Novel Streptomycete Antimicrobial Compounds: Activity against Pathogens from Domestic Rainwater Harvesting Systems

By

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Declaration

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Summary

Due to increased urbanisation, frequent droughts and poor water sources in rural communities, the scarcity of water has become a major concern. Water is an essential part of life – in order to meet the ever growing demand for water, it is imperative to search for alternative water sources. Domestic rainwater harvesting (DRWH) is considered a potential source of water and is commonly used in most parts of the world. However, the microbial quality of harvested rainwater poses a serious health risk to the consumer. Pathogenic bacteria such as *Staphylococcus* spp., *Salmonella* spp., *Pseudomonas aeruginosa* and *Klebsiella* spp., amongst others, have been associated with rainwater harvesting systems and the poor quality of the water is mainly attributed to faecal contamination caused by mammals, birds and reptiles. Generally, these pathogenic strains are highly virulent and are often resistant to common antimicrobials utilised for the treatment of diseases caused by these pathogens. An increase in drug-resistant infectious diseases presents a significant challenge to antimicrobial therapies. Urgent developments of novel antimicrobial agents are required to treat a wide spectrum of targets which include bacteria, fungi, viruses and parasites.

Actinomycetes are a group of soil microorganisms known for the production of bio-active compounds. *Streptomyces pharetrae* CZA14^T is one such actinomycete which was first described in 2005. Preliminary tests revealed that the strain has the ability to produce bio-active compounds. Subsequently, the genome of CZA14^T was sequenced and submitted to an online secondary metabolite prediction software, antiSMASH, to predict secondary metabolite biosynthetic gene clusters (smBGCs) which then govern the expression of the compounds of interest. Fifty-five smBGCs encoding for secondary metabolites of interest were predicted by antiSMASH, of which, 23 were non-ribosomal peptide synthetases (NRPSs), seven were lantipeptide/lassopeptides/bacteriocins, eight were polyketide synthases (PKSs) and the remaining 17 biosynthetic gene clusters include genes for siderophores, terpenes, butyrolactones, ectoines, melanin and genes labelled as "other".

In the current study, previously identified optimal production media and fermentation conditions for maximum antimicrobial production by CZA14^T were scaled-up from 0.5 L, 1 L and 2 L baffled flasks, to fermentation in a 3 L airlift reactor (ALR) and a 5 L continuous stirred tank reactor (CSTR). Solvent extractions were performed on both the supernatant and mycelia to test for extra- and intra-cellular production of bio-active compounds. Fermentation samples

were also collected from the ALR at days 3, 6, 9 and 12, and used for molecular studies for which RNA extraction was performed and cDNA was synthesised and sequenced. Using molecular techniques, it was determined that CZA14^T possess the genes for the expression of a Type-II PKS – curamycin A. Sequence information indicated that the Type II PKS involved in the production of curamycin A was expressed under the fermentation conditions used in the ALR. This Type-II PKS corresponded to the predicted Type-II PKS from contig 196, cluster 24 of the CZA14^T genome.

Solvent extracts were analysed using Thin Layer Chromatography (TLC) and the inhibitory efficacy of the crude and partially purified extracts were determined using filter disk diffusion and bioautography assays against a range of ATCC clinical test strains and environmental DRWH system isolates. Compounds separated by TLC showed bands with similar R$_f$ values when visualised under UV light throughout all fermentation processes, but the focus of the study was mostly on the extracts prepared from the fermentation in the ALR. Compounds produced by CZA14^T exhibited a broad spectrum of bio-activity against all the test strains used in this study, displaying both anti-bacterial and anti-fungal activity. Compounds produced by CZA14^T in the ALR displayed various chemical and physicochemical characteristics as determined by different staining methods of the developed TLC plates and UV/Vis spectroscopy of the biologically active compounds.

The crude compounds were partially purified using silica column chromatography and the fractions collected were analysed by liquid chromatography-mass spectrometry (LC-MS). Furthermore, the ALR fermentation broth was subjected to ammonium sulphate precipitation, dialysis and organic solvent extraction. This yielded a set of bio-active compounds with activity against various ATCC strains and DRWH system isolates. This sample was also subjected to LC-MS analyses and results showed the presence of a mass (1374.8 m/z) that corresponds to that of curamycin A, which serves as additional confirmation that the smBGC that encodes for curamycin A, is expressed under the fermentation conditions used in the ALR. Future studies will focus on the purification and structural elucidation of the bio-active compounds produced by CZA14^T, including curamycin A.

Key words: antimicrobial, polyketide synthases (PKSs), curamycin, broad-spectrum bio-activity

Opsomming

Toenemende verstedeliking, gereelde droogtes, swak gehalte water en skaars waterbronne, veral in landelike gebiede, is 'n bron van kommer wat voorsiening van vars water aanbetref. Water is noodsaaklik vir lewe en om die groeiende vraag na water te voorsien, is dit noodsaaklik om alternatiewe waterbronne te ondersoek. Die opvang van reënwater word as 'n potensiële alternatiewe bron van water beskou en word algemeen in baie dele van die wêreld as 'n water bron gebruik. Daar is egter 'n ernstige gesondheidsrisiko verbonde aan die gebruik van versamelde reënwater, veral as dit as drink-water bron gebruik word. Die rede hiervoor is dat patogene soos *Staphylococcus* spp., *Salmonella* spp., *Pseudomonas aeruginosa* en *Klebsiella* spp. bewys is om teenwoordig te wees in reënwater tenks, en die kontaminasie van die tenks met hierdie patogene word dan algemeen toegeskryf aan fekale besmetting van die opvang area van die tenks deur klein soogdiere, voëls en reptiele. Hierdie patogene is oor die algemeen hoogs virulent en weerstandig teen antimikrobiese middels wat huidiglik gebruik word om siektes te behandel wat deur hierdie organismes veroorsaak word. 'n Toename in antimikrobiese weerstandige infeksies en siektes plaas druk op antimikrobiese terapie vir die suksesvolle behandeling van hierdie infeksies en siektes. Daarom is dit noodsaaklik om nuwe antimikrobiese middels te ondersoek wat effektief sal wees teen 'n wye spektrum van bakterieë, fungi, virusse en parasiete.

Aktinomisete is 'n groep grond mikro-organismes wat bekend is vir die produksie van bio-aktiewe verbindings. *Streptomyces pharetrae* CZA14^T is een so aktinomiseet wat vir die eerste keer in 2005 beskryf is. Voorlopige toetse het getoon dat CZA14^T die vermoë het om bio-aktiewe verbindings te produseer. Die genoom volgorde van CZA14^T is daarom bepaal. Die genoom is toe gebruik om die teenwoordigheid van sekondêre metaboliet biosintetiese geen klusters (smBGKs) te voorspel, wat beheer uitoefen oor die uitdrukking van bio-aktiewe verbindings. Dit is gedoen deur gebruik te maak van die sagteware AntiSMASH. Vyf-en-vyftig smBGCs is geïdentifiseer deur AntiSMASH, waarvan 23 kodeer vir nie-ribosomale peptied sintetases (NRPSs), sewe kodeer vir lantipeptiede / lassopeptiede / bakteriosiene, agt kodeer vir poliketide sintases (PKSs) en die oorblywende 17 biosintetiese geen klusters kodeer vir siderofore, terpene, butyrolaktone, ektoines, melanien en gene wat as "ander" beskryf is.

In die huidige studie is 'n medium en fermentasie kondisies gebruik wat voorheen bewys is om optimaal te wees vir die produksie van antimikrobiese verbindings deur CZA14^T. Die eksperimente is opgegradeer van 0.5 L, 1 L en 2 L gekeepte fles fermentasies na fermentasie in 'n 3 L "airlift reactor (ALR)" en 5 L aanhoudend gemengde tenk reaktor (CSTR). Ekstraksies is uitgevoer op beide die miselium en supernatant om te toets vir intra- en ekstrasellulêre produksie van bio-aktiewe verbindings. Fermentasie monsters is ook geneem uit die ALR op dag 3, 6, 9 en 12, en daarna gebruik vir molekulêre analises naamlik: RNA ekstraksies, cDNA sintese en cDNA volgorde bepaling. Deur die gebruik van molekulêre tegnieke, is vasgestel dat CZA14^T gene besit vir die uitdrukking van 'n Tipe-II PKS kuramisin A. Volgorde inligting het aangedui dat die Tipe-II PKS, onder die gespesifiseerde fermentasie kondisies in die ALR, uitgedruk is. Hierdie Tipe-II PKS het ooreengestem met die voorspelde Tipe-II PKS van kontik 196, kluster 24 van die genoom van CZA14^T.

Oplosmiddel ekstraksies is ontleed met behulp van dunlaagchromatografie (DLC) en die inhiberende doeltreffendheid van die kru- en gedeeltelike gesuiwerde ekstraksies is bepaal deur gebruik te maak van diffusie filter skyfies en bioautografiese toetse. Die ekstraksies is getoets teen 'n reeks ATCC kliniese isolate en omgewings-isolate wat vanuit reënwater tenks geïsoleer is. Die verbindings wat met behulp van DLC geskei is, het deurentyd dieselfde bande getoon (onder UV-lig gevisualiseer) met ooreenstemmende R_f-waardes vir die fermentasie-tydperk. Die fokus van die studie was egter op die ekstraksies wat vanuit die fermentasie-produk van die ARL voorberei is. Die verbindings wat deur CZA14^T geproduseer is, was aktief teen al die toets organismes wat in hierdie studie gebruik is en het dus beide anti-bakteriële en anti-fungi aktiwiteit getoon. Verbindings wat deur CZA14^T geproduseer is in die ALR, het verskeie chemiese en fisiese-chemiese eienskappe vertoon, soos bepaal deur verskillende kleuringsmetodes van die ontwikkelde DLC plate en UV / Vis spektroskopie van die biologiese aktiewe verbindings.

Die kru-verbindings is gedeeltelik gesuiwer met behulp van silika kolomchromatografie en die versamelde breukdele is ontleed deur vloeistofchromatografie-massaspektrometrie (LC-MS). Daarbenewens is die ALR fermentasie mengsel onderwerp aan ammoniumsulfaat presipitasie, dialise en organise oplosmiddel ekstraksie. 'n Stel bio-aktiewe verbindings, wat aktiwiteit teen 'n verskeidenheid ATCC – en reënwater isolate getoon het, is op hierdie manier geïdentifiseer. Hierdie monster is ook blootgestel aan LC-MS analise en resultate het die teenwoordigheid van

'n massa (1374,8 m/z), wat ooreenstem met dié van kuramisin A, getoon. Hierdie resultate dien as addisionele bevestiging dat die smBGC wat kodeer vir kuramisin A uitgedruk word tydens die ALR fermentasie kondisies. Toekomstige studies sal fokus op die suiwering en strukturele ontleding van die bio-aktiewe verbindings wat deur CZA14^{T} geproduseer word, insluitende kuramisin A.

Sleutel woorde: antimikrobiese, polyketide sintases (PKSs), kuramisin, breë spektrum bio-aktiwiteit

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Glossary

Clarification of basic terms and concepts

Actinomycete:	Filamentous, Gram positive bacterium, typically with a high GC content in its DNA
Antimicrobial:	A natural or synthetic substance capable of inhibiting the growth of a microorganism
Antimicrobial Resistance:	Ability of an organism to resist the effects of a pharmacological compound/drug
Bio-active Compound:	A compound that has an effect on a living cell, tissue or organism
Bactericidal Activity:	An agent/compound capable of killing a bacterium
Bacteriostatic Activity:	An agent/compound which prevents the growth of a bacterium
Gene:	A section/region of DNA that encodes a protein product or functional RNA
Genome:	A particular organism's complete genetic material/make-up
Nosocomial infection:	An infection acquired in hospitals
Secondary Metabolite:	Compounds which are not directly involved in the normal development, growth or reproduction of an organism
Zone of Inhibition:	Area of clearing around an actinomycete colony or filter disk impregnated with a bio-active compound when exposed to test strains

List of Abbreviations

Abbreviation	Meaning
A	Adenylation
ACP	Acyl Carrier Protein
ALR	Airlift Reactor
AMR	Antimicrobial Resistance
AntiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
A/S	Ammonium Sulphate
AT	Acyltransferase
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C	Condensation
CoA	Coenzyme A
CSTR	Continuous Stirred Tank Reactor
Cy	Cyclase
DAEC	Diffusely Adherent *Escherichia coli* (*E. coli*)
DH	Dehydrase
DNA	Deoxyribonucleic acid
DRWH	Domestic Rainwater Harvesting
DTT	Dithriothreitol
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ER	Enoylreductase

KR	Ketoreductase
KS	Ketosynthase
KSα	α-ketoacyl synthase
KSβ	β-ketoacyl synthase
LC/MS	Liquid Chromatography/Mass Spectrometry
M	Methylation
MHA	Mueller Hinton Agar
MIC	Minimal Inhibitory Concentration
MLC	Minimum Lethal Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MT	Methyltransferase
MTT	Thiazolyl Blue Tetrazolium Blue
NRPS	Non-Ribosomal Peptide Synthetases
Nt	Nucleotides
OD	Optical Density
PCR	Polymerase Chain Reaction
pHMMs	profile Hidden Markov Models
PKS	Polyketide Synthases
RNA	Ribonucleic acid
RW	Rainwater
SM	Secondary Metabolite
smBGCs	secondary metabolite Biosynthetic Gene Clusters
smCOGs	secondary metabolite Clusters of Orthologous Groups
SNF	Supernatant Filtrate
TE	Thioesterase
TLC	Thin Layer Chromatography
UV	UltraViolet
VRE	Vancomycin-Resistant enterococci
WHO	World Health Organisation

List of Symbols

Symbol	Meaning
° C	Degrees Celsius
α	Alpha
β	Beta

CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1. Background Introduction

Water is imperative to the survival of all living organisms. The provision of adequate, accessible and safe water sources to the entire global population is thus essential. Currently a number of the world's population, particularly in sub-Saharan Africa, struggle to obtain access to a safe drinking-water source and often results in zoonotic disease outbreaks. Rainwater harvesting (RWH) has been earmarked as an alternative and sustainable water source and has been adopted in many countries worldwide. However, numerous pathogenic microorganisms have been isolated from RWH tanks, which could negatively affect the health of the consumer. This problem is exacerbated as microorganisms isolated from the environment often build up or acquire resistance to various classes of antibiotics.

1.2. Motivation for study

The growing prevalence of infections associated with poor drinking water quality and the emergence of new pathogenic organisms that are resistant to current antimicrobial therapies, together with a decrease in the number of compounds entering the market for treatment of these diseases, presents a challenge. It is therefore imperative to search for new, efficient and safe antimicrobials with novel mechanisms of action that are active against a broad range of pathogens.

Streptomyces pharetrae CZA14[T] used in this study has the potential to produce a number of biologically active compounds when conditions for production are optimised. In a previous study, the genome of strain CZA14[T] was sequenced and annotated. The genome of CZA14[T] was analysed during a study that was conducted in fulfilment of the academic requirements for an Honours degree (Kim Durrell, UWC, 2013). The bioinformatics online tool, antiSMASH, detected 55 biosynthetic gene clusters indicating the potential of this strain for antimicrobial production. The optimised parameters for the production of the maximum number of compounds was also determined during this study and served as the basis for the scale-up production of antimicrobials that was the focus of the current study.

1.3. Aims and Objectives

The aim of this study was to scale-up the production of the bio-active compounds produced by strain CZA14^T and to test their bio-activity against a range of American Type Culture Collection (ATCC) strains and domestic rainwater harvesting (DRWH) system isolates. The specific objectives of the study were therefore the following:

a)   The optimisation of medium-scale (3 L and 5 L) production of secondary metabolites produced by *S. pharetrae* CZA14^T;

b)   To test the inhibitory efficacy of crude antimicrobial extracts against a number of known pathogenic bacteria (ATCC test strains);

c)   To test the inhibitory efficacy of crude antimicrobial extracts against the pathogenic bacteria isolated from the DRWH systems; and

d)   The partial purification of selected compounds and the determination of their antimicrobial profiles.

1.4. Methodology

Phase 1: A pre-determined production medium for optimal antimicrobial production were scaled-up to medium- and larger-scale production in baffled flasks (2 L size) and/or fermentation vessels (3 L and 5 L capacity).

Phase 2: Inhibitory efficacy of antimicrobials produced by strain CZA14^T during fermentation processes in phase 1 were tested against a wide range of known pathogenic microorganisms (e.g. bacteria and microfungi/yeasts) to determine bio-activity potential.

Phase 3: Purification techniques suitable for the separation of compounds produced by strain CZA14^T were determined and applied in this study. Furthermore, LC/MS analysis was performed to determine the exact mass of compounds produced by strain CZA14^T.

1.5. Structure of Thesis

This thesis consists of an introduction and literature review (Chapter 1 and 2), four experimental chapters (Chapters 3, 4, 5 and 6), and one chapter concluding the thesis. Chapter 3 focused on the production of secondary metabolites produced by *S. pharetrae* CZA14^T used in this study in varying fermentation vessels. Chapter 4 focused on the antimicrobial potential of

strain CZA14^T against a range of ATCC test strains and DRWH isolates, as well as, the partial identification of DRWH system isolates using 16S rRNA gene sequencing. Chapter 5 focused on the partial purification of selected compounds produced by strain CZA14^T. Lastly, Chapter 6 focused on the biosynthetic potential of strain CZA14^T; molecular and bioinformatics based techniques were applied.

CHAPTER 2:

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1.   Chapter Summary

The literature review covers aspects of domestic rainwater harvesting (DRWH), the microbial quality of water found in DRWH tank systems, the diseases associated with the consumption of rainwater collected in these systems, and ways to curb the spread of diseases caused by pathogens found in DRWH systems. The chapter concludes with information on antimicrobials, and actinomycetes as a source of these compounds.

2.2.   Introduction

Over the past few decades an increased incidence of infectious diseases caused by microorganisms resistant to conventional treatment has been observed with one of the leading causes attributed to waterborne outbreaks (WHO: Antimicrobial Resistance, 2012). These infections may result in prolonged disease and even untreatable diseases, which eventually result in death. Poor sanitation along with inadequate drinking-water quality is of global concern as it is amongst the world's major causes of preventable morbidity and in turn, mortality (OECD and WHO, 2003).

In order to alleviate this problem, the Millennium Development Goals (MDGs) were established by the United Nations (UN) in the year 2000. One-hundred and three UN member states and at least 23 international organisations adopted this strategy with South Africa being one of its signatories. The target of the MDGs was to halve the proportion of people who are without sustainable water sources and basic sanitation by the year 2015 (Millennium Project, 2006). According to the 2012 projected results in the WHO and the UN International Children's Emergency Fund (UNICEF) 2014 update report, more than two billion people worldwide have received an improved drinking-water source since 1990. One-hundred and sixteen countries have reached the water target set out by the MDGs, while 77 countries have met the MDGs target for sanitation; furthermore, 56 countries have already met the MDGs target for both water and sanitation (WHO and UNICEF, 2014).

However, according to the 2014 WHO/UNICEF update report, at least 11% of the world's population remain without access to an improved source of drinking-water with sub-Saharan

Africa having the lowest drinking-water coverage as depicted in Figure 2.1 (WHO and UNICEF, 2014).
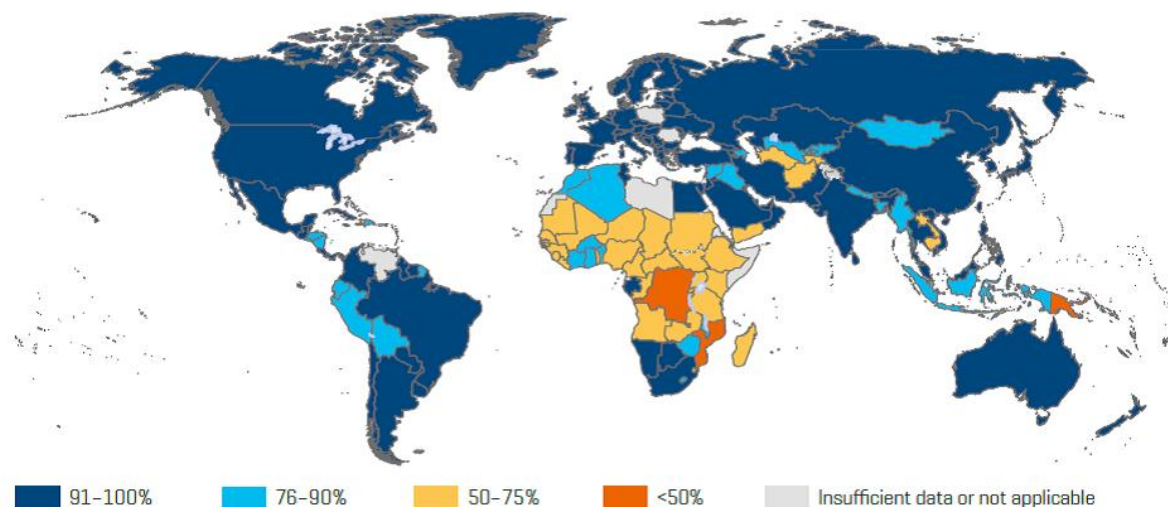

Figure 2.1: Proportion of the world's population that are using an improved drinking-water source in 2012 (WHO and UNICEF, 2014).

Currently, more than 663 million people world-wide still lack access to an improved drinking-water source (United Nations, 2015), with more than 300 million people in sub-Sahara Africa still utilising inadequate water supplies. In addition, almost 173 million people rely on untreated groundwater with well over 90% of these people living in rural areas.

A post-2015 development agenda was devised, the Water, Sanitation and Hygiene (WASH) report, with targets to obtain universal access to basic drinking water, sanitation and hygiene for schools, households and health facilities; and to halve the proportion of the world's population without access to safe and manageable drinking water and sanitation services by the year 2030 (WHO and UNICEF, 2014). Improved water and sanitation can significantly reduce water-borne illness associated with morbidities. With an increasing population and the looming negative effects of climate change, water conservation is key for survival. Many countries world-wide are searching for alternative water sources to ensure sustainability, and rainwater harvesting (RWH) serves as an alternative for the world to meet the MDGs by 2030 (WHO and UNICEF, 2014).

RWH is a technology used for the concentration, collection and storage of rainwater from land surfaces, rooftops or rock catchments (Abdel Khaleq and Ahmed, 2007). There are three main types of RWH systems namely:

1) *In situ* RWH – it involves water storage in the soil profile by the entrapment of the rainwater where it falls;

2) External water harvesting - involves the collection of rainwater that has run off a surface elsewhere and is stored offsite; both *in situ* RWH and external RWH is used for agricultural use; and

3) Domestic RWH – is associated with the collection of run-off water from roofs, courtyards and streets.

The main focus of this study is on DRWH and diseases commonly associated with the water collected and stored in these systems.

2.3.   Domestic Rainwater Harvesting Systems

Domestic rainwater harvesting can be defined as the collection of rainwater which has runoff from ground or roof-top surfaces and is used as a domestic water supply for purposes such as drinking, cooking and washing (Worm and van Hattum, 2006). There are a number of advantages for the collection and storage of rainwater (RW) for domestic use, which include:

1) It is a simple, low cost technology that makes use of inexpensive material to store RW;

2) In rural areas in particular, water sources are traditionally situated a distance away from the community, DRWH provides point-of-use for added convenience;

3) RW is a renewable resource with low impact to the environment; and

4) RW may be used as a supplementary water resource along with more 'conventional' water supply technologies to balance existing water supplies in rural communities and even in areas of the world where adequate water resources are available to combat ever growing water demands.

Many countries including Australia, Canada, Denmark, Germany, India, Japan, New Zealand, Thailand and the United States have recognised RWH as an alternative water resource for non-potable uses (Despins *et al.*, 2009). The Department of Science and Technology (DST) and the Department of Water Affairs (DWA; now Department of Water and Sanitation) has distributed domestic rainwater tanks throughout Limpopo, KwaZulu-Natal, the Eastern Cape, the Free State

and the Western Cape (de Lange, 2006). However, only 0.4% of households are currently equipped with DRWH systems (Statistics South Africa, 2010).

Although DRWH is a simple, low cost technology that conserves water and is convenient, flexible, adaptable, and provides better water quality than groundwater, there are some disadvantages associated with DRWH systems. The system is prone to contamination via air pollution, animal and bird droppings, insect, dirt, and organic matter that could pose a serious health risk. The greatest challenge for the 21st century is microbial pathogens within the water that easily goes undetected due to the lack of available methodology to quantify these organisms and the lack of knowledge of RW consumers.

2.3.1. Microbial Quality of Water found in DRWH systems

Waterborne pathogens such as *Legionella* species (spp.), some mycobacterial species, *Naegleria fowleri*, *Pseudomonas aeruginosa* and "enteric" pathogens such as *Salmonella*, *Campylobacter*, *Giardia* and *Cryptosporidium* species associated with faecal matter, are commonly found in polluted water and are easily adaptable to the low nutrient, chemical, physical and biological conditions found in water. Water plays an essential role for these pathogens by acting as a vehicle of transmission. The lifespan of the pathogen and the period of time it survives in water is greatly dependent on the pathogen itself (Percival *et al*., 2014). For example, pathogens such as *Cryptosporidium* spp. and *Giardia* spp. have extended periods of survival in water because of their resistant cysts/oocysts; whereas pathogens such as *Escherichia coli* O157 and *Vibrio cholera* were previously believed to be host-dependent for replication and their growth in water, was only exhibited under special conditions (Vital *et al*., 2007; 2008).

Fundamentally, harvesting RW prior to contamination is key. However, this is difficult to achieve as pollution may take place through cross-contamination of raindrops through polluted air, on the collection surface or in the storage container. It is thus important to treat harvested rainwater before consumption to reduce the occurrence of infection. The most commonly used techniques used to treat RW is by coagulation, flocculation, sedimentation, filtration and disinfection (WHO, 1982). DRWH systems may also be fitted with first flush filters to clean the catchment area inside of the storage tank and to divert the first few millilitres of RW to improve water quality (Mwenge Kahinda *et al*., 2007). These type of devices may not always be made available to rural communities and most DRWH users make use of their own storage tank

systems and disinfect the RW by boiling or chlorination to kill any bacteria present in the RW (Dillaha and Zolan, 1985). Chlorination in turn also has its disadvantages as it may form dangerous by-products when interacting with organic matter found in sludge which settles at the bottom of the water storage tanks. While chlorination and boiling inactivates many waterborne pathogens, it does not kill *Mycobacterium* spp. and *Cryptosporidium parvum* oocysts (Gordon *et al*., 1995).

Most waterborne pathogens form biofilms to protect themselves against stress factors such as organism density, nutrient depletion, changes in temperature and other abiotic and biotic factors (Al-Azemi *et al*., 2010; Garny *et al*., 2009). Biofilms are slimy coatings which form under unhygienic practices; they are formed by the combination of bacterial organisms which excrete exopolysaccharides and attach themselves to surfaces for further replication. These biofilms can also form on the water surfaces as 'floating biofilms' and can offer refuge to viruses and protozoa which cannot actively form biofilms. The biggest challenge associated with these biofilms is sampling and detection of waterborne pathogens as biofilms are considered the place in which different species form close contact with each other. This enables these organisms to communicate via signals, internalise smaller microorganisms and transfer genetic material with each other which in turn helps in the survival of the pathogen in water. Increased resistance to disinfection and other stress factors are often observed when biofilms are present making it problematic to treat water containing these biofilms (Percival *et al.*, 2014).

In general, waterborne pathogens are transmitted via the faecal-oral route. Other means of transmission can be through healthy hair, skin, urine, wounds, mucus, sputum and saliva; this is most likely to occur by inhalation of aerosols. Assessing the safety of harvested RW by testing for numerous pathogens would thus be technically and economically impractical. Currently, there are no standards put into place for RW quality in South Africa. Guidelines for assessing the microbial quality of potable water involve the use of indicator organisms to monitor the quality of water by detecting the presence or absence of pathogens in the water. The downside of testing for indicator organisms is that there is no single indictor organism which meets all of the water specification requirements. A combination of indicators are used to allow for reliable indication of probable risks of infection – these include heterotrophic bacteria, total coliforms, total faecal coliforms, *E. coli*, and somatic- and male-specific coliphages. The microbial requirements for drinking water harvested from DRWH systems are presented in Table 2.1 (Kempster *et al*., 1997; SABS, 2001; DWAF, 1996).

