MONASH University

Identification and Characterization of Pyochelin Produced by a Novel Bacterium *Burkholderia paludis*

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Abstract

The increase in prevalence of antimicrobial-resistant bacteria (ARB) in the past decade is a serious global threat, hence there is a need for new antimicrobial compounds to combat infections caused by ARB. One strategy to look for antimicrobial compounds is by prospecting for novel microorganisms - the exploration for novel microorganisms in unique ecological niches. An antimicrobial-producing *Burkholderia* species (MSh1) was previously isolated from tropical peat swamp forest soil in Malaysia and was found to exhibit antimicrobial activity against gram-positive and gram-negative bacteria. The bacterium, along with its antimicrobial compounds was thus further characterized in this study.

The whole genome of *Burkholderia* sp. MSh1 was sequenced and annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotations using Subsystems Technology (RAST). *Burkholderia* sp. MSh1 was characterized by using a polyphasic taxonomy approach and it can be concluded MSh1 represents a novel species within the *Burkholderia cepacia* complex (Bcc), for which the name *Burkholderia paludis* sp. nov. is proposed. The type strain is MSh1 (=DSM 100703 =MCCC 1K01245).

The antimicrobial production of *B. paludis* MSh1 was optimized using the Plackett-Burman design (PBD) followed by the conventional method of one-factor-at-a-time (OFAT) experiments. The optimized media contained 5 g/L glycerol, 15 g/L peptone and 1 % v/v minerals. The culture (1% v/v inoculum size) was incubated for four days at 30°C, 200 rpm. Validation of the experimental design was accomplished by using the optimized conditions, which gave 105.00 ± 5.47 U/mL of antimicrobial activity against *Enterococcus faecalis* ATCC

700802 (4.4-fold increase), as compared to using the conventional NB, which gave 23.33 ± 5.16 U/mL of antimicrobial activity.

The antimicrobial compound (pyochelin) was isolated and purified from the supernatant of *B. paludis* MSh1 culture grown in the optimized media via the bioassay-guided isolation method. Pyochelin had MIC values (MBC values) of 3.13 µg/mL (6.26 µg/mL) and 6.26 µg/mL (25.00 µg/mL) against three *Enterococcus* isolates (*E. faecalis* ATCC 700802, *E. faecalis* ATCC 29212 and *E. faecalis* JH-22) and four *Staphylococcus* isolates *(S. aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213). Pyochelin was able to inhibit *E. faecalis* ATCC 700802 in a time and dose dependent manner via killing kinetics assay. Pyochelin enhanced the production of reactive oxygen species (ROS) over time, which subsequently caused a significant increase in malondialdehyde production (a marker for lipid peroxidation), and ultimately leading to cell death by disrupting the integrity of the bacterial membrane (validated via BacLight assay).

This study has fully characterized a novel Bcc species, *B. paludis* isolated from Malaysian tropical peat swamp forest soil, along with its antimicrobial compound(s) for the first time. Extraction of the crude fermentation (under optimized conditions) and purification of the active fraction has revealed the antimicrobial compound was pyochelin. The study has also revealed the mechanism of action of pyochelin as an antimicrobial agent; and has shown that pyochelin might be able to treat infections caused by *S. aureus* and *E. faecalis*. However further characterization work on the mechanism of action using molecular techniques, synergism studies with currently available antibiotics and cytotoxic evaluation on normal cell lines should be conducted to confirm the potential of pyochelin as an antimicrobial agent in the future.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the test of the thesis.

This thesis includes three original papers published in peer reviewed journal and one paper currently under review for publication. The core theme of the thesis is "Identification and characterization of pyochelin produced by a novel bacterium *Burkholderia paludis*".

The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Science, Monash University Malaysia, under the supervision of Dr. Lee Sui Mae, Dr. Cheow Yuen Lin and Dr. Lee Learn Han.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, 3, 4, 5 and 6, my contribution to work involved the following:

Thesis chapter	Publication title	Publication Status	Nature and % of Student's Contribution	Co-author Name(s), Nature and % Co-author's Contribution
Chapter 2	Draft genome sequences of two antimicrobial-producing *Burkholderia* sp. strains, MSh1 and MSh2, isolated from Malaysian tropical peat swamp forest soil	Published	Experimental design, data interpretation and manuscript preparation (89%)	1. Aw Yoong Kit, experimental design and manuscript review (1%) (Monash student) 2. Gan Hang Ming, experimental design and manuscript review (1%) 3. Catherine Yule, experimental design and manuscript review (1%) 4. Lee Sui Mae, experimental design, data analysis and manuscript review (8%)
Chapter 3 and 5	*Burkholderia paludis* sp. nov., an antibiotic-siderophore producing novel *Burkholderia cepacia* complex species, isolated from Malaysian tropical peat swamp soil	Published	Experimental design, data interpretation and manuscript preparation (85%)	1. Aw Yoong Kit, experimental design and manuscript review (1%) (Monash student) 2. Catherine Yule, experimental design and manuscript review (2%) 3. Cheow Yuen Lin, experimental design and manuscript review (2%) 4. Lee Learn Han, experimental design and manuscript review (2%) 5. Lee Sui Mae, experimental design, data analysis and manuscript review (8%)
Chapter 4	Optimization of anti-enterococcal compounds produced by a novel bacterium, *Burkholderia paludis* MSh1, towards the improvement of its therapeutic potential	Under review	Experimental design, data interpretation and manuscript preparation (90%)	1. Cheow Yuen Lin, experimental design and manuscript review (2%) 2. Lee Sui Mae, experimental design, data analysis and manuscript review (8%)
Chapter 6	The role of reactive oxygen species in the antimicrobial activity of pyochelin	Published	Experimental design, data interpretation and manuscript preparation (90%)	1. Cheow Yuen Lin, experimental design and manuscript review (2%) 2. Lee Sui Mae, experimental design, data analysis and manuscript review (8%)

Dr. Lee Sui Mae, the main supervisor of the project was involved in the experimental design and critically revising the papers so as to contribute to the interpretation of the results with myself, the candidate. Dr. Cheow Yuen Lin was involved in co-supervision on the experimental design and manuscript reviewing of Paper II, III and IV. Dr. Lee Learn Han was involved in co-supervision on the experimental design and manuscript reviewing of Paper II. Professor Catherine Yule and Mr. Aw Yoong Kit contributed to the experimental design and manuscript reviewing of Paper I and II. The use and part of experimentation of Next-generation sequencing (NGS) technology using Illumina Miseq, reported in Paper I was done by Dr. Gan Hang Ming, a research fellow from School of Science, Monash University Malaysia. The liquid chromatography-mass spectrometry (LC-MS) analysis was done by Ms. Nurziana Sharmilla from the School of Medicine, Monash University Malaysia and interpretation of data through the use of services provided by School of Medicine was conducted by myself, the candidate.

I have renumbered sections of submitted of published papers in order to generate a consistent presentation within the thesis.

Student signa :: [redacted] Ong Kuan Shion Date: 3/10/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contribution to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main supervisor signature: [redacted] Dr. Lee Sui Mae Date: 3/10/2017

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List of abbreviations

ARB	Antimicrobial-resistant bacteria	bp	base pair
ATCC	American Type Culture Collection	g	gram
BLAST	Basic Local Alignment Search Tool	*g*	G force
Bcc	*Burkholderia cepacia* complex	µm	micrometer
CLSI	Clinical and Laboratory Standard Institute	mL	milliliter
DNA	Deoxyribonucleic acid	M	molar
HCl	Hydrochloric acid	rpm	revolutions per minute
IMR	Institute of Medical Research	µL	microliter
MBC	Minimum bactericidal activity	µg	microgram
MHA	Mueller-Hinton agar	min	minutes
MHB	Mueller-Hinton broth	sec	seconds
MIC	Minimum inhibitory concentration	mg/mL	milligram/milliliter
MRSA	Methicillin-resistant *Staphylococcus aureus*	µg/mL	microgram/milliliter
NA	Nutrient agar	U	unit
NaOH	Sodium hydroxide	v/v	volume/volume
NB	Nutrient broth	w/v	weight/volume
NCBI	National Center for Biotechnology Information		
PCR	Polymerase chain reaction		
rRNA	Ribosomal ribonucleic acid		
SEM	Scanning electron microscopy		
TSA	Tryptone soy agar		
TSB	Tryptone soy broth		
TPSF	Tropical peat swamp forest		

Chapter 1:

Literature review

1.1 Antimicrobials

Antimicrobials are agents that are able to destroy, inhibit or prevent pathogenic action of microbes (Banin et al., 2017). They are generally categorized into four groups based on their mechanism of action: (1) cell wall biosynthesis inhibitors (beta-lactams), (2) protein synthesis inhibitors (chloramphenicol, tetracyclines, aminoglycosides and macrolides), (3) nucleic acid synthesis inhibitors (quinolones) and (4) membrane disruptors (lipopeptides) (Fischbach and Walsh, 2009). With the advancement in drug discovery, compounds with new antimicrobial mechanisms have been discovered, for instance the dihydrofolate reductase inhibitor (trimethoprim), para-amino benzoic acid (PABA) inhibitor (abyssomicin), and more recently the lipid II biosynthesis inhibitor (teixobactin) (Capasso and Supuran, 2014, Khan et al., 2014, Ling et al., 2015). These existing antimicrobial compounds are commonly used to treat bacterial infections, and as prophylaxis to prevent initial or recurrence of infection (Banin et al., 2017).

1.2 Antimicrobial resistant bacteria (ARB)

Bacteria are capable of evolving to survive under unfavorable conditions and this is an inevitable natural phenomenon. They are able to gain resistance towards antimicrobial compounds intrinsically or by acquiring the ability via conjugation giving rise to antimicrobial-resistant bacteria (ARB) (Munita and Arias, 2016). The extensive use of antimicrobial agents to control microbial infections has unprecedentedly accelerated this process and has led to an increase in emergence and prevalence of ARB (Mishra et al., 2012).

Staphylococcus aureus is a major example of an ARB that causes life-threatening infections. The first line therapy for *S. aureus* infection is usually beta-lactam antimicrobial

agents (Jovetic et al., 2010). Unfortunately, the emergence of methicillin-resistant *S. aureus* (MRSA) strains essentially indicates that they are resistant to all currently available beta-lactam antimicrobial agents, except for the fifth generation cephalosporins (e.g. ceftobiprole) (Farrell et al., 2014, Gorwitz et al., 2008). This limits the treatment options to non-beta lactam antimicrobial agents, for instance tigecycline (protein synthesis inhibitor), vancomycin (glycopeptide), daptomycin (lipopeptide) and linezolid (protein synthesis inhibitor) to treat MRSA infections (Farrell et al., 2014, Rivera and Boucher, 2011). Despite the stringent control in the administration of these drugs, bacterial resistance has occurred, leading to the emergence of vancomycin-resistant, daptomycin-resistant and linezolid resistant *S. aureus* (Epand et al., 2008, Holmes and Jorgensen, 2008, Locke et al., 2010). These incidents have shown that *S. aureus* has gained resistance even to the last few options of antimicrobial therapy.

Enterococcus faecium and *Enterococcus faecalis* are another example of ARB known to be difficult to treat due to their intrinsic resistance and their ability to acquire resistance through horizontal gene transfer (Arias and Murray, 2012, Munita and Arias, 2016). Enterococci are the third most common cause of healthcare-associated bloodstream infections (BSI) in the United States (Balli et al., 2014). As some strains of *E. faecium* and *E. faecalis* are resistant to beta-lactam and glycopeptide antimicrobial agents, aminoglycoside are usually used together synergistically with either of the two classes of antimicrobial agents (Chang et al., 2010). However, aminoglycoside-resistant enterococci can arise when the ribosome binding site is mutated or altered, hence losing the synergistic effect with either class of the drug (Arias and Murray, 2012). Until recently, vancomycin is virtually the only antimicrobial that could be relied on to treat multidrug-resistant enterococci infections (Balli et al., 2014, Rivera and Boucher, 2011). As vancomycin is widely used to treat infections caused by MRSA and other gram-

positive bacteria such as *Clostridium difficile*, some enterococci isolates have gained resistance towards vancomycin (Rivera and Boucher, 2011). Vancomycin-resistant *E. faecium* and *E. faecalis* nowadays account for approximately one-third of the enterococcal healthcare-associated infections in the USA and for more than 20% of such infections in some European countries (Balli et al., 2014).

A similar pattern of antimicrobial resistance can be seen in other bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Peleg and Hooper, 2010). Infections caused by these gram-negative bacteria are usually treated with cephalosporins. Cephalosporins may no longer effective, thus leading to an increase use of carbapenems which were previously reserved only for extreme cases (Brown, 2015). Some gram-negative bacteria have now adapted by producing carbapenemases enzymes that can degrade carbapenems, such as the *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* and *Escherichia coli* (Huang et al., 2017, Stoesser et al., 2017). Recently, New Delhi metallo-beta-lactamase (NDM) producing bacteria were isolated and they can produce enzymes that can degrade numerous beta-lactam antimicrobial agents (Jain et al., 2014, Jamal et al., 2016, Walsh et al., 2011). Furthermore in some reported cases, carbapenem-resistant bacteria (CRB) are becoming resistant to beta-lactamase inhibitors, for example clavulanic acid and avibactam. CRB with beta-lactamase producing ability is virtually resistant to all antibiotic classes and is currently a serious threat to many developed countries, which include the United States, Europe and China (Brown, 2015, Gulay et al., 2013).

This is an alarming issue as the rapid emergence of ARB will eventually limit treatment options and increase mortality (Davies and Davies, 2010). According to the Director General of

the World Health Organization (WHO), we are heading towards a post-antibiotic era in which common infections and injuries could once again kill (WHO, 2012). Therefore, there is an urgent need for antimicrobial agents to combat these ARB infections.

1.3 Prospecting of novel microorganisms for antimicrobial compounds

One strategy to look for antimicrobial compounds is by prospecting for novel microorganisms - the exploration for novel microorganisms in unique ecological niches (Imhoff et al., 2011) . Microbes thriving in extreme and isolated environments can evolve due to selection pressure, giving rise to potentially novel species. Subsequently, they produce antimicrobial compounds to gain an upper hand in competing for resources and colonization of new habitats (Hibbing et al., 2010, Traxler and Kolter, 2015). Competition is likely to be most intense where sources such as nutrients are in short supply or where environment conditions are extreme (Hibbing et al., 2010, Imhoff et al., 2011). This suggests that novel microorganisms with antimicrobial producing ability could be prospected from unique ecological niches and thus, a tropical peat swamp forest in Malaysia was chosen previously as a potential bioprospecting location for this purpose (Ong et al., 2015).

1.4 Tropical peat swamp forest (TPSF)

Tropical peat swamp forests (TPSFs) are unique wetland ecosystems periodically inundated with fresh water from rainfall (Yule, 2010, Yule and Gomez, 2009). Out of 30–45 million hectares of global wetland in the world, about 18.1 million hectares are TPSFs widely distributed around Southeast Asia (Hirano et al., 2007, Page et al., 2010) (Figure 1.1). TPSFs contribute to almost 20% of the total global organic carbon and form one of the largest global

terrestrial organic carbon sinks. Carbon is stored in peat layers (partially decomposed organic matter) up to 25 m deep and will be released upon disturbance (such as clearing, drainage and fire) as fine particulates and greenhouse gases such as methane and carbon dioxide to the atmosphere. The greenhouse gases produced are the direct result of the decomposition or combustion of organic matter (Jauhiainen et al., 2005). Furthermore, TPSFs hold a key role in regional hydrology (movement and distribution of water). This is because peat holds 5–10 times its weight in water. This is important as the water stored can act as a buffer in reducing water velocity thus minimizing the impact of downstream flooding (Yule, 2010).

Figure 1.1: Locations (in red) of tropical peat swamp forests in Southeast Asia (Page et al., 2010).

TPSFs grow on a substrate formed by the accumulation of layers of peat. These are usually dome-shaped due to the buildup of peat, leading to poor drainage and permanent water logging followed by substrate acidification which contributes to the acidic nature of TPSF, having a pH range of 2.9 to 4.5 (Yule, 2010, Yule and Gomez, 2009). The leaching of tannins

and humic acids (20.2 ± 2.3 mg/L) from the leaves and other detritus of endemic plants further causes the dark brown water in TPSFs (Beamish et al., 2003) (Figure 1.2). The dark brown water reduces photosynthesis which combined with the high tropical temperature and very slow movement of water create an anoxic environment which then reduces the decomposition rate of organic matter (decay rates of 0.0006-0.0016 k/day for endemic plants), hindering nutrient recycling thus forming a highly concentrated carbon reservoir (Yule, 2010). In addition, TPSFs are ombrotrophic, receiving nutrients and water solely from rainfall and dust (Yule, 2010, Yule and Gomez, 2009). The lack of nutrient input and slow decomposition rate results in a low nutrient environment in TPSFs.

Figure 1.2: The acidic dark brown water characteristic of a Malaysian tropical peat swamp forest.

Despite the extremely harsh conditions in TPSF, Kanokratana et al. (2011) deduced bacteria constituted the most abundant microbial group in a Thailand TPSF. From the bacterial sequences identified, Proteobacteria was the largest phylum (37.9% of total bacteria), followed by Acidobacteria (35.0% of total bacteria). Other key minor bacterial phyla include Verrumicrobia (5.7%), Planctomycetes (9.6%), Actinobacteria (2.5%), Bacteroidestes (1.1%), Nitrospirae (1.8%), Firmicutes (0.4%) and other unclassified bacteria (6.0%). This was supported by Jackson et al. (2009) who showed that TPSFs are dominated by Proteobacteria and Acidobacteria (more than 50% of the total bacteria population). In addition, Crenarchaeota belonging to the Archaea kingdom was isolated from North Selangor peat swamp forest at a depth of 20 to 50 cm (Yule, 2010). Moreover, a new genus of aeroaquatic fungus *Polyancora* was isolated by Voglmayr and Yule (2006) and was named *Polyancora globosa*. Furthermore, a novel species of actinomycete *Micromonospora* sp. was discovered from southern Thailand tropical peat soil (Thuwai et al., 2005). This greatly supports the proposition there is a huge diversity of microbes in tropical peat swamp forests and hence they are potential locations for the discovery of novel microorganisms which may produce antimicrobial compounds.

1.5 Previous study on *Burkholderia* sp. MSh1 and MSh2

Two antimicrobial producing bacteria, *Burkholderia* sp. MSh1 and MSh2 were successfully isolated from Southeast Pahang tropical peat swamp soil (3° 01’ 19.56” N; 103° 39’ 29.67” E) in Malaysia, in March 2013. During sampling, the water pH was 3.44 ± 0.09 with a total dissolved solid reading of 94.25 ± 3.54 ppm and 6.30 ± 0.60% dissolved oxygen, in which these characteristics are consistent with a literature (Yule and Gomez, 2009). The two isolates were identified as *Burkholderia* species via the 16S rRNA gene sequence analysis with a

maximum identity of 99%. It was found the isolates had broad spectrum antimicrobial activity against several gram-positive (*Bacillus cereus* ATCC 14579, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 700802, *S. aureus* ATCC 700802, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213) and gram-negative bacteria (*K. pneumoniae* ATCC 10031, *Salmonella enterica* ATCC 14028 and *Shigella flexneri* ATCC 12022). The antimicrobial activity of the crude cell-free supernatant was stable at a pH range of 3.0 to 13.0 and temperatures of -20°C to 100°C. The antimicrobial activity of the supernatant remained unchanged after treating with three proteolytic enzymes: α-chymotrypsin, proteinase K and trypsin indicating the antimicrobial compounds produced may not be proteinaceous (Ong et al., 2015). These preliminary results showed that *Burkholderia* sp. MSh1 and MSh2 may be novel bacterial species and can produce non-proteinaceous antimicrobial compounds. Therefore warrants the need to characterize the isolates using whole genome sequencing, coupled with a polyphasic taxonomic approach which includes phenotypic and genotypic characterization.

1.6 *Burkholderia* species

The *Burkholderia* genus consists of a group of ubiquitous bacteria that occur in aquatic environments, soil, plant rhizosphere and animals, including humans (Coenye and Vandamme, 2003, Vandamme and Dawyndt, 2011). *Burkholderia* are mesophilic gram-negative rods, oxidase positive, motile microorganisms (Coenye and Vandamme, 2003, Vandamme and Dawyndt, 2011, Vanlaere et al., 2009). They are known to be versatile and diverse, and can tolerate and thrive under extreme conditions, such as environments with low pH, high salt or even the presence of pollutants (e.g. hydrocarbon and pesticides) (Goris et al., 2004, Zhang et al., 2000).

Burkholderia can be characterized phenotypically by their pigmentation, presence of hydroxyl fatty acids of 14, 16 and 18 carbon atoms, possession of distinct polar lipids, and by having Q-8 cellular respiratory quinones (Vandamme and Dawyndt, 2011, Vanlaere et al., 2009). The genus can be further divided into three groups: *Burkholderia cepacia* complex (Bcc), *Paraburkholderia* and *Caballeronia* (Dobritsa and Samadpour, 2016). Bcc species have diverse ecological roles and have been used in biocontrol and bioremediation (El-Banna and Winkelmann, 1998, Goris et al., 2004, Zhang et al., 2000); while the *Paraburkholderia* species such as *Paraburkholderia bryophila and Paraburkholderia megapolitana*, are usually plant growth promoters as they are able to fix nitrogen and supply nutrients to their plant hosts (Dobritsa and Samadpour, 2016, Sawana et al., 2014, Vandamme et al., 2007).

Bcc is a group of closely related *Burkholderia* species. They share a high degree of 16S rRNA (98-100%) and *recA* (94-95%) gene sequence similarity which makes them difficult to be differentiated using conventional molecular techniques (Coenye et al., 2001). To differentiate different species of Bcc, multilocus sequence analysis (MLSA) is usually adopted as the technique provides the discriminatory power needed for both identification and differentiation of Bcc species (Spilker et al., 2009).

Several Bcc species (*B. cepacia* and *Burkholderia pyrrocinia*) can be used for biocontrol agents as they can produce secondary metabolites to repress soil borne pathogens. An example is pyrrolnitrin, which targets the electron transport chain of gram-positive bacteria and fungi (El-Banna and Winkelmann, 1998). Some Bcc species (*Burkholderia unamae*) can act as plant growth promoters (Caballero-Mellado et al., 2004). They can also be used for bioremediation of recalcitrant xenobiotics, for instance *Burkholderia xenovorans* has the ability to break down

chlorinated toxic phenolic compounds commonly found in pesticides and herbicides (Goris et al., 2004, Zhang et al., 2000).

1.7 Secondary metabolites

Bacteria are known to produce a wide variety of secondary metabolites which includes enzymes, antimicrobial compounds and siderophores. They are usually produced in low quantities as they are metabolically expensive. Secondary metabolites are non-essential for bacterial growth but do provide diverse survival functions in nature (Martin et al., 2005, Sanchez et al., 2010, Yim et al., 2007). The production of secondary metabolites can be manipulated by altering their nutrient requirements and physical growth parameters (Wang et al., 2008). However, this phenomenon is highly dependent on the ability of the bacteria to metabolize different sources which might up-regulate or down-regulate the essential genes needed for the biosynthesis of the secondary metabolites (Sanchez et al., 2010). Hence, optimization studies involving conventional methods (one-factor-at-a-time experiments) or statistical methods (Plackett-Burman design; response surface methodology) are usually adopted to enhance the metabolite production prior to isolation and purification of the antimicrobial compounds (Singh et al., 2017). These studies allow accurate selection of nutrients and physical parameters which ensures maximum production at a lower cost.

1.8 Secondary metabolites produced by Bcc species

Many secondary metabolites with antimicrobial activity, produced by the Bcc species have been identified and they usually possess antifungal and/or antibacterial activity (Table 1.1). As the antimicrobial compounds produced by *Burkholderia* sp. MSh1 and MSh2 have not been

described thus far, one of the main objectives of the present study is to identify and characterize the compounds with antimicrobial activity.

Table 1.1: Examples of antimicrobial compounds produced by Bcc species.

Compounds	*Burkholderia* species	Antimicrobial activity	References
Burkholdines	*Burkholderia ambifaria* 2.2N	Antifungal	Tawfik et al. (2010)
Cepacidin A	*Burkholderia cepacia*	Antifungal	Lee et al. (1994)
Cepacins A and B	*Burkholderia cepacia* SC 11	Antibacterial	Parker et al. (1984)
Cepafungin	*Burkholderia* sp.	Antifungal, anti-cancer	Shoji et al. (1990)
Cepalycin	*Burkholderia cepacia*	Antifungal	Abe and Nakazawa (1994)
Gladiolin	*Burkholderia gladioli*	Anti-mycobacterium	Song et al. (2017)
Iminopyrrolidines	*Burkholderia plantari* #9424 ICMP	Antibacterial	Mitchell and Teh (2005)
Occidiofungin	*Burkholderia contaminans* MS14	Antifungal	Lu et al. (2009)
Pyochelin	*Burkholderia cepacia*	Antibacterial	Adler et al. (2012)
Pyrazoles derivatives	*Burkholderia glumae* #3729 ICMP	Antibacterial	Mitchell et al. (2008)
Pyrrolnitrin	*Burkholderia cepacia*	Antifungal, antibacterial	El-Banna and Winkelmann (1998)
Vietnamycin	*Burkholderia vietnamiensis*	Antibacterial	Rowe et al. (2016)
Xylocandin	*Burkholderia cepacia*	Antifungal	Meyers et al. (1987)

1.9 Research objectives

Burkholderia sp. MSh1 and MSh2 were previously identified to be potentially novel species based on the phylogenetic analysis of the bacterial 16S rRNA gene sequence. Although the isolates showed broad spectrum antimicrobial activity, the identity of the secondary metabolites produced are unknown. Hence, the objectives of this research are:

i. To perform whole genome sequencing on *Burkholderia* sp. MSh1 and MSh2 (Chapter 2)
ii. To identify *Burkholderia* sp. MSh1 via a polyphasic taxonomic approach (Chapter 3)
iii. To optimize the antimicrobial activity of *Burkholderia paludis* MSh1 (Chapter 4)
iv. To extract, purify and identify the antimicrobial compound(s) produced by *B. paludis* MSh1 (Chapter 5)
v. To characterize the antimicrobial activity of the purified compound(s) produced by *B. paludis* MSh1 (Chapter 6)

Chapter 2:

Draft genome sequences of *Burkholderia* sp. strains, MSh1 and MSh2, isolated from Malaysian tropical peat swamp forest soil

The work presented in this chapter represents the following peer reviewed publication (attached in Appendix 7a):

Ong, K. S., Aw, Y. K., Gan, H. M., Yule, C. M. and Lee, S. M. (2014). Draft genome sequences of two antimicrobial-producing *Burkholderia* sp. strains, MSh1 and MSh2, isolated from Malaysian tropical peat swamp forest soil. Genome Announcements 2(5):e01032-14.

2.1 Introduction

Extensive use of antimicrobial compounds has led to an increase in infections caused by antimicrobial-resistant bacteria (ARB). The increase in morbidity and mortality from ARB infection coupled with limited treatment options has prompted the need for antimicrobial compounds (Davies and Davies, 2010). Microorganisms are able to produce antimicrobial compounds in nutrient-poor and extreme environments to gain an advantage in competing for resources (Hibbing et al., 2010). Hence, a Malaysian tropical peat swamp forest was chosen as a potential location for antimicrobial compounds due to its low nutrient level and low pH conditions (Yule, 2010). Two antimicrobial-producing bacteria were successfully isolated and identified as *Burkholderia* sp. using 16S rRNA gene sequence analysis (Ong et al., 2015).

Burkholderia sp. MSh1 and MSh2 are oxidase positive, non–spore forming, gram-negative bacteria isolated from tropical peat swamp forest soil from Southeast Pahang, Malaysia. They were isolated due to their ability to produce antibacterial compounds that were active against several ARB such as methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 700699 and *Enterococcus faecalis* ATCC 700802 with minimum inhibitory concentration (MIC) values of the crude extract at 1.250 mg/mL and 0.313 mg/mL, respectively (Ong et al., 2015).

Two antimicrobial-producing *Burkholderia* isolates, MSh1 and MSh2 were subjected to whole genome sequencing to produce a draft genome sequence. The draft genomes will be used to determine the relationship between MSh1 and MSh2 in this chapter, and will be subsequently be used for further genomic analysis which will be discussed in Chapter 3. Annotations were performed to predict and determine the presence of different antimicrobial biosynthetic gene clusters in the draft genome sequences.

2.2 Materials and Methods

2.2.1 Genomic DNA extraction

The genomic DNA (gDNA) of *Burkholderia* sp. MSh1 and MSh2 were isolated from a 2-day-old culture in nutrient broth (NB) (Merck, Germany) using a GF-1 nucleic acid extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions. The purity and concentration of the gDNA were determined using Agilent 2100 Bioanalyzer.

2.2.2 Whole genome sequencing, genome assembly and annotation

The gDNA were converted into an Illumina-compatible next-generation sequencing library using Nextera XT (Illumina, San Diego, CA). The library was sequenced on the Illumina MiSeq (150-bp paired-end reads) at the Monash University Malaysia Genomics Facility. The raw reads were then trimmed and assembled *de novo* (default settings) using CLC Genomics Workbench 6 (CLC Bio, Denmark) to merge overlapping sequence reads into contiguous sequences (contigs). Gene annotations were performed using National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotations using Subsystems Technology (RAST).

2.2.3 Genome-to-genome distance calculator and average nucleotide identity

GGDC was performed to predict the DNA-DNA hybridization value between *Burkholderia* sp. MSh1 with *Burkholderia* sp. MSh2. GGDC was performed at http://ggdc.dsmz.de/distcalc2.php using the standard parameters (GGDC 2.0 BLAST+) (Meier-Kolthoff et al., 2013). The genome sequence of *Burkholderia* sp. MSh1 was queried with *Burkholderia* sp. MSh2. Results were expressed as similarity percentage ± confidence interval.

ANI was performed to estimate the mean values between homologous genomic regions shared by MSh1 and MSh2 using the whole genome sequence obtained. ANI was performed at http://enve-omics.ce.gatech.edu/ani using the standard parameters (Goris et al., 2007). Results were expressed as ANI percentage ± standard deviation.

2.2.4 Deposition

The draft genome sequences of *Burkholderia* sp. MSh1 and MSh2 were deposited in DDBJ/EMBL/GenBank under the accession numbers JPGL00000000 and JPGM00000000, respectively.

2.3 Results and Discussion

The draft genome of *Burkholderia* sp. MSh1 was assembled into 172 contigs with 67.08% G+C content and a total length of 8,633,651 bp (N_{50} = 110,286 bp), while *Burkholderia* sp. MSh2 had 167 contigs with 67.13% G+C content and an accumulated length of 8,723,138 bp (N_{50} = 129,187 bp). The GGDC and ANI values between *Burkholderia* sp. MSh1 and *Burkholderia* sp. MSh2 were 95.10 ± 1.38% and 99.43 ± 1.04%, respectively. The results indicate that *Burkholderia* sp. MSh1 and *Burkholderia* sp. MSh2 are the same species within the *Burkholderia* genus, as the threshold for species delineation for GGDC and ANI is 70% and 96%, respectively (Goris et al., 2007, Wayne et al., 1987).

Using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), 6,963 coding DNA sequences (CDSs), 10 rRNAs, and 64 tRNAs were annotated for *Burkholderia* sp. MSh1, while 7,072 CDSs, 9 rRNAs, and 59 tRNAs were annotated for *Burkholderia* sp. MSh2. Several antimicrobial biosynthesis genes were predicted in the genome of both *Burkholderia* sp. MSh1

and MSh2, including polyketide cyclase (KFG96276 and KEZ06389), antibiotic biosynthesis monooxygenase (KFG97610 and KEZ06948), mitomycin antibiotic biosynthesis protein (KFG98191 and KEZ04328), colicin V production protein (KFG93602 and KEZ04863), and phenazine biosynthesis protein (PhzC/PhzF) (KFG92381 and KEZ02706).

Based on RAST, some of the known antimicrobials produced by *Burkholderia* species were absent, for instance, pyrrolnitrin (*Burkholderia pyrrocinia*), rhizobitoxin (*Paraburkholderia andropogonis*), and pyoluteorin (*Burkholderia cepacia*) (Aziz et al., 2008, Mitchell, 1994, Souza and Raaijmakers, 2003). This indicates the antimicrobial compounds produced by the two isolates may be different from the aforementioned compounds. Therefore, further purification and identification of the antimicrobial compounds are required, which will be discussed in Chapter 5.

2.4 Conclusions

We report the draft genome sequences of two antimicrobial-producing isolates, *Burkholderia* sp. MSh1 and MSh2 which were isolated from tropical peat swamp forest soil. Putative genes related to different antimicrobial production have been annotated in both genome sequences. As *Burkholderia* sp. MSh1 and MSh2 showed high similarity in terms of their genetic content and phenotypic appearances, only *Burkholderia* sp MSh1 will be further characterized in the subsequent chapters of this thesis.

Chapter 3:

Burkholderia paludis sp. nov., a novel *Burkholderia cepacia* complex bacterium isolated from Malaysian tropical peat Swamp soil

The work presented in this chapter represents a part of the following peer reviewed publication (attached in Appendix 7b):

Ong, K. S., Aw. Y. K., Lee, L. H., Yule, C. M., Cheow, Y. L. and Lee, S. M. (2016). *Burkholderia paludis* sp. nov., an antibiotic-siderophore producing novel *Burkholderia cepacia* complex species, isolated from Malaysian tropical peat swamp soil. Frontiers in Microbiology 7: 2046.

3.1 Introduction

The *Burkholderia cepacia* complex (Bcc) consists of a group of ubiquitous bacteria that occur in aquatic environments, plant rhizospheres and animals, including humans (Coenye and Vandamme, 2003, Peeters et al., 2013, Vanlaere et al., 2009). Certain Bcc species, for example *B. cepacia*, can be used for biocontrol as they can produce antifungal compounds to repress soil borne pathogens (Caballero-Mellado et al., 2004). An example of one such compound is pyrrolnitrin which targets the electron transport chain of both gram-positive bacteria and fungi (El-Banna and Winkelmann, 1998). Bcc species share a high degree of 16S rRNA (98–100%) and *recA* (94–95%) gene sequence similarity, and moderate levels of DNA-DNA hybridization (30–50%) (Coenye et al., 2001). To differentiate different species of Bcc, multilocus sequence analysis (MLSA) is usually adopted as this taxonomic technique provide the discriminatory power needed for identification and differentiation of Bcc species (Peeters et al., 2013, Spilker et al., 2009, Vanlaere et al., 2009).

The objective of this Chapter was to characterize MSh1 using a polyphasic taxonomic approach which include a comprehensive analysis on the phenotypic, genotypic and chemotaxonomic profile.

3.2 Material and methods

3.2.1 Isolation and maintenance of isolate

MSh1 was maintained on nutrient agar (NA) at 30°C aerobically and in 25% (v/v) glycerol in nutrient broth (NB) (Merck, Germany) at -80°C for long term preservation.

3.2.2 Sequence and phylogenetic analysis

The genomic DNA of MSh1 was isolated using GF-1 nucleic acid extraction kit (Vivantis, Malaysia). The 16S rRNA gene sequence of MSh1 was amplified using the universal primers 27f (5' – AGA GTT TGA TCC TGG CTC AG -3') and 1492r (5' – ACG GCT ACC TTG TTA CGA CTT -3') (Kane et al., 1993). Polymerase chain reaction (PCR) was set up as follows: 150 ng (5 µL) of DNA extract, 10 µL of 5× MyTaq Red Reduction Buffer, 5 µM of forward primer, 5 µM of reverse primer and 1.25 U of MyTaq DNA polymerase. The reaction volume was made up to 50 µL using sterile milliQ water (Millipore, Germany). The PCR included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 45 sec and elongation at 72°C for 45 sec. PCR products were separated on a 1.5% (w/v) agarose gel in TAE buffer, and the bands were visualized with 1× GelRed. Amplification products were purified and cloned into pJET1.2 vector (FirstBase, Malaysia). The 16S rRNA gene sequence of MSh1 was aligned with sequences of closely related type strains of the genus *Burkholderia* retrieved from the GenBank/EMBL/DDBJ and EzBioCloud.net databases using CLUSTAL-X software (Thompson et al., 1997). The alignment was manually verified and adjusted prior to the construction of phylogenetic tree using the neighbor-joining (Saitou and Nei, 1987) algorithm with the MEGA version 6.0 software (Tamura et al., 2011). The stability of the resultant tree topologies were evaluated by using the bootstrap resampling method (Felsenstein, 1985). The evolutionary distances for the neighbor-joining algorithm were computed using the Kimura's two-parameter model (Kimura, 1980).

3.2.3 DNA-DNA hybridization (DDH)

DDH tests were carried out by the Identification Service of Deutsche SammLung von Mikroorganisen und Zellkulturen (DSMZ, Germany) to evaluate the DNA–DNA relatedness between MSh1, *B. arboris* R-24201, *B. cenocepacia* J2315 and *B. lata* 383 (closely related strains based on the phylogenetic analysis of the 16S rRNA gene sequence) using the optical renaturation rate method as described by De Ley et al. (1970) under consideration of the modifications described by (Huss et al., 1983).

3.2.4 Multilocus sequence analysis (MLSA)

MLSA was performed on MSh1 based on the method described by Spilker et al. (2009). A phylogenetic tree of the concatenated sequence (2773 bp) of seven housekeeping gene fragments [*atp*D (443 bp), *glt*B (400 bp), *gyr*B (454 bp), *rec*A (393 bp), *lep*A (397 bp), *pha*C (385 bp) and *trp*B (301 bp)] was constructed using MEGA6.0 (Tamura et al., 2011). The mean number of nucleotide substitution per site (i.e. the percentage of divergence of concatenated allele sequences) between established Bcc type strains and MSh1 was calculated using the DnaSP v5.10 (Librado and Rozas, 2009) based on the Jukes-Cantor method (Jukes and Cantor, 1969). Nucleotide sequences of each allele, allelic profiles and sequence types of MSh1 from the present study are available on the Bcc PubMLST database (http://pubmLst.org/bcc).

