar color.

The positive control was represented by Desferal, an iron-chelator (Novartis, 2011) while SSM acted as the negative control. The 24-hour culture supernatant contained a higher concentration of siderophores as the orange halo formed was the largest among the 24-, 48- and 72-hour supernatants. Tailor and Joshi (2011) also proved that 24-hour culture yielded the highest level of siderophore and the level decreased gradually over time. However, the amounts of siderophores from all three supernatants were low as the intensity and the diameter of the halos were much smaller than that for desferal. According to Ghodsalavi, et al. (2013), the diameter of orange halo formed is related to the siderophore production level.

5.3 Purification of Siderophores

Prior to the purification process, the culture supernatant was ferrated by adding ferric chloride. This step is important as iron-siderophore complex shows great stability than the free siderophore (Cody and Gross, 1987). Based on the high stability of iron-siderophore complex, siderophore extraction and purification was carried out in the form of iron-siderophore complex rather than free siderophore. Upon the addition of ferric chloride, the yellow-green supernatant turned brown.

As stated by Meyer and Abdallah (1978), the change to brown color with the loss of fluorescence is the characteristic of iron complexation on siderophores.

Ferrated siderophores gave a peak at about 400 nm when scanning through a UV-VIS spectrophotometer. The close proximity of the two peaks from ferrated siderophores and crude siderophores (400 nm and 404 nm, respectively) indicated the conservation of the siderophore property. Another characteristic of ferrated siderophores can be based on the absorption at 450 nm. The spectrum of ferrated siderophore gave a much higher absorbance at 450 nm as compared to the spectrum of crude siderophore. Meyer and Abdallah (1978) reported that free pigment showed zero absorbance at 450 nm while the increase in iron concentration added to the pigment increase the absorbance at 450 nm. The absorbance of siderophores at 450 nm is also known as 'iron shoulder' as Haas, et al. (1991) stated that the shoulder indicates the presence of ferripyoverdine.

5.3.1 Ammonium Sulfate Precipitation

Ferrated siderophore was centrifuged and concentrated to a lower volume. This was to ease the purification process with a smaller volume to work on. Ammonium sulfate was used to remove protein contaminants as it is a soluble salt and usually does not interfere with the subject under study (Abdul-Razzaq and Abdul-Razzaq, 2013). It works on a basic principle that protein precipitates out at high salt concentration especially near 100% saturation (Clark, 2002). This is

because at high salt concentration, the salt requires more water to dissolve into ionized form (Education Portal Life Science, n.d.). Ionized salt has higher affinity towards water than protein and this causes the protein to interact via hydrophobic interaction of non-polar amino acids over hydrophilic interaction (Education Portal Life Science, n.d.). Hence, most of the contaminating proteins were removed by treatment of 100% saturation of ammonium sulfate to the ferrated supernatant. The contaminants were removed by centrifugation, yielding purer ferrated siderophores.

5.3.2 Phenol-chloroform Extraction

Phenol-chloroform extraction was carried out to eliminate salt material in the ferrated siderophore supernatant. Neilands (1995) reviewed that siderophores can be driven into organic phase such as phenol-chloroform and benzyl alcohol to remove salt. The ferrated siderophore supernatant was treated with phenol-chloroform [1:1, (w/v)] for a total of three times. This step generally removed the salt material present in the supernatant by first depositing the ferrated siderophore in organic phase. As the hexadentate ligand of siderophore consists of oxygen donor atoms, these oxygen atoms interact strongly with ferric ions by forming ionic bond (Miethke and Marahiel, 2007). This type of interaction can also happen between the carbonyl group of phenol and ferric ion by ionic interaction as Hrubý, Hradil and Beneš (2004) found that phenol has strong interaction with metal complexes such as cooper (II) and iron (III) complexes through ion bonding.

As a result, while the siderophore was in the phenol-chloroform phase, those unwanted salt materials were removed along with the aqueous phase.