Table 2.1: The microbiological requirements for drinking water in South Africa

Indicator Organism	Units	Allowable Compliance
Heterotrophic plate count	Colony forming units (CFU) 1 mL^{-1}	100
Total coliform bacteria	CFU 100 mL^{-1}	10
Faecal coliform bacteria	CFU 100 mL^{-1}	1
Escherichia coli	CFU 100 mL^{-1}	0
Somatic bacteriophages	CFU 10 mL^{-1}	1
Enteric viruses	Plaque forming units (PFU) 100	1
Protozoan parasites (*Giardia*/ *Cryptosporidium*)	Count 100	0

Key: Total coliform bacteria – Primary indicator of general hygienic quality of harvested rainwater; Total faecal coliform bacteria – primary indicator of faecal pollution of the harvested rainwater; *Escherichia coli* – very specific indicator of faecal pollution of the harvested rainwater; Bacteriophages – the incidence and survival of bacterial viruses in harvested rainwater; somatic coliphages – bacteriophages which infect and replicate in *E. coli* and related species; male- specific (F-RNA) coliphages – very specific indicator of sewage pollution, these coliphages cannot replicate in water bodies.

Limited information about the microbial and chemical quality of harvested RW is available. Indicator organisms were used to determine the microbial quality of harvested RW worldwide and the results of these studies are represented in Table 2.2. Ahmed *et al*. (2011) reported the percentage of positive samples for faecal indicator bacteria and total bacteria in water samples collected from harvested RW from data collected from 18 different research studies (Table 2.2); de Kwaadsteniet *et al*. (2013) contributed to the data with an additional six more studies found in literature.

Table 2.2: Percentage of DRWH system samples testing positive (> 1 CFU 100 mL^{-1}) for total faecal indicators and total bacteria (adapted from Ahmed *et al*., 2011; de Kwaadsteniet *et al*., 2013). The number of sample selected is indicated in brackets.

Country	Total Coliforms	Faecal coliforms	*E. coli*	Enterococci	Total bacteria	Reference
Australia	52 (100)	38 (100)	*	*	*	Verrinder and Keleher, 2001
	90 (49)	*	33 (49)	73 (49)	*	Spinks *et al*., 2006
	*	*	63 (27)	78 (27)	*	Ahmed *et al*., 2008
	*	*	58 (100)	83 (100)	*	Ahmed *et al*., 2010
	*	*	63 (15)	92 (22)	*	Ahmed *et al*., 2012a
	91 (46)	78 (41)	57 (67)	82 (67)	100 (67)	CRC for water quality and treatment, 2006
	*	83 (6)	*	*	*	Thomas and

						Greene, 1993
	63 (81)	63 (81)	*	*	100 (77)	Evans *et al.*, 2006
Canada	31 (360)	14 (360)	*	*	*	Despins *et al.*, 2009
Greece	80 (156)	*	41 (156)	29 (156)	*	Sazakil *et al.*, 2007
Denmark	*	*	79 (14)	*	100 (14)	Albrechtsen, 2002
Micronesia	43 (155)	70 (176)	*	*	*	Dillaha and Zolan, 1985
New Zealand	*	56 (125)	*	*	*	Simmons *et al.*, 2001
Nigeria	100 (6)	#	*	#	100 (6)	Uba and Aghogho, 2000
South Korea	92 (90)	*	72 (90)	*	*	Lee *et al.*, 2010
Thailand	*	*	40 (86)	*	*	Pinfold *et al.*, 1993
USA	93 (30)	*	3 (30)	*	100 (30)	Lye, 1987
US Virgin Islands	57 (45)	36 (45)	*	*	86 (45)	Crabtree *et al.*, 1996
US Virgin Islands	*	59 (17)	*	*	*	Rushin and Krishna, 1990
Bermuda	90 (102)	*	66 (102)	*	*	Levesque *et al.*, 2008
Palestine	95 (100)	57 (100)	*	*	*	Al-Salaymeh *et al.*, 2011
	49 (255)	*	17 (255)	*	*	Abo-Shehada *et al.*, 2004
Hawaii, USA	*	89 (9)	*	*	*	Fujioka *et al.*, 1991
Zambia	100 (5)	100 (5)	*	*	*	Handia, 2005

*** Not reported, *#* not determined**

The compilation of research data by Ahmed *et al.* (2011) highlights the poor correlation between pathogenic bacteria and faecal indicators. According to Simmons *et al.* (2001), 30% and 57% of the 155 and 176 RWH samples surveyed in Micronesia had no detectable numbers of faecal coliforms and total coliforms, respectively. Lye (1987) also reported good quality RW according to WHO drinking water standards (total coliform numbers should be <10 CFU 100 mL^{-1}) in 95% of water samples collected from a particular water origin in the USA. In Kentucky, USA, 3% of samples had faecal coliform numbers < 10 CFU 100 mL^{-1}. In contrast, a number of studies revealed higher numbers of faecal coliforms in harvested RW which does not meet the drinking water requirements of the WHO. In a study by Lee *et al.* (2010), in South Korea, 72% and 92% of harvested RW samples tested positive for *E. coli* and total coliforms, respectively, with all samples containing indicators above WHO standards. In Victoria, Australia, out of the 49 RW tanks surveyed, 73% of samples tested positive for enterococci and 33% of samples tested positive for *E. coli*. These numbers exceeds the Australian Drinking Water Guidelines of zero CFU 100 mL^{-1} (ADWG, 2004). Albrechtsen (2002) determined that the harvested RW in

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Danish RW systems are not safe for human consumption: it was determined that the water contained high numbers of *E. coli* ranging from 4 – 900 CFU 100 mL^{-1} in 11 out of 14 systems tested. High numbers of enterococci and *E. coli* ranging from 5 – 200 CFU 100 mL^{-1} and 4 – 800 CFU 100 mL^{-1}, respectively, was reported by Ahmed *et al*. (2008) in RW collected from systems in Queensland, Australia. Twenty-seven samples were tested during this study – 78%, 63% and 48% were positive for enterococci, *E. coli* and *Clostridium perfringens*, respectively.

In South Africa, a study by Dobrowsky *et al*. (2014) to determine the prevalence of virulence genes associated with *E. coli* was performed. Isolates were sampled from RW tanks in Kleinmond, South Africa, during the low- and high-rainfall periods. Ninety two organisms were confirmed as *E. coli* species, with 6% of these being positively identified as *E. coli* 0157:H7 (16S rRNA gene sequencing). Out of the 80 samples which were collected from 10 DRWH systems, 3% of the samples tested positive for virulent pathogenic *E. coli* namely enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), and 16% of the samples tested positive for enteroaggregative *E. coli* (EAEC). In another study by Dobrowsky *et al*. (2014), 29 RW tanks situated in Kleinmond, South Africa, were monitored for the presence of pathogenic bacteria during periods of high-rainfall. One hundred and sixteen samples were collected from the DRWH tanks during the sampling period. 16S rRNA gene sequencing confirmed the identity of the potential pathogenic bacteria in the RW samples with 19% of the sample being positive for *Pseudomonas* spp. Other dominant genera detected, were *Aeromonas* (16% of samples) and *Enterobacter* (11% of the samples). Other bacteria from various other genera were also isolated, namely *Legionella* spp., *Salmonella* spp., *Shigella* spp., *Yersinia* spp., and *Giardia* spp.

2.4.   Diseases associated with water from DRWH systems

A number of infectious diseases are linked to the consumption of water that is known to be contaminated with faecal matter (Leclerc *et al*., 2002; Theron and Cloete, 2002). Enteric pathogens are responsible for waterborne diseases and include bacteria, parasites and viruses – these pathogens are transmitted via the faecal–oral route (Grabow, 1996; Leclerc *et al*., 2002; Theron and Cloete, 2002). Table 2.3 shows a number of bacteria, parasites and viruses associated with waterborne diseases.

Typically, infections can be caused by bacteria ranging between 10^7-10^8 cells/mL while some enteric bacteria can cause diseases at low doses of 10^1 cells/mL (Edberg *et al*., 2000; Leclerc *et al*., 2002). A number of the organisms presented in Table 2.3 are highly resistant to most

antiseptics and disinfectants used to treat water for domestic use (Kümmerer, 2004; Xi *et al*., 2009). A recent study by Dobrowsky *et al.* (2015) showed the importance of sterilising harvested RW prior to consumption. The study involved solar pasteurisation of RW at temperatures ranging from 55°C – 91°C. The study was conducted on the Welgevallen experimental farm of Stellenbosch University, South Africa, whereby RWH tanks were set-up with Apollo^{TM} solar pasteurisation systems attached to it. The pasteurisation resulted in the reduction of indicator organisms – *E. coli*, heterotrophic bacteria and total coliforms were all below the detection limit. However, *Legionella* spp., *Yersinia* spp., and *Pseudomonas* spp. were still detected in the harvested rainwater samples treated at temperatures above 72°C.

Table 2.3: Diseases associated with waterborne pathogens (Bifulco *et al*., 1989; Grabow, 1996; WHO, 1996; Guerrant, 1997; Leclerc *et al*., 2002; Theron and Cloete, 2002; Yatsuyanagi *et al*., 2003; NRC, 2004)

Pathogen		Diseases
Bacteria	*Campylobacter* spp.	Acute gastroenteritis and diarrhoea
	Enteropathogenic *E. coli*	Diarrhoea
	E. coli 0157:H7	Haemolytic syndrome and bloody diarrhoea
	Salmonella spp.	Diarrhoea, Typhoid fever
	Shigella spp.	Diarrhoea, Dysentery
	Vibrio cholera	Diarrhoea, Cholera
	Yersinia spp.	Gastrointestinal infections, Diarrhoea
Protozoan parasites	*Crytosporidium parvum*	Diarrhoea, cryptosporidiosis
	Entamoeba hystolytica	Amoebic dysentery
	Giardia spp.	Diarrhoea, Giardiasis
Viruses	Adenoviruses	Conjunctivitis, respiratory disease, diarrhoea
	Astroviruses	Diarrhoea
	Coxsackie viruses (Enterovirus)	Meningitis, diabetes, respiratory, vomiting, skin rashes, diarrhoea
	Echovirus 68-71	Diarrhoea, rashes, acute enteroviral haemorrhagic conjunctivitis, meningitis, respiratory diseases
	Hepatitis viruses (A; E)	Hepatitis (jaundice), gastroenteritis
	Caliciviruses	Vomiting, diarrhoea
	Polioviruses (Enteroviruses)	Poliomyelitis
	Rotaviruses	Vomiting, diarrhoea
	Small round structured viruses	Vomiting, diarrhoea
Emerging	*Acinetobacter* spp.	Meningitis, endocarditis, septicaemia

opportunistic pathogens	*Aeromonas* spp.	Gastroenteritis, diarrhoea
	Cyclospora spp.	Abdominal cramping, fever, diarrhoea
	Isospora spp.	Diarrhoea
	Legionella spp.	Pontiac fever, Legionnaires disease
	Microsporidia spp.	Diarrhoea, gastrointestinal infection
	Non-tuberculosis mycobacteria	Cervical lymphadenitis, skin infections, nontuberculosis *Mycobacterium* disease
	P. aeruginosa	Wound and eye infections, septicaemia

Over the past few years, several outbreaks of diseases caused by pathogens were reported that cannot be inhibited by conventional water treatment. In 1987 and 1983, an outbreak of waterborne *Campylobacter* spp. in two communities in Canada and the USA were reported, respectively (Alarly and Nadeau, 1990; Sacks *et al*., 1986). In a more recent study, waterborne *E. coli* 0157:H7 resulted in infection of 2 300 hundred people in 2000 in Walerton, Canada (Hrudey *et al*., 2003). Due to flooding that occurred in Bangladesh, 67 718 people reported having diarrhoea of which nine people died as a result of waterborne diseases (International Water Association, 2004). Similarly, these disease causing pathogens have been reported in harvested RW used for domestic use (Table 2.4).

Table 2.4: Cases of diseases reported in literature which is associated with the consumption of untreated roof-harvested rainwater (adapted from Ahmed *et al*., 2011).

Country	Disease causing pathogens	Types of disease	No. of people affected	Reference
Australia	*Campylobacter* spp.	Diarrhoea, vomiting	1	Broadribb *et al*., 1995
Australia	*Campylobacter fetus*	Diarrhoea, abdominal pain	23	Merritt *et al*., 1999
Australia	*C. botulinum*	N/S	3	Murrell and Stewart, 1983
New Zealand	*L. pneumophila*	Legionnaires disease	1	Simmons *et al*., 2008
U.S Virgin Islands	*L. pneumophila* Serogroup 1	Legionnaires disease	27	Schlech *et al*., 1985
West Indies	*S. arechevalata*	Diarrhoea, headache, fever	48	Koplan *et al*., 1978
Australia	*S. typhimurium* phage 9	Vomiting, diarrhoea, abdominal pain, nausea	27	Franklin *et al*., 2009
New Zealand	*S. typhimurium* phage 1	Diarrhoea	2	Simmons and Smith, 1997

N/S, not specified

Other bacteria commonly isolated from harvested RW belong to the genera *Bacillus*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Staphylococcus* and

Salmonella. Efuntoye and Apanpa (2010), reported that out of all species isolated, *E. coli* was the most predominant species constituitng 36.5% of all bacteria isolated. *Klebsiella aerogenes, Staphylococcus aureus* and *P. aeruginosa* constituted 26%, 14.6% and 7.3% of all isolates, respectively. *Bacillus cereus* was also isolated from 200 samples collected from well water in Ayo-Iwoye, Nigeria (Akharaiyi *et al*., 2007), but in low quantity. *S. aureus, P. aeruginosa* and *E. coli* were isolated from water stored in storage tanks in homes in Tanke area, Ilorin, Nigeria (Sule *et al*., 2011). Hussain *et al*. (2015) also reported on the isolation of *E. coli, P. aeruginosa, S. aureus* and *Salmonella typhi* from thirteen environmental samples collected from RW that collected on ground surfaces during the rainy season and which was used for domestic purposes. In addition, Ofukwu *et al*. (2014) collected 126 samples from reservoirs in Ogbadibo, Nigeria, and found that 104 of those samples were positive for *Enterobacter* spp., 83 of the samples contained *Klebsiella* spp., 29 out of 126 samples contained *E. coli*, and 15 samples were positive for *Salmonella* spp.

The aforementioned genera/species were the focus of the study reported in this thesis as very high rates of resistance to first- and second-line antimicrobials used to treat infections caused by these organisms have recently been observed. A brief profile of each these microorganisms are detailed below.

Bacillus cereus

Bacillus cereus is an aerobic-to-facultative, Gram-positive spore-forming, rod-shaped bacterium that is ubiquitous in nature and bears a close genetic and phenotypic relationship to a number of other *Bacillus* spp., particularly *Bacillus anthracis* (Ash *et al*., 1991). *B. cereus* is typically known as a foodborne pathogen, but is commonly detected in sources of water (Jensen *et al*., 2003). The consumption of water and food contaminated with *B. cereus* may lead to human intestine transient colonisation (Ghosh, 1978). *B. cereus* is a notorious pathogen for nosocomial infections (Avashia *et al*., 2007; Barrie *et al*., 1992; Bryce *et al*., 1993; Van Der Zwet *et al*., 2000). Environments such as fog droplets, dust, plants, sediments, soil, and water in clouds have also been identified as a typical source of *B. cereus* – commonly polluting ground and roof-harvested RW used for domestic purposes and irrigation (Amato *et al*., 2007; DeLeon-Rodriguez *et al*., 2013). A number of *B. cereus* strains have been reported to be resistant to β-lactam type antibiotics, especially penicillin, and have been implicated in a number of eye infections such as endophthalmitis.

Enterobacter cloacae

Enterobacter cloacae occur in the gastrointestinal tract of 40-80% of healthy humans where it acts as normal gut flora and is ubiquitous in nature. However, *Enterobacter* spp. are opportunistic pathogens and are typically implicated in blood infections acquired in a hospital setting (Diekma *et al*., 2002). Members of the family *Enterobacteriaceae* are rod-shaped or cocco-bacilli, Gram-negative, facultative anaerobes that contribute to approximately 50% of isolates associated with hospital acquired infections (Karlowsky *et al*., 2003). Entire families of antimicrobial agents (such as penicillins and aminoglycosides) are ineffective in the treatment of diseases caused by this bacterium. Intrinsic resistance to narrow-spectrum cephalosporins and ampicillin are exhibited by *E. cloacae* (Diekma *et al*., 2002).

Enterococcus faecalis

Enterococci commonly inhabit the oral cavity, the vagina and the gastrointestinal tract in human beings as normal bodily bacterial flora. However, virulent strains are capable of causing diseases such as urinary tract infection, infection of the abdomen, biliary tract, endocardium, bloodstream, and burn wounds, to name a few (Jett *et al*., 1994). *E. faecalis* are small cocci, Gram-positive bacteria with high levels of antibiotic resistance caused by intrinsic factors. In RW, *Enterococcus* spp. are commonly used as faecal indicators when testing the microbiological quality of water used for domestic purposes. *E. faeccalis* are highly prevalent among clinical isolates and human wastewater, and are often resistant to antibiotics such as vancomycin, which is the first-line drug used in the treatment of diseases caused by this bacterium (Ahmed *et al*., 2012b).

Escherichia coli

Gram-negative, short rod-shaped *E. coli* are ubiquitous in nature and are generally linked to faecal contamination. *E. coli* is largely present in normal intestinal flora in human beings and animals – in some parts of the body however, *E. coli* can pose a health threat. Some conditions caused by *E. coli* include bacteraemia and urinary tract infections (Cotruvo *et al*., 2004). Acute diarrhoea is caused by a limited number of *E. coli* classified as enteropathogenic strains, these include: enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Natano and Kaper, 1998).

Klebsiella pneumoniae

Klebsiella spp. are non-motile bacilli, Gram-negative bacteria belonging to the family *Enterobacteriaceae. K. pneumoniae* is predominantly isolated from clinical and faecal specimens and amount to approximately 60 – 80% of all isolates. *Klebsiella* spp. thrive in water environments and increase to high numbers in nutrient-rich waters, such as sugar cane processing operations, waste from pulp mill and textile finishing plants. This bacterium is also known to grow well in drinking-water distribution systems and easily colonise washers found in taps. Nosocomial infections are caused by *Klebsiella* when water contaminated with this bacterium is consumed or via inhalation of faecal coliform aerosols (Ainsworth, 2004).

Pseudomonas aeruginosa

P. aeruginosa is ubiquitous in nature – colonising humans, soil, plants and industrial surfaces. *P. aeruginosa* forms part of the family *Pseudonadaceae* and is an aerobic, rod-shaped, Gram-negative, polarly flagellated bacterium. This organism is easily identified on optimal medium by its non-fluorescent pyocyanin bluish pigment or fluorescent green pyoverdin pigment (De Victoria and Galván, 2001). *P. aeruginosa* is considered harmless, inhabiting the pharyngeal and nasal mucosal surfaces as natural flora. However, if the bacterium penetrates to submucosal tissue or if individuals' immune systems are compromised, *P. aeruginosa* may persist as an opportunistic pathogen. Infections caused by *P. aeruginosa* are treated using a broad spectrum of antibiotics such as β-lactams, fluoroquinolones and aminoglycosides. However, due to changes in the resistance mechanism of the organism, strains of *P. aeruginosa* has become multi-drug resistant with no new drugs to treat diseases caused by this bacterium presently available on the market (Page and Heim, 2009).

Salmonella enterica

Salmonella spp. form part of the *Enterobactemiaceae* family. They are Gram-negative, motile bacilli that produce hydrogen sulphide but, do not ferment lactose. *S. enterica* consists of six subspecies namely: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *diarizonae*, and *S. enterica* subsp. *indica*. The differences in antigen surfaces [somatic (O) and flagella (H) antigens] allow for differentiation of the *Salmonella* species. *Salmonella* is involved in a number of clinical manifestations, namely: bacteraemia, typhoid or enteric fever, gastroenteritis and septicaemia.

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Salmonella spp. are ubiquitous in nature – they gain access to water systems by means of faecal contamination through livestock, sewage discharges, and wild animals. Livestock are typically infected by *Salmonella* spp., but a wide range of animals such as pigs, sheep, birds, poultry and even reptiles can also be infected by *Salmonella* spp. Humans may become infected via the spread of the bacterium by the faecal-oral route through the consumption of food and water contaminated with *Salmonella* (Kim *et al*., 2006; Koplan *et al*., 1978)

Staphylococcus aureus

Staphylococcus aureus is a facultative anaerobic, Gram-positive, cocci, non-spore forming, coagulase- and catalase-positive microorganism. The genus *Staphylococcus* comprises of approximately fifteen different species. *S. aureus* is associated with a normal gut flora and skin of humans; however, disease may arise via two varying mechanisms. One mechanism of action is via the multiplication and spread of the microbe in tissues, and the other mechanism of action is based on the microorganism's ability to produce extracellular toxins and enzymes. *Staphylococcus* spp. are widespread in nature, but is predominantly found on the mucous membranes and skin of animals. Staphylococci are commonly detected in sewage and water environments mainly due to the release of the organism from the gastrointestinal tract (Antal, 1987).

The discovery of antimicrobials have revolutionised medicine and have saved countless lives since its discovery. However, the use of these drugs resulted in the rapid appearance of resistant organisms. In order to combat resistance, new antimicrobials are required with broad spectrum mechanisms of action; this topic is further elaborated upon in the following section.

2.5.   Antimicrobials

Antimicrobials are low molecular weight compounds produced during secondary metabolism of a producing organism (Demain, 1992). The term 'antimicrobials' may be used interchangeably with the term 'secondary metabolite/s' in this study; however, it is not a synonym of the term. The term 'antimicrobial' is a collective term to describe antibacterials, antivirals, antifungals and antiprotozoals. Antimicrobial products include antibiotics, toxins, pigments, pheromones, effectors of symbiosis and ecological competition, growth promoters of plants and animals, anticancer agents, enzyme inhibitors, receptor agonists and antagonists, and immune-modulating agents (Demain, 1992). Antimicrobials can be defined as both natural and synthetic

agents, which may inhibit growth or even destroy microorganisms at particular concentrations (Waksman, 1973). Antimicrobial products in natural or semisynthetic form are grouped into a single class based on similarities in its chemical structure and is named after a fundamental chemical property or after the first discovered member (Todar, 2009).

2.5.1. Antimicrobial Resistance (AMR)

Bacterial resistance can be caused by various means, such as intrinsic factors whereby genes existing in the genome of a particular bacterium could create a resistance phenotype. Other means, which is important to this study, is resistance as a result of anthropogenic activities (Figure 2.2).

As early as the 1940s, an increase in the number of antibiotics selected for human applications have been produced for clinical use and further released into the environment where it is widely disseminated. A number of types of anthropogenic activities which includes the use of antibiotics in aquaculture and agriculture, waste disposal and other non-human applications of antibiotics (Figure 2.2) generates major reserves of environmental resistance (Doyle, 2006); as well as virulence genes and organisms which contain them (Moura *et al*., 2010). It was estimated that millions of antibiotic compounds in metric tons have been distributed over the last half century, into the biosphere (Gottlieb, 1976). This provides an endless maintenance and selection pressure of resistant organisms in all environments, particularly rivers, reservoirs, ground and rain harvested water used for domestic purposes. As a result, putative antibiotic resistance genes are omnipresent in all natural environments.
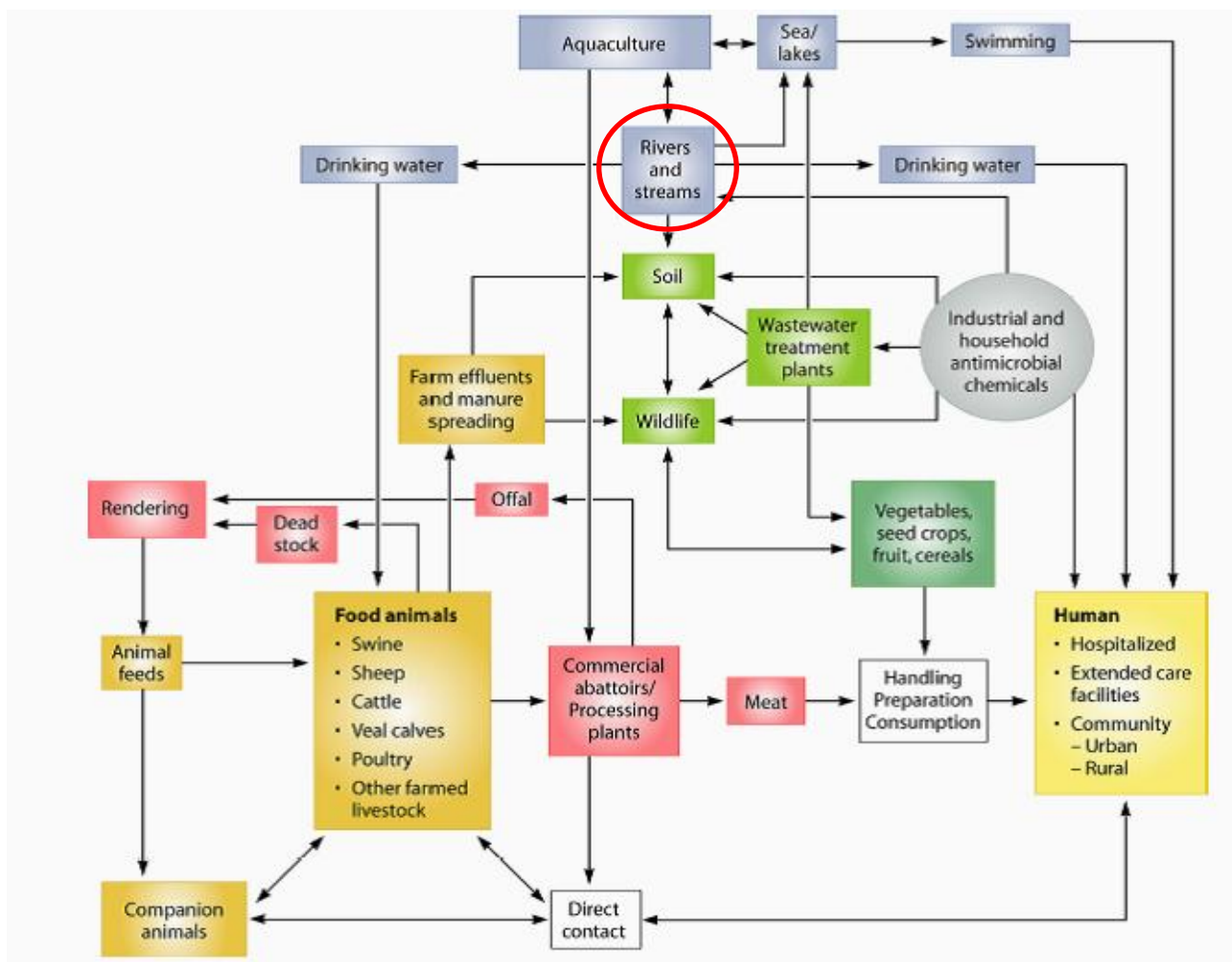
Figure 2.2: Dissemination of antibiotic resistance due to antibiotic resistance within the community, wastewater treatment, hospital, agriculture and associated environments (Doyle, 2006; Liebert *et al*., 1999).

The expression of resistant genes is genetically regulated by the action of the antibiotics, and bacterial cells expend a great deal of genetic space and energy to actively resist antimicrobial agents (Wright, 2005). Bacterial cells achieve resistance against secondary metabolites (SMs) via a number of routes (Figure 2.3).