3.2.5 PCR amplification of *prnD* (pyrrolnitrin) gene

The synthesis of pyrrolnitrin is regulated mainly by four genes *prnA*, *prnB*, *prnC* and *prnD*, where *prnD* being the most crucial gene. The gene product of *prnD* catalyses the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Souza and

Raaijmakers, 2003). As pyrrolnitrin is the only reported compound with antimicrobial activity against gram-positive bacteria known to be produced by Bcc (El-Banna and Winkelmann, 1998), this assay was carried out to avoid the rediscovery of a known compound (pyrrolnitrin). Hence in order to assess the ability of MSh1 to produce pyrrolnitrin, the *prnD* gene was amplified with a forward primer PRND1 (5' – GGG GCG GGC CGT GGT GAT GGA – 3') and a reverse primer PRND2 (5' – YCC CGC SGC CTG YCT GGT CTG – 3') (Souza and Raaijmakers, 2003). PCR were set up as follows: 5 µL of DNA extract, 10 µL of 5× MyTaq Red Reduction Buffer, 5 µM of forward primer, 5 µM of reverse primer and 1.25 U of MyTaq DNA polymerase. The reaction volume was made up to 50 µL using sterile milliQ water (Millipore, Germany). The PCR included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 68°C for 30 sec and elongation at 72°C for 30 sec. Genomic DNA of pyrrolnitrin producer *B. lata* 383 was used as a positive control and non-pyrrolnitrin producer *B. cenocepacia* J2315 was used as a negative control. PCR products were separated on a 1.5% (w/v) agarose gel in TAE buffer, and the bands were visualized with 1× GelRed.

3.2.6 Phenotypic and biochemical characterization

Cellular morphological characteristics of MSh1 were observed by using a Hitachi S-4800 field-emission scanning electron microscopy (FE-SEM) at 25000× magnification after two days of growth in NB at 30°C. Colony morphology of MSh1 was examined after two days of growth on NA at 30°C. Cell motility was measured using hanging drop method after incubation for two days at 30°C aerobically in NB (Robbie, 1945). The optimal temperature for growth was measured at 4, 15, 28, 30, 37, 40 and 45°C in NB for two days. The pH range for growth was tested with in NB adjusted with HCl or NaOH to pH 3–11, at intervals of 0.5 pH units. Sodium chloride (NaCl) tolerance at 0–5% (w/v) at interval of 0.5% (w/v) was determined in NB for two

days. Anaerobic growth was tested in NB for up to five days in a 2.5 L jar containing an Anaero Pack (Thermo Scientific, USA). Phenotypic characteristics including Gram staining, catalase and oxidase activity of MSh1 were examined using the methods described by Buck (1982) and Cappuccino and Sherman (2002) after two days of growth on NA at 30°C. Physiological and biochemical properties were further determined using API 50CH, API 20NE and API ZYM strips (bioMerieux, France) according to the manufacturer's instructions. All tests were conducted in duplicate. The API 50CH and API 20NE tests were read after 24–48 hours at 30°C, while the API ZYM tests were read after four hours of incubation at 30°C.

3.2.7 Chemotaxonomic characterization

The cellular fatty acids analysis of MSh1, *B. arboris* R-24201, *B. cenocepacia* J2315 and *B. lata* 383 was performed by the Identification Service of DSMZ (Braunschweig, Germany). The cell mass of MSh1 and closely related type strains were harvested from NB after incubation at 30°C aerobically for two days. Extraction and analysis of the cellular fatty acids were performed according to the standard protocols of the Sherlock Microbial Identification System (MIDI) (Miller, 1982), analyzed using an Agilent 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column. Peaks were integrated and identified using the peak-naming table TSBA40 at the DSMZ. Polar lipids were extracted from 200 mg freeze-dried bacterial cell material using chloroform: methanol: 0.3% (w/v) aqueous NaCl mixture 1:2:0.8 (v/v/v) as described by (Tindall, 1990). The extracted polar lipids were separated by two dimensional silica gel thin layer chromatography with chloroform: methanol: water (65:25:4, v/v/v) as mobile phase for the first direction, followed by chloroform: methanol: acetic acid: water (80:12:15:4, v/v/v/v) as the mobile phase for the second direction (Tindall et al., 2007). Cellular ubiquinones were extracted and purified as described previously by Tindall (1990). The

different quinone classes were firstly separated using thin layer chromatography on silica gel using hexane-tert-butylmethylether (9:1, v/v) as a solvent and then further purified with reversed-phase HPLC using methanol:heptane (9:1, v/v) as the eluent. The purified quionones were compared to standards at the DSMZ.

3.2.8 GGDC and ANI analysis

The GGDC and ANI analysis were performed as according to Section 2.2.3 of this thesis.

3.2.9 Genome analysis of secondary metabolites gene clusters using antiSMASH

The whole genome of MSh1 was screened for biosynthetic gene clusters responsible for the synthesis of secondary metabolites using Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) 2.0) (Blin et al., 2013). The antiSMASH 2.0 program analyses the whole genome sequence for homologues to known secondary metabolites via BLAST search and annotates them based on different biosynthetic gene clusters, for example polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), terpene and bacteriocins.

3.2.10 Nucleotide sequence accession number

The 16s rRNA gene sequence of MSh1 has been deposited in GenBank/EMBL/DDBJ under the accession number KT159931. The gene sequences of each MLSA loci have been deposited at the Bcc PubMLST database with sequence typing (ST) 1043; and GenBank/EMBL/DDBJ under the accession number KU301866–301872.

3.3 Results and discussion

3.3.1 16S rRNA gene sequence analysis

The 16S rRNA gene sequence was obtained for MSh1 (1497 bp; GenBank/EMBL/DDBJ accession number KT159931) and a phylogenetic tree was constructed (Fig. 1). Phylogenetic analysis demonstrated MSh1 is closely related to *B. arboris* R-24201 and *B. lata* 383, as they formed a distinct clade (Figure 3.1). Pairwise comparison of the 16S rRNA gene sequence of MSh1 with Bcc type strains revealed similarity levels between 97.1 to 99.9% (data not shown). It was observed that MSh1 was distinctly different from the non-Bcc species which include *Caballeronia sordidicola* S5-B, *Caballeronia terrestris* LMG 22937 and *Caballeronia telluris* LMG 22936, as the non-Bcc species formed a different clade from the Bcc representatives (Figure 3.1). Therefore, only the Bcc species will be used as a comparison for the subsequent analysis.

3.3.2 DNA-DNA hybridization (DDH)

The DNA–DNA relatedness values between MSh1 with its close neighbour based on 16S rRNA phylogenetic analysis: *B. arboris* R-24201 (29.0 ± 3.7%), *B. cenocepacia* J2315 (32.8 ± 2.2%) and *B. lata* 383 (19.5 ± 1.8%) were below the 70% threshold value for species delineation (Wayne et al., 1987).

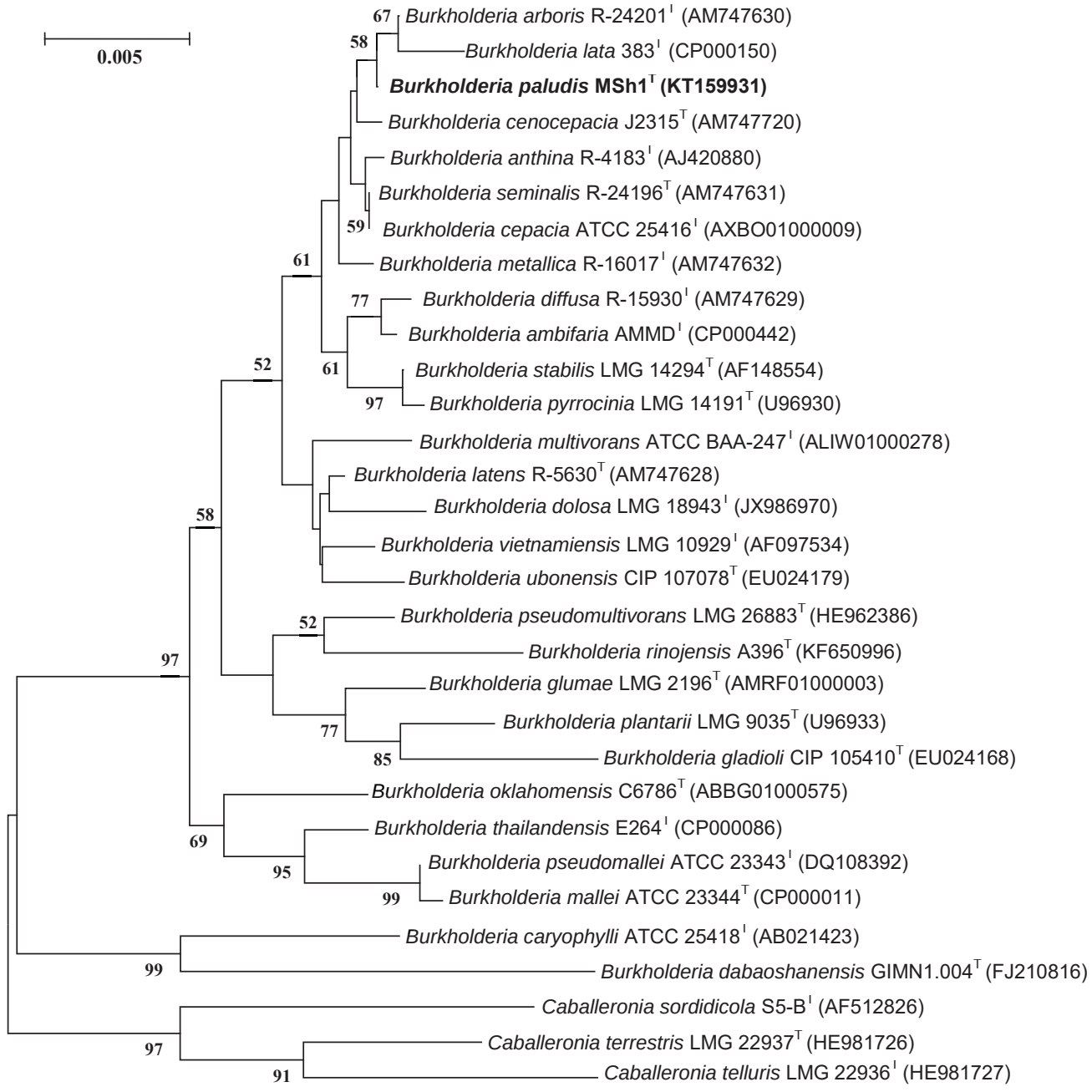

Figure 3.1: Neighbour-joining tree based on 16S rRNA gene sequences showing relationship between MSh1 and representatives of some other related taxa. Bootstrap values (> 50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 5 substitutions per 1000 nucleotide positions.

3.3.3 Multilocus sequence analysis (MLSA)

Phylogenetic analysis of concatenated allele sequences demonstrated MSh1 diverged from the other closely related Bcc type strains, supported by a bootstrap value of 86% (Figure 3.2). It was observed that the closest relatives of MSh1 shown in Figure 3.2 (*B. pyrrocinia* LMG 21823, *B. stabilis* LMG 14294 and *B. cenocepacia* IIIC LMG 19230) were different from the 16S rRNA gene phylogenetic tree (Figure 3.1). The different patterns observed in both figures are attributed to the differences in the length and type of gene sequences used for species identification of *Burkholderia*. Figure 3.1 was built based on the sequence of a single bacterial gene - 16S rRNA gene (1497 bp), while Figure 3.2 was constructed using the concatenation of seven housekeeping genes (2773 bp). Furthermore, as Bcc species share a high degree of 16S rRNA gene sequence similarity, this technique can only identify down to genus – Bcc and non-Bcc species, and is unable to accurately resolve the phylogenetic relationships of species within the Bcc. Thus MLSA is usually adopted as this technique provides the discriminatory power needed for identification and differentiation of Bcc species (Peeters et al., 2013). MLSA data were used to assign allele types and allelic profiles. The allelic profile was as follows: *atpD*, 302; *gltB*, 11; *gyrB*, 50; *recA*, 350; *lepA*, 288; *phaC*, 249; and *trpB*, 345 (GenBank/EMBL/DDBJ accession number KU301866-301872). MSh1 showed distinct allelic profile when compared with those closely related type strains (Appendix 1; Table A1.1). The average concatenated allele sequence divergence of MSh1 towards its nearest neighbour *B. stabilis* LMG 14294 (5.23%), *B. pyrrocinia* LMG 14191 (5.81%) and *B. cenocepacia* IIIC 19230 (6.87%) were above the 3% cut-off value hence indicating MSh1 is a novel species within the Bcc (Appendix 1; Table A1.2) (Peeters et al., 2013, Vanlaere et al., 2009).

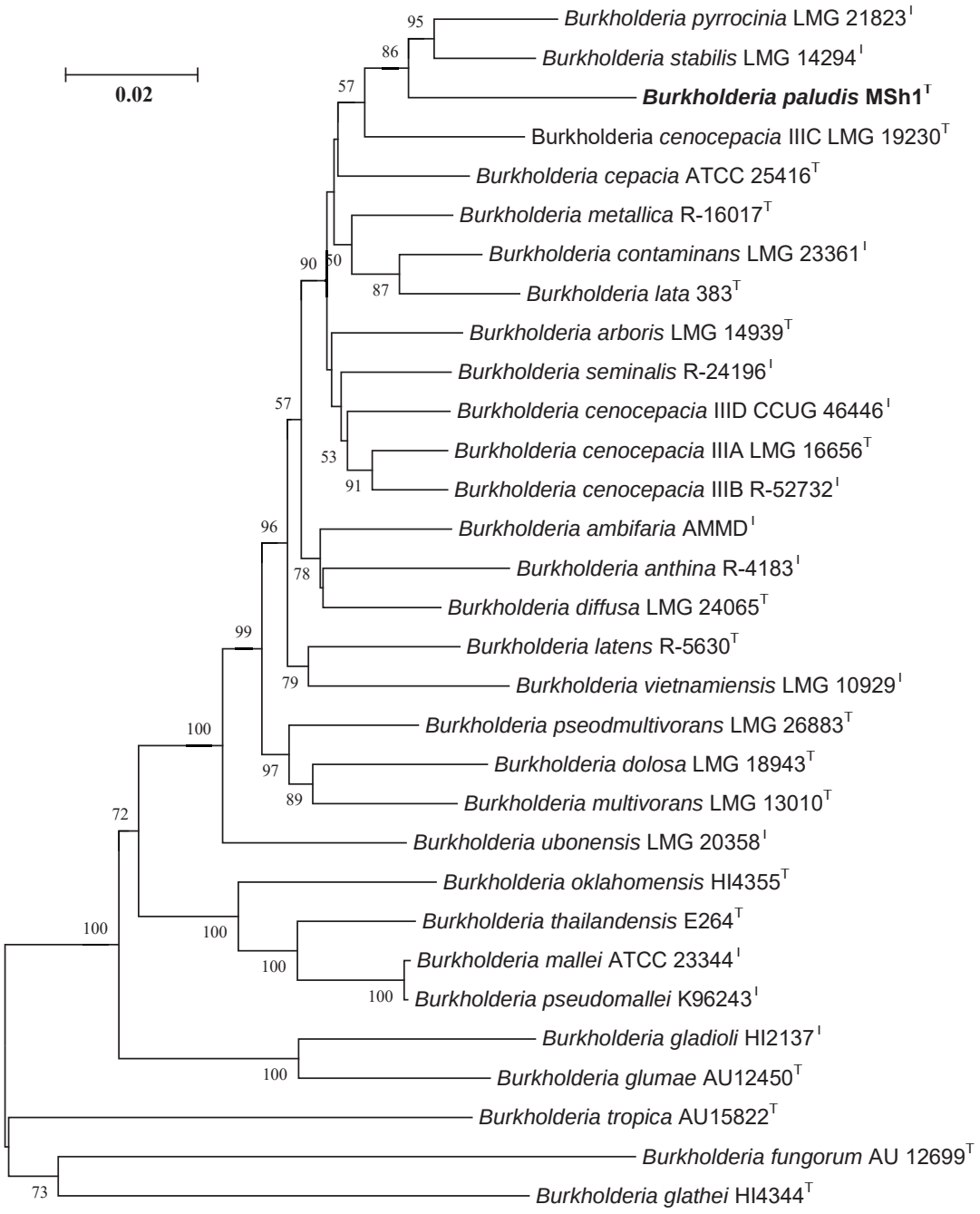

Figure 3.2: Phylogenetic tree based on the concatenated sequences of seven housekeeping gene fragments of established Bcc species and MSh1. Bootstrap values (> 50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 2 substitutions per 100 nucleotide positions.

3.3.4 Phenotypic and biochemical characterization

MSh1 is a gram-negative, facultative anaerobic, motile, rod-shaped, 0.6–0.8 μm × 1.6–2.1 μm bacterium (Figure 3.3). Colonies produced by MSh1 on NA are round, yellow coloured with a smooth surface and 2–3 mm in diameter. Growth of MSh1 occurs at 15–40°C (optimum 30°C) and pH 4.0–10.0 (optimum pH 7.0) in NB. Growth occurs with 0–2.5% (w/v) NaCl (optimum without NaCl).

MSh1 cells are positive for oxidase, glucose fermentation, arginine dihydrolase, urease, esculin hydrolysis and assimilation of glucose, but negative for nitrate/nitrite reduction, indole production, gelatin hydrolysis, β-galactosidase, assimilation of arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid (API 20NE).

In API ZYM tests, MSh1 cells are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and n-acetyl- β-glucosaminidase, but negative for valine arylamidase, cystine arylamidase, trypsin, a-chymotrypsin, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, αmannosidase and α-fucosidase. The following carbon sources are utilized in the API 50CH: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-glucoside, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, D-turanose, D-xylose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol; the other substrates, L-xylose, adonitol, α-methyl-D-xiloside, L-sorbose, rhamnose, α-methyl-D-mannoside, α-methyl-D-

glucosamine, amygdalin, melibiose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, β-gentiobiose, gluconate, 2-keto-gluconate and 5-keto-gluconate , are not utilized.

Table 3.1 shows MSh1 can be differentiated biochemically from the closely related members of its genus *Burkholderia*. MSh1 differs from the other Bcc species by the ability to acidify adonitol, having arginine dihydrolase activity and inability to assimilate N-acetyl-glucosamine (Coenye et al., 2001, Henry et al., 2001, Peeters et al., 2013, Vandamme et al., 2002, Vandamme et al., 1997, Vandamme et al., 2000, Vanlaere et al., 2009, Vanlaere et al., 2008).

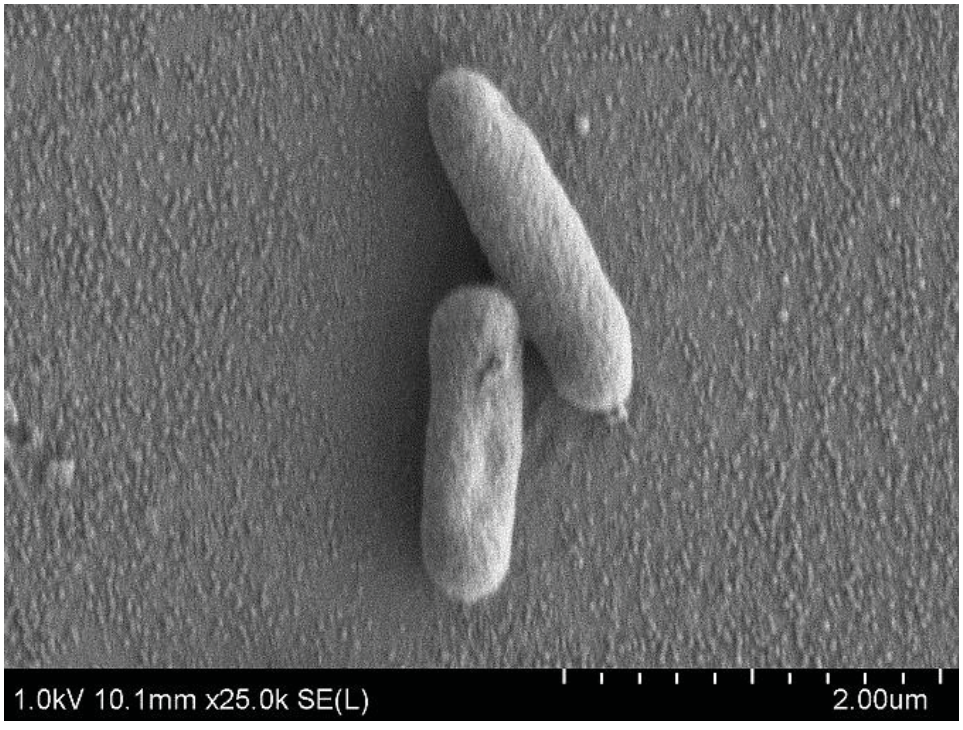

Figure 3.3: SEM image of MSh1. Image was taken under 25000× magnification at 1 kV. The bacterial cells sizes are approximately 0.6–0.8 µm × 1.6–2.1 µm.

3.3.5 Chemotaxonomic characterization

Chemotaxonomic analysis revealed the major cellular fatty acids were $C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%) and $C_{19:0}$ cyclo ω8c (16.1%).The fatty acid profile of MSh1 was consistent with these of closely related type strains such as *B. arboris* R-24201, *B. cenocepecia* J2315 and *B. lata* 383 which contained fatty acid $C_{16:0}$ (28.2%–36.5%), $C_{17:0}$ cyclo (22.5%–26.1%) and $C_{19:0}$ cyclo ω8c (13.7%–19.8%) (Table 3.1). The ubiquinone Q-8 was detected. The polar lipids consisted phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG), aminolipid and aminophospholipid (Figure 3.4). The fatty acid profile, polar lipid profile and major isoprenoid quinone of MSh1 were consistent with *Burkholderia* type strains (Gillis et al., 1995).

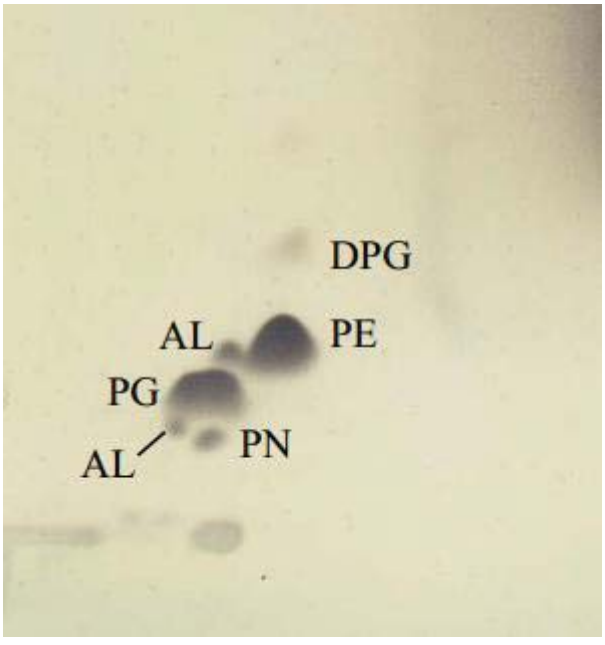

Figure 3.4: Thin layer chromatogram of polar lipid detected in MSh1. AL: aminolipid; PN: aminophospholipid; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; DPG: diphosphatidylglycerol.

Table 3.1: Differentiation characteristics of MSh1 and type strains of closely related species of Bcc.

Species: 1, MSh1; 2, B. *arboris* R-24201; 3. *B. cenocepacia* J2315; 4, *B. lata* 383; 5, *B. cepacia*; 6, *B. stabilis*; 7, *B. vietnamiensis*; 8, *B. dolosa*; 9, *B. ambifaria*; 10, *B. anthina*; 11, *B. pyrrocinia*, 12, B. *ubonensis*; 13, *B. latens*; 14, *B. diffusa*; 15, *B. seminalis*; 16, *B. metallica*; 17, *B. contaminans*; 18; *B. multivorans*; 19, *B. pseudomultivorans*; 20, *B. gladioli*

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Acidification of:																				
Sucrose	+	+	+	-	+	-	+	-	+	v	+	+	+	+	+	+	+	-	-	-
Adonitol	-	+	+	+	v	+	-	+	+	v	+	-	+	v	+	+	+	+	+	+
Assimilation of:																				
N-acetyl-glucosamine	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enzymatic activity of:																				
Arginine decarboxylation	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Lysine decarboxylation	+	+	+	+	+	+	+	-	+	v	+	-	+	+	v	+	+	v	+	-
β-galactosidase	-	+	+	-	+	-	+	+	+	v	+	-	+	+	+	+	+	+	+	+
Fatty acid content:																				
$C_{16:0}$ (%)	31.7	29.8	36.5	28.2	26.8	25.6	19.5	26.1	26.3	28.4	22.9	16.6	17.8	21.2	28.1	26.4	21.9	28.9	20.9	29.0
$C_{17:0}$ cyclo (%)	26.6	22.5	26.1	23.5	17.9	17.8	14.0	16.0	11.3	4.6	13.5	5.7	12.6	1.6	11.7	10.4	5.2	18.2	8.0	17.2
$C_{19:0}$ cyclo ω8c (%)	16.5	13.7	17.6	19.8	12.5	15.3	5.8	14.8	4.8	1.3	4.7	2.4	9.5	0.4	1.6	3.0	1.8	9.7	4.2	10.0

Data for MSh1, *B. arboris* R-24201, *B. cenocepacia* J2315 and *B. lata* 383 are from this study. Data for the other Bcc species were obtained from Coenye et al. (2001), Henry et al. (2001), Peeters et al. (2013), Vandamme et al. (2002), Vandamme et al. (1997), Vandamme et al. (2000), Vanlaere et al. (2009) and Vanlaere et al. (2008). Data for fatty acid content are percentages of whole-cell fatty acid content. +, >90% of all isolate positive; v, 10-90% position; -, <10% of strains positive.

3.3.6 GGDC and ANI

GGDC is an *in-silico* genome-to-genome comparison tool used to calculate the intergenomic distances and relatedness of MSh1 with known type strains (Meier-Kolthoff et al., 2013). The data obtained is converted to similarity values analogous to DDH hence similarity value of 70% is generally regarded as a threshold for new species determination (Meier-Kolthoff et al., 2013). It was found that the similarity values between MSh1 with related Bcc species are less than 70%, thus MSh1 may represent a novel species. This is further supported by the ANI results which revealed MSh1 had ANI value lower than the 96% threshold for new species (Goris et al., 2007) (Appendix 1; Table A1.3).

3.3.7 Genome analysis of secondary metabolites gene clusters using antiSMASH

Antimicrobial compounds produced by bacteria are usually secondary metabolites which are regulated by biosynthetic gene clusters. The gene clusters can be annotated using antiSMASH 2.0 (Blin et al., 2014, Blin et al., 2013). Analysis showed the genome of MSh1 had 43 gene clusters responsible for the biosynthesis of secondary metabolites which include the polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), bacteriocin and terpene biosynthetic genes. MSh1 (43 gene clusters) possesses a higher number of biosynthetic gene clusters when compared to other related *Burkholderia* species (9 to 25 gene clusters) despite having a similar genome size (Table 3.2). This may indicate some of the putative biosynthetic gene clusters may be new as antiSMASH was unable to annotate due to the lack of similarity between query sequences from the database. The results revealed the antimicrobial compounds produced by MSh1 may either be new or under-studied.

Table 3.2: Comparison of gene clusters responsible for secondary metabolites biosynthesis between MSh1 with related *Burkholderia* species.

Burkholderia species	Genome size (Mbp)	PKS	NRPS	Hybrid	Bacteriocin	Putative	Others	Total
Burkholderia sp. MSh1	8.63	1	1	1	4	29	7	43
B. ambifaria AMMD	7.64	1	2	2	2	0	12	19
B. cenocepacia J2315	8.06	1	3	0	1	0	8	13
B. cepacia GG4	6.47	1	1	0	1	0	5	8
B. lata 383	8.68	1	2	0	2	0	8	13
B. multivorans ATCC 17616	7.01	1	1	0	1	0	10	13
B. pyrrocinia CH-67	8.04	1	3	2	3	0	10	19
B. ubunensis Bu	6.93	0	4	1	3	0	17	25
B. vietnamiensis G4	8.39	0	1	0	1	0	7	9

PKS: polyketide synthase; NRPS: non-ribosomal polyketide synthethase; hybrid: combination of PKS and NRPS; putative: no annotation available; others: terpene, ectoine, butyrolctone, phenazine, phosphonate and hserlactone.

The genus *Burkholderia* is well known for producing a wide range of secondary metabolites which include siderophores (Asghar et al., 2011), antifungal (Lu et al., 2009, Tawfik et al., 2010), antibacterial (El-Banna and Winkelmann, 1998, Mitchell et al., 2008, Mitchell and Teh, 2005), antitumor (He et al., 2014, Klausmeyer et al., 2011) and exotoxins (Jeong et al., 2003, Partida-Martinez and Hertweck, 2007). Many of these secondary metabolites are regulated by complex synthesis mechanisms within the bacteria itself which include the polyketide synthase (PKS) and non-ribosomal polyketide synthase (NRPS). The assembly of a polyketide via the PKS system begins by priming the starter molecule to a catalytic domain, followed by

chain elongation with extender units by different kind of enzymes. As for the NRPS system, a NRP will firstly be generated and then linked with other molecules for instance a phosphate group, methyl group or fatty acids (Amoutzias et al., 2008, Donadio et al., 2007).

It was found that PKS and NRPS gene clusters are present in the genome of MSh1, indicating the antimicrobial compounds produced might be assembled by either of these two gene clusters. Moreover, MSh1 may be able to produce a more complex antimicrobial compound as a hybrid PKS-NRPS was annotated by antiSMASH. One example of such an antimicrobial compound is a lipopeptide class compound (Sorensen et al., 2014). Lipopeptides are antimicrobial compounds, known to disrupt the surface membrane charges of its target. There have been only two reported lipopeptides (burkholdine and occidiofungin) produced by Bcc species and they only possess antifungal activity (Lu et al., 2009, Tawfik et al., 2010). Bacteriocins are small peptides with narrow-spectrum antimicrobial properties (Cotter et al., 2013). Capistruin, is an example of antimicrobial peptide produced by a *Burkholderia* species. The antimicrobial activity is however only limited to several gram-negative bacteria which include *Burkholderia* species, *Psuedomonas aeruginosa* and *Escherichia coli* (Knappe et al., 2008). This suggests MSh1 may be producing a potentially new lipopeptide or bacteriocin that can target bacteria and yeast. The data obtained from antiSMASH provided a preliminary insight into the secondary metabolite gene clusters present in the genome of MSh1 and it is certain that MSh1 can indeed produce antimicrobial compounds. As many secondary metabolite biosynthetic gene clusters were present in the genome, the broad-spectrum activity observed might be due to the presence of multiple antimicrobial compounds. Therefore extraction of the antimicrobial compounds produced by MSh1 was performed in the subsequent chapter.

3.4 Conclusions

A novel gram-negative rod-shaped bacterium, designated MSh1, was isolated from Southeast Pahang tropical peat swamp forest soil in Malaysia and characterized using a polyphasic taxonomy approach. The predominant cellular fatty acids (>10.0%) were $C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%) and $C_{19:0}$ cyclo ω8c (16.1%). The polar lipids detected were phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The predominant ubiquinone was Q-8. The results confirmed MSh1 belongs to the genus *Burkholderia*. MSh1 can be differentiated from other Bcc species by phylogenetic analysis of 16S rRNA gene sequence, MLSA, ANI and several biochemical tests. DNA-DNA relatedness values between MSh1 and closely related type strains were below the 70% threshold value. Based on this polyphasic study, it can be concluded MSh1 represents a novel species within the Bcc, for which the name *Burkholderia paludis* sp. nov. is proposed. The type strain is MSh1 (=DSM 100703 =MCCC 1K01245).

Chapter 4:

Optimization of antimicrobial compounds produced by *Burkholderia paludis* MSh1

The work presented in this chapter represents the following manuscript submitted for peer-review:

Ong, K. S., Cheow, Y. L. and Lee, S. M. (submitted). Optimization of anti-enterococcal compounds produced by a novel bacterium, *Burkholderia paludis* MSh1, towards the improvement of its therapeutic potential.

4.1 Introduction

Antimicrobial compounds produced by microbes are usually secondary metabolites which are non-essential for their growth or reproduction but provide diverse survival functions in nature (Martin et al., 2005, Sanchez et al., 2010, Yim et al., 2007). The production of secondary metabolites can be manipulated by several factors such as nutrient requirements and physical parameters (Wang et al., 2008). Different bacteria have different preference for nutrient sources in producing antimicrobial compounds. For instance, glycerol promotes the production of antimicrobial compounds in *Xenohabdus bovienii*, but represses the production of an antimicrobial compound (carbapenem) in *Erwinia caratovora* (Sanchez et al., 2010, Wang et al., 2011). The phenomenon is highly dependent on the ability of the bacterial cells to metabolize different nutrient sources which might up-regulate or down-regulate essential genes needed for the biosynthesis of antimicrobial compounds (Sanchez et al., 2010). Hence, an accurate selection of nutrients required for the production of antimicrobial compounds is essential.

The selection of nutrients usually involves carbon sources, nitrogen sources and essential elements such as mineral salts. These nutritional requirements can be manipulated by either conventional methods or statistical methods (Singh et al., 2011). Conventional method, also known as one-factor-at-a-time (OFAT) experiment involves changing one independent factor at a time whilst keeping other factors at a constant level (Irfan et al., 2014, Xu et al., 2003). The approach is simple and essentially selects the significant factors affecting the production of antimicrobial compounds. However, the approach is time-consuming as the number of runs will increase with increasing number of factors. Therefore to screen for multiple factors within a

short duration with lower total number of runs, statistical methods are commonly utilized (Chauhan et al., 2007, Srinivas et al., 1994).

Different types of statistical methods can be applied for such optimization experiments, for instance the Plackett-Burman design (PBD) (Hassan and Schrum, 1994). PBD is a two level fractional factorial design, frequently used to screen for main factors from a large number of factors, in a k + 1 experiments (where k is the total number of factors). The design is orthogonal in nature, providing pure effect of each factor as there will be no confounding interactions among the factors (Chauhan et al., 2007, Krishnan et al., 1998, Reddy et al., 2008). To analyze multiple factors in a shorter duration, PBD has been adopted in this study.

Scaling-up of antimicrobial compounds production is governed not only by nutrients, but also physical parameters such as incubation duration, incubation temperature, initial pH of culture medium, agitation speed and inoculum size (El-Hadi et al., 2014, Irfan et al., 2014, Song et al., 2012). Therefore, optimal conditions for expression of high antimicrobial activity must firstly be determined in laboratory scale via flask level fermentation process followed by pilot- and industrial-level fermenters.

A number of reports have already been published regarding the role of different nutrients on enzyme (lipase) production by *Burkholderia* species (Gupta et al., 2007, Shu et al., 2012), but there is no published study regarding the effect of nutritional and physical parameters on the antimicrobial activity of any *Burkholderia* species. Thus, the objective of this Chapter was to optimize the antimicrobial activity of *B. paludis* MSh1 through statistical (PBD) and conventional (one-factor-at-a-time; OFAT) approach at flask level fermentation.

4.2 Material and Methods

4.2.1 Bacterial isolates and growth conditions

B. paludis MSh1 was maintained on NA (Merck, Germany) at 30°C aerobically for 24 hours aerobically; while *E. faecalis* ATCC 700802 (a vancomycin-resistant isolate) was maintained on tryptone soy agar (TSA) (Merck, Germany) at 37°C aerobically for 24 hours. *B. paludis* MSh1 and *E. faecalis* ATCC 700802 were kept in 25% (v/v) glycerol (Merck, Germany) in NB and TSB respectively, at -80°C for long term preservation.

4.2.2 Inoculum preparation

The inoculum was prepared by inoculating pure culture of *B. paludis* MSh1 from NA into 20 mL of NB (Merck, Germany) in a 100 mL Erlenmeyer flask and incubated at 30°C aerobically for 24 hours, at 200 rpm (Smith A3555, Progressive Scientific). Cells were harvested by centrifugation at 15000 × *g* for 10 min in a bench-top centrifuge (Eppendorf, 5810R). The pellet was resuspended in sterile distilled water and adjusted to 0.5 McFarland standard at OD_{625} (~10^8 CFU/mL) using a light spectrophotometer (Prim Light, Secomam). Two hundred µL of the adjusted culture was inoculated into 19.80 mL of fermentative media (prepared in subsequent experiments) with final adjusted inoculum ~10^6 CFU/mL.

4.2.3 Experimental design

4.2.3.1 Plackett-Burman design (PBD)

For the selection of significant factors for antimicrobial compounds production, a variety of mineral salts, carbon sources, nitrogen sources, and cultivation factors were tested and

identified via the PBD experiment. A total of 19 factors were included for selection, with each factor represented at two levels (Table 4.1).

Table 4.1: Nineteen experimental factors at two levels used to determine the antimicrobial activity of *B. paludis* MSh1 using PBD.

Factors	Medium components	Units	+ Values	- Values	Supplier
X1	Manganese chloride	g/L	0.050	0.005	ICN Biomedical, USA
X2	Magnesium chloride	g/L	0.200	0.020	R&M Chemicals, Malaysia
X3	Iron sulphate	g/L	0.050	0.005	Friendemann Schmidt, UK
X4	Potassium hydrogen phosphate	g/L	2.000	0.200	Friendemann Schmidt, UK
X5	Potassium dihydrogen phosphate	g/L	0.250	0.025	J. Kollin Chemical, USA
X6	Sodium chloride	g/L	5.000	0.500	Fisher Scientific, USA
X7	Calcium chloride	g/L	5.000	0.500	R&M Chemicals, Malaysia
X8	Copper sulphate	g/L	0.050	0.005	Systerm, Malaysia
X9	Zinc sulphate	g/L	0.050	0.005	Friendemann Schmidt, UK
X10	Glucose	g/L	15.000	5.000	Merck, Germany
X11	Glycerol	g/L	15.000	5.000	R&M Chemicals, Malaysia
X12	Beef extract	g/L	15.000	5.000	Oxoid, UK
X13	Peptone	g/L	15.000	5.000	Becton Dickinson, USA
X14	Incubation duration	days	4	2	-
X15	Incubation temperature	°C	37	30	-
X16	Initial pH	pH	8	6	-
X17	Agitation	rpm	150	0	-
X18	Flask size	mL	250	100	-
X19	Inoculum size	% (v/v)	5	1	-

The type and concentration of each factor were selected and modified based on studies conducted by Chauhan et al. (2007), Krishnan et al. (1998) and Reddy et al. (2008). Table 4.2 represents the PBD for 20 runs with two levels of concentrations for each factor. Each run was performed in triplicate (n = 3). The effects and significance of each factor on antimicrobial activity were analyzed and determined using the program Minitab 17 (Minitab, Inc. State College, Pennsylvania). If the factors showed significance ($P < 0.05$) and its effect was negative, it indicated that the factor had significant effect on the antimicrobial production at the lower level in PBD experiment. Conversely, if the effect was positive, the antimicrobial activity is affected by the factor at higher level (Chauhan et al., 2007, Reddy et al., 2008).

