Involvement of small molecules in the interaction between *Pseudomonas aeruginosa* and *Scedosporium aurantiacum*

By
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Candidate's declaration

I, Bhavin Popatlal Pethani (MQ Student ID: 43171354), certify that this thesis entitled as "Involvement of small molecules in the interaction between *Pseudomonas aeruginosa* and *Scedosporium aurantiacum*" is a presentation of my original research work carried out as part of the Master of Research program (Molecular Science) at Macquarie University. It has not previously been submitted for a degree nor as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of this thesis itself has been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by Macquarie University Biosafety Committee, The reference numbers of biosafety approvals for *S. aurantiacum* and *P. aeruginosa* are as follows:

1. *Scedosporium aurantiacum*: 5201200092
2. *Pseudomonas aeruginosa*: VIV030712BHA

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Abstract

Pathogenic bacteria and filamentous fungi frequently coexist in airways of individuals with cystic fibrosis (CF) and increase morbidity and mortality rates. In this thesis, interactions were studied between the opportunistic bacterium *Pseudomonas aeruginosa*, and emerging fungal pathogen *Scedosporium aurantiacum*, which are prominent causes of CF infection in Australia. Co-cultivation of *P. aeruginosa* and *S. aurantiacum* on lung mimicking agar-medium revealed inhibitory effects of *P. aeruginosa* on fungal growth. The nature of interaction was explored using heat killed cells, cell lysate and culture supernatant of *P. aeruginosa*, indicating the necessity of live *P. aeruginosa* cells and/or intra and extracellular small molecules to inhibit the growth of *S. aurantiacum*. To identify molecular basis of interaction, small molecules were extracted using various solvents from cell lysates and culture supernatants of selected strains of *P. aeruginosa* and *S. aurantiacum*. The inhibitory activity of solvent fractions of *P. aeruginosa* against *S. aurantiacum* indicated presence of small bioactive molecules produced by the bacterium. The solvent fractions were partially characterised and fractionated using ultraviolet-visible spectroscopy and high performance liquid chromatography. The work contributes to characterisation of microbial interactions that may affect the lungs of people with CF, and the search for effective management of the disease. (199 words)

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- **Bhavin Pethani**

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List of abbreviations

1-OHPHZ - 1-Hydroxyphenazine

2-OHPCA - 2-Hydroxyphenazine-1-Carboxylic Acid

3-Oxo-C_{12}-HSL - N-(3-Oxododecanoyl)-L-Homoserine Lactone

AHL - Acylated Homoserine Lactones

ASL - Airway Surface Liquid

ATCC - American Type Culture Collection

C_4-HSL - N-Butyryl-L-Homoserine Lactone

CF - Cystic Fibrosis

CFTR - Cystic Fibrosis Transmembrane Conductance Regulator

COPD – Chronic Obstructive Pulmonary Diseases

CSLD - Chronic Suppurative Lung Disease

HPLC - High Performance Liquid Chromatography

HSL – Homoserine Lactones

ICAM - Intracellular Adhesion Molecule

KCl - Potassium Chloride

LB - Luria-Bertani Broth

LB Agar - Luria-Bertani Agar

$Mgcl_2\,6H_2O$ - Magnesium Chloride Hexa Hydrate

MIC Minimum Inhibitory Concentration

NaCl - Sodium Chloride

OD - Optical Density

PCA - Phenazine-1-Carboxylic Acid

PDA - Potato Dextrose Agar

PQS - *P. Aeruginosa* Quorum Sensing

PYO - 1-Hydroxy-5-Methylphenazine / Pyocyanin

QS - Quorum Sensing

RIA - Relative Inhibition Activity

RP - Relative Polarity

RPM - Revolution per Minute

RT- Room Temperature

SCFM - Synthetic Cystic Fibrosis Medium

T3SS - Type III Secretion System

TFA - Trifluoroacetic Acid

TLR - Toll-Like Receptor

UV-Vis - Ultraviolet-Visible

ZOI - Zone Of Inhibition

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Chapter 1

Introduction

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Lung diseases are one of the most threatening medical conditions in the world. Millions of people suffer from lung diseases which alter their normal airway physiology or lung structure by means of physiological alterations, infections and genetic disorders (Pauwels *et al.*, 2012). The lung system is a complex apparatus, expanding and relaxing thousands of times a day to maintain adequate inhalation of oxygen and exhalation of carbon dioxide. Therefore, altered lung physiology and structure can lead to impaired respiratory system. Various lung diseases such as asthma, cystic fibrosis, bronchitis, emphysema and other chronic obstructive pulmonary diseases (COPD) are most likely to found across wide groups of global populations and well-studied as they result in chronic clinical manifestations (Mannino and Buist, 2007). Apart from the other lung diseases, cystic fibrosis has held the attention of medical research for the last few decades due to its increasing morbidity and mortality rates.

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is one of the most prevalent autosomal recessive diseases affecting not only to the lungs, but also other organs such as pancreas, intestine and liver (Cohen and Prince, 2012). CF is categorized as a single gene mutation in the gene coding for the protein called cystic fibrosis transmembrane conductance regulator (CFTR) (Kerem *et al.*, 1989). The CFTR protein acts as a cyclic adenosine monophosphate-regulated chloride ion channel. Deletion of a phenylalanine residue in the 508^{th} position (called ΔF508 mutation) of CFTR protein accounts for >70% of all CF mutations (Schroeder *et al.*, 1995). The gene mutation results in partial or complete loss of activity in the CFTR protein on epithelial cell surface. Impaired CFTR function results in electrolytic imbalance (such as Cl^-, Na^+ and HCO_3^-) over epithelial surface leading to increased water absorbance by cells, resulting in water depletion from the airway surface liquid (ASL) (Stutts *et al.*, 1995). Reduced ASL water level and electrolytic imbalance causes airway dehydration and formation of highly viscous mucous, which in turn leads to impaired mucociliary clearance (Cohen and Prince, 2012). The viscous mucous contains many individual constituents such as water, pus, cellular debris, serous fluid, inorganic salts, mucins and many other proteins. Accumulation of hyper viscous mucous entraps microbes such as bacteria, fungal spores and viruses from the air and provides shelter to them. The accumulation of hyper viscous mucous also facilitates susceptibility to acquire microbial infections, which together promote chronic airway inflammation (Gibson *et al.*, 2003). All these processes ultimately result in gradual deterioration of the lung structure and function.

1.1.1 Inflammation of CF airways

Prevalent bacterial infections and chronic lung inflammation are hallmark of CF progression in early life of CF patients. Recently, research conducted on newborn pig CF model revealed that bacterial infection is most likely due to high inflammation (Stoltz *et al.*, 2010). Eventually, these pathological and physiological conditions (infection and inflammation), probably become the main cause of morbidity and mortality in CF (Lyczak *et al.*, 2002). Therefore, they are the major target for therapeutic interventions. Till date, the exact mechanism of the chronic inflammation and bacterial infection caused by CFTR dysfunction is not exactly clear. It has been postulated that the pro-inflammatory phenotype of lung cells is caused due to overexpression of tumour necrosis factor alpha (TNF-α), Interleukin-1, Interleukin-8 and several other inflammatory cytokines and chemokines in CF lungs (Armstrong *et al.*, 1997;Dakin *et al.*, 2002). This causes excessive influx of neutrophils into the airways, which generates an inflammatory response. The inflammatory response is essential to tackle pathogenic infection, but in excess, it causes disparaging effects on host lungs (Elizur *et al.*, 2008). As the disease progresses, the inflammatory responses turn systemic and recurrent. This sequential process of inflammation also plays an important role in bacterial infection and adaptation in the respiratory tract of CF individuals.

1.1.2 Infections in CF airways

Lungs of CF patients are often infected by bacteria throughout their life span. Studies have suggested the importance of bacterial infections as a determinant of host health (Khan *et al.*, 2012). This is particularly true concerning the CF airway where, besides *P. aeruginosa*, a diverse microbiome exists within both, paediatric and adult CF populations (Harrison, 2007). Cross-sectional profiling of the CF lung has revealed the complex microflora during early stages of CF and this complexity of microbial diversity is thought to be decreasing with age (Hunter *et al.*, 2012). Antibiotic treatments have been reported to bring changes in this microbial community. However, arrival of *P. aeruginosa* appears to result in a stochastic shift in the composition of existing microbial community and additional environmental factors also help to shape the microbiome in the respiratory tract of CF individuals (Hunter *et al.*, 2012). Therefore, defining the effect of *P. aeruginosa* on the overall microbiome and other specific CF pathogens may hold value in understanding the etiology of disease progression.

In terms of bacterial infections, *Staphylococcus aureus* and *Hemophilus influenzae* are common colonizers in the airways of CF patients during childhood and have been isolated

from the expectorated viscous mucous secretions (also called as sputum) from CF individuals (Armstrong *et al.*, 1995). Later on during adulthood, *P. aeruginosa* is found to be the most common pathogen identified from nearly 82% of CF patients at the age of 18 years (LiPuma, 2010). Inherently, occurrence of Pseudomonal infections diverges significantly with respect to the age groups and race of CF patients (Valerius *et al.*, 1991). It is also important to state that other bacterial species such as *Stenotrophomonas maltophilia, Achromobacter xylosoxidans* and *Burkholderia cepacia* and numerous fungi are also commonly observed in the CF lungs (Le Bourgeois *et al.*, 2011). These bacteria and fungi are frequently co-isolated from the sputum, but little is known about co-existence and interaction of these pathogens in the context of CF lung (Gilligan, 1991;Harrison, 2007).

The nutrient rich environment of ASL is the primary site of infection for these pathogens as the high viscous mucous cannot be cleared by normal defence mechanisms such as mucociliary clearance, inflammatory response and hypoxic environment (Ramphal and Arora, 2001). Inefficiency of the defence mechanisms increases the tendency of infections in the CF lungs. After colonization within the CF lung, the bacterial pathogens form biofilms, which are considered as a complex network of bacterial communities (Verkman *et al.*, 2003). As infection transits from an early to late stage of the diesease, the microbial environment shifts from bacterial infections to mixed infection, i.e. bacterial community with one or more bacterial and fungal pathogens (Sibley *et al.*, 2008).

Major fungal colonisers of CF lungs are filamentous fungi such as *Aspergillus fumigatus*, *Scedosporium* spp. *Aspergillus terreus, Exophiala dermatitidis* and the yeast *Candida albicans,* but their clinical significance is remains to be defined (Bakare *et al.*, 2003). During chronic fungal colonization, these fungi are thought to contribute to an inflammatory reaction, similarly to the chronic bacterial infections. Moreover, due to their propensity to disseminate and poor susceptibility to current antifungals, they cause severe, and sometimes fatal infections in immunocompromised hosts such as lung transplant recipients (Fridkin and Jarvis, 1996). Therefore, numerous questions arise concerning fungal colonization in the CF airway as many filamentous fungi found from CF sputum are environmental and rarely classified as clinical pathogens (Richardson and Lass-Flörl, 2008). Thus, basic research on the ecology and etiology of fungal lung colonisation and these interactions between fungi with other CF pathogens, their biochemistry and pathogenic mechanisms is required. This effort could provide significant information to define prophylactic measures or to develop more efficient drugs against fungal infections.

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a gram negative γ-proteobacerium and an opportunistic pathogen displaying high level of antibiotic resistance. It is capable of infecting many hosts such as plants and animals, including humans (Stover *et al.*, 2000). In humans, it causes infection in injured, immunodeficient, or otherwise immunocompromised hosts. Pseudomonal infections cause damage to epithelial cells in the respiratory tract, lungs, skin and eyes (Amitani *et al.*, 1991). The infections range from localized infections such as *Pseudomonas* keratitis, to chronic localized infections, to acute systematic infections in certain neoplasia and burns patients (Lyczak *et al.*, 2000). *P. aeruginosa* is commonly found in soil, water supplies and as the skin microflora, hence creates a high risk of exposure to CF patients, burns patients, and immunodeficients individuals.

P. aeruginosa is an exceptional example of genetic and metabolic flexibility among all opportunistic pathogens. The genetic flexibilities result in altered productions of secondary metabolites which may or may not contribute towards its pathogenesis (Gómez and Prince, 2007). Many of these products such as phenazine pigments, exotoxin A, lipase, elastase and several other proteases, are extracellular virulence factors causing infections which cause host cell or tissue damage (Valerius *et al.*, 1991).

1.2.1 *P. aeruginosa* virulence factors in establishment and maintenance of infection

The human lung provides a hostile environment for *P. aeruginosa* to initiate an infection cycle. Thus, *P. aeruginosa* possesses a cache of intra- and extra-cellular virulence factors to alter host physiology and to overcome host defence mechanisms.

Bacterial appendages such as flagellae, pili and cellular lipopolysaccharides, are not only important for motility and cellular adhesion, but also serve as activators of Toll-like receptor 5 (TLR5), TLR2 and TLR4, to activate immune response (Smith *et al.*, 2006). In addition, LepA, a protease secreted by *P. aeruginosa*, cleaves protease activated receptors 1, 2, and 4 to activate the nuclear factor NF-κB and rise inflammation level (Kida *et al.*, 2008). Moreover, phenazine pigments, secreted redox-active molecules, act as a terminal electron acceptor during cellular respiration. Therefore, they are believed to play a significant role in *Pseudomonas* defence against the host (Price-Whelan *et al.*, 2006). Thus, it seems that the phenazine molecules adversely impact several eukaryotic cellular processes such as cell

respiration, electron transport chain. Indeed, these phenazine pigments are also correlated with a poorer CF prognosis.

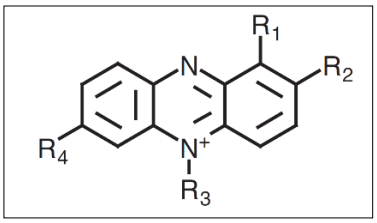
P. aeruginosa halts protein expression in the host cells and kills the host cells by using the ADP-ribosylating protein ExoA. Besides, the Type III secretion system (T3SS) effector proteases ExoS, ExoT and ExoY inhibit actin polymerization in eukaryotic cells and also prevent phagocytosis (Kaufman *et al.*, 2000). In addition, the ExoY increases the membrane permeability of eukaryotic cells, while ExoU, a phospholipase, damages cell membrane to cause cell lysis (Sayner *et al.*, 2004). Together, all of these proteins and pigments dramatically alter the physiology of epithelial cells and the structure of microbial community in the host lung by disrupting cell polarity, causing cell damage. They are also helpful to prevent phagocytosis and clearance of *P. aeruginosa* against host immune response (Fick, 1989). Therefore, these virulence factors allow this opportunistic pathogen to inaugurate an infection cycle in the CF lung.

Once the *Pseudomonas* has escaped from mucociliary clearance and initiated an infection cycle, it must bring upon changes to reduce immune activation and obstruct clearance mechanisms to persevere location in the lung. Hence, *P. aeruginosa* has a set of exo-proteases. These proteases are elastases: LasA and LasB. LasA is lysine-specific endopeptidase, which is shown to be important for survival of the bacterium (Lyczak *et al.*, 2000). Along with LasA, LasB cleaves the abundant lung protein elastin, which is required for normal elasticity of lung. Reduction in lung elasticity worsens the disease and turns to be painful to the host. Protease IV degrades surfactant proteins A, B and D, which are important to maintain surface tension and innate immunity of lungs; their impairment makes airways more vulnerable to other infections (Malloy *et al.*, 2005;Alcorn and Wright, 2004). Additionally, *P. aeruginosa* forms alginate, a polysaccharide, which stimulates mucin production and bacterial biofilm formation, thereby limiting immune recognition and clearance (Worlitzsch *et al.*, 2002;May *et al.*, 1991). The expression of all of the virulence factors listed above is thought to be critical for *P. aeruginosa* to establish and maintain an infection. This project has special emphasis on small organic molecules such as phenazine pigments as *P. aeruginosa* virulence factors.

1.2.1.1 Phenazine pigments of *P. aeruginosa*

Phenazines are heterocyclic compounds, which are naturally produced and diffused in to the growth surrounding by *P. aeruginosa* and several other gram-negative bacteria. Phenazine

core ring structure is nitrogen containing tri-aromatic ring structure (Figure 1.1), which is substituted at different points around their rings to add different side groups. Table 1.1 shows the different phenazine derivatives isolated from *P. aeruginosa* (Price-Whelan *et al.*, 2006). Figure 1.2 shows how small modifications of the core phenazine structure impart a full spectrum of colours, ranging from the deep red of 5-methyl-7-amino-1-carboxyphenazinum betaine (aeruginosin A, Figure 1.2B), bright orange of 2-hydroxyphenazine-1-carboxylic acid (2-OHPCA, Figure 1.2A and 1.2B) and the lemon yellow of phenazine-1-carboxylic acid (PCA, Figure 1.2B), to greenish blue of 1-hydroxy-5-methylphenazine (pyocyanin, Figure 1.2B) (Price-Whelan *et al.*, 2006).



No.	Name	R1	R2	R3	R4
1	7-imino-5-methylphenazine-1-carboxylate (Aeruginosin A)	-COOH	-	$-CH_3$	$-NH_2$
2	Phenazine-1-carboxylic acid (PCA)	-COOH	-	-	-
3	1-hydroxy-5-methylphenazine (Pyocyanin-PYO)	-OH	-	$-CH_3$	-
4	2-hydroxyphenazine-1-carboxylic acid (2-OHPCA)	-COOH	-OH	-	-
5	Phenazine-1-carboxamide (PCN)	$-CONH_2$	-	-	-
6	1-hydroxyphenazine (1-OHPHZ)	-OH	-	-	-

Figure 1.1: Core ring structure of phenazine pigments and characteristics of some phenazine derivatives extracted by *Pseudomonas* with respect to their side groups,
- represents absence of side chain (Price-Whelan *et al.*, 2006).

The presence of combinations and variety of functional groups added to core phenazine structure determines the redox potential and solubility of these compounds, thus affecting their biological activity (Kerr, 2000). The antagonistic effects of almost all of these derivatives are usually attributed to one general characteristic: redox activity (Hassan and Fridovich, 1980). The effects of some of these derivatives are discussed here in detail.

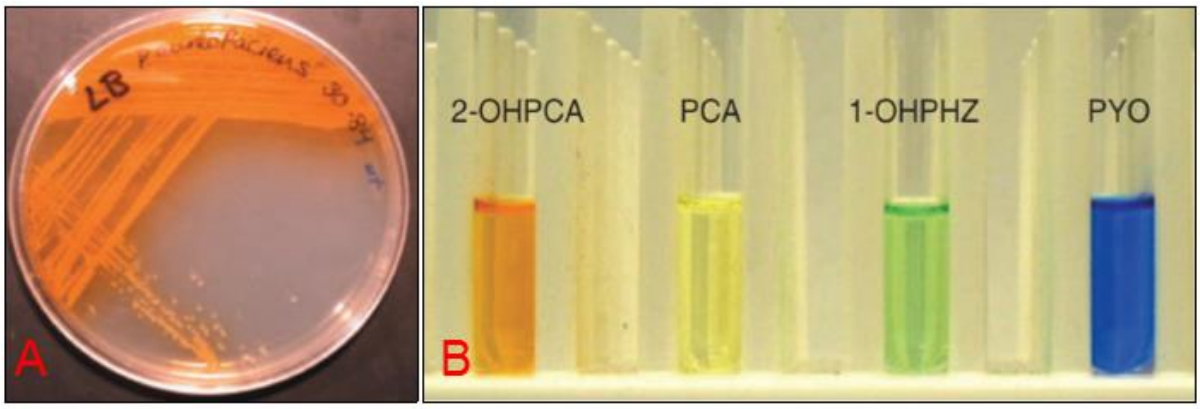


Figure 1.2: Colourful spectrum of phenazine compounds, diffusible molecules of *Pseudomonas*. (A) Streak plate of the biocontrol strain *P. aureofaciens* 30-84. The phenazine 2-OHPCA turns the agar bright orange. (B) Aqueous solutions of some of the phenazines produced by various *Pseudomonas* strain; 2-OHPCA, PCA, 1-OHPHZ and PYO represent orange, lemon yellow, green and blue colour, respectively (Price-Whelan *et al.*, 2006).

1.2.1.2 *P. aeruginosa* pigments as a virulence factors

Many *P. aeruginosa* strains produce the blue-green and lemon yellow coloured phenazine derived pigments named as pyocyanin (PYO) and phenazine-1-carboxylic acid (PCA), respectively. PYO imparts a greenish hue to the sputum of CF individuals with chronic *P. aeruginosa* lung infection (Liu and Nizet, 2009). *P. aeruginosa* PYO and PCA demonstrate a paradoxical pro-oxidant property. Previous research has revealed that PYO or PCA deficient *P. aeruginosa* mutants are significantly attenuated in both acute and chronic mouse models of lung infection (Britigan *et al.*, 1999).

PYO is a zwitterion that can easily penetrate biological membranes and directly accept electrons from biological reducing agents such as NADPH and reduced glutathione (Lau *et al.*, 2004). Then, PYO transfers the electrons to oxygen to generate reactive oxygen species (ROSs) such as H_2O_2 and $^1O_2$ (singlet oxygen) at the cost of host antioxidant systems such as glutathione and catalase (Britigan *et al.*, 1999). Many ROSs employ a direct effect on NF-kB and other signalling pathways to boost inflammatory cytokine secretion (McDonald *et al.*, 2001). For example, PYO elevates the release of the neutrophil chemokine IL-8 from lung epithelial cells and upregulates *in vitro* and *in vivo* expression of ICAM-1 (intracellular adhesion molecule) neutrophil receptor; it causes pro-inflammatory effects in infected individuals (Kerr, 2000;Price-Whelan *et al.*, 2006).

Other research in this area has shown distinguished toxic properties of PYO and PCA to a broad array of target organisms such as bacteria, fungi, insects and nematodes (Laursen and

Nielsen, 2004; Liu and Nizet, 2009). Inhibition of cellular respiration is a crucial mechanism of PYO and PCA toxicity to bacterial and eukaryotic cells (Laursen and Nielsen, 2004; Liu and Nizet, 2009). Alteration in the redox cycle and increased oxidative stress from PYO seem to be central in causing detrimental effects on host cells. For instance, in human airway epithelial cells, PYO and PCA can disrupt Ca^{2+} homeostasis by oxidant-dependent increase in inositol trisphosphate level and abnormal release of Ca2+ from intracellular space (Denning *et al.*, 1998). Since Ca2+ is essential for regulation of ion transport, mucus secretion and ciliary beat, therefore, Ca^{2+} disruption can have significant role in Pseudomonal infections. Other potential toxic effects of PYO and PCA include perturbance of cellular respiration, inhibition of epidermal cell growth, prostacyclin release from lung endothelial cells and imbalance of protease-anti protease activity in the CF lung (Price-Whelan *et al.*, 2006).

Although no therapeutic strategies directly target PYO or PCA, antioxidant therapies have been proven useful. Recently, several authors have suggested inhibition of PYO and PCA synthesis as a therapeutic strategy, although such studies have to be conducted.

1.2.1.3 Pyoverdine as *P. aeruginosa* virulence factor

Pyoverdine is a siderophore compound used for chelating free iron from the environment in *P. aeruginosa* (Zaborin *et al.*, 2009). Pyoverdine is made of a hydroxyquinoline like chromophore moiety attached to a short peptide chain (6–12 amino acids) (Jones *et al.*, 2009). The hydroxyquinoline moieties impart a yellowish-green colour and strong fluorescence properties to pyoverdine, with excitation maximum at~ 405 nm and emission maximum ~ 450-470 nm.

Pyoverdine has been also shown to play a role in *P. aeruginosa* virulence. This research stated that pyoverdine regulates the secretions of other *P. aeruginosa* virulence factors, exotoxins A and several endoproteases and its own secretion (Lamont *et al.*, 2009; Martin *et al.*, 2011). In previous studies, pyoverdine has been purified from six CF sputums, which indicated its role in infections of *P. aeruginosa* in human host. These in vivo observations could be linked to the inability of pyoverdine-deficient mutants of *P. aeruginosa* to infect animal models of lung diseases. Therefore, it indicated the role of pyoverdine in vivo survival and growth of *P. aeruginosa* (Zaborin et al., 2009). Therefore, pyoverdine seems an attractive target for therapeutics design. As yet, There are no therapeutic strategies aimed to block pyoverdine and its synthesis pathways.