Route 1: Pumping proteins associated with the membrane of the cell are involved in the efflux of the antimicrobial agent from the cell; Route 2: the antibiotic target is modified through the mutation of important binding elements (i.e. ribosomal RNA) or by reprogramming the biosynthetic pathways (e.g. to gain resistance against glycopeptide antibiotics); and Route 3: modifying enzymes are synthesised to selectively target and destroy the active site of the antimicrobial agent (Wright, 2005).
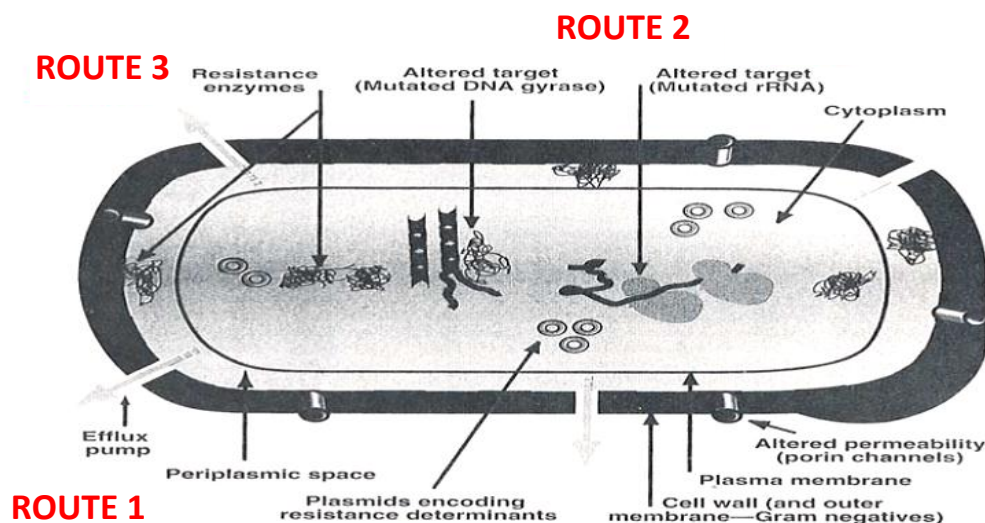
Figure 2.3: Schematic representation of resistance mechanisms elucidated by a bacterial cell (adapted from Kotra *et al.*, 2000).

2.5.2. Actinomycetes as a novel source of antimicrobials

The discovery of the first known antimicrobial produced by a *Streptomyces* spp., was streptothricin in 1942, two years after which streptomycin was discovered. Figure 2.4 shows the key findings and dates of antimicrobial agents produced by actinobacterial species, mainly *Streptomyces* spp. (Watve *et al.*, 2001; de Lima Procópio *et al.*, 2012), showing the diversity of classes of antimicrobials produced by this genus.

Gram-positive actinomycetes have been identified as the most prolific known producer of antimicrobials to-date, accounting for the bulk of SM production (Lam, 2006). Actinomycetes belong to the order *Actinomycetales,* they are predominantly soil bacteria which form filaments or branching hyphae and asexual spores for reproduction (Goodfellow, 1988). Actinomycetes, predominantly the genus *Streptomyces* [which contains more than 600 species (Parte, 2015)] with a high G+C content of approximately 57-75 % (Lo *et al.*, 2002) account for more than 70 % of known antimicrobials (Lam, 2006). Approximately 15 % of industrially important metabolites are also produced by the related members of actinomycetes namely: *Actinomadura*, *Actinoplanes*, *Micromonospora*, *Nocardia*, *Streptosporangium*, *Streptoverticullum* and *Thermoactinomycetes* (Okudoh, 2001). This is accomplished as actinomycetes transcribe more than 3300 genes in its circular genomes. Most of these genes encodes for SMs and complex differentiation of morphology (Hopwood *et al.*, 1985). Antimicrobial resistance and antimicrobial biosynthetic gene transfer among species within the various actinomycete genera is achieved by linear plasmids (large extra-chromosomal elements of DNA) (Kinashi, 1994).

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	Platensimycin[2006] *S. platensis*
	Daptomycin[2003] *S. roseosporus*
2000	Linezolid[2000] Synthetic
	Mupirocin[1985] *Pseudomonas fluorescens*
1970	Ribostamycin[1970] *S. ribosidificus*
	Fosfomycin[1969] *S. fradiae*
	Trimethoprim[1968] Synthetic
	Gentamicin[1963] *Micromonospora purpurea*
	Fusidic acid[1963] *Fusidium coccineum*
1960	Nalidixic acid[1962] Synthetic
	Tinidazole[1959] Synthetic
	Kanamycin[1957] *S. kanamyceticus*
	Rifamycin[1957] *Amycolatopsis mediterranei*
	Noviobiocin[1956] *S. niveus*
	Vancomycin[1956] *S. orientalis*
	Cycloserine[1955] *S. garyphalus*
	Lincomycin[1952] *S. lincolnensis*
	Erithromycin[1952] *Saccharopolyspora erythraea*
	Virginiamycin[1952] *in S. pristinaespiralis S. virginiae*
	Isoniazid[1951] Synthetic
	Viomycin[1951] *S. vinaceus e S. capreolus*
	Isoniazid[1951] Synthetic
	Viomycin[1951] *S. vinaceus e S. capreolus*
	Nystatin[1950] *S. noursei*
1950	Tetracycline[1950] *S. aureofaciens*
	Neomycin[1949] *S. fradiae*
	Chloramphenicol[1949] *S. venezuelae*
	Polymyxin[1947] *Bacillus polymyxa*
	Nitrofurantoin[1947] Synthetic
	Cephalosporins[1945] *S. clavuligerus*
	Bacitracin[1945] *Bacillus licheniformis*
	Cephalosporins[1945] *S. clavuligerus*
	Streptomycin[1944] *S. griseus*
1940	Penicillin[1941] *Penicillium chrysogenum*

Figure 2.4: Antimicrobial compounds discovered since 1941. Twenty-two of the 34 commercial compounds listed here have been and are currently produced by actinomycetes (de Lima Procópio *et al*., 2012).

Some classes of antimicrobial compounds discovered so far are presented in Figure 2.5, most of which are produced by members of the genus *Streptomyces* and are discussed in a greater detail below.
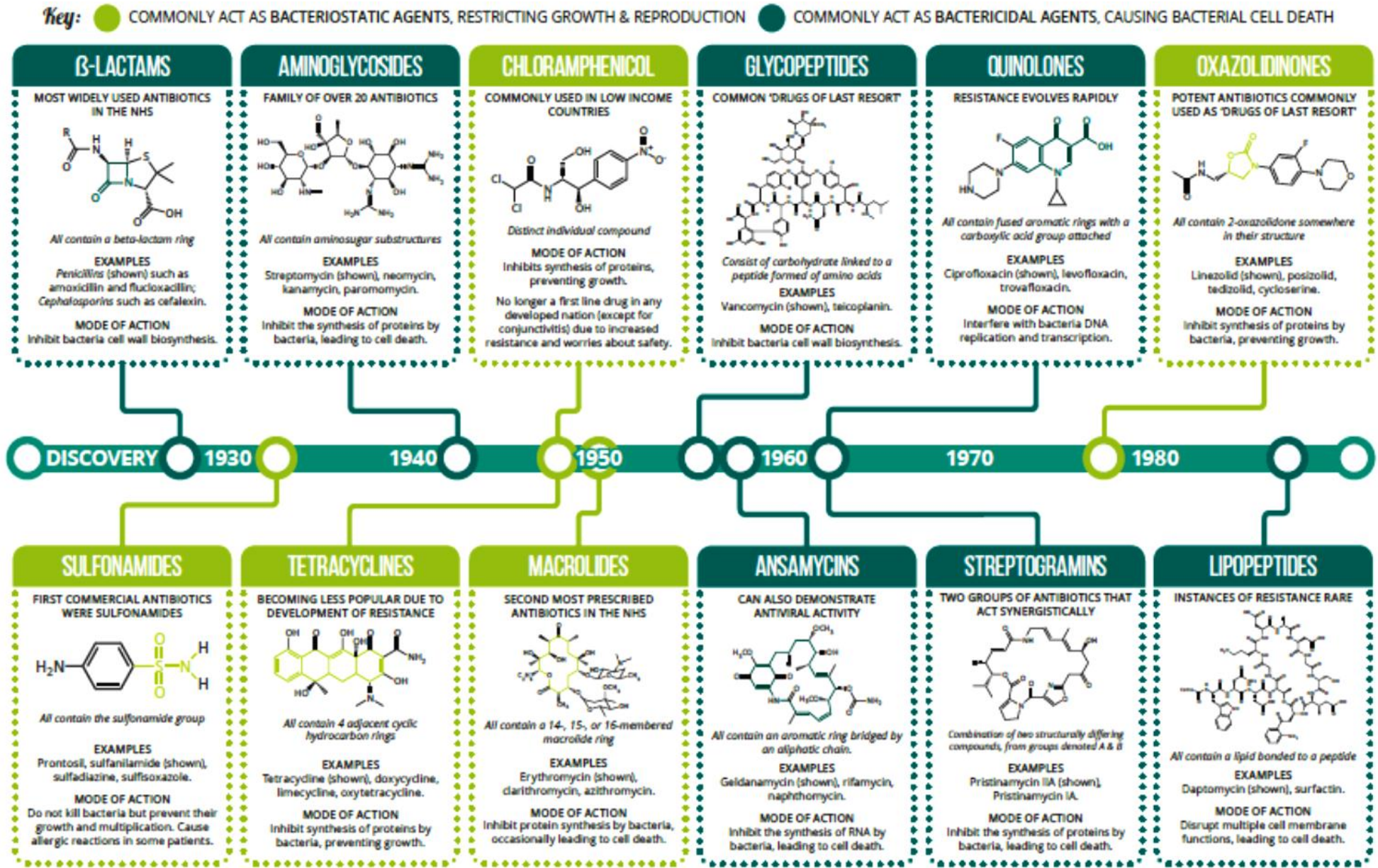
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Key: ● COMMONLY ACT AS BACTERIOSTATIC AGENTS, RESTRICTING GROWTH & REPRODUCTION ● COMMONLY ACT AS BACTERICIDAL AGENTS, CAUSING BACTERIAL CELL DEATH

ß-LACTAMS

MOST WIDELY USED ANTIBIOTICS IN THE NHS

All contain a beta-lactam ring

EXAMPLES
Penicillins (shown) such as amoxicillin and flucloxacillin; Cephalosporins such as cefalexin.

MODE OF ACTION
Inhibit bacteria cell wall biosynthesis.

AMINOGLYCOSIDES

FAMILY OF OVER 20 ANTIBIOTICS

All contain aminosugar substructures

EXAMPLES
Streptomycin (shown), neomycin, kanamycin, paromomycin.

MODE OF ACTION
Inhibit the synthesis of proteins by bacteria, leading to cell death.

CHLORAMPHENICOL

COMMONLY USED IN LOW INCOME COUNTRIES

Distinct individual compound

MODE OF ACTION
Inhibits synthesis of proteins, preventing growth.

No longer a first line drug in any developed nation (except for conjunctivitis) due to increased resistance and worries about safety.

GLYCOPEPTIDES

COMMON 'DRUGS OF LAST RESORT'

Consist of carbohydrate linked to a peptide formed of amino acids

EXAMPLES
Vancomycin (shown), teicoplanin.

MODE OF ACTION
Inhibit bacteria cell wall biosynthesis.

QUINOLONES

RESISTANCE EVOLVES RAPIDLY

All contain fused aromatic rings with a carboxylic acid group attached

EXAMPLES
Ciprofloxacin (shown), levofloxacin, trovafloxacin.

MODE OF ACTION
Interfere with bacteria DNA replication and transcription.

OXAZOLIDINONES

POTENT ANTIBIOTICS COMMONLY USED AS 'DRUGS OF LAST RESORT'

All contain 2-oxazolidone somewhere in their structure

EXAMPLES
Linezolid (shown), posizolid, tedizolid, cycloserine.

MODE OF ACTION
Inhibit synthesis of proteins by bacteria, preventing growth.

DISCOVERY — 1930 — 1940 — 1950 — 1960 — 1970 — 1980

SULFONAMIDES

FIRST COMMERCIAL ANTIBIOTICS WERE SULFONAMIDES

All contain the sulfonamide group

EXAMPLES
Prontosil, sulfanilamide (shown), sulfadiazine, sulfisoxazole.

MODE OF ACTION
Do not kill bacteria but prevent their growth and multiplication. Cause allergic reactions in some patients.

TETRACYCLINES

BECOMING LESS POPULAR DUE TO DEVELOPMENT OF RESISTANCE

All contain 4 adjacent cyclic hydrocarbon rings

EXAMPLES
Tetracycline (shown), doxycycline, limecycline, oxytetracycline.

MODE OF ACTION
Inhibit synthesis of proteins by bacteria, preventing growth.

MACROLIDES

SECOND MOST PRESCRIBED ANTIBIOTICS IN THE NHS

All contain a 14-, 15-, or 16-membered macrolide ring

EXAMPLES
Erythromycin (shown), clarithromycin, azithromycin.

MODE OF ACTION
Inhibit protein synthesis by bacteria, occasionally leading to cell death.

ANSAMYCINS

CAN ALSO DEMONSTRATE ANTIVIRAL ACTIVITY

All contain an aromatic ring bridged by an aliphatic chain.

EXAMPLES
Geldanamycin (shown), rifamycin, naphthomycin.

MODE OF ACTION
Inhibit the synthesis of RNA by bacteria, leading to cell death.

STREPTOGRAMINS

TWO GROUPS OF ANTIBIOTICS THAT ACT SYNERGISTICALLY

Combination of two structurally differing compounds, from groups denoted A & B

EXAMPLES
Pristinamycin IIA (shown), Pristinamycin IA.

MODE OF ACTION
Inhibit the synthesis of proteins by bacteria, leading to cell death.

LIPOPEPTIDES

INSTANCES OF RESISTANCE RARE

All contain a lipid bonded to a peptide

EXAMPLES
Daptomycin (shown), surfactin.

MODE OF ACTION
Disrupt multiple cell membrane functions, leading to cell death.

Figure 2.5: An overview of various antimicrobial classes produced by *Streptomyces* spp. http://www.compoundchem.com.

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2.5.3. Antimicrobials produced by actinomycetes

β-lactams

β-lactam-type SMs consist of penicillins such as flucloxacillin and amoxicillin, and cephalosporins such as cephalexin (Figure 2.6). β-lactams consist of a β-lactam ring structure – penicillin's ring structure contain a fused thiazolindine ring and is termed 6-aminopenicillanic acid. The spectrum of activity of penicillins is dependent on the amide-bonded side chains which is different for each common backbone it is connected to (Batchelor *et al.*, 1965). Penicillin's mechanisms of action is via the inhibition of the synthesis of important structural components of the cell wall of the target bacterium (such as peptidoglycan – which is absent in mammalian cells).

Penicillins are considered the most efficacious and safest class of antibiotics for the use against bacterial infections due to the metabolism of the host cell being unaffected. Penicillins exhibit poor antibacterial activity against aerobic rod-shaped Gram-negative microbes, however they have a broad spectrum of activities against Gram-positive microorganisms. Penicillins are widely used to treat infections caused by susceptible staphylococci, streptococci, *Neisseria*, clostridia and *Pasteurella multocida* (Park and Strominger, 1957). *Streptomyces lavendulae* has been reported as a producer of high levels of penicillin V acylase (Torres *et al.*, 1999). Cephalosporins also contain a β-lactam ring consisting of a fused 6-membered dihydrothiazine ring. The modes of action of cephalosporins are by interference of the synthesis of the bacterial cell wall. Cephalosporins are commonly used to treat infections caused by Gram-positive and Gram-negative bacteria such as *Proteus mirabilis, Klebsiella* spp. and *E. coli*. Cephalosporins have been reported to be produced by *Streptomyces clavuligeris* (Rius and Demain, 1997; Xiao *et al.*, 1991; Depestel *et al.*, 2003).
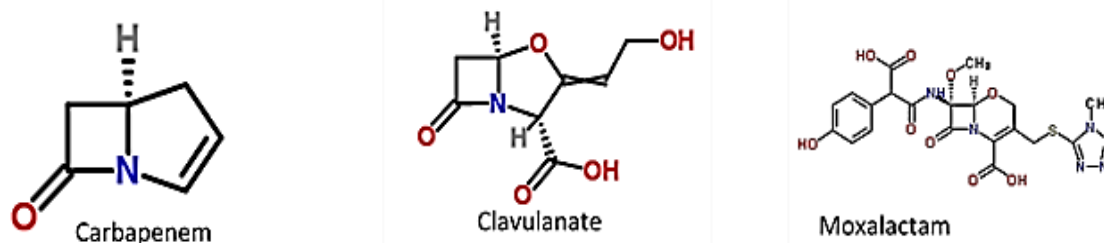
Figure 2.6: Examples of β-lactam type antibiotics (Williams, 2015).

Aminoglycosides

Aminoglycosides are comprised of a dibasic cyclitol with one or more aminated sugars attached to it via glycosidic linkages (Figure 2.7) (Mingeot-Leclercq *et al.,* 1999). Aminoglycosides' mechanism of action is via binding directly to the ribosomal RNA causing translocation inhibition and a decrease translation accuracy of the ribosome (Davies *et al*., 1965; Davies and Davis, 1968). In Gram-negative bacteria, aminoglycosides cause protein synthesis inhibition which results in bactericidal activity which is concentration-dependent (Bryan and van der Elzen, 1977). Aminoglycosides are typically isolated from *Streptomyces* spp. such as *Streptomyces kanamyceticus, Streptomyces tenjimariensis* and *Streptomyces spectabilis*, and some *Micromonospora* spp. When isolated from *Streptomyces* spp. the aminoglycosides name ends with the suffix – "mycin", whereas aminoglycosides derived from *Micromonospora* spp. have names ending with the suffix – "micin" (Benveniste and Davies, 1973).
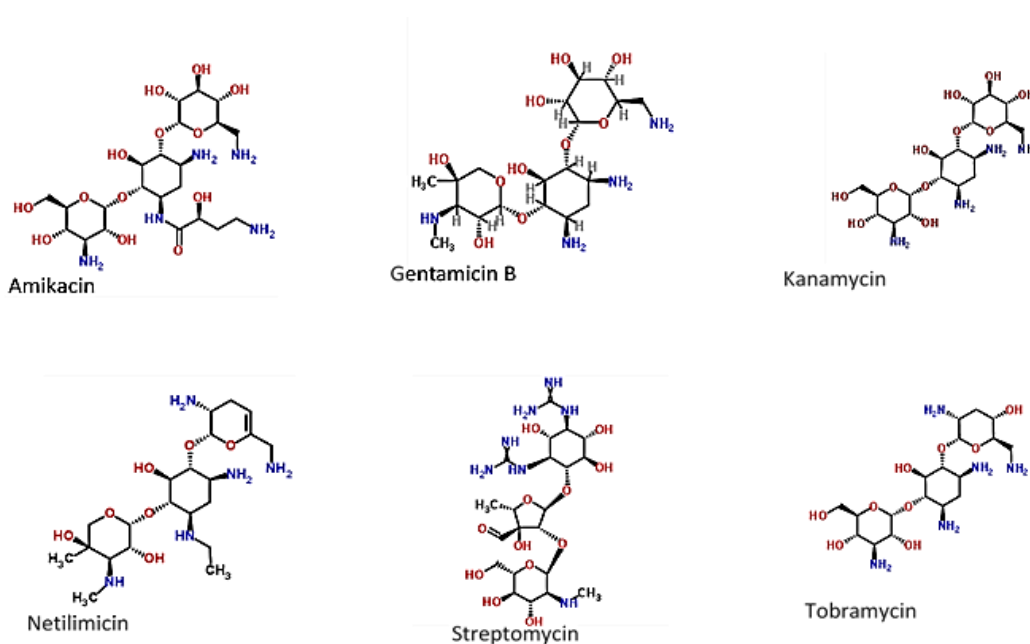
Figure 2.7: Examples of aminoglycoside antibiotics (Williams, 2015).

Quinolones

Quinolones are heterocyclic in structure consisting of a bicyclic core which aids in the inhibition of bacterial topoisomerase (Figure 2.8) (Hardman *et al*., 1996). Depending on the concentration of this class of antibiotics, quinolones exhibits bactericidal activity. Quinolones target the topoisomerase IV and DNA gyrase of a bacterium – these enzymes are important for

transcription and DNA replication, respectively. Limited activity was exhibited by early quinolones, such as nalidixic acid, and was mainly used for urinary tract infections caused by Gram-negative bacteria. Next generation quinolones – the fluoroquinolones (ofloxacin, norfloxacin, ciprofloxacin, enoxacin and lomefloxacin) show wide range activity against other Gram-negative bacteria. Enhanced activities against a number of Gram-positive and Gram-negative microorganisms were displayed by newer fluoroquinolones such as trovafloxacin, levofloxacin, sparfloxacin and grepafloxacin. Quinolones show potent activity against *Helicobacter pylori* and have been reportedly isolated from an actinomycete strain, a *Pseudonocardia* sp. (Smith, 1986; Dekker *et al.*, 1998; Siegmund *et al.*, 2005).
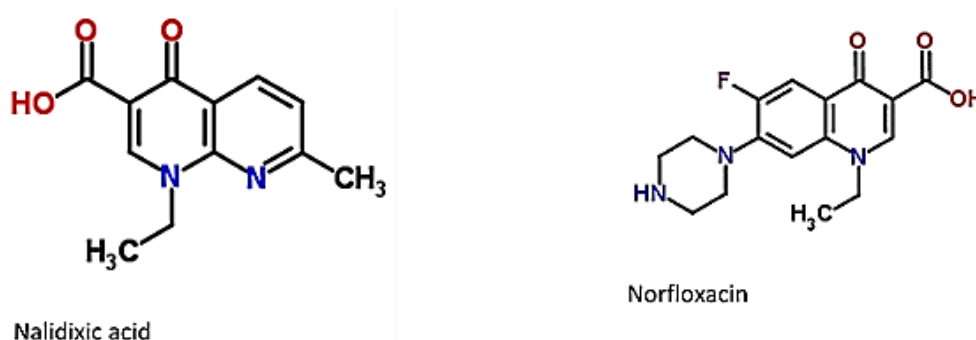
Figure 2.8: Structures of quinolone type antibiotics (Williams, 2015).

Tetracyclines

Tetracyclines are comprised of four (tetra) hydrocarbon rings (cycle) and consist of a linear fused tetracyclic nucleus whereby a number of functional groups attach (Figure 2.9). Tetracyclines act by inhibiting protein synthesis in cells by blocking the aminoacyl-tRNA from attaching itself to the ribosomal acceptor (A) site. Tetracyclines were first discovered in 1945 by Benjamin Duggar from the producing strain *Streptomyces aureofaciens* and has a broad spectrum activity against Gram-positive and Gram-negative organisms associated with especially rickettsiae, mycoplasms and chlamydiae (Darken *et al.*, 1960; Blackwood *et al.*, 1963; Chopra and Roberts, 2001).

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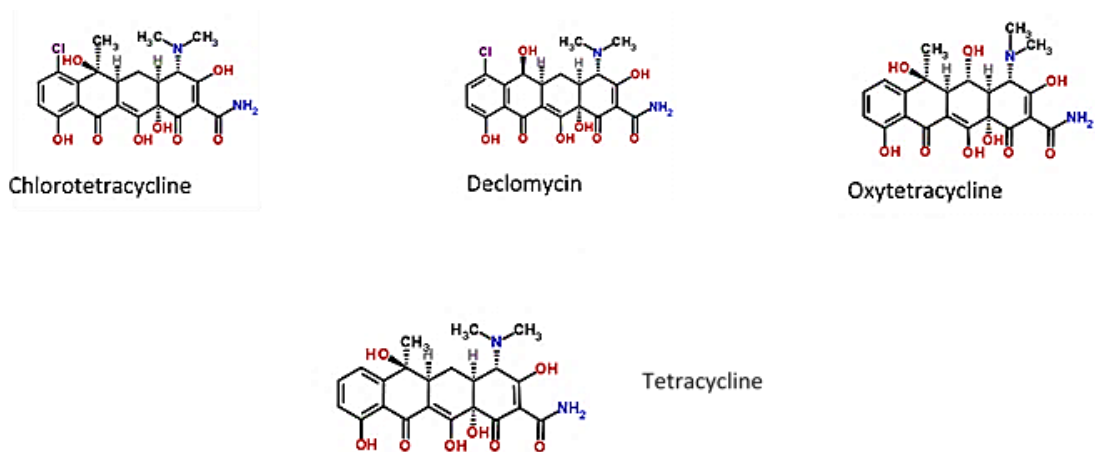
Figure 2.9: Examples of tetracycline antibiotics (Williams, 2015).

Macrolides

Macrolides are easily recognised by their large lactone ring which contains 12-16 atoms linked via glycosidic bonds (Figure 2.10) (Mazzei *et al*., 1993). Macrolides are typically used in the treatment of infections of the skin and soft tissue, genital tract and respiratory tract caused by Gram-positive microorganisms, some Gram-negative microorganisms, anaerobic bacteria and mycoplasma. Macrolides act by inhibiting the protein synthesis on ribosomes in bacteria. They are produced by a number of *Streptomyces* spp., particularly *Streptomyces venezuelae* (Xue *et al*., 1998) and are commonly used as a replacement antibiotic for penicillin in penicillin-sensitive patients (Brisson-Noel *et al*., 1988). Some commonly used macrolides are azithromycin, erythromycin and its related agents, dirithromycin, clarithromycin, flurithromycin, roxithromycin, josamycin, kitasamycin, rokitamycin, mirosamycin, mycinamycin, oleandomycin, spiramycin, tylosin and rosaramicin (Alvarez-Elcoro and Enzler, 1999).
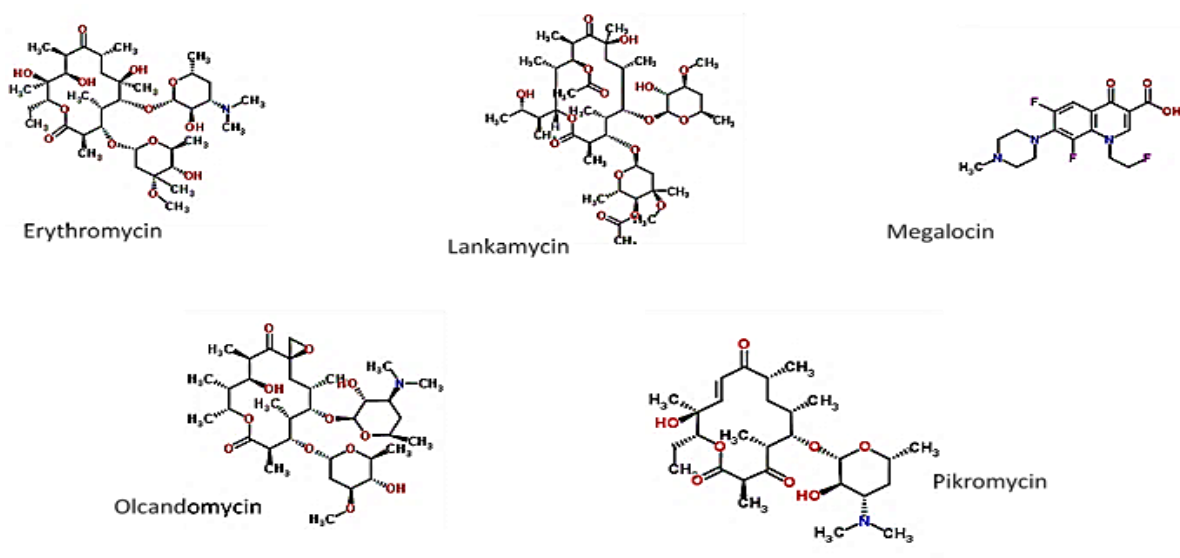
Figure 2.10: Examples of structures of macrolide antibiotics (Williams, 2015).

Another class of natural products produced by *Streptomyces* spp. not mentioned in Figure 2.5 are the polyketides.