The organic phase retained earlier was then treated with diethyl ether and distilled water. Diethyl ether is an organic solvent and causes the phenol-chloroform layer to be sufficiently non-polar (Lammers, 1982). This increase in organic phase density then re-extracted the ferrated siderophore back into the distilled water aqueous phase. The low density (0.714 g/cm^3) of diethyl ether (Chemical Book, 2010) formed the upper layer and the distilled water layer with a higher density formed the bottom layer in the separatory funnel. After that, the re-extraction of the upper organic layer by distilled water was performed to completely remove the ferrated siderophore from organic phase. This was judged by the turning to a lighter brown color of diethyl ether layer at the upper site. The last step was the washing of the aqueous layer with diethyl ether to remove the phenol residual (Cody and Gross, 1987).

5.3.3 Ion-exchange Chromatography

Ion-exchange chromatography was carried out to further purify the ferrated siderophore by removing impurities. Cody and Gross (1987) showed that precursor of pyoverdine and breakdown products also appeared along with the ferrated pyoverdine through ion-exchange chromatography. CM Sephadex C-25 was used as the weak cation exchanger and consists of negatively charged support

solid or beads to separate positively charge compound (Pharmacia Biotech, n.d.; Tosoh, n.d.). The beads of CM Sephadex C-25 are more rigid and less likely to swell; hence this exchanger is suitable for small molecules with sizes up to 30,000 MW (Pharmacia Biotech, n.d.). According to Cody and Gross (1987), the size of *Pseudomonas syringae* ferrated pyoverdine was 1,175 MW. Therefore, the beads were suitable to separate ferrated siderophore from impurities. In addition, the column was eluted with pyridine-acetate buffer in pH 5. Pyridine acetate buffer is popular among cation exchange chromatography as it is volatile and can certainly separate from the eluents (Triantaphyllopoulos, 1973). The buffer was adjusted to acidic buffer as siderophores are unstable under alkaline conditions (Meyer and Abdallah, 1978).

Spectra of eluents were analyzed and selected based on the maxima wavelength yielded. Only those spectra with a peak at about 400 nm (eluents 2 to 19) were pooled together. This is because the eluents gave the characteristic peak of ferrated siderophores as determined earlier and also these eluents appeared as brown color which indicated the color of ferric complexes. Other eluents that did not yield the characteristic peak were discarded as these eluents contained impurities. According to Meyer and Abdallah (1978), a portion of eluents that was eluted before the collected eluents contained degraded pigment products.

5.3.4 Preparation of Iron-free Siderophores

Ferric ions bound on siderophores were removed by treatment with 5% (w/v) 8-hydroxyquinoline/chloroform. 8-hydroxyquinoline is a strong and insoluble iron chelator with bidentate ligand (Pierre, Baret and Serratrice, 2003; Manthey, Crowley and Luster, 1994). The ligands compete for ferric ions with ferrated siderophores, resulting in removal of iron from siderophore. This was observed as the siderophore-containing solution turned from darker brown to lighter brown color. This showed that iron was partially removed from siderophore and not completely removed as the significant yellow-green fluorescent color of free siderophore was not observed. The color of ferric 8-hydroxyquinoline complex was greyish green and was extracted to the chloroform organic layer (Manthey, Crowley and Luster, 1994). The residual 8-hydroxyquinoline in the aqueous layer was then removed by chloroform (Meyer and Abdallah, 1978). As stated by Meyer and Abdallah (1978), the preparation of iron-free siderophore and transfer of sample to gel filtration chromatography column should be conducted quickly as the iron-free siderophores are unstable and may decompose easily.

Another important step in this procedure was to adjust the pH of the 8-hydroxyquinoline/chloroform and the aqueous layer yielded to pH 4. Metal binding of 8-hydroxyquinoline is pH dependent. Ferric ion generally binds to 8-hydroxyquinoline in the range of pH 3 to 4 (Lohan, Aguilar-Islas and Bruland,

2006). Besides, the free siderophores are more stable under acidic condition (Meyer and Abdallah, 1978).

5.3.5 Gel Filtration Chromatography

Gel filtration chromatography was carried out to separate impurities from deferrated siderophores based on size. Sephadex G-25 was used as the beads of the chromatography can retain molecules with molecular weight up to 5,000 MW (Amersham Biosciences, n.d.). According to Storey (2005), siderophores have low molecular weight ranging from 350 to 1500 Daltons. Therefore, the beads were suitable as they retained the siderophores and allowed large molecular weight impurities to pass through the column before the siderophores were eluted.