1.3 *Scedosporium spp.*

Scedosporium spp. are ubiquitous filamentous fungi with worldwide distribution, present in soil, sewage and polluted waters (Pfaller and Diekema, 2004). *Scedosporium* spp. are emerging fungal pathogens in the world, including Australia (Cortez *et al.*, 2008). The genus *Scedosporium* consists of three clinically important species: *S. apiospermum*, *S. prolificans* and *S. aurantiacum.* Scedosporiosis represents a broad spectrum of clinical diseases caused by the *Scedosporium spp.* that can be colonizers of damaged bronchopulmonary segments, as found in pulmonary tuberculosis, CF or bronchiectasis of lungs of any clinical etiology. *Scedosporium* infections can be localized, extend up to the surrounding tissues (known as deep extension), or distribute hematogenously to distantly located organs (Husain *et al.*, 2005). Tissue infections include post trauma infections to tendons, ligaments and bones and clinical infections such as pneumonia, brain abscess, endophthalmitis (Guerrero *et al.*, 2001). These disseminated infections are frequently seen among immunocompromised patients; however, cases of disseminated infections have been reported in immuno-competent individuals (Morio *et al.*, 2010;Symoens *et al.*, 2006).

Scedosporium spores can infect an individual *via* entry through the respiratory tract, ulcerative lesions in the gastrointestinal tract, surgical wounds and inoculation from trauma. When spores reach the target organ, they produce hyphae and eventually sporulate in the target tissue, enter into the bloodstream and spread further to other organs, particularly the lungs, kidneys and the brain (Cortez *et al.*, 2008;Roilides *et al.*, 2007). Despite the antifungal treatments, most of these disseminated infections are fatal, if not removed surgically. The therapeutic approach to patients with *Scedosporium* infection involves complete surgical removal of the infection lesion with or without antifungal therapy. *Scedosporium* infections have been treated with certain antifungals such as amphotericin B, miconazole, ketoconazole, itracinaxole and variconazole with varying clinical responses (Odds *et al.*, 1998). Studies have suggested that certain *S. prolificans* and *S. apiospermum* strains are resistant *in vivo* to many antifungal drugs and making it difficult to treat *Scedosporium* infections. Such species of *S. prolificans* require higher concentration of most of the drugs tested; the concentration could be as high as 20mg/kg/day of Liposomal amphotericin B or Caspofungin in mice model, and 25mg/kg/day of Albaconazole (UR-9825) in rabbit model, to treat invasive fungal infection (Cortez *et al.*, 2008). In addition, treatment of invasive infection caused by *S. apiospermum* requires dose of Amphotericin B + Voriconazole in concentration of 40mg/kg/day in mice model. Given the demand for more aggressive antifungal therapy to

manage *Scedosporium* infections and the *in vivo* resistance of *Scedosporiu*m *spp.* to the common antifungal agents, new, more active antifungal drugs are required.

1.3.1 *S. aurantiacum*

S. aurantiacum has been proposed as the anamorph (sexually reproducing) state of the ascomycete fungus *Pseudallescheria boydii* (Cortez *et al.*, 2008)). Among all *Scedosporium* spp., *S. aurantiacum* is a fast growing fungus with greyish-white colonies (Figure 1.3). Some of the *S. aurantiacum* isolates produce a light yellow diffusible pigment on solid media.

(A) (B)

Figure 1.3: Colonies of *S. aurantiacum* on potato dextrose agar after 96 hours of incubation at 37°C.
(A) Clinical isolate of *S. aurantiacum* WM08.202, (B) Environmental isolate of *S. aurantiacum* WM10.136 (Kaur *et al.*, 2014).

S. aurantiacum infections are represented in 2.3% of pulmonary mycosis in lung transplantation patients and 8% in the lower respiratory tract of the patients with chronic suppurative lung disease (CSLD) and CF (Blyth *et al.*, 2010). A survey Heath *et al.*(2009) identified a significant number of *S. aurantiacum* in the greater Sydney region of Australia and its ability to cause a wide range of invasive infections. Therefore, it increases a public health concern and need for understanding of the infection mechanisms.

Genetic studies of clinical *Scedosporium apiospermum* isolates (other than *S. aurantiacum*) have revealed patient to patient uniqueness in terms of antifungal resistance over time and antifungal treatment (Pihet *et al.*, 2009). This shows the flexibility of the *Scedosporium* genome for adaptation in a clinical setting with respect to various antifungal treatments (Morio *et al.*, 2010). Apart from this knowledge, there are few studies of *S. aurantiacum* infections, but no significant published work on developing a therapeutics approach against *Scedosporium* infections.

This project is a new initiative to define the effect of small molecules from *P. aeruginosa* on *S. aurantiacum,* which may have significant potential to define therapeutic intervention to treat *S. aurantiacum* infections.

1.4 Interactions between bacteria and fungi

The presence of numerous bacteria and fungi within the CF lung has been identified, but the study of interactions between such microbes leading to an escalation in pathogenicity in a mixed microbial environment, is still lacking. As earlier studies have shown that patient health and mortality are negatively impacted by mixed infections, the understanding the mode of interaction is of academic and clinical importance.

High prevalence of *P. aeruginosa* and fungal colonization (such as *Aspergillus* spp., *Scedosporium* spp., *Candida* spp.) in the airways of CF individuals causes the formation of mixed microbial biofilms (Lyczak *et al.*, 2002). Biofilm embedded bacterial or fungal cells escape from harsh antimicrobials resulting in high resistance to antimicrobial drug therapies making it difficult to eradicate the infection. These biofilms usually act as a microbial reservoir to cause and maintain serious life-threatening infection (Schaefer *et al.*, 2000).

Along with chronic bacterial infections, frequent fungal colonization and mycosis progressively declines lung function, though both the aetiology and pathophysiology of polymicrobial infection remains poorly understood (Gibson *et al.*, 2003). Till date, studies into interactions between *P. aeruginosa* with *Aspergillus* spp. have demonstrated substantial importance of these mixed microbial interactions in inflammation and disease progression, but no information is available concerning *Scedosporium* spp.

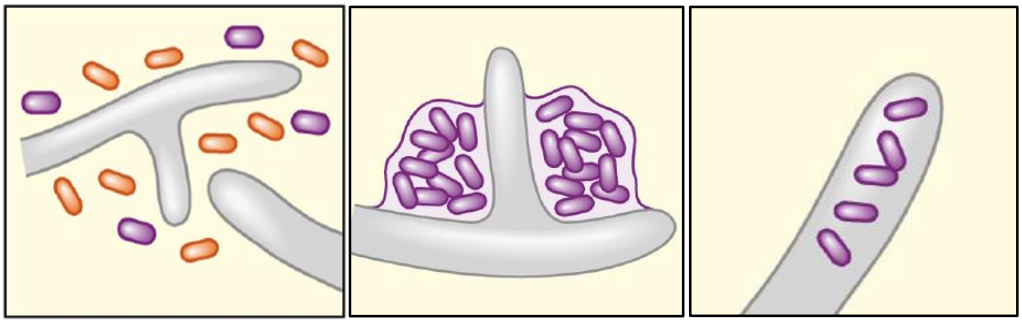
1.4.1 Physical interactions between bacteria and fungi

Physical interaction between bacteria and fungi involves direct contact with one another which may affect growth and behaviour of interacting organisms. For the *P. aeruginosa*, there are numerous potential benefits from such association with fungi (Frey-Klett *et al.*, 2011).

P. aeruginosa can utilise fungal cell wall and fungi-secreted products as a source of nutrients for bacteria to scavenge from (Boer *et al.*, 2005). *P. aeruginosa* facilitate the secretion of antifungal compounds in the area of fungal growth to enhance antagonistic activity against fungus (Cardoza *et al.*, 2006) and also use the fungal movement within the lung environment

to assist their own growth into new lung niches (Hibbing *et al.*, 2009). Fungal bacterial synergy can also be used to breakdown the complex substrates from the environment (Boer *et al.*, 2005).

Frey Klett *et al.* (2011) reviewed several mechanisms by which bacteria-fungi physical interactions occur (Figure 1.4). These mechanisms include planktonic association (Figure 1.4A), mixed biofilm formation (Figure 1.4B) and intrahyphal colonisation (Figure 1.4C). These interactions between bacteria and fungi have also been found to occur on several medical devices such as catheters, prostheses and mechanical ventilators (Pierce, 2005). Microbes organised in the typical structures (depicted in Figure 1.4) show enhanced resistance to antimicrobial therapies, leading to poorer outcome of the antimicrobial treatments.



(A) (B) (C)

Figure 1.4: Physical interaction between bacteria and fungi. (A) Planktonic association, (B) Mixed biofilm, (C) intrahyphal colonisation (Frey-Klett *et al.*, 2011).

Mowat *et al.* (2010) have shown adherence of *P. aeruginosa* PAO1 (ATCC 15692) cells to the *A. fumigatus* hyphae during co-growth studies. In this case, mature fungal biomass was minimally affected by *P. aeruginos*a co-growth, compared to the vegetative hyphae and fungi only control. It suggested the inhibitory effect of *P. aeruginosa* on vegetative growth of *A. fumigatus,* but not on mature fungal growth.

In the nutrient rich competitive environment of human lung, bacteria take an advantage of growing over the fungus by exploiting the fungi for nutrients (Hibbing *et al.*, 2009). For example, *P. aeruginosa* adheres to hyphal form of *C. albicans* and causes cell death, but it is not capable of doing this with the yeast form of *C. albicans*. Other bacterial species such as *Burkholderia cenocepacia* are also capable of showing this type of antagonistic interactions (Jarosz *et al.*, 2011).

1.4.2 Chemical interactions between bacteria and fungi

Within surroundings such as the complex microbiome of the CF lung, competition between numerous microbial species is common and facilitated by cell-to-cell signalling/quorum sensing (QS) or extracellular secretions. In gram-negative bacteria (i.e. *P. aeruginosa*), QS is mediated by certain signalling molecules such as acylated homoserine lactones (AHL) (Nikolaev and Plakunov, 2007). Main function of QS is to regulate expression of the genes involved in biofilm formation, to respond to fluctuations in cell-population density. For instance, QS in *P. aeruginosa* controls certain processes such as biofilm formation, swarming motility and cell aggregation. QS associated molecules such as N-butyryl-L-homoserine lactone (C_4-HSL) and N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) have been found from the CF sputum (Miller and Bassler, 2001).

P. aeruginosa utilises the *LasI/LasR* (*RhlI/RhlR*) QS system. The impact of this QS system was shown by Ramage group in 2010 at in University of Glasgow, UK (Ramage *et al.*, 2010). They reported the role of *LasI/LasR* QS system in the inhibition of *A. fumigatus* biofilm formation (Mowat *et al.*, 2010). In addition, A study on *LasI* and *LasR* knockout mutants of *P. aeruginosa* PA01 (named as PA01:*ΔLasI and* PA01:*ΔLasR)* showed that, PA01:*ΔLasI* were unable to produce homoserine lactones (HSL), and PA01:*ΔLasR*, which was able to synthesize HSL, but none of them could respond to presence HSL. Direct co-culture of *P. aeruginosa* PA01:*ΔLasI and* PA01:*ΔLasR* with *A. fumigatus* did not inhibit biofilm growth, where wild-type PA01 inhibited the formation of fungal biofilm (Mowat *et al.*, 2010).

Another well studied example of virulence up regulation is secretion of homoserine lactones by *P. aeruginosa* in the presence of *C. albicans* (Gibson et al., 2009). Expression of *P. aeruginosa* genes encoding the formation of phenazine pigments are up regulated in the presence of *C. albicans* (Gibson et al., 2009). A supporting study with *P. aeruginosa* PAO1 has showed occurrence of high levels of phenazine pigments in the presence of *C. albicans* or farnesol. Farnesol is an intra-kingdom signalling molecule secreted by *C. albicans*. Studies also indicated that phenazine pigments are associated with antifungal activity against *C. albicans.* Studies indicate that phenazine pigments are associated with antifungal activity against *C. albicans* (Bandara *et al.*, 2013;Gibson *et al.*, 2009). These virulence factors, phenazine pigments, are also capable of damaging host lung tissues. Therefore, up regulation of phenazines within respiratory tract of CF patients can contribute towards increased morbidity not only in fungal cells but also host tissues, leading to increased lung tissue deterioration (Gibson *et al.*, 2009).

In summary, interactions can be either beneficial, detrimental or neutral to the microbes involved (Peleg *et al.,* 2010) as bacteria or fungi can secrete compounds which enhance growth and survival of the other, up-regulate the synthesis of virulence factors, or negatively influence each other's survival causing inhibition of the activity of certain virulence factors (Sachs *et al.,* 2011). The majority of clinically isolated bacterial pathogens show an antagonistic effect on other pathogens such as bacteria and fungi. However, synergistic interactions between clinical pathogens are rarely found, although the majority of them enhance overall virulence of mixed infections (Peleg *et al.,* 2010).

1.4.3 Examples of bacterial-fungal interactions and their impact on virulence

Although research in the field of bacterial fungal interactions is not well developed, a number of interaction studies have been conducted using various species of bacteria and fungi to explore how these interactions affect the microbes and the host.

Prolonged bacterial/fungal infection could be a factor influencing interactions between bacteria and fungi, i.e. bacteria enter into the environment which is already colonized by fungi or vice versa. For example, introduction of *C. albicans* into burn wound mice model, previously infected by *P. aeruginosa*, showed an increase in mortality rate within the mice (Peleg *et al.,* 2010). Mice treated with a sublethal inoculum of *P. aeruginosa* before being treated with a sublethal inoculum of *C. albicans* showed 60% mortality, compared to 10% in those mice treated with a sublethal inoculum of *C. albicans* only. Post follow up from these studies stated that the death of mice was due to *C. albicans*, but the *P. aeruginosa* virulence factor *LasB* and phenazine pigments were also played a role in causing up-regulation of virulence in this mixed infection.

All interactions between fungi and bacteria do not involve growth inhibition and physiological alteration of fungi. As stated previously that *P. aeruginosa* inhibits the growth of *C. albicans*, but Leclair and Hogan showed that secretion of the extracellular signalling molecule 3-oxo-C12-homoserine lactone (3-OC_{12}HSL) from *P. aeruginosa* promoted the survival of *C. albicans* (Leclair and Hogan 2010). This molecule inhibits bacterial killing of *C. albicans*, as *P. aeruginosa* requires a hyphal form of the yeast *albicans*. This mechanism indirectly assists *C. albicans* survival. During hyphae formation, *C. albicans* also utilises the Ras1-cAMP-controlled signalling pathway to induce oxidative stress resistance which provides increased resistance against host phagocytosis (Hoffman *et al.,* 2009). In terms of

further research studies, the interaction studies of fungi other than *C. albicans* and *A. fumigatus* with *P. aeruginosa* are of particular interest. As *Scedosporium* spp. are second most fungal pathogens within CF lung, it provides a good target for further studies.

1.5 Techniques used in interactions studies

In order to determine the type of interactions and associated molecules, numerous microbiological and analytical techniques are available. Disregarding the types of microbes, the primary screening of microbes for their interaction and/or antimicrobials, is generally conducted using solid plate assays. For example, Kerr *et al.* (2013) reported a growth diffusion method to study interaction of bacteria and fungi on solid plate. Using this method, the involvement of enzymes or other extracellular molecules can be predicted. Enzymes and their activity can be determined using colorimetric assays whereas assessment of secondary molecules such as organic molecules require extraction using solvents followed by identification and characterisation of extracted compounds. Some techniques widely used for the interaction studies are discussed below.

1.5.1 Interaction studies of microbes on solid media

Primary screening of interactions between bacteria and fungi is usually conducted on a solid medium to determine visible phenotypic changes on microbial growth. The testing can show an inhibitory or stimulative effect. Interactions can be further studied in order to define the nature of interaction that can be physical or chemical. Physical interactions are tested using live whole organisms, whereas cell lysates, cellular fractions and culture supernatants can be used to determine chemical interactions mediated by compounds produced. The cell lysate represents the intracellular content of the cell, whereas culture supernatant represents secreted extracellular compounds. Further, cellular and subcellular fractions may contain biomolecules that show inhibitory or stimulative effects.

The agar diffusion method is commonly used for qualitative determination of effects of cellular or subcellular fractions (inhibition/no inhibition/growth stimulation) (Ventura *et al.*, 2012). It involves the application of cellular or subcellular fractions in different concentrations to wells or paper discs, punched in/or placed on to agar plates seeded with the test microbial strain. Diffusion of biomolecules from these fractions into the agar medium leads to inhibition or growth stimulation of microbial growth in the vicinity of the test fraction indicated by formation (or non-formation) of clearing zones (Ventura *et al.*, 2012). The

diameter of these zones can be used for rough quantitation and comparative analysis of test samples. Alternatively, cross streaking of two different microbes on a solid medium can be used to detect inhibitory/non-inhibitory effects.

In order to determine the degree of inhibition in liquid medium, microbes such as bacteria and fungi are co-grown and growth of both microbes are measured as optical density (OD) of the growth or producing viable cell count. However, viable count is labour extensive for a large number of samples whereas the measurement of OD does not provide reliable information during co-growth studies. Measurement of fungal growth using OD also does not provide accurate information about cell density as fungi form aggregates of hyphae, resulting in non-uniformed growth. Therefore, the agar diffusion method and sometimes cross streaking are preferred for primary screening of interactions. If primary screening reveals inhibitory effects, organic molecules can be extracted using solvent extraction methods for further analysis.

1.5.2 Solvent extraction of molecules from bacterial samples

Solvent extraction methods are most commonly used to prepare molecular extracts from the bacterial samples due to their ease of use, efficiency and wide applicability (Dai and Mumper, 2010). Generally, the yield of metabolite extraction depends on the varying polarity of samples, chemical nature and composition of molecules, extraction time and temperature, and sample-to-solvent ratio. Bacterial samples contain aromatic compounds ranging from simple to highly polymerised compounds of different quantities in relation to other cellular components such as proteins, lipids and carbohydrates (Harvey *et al.*, 2010). Therefore, there is no universal procedure for extraction of all bacterial molecules (Sarker and Nahar, 2012).

A mixture of molecules extracted in solvent depends on the solvent system used for extraction. It may contain some unwanted substances such as salt, sugars and fats from the sample. Therefore, additional steps such as deionisation may be used to remove salt and sugars. Solvents, such as n-hexane, petroleum ether, ethyl acetate, methanol, 1-butanol, chloroform, diethyl ether and their combinations have been used for the extraction of heterocyclic compounds from bacterial samples (Wu *et al.*, 2014). Unwanted salts from the extraction samples are usually removed with deionised water using liquid-liquid extraction method. Extracted molecules are characterised and tested against the test organism.

1.5.3 Testing the effects of extracted molecules on microbial growth

MIC is defined as the lowest concentration able to inhibit any visible microbial growth in solid or liquid culture medium (Klančnik *et al.*, 2010) and generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. MIC on solid culture plates is determined using the agar diffusion method as discussed in 1.5.1. High throughput techniques such as the use of microtitre plates containing the growth medium, microbial culture and the test metabolite, are commonly used. In this method, serial dilutions are made of the molecules in the growth medium (Klančnik *et al.*, 2010). The test organisms are then added to the dilutions of the molecules, incubated, and scored visually for growth. This procedure is a standard assay for antimicrobial activity testing. Clinically, MICs are important to confirm antimicrobial resistance and to detect the activity of new antimicrobial agents (Van Hal *et al.*, 2012).

1.5.4 Characterization and fractionation of molecular fractions

Following molecular extraction and establishing a growth inhibitory effect of a metabolite mixture, identification of the dominant chemical groups require designing a chromatography protocol for fractionation and for structural elucidation of the compounds. If the molecules are known, it is relatively straightforward to identify them using literature or direct chromatographic comparison with the standard sample (Sarker and Nahar, 2012). However, if the molecules are unknown and complex, combination of physical, chemical and spectroscopic data analyses is required for identification (Van der Hooft *et al.*, 2013). Information on the chemical compounds produced by microbial genus or the family under investigation can also provide important information regarding the possible chemical classes of the molecules. For example, *P. aeruginosa* produces a wide range of organic compounds such as phenazines and Homoserine Lactones (HSL).

Spectroscopic techniques are employed for structural determination of molecules (Imhoff *et al.*, 2011). Ultraviolet-visible (UV-vis) spectroscopy provides information on the chromophores present in the molecule. Some complex mixtures such as isoquinoline alkaloids and flavonoids can be primarily characterized from their characteristic absorption peaks (Giessen *et al.*, 2012). In addition, infrared spectroscopy (IR) provides information on different functional groups such as -C=O, -OH, -NH2 or aromatic rings present in a metabolite. Once the fractionation protocol is established for separation of molecules,

separation can be achieved using various chromatographic techniques such as such as circular centrifugal chromatography, size exclusion chromatography and high performance liquid chromatography (HPLC). Advanced analytical techniques such as LC-NMR, LC-MS-NMR offer combined separation and structural elucidation of molecules.

1.6 Aims

Despite the notable progression in research on microbial infections in CF, there is a need for more research to be conducted to elucidate the uncovered factors involved in mixed infections. One such factor is interaction between bacteria and fungi in the CF lung. This project addresses a hypothesis that during co-growth of *P. aeruginosa* with *S. aurantiacum*, *P. aeruginosa* shows an inhibitory effect on *S. aurantiacum* in CF under lung mimicking conditions. Once inhibitory activity of *P. aeruginosa* is established against *S. aurantiacum*, chemical nature of interaction will be revealed by testing cell lysates and culture supernatants against *S. aurantiacum* and extracting small molecules from cell lysates and culture supernatants from selected *P. aeruginosa* strains using solvent extraction.

The aims of the study were as follows:

1. To determine the nature of interactions between *P. aeruginosa* and *S. aurantiac*um initial testing for inhibition is carried out on agar plates.
2. To evaluate the effect of *P. aeruginosa* cell lysate and culture supernatant on the growth of *S. aurantiacum* (inhibition/no-inhibition).
3. To extract and characterise small molecules from cell lysates and culture supernatants of *P. aeruginosa* using solvent extraction, MIC and spectroscopic analysis.
4. To explore the role of small molecules in growth inhibition.

Chapter 2

Materials and methods

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2.1 Growth and maintenance of microbial strains and sample preparation

2.1.1 *Pseudomonas aeruginosa* strains

Four *P. aeruginosa* clinical (PASS) strains were used along with *P. aeruginosa* PAO1 as reference strain (Table 2.1). All strains were readily available in the Nevalainen group, Department of chemistry and Biomolecular sciences, Macquarie University, Sydney, Australia, as part of the ARC Super Science Fellowships Project. The strains were stored in a Revco medium (3.73% KCl, 0.81% $MgCl_2$ $6H_2O$, 1.21% Tris base, 50% Glycerol, pH 8.5) at -80°C. For experimental purposes, all *P. aeruginosa* strains were periodically grown on overnight on Luria-Bertani (LB) agar plates (0.6% Trypton, 0.3% Yeast Extract, 0.6% NaCl, 2 % agar, pH 7) at 37°C and plates were stored at 4°C, if required.

Table 2.1: *P. aeruginosa* strains used in the current research.