Polyketides

Polyketide synthases (PKS) are enzymes involved in the production of peptide polyketide (PK) antibiotics. PKs are commonly biosynthesised in an assembly line fashion from acyl–coenzyme A (CoA) monomers and amino (also termed hydroxyl) acids.

Bacterial derived PKs are subdivided into two major pathways namely: type I PKS – which consist of large multifunctional proteins with each active site capable of catalysing one cycle of PK modification and elongation; and type II PKSs which are very similar to non-ribosomal peptide synthetase (NRPS) pathways in terms of their assembly. However, Type II PKSs contain an active site which is situated on either mono- or bi-functional proteins. Genes clustered in large operons of approximately 150 kb code for large enzymatic complexes. These operons code for biosynthetic genes as well as other genes such as regulators, export systems and mechanisms of resistance (Hertweck *et al.*, 2007). Type III PKSs are commonly found in plants, but several fungi and bacteria have also been found to possess type III PKSs. Type III PKS is made up of one multi-modular protein for the synthesis of molecules; this type of polyketide is not known for exceptional bio-activity and will not be further discussed in this review (Moore *et al.*, 2002).

PKs represent the largest family of natural products; pathways are ubiquitous among actinobacteria and fungi, but are also found in higher animals and plants. PK compounds contribute a high economic importance, one example of this is in the case of doxorubicin (Adriamycin), an anthracycline antibiotic used as an anti-tumour drug, which has generated well over 156 million dollars in 1993 (Dickens, 1996).

Modules within each PKS consist of two or more core domains (Figure 2.11). These domains are namely: acyltransferase (AT) domain, which transfer the appropriate extender unit to the domain called an acyl carrier protein (ACP), where the formation of a thioester bond occurs to fix the extending polyketide to the synthase and a domain called ketosynthase (KS). The KS domain catalyses a condensation reaction which occurs between the extender unit situated on the ACP domain of the module of interest, and the intermediate polyketide, which is bound to the ACP domain of the module preceding it (Meurer, 1997). The growing polyketide molecule is modified with the addition of secondary domains namely dehydratase (DH), enoylreductase

(ER), ketoreductase (KR), methylation (M) and methyltransferase (MT) domains (Meurer, 1997). A cyclase (Cy) domain is often featured in the type II PKS molecule which commonly leads to aromatic structure formation (Meurer, 1997). The final module consists of a thioesterase (TE) domain which catalyses the termination and release of the final product from the enzyme (Meurer, 1997).
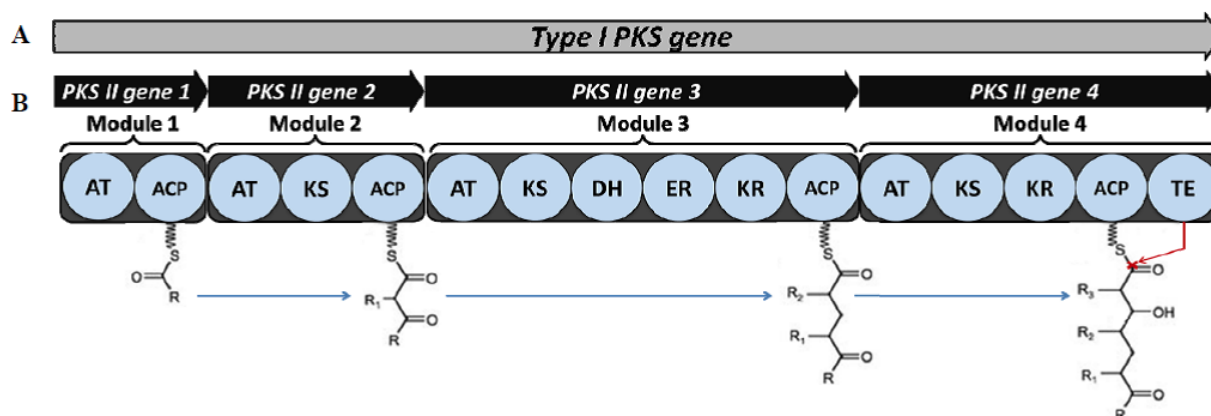
Figure 2.11: A hypothetical polyketide synthase consisting of four distinct modules, represented schematically. A) PKS composed of one multi-modular protein which is encoded by a gene made up of the four enzymatic modules, this is characteristic of type I PKS. B) Type II PKS has each module situated on a singular monofunctional protein. Key: Acyltransferase (AT), acyl carrier protein (ACP), ketosynthase (KS), dehydratase (DH), ketoreductase (KR) and thioesterase (TE)

Interestingly, the gene cluster for hedamycin produced by Streptomyces griseoruber represents an organisation of module domains. Hedamycin, an antitumor polyketide, is assembled by type I / type II hybrid PKS and causes intercalation between DNA nucleotides. Upon characterisation of this anti-tumour agent it was seen that the gene cluster within hedamycin contains two type I and six type II genes (Billign *et al*., 2004), showing the versatility of polyketides as antimicrobials. This versatility may be further exhibited by their diversity in terms of biological activities and structures. The antimicrobial, erythromycin for example, inhibits the synthesis of proteins by binding to the 50S subunit situated on the bacterial 70S RNA complex (Brisson-Noel *et al*., 1988). The insecticidal and anti-parasitic drug avermectin's inhibitory mechanism is caused by the selective binding to glutamate-gated chloride ion channels in nerve cells and muscle in vertebrates. This in effect increases the permeability of the cell membrane to chloride ions and as a result the cell becomes hyperpolarised which leads to paralysis and death of the parasite (Shoop *et al*., 1995). The antifungal, nystatin, causes inability of the cell

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membrane to function as a selective barrier when bound to sterols found in the cell membrane, resulting in the loss of essential cellular components (Rècamier *et al.*, 2010).

Even though there has been much success with the discovery and advances in the production of antimicrobials, diseases caused by pathogenic bacteria still remain one of the leading causes of death worldwide. Seventeen million annual deaths are as a result of bacterial infections which mainly affect the elderly and children (Watve *et al.*, 2001). This is due to failure in treatment options when first- and second-line antibiotics cannot treat the infection caused by pathogenic bacteria as a result of resistance built up by the bacterium or due to the unavailability of medications particularly in rural areas worldwide. Consequently, more toxic antibiotics are used to treat resistant bacteria which frequently is less effective and often more expensive. The effective treatment and prevention of an ever-increasing spectrum of infections caused by bacteria, viruses, fungi and parasites are thus threatened by antimicrobial resistance (AMR) (CDC, 2013).

2.5.4. Production of Antimicrobials

SMs produced by actinomycetes depend on a number of biochemical, physical and environmental factors (Table 2.5). Components added to media which may elicit or enhance the production of a wide array of SMs is not known. However, there are indications that additives that limit the release of nutrients that can be easily assimilated, promote the induction of novel SMs by the entrapment of inhibitory compounds. Compounds such as magnesium phosphate, which is a natural zeolite, can be included as these compounds form complexes with ammonium salts (Shapiro, 1989; Masuma *et al.*, 1983). Similarly, research has shown that organic sources of nitrogen such as soya-based fermentation media can be used in the induction of certain classes of SMs (Hessler *et al.*, 1997; Gouveia *et al.*, 1999).

Table 2.5: Factors affecting the production of secondary metabolites in actinomycetes

Media Composition	Fermentation Conditions
Carbon source	Agitation
Inducers	Carbon dioxide
Inhibitors	Fermentation vessel
Inorganic phosphates	Ionic strength
Inorganic salts	Osmotic pressure
Nitrogen source	Oxidation-reduction potential (E_h)
Precursors	Oxygen-transfer
Trace salts or metals	pH
	Temperature
	Rheology

a)   Effect of Fermentation Media Components on Secondary Metabolite Production

Species-specific variation is displayed in actinomycetes when exposed to carbon sources; this affects the cell growth as well as secondary metabolism (Dekleva *et al*., 1985; Demain, 1989; Platas *et al*., 1999). Certain carbon sources may cause negative repressive effects on the production of SMs, mainly due to repression of gene expression typically associated with carbon catabolites. Carbon sources which are rapidly assimilated (e.g. glucose) are good components for cell growth, but may also repress the production of essential enzymes involved in the biosynthesis of SMs. Oils (e.g. methyloleate, soybean), oligosaccharides (e.g. lactose), polyalcohols (e.g. glycerol), and polysaccharides (e.g. starch), are commonly used in combination as non-repressing carbon sources (Trilli, 1990; Demain and Fang, 1995). These carbon sources are used as it is more slowly released by hydrolysis (Demain and Fang, 1995).

Fermentations for the production of SMs commonly include organic nitrogen sources, as these compounds are easily broken down into small units for the ease of transportation into bacterial cells (e.g. ammonia (NH_4^+) and amino acids) (Dunn, 1985). The NH_4^+ ion is favoured as an inorganic nitrogen source in actinomycete fermentations; these sources of ammonia are added as ammonium chloride (NH_4Cl) or ammonium sulphate ($(NH_4)_2SO_4$) (Dunn, 1985). However, excessive amounts of easily assimilated nitrogen sources causes repression of the formation of SMs, and is generally caused by amino acids and ammonium salts (Ōmura and Tanaka, 1984). This problem may be alleviated by using complex nitrogen sources such as soyabean meal or peptones (Bhattacharyya *et al*., 1998; Demain and Fang, 1995).

The regulation of inorganic phosphate (PO_4^{3-}) is known to influence the production of numerous SMs produced by actinomycetes. However, the biosynthesis of SMs in liquid media are inhibited or repressed by phosphate concentrations above 1mM (Martin, 1989). In some cases, excess phosphate and glucose act in a synergistic manner causing the repression of SM biosynthesis.

The quantity and types of SMs produced by actinomycetes are influenced by micronutrients/trace elements (Weinberg, 1989). Although, trace elements are essential for biological activities to occur within the cell, excess amounts of these micronutrients may be toxic and elements such as manganese (Mn^{2+}) and chromium (Cr^{2+}) in high concentrations can cause an inhibitory effect against the production of SMs, thus a small range of the amounts of micronutrients is beneficial for SM production (Weinberg, 1989).

b)   Effect of Fermentation Conditions on Secondary Metabolite Production

Shifts in temperature

The expression of biosynthetic genes involved in secondary metabolism and the rate of decay of SM products are influenced by temperature (Votruba and Vaněk, 1989). The temperature at which SMs are produced is independent of the temperature at which the microorganism grows. Typically, the range associated with optimal growth temperature can be as wide as 20°C whereas the optimal temperature for the production of SMs may be as narrow as 5-10°C (Iwai and Ōmura, 1982). Take for instance, streptomycin produced by *Streptomyces griseus*, an increase in temperature of 1°C at the threshold temperature produces a reduction of antibacterial agent production by 80% (Dunn, 1985).

The effects of pH

The rate of growth, mycelium morphology and SM production by actinomycetes are also affected by the pH of the medium (Braun and Vecht-Lifshitz, 1991). Actinomycetes have a wide pH optima range for growth, whereas SM production is influenced by high pH and can only tolerate a narrow pH range within 0.2 pH units (James *et al*., 1991; Chen *et al*., 1999). According to Hayakawa *et al*., (1995), the increase in pH value from pH 5.5 to pH 7.5 increased the antimicrobial activity of some *Microbispora* species, a relatively rare actinomycetes genus.

2.6.   Developing novel antimicrobials

As a result of poor antimicrobial stewardship and the increase in antimicrobial resistance due to the natural process of evolution by pathogenic bacteria or opportunistically infectious bacteria – new antimicrobials with new mechanisms of action are required to keep up with resistant microorganisms, along with new diagnostic tests to track the development of resistance. The number of new antibacterial agents from the year 1980 – 2012 that has been developed and approved for clinical and agricultural use has decreased steadily over the past three decades, providing fewer options in the treatment of resistant bacteria (Figure 2.12).
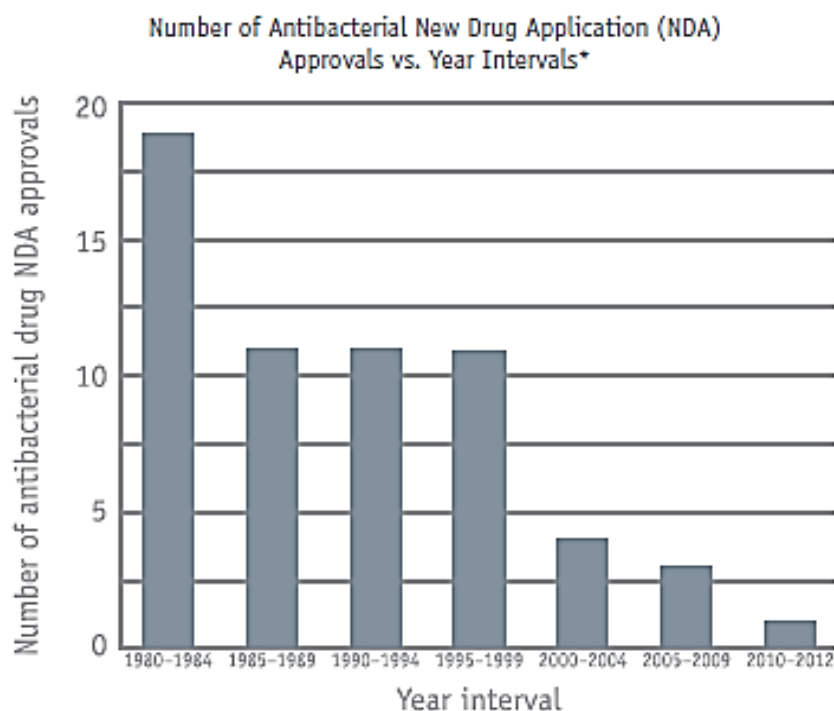
Figure 2.12: *Intervals from 1980–2009 are 5-year intervals; 2010–2012 is a 3-year interval. Drugs are limited to systemic agents. Data courtesy of FDA's Center for Drug Evaluation and Research (CDER).

The most successful products to date, have been produced by soil-dwelling actinomycetes. The most famous example involved the collection of soil from a golf course in Tokyo in the early 1970s and the isolation of the avermectin producing strain, Stretomyces avermitilis by professor Satoshi Ōmura of the Kitasato institute. Other examples include spinosad produced by Saccharopolyspora spinosa isolated from soil in a defunct sugar mill rum still in the Virgin Islands (Waldron *et al.*, 2001); and erythromycin, produced by Saccharopolyspora erythraea in 1949 in Turkey (Eisenstein *et al.*, 2010).

In the first 30 years (1940s – 1970s) of antimicrobial discovery, the Waksman group at Rutgers developed systematic screening of actinomycetes which led to the discovery of streptothricin, actinomycin and, most notably, streptomycin. Due to their discoveries, pharmaceutical companies began screening extracts of fungi and actinomycetes for compounds with bioactivities primarily against pathogenic bacteria. This process involved the isolation of microorganisms of interest, fermentation, isolation of prodct and then testing of the fermentation broths and purified compounds against various test organisms using phenotypic screening in terms of bioassays (Katz and Baltz, 2015). Although this approach has led to finding a number of novel medicinally important metabolites, this approach is often very taxing due to

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its high rate for rediscovery of natural products that are already known (Newman and Cragg, 2007).

New approaches are required to accelerate the discovery of novel antimicrobial agents to avoid reverting to a pre-antibiotic era. These strategies are presented in Table 2.6. According to Mian and Davies (2010), a collective study of the genomics, microbiology, biochemistry, genetics and modern natural product biochemistry will be required to find novel therapeutic agents in the next decade. Baltz (2006) also suggested that whole cell screening of the natural product be revisited but in a miniaturized version in order to increase throughput over conventional screening.

The discovery of novel microbial natural products is costly, and currently, commercial attention has turned towards reducing expenses by the screening of novel groups of actinomycetes or by re-screening of culture collections which already exists (Kurtböke, 2012).

Table 2.6: Approaches to accelerate the discovery of novel antimicrobial agents from actinomycetes (Baltz, 2006)

High-throughput whole cell screening of terrestrial microorganisms
Global soil sampling
Pooling of soils and extraction of spores
Miniaturized fermentations starting with spores
Screening organisms resistant to common antibiotics
Improved throughput with automation
Enrichments and selections for uncommon terrestrial and marine microbes
Antibiotics and taxon selective media
Untapped random and exotic soils
Untapped marine sediments
Genome mining
Sequencing multiple common microbes
Sequencing rare and slow growing microbes
Expression of new pathways in robust bacterial hosts
Combinatorial biosynthesis
NRPS pathways
PKS pathways
Glycosylations and other modifications

In the current study, genome mining will be employed to explore the genome of a slow growing microbe – *Streptomyces pharetrae* CZA14[T]. *S. pharetrae* CZA14[T] was identified as a source of novel metabolites and forms part of the culture collection of the Biocatalysis and Technical Biology Research Group, CPUT, Western Cape Province, South Africa. Strain CZA14[T] was isolated from soil collected from the base of a giant quiver tree (Aloe pillansii) situated in the

Karoo Desert National Botanical Gardens in Worcester, Western Cape Province in South Africa (le Roes and Meyers, 2005). It was identified as a member of the genus Streptomyces by 16S rRNA gene sequencing as well as chemotaxonomic techniques. This strain was found to represent the type strain of a new species for which the name S. pharetrae was proposed: CZA14^T (=DSM 41856^T = NRRL 24333^T) (le Roes and Meyers, 2005). Standard screening against various test strains for antibacterial activity showed weak antibiosis against Mycobacterium aurum A+, Bacillus coagulans ATCC 7050T and Acinetobacter calcoaceticus C91 (le Roes and Meyers, 2005). *S. pharetrae* CZA14^T is a Gram-positive, aerobic actinomycete, capable of producing spores and melanin, which is associated with secondary metabolite activity (le Roes and Meyers, 2005).

The genome of this actinomycete was previously sequenced; and the biosynthetic gene clusters encoding for secondary metabolites were detected using a bioinformatics programme called antiSMASH. Some of these genes were expressed during fermentation experiments performed during the course of this study. PKS pathways in particular and bio-active compounds were tested against multi-drug resistant organisms. The outcomes of these experiments are presented in chapters 3-6.

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CHAPTER 3:

Optimisation of medium to larger scale production (0.5 L – 5 L) of secondary metabolites produced by strain CZA14^T
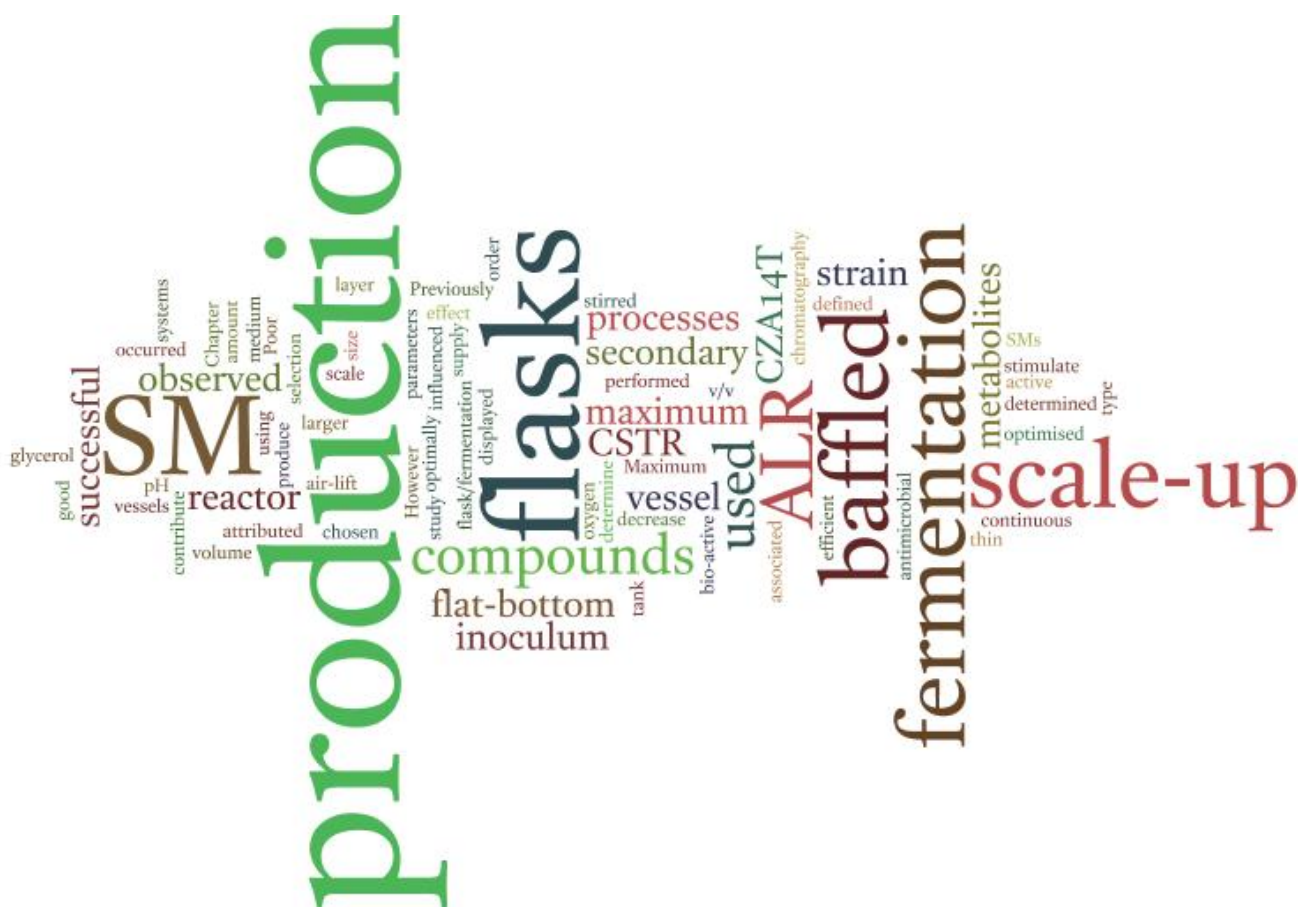
Abstract

The production of secondary metabolites is influenced by the fermentation vessel and fermentation parameters used during production. A successful scale-up of fermentation processes is associated with a good selection of fermentation systems. In this study, flat-bottom flasks, baffled flasks, a continuous stirred tank reactor (CSTR) and an airlift reactor (ALR) were used to determine the effect of flask/fermentation vessel type on the production of secondary metabolites (SMs) by strain CZA14[T] and to contribute to a successful scale-up of SM production. Previously defined antimicrobial production medium (153 + glycerol, pH 6.0) was used for the production of bio-active compounds by strain CZA14[T]. In this study, poor SM production was displayed in flat-bottom flasks (as determined by thin layer chromatography) and further scale-up processes were performed using baffled flasks. The inoculum volume was optimised in order to stimulate maximum SM production at a larger scale. A 15% (v/v) inoculum size was selected to optimally produce maximum amount of SM compounds during scale-up. Maximum SM production was observed in 2 L baffled flasks over the 1 L baffled flasks. A decrease in SM production occurred upon scale-up from 2 L baffled flasks to CSTR (5 L) and ALR (3 L).

Key words: Fermentation vessels, Flat-bottom flasks, baffled flasks, CSTR, ALR

Introduction

Streptomyces species are Gram-positive, mostly filamentous soil bacteria which grow as vegetative mycelia with branching aerial hyphae that emerge from the surface of the colony (Madigan *et al.*, 2000). Clinically important antimicrobials are commonly produced by streptomycetes and as a result, several studies are directed toward the isolation of new *Streptomyces* species from various habitats (Mansour, 2003; Chater, 2006; Bon *et al.*, 2008). The production of antimicrobials can be enhanced or initiated by manipulating growth/culture conditions (Omura and Tanaka, 1984). To fully understand the biosynthetic potential of micro-organisms of interest, solid-state/substrate and shaken-flask/submerged fermentations are commonly performed in parallel. Submerged fermentation was the focus of the current study.

Fermentation under submerged conditions aims to offer a homogenous environment with specific stress factors and limitations. Initial fermentation systems and conditions are selected to fulfil the requirements of a particular ecological or taxonomic group of micro-organisms, which in the current study was an actinomycete. Besides considering the suitable processes for a specific organism group, it is also essential to consider whether the fermentation process and production of antimicrobials can be replicated, as well as the subsequent ease of scale-up by number or volume. In order to optimise the probability that fermentation at a larger-scale can be repeated, the initial fermentation process parameters are maintained. A set of standard parameters are defined for small-scale fermentations in order to achieve successful re-fermentation and is further translated into a set of standard process parameters for the scale-up to medium and larger scale fermentation (Whitaker, 1992).

In the current study, various concentrations of carbon and nitrogen sources were supplemented into culture media for optimal antimicrobial production. Angular baffled flasks were used to provide effective mixing of fermentation broth over a range of viscosities, while simultaneously limiting the splashing of the broth that promotes wall growth in the flask (Katzer *et al.*, 2001). In a study by Katzer *et al.* (2001), similar culture morphology in terms of shape and size of pellets in shake-flask fermentation was observed compared to fermentation in stirred vessels. This may aid in the high success rate for re-fermentation under submerged conditions, which is generally 80%. In terms of other bioreactor vessels such as the airlift bioreactor used in the current study, aeration plays a major role in the production of high yields

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of secondary metabolites. Adequate oxygen supply is a major problem when using shake flasks (Buchs, 2001). Shake flasks with angular baffles or indents are used for actinomycetes, as these indentations help in increasing oxygen transfer. The oxygen supply in stirred tank reactors are mostly better than baffled shake flasks, but mechanical shearing may take place in stirred vessels due to the impellers fitted inside of the stirred vessel which in turn may affect antimicrobial production. The airlift bioreactor was used to overcome the aforementioned limitations (Merchuk. 2002). An insignificant difference between shake-flask fermentation and fermentation in stirred-vessels upon scale-up suggests comparability of process conditions in these fermentation vessels, while results may vary in other bioreactor types. In the current chapter, secondary metabolite (SM) production by a *Streptomyces* species was scaled up from fermentation in 0.5 L baffled flasks to 3 L air lift reactor and 5 L continuous stirred tank reactor volumes (Figure 3.1).

Optimisation of the effect of aeration on the production of secondary metabolites (SMs) by strain CZA14[T] was performed using various fermentation vessels. The scale of SM production was gradually increased with each experiment in different fermentation vessels to test if any product loss would occur upon scale-up. SM production was performed in 2L baffled flasks, an airlift reactor and a continuous stirred tank reactor. Compounds were extracted from the fermentation broth from each vessel type using ethyl acetate and visualised as bands under short-wave (265 nm) and long-wave (365 nm) ultraviolet light (Figure 3.1).
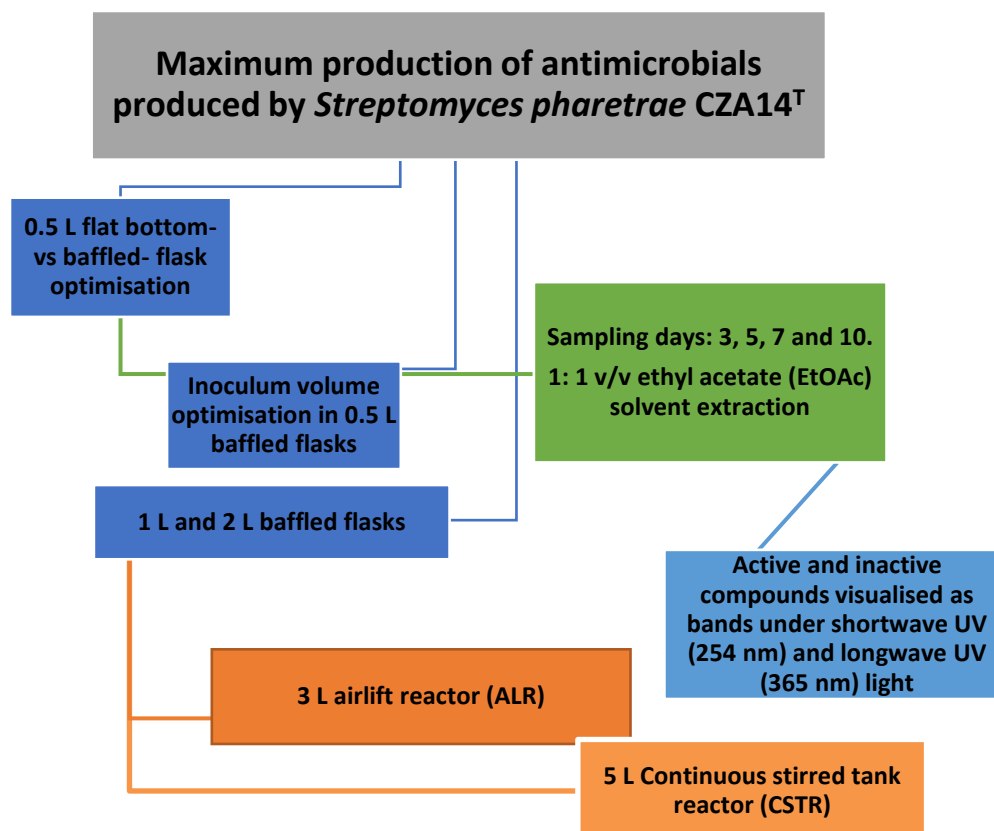
Figure 3.1: Flow-chart of methods used for the maximum production of antimicrobials produced by *Streptomyces pharetrae* CZA14[T].