4.2.3.2 Optimization of nutritional and physical parameters using one-factor-at-a-time (OFAT) experiments

The significant factors which were determined using PBD were subjected to OFAT experiments. In the conventional optimization approach, different nutritional and physical parameters were optimized by maintaining all factors at a constant level in the basal medium, except the one under study. The carbon source, nitrogen sources and physical parameters were selected based on studies conducted by El-Hadi et al. (2014), Irfan et al. (2014), Song et al. (2012) and Wang et al. (2011). The carbon sources tested were glucose (Merck, Germany), galactose (ACROS organics, USA), fructose (Fisher Scientific, USA), xylose (Merck, Germany), dextrose (Oxoid, UK), maltose (Fisher Scientific, USA), sucrose (Fisher Scientific, USA), lactose (Merck, Germany), glycerol (R&M Chemicals, Malaysia), xylitol (ACROS organics, USA), sorbitol (BioBasic, Canada), starch (Fisher Scientific, USA), xylan (Sigma-Aldrich, USA) and cellulose (ACROS organics, USA) at 5 g/L. For the effect of different nitrogen sources,

peptone (Becton Dickinson, USA), tryptone (Becton Dickinson, USA), casein (ACROS organics, USA), beef extract (Oxoid, UK), yeast extract (Becton Dickinson, USA), malt extract (Oxoid, UK), urea (Merck, Germany), sodium nitrate (J. Kollin Chemical, USA), ammonium chloride (Fisher Scientific, USA), ammonium sulphate (R&M Chemicals, Malaysia), ammonium hydrogen phosphate (R&M Chemicals, Malaysia), ammonium acetate (Merck, Germany), ammonium citrate (Merck, Germany) and ammonium nitrate (Duchefa Biochemie, Netherlands) were studied at 15 g/L. After optimization of carbon and nitrogen sources along with their concentrations (5–30 g/L), various concentration levels of minerals (0.5–5% v/v) were also studied. Agitation speed (0–250 rpm), incubation duration (24-144 hours), incubation temperature (25–37°C), initial pH of the medium (4.0–9.0; adjusted with 1 M hydrochloric acid [R&M Chemicals, Malaysia] or sodium hydroxide [R&M Chemicals, Malaysia] before sterilization) and inoculum size (0.5–5% v/v) were the physical parameters studied for their effect on bacterial growth and antimicrobial activity. Each experiment was performed in triplicate (n = 3).

4.2.4 Assay for antimicrobial activity

The antimicrobial activity was measured by disc-diffusion assay based on the method described by Wang et al. (2011), with slight modifications. *E. faecalis* ATCC 700802 was used as the test microorganism for the entire optimization study because the isolate was shown to be more susceptible to the antimicrobial compounds produced by *B. paludis* MSh1 (Ong et al., 2015). Briefly, 100 µl of 0.5 McFarland (OD_{625} 0.08–0.11) adjusted culture in tryptone soy broth (TSB) (Merck, Germany) was spread plated on TSA. One mL of culture supernatant of *B. paludis* MSh1 was centrifuged at 15000 × *g* for 10 min to remove the pellet and subjected to

centrifugal evaporator (Eppendorf, 5418R) to concentrate the culture supernatant. The dried extract was reconstituted in sterile distilled water to obtain 10 mg/mL extract. One hundred µL of the extracts were placed on 6 mm Whatman disk filter and air dried. The dried discs were placed on the TSA inoculated with *E. faecalis* ATCC 700802 and incubated at 37°C aerobically for 24 hours. The antimicrobial activity was determined by measuring the zones of inhibition (from the edge of the disc to the margin of the zone of inhibition). Antimicrobial activity was expressed as units of activity per mL supernatant of the cultures, where 1 U was defined as a 1.0 mm annular clearing around the disc × 10 (dilution factor) (Wang et al., 2011). The increase in zone of inhibition (antimicrobial activity) is proportional to the increase in antimicrobial production (Maxwell et al. 1994). Fifty µg/mL of chloramphenicol (Calbiochem, Malaysia) was used as a positive control; while sterile distilled water was used as a negative control.

4.2.5 Measurement of growth

The bacterial cell growth of *B. paludis* MSh1 was measured by optical density at 625 nm and the biomass concentrations [dry cell weight (DCW): g/L] were determined using a calibration curve. Fixed volume biomass suspension dilutions were centrifuged at 15000 × *g* for 10 min (Eppendorf, 5810R). The cell pellets were resuspended in sterile distilled water and the optical densities were measured at 625 nm. The biomass suspensions were subjected to centrifugation at 15000 × *g* for 10 min, and the cell pellets were dried at 60°C for 24 hours. The calibration curve was then plotted using the optical density at 625 nm and biomass concentration (DCW: g/L) (Appendix 2; Figure A2.1).

4.2.6 Statistical analysis

Regression analysis on the 19 factors (used in the PBD experiments) affecting the antimicrobial activity of *B. paludis* MSh1 was determined using Minitab 17. Statistical analysis to determine significance of results for the OFAT experiments and validation of experimental design were performed using one way analysis of variance (ANOVA) followed by Tukey's test and independent-sample *t*-test, respectively at the significance level of $\alpha = 0.05$. Statistical analysis was performed using IBM SPSS Statistics 20. Experiments were conducted in triplicate and the results were expressed as averages ± standard deviation followed by corresponding letters which indicate the significant differences ($P < 0.05$).

4.3 Results and discussion

4.3.1 Effect of different factors on antimicrobial activity (Plackett-Burman design)

The antimicrobial activity and DCW of each flask are shown in Table 4.2. The factors were screened at the confidence level of $P = 0.05$ on the basis of their effects. The results obtained from the PBD are summarized in Table 4.3.

Peptone (nitrogen source) and glycerol (carbon source) significantly enhanced the antimicrobial activity of *B. paludis* MSh1. Among the phosphate sources used, both phosphates (potassium hydrogen phosphate and potassium dihydrogen phosphate) were not significant on antimicrobial production. Manganese chloride, iron sulphate, calcium chloride, sodium chloride and zinc sulphate were significant ($P < 0.05$) with positive effect in affecting the antimicrobial activity of *B. paludis* MSh1 against *E. faecalis* ATCC 700802, thus its higher concentration will be used for further optimization studies. Copper sulphate was significant ($P < 0.05$) with negative effect on antimicrobial activity; therefore its lower concentration will be used for further optimization studies in the future. Magnesium chloride was found to be insignificant hence will be eliminated from further study. Trace elements that showed significant effect on the antimicrobial activity were reported to be essential for bacteria survival as they could reduce oxidative stress by regulating the biosynthesis of superoxide dismutase and act as co-factors for several crucial metabolic enzymes (Bunch and Harris, 1986, Hassan and Schrum, 1994). Apart from the media components, physical parameters such as incubation duration, incubation temperature, initial pH, agitation and inoculum size were significant ($P < 0.05$) on the antimicrobial production (Table 4.3).

Table 4.2: The observed antimicrobial activity and DCW of 20 runs of experiment with two levels of concentration for each factor.

Run order	Factors																			Antimicrobial activity ± SD (U/mL)	DCW ± SD (g/L)
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17	X18	X19		
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	43.33 ± 5.77	1.613 ± 0.173
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	6.67 ± 5.77	0.763 ± 0.097
3	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	40.00 ± 10.00	2.139 ± 0.067
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	53.33 ± 5.77	1.442 ± 0.015
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	16.67 ± 5.77	1.188 ± 0.058
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	6.67 ± 5.77	0.835 ± 0.101
7	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	60.00 ± 10.00	1.702 ± 0.157
8	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	10.00 ± 10.00	1.158 ± 0.115
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	13.33 ± 5.77	0.662 ± 0.045
10	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	123.33 ± 5.77	2.306 ± 0.061
11	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	70.00 ± 10.00	1.979 ± 0.171
12	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	6.67 ± 5.77	1.226 ± 0.105
13	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	43.33 ± 5.77	2.084 ± 0.155
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	103.33 ± 5.77	2.398 ± 0.045
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	6.67 ± 5.77	1.616 ± 0.158
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	6.67 ± 5.77	1.105 ± 0.132
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	6.67 ± 5.77	0.958 ± 0.127
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	126.67 ± 5.77	2.126 ± 0.096
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	6.67 ± 5.77	0.882 ± 0.028
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	16.67 ± 5.77	0.563 ± 0.078

Example of preparation for Run 1: Manganese chloride (X1: 0.050 g/L), magnesium chloride (X2: 0.020 g/L), iron sulphate (X3: 0.050 g/L), potassium hydrogen phosphate (X4: 2.000 g/L), potassium dihydrogen phosphate (X5: 0.025 g/L), sodium chloride (X6: 0.050 g/L), calcium chloride (X7: 0.050 g/L), copper sulphate (X8: 0.005 g/L), zinc sulphate (X9: 0.050 g/L), glucose (X10: 5.000 g/L), glycerol (X11: 15.000 g/L), beef extract (X12: 5.000 g/L), peptone (X13: 15.000 g/L), incubation duration (X14: 4 days), incubation temperature (X15: 37°C), initial pH (X16: pH 8), agitation (X17: 0 rpm), flask size (X18: 100 mL) and inoculum size (X19: 1 % v/v).

Table 4.3: Estimated effect, regression coefficient, standard error (SE) of regression coefficient, *t*-values, *P*-values and significance of 19 factors on the antimicrobial activity of *B. paludis* MSh1.

Factors	Effect	Coefficient	SE Coefficient	*t*-value	*P*-value	Significant
X1: Manganese chloride	4.67	2.33	0.89	2.62	0.012	Yes
X2: Magnesium chloride	0.00	0.00	0.89	0.00	1.000	No
X3: Iron sulphate	15.33	7.67	0.89	8.61	<0.001	Yes
X4: Potassium hydrogen phosphate	0.67	0.33	0.89	0.37	0.710	No
X5: Potassium dihydrogen phosphate	2.00	1.00	0.89	1.12	0.268	No
X6: Sodium chloride	6.67	3.33	0.89	3.75	<0.001	Yes
X7: Calcium chloride	22.67	11.33	0.89	12.73	<0.001	Yes
X8: Copper sulphate	-32.67	-16.33	0.89	-18.35	<0.001	Yes
X9: Zinc sulphate	30.00	15.00	0.89	16.85	<0.001	Yes
X10: Glucose	33.30	1.67	0.89	1.87	0.069	No
X11: Glycerol	-10.67	-5.33	0.89	-5.99	<0.001	Yes
X12: Beef extract	2.00	1.00	0.89	1.12	0.268	No
X13: Peptone	39.33	19.67	0.89	22.10	<0.001	Yes
X14: Incubation duration	9.33	4.67	0.89	5.24	<0.001	Yes
X15: Incubation temperature	-29.33	-14.67	0.89	-16.48	<0.001	Yes
X16: Initial pH	-12.67	-6.33	0.89	-7.12	<0.001	Yes
X17: Agitation	10.00	5.00	0.89	5.62	<0.001	Yes
X18: Flask size	2.67	1.33	0.89	1.50	0.142	No
X19: Inoculum size	-20.00	-10.00	0.89	-11.24	<0.001	Yes

Factors that show significance (P < 0.05) with negative value, indicates that the factor has significant effect on the antimicrobial production at lower level in PBD experiment. On the other hand, if the effect is positive, the antimicrobial activity is affected by the factor at higher level.

Based on Table 4.2, run number 18 showed maximum antimicrobial activity as compared to the other runs (Table 4.2). It was observed that the antimicrobial activity of *B. paludis* MSh1 against *E. faecalis* ATCC 700802 increased proportionally with the DCW, indicating that the production of antimicrobial compounds may be influenced by the amount of bacterial cells present in the culture medium.

This study demonstrated that the antimicrobial activity of *B. paludis* MSh1 can be affected by different nutritional components. As shown in Table 4.3, the carbon source glycerol was found to be significant, while glucose was found to be insignificant which suggests catabolite repression on the production of antimicrobial compounds. It was also found peptone, but not beef extract significantly increased the antimicrobial activity of *B. paludis* MSh1 against *E. faecalis* ATCC 700802. A similar result was obtained by Wang et al. (2011), in which *Xenorhabdus bovienii* prefers soytone and glycerol as the nitrogen and carbon source over a selection of different sources in improving the antimicrobial activity against *Bacillus cereus*. Mao et al. (2007) reported peptone and glycerol affect the production of candicidin by a *Streptomyces* species using a statistical approach. These studies support the importance of selecting appropriate media components for the production of microorganism secondary metabolites (antimicrobial compounds).

4.3.2 Optimization of nutritional and physical parameters via OFAT experiments

OFAT experiments were performed to further optimize the carbon and nitrogen sources that could significantly increase the antimicrobial activity of *B. paludis* MSh1. Maximum antimicrobial activity (96.67 ± 5.77 U/mL) and DCW was observed with glycerol after four days of incubation (Figure 4.1A and Figure 4.1B). Other carbon sources showed lower antimicrobial activity: dextrose (73.33 ± 5.77 U/mL) > sorbitol (53.33 ± 5.77 U/mL) > maltose (36.67 ± 5.77 U/mL) and cellulose (36.67 ± 5.77 U/mL). It was shown that the other carbon sources did not favor the production of antimicrobial compounds which contribute to the antimicrobial activity and this may be due to catabolite repression (Singh et al., 2017).

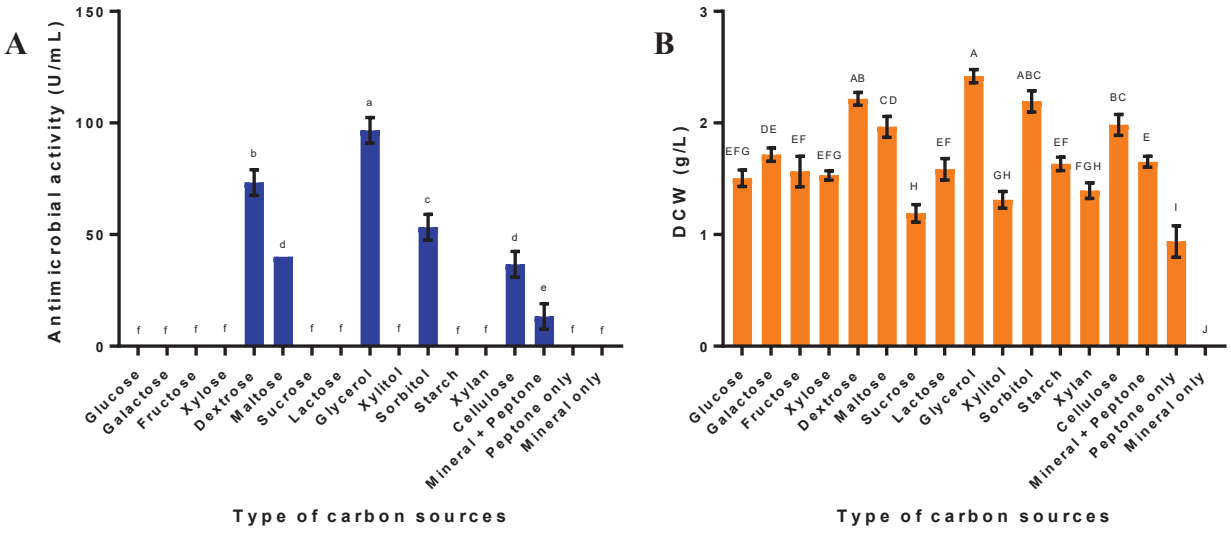

Figure 4.1: Effect of different carbon sources on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Controls: mineral and peptone only, peptone only, and mineral only.

The concentration of glycerol was further optimized from a range of 5 to 30 g/L. It was shown that there was no significant difference on the antimicrobial activity and DCW of *B. paludis* MSh1 (Figure 4.2A and Figure 4.2B) despite the range of concentration tested; hence the minimum concentration of 5 g/L glycerol was selected for further optimization due to cost.

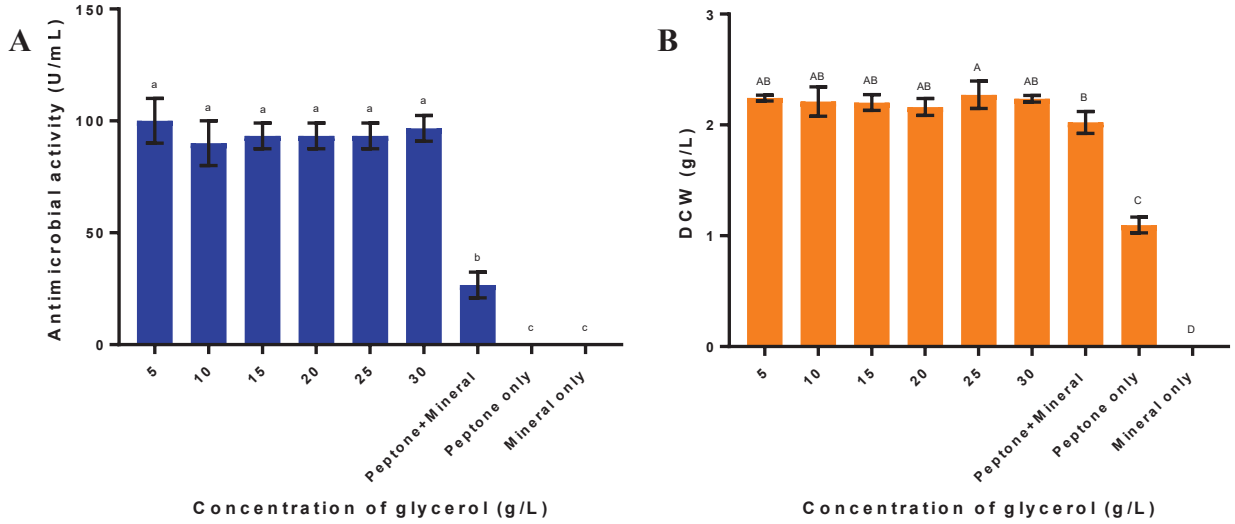

Figure 4.2: Effect of different concentrations of best carbon source (glycerol) on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Controls: peptone and mineral only, peptone only, and mineral only.

Each organic nitrogen source studied supported bacterial growth except for urea, while all inorganic nitrogen sources inhibited bacterial growth thus having no antimicrobial activity. Maximum antimicrobial activity can be observed for peptone (100.00 ± 10.00 U/mL), followed by tryptone (43.33 ± 5. 77 U/mL), malt extract (43.33 ± 5.77 U/mL) and beef extract (23.33 ± 5.77 U/mL) (Figure 4.3A). This indicates that one of the amino acids in peptone may be the precursor for the antimicrobial compounds of *B. paludis* MSh1. The DCW of *B. paludis* cultured in the media containing peptone was significantly higher when compared to the other organic nitrogen sources which include tryptone, casein, beef extract, yeast extract and malt extract (Figure 4.3B).

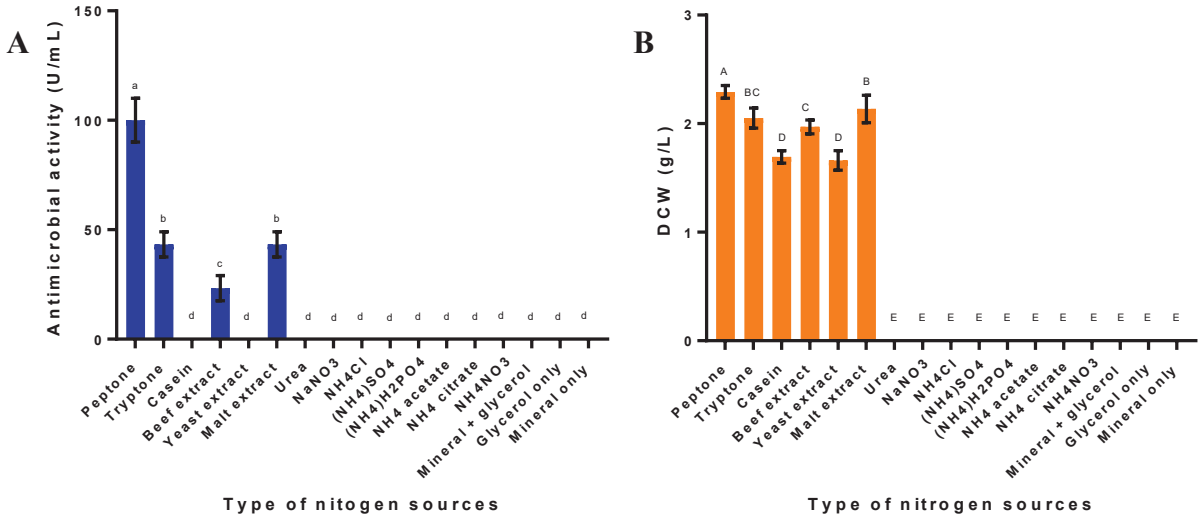

Figure 4.3: Effect of different nitrogen sources on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Controls: mineral and glycerol only, glycerol only, and mineral only.

Complex organic nitrogen sources are generally required by microbes to produce enzymes or antimicrobial compounds; however, the requirement of the specific nitrogen source varies from microbe to microbe. The concentration of peptone which yielded maximum antimicrobial activity and DCW was at 15 to 25 g/L (Figure 4.4A and Figure 4.4B). However it was observed that the antimicrobial activity was significantly reduced at 30 g/L of peptone. This may be due to the repression of the antimicrobial biosynthetic gene clusters when amino acids are in excess (Singh et al., 2017). Therefore, the lower concentration of peptone (15 g/L) was used for further optimization.

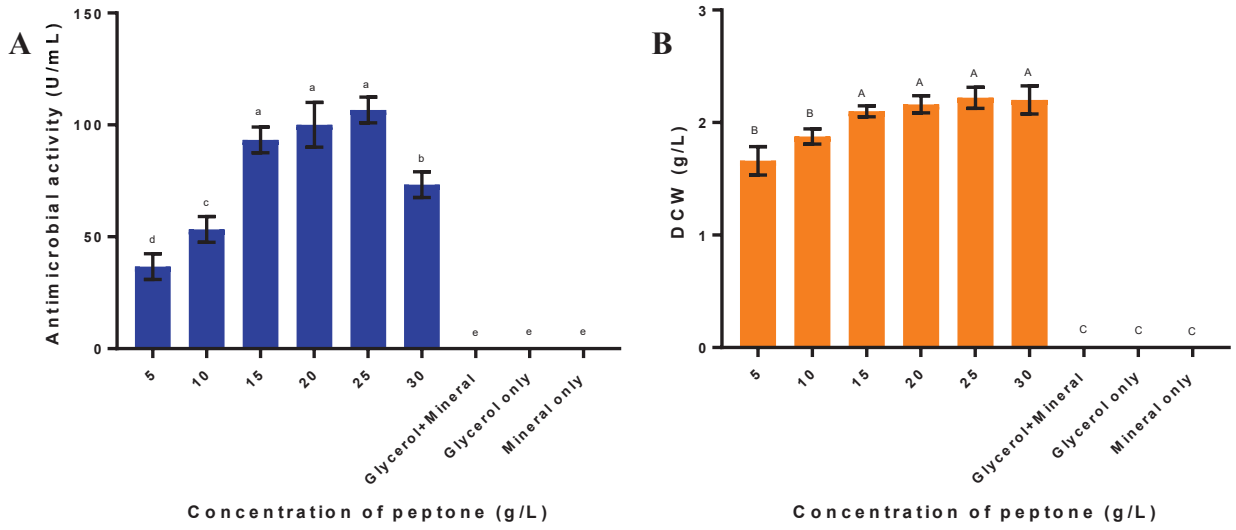

Figure 4.4: Effect of different concentrations of best nitrogen source (peptone) on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Controls: glycerol and minerals only, glycerol only, and mineral only.

The composition of minerals include manganese chloride (0.050 g/L), iron sulphate (0.050 g/L), sodium chloride (0.500 g/L), calcium chloride (0.500 g/L), copper sulphate (0.005 g/L) and zinc sulphate (0.050 g/L), as determined earlier by PBD. The maximum antimicrobial activity was achieved at 1% (v/v) minerals (93.33 ± 5.77 U/mL). As the concentration of minerals increase above 1.0% (v/v), the antimicrobial activity decreases (Figure 4.5A). The DCW of *B. paludis* MSh1 in 2.0% (v/v) and 2.5% (v/v) minerals was significantly higher than 2.5% (v/v) and 3% (v/v), but insignificant at 0.5–1.5% (v/v) (Figure 4.5B). Therefore 1.0% (v/v) minerals, which showed maximum antimicrobial activity with insignificant effect on the DCW of *B. paludis* MSh1, were used for subsequent optimization experiments.

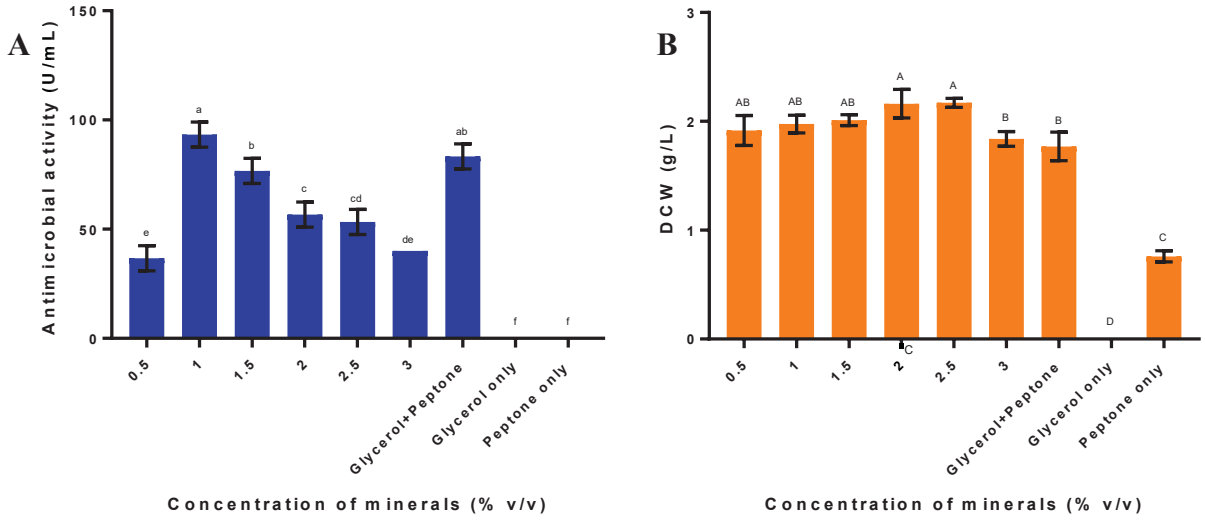

Figure 4.5: Effect of different concentrations of minerals on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Controls: glycerol and peptone only, glycerol only, and peptone only.

Apart from nutritional composition, physical parameters possess significant effect on the antimicrobial activity of *B. paludis* MSh1. For instance, the antimicrobial activity increased progressively and reached maximum antimicrobial activity at an agitation speed of 200 rpm (100.00 ± 10.00 U/mL). Any change in agitation speed (>200 rpm) decreased the antimicrobial activity of *B. paludis* MSh1 against *E. faecalis* ATCC 700802 (Figure 4.6A). With the increase of agitation speed, there may be an increase in aeration of culture media, which enhanced the supply of dissolved oxygen and metabolism of the bacterial cells. The decrease in antimicrobial activity at 250 rpm might due to compound degradation caused by mechanical damage (Singh et al., 2011). The DCW of *B. paludis* MSh1 was significantly higher at 250 rpm when compared to 0 and 50 rpm (Figure 4.6B). However, as the maximum antimicrobial activity of *B. paludis* MSh1 was observed at 200 rpm, this speed was therefore used for further optimization.

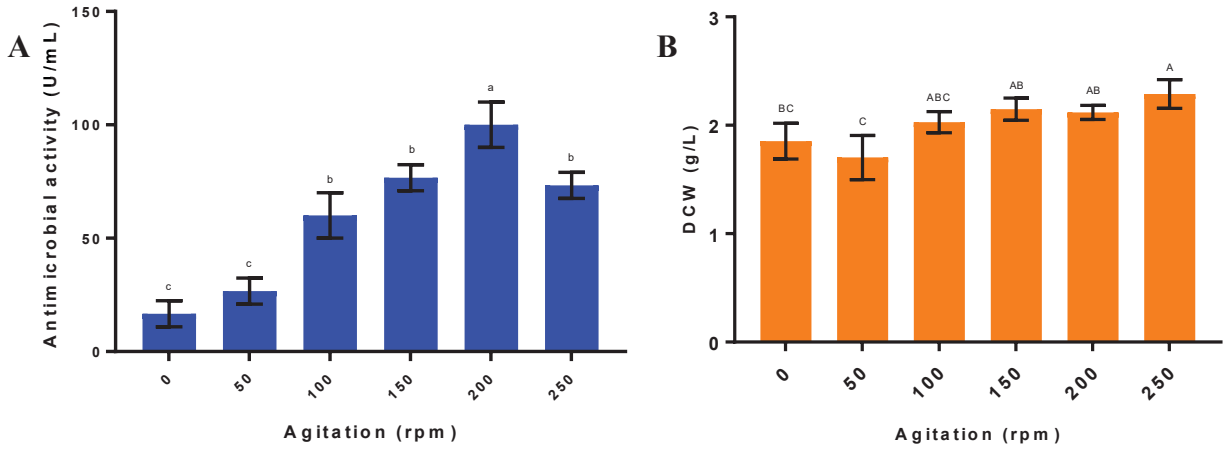

Figure 4.6: Effect of different agitation speed on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups. Control: 0 rpm (no agitation).

In any bioprocess, specific temperature requirement and fermentation duration is one of the most important physical parameters. The optimal incubation temperature for maximum antimicrobial activity was at 30°C. *B. paludis* MSh1 was able to grow from 25–37°C. However, positive antimicrobial activity was observed only from 27–35°C, with maximum antimicrobial activity at 30°C (96.66 ± 11.55 U/mL) (Figure 4.7A). The DCW of *B. paludis* MSh1 was significantly higher at 37°C when compared to 25–30°C (Figure 4.7B). Despite the high DCW, no antimicrobial activity was observed at 37°C, suggesting that temperature may repress the production of the antimicrobial compounds. Since *B. paludis* MSh1 showed the highest antimicrobial activity at 30°C, this temperature was kept constant for the following experiments.

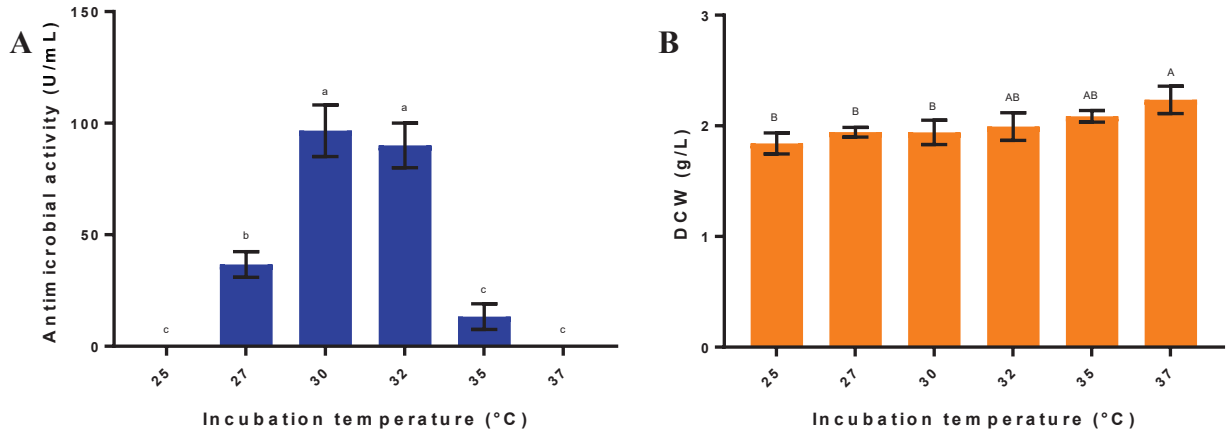

Figure 4.7: Effect of different incubation temperatures on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups.

The antimicrobial activity of *B. paludis* MSh1 was significantly higher at 96–144 hours when compared to 24–72 hours (Figure 4.8A). This is because the growth of *B. paludis* MSh1 was in its exponential phase up to 48 hours; thereafter entered the stationary phase at 72 hours (Figure 4.8B). This also indicated that the antimicrobial activity against *E. faecalis* ATCC 700802 increased proportionally with the DCW, and this is consistent with the results obtained earlier from the PBD experiments. Microorganisms will tend to produce compounds, such as enzymes and antimicrobial compounds at late exponential phase (El-Hadi et al., 2014, Irfan et al., 2014, Song et al., 2012).

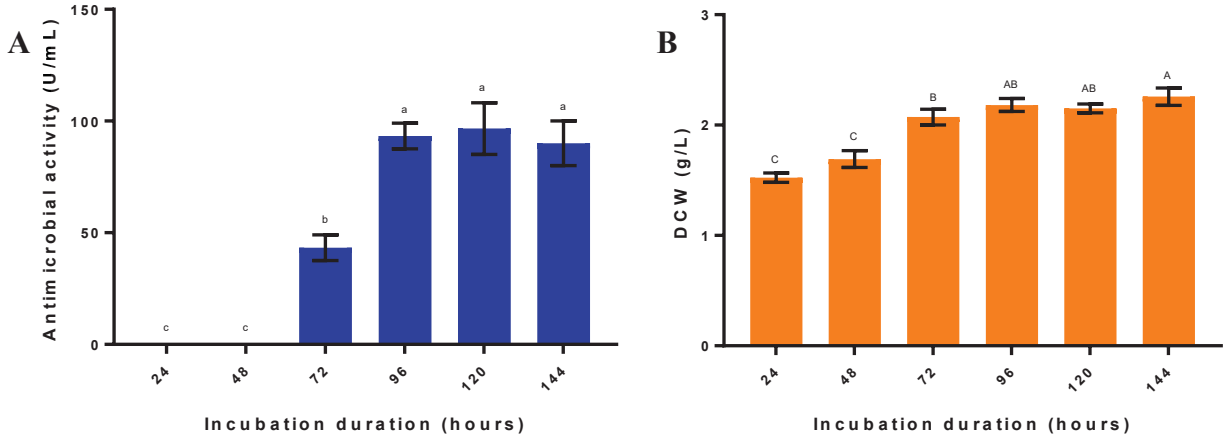

Figure 4.8: Effect of different incubation durations on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated at 30ºC at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups.

To determine the effect of different pH of the culture media on the antimicrobial activity of *B. paludis* MSh1, the culture media was adjusted to a range of pH from 4 to 9. *B. paludis* MSh1 was able to grow in the selected pH range (4.0–9.0), but its antimicrobial activity against *E. faecalis* ATCC 700802 was restricted to pH 5.0 to 8.0, with pH 6.0 (100.00 ± 10.00 U/mL) having the maximum antimicrobial activity (Figure 4.9A). It was observed that there was no antimicrobial activity at pH 4 and 9, and this might due to the poor bacterial growth (low DCW) which ultimately affects the production of antimicrobial compounds (Figure 4.9B).

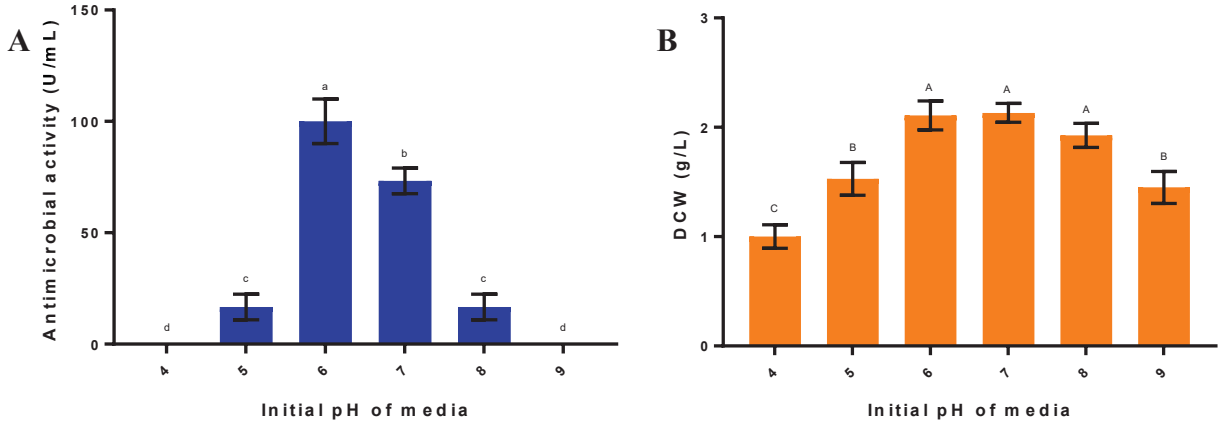

Figure 4.9: Effect of different initial pH of medium on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture (1% v/v inoculum size) was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask (n = 3). Different letters represent significant differences ($P < 0.05$) between groups.

As for the effect of inoculum size on the antimicrobial activity, 1.0% (v/v) inocula showed highest antimicrobial activity (100.00 ± 10.00 U/mL) (Figure 4.10A). The smaller inoculum size (0.5% v/v) showed significantly lower antimicrobial activity and this may be due to an increase in lag phase as lower count of bacteria were present to kick-start the process of antimicrobial production. Conversely high inoculum size (4.0–5.0% v/v) may be lead to improper inoculum to substrate ratio, causing nutritional imbalance which ultimately represses antimicrobial compounds production (El-Hadi et al., 2014). The DCW of *B. paludis* MSh1 with an inoculum size of 5.0% v/v was significantly higher than 0.5% v/v, but not significantly different when compared to 1.0–4.0% v/v (Figure 4.10B). As 1.0% v/v inoculum size of *B. paludis* MSh1 was the lowest concentration to give the maximum antimicrobial activity, it was thus used for subsequent experiments.