***P. aeruginosa* Isolate**	**Biological and geographic origin**	**Pigments on LB agar**
PASS1	40 year old female CF patient, sputum sample, Sydney, NSW, Australia	Light green colony with greenish halo
PASS2	27 year old male patient, sputum sample, Sydney, NSW, Australia	Orange colony with orange halo
PASS3	23 year old male patient, sputum sample, Sydney, NSW, Australia	Green colony with light green halo
PASS4	23 year old female patient, sputum sample, Sydney, NSW, Australia	Dark greenish-blue colony with bluish halo
PAO1 (ATCC 15692)	Wound exudate, Melbourne, VIC, Australia (1955)	Green with greenish yellow halo

2.1.1.1 Standardisation of the *P. aeruginosa* culture

A loopful of *P. aeruginosa* was taken from LB plates grown overnight and inoculated into 10 ml sterile synthetic cystic fibrosis medium (SCFM)(Section 2.2). Cultures were incubated at 37°C in a shaking incubator (Scientifix NB-205Q) at 180 rpm, until optical density (O.D.) of 1.0 at 600nm was reached, which corresponds to 1×10^8 cells/ml. O.D. was measured using

Eppendorf Biophotometer. For confirmation, total number of the cells was counted using a standard plate count method (Allen *et al.*, 2004). For all experimental purposes, 10 µl of the *P. aeruginosa* culture was used in order to produce the desired standardised *P. aeruginosa* cell concentration of 1×10^6 cells per inoculum.

2.1.2 *Scedosporium aurantiacum* strains

Two clinical and two environmental strains of *S. aurantiacum* were provided by Professor Wieland Meyer, Westmead hospital, Westmead, NSW 2145, Australia (Table 2.2). The strains were maintained as conidial suspensions in a Revco medium at -80°C. The fungal strains were grown on Potato Dextrose Agar Plates (Difco) at 37°C for 5 days.

Table 2.2: *S. aurantiacum* strains used in the current research.

***S. aurantiacum* isolate**	**Biological and geographic origin**	**Virulence level**[1]
WM06.482	CF lung isolate, Sydney, Australia	High
WM09.24	Environmental isolate, Sydney, Australia	High
WM08.202	Wound exudate isolate, Spain	Low
WM10.136	Environmental isolate, Austria	Low

2.1.2.1 Standardisation of the conidial count

Fungal conidia were harvested from a 5 days old PDA plate in sterile salt solution (0.9% NaCl and 0.01% Tween 80 in milliQ water**)** and filtered through sterile 5 ml pipette tip containing cotton. Following this, the number of conidia in the filtrate were counted using a Neubauer haemocytometer (BLAUBRAND®, Germany); conidial suspension was diluted if needed. Further in order to achieve a concentration of 2×107 conidia/ml, conidial suspension was diluted with the sterile salt solution (0.9% NaCl and 0.01% Tween 80 in milliQ water). The confirmation of the conidial count was carried out using viable count (Pfaller *et al.*, 1988). Fresh conidia were harvested from the PDA plates approximately every three weeks, or more frequently as required. Ten µl of the conidial suspension containing 2×10^5 conidia was used for further experimental purposes.

[1] Tested in a BALB/c (albino) mice model, (Harun *et al.*, 2010).

2.2 Preparation of synthetic cystic fibrosis medium (SCFM)

SCFM was prepared using a range of free amino acids, glucose and lactate in order to mimic the physiological conditions of CF lung (Palmer *et al.*, 2007) (Refer supplementary table 1). To prepare SCFM agarose plates, 2%w/v agarose was mixed with 10 ml sterile distilled water and heated until boiled, then kept in a waterbath set at 70°C. An appropriate amount of molten agarose was mixed with SCFM, mixed and poured on plates.

2.3 Preparation of *Pseudomonas aeruginosa* samples

2.3.1 Heat killed cells

One ml of the *P. aeruginosa* standard culture was collected in a 1.5 ml sterile eppendorf tube and heat treated at 80°C for 60 minutes using Eppendorf Thermomixer® comfort. Ten µl aliquots of culture were withdrawn after every 10 minutes, plated on LB and incubated overnight at 37°C. Plates were observed for the growth of *P. aeruginosa* to check the efficiency of the heat treatment.

2.3.2 Culture supernatant

Ten µl of *P. aeruginosa* strains were inoculated in 50 ml of SCFM and grown for 48 hours at 37°C. Culture medium was centrifuged at 7500 rpm for 30 minutes using Sigma 3-18K centrifuge and the supernatant was collected without disturbing the cell pellet. The collected supernatants were filtered through Minisart 0.22µm syringe filter and stored in the dark at 4°C until used.

2.3.3 Cell lysate

50µl of standardised *P. aeruginosa* culture prepared as in 2.3.2 was centrifuged at 7500 rpm for 30 minutes using Sigma 3-18K centrifuge. The supernatant was discarded and cell pellet was resuspended in an equal amount of MilliQ water and chilled at -20°C. Chilled *P. aeruginosa* suspensions were solicited in 10 passes of 30 seconds for 20 minutes, at 50% amplitude using a BRANSON 102C-CE sonication probe in a sound proof cabinet. The sonicated suspensions were centrifuged at 10,000rpm for 30 minutes at 4°C using Sigma 6K15 centrifuge, to remove heavy cellular matter. Following centrifugation, suspensions were filtered through a Minisart 0.22µm syringe filter to remove the remaining cell debris.

2.4. Interaction between *P. aeruginosa* and *S. aurantiacum* using solid plate assay: Opposite end inoculation method

In order to reveal interactions between the bacterium and the fungus, 10 µl of all strains of *P. aeruginosa* standardised culture and 10 µl of all strains of *S. aurantiacum* conidial suspension containing 2×10^5 conidia were inoculated at the opposite ends of the SCFM agarose plates (1 cm away from the edge) in pairwise combinations. Plates were inoculated at 37°C for 12 days. Fungal growth was observed as inhibition/no-inhibition. Interaction of five strains of *P. aeruginosa* against four strains of *S. aurantiacum* resulted in 20 *P. aeruginosa* vs *S. aurantiacum* combinations for this initial interaction study using solid medium.

2.4.1 Interaction between *P. aeruginosa* and clinical strains of S. *aurantiacum*: centre inoculation method

To determine relative inhibition activity (RIA) of all strains of *P. aeruginosa* against clinical strains of *S. aurantiacum*, 100 µl of 1/10 diluted *S. aurantiacum* standard conidial suspension containing 2×10^5 conidia were uniformly spread over SCFM agarose plates and cultures incubated at room temperature (RT) for 15 minutes. Later, 10 µl of *P. aeruginosa* cell suspension was inoculated in the centre of the SCFM agarose plate. *S. aurantiacum* only, *P. aeruginosa* only and un-inoculated SCFM plates were used as fungal, bacterial and medium control, respectively. Plates were incubated at 37°C for 5 days. Diameter of the bacterial colony (D) and zone of inhibition (d) were measured (in millimetres) to calculate relative inhibition activity (RIA) for each bacterial isolate against clinical strains of *S. aurantiacum* using formula introduced by Bradner *et al.*, (1999). This experiment was done in triplicate.

$$\text{Relative inhibition activity} = \frac{(D)^2-(d)^2}{(d)^2}$$

Where: D = diameter of zone of inhibition (in mm)

d = diameter of colony or well (in mm)

For comparison, 100µl of 1/10 diluted *S. aurantiacum* conidial suspension containing 2×10^5 conidia was spread on a sterile SCFM agarose plate as in 2.4.1. Later, 10µl or 100µl of heat killed *P. aeruginosa* cells were inoculated in the centre of the SCFM agarose plate. *S. aurantiacum* only, heat killed *P. aeruginosa* cells only and un-inoculated SCFM plates served as fungal, bacterial and medium control, respectively. Plates were incubated at 37°C for

5 days. Diameter of the heat killed bacterial inoculum and zone of inhibition were measured to calculate RIA.

2.5 Chemical interaction *P. aeruginosa* and *S. aurantiacum* using agar well diffusion method

To determine the nature interaction mediated by chemical compounds, *P. aeruginosa* cell lysate and culture supernatants were tested against *S. aurantiacum*. As described in 2.4.1, 100µl of 1/10 diluted *S. aurantiacum* conidial suspension containing 2×10^5 conidia was uniformly spread over an SCFM plate using a sterile spreader and incubated at RT for 15 minutes. Wells were dug using a sterile cork borer (8 mm and 10 mm, well diameter (d)). Different cork borers were used for different *S. aurantiacum* strains. Later, 10µl (in 8 mm wells) or 100µl/200 µl (in 10 mm wells) of *P. aeruginosa* cell lysates or culture supernatants were plated in the well. *S. aurantiacum* only, *P. aeruginosa* cell lysate only or culture supernatants only and un-inoculated SCFM plates were used as fungal, bacterial and medium control, respectively. Plates were incubated at 37°C for 5 days. Zone of inhibition (D, in mm) was measured and RIA calculated as described in 2.4.1.

2.6 Extraction of molecules from *P. aeruginosa* strains

To standardise the protocols for the extraction of molecules form *P. aeruginosa* strains during this pilot project, only *P. aeruginosa* strains PASS1 and PAO1 were used. Molecules from the cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1 (referred as *P. aeruginosa* cellular fractions here), were extracted using liquid-liquid extraction method. Fifty ml of *P. aeruginosa* cellular fractions were mixed with equal amount of organic solvents in a 500 ml separatory funnel and mixed by shaking for at least 10 minutes. Once the two phases had settled, organic phase was collected and aqueous phase was again put through the same solvent treatment. The serial extraction procedure was repeated thrice for petroleum ether, ethyl acetate and 1-butanol each. Alltogether, four *P. aeruginosa* cellular fractions were extracted using three organic solvents resulting into twelve fractions (hereafter referred as molecular factions) from *P. aeruginosa*.

All molecules fractions were evaporated using BUCHI Rotavapor R-200 at 40°C and 200 millibar pressure and residual material was resuspended in 5 ml of same solvent to achieve 10× concentration of the molecules. These concentrated molecular fractions were deionised using milliQ water. The activity of the deionised molecular fractions of *P. aeruginosa* were

tested against clinical strains of *S. aurantiacum* using agar well diffusion method as described in 2.5.2. Zone of inhibition (D, in mm) was measured and RIA calculated as described in 2.4.1, if inhibition was observed.

2.7 MIC of extracted molecules from *P. aeruginosa* against clinical strains of *S. aurantiacum*

As molecules extracted in 1-butanol showed inhibition against clinical strains of *S. aurantiacum*, the further study used only molecular fractions extracted with 1-butanol from the cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1. MICs of these four molecular fractions from *P. aeruginosa* were determined using the broth dilution method in a 96-well microtitre plate against clinical strains of *S. aurantiacum*. Up to 0-100 µl/ml serial dilution of 1-butanol extracted molecules from *P. aeruginosa* were made in SCFM. In order to achieve 2×10^5 conidia/ml, 3.3 µl of *S. aurantiacum* conidial suspension was inoculated in the serial dilutions of extracted molecules fractions from *P. aeruginosa*. The microtitre plates were incubated at 37°C for 6 days without shaking and fungal growth was measured as OD_{590} using BMG Labtech PHERAstar FS at 0, and 6^{th} day of incubation. After incubation, a loopful from each dilution was inoculated on PDA plates to determine nature of growth inhibition (fungicidal or fungistatic). The lowest concentration/volume of extracted molecules which showed no *S. aurantiacum* growth on PDA plates was regarded as the MIC (Vipra *et al.*, 2013).

For all MIC assays, cultivation medium inoculated with *S. aurantiacum* only and un-inoculated medium served as positive and negative control, respectively. Along with serial dilutions of 1-butanol extracted molecules, 0-100 µl/ml serial dilutions of 1-butanol were used as 1-butanol control.

2.8 Spectroscopic analysis of 1-butanol extracted molecules from *P. aeruginosa*

Absorbance of all 1-butanol molecular fractions from *P. aeruginosa* were measured in triplicate in the range of 220-1000 nm against 1-butanol as blank (BMG Labtech PHERAstar FS). The average of all the replicates of 1-butanol molecules fractions were plotted as Absorbance vs wavelength using Microsoft Excel 2013.

2.9 Fractionation of 1-butanol extracted molecules from *P. aeruginosa* using HPLC

HPLC separation of molecules from 1-butanol extracted molecules of *P. aeruginosa* was conducted using Agilent Zorbax SB-C18 column attached to Shimadzu SCL series HPLC instrument with isocratic gradients reported by Wilson *et al.,* (1987).

Elution gradient 1: Acetonitrile/water/trifluoroacetic acid (TFA) in linear gradient from 100:0:0.05 vol/vol/vol to 60:40:0.05vov/vol/vol, for 60 minutes followed by 10 minute liner gradient of the same at 0.2 ml/min flow rate (Wilson *et al.*, 1987).

Elution gradient 2: 0 to 40% isopropanol in aqueous acetic acid (2.5%) in 65 minutes of linear gradient from followed by 15 minute of 40% isopropanol in aqueous acetic acid (2.5%) at 0.2 ml/min flow rate (Wilson *et al.*, 1987). In this gradient, acid concentration was reduced up to 2.5% from 5.0% to avoid harsh acid treatment to the complex mixture of molecules in the 1-butanol fraction.

Fractions of 1 ml each, were collected resulting in 12 and 13 fractions in case of elution gradient 1 and 2, respectively.

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Chapter 3

Results

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3.1 Interactions between *Pseudomonas aeruginosa* and *Scedosporium aurantiacum*

To determine the type and nature of the interactions between *P. aeruginosa* and *S. aurantiacum*, a series of screening assays were performed. The study began with visual observation of interactions, followed by determination of the nature of the interactions: physical involving live cells or chemical mediated by small molecules.

3.1.1. Interactions between *P. aeruginosa* and *S. aurantiacum* on solid media: opposite end growth and growth inhibition

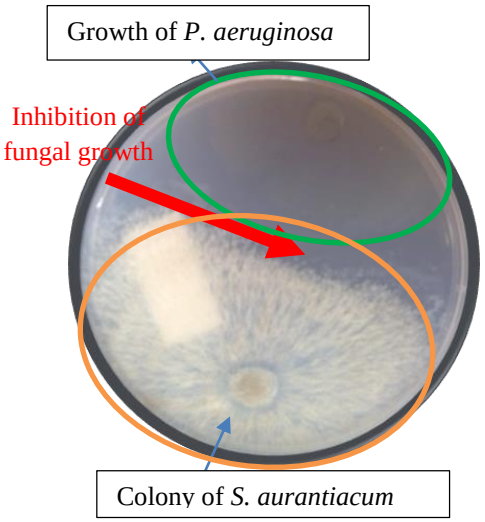

Figure 3. 1: Inhibition of the growth of the fungal colony during co-growth (opposite end inoculation) of *P. aeruginosa* PASS1 and *S. aurantiacum* WM06.482 on SCFM agarose plate after 8 days of incubation at 37°C.

Screening of interaction between five *P. aeruginosa* strains and four *S. aurantiacum* strains was conducted on SCFM agarose plate. *P. aeruginosa* (clinical and reference strains, Table 2.1) and *S. aurantiacum* (clinical and environmental strains, Table 2.2) were inoculated at opposite ends of the same plate at the same time, their growth was monitored up to 10 days and images were taken at the interval of two days. Inhibition of growth of *S. aurantiacum* was indicated by a clear zone of inhibition on the edge of the fungal colony, where *P. aeruginosa* was in close contact of the *S. aurantiacum* colony, but not touching (Figure 3.1 & 3.2). For example, when the clinical strains of *P. aeruginosa* (PASS1) and *S. aurantiacum* (WM06.482) were grown on SCFM agarose plate, a zone of no growth was present between the bacterial and fungal colonies (Figure 3.1). Similarly, when *S. aurantiacum* WM08.202 and *P. aeruginosa* PASS2 were co-grown on the same solid medium, visible inhibition of *S. aurantiacum* was found as a partial clear zone at the periphery of the fungal colony (Figure 3.2). In order to investigate the inhibitory effect of *P. aeruginosa* against *S. aurantiacum*, a total of 20 strain combinations[1] of *P. aeruginosa* vs *S. aurantiacum* were tested and the results were recorded as inhibition/no-inhibition of fungal growth as shown in Table 3.1.

[1] 5 *P. aeruginosa* strains × 4 *S. aurantiacum* strains = 20 *P. aeruginosa* vs *S. aurantiacum*.

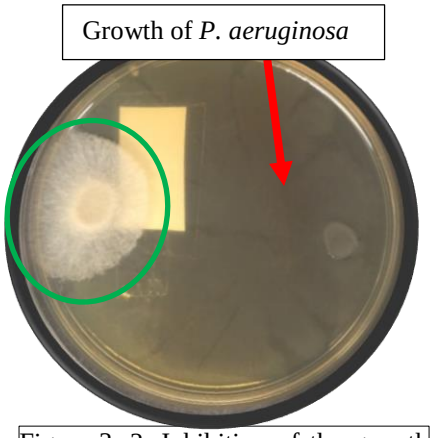

Figure 3. 2: Inhibition of the growth of the fungal colony during co-growth (opposite end inoculation) of *P. aeruginosa* PASS1 and *S. aurantiacum* WM08.202 on an SCFM agarose plate after 8 days of incubation at 37°C.

The majority of the *P. aeruginosa* strains showed an inhibitory effect against *S. aurantiacum*, exhibited as gradual disappearance/degradation of fungal hyphae due to bacterial growth. Among all twenty co-growth combinations, only two combinations did not show inhibitory effects on fungi. These were *P. aeruginosa* PASS2 against *S. aurantiacum* WM06.482, and *P. aeruginosa* PASS4 against *S. aurantiacum* WM08.202. Growth of P. aeruginosa PASS2 and PASS4 did not show any effect on the growth of S. aurantiacum WM06.482 and WM08.202, respectively. Bacteria and fungi started to grow simultaneously, but surprisingly fungi didn't show any inhibition against bacterial growth.

When *P. aeruginosa* PASS1 was co-grown with *S. aurantiacum* clinical strains WM06.482 and WM08.202, fungal growth was inhibited. In the case of *S. aurantiacum* WM06.482, fungi and *P. aeruginosa* were growing robustly for the first four days of incubation. After that, density of fungal growth started to decrease from the edge of the colony, where both organisms were in close contact with each other but did not touch each other (Figure 3.1). In the case of *S. aurantiacum* WM08.202, *P. aeruginosa* PASS1 covered the whole plate within four days of incubation, whereas *S. aurantiacum* appeared as an intermediate sized colony at the edge of the SCFM plate. Surprisingly, *S. aurantiacum* started growing back (upwards) after the fifth day of incubation (Figure 3.2). During co-growth of *P. aeruginosa* PASS2 with the *S. aurantiacum* low virulent clinical strain, relatively less inhibition of fungal growth was found (Figure 3.3)

Table 3.1: Changes in fungal growth (inhibition/no inhibition), when *P. aeruginosa* and *S. aurantiacum* were co-grown on SCFM agarose plates. Ten µl of culture of *P. aeruginosa* and *S. aurantiacum* suspensions were inoculated at opposite ends of SCFM agarose plates. Images taken during the 8th day of co-growth studies are presented in supplementary Table 2.

***S. aurantiacum* strains**	**Inhibition/no inhibition of *S. aurantiacum* growth by the indicated *P. aeruginosa* strains**				
	PASS1	PASS2	PASS3	PASS4	PAO1
WM06.482 (clinical strain)	Inhibition	No Inhibition	Inhibition	Inhibition	Inhibition
WM08.202 (clinical strain)	Inhibition	Inhibition	Inhibition	No Inhibition	Inhibition
WM09.24 (environmental strain)	Inhibition	Inhibition	Inhibition	Inhibition	Inhibition
WM10.136 (environmental strain)	Inhibition	Inhibition	Inhibition	Inhibition	Inhibition

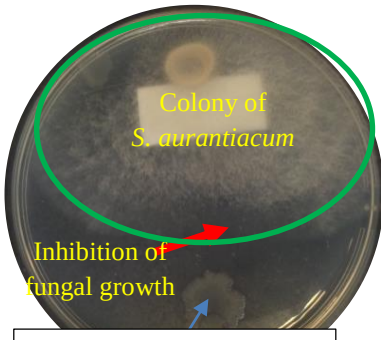

Figure 3. 3: Inhibition of the growth of the fungal colony during co-growth (opposite end inoculation) of *P. aeruginosa* PASS2 and *S. aurantiacum* WM08.202 on an SCFM agarose plate after 8 days of incubation at 37°C.

On the other hand, *P. aeruginosa* PASS2 and PASS4 did not inhibit the growth of the clinical strains of *S. aurantiacum* WM06.482 and WM08.202, respectively.

Further, the co-growth study of environmental strains of *S. aurantiacum* with *P. aeruginosa* revealed inhibition of the fungus in all possible combinations. The fungal growth inhibition was quite similar to all other cases discussed above. However, clinical strain of *P. aeruginosa* PASS1 and PASS2 both showed least inhibition against environmental strains of *S. aurantiacum* WM09.24 and WM10.136, based on visual observation

With this in mind and due to clinical relevance of clinical strains of *S. aurantiacum*, an interaction study was conducted to determine the relative inhibition activity of *P. aeruginosa* against the clinical strains of *S. aurantiacum* on solid medium.

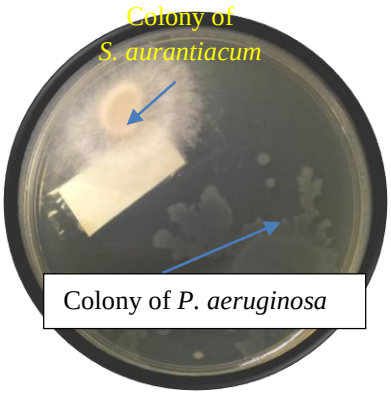

Figure 3. 4: No inhibition of the growth of the fungal colony during co-growth (opposite end inoculation) of *P. aeruginosa* PASS4 and *S. aurantiacum* WM08.202 on an SCFM agarose plate after 8 days of incubation at 37°C.

3.1.2 Interactions between *P. aeruginosa* and *S. aurantiacum* on solid media: centre inoculation

Only clinical strains of *S. aurantiac*um were tested against all four strains of *P. aeruginosa* and reference strain to determine the RIA between these two pathogens. In this case, 10 µl of *P. aeruginosa* culture was spotted in the middle of an SCFM agarose plate previously seeded with 10 µl of the conidial suspension of *S. aurantiacum* as described in 2.4.1. Plates were incubated at 37°C for 96 hours and results were recorded as *P. aeruginosa* colony diameter and diameter of the zone of inhibition against *S. aurantiacum* (Figure 3.5). Figure 3.5 shows a slimy *P. aeruginosa* PASS3 colony in centre of the SCFM agarose plate and a semi-transparent zone of inhibition around the colony against white growth of *S. aurantiacum* over the plate. The RIA values were calculated using a formula described in Materials and Methods (2.4.1) and also a statistical analysis was carried out to determine standard deviation within the replicates (n=3)

(refer Supplementary Table 2) Relative inhibition activity of *P. aeruginosa* against clinical strains of *S. aurantiacum* is shown in Figure 3.6.

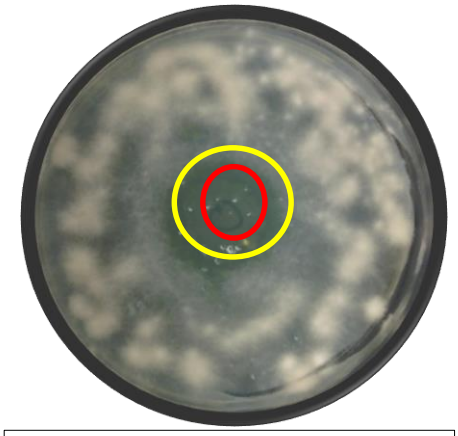

Figure 3. 5: Inhibition of the growth of the fungal colony during co-growth (centre inoculation) of *P. aeruginosa* PASS3 and *S. aurantiacum* WM08.202 on an SCFM agarose plate after 96 hours of incubation at 37°C. Bacterial colony is shown in a red circle whereas zone of inhibition is shown in a yellow circle against white fungal growth.