Materials and Methods

3.1. Materials

Most of the chemicals used in this study were purchased from Merck Millipore or Sigma-Aldrich unless otherwise stated. All solvents used were of analytical grade.

3.2. Methods

3.2.1. Maintenance of *Streptomyces pharetrae* CZA14[T]

S. pharetrae CZA14[T] previously isolated by le Roes and Meyers (2005), was stored as spore and mycelial suspension stock cultures in 20% (v/v) glycerol at -20°C and the strain was maintained on International *Streptomyces* Project (ISP) #2 agar (g/L): 4 glucose, 10 malt extract, 4 yeast extract and 20 bacteriological agar, pH 7.2 (Shirling and Gottlieb, 1966).

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3.2.2. Preparation of Pre-Cultures

Strain CZA14[T] was cultured on ISP #2 agar (refer to section 3.2.1) and incubated at 30°C for 7–12 days (Figure 3.2). Approximately 2-3 loopfulls of spores were inoculated into 5 mL sterile deionised water (dH₂O) to produce a spore suspension and 500 μL spore suspension per 10 mL medium was used to inoculate liquid ISP #2 medium. Inoculated liquid medium was incubated at 30°C, shaking at 160 rpm on a Labcon orbital shaker (Advanced African Technology) for approximately 3-4 days before it was used to inoculate experimental flasks.

Figure 3.2: *Streptomyces pharetrae* CZA14[T] on ISP #2 agar after 14 days of incubation at 30 °C.

3.2.3. Medium-scale Secondary Metabolite Production

3.2.3.1. Flat-bottom flask VS baffled flask optimisation in 0.5 L flask volume

SM production volume was scaled-up from 50 mL to 100 mL small-scale media volume in 0.5 L flat-bottom and baffled flasks (20% [v/v] media volume/flask volume; 5% [v/v] inoculum volume/media volume). Previously determined optimal production medium 153 + glycerol (g/L: 20 glycerol, 5 peptone, 5 meat extract, 3 CaCO₃, pH 6.0; unpublished results: Durrell, 2013) was used throughout the duration of this study. Pre-cultures were prepared as described in section 3.2.2 and were used to inoculate experimental flasks (in duplicate) containing sterile production medium. Un-inoculated control flasks (in duplicate) were prepared, and both controls and inoculated experimental flasks were placed at optimal submerged fermentation conditions (30°C, 160 rpm). Fermentation was conducted over a period of 10 days and 5 mL samples were collected at days 3, 5, 7 and 10. Solvent extraction was performed as described in section 3.2.5 and thin layer chromatography (TLC) (see section 3.2.6) was used to determine which flask type was suitable for maximum SM production.

3.2.3.2. Optimisation of inoculum volume in 0.5 L baffled flasks

It was determined that baffled flasks were best suited for maximum SM production by strain CZA14^T and these flasks were further used in optimisation studies. Various inoculum volumes were tested to determine optimal inoculum volume for maximum SM production by strain CZA14^T, medium volume was kept constant (20% [v/v]) while the inoculum volume varied by 5%, 10%, 15% and 20% [v/v]. Pre-cultures were prepared as in section 3.2.2 and were inoculated into the main experimental flasks (in duplicate) at varying inoculum volumes as indicated above. Un-inoculated control flasks (in duplicate) were prepared, and both controls and inoculated experimental flasks were placed at optimal submerged fermentation conditions (30°C, 160 rpm). Fermentation was conducted over a period of 10 days and 5 mL samples were collected at days 3, 5, 7 and 10. Solvent extraction was performed as described in section 3.2.5 and TLC (section 3.2.6) was used to determine optimal inoculum volume for maximum SM production.

3.2.3.3. 1 L and 2 L baffled flask optimisation

SM production was further scaled-up from 100 mL to 200 mL and 400 mL media volume in 1 L and 2 L baffled flasks, respectively (20%, media volume; 15%, inoculum volume) (Figure 3.3). Pre-cultures were prepared as described in section 3.2.2 and were used to inoculate experimental flasks (in duplicate) containing sterile optimal production medium. Un-inoculated control flasks (in duplicate) were prepared, and both controls and inoculated experimental flasks were placed at optimal submerged fermentation conditions (30°C, 160 rpm). Fermentation was conducted over a period of 10 days, and 10 mL and 20 mL samples were collected from 1 L and 2 L baffled flask fermentation broth respectively, at days 3, 5, 7 and 10. Solvent extraction was performed as described in section 3.2.5 and TLC (section 3.2.6) was used to determine maximum SM production.
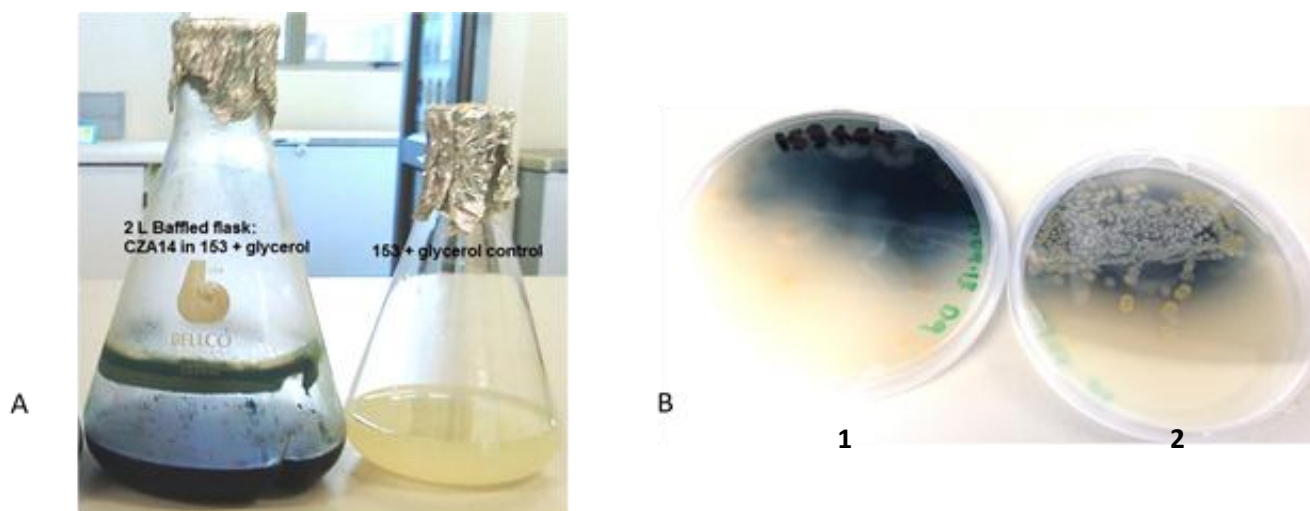
Figure 3.3: Strain CZA14[T] cultured in A) 2 L baffled flask containing 153 + glycerol (on the left), 153 + glycerol control (on the right); and B) on 153 + glycerol agar (1) substrate mycelium, (2) aerial mycelium.

3.2.4. Larger-Scale Secondary Metabolite production

During the scale-up procedure from 2 L baffled flasks to 3 L airlift reactor and 5 L continuous stirred tank reactor, both fermentations were performed twice to determine the reproducibility of compounds produced by CZA14[T]. The results from the second round of fermentations are presented in the study.

3.2.4.1. 3 L Airlift reactor (ALR) fermentation optimisation

Figure 3.4 shows the airlift reactor (ALR) used in the current study. This ALR is an internal-loop concentric tube reactor consisting of a riser, downcomer, base and gas separator. A 4 day old pre-culture was prepared (refer to section 3.2.2) and used to seed (15% v/v inoculum) sterile production medium, modified 153 + glycerol (g/L: glycerol 40, peptone 10, beef/meat extract 10 and $CaCl_2$ 6, pH 6.0).

Prior to inoculation of the production medium, an air compressor was used to facilitate the input of air into the fermenter at aeration ~ 3 L. min^{-1} (or ~ 3 vvm - where air flow rate is at 1 volume of air/ volume of liquid/minute). The temperature was set to 30°C using manual heaters; the pH was neither controlled nor monitored anytime during this experiment. Sterile antifoam 204 (Sigma) was added as needed at 0.2 µL of antifoam per 1 mL of culture. Thirty millilitre samples were collected daily for 0-20 days; the broth culture was made up to volume after each sampling session using sterile dH_2O. The cell mass production vs SM production was monitored with every sampling session (refer to section 3.2.4.1.1).

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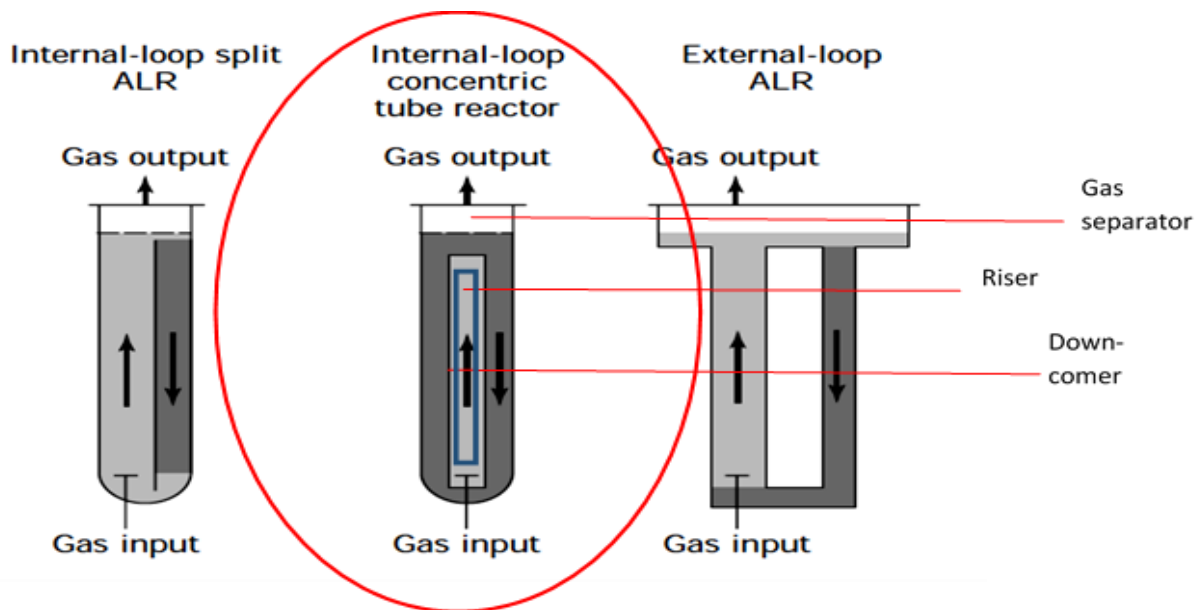
Figure 3.4: Different types of air lift reactors – the internal loop concentric tube airlift reactor was used in this study (Merchuk and Gluz, 2002).

3.2.4.1.1. Monitoring the cell mass production

To monitor growth, 1.5 mL Eppendorf tubes were dried at 60°C overnight (O/N) and placed into a desiccator O/N to remove any moisture. The tubes were weighed using an analytical balance to obtain the mass of individual tubes used during this experiment. Five hundred microliter aliquots were removed daily from the main culture and were centrifuged at 10 000 rpm for 10 minutes. The cell mass was dried at 60°C O/N and placed in a desiccator, after which the respective tubes were weighed and the mass was recorded. The initial weight of the tubes was subtracted from the final weight of the tubes containing dried cell mass to determine the actual mass. A growth curve was constructed to determine optimal growth for antimicrobial production.

3.2.4.2. Continuous stirred tank reactor (CSTR) fermentation optimisation

BIOFLO 3000 fermenter

Figure 3.5 shows the components of the BIOFLO 300 continuous stirred tank reactor (CSTR) used in the current study. The fermenter had a removable baffle assembly with six Rushton impellers along the rotor shaft and was equipped with automated monitoring systems for control over agitation, pH, temperature, and dissolved oxygen concentrations. Only the

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agitation and temperature were controlled in this study. The temperature was maintained within set limits by circulating water for cooling and with an electric jacket wrapped around the vessel for increasing the temperature when needed. The working maximum volume of the fermenter was 5 L, and 4.25 L of optimal antimicrobial production medium was prepared (at double the concentration to ensure enough nutrients were available throughout the duration of the fermentation); also $CaCO_3$ was replaced with $CaCl_2$, as $CaCO_3$ is insoluble in fermentation processes and will cause difficulty during purification by liquid solvent extraction of products.

The fermenter, its components and media, was sterilised by autoclaving at 121°C for 20 minutes and a 4 day old pre-culture (section 3.2.2) served as an inoculum to seed the production medium (15%, v/v inoculum) after the media has cooled. Prior to inoculation of the production medium an air compressor was used to facilitate the input of air into the fermenter at an aeration of ~ 4 vvm. The temperature was set to 30°C and the agitation was set at 150 rpm. Sterile antifoam 204 (Sigma) was added as needed at 0.2 μL of antifoam per 1 mL of culture. Forty millilitre samples were collected daily for 0-20 days. The broth culture was made up to volume after sampling sessions using sterile dH_2O. A sterile media control was placed on a shaker at 30 °C, 150 rpm for the same duration as the fermentation. The cell mass production vs antimicrobial production was monitored with every sampling session (see section 3.2.4.1.1).
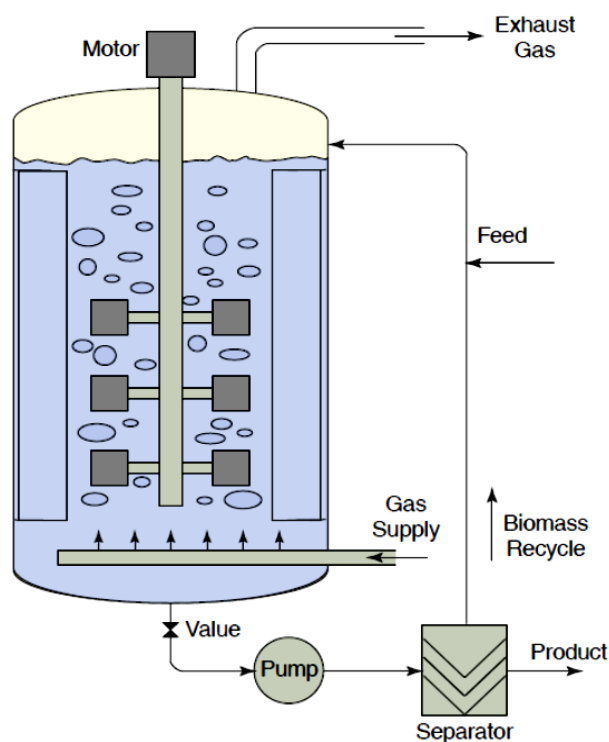
Figure 3.5: Graphic representation of a continuous stirred tank reactor (Williams, 2002).

3.2.5. Medium to Larger Scale Fermentation: Solvent Extraction

For the 50 mL, 100 mL and 200 mL culture flask volumes, 2 mL, 5 mL and 10 mL samples were collected, respectively. Samples were collected at days 3, 5, 7 and 10; and for ALR and CSTR fermentation, 30 mL and 40 mL samples were collected, respectively at days 0 - 20. SNF and mycelial mass were separated by centrifugation at 10 000 rpm for 10 minutes. Ethyl acetate (EtOAc) (1:1, v/v) was used to extract metabolites from both the supernatant filtrate (SNF) (extracellular) and mycelia (intracellular). For complete SM extraction, the samples were incubated at 37°C with shaking at 160 rpm, O/N. The organic phase was placed into sterile universal glass bottles and evaporated to dryness under a fume hood. Extracts were reconstituted with EtOAc to a 50x concentrate and used in bio-activity assays to determine bio-activity profiles and band profiling by TLC (section 3.2.6).

3.2.6. Thin Layer Chromatography (TLC)

TLC was used for the separation of secondary metabolites isolated from fermentation broth. Five microlitres of 50x concentrated extract was spotted onto the base line created on a silica gel plate and dried with the use of a handheld hairdryer. Silica gel 60 with an aluminium back support and a fluorescent indicator (F_{254}) TLC plates were used as the solid phase, while the mobile phase consisted of chloroform: EtOAc: formic acid: n-hexane at a ratio of 5:4:1:0.02 (v/v/v/v). The TLC plates were developed for ± 10-20 minutes in an airtight glass tank. The plate/s was/were dried under a fume hood for 1-2 hours. Bands were visualised at a wavelength of 254 nm and 365 nm using a UV light and their retention time (R_f) values were calculated as follows:

$$Rf = Distance\ from\ start\ (baseline)\ to\ centre\ of\ substance\ spot\ (mm)$$
$$\div\ Distance\ from\ start\ (baseline)\ to\ solvent\ front\ (mm)$$

Standard deviation (SD) were ±0 for all of the results in this study as duplicate samples represented experimental replicates and samples showed the same number of SM bands under UV light.

3.3.   RESULTS

3.3.1. Medium-scale antimicrobial production

Strain CZA14^T used in the current study has the potential to produce a number of bio-active compounds when conditions for production are optimised. Previously identified optimal medium (153 + glycerol, pH 6.0) and fermentation conditions (30°C, 160 rpm) for maximum SM production by CZA14^T were scaled-up from medium to larger scale production in 0.5 L, 1 L, and 2 L baffled flasks to 3 L airlift reactor (ALR) and 5 L continuous stirred tank reactor (CSTR) fermentation vessels. The effect of aeration and type of fermentation vessel was investigated and SM production was monitored over time.

3.3.1.1.   Flat-bottom flask vs baffled flask optimisation in 0.5 L flask volume

The results relating to the maximum production of SMs by CZA14^T cultured in 153 + glycerol in flat bottom vs baffled flasks are presented in Table 3.1. Compounds were separated on TLC using a four component solvent system and bands were visualised under 254 nm and 365 nm UV light, respectively (Appendix A, Figures A1 – A4). R_f values were calculated (example in Appendix B, Figure B1) for compounds produced extra- and intra-cellularly and are presented in Table 3.1.

Using a standard 5% inoculum volume, 0.5 L flat-bottom Erlenmeyer and baffled flasks were compared to determine which flask type resulted in the maximum number of SM production as visualised by TLC. Optimal fermentation flask type for maximum SM production were displayed using baffled flasks with CZA14^T cultured in 153 + glycerol media, pH 6.0 at 30°C, 160 rpm over a period of 3-10 days. A maximum production of seven compounds was produced extracellularly at day 7, whereas eight compounds were produced intracellularly at day 10 in flat-bottom flasks. Four out of the seven compounds were produced extra- and intra-cellularly over a period of 3 – 10 days and had similar R_f values. Compounds with similar R_f values were also produced extra- and intra-cellularly in baffled flasks. While a number of similar compounds were produced in flat-bottom flasks and baffled flasks, as observed using UV Vis visualisation more compounds were observed for the baffled flasks compared to the flat bottom flasks (Figure 3.6). A maximum production of seven compounds at day 5 was produced extracellularly, whereas nine compounds at day 3 were produced intracellularly.

Table 3.1: R_f values of bands visualised under 254 nm UV light on a developed TLC of 0.5 L flat-bottom- and baffled- flask supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production) EtOAc extracts.

Day	Flat-bottom flask (R_f value)		No. of bands	
	SNF	CELL	SNF	CELL
3	0.95, 0.80, 0.67, 0.39, 0.28	0.97, 0.90, 0.83, 0.25	5	4
5	0.95, 0.80, 0.67, 0.40, 0.28	0.96, 0.78, 0.38, 0.25	5	4
7	0.97, 0.80, 0.71, 0.66, 0.53, 0.41, 0.28	0.94, 0.78, 0.38, 0.25	7	4
10	0.79, 0.71, 0.66, 0.40	0.95, 0.90, 0.77, 0.39, 0.26, 0.12, 0.08, 0.04	4	8
Day	Baffled flasks (R_f value)		No. of bands	
	SNF	CELL	SNF	CELL
3	0.97, 0.80, 0.66, 0.41	0.96, 0.86, 0.76, 0.38, 0.25, 0.19, 0.12, 0.07, 0.03	4	9
5	0.96, 0.80, 0.67, 0.42, 0.29, 0.21, 0.13	0.96, 0.90, 0.76, 0.38, 0.25, 0.12, 0.08, 0.04	7	8
7	0.80, 0.72, 0.66, 0.40	0.96, 0.90, 0.76, 0.38, 0.25	4	5
10	0.97, 0.80, 0.70, 0.66, 0.40, 0.28	0.97, 0.91, 0.78, 0.68, 0.39, 0.27, 0.04	6	7

3.3.1.2. Optimisation of inoculum volume in 0.5 L baffled flasks

The maximum number of compounds produced was observed in baffled flask submerged fermentation, this prompted inoculum volume optimisation tests in 0.5 L baffled flasks to determine the optimal inoculum volume for maximum antimicrobial production. The results relating to optimal inoculum volume for the maximum production of SMs by CZA14[T] cultured in 153 + glycerol in 0.5 L baffled flasks are presented in Figure 3.6. Compounds were separated on TLC using a four component solvent system and bands were visualised under 254 nm and 365 nm UV light (Appendix C, Figures C1 – C8). R_f values were calculated for compounds produced extra- and intra-cellularly and are presented in Table 3.2.
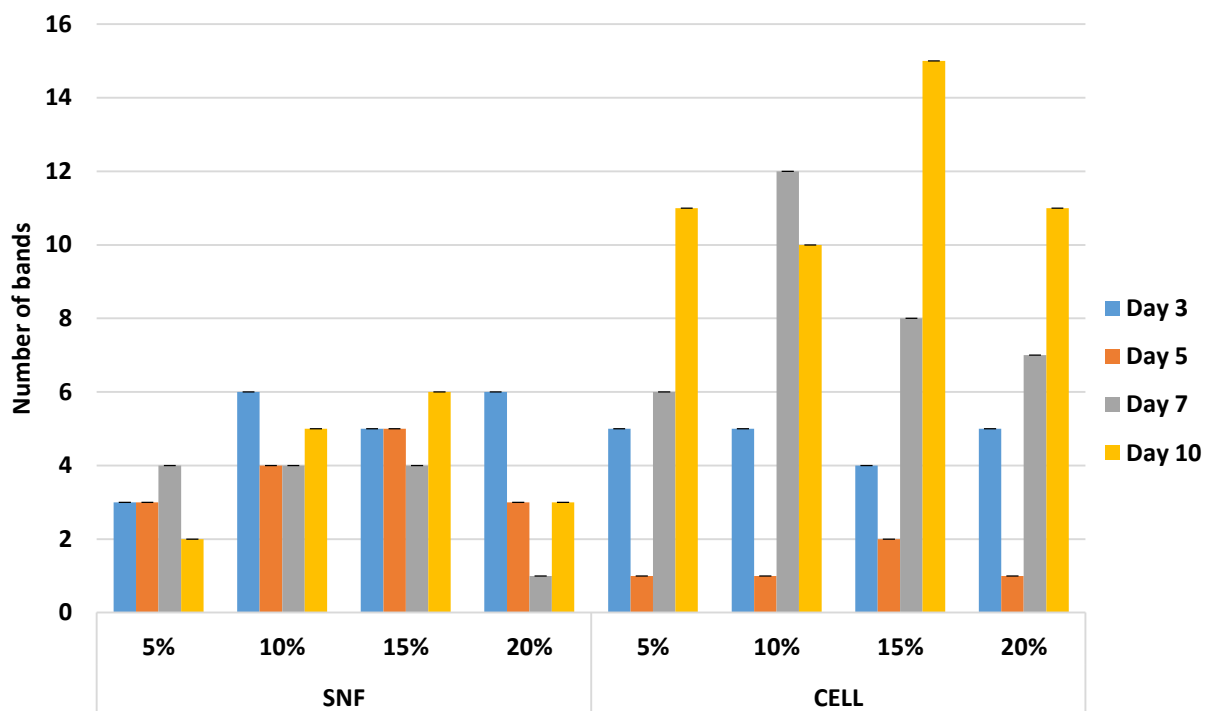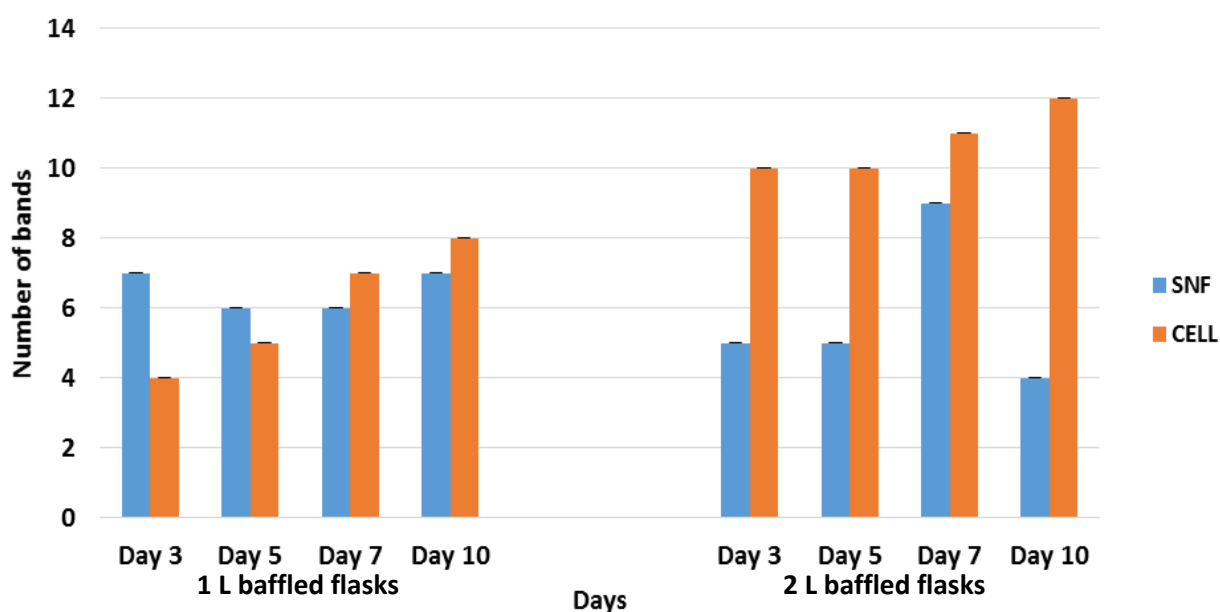
Figure 3.6: Column graph representing number of bands visualised under 254 nm short-wave UV light on a developed TLC of 0.5 L baffled flask supernatant filtrate (SNF) – extracellular production and cell mass (cell) – intracellular production EtOAc extracts from experiments at different inoculum volumes (% v/v).

The inoculum volume was optimised in 0.5 L baffled flasks. Compounds with similar R_f values were observed throughout this experiment at varying inoculum volumes. The maximum number of compounds produced was observed during intracellular production using 15 %, v/v inoculum volume and on day 10. This % v/v inoculum volume was used throughout the duration of this study.