Eluents with the characteristic peaks at about 404 nm (eluents 9 to 17) were collected and pooled together. These eluents gave the characteristic deferrated pyoverdine peak (404 nm) as determined previously. Although no yellow-green fluorescent color was obtained, the color of these eluents was yellowish-brown in color which appeared to be much lighter in brown color as compared to before iron removal (Figure 4.8). The other eluents were discarded as no characteristic peak of free pyoverdine was observed from the spectra. Gel filtration chromatography was proven to be enough for partial purification as Meyer and Abdallah (1978) yielded only a single peak corresponded to the free pyoverdine from this procedure.

5.3.6 Detection of Iron-free Siderophores

CAS agar diffusion assay was once again carried out to detect the presence of iron-free siderophore after going through the purification processes. As usual, the well filled with the positive control, desferal (2.5 mM), formed a large orange halo, with 1.3 cm. There was no orange halo observed around the wells of the two negative controls, which were the distilled water as the partially purified sample's control and SSM as the crude sample's control, respectively. Paritally purified siderophore formed a larger halo than the 24-hour crude siderophore (Figure 4.11). The diameter of the orange halo for purified siderophore was 0.9 cm while the crude siderophore only formed 0.7 cm diameter of the orange halo. This indicated that the activity of purified siderophore to scavenge iron from the CAS-ferric-HDTMA complex was higher as compared to the crude sample.

5.4 Antimicrobial and Growth-promoting Activities of Siderophores

The growth of all Gram-positive bacteria tested were not inhibited by the siderophores of *P. otitidis* B1 and only one bacterial strain, *S. aureus* ATCC 25933, showed growth promoting activity upon incubating with the partially purified siderophore. According to D'Onofrio, et al. (2010), siderophore produced by *Micrococcus luteus* was able to promote the growth of uncultured bacteria. Several Gram-positive bacteria possess one or more foreign iron-siderophore complex-binding proteins. For example, *Bacillus subtilis* utilizes ferrioxamine and ferricrocin-binding proteins; *S. aureus* consists of two types of ferric

hydroxamate-binding proteins, FhuD1 and FhuD2 (Fukushima, et al., 2013). This enables *S. aureus* to uptake iron from other sources of siderophores such as hydroxamate that are not synthesized by *S. aureus* (Sebulsky, et al., 2000). Hence, the growth-promoting activity observed in *S. aureus* plate was most probably due to this ability in recognizing a variety of outer source ferric siderophores. In contrast, the other three Gram-positive bacteria may not own receptor proteins that are capable of binding the siderophore from *P. otitidis* B1 and thus no growth promoting activity was observed.

However, the siderophore of *P. otitidis* B1 did not exhibit antimicrobial activity to all the Gram-positive bacterial species. This may be due to the iron-binding affinity of *P. otitidis* B1's siderophore was lower than the tested bacteria' siderophore (Eldik and Hubbard, 2009). Another reason might be the great losss or degradation of the siderophores after going through all the purification processes.

On the other hand, all the tested Gram-negative bacteria were neither inhibited or growth promoted by the siderophores of *P. otitidis* B1. The outer membranes of Gram-negative bacteria consist of ferric siderophore specific receptors (Miethke and Marahiel, 2007). Due to this high specificity, the tested Gram-negative bacteria might be unable to recognize the siderophore of *P. otitidis* B1 and therefore, no growth promoting activity was observed.

In addition, the inhibitory effect of the siderophore of *P. otitidis* B1 on the tested Gram-negative bacteria was not detected. One of the reasons might be the same as Gram-positive bacteria, where the iron-binding affinities of the tested Gram-negative bacterial siderophores were higher than *P. otitidis* B1. Another possible explanation was the concentration of purified siderophore used. As the amount of the partially purified siderophore was only 0.65 mg, it might be insufficient for growth promotion or inhibition on the test bacteria. Stintzi, et al. (2000) suggested that increase in the concentration of iron-free siderophore causes higher rate of iron sequestering. High proportion of siderophore breakdown might also contribute to the absence of antimicrobial activities.