In terms of inhibition against the *S. aurantiacum* WM06.482, average RIA values were 6.64, 5.00, 5.25 and 1.44 for *P. aeruginosa* strains PASS1, PASS2, PASS3, and PASS4, respectively. In contrast to that *P. aeruginosa* PASS1, PASS2, PASS3 and PASS4 showed average RIA values of 2.70, 3.21, 1.30, and 2.75, respectively, against *S. aurantiacum* strain WM08.202. Highest relative inhibition activity is shown by *P. aeruginosa* PASS1 and PASS2 against *S. aurantiacum* strains WM06.482 and WM08.202, respectively. The *P. aeruginosa* control PAO1 showed the least RIA values, 1.21 and 0.93 against *S. aurantiacum* WM06.482 and WM08.202, respectively.

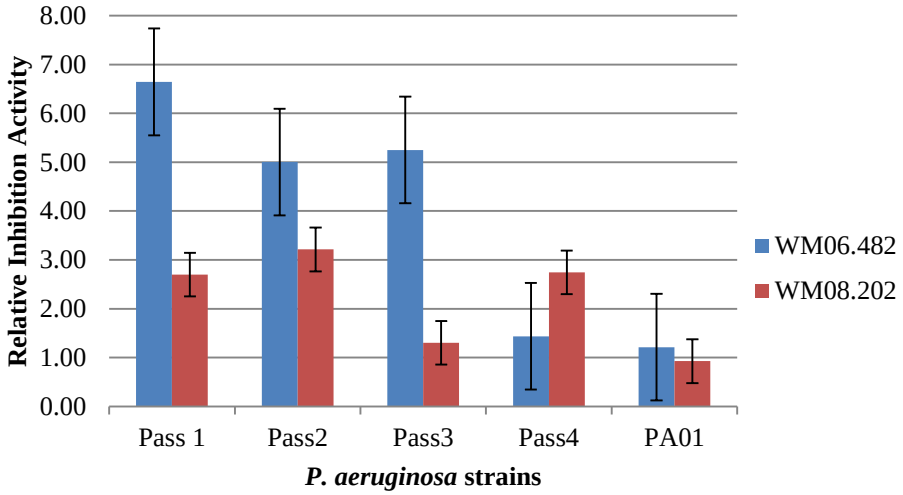

Figure 3. 6: Relative inhibition activity (RIA) displayed by the clinical and reference *P. aeruginosa* strains against clinical strains of *S. aurantiacum* on SCFM agarose plates. RIA was calculated from the averaged values of three replicates as described in Material and Methods (2.4.1). Error bars represents the standard deviation among the technical replicates. Maximum standard deviation between measurements was 1.5% (n=3). (For analysis of the data refer to Supplementary Table 3).

To summarise, all the *P. aeruginosa* strains (except PASS4) showed high RIA values against *S. aurantiacum* WM06.482 compared to WM08.202, representing higher inhibition of the high virulent clinical strain of *S. aurantiacum* (WM06.482). Figure 3.6 points to a higher inhibition activity of the clinical strains of *P. aeruginosa* compared to the reference strain PAO1. Findings of this experiment confirmed the inhibitory activity of *P. aeruginosa* against *S. aurantiacum*.

Figure 3. 6: Inhibition of fungal growth during co-growth (centre inoculation) of heat killed cells of *P. aeruginosa* PASS2 and *S. aurantiacum* WM10.136 on solid media (SCFM agarose plate) after 96 hours of incubation at 37°C. Heat killed bacteria is shown in a red circle whereas zone of inhibition is shown in a yellow circle against white fungal growth.

In order to determine the nature of interaction, heat killed *P. aeruginosa* cells were tested against all *S. aurantiacum* strains. In this study, 10µl and 100µl of heat killed cells of clinical (representing PASS1, PASS2, PASS3 and PASS4) and reference (PAO1) strains of *P. aeruginosa* were spotted in the centre of SCFM agarose plate, previously seeded with conidial suspension of each four strains of *S. aurantiacum*. Plates were incubated at 37°C for 96 hours and results were recorded as inhibition or no inhibition of *S. aurantiacum* growth.

Majority of the heat killed *P. aeruginosa* did not show any significant inhibitory effect *P. aeruginosa* on *S. aurantiacum*, except *P. aeruginosa* PASS2. 100µl aliquot of *P. aeruginosa* PASS2 showed an inhibitory effect against the *S. aurantiacum* environmental strain WM10.136. In this case heat killed cells were spotted in a 10 mm area in the centre of the SCFM plate, which showed a zone of inhibition of 17 mm giving an RIA value of 1.89.

These findings revealed that to have an inhibitory effects on *S. aurantiacum*, live cells of *P. aeruginosa* are generally required. In the case of *P. aeruginosa* PASS2 vs *S. aurantiacum* WM10.136, intracellular molecules or any other virulence determinants could be present which have an inhibitory effect against *S. aurantiacum* WM10.136. The study was then broadened to examine the inhibitory effects of intracellular molecules and culture supernatants.

3.1.3 Chemical interactions between *P. aeruginosa* and *S. aurantiacum*

Chemical interaction can be mediated by intra or extracellular (small) molecules. Thus, cell lysates and culture supernatants of all strains of *P. aeruginosa* were tested against *S. aurantiacum* using an agar well diffusion method as described in 2.5 and RIA was established. The effects of *P. aeruginosa* cell lysates are discussed first.

Aliquots of 10µl and 100µl of cell lysates from all *P. aeruginosa* strains were tested against all *S. aurantiacum* strains. The aliquot of 10 µl of cell lysate from the *P. aeruginosa* strains tested did not show any inhibition of *S. aurantiacum*, whereas aliquots of 100 µl showed strong inhibitory activity. Thus, inhibitory effects of 100 µl aliquots are presented here and shown in Figure 3.8.

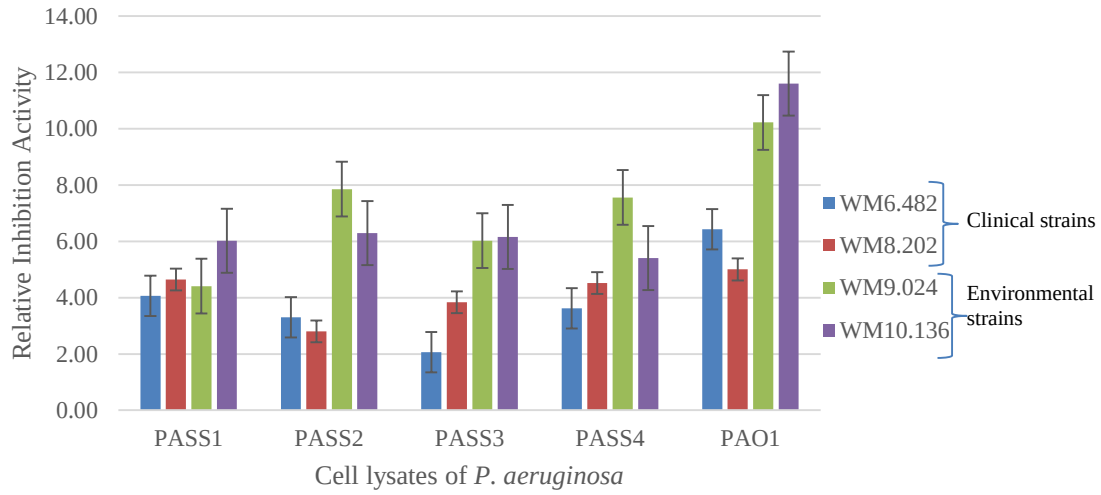

Figure 3.8: Relative inhibition activities (RIAs) of cell lysates (100µl) from the clinical and PAO1 reference strains of *P. aeruginosa* against *S. aurantiacum* on SCFM agarose plates. Relative inhibition index was calculated from the averaged values of three replicates as described in Material and Methods. Error bars represents the standard deviation among the technical replicates. Maximum standard deviation between measurements was 1.5% (n=3) (For analysis of the data refer to Supplementary Table 4).

After 96 hours of incubation, the resulting RIA values against *S. aurantiacum* WM06.482 were 4.06, 3.31, 2.06 and 3.62 for cell lysates of *P. aeruginosa* PASS1, PASS2 and PASS3 and PASS4, respectively (Figure 3.8). Figure 3.8 represents the inhibitory activity of cell lysate of *P. aeruginosa* clinical strain PASS1 against *S. aurantiacum* WM08.202 after 96 hours incubation at 37°C. *P. aeruginosa* strains PASS2 and PASS3 showed minimum inhibition on clinical strains of *S. aurantiacum*: WM08.202 and WM06.482, respectively. *P. aeruginosa* strains PASS2 and PASS4 showed the highest inhibition against the

S. aurantiacum high virulent environmental strain WM09.24 (Figure 3.8). Among all *P. aeruginosa* clinical strains, PASS1 showed highest inhibition of *S. aurantiacum* clinical strains, WM06.482 and WM08.202 (Figure 3.9) as shown in co-growth experiment. Surprisingly, cell lysate of *P. aeruginosa* reference strain PAO1 showed highest inhibition against all strains of *S. aurantiacum*.

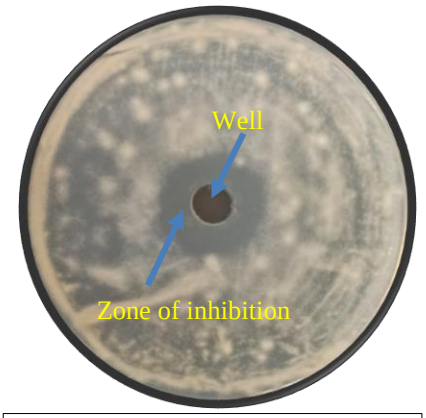

Figure 3. 9: Inhibitory activity of *P. aeruginosa* PASS1 cell lysate (100µl) against *S. aurantiacum* WM08.202 on an SCFM agarose plate using agar well diffusion method after 96 hours of incubation at 37°C.

Cell lysate of *P. aeruginosa* PASS4 revealed least inhibitory activity against *S. aurantiacum* clinical strains (WM06.482, RIA, 3.62 and WM08.202, RIA, 4.52). In contrast, *P. aeruginosa* strain PASS1 showed overall equal inhibitory effects on all *S. aurantiacum* strains, except for WM10.136. Compared to all the *P. aeruginosa* strains, the control strain PAO1 showed highest inhibition against *S. aurantiacum* strains WM10.136 (RIA 11.60) and WM09.24 (RIA 10.22), whereas least inhibition was shown against *S. aurantiacum* clinical strain WM08.202 (RIA, 5.00). Figure 3.8 shows lowest inhibitory activity of all strains of *P. aeruginosa* against clinical strains of *S. aurantiacum* and high inhibition against the environmental strains of *S. aurantiacum*. Again, the cell lysate of *P. aeruginosa* reference strain PAO1 showed highest inhibition against all strains of *S. aurantiacum*. In contrast to this, the same strain showed lowest inhibition against clinical and environmental strains of *S. aurantiacum*, during co-growth studies (Figure 3.8).

These results provided evidence for the inhibitory activity of *P. aeruginosa* cell lysate against *S. aurantiacum*. Therefore, it could be deduced that the cell lysates of *P. aeruginosa* strains may contain (small) molecules, which could possess antifungal properties. Since, *P. aeruginosa* also secretes certain virulence factors, which may have inhibitory activity against *S. aurantiacum* (Liu and Nizet, 2009), culture supernatants were tested against *S. aurantiacum* for their inhibitory activity.

In order to determine effects of the culture supernatants of *P. aeruginosa*, aliquots of 10 µl, 100µl and 200µl of culture supernatants from all *P. aeruginosa* strain were tested against all four strains of *S. aurantiacum* and RIA was established. Aliquots of 10 µl, 100 µl of culture supernatants did not show any inhibitory activity against *S. aurantiacum*, but aliquots of

200 µl of the culture supernatant did. Thus, data of inhibitory effects of 200µl aliquots are presented here as shown in figure 3.10.

When results of culture supernatant assays were recorded after 96 hours of incubation, *P. aeruginosa* reference strain PAO1 showed highest inhibition against all *S. aurantiacum* strains, except for WM08.202. Similarly to the cell lysate, PAO1 culture supernatant showed highest inhibition against *S. aurantiacum* WM10.136 and WM09.24 with RIA values of 9.89 and 8.15, respectively. Again it is seen that the environmental strains of *S. aurantiacum* are more susceptible to inhibition by *P. aeruginosa* whereas clinical strains of *S. aurantiacum* strains are less susceptible to inhibition, overall.

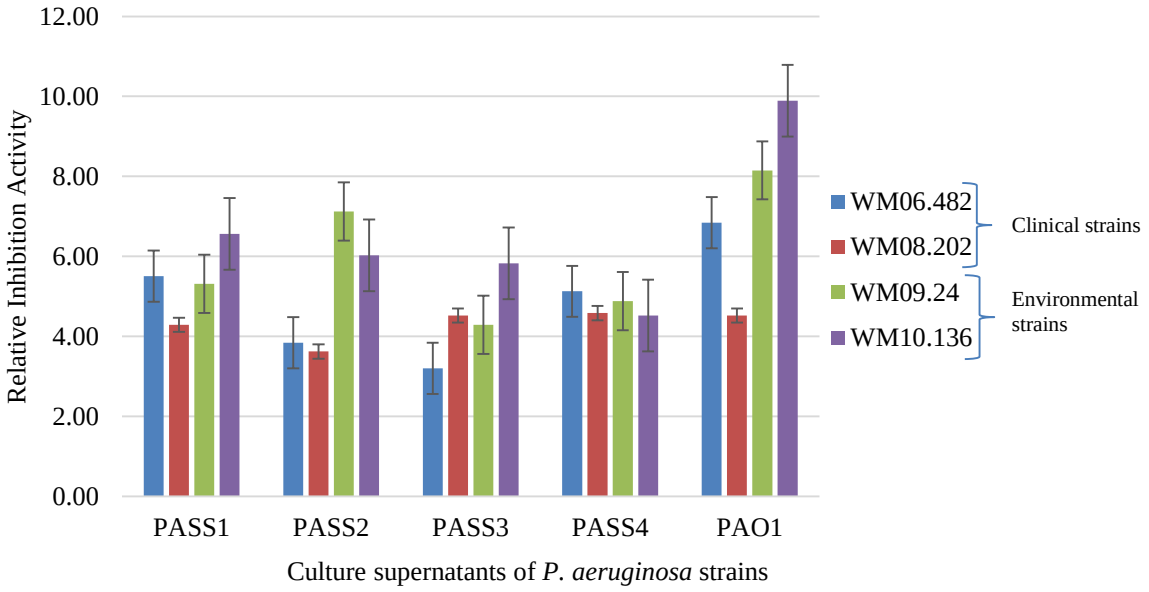

Figure 3.10: Relative inhibition activities (RIAs) of culture supernatants (200µl) from the clinical and control strains of *P. aeruginosa* against *S. aurantiacum* on SCFM agarose plates. Relative inhibition index was calculated from the averaged values of three replicates as described in Material and Methods. Error bars represents the standard deviation among the technical replicates. Maximum standard deviation between measurements was 2% (n=3) (For analysis of the data refer Supplementary Table 5).

Among all clinical strains of *P. aeruginosa*, PASS1 showed highest inhibition against all *S. aurantiacum* strains, except WM09.24. In case of WM09.24, PASS2 showed highest inhibition. Moreover, *P. aeruginosa* PASS1 and PASS4 showed similar average effects, against high virulent clinical strain of *S. aurantiacum* WM06.482. In addition, *P. aeruginosa* strains PASS2 and PASS3 showed relatively less inhibitory activity against clinical strains of *S. aurantiacum*, when compared to other *P. aeruginosa* strains.

These results also showed presence of the inhibitory activity of *P. aeruginosa* culture supernatants against *S. aurantiacum*. Thus, it can be assumed that the culture supernatants of *P. aeruginosa* contain compounds with antifungal properties. There was no physical contact between *P. aeruginosa* and *S. aurantiacum* during co-growth studies on SCFM agarose plates, even though *P. aeruginosa* showed inhibitory effects on the fungal growth. It however seems that during co-growth studies something happens that induces the formation of molecules with antifungal properties from *P. aeruginosa* strains (except PASS2) Therefore, it can be assumed that the interactions between *P. aeruginosa* and *S. aurantiacum* were mediated by intra and extracellular compounds.

Among all clinical strains of *P. aeruginosa*, PASS1 showed the highest inhibition effects against clinical strains of *S. aurantiacum* during both, co-growth studies and testing of cell lysate and culture supernatant. Therefore, *P. aeruginosa* PASS1 was selected for further studies along with PAO1 as a reference. As clinical strains of *S. aurantiacum* cause increased morbidity and mortality, they were used for further antifungal assays. In order to identify potential effects of small molecules, cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1 were subjected to solvent extraction.

3.2 Solvent extraction of molecules from *P. aeruginosa* (PASS1 and PAO1) cell lysates and culture supernatants

Possible consequence of the *P. aeruginosa* interactions with *S. aurantiacum* is the up regulation of virulence factors leading to increased pathogenicity in the host. Small organic molecules are one example of those virulence factors (Liu and Nizet, 2009). As *P. aeruginosa* cell lysates and culture supernatant showed inhibitory activity against *S. aurantiacum*, molecules from cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1 were extracted using solvents. Three different solvents, petroleum ether, ethyl acetate and 1-butanol were used in a sequential manner to achieve a wide range of molecules based on their polarities. The solvents were evaporated using a Rotavapor at 40°C and 200 millibar pressure and residual material was resuspended in 5 ml of same solvent to achieve 10× concentration of molecular fractions. Activity of 100µl aliquots of each fraction was tested against clinical strains of *S. aurantiacum* using the agar diffusion method and RIA was established. The assays of these molecular fractions revealed non inhibitory activity of molecules extracted within petroleum ether and ethyl acetate. In contrast, molecules fractions extracted in 1-butanol from both *P. aeruginosa* cell lysates and culture supernatants showed

inhibitory activity against clinical strains of *S. aurantiacum*. The results of the antifungal plate assay using 1-butanol molecular extracts are presented in Table 3.2.

Table 3.2: Antifungal activity of molecular fractions extracted in 1-butanol (10X) from *P. aeruginosa* PASS1 and PAO1 cell lysates and culture supernatants, shown as of zone of inhibition (ZOI) and relative inhibition activity (RIA) against clinical strains of *S. aurantiacum* using agar diffusion assays.
(Well diameter = 10 mm, n=3)

***S. aurantiacum* WM06.482**					
***P. aeruginosa* molecular fraction extracted in 1-butanol**	PASS1 cell lysate	PAO1 cell lysate	PASS1 culture supernatant	PAO1 culture supernatant	1-Butanol control
Mean zone of inhibition (in mm)	20.5	26.67	22.33	23.67	No inhibition
Relative inhibition activity	3.20	6.11	3.99	4.60	-
***S. aurantiacum* WM08.202**					
***P. aeruginosa* molecular fraction extracted in 1-butanol**	PASS1 cell lysate	PAO1 cell lysate	PASS1 culture supernatant	PAO1 culture supernatant	1-Butanol control
Mean zone of inhibition (in mm)	22.33	27.5	21.5	25.33	No inhibition
Relative inhibition activity	3.99	6.56	3.62	5.42	-

In case of 1-butanol control, small visible zone of inhibition was seen up to 3 day into incubation, but further incubation showed no zone of inhibition and revealed actively growing clinical strains of *S. aurantiacum*. From table 3.2 indicates that the molecules from cell lysates of *P. aeruginosa* reference strain PAO1 showed relatively high inhibitory activity against clinical strains of *S. aurantiacum*, compared to molecules from cell lysates of *P. aeruginosa* clinical strain PASS1 (Table 3.2). Moreover, all the 1-butanol extracted molecular fractions of *P. aeruginosa* showed high inhibition of *S. aurantiacum* WM08.202 in comparison to *S. aurantiacum* WM06.482. The 1-butanol molecular fraction from *P. aeruginosa* PAO1 cell lysate showed highest inhibition of both clinical strains of *S. aurantiacum*, indicating presence of potential small molecules that inhibit *S. aurantiacum* growth. Since, only the 1-butanol fractions showed inhibitory effect on *S. aurantiacum*, the 1-butanol fractions were used for further studies.

3.3 Determination of MIC of 1-butanol extracted molecular fractions from *P. aeruginosa*

Following extraction of molecules, 1-butanol fractions were assayed to determine a minimal inhibitory concentration (MIC) against the clinical strains of *S. aurantiacum.* As described in 2.7, MIC was performed using a growth medium dilution method. Serial dilutions of all of the 1-butanol fractions were made in the range of 10-100 µl/ml concentration and 3.3 µl of standard conidial suspensions of *S. aurantiacum* clinical strains were inoculated then incubated at 37°C for 6 days, and OD_{590} was used to measure fungal growth. For all MIC assays, medium inoculated with *S. aurantiacum* only and un-inoculated medium served as positive and negative control, respectively. The results obtained showed a slight increase in the OD_{590} in up to certain dilutions of the 1-butanol extracted molecular fractions as shown in Table 3.3. The OD_{590} of all positive controls increased with incubation time, whereas the OD_{590} of negative control and 1-butanol controls remained relatively constant during the incubation time, therefore no growth in negative control.

Table 3.3: Lowest dilution (µl/ml) of *P. aeruginosa* 1-butanol molecular fractions representing *in vitro* inhibition of *S. aurantiacum* growth using micro titre plate assay (n=3).

1-butanol fraction against *S. aurantiacum*	**PASS1 cell lysate**	**PAO1 cell lysate**	**PASS1 culture supernatant**	**PAO1 culture supernatant**
WM06.482	20	10	40	10
WM08.202	10	10	10	10

Results of the MIC assays showed that the low virulent clinical strain of *S. aurantiacum* required only 10 µl/ml of any of the 1-butanol molecular fractions from *P. aeruginosa*. On the other hand, 20 µl/ml and 40 µl/ml concentrations of 1-butanol extracted *P. aeruginosa* PASS1 cell lysate and culture supernatant, respectively, was required to inhibit the growth of *S. aurantiacum* WM06.482. In contrast, only 10µl/ml concentration of any of the 1-butanol extracted molecules of *P. aeruginosa* PAO1 (cell lysate or culture supernatant) was required to inhibit growth of *S. aurantiacum* WM06.482. Thus, it was assumed that the 1-butanol extracts of molecules from *P. aeruginosa* PAO1 have more potential to show inhibitory activity against *S. aurantiacum* or they are higher in concentration compared to 1-butanol extracts of molecules from *P. aeruginosa* PASS1.

Following the MIC assays, aliquots from all the dilutions were taken using a sterile wire loop and streaked on PDA plates to check viability of the fungal inoculum. Upon 96 hours of incubation, results were recoded. Results showed (normally) growing fungi from positive controls and from 1-butanol controls in case of both clinical strains of *S. aurantiacum*. In case of 1-butanol control OD_{590} remained almost unaffected indicating no fungal growth, even though fungi revived, when aliquots of from 1-butanol were streaked on PDA plates. So, it could be said that 1-butanol had a fungistatic effect on the clinical strains of *S. aurantiacum*. In contrast to this, negative controls showed no fungal growth on PDA plates. In case of *S. aurantiacum* WM08.202, no fungal growth was found from any serial dilution of any of the 1-butanol molecular fractions of *P. aeruginosa,* both strains. This means that the MIC of all of the 1-butanol molecular fractions of *P. aeruginosa*, was 10 µl/ml for *S. aurantiacum* WM08.202. On the other hand, absence of fungal growth from all serial dilutions of the 1-butanol molecular fractions of *P. aeruginosa* PAO1, representing MICs of 10 µl/ml and fungicidal effect against *S. aurantiacum* WM06.482 (refer to Supplementary Table 6). *S. aurantiacum* WM06.482 was only re-grown from the 10 µl/ml dilutions and 10-30 µl/ml dilutions of 1-butanol molecular extracts of the PASS1 cell lysate and culture supernatant molecular fractions, indicating MICs of 20 and 40 µl/ml, respectively. In addition, absence of fungal growth on PDA indicates the fungicidal activity of molecular fractions of *P. aeruginosa* PASS1. Thus, it is assumed that 1-butanol as such has fungistatic effect, whereas 1-butanol extracted molecular fractions has fungicidal effects against clinical isolates of *S. aurantiacum*.