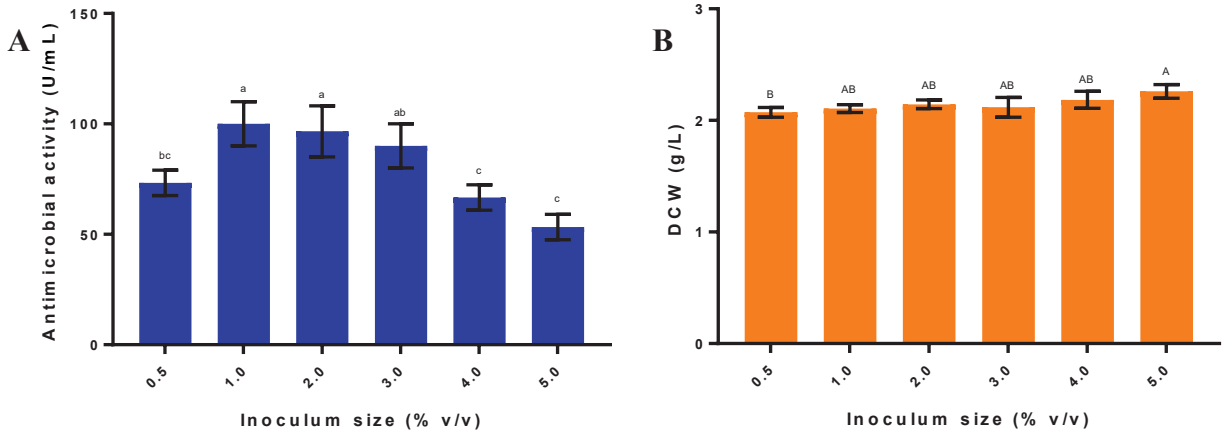

Figure 4.10: Effect of different inoculum sizes on the (A) antimicrobial activity and (B) DCW of *B. paludis* MSh1. The culture was incubated for four days (30ºC) at 200 rpm in a 100 mL Erlenmeyer flask containing media adjusted to pH 6 (n = 3). Different letters represent significant differences ($P < 0.05$) between groups.

Overall, the near-optimized culture media contained 5 g/L glycerol, 15 g/L peptone and 1% (v/v) minerals. The culture media was adjusted to pH 6 before sterilization, inoculated with 1% (v/v) bacterial culture, and incubated at 30°C for 96 hours (at 200 rpm) to achieve maximum antimicrobial activity. Validation of experimental design was accomplished by using the near-optimized culture media which gave 105.00 ± 5.47 U/mL of antimicrobial activity, as compared to using the conventional NB, which gave 23.33 ± 5.16 U/mL of antimicrobial activity against *E. faecalis* ATCC 700802. The antimicrobial activity was significantly increased by 4.4-fold ($P < 0.05$), indicating the optimized medium favors higher production of antimicrobial compounds.

4.4 Conclusions

Thirteen factors screened were found to be significant in increasing the antimicrobial activity of *B. paludis* MSh1 using the PBD. The optimized factors using OFAT experiments were found to be: glycerol (5 g/L), peptone (15 g/L), minerals (1 % v/v), agitation speed (200 rpm), incubation temperature (30°C), incubation duration (96 hours), initial culture media (pH 6) and inoculum size (1% v/v). The antimicrobial activity in the optimized media was significantly higher by 4.4-fold than the conventional NB against *E. faecalis* ATCC 700802.

Chapter 5:

Extraction, isolation and purification of antimicrobial compounds produced by *Burkholderia paludis* MSh1

The work presented in this chapter represents a part of the following peer reviewed publication (attached in Appendix 7b):

Ong, K. S., Aw. Y. K., Lee, L. H., Yule, C. M., Cheow, Y. L. and Lee, S. M. (2016). *Burkholderia paludis* sp. nov., an antibiotic-siderophore producing novel *Burkholderia cepacia* complex species, isolated from Malaysian tropical peat swamp soil. Frontiers in Microbiology 7: 2046.

5.1 Introduction

The misuse of antimicrobial compounds to treat infections has led to an increase in the prevalence of antimicrobial resistant bacteria (ARB), often associated with nosocomial (hospital acquired) infections (Mishra et al., 2012). Nosocomial infections constitute the sixth leading cause of death in the United States and more than 70% of nosocomial pathogens are resistant to at least one of the common antimicrobial drugs used (Iowa Department of Public Health). This is an alarming issue because ARB infections often result in increased morbidity and mortality, limited treatment options and cause increased healthcare costs (Ammerlaan et al., 2016). Thus there is a need for potentially useful antimicrobial compounds with antimicrobial activity to combat ARB infections.

One strategy to look for antimicrobial compounds is bioprospecting of novel microorganisms (Imhoff et al., 2011). Microorganisms thriving in extreme and isolated environments have the potential to produce antibiotics to gain an advantage in competing for resources and colonization of new habitats (Hibbing et al., 2010, Traxler and Kolter, 2015). As a result, tropical peat swamp forests in Malaysia were chosen as potential locations to prospect for microorganisms with the ability to produce antimicrobial compounds. Tropical peat swamp forests are unique wetland ecosystems characterized by their acidic (pH range of 2.9 to 4.5) and waterlogged conditions. They are ombotrophic hence receiving nutrients solely from rain and atmospheric deposition which results in an extremely nutrient poor environment (Yule, 2010). Despite being such a harsh environment, we successfully isolated an antimicrobial-producing bacterium which was identified as a novel species *Burkholderia paludis* (Ong et al., 2016, Ong et al., 2015).

Based on current literature, *Burkholderia* species are known to produce narrow-spectrum antimicrobial compounds such as iminopyrrolidines (produced by *Burkholderia plantari*) and occidiofungin (*Burkholderia contaminans*) which only targets gram-negative *Erwinia amylovora* and fungi respectively (Lu et al., 2009, Tawfik et al., 2010). However *B. paludis* MSh1 exhibit a different spectrum of antimicrobial activity, which showed antimicrobial activity against gram-positive bacteria, particularly strains of *Staphylococcus aureus* and *Enterococcus faecalis* (Ong et al., 2015). Thus the main objective of this Chapter was to extract, isolate and purify the antimicrobial compounds produced by *B. paludis* MSh1.

5.2 Material and Methods

5.2.1 Test microorganism isolates and culture conditions

Test microorganism isolates used in the study included *Bacillus cereus* ATCC 14579, *B. subtilis* ATCC 8188, *E. faecalis* ATCC 700802, *E. faecalis* ATCC 29212, *E. faecalis* JH-22, *S. aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213, *Aeromonas hydrophila* ATCC 49140, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Proteus mirabilis* ATCC 49140, *Proteus vulgaris* (Institute of Medical Research, Malaysia), *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas aeruginosa* ATCC BAA-47, *Salmonella* Typhimurium ATCC 14028 and *Shigella flexneri* ATCC 12022. Isolates were cultured on Muller Hinton broth (MHB) (Oxoid, UK) at 37°C aerobically and maintained at -80°C in MHB with 25% (v/v) glycerol.

5.2.2 Culture conditions and maintenance of *B. paludis* MSh1

B. paludis MSh1 was maintained on NA at 30°C aerobically and in 25% (v/v) glycerol in NB (Merck, Germany) at -80°C for long term preservation.

5.2.3 Preparation of crude cell-free supernatant

B. paludis MSh1 was first grown in the optimized media (containing 5 g/L glycerol, 15 g/L peptone and 1% v/v minerals) for four days at 30°C. The cultures were centrifuged at 8000 × *g* for 15 min to remove the bacterial cell pellet. The crude cell-free supernatant was concentrated under reduced pressure and subjected to sequential solvent fractionation.

5.2.4 Sequential solvent fractionation and purification of the crude extract

Sequential solvent fractionation was performed on the crude extract to fractionate the extract into different fractions with different polarity. The crude extract was extracted with hexane (HEX) (Merck, Germany), dichloromethane (DCM) (Merck, Germany) and ethyl acetate (EtOAc) (Merck, Germany) sequentially. Each extraction step was performed three times and combined into one fraction before lyophylization in a Freezone 4.5 Plusfreeze dryer (Labconco, USA). The lyophilized active fraction was dissolved in methanol and partially purified on a gravity C_{18} column (Merck, Germany), followed by a reversed-phase HPLC using a Cosmosil $5C_{18}$-MS-II, 20 × 250 mm, 5 µm column (Nacalai, USA). All processes were monitored by a bioassay (broth microdilution).

5.2.5 Identification of the antimicrobial compounds from *B. paludis* MSh1

Structural determination of the antimicrobial compound was performed by spectroscopic techniques and literature comparison. The antimicrobial compound was analyzed by thin-layer chromatography on a silica 60 plate (Merck, Germany) with chloroform-acetic acid-ethanol at 95:5:2.5 (v/v) as the mobile phase, followed by spraying of an iron reagent (0.1 M $FeCl_3$ in 0.1 M HCl). Liquid chromatography-mass spectrometry (LC-MS) was performed with an Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer (dual ESI source) equipped with an Agilent Zorbax Eclipse XDB-C_{18} column. The ultraviolet/visible absorption spectrum was recorded with the photodiode array detector equipped with the above-mentioned LC. The mobile phase was composed of water (A, 0.5% formic acid) and acetonitrile (B, 0.5% formic acid), the gradient program of which was 0–12.00 min 90% A and 10% B and 12.00–15.00 min 100% B. The flow rate of the mobile phase was 0.3 mL/min, and the column temperature was set to 25ºC. The injection volume was 10 µL.

5.2.6 Antimicrobial testing via broth microdilution

The antimicrobial activity of each fraction was evaluated using a broth microdilution assay to determine the minimum inhibitory concentration (MIC) of an antimicrobial compound as described by the Clinical and Laboratory Standard Institute (CLSI). MIC is defined as the lowest concentration of an antimicrobial to inhibit the visible growth of a microorganism after overnight incubation. Briefly, the test microorganisms were grown in MHB at 37°C aerobically for 24 hours and adjusted to 0.5 McFarland standard (OD_{625} 0.08–0.11). The adjusted cultures were then diluted 100× in MHB and used as inocula. The extracts were serially diluted using sterile MHB in a 96-well flat bottomed microtiter plate. One hundred µl of test microorganisms

corresponding to approximately 10^6 colony forming units (CFU) was added to each well. Determination of MIC was performed in triplicate. The antibiotic control was vancomycin. The negative control contained MHB with test microorganisms. The blank control consisted only of MHB. The microtiter plate was incubated at 37°C aerobically for 16–20 hours and the MIC was determined by the concentration of extract (µg/mL) where no visible growth was observed.

5.2.7 Scanning electron microscopy (SEM)

SEM was performed based on the method described by Pilsczek et al. (2010) with modification, to determine the effect of the extract on the cellular morphology of the bacteria. *E. faecalis* ATCC 700802 was grown in MHB at 37°C aerobically for 24 hours and the turbidity was adjusted to 0.5 McFarland standard. The MIC of the extract was added to the adjusted bacterial culture. An untreated control was used as a negative control, while treatment with MIC of vancomycin (64 µg/mL) was used as an antibiotic control. All samples were incubated at 37°C for four hours. The cultures were then centrifuged at 5000 × *g* for three min and the supernatant was discarded. The bacterial pellet was washed with phosphate buffered saline (PBS) and subjected to centrifugation at 5000 × *g* for three min. This washing process was repeated three times. The washed pellets were reconstituted in minimal volume of PBS, placed onto glass slides (5 mm × 5 mm) and allowed to air dry for 30 min. The slides were fixed using 2.5% (v/v) glutaraldehyde (Sigma-Aldrich, UK) in PBS for four hours and washed three times with PBS to remove excess glutaraldehyde. The slides were then serially dehydrated with increasing concentration of ethanol and kept in a desiccator overnight. The slides were spur-coated with platinum using Q150R rotary-pumped sputter coater before observed using SU8010 FE-SEM (Hitachi, Japan).

5.3 Results and Discussion

5.3.1 Extraction of the antimicrobial compounds produced by *B. paludis* MSh1

The MIC values for *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 700802 were 20 μg/mL and 39 μg/mL respectively, which were lower as compared to *S. aureus* ATCC 29213 (313 μg/mL), *S. aureus* ATCC 700699 (156 μg/mL) and *E. coli* ATCC 25922 (2500 μg/mL) (Table 5.1). The results shown were consistent with a previous study conducted by Ong et al. (2015).

Table 5.1: The minimum inhibitory concentration (MIC) of different fractions and crude extract against five test microorganisms.

Test microorganisms	MIC (μg/mL)				
	CE	HEX	DCM	EtOAc	Water
Enterococcus faecalis ATCC 29212	20	>5,000	20	313	156
Enterococcus faecalis ATCC 700802	30	>5,000	39	313	313
Staphylococcus aureus ATCC 29213	313	>5,000	313	2,500	1,250
Staphylococcus aureus ATCC 700699	156	>5,000	156	1,250	1,250
Escherichia. coli ATCC 25922	2,500	>5,000	2,500	1,250	1,250

CE: Crude extract; HEX: Hexane fraction; DCM: Dichloromethane fraction; EtOAc: Ethyl acetate.

Thus far, there has only been one antimicrobial compound isolated from *Burkholderia* species that has inhibitory effect against gram-positive bacteria and fungus (pyrrolnitrin). Based

on the PCR performed in Section 3.2.5, the *prnD* gene was not found in MSh1 which further substantiate MSh1 may be producing other types of antimicrobial compounds. Furthermore, the genes that code for *prnA*, *prnB*, *prnC* and *prnD* were absent from the draft genome of MSh1 (obtained from Chapter 2).

Sequential solvent fractionation was performed to fractionate and partially purify the crude extract. A similar method was adopted from Tawfik et al. (2010) to isolate antifungal burkholdines produced by *B. ambifaria* 2.2N. In the present study, four different solvents were used: hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and water to extract the antimicrobial compounds produced by MSh1. HEX is a non-polar solvent commonly used to remove non-polar compounds from the crude extract. DCM and EtOAc both have middle-range polarity, thus is able to attract a wider range of compounds with different polarity (Moreau et al., 2003). Water being the most polar solvent will retain any compounds with higher polarity (Wang and Weller, 2006). It was shown the HEX fraction had no antimicrobial activity against all test microorganisms up to 5 mg/mL. The DCM fraction had the best antimicrobial activity against all the test microorganisms (when compared to the EtOAc and water fractions) as the MIC values were comparable or similar to the crude extract (Table 5.1). Thus, further purification was conducted on the DCM fraction using C_{18} column chromatography which led to the isolation of an antimicrobial compound.

5.3.2 Identification of the antimicrobial compounds produced by *B. paludis* MSh1

Chromatographic analysis of the pure compound on a TLC plate with chloroform-acetic acid-ethanol at 90:5:2.5 (v/v) as the mobile phase showed one spot (R_f 0.35) with yellow-green fluorescent band that turned red-brown after spraying with the iron reagent (indication of phenol

group). Analysis of the compound by analytical HPLC indicated one peak with three maxima at 210, 270 and 320 nm.

The mass of the compound (*m/z* 325, $[M+H]^+$) was determined by liquid chromatography-electrospray ionization-mass spectrometer (LC-ESI-MS). The ESI-MS indicated that the molecular formula of the compound was $C_{14}H_{16}N_2O_3S_2$. The interpretation of ESI-MS and UV spectrum results of the antimicrobial compound was found to be identical to the commercially purchased pyochelin which is in good agreement with previous literatures (Adler et al., 2012, Cox et al., 1981) (Figure 5.1).

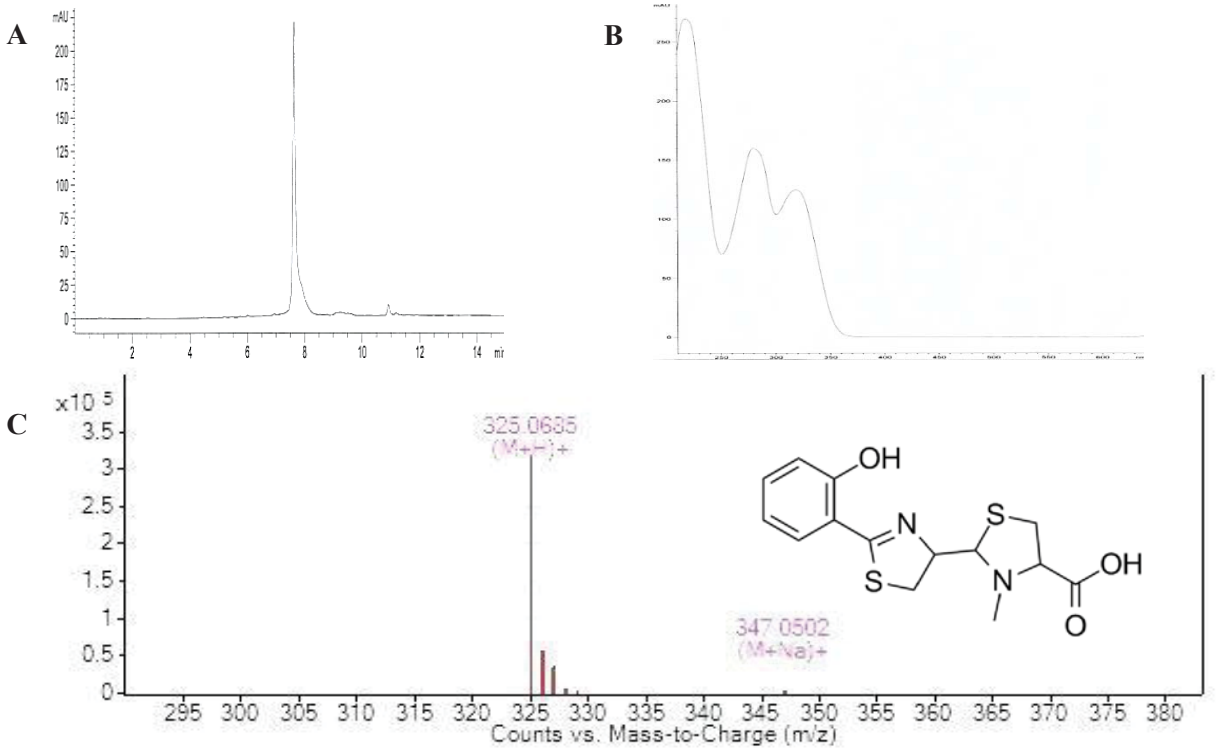

Figure 5.1: (A) HPLC chromatogram, (B) UV spectra and (C) LC-ESI-MS chromatogram of the extracted antimicrobial compound (pyochelin).

5.3.3 Antimicrobial activity of pyochelin

Pyochelin is a type of siderophore commonly produced by the *Pseudomonas* genus (Buysens et al., 1996, Cox et al., 1981, Lim et al., 2016). Siderophores can solubilize ferric ion, hence is an iron chelating growth factor for many bacteria. Only three *Burkholderia* species (*B. arboris*, *B. cenocepacia* and *B. contaminans*) have been known to produce pyochelin (Dang et al., 2011, Deng et al., 2015, Schwagner et al., 2012). As pyochelin is a siderophore, its antimicrobial properties were not extensively studied. Pyochelin can inhibit or kill bacteria by catalyzing the generation of reactive oxygen species (ROS) (Adler et al., 2012). However this is bacteria dependent as shown in Table 5.2.

The gram-negative bacteria tested, belonging to the Enterobacteriaceae family, were found to be resistant to pyochelin and this is consistent with a study conducted by Adler et al. (2012). The resistant profile shown by the Enterobacteriaceae was due to the production of catecholate siderophores such as enterobactin. These catecholate siderophores can act as hydrogen atom donors and efficiently terminate radical chain reactions hence rendering pyochelin ineffective.

The inhibitory effect of pyochelin on the four *S. aureus* and three *E. faecalis* isolates may be due to presence of ROS generated by pyochelin. Nevertheless, the literature has not reported on the downstream effect of the ROS produced by pyochelin, as it may target the DNA/RNA, electron transport chain or bacterial cell membrane. Moreover, the nutrient availability of these isolates may be compromised as pyochelin can chelate the vital metal ions present in the environment. Sensitive isolates might be starved and ultimately led to death, as they lack the receptors to mediate the entry of metal-bounded pyochelin into the bacterial cells.

Table 5.2: The minimum inhibitory concentration (MIC) of the extracted pyochelin and vancomycin (antibiotic control) against eighteen test microorganisms.

Gram stain	Test microorganisms	MIC (μg/mL)	
		Pyochelin	Vancomycin
Gram-positive	*Bacillus cereus* ATCC 14579	>100.00	0.50
	Bacillus subtilis ATCC 8188	>100.00	0.50
	Enterococcus faecalis ATCC 700802	3.13	64.00
	Enterococcus faecalis ATCC 29212	3.13	4.00
	Enterococcus faecalis JH-22	3.13	4.00
	Staphylococcus aureus ATCC 700699	6.26	1.00
	Staphylococcus aureus ATCC 43300	6.26	1.00
	Staphylococcus aureus ATCC 6538P	6.26	1.00
	Staphylococcus aureus ATCC 29213	6.26	1.00
Gram-negative	*Aeromonas hydrophila* ATCC 49140	>100.00	>100.00
	Escherichia coli ATCC 25922	>100.00	>100.00
	Klebsiella pneumoniae ATCC 10031	>100.00	>100.00
	Proteus mirabilis ATCC 49140	>100.00	>100.00
	Proteus vulgaris IMR	>100.00	>100.00
	Pseudomonas aeruginosa ATCC 10145	>100.00	>100.00
	Pseudomonas aeruginosa ATCC BAA-47	>100.00	>100.00
	Salmonella Typhimurium ATCC 14028	>100.00	>100.00
	Shigella flexneri ATCC 12022	>100.00	>100.00

Hence as a preliminary study, we investigated the effect of pyochelin on the cellular morphology of *E. faecalis* ATCC 700802 via SEM. Pyochelin did not affect the cellular morphology of *E. faecalis* ATCC 700802, when compared to the antibiotic control in which the morphology of the bacteria were distorted when treated with 64 μg/mL (MIC) of vancomycin

(Figure 5.2). This result supports ROS generated by pyochelin may target the intracellular components of *E. faecalis* ATCC 700802. Further work on the effect of pyochelin on the production of ROS and bacterial membrane integrity of *E. faecalis* ATCC 700802 had been performed, and will be discussed in Chapter 6.

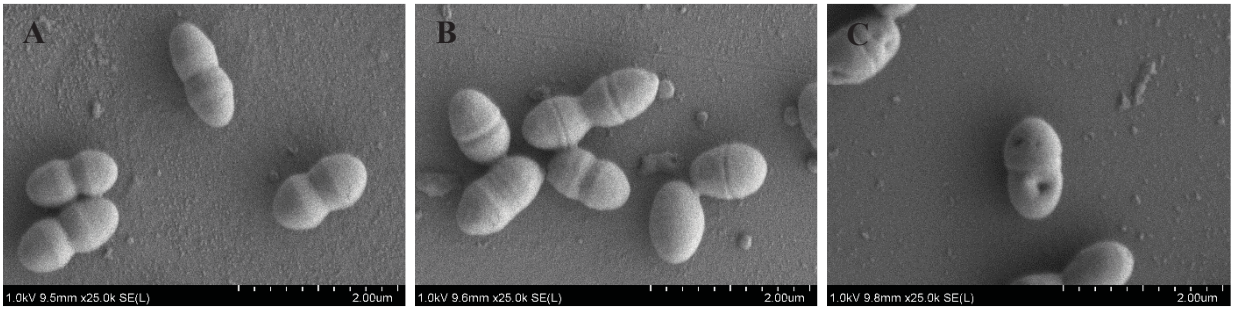

Figure 5.2: FE-SEM images of *E. faecalis* ATCC 700802 showing (A) negative control, (B) treated with 3.13 µg/mL (MIC) of the extracted pyochelin and (C) treated with 64 µg/mL (MIC) of vancomycin (antibiotic control). Images were taken under 5000× magnification at 1 kV.

5.4 Conclusions

An antimicrobial compound was successfully isolated and purified from the crude extract. The interpretation of the ESI-MS and UV spectrum results of the pure compound was found to be identical to pyochelin which is in good agreement with previous literatures. Pyochelin was found to be effective in inhibiting the growth of four *S. aureus* and three *E. faecalis* isolates, with MIC values of 6.26 µg/mL and 3.13 µg/mL, respectively via broth microdilution. This study has revealed pyochelin might potentially be used to ARB infections.

Chapter 6:

Antimicrobial characterization of pyochelin

The work presented in this chapter represents the following peer reviewed publication (attached in Appendix 7c):

Ong, K. S., Cheow, Y. L. and Lee, S. M. (2017). The role of reactive oxygen species in the antimicrobial activity of pyochelin. Journal of Advanced Research 8(4): 393-398.

6.1 Introduction

The increase in prevalence and emergence of ARB is an alarming concern. ARB infections often result in increased morbidity and mortality and healthcare costs. *E. faecalis* and *E. faecium* are examples of ARB that is difficult to treat due to its intrinsic resistance and ability to acquire resistance through mutation or horizontal gene transfer (Arias and Murray, 2012). As vancomycin is the last line of defense to combat *E. faecalis* and *E. faecium* infections, strains that are resistant to this antibiotic are a threat (Munita and Arias, 2016).

S. aureus is another example of ARB that causes life-threatening infections. The emergence of MRSA strains limits the treatment options to non-beta lactam antimicrobial agents such as tigecycline, vancomycin, daptomycin and linezolid to treat MRSA infections (Farrell et al., 2014, Jovetic et al., 2010). But however recently there is an increase in prevalence of strains being resistant towards these last few antibiotic options (Gorwitz et al., 2008, Holmes and Jorgensen, 2008). The limited treatment options available to treat these ARB infections have prompted the urgent need for antimicrobial compounds.

One strategy to look for antimicrobial compounds is by prospecting for novel microorganisms (Imhoff et al., 2011). Bacteria thriving in these environments may produce antimicrobial compounds to gain an advantage in competing for resources and colonization of new habitats (Traxler and Kolter, 2015). As a result, a tropical peat swamp forest in Malaysia, characterized by its acidic (pH range of 2.9 to 4.5), ombotrophic and waterlogged conditions was previously chosen as a bioprospecting location for antimicrobial compounds (Yule, 2010). Despite being such a harsh environment, Ong et al. (2016) successfully isolated a novel

bacterium *Burkholderia paludis* which showed potent antimicrobial activity towards *S. aureus* and *E. faecalis*. The antimicrobial compound was identified as pyochelin.

Pyochelin is a type of siderophore commonly produced by the *Pseudomonas* and *Burkholderia* genus. The biosynthetic gene clusters of pyochelin, along with its iron-solubilizing ability are well characterized. However, pyochelin has demonstrated other biological activity recently other than being only a chelating compound. The compound can particularly inhibit *S. aureus* in a study conducted by Adler et al. (2012) and this finding was further substantiated by another study performed by Ong et al. (2016). Pyochelin is not only effective in inhibiting non-antimicrobial-resistant isolates of *S. aureus* and *E. faecalis*, but also resistant isolates at 6.26 μg/mL and 3.13 μg/mL, respectively. It has been postulated pyochelin can inhibit bacterial growth by enhancing the production of reactive oxygen species (ROS) in the cells, which consequently inhibit certain essential biological processes. The mechanism of action of pyochelin as an antimicrobial compound is however not well characterized. The present study aimed to characterize the antimicrobial property of pyochelin.

6.2 Material and methods

6.2.1 Culture conditions and maintenance of bacterial isolates

Test microorganism isolates used in the study included *Enterococcus faecalis* ATCC 700802, *E. faecalis* ATCC 29212, *E. faecalis* JH-22, *Staphylococcus aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213. All isolates were cultured on Mueller-Hinton agar (MHA) (Oxoid, UK) at 37°C aerobically and maintained at -80°C in MHB (Oxoid, UK) with 25% (v/v) glycerol (Merck, Germany). As for *B. paludis*

MSh1, the isolate was maintained on NA (Merck, Germany) at 30°C aerobically and in 25% (v/v) glycerol in NB (Merck, Germany) at -80°C for long term preservation.

6.2.2 Extraction of pyochelin from *B. paludis* MSh1

Extraction of pyochelin from *B. paludis* MSh1 was performed according to the methodology described by Ong et al. (2016), with slight modification. Briefly, *B. paludis* MSh1 was cultured on NA containing 5 g/L of glycerol and incubated for four days at 30°C. The media and the bacteria was extracted using methanol (Merck, Germany) and subsequently fractionated using dichloromethane (DCM) (Merck, Germany). The DCM fraction was purified on a gravity C_{18} column, followed by further purification using a reverse-phased preparative high performance liquid chromatography (HPLC). The purity of pyochelin was compared with a standard purchase from Santa Cruz Biotechnology, USA (Appendix 3; Figure A3.1).

6.2.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pyochelin

The MIC of pyochelin was determined using broth microdilution assay as described by the Clinical and Laboratory Standard Institute (CLSI). The MIC is defined as the lowest concentration of an antimicrobial compound to inhibit the visible growth of a microorganism after 16–20 hours of incubation (CLSI, 2012). Briefly, the test microorganisms were grown in MHB at 37°C aerobically for 20 hours and adjusted to 0.5 McFarland standard (OD_{625} 0.08–0.11), corresponding to 1.5×10^8 colony forming unit (CFU)/mL. The adjusted cultures were then diluted 100× in MHB and used as inocula. The extracts were two-fold serially diluted using sterile MHB in a 96-well flat bottomed microtiter plate. One hundred µL of the adjusted test

microorganisms was added to each well. Determination of MIC was performed in triplicate. The antibiotic control was vancomycin (Calbiochem, Malaysia). The negative control contained MHB with test microorganisms. The blank control consisted only of MHB. The microtiter plate was incubated at 37°C aerobically for 16–20 hours and the MIC was determined by the concentration of extract (μg/mL) where no visible growth was observed. All clear wells containing cultures with no visible growth was streaked out onto MHA to determine the minimum bactericidal concentration (MBC). MBC is defined as the lowest concentration of antimicrobial compound that will prevent the growth of a microorganism after subculture on to an antibiotic-free media. The lowest concentration of pyochelin that showed absence of growth was determined as the MBC (CLSI, 2012).

6.2.4 Killing-kinetics studies

A killing kinetic study was performed to determine the effect of different concentrations of pyochelin on *E. faecalis* ATCC 700802 for 24 hours. As the *Enterococcus* isolates were shown to be more susceptible to pyochelin as compared to the *Staphylococcus* isolates, further characterization on the antimicrobial activity of pyochelin was conducted on an *Enterococcus* strain, with particular interest of *E. faecalis* ATCC 700802 due to its vancomycin-resistant property. The killing kinetics assay was performed according to the method described by Pag et al. (2004) and Yan et al. (2013). Different concentrations of pyochelin corresponding to 1×, 2× and 4× the MIC determined by broth microdilution were added into 100× diluted 0.5 McFarland adjusted bacteria culture (1.5×10^6 CFU/mL) in 0.85% (w/v) saline (Fisher Scientific, USA) and incubated at 37°C. Untreated bacterial culture was served as a negative control. The viable count was monitored up to 24 hours. Aliquots were taken at defined intervals (0 hour, 2nd hour, 4th

hour, 8th hour and 24th hour) and appropriately diluted in 0.85% (w/v) saline. One hundred µL of each of the dilutions was plated in triplicate on MHA. The plates were incubated at 37ºC and the cell viability was assessed by enumerating the colony forming unit (CFU) per mL after 24 hours. Killing kinetic studies of pyochelin on *E. faecalis* ATCC 700802 were performed under three different conditions: (1) exponential phase culture with agitation at 200 rpm (Smith A3555, Progressive Scientific); (2) stationary phase culture with agitation at 200 rpm (Smith A3555, Progressive Scientific); and (3) exponential phase culture at anaerobic condition. The anaerobic cultures were cultured in an anaerobic jar (Labozone, France) with AnaeroGen pack (Oxoid, UK).

6.2.5 Detection of reactive oxygen species (ROS)

The production of ROS by *E. faecalis* ATCC 700802 after treatment with pyochelin was evaluated using a peroxynitrite indicator, 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK), which can detect a broad range of ROS including nitric oxide and hydrogen peroxide (Arakha et al., 2015). The adjusted bacterial culture (0.5 McFarland exponential phase bacteria culture) were treated with different concentrations of pyochelin corresponding to 1, 2 and 4× MIC in presence of DCFH-DA at a final concentration of 5 µM in 0.85% (w/v) saline and incubated at 37°C aerobically at 200 rpm (Smith A3555, Progressive Scientific) for 24 hours. Untreated bacterial culture was served as a negative control. The fluorescence emission of DCFH-DA was measured at 525 nm using a Tecan microtitre plate reader with an excitation wavelength of 485 nm (Han et al., 2016). The background fluorescence of 0.85% (w/v) saline and auto-florescence of the bacterial cells incubated without the probe was measured to calculate the net fluorescence emitted from the assay itself. Experiment was conducted in triplicate (n = 3).

6.2.6 Determination of malondialdehyde (MDA)

Malondialdehyde (MDA) is a natural by-product of lipid peroxidation of polyunsaturated fatty acids caused by ROS, thus is commonly used as a marker for oxidative stress. The production of MDA was quantified by using the OxiSelect^{TM} TBARS Assay kit according to manufacturer's protocol (Cell Biolabs Inc., USA). Briefly, the adjusted bacterial culture (0.5 McFarland adjusted exponential phase bacteria culture) were treated with different concentrations of pyochelin corresponding to 1, 2 and 4× the MIC at 37°C aerobically whereas the control was incubated in 0.85% (w/v) saline alone for 24 hours. One hundred µl of the SDS lysis solution were added to 100 µl aliquot of the treated culture and incubated for five min at room temperature. The mixtures were then incubated at 95ºC for 60 min in presence of thiobarbituric acid (TBA) reagent. Each of the mixture was cooled to room temperature in an ice bath for 5 min and centrifuged at 3000 × *g* for 15 min (Eppendorf, 5810R). The supernatants were then collected and the absorbance values were read at 532 nm. The concentrations of MDA in each treatment were calculated based on the standard curve of absorbance against MDA concentration (Appendix 4; Figure A4.1). This assay was performed in triplicates (n = 3).

6.2.7 Membrane integrity assay

As the bacterial membrane is composed of phospholipilid bilayer, the production of ROS prior to pyochelin treatment might oxidize the lipid content on the cell membrane, hence affecting the bacterial membrane integrity. Therefore, the effect of pyochelin on the membrane integrity of *E. faecalis* ATCC 700802 was determined by using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen) according to a protocol from Ong et al. (2015). The adjusted bacterial cultures were treated with different concentrations of pyochelin

corresponding to 1×, 2× and 4× the MIC at 37°C aerobically at 200 rpm (Smith A3555, Progressive Scientific) whereas the control was incubated with 0.85% (w/v) saline alone for 24 hours. After incubation, the treated cultures were pelleted by centrifugation (10000 × *g*, 15 min) at room temperature, washed twice and resuspended in 0.85% (w/v) saline. One hundred µL of the 2× staining solution were added into 100 µl of the bacteria suspension, and incubated in the dark for 15 min. At the end of the incubation period, green fluorescence (SYTO 9) was read at 530 nm while the red fluorescence (propidium iodide) was read at 645 nm with an excitation wavelength of 485 nm. This kit utilizes a mixture of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. The SYTO 9 stain generally labels all bacteria in a population including those with intact membranes and those with damaged membranes. In contrast, PI is impermeable to bacterial cells with an intact cell membrane due to its large molecular size (Stocks, 2004). Thus, bacteria with intact cell membranes will be stained fluorescent green, whereas bacteria with damaged membranes will be stained fluorescent red. The percentage of live bacteria was determined by referring to a standard curve of G/R ratio versus percentage of live *E. faecalis* ATCC 700802 which was pre-plotted earlier (Appendix 5; Figure A5.1). This assay was performed in six replicates (n = 6).

6.2.8 Statistical analysis

The significance of results for the killing kinetics studies, detection of ROS and quantification of MDA were performed using independent-sample *t*-test at the significance level of $\alpha = 0.05$. The significance of results for membrane integrity assay was performed using Wilcoxon test at the significance level of $\alpha = 0.05$ (Kolmogoroff-Smirnow test was used to analyze the normal distribution). Statistical analysis was performed using IBM SPSS Statistics 20.

6.3 Results and Discussion

6.3.1 MIC, MBC and killing kinetics studies of pyochelin

The MIC values of pyochelin against the *Enterococcus* (*E. faecalis* ATCC 700802, *E. faecalis* ATCC 29212, *E. faecalis* JH-22) and *Staphylococcus* isolates (*S. aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213) were 3.13 µg/mL and 6.26 µg/mL respectively; while the MBC values were 6.26 µg/mL and 25.00 µg/mL respectively (Table 6.1). It was shown that the *Enterococcus* isolates are more susceptible to pyochelin when compared to the *Staphylococcus* isolates. Nonetheless pyochelin is bactericidal against both *Enterococcus and Staphylococcus* isolates as the MBC values were no more than 4× the MIC values (Pankey and Sabath, 2004). The low MIC values of pyochelin against the *E. faecalis* and *S. aureus* isolates is an advantage as it is comparable or lower than the currently available antibiotics which have MIC values of 4–32 µg/mL (CLSI, 2012).

Killing kinetics was performed to evaluate the effect of different concentrations of pyochelin on *E. faecalis* ATCC 700802 for 24 hours. Two phases of bacterial culture were used in this study: exponential phase and stationary phase. Exponential phase culture consists of actively growing cells which consume readily available oxygen and nutrients for growth. On the other hand, stationary phase culture comprises mostly of mature non-dividing cells which are metabolically inactive (Roostalu et al., 2008). Different types of antibiotics work differently depending on their mechanism of action. For instance, lipopeptides (membrane disruptors) inhibits bacterial growth (both exponential phase and stationary phase culture) instantly by puncturing their cell wall (Steinbuch and Fridman, 2016); while beta-lactams (cell wall

biosynthesis inhibitors) only inhibit actively growing bacterial cells in a time-dependent manner, but they are effective at both aerobic and anaerobic conditions (Holten and Onusko, 2000).