Table 3.2: R_f values of bands visualised under 254 nm short-wave UV light on a developed TLC of 0.5 L baffled flask supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production) EtOAc extracts from experiments at different inoculum volumes (X % v/v)

Day	5 % (v/v) inoculum volume (R_f value)	
	SNF	CELL
3	0.76, 0.69, 0.56	0.96, 0.78, 0.43, 0.29, 0.06
5	0.94, 0.73, 0.57	0.95
7	0.74, 0.71, 0.58, 0.38	0.95, 0.92, 0.86, 0.68, 0.64, 0.38
10	0.75, 0.62	0.95, 0.90, 0.85, 0.70, 0.64, 0.57, 0.39, 0.27, 0.15, 0.10, 0.06
Day	10 % (v/v) inoculum volume (R_f value)	
	SNF	CELL
3	0.92, 0.69, 0.64, 0.56, 0.38	0.96, 0.78, 0.43, 0.29, 0.06
5	0.24, 0.94, 0.89, 0.76, 0.63	0.95
7	0.73, 0.71, 0.58, 0.38	0.96, 0.93, 0.90, 0.85, 0.68, 0.64, 0.45, 0.38, 0.26, 0.14, 0.10, 0.06
10	0.94, 0.75, 0.71, 0.66, 0.59	0.95, 0.92, 0.90, 0.69, 0.64, 0.56, 0.26, 0.14, 0.09, 0.06
Day	15 % (v/v) inoculum volume (R_f value)	
	SNF	CELL
3	0.92, 0.69, 0.64, 0.56, 0.38	0.95, 0.62, 0.39, 0.29
5	Smear	0.95, 0.47
7	0.73, 0.71, 0.58, 0.38	0.95, 0.90, 0.85, 0.64, 0.56, 0.54, 0.38, 0.26
10	0.73, 0.68, 0.58, 0.42, 0.29, 0.05	0.97, 0.92, 0.89, 0.66, 0.63, 0.58, 0.57, 0.54, 0.53, 0.51, 0.42, 0.38, 0.25, 0.09, 0.06
Day	20 % (v/v) inoculum volume (R_f value)	
	SNF	CELL
3	0.92, 0.69, 0.67, 0.64, 0.56, 0.38	0.94, 0.63, 0.41, 0.38, 0.29
5	0.71, 0.59, 0.38	0.95
7	0.38	0.95, 0.90, 0.85, 0.64, 0.56, 0.38, 0.26
10	0.73, 0.68, 0.60	0.99, 0.95, 0.90, 0.69, 0.64, 0.59, 0.56, 0.41, 0.38, 0.09, 0.06

3.3.1.3.   1 litre and 2 litre baffled flask optimisation

A 15 %, v/v, optimal inoculum volume for maximum SM production by CZA14^T was used during the rest of this study (in conjunction with 20 %, v/v media volume). SM production was further scaled-up to 1 L and 2 L baffled flask submerged fermentation. Maximum SM production was monitored over a period of 3-10 days by separating compounds on TLC (Appendix D, Figures D1 – D4) to determine the success of the fermentation. R_f values were calculated for compounds produced extra- and intra-cellularly, and are presented in Figure 3.7 and Table 3.3.

Figure 3.7: Column graph representing number of bands visualised under 254 nm short wave UV light on a developed TLC of 1 litre and 2 litre baffled flask EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production).

Table 3.3: R_f values of bands visualised under 254 nm short wave UV light on a developed TLC of 1 litre and 2 litre baffled flask EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production)

Day	1 Litre baffled flask (R_f value)	
	SNF	CELL
3	0.73, 0.67, 0.62, 0.56, 0.51, 0.38, 0.26	0.92, 0.54, 0.49, 0.24
5	0.73, 0.67, 0.56, 0.51, 0.38, 0.26	0.92, 0.54, 0.51, 0.37, 0.25

7	0.73, 0.67, 0.56, 0.51, 0.37, 0.26	0.92, 0.54, 0.51, 0.37, 0.25, 0.10, 0.06
10	0.73, 0.67, 0.56, 0.51, 0.44, 0.37, 0.26	0.92, 0.73, 0.56, 0.51, 0.37, 0.25, 0.10, 0.06
Day	2 Litre baffled flasks (R_f value)	
	SNF	CELL
3	0.74, 0.67, 0.54, 0.37, 0.24	0.90, 0.85, 0.71, 0.63, 0.59, 0.37, 0.25, 0.10, 0.08, 0.05
5	0.73, 0.66, 0.55, 0.38, 0.24	0.90, 0.85, 0.71, 0.63, 0.59, 0.36, 0.25, 0.10, 0.08, 0.05
7	0.87, 0.73, 0.66, 0.61, 0.55, 0.52, 0.38, 0.25, 0.10	0.90, 0.85, 0.71, 0.63, 0.60, 0.52, 0.36, 0.25, 0.10, 0.08, 0.05
10	0.92, 0.72, 0.54, 0.52	0.90, 0.85, 0.71, 0.65, 0.62, 0.52, 0.45, 0.37, 0.24, 0.10, 0.08, 0.05

In accordance with the results at 0.5 L scale, the maximum number of compounds produced was observed during intracellular production after 10 days of fermentation at 1 L and 2 L scale. While the number of bands increased upon scale-up, most of these bands were also present at 0.5 L scale; furthermore the majority of the bands detected during extracellular production were also present intracellularly.

3.3.2. Larger-scale secondary metabolite production

After successful medium-scale SM production, larger-scale production in 3 L and 5 L fermentation vessels was tested. The larger-scale production of SMs depends greatly on the fermentation processes. During fermentation, large volumes of the antimicrobial-producing organism are grown to produce the SMs. The whole process of finding a new compound can take years and the manufacturer should be able to produce the compound in high yields during the fermentation process and the isolation of the compound should be feasible. Extensive research is required prior to commercially scaling up the production of a new antimicrobial. Sections 3.3.2.1 and 3.3.2.2 gives an insight of the capabilities of strain CZA14[T] to produce SM compounds at a larger-scale.

3.3.2.1.   Airlift reactor (ALR) fermentation optimisation

Figures 3.8a and b shows the changes in culture fermentation broth colour after 3 days of growth in the airlift reactor used in this study (a) and after 7-10 days of growth of CZA14[T] (b).
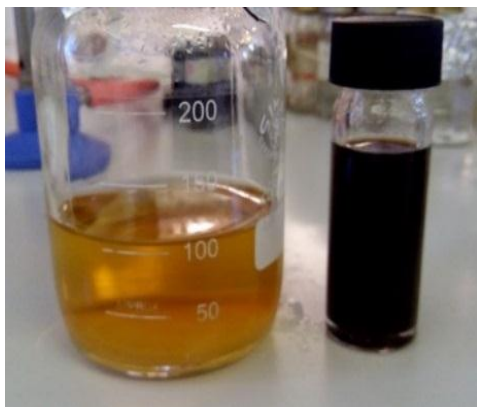
Figure 3.8a: Control media on the left, experimental broth on the right after 3 days of fermentation of CZA14[T] in an ALR in 153 + glycerol medium, pH 6.0 at 30°C.

Figure 3.8b: 3 L Airlift Bioreactor after 10 days of fermentation of CZA14[T] in an ALR in 153 + glycerol medium, pH 6.0 at 30°C.

The results relating to the maximum production of SMs by CZA14[T] cultured in 153 + glycerol in an airlift reactor (ALR) is presented in Figure 3.9; furthermore the effect of cell mass production on SM production is presented in Figure 3.10. Compounds were separated on TLC using a four component solvent system and bands were visualised under 254 nm and 365 nm UV light (Appendix E, Figure E1). R_f values were calculated for compounds produced extra- and intra-cellularly and are presented in Table 3.4.
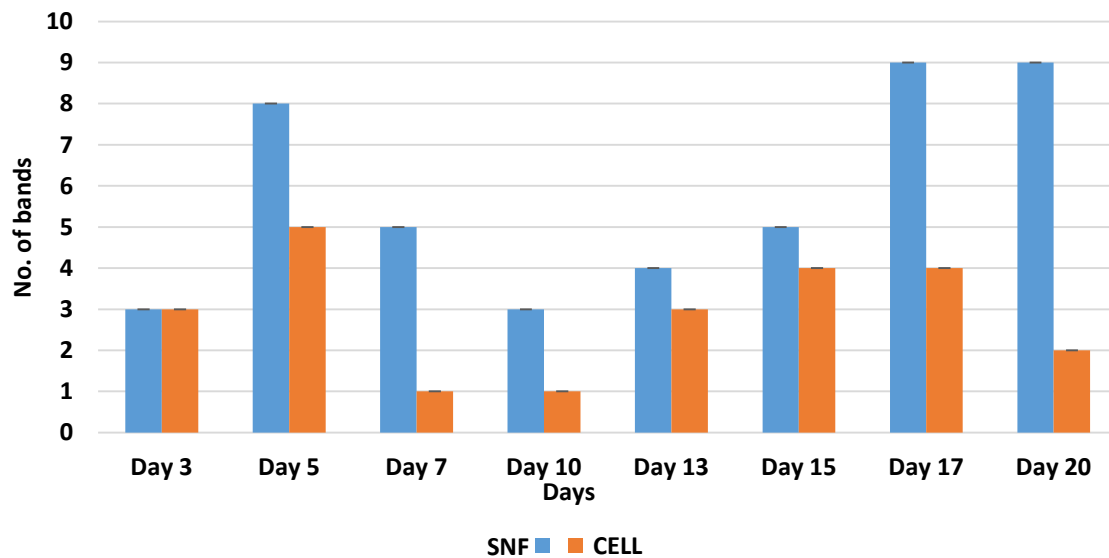
Figure 3.9: Column graph representing number of bands visualised under 254 nm short wave UV light on a developed TLC of EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production) extracted from fermentation broth collected from an airlift bioreactor (ALR).

	Day 0	Day 2	Day 5	Day 7	Day 10	Day 13	Day 15	Day 17	Day 20
SNF	0	3	8	5	3	4	5	9	9
CELL	0	3	5	1	1	3	4	4	2
SD	0.000	0.009	0.021	0.009	0.001	0.000	0.004	0.012	0.018
Cell Density	1.012	1.022	1.032	1.022	1.019	1.018	1.023	1.016	1.030
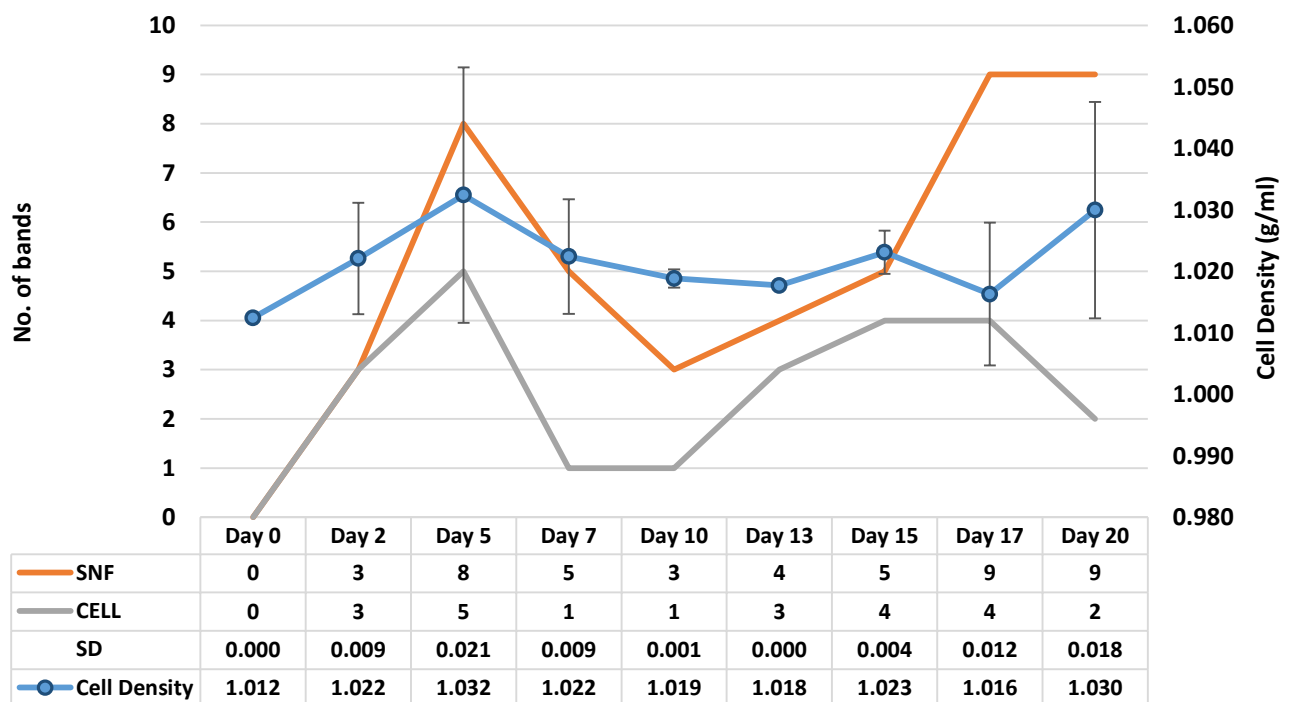
Figure 3.10: Line graph of cell mass production vs antimicrobial production over a period of 0-20 days during fermentation of CZA14[T] in a 3L airlift bioreactor

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Table 3.4: R_f values of bands visualised under 254 nm short wave UV light on a developed TLC of ALR EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production).

Day	ALR (R_f value)	
	SNF	**CELL**
3	0.94, 0.38, 0.26	0.94, 0.26, 0.10
5	0.94, 0.85, 0.77, 0.73, 0.38, 0.26, 0.13, 0.10	0.94, 0.38, 0.26, 0.13, 0.10
7	0.85, 0.38, 0.26, 0.13, 0.10	0.90
10	0.85, 0.38, 0.26	0.90
13	0.94, 0.85, 0.38, 0.26	0.90, 0.38, 0.26
15	0.94, 0.85, 0.77, 0.38, 0.26	0.94, 0.77, 0.38, 0.26
17	0.94, 0.90, 0.85, 0.77, 0.73, 0.64, 0.59, 0.38, 0.26	0.94, 0.77, 0.38, 0.26
20	0.94, 0.90, 0.85, 0.77, 0.73, 0.64, 0.59, 0.38, 0.26	0.94, 0.26

All compounds produced intracellularly were also produced extracellularly. A maximum production of eight and nine compounds was produced extracellularly at day 5 and days 17-20, respectively, whereas five compounds were produced intracellularly at day 5. Cell growth was monitored throughout the fermentation alongside SM production by CZA14[T] to determine the effect of biomass on the number of SMs produced. An increase in biomass production occurred at day 5 and decreased thereafter. An unexpected increase occurred at day 15 followed by a decrease at day 17 and a sudden increase again at day 20. SM production follows a similar trend compared to the growth curve of CZA14[T]. Cell growth was the same across the time period of the experiment, no real changes occurred.

3.3.2.2. Continuous stirred tank reactor (CSTR) fermentation optimisation

Figure 3.11 shows the BIOFLO 3000 continuous stirred tank reactor (CSTR) used in this study.

Figure 3.11: BIOFLO 3000 Continuous Stirred Tank Reactor.

The results relating to the maximum production of SMs by CZA14[T] cultured in 153 + glycerol in a continuous stirred tank reactor (CSTR) is presented in Figure 3.12; furthermore the effect of cell mass production on SM production is presented in Figure 3.13. Compounds were separated on TLC using a four component solvent system and bands were visualised under 254 nm and 365 nm UV light (Appendix F, Figure F1). R_f values were calculated for compounds produced extra- and intra-cellularly and are presented in Table 3.5.
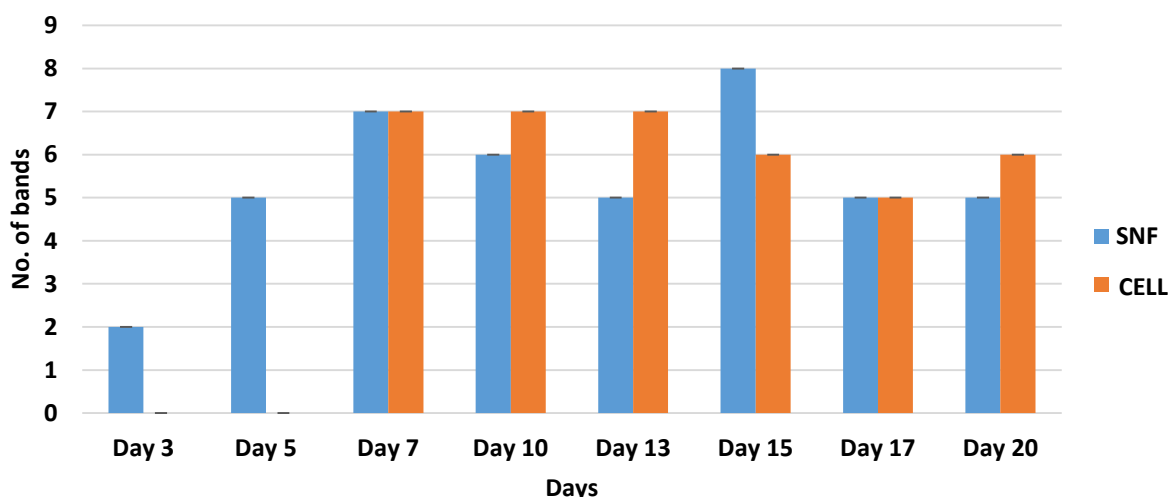
Figure 3.12: Column graph representing number of bands visualised under 254 nm short wave UV light on a developed TLC of EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production) extracted from fermentation broth collected from continuous stirred tank reactor (CSTR).

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	Day 0	Day 3	Day 5	Day 7	Day 10	Day 13	Day 15	Day 17	Day 20
SNF	0	2	5	7	6	5	8	5	5
CELL	0	0	0	7	7	7	6	5	6
SD	0.001	0.002	0.001	0.014	0.009	0.007	0.004	0.005	0.010
Cell Density	1.013	1.024	1.025	1.042	1.027	1.023	1.021	1.029	1.023
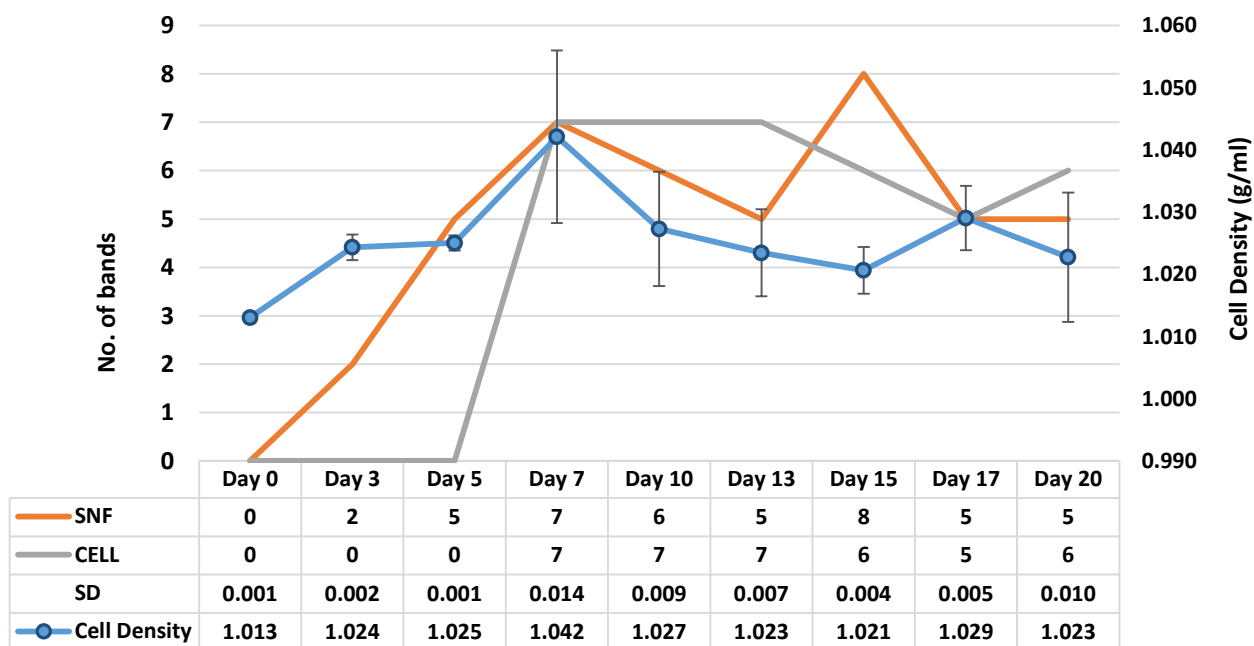
Figure 3.13: Line graph of cell mass production vs antimicrobial production over a period of 0-20 days during fermentation of CZA14[T] in a continuous stirred tank reactor.

Table 3.5: R_f values of bands visualised under 254 nm short wave UV light on a developed TLC of ALR EtOAc extracts from the supernatant filtrate (SNF) (extracellular production) and cell mass (cell) (intracellular production)

Day	CSTR (R_f value)	
	SNF	**CELL**
3	0.59, 0.56	NA
5	0.90, 0.60, 0.56, 0.38, 0.26	NA
7	0.90, 0.69, 0.60, 0.56, 0.49, 0.38, 0.26	0.94, 0.90, 0.69, 0.60, 0.56, 0.38, 0.26
10	0.90, 0.69, 0.60, 0.56, 0.49, 0.38	0.94, 0.90, 0.69, 0.60, 0.56, 0.38, 0.26
13	0.90, 0.69, 0.60, 0.56, 0.49	0.94, 0.90, 0.69, 0.60, 0.56, 0.38, 0.26
15	0.90, 0.60, 0.55, 0.51, 0.49, 0.42, 0.38, 0.26	0.94, 0.90, 0.55, 0.38, 0.26, 0.17

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17	0.90, 0.55, 0.48, 0.38, 0.26	0.94, 0.55, 0.38, 0.26, 0.17
20	0.90, 0.55, 0.48, 0.38, 0.26	0.94, 0.55, 0.42, 0.38, 0.26, 0.17

A maximum of seven and eight compounds were produced extracellularly at days 7 and 15, respectively. Whereas seven compounds were produced intracellularly at days 7-13. Five out of the seven compounds produced intracellularly had similar R_f values to compounds produced extracellularly. Three out of the eight compounds produced during fermentation using the CSTR had similar R_f values compared to compounds produced during fermentation with the ALR. An increase in biomass occurred at day 7 and decreased thereafter, followed by an increase in biomass at day 17. Intra- and extra-cellular SM production follows a similar line pattern compared to the CZA14[T] growth curve, however a sudden increase in extracellularly produced compounds occurred at day 15.

3.4. DISCUSSION

Previously defined optimal media and fermentation conditions were selected based on the premise that SM production by actinomycetes are enhanced or initiated by manipulating fermentation media components and conditions (Zhang and Greasham, 1999). The media type (153 + glycerol) used in the current study consists of glycerol, meat extract, peptone and $CaCO_3$. Glycerol was used as a carbon source to provide a stabilising effect on biosynthetic enzymes and SMs which in turn causes an increase in SM yield in actinomycetes (Amoyo *et al*., 2000; Chen *et al*., 2002). Meat extract and peptone were used as nitrogen sources. The combination of these two components was to reduce the problems associated with easily assimilated nitrogen sources, which may repress SM production caused by amino acids and ammonium salts (Omura and Tanaka, 1984). $CaCO_3$ was used as a micronutrient, which is essential for biological activities; excess amounts of this micronutrient may be toxic and was used in small concentrations. Maximum SM production by CZA14[T] in 153 + glycerol was achieved at media pH of 6.0. Generally actinomycetes produce SMs best at optimal growth pH and in this case, CZA14[T] has a growth optima of pH 7.0 (Le Roes and Meyers, 2005). The optimal fermentation conditions for the growth and maximum SM production by CZA14[T] was at 30°C, shaking at 160

rpm. It has been reported that actinomycete producing SMs are mesophilic and have optimal SM production as well as growth optima at about 28°C (Iwai and Omura, 1982).

Submerged fermentation was used to produce SMs by CZA14^T. The primary concerns in utilising submerged fermentations for SM production by filamentous actinomycetes are the fermentation vessel and the speed of the shaker to provide adequate oxygen supply (Whittaker, 1992). SM production in 0.5 L flat-bottom Erlenmeyer flasks and baffled-flasks (Table 3.1) were compared to determine the best flask type for maximum SM production. These flasks were shaken at 160 rpm – the speed at which the shaker is moving should be adequate enough to provide sufficient oxygen supply without shearing the mycelia (required for SM production) which is associated with high agitation rates (Whittaker, 1992). One band visualised under UV on a developed TLC plate could constitute one or more compounds, but to quantify the production of SMs the number of bands visualised under UV was used as a measurement of the success of a particular fermentation and is referred to as a SM compound. Comparison of the two flask types showed that higher amounts of SM compounds were produced in baffled flasks (Table 3.1). Seven compounds were produced extracellularly at day 5 and nine compounds were produced intracellularly as early as day 3 in baffled flasks, whereas seven compounds were produced extracellularly at day 7 and eight compounds were produced intracellularly at day 10 in flat-bottom flasks (Appendix A, Figure A1 – A4). SM production in flat-bottom flasks was different to that of baffled flasks. This could be due to the level of dissolved oxygen present within these respective flask types. Shake flasks with angular baffles or indents are used for actinomycetes as these indentations help in increasing oxygen transfer and also aids in efficient mixing (Katzer *et al*., 2001).

A number of similar compounds produced in flat-bottom flasks were also produced using baffled flasks. Minor variations in R_f values may have occurred throughout this study due to slight fluctuations in the solvent system, temperature and technical error during measuring the R_f values of the compounds. Compounds with identical R_f values do not necessarily imply that compounds are identical and further analysis (by more sensitive equipment such as LC/MS) should be performed before those conclusions can be made. Based on the results obtained, baffled flasks were further used in the optimisation experiments.

Other factors influencing antimicrobial production by actinomycetes is the size of the inoculum. Whittaker (1991) investigated the influence of the concentration of viable spores in the

inoculum on the morphological form of actinomycetes. He found that a high concentration of viable spores produces a dispersed growth form compared to pellet formation when spores are at low concentration in the inoculum. In this study, the concentration of spores was not taken into account. A number of compounds with similar R_f values were produced using 5% - 20%, v/v inoculum volumes (Figure 3.6; Table 3.2; Appendix C, Figures C1 – C8). The maximum number of SMs produced was observed using a 15%, v/v inoculum size at days 7 and 10 which was three times higher than the initial inoculum volume previously used. It was thus found that the increase in SM production was directly proportional to the increase in inoculum size. However, fewer SMs were produced with a 20% inoculum volume – this may be due to nutrient limitation. A study by Tresner *et al*. (1967), reported the higher degree of fragmentation of the mycelia of *Streptomyces aureofaciens* when inoculum volume was increased from 1 to 15%. Sufficient mycelium fragmentation results in abundant spore formation in actinomycetes – spore production in actinomycetes are associated with SM production.

Baffled flasks were used to achieve a less fragmented mycelial morphology – mycelial fragmentation results in a loss in SM production. These flasks however cause splashing of the culture against the flask wall at high shaker speeds as observed in Figure 3.3. Filamentous mycelial morphology with little to no fragmentation results in more abundant spore formation. Under these conditions of growth (baffled flasks), CZA14[T] produces a blue-green diffusible pigment and spores between 1-2 days of growth (Figure 3.3). In this study, CZA14 uses nutrients quickly and produces a diffusible pigment at the beginning of the fermentation. Limitation of nutrients, specifically nitrogen causes the production of spores, melanin or diffusible pigment production due to stress; the reproductive phase is coordinated with the secretion of SMs which protect the colony against invading bacteria during aerial growth and sourcing of new nutrient sources (Behal, 2000).