The data obtained, showed that *S. aurantiacum* WM08.202, WM06.482 requires the higher concentration of a certain molecular fraction to show inhibition of growth. This supports the currently available data regarding the virulence status of these *S. aurantiacum* clinical strains. Further, the findings revealed higher MIC values of molecules extracted in 1-butanol from *P. aeruginosa* PAAS1 (both, cell lysate and culture supernatant) against *S. aurantiacum* WM06.482, when compared to *P. aeruginosa* PAO1. Moreover, in contrast to *P. aeruginosa* PASS1 molecular, molecules of *P. aeruginosa* PAO1 had high potential to inhibit *S. aurantiacum* WM06.482 indicating high virulence of *P. aeruginosa* PAO1 in comparison of PASS1.

Once the MIC was determined, spectroscopic analysis was carried out to achieve an idea of the presence of particular functional groups in the molecular fractions extracted in 1-butanol from *P. aeruginosa* PASS1 and PAO1.

3.4 Spectroscopic analysis of the *P. aeruginosa* molecular fractions

All of molecular fractions extracted in 1-butanol from *P. aeruginosa* PASS1 and PAO1 were analysed using UV-Visible spectrophotometer to check presence of particular functional groups within them. Here, 1-butanol served as blank.

The results from spectroscopic analysis (220-470nm) of the molecular fractions extracted in 1-butanol from *P. aeruginosa* cell lysate and culture supernatant are shown in Figure 3.11. Molecular fractions from the culture supernatant absorbed maximally in range of 275-355 nm wavelength, compared to cell lysate fractions that absorbed maximally in the range of 265-290 nm wavelength. There was no significance absorbance in the range of 470-1000 nm.

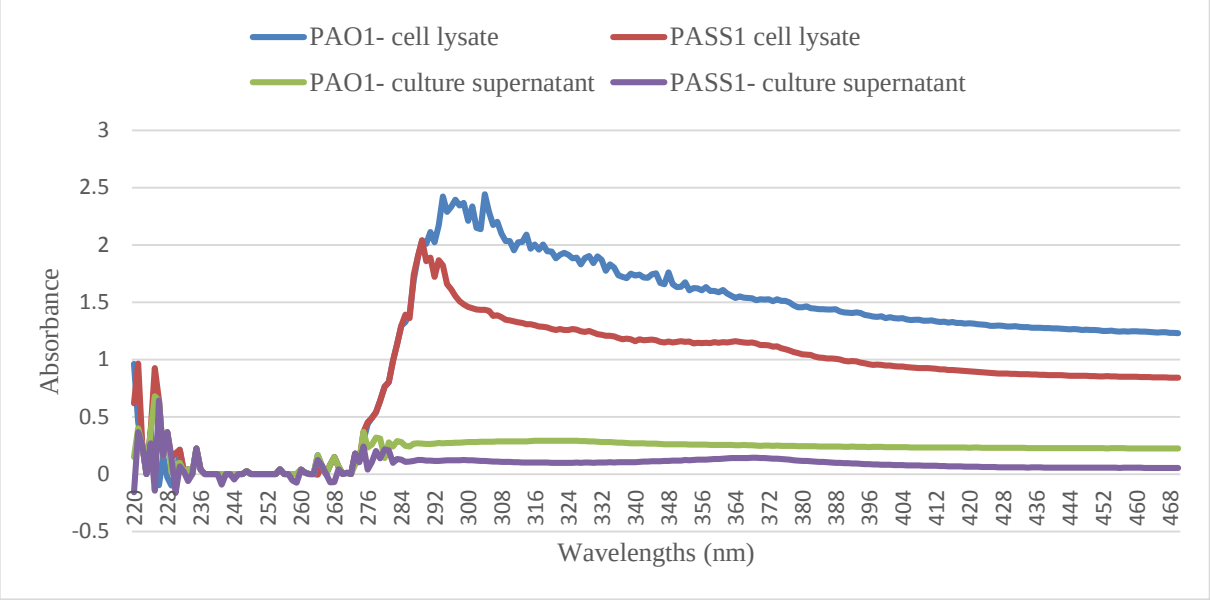

Figure 3.11: Spectroscopic analysis (range 220-470nm) of 1-butanol metabolic fraction of *P. aeruginosa* PASS1 and PAO1, cell lysate and culture supernatant.

When the overall absorbance of the molecular fraction extracted in 1-butanol from the *P. aeruginosa* PASS1 cell lysate was compared with PAO1 cell lysate, PAO1 molecular fraction showed relatively higher absorbance. Similarly, there was a difference in the absorbance pattern of PASS1 culture supernatant compared to PAO1 culture supernatant. Table 3.4 shows the wavelengths where the molecular fractions of *P. aeruginosa* cell lysate and culture supernatant had an increased absorbance.

Table 3.4: Wavelengths of UV-visible spectra (220-470nm), where the 1-butanol extracted molecular fraction of *P. aeruginosa* PASS1 and PAO1 absorbs higher compared to other wavelengths.

Molecular fraction	Wavelengths where fractions had higher absorbance
PASS1 cell lysate	221, 225, 228, 231, 288, 293 304
PAO1 cell lysate	220, 224, 227, 231, 288, 293, 300, 304, 345
PASS1 Culture supernatant	221, 224, 228, 235, 264, 275
PAO1 Culture supernatant	221, 225, 228, 235, 264, 275

From the comparative analysis of higher absorbance in the *P. aeruginosa* molecular fractions displayed in Figure 3.11 and Table 3.5, it was found that cell lysate and culture supernatant molecular fractions from both *P. aeruginosa* strains PASS1 and PAO1, had almost similar absorption spectra. PASS1 and PAO1 cell lysates absorbed optimally at 231nm, 288nm, 293nm and 304nm, whereas PASS1 and PAO1 culture supernatants absorbed optimally at 221nm, 228nm, 235nm, 264nm and 275nm (Table 3.4). *P. aeruginosa* PAO1 cell lysate molecular fraction also absorbed comparatively higher at 220nm, 300nm and 345nm, when compared to *P. aeruginosa* PASS1 cell lysate molecular fractions.

3.5 Fractionation of the *P. aeruginosa* molecular fractions

As the bacterial cell possess a variety of molecules, fractionation and separation of these molecules is necessary in order to determine their effects on *S. aurantiacum*. Therefore, to achieve possible separation of molecules present in all of the 1-butanol molecular fractions, fractionation of the extracted molecules was done using HPLC. In order to have a good separation of pyocyanin and 1-hydroxyphenazine (if present), two isocratic gradients of acetonitrile and isopropanol were used (Wilson *et. al.*1987). In addition, as shown in Table 3.4 that the majority of the 1-butanol molecular fractions from *P. aeruginosa* PASS1 and PAO1 absorbs optimally at 220nm, 275-280nm and 354nm, elution was monitored at 220nm, 280nm and 354nm in UV range.

First elution gradient using acetonitrile / water / trifluoroacetic acid (100:0:0.05 vol/vol/vol to 60:40:0.05vov/vol/vol in a liner gradient) elution was conducted for all 1-butanol extracted molecular fractions but none of them show separation of molecules. In addition, elution gradient using 0 to 40% isopropanol in aqueous acetic acid (2.5% vol/vol) showed partial separation of molecules from all of *P. aeruginosa* molecular fractions extracted in 1-butanol (Refer supplementary Table 7 and 8). The chromatogram of 1-butanol molecular fractions

from *P. aeruginosa* PASS1 and PAO1 cell lysates showed a similar elution pattern, but at certain point the area of peaks were different, which possibly represented the different concentration of the similar or similar type of molecules in the cell lysate fraction of *P. aeruginosa* PASS1 and PAO1. Similar pattern also was found in the chromatogram of 1-butanol molecules fractions form *P. aeruginosa* PASS1 and PAO1 culture supernatants which did not show good separation of the molecules. Therefore, on the basis of these primary studies of fractionation, further studies will be conducted in the future to strain pure compounds from all the 1-butanol molecular fractions from cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1.

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Chapter 4

Discussion

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Cystic fibrosis (CF) is the most common genetic disorder amongst the people of Caucasian background, leaving the affected vulnerable to chronic mixed microbial infections throughout their life. As stated in Chapter 1, the respiratory tract and lungs of the individuals with CF are frequently found to be infected by bacteria and fungi (Bakare *et al.*, 2003; Davies *et al.*, 2007). Inhibitory effects of *P. aeruginosa* against *S. aurantiacum* are represented in this study. It has been shown previously that co-infection of *P. aeruginosa* with fungi within the lungs of CF individuals involves *in vivo* interactions between two pathogens (Bandara *et al.*, 2013; Gibson *et al.*, 2009). The interactions of these microbes with each other may result in phenotypic changes in their growth and physiology amongst other changes. Therefore, improving the understanding of the nature of their interactions may help provide insight into improving CF therapies (Mowat *et al.*, 2010).

It has been previously shown that *P. aeruginosa* can inhibit the growth of the filamentous fungus *A. fumigatus* and the yeast *C. albicans*. Studies of interactions between the human opportunistic pathogens *P. aeruginosa* and *C. albicans* showed that they affected virulence traits in both organisms (Keçeli *et al.*, 2012). It has also been shown that the culture supernatant from four *P. aeruginosa* strains strongly inhibited biofilm formation of *C. albicans in vitro* (Holcombe *et al.*, 2010).

In the context of *P. aeruginosa* and its interactions with fungi in the lungs of CF individuals, inhibition of the growth of the fungus possibly occurs through the action of small secreted heat-stable molecules (i.e. pyocyanin, 1-hydroxyphenazine, decanol, decanoic acid and dodecanol) (Mowat *et al.*, 2010). This was indicated by showing that filtered culture supernatant from *P. aeruginosa* caused inhibitory effect on *A. fumigatus*, as live *P. aeruginosa* cells did. A study conducted by Ramage group (2010) demonstrated similar effects of live *P. aeruginosa* on live *A. fumigatus* upon co-growth in liquid medium, leading to significant reduction (14.5%) in the growth of *A. fumigatus*. Despite this, *P. aeruginosa* did not show inhibition against preformed biofilms of *A. fumigatus* (Mowat *et al.*, 2010).

With this in mind, the main aim of the current study was to explore the molecular basis of interaction between *P. aeruginosa* and *S. aurantiacum* in lung mimicking conditions. *P. aeruginosa* produces a vast range of virulence factors that include proteases, lipases and pigments such as pyocyanin and related organic molecules. Small molecules from *P. aeruginosa* were selected as a focus of this work. These small molecules are produced by the majority of *P. aeruginosa* strains and include pigments such as PYO, PCA and pyoverdine

(Liu and Nizet, 2009). These pigments are known to have potential pathogenic effects due to their ability to halt cell respiration and protein expressions of eukaryotic cells (i.e. fungi and host cells) (Kerr, 2000). Heterocyclic pigments produced by *P. aeruginosa* have been well studied, but numerous others remain to be explored as *P. aeruginosa* has great metabolic flexibility

4.1 Interactions between live cells of *P. aeruginosa* and *S. aurantiacum* on solid media: opposite end growth and growth inhibition

During early stages of the project, interaction between *P. aeruginosa* and *S. aurantiacum* was studied on the SCFM agarose plates. During all of these experiments, plates were prepared using 20 ml of SCFM agarose medium to avoid nutritional variation in the growth environment. The assays were performed as stated in section 2.4 of chapter 2. In this study, the standardised inoculum avoided significant variation in the amount of bacterial and fungal cells that were applied to agarose plates prior to studying growth and inhibition of the various *P. aeruginosa* and *S. aurantiacum* strains.

In this study, *P. aeruginosa* inhibited the growth of *S. aurantiacum* where they grew close, but were not in physical contact with each other. In most cases, the inhibition zone was seen at the edges of the colony where there were growing fungal mycelia, but did not infect interior of the colony. Moreover, during co-growth of these microbes, colour intensity of pigments of *P. aeruginosa* decreased with increasing incubation time. In comparison, when *P. aeruginosa* was cultivated alone, the colour intensity of *P. aeruginosa* pigments remained stable during the incubation period and diffused over the SCFM plate, turning it green. This indicated that it was possible that extracellular secretions of *P. aeruginosa* were involved in inhibition of the growth of fungus. The inhibition of *S. aurantiacum* growth by *P. aeruginosa* was similar to the results presented by Ramage group (2010) that when *P. aeruginosa* and *A. fumigatus* were co-grown, *P. aeruginosa* inhibited the growth of the fungus (Mowat *et al.*, 2010).

During co-growth of clinical isolate of *P. aeruginosa* PASS4 and *S. aurantiacum* WM06.482 (high virulent clinical isolate), the bacteria degraded half of the fungal colony within six days of incubation. On the other hand, the same *P. aeruginosa* isolate did not inhibit growth of *S. aurantiacum* WM08.202 (low virulent clinical isolate). Here, *S. aurantiacum* strains showed variation in terms of interaction with *P. aeruginosa*, which might result from different microbial physiology and possibly antimicrobial resistance. On the other hand, *P. aeruginosa*

PASS1 showed partial inhibition of *S. aurantiacum* WM06.48 growth (Figure 3. 1), while its co-growth with *S. aurantiacum* WM08.202 showed a stronger inhibitory effect.

In the case of *P. aeruginosa* PASS1/*S. aurantiacum* WM08.202 co-growth, bacteria covered the majority of the growth medium and there was only an intermediate sized fungal colony (Figure 3. 2). Here, the bacteria halted the expansion of fungal growth on the surface of the medium, resulting in to the upwards growth of *S. aurantiacum*. The upwards growth could be a survival strategy for fungi in mixed microbial environments.

The recent studies concerning co-growth of *P. aeruginosa* and *Scedosporium* species. For example, in the study conducted by Mayer group (2013), average 76% and 80% of the *P. aeruginosa* strains demonstrated inhibitory effects on *S. aurantiacum* and *S. prolificans,* respectively (Shilpa *et al.*, 2013). In this case, co-growth of methanol killed *P. aeruginosa* and *Scedosporium* strains in liquid culture revealed actively growing fungi. For *A. fumigatus, C. albicans, S. prolificans, S. aurantiacum* and other majority of the fungi, biofilm formation is one of the key aspect of their infection cycle (Ramage *et al.*, 2010;Ramage *et al.*, 2009). Therefore, the ability of *P. aeruginosa* to antagonize fungal growth has clear biomedical implication.

As stated in Chapter 1, *P. aeruginosa* pigments such as PYO and PCA hold potential to generate ROSs and halt cell respiration and could be predicted to cause inhibition of growth of *S. aurantiacum*. *P. aeruginosa* are also reported to secrete chitinase (Folders *et al.*, 2001) which degrade chitin of fungal cell walls. Chitin is major constituent of fungal cell wall providing shape and rigidity of fungal cells. Thus, it can weaken the fungal cell wall resulting in the cell damage. In addition, degraded chitin can help *P. aeruginosa* to survive in nutrition depleting conditions in SCFM agarose assay plates during prolonged incubation during its co-growth with fungi (Hibbing *et al.*, 2009). However, it may not be the same interaction during the infection in nutrient rich environment in the lungs of CF individuals. Thus, it could be possible that fungi can escape from the harsh secretions of phenazine, proteases and lipases from *P. aeruginosa* and might move to other ecological niche, such as in alveoli or within the airway/lung cavities and form fungal balls. As a result, they can be co-isolated from the sputum but may not co-exist in the same ecological niche.

Further, when *S. aurantiacum* environmental strains WM09.24 and WM10.136 were co-grown with all respective *P. aeruginosa* clinical strains, a similar degree of inhibition was shown on WM09.24 and WM08.202 growth (Supplementary Table 2) which however was

lower than clinical isolates WM06.482 and WM10.136. Substrate utilising patterns of these *S. aurantiacum* strains using biolog studies showed that low virulence environmental isolate of *S. aurantiacum* WM10.136 showed high respiration for majority of the substrates tested (Kaur *et al.* in preparation) . These substrates include various carbon source such as D-glucose, Stachyose, Sucrose, D-maltose, D-cellobiose, 3-O-Methyl-D-Glucose, D-Fucose, D-Salicin, D-Turanose, N-Acetyl-Neuraminic acid, N-Acetyl-b-D-Mannosamine and showed metabolic flexibility of environmental strains of *S. aurantiacum*. Previous research showed that *Scedosporium* spp. can use certain aromatic compounds such phenol, *p*-cresol, phenyl-benzoate, and toluene as a source of carbon (Claußen and Schmidt, 1998;Clauβen and Schmidt, 1999;García-Peña *et al.*, 2001). Thus, it wouldn't be surprising to assume that environmental strains of *S. aurantiacum* may utilise/tolerate molecules from *P. aeruginosa* up to certain concentrations, but when concentration of those unknown molecules exceeds in the growth environment, fungal growth is possibly inhibited.

4.1.1 **Interactions between *P. aeruginosa* and clinical strains of *S. aurantiacum* using centre inoculation method**

As the interactions between *P. aeruginosa* and *S. aurantiacum* are of clinical importance, another interaction assay was conducted to confirm antagonistic activity and determine relative inhibitory activity of *P. aeruginosa* clinical strains against clinical strains of *S. aurantiacum* as mentioned in section 2.4.1 of chapter 2.

In terms of inhibitory effects on *S. aurantiacum* WM06.482, the highest RIA value was shown by *P. aeruginosa* PASS1, whereas the lowest RIA value was shown by control *P. aeruginosa* strain, PAO1 (Figure 3.6). *P. aeruginosa* PASS2 and PAO1 were shown to have the highest and lowest inhibition against *S. aurantiacum* WM08.202. However, *P. aeruginosa* PASS1 showed about 2.5 times higher RIA against *S. aurantiacum* WM06.482 in comparison of WM08.202. It could be expected that *P. aeruginosa* would show higher degree of inhibition against low virulent strain of *S. aurantiacum* WM08.202 compared to high virulent strain of *S. aurantiacum* WM06.482. However, findings of this assay showed opposite results where high virulent strain was highly inhibited, whereas the inhibition of the low virulent strain was lower. Thus, the higher degree of inhibition against high virulent fungi could be as result of due the presence of fungi near *P. aeruginosa* growth. As stated in Chapter 1, presence of farnesol from *C. albicans* up regulates the phenazine pigment

producing genes in *P. aeruginosa*, resulting in to high pigment production and show higher inhibition against fungus.

4.1.2 Inhibition against *S. aurantiacum*: Live or dead cells of *P. aeruginosa*

In order to determine, if interactions were induced by live cells of *P. aeruginosa* or something else, *P. aeruginosa* cells were killed by a heat treatment. During the treatment aliquots were taken at each 5 minutes and streaked on LB agar plate to check the killing time. After 24 hours of incubation, plates showed growth of *P. aeruginosa* only in plates with aliquots taken up to 20 minutes, whereas no growth found in 25 and 30 minutes aliquots for all the *P. aeruginosa* strains. For bacterial killing, alcohol treatment is also used, but it can wash off the lipids present in the cell surface and increase porosity of the bacterial cell wall and release cellular content. Moreover, alcohols are known to have adverse effects on fungal morphogenesis, resulting impaired growth. Thus, if the alcohol treated bacterial samples are used it may lead to false positive reports, therefore, heat killing of bacterial cells was used for this study.

When suspension of heat killed cells of all of the *P. aeruginosa* strains were tested against all the *S. aurantiacum* strains, only PASS2 showed an inhibitory effects against the environmental isolate of *S. aurantiacum* WM10.136. *P. aeruginosa* PASS2 is the least virulent strain and has smallest genome among all clinical strains of *P. aeruginosa*. It also lacks the clusters of biofilm forming and pigment (pyocyanin and pyoverdine, both) forming genes (Penesyan *et al.*, 2013). This could partially explain why PASS2 has not shown strong inhibitory activity against *S. aurantiacum* strains during co-growth studies of live cells of bacteria and fungi. In this case, it could be possible that in place of deletion of biofilm and pigment forming gene clusters, PASS2 might have acquired new virulence factors as changes in the genome of *P. aeruginosa* are quite common in clinically strains. However, findings from this experiment suggested (bio-) chemical interaction between *P. aeruginosa* and *S. aurantiacum* which could be mediated by enzymes and small molecules. Therefore, cell lysates and culture supernatants of *P. aeruginosa* were used to test effects of intra- and extracellularly secreted small organic molecules, respectively, against *S. aurantiacum*.

4.1.3 Study of chemical interactions between *P. aeruginosa* and *S. aurantiacum*

4.1.3.1 Effects of *P. aeruginosa* cell lysates on *S. aurantiacum*

In order to determine interactions mediated by bio-chemicals, cell lysates of all strains of *P. aeruginosa* were tested against all strains of *S. aurantiacum* using the agar well diffusion method as mentioned in section 2.5 of chapter 2.

Cell lysates of *P. aeruginosa* strains showed highest inhibitory effects on environmental strains of *S. aurantiacum*, compared to clinical strains. In context to clinical strains of *S. aurantiacum*, 60% (3/5) of the *P. aeruginosa* strains showed lowest inhibition of *S. aurantiacum* WM06.482 and the remaining 40%, (2/5) showed lowest inhibition of *S. aurantiacum* WM08.202 (Figure 3.8). In terms of these clinical strains of *S. aurantiacum*, there was no significant inhibition pattern found. Surprisingly, *P. aeruginosa* PASS2 showed second highest inhibition against *S. aurantiacum* WM09.24 and WM10.136. Among all clinical strains of *P. aeruginosa*, PASS1 showed highest degree of inhibition against clinical strains of *S. aurantiacum* WM10.136. Therefore, it could be the case that *P. aeruginosa* PASS1 possesses potential intracellular biomolecules with fungal antifungal activity. This study confirmed inhibitory activity of *P. aeruginosa* cell lysates on *S. aurantiacum*, which could be mediated by proteases and small organic molecules.

4.1.3.2 Effects of *P. aeruginosa* culture supernatants on *S. aurantiacum*

P. aeruginosa is reported to secrete a wide range of secondary molecules, which are reported to have potential inhibitory effects on several bacteria and fungi. Recently, a study of interactions between *P. aeruginosa* and *C. albicans* showed the increased virulence of *P. aeruginosa* when it was co-grown with *C. albicans*. It has been emphasised that inhibitory activity of *P. aeruginosa* against *C. albicans*, was especially due to its pigment which is secreted in extracellular space (Keçeli *et al.*, 2012). Kerr *et al*., (1999) showed inhibitory effect of *P. aeruginosa* against *C. albicans* and *A. fumigatus*. These antagonistic interactions were also thought to be mediated by organic molecules such as pyocyanin and 1-hydroxy phenazines, which are secreted in to extracellular space (Kerr *et al.*, 1999).

So, the logic is that the culture supernatants of *P. aeruginosa* contain several small molecules which may have potential to inhibit fungi. Thus, the culture supernatants of all *P. aeruginosa* strains were tested against all strains of *S. aurantiacum*. Similarly to the previous experiment using cell lysates of *P. aeruginosa*, culture supernatant of *P. aeruginosa*

also showed highest inhibitory effects against environmental strains of *S. aurantiacum*. Among all clinical strains of *P. aeruginosa*, culture supernatant from PASS1 showed the highest inhibition of *S. aurantiacum* WM06.482 and WM10.136, whereas culture supernatant from PASS3 showed least inhibition against *S. aurantiacum* WM06.482 and WM09.24 (Figure .10). In addition, culture supernatants from *P. aeruginosa* PASS4 showed similar average inhibitory activity against all *S. aurantiacum* strains. This study showed inhibitory effects of *P. aeruginosa* culture supernatants against *S. aurantiacum* and indicated the presence of virulence factors with antifungal properties in *P. aeruginosa* culture supernatants.