Table 6.1: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of pyochelin against different test microorganisms.

Test microorganisms	MIC (µg/mL)	MBC (µg/mL)
Enterococcus faecalis ATCC 700802	3.13	6.26
Enterococcus faecalis ATCC 29212	3.13	6.26
Enterococcus faecalis JH-22	3.13	6.26
Staphylococcus aureus ATCC 700699	6.26	25.00
Staphylococcus aureus ATCC 43300	6.26	25.00
Staphylococcus aureus ATCC 6538P	6.26	25.00
Staphylococcus aureus ATCC 29213	6.26	25.00

Pyochelin inhibits growth of exponential phase *E. faecalis* ATCC 700802 in a dose and time dependent manner (Figure 6.1A). *E. faecalis* ATCC 700802 culture treated with 3.13 µg/mL (1× MIC) of pyochelin achieved 3 log reduction after 24 hours; while bacterial culture treated with 6.26 µg/mL (2× MIC) and 12.52 µg/mL (4× MIC) of pyochelin achieved 6 log reduction after 24 hours. However, a different scenario was observed when stationary phase *E. faecalis* ATCC 700802 was treated with pyochelin as there was only 2 log reduction after incubated for 24 hours at 4× MIC aerobically (Figure 6.1B). This result has revealed pyochelin work best only on actively growing bacterial cells. Nevertheless, pyochelin is different from the beta-lactams as it is ineffective against bacterial cells incubated under anaerobic condition (Figure 6.1C), suggesting that oxygen might play an important role in the bactericidal effect of pyochelin on *E. faecalis* ATCC 700802.

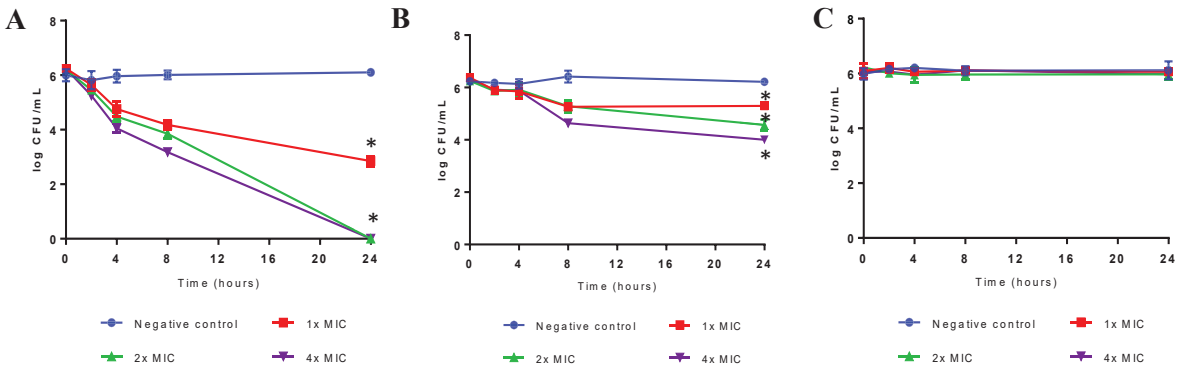

Figure 6.1: Effect of different concentrations of pyochelin against (A) exponential phase *E. faecalis* ATCC 700802 (incubated aerobically); (B) stationary phase *E. faecalis* ATCC 700802 (incubated aerobically); (C) exponential phase *E. faecalis* ATCC 700802 (incubated anaerobically) at 37ºC for 24 hours. Results are expressed as mean log CFU/mL ± SD plotted against time (n = 3). Asterisk represents significant difference ($P < 0.05$) between each treatment with the negative control at 24 hours.

6.3.2 Effect of pyochelin on the enhancement of ROS production and membrane integrity

The production of ROS in healthy untreated bacterial cells is a natural side effect of aerobic respiration. These ROS can damage the RNA/DNA pool and also oxidizes lipid contents. Thus to protect themselves against the detrimental effect of ROS, bacteria are capable of producing enzymes (catalase and superoxide dismutase) to detoxify the ROS and having regulatory mechanisms (SoxRS, OxyRS and SOS regulons) to counteract the damage (Gasser et al., 2016, Van Acker et al., 2016).

Pyochelin can inhibit *S. aureus* ATCC 25923 and *Xanthomas* species by catalyzing the Haber-Weiss reaction, hence leading to oxidative stress and bacterial cell death (Adler et al., 2012). Therefore, it was postulated that a similar scenario can be observed, in which the formation of ROS will be enhanced in *E. faecalis* ATCC 700802 bacterial cells treated with pyochelin. The accumulation of ROS can damage the iron-sulphur clusters, thereby releasing ferrous ion. This iron can react with hydrogen peroxide in the Fenton reaction, causing a chain reaction, generating hydroxyl radicals which can directly damage intracellular DNA, lipids and proteins (Van Acker et al., 2016). To determine the effect of pyochelin on the enhancement of ROS production, *E. faecalis* ATCC 700802 was treated with different concentrations of pyochelin in presence of DCFH-DA, an unspecific probe for ROS. It was shown that the ROS production in *E. faecalis* ATCC 700802 was enhanced in a dose dependent manner when treated with pyochelin (Figure 6.2). This suggests that the enhanced production of ROS has an indirect effect on the growth of *E. faecalis* ATCC 700802.

As one of the side effects of increased production of ROS is lipid peroxidation, an example of the by-product in this process (malondialdehyde; MDA) was quantified in this study. The concentration of MDA in the treated *E. faecalis* ATCC 700802 culture was increased significantly with increasing concentrations of pyochelin. This indicates that the accumulation of ROS (Figure 6.2) in *E. faecalis* ATCC 700802 after treated with pyochelin has caused an increase in lipid peroxidation (Figure 6.3).

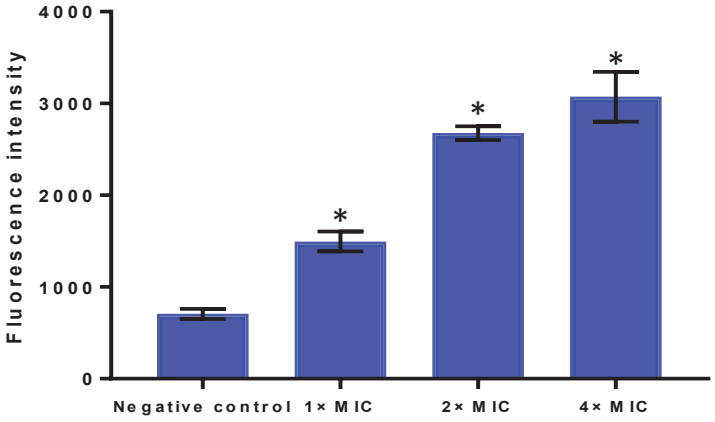

Figure 6.2: Quantitation of ROS production by exponential phase *E. faecalis* ATCC 700802 after 24 hours treatment with different concentrations of pyochelin using the DCFA-DA probe. Results are expressed as mean fluorescence intensity ± SD (n = 3). Asterisk represents significant difference ($P < 0.05$) between each treatment with the negative control.

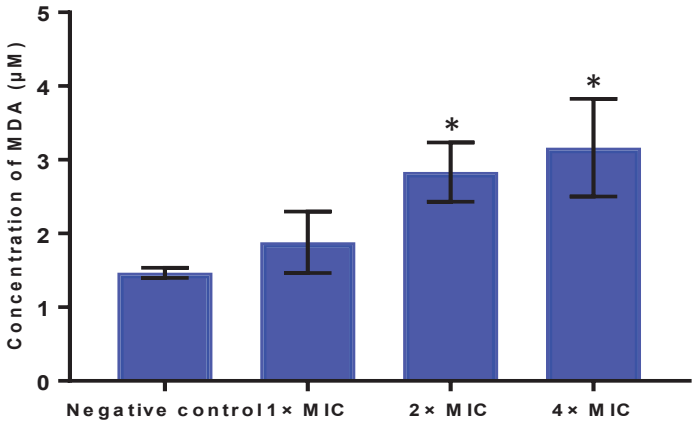

Figure 6.3: Quantification of MDA production in exponential phase *E. faecalis* ATCC 700802 after 24 hours treatment with different concentrations of pyochelin. Results are expressed as mean ± SD (n = 3). Asterisk represents significant difference ($P < 0.05$) between each treatment with the negative control.

Since lipid is an essential macromolecule to the bacterial cell membrane, the membrane integrity of *E. faecalis* ATCC 700802 was evaluated using the Live/Dead BacLight Bacterial Viability Kits. It was found that the percentage live bacteria of *E. faecalis* ATCC 700802 was 52.05% at 8 hours and 50.35% at 24 hours when treated with 1× MIC of pyochelin (Figure 6.4). This is because the enhanced generation of ROS at 1× MIC by pyochelin is not sufficient to eliminate the entire bacterial population. Bacterial cells are capable of lowering their metabolic activity at sub-lethal ROS concentration, hence allowing the cell's regulatory mechanisms to repair the damaged protein or DNA clusters and concurrently producing more enzymes to detoxify the detrimental effect of ROS (Keren et al., 2013). The results shown is consistent with the killing kinetics data as there was only 3 log reduction at 1× MIC of pyochelin after 24 hours.

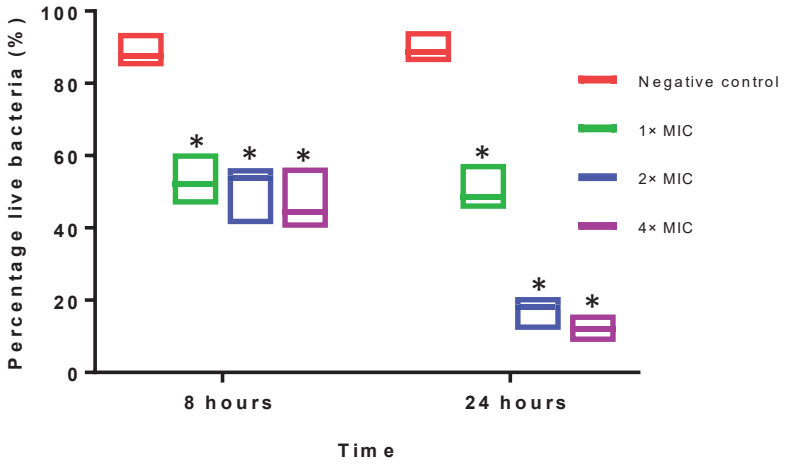

Figure 6.4: Percentage of live exponential phase *E. faecalis* ATCC 700802 at 8 hours and 24 hours after treatment with different concentrations of pyochelin using the Live/Dead BacLight Bacterial Viability Kit. Results are expressed as median with range (n = 6). Asterisk represents significant difference ($P < 0.05$) between each treatment with the negative control at each time-point using Wilcoxon test.

Furthermore, the MDA concentration of *E. faecalis* ATCC 700802 treated at 1× MIC of pyochelin was not statistically significant compared to the untreated control, indicating that the ROS level generated in presence of 1× MIC of pyochelin did not trigger significant lipid peroxidation, hence the higher percentage of live bacteria. Nevertheless, the percentage live bacteria of *E. faecalis* ATCC 700802 decreases in a time dependent manner when treated with higher concentrations of pyochelin (2× and 4× MIC) (Figure 6.4) and this is in agreement with the data obtained from the killing kinetics study.

The result substantiates pyochelin can enhance the production of ROS, which later affects the membrane integrity of *E. faecalis* ATCC 700802, leading to bacterial cell death. Furthermore, the lipophilicity of pyochelin may play an important role in affecting the membrane fluidity or membrane potential (proton motive force), thus allowing the initial entry of pyochelin into the bacterial cells to exert its antimicrobial effect (Mingeot-Leclercq and Decout, 2016). A similar pattern can be observed from other studies conducted using aspidin BB (an alkaloid), metal oxide nanoparticles and synthesized pyrimidine derivatives, as these compounds exert their antibacterial properties by inducing the generation of ROS as well (Dizaj et al., 2014, Li et al., 2014, Suresh et al., 2016).

The killing mechanism shown in the study may potentially be useful in combating antimicrobial resistance, as it involves the bacterial cell's redox reaction which directly influences the survival of the cells (Keren et al., 2013, Paiva and Bozza, 2014). However sequential passaging of the bacterial culture with sub-MIC of pyochelin should be performed in the future to evaluate the development of resistance of *E. faecalis* ATCC 700802 towards pyochelin over generations (Ling et al., 2015). Nevertheless, this is the first study to characterize

the potential of pyochelin as an antimicrobial compound against vancomycin-resistant *E. faecalis*. Further work such as *in vitro* cytotoxic evaluation of pyochelin using normal human cell lines and potentiation of pyochelin with existing antibiotics should be conducted. Furthermore, different isolates of *E. faecalis* or other test microorganisms such as the *S. aureus* isolates should be tested to further support pyochelin as a potential therapeutic option against ARB infections.

6.4 Conclusions

Pyochelin was found to be effective in inhibiting the growth of three *E. faecalis* isolates and four *S. aureus* isolates, with MIC values (MBC values) of 3.13 μg/mL (6.26 μg/mL) and 6.26 μg/mL (25.00 μg/mL) respectively via broth microdilution. Pyochelin is able to enhance the production of ROS, subsequently causing an increase in MDA production and a decrease in membrane integrity of *E. faecalis* ATCC 700802 after 24 hours. The study has shown that pyochelin might potentially be useful in treating infections caused by ARB, particularly *E. faecalis* in the future.

Chapter 7:

Overall conclusions and future work

7.1 Overall conclusions

Burkholderia sp. MSh1 was previously isolated from Southeast Pahang tropical peat swamp soil in March, 2013. It was identified as *Burkholderia* species via the 16S rRNA gene sequence analysis with a maximum identity of 99%. The isolate had broad spectrum antimicrobial activity against several gram-positive and gram-negative bacteria. Hence, *Burkholderia* sp. MSh1 was further studied in this project.

The whole genome of *Burkholderia* sp. MSh1 was sequenced using the Illumina MiSeq (150-bp paired-end reads) at the Monash University Malaysia Genomics Facility. Using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), 6,963 coding DNA sequences (CDSs), 10 rRNAs, and 64 tRNAs were annotated for *Burkholderia* sp. MSh1. Several antimicrobial biosynthesis genes were predicted in the genome of *Burkholderia* sp. MSh1 including polyketide cyclase (KFG96276 and KEZ06389), antibiotic biosynthesis monooxygenase (KFG97610 and KEZ06948), mitomycin antibiotic biosynthesis protein (KFG98191 and KEZ04328), colicin V production protein (KFG93602 and KEZ04863), and phenazine biosynthesis protein (PhzC/PhzF) (KFG92381 and KEZ02706). Based on RAST, some of the known antimicrobials produced by *Burkholderia* species were absent, for instance, pyrrolnitrin (*B. pyrrocinia*), rhizobitoxin (*B. andropogonis*), and pyoluteorin (*B. cepacia*).

Burkholderia sp. MSh1 was further characterized using a polyphasic taxonomy approach. The predominant cellular fatty acids [$C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%) and $C_{19:0}$ cyclo ω8c (16.1%)], polar lipids (phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol) and ubiquinone (Q-8) of this strain was consistent with the genus *Burkholderia*. MSh1 can be differentiated from the other Bcc species by phylogenetic analysis of

16S rRNA gene sequence (GenBank/EMBL/DDBJ KT159931), MLSA (GenBank/EMBL/DDBJ KU301866-301872), ANI and biochemical tests. DNA-DNA relatedness values between MSh1 and closely related type strains were below the 70% threshold value. Based on the polyphasic study, it can be concluded MSh1 represents a novel species within the Bcc, for which the name *Burkholderia paludis* (pa.lu.dis. L. gen. n. *paludis* of the swamp) is proposed. The type strain is MSh1 (=DSM 100703 =MCCC 1K01245).

The antimicrobial activity of *B. paludis* MSh1 was optimized using both statistical (Plackett-Burman design; PBD) and conventional (one-factor-at-a-time; OFAT) approach. PBD was used to screen for main factors affecting the antimicrobial activity of *B. paludis*. The analysis revealed that peptone, glycerol, minerals (manganese chloride, iron sulphate, sodium chloride, calcium chloride, copper sulphate and zinc sulphate) and physical parameters (incubation duration, incubation temperature, initial pH, agitation and inoculum size) were the significant factors influencing the antimicrobial activity of *B. paludis* MSh1. These factors were further optimized using the conventional method of one-factor-at-a-time (OFAT) experiments. The optimized factors using OFAT experiments were found to be: glycerol (5 g/L), peptone (15 g/L), minerals (1% v/v), agitation speed (200 rpm), incubation temperature (30°C), incubation duration (96 hours), initial culture media (pH 6) and inoculum size (1% v/v). The antimicrobial activity in the nearly-optimized culture medium was 105.00 ± 5.47 U/mL, which was approximately significantly higher (4.4-fold) than that without optimization (NB only) against *E. faecalis* ATCC 700802 (23.33 ± 5.16 U/mL).

B. paludis MSh1 was first cultured in the optimized media. The culture supernatant was then fractionated with hexane, dichloromethane followed by ethyl acetate sequentially with increasing polarity. The dichloromethane fraction was found to have the best antimicrobial activity via broth microdilution and hence was subjected for further purification. Purification of the dichloromethane fraction using C_{18} gravity column chromatography and reversed-phase preparative HPLC had yielded a compound with antimicrobial activity.

The interpretation of ESI-MS and UV spectrum results of the pure compound was found to be identical to pyochelin which is in good agreement with previous literatures. Pyochelin was found to be effective in inhibiting the growth of four *S. aureus* and three *E. faecalis* isolates, with MIC values of 6.26 µg/mL and 3.13 µg/mL respectively via broth microdilution. Pyochelin was able to enhance the production of ROS, subsequently causing an increase in MDA production and a decrease in membrane integrity of *E. faecalis* ATCC 700802 after 24 hours. This study has shown that pyochelin can be used as an antimicrobial compound to treat infections caused by ARB, particularly *S. aureus* and *E. faecalis*.

7.2 Future work

Several approaches can be conducted in the future as a continuation of this study.

1. Complete genome sequence of *B. paludis* MSh1

At present, the genome of *B. paludis* MS1 had been sequenced, assembled and represented in contiguous sequences (contigs). The draft genome is sufficient to annotate common gene clusters; however problems reside in the gap between the contigs and this remains a challenging task. These gaps are usually low-coverage regions that may contain essential functions for the organisms studied (Dayarian et al., 2010); hence gap-closure is needed to establish a complete genome sequence. There are several methods to accomplish this. Firstly is by using GapFiller, an automated program which locates sequence pair-ends and generates overlapping short sequences corresponding to the gaps between contigs. The main drawback of using this software is that mate pair libraries can produce large insert distributions thus making it hard to define the correct distance between the pairs. Besides that, the background noise generated in mate pair data can lead to incorrect scaffolds and consequently affecting the gap-closure (Boetzer and Pirovano, 2012). The next option is by using third generation sequencing technologies, for instance PacBio and IonTorrent systems which are capable of generating reads with large inserts, thus providing valuable paired read information for the assembly and scaffolding process. This method increases the sequence coverage and reduces the number of contigs. Many complete genome sequences of different organisms had been assembled, for example *Azospirillum thiophilum* BV-S, *Salmonella enterica* serovar Typhimurium NC983 and *Rhodococcus* sp. UM008 (Fomenkov et al., 2016, Troxell et al., 2016, Wiens et al., 2016). These complete genome sequences can be used to bio-mine useful secondary metabolites, as reported

by Esmaeel et al. (2016) and Kodani et al. (2016) which dealt with *Burkholderia* species and *Actinomadura atramentaria* respectively.

2. Optimization of pyochelin production via response surface methodology (RSM)

Optimization of pyochelin production can be carried out using response surface methodology (RSM). RSM is an empirical mathematical tool used to optimize a set of factors to achieve a maximum level of outcome. Each factor (such as pH, temperature, concentration of carbon source and nitrogen source) can be deliberated in three levels denoted as -1 (low level), 0 (center level) and +1 (high level) through multiple experimental runs. The experimental values will then be compared with the predicted values determined by RSM. Statistical analysis will be performed using multiple regressions, canonical analysis and three-dimensional surface plots to validate the significance of selected parameters on the outcome (Bezerra et al., 2009). The increased in production of pyochelin will be useful for further characterization work.

3. Antimicrobial activity characterization of pyochelin

The bactericidal activity demonstrated by pyochelin was due to the enhanced generation of ROS. These ROS cause lipid peroxidation which ultimately leads to bacterial cell death. To protect themselves against the deleterious effects of ROS, aerobic bacteria are equipped with enzymes (catalases and superoxide dismutases) that can detoxify ROS and regulatory mechanisms such as *hypR* (hydrogen peroxide regulator) and *soxR* (redox-sensitive transcriptional activator) to counteract any collateral damage (Verneuil et al., 2004, Yan et al., 2015, Zhao et al., 2015). Despite having the protective mechanism as aforementioned, the growth of *E. faecalis* and *S. aureus* isolates were still affected, suggesting that pyochelin might indirectly have a negative impact on these enzymes or regulons. Hence in order to evaluate the

effect of pyochelin on these sensitive bacterial isolates, qPCR can be performed to assess the regulations of these genes.

4. Cytotoxic evaluation of pyochelin

Antimicrobial compounds can be applied either topically on skin cells or ingested depending on the site of infection and solubility of the compounds. Topical antimicrobials can be removed via surface washing, while ingested antimicrobials will be metabolized in the liver and excreted by the kidney cells. Hence, different representative cell lines such as the human epidermal keratinocytes (HEK cells), THLE-2 liver cells and TK-173 kidney cells are required to evaluate the cytotoxicity of pyochelin via MTT assay (Boonkaew et al., 2014, Eltoweissy et al., 2016, Kraijka-Kuzniak et al., 2013). These cells will first be cultured in their respective growth medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37ºC in a humidified atmosphere with 5% CO_2. Various concentrations of pyochelin will then be added to the cultured cells for 24 hours. The viability of the cells will then be determined by adding the tetrazolum dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The reduction of the yellow tetrazolum salt into purple formazan by the cellular NAD(P)H-dependent oxidoreductase enzyme, indirectly reflects the number of metabolically viable cell present (Berridge et al., 2005). An antimicrobial with low cytotoxic profile can be a promising therapeutic candidate in the future.

5. Potentiation of antibiotics with pyochelin

Pyochelin was previously reported to able to potentiate the inhibitory effect of gallium against *Pseudomonas aeruginosa* (Frangipani et al., 2014). Their findings demonstrated that pyochelin facilitates the entrance of gallium into the cell through specific uptake machinery. A

similar approach can be used to potentiate antibiotics using pyochelin via checkerboard assay. This assay was designed to determine the effect of two combinational antimicrobial agents at different concentrations. The results will then be interpreted as fractional inhibitory concentration (FIC). The FIC is the concentration of an antimicrobial agent that kills when used in combination with another agent divided by the concentration that has the same effect when used alone. Thus a FIC index of < 0.5 represents synergism, a FIC index between 0.5 and 4.0 is regarded as indifference, while combination of antimicrobial agents with FIC index > 4.0 is considered as antagonistic (Aleksic et al., 2014, Schurch et al., 2014, Wang et al., 2016).

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Appendix 1:

Allelic profile and GGDC similarity values of MSh1 with known Bcc type strains

Table A1.1: Allelic profile of seven house-keeping genes of MSh1 compared with 30 Bcc type strains.

Burkholderia type strains	Allellic profile						
	*atp*D	*glt*B	*gyr*B	*rec*A	*lep*A	*pha*C	*trp*B
Burkholderia pyrrocinia LMG 14191^T	70	88	250	108	98	28	86
Burkholderia stabilis LMG 14294^T	26	18	14	21	70	10	16
***Burkholderia paludis* MSh1^T**	**302**	**11**	**50**	**350**	**288**	**249**	**387**
Burkholderia cenocepacia IIIC LMG 19230^T	65	49	41	47	33	36	44
Burkholderia cepacia ATCC 25416^T	5	4	44	4	4	4	48
Burkholderia metallica AU0553^T	239	189	268	187	202	153	242
Burkholderia contaminans LMG 23361^T	64	80	76	89	105	97	70
Burkholderia lata 383^T	63	46	38	44	30	33	42
Burkholderia arboris LMG 14939^T	60	43	36	42	27	72	72
Burkholderia seminalis R-24196^T	203	161	386	144	286	123	240
Burkholderia cenocepacia IIID CCUG 46446^T	148	172	216	161	24	30	38
Burkholderia cenocepacia IIIA J2315^T	15	11	9	14	11	6	12
Burkholderia cenocepacia IIIB R-52732^T	16	98	603	365	417	41	404
Burkholderia ambifaria AMMD^T	35	25	123	98	103	59	49
Burkholderia anthina R-4183^T	43	33	27	33	20	21	30
Burkholderia diffusa R-15930^T	98	42	68	87	53	50	41
Burkholderia latens R-5630^T	96	169	209	150	176	99	150
Burkholderia vietnamiensis LMG 10929^T	27	19	15	23	35	56	17
Burkholderia pseodmultivorans CCUG 62895^T	150	204	205	250	171	163	304
Burkholderia dolosa LMG 18943^T	30	21	18	24	72	13	20
Burkholderia multivorans ATCC BAA-247^T	11	60	117	81	37	86	97
Burkholderia ubonensis CIP 107078^T	137	229	200	216	243	177	153
Burkholderia oklahomensis HI4355^T	270	296	434	279	332	236	313
Burkholderia thailandensis E264^T	253	278	411	261	311	220	277
Burkholderia mallei ATCC 23344^T	95	117	128	46	32	34	43
Burkholderia pseudomallei K96243^T	95	116	128	45	31	34	43
Burkholderia gladioli HI2137^T	223	309	417	265	318	225	312
Burkholderia glumae AU12450^T	252	305	394	251	309	219	269
Burkholderia tropica AU15822^T	255	280	413	263	313	223	279
Burkholderia fungorum AU 12699^T	249	275	406	256	305	216	274
Burkholderia glathei HI4344^T	264	291	429	274	326	233	288

Table A1.2: Percentage of divergence of concatenated allele sequences between MSh1 with 30 Bcc type strains.

Burkholderia type strains	Divergence (%)
Burkholderia pyrrocinia LMG 14191^T	5.81
Burkholderia stabilis LMG 14294^T	5.23
***Burkholderia paludis* MSh1^T**	-
Burkholderia cenocepacia IIIC LMG 19230^T	6.87
Burkholderia cepacia ATCC 25416^T	6.44
Burkholderia metallica AU0553^T	6.71
Burkholderia contaminans LMG 23361^T	6.08
Burkholderia lata 383^T	5.50
Burkholderia arboris LMG 14939^T	6.51
Burkholderia seminalis R-24196^T	6.70
Burkholderia cenocepacia IIID CCUG 46446^T	6.63
Burkholderia cenocepacia IIIA J2315^T	6.59
Burkholderia cenocepacia IIIB R-52732^T	7.11
Burkholderia ambifaria AMMD^T	6.99
Burkholderia anthina R-4183^T	8.27
Burkholderia diffusa R-15930^T	7.11
Burkholderia latens R-5630^T	7.74
Burkholderia vietnamiensis LMG 10929^T	8.99
Burkholderia pseodmultivorans CCUG 62895^T	7.46
Burkholderia dolosa LMG 18943^T	8.51
Burkholderia multivorans ATCC BAA-247^T	8.14
Burkholderia ubonensis CIP 107078^T	8.18
Burkholderia oklahomensis HI4355^T	11.31
Burkholderia thailandensis E264^T	11.35
Burkholderia mallei ATCC 23344^T	10.93
Burkholderia pseudomallei K96243^T	10.89
Burkholderia gladioli HI2137^T	15.66
Burkholderia glumae AU12450^T	10.01
Burkholderia tropica AU15822^T	15.35
Burkholderia fungorum AU 12699^T	18.20
Burkholderia glathei HI4344^T	10.42

Table A1.3: GGDC similarity values and ANI between MSh1 to related Bcc species.

Bcc species	*Burkholderia paludis* MSh1		
	Wetlab DDH (% ± standard deviation)	GGDC (% Similarity ± confidence interval)	ANI (% ± standard deviation)
Burkholderia arboris R-24201^T	29.0 ± 3.7	n/a	n/a
Burkholderia cenocepacia J2315^T	32.8 ± 2.2	42.40 ± 2.53	92.28 ± 3.33
Burkholderia lata sp. 383^T	19.5 ± 1.8	43.70 ± 2.54	92.55 ± 3.33

n/a: not available as the genome of *Burkholderia arboris* R-24201^T has not been sequenced

Appendix 2:

Calibration curve of absorbance at OD_{625} against the dry cell weight (g/L) of *B. paludis* MSh1

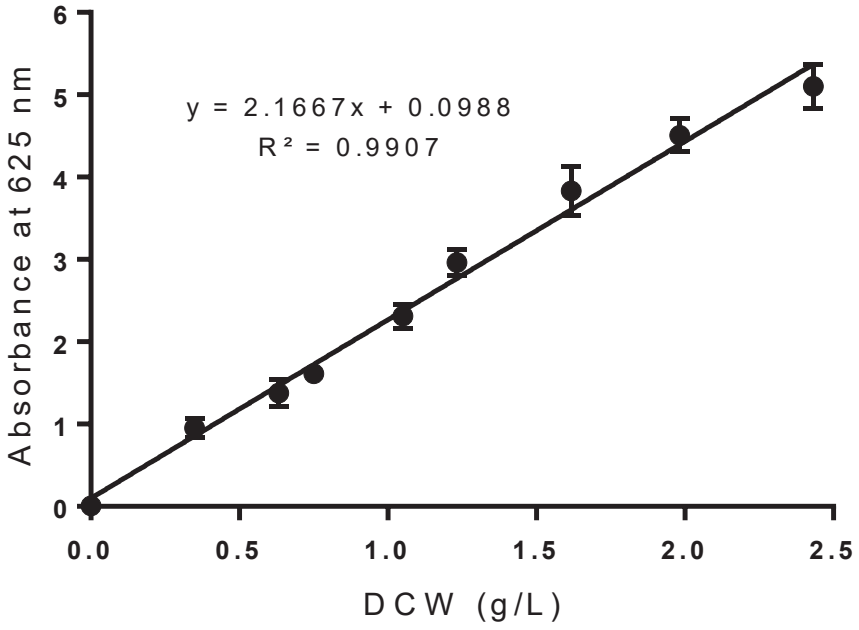

Figure A2.1: Calibration curve of absorbance at OD625 against the dry cell weight (g/L) of *B. paludis* MSh1.

Appendix 3:

HPLC, UV spectra and LC-ESI-MS chromatogram of extracted pyochelin and commercial pyochelin standard

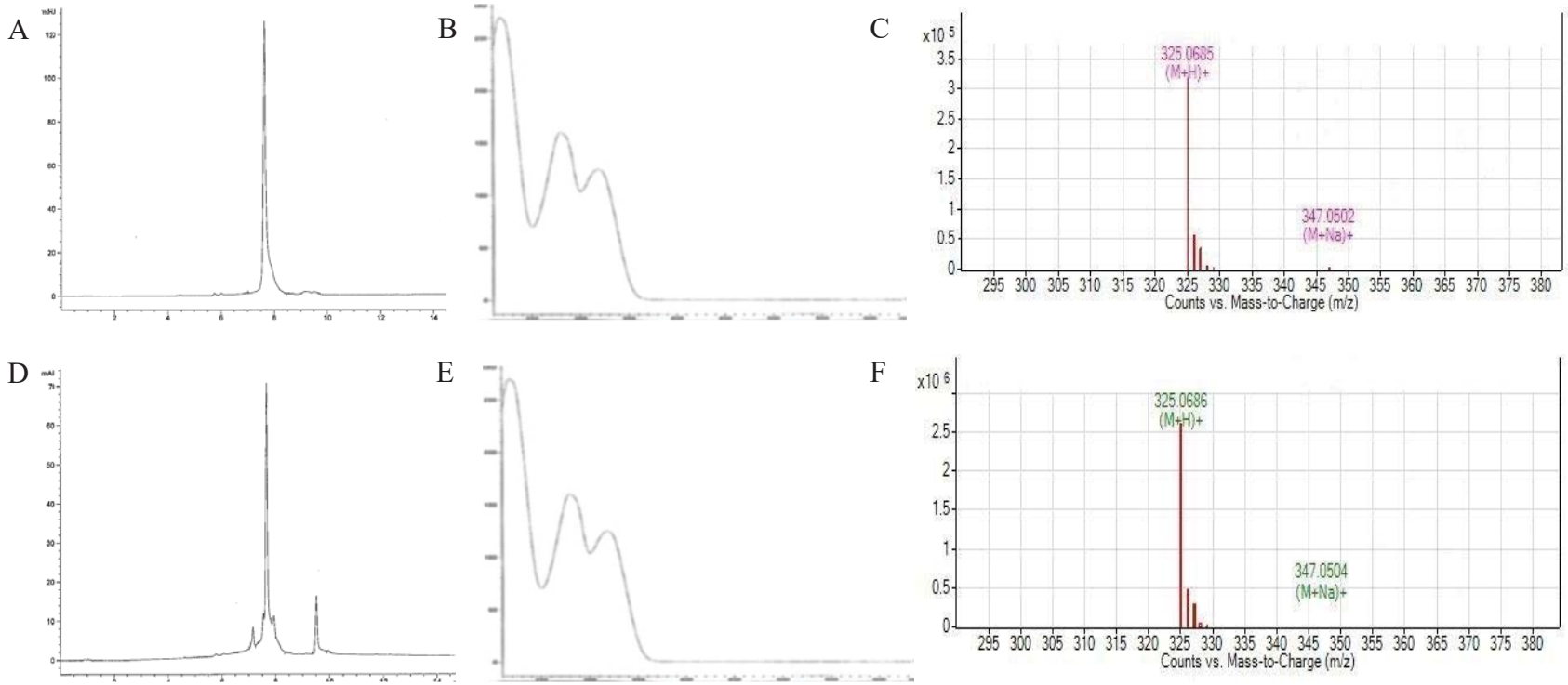

Figure A3.1: HPLC, UV spectra and LC-ESI-MS chromatogram of extracted pyochelin (A, B, C) with commercial pyochelin standard (D, E, F).

Appendix 4:

Standard curve of absorbance against different concentrations of MDA

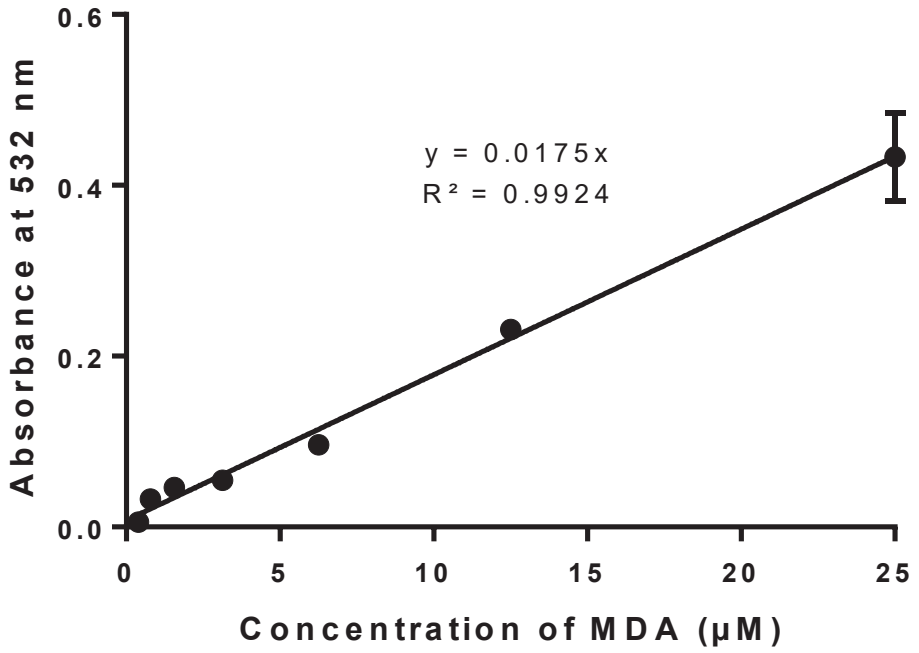

Figure A4.1: Standard curve of absorbance against different concentrations of MDA.

Appendix 5:

Standard curve of G/R ratio against percentage live *E. faecalis* ATCC 700802 bacterial cells

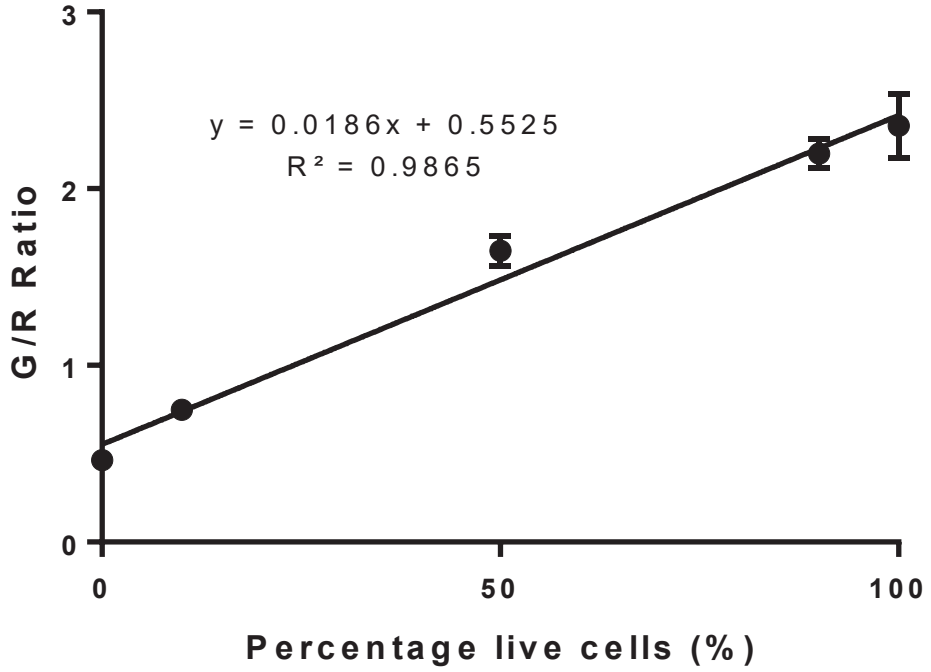

Figure A5.1: Standard curve of G/R ratio against percentage live *E. faecalis* ATCC 700802 bacterial cells.