5.5 Future Prospects

Partial purification of siderophore may not yield satisfying result as compared to higher purity siderophore. The ferrated siderophores and deferrated siderophores should be subjected to ion-exchange chromatography and gel filtration chromatography, respectively, for another time to obtain better separated product. This was demonstrated by Cody and Gross (1987). Recent researches rely more on modern technique such as high performance liquid chromatography (HPLC) to gain a higher purity of siderophore (Storey, 2005; Patel, et al., 2009).

For detection of siderophore, more specific detection method could be applied. The well-established Arnow and Csàky tests could be used to determine the type of siderophore produced by *P. otitidis* B1. These tests enable the identification of catecholate and hydroxamate siderophore if any of these siderophores are present in the *P. otitidis* B1 culture (Cody and Gross, 1987; Bhattacharya, 2010). Characterization of siderophore produced can be done by mass spectrometry (MS) analysis. Several works had been incorporating the HLPC with electrospray ion-MS (ESI-MS) to determine the molecular weight of siderophore right after the purification step (Presswood, 2010; Sullivan, et al., 2008). Furthermore, the structural composition of siderophore can be determined using nuclear magnetic resonance (NMR) (Storey, 2005; Presswood 2010).

Molecular characterization of genes involved in regulation and production of siderophore can be conducted for further understanding the mechanism of *P. otitidis* B1 in acquiring iron under iron-limiting condition. Visca, et al. (2002) studied the *Pseudomonas aeruginosa* gene transcription sequence, PvdS, which corresponded for pyoverdine production.

Last but not least, the antimicrobial and growth-promoting activities of siderophores extracted from *P. otitidis* B1 can be further studied by testing on other species of bacteria. Moreover, the potential antifungal activities of *P. otitidis* B1 can be evaluated too.

CHAPTER 6

CONCLUSIONS

Fluorescent yellow-green pigments were produced after *P. otitidis* B1 was incubated for 24 hours in simple succinate medium (SSM). The colored pigments contained siderophore activity when tested through the chrome azurol sulphonate (CAS) agar diffusion assay. Upon scanning the supernatant of the culture bacteria through UV-VIS spectrophotometer, a peak at 404 nm was formed which corresponded to the characteristic absorption wavelength of the pyoverdine type of siderophores.

Prior to the siderophore extraction, ferric chloride was added to the culture in order to form the ferric-siderophore complexes. Formation of complex is able to ensure stability of the siderophore throughout the purification process. Ferric-siderophore complex was characterized by forming a peak at 400 nm when scanning through a UV-VIS spectrophotometer. The properties of free siderophore was able to conserve to the end of the purification procedure as the free siderophore gave the characteristic peak at 404 nm and was able to be detected by the CAS agar diffusion assay.

The partially purified siderophores of *P. otitidis* B1 did not inhibit the growth of all the Gram-positive and Gram-negative bacteria tested. However, the growth of *S. aureus* ATCC 25933 was enhanced by the partially purified siderophore. Studies on growth promotion should be further investigated to shed light on the growth promoting mechanisms.

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Appendix A

Apparatus and Equipment

Table A: List of apparatus and equipment with their respective manufacturers.

Apparatus or Equipment	Manufacturer
Autoclave machine	Hirayama
Beakers (1000 ml, 500 ml, 250 ml)	NICE, GQ
Blank sterile discs	Liofilchem
Bunsen burner	Campingaz
Burette	Favorit
Centrifuge machine (15 ml)	Dynamica
Centrifuge machine (250 ml)	Beckman Coulter
Centrifuge tubes (15 ml)	Greiner
Centrifuge tubes (50 ml)	Greiner
Centrifuge tubes (250 ml)	Beckman Coulter
Conical flasks (100 ml)	NICE
Conical flasks (250 ml)	NICE, GQ
Conical flasks (1 L)	NICE
Cotton swabs	Fisher Scientific
Deionized water dispenser	Sartorius
Double-beam UV-VIS spectrophotometer	PerkinElmer
Freeze-drying machine	ScanVac
Freezer	Panasonic
Fridge	LinDen
Hotplate stirrer	Stuart
Incubator	Memmert
Laminar flow hood	Camfil Farr
Membrane filter (0.45 μm)	Pall Life Sciences
Microcentrifuge machine	Sartorius
Microcentrifuge tubes	Axygen
Micropipette tips	Axygen
Micropipettes	Biohit
Parafilm	Pechiney
pH meter	Eutech Instruments
Rotary evaporator	Büchi
Schott bottles	Schott Duran
Separatory funnel	Favorit
Shaking incubator	LabTech
Syringes	Terumo
Weighing scale	Ohaus

Appendix B

Chemicals and Reagents

Table B: List of chemicals, reagents and pre-mixed media with their respective manufacturers.