As stated earlier, Mowat *et al.*, (2010) suggested that small diffusible extracellular molecules of *P. aeruginosa* (i.e. PCA, PYO, decanol, decanoic acid and dodecanol) influence conidial germination and biofilm formation of *A. fumigatus*. In addition, another study using *Cryptococcus spp*. (encapsulated yeast) showed that *P. aeruginosa* inhibited the growth of *C. neoformans* by producing antifungal molecules such as pyocyanin and 2-heptyl-3,4-dihydroxyquinoline (a PQS molecule) (Rella *et al.*, 2012).

To summarise, it can be concluded that cell lysates and culture supernatant of *P. aeruginosa* had inhibitory activity against *S. aurantiacum*. Thus, the inhibitory effects of live cells of *P. aeruginosa* appears to be due to cell content and extracellular secretions (cell lysate and culture supernatant.

When effects of *P. aeruginosa* cell lysate and culture supernatants were compared with co-growth of live *P. aeruginosa* and *S. aurantiacum* using centre inoculation method, patterns of growth inhibition of clinical strains of *S. aurantiacum* were found to be similar. Both of these above mentioned experiments showed the highest inhibition of clinically high virulent isolate of *S. aurantiacum* WM06.482, when compared to clinically low virulent isolate of *S. aurantiacum* WM08.202. From this similar pattern observed, it can be stated that *P. aeruginosa* extracellular molecules contribute to increased inhibition of the growth of *S. aurantiacum* WM06.482.

However, when interactions between live cells of *P. aeruginosa* and *S. aurantiacum* (as discussed in 4.1.1) showed higher inhibitory activity compare to inhibitory effects showed by *P. aeruginosa* cell lysates and culture supernatants. In this case, it may be possible that the specific growth phase dependent molecules mediate fungal growth inhibition. Future experiments will be designed to harvest bacterial cultures over several time points and will be tested against the fungus to check the presence/absence of growth phase dependent

molecules/defensive compounds. In this case, it also could be possible that when live cell interactions were studied, *P. aeruginosa* and *S. aurantiacum* were growing on the same SCFM agarose plate, where presence of fungus within the same growth environment may act as inducer to up regulate virulence factors of *P. aeruginosa*. In contrast to this, *P. aeruginosa* was grown alone in liquid culture to harvest cell (to prepare cell lysate) and culture supernatant showing absence of fungus. This indicates the increased inhibitory activity of *P. aeruginosa* in presence of the fungus, which was showed as high inhibition of the growth of *S. aurantiacum.* In opposite situation, *P. aeruginosa* reference strain PAO1 showed least inhibitory activity against all *S. aurantiacum* isolates during co-growth studies. In this case, possible nutritional stress bacteria may have altered the production of bacterial secondary metabolites which may have shown inhibition of fungal growth. On other hand, *P. aeruginosa* cell lysate and culture supernatants showed inhibition of fungal growth. It showed the factor causing fungal growth inhibition were secreted when *P. aeruginosa* has not grown in nutritional stress. It can be also suspected that their production was supressed in presence of fungus in the growth medium. It seems unreasonable, but that may happen. *P. aeruginosa* PAO1 showed comparative less inhibition of *S. aurantiacum* in the growth assays, but extracts such as lysates and supernatant from this strain were most inhibitory. Thus, it may be predicted that the inhibitory effects may be due to specific growth phase dependent molecules. These molecules may have been suppressed or may not been produced during co-growth studies. This hypothesis can be validated during future studies.

However, it logical that to survive in the competitive environment, *P. aeruginosa* cell population increases to overcome fungal growth. In stressed or competitive growth environments such as co-growth studies and mixed infections, *P. aeruginosa* are highly interactive and showing higher rate of cell-to-cell communication in order to control bacterial population-dependent gene expression (Das and Manefield, 2012). *P. aeruginosa* QS provides improved access to nutrients and specific environmental niches, promotes collective defence, and facilitates survival through differentiation into certain morphological forms (i.e. biofilms) and over expression of the virulence factors to combat environmental threats (Hall-Stoodley *et al.*, 2004). *P. aeruginosa* QS (PQS) molecules such as C_4-HSL and 3-oxo-C_{12}-HSL and *N*-acyl-homoserine lactone (AHL) regulates the production of certain virulence determinants including elastase, rhamnolipids, and pyocyanin (Mavrodi *et al.*, 2001).

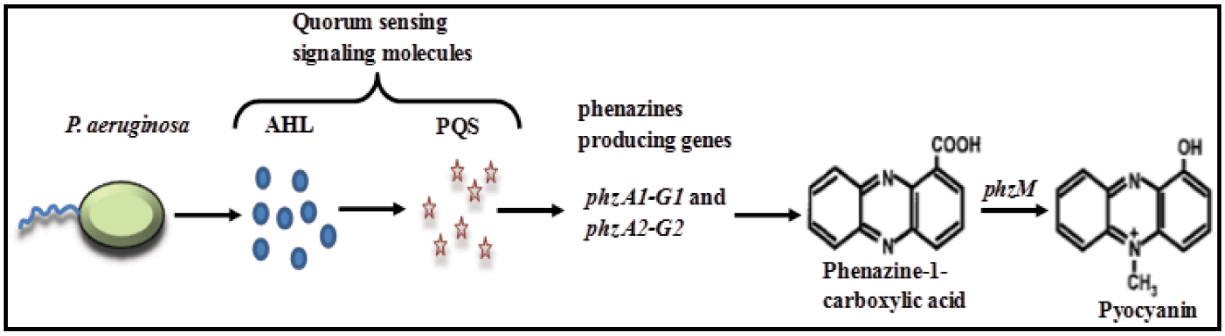


Figure 4.1: Schematic representation of quorum sensing regulated expression of genes encoding phenazine-1-carboxylic acid (PCA) and pyocyanin production in *P. aeruginosa* (Das and Manefield, 2012).

The schematic representation of pyocyanin biosynthetic pathway is presented in Figure 4.1. It shows that presence of the AHL and PQS of *P. aeruginosa* in growth medium results in expression of phenazine producing genes, such as *phz* A1-G1 and *phz* A2-G2. These genes in turn produces phenazine-1-carboxylic acid (PCA) and the expression of *phz*M results in production of pyocyanin from phenazine-1-carboxylic acid (PCA) (Figure 4.1) (Mavrodi *et al.*, 2001).

Therefore, it can be assumed that presence of fungus in growth medium during co-growth studies accelerates QS which increases AHL and PQS levels. This increase in AHL and PQS levels may increase production of PCA and PYO, which could result in higher inhibition of *S. aurantiacum*. Further studies could be conducted to determine the altered level of AHL and PQS and their effects on fungal growth inhibition. This interaction can be important to *P. aeruginosa* to survive and to get better access to nutrients in nutrient limiting conditions in SCFM agarose plates.

From the sets of studies discussed above, it was clear that the antagonistic interactions between *P. aeruginosa* and *S. aurantiacum* were chemical interactions mediated by intra and extracellular molecules from *P. aeruginosa*. In all above mentioned assays to study interactions of *P. aeruginosa,* PASS1 showed highest inhibition activity against clinical strains of *S. aurantiacum,* among all *P. aeruginosa* strains. Therefore, *P. aeruginosa* PASS1 was selected for further studies against PAO1 as reference strain.

4.2 Solvent extraction of molecules from cell lysates and culture supernatants of *P. aeruginosa*

In order to achieve extraction of extracellularly secreted organic molecules solvent extraction method was used. A bacterial cell possesses wide range of organic molecules. Therefore,

there is no universal protocol for extraction of the molecules from bacteria. In terms of isolation of pyocyanin and 1-hydroxyphenazine from the culture supernatant of *P. aeruginosa*, chloroform extraction protocols have been established and well-studied (Wilson *et al.*, 1987). In addition, several research studies presented methods to purify pyoverdine using numerous chromatographic techniques. However, to extract wide range of molecules along with pyocyanin, 1-hydroxyphenazine and pyoverdine, three different solvents were used to extract broader range of organic molecules based on their polarities. To extract organic molecules on the basis of increasing polarities, solvents were used in ascending order of values of their relative polarity (RP) in series of petroleum ether (C_6H_{14}, RP-0.117), ethyl acetate ($C_4H_8O_2$, RP-0.228) and 1-butanol ($C_4H_{10}O$, RP-0.586).

As Wilson *et.al,* (1987) used chloroform to isolate pyocyanin and 1-hydroxyphenazine, in this study chloroform was replaced with ethyl acetate. There is comparatively smaller difference in the relative polarities of these solvents. Thus, pyocyanin and molecules with similar polarity could be extracted using ethyl acetate. Majority of the *P. aeruginosa* pigments are water soluble. Therefore, if they are not extracted within ethyl acetate, these pigments are more likely to be extracted in 1-butanol as 1-butanol has high polarity compared to ethyl acetate. In addition, optimum extraction of molecules was acquired using 3 times extraction with each solvent. All the fractions of same solvents for each solvent were mixed and evaporated using rotavapor at 40°C and 200 millibar pressure and resuspended in appropriate volume of the same solvent to achieve 10× concentration of molecules in the fractions. As *P. aeruginosa* cell lysates and culture supernatants showed inhibitory activity against *S. aurantiacum* (as discussed in 4.2.1. and 4.2.2), small molecules were extracted from the fifty millilitres of cell lysate and culture supernatant from *P. aeruginosa* PASS1 and PAO1 (four samples) resulting in 12 fractions. All of these fractions prepared as mentioned in section 2.6 of chapter 2.

To determine the activity of the extracted molecules, 100µl aliquots of all the molecules fractions were tested against clinical strains of *S. aurantiacum* using agar well diffusion method to test activity of extracted molecules. Petroleum ether and ethyl acetate controls did not show inhibitory effects against clinical strains of *S. aurantiacum*. However, 1-butanol control showed inhibitory activity as zone of inhibition around well for initial 3 days of incubation at 37°C. Surprisingly, after 5 days of incubation at 37°C, fungi was found to be growing around well, which was inoculated with 100 µl of 1-butanol. The presence of 1-butanol in the well shows inhibition in fungal growth, but once 1-butanol is gradually evaporated, the fungus starts growing which indicates the fungi static effect of 1-butnaol on

S. aurantiacum. Further from all the fractions, only the molecules extracted in 1-butanol showed inhibitory activity against *S. aurantiacum* after 6 days of incubation at 37°C. This result indicates higher inhibition activity of molecular fraction from culture supernatants of *P. aeruginosa* compared to molecular fractions from cell lysates. In addition, reference strain of *P. aeruginosa* PAO1 showed higher inhibition of clinical strains of the *S. aurantiacum*, when compared with clinical isolate of *P. aeruginosa* PAO1. These findings were identical to the previously discussed interaction assays where among all strains of *P. aeruginosa*, PAO1 showed highest inhibition activity against all *S. aurantiacum* strains. In addition, this experiment showed higher degree of inhibition in *S. aurantiacum* WM08.202 (clinical low virulent isolate) compared to WM06.482 (clinical high virulent isolate). As stated earlier that *P. aeruginosa* pigments are more likely to be extracted within 1-butanol fraction. The pigments such as PYO, PCA and 1-hydroxyphenazinnes could have been present within molecules fractions extracted with 1-butnaol from cell lysate and culture supernatant of *P. aeruginosa* PASS1 and PAO1. Possibly, they might have shown inhibitory activity against *S. aurantiacum*. Once the activity of molecular fractions was confirmed, the MIC of the molecules fractions was determined. It was to standardise amount of fraction required to inhibit fungal growth on plates which could be used to determine activity of purified or characterised metabolites in future experiments.

4.3 Determination of MIC of 1-butanol extracted molecular fractions from *P. aeruginosa*

A 96 well plate assay conducted by Kerr *et al.,* (1999) used pyocyanin and 1-hydroxyphenzine to the Brain heart infusion agar containing the fungal indicator stains and found that pyocyanin caused inhibition of both, *A. fumigatus* and *C. albicans*. However, whilst dilution based assays of pyocyanin revealed that >19µg/ml and 35 µg/ml were the minimal concentration of pyocyanin to show *in vitro* inhibition of *A. fumigatus* and *C. albicans*, respectively (Kerr *et al.*, 1999). Similarly, MIC well plate assay was conducted using 1-butanol extracted molecular fraction from cell lysate and culture supernatant of *P. aeruginosa* strains with SCFM medium in microtitre plate. This assay showed inhibition of both of the clinical isolate of *S. aurantiacum* from these molecular fraction. All molecular fraction (cell lysate and culture supernatant) from *P. aeruginosa* PAO1 showed MIC of 10µl/ml against both of the clinical strains of *S. aurantiacum*. On other hand, 1-butnaol extracted molecular fraction from *P. aeruginosa* showed MIC of 10µl/ml against *S. aurantiacum* WM08.202. It showed that that the concentration extracted molecule within 1-butanol fractions from cell lysates and culture supernatant of *P. aeruginosa* PAO1 was higher compared to *P. aeruginosa* PASS1. In contrast, molecular fraction of cell lysate and

culture supernatant of *P. aeruginosa* PASS1 showed MICs of 20 and 40µl/ml against *S. aurantiacum* WM06.482.

In order to determine mode of action of these molecular fraction, fungi were regrown on PDA agar to check whether fungi was only inhibited or if the cultures were killed. If molecular fraction only inhibited the growth, fungi will revive on the medium, and if killed, it will not revive on the medium.

When the fungi were grown from all of these assay wells on PDA, both clinical strains of *S. aurantiacum* were grown from the 1-butanol controls confirming only growth inhibition (fungistatic effect) of 1-butanol on clinical strains of *S. aurantiacum*. After five days of incubation at 37°C, fungi were found to be growing from the 1-butnaol controls. Moreover, *S. aurantiacum* WM06.482 was only revived from 10µl/ml and 10-30 µl/ml dilutions of molecular fraction extracted within 1-butanol from the cell lysate and culture supernatant of *P. aeruginosa* PASS1, respectively. On the other hand, fungal inoculum from the rest of the wells did not show fungal growth on PDA plate which indicated dead fungal conidia as a result of fungicidal effect of all of these molecules fractions.

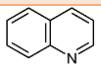
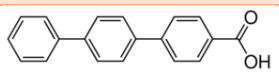
To summarise, it can be assumed that 1-butanol has fungi static effects, but molecules from cell lysates and culture supernatants imparted fungicidal effects to the fractions. Therefore, in order to purify and characterize the molecules within this fractions, spectroscopic analysis and HPLC were used.

4.4 Spectroscopic analysis of the 1-butanol extracted molecules fractions of *P. aeruginosa*

Spectroscopic analysis was used to identify the probable maximum absorbance of these 1-butanol extracted molecular fraction which can help to determine possible functional groups within them. Generally, the spectroscopic analysis of the crude fractions does not provide structural information but certainly provides information of their absorbance maxima and probable functional groups present in it. In this study, these 1-butanol extracted molecular fraction were analysed within the range of 220-1000nm wavelengths. The findings from these experiment showed maximum absorbance in range of 220-470nm indicating complex absorbance pattern in range of 220-350nm. From these range, certain wavelengths showed maximum absorbance such as 220, 228, 275, 288, 304 and 345 (Table 4.1). On the basis of

these absorbance maxima, Pyrrole, Qunoline, Thiophene, p-terphenyl and Naptheyl groups were suspected to be present in these molecules fractions (Table 4.1).

Table 4.1: Some of the predicted cyclic and heterocyclic compounds from the absorption spectra where these compounds also absorb maximally at the given wavelengths, their structure and λmax in alcoholic solvent are shown.

Name	Structure	λmax
Pyrrole		217-220nm
Quinoline		227nm
Thiophene		231nm
p-terphenyl		275 nm
Naphthyl group/ napthelene		315nm

Presence of pyrrole and quinolone derivatives could be predicted in all of the 1-butanol extracted molecular fractions of *P. aeruginosa (Van Pee et al., 1983).* Quinolone derivatives could be the *P. aeruginosa* cell-to-cell communication signals. 1-butanol molecular fraction of *P. aeruginosa* cell lysate could have thiophene and related derivatives or several other heterocyclic compounds absorbing maximally at 231nm. On the other hand, *P. aeruginosa* culture supernatant may contain P-terphenyl or its derivative or any other heterocyclic compounds absorbing maximally at 275nm. Several of these compounds could be intermediate compounds of biosynthetic pathways to produce pigments and other secondary organic molecules, which may have inhibitory activity against clinical strains of *S. aurantiacum*. Upon prediction of the possible functional groups in the crude 1-butanol extracted molecule fractions, it is necessary to fractionate those fractions using chromatographic techniques to reduce complexity of mixture of molecules.

As research suggested that *P. aeruginosa* QS system uses quinoline signals and several *Pseudomonas* spp. are reported to produce five brominated amino- and three brominated nitrophenyl pyrrole (Van Pee *et al.*, 1983), possibilities of quinoline and pyrrole in molecules extracts seem reasonable. On the other hand, thiophene and p-terphenyl are not reported to be produced by any *P. aeruginosa* strains but they are reported to have antibacterial activity (Tian *et al.*, 2013). Last, *P. aeruginosa* is not reported to produce Naphthyl derivatives, but

substitution of some functional groups and the any of the ring structure of phenazines can mimic as Naphthyl derivative and show similar absorbance pattern. Results obtained indicated that these molecules fractions absorb maximally at 220, 275, 288 and 354 nm of wavelengths. Thus, HPLC experiments was be monitored at 220, 280 and 354nm of wavelengths.

4.5 Fractionation of the 1-butanol extracted molecules fractions of *P. aeruginosa*

Once the possible functional groups and absorbance maxima were defined, the molecular fraction were further fractionated using HPLC to achieve separation of molecules. As the molecular fractions were thought to be a complex mixture of numerous molecules, it was not easy task to separate them using any reported HPLC protocol. Wilson *et al.*, (1987) reported elution of pyocyanin and 1-hydroxyphenazine using isocratic elution gradients using acetonitrile/water/TFA and isopropanol with 5% acetic acid to isolate in C-18 resin. Therefore, all of above mentioned molecular fractions were used for HPLC analysis to achieve separation using elution gradients mentioned by Wilson *et al.*, (1987). It should be noted that to avoid harsh acid treatment to the crude mixture of molecules in dilution gradient, acetic acid concentration was reduced up to 2.5% while using elution gradient of iso-propanol. Moreover, the elution of molecules from all the molecular fraction using acetonitrile/water/TFA did not show any separation. Elution gradient using isopropanol with 2.5% acetic acid showed partial separation. Chromatograms are shown in supplementary Tables 7 and 8 for the 1-butanol fractions of the *P. aeruginosa* culture supernatants and cell lysates, respectively.

However, any elution gradient did not show good separation of the molecules from any molecular fraction. Therefore, optimisation of the isocratic elution gradient using isopropanol was needed or possibly another elution gradient could be required. However, due to short time span of the project, optimisation of elution gradient was not became possible. Therefore, further studies will optimise the elution gradient to achieve separation of molecules followed by identification and characterisation using LC-MS and NMR.

Chapter 5

Summary

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In conclusion, the main findings of this investigation were as follows:

1. *P. aeruginosa* showed inhibitory activity against *S. aurantiacum*. The reference strain *P. aeruginosa*, PAO1 showed higher inhibition against all *S. aurantiacum* strains when compared with clinical strains of *P. aeruginosa* which indicated comparative higher virulence of *P. aeruginosa* PAO1 against *S. aurantiacum*.

2. Antagonistic interactions between *P. aeruginosa* and *S. aurantiacum* were mediated by intracellular and extracellular molecules from *P. aeruginosa*.

3. Relative inhibitory activity of *P. aeruginosa* was increased o induced by physical proximity of fungal growth in the proximity of *S. aurantiacum* supporting increased cell-to-cell communication.

4. Inhibitory activity of molecules extracted within 1-butanol from the cell lysate and culture supernatant confirmed inhibitory activity of intra and extracellular metabolites from *P. aeruginosa* PASS1 and PAO1.

5. A microtitre plate assay to determine MIC of the extracted molecules showed fungicidal effect of molecules extracted in 1-butanol from cell lysate and culture supernatant of *P. aeruginosa* PASS1 and PAO1.

6. Spectroscopic studies revealed higher concentrations of molecules extracted from cell lysates and culture supernatants of *P. aeruginosa* PAO1 compared to PASS1, which was assumed to show higher inhibition against *S. aurantiacum* throughout the presented study.

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Supplementary data

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Supplementary Table 1: Preparation of synthetic cystic fibrosis medium (987ml).

Step1: Prepare following solutions

Preparation of Buffer Base

Sr. No.	Component	Molecular weight	Amount (g)	Water (ml)	Enough for (litres)
1	0.2M NaH2 PO4	137.99	2.75	100	15.38461538
2	0.2M Na2 HPO4	141.96	2.82	100	16
3	1M KNO3	101.1	0.505	5	14.36781609
4	1M NaOH	40	2	50	50
5	0.5M NaOH	40	1	50	50
6	0.2M NaOH	40	0.08	10	10
7	1M HCl	-	4.79 HCl	45.2	50

Preparation of amino acid stocks

Sr. No	Component	Molecular weight	Amount (g)	Water (ml)	Enough for (liters)	Comment
1	100mM L-Aspartic acid	133.1	0.399	**30** 0.5MNaoH	3.60	
2	100mM L-Threonine	119.12	0.476	40	3.73	
3	100mM L-Serine	105.09	0.525	50	3.46	
4	100mM L-Glutamic acid hydrochloride	183.59	0.917	50 1M HCl	3.20	
5	100mM L-Proline	115.13	0.575	50	3.01	
6	100mM Glycine	75.07	0.3	40	3.33	
7	100mM L-Alanine	89.09	0.445	50	2.81	
8	100mM L-Cystine	240.3	0.192	8	5.00	0.6ml 1M NaoH
9	100mM L-Valine	117.15	0.468	40	3.58	
10	100mM L-Methionine	149.21	0.298	20	3.16	
11	100mM L-Isoleucine	131.17	0.524	40	3.57	
12	100mM L-Leucine	131.17	0.655	50	3.11	
13	100mM L-Tyrosine	181.19	0.543	**30** 1MNaoH	3.70	
14	100mM L-Phenylalanine	165.19	0.33	20	3.77	
15	L-Ornithine monohydrochloride	168.62	0.505	30	4.44	
16	100mM L-Lysine	146.19	0.73	50	2.35	
17	100mM L-Histidine monohydrochloride monohydrate	209.63	0.419	20	3.85	
18	100mM L-Tryptophan	204.23	0.02	**1** 0.2MNaoH	7.60	
19	100mM L-Arginine	174.2	0.174	10	3.27	

Preparation of salt stock solutions

Sr. No.	Component (Sterilize after preparation)	Molecular weight	Amount (g)	Water (ml)	Enough for (liters)
1	1M CaCl 2	110.98	1.109	10	5.70
2	1M MgCl 2	95.211	0.285	3	4.95
3	3.6 mM FeSO 4 7H 2 O	278.01	0.004	4	4
	OPTIONAL				
1	1M D-Glucose	180.16	1.801	10	3.33
2	1M L-Lactate	90.08	2.7	30	3.23

Step2: Mix following components to make final buffer solution (total volume: 792.69ml)

Sr. No.	Component	Volume to add for 987 ml in ml	Amount to added (g)	Volume to add for 1974 ml in ml
1	Autoclaved distilled water	779.6	None	1559.2
2	0.2M NaH2 PO4 solution	6.5	None	13
3	0.2M Na2 HPO4 solution	6.25	None	12.5
4	1M KNO3 solution	0.348	None	0.696
5	NH4 Cl	None	0.122	0.244
6	KCl	None	1.114	2.228
7	NaCl	None	3.03	6.06
8	10 mM MOPS	None	1.631	3.262

Step3: Add following volume of 100mM amino acid solution to buffer solution (total volume: 984.2 ml)

Sr. No	Component	Volume to add for 987 ml in ml	Volume to add for 1974 ml in ml
1	100mM L-Aspartic acid	8.27	16.54
2	100mM L-Threonine	10.72	21.44
3	100mM L-Serine	14.46	28.92
4	100mM L-Glutamic acid hydrochloride	15.49	30.98
5	100mM L-Proline	16.61	33.22
6	100mM Glycine	12.03	24.06
7	100mM L-Alanine	17.8	35.6
8	100mM L-Cystine	1.6	3.2
9	100mM L-Valine	11.17	22.34
10	100mM L-Methionine	6.33	12.66
11	100mM L-Isoleucine	11.2	22.4
12	100mM L-Leucine	16.09	32.18
13	100mM L-Tyrosine	8.02	16.04
14	100mM L-Phenylalanine	5.3	10.6
15	L-Ornithine monohydrochloride	6.76	13.52
16	100mM L-Lysine	21.28	42.56
17	100mM L-Histidine monohydrochloride monohydrate	5.19	10.38
18	100mM L-Tryptophan	0.13	0.26
19	100mM L-Arginine	3.06	6.12
	Total volume (ml)	**191.51**	383.02

Step 4 Adjust the pH to 6.8

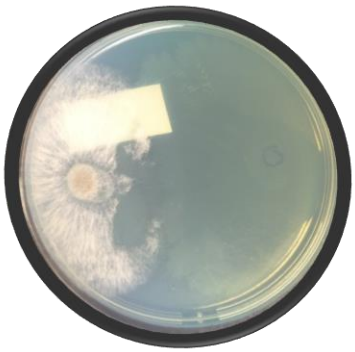
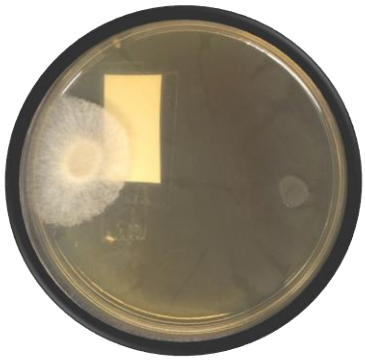
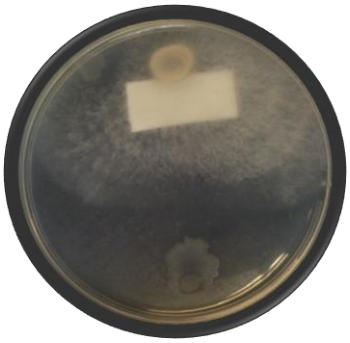
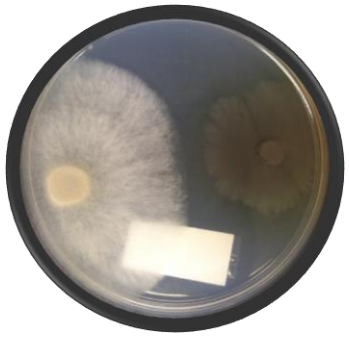
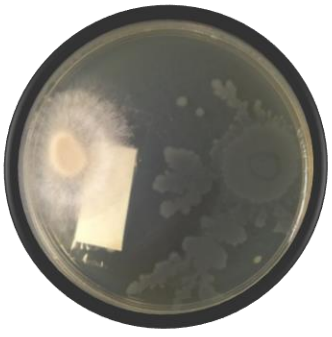
Step 5 Sterilize the medium using 0.22 filter.