Appendix 6:

Statistical analysis

Table A6.1: Descriptive data and ANOVA showing the effect of different carbon sources on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	glucose	3	1.2037	.05974	.03449	1.0553	1.3521	1.16	1.27
	galactose	3	1.3727	.04908	.02834	1.2507	1.4946	1.32	1.40
	fructose	3	1.2517	.10870	.06276	.9816	1.5217	1.15	1.36
	maltose	3	1.5717	.07433	.04292	1.3870	1.7563	1.49	1.63
	sucrose	3	.9533	.06329	.03654	.7961	1.1105	.88	1.01
	lactose	3	1.2683	.07801	.04504	1.0745	1.4621	1.20	1.35
	glycerol	3	1.9353	.04718	.02724	1.8181	2.0525	1.88	1.97
	xylose	3	1.2240	.03360	.01940	1.1405	1.3075	1.19	1.26
	starch	3	1.3073	.04786	.02763	1.1884	1.4262	1.27	1.36
	dextrose	3	1.7727	.04636	.02677	1.6575	1.8878	1.73	1.82
	xylitol	3	1.0497	.06058	.03498	.8992	1.2002	.98	1.09
	sorbitol	3	1.7547	.07564	.04367	1.5668	1.9426	1.71	1.84
	xylan	3	1.1157	.05558	.03209	.9776	1.2537	1.05	1.16
	cellulose	3	1.5860	.07550	.04359	1.3985	1.7735	1.52	1.67
	mineral+peptone	3	1.3210	.03874	.02237	1.2248	1.4172	1.28	1.36
	peptone only	3	.7513	.11217	.06476	.4727	1.0300	.63	.85
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	51	1.2611	.44123	.06178	1.1370	1.3852	.00	1.97
Activity	glucose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	galactose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	fructose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	maltose	3	36.6667	5.77350	3.33333	22.3245	51.0088	30.00	40.00
	sucrose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	lactose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol	3	96.6667	5.77350	3.33333	82.3245	111.0088	90.00	100.00
	xylose	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	starch	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	dextrose	3	73.3333	5.77350	3.33333	58.9912	87.6755	70.00	80.00
	xylitol	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	sorbitol	3	53.3333	5.77350	3.33333	38.9912	67.6755	50.00	60.00
	xylan	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	cellulose	3	36.6667	5.77350	3.33333	22.3245	51.0088	30.00	40.00
	mineral+peptone	3	13.3333	5.77350	3.33333	-1.0088	27.6755	10.00	20.00
	peptone only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	51	18.2353	29.91360	4.18874	9.8220	26.6486	.00	100.00

Table A6.1: Descriptive data and ANOVA showing the effect of different carbon sources on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison (continued).

DCW

Tukey HSD

Carbon	N	Subset for alpha = 0.05									
		1	2	3	4	5	6	7	8	9	10
mineral only	3	.0000									
peptone only	3		.7513								
sucrose	3			.9533							
xylitol	3			1.0497	1.0497						
xylan	3			1.1157	1.1157	1.1157					
glucose	3				1.2037	1.2037	1.2037				
xylose	3				1.2240	1.2240	1.2240				
fructose	3					1.2517	1.2517				
lactose	3					1.2683	1.2683				
starch	3					1.3073	1.3073				
mineral+peptone	3						1.3210				
galactose	3						1.3727	1.3727			
maltose	3							1.5717	1.5717		
cellulose	3								1.5860	1.5860	
sorbitol	3								1.7547	1.7547	1.7547
dextrose	3									1.7727	1.7727
glycerol	3										1.9353
Sig.		1.000	1.000	.228	.147	.073	.180	.053	.104	.090	.115

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Carbon	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
glucose	3	.0000					
galactose	3	.0000					
fructose	3	.0000					
sucrose	3	.0000					
lactose	3	.0000					
xylose	3	.0000					
starch	3	.0000					
xylitol	3	.0000					
xylan	3	.0000					
peptone only	3	.0000					
mineral only	3	.0000					
mineral+peptone	3		13.3333				
maltose	3			36.6667			
cellulose	3			36.6667			
sorbitol	3				53.3333		
dextrose	3					73.3333	
glycerol	3						96.6667
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table A6.2: Descriptive data and ANOVA showing the effect of different concentrations of glycerol on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	5	3	2.2427	.02566	.01481	2.1789	2.3064	2.22	2.27
	10	3	2.2117	.13231	.07639	1.8830	2.5403	2.10	2.36
	15	3	2.2017	.07123	.04113	2.0247	2.3786	2.13	2.28
	20	3	2.1623	.07695	.04443	1.9712	2.3535	2.11	2.25
	25	3	2.2713	.12378	.07147	1.9638	2.5788	2.13	2.36
	30	3	2.2360	.02946	.01701	2.1628	2.3092	2.20	2.26
	peptone+mineral	3	2.0230	.09853	.05689	1.7782	2.2678	1.92	2.11
	peptone only	3	1.0970	.07151	.04128	.9194	1.2746	1.02	1.17
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.8273	.75097	.14452	1.5302	2.1244	.00	2.36
Activity	5	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	10	3	90.0000	10.00000	5.77350	65.1586	114.8414	80.00	100.00
	15	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	20	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	25	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	30	3	96.6667	5.77350	3.33333	82.3245	111.0088	90.00	100.00
	peptone+mineral	3	26.6667	5.77350	3.33333	12.3245	41.0088	20.00	30.00
	peptone only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	65.9259	42.17721	8.11701	49.2412	82.6107	.00	110.00

DCW

Tukey HSD

Carbon	N	Subset for alpha = 0.05			
		1	2	3	4
mineral only	3	.0000			
peptone only	3		1.0970		
peptone+mineral	3			2.0230	
20	3			2.1623	2.1623
15	3			2.2017	2.2017
10	3			2.2117	2.2117
30	3			2.2360	2.2360
5	3			2.2427	2.2427
25	3				2.2713
Sig.		1.000	1.000	.075	.776

Means for groups in homogeneous subsets are displayed.

Table A6.2: Descriptive data and ANOVA showing the effect of different concentrations of glycerol on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison (continued).

Activity

Tukey HSD

Carbon	N	Subset for alpha = 0.05		
		1	2	3
peptone only	3	.0000		
mineral only	3	.0000		
peptone+mineral	3		26.6667	
10	3			90.0000
15	3			93.3333
20	3			93.3333
25	3			93.3333
30	3			96.6667
5	3			1.0000E2
Sig.		1.000	1.000	.611

Means for groups in homogeneous subsets are displayed.

Table A6.3: Descriptive data and ANOVA showing the effect of different nitrogen sources on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	peptone	3	1.7747	.04500	.02598	1.6629	1.8865	1.73	1.82
	tryptone	3	1.5890	.07192	.04153	1.4103	1.7677	1.51	1.66
	casein	3	1.3113	.04398	.02539	1.2021	1.4206	1.26	1.35
	beef extract	3	1.5253	.04895	.02826	1.4037	1.6469	1.47	1.56
	yeast extract	3	1.2867	.06987	.04034	1.1131	1.4602	1.22	1.36
	malt extract	3	1.6530	.09714	.05608	1.4117	1.8943	1.58	1.76
	urea	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nano3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4cl	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	(nh4)so4	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	(nh4)h2po4	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4 acetate	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4 citrate	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4no3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral+glycerol	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	51	.5376	.74344	.10410	.3286	.7467	.00	1.82
Activity	peptone	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	tryptone	3	43.3333	5.77350	3.33333	28.9912	57.6755	40.00	50.00
	casein	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	beef extract	3	23.3333	5.77350	3.33333	8.9912	37.6755	20.00	30.00
	yeast extract	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	malt extract	3	43.3333	5.77350	3.33333	28.9912	57.6755	40.00	50.00
	urea	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nano3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4cl	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	(nh4)so4	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	(nh4)h2po4	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4 acetate	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4 citrate	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	nh4no3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral+glycerol	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	51	12.3529	26.65245	3.73209	4.8568	19.8491	.00	110.00

Table A6.3: Descriptive data and ANOVA showing the effect of different nitrogen sources on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison (continued).

DCW

Tukey HSD

Nitrogen	N	Subset for alpha = 0.05				
		1	2	3	4	5
urea	3	.0000				
nano3	3	.0000				
nh4cl	3	.0000				
(nh4)so4	3	.0000				
(nh4)h2po4	3	.0000				
nh4 acetate	3	.0000				
nh4 citrate	3	.0000				
nh4no3	3	.0000				
mineral+glycerol	3	.0000				
glycerol only	3	.0000				
mineral only	3	.0000				
yeast extract	3		1.2867			
casein	3		1.3113			
beef extract	3			1.5253		
tryptone	3			1.5890	1.5890	
malt extract	3				1.6530	
peptone	3					1.7747
Sig.		1.000	1.000	.831	.825	1.000

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Nitrogen	N	Subset for alpha = 0.05			
		1	2	3	4
casein	3	.0000			
yeast extract	3	.0000			
urea	3	.0000			
nano3	3	.0000			
nh4cl	3	.0000			
(nh4)so4	3	.0000			
(nh4)h2po4	3	.0000			
nh4 acetate	3	.0000			
nh4 citrate	3	.0000			
nh4no3	3	.0000			
mineral+glycerol	3	.0000			
glycerol only	3	.0000			
mineral only	3	.0000			
beef extract	3		23.3333		
tryptone	3			43.3333	
malt extract	3			43.3333	
peptone	3				1.0000E2
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table A6.4: Descriptive data and ANOVA showing the effect of different concentrations of peptone on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	5	3	1.2453	.09529	.05502	1.0086	1.4820	1.14	1.32
	10	3	1.4073	.05140	.02968	1.2796	1.5350	1.35	1.45
	15	3	1.5753	.03774	.02179	1.4816	1.6691	1.53	1.60
	20	3	1.6223	.05787	.03341	1.4786	1.7661	1.58	1.69
	25	3	1.6660	.07019	.04053	1.4916	1.8404	1.60	1.74
	30	3	1.6517	.09452	.05457	1.4169	1.8865	1.56	1.74
	glycerol + mineral	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.0187	.74651	.14367	.7234	1.3140	.00	1.74
Activity	5	3	36.6667	5.77350	3.33333	22.3245	51.0088	30.00	40.00
	10	3	53.3333	5.77350	3.33333	38.9912	67.6755	50.00	60.00
	15	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	20	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	25	3	1.0667E2	5.77350	3.33333	92.3245	121.0088	100.00	110.00
	30	3	73.3333	5.77350	3.33333	58.9912	87.6755	70.00	80.00
	glycerol + mineral	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	mineral only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	51.4815	42.94036	8.26388	34.4948	68.4681	.00	110.00

DCW

Tukey HSD

Nitrogen	N	Subset for alpha = 0.05		
		1	2	3
glycerol + mineral	3	.0000		
glycerol only	3	.0000		
mineral only	3	.0000		
5	3		1.2453	
10	3		1.4073	
15	3			1.5753
20	3			1.6223
30	3			1.6517
25	3			1.6660
Sig.		1.000	.059	.615

Means for groups in homogeneous subsets are displayed.

Table A6.4: Descriptive data and ANOVA showing the effect of different concentrations of peptone on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison (continued).

Activity

Tukey HSD

Nitrogen	N	Subset for alpha = 0.05				
		1	2	3	4	5
glycerol + mineral	3	.0000				
glycerol only	3	.0000				
mineral only	3	.0000				
5	3		36.6667			
10	3			53.3333		
30	3				73.3333	
15	3					93.3333
20	3					1.0000E2
25	3					1.0667E2
Sig.		1.000	1.000	1.000	1.000	.129

Means for groups in homogeneous subsets are displayed.

Table A6.5: Descriptive data and ANOVA showing the effect of different concentrations of mineral on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	0.5	3	1.9167	.13695	.07907	1.5765	2.2569	1.80	2.07
	1	3	1.9757	.08075	.04662	1.7751	2.1763	1.90	2.06
	1.5	3	2.0120	.05027	.02902	1.8871	2.1369	1.95	2.04
	2	3	2.1623	.13089	.07557	1.8372	2.4875	2.01	2.25
	2.5	3	2.1700	.04115	.02376	2.0678	2.2722	2.13	2.21
	3	3	1.8387	.06790	.03920	1.6700	2.0073	1.78	1.92
	glycerol+peptone	3	1.7697	.13137	.07584	1.4433	2.0960	1.63	1.90
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	peptone only	3	.7597	.05093	.02941	.6331	.8862	.72	.82
	Total	27	1.6227	.71631	.13785	1.3394	1.9061	.00	2.25
Activity	0.5	3	36.6667	5.77350	3.33333	22.3245	51.0088	30.00	40.00
	1	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	1.5	3	76.6667	5.77350	3.33333	62.3245	91.0088	70.00	80.00
	2	3	56.6667	5.77350	3.33333	42.3245	71.0088	50.00	60.00
	2.5	3	53.3333	5.77350	3.33333	38.9912	67.6755	50.00	60.00
	3	3	40.0000	.00000	.00000	40.0000	40.0000	40.00	40.00
	glycerol+peptone	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	glycerol only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	peptone only	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	39.6296	33.22204	6.39358	26.4874	52.7718	.00	100.00

DCW

Tukey HSD

Mineral	N	Subset for alpha = 0.05			
		1	2	3	4
glycerol only	3	.0000			
peptone only	3		.7597		
glycerol+peptone	3			1.7697	
3	3			1.8387	
0.5	3			1.9167	1.9167
1	3			1.9757	1.9757
1.5	3			2.0120	2.0120
2	3				2.1623
2.5	3				2.1700
Sig.		1.000	1.000	.069	.051

Means for groups in homogeneous subsets are displayed.

Table A6.5: Descriptive data and ANOVA showing the effect of different concentrations of minerals on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison (continued).

Activity

Tukey HSD

Mineral	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
glycerol only	3	.0000					
peptone only	3	.0000					
0.5	3		36.6667				
3	3		40.0000	40.0000			
2.5	3			53.3333	53.3333		
2	3				56.6667		
1.5	3					76.6667	
glycerol+peptone	3					83.3333	83.3333
1	3						93.3333
Sig.		1.000	.992	.054	.992	.721	.253

Means for groups in homogeneous subsets are displayed.

Table A6.6: Descriptive data and ANOVA showing the effect of different agitation speed on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	0	3	1.5343	.13600	.07852	1.1965	1.8722	1.40	1.67
	50	3	1.4090	.16865	.09737	.9900	1.8280	1.23	1.56
	100	3	1.6777	.08035	.04639	1.4781	1.8773	1.58	1.73
	150	3	1.7763	.08495	.04905	1.5653	1.9874	1.68	1.84
	200	3	1.7513	.05445	.03143	1.6161	1.8866	1.69	1.80
	250	3	1.8927	.10861	.06271	1.6229	2.1625	1.79	2.00
	Total	18	1.6736	.18986	.04475	1.5791	1.7680	1.23	2.00
Activity	0	3	16.6667	5.77350	3.33333	2.3245	31.0088	10.00	20.00
	50	3	26.6667	5.77350	3.33333	12.3245	41.0088	20.00	30.00
	100	3	60.0000	10.00000	5.77350	35.1586	84.8414	50.00	70.00
	150	3	76.6667	5.77350	3.33333	62.3245	91.0088	70.00	80.00
	200	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	250	3	73.3333	5.77350	3.33333	58.9912	87.6755	70.00	80.00
	Total	18	58.8889	30.46481	7.18062	43.7391	74.0387	10.00	110.00

DCW

Tukey HSD

Agitation	N	Subset for alpha = 0.05		
		1	2	3
50	3	1.4090		
0	3	1.5343	1.5343	
100	3	1.6777	1.6777	1.6777
200	3		1.7513	1.7513
150	3		1.7763	1.7763
250	3			1.8927
Sig.		.100	.159	.247

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Agitation	N	Subset for alpha = 0.05		
		1	2	3
0	3	16.6667		
50	3	26.6667		
100	3		60.0000	
250	3		73.3333	
150	3		76.6667	
200	3			1.0000E2
Sig.		.589	.138	1.000

Means for groups in homogeneous subsets are displayed.

Table A6.7: Descriptive data and ANOVA showing the effect of different incubation temperature on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	25	3	1.5770	.08155	.04709	1.3744	1.7796	1.51	1.67
	27	3	1.6637	.03754	.02167	1.5704	1.7569	1.62	1.70
	30	3	1.6613	.09400	.05427	1.4278	1.8948	1.57	1.76
	32	3	1.7073	.10666	.06158	1.4424	1.9723	1.62	1.82
	35	3	1.7853	.04461	.02576	1.6745	1.8962	1.74	1.82
	37	3	1.9133	.10453	.06035	1.6537	2.1730	1.80	2.00
	Total	18	1.7180	.13037	.03073	1.6532	1.7828	1.51	2.00
Activity	25	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	27	3	36.6667	5.77350	3.33333	22.3245	51.0088	30.00	40.00
	30	3	96.6667	11.54701	6.66667	67.9823	125.3510	90.00	110.00
	32	3	90.0000	10.00000	5.77350	65.1586	114.8414	80.00	100.00
	35	3	13.3333	5.77350	3.33333	-1.0088	27.6755	10.00	20.00
	37	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	18	39.4444	41.65294	9.81769	18.7309	60.1580	.00	110.00

DCW

Tukey HSD

Temperature	N	Subset for alpha = 0.05	
		1	2
25	3	1.5770	
30	3	1.6613	
27	3	1.6637	
32	3	1.7073	1.7073
35	3	1.7853	1.7853
37	3		1.9133
Sig.		.080	.084

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Temperature	N	Subset for alpha = 0.05		
		1	2	3
25	3	.0000		
37	3	.0000		
35	3	13.3333		
27	3		36.6667	
32	3			90.0000
30	3			96.6667
Sig.		.262	1.000	.849

Means for groups in homogeneous subsets are displayed.

Table A6.8: Descriptive data and ANOVA showing the effect of different incubation duration on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	1	3	1.2607	.03453	.01994	1.1749	1.3464	1.22	1.28
	2	3	1.3993	.06274	.03622	1.2435	1.5552	1.34	1.47
	3	3	1.7143	.05896	.03404	1.5679	1.8608	1.67	1.78
	4	3	1.8047	.04981	.02876	1.6809	1.9284	1.77	1.86
	5	3	1.7787	.03402	.01964	1.6942	1.8632	1.74	1.80
	6	3	1.8673	.06484	.03744	1.7063	2.0284	1.80	1.93
	Total	18	1.6375	.23625	.05569	1.5200	1.7550	1.22	1.93
Activity	1	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	3	3	43.3333	5.77350	3.33333	28.9912	57.6755	40.00	50.00
	4	3	93.3333	5.77350	3.33333	78.9912	107.6755	90.00	100.00
	5	3	96.6667	11.54701	6.66667	67.9823	125.3510	90.00	110.00
	6	3	90.0000	10.00000	5.77350	65.1586	114.8414	80.00	100.00
	Total	18	53.8889	43.67512	10.29433	32.1698	75.6080	.00	110.00

DCW

Tukey HSD

Duration	N	Subset for alpha = 0.05		
		1	2	3
1	3	1.2607		
2	3	1.3993		
3	3		1.7143	
5	3		1.7787	1.7787
4	3		1.8047	1.8047
6	3			1.8673
Sig.		.061	.342	.360

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Duration	N	Subset for alpha = 0.05		
		1	2	3
1	3	.0000		
2	3	.0000		
3	3		43.3333	
6	3			90.0000
4	3			93.3333
5	3			96.6667
Sig.		1.000	1.000	.849

Means for groups in homogeneous subsets are displayed.

Table A6.9: Descriptive data and ANOVA showing the effect of different initial pH of media on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	4	3	1.0013	.10819	.06246	.7326	1.2701	.90	1.11
	5	3	1.5290	.15029	.08677	1.1557	1.9023	1.36	1.64
	6	3	2.1093	.13268	.07660	1.7797	2.4389	1.96	2.23
	7	3	2.1323	.08729	.05040	1.9155	2.3492	2.06	2.23
	8	3	1.9257	.11012	.06358	1.6521	2.1992	1.80	2.02
	9	3	1.4500	.14663	.08465	1.0858	1.8142	1.30	1.59
	Total	18	1.6913	.42982	.10131	1.4775	1.9050	.90	2.23
Activity	4	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	5	3	16.6667	5.77350	3.33333	2.3245	31.0088	10.00	20.00
	6	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	7	3	73.3333	5.77350	3.33333	58.9912	87.6755	70.00	80.00
	8	3	16.6667	5.77350	3.33333	2.3245	31.0088	10.00	20.00
	9	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	18	34.4444	39.73770	9.36627	14.6834	54.2055	.00	110.00

DCW

Tukey HSD

pH	N	Subset for alpha = 0.05		
		1	2	3
4	3	1.0013		
9	3		1.4500	
5	3		1.5290	
8	3			1.9257
6	3			2.1093
7	3			2.1323
Sig.		1.000	.966	.380

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

pH	N	Subset for alpha = 0.05			
		1	2	3	4
4	3	.0000			
9	3	.0000			
5	3		16.6667		
8	3		16.6667		
7	3			73.3333	
6	3				1.0000E2
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table A6.10: Descriptive data and ANOVA showing the effect of different inoculum size on the DCW and antimicrobial activity of *B. paludis* MSh1, follow by Tukey's Post Hoc comparison.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DCW	0.5	3	1.6550	.02987	.01724	1.5808	1.7292	1.63	1.69
	1	3	1.6837	.02810	.01622	1.6139	1.7535	1.66	1.71
	2	3	1.7153	.03089	.01784	1.6386	1.7921	1.68	1.74
	3	3	1.6940	.07111	.04105	1.5174	1.8706	1.64	1.77
	4	3	1.7473	.06093	.03518	1.5960	1.8987	1.68	1.80
	5	3	1.8070	.04949	.02857	1.6841	1.9299	1.76	1.86
	Total	18	1.7171	.06473	.01526	1.6849	1.7492	1.63	1.86
Activity	0.5	3	73.3333	5.77350	3.33333	58.9912	87.6755	70.00	80.00
	1	3	1.0000E2	10.00000	5.77350	75.1586	124.8414	90.00	110.00
	2	3	96.6667	11.54701	6.66667	67.9823	125.3510	90.00	110.00
	3	3	90.0000	10.00000	5.77350	65.1586	114.8414	80.00	100.00
	4	3	66.6667	5.77350	3.33333	52.3245	81.0088	60.00	70.00
	5	3	53.3333	5.77350	3.33333	38.9912	67.6755	50.00	60.00
	Total	18	80.0000	18.78673	4.42807	70.6576	89.3424	50.00	110.00

DCW

Tukey HSD

Size	N	Subset for alpha = 0.05	
		1	2
0.5	3	1.6550	
1	3	1.6837	1.6837
3	3	1.6940	1.6940
2	3	1.7153	1.7153
4	3	1.7473	1.7473
5	3		1.8070
Sig.		.246	.072

Means for groups in homogeneous subsets are displayed.

Activity

Tukey HSD

Size	N	Subset for alpha = 0.05		
		1	2	3
5	3	53.3333		
4	3	66.6667		
0.5	3	73.3333	73.3333	
3	3		90.0000	90.0000
2	3			96.6667
1	3			1.0000E2
Sig.		.110	.229	.704

Means for groups in homogeneous subsets are displayed.

Table A6.11: Independent-sample *t*-test showing the difference in antimicrobial activity of *B. paludis* MSh1 between the near-optimized culture medium and NB.

Group Statistics

	Media	N	Mean	Std. Deviation	Std. Error Mean
Activity	1	6	1.0500E2	5.47723	2.23607
	2	6	23.3333	5.16398	2.10819

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Activity	Equal variances assumed	.625	.448	26.574	10	.000	81.66667	3.07318	74.81919	88.51414
	Equal variances not assumed			26.574	9.966	.000	81.66667	3.07318	74.81598	88.51735

Table A6.12: Independent-sample *t*-test showing the effect of different concentrations of pyochelin on the log CFU/mL of exponential phase *E. faecalis* ATCC 700802 after 24 hours, incubated aerobically.

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.1033	.14012	.08090
	1x MIC	3	2.8500	.20881	.12055

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	1.119	.350	22.409	4	.000	3.25333	.14518	2.85024	3.65642
	Equal variances not assumed			22.409	3.498	.000	3.25333	.14518	2.82635	3.68032

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.1033	.14012	.08090
	2x MIC	3	.0000	.00000	.00000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	4.614	.098	75.445	4	.000	6.10333	.08090	5.87873	6.32794
	Equal variances not assumed			75.445	2.000	.000	6.10333	.08090	5.75526	6.45141

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.1033	.14012	.08090
	4x MIC	3	.0000	.00000	.00000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	4.614	.098	75.445	4	.000	6.10333	.08090	5.87873	6.32794
	Equal variances not assumed			75.445	2.000	.000	6.10333	.08090	5.75526	6.45141

Table A6.13: Independent-sample *t*-test showing the effect of different concentrations of pyochelin on the log CFU/mL of stationary phase *E. faecalis* ATCC 700802 after 24 hours, incubated aerobically.

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.2133	.09504	.05487
	1x MIC	3	5.2967	.15144	.08743

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	1.373	.306	8.880	4	.001	.91667	.10323	.63007	1.20327
	Equal variances not assumed			8.880	3.364	.002	.91667	.10323	.60738	1.22596

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.2133	.09504	.05487
	2x MIC	3	4.4933	.10017	.05783

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	.087	.783	21.575	4	.000	1.72000	.07972	1.49866	1.94134
	Equal variances not assumed			21.575	3.989	.000	1.72000	.07972	1.49842	1.94158

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.2133	.09504	.05487
	4x MIC	3	4.0033	.11719	.06766

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	.383	.570	25.369	4	.000	2.21000	.08711	1.96813	2.45187
	Equal variances not assumed			25.369	3.836	.000	2.21000	.08711	1.96401	2.45599

Table A6.14: Independent-sample *t*-test showing the effect of different concentrations of pyochelin on the log CFU/mL of exponential phase *E. faecalis* ATCC 700802 after 24 hours, incubated anaerobically.

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.0833	.31660	.18279
	1x MIC	3	6.0667	.10599	.06119

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	2.238	.209	.086	4	.935	.01667	.19276	-.51851	.55185
	Equal variances not assumed			.086	2.443	.938	.01667	.19276	-.68407	.71741

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.0833	.31660	.18279
	2x MIC	3	5.9700	.20518	.11846

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	.522	.510	.520	4	.630	.11333	.21782	-.49142	.71809
	Equal variances not assumed			.520	3.428	.635	.11333	.21782	-.53337	.76003

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
CFU	Negative control	3	6.0833	.31660	.18279
	4x MIC	3	5.9800	.13892	.08021

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CFU	Equal variances assumed	1.411	.301	.518	4	.632	.10333	.19961	-.45087	.65754
	Equal variances not assumed			.518	2.743	.644	.10333	.19961	-.56700	.77367

Table A6.15: Independent-sample *t*-test showing the effect of different concentrations of pyochelin on the production of ROS on *E. faecalis* ATCC 700802 after 24 hours.

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Value	Negative control	3	7.2033E2	34.53018	19.93601
	1x MIC	3	1.4623E3	71.69612	41.39377

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Value	Equal variances assumed	3.002	.158	-16.150	4	.000	-742.00000	45.94441	-869.56213	-614.43787
	Equal variances not assumed			-16.150	2.880	.001	-742.00000	45.94441	-891.70927	-592.29073

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Value	Negative control	3	7.2033E2	34.53018	19.93601
	2x MIC	3	2.6770E3	74.48490	43.00388

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Value	Equal variances assumed	3.502	.135	-41.280	4	.000	-1956.66667	47.40019	-2088.27069	-1825.06265
	Equal variances not assumed			-41.280	2.822	.000	-1956.66667	47.40019	-2113.05377	-1800.27957

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MIC1x	1	3	7.2033E2	34.53018	19.93601
	4	3	3.0400E3	215.92360	124.66355

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MIC1x	Equal variances assumed	6.213	.067	-18.374	4	.000	-2319.66667	126.24755	-2670.18607	-1969.14727
	Equal variances not assumed			-18.374	2.102	.002	-2319.66667	126.24755	-2838.32155	-1801.01178

Table A6.16: Independent-sample *t*-test showing the effect of different concentrations of pyochelin on the concentration of MDA after 24 hours.

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	Negative control	3	1.4667	.06817	.03936
	1x MIC	3	1.9390	.31859	.18394

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration	Equal variances assumed	7.275	.054	-2.511	4	.066	-.47238	.18810	-.99463	.04987
	Equal variances not assumed			-2.511	2.183	.118	-.47238	.18810	-1.22011	.27535

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	Negative control	3	1.4667	.06817	.03936
	2x MIC	3	2.9295	.33370	.19266

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration	Equal variances assumed	5.117	.086	-7.439	4	.002	-1.46286	.19664	-2.00883	-.91689
	Equal variances not assumed			-7.439	2.167	.014	-1.46286	.19664	-2.24954	-.67617

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	Negative control	3	1.4667	.06817	.03936
	4x MIC	3	3.1638	.66331	.38296

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration	Equal variances assumed	6.978	.057	-4.408	4	.012	-1.69714	.38498	-2.76601	-.62827
	Equal variances not assumed			-4.408	2.042	.046	-1.69714	.38498	-3.32116	-.07312

Table A6.17: Wilcoxon test showing the effect of different concentrations of pyochelin on the membrane integrity of *E. faecalis* ATCC 700802 after 8 hours.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	88.2678	3.15707	84.74	93.98
MIC1x	6	52.0481	4.98682	46.50	60.58

Test Statistics[b]

	MIC1x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	88.2678	3.15707	84.74	93.98
MIC2x	6	51.2499	5.78386	41.03	56.50

Test Statistics[b]

	MIC2x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	88.2678	3.15707	84.74	93.98
MIC4x	6	46.6176	6.17471	40.02	56.75

Test Statistics[b]

	MIC4x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Table A6.18: Wilcoxon test showing the effect of different concentrations of pyochelin on the membrane integrity of *E. faecalis* ATCC 700802 after 24 hours.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	89.3621	2.86524	85.89	94.42
MIC1x	6	50.3579	5.40053	45.33	57.75

Test Statistics[b]

	MIC1x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	89.3621	2.86524	85.89	94.42
MIC2x	6	17.5575	3.18262	11.80	20.89

Test Statistics[b]

	MIC2x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Negative_Control	6	89.3621	2.86524	85.89	94.42
MIC4x	6	12.0339	3.35961	8.48	16.02

Test Statistics[b]

	MIC4x - Negative_ Control
Z	-2.201[a]
Asymp. Sig. (2-tailed)	.028

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Appendix 7:

Publications related to thesis

Appendix 7(a):

Draft genome sequences of two antimicrobial-producing *Burkholderia* sp. strains, MSh1 and MSh2, isolated from Malaysian tropical peat swamp forest soil

genomeA
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Draft Genome Sequences of Two Antimicrobial-Producing *Burkholderia* sp. Strains, MSh1 and MSh2, Isolated from Malaysian Tropical Peat Swamp Forest Soil

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We report the draft genome sequences of two antimicrobial-producing isolates, *Burkholderia* sp. strains MSh1 and MSh2, which were isolated from tropical peat swamp forest soil. Putative genes related to different antimicrobial production have been annotated in both genome sequences.

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Extensive use of antimicrobials has led to an increase in infections caused by antimicrobial-resistant bacteria (ARB). The increase in mortality rates from infection coupled with limited treatment options has prompted the need for new antimicrobials (1). It is hypothesized that bacteria produce antimicrobial compounds in nutrient-poor and extreme environments to gain an advantage in competing for resources (2). Hence, a Malaysian tropical peat swamp forest was chosen as a potential location for new antimicrobial compounds due to its low nutrient level and low pH conditions (3). Two antimicrobial-producing bacteria were successfully isolated and identified as *Burkholderia* sp. using 16S rRNA gene analysis (4).

Both *Burkholderia* sp. strains MSh1 and MSh2 are oxidase positive, non–spore forming, Gram-negative bacteria isolated from tropical peat swamp forest soil from Southeast Pahang, Malaysia. Both were isolated due to their ability to produce antibacterial compounds that were active against several drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 700699 and vancomycin-resistant enterococci (VRE) ATCC 700802 with MIC values of the crude acetonitrile extract at 1.250 mg/mL and 0.313 mg/mL, respectively.

The genomic DNA of *Burkholderia* sp. MSh1 and MSh2 were isolated from a 2-day-old culture on nutrient agar using a GF-1 nucleic acid extraction kit (Vivantis, Malaysia) and subsequently converted into an Illumina-compatible next-generation sequencing library using Nextera XT (Illumina, San Diego, CA). The library was then sequenced on the Illumina MiSeq (150-bp paired-end reads) at the Monash University Malaysia Genomics Facility. The raw reads were trimmed and assembled *de novo* (default settings) using CLC Genomics Workbench 6 (CLC Bio, Denmark). The draft genome of *Burkholderia* sp. MSh1 was assembled into 172 contigs with 67.08% G+C content and a total length of 8,633,651 bp (N_{50} = 110,286 bp), while *Burkholderia* sp. MSh2 had 167 contigs with 67.13% G+C content and an accumulated length of 8,723,138 bp (N_{50} = 129,187 bp).

Using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), 6,963 coding DNA sequences (CDSs), 10 rRNAs, and 64 tRNAs were annotated for *Burkholderia* sp. MSh1, while 7,072 CDSs, 9 rRNAs, and 59 tRNAs were annotated for *Burkholderia* sp. MSh2. Several antimicrobial biosynthesis genes were predicted in the genome of both *Burkholderia* sp. MSh1 and MSh2, including polyketide cyclase (KFG96276 and KEZ06389), antibiotic biosynthesis monooxygenase (KFG97610 and KEZ06948), mitomycin antibiotic biosynthesis protein (KFG98191 and KEZ04328), colicin V production protein (KFG93602 and KEZ04863), and phenazine biosynthesis protein (PhzC/PhzF) (KFG92381 and KEZ02706).

Based on RAST, some of the known antimicrobials produced by *Burkholderia* species were absent, for instance, pyrrolnitrin (*B. pyrrocinia*), rhizobitoxin (*B. andropogonis*), and pyoluteorin (*B. cepacia*) (5–7). This indicates that the antimicrobial compounds produced by these two isolates might be new and hence have the potential to treat infection caused by antimicrobial-resistant bacteria. However, further purification and identification of the antimicrobial compounds are required.

Nucleotide sequence accession numbers. The draft genome sequences of *Burkholderia* sp. MSh1 and MSh2 were deposited in DDBJ/EMBL/GenBank under the accession numbers JPGL00000000 and JPGM00000000, respectively. The versions described in this paper are the first versions, JPGL00000000.1 and JPGM00000000.1.

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Appendix 7(b):

Burkholderia paludis sp. nov., an antibiotic siderophore producing novel *Burkholderia cepacia* complex species, isolated from Malaysian tropical peat swamp soil

frontiers in Microbiology

ORIGINAL RESEARCH
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Burkholderia paludis sp. nov., an Antibiotic-Siderophore Producing Novel *Burkholderia cepacia* Complex Species, Isolated from Malaysian Tropical Peat Swamp Soil

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A novel Gram negative rod-shaped bacterium, designated strain MSh1^T, was isolated from Southeast Pahang tropical peat swamp forest soil in Malaysia and characterized using a polyphasic taxonomy approach. The predominant cellular fatty acids (>10.0%) were $C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%), and $C_{19:0}$ cyclo ω8c (16.1%). The polar lipids detected were phosphatidylglycerol, phosphatidylethanolamine, and diphosphatidylglycerol. The predominant ubiquinone was Q-8. This revealed that strain MSh1^T belongs to the genus *Burkholderia*. The type strain MSh1^T can be differentiated from other *Burkholderia cepacia* complex (Bcc) species by phylogenetic analysis of 16S rRNA gene sequence, multilocus sequence analysis (MLSA), average nucleotide identity (ANI) and biochemical tests. DNA–DNA relatedness values between strain MSh1^T and closely related type strains were below the 70% threshold value. Based on this polyphasic study of MSh1^T, it can be concluded that this strain represents a novel species within the Bcc, for which the name *Burkholderia paludis* sp. nov. is proposed. The type strain is MSh1^T (=DSM 100703^T =MCCC 1K01245^T). The dichloromethane extract of MSh1^T exhibited antimicrobial activity against four Gram positive bacteria (*Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 700802, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 700699) and a Gram negative bacteria (*Escherichia coli* ATCC 25922). Further purification work has led to the isolation of Compound 1, pyochelin. Pyochelin demonstrated antimicrobial activity against four *S. aureus* strains and three *E. faecalis* strains with MIC-values of 3.13 μg/ml and 6.26 μg/ml, respectively. SEM analysis showed that the cellular morphology of *E. faecalis* ATCC 700802 was not affected by pyochelin; suggesting that it might target the intracellular components. Pyochelin, a siderophore with antimicrobial activity might be useful in treating bacterial infections caused by *S. aureus* and *E. faecalis*, however further work has to be done.

Keywords: antimicrobial, *Burkholderia cepacia* complex, multilocus sequence analysis (MLSA), polyphasic taxonomy, tropical peat swamp forest

INTRODUCTION

The misuse of antimicrobial compounds to treat infections has led to an increase in the prevalence of antimicrobial resistant bacteria (ARB), often associated with nosocomial (hospital acquired) infections (Mishra et al., 2012). It was reported that nosocomial infections constitute the sixth leading cause of death in the United States and more than 70% of these nosocomial pathogens are resistant to at least one of the common antimicrobial drugs used to treat them (Iowa Department of Public Health). This is an alarming issue because ARB infections often result in increased mortality rates, limit treatment options and cause increased healthcare costs (Ammerlaan et al., 2016). Thus, there is a need for new antimicrobial compounds to combat ARB infections.