Chemicals, reagents or pre-mixed media	Manufacturer
Agarose	NextGene
Ammonium sulfate	Merck Chemicals
Barium chloride	Systerm Chemicals
CAS (Chrome azurol sulphonate) reagent	Nacalai Tesque
Chloroform	Merck Chemicals
CM Sephadex C-25	GE Healthcare
Desferal (DFX)	Calbiochem
Diethyl ether	Merck Chemicals
Dipotassium phosphate	UNI-CHEM
Ferric chloride	R & M Chemicals
Ferric chloride hexahydrate	Systerm Chemicals
Glacial acetic acid	Systerm Chemicals
Glycerol	Fisher Scientific
HDTMA (Hexadecyltrimethylammonium)	R & M Chemicals
Hydrochloric acid	RCI Labscan
Luria-Bertani (LB) agar	Merck Chemicals
LB broth	Conda
Magnesium sulphate heptahydrate	J. Kollin Chemicals
Müeller-Hilton agar	Oxoid
Phenol	Nacalai Tesque
PIPES (1,4-Piperazinediethanesulphonic acid)	Acros Organics
Potassium dihydrogen phosphate	QRëc Chemicals
Pyridine	Merck Chemicals
Sephadex G-25	GE Healthcare
Sodium chloride	Merck Chemicals
Sodium hydroxide	Merck Chemicals
Succinic acid	HmbG Chemicals
Sulfuric acid	Merck Chemicals

Appendix C

Agar, Media, Buffers and Solutions

i. Agar and Media

Luria-Bertani (LB) Agar

Pre-mixed LB agar powder	37 g

1000 ml of distilled water was added and was autoclaved at 121°C for 15 minutes.

Luria-Bertani (LB) Broth

Pre-mixed LB broth powder	25 g

1000 ml of distilled water was added and was autoclaved at 121°C for 15 minutes.

Müeller-Hinton (MH) Agar

Pre-mixed MH agar powder	38 g

1000 ml of distilled water was added and was adjusted to pH 7 prior to autoclave at 121°C for 15 minutes.

Simple Succinate Medium (SSM)

Ammonium sulfate	1.0 g
Dipotassium phosphate	6.0 g
Magnesium sulphate heptahydrate	0.2 g
Potassium dihydrogen phosphate	3.0 g
Succinic acid	4.0 g

1000 ml of distilled water was added and was adjusted to pH 7 prior to autoclave at 121°C for 15 minutes.

ii. Buffers and Solutions

[1:1. (w/v)] Phenol-chloroform Solution

Phenol	50 g
Chloroform	50 ml

This solution was prepared prior to use to prevent oxidation of phenol.

5% (w/v) 8-Hydroxyquinoline/chloroform Solution

8-Hydroxyquinoline	50 g
Chloroform	1000 ml

This solution was prepared prior to use and was stored at 4°C.

Iron (III) Solution

1mM Ferric chloride hexahydrate	2.703 mg
10 mM Hydrochloric acid	10 ml

The mixture was prepared to make up a 10 ml iron (III) solution.

0.5 McFarland Standard

1.175% Barium chloride	0.5 ml
1% Sulfuric acid	99.5 ml

The solution was prepared and stored in dark to prevent oxidation.

0.5 M Pyridine-acetate Buffer (pH 5)

Pyridine	40 ml
Glacial acetic acid	30 ml

The mixture was top up to 1000 ml and the solution was adjusted to pH 5. However, only 100 ml of the mixture solution was used each time as the concentration needed was 50 mM.

Appendix D

Ammonium Sulfate Precipitation Table

Final concentration of Ammonium Sulfate, (% Saturation)

Initial Concentration of Ammonium Sulfate, (% Saturation)

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

Figure D: Ammonium sulfate precipitation table. The figure shows the weight (g) of ammonium sulfate to be added to one liter of solution to produce a desired change in the concentration (% saturation) of ammonium sulfate (Education Portal Life Science, n.d.).