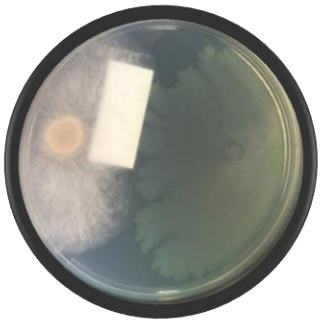
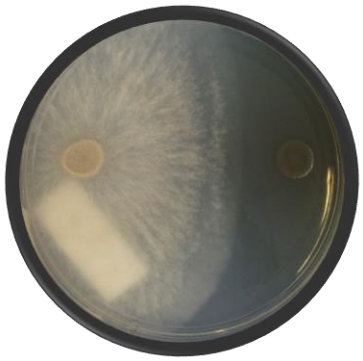
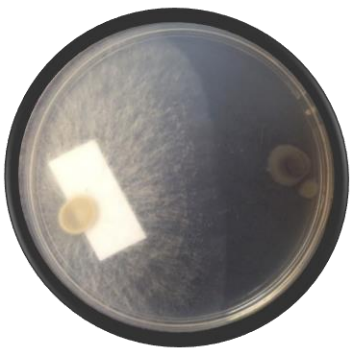
Step 6 Add salts solutions to the medium to make final SCFM (total VOLUME: 987.56ml)

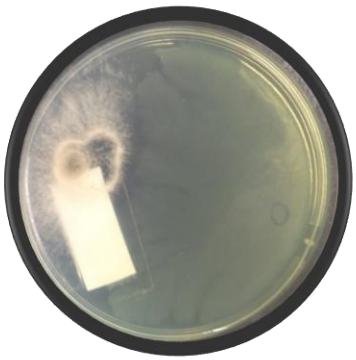
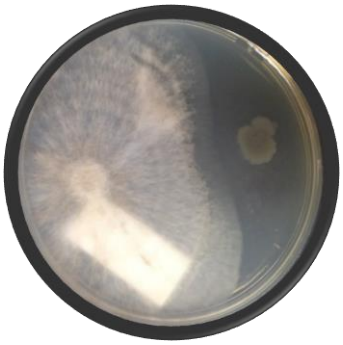
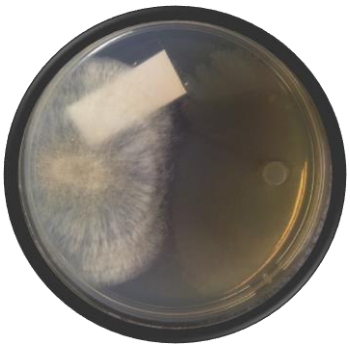
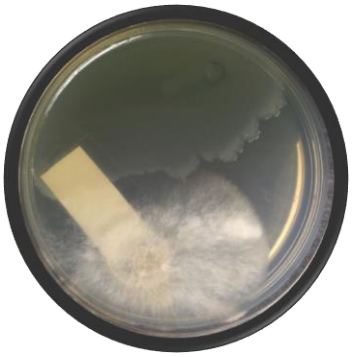
Sr. No	Component	Volume to add for 987 ml in ml	Volume to add for 1974 ml in ml
1	1M CaCl 2	1.754	3.508
2	1M MgCl 2	0.606	1.212
3	3.6 mM FeSO 4 7H 2 O	1	2
4	1M D-Glucose	3	6
5	1M L-Lactate	9.3	18.6

Supplementary Table 2: Phenotypic interactions between *P. aeruginosa* (clinical and control strains) and *S. aurantiacum* (clinical and environmental strains) on the surface of SCFM agarose plates. (10µl of standardised culture of *P. aeruginosa* and *S. aurantiacum* conidial suspension were inoculated on opposite ends of SCFM agarose plates. Images of SCFM plates taken at 8th day of incubation. n=1.)

S. aurantiacum strain	*P. aeruginosa* strain	Image (Day 8)	Inhibition/no inhibition of fungal growth
WM-6-482	Pass 1		Inhibition
WM-6-482	Pass 2		No Inhibition
WM-6-482	Pass 3		Inhibition
WM-6-482	Pass 4		Inhibition

WM-6-482	PA01		Inhibition
WM-8-482	Pass 1		Inhibition
WM-8-482	Pass 2		Inhibition
WM-8-482	Pass 3		Inhibition
WM-8-482	Pass 4		No Inhibition

WM-8-482	PA01		Inhibition
WM-9-124	Pass 1		Inhibition
WM-9-124	Pass 2		Inhibition
WM-9-124	Pass 3		Inhibition
WM-9-124	Pass 4		Inhibition

WM-9-124	PA01		Inhibition
WM-10-136	Pass 1		No Inhibition
WM-10-136	Pass 2		Inhibition
WM-10-136	Pass 3		Inhibition
WM-10-136	Pass 4		Inhibition

WM-10-136	PA01		Inhibition

Supplementary Table 3: Analysis of the data of growth inhibition assays (centre inoculation method) of *P. aeruginosa* against *S. aurantiacum* using SCFM plates (n=3)

***S. aurantiacum* WM06.482**				
P. aeruginosa	Diameter of colony	Diameter of zone of inhibition	Inhibition index	Standard deviation
Pass 1	8.50	23.50	6.64	9.24
Pass2	10.00	24.50	5.00	10.13
Pass3	11.00	27.50	5.25	11.55
Pass4	20.50	32.00	1.44	15.44
PA01	20.50	30.50	1.21	14.89
***S. aurantiacum* WM08.202**				
P. aeruginosa	Diameter of colony	Diameter of zone of inhibition	Inhibition index	Standard deviation
Pass 1	9.75	18.75	2.70	0.75
Pass2	9.50	19.50	3.21	0.50
Pass3	21.25	32.25	1.30	1.25
Pass4	15.50	30.00	2.75	3.00
PA01	24.50	34.00	0.93	1.00

Supplementary Table 4: Analysis of the data of the effect of *P. aeruginosa* cell lysate (100µl) against *S. aurantiacum* (zone of inhibition, relative inhibition index and standard deviation (n=3))

***S. aurantiacum* WM06.482**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	22.5	20.75	17.5	21.5	27.25
Relative inhibition activity	4.06	3.31	2.06	3.62	6.43
Standard deviation (%)	0.5	0.25	0.5	0.5	0.25
***S. aurantiacum* WM08.202**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	23.75	19.5	22	23.5	24.5
Relative inhibition activity	4.64	2.80	3.84	4.52	5.00
Standard deviation (%)	0.75	0.5	0.5	0.5	0.5
***S. aurantiacum* WM09.024**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	23.25	29.75	26.5	29.25	33.5
Relative inhibition activity	4.41	7.85	6.02	7.56	10.22
Standard deviation (%)	0.25	0.75	1.5	0.75	1.5
***S. aurantiacum* WM10.136**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	26.5	27	26.75	25.3	35.5
Relative inhibition activity	6.02	6.29	6.16	5.40	11.60
Standard deviation (%)	0.5	1	0.75	1.3	0.5

Supplementary Table 5: Analysis of the data of the effect of *P. aeruginosa* culture supernatant (200µl) against *S. aurantiacum* (zone of inhibition, relative inhibition index and standard deviation (n=3))

***S. aurantiacum* WM06.482**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	25.50	22.00	20.50	24.75	28.00
Relative inhibition activity	5.50	3.84	3.20	5.13	6.84
Standard deviation (%)	0.50	1.00	0.50	0.75	0.50
***S. aurantiacum* WM08.202**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	23.00	21.50	23.50	23.63	23.50
Relative inhibition activity	4.29	3.62	4.52	4.58	4.52
Standard deviation (%)	0.50	0.50	1.00	1.13	0.50
***S. aurantiacum* WM09.024**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	25.13	28.50	23.00	24.25	30.25
Relative inhibition activity	5.31	7.12	4.29	4.88	8.15
Standard deviation (%)	0.63	0.50	1.00	0.75	0.75
***S. aurantiacum* WM10.136**					
P. aeruginosa	PASS1	PASS2	PASS3	PASS4	PAO1
Mean diameter of zone of inhibition	27.50	26.50	26.13	23.50	33.00
Relative inhibition activity	6.56	6.02	5.83	4.52	9.89
Standard deviation (%)	0.50	0.50	0.38	0.50	1.00

Supplementary Table 6: Revival of *S. aurantiacum* form all MIC dilutions from 1-butanol extracted molecules fractions from cell lysates and culture supernatants of *P. aeruginosa* PASS1 and PAO1 on PDA plates at 37°C for 7 days. n=3. Keys: + : successful revival/growth on PDA plate, - : no revival/growth on PDA plate, P = Positive control, N= negative control).

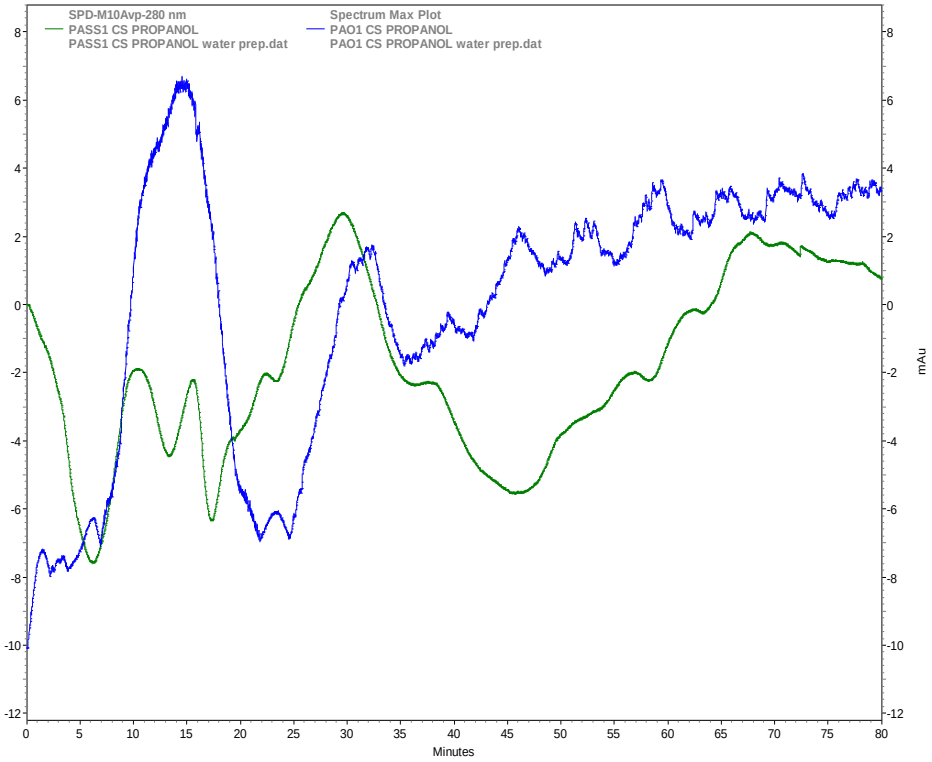
Concentration of metabolic fraction (µl/ml) against *S. aurantiacum*	10	20	30	40	50	60	70	80	90	100	P	N
	PASS1 cell lysate											
WM06.482	+	-	-	-	-	-	-	-	-	-	+	-
WM08.202	-	-	-	-	-	-	-	-	-	-	+	-
	PAO1 cell lysate											
WM06.482	-	-	-	-	-	-	-	-	-	-	+	-
WM08.202	-	-	-	-	-	-	-	-	-	-	+	-
	PASS1 culture supernatant											
WM06.482	+	+	+	-	-	-	-	-	-	-	+	-
WM08.202	-	-	-	-	-	-	-	-	-	-	+	-
	PAO1 culture supernatant											
WM06.482	-	-	-	-	-	-	-	-	-	-	+	-
WM08.202	-	-	-	-	-	-	-	-	-	-	+	-
	1-butanol control											
WM06.482	+	+	+	+	+	+	+	+	+	+	+	-
WM08.202	+	+	+	+	+	+	+	+	+	+	+	-

Supplementary Table 7: An HPLC chromatogram of 1-butanol fractions of *P. aeruginosa* culture supernatant derived using 0 to 40% isopropanol in aqueous acetic acid (2.5%) in 65 minutes of linear gradient and monitored at (A) 280nm and (B) 354 nm.

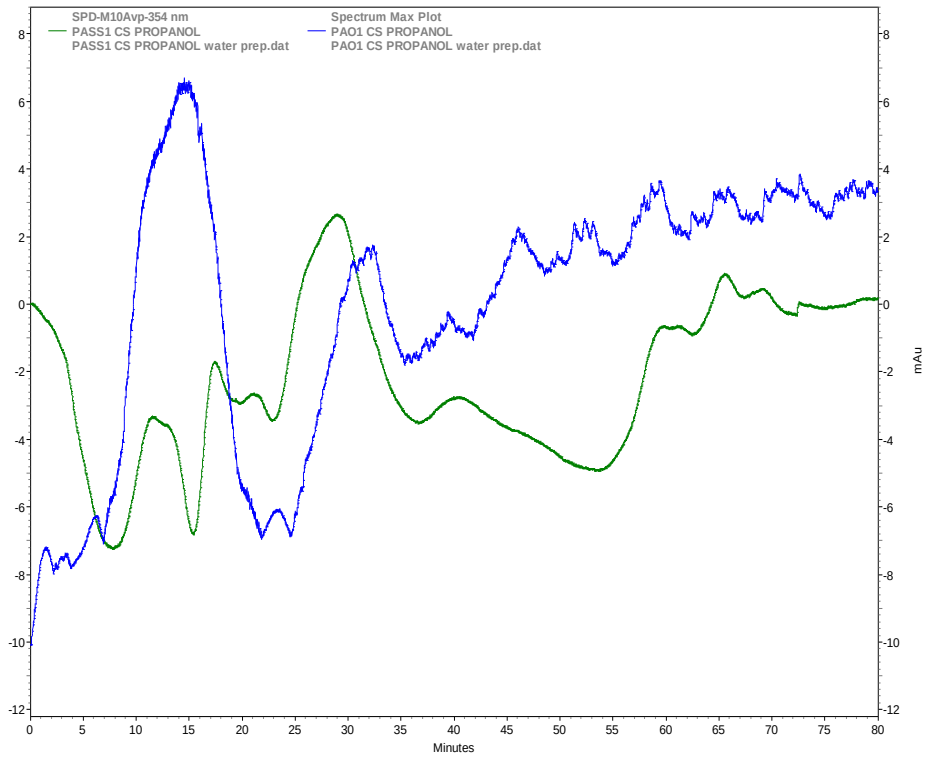
Green: 1-butanol fraction of *P. aeruginosa* PASS culture supernatant

Blue: 1-butanol fraction of *P. aeruginosa* PAO1 culture supernatant

(A)



(B)

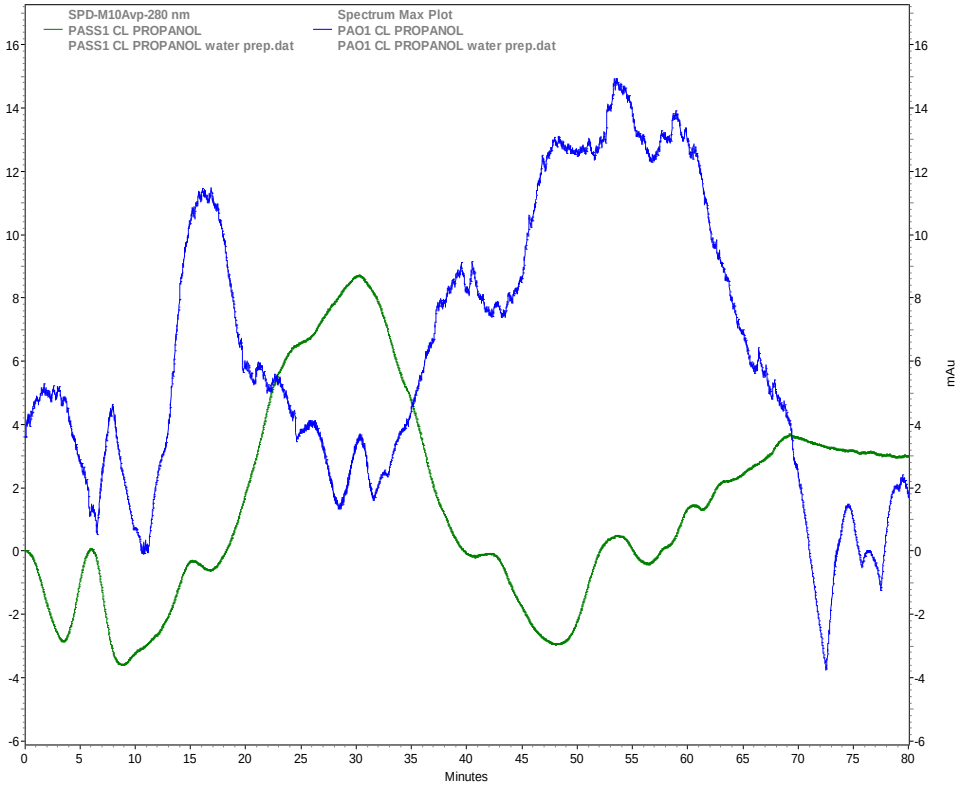


Supplementary Table 8: An HPLC chromatogram of 1-butanol fractions of *P. aeruginosa* cell lysate derived using 0 to 40% isopropanol in aqueous acetic acid (2.5%) in 65 minutes of linear gradient and monitored at (A) 280nm and (B) 354 nm.

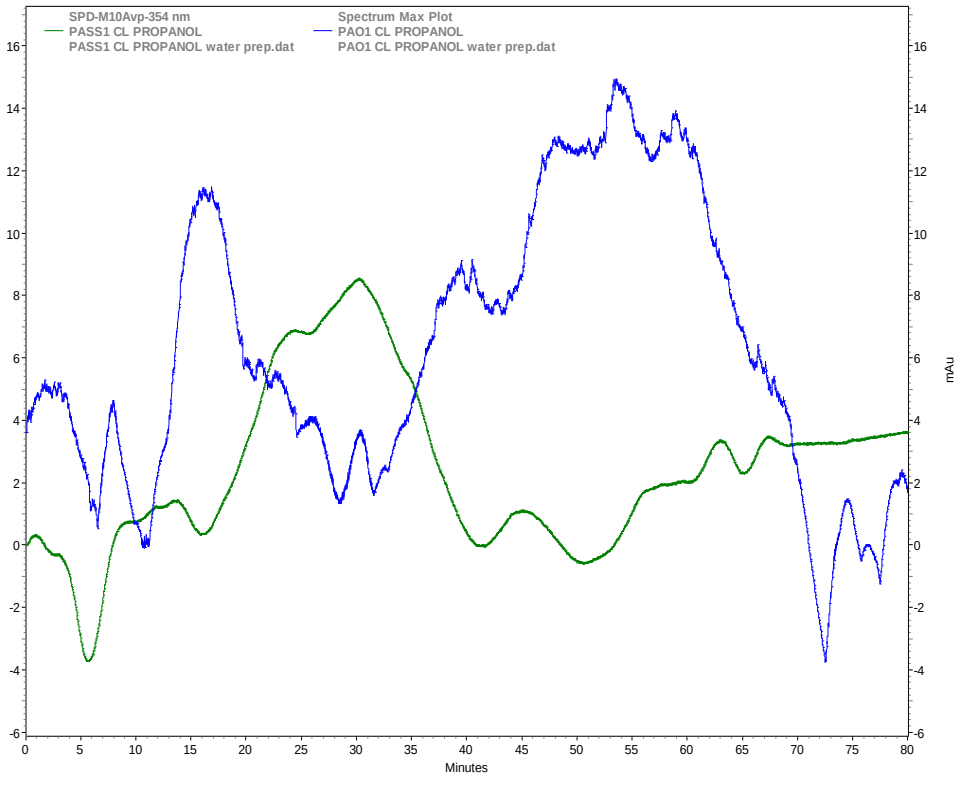
Green: 1-butanol fraction of *P. aeruginosa* PASS cell lysate

Blue: 1-butanol fraction of *P. aeruginosa* PAO1 cell lysate

(A)



(B)



Biosafety Approvals

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Biosafety Approval: *P. aeruginosa* (VIV030712BHA)

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SECTION A

MACQUARIE UNIVERSITY

Biohazard Risk Assessment Form – NON GMO

Notification Number:	*VIV030712BHA*

Investigator completing assessment :	**Vignesh Venkatakrishnan**	**Date of assessment:**	**03/07/2012**
Department:	**Chemistry and Biomolecular Science**	**Name of Supervisor submitting this assessment:**	**Prof. Nicolle Packer**
Contact number/email:	**9850-8200/ vignesh.venkatakrishnan@mq.edu.au**		

Reason for this assessment

☒ **New research** ☐ **New information relating to existing research** ☐ **other**_____

Exact location(s) of research:

E8A 109, E8C 320, E8C 326

Control measures: Eliminate risk ☐ **Substitute the hazard** ☐ **Isolate the hazard** ☒ **Implement engineering controls** ☐ **Administration** ☐ (e.g. Training) **PPE**☐

All samples are sealed in a box and carried to cobalt cave for gamma irradiation.