One strategy to discover new antimicrobials is bioprospecting—the exploration for new compounds in unique ecological niches (Imhoff et al., 2011). The discovery of abyssomicins, novel antibiotics produced by *Verrucosispora* sp. from South China Sea sediments is a successful example of bioprospecting (Wang et al., 2013). Bacteria producing antibiotics may have an advantage in competing for resources and colonization of new habitats in natural environments. Novel microorganisms thriving in extreme and isolated environments have the potential to develop unique biosynthesis gene clusters giving rise to novel compounds (Bull and Stach, 2007; Hibbing et al., 2010). Consequently, tropical peat swamp forests in Malaysia were chosen as potential bioprospecting locations for novel antimicrobial compounds. Tropical peat swamp forests are unique wetland ecosystems characterized by their acidic (pH range of 2.9 to 4.5) and waterlogged conditions. They are ombotrophic hence receiving nutrients solely from rain and atmospheric deposition which results in an extremely nutrient poor environment (Yule, 2010). Despite being such a harsh environment, we successfully isolated an antimicrobial-producing bacterium, belonging to the *Burkholderia cepacia* complex (Bcc) in a previous study (Ong et al., 2015).

The Bcc consists of a group of diverse, ubiquitous bacteria that occur in aquatic environments, plant rhizospheres and animals, including humans (Coenye and Vandamme, 2003; Vanlaere et al., 2009; Peeters et al., 2013). Certain Bcc species, for example *B. cepacia*, can be used for biocontrol as they can produce antifungal compounds to repress soil borne pathogens (Caballero-Mellado et al., 2004). An example of one such compound is pyrrolnitrin which targets the electron transport chain of both Gram positive bacteria and fungi (El-Banna and Winkelmann, 1998). Bcc species share a high degree of 16S rRNA (98–100%) and recA (94–95%) gene sequence similarity, and moderate levels of DNA–DNA hybridization (30–50%) (Coenye et al., 2001). In order to differentiate different species of Bcc, multi-locus sequence analysis (MLSA) are usually adopted as these taxonomic techniques provide the discriminatory power needed for both identification and differentiation of Bcc species (Spilker et al., 2009; Vanlaere et al., 2009; Peeters et al., 2013).

Abbreviations: Bcc, Burkholderia cepacia complex; MLSA, multilocus sequence analysis.

In this paper, we report a novel antimicrobial-producing Bcc species, isolated from the peat soil of the Southeast Pahang tropical peat swamp forest reserve in Malaysia. This polyphasic taxonomic study revealed that strain MSh1^T represents a novel Bcc species, for which the name *B. paludis* is proposed. Based on current literature, *Burkholderia* species are known to produce narrow-spectrum antimicrobial compounds such as iminopyrrolidines (produced by *B. plantari*) and occidiofungin (*B. contaminans*) which only targets Gram negative *Erwinia amylovora* and fungi, respectively (Lu et al., 2009; Tawfik et al., 2010). In this study, strain MSh1^T exhibit a different spectrum of antimicrobial activity, which targets Gram positive bacteria only, particularly strains of *S. aureus* and *E. faecalis*.

MATERIALS AND METHODS

Test Microorganism Strains and Culture Conditions

Test microorganism strains that were used in this study include *Bacillus cereus* ATCC 14579, *Bacilus subtilis* ATCC 8188, *Enterococcus faecalis* ATCC 700802, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* JH-22, *Staphylococcus aureus* ATCC 700699, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* ATCC 29213, *Aeromonas hydrophila* ATCC 49140, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Proteus mirabilis* ATCC 49140, *Proteus vulgaris* (Institute of Medical Research, Malaysia), *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas aeruginosa* ATCC BAA-47, *Salmonella* Typhimurium ATCC 14028, and *Shigella flexneri* ATCC 12022. Strains were cultured on Muller Hinton borth (MHB) (Oxoid, UK) at 37°C and maintained at −80°C in MHB with 25% (v/v) glycerol.

Isolation and Maintenance of Isolate

Strain MSh1^T was previously isolated from surface peat collected from the Southeast Pahang tropical peat swamp forest reserve (3° 01′ 19.56″ N; 103° 39′ 29.67″ E) in Malaysia on March 2013. Strain MSh1^T was maintained on nutrient agar (NA) at 30°C and in 25% (v/v) glycerol in nutrient broth (NB) (Merck, Germany) at −80°C for long term preservation.

Sequence and Phylogenetic Analysis

The genomic DNA of strain MSh1^T was isolated using GF-1 nucleic acid extraction kit (Vivantis, Malaysia). The 16S rRNA gene sequence of strain MSh1^T was amplified using the universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-ACG GCT ACC TTG TTA CGA CTT 3′) (Kane et al., 1993). PCR were set up as follows: 150 ng (5 μL) of DNA extract, 10 μL of 5x MyTaq Red Reduction Buffer, 5 μM of forward primer, 5 μM of reverse primer, and 1.25 U of MyTaq DNA polymerase. The reaction volume was made up to 50 μL using sterile milliQ water (Millipore, Germany). The PCR included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 45 s and elongation at 72°C for 45 s. PCR products were separated on a 1.5% (w/v) agarose gel and the bands were visualized with 1x GelRed. Amplification products were purified

and cloned into pJET1.2 vector (FirstBase, Malaysia). The 16S rRNA gene sequence of strain MSh1^T was aligned with sequences of closely related type strains of the genus *Burkholderia* retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al., 1997). The alignment was manually verified and adjusted prior to the construction of phylogenetic tree using the neighbor-joining (Saitou and Nei, 1987) algorithm with the MEGA version 6.0 software (Tamura et al., 2011). The stability of the resultant tree topologies were evaluated by using the bootstrap resampling method (Felsenstein, 1985). The evolutionary distances for the neighbor-joining algorithm were computed using the Kimura's two-parameter model (Kimura, 1980).

DNA–DNA Hybridization (DDH)

DDH-tests were carried out by the Identification Service of Deutsche Sammlung von Mikroorganisen und Zellkulturen (DSMZ, Germany) to evaluate the DNA–DNA relatedness between strain MSh1^T, *B. arboris* R-24201^T, *B. cenocepacia* J2315^T, and *B. lata* 383^T using the optical renaturation rate method as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983).

Multilocus Sequence Analysis (MLSA)

MLSA was performed on strain MSh1^T based on the method described by Spilker et al. (2009). A phylogenetic tree of the concatenated sequence (2773 bp) of seven housekeeping gene fragments [*atp*D (443 bp), *glt*B (400 bp), *gyr*B (454 bp), *rec*A (393 bp), *lep*A (397 bp), *pha*C (385 bp) and *trp*B (301 bp)] was constructed using MEGA6.0 (Tamura et al., 2011). The mean number of nucleotide substitution per site (i.e., the percentage of divergence of concatenated allele sequences) between established Bcc type strains and strain MSh1^T was calculated using the DnaSP v5.10 (Librado and Rozas, 2009) based on the Jukes-Cantor method (Jukes and Cantor, 1969). Nucleotide sequences of each allele, allelic profiles and sequence types of strain MSh1^T from the present study are available on the Bcc PubMLST database (http://pubmlst.org/bcc).

PCR Amplification of *prnD* (Pyrrolnitrin) Gene

The synthesis of pyrrolnitrin is regulated mainly by four genes *prnA*, *prnB*, *prnC* and *prnD*, where *prnD* being the most crucial gene. The gene product of *prnD* catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Souza and Raaijmakers, 2003). As pyrrolnitrin is the only reported compound with antimicrobial activity against Gram positive bacteria known to be produced by Bcc (El-Banna and Winkelmann, 1998), this assay was carried out to avoid the rediscovery of a known compound (pyrrolnitrin). Hence in order to assess the ability of strain MSh1^T to produce pyrrolnitrin, the *prnD* gene was amplified with a forward primer PRND1 (5′-GGG GCG GGC CGT GGT GAT GGA-3′) and a reverse primer PRND2 (5′-YCC CGC SGC CTG YCT GGT CTG-3′) (Souza and Raaijmakers, 2003). PCR were set up as follows: 5 μL of DNA extract, 10 μL of 5x MyTaq Red Reduction Buffer, 5 μM of forward primer, 5 μM of reverse primer, and 1.25 U of MyTaq DNA polymerase. The reaction volume was made up to 50 μL using sterile milliQ water (Millipore, Germany). The PCR included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 68°C for 30 s, and elongation at 72°C for 30 s. Genomic DNA of pyrrolnitrin producer *B. lata* 383^T was used as a positive control and non-pyrrolnitrin producer *B. cenocepacia* J2315^T was used as a negative control. PCR products were separated on a 1.5% (w/v) agarose gel and the bands were visualized with 1x GelRed.

Phenotypic and Biochemical Characterization

Cellular morphological characteristics of strain MSh1^T were observed by using a Hitachi S-4800 field-emission scanning electron microscopy (FE-SEM) at 25,000× magnification after 2 days of growth in nutrient broth (NB) at 30°C. Colony morphology of strain MSh1^T was examined after 2 days of growth on NA at 30°C. Cell motility was measured using hanging drop method after incubation for 2 days at 30°C in NB (Robbie, 1945). The optimal temperature for growth was measured at 4, 15, 28, 30, 37, 40, and 45°C in NB for 2 days. The pH range for growth was tested with in NB adjusted with HCl or NaOH to pH 3–11, at intervals of 0.5 pH units. NaCl tolerance at 0–5% (w/v) at interval of 0.5% (w/v) was determined in NB for 2 days. Anaerobic growth was tested in NB for up to 5 days in a 2.5 l jar containing an AnaeroPack-Anaero (Thermo Scientific, USA). Phenotypic characteristics including Gram staining, catalase, and oxidase activity were examined using the methods described by Buck (1982) and Cappuccino and Sherman (2002) after 2 days of growth on NA at 30°C. Physiological and biochemical properties were further determined using API 50CH, API 20NE, and API ZYM strips (bioMerieux, France) according to the manufacturer's instructions. All tests were conducted in duplicate. The API 50CH and API 20NE-tests were read after 24–48 h at 30°C, while the API ZYM-tests were read after 4 h of incubation at 30°C.

Chemotaxonomic Characterization

The cellular fatty acids analysis of strain MSh1^T, *B. arboris* R-24201^T, *B. cenocepacia* J2315^T, and *B. lata* 383^T was carried out by the Identification Service of DSMZ (Braunschweig, Germany). The cell mass of strain MSh1^T and closely related type strains were harvested from NB after incubation at 30°C for 2 days. Extraction and analysis of the cellular fatty acids were performed according to the standard protocols of the Sherlock Microbial Identification System (MIDI) (Miller, 1982), analyzed using an Agilent 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column. Peaks were integrated and identified using the peak-naming table TSBA40 at the DSMZ. Polar lipids extracted from 200 mg freeze-dried cell material using chloroform: methanol: 0.3% (w/v) aqueous NaCl mixture 1:2:0.8 (v/v/v) as described by Tindall (1990). The extracted polar lipids were then separated by two dimensional silica gel thin layer chromatography with chloroform: methanol: water (65:25:4, v/v/v) as mobile phase for the first direction, followed by chloroform: methanol: acetic acid: water (80:12:15:4, v/v/v/v) as the mobile phase for the second direction (Tindall et al., 2007). Cellular ubiquinones were extracted and purified as

described previously by Tindall (1990). The different quinone classes were firstly separated using thin layer chromatography on silica gel using hexane-tert-butylmethylether (9:1, v/v) as a solvent and then further purified with reversed-phase HPLC using methanol:heptane (9:1, v/v) as the eluent. The purified quionones were compared to standards at the DSMZ.

Genome-To-Genome Distance Calculator (GGDC) and Average Nucleotide Identity (ANI)

GGDC was performed to predict the DNA–DNA hybridization value between strain MSh1^T with related *Burkholderia* species. GGDC was performed at http://ggdc.dsmz.de/distcalc2.php using the standard parameters (GGDC 2.0 BLAST+) (Meier-Kolthoff et al., 2013). The genome sequence of strain MSh1^T (DDBJ/EMBL/GenBank JPGL00000000) (Ong et al., 2014) was queried with related Bcc species. Results were expressed as similarity percentage ± confidence interval. ANI was performed to estimate the mean values between homologous genomic regions shared by strain MSh1^T with related Bcc species using the whole genome sequence obtained. ANI was performed at http://enve-omics.ce.gatech.edu/ani using the standard parameters (Goris et al., 2007). Results were expressed as ANI percentage ± standard deviation.

Genome Analysis of Secondary Metabolites Gene Clusters Using Antismash

The whole genome of strain MSh1^T was screened for biosynthetic gene clusters responsible for the synthesis of secondary metabolites using Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) 2.0) (Blin et al., 2013). The antiSMASH 2.0 program analyses the whole genome sequence for homologs to known secondary metabolites via BLAST search and annotates them based on different biosynthetic gene clusters, for example polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), terpene and bacteriocins.

Methanol Extraction of MSh1^T

Strain MSh1^T was first grown on nutrient agar supplemented with 5 g/l of glycerol for 5 days at 30°C. The whole agar including the bacteria was extracted three times with 100% methanol (Merck, Germany) (Isnansetyo and Kamei, 2003). The crude methanol extract was lyophilized using a Freezone 4.5 Plusfreeze Dryer (Labconco, USA).

Sequential Solvent Fractionation and Purification of the Crude Methanol Extract

Sequential solvent fractionation was performed on the crude methanol extract to fractionate the extract into different fractions with different polarity. Five grams of crude methanol extract was first dissolved in 500 ml of distilled water, and then extracted with hexane (HEX), dichloromethane (DCM) and ethyl acetate (EtOAc) sequentially. Each extraction step was performed three times and combined into one fraction before lyophylization in a Freezone 4.5 Plusfreeze dryer (Labconco, USA). The lyophilized active fraction was dissolved in methanol and partially purified on an open C_{18} column (Merck, Germany), followed by reversed-phase HPLC using a Cosmosil 5C18-MS-II, 20 × 250 mm, 5 μm column (Nacalai, USA), to yield compound 1. All processes were monitored by bioassay.

Identification of the Antimicrobial Compound(s) from Strain MSh1^T

Structural determination of the antimicrobial compound was performed by spectroscopic techniques and literature comparison. The antimicrobial compound was analyzed by thin-layer chromatography on a silica 60 plates (Merck, Germany) with chloroform-acetic acid-ethanol at 95:5:2.5 (v/v) as the mobile phase, followed by spraying of an iron reagent (0.1 M $FeCl_3$ in 0.1 M HCl). LC-MS was performed with an Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer (dual ESI source) equipped with an Agilent Zorbax Eclipse XDB-C18 column. The ultraviolet/visible absorption spectrum was recorded with the photodiode array detector equipped with the above-mentioned HPLC. The mobile phase was composed of water (A, 0.5% formic acid) and acetonitrile (B, 0.5% formic acid), the gradient program of which was 0–12.00 min 90% A and 10% B and 12.00–15.00 min 100% B. The follow rate of the mobile phase was 0.3 ml/min, and the column temperature was set to 25°C. The injection volume was 10 μl.

Antimicrobial Testing Via Broth Microdilution

The antimicrobial activity of each fraction was evaluated using a broth microdilution assay to determine the minimum inhibitory concentration (MIC) of an antimicrobial compound as described by the Clinical and Laboratory Standard Institute (CLSI). MIC is defined as the lowest concentration of an antimicrobial to inhibit the visible growth of a microorganism after overnight incubation. Briefly, the test microorganisms were grown in MHB at 37°C for 24 h and adjusted to 0.5 McFarland standard (OD_{625} 0.08–0.11). The adjusted cultures were then diluted 100 times in MHB and used as inocula. The extracts were serially diluted using sterile MHB in a 96-well flat bottomed microtiter plate. One hundred micro liters of test microorganisms corresponding to approximately 10^5 colony forming units (CFU) was added to each well. Determination of MIC was performed in triplicate. The positive control for bacteria was 100 μg/ml chloramphenicol. The negative control contained MHB with test microorganisms. The blank control consisted only of MHB. The microtiter plate was incubated at 37°C aerobically for 24 h and the MIC was determined by the concentration of extract (μg/ml) where no visible growth was observed.

Scanning Electron Microscopy (SEM)

SEM was performed based on the method described by Pilsczek et al. (2010) with modification, to determine the effect of the extract on the cellular morphology of the bacteria. *E. faecalis* ATCC 700802 was grown in MHB at 37°C for 24 h and the turbidity was adjusted to 0.5 McFarland standard. The MIC of the extract was added to the adjusted bacterial culture. An untreated control was used as a negative control, while treatment with 100 μg/mL chloramphenicol was used as the positive control.

All samples were incubated at 37°C for 4 h. The cultures were then centrifuged at 5000 × *g* for 3 min and the supernatant was discarded. The bacterial pellet was washed with phosphate buffered saline (PBS) and subjected to centrifugation at 5000 × g for 3 min. This washing process was repeated three times. The washed pellets were reconstituted in minimal volume of PBS, placed onto glass slides (5 × 5 mm) and allowed to air dry for 30 min. The slides were fixed using 2.5% (v/v) gluteraldehyde in PBS for 4 h and washed three times with PBS to remove excess glutaraldehyde. The slides were then serially dehydrated with increasing concentration of ethanol and kept in a desiccator overnight. The slide was spur-coated with platinum using Q150R rotary-pumped sputter coater before observed using SU8010 FE-SEM (Hitachi, Japan).

Nucleotide Sequence Accession Number

The 16s rRNA gene sequence of strain MSh1^T has been deposited in GenBank/EMBL/DDBJ under the accession number KT159931. The gene sequences of each MLSA loci have been deposited at the Bcc PubMLST database with sequence typing (ST) 1043; and GenBank/EMBL/DDBJ under the accession number KU301866–301872.

RESULTS

16S rRNA Gene Sequence Analysis

The 16S rRNA gene sequence was obtained for strain MSh1^T (1497 bp; GenBank/EMBL/DDBJ accession number KT159931) and a phylogenetic tree was constructed (**Figure 1**). Phylogenetic analysis demonstrated that strain MSh1^T is closely related to *B. arboris* R-24201^T and *B. lata* 383^T, as they formed a distinct clade (**Figure 1**). Pairwise comparison of the 16S rRNA gene sequence of strain MSh1^T with those Bcc type strains revealed similarity levels between 97.1 and 99.9% (data not shown).

DDH

The DNA–DNA relatedness values between strain MSh1^T with its close neighbor based on 16S rRNA phylogenetic analysis: *B. arboris* R-24201^T (29.0 ± 3.7%), *B. cenocepacia* J2315^T (32.8 ±

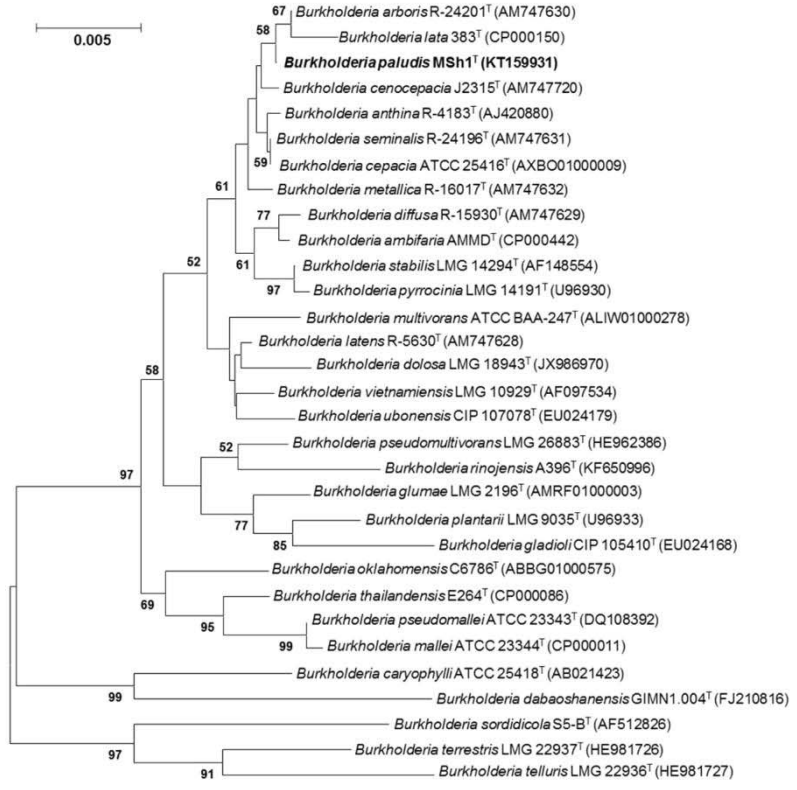

FIGURE 1 | Neighbor-joining tree based on 16S rRNA sequences showing relationship between strain MSh1^T and representatives of some other related taxa. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 5 substitutions per 1000 nucleotide positions.

2.2%) and *B. lata* 383^T (19.5 ± 1.8%) were significantly below the 70% threshold value for species delineation (Wayne et al., 1987).

MLSA

Phylogenetic analysis of concatenated allele sequences demonstrated that strain MSh1^T diverged from the other closely related Bcc type strains, supported by a bootstrap value of 86% (**Figure 2**). MLSA data were used to assign allele types and allelic profiles. The allelic profile were as follows: *atpD*, 302; *gltB*, 11; *gyrB*, 50; *recA*, 350; *lepA*, 288; *phaC*, 249; and *trpB*, 345 (GenBank/EMBL/DDBJ accession number KU301866–301872). Strain MSh1^T showed distinct allelic profile when compared with those closely related type strains (Table S1). For each established Bcc species, all allele types and allelic profiles were exported from the Bcc MLST database. The average concatenated allele sequence divergence of strain MSh1^T toward its nearest neighbor *B. stabilis* LMG 14294^T (5.23%), *B. pyrrocinia* LMG 14191^T (5.81%) and *B. cenocepacia* IIIC 19230^T (6.87%) were above the 3% cut-off value hence indicating that strain MSh1^T is a novel species within the Bcc (Table S2) (Vanlaere et al., 2009; Peeters et al., 2013). Moreover, these data further substantiated the results obtained from DDH which confirmed that strain MSh1^T is a novel species within the Bcc as the concatenated allele divergences between strain MSh1^T and its close neighbor,

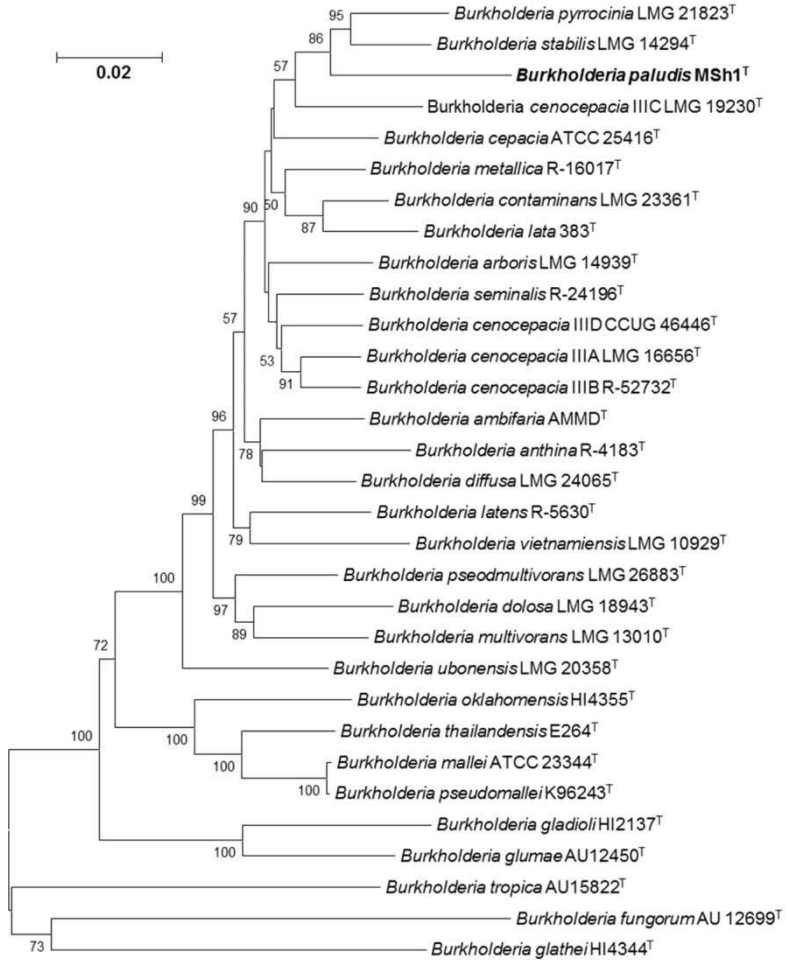

FIGURE 2 | Phylogenetic tree based on the concatenated sequences of seven housekeeping gene fragments of established Bcc species and strain MSh1^T. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 2 substitutions per 100 nucleotide positions.

B. arboris R-24201^T, *B. cenocepacia* J2315^T and *B. lata* 383^T were more than 3%.

Phenotypic and Biochemical Characterization

Strain MSh1^T was Gram negative, facultative anaerobic, motile, rod-shaped, 0.6–0.8 × 1.6–2.1 μm bacterium (**Figure 3**). Colonies produced by the strain MSh1^T on NA were round, yellow colored with a smooth surface and 2–3 mm in diameter. Growth of strain MSh1^T occurs at 15–40°C (optimum 30°C) and pH 4.0–10.0 (optimum pH 7.0) in NB. Growth occurs with 0–2.5% NaCl (optimum without NaCl). **Table 1** shows that strain MSh1^T can be differentiated biochemically from the closely related members of the genus *Burkholderia*. It was shown that strain MSh1^T differs from the other Bcc species by the ability to acidify adonitol, having arginine dihydrolase activity and inability to assimilate N-acetyl-glucosamine (Vandamme et al., 1997, 2000, 2002; Coenye et al., 2001; Henry et al., 2001; Vanlaere et al., 2008, 2009; Peeters et al., 2013).

Chemotaxonomic Characterization

Chemotaxonomic analysis revealed that the major cellular fatty acids were $C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%) and $C_{19:0}$ cyclo ω8c (16.1%). The fatty acid profile of MSh1^T was consistent with these of closely related type strains such as *B. arboris* R-24201^T, *B. cenocepecia* J2315^T, and *B. lata* 383^T which contained fatty acid $C_{16:0}$ (28.2–36.5%), $C_{17:0}$ cyclo (22.5–26.1%) and $C_{19:0}$ cyclo ω8c (13.7–19.8%) (**Table 1**). The ubiquinone Q-8 was detected. The polar lipids consisted phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG), aminolipid and aminophospholipid (**Figure 4**). The fatty acid profile, polar lipid profile and major isoprenoid quinone of strain MSh1^T were consistent with *Burkholderia* type strains (Gillis et al., 1995).

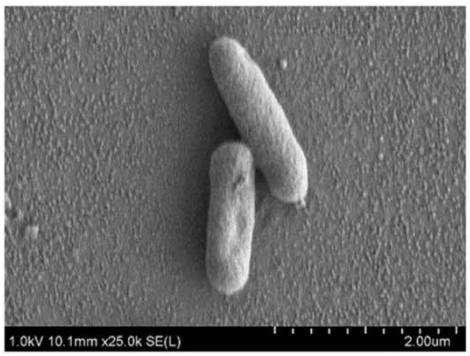

FIGURE 3 | SEM images of *Burkholderia paludis* MSh1^T. Images were taken under 25,000× magnification at 1 kV. The bacterial cells sizes are approximately 0.6–0.8 × 1.6–2.1 μm.

Genome-To-Genome Distance Calculator (GGDC) and Average Nucleotide Identity (ANI)

GGDC is an *in silico* genome-to-genome comparison tool used to calculate the intergenomic distances and relatedness of strain MSh1^T with known type strains (Meier-Kolthoff et al., 2013). The data obtained is converted to similarity values analogous to DNA–DNA hybridization (DDH) hence similarity value of 70% is generally regarded as a threshold for new species determination (Meier-Kolthoff et al., 2013). It was found that the similarity values between strain MSh1^T with related known Bcc species are less than 70%, thus suggesting that this isolate might represent new species. This is further supported by the ANI results which revealed that strain MSh1^T had ANI-value lower than the 96% threshold for new species (Goris et al., 2007; Table S3).

Genome Analysis of Secondary Metabolites Gene Clusters Using Antismash

Antimicrobial compounds produced by bacteria are usually secondary metabolites which are regulated by biosynthetic gene clusters. These gene clusters can be annotated using antiSMASH 2.0 (Blin et al., 2013, 2014). Analysis showed that the genome of strain MSh1^T had 43 gene clusters responsible for the biosynthesis of secondary metabolites which include the PKS, NRPS, bacteriocin, and terpene biosynthetic genes. Besides that, it was found that strain MSh1^T (43 gene clusters) possess a higher number of biosynthetic gene clusters compared to other related *Burkholderia* species (9 to 25 gene clusters) despite having similar genome size (**Table 2**). This might indicate that some of the putative biosynthetic gene clusters might be new as antiSMASH was unable to annotate due to the lack of similarity between query sequences from the database. These results revealed that the antimicrobial compounds produced by strain MSh1^T might either be new or under-studied.

Antimicrobial Activity of MSh1^T

The methanol extract of MSh1^T demonstrated antimicrobial activity four Gram positive bacteria and a Gram negative bacterium (**Table 3**). It was found that the Gram positive bacteria had lower MIC-value when compared to the Gram negative bacteria. Sequential solvent fractionation of the methanol crude extract yielded four different fractions with different polarities (HEX fraction, DCM fraction, EtOAc fraction and water fraction). The DCM fraction showed similar antimicrobial activity (MIC 0.020 mg/ml) to the crude methanol extract, indicating that the antimicrobial compounds had successfully been fractionated from the original extract. Further purification of the DCM extract has led to the isolation of Compound 1, pyochelin. Antimicrobial testing of Compound 1 was performed with 13 other test microorganisms via broth microdilution. Compound 1 demonstrated antimicrobial activity against four *Staphylococcus* strains and three *Enterococcus* strains; but not *Bacillus subtilis* ATCC 8188, *B. cereus* ATCC 14579 and all other

TABLE 1 | Differentiation characteristics of strain MSh1^T and type strains of closely related species of Bcc.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ACIDIFICATION OF																				
Sucrose	+	+	+	−	+	−	+	−	+	v	+	+	+	+	+	+	+	−	−	−
Adonitol	−	+	+	+	v	+	−	+	+	v	+	−	+	v	+	+	+	+	+	+
ASSIMILATION OF																				
N-acetyl-glucosamine	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ENZYMATIC ACTIVITY OF																				
Arginine decarboxylation	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Lysine decarboxylation	+	+	+	+	+	+	+	−	+	v	+	−	+	+	v	+	+	v	+	−
β-galactosidase	−	+	+	−	+	−	+	+	+	v	+	−	+	+	+	+	+	+	+	+
FATTY ACID CONTENT																				
$C_{16:0}$(%)	31.7	29.8	36.5	28.2	26.8	25.6	19.5	26.1	26.3	28.4	22.9	16.6	17.8	21.2	28.1	26.4	21.9	28.9	20.9	29.0
$C_{17:0}$cyclo (%)	26.6	22.5	26.1	23.5	17.9	17.8	14.0	16.0	11.3	4.6	13.5	5.7	12.6	1.6	11.7	10.4	5.2	18.2	8.0	17.2
$C_{19:0}$ cyclo ω8c (%)	16.5	13.7	17.6	19.8	12.5	15.3	5.8	14.8	4.8	1.3	4.7	2.4	9.5	0.4	1.6	3.0	1.8	9.7	4.2	10.0

Species: 1, strain MSh1^T; 2, B. arboris R-24201^T; 3. B. cenocepacia J2315^T; 4, B. lata 383^T; 5, B. cepacia; 6, B. stabilis; 7, B. vietnamiensis; 8, B. dolosa; 9, B. ambifaria; 10, B. anthina; 11, B. pyrrocinia, 12, B. ubonensis; 13, B. latens; 14, B. diffusa; 15, B. seminalis; 16, B. metallica; 17, B. contaminans; 18; B. multivorans; 19, B. pseudomultivorans; 20, B. gladioli.

Data for strain MSh1^T, B. arboris R-24201^T, B. cenocepacia J2315^T, and B. lata 383^T are from this study. Data for the other Bcc species were obtained from Coenye et al. (2001), Henry et al. (2001), Peeters et al. (2013), Vandamme et al. (2002), Vandamme et al. (1997), Vandamme et al. (2000), Vanlaere et al. (2009), and Vanlaere et al. (2008). Data for fatty acid content are percentages of whole-cell fatty acid content. +, >90% of all isolate positive; v, 10–90% position; −, <10% of strains positive.

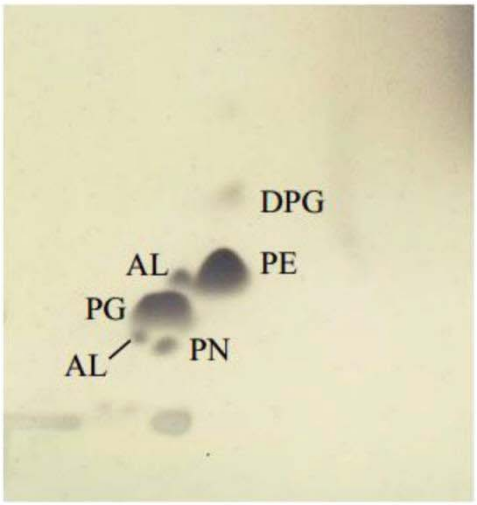

FIGURE 4 | Thin layer chromatogram polar lipid detected in *Burkholderia paludis* MSh1^T. AL, aminolipid; PN, aminophospholipid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol.

Gram negative bacteria tested. SEM analysis showed that the cellular morphology of *E. faecalis* ATCC 700802 was not affected by pyochelin.

DISCUSSION

The taxonomic position of strain MSh1^T was determined and characterized using a polyphasic approach. The 16S rRNA sequence of strain MSh1^T revealed that it belonged to the genus *Burkholderia* and was closely related to the Bcc. Phylogenetic analysis further showed that strain MSh1^T clades closely to *B. arboris*, *B. lata* and *B. cenocepacia*. Despite having high 16S rRNA gene sequence similarity (data not shown), phylogenetic analysis indicated that strain MSh1^T is situated on a different branch in the tree, signifying the possibility of being a novel species. As members of Bcc are known to have high similarity in terms of 16S rRNA gene sequence, other molecular taxonomy techniques have to be used to differentiate and identify them (Coenye and Vandamme, 2003; Spilker et al., 2009; Peeters et al., 2013). Hence, DDH and MLSA were performed in this study. The DDH results which showed that the DNA–DNA relatedness value between strain MSh1^T and its closely related neighbors (based on 16S rRNA phylogenetic analysis) were below the 70% threshold value for species delineation (Wayne et al., 1987). This was further supported by the average concatenated allele sequence divergence (obtained from the MLSA analysis) of strain MSh1^T toward other Bcc species which were above the 3% threshold (Librado and Rozas, 2009). Moreover, based on the biochemical profile of strain MSh1^T, it can be distinguished from most Bcc by its inability to assimilate N-acetyl-glucosamine. Furthermore, this study has used a combination of both polyphasic and genome comparison (via next generation sequencing) to determine the species delineation of strain MSh1^T. For every technique used, each result revealed that strain MSh1^T is indeed a novel species within the Bcc.

TABLE 2 | Comparison of gene clusters responsible for secondary metabolites biosynthesis between strain MSh1 with related *Burkholderia* species.

Burkholderia strains	Genome size (Mbp)	PKS	NRPS	Hybrid	Bacteriocin	Putative	Others	Total
Burkholderia sp. MSh1	8.63	1	1	1	4	29	7	43
B. ambifaria AMMD	7.64	1	2	2	2	0	12	19
B. cenocepacia J2315	8.06	1	3	0	1	0	8	13
B. cepacia GG4	6.47	1	1	0	1	0	5	8
B. lata sp. 383	8.68	1	2	0	2	0	8	13
B. multivorans ATCC 17616	7.01	1	1	0	1	0	10	13
B. pyrrocinia CH-67	8.04	1	3	2	3	0	10	19
B. ubunensis Bu	6.93	0	4	1	3	0	17	25
B. vietnamiensis G4	8.39	0	1	0	1	0	7	9

PKS, polyketide synthase; NRPS, non-ribosomal polyketide synthethase; hybrid, combination of PKS and NRPS.

TABLE 3 | The minimum inhibitory concentration (MIC) of different fractions and crude methanol extract against the five test microorganism strains.

Test microorganism	MIC (mg/ml)				
	CME	HEX	DCM	EtOAc	Water
E. faecalis ATCC 29212	0.020	>5.000	0.020	0.313	0.156
E. faecalis ATCC 700802	0.039	>5.000	0.039	0.313	0.313
S. aureus ATCC 29213	0.313	>5.000	0.313	2.500	1.250
S. aureus ATCC 700699	0.156	>5.000	0.156	1.250	1.250
E. coli ATCC 25922	2.500	>5.000	2.500	1.250	1.250

CME, Crude methanol extract; HEX, Hexane fraction; DCM, Dichloromethane fraction; EtOAc, Ethyl acetate.

The genus *Burkholderia* is well known for producing a wide range of secondary metabolites which include siderophores (Asghar et al., 2011), antifungal (Lu et al., 2009; Tawfik et al., 2010), antibacterial (El-Banna and Winkelmann, 1998; Mitchell and Teh, 2005; Mitchell et al., 2008), antitumor (Klausmeyer et al., 2011; He et al., 2014), and exotoxins (Jeong et al., 2003; Partida-Martinez and Hertweck, 2007). Secondary metabolites are compounds which are non-essential for microbial growth or reproduction but provide diverse survival functions in nature (Martin et al., 2005). Many of these secondary metabolites are regulated by complex synthesis mechanisms within the bacteria itself which include the PKS and NRPS. The assembly of a polyketide via the PKS system begins by priming the starter molecule to a catalytic domain, followed by chain elongation with extender units by different kind of enzymes. As for the NRPS system, a NRP will firstly be generated and then linked with other molecules for instance a phosphate group, methyl group or fatty acids (Donadio et al., 2007; Amoutzias et al., 2008). In this present study, antiSMASH was used to annotate the secondary metabolite biosynthetic gene clusters present in the draft genome of strain MSh1^T. It was found that strain MSh1^T has a higher number of secondary metabolite biosynthetic gene clusters as compared to other known antimicrobial producing Bcc species, for example *B. cepacia* and *B. pyroccinia* (Souza and Raaijmakers, 2003). Both PKS and NRPS gene clusters are present in the genome, indicating the antimicrobial compounds produced might be assembled by either of these two gene clusters. Moreover, strain MSh1^T might be able to produce a more complex antimicrobial compound as a hybrid PKS-NRPS was annotated by antiSMASH. One example of such an antimicrobial compound is a lipopeptide class compound (Sorensen et al., 2014). Lipopeptides are antimicrobial compounds, known to disrupt the surface membrane charges of its target. Thus far, there have been only two reported lipopeptides (burkholdine and occidiofungin) produced by Bcc species and they only possess antifungal activity (Lu et al., 2009; Tawfik et al., 2010). Bacteriocins are small peptides with narrow-spectrum antimicrobial properties (Cotter et al., 2013). Capistruin, is an example of antimicrobial peptide produced by a *Burkholderia* species. Nevertheless, the antimicrobial activity is only limited to several Gram negative bacteria which include *Burkholderia* species, *Psuedomonas aeruginosa,* and *E. coli* (Knappe et al., 2008). The data obtained from antiSMASH provided a preliminary insight into the secondary metabolite gene clusters present in the genome of strain MSh1^T and it is certain that strain MSh1^T can indeed produce antimicrobial compounds. As many secondary metabolite biosynthetic gene clusters were present in the genome, the antimicrobial activity observed might be due to the presence of multiple compounds. Therefore, extraction of the antimicrobial compounds produced by strain MSh1^T was performed in this study.