More compounds were produced intracellularly than extracellularly in the 1 L and 2 L baffled flasks (Figure 3.7; Table 3.3). A number of different compounds were produced during extracellular production compared to intracellularly produced compounds in 1 L and 2 L baffled flasks. More compounds were produced in the 2 L baffled flasks with a maximum of nine compounds produced extracellularly at day 7 and twelve compounds produced intracellularly at day 10 (Appendix D, Figures D3 – D4). Six to seven compounds were produced extracellularly in 1 L baffled flasks over a period of 3–10 days and 5 to 8 compounds were produced intracellularly over a period of 3-10 days (Appendix D, Figures D1 – D2). This could be due to

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increased oxygen uptake rate in 2 L baffled flasks compared to 1 L baffled flasks. In 2 L baffled flasks the bottom of the flask has a bigger surface area than that in 1 L baffled flasks causing more improved mixing without causing heavy splashing. Adequate mixing in 2 L baffled flasks helps to achieve similar pellet shape and size that is observed in stirred fermentations for optimal SM production.

Throughout the scale-up of fermentation vessels from 0.5 L flat-bottom flasks to 2 L baffled flasks compounds detected under short-wave UV light (254 nm) had similar R_f values of those compounds detected under long-wave UV light (365 nm). Most of these compounds fluoresced blue, yellow and orange in colour under 365 nm UV light depending on the flask type CZA14[T] was cultured in.

A decrease in intracellular SM production occurred upon scale-up to 3 L airlift reactor (ALR), however the extracellular SM production is on par with SM production in 2L baffled flasks. A number of different compounds were produced by CZA14[T] in the ALR compared to other fermentation vessels in this study and could be due to sufficient biomass production. Biomass production is related to good gas/liquid mass-transfer at increased concentrations of dissolved substances. While a limitation in oxygen supply may be detrimental to biomass production, low biomass production may also be attributed to large amounts of nutrients inhibiting SM production. A fed batch system technique was used to overcome this problem where a certain volume of culture medium is first inoculated with the SM producing organism and over time another dosage of nutrients is supplied to the fermenter (in this study, sterile water was used to supplement the fermentation) for prolonged cultivation to increase SM production (Behal, 2000). This was performed as dry air is introduced into the system causing evaporation of the fermentation broth; water was used to maintain a constant volume of fermentation broth in the airlift reactor to aid in sufficient biomass production. This technique was used in both the ALR and continuous stirred tank reactor (CSTR) used in the study. However, the number of SMs produced in the CSTR was lower than that produced in the ALR and 2 L baffled flasks. This could be as a result of the mechanical shearing of the mycelial cells by the impellers in the CSTR. No diffusible pigment was observed during fermentation with the CSTR, the media changed from tan to brown suggesting low melanin production. Interestingly, during fermentation with the ALR, a dark red-brown pigment was produced from 3 – 20 days of growth (Figure 3.8a and b). It appears as if melanin is being produced after 3 days of growth and red and yellow compounds were observed after preparation of solvent extracts of samples taken after 7 – 10 days of

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growth during the production of the red culture pigment, with the compounds becoming lighter in colour after 10 days of SM production.

A growth curve of strain CZA14[T] was performed to determine effect of biomass on the number of SMs produced in the ALR and CSTR. In the ALR, an increase in biomass production occurred around day 5 and decreased thereafter with an increase in SM products following the same development. This translates to 5 days of log growth after which the culture entered stationary phase and began death phase after day 15. In the CSTR, after 7 days of log growth the culture entered stationary phase and began death phase after day 17. In both the ALR and CSTR, variation in the biomass and production occurred around day 15 – day 20. This could be attributed to the phenomenon of autophagy – this involves a cellular process in which proteins, organelles and lipids are targeted towards lysosomes for degradation. These are reused to fulfil energy requirements of the cell under nutrient-limiting conditions (Huang and Brumell, 2014).

The scale-up from flask-type fermentation vessels to bioreactor systems were moderately successful. ALR and CSTR systems present their own advantages and limitations, but by definition, involve similar processes. Most bioreactor systems allow for improved cultivation of the microorganism of interest in a controlled manner over shake-flasks; these systems involve the conversion of materials via specific reactions into valuable by-products. The CSTR was selected as it results in continuous production and output. This system allows for automation of processes so it reduces labour expense. Production time used to empty, fill and sterilise the reactor is reduced. Due to operating parameters, the quality of the products formed is consistent. CSTR has a number of limitations associated with its use such as, negligible robustness due to slight process variation – it is an automated system. Uniform raw material quality is required to guarantee that the process remains continuous. The costs associated with purchasing and maintaining these systems are high. Higher risk of cell mutation and contamination of the culture is associated with the CSTR due to cultivation periods required within the CSTR (Williams, 2002).

The ALR was selected for further study as it provides more advantages over the CSTR (as observed in this study). Some of these advantages include less maintenance, easier sterilisation and reduced risk of defects as the design of the ALR is simpler and contains no agitator or moving parts. The risk of shearing of the mycelia is lower in the ALR, which results in greater robustness. It also provides low energy input due to large, specific interfacial contact-area.

Efficient mixing and well-controlled flow provide well-defined habitation time for all growth phases and enhanced oxygen transfer in solution results in an increase in mass-transfer rate. Lastly, the ALR provides greater heat-removal compared to the CSTR (Williams, 2002).

3.5.   CONCLUSION

Figure 3.14 presents a summary of the use of various fermentation vessels and the maximum SM production in those vessels.
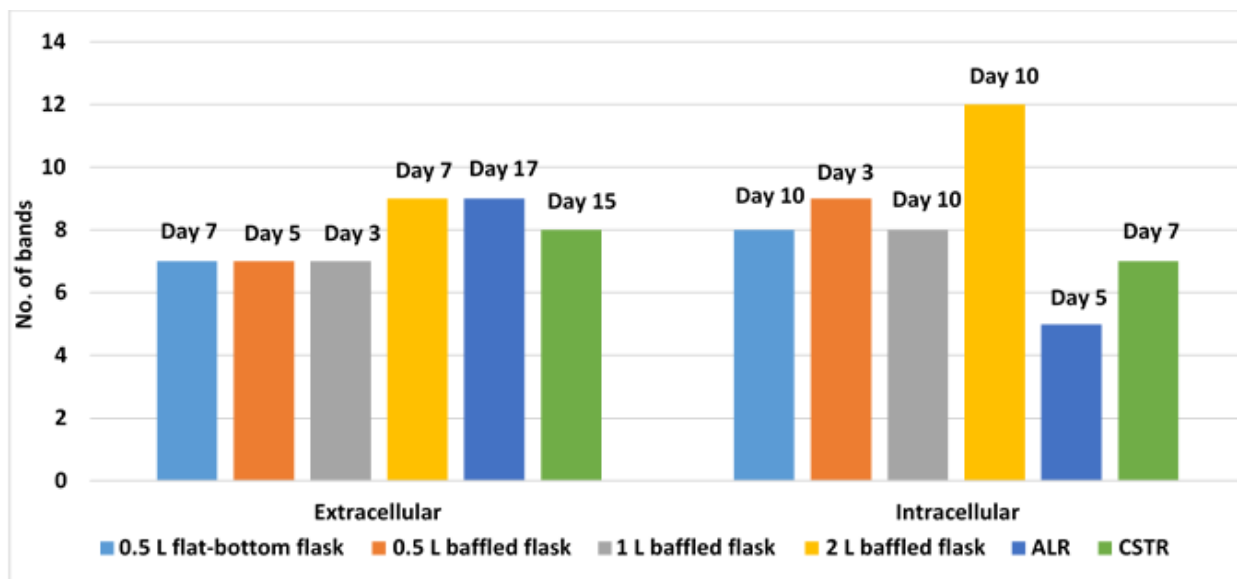
Figure 3.14: Summary of maximum secondary metabolite production in various fermentation vessels.

0.5 L flat-bottom flasks were compared to 0.5 L baffled flasks. Overall, more SM compounds were produced intracellularly in baffled flasks at a faster rate compared to production in flat-bottom flasks. This was attributed to poor oxygen supply in flat-bottom flasks.

The production was successfully scaled-up from 0.5 L baffled flasks to 1 L and 2 L baffled flasks. More SM compounds were produced extra- and intra-cellularly in 2 L baffled flasks compared to 1 L baffled flasks. It was concluded that this could be a result of reduced pellet formation (which limits SM production) in the 2 L baffled flasks. Dispersed mycelium formation occurred in 2 L baffled flasks in this study due to larger surface area of the flask causing higher aeration during agitation of the culture medium and in turn resulting in an increase in production of SM products.

Extracellular SM production in the ALR is comparable with extracellularly produced compounds in the 2 L baffled flasks. Intracellular compound production, however, was less than half of that

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produced in 2 L baffled flasks. CZA14^{T} produced a number of compounds in the CSTR of similar R$_f$ values to those produced in the ALR, but fewer compounds compared to both the ALR and 2 L baffled flasks. Optimization of the CSTR could be performed in future as a number of the features and culture conditions are automated and is easier to control compared to other bioreactors.

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CHAPTER 4:

Testing the inhibitory efficacy of crude extracts against a number of known pathogenic bacteria
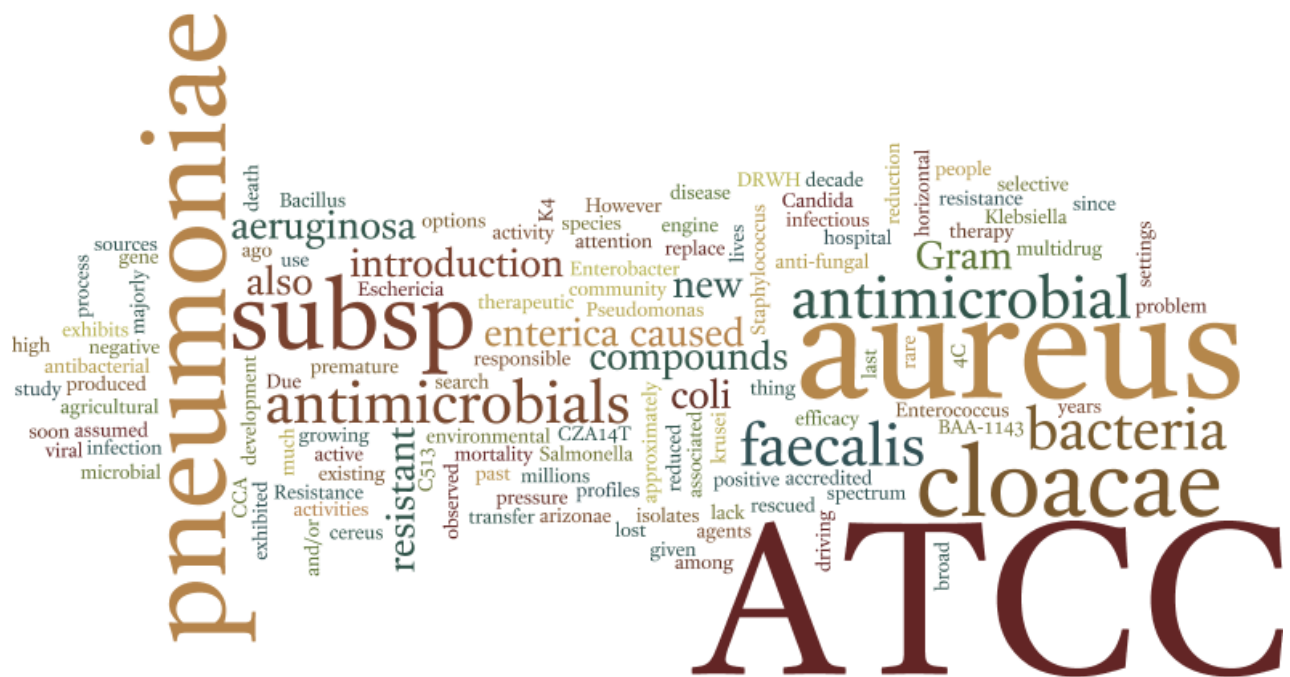
Abstract

The introduction of antimicrobial therapy approximately 70 years ago has since saved the lives of millions of people and majorly reduced premature death caused by microbial infection. Due to this reduction it was assumed that high mortality associated with infectious disease was a thing of the past. However, resistance profiles were observed soon after the introduction of antimicrobial compounds. Resistance caused by horizontal gene transfer between and among species of both Gram-positive and Gram-negative bacteria were responsible for the development of multidrug resistant bacteria. The driving engine of this process can be accredited to the selective pressure of the use of antimicrobials in community, hospital and agricultural settings. The lack of new therapeutic options to replace those antimicrobial agents that have lost their efficacy against resistant bacteria is a growing concern. The search for new antimicrobials from rare and/or existing sources such as strains in culture collections have been given much attention over the last decade. In this study, antimicrobials produced by CZA14^{T} exhibited broad spectrum antibacterial activities against *Bacillus cereus* ATCC 10876, *Enterobacter cloacae* subsp. *cloacae* ATCC BAA-1143, *Enterococcus faecalis* ATCC 29212, *Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 29213, *E. faecalis* ATCC 51299, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700603, *Salmonella enterica* subsp. *arizonae* ATCC 13314, and *S. aureus* subsp. *aureus* ATCC 33591. It also exhibited anti-fungal activity against *Candida krusei* ATCC 34135. These compounds were also active against environmental domestic rainwater harvesting (DRWH) system isolates, *E. coli* K4 CCA, *E. cloacae* 11, *E. cloacae* 20, *E. cloacae* 22, *E. faecalis* C513, *K. pneumoniae* 1, *K. pneumoniae* 2, *K. pneumoniae* 4, *K. pneumoniae* 5, *P. aeruginosa, S. enterica* 4C, *S. aureus* 1, *S. aureus* 2, *S. aureus* 4, and *S. aureus* 5.

Key words: Broad-spectrum, antimicrobial therapy, bio-activity, ATCC, DRWH

Introduction

Antimicrobial resistance poses a major health risk due to infections caused by resistant bacteria. Over time, a number of these pathogenic bacteria have gained resistance to multiple classes of antimicrobials available on the market. Reduced amount of effective antimicrobial agents against common infections currently threaten our ability to fight against infectious diseases and to manage complications caused by these infections, especially in immuno-compromised patients. The development of new antimicrobial compounds and new diagnostic tests are desperately needed for use against resistant bacteria.

There are a number of types of microbiological tests used to determine the inhibitory effects of an antimicrobial agent on a particular test organism. These tests are commonly referred to as antimicrobial assays. A biological assay is a practical method whereby the effectiveness of a material of unknown bio-activity is assessed by comparing the efficacy in a biological system against a reference standard of known potency. Ideally, a homogenous biological system is required so that the reference standard and unknown would be reacting under similar conditions (Hewitt, 2003).

Infections caused by bacteria and the comparative analysis of antimicrobials are successfully managed by accurately determining the bacterial susceptibility to antimicrobial agents. A number of techniques can be employed to do this, such as diffusion, dilution and bioautography assays (Mishra *et al*., 2006). The agar diffusion method is used for the quantitative detection of inhibitory agents. The method can be performed by means of disk diffusion, cylinder diffusion and the hole-plate assay. The most commonly used diffusion method is the disk diffusion assay (used in this study), whereby an antimicrobial substance is placed on a filter disk and allowed to diffuse into the agar medium containing the test organism of interest. This leads to the inhibition of the growth of the organism in the area of the source antimicrobial to form clear 'zones' without any bacterial lawn/growth. The effectiveness of antimicrobial agents can be evaluated by means of their ability to suppress the growth of the test organism described by dilution method – minimal inhibitory concentration (MIC). The minimum lethal concentration (MLC) is characterized by the lowest concentration of an antimicrobial agent to kill the test organism of interest (Macias *et al*., 1994; Abraham *et al*., 1992). Lastly, the quantitative bioautography assay may be performed by means of contact,

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immersion and direct bioautography. In this study, direct thin layer chromatography (TLC) – bioautography was used for the detection of compounds in the form of bands (e.g. visualisation by UV) and/or a chromogenic spray substance, and bio-activity. The aforementioned assays provide a quick screen for novel antimicrobial compounds by bioassay-guided isolation (Moreno *et al.*, 1999).

In the search for new antimicrobials with novel mechanisms of action, a comparative study between clinical test strains obtained from the American Type Culture Collection (ATCC) and environmental strains isolated from domestic rainwater harvesting (DRWH) systems was performed to determine the antimicrobial profiles of secondary metabolite (SM) producing strain CZA14^T using a number of bioassay strategies. SMs produced by CZA14^T were also tested against clinical yeast test strains to establish antimicrobial properties.

This chapter describes the bio-activity profiles of crude compounds extracted from fermentation broth after fermentation of CZA14^T in 2 L baffled flasks, airlift reactor (ALR) and continuous stirred tank reactor (CSTR). This chapter deals with the second and third objective of this study – to test the inhibitory efficacy of crude extracts against clinical ATCC test strains and environmental bacterial pathogens isolated from DRWH systems; the methodology employed to achieve these objectives are outlined in Figure 4.1.

The agar overlay assay was used to determine the bio-activity potential of CZA14^T against various Gram positive and Gram negative ATCC test strains and DRWH system isolates. The inhibitory efficacy of crude extracts produced by CZA14^T in various fermentation vessels was tested using standard filter disk-diffusion assays against the ATCC test strains and DRWH system isolates; where a clear "zone of inhibition" indicates the killing potential of the compounds produced by CZA14^T. The results obtained during standard filter disk-diffusion assays were confirmed using bioautography assays (Figure 4.1).

Inhibitory efficacy of crude extracts against American Type Culture Collection (ATCC) and Domestic Rainwater Harvesting (DRWH) test strains

Determining bio-activity potential of *Streptomyces pharetrae* sp. strain CZA14ᵀ using the agar-overlay method against ATCC test strains and DRWH isolates

Bioassays: Filter disk diffusion assay and bioautography to determine active compounds against ATCC test strains and DRWH isolates

DNA isolation of DRWH test strains for the identification of these strains to genus level using phylogenetic analysis:

Chromas

DNAMAN

BLAST search via NCBI database

ATCC: *Bacillus cereus* ATCC 10876, *Enterobacter cloacae subsp. Cloacae* ATCC BAA-1143, *Enterococcus faecalis* ATCC 29212, *Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus subsp. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumonia subsp. Pneumonia* ATCC 700603, *Salmonella enterica subsp. arizonae* ATCC 13314, *Staphylococcus aureus subsp. aureus* ATCC 33591, Yeast strains: *Candida albicans* ATCC 24433, *Candida albicans* ATCC 90028, *Candida krusei* ATCC 14243, *Candida krusei* ATCC 34135, *Candida tropicalis* ATCC 750

DRWH isolates: *Bacillus cereus, Escherichia coli* K4 CCA, *Escherichia coli* Mi CCA, *Enterobacter cloacae* 11, *Enterobacter cloacae* 19, *Enterobacter cloacae* 20, *Enterobacter cloacae* 22, *Enterobacter cloacae* 23, *Enterococcus faecalis* C513, *Klebsiella pneumonia* 1, *Klebsiella pneumonia* 2, *Klebsiella pneumonia* 3, *Klebsiella pneumonia* 4, *Klebsiella pneumonia* 5, *Klebsiella pneumonia* 6, *Klebsiella pneumonia* 7, *Klebsiella pneumonia* 8, *Pseudomonas aeruginosa, Salmonella enterica* 4C, *Salmonella enterica* 5J, *Staphylcoccus aureus* 1, *Staphylcoccus aureus* 2, *Staphylcoccus aureus* 3, *Staphylcoccus aureus* 4, *Staphylcoccus aureus* 5, *Staphylcoccus aureus* 6, and *Staphylcoccus aureus* 7

Figure 4.1: Flow-chart of methods used to determine the inhibitory efficacy of crude extracts from the supernatant filtrate (SNF) and cell mass (cell) extracted from fermentation broth from 2L baffled flasks, ALR and CSTR.

Materials and Methods

4.1. Materials

Most of the chemicals used in this study were purchased from Merck Millipore or Sigma-Aldrich unless otherwise stated. All solvents used were analytical grade.

4.2. Methods

4.2.1. The overlay technique to determine CZA14ᵀ bio-activity potential against clinical ATCC control test strains and environmental DRWH isolates

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4.2.1.1. Base layer agar for preparation of susceptibility testing

Strain CZA14[T] was inoculated onto ISP #2 agar (g/L: 4 glucose, 10 malt extract, 4 yeast extract and 20 bacteriological agar, pH 7.2) for ± ten days at 30°C using the actinomycete streak technique (unpublished data: Kirby and le Roes-Hill, 2009). Mature CZA14[T] agar cultures were used to stab inoculate, 153 + glycerol (g/L: 10 glycerol, 5 peptone, 5 meat extract, 3 $CaCO_3$, and 20 agar bacteriological, pH 6.0) agar plates using sterile toothpicks. The plates were inverted and incubated at optimal temperature (30°C) for eleven days.

4.2.1.2. Overlay layer – Part 1 - inoculation of test bacteria

The test bacteria used in this study were inoculated onto optimal solid growth media, inverted and incubated for 24-48 hours at optimal growth temperature (Table 4.1). A loopful of test bacteria was inoculated (using aseptic techniques) into 10 mL sterile liquid optimal growth medium and incubated overnight at optimal growth temperature (Table 4.1) with shaking (160 rpm, 16-20 hours). Overnight cultures were Gram-stained using a standard Gram-staining procedure prior to use to ensure no contamination of the test cultures took place. Test strains were stored as cell suspension stock cultures in 20% (v/v) glycerol at -20°C and further maintained on optimal growth media (Table 4.1). The Optical Density (OD) of the pure test cultures were measured at 600 nm using a RayLeigh UV-9200 spectrophotometer (sterile growth media was used as a blank) and the test cultures were diluted accordingly. The following dilutions were performed: fast growing bacteria such as *Escherichia coli* were diluted 1-in-10 and slow growing bacteria such as *Enterococcus faecalis* (and most enteric bacteria) were diluted 1-in-4.

4.2.1.3. Overlay layer – Part 2 - preparing the sloppy agar

Optimal growth medium sloppy agar (0.7%, w/v agar) was prepared by dissolving all ingredients in dH_2O in the microwave at full power and aliquoting the molten agar into McCartney bottles in 6 mL volumes using a 10 mL glass pipette. Metal caps with rubber stoppers were placed onto each McCartney bottle and sterilized by autoclaving the media for 15 minutes at 121°C. After autoclaving the media the bottles were placed at 60°C in a water bath to bring the media to temperature in order to add the test culture to the sterile sloppy agar medium.

4.2.1.4. Overlay layer – Part 3 - overlaying

To ensure that the same concentration of cells are in the sloppy agar when repeating or performing overlay experiments the OD_{600} was taken into account for each test culture. An

empirical formula varying for different test bacteria was used to determine the number of microliters (µLs) added to each sloppy agar tube.

The number of µLs of test bacteria used per 6 ml overlay was such that when multiplying the number of µLs of bacterial culture used per sloppy agar by the OD_{600} of the broth culture approximate values of : 160 $OD_{600\text{-}µL}$ for *M. aurum*, 4 $OD_{600-µL}$ for *E. coli*, and 160 $OD_{600-µL}$ for *E. faecalis* was achieved.

The calculated amount of test bacteria was added into a McCartney bottle containing optimal growth sloppy agar medium and vortexed gently to ensure homogenity. The 6 mL volume sloppy agar containing the test bacteria was poured onto the surface of the stab-inoculated CZA14^T plate cautiously to avoid pouring directly onto the streptomycete colony (to avoid dispersion of spores all over the agar plate). The sloppy agar was allowed to set, the plates were inverted and incubated overnight at optimal temperature of the test strains (Table 4.1) and zones of inhibition were recorded to determine bio-activity potential of strain CZA14^T.

4.2.2. DNA isolation of Domestic Rainwater Harvesting test strains, PCR and phylogenetic analysis

The DNA isolation method was adapted from Cheng and Jiang (2006) whereby the test strains of interest were grown in optimal media and under optimal conditions (Table 4.1). Cells were harvested from 1 mL of the cell suspension by centrifuging at 10 000 rpm for 2 minutes. The supernatant was discarded and the cells were washed twice with 400 µL STE buffer (100 mM NaCl, 10 mM tris-HCl, 1 mM EDTA, pH 8.0), centrifuging at 10 000 rpm for 2 minutes between each wash step. The buffer was discarded and the cells were resuspended in 200 µL Tris-EDTA (TE) buffer (10 mM tris-HCl, 1mM EDTA, pH 8.0). Resuspended cells were extracted with 100 µL tris-saturated phenol (pH 8.0) by lysing the cells using a vortex for 1 minute.

Table 4.1: Optimal growth conditions and medium for optimal growth of test strains of interest

American Type Culture Collection (ATCC) strains	Culture Method
Bacillus cereus ATCC 10876	**Medium:** Nutrient agar/broth **Growth Conditions** **Temperature:** 30°C **Atmosphere:** Aerobic
Enterobacter cloacae subsp. cloacae ATCC BAA-1143, *Enterococcus faecalis* ATCC 29212, *Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* subsp*. aureus* ATCC 29213	**Medium:** Trypticase Soy Agar/Broth **Growth Conditions** **Temperature:** 37°C **Atmosphere:** Aerobic

Enterococcus faecalis ATCC 51299	**Medium:** Brain Heart Infusion agar/broth with vancomycin 4 mcg/ml **Growth Conditions** **Temperature:** 37°C **Atmosphere:** Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* ATCC 700603, *Salmonella enterica* subsp. *arizonae* ATCC 13314, and *Staphylococcus aureus* subsp. *aureus* ATCC 33591.	**Medium:** Nutrient agar/ broth **Growth Conditions** **Temperature:** 37°C **Atmosphere:** Aerobic
Yeast strains: *Candida albicans* ATCC 24433, *Candida albicans* ATCC 90028, *Candida krusei* ATCC 14243, *Candida krusei* ATCC 34135, *Candida tropicalis* ATCC 750	**Medium:** Nutrient agar/ broth **Growth Conditions** **Temperature:** 30°C **Atmosphere:** Aerobic
*Domestic Rainwater Harvesting (DRWH) Strains	**Culture Method**
Bacillus cereus	**Medium:** Nutrient agar/broth **Temperature:** 30 °C
Escherichia coli K4 CCA, *Escherichia coli* Mi CCA, *Enterobacter cloacae* 11, *Enterobacter cloacae* 19, *Enterobacter cloacae* 20, *Enterobacter cloacae* 22, *Enterobacter cloacae* 23, *Enterococcus faecalis* C513, *Klebsiella pneumoniae* 1, *Klebsiella pneumoniae* 2, *Klebsiella pneumoniae* 3, *Klebsiella pneumoniae* 4, *Klebsiella pneumoniae* 5, *Klebsiella pneumoniae* 6, *Klebsiella pneumoniae* 7, *Klebsiella pneumoniae* 8, *Pseudomonas aeruginosa, Salmonella enterica* 4C, *Salmonella enterica* 5J, *Staphylcoccus aureus* 1, *Staphylcoccus aureus* 2, *Staphylcoccus aureus* 3, *Staphylcoccus aureus* 4, *Staphylcoccus aureus* 5, *Staphylcoccus aureus* 6, and *Staphylcoccus aureus* 7	**Medium:** Nutrient agar/broth **Temperature:** 37 °C

*DRWH strains were previously isolated from RWH systems by members of the Khan laboratory, Stellenbosch University. These test strains were characterised phenotypically and biochemically to determine the genus it belongs to. Note: Media were prepared as per the manufacturer's instructions.