Supporting documents which must be read in conjunction with this assessment. (e.g. Safe Working Procedures, Safety Data Sheets, Guidelines/Protocols)

What is the type of the biological material?

Bacteria ☒ **Fungi** ☒**Virus** ☐ **Cell Line** ☐ **Tissue** ☐ **Parasite** ☐ **Animal** ☐ **Plant** ☐ **Soil** ☐ **Toxin** ☐ **Prions** ☐ **Nucleic Acid**☐ **other** ☐_____

What is the name of the biological agent?

Pseudomonas aeruginosa, Burkholderia cenocepacia **(bacteria) and fungi** ***Aspergillus fumigatus*** **and** ***Scedosporium prolificans*** **that are cultured from the sputum samples.**

List the Personal Protective Equipment required:

Gloves ☒_____(e.g. chemical resistant) **Eye protection** ☒**safety glasses(**e.g. safety glasses/goggles) **Clothing_Coveralls**_____ ☒ (e.g. button up lab coat/coveralls/apron)

Footwear ☒_____(e.g. Enclosed/Gumboots/overshoe covers) **Respiratory Protection** ☐_ (e.g.PF2 face mask) **Other** ☐_____

The University does not hold risk group 4 microorganisms

What are the risks associated with this Biological Agent. (Can be more than one risk group depending on method)			
Risk Group	**Details of Biohazards**	**Biosafety level**	**Risk Reduction Measures** (must be followed by the researcher)
Group 1- Low Individual and community risk (Microorganism that is unlikely to cause human, plant or animal disease)		(e.g.BSL1/PC1)	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ2243:3:2010 and university guidelines (see supporting documents - Section A above)
Group 2- Moderate individual risk, limited community risk (Microorganism that is unlikely to be a significant risk to laboratory workers, the community/livestock/environment. Laboratory exposures may cause infection but effective treatment and preventative measures are available and the risk of spread is limited).	*Pseudomonas aeruginosa, Aspergillus fumigatus, Scedosporium prolificans, Burkholderia cenocepacia*	PC2 level	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 2** (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above)
Group 3 -High individual risk, limited community risk (Microorganisms that usually causes serious human or animal disease and may present a significant risk to laboratory workers. It could present a limited to moderate risk if spread in the community or the environment, but there are usually effective preventative measures or treatment available).			1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 3** (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision

			and understands safe laboratory practice according to AS/NZ 243:3:2010 and university guidelines (see supporting documents - Section A above)

Process and equipment to be used	You must include: - 1 Description and quantity of any chemicals, gasses, substances and radiation used. 2 Any aerosols produced and any controls necessary to ensure the health and safety of investigators and others 3 Any alternative and/or additional control measures to those identified above and explain why these are necessary. 4 Safe Work Procedure if there is no existing SWP* 5 Explain why risks cannot be eliminated. 6 Waste disposal method.

1. **Pathogens**: The CF sputum samples are obtained from the Westmead Hospital are transported in a ziplock bag from Westmead to Macquarie using a sealed container with dry ice. The sample is then opened and an aliquot is transferred to Eppendorf tube in the laminar flow hood in the PC2 lab E8A109. The samples are then cultured on to Luria Broth agar plates and Horse blood agar plates to isolate the pathogens *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Aspergillus fumigatus*. The cultured organisms would be added to REVCO media and stored in the freezer. None of the above mentioned work is performed at Westmead Hospital. Everything regarding handling the pathogens are carried out at Macquarie University PC2 laboratory E8A 109.

2. **Radiations used**: The CF sputum samples are sealed in a box and carried to irradiation facility in E8A basement in a sealed container where they are irradiated for 2 hour at measurement of approx 2000 grays in the Cobalt Cave to kill any pathogens present in the sample as tested by lack of growth. This sample would then be brought to E8C 320 laboratory for further work. Proper induction has been obtained for the use of Cobalt cave.

3. It is a safe measure to irradiate the organisms which should completely kill all the pathogens and would be safe to be brought to the laboratory for further work related to glycomics work.

4. Before starting the experiment, the lab bench would be wiped using 4% Pyroneg to remove any spills from bench surface and then sterilized with 70% ethanol. Safety glasses and lab coat will be worn in PC2 lab at all times.

   Once the work is completed, the bench will be cleaned and the bench will be wiped using 4% Pyroneg and 70% ethanol to decontaminate it.

5. The risks involved could well be reduced by following the above mentioned steps and every measure has been taken for the safety of others working in the lab.

6. **Waste disposal method**: Everything that has been in contact with the pathogens (consumables as loops, tips, test tubes) will be thrown in to the bin for autoclaving or sterilizing. The bench will be wiped using 4% pyroneg and 70% ethanol to decontaminate it. All the wastes would be sealed and would be carefully taken for autoclaving and it will be disposed accordingly.

SECTION B	
MACQUARIE UNIVERSITY	**Biohazard Safety Committee – Risk Assessment Decision**
	Notification Number: *VIV030712BHA*

Important Information

For **non GMO** investigations email this assessment to **biohazard@mq.edu.au** for approval by the **Biohazard** Safety Committee.

Individual Responsibilities

By submitting this assessment the **Supervisor** identified in Section A, confirms that any supporting documents, training, guidance, instruction or protocols issued by the University will been followed so far as reasonably practicable to ensure the work is carried out without risk to health, safety or the environment. The named **Supervisor** confirms that the investigator has received appropriate training and instruction or will have adequate supervision and understands safe laboratory practice according to AS/NZ 2243:3:2010 and university guidelines.

Decision to be completed by the Biohazard Safety Committee:

The Committee has agreed that this risk assessment is sufficient for investigations to commence? **Yes** ☒ **No** ☐ **Further action required** ☒

Further Action/Comments:
- In relation to Point 4 *(Before starting the experiment, the lab bench would be wiped using 4% Pyroneg to remove any spills from bench surface and then sterilized with 70% ethanol. Safety glasses and lab coat will be worn in PC2 lab at all times. Once the work is completed, the bench will be cleaned and the bench will be wiped using 4% Pyroneg and 70% ethanol to decontaminate it.)* – all "work" should be conducted in the Class II cabinets, NOT on the bench, and any spills on the bench should be cleaned up by the previous user with 1% available chlorine.
- Place a 'tick' in Engineering Methods, Administration, and PPE.
- Correct the procedure to match pathogen risk group with the right containment (BSC Class II, PC2 Lab).

Name of Approver (Committee Rep):	Michael Gillings (Chairperson, Biohazard Committee)
Extension and email address:	Michael.gillings@mq.edu.au ext: 8199
Date Approved and submitted to Health and Safety Unit:	4th September, 2012

Biosafety Approval: *S. aurantiacum* (5201200092)

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OFFICIAL USE ONLY
SURNAME:
REF. NO.:

MACQUARIE UNIVERSITY

BIOSAFETY COMMITTEE

NOTIFICATION OF A 'NOTIFIABLE LOW RISK DEALING' (NLRD)

COVER SHEET

(Nov 2011 Version)

IMPORTANT INFORMATION FOR INVESTIGATORS

- **Regulations and Guidelines**

  The Committee strongly suggests that researchers be familiar with the Gene Technology Regulations Act (2000) and the Gene Technology Regulations (2001), as well as the Office of the Gene Technology Regulator (OGTR) website at www.ogtr.gov.au

  These are all available via the Institutional Biosafety Committee's website at: http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics

- **Commercial in confidence**

  If information you have provided on the OGTR Notification form is considered by you to be commercial in confidence, this should be noted on the OGTR Notification form and you should also complete and submit the *OGTR Application for declaration that specified information is confidential commercial information.*

  http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/forms-1

  On receipt of such an application, the OGTR will assess whether the information should be treated as confidential commercial information in accordance with the processes set out under the *Gene Technology Act 2000.*

- **Standard Conditions are applied by the OGTR to all Notifiable Low Risk Dealings (NLRDs)**

  NLRDs, when undertaken, must comply with the following requirements:

  - The NLRDs must be conducted in a facility certified by the Regulator to at least PC2 and of appropriate design for containing the particular type of GMO;

  - The dealings must be properly supervised and a record of the details of the dealings retained;

- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMO* available at:

  http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1; and

- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2010 (Safety in laboratories: microbiology).

- **For your information – Location of PC2 Laboratories and PC2 Glasshouse**

PC2 Laboratory	: Room 109, Building E8A
PC2 Glasshouse	: Room 413, Building F5A
APAF PC2 Laboratory	: Laboratory Room 323, Building E8C

Please note that as at January 2010 all applications must be submitted by academic staff. Students are no longer to be listed as Chief Investigators on research protocols. This includes Honours and all Postgraduate research students.

The completed cover sheet as well as your NLRD form and risk assessment form should be submitted to biosafety@mq.edu.au
Please forward a signed hard copy to Research Office, Level 3, Research HUB, Building C5C.

(1) Brief title of research project:

Molecular toolbox for the studies into the filamentous fungus *Scedosporium aurantiacum.*

(2) Chief Investigator Details

Name:	Helena Nevalainen
Title: (Prof, Dr. etc.)	Professor
MQ Staff no. (Mandatory)	93500068
Qualifications:	Doctor of Philosophy
Positions held:	Head of Department and Professor in Biotechnology
Faculty:	Science
Department/Unit:	Chemistry and Biomolecular Sciences
Full mailing address:	Herring Road 2109 Macquarie University NSW
Tel No. (W):	9850 8135
Tel No: (H):	
Mobile No:	0403162038
Fax number:	98508313
E-mail address:	helena.nevalainen@mq.edu.au

(3) CERTIFICATION TO BE FILLED OUT BY CHIEF INVESTIGATOR

Please fill out one of the following:

(i) I attended a Biosafety workshop on (date): .....

(ii) I intend to attend the Biosafety workshop planned for (date):

26/07/2012 (tentative)

GENE TECHNOLOGY REGULATIONS:

Please check your project against the Gene Technology Regulations to ensure that the dealings to be conducted as part of the above project:

(a) are dealings of a type included in Part 1 of Schedule 3 of the Regulations
(b) are NOT dealings of a type included in Part 2 of Schedule 3 of the Regulations
(c) meet the conditions of NLRDs set out in Part 3 Division 2 of the Gene Technology Regulations

I confirm that I have read and understood the relevant Guidelines (available via: http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/policy and that this project conforms in all respects with said Acts, Regulations and Guidelines. As Chief Investigator I also confirm that I will ensure that all personnel working on this project are appropriately trained and conversant with all relevant requirements, Acts, Regulations and Guidelines.

Helena Nevalainen
Name of Chief Investigator

Date: 29/2-12

Helena Nevalainen
Signature of Chief Investigator

Australian Government

Department of Health and Ageing
Office of the Gene Technology Regulator

Record of Assessment
Notifiable Low Risk Dealings (NLRDs)

As of 1 September 2011, the Gene Technology Amendment Regulations 2011 (Commonwealth) amend the Gene Technology Regulations 2001. These changes will affect the way Institutional Biosafety Committees (IBCs) record their assessment of Notifiable Low Risk Dealings (NLRDs). The new regulation 13B(a) requires an IBC that has assessed a proposal as being a NLRD to make a record of its assessment, in a form approved by the Regulator, and specifies the information that this record must contain.

IBCs **must** make a NLRD record of assessment (RoA). IBCs have the option of using this model form to make a RoA of a NLRD as required by 13B(a) of the Regulations. Alternately, IBC may choose to record this information using a different recording system/format. However, it is important to note that, at a minimum, all the information specified in 13B(a) must be included in the RoA. If the record contains all the information specified in Regulation 13B(a)(i)-(x), then this record will be considered to be in a form approved by the Regulator.

A document titled *Guidance for making Records of Assessment of NLRDs* has been prepared to assist IBCs with preparing RoAs of NLRDs (Reg 13B). It is strongly encouraged that the guidance document is used in conjunction with this model form to ensure accurate and comprehensive information is recorded.

Regulation 13B (a)	
(i) What is the identifying name or title of the proposed dealing (as given by the person or organisation proposing to undertake the dealing)?	Molecular toolbox for studies into filamentous fungus *Scedosporium aurantiacum*
(ii) Describe the dealing to be undertaken: *(Consider the breadth and scope of the all activities in relation to the dealings including any importation, transport, storage or disposal of the GMO – refer to Section 10 of the Gene Technology Act 2000 for a definition of "deal with" in relation to dealing with a GMO)*	*The *Scedosporium* strains provided by A/Prof Wieland Meyer at Westmead hospital will be stored and cultured on microbiological media (e.g. Sabouroud's agar or potato dextrose agar) under aseptic conditions in the PC2 lab. *This will be followed by microscopic examination of the hyphae and testing of the organism for antibiotic susceptibility (identifying their minimum inhibitory concentrations) in the PC2 lab to find a suitable antibiotic marker for genetic transformation. *The proteins secreted by the organism will be extracted and separated on a 2D gel to compare protein amounts and profiles to identify a suit of strong gene promoters to be used for recombinant expression of gene products of interest. Gene promoters will also be identified from the genome

	sequencing carried out for *Scedopsorium* strain. *Construction of vectors containing a gene promoter, secretion signal sequence (when applicable), transcription terminator sequence and a transformation marker will be carried out using different PCR techniques. *Different fungal transformation methods such as protoplast transformation will be trialed to introduce the DNA containing appropriate genes into *Scedosporium* under sterile conditions following laboratory safety guidelines. *Inactivation of genes of interest will be carried out using the developed molecular tools. The effect of these changes will be determined on biofilm formation or interactions with bacteria such as *Pseudomonas* using fluorescent imaging, biochemical and molecular methods.
(iii) Is the proposed dealing an NLRD in Part 1 or Part 2 of Schedule 3	Yes ☒ *If the proposed dealing is NOT listed in either Part 1 or Part 2 the dealing is either exempt or should be a Dealing Not involving Intentional Release (DNIR) i.e. a licensable dealing. This RoA is not required for exempt or licensable dealings.*

Regulation 13B (a)	
(iv) If yes to the above question, what kind of dealing is it? (As mentioned in the relevant Part of Schedule 3) *(As the NRLD may involve a number of scheduled dealings, all the kinds should be listed)*	The dealing has been described in part 2 of schedule 3 of the Gene Technology regulations 2001. It involves knocking out the genes in *Scedosporium* which is unlikely to increase the capacity of the GMO to cause harm compared to the parent organism before genes were knocked out.
(v) Date of IBC assessment *(Preferably in dd/mm/yyyy format)*	
(vi) List the persons (or classes of persons) considered by the IBC to have appropriate training and experience to undertake the dealing	1. Prof. Helena Nevalainen 2. Dr. Junior Te'o 3. Dr. Anwar Sunna 4. Prof. Ian Paulsen 5. Dr. Anahit Penesyan
(vii) List the facilities (or	1. Building E8A Room 109 (PC2 Facility) for

classes of facilities) the IBC considers to be of the appropriate physical containment level and type for the dealing	all live work. 2. Building E8C room 322 for DNA work 3. APAF PC2 laboratory in E8C 323 for occasional work if required.
(viii) Record the name of the IBC that assessed the proposal	Macquarie University Biosafety Committee
(ix) Record the name of the person or accredited organization that submitted the proposal	Prof. Helena Nevalainen Jashanpreet Kaur
(x) Record the name of the person or accredited organization proposing to undertake the dealing	Prof. Helena Nevalainen Jashanpreet Kaur

IBC Chair or delegate with authority to sign

Printed name:	Signature:
Job title:	Date:

Post-Assessment Checklist for Record of Assessment of Notifiable Low Risk Dealings (NLRDs)

As required by regulation 13B(b), the IBC **must** give a copy of the record of assessment of NLRDs to the person or accredited organisation that submitted the proposal to the IBC. The IBC can choose to use this post-assessment section (or an alternative system) to demonstrate that this has occurred.

Regulation 13B (b)	
Have you given a copy of the record of assessment to the person or accredited organisation that submitted the proposal to the Committee?	Yes ☐ ..... (printed name) ..... (job title) ..... (signature of IBC representative) (date)

I (the person/organisation that submitted the NLRD proposal) have received a copy of the Record of Assessment	..... (printed name) ..... (signature) (date)

MACQUARIE UNIVERSITY

Risk Assessment Form - Research

Risk Assessment Number:

General Information -

☐ Chemical	☐ GMO/OGTR	X☐ Biohazard	☐ Radiation	☐ Laser	☐ other: _____

Relevant reading (SWP, Msds etc)

What is being assessed - Risks associated with handling of pathogenic fungal strain.

Descripton of Task/Activity:	The experiments entail standard molecular biology techniques which carry no special risk and are all conducted in accordance with the safety SOP held in house.

Where is the activity/task undertaken -

Location/building:	E8A	Date of Assessment:	
Floor Number:	1	Faculty/Office:	Science
Room Number:	109	Department/unit:	CBMS

Who undertook the assessment -

Assessed by:	*Health & Safety Rep:*		*Employee(s):*	Jashanpreet Kaur	
Approved by:	*Head of Department:*	Prof. Helena Nevalainen	*Signature:*	H. Nevalainen	*Date:* 29/2-12

No:	Hazard and consequences	Risk Rating	Control Measures	Residual Risk	Action Required	Responsibility	Date to be completed
1	Pathogenic Fungal strain (*Scedosporium aurantiacum*)	2	Working in the PC2 lab.	2	Follow SOP (Attached)	Prof. Helena Nevalainen	
	Can cause infection		Personal protective equipment.		Use custom made fungal cabinets.		
			Safe work practices and training		Internal training and guidance.		

No:	Hazard and consequences	Risk Rating	Control Measures	Residual Risk	Action Required	Responsibility	Date to be completed

Notes: (Include justification why the referenced hazard has not been eliminated)
There are some risks but adequate controls are in place.

RISK MATRIX – use the risk rating calculator to calculate the risk rating

1. Assess the likelihood

Rating	Score	Description
Almost Certain	**5**	High likelihood of risk event happening several times within the next year.
Probable	**4**	A risk event is likely to occur more than once in the next 12 months.
Possible	**3**	Would not surprise if risk event occurred, and will probably occur at some time in the coming 2 to 5 years.
Unlikely	**2**	The risk event could occur at some time but is unlikely.
Rare	**1**	Within the realms of possibility but extremely unlikely to occur. Occurs once in 10 years.

2. Assess the Consequence

Rating	Score	Criteria
Catastrophic	**5**	• Multiple deaths and injuries • Severe environmental damage • Long term cessation of core activities (months) • Destruction or long-term unavailability of infrastructure, systems and resources directly impacting operations • Financial loss not covered by insurance (more than $5 million)
Major	**4**	• Single death and/or multiple injuries • Short term cessation of core activities (weeks) • Financial loss not covered by insurance ($2.5 - $5 million)
Moderate	**3**	• Injuries requiring off campus medical treatment • Significant disruption to core activities (days) • Financial loss not covered by insurance ($500,000 - $2.5 million)
Minor	**2**	• Injuries requiring on campus medical treatment • Short-term disruption to core activities (days) • Long-term disruption to non-core activities (weeks) • Financial loss not covered by insurance ($50,000 - $500,000)
Insignificant	**1**	• Minor injuries • Minimal impact on operations • Minimal financial loss (less than $50,000)

3. Risk Assessment Matrix

Risk rating as a function of consequence and likelihood scores.

Consequence						
	5	MEDIUM	HIGH	CRITICAL	CRITICAL	CRITICAL
	4	LOW	MEDIUM	HIGH	CRITICAL	CRITICAL
	3	LOW	LOW	MEDIUM	HIGH	CRITICAL
	2	VERY LOW	LOW	LOW	MEDIUM	HIGH
	1	VERY LOW	VERY LOW	LOW	LOW	MEDIUM
		1	2	3	4	5
		Likelihood				

Hierarchy of Control

Eliminate the hazard at source	▲ Most Desirable
Substitute for less hazardous equipment or substances	
Use engineering controls (guards)	
Isolate the hazard from anyone who can be harmed	
Administrative procedures and training	
Supply Personal Protective Equipment	▼ Less Desirable

Source: Manager, Health & Safety
Created: 2011
Document No:
Revised: N/A
Version No: 1

SAFE OPERATING PROCEDURE

Explanatory note: Safe operating procedures need to be developed and documented. These operating procedures define how a task should be done in a safe manner. The development of the procedure must involve consultation with members of staff who carry out the task in the workplace (refer to Section 27.02)

Task: **Working with potential human pathogens and known human pathogens in PC2 laboratory (E8A109)**

SOP No: Version: 1 Issue Date: **19.12.11**

Division / Office: **Faculty of Science** Department: **CBMS**

Supervisor / Manager: **Professor Helena Nevalainen**

Other Contacts: **Prof. Ewa Goldys, Dr Liisa Kautto, Jashanpreet Kaur**

HAZARDS	Working with potential human pathogens and known human pathogens. It is safe to work with if a proper protection and aseptic techniques are used. After spills of bacteria and fungi containing liquids, proper sterilisation is required. All material in contact with those pathogens will be autoclaved or if not autoclave able, then wiped with 4% pyroneg and spayed with 70% ethanol. Hands need to be washed and sterilised after work with 10% glycerol+70% ethanol.
PROTECTIVE EQUIPMENT & EMERGENCY EQUIPMENT	The potential pathogen and known pathogen strains will be plated and inoculated in PC2 Lab and work will be done only on the nominated area/ bench. Aseptic work technique will be followed. The bench will be wiped with 4% pyroneg to remove any spills from bench surface and then sterilised with 70% ethanol. Safety classes and lab coat for PC2 lab will be worn all the time.
BEFORE YOU START	Safety glasses and lab coat for PC2 lab will be worn all the time. Make sure you have 4% pyroneg and 70% ethanol nearby to immediately remove and sterilise any spills.
NEVER	Never leave the bench before cleaning/sterilising with 4% pyroneg and 70% ethanol. Never leave PC2 lab without washing your hands and using the glycerol-ethanol solution to sterilise them.
JOB STEPS	<u>Culturing and preparing bacteria and fungal cells:</u> Both bacterial and fungal strains will be cultured in PC2 lab either on the plate or liquid cultures and incubated in the incubaters used for the respective pathogen. Those microbial cultured isolates will be stored for further use in appropriate medium at -80°C. All consumables in contact with the pathogens (loops, tubes, tips etc.) will be placed into the dedicated bins. When harvesting cells, all work steps will be conducted with special care avoiding spills and working aseptically. After harvesting cells plates will be sealed with tape and placed into the bin for autoclaving / sterilising if not used anymore. Glass bottles used for cell cultures will be sealed and autoclaved as normal autoclave waste. After autoclaving bottles will be washed and can be reused. Any bacteria washing steps are done within the PC2 lab with the Eppendorf centrifuge located nearby on the bench working aseptically. <u>Preparation of bacterial/ fungal cells for imaging/ detection:</u> Preparation of bacteria and fungal cells or bacteria/ fungal containing materials for imaging / detection will be done aseptically at PC2 lab. Pathogen containing plates or slides will be sealed. Slides/ plates will be placed into clean container, which then will be wiped out side with 70% ethanol before carried out from PC2 lab. The measurements of bacteria or fungi containing slides / plates performed in other laboratories will be carried out with extreme care to minimise the possibilities to contaminate bench areas or equipments. All samples taken out of the PC2 lab to the microscope facility in E7B 113 and 109 or F7B-0 will be mounted on microscope slides and sealed for their examination so that there is no chance for the micro-organism to escape in the environment. All places where slides/ plates need to be placed for measurement will be cleaned afterwards with 70% ethanol. The slides will be returned to E8A109 for appropriate dispose after examination.

WHEN YOU FINISH	The bench will be cleaned. Everything that has been in contact with the pathogens (consumables as loops, tips, test tubes) will be thrown in to the bin for autoclaving or sterilizing. The bench will be wiped using 4% pyroneg and 70% ethanol to decontaminate it. All the wastes will be sealed and will be carefully taken for autoclaving .

Approved by: Idelsa Benavides DATE 29/2-12