Since the antimicrobial activity of strain MSh1^T was determined with agar overlay assay in the previous study (Ong et al., 2015), this showed that the antimicrobial compounds produced could be extracted from the agar itself. Therefore, agar extraction (solid-liquid extraction) was carried out. This technique was adopted from a study conducted by Isnansetyo and Kamei (2003) in which they successfully extracted an antimicrobial compound produced by *Pseudoalteromonas phenolica* cultured on marine solid media. It was shown that *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 700802 were highly sensitive to the antimicrobial compounds with MIC-value of 0.020 mg/ml and 0.039 mg/ml, respectively, as compared to *S. aureus* ATCC 29213 (0.313 mg/ml), *S. aureus* ATCC 700699 (0.156 mg/ml) and *E. coli* ATCC 25922 (2.500 mg/ml) (consistent

with Ong et al., 2015). To the best of our knowledge, there has only been one antimicrobial compound with inhibitory effect against Gram positive bacteria and fungus (pyrrolnitrin), isolated from *Burkholderia* species, and based on the PCR performed in this study, the *prnD* gene was not found in strain MSh1^T which further substantiate that it might be producing other types of antimicrobial compounds. Furthermore, the genes that code for *prnA*, *prnB*, *prnC* and *prnD* were absent from the draft genome of strain MSh1^T.

Sequential solvent fractionation was performed to fractionate and partially purify, at the same time removing impurities present in the crude methanol extract. A similar method was adopted from Tawfik et al. (2010) to isolate antifungal burkholdines produced by *B. ambifaria* 2.2N. In this study, four different solvents were used: hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and water. HEX is a non-polar solvent commonly used to remove non-polar compounds from the crude extract. DCM and EtOAc both have middle-range polarity, thus is able to attract a wider range of compounds with different polarity (Moreau et al., 2003). Water being the most polar solvent will retain any compounds with highly polarity (Wang and Weller, 2006). It was shown that the HEX fraction had no antimicrobial activity against all test microorganisms up to 5 mg/mL. The DCM fraction had the best antimicrobial activity against all the test microorganism strains (when compared with the EtOAc and water fractions) as the MIC-values were comparable or similar to the crude methanol extract (**Table 3**). Thus, further purification was conducted on the DCM fraction using C_{18} which led to the isolation of Compound 1. Chromatographic analysis of Compound 1 on a TLC plate with chloroform-acetic acid-ethanol at 90:5:2.5 (v/v) as the mobile phase showed one spot (R_f 0.35) with yellow-green fluorescent band that turned red-brown after spraying with the iron reagent. Analysis of Compound 1 by analytical HPLC indicated that the compound contained one peak with three maxima at 210, 270, and 320 nm. The mass of Compound 1 (*m/z* 325, $[M+H]^+$) was determined by liquid chromatography-electrospray ionization-mass spectrometer (LC-ESI-MS). The ESI-MS indicated that the molecular formula of the compound was $C_{14}H_{16}N_2O_3S_2$. The interpretation of ESI-MS and UV spectrum of Compound 1 were found to be identical to pyochelin which was in good agreement with previous literature (Cox et al., 1981; Adler et al., 2012) (Figures S1, S2).

Pyochelin is a type of siderophore commonly produced by the genus *Pseudomonas* (Cox et al., 1981; Buysens et al., 1996; Lim et al., 2016). Siderophores can solubilize ferric ion, hence is an iron chelating growth factor for many bacteria. Thus far, only three *Burkholderia* species (*B. arboris*, *B. cenocepacia* and *B. contaminans*) have been known to produce pyochelin (Dang et al., 2011; Schwagner et al., 2012; Deng et al., 2015). As pyochelin is a siderophore, its antimicrobial properties were not extensively studied. Pyochelin can inhibit or kill bacteria by catalyzing the generation of reactive oxygen species (ROS) (Adler et al., 2012). However, this is bacteria dependent as shown in **Table 4**. The Gram negative bacteria (typically the *Enterobacteriaceae*) were found to be resistant to Compound 1 (pyochelin) and this is consistent with the study conducted by Adler et al. (2012). The resistant profile shown by the *Enterobacteriaceae* was due to the production of catecholate siderophores such as enterobactin. These siderophores can act as hydrogen atom donors and efficiently terminate radical chain reactions; hence rendering pyochelin ineffective. The inhibitory effect of pyochelin on the four *S. aureus* and three *E. faecalis* strains might due to presence of ROS generated by pyochelin. Nevertheless, no literature has reported on the downstream effect of the ROS produced by pyochelin, as it might target the DNA/RNA replication, electron transport chain or bacterial cell membrane. Moreover, the nutrient availability of these strains might be compromised as pyochelin can chelate the vital metal ions present in the environment. Consequently, these sensitive strains might be starved and ultimately led to death, as they lack the receptors to mediate the entry of metal-bounded pyochelin into the bacterial cells. Hence as a preliminary study, we investigated the effect of pyochelin on the cellular morphology of *E. faecalis* ATCC 700802 via SEM. Pyochelin did not affect the cellular morphology of *E. faecalis* ATCC 700802, when compared to the positive control in which the morphology of the bacteria were distorted when treated with 100 μg/ml of chloramphenicol (**Figure 5**). This result supports that the ROS generated by pyochelin might target the intracellular components of *E. faecalis* ATCC 700802. Further work on the effect of pyochelin on the DNA/RNA replication will be validated via qPCR in the future. Moreover, the antimicrobial activity of pyochelin was not only restricted to normal strains of *S. aureus* and *E. faecalis*, but it also affects the antimicrobial-resistant strains, for instance methicillin-resistant *S. aureus*

TABLE 4 | The minimum inhibitory concentration (MIC) of Compound 1 (pyochelin) against 18 test microorganisms.

Gram stain	Bacteria strains	MIC (μg/ml)
Gram positive	*Bacillus cereus* ATCC 14579	> 100.00
	Bacillus subtilis ATCC 8188	> 100.00
	Enterococcus faecalis ATCC 700802	3.13
	Enterococcus faecalis ATCC 29212	3.13
	Enterococcus faecalis JH-22	3.13
	Staphylococcus aureus ATCC 700699	6.26
	Staphylococcus aureus ATCC 43300	6.26
	Staphylococcus aureus ATCC 6538P	6.26
	Staphylococcus aureus ATCC 29213	6.26
Gram negative	*Aeromonas hydrophila* ATCC 49140	> 100.00
	Escherichia coli ATCC 25922	> 100.00
	Klebsiella pneumoniae ATCC 10031	> 100.00
	Proteus mirabilis ATCC 49140	> 100.00
	Proteus vulgaris IMR	> 100.00
	Pseudomonas aeruginosa ATCC 10145	> 100.00
	Pseudomonas aeruginosa ATCC BAA-47	> 100.00
	Salmonella Typhimurium ATCC 14028	> 100.00
	Shigella flexneri ATCC 12022	> 100.00

FIGURE 5 | FE-SEM images of *E. faecalis* ATCC 700802 showing (A) negative control, **(B)** treated with MIC of Compound 1 and **(C)** treated with 100 μg/ml of chloramphenicol (positive control). Images were taken under 5000x magnification at 1 kV.

ATCC 700699, methicillin-resistant *S. aureus* ATCC 43300 and vancomycin-resistant *E. faecalis* ATCC 700802. However, this has to be validated by increasing the number of test strains used. Additional work is on-going to further elucidate the downstream effect of pyochelin on these sensitive strains, and also to determine its potential synergistic action with antibiotics. Nevertheless, this study has provided an insight that the infamous pyochelin might potentially be used to treat infections caused by ARB.

Based on the results of phylogenetic analysis, DDH, MLSA, GGDC, ANI, phenotypic, chemotaxonomic and biochemical characterization, strain MSh1^T was assigned to a novel species in the genus *Burkholderia*, for which the name *Burkholderia paludis* sp. nov. is proposed.

Description of *Burkholderia paludis* sp. nov.

Burkholderia paludis (pa.lu.dis. L. gen. n. *paludis* of the swamp). Cells are Gram negative, facultative anaerobic, motile rods, about 0.6–0.8 μm wide and 1.6–2.1 μm long. Colonies are regular, circular, convex, translucent, moist and 1.0–3.0 mm in diameter after 2 days of cultivation at 30°C on NA. Grows at 15–40°C (optimum 30°C) and pH 4.0–10.0 (optimum pH 7.0) in NB. Grows at 0–2.5% NaCl (optimum without NaCl). Cells are positive for oxidase, glucose fermentation, arginine dihydrolase, urease, esculin hydrolysis and assimilation of glucose, but negative for nitrate/nitrite reduction, indole production, gelatin hydrolysis, β-galactosidase, assimilation of arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid (API 20NE).

In API ZYM-tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and n-acetyl-β-glucosaminidase, but negative for valine arylamidase, cystine arylamidase, trypsin, a-chymotrypsin, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, αmannosidase and α-fucosidase. The following carbon sources are utilized in the API 50CH: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-glucoside, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, D-turanose, D-xylose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol; the other substrates, L-xylose, adonitol, α-methyl-D-xiloside, L-sorbose, rhamnose, α-methyl-D-mannoside, α-methyl-D-glucosamine, amygdalin, melibiose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, β-gentiobiose, gluconate, 2-keto-gluconate and 5-keto-gluconate, are not utilized.

The fatty acid profile is composed mainly of $C_{16:0}$ (31.7%), $C_{17:0}$ cyclo (26.6%), and $C_{19:0}$ cyclo ω8c (16.1%). The major compounds in the polar lipid profile are phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The predominant ubiquinone is Q-8. Strain MSh1^T is able to produce antimicrobial compounds active against four strains of *S. aureus* and three strains of *E. faecalis*.

The type strain is MSh1^T (=DSM 100703^T =MCCC 1K01245^T), isolated from surface peat from the Southeast Pahang tropical peat swamp forest reserve, Malaysia. The 16S rRNA and MLSA gene sequence of strain MSh1^T has been deposited in GenBank/EMBL/DDBJ under the accession number KT159931 and KU301866–301872, respectively.

ACCESSION NUMBERS

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MSh1^T is KT159931. The GenBank/EMBL/DDBJ accession number for the MLSA gene sequences of strain MSh1^T are KU301866–301872.

AUTHOR CONTRIBUTIONS

KSO performed the laboratory experiments, data analysis and the manuscript write-up. SML supervised the entire study. LHL and YLC co-supervised the study. SML, KSO and YKA contributed to the experimental designs. LHL and KSO contributed to the polyphasic taxonomy. CMY, SML and YKA apprehended the idea of bioprospecting in tropical peat swamp forest. All authors proofread and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02046/full#supplementary-material

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Appendix 7(c):

The role of reactive oxygen species in the antimicrobial activity of pyochelin

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Original Article

The role of reactive oxygen species in the antimicrobial activity of pyochelin

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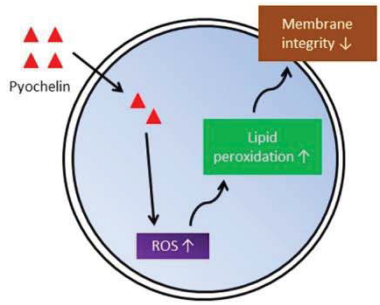
GRAPHICAL ABSTRACT



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ABSTRACT

The increase in prevalence of antimicrobial-resistant bacteria (ARB) is currently a serious threat, thus there is a need for new antimicrobial compounds to combat infections caused by these ARB. An antimicrobial-producing bacterium, *Burkholderia paludis* was recently isolated and was able to produce a type of siderophore with antimicrobial properties, later identified as pyochelin. The chelating ability of pyochelin has been well-characterized but not for its antimicrobial characteristics. It was found that pyochelin had MIC values (MBC values) of 3.13 μg/mL (6.26 μg/mL) and 6.26 μg/mL (25.00 μg/mL) against three *Enterococcus* strains and four *Staphylococcus* strains. Pyochelin was able to inhibit *E. faecalis* ATCC 700802 (a vancomycin-resistant strain) in a time and dose dependent manner via killing kinetics assay. It was demonstrated that pyochelin enhanced the production of intracellular reactive oxygen species (ROS) over time, which subsequently caused a significant increase in malondialdehyde (MDA) production (a marker for lipid peroxidation) and ultimately led to cell death by disrupting the integrity of the bacterial membrane (validated via BacLight assay). This study has revealed the mechanism of action of pyochelin as an antimicrobial agent for the first time and has shown that pyochelin might be able to combat infections caused by *E. faecalis* in the future.

Introduction

The increase in prevalence and emergence of antimicrobial resistant bacteria (ARB) is an alarming concern. This is because ARB infections often result in increased mortality rates and cause

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increased healthcare costs. *Enteroccocus faecalis* is a major example of ARB that is difficult to treat due to its intrinsic resistance and ability to acquire resistance through mutation or horizontal gene transfer [1,2]. As vancomycin is the last line of defence to combat enterococci infections, strains that are resistant to this antibiotic are a threat. Vancomycin-resistant enterococci (VRE) account for approximately one-third of the enterococcal healthcare-associated infections in the USA and for more than 20% of such infections in some European countries [3]. Besides that, *Staphylococcus aureus* is another example of ARB that causes life-threatening infections. The first line therapy for *S. aureus* infection is usually beta-lactam antibiotics [4]. Unfortunately, the emergence of methicillin-resistant *S. aureus* (MRSA) strains essentially indicates that they are resistant to all currently available beta-lactam antimicrobial agents. This limits the treatment options to three non-beta lactam antimicrobial agents such as vancomycin, daptomycin and linezolid to treat MRSA infections, but however recently there is an increase in prevalence of *S. aureus* strains having resistance towards these last few antibiotic options [5,6]. Due to the limited treatment options available to treat these ARB infections, new antimicrobial compounds are needed to combat this issue.

One strategy is bioprospecting, which is defined as the exploration for potentially new bioactive compounds in unique and extreme ecological niches to treat ARB infections [7]. Bacteria thriving in these environments might produce antimicrobial compounds to gain an advantage in competing for resources and colonization of new habitats. As a result, a tropical peat swamp forest in Malaysia, characterized by its acidic (pH range of 2.9–4.5), ombotrophic and waterlogged conditions was previously chosen as a bioprospecting location for antimicrobial compounds [8]. Despite being such a harsh environment, Ong et al. [9] successfully isolated a novel bacterium *Burkholderia paludis* which showed potent antimicrobial activity towards methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE). The antimicrobial compound was later identified to be pyochelin.

Pyochelin is a type of siderophore commonly produced by the genus *Pseudomonas* and *Burkholderia*. The biosynthetic gene clusters of pyochelin, along with its iron-solubilizing ability are well characterized. However, pyochelin has demonstrated other biological activity recently other than being only a chelating compound. This compound can particularly inhibit *S. aureus* in a study conducted by Adler et al. [10] and this activity was further substantiated by another study performed by Ong et al. [9]. It was demonstrated that pyochelin is not only effective in inhibiting non-antimicrobial-resistant strains of *S. aureus* and *E. faecalis*, but also the resistant strains at 6.26 μg/mL and 3.13 μg/mL, respectively. It was postulated that pyochelin can inhibit bacterial growth by enhancing the production of reactive oxygen species (ROS) in the cells, which consequently inhibit certain essential biological processes. Nevertheless the mechanism of action of pyochelin as an antimicrobial compound is not well characterized. Thus this study aims to characterize the antimicrobial property of pyochelin.

Material and methods

Culture conditions and maintenance of bacterial strains

Test microorganism strains that were used in this study include *Enterococcus faecalis* ATCC 700802, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* JH-22, *Staphylococcus aureus* ATCC 700699, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538P and *Staphylococcus aureus* ATCC 29213. Strains were cultured on Mueller-Hinton agar (MHA) (Oxoid, UK) at 37 °C and maintained at −80 °C in MHB (Oxoid, UK) with 25% (v/v) glycerol (Merck, Germany). As for *B. paludis* MSh1, it was maintained on nutrient agar (NA) (Merck, Germany) at 30 °C and in 25% (v/v) glycerol in nutrient broth (NB) (Merck, Germany) at −80 °C for long term preservation.

Extraction of pyochelin from B. paludis MSh1

The extraction of pyochelin from *B. paludis* MSh1 was performed according to methodology described by Ong et al. [9]. Briefly, *B. paludis* MSh1 was grown on NA containing 5 g/L of glycerol and incubated for 5 days at 30 °C. The whole media with the bacteria was extracted using methanol (Merck, Germany) and subsequently fractionated using dichloromethane (DCM) (Merck, Germany). The DCM fraction was purified on an open C_{18} column, followed by further purification using preparative high performance liquid chromatography (HPLC). The purity of pyochelin was compared with a standard purchase from Santa Cruz Biotechnology, USA.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pyochelin

The MIC of pyochelin was determined using a broth microdilution assay as described by the Clinical and Laboratory Standard Institute (CLSI). The MIC is defined as the lowest concentration of an antimicrobial to inhibit the visible growth of a microorganism after 16–20 h incubation [11]. Briefly, the test microorganisms were grown in MHB at 37 °C for 20 h and adjusted to 0.5 McFarland standard (OD_{625} 0.08–0.11), corresponding to 1.5×10^8 colony forming unit (CFU)/mL. The adjusted cultures were then diluted 100 times in MHB and used as inocula. The extracts were twofold serially diluted using sterile MHB in a 96-well flat bottomed microtiter plate. One hundred μL of the adjusted test microorganisms was added to each well. Determination of MIC was performed in triplicate. The positive control for bacteria was 200 μg/mL chloramphenicol (Calbiochem, Malaysia). The negative control contained MHB with test microorganisms. The blank control consisted only of MHB. The microtiter plate was incubated at 37 °C aerobically for 16–20 h and the MIC was determined by the concentration of extract (μg/mL) where no visible growth was observed. All clear wells containing cultures with no visible growth was streaked out onto MHA to determine the minimum bactericidal concentration (MBC). MBC is defined as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media. The lowest concentration of pyochelin that showed absence of growth was determined as the MBC level [11].

Killing-kinetics studies

A killing kinetic study was performed to determine the effect of different concentrations of pyochelin on *E. faecalis* ATCC 700802 for 24 h. As the *Enterococcus* strains were shown to be more susceptible to pyochelin as compared to the *Staphylococcus* strains, further characterization on the antimicrobial activity of pyochelin was conducted on an *Enterococcus* strain, with particular interest of *E. faecalis* ATCC 700802 due to its vancomycin-resistant property. The killing kinetics assay was performed according to the method described by Pag et al. [12] and Yan et al. [13]. Different concentrations of pyochelin corresponding to 1×, 2× and 4× the MIC determined by broth microdilution were added into 100× diluted 0.5 McFarland adjusted bacteria culture (1.5×10^6 CFU/mL) in 0.85% (w/v) saline (Fisher Scientific, USA) and incubated at 37 °C. Untreated bacterial culture was served as a negative control. The viable count was monitored up to 24 h. Aliquots were taken at

defined intervals (0 h, 2nd hour, 4th hour, 8th hour and 24th hour) and appropriately diluted in 0.85% (w/v) saline. One hundred microliters of each of the dilutions was plated in triplicate on MHA. The plates were incubated at 37 °C and the cell viability was assessed by enumerating the colony forming unit (CFU) per millilitre after 24 h. Killing kinetic studies of pyochelin on *E. faecalis* ATCC 700802 were performed under three different conditions: (1) exponential phase culture with agitation at 200 rpm (Smith, A3555, Progressive Scientific); (2) stationary phase culture with agitation at 200 rpm (Smith, A3555, Progressive Scientific); and (3) exponential phase culture at anaerobic condition. The anaerobic cultures were cultured in an anaerobic jar (Labozone, France) with AnaeroGen pack (Oxoid, UK).

Detection of reactive oxygen species (ROS)

The production of ROS by *E. faecalis* ATCC 700802 after treatment with pyochelin was evaluated using a peroxynitrite indicator, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK), which can detect a broad range of ROS including nitric oxide and hydrogen peroxide [14]. The adjusted bacterial culture (0.5 McFarland exponential phase bacteria culture) were treated with different concentrations of pyochelin corresponding to 1, 2 and 4 times MIC in presence of DCFH-DA at a final concentration of 5 μM in 0.85% saline and incubated at 37 °C aerobically at 200 rpm (Smith, A3555, Progressive Scientific) for 24 h. Untreated bacterial culture was served as a negative control. The fluorescence emission of DCFH-DA was measured at 525 nm using a Tecan microtitre plate reader with an excitation wavelength of 485 nm [15]. The background fluorescence of 0.85% saline and autofloresecence of the bacterial cells incubated without the probe was measured to calculate the net fluorescence emitted from the assay itself. Experiment was conducted in triplicate.

Determination of malondialdehyde (MDA)

Malondialdehyde (MDA) is a natural by-product of lipid peroxidation of polyunsaturated fatty acids caused by ROS, thus is commonly used as a marker for oxidative stress. The production of MDA was quantified by using the OxiSelect™ TBARS Assay kit according to manufacturer's protocol (Cell Biolabs Inc., USA). Briefly, the adjusted bacterial culture (0.5 McFarland adjusted exponential phase bacteria culture) were treated with different concentrations of pyochelin corresponding to 1, 2 and 4 times the MIC at 37 °C aerobically whereas the control was incubated with 0.85% (w/v) saline alone for 24 h. One hundred μl of the SDS lysis solution were added to 100 μl aliquot of the treated culture and incubated for 5 min at room temperature. The mixtures were then incubated at 95 °C for 60 min in presence of thiobarbituric acid (TBA) reagent. Each of the mixture was cooled to room temperature in an ice bath for 5 min and centrifuged at 3000*g* for 15 min (Eppendorf, 5810R). The supernatants were then collected and the absorbances were read at 532 nm. The concentrations of MDA in each treatment were calculated based on the standard curve of absorbance against MDA concentration. This assay was performed in triplicates.

Membrane integrity assay

As the bacterial membrane is composed of phospholipilid bilayer, the production of ROS prior to pyochelin treatment might oxidize the lipid content on the cell membrane, hence affecting the bacterial membrane integrity. Therefore, the effect of pyochelin on the membrane integrity of *E. faecalis* ATCC 700802 was determined by using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen) according to a protocol from Ong et al. [16]. The adjusted bacterial cultures were treated with different concentrations of pyochelin corresponding to 1×, 2× and 4× the MIC at 37 °C aerobically at 200 rpm (Smith, A3555) whereas the control was incubated with 0.85% (w/v) saline alone for 24 h. After incubation, the treated cultures were pelleted by centrifugation (10,000*g*, 15 min) at room temperature, washed twice and resuspended in 0.85% (w/v) saline. One hundred microliters of the 2× staining solution were added into 100 μl of the bacteria suspension, and incubated in the dark for 15 min. At the end of the incubation period, green fluorescence (SYTO 9) was read at 530 nm while the red fluorescence (propidium iodide) was read at 645 nm with an excitation wavelength of 485 nm. This kit utilizes a mixture of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. The SYTO 9 stain generally labels all bacteria in a population including those with intact membranes and those with damaged membranes. In contrast, PI is impermeable to bacterial cells with an intact cell membrane due to its large molecular size [17]. Thus, bacteria with intact cell membranes will be stained fluorescent green, whereas bacteria with damaged membranes will be stained fluorescent red. The percentage of live bacteria was determined by referring to a standard curve of G/R ratio versus percentage of live *E. faecalis* ATCC 700802 which was pre-plotted earlier. This assay was performed in triplicates.

Statistical analysis

The significance of results for the killing kinetics studies, detection of ROS and quantification of MDA were performed using paired-sample *t*-test at the significance level of $\alpha = 0.05$. The significance of results for membrane integrity assay was performed using Wilcoxon test at the significance level of $\alpha = 0.05$ (Kolmogoroff-Smirnow test was used to analyse the normal distribution). Statistical analysis was performed using IBM SPSS Statistics 20.

Results and discussion

MIC, MBC and killing kinetics studies of pyochelin

Pyochelin is a type of siderophore commonly produced by the genus *Pseudomonas* [10]. It has also been reported to be produced by certain *Burkholderia* species such as *B. arboris*, *B. contaminans* and *B. cenocepacia* [18–20]. Siderophores are important to bacteria as they are able to scavenge ferric ion in the nature for essential biological functions such as DNA synthesis [21]. Pyochelin has been extensively studied from a molecular perspective, as well as its chelating abilities. However this study had shown that pyochelin possesses other biological activity.

The MIC values of pyochelin against the *Enterococcus* strains (*E. faecalis* ATCC 700802, *E. faecalis* ATCC 29212, *E. faecalis* JH-22) and *Staphylococcus* strains (*S. aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213) were 3.13 μg/mL and 6.26 μg/mL respectively; while the MBC values were 6.26 μg/mL and 25.00 μg/mL respectively (Table 1). It was

Table 1
MIC and MBC of pyochelin against different test microorganisms.

Test microorganisms	MIC (μg/mL)	MBC (μg/mL)
E. faecalis ATCC 700802	3.13	6.26
E. faecalis ATCC 29212	3.13	6.26
E. faecalis JH-22	3.13	6.26
S. aureus ATCC 700699	6.26	25.00
S. aureus ATCC 43300	6.26	25.00
S. aureus ATCC 6538P	6.26	25.00
S. aureus ATCC 29213	6.26	25.00

shown that the *Enterococcus* strains are more susceptible to pyochelin when compared to the *Staphylococcus* strains. Nonetheless pyochelin is bactericidal against both *Enterococcus and Staphylococcus* strains as the MBC values were no more than 4× the MIC values [22]. The low MIC values of pyochelin against the *Enterococcus faecalis* and *Staphylococcus aureus* strains is an advantage as it is comparable or lower than the currently available antibiotics which have MIC values of 4–64 μg/mL [11].

Killing kinetics was performed to evaluate the effect of different concentrations of pyochelin on *E. faecalis* ATCC 700802 for 24 h. Two phases of bacterial culture were used in this study: exponential phase and stationary phase. Exponential phase culture consists of actively growing cells which consume readily available oxygen and nutrients for growth. On the other hand, stationary phase culture comprises mostly of mature non-dividing cells which are metabolically inactive [23]. Different types of antibiotics work differently depending on their mechanism of action. For instance, lipopeptides (membrane disruptors) inhibits bacterial growth (both exponential phase and stationary phase culture) instantly by puncturing their cell wall [24]; while beta lactams (cell wall biosynthesis inhibitor) only inhibit actively growing bacterial cells in a time-dependent manner, but they are effective at both aerobic and anaerobic conditions [25].

Pyochelin inhibits growth of exponential phase *E. faecalis* ATCC 700802 in a dose and time dependent manner (Fig. 1A). *E. faecalis* ATCC 700802 culture treated with 3.13 μg/mL (1× MIC) of pyochelin achieved 3 log reduction after 24 h; while bacterial culture treated with 6.26 μg/mL (2× MIC) and 12.52 μg/mL (4× MIC) of pyochelin achieved 6 log reduction after 24 h. However, a different scenario was observed when stationary phase *E. faecalis* ATCC 700802 was treated with pyochelin as there was only 2 log reduction after incubated for 24 h at 4× MIC aerobically (Fig. 1B). This result has revealed that pyochelin work best only on actively growing bacterial cells. Nevertheless, pyochelin is different from the beta lactams as it is ineffective against bacterial cells incubated under anaerobic condition (Fig. 1C), suggesting that oxygen might play an important role in the bactericidal effect of pyochelin on *E. faecalis* ATCC 700802.

Effect of pyochelin on the enhancement of ROS production and membrane integrity

It was hypothesized that in presence of pyochelin, the formation of ROS was enhanced in *E. faecalis* ATCC 700802 which can damage the iron-sulphur clusters, thereby releasing ferrous ion. This iron can react with hydrogen peroxide in the Fenton reaction, causing a chain reaction, generating hydroxyl radicals which can directly damage intracellular DNA, lipids and proteins [26]. Hence to validate the hypothesis, the intracellular ROS in *E. faecalis* ATCC 700802 was quantified prior to pyochelin treatment in the subsequent experiments.

The production of ROS in healthy untreated bacterial cells is a natural side effect of aerobic respiration. These ROS can damage the RNA/DNA pool and also oxidizes lipid contents. Thus to protect themselves against the detrimental effect of ROS, bacteria are capable of producing enzymes (catalase and superoxide dismutase) to detoxify the ROS and having regulatory mechanisms (SoxRS, OxyRS and SOS regulons) to counteract the damage [26,27]. To determine the effect of pyochelin on the enhancement of ROS production, *E. faecalis* ATCC 700802 was treated with different concentrations of pyochelin in presence of DCFH-DA, an unspecific probe for ROS. It was shown that the ROS production in *E. faecalis* ATCC 700802 was enhanced in a dose dependent manner when treated with pyochelin (Fig. 2). This suggests that the enhanced production of ROS has an indirect effect on the growth of *E. faecalis* ATCC 700802.

As one of the side effects of increased production of ROS is lipid peroxidation, an example of the by-product in this process (malondialdehyde; MDA) was quantified in this study. The concentration of MDA in the treated *E. faecalis* ATCC 700802 culture was

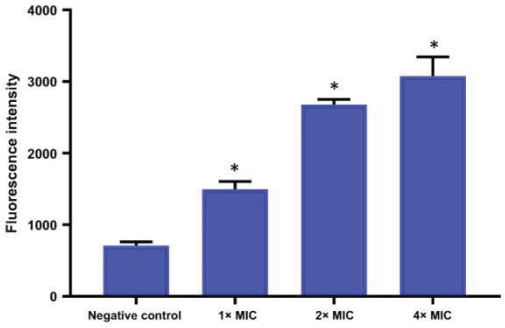

Fig. 2. Quantitation of intracellular ROS production by *E. faecalis* ATCC 700802 after 24 h treatment with different concentrations of pyochelin using the DCFA-DA probe. Results are expressed as mean fluorescence intensity ± SD (n = 3). Asterisk represents significant difference ($P = 0.05$) between each treatment with the negative control.

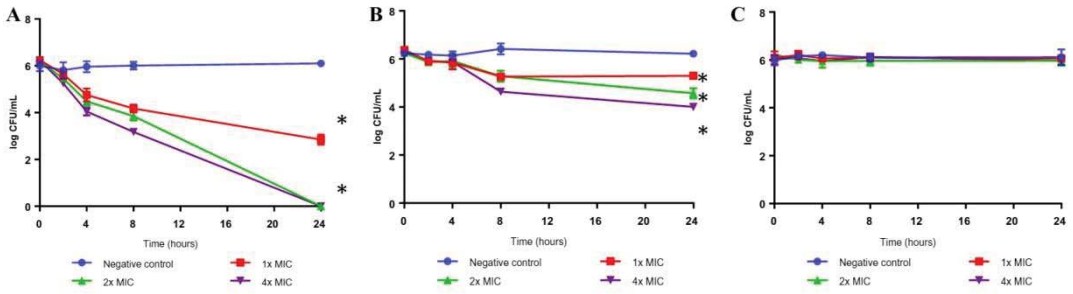

Fig. 1. Effect of different concentrations of pyochelin against (A) exponential phase *E. faecalis* ATCC 700802 (incubated aerobically); (B) stationary phase *E. faecalis* ATCC 700802 (incubated aerobically); (C) exponential phase *E. faecalis* ATCC 700802 (incubated anaerobically) at 37 °C for 24 h. Results are expressed as mean log CFU/mL ± SD plotted against time (n = 3). Asterisk represents significant difference ($P = 0.05$) between each treatment with the negative control at 24 h. As the responding data covers a range from 0 to 10^6, the geometric sequence of the responding data (representing bacterial growth and bacterial cell death) has been transformed into a logarithmic plot of $\log_{10}$ CFU/mL against time. Example: the number of bacteria (negative control) at 24 h is 1.3×10^6 CFU/mL, hence after transformation (log10 1.3×10^6), the value is 6.11.

increased significantly with increasing concentrations of pyochelin. This indicates that the enhanced production of ROS (Fig. 3) in *E. faecalis* ATCC 700802 prior to treatment with pyochelin has caused an increase in lipid peroxidation (Fig. 3).

Since lipid is an essential macromolecule to the bacterial cell membrane, the membrane integrity of *E. faecalis* ATCC 700802 was evaluated using the Live/Dead BacLight Bacterial Viability Kits. It was found that the percentage live bacteria of *E. faecalis* ATCC 700802 was 52.05% at 8 h and 50.35% at 24 h when treated with 1× MIC of pyochelin (Fig. 4). This is because the enhanced generation of ROS at 1× MIC by pyochelin is not sufficient to eliminate the entire bacterial population. It was previously reported that bacterial cells are capable of lowering their metabolic activity at sublethal ROS concentration, hence allowing the cell's regulatory mechanisms to repair the damaged protein or DNA clusters and concurrently producing more enzymes to detoxify the detrimental effect of ROS [28]. The results shown is consistent with the data obtained from the killing kinetics study as there was only 3 log reduction at 1× MIC of pyochelin after 24 h. Furthermore, the MDA concentration of *E. faecalis* ATCC 700802 treated at 1× MIC of pyochelin was not statistically significant compared to the untreated control, indicating that the ROS level generated in presence of 1× MIC of pyochelin did not trigger significant lipid peroxidation, hence the higher percentage of live bacteria. Nevertheless, the percentage live bacteria of *E. faecalis* ATCC 700802 decreases in a time dependent manner when treated with higher concentrations of pyochelin (2× and 4× MIC) (Fig. 4) and this is in agreement with the data obtained from the killing kinetics study.

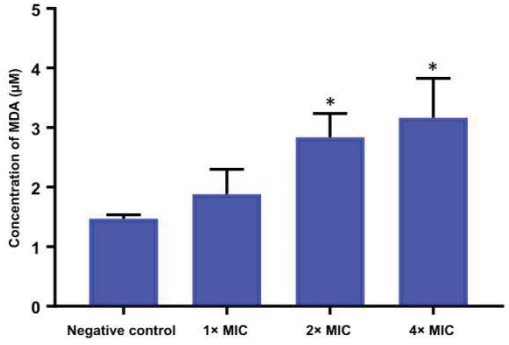

Fig. 3. Quantification of MDA production in *E. faecalis* ATCC 700802 after 24 h treatment with different concentrations of pyochelin. Results are expressed as mean ± SD (n = 3). Asterisk represents significant difference ($P = 0.05$) between each treatment with the negative control.

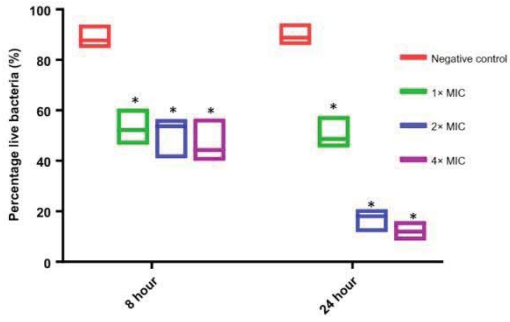

Fig. 4. Percentage of live *E. faecalis* ATCC 700802 at 8 h and 24 h after treatment with different concentrations of pyochelin using the Live/Dead BacLight Bacterial Viability Kit. Results are expressed as median with range (n = 6). Asterisk represents significant difference ($P = 0.05$) between each treatment with the negative control at each time-point using Wilcoxon test.

This result substantiates that pyochelin can enhance the intracellular production of ROS, which later affects the membrane integrity of *E. faecalis* ATCC 700802, leading to bacterial cell death. Furthermore, the lipophilicity of pyochelin might play an important role in affecting the membrane fluidity or membrane potential (proton motive force), thus allowing the initial entry of pyochelin into the bacterial cells to exert its antimicrobial effect [29]. A similar pattern can be observed from other studies conducted using aspidin BB (an alkaloid), metal oxide nanoparticles and synthesized pyrimidine derivatives, as these compounds exert their antibacterial properties by inducing the generation of ROS as well [30–32]. The killing mechanism shown in this study might potentially be useful in combating antimicrobial resistance, as it involves the bacterial cell's redox reaction which directly influences the survival of the cells [28,33]. However sequential passaging of the bacterial culture with sub-MIC of pyochelin should be done in the future to evaluate the development of resistance of *E. faecalis* ATCC 700802 towards pyochelin over generations [34]. Nevertheless, this is the first study to characterize the potential of pyochelin as an antimicrobial compound against vancomycin-resistant *Enteroccocus* (VRE). Further work such as *in vitro* cytotoxic evaluation of pyochelin using normal human cell lines and potentiation of pyochelin with existing antibiotics should be conducted. Furthermore, different strains of *Enterococcus faecalis* or other test microorganisms such as the *Staphylococcus aureus* strains should be tested to further support pyochelin as a potential therapeutic option against ARB infections.

Conclusions

Pyochelin was found to be effective in inhibiting the growth of three *E. faecalis* strains and four *S. aureus*, with MIC values (MBC values) of 3.13 μg/mL (6.26 μg/mL) and 6.26 μg/mL (25.00 μg/mL) respectively via broth microdilution. Pyochelin is able to enhance the production of intracellular ROS, subsequently causing an increase in MDA production and a decrease in membrane integrity of *E. faecalis* ATCC 700802 (VRE) after 24 h. This study has provided an insight that pyochelin might potentially be useful in treating infections caused by ARB, particularly VRE in the future.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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