The samples were centrifuged at 12 000 rpm for 5 minutes to separate the aqueous phase from the organic phase and ~ 160 μL of the upper phase was transferred into a sterile 1.5 mL Eppendorf tube after which 40 μL of TE buffer was added to the tube (this step was repeated 2–3 times). One hundred μL of chloroform: isoamyl alcohol (24:1) was added to the extracted samples and centrifuged at 12 000 rpm for 5 minutes (this step was repeated three times). The samples were then precipitated with an equal volume of isopropanol and the lysate was further purified by chloroform extraction until a white interface was no longer present. Approximately 180 μL of the upper phase was transferred into a sterile Eppendorf tube and 20 μL of TE was added to the sample. One hundred μL RNase A (100 μg/mL) was added to the samples and incubated at 37°C on a heating block for 30 minutes. 100 μL of chloroform was added to the

tube and centrifuged at 12 000 rpm for 5 minutes. ~ 150 µL of upper aqueous phase was transferred to a sterile 1.5 mL Eppendorf tube; this contained the gDNA that was used as a template for PCR with 16S rRNA primer sets (16S-F: 5' – AGAGTTTGATCITGGCTCAG - 3', 16S-R: 5' – ACGGITACCTTGTTACGACTT – 3') (de Bruijn, 2011).

Conditions for amplification of the 16S rRNA gene were: 96°C for 2 minutes (initial denaturation), 30 cycles of 96°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 2 minutes (extension) and a final extension of 72°C for 5 minutes. PCR products were purified according to the manufacturer's instructions using the Stratec Molecular MSB spin PCRapace kit. Samples were electrophoresed on a 1% (w/v) 1 X tris-acetate-EDTA (TAE) agarose gel (100 volts (V) for 1 hour (hr) and viewed under UV (Appendix G, Figures G1 and G2). The product of samples with low intensity bands were re-amplified using the same conditions and primer sets and samples were sequenced at Inqaba Biotec (http://www.inqababiotec.co.za/) for sequencing. Chromatograms received from Inqaba Biotec were edited in Chromas (the Chromas Lite program (Technelysium) can be downloaded from: http://www.technelysium.com.au/chromas.html), exported as a FASTA format file and a consensus sequence was produced using the multiple sequence alignment tool in DNAMAN (Lynnon Biosoft; www.lynnon.com). For those sequences where no successful consensus sequence was produced, the sequence generated using the forward primer only was used to identify the organism of interest. Forward/consensus sequences were uploaded onto the EzTaxon website (http://www.eztaxon.org/; Chun *et al*., 2007) to determine the identity of the DRWH system isolates. The EzTaxon database makes use of basic local alignment search tool (BLAST) (using the BLASTn function) using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare nucleotide sequences to sequence databases. Phylogenetic analysis was performed on the sequences from the BLAST and EzTaxon analysis, and *Corynebacterium diphtheria* strain ATCC 27010 was used as an out-group. The sequences were aligned using the ClustalW function of MEGA6 (http://www.megasoftware.net/mega.html) and the overhanging parts were deleted to produce sequences of equal length. A neighbour-joining phylogenetic tree was produced using MEGA6 (bootstrap setting of 1000) and the relationship of the strains were confirmed by creating phylogenetic trees using the minimum evolution and/or maximum parsimony functions. Conserved regions were indicated with an asterisk at the node of the branching

region of the tree. The degree of similarity between the tested sequences is determined by conserved regions.

4.2.3. Bioassay methods

4.2.3.1. Filter disk assay of crude extract from the supernatant filtrate (SNF) and cell mass extracted from fermentation broth from 2L baffled flasks, CSTR and ALR.

Antimicrobial activity of EtOAc culture extracts were determined using filter-disc diffusion agar assay method (Eckwall and Schottel, 1997). Sterile Whatman filter paper discs (Cat. No. 2501-150) were spotted with 10 µL EtOAc culture extracts (prepared as described in Chapter 3) and allowed to dry. Test strains were prepared (refer to section 4.2.1.2), and 0.1 mL 0.5 OD_{600} culture were spread plated onto Mueller Hinton agar (MHA) medium (g/L: 2 beef/meat extract, 17.5 casein hydrolysate, 1.5 starch and 17 bacteriological agar, pH 7.3) and left for 5–7 minutes. Impregnated filter disks were placed onto the surface of an inoculated MHA plate. The agar plates were incubated at optimal temperature (Table 4.1) for 24-36 hours and the diameter of zones of growth inhibition were measured. The assay was performed in duplicate and control plates contained a sterile filter disk control, EtOAc control, commercial antibiotic controls (ampicillin, chloramphenicol and vancomycin at 1 mg/mL) and test organism control without a filter disk.

4.2.3.2. Bioautography assay of crude extracts from the SNF and cell mass obtained from fermentation broth from 2 L baffled flasks, CSTR and ALR.

The bioautography assay was adapted from Betina (1973). Compounds of interest were analysed using TLC and visualised under ultraviolet (UV) light. Silica gel G F_{254} TLC plates were developed in a solvent system containing chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02, v/v/v/v) and allowed to dry O/N under a fume hood. Test bacteria were prepared (refer to section 4.2.1.2) and applied to the surface of the TLC plates with sterile absorbent cotton wool. Sealable plastic containers were lined with moist paper towel and the TLC plates covered with test bacteria were placed in containers and incubated O/N at optimal temperature (Table 4.1). A final concentration of 0.25 w/v thiazolyl blue tetrazolium blue (MTT) was prepared by dissolving MTT in a phosphate buffered saline solution (g/l): 2.26 Na_2HPO_4, 2.27 KH_2PO_4 and 8.00 NaCl at pH 7.0. After incubation, MTT was applied to the surface of the TLC plates using sterile non-absorbent cotton wool and the plates were incubated for ± 2-3 hours at optimal temperature (Table 4.1). This method relies on a staining using tetrazolium salts for visualisation of active bands. Living organisms have the ability to perform dehydrogenase

reactions which converts the tetrazolium salt into formazan which is intensely coloured (in this case, purple). The presence of antimicrobial activity results in a cream spot appearing around a purple background on the surface of the TLC plate, indicating the lack of dehydrogenase activity and hence the death of the test strain.

4.3.   RESULTS

4.3.1. The overlay technique to determine CZA14^T bio-activity potential against clinical ATCC control test strains and environmental DRWH isolates

The bio-activity potential of CZA14^T was tested by performing an agar overlay method. CZA14^T showed antimicrobial activity against a broad range of ATCC and DRW isolated Gram-positive and Gram-negative bacteria as well as against a yeast strain (Table 4.2). CZA14^T exhibited broad spectrum bio-activities against all of the bacterial clinical ATCC test strains, a number of DRWH isolates and one of the yeast clinical ATCC test strains.

Table 4.2: Antimicrobial activity of *Streptomyces pharetrae* CZA14^T on 153 + glycerol agar, pH 6.0 at 30°C after 10 days of growth against bacterial and yeast ATCC strains as well as environmental DRWH system isolated bacterial pathogens

Bacterial ATCC Strains	Average (mm²)	Yeast ATCC Strains	Average (mm²)	Environmental DRWH system isolates	Average (mm²)
B. cereus ATCC 10876	137	*C. albicans* ATCC 24433	NI	*B. cereus*	1247
E. cloacae ATCC BAA-1143	253	*C. albicans* ATCC 90028	NI	*E. coli* Mi CCA	NI
E. faecalis ATCC 29212	76	*C. krusei* ATCC 14243	NI	*E. coli* K4 CCA	106
E. faecalis ATCC 51299	81	*C. krusei* ATCC 34135	176	*E. cloacae* 11	110
E. coli ATCC 25922	262	*C. tropicalis* ATCC 750	NI	*E. cloacae* 19	NI
K. pneumoniae ATCC 700603	54			*E. cloacae* 20	275
P. aeruginosa ATCC 27853	311			*E. cloacae* 22	123
S. enterica ATCC 13314	94			*E. cloacae* 23	NI
S. aureus ATCC 29213	427			*E. faecalis* C513	NI
S. aureus ATCC 33591	556			*S. enterica* 4C	2133
				S. enterica 5J	NI
				P. aeruginosa	NI
				S. aureus (1)	1376
				S. aureus (2)	1866
				S. aureus (3)	ND
				S. aureus (4)	368
				S. aureus (5)	263

				S. aureus (6)	NI
				S. aureus (7)	NI
				K. pneumoniae (1)	64
				K. pneumoniae (2)	NI
				K. pneumoniae (3)	204
				K. pneumoniae (4)	NI
				K. pneumoniae (5)	NI
				K. pneumoniae (6)	NI
				K. pneumoniae (7)	NI
				K. pneumoniae (8)	NI

Key (Table 4.2): VW (very weak): <100 mm^2; W (weak), 100-1000 mm^2; M (moderate), 1001-2000 mm^2; S (strong), 2001-3000 mm^2; VS (very strong), >3000 mm^2; NI: no inhibition.

4.3.2. DNA isolation of Domestic Rainwater Harvesting; PCR and phylogenetic analysis

The 16S rRNA genes of the environmental strains isolated from DRWH systems were amplified using PCR and sequenced. Only strains against which CZA14^T showed the best antimicrobial activity during agar overlay bioassay were included in this experiment. The F1 primer sequences and consensus sequences were uploaded onto the EzTaxon server and the BLASTn function was used to identify the organisms most closely related to the DRWH isolate of interest. The results are presented in the form of a phylogenetic tree in Figure 4.2.

The identity of the DRWH system isolates used in this study was determined using 16S rRNA gene sequencing. *S. aureus* 2 was confirmed as *Staphylococcus argenteus* MSHR1132^T with 100 % identity. *S. aureus* 4 was identified as *S. aureus* DSM 20231^T with a 100 % identity. *E. faecalis* C513 was identified as *E. faecalis* ATCC 19433 with a 97 % identity. *P. aeruginosa* was confirmed to be a *P. aeruginosa* strain with an identity of 97 %. *B. cereus* was identified as an *Ochrobactrum* spp. with an identity of 87 %. *S. enterica* 4C strain isolated from a DRWH system was identified as *Providencia rettgeri* strain DSM 4542^T with a 97 % identity rating. *K. pneumoniae* 3 was identified as *Raoultella ornothinolytica* strain JCM 6096^T (formerly known as *Klebsiella ornothinolytica*) with an identity of 97 %; *K. pneumoniae* 3 is further grouped with three *Klebsiella* species with two of the three being *K. pneumoniae*. *E. cloacae* 11 was identified as *Kosakonia cowanii* CIP 107300^T, also known as *Enterobacter* sp. with an identity of 93 %. *E. cloacae* 20 and *E. cloacae* 22 were grouped with the *E. cloacae* species with a 28 % and 46 % sequence identity, respectively. *E. coli* K4 CCA, *S. aureus* 1, *S. aureus* 5 and *K. pneumoniae* 1 are related to *Klebsiella* and *Enterobacter* lineage which are of the family *Enterobacteriaceae* and could not be accurately classified due to poor sequence quality.
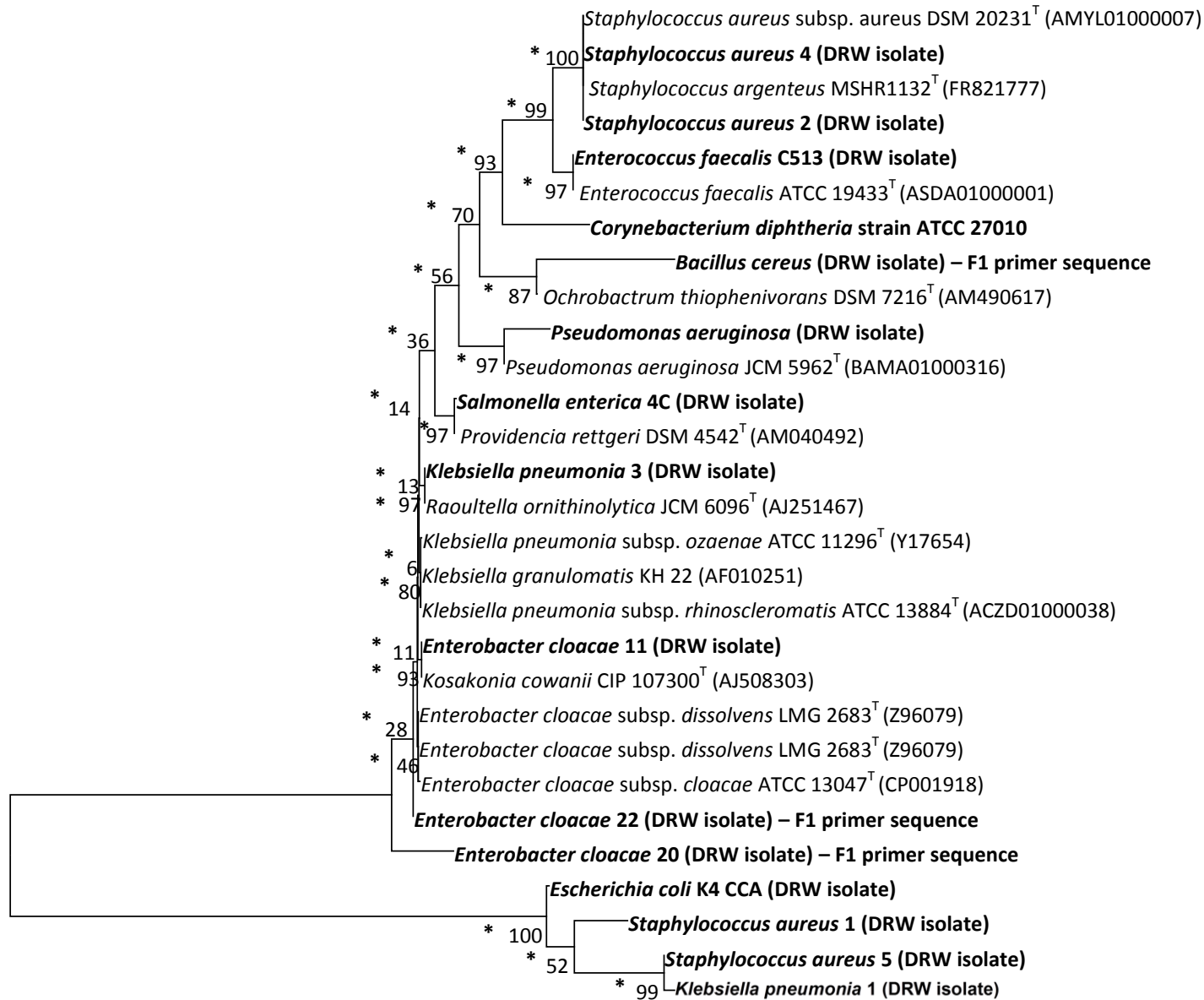
Figure 4.2: Neighbour-joining tree, bootstrap test of phylogeny of domestic rainwater isolates, 768 bp in length. An asterisk at the node of the tree is [91] indicative of conserved relationships between species. The more conserved the groupings are, the greater the degree of similarity between sequences tested.

4.3.3. Bioassay method

4.3.3.1. Filter disk assay of crude extract from the supernatant filtrate and cell mass extracted from fermentation broth from 2 L baffled flasks, CSTR and ALR.

Filter disk diffusion assay was performed to determine the diffusibility and activity of the compounds of interest. Experiments were performed in duplicate. Sterile filter disks and EtOAc controls were included in the assay, as well as antibiotic controls (10 µl of 1 mg/ml stock) – vancomycin, ampicillin and chloramphenicol. The results are presented in Figure 4.3 (against ATCC clinical control strains) and Figure 4.4 (against environmental DRWH isolates).
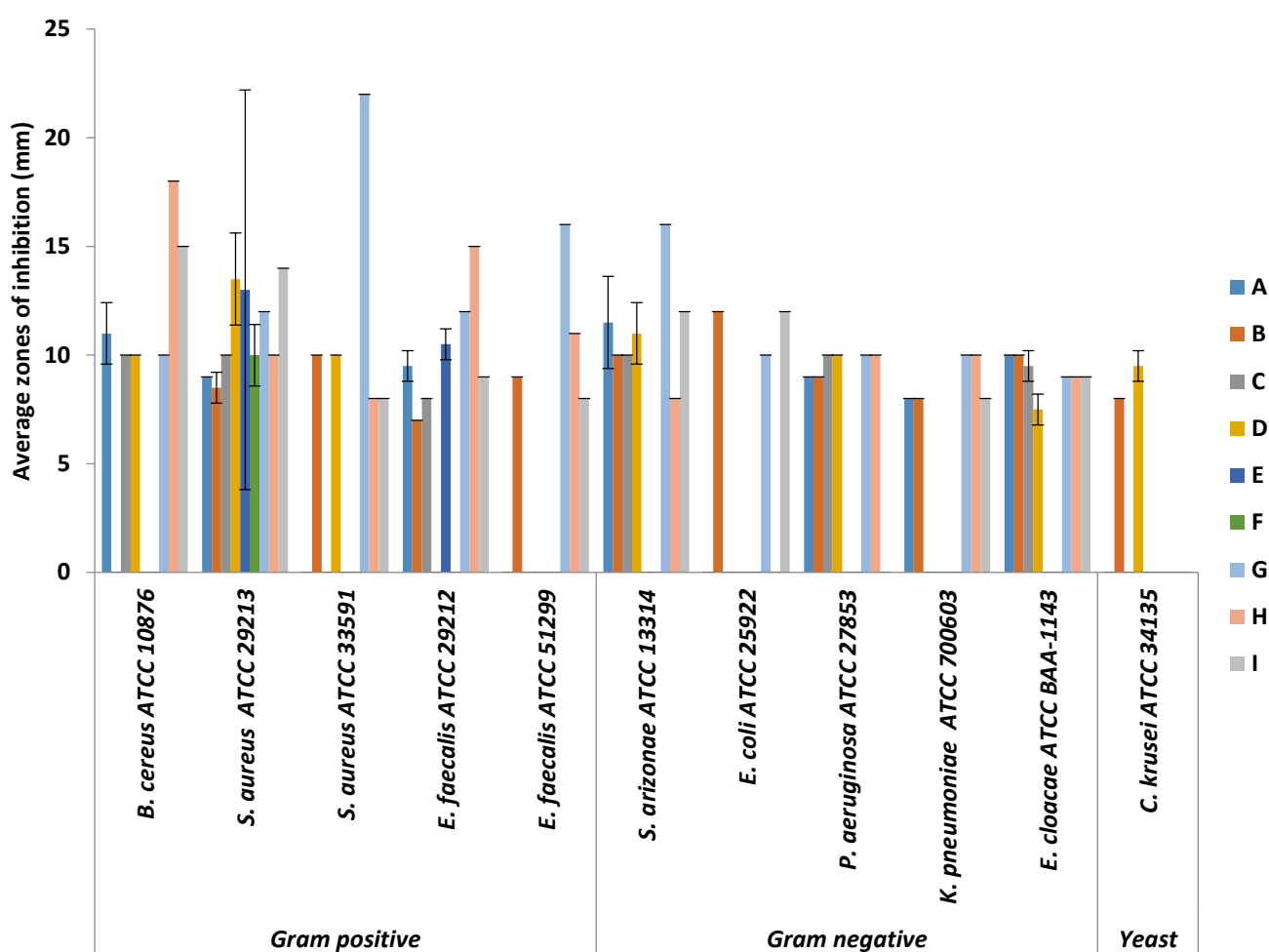
Figure 4.3: Filter disk assay of various extracts tested against ATCC clinical control strains.

Key: *Experiment* - A = EtOAc extract from SNF from ALR fermentation; B = EtOAc extract from cell mass from ALR fermentation; C = EtOAc extract from SNF from CSTR fermentation; D = EtOAc extract from cell mass from CSTR fermentation; E = EtOAc extract from SNF from 2L baffled flask fermentation; F = EtOAc extract from cell mass from 2 L baffled flask fermentation. *Controls* – G = ampicillin; H = vancomycin; I = chloramphenicol; sterile filter disk; and EtOAc control filter disk.
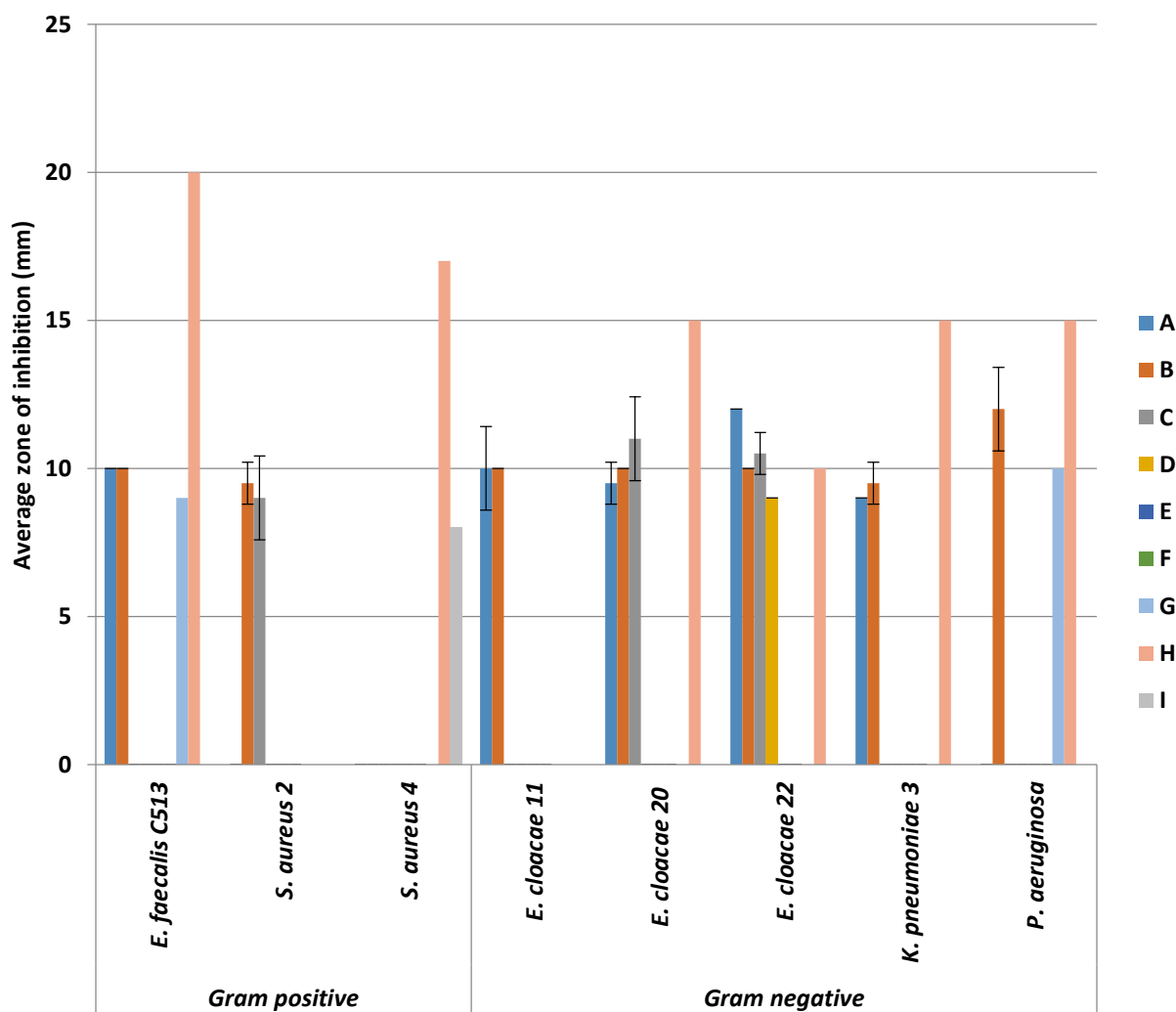
Figure 4.4: Filter disk diffusion assay of various extracts tested against environmental DRWH strains.

Key: *Experiment* - A = EtOAc extract from SNF from ALR fermentation; B = EtOAc extract from cell mass from ALR fermentation; C = EtOAc extract from SNF from CSTR fermentation; D = EtOAc extract from cell mass from CSTR fermentation; E = EtOAc extract from SNF from 2L baffled flask fermentation; F = EtOAc extract from cell mass from 2 L baffled flask fermentation. *Controls* – G = ampicillin; H = vancomycin; I = chloramphenicol; sterile filter disk; and EtOAc control filter disk.

Crude extracts from various fermentation vessels (2 L baffled flasks, ALR and CSTR), exhibited bio-activity against all of the bacterial and yeast clinical ATCC test strain/s). However, these crude extracts were active against all of the DRWH isolates except *S. aureus* 4 and *K. pneumoniae* 1. Larger zones of inhibition were detected against the DRWH system isolates, with broader range of bio-activities exhibited by the crude extracts from the ALR.

4.3.3.2. **Bioautography assay of crude extracts from the SNF and cell mass extracted from fermentation broth from 2 L baffled flasks, CSTR and ALR.**

Bioautography assay was performed to determine the antimicrobial profiles of the crude extracts against a number of ATCC clinical control strains and DRW isolated bacteria. Experiments were performed in duplicate and results are presented in Tables 4.3 and 4.4, respectively.

Table 4.3: R_f values of bands which exhibited inhibition against ATCC test strains during bioautography assay

Gram positive ATCC test strains		Gram negative ATCC test strains	
Test strain	R_f values of active compounds: samples A-F (in duplicate SD±0)	Test strain	R_f values of active compounds: samples A-F (in duplicate SD±0)
B. cereus ATCC 10876	[B]0.94, [D]0.91, [A,B]0.61, [C]0.56, [A,B]0.26, [E,F]N/I	*S. enterica* ATCC 13314	[B]0.84, [A]0.52, [A,B]0.47, [A]0.26, [C,D, E, F]N/I
S. aureus ATCC 29213	[A,B,C,E.F]0.94, [A,B,D,F]0.91, [B]0.52	*E. coli* ATCC 25922	[B]0.94, [D,F]0.91, [A]0.61 - 0.26, [A,B]0.47, [C,E]N/I
S. aureus ATCC 33591	[A,B,D]0.94, [F]0.91, [A,B]0.84, [B]0.62, [A,B]0.52 - 0.26	*P. aeruginosa* ATCC 27853	[B]0.84, [B]0.52, [A,C,D,E,F]N/I
E. faecalis ATCC 29212	[E]0.91, [A,B,D]0.84, [A,B]0.57 - 0.26, [C,D,F]N/I	*K. pneumoniae* ATCC 700603	[B]0.91, [B,C]0.52, [A,D,E,F]N/I
E. faecalis ATCC 51299	[D]0.94, [B,D]0.91, [B]0.84, [D]0.55, [A,B]0.47, [C,E,F]N/I	*E. cloacae* ATCC BAA-1143	[B,C,D]0.91, [B]0.57 - 0.26, [A,E,F]N/I
Test strain		R_f values of active compounds: samples A-F (in duplicate SD±0)	
Yeast strain: *C. krusei* ATCC 34135		[B,D,F]0.94, [A,B]0.52 - 0.26, [C,E]N/I	

Key: A: EtOAc extract from ALR fermentation, SNF; B: EtOAc extract from ALR fermentation, cell mass; C: EtOAc extract from CSTR fermentation, SNF; D: EtOAc extract from CSTR fermentation, cell mass; E: EtOAc extract from 2L baffled flask fermentation, SNF; and F: EtOAc extract from 2 L baffled flask fermentation, cell mass. N/I: No inhibition

Table 4.4: R_f values of bands which exhibited inhibition against DRWH isolates during bioautography assay

Gram positive DRWH isolates		Gram negative DRWH isolates	
Test strain	**R_f values of active compounds: samples A-F (in duplicate SD±0)**	**Test strain**	**R_f values of active compounds: samples A-F (in duplicate SD±0)**
E. coli K4 CCA (*Enterobacteriaceae*)	A,B0.94, B,D0.91, A,B0.84, B0.81, A,B0.47 - 0.26, C,E,FN/I	*B. cereus* (*Ochrobactrum* spp.)	A,B,F0.94, A,B0.47 - 0.26, C,D,EN/I
E. faecalis C513	B0.94, B0.91, B0.84, A0.81, A,B0.52 - 0.26, C,D,E,FN/I	*E. cloacae* 11	F0.94, A,B,D,E0.91, B,D,F0.84, A0.81, A,B0.52 - 0.26, CN/I
S. aureus 1 (*Enterobacteriaceae*)	A,B,D,F0.94, B,D0.91, F0.84, A,B0.84 – 0.34, C,EN/I	*E. cloacae* 20 (*Enterobacteriaceae*)	A,B0.52 - 0.26, C,D,E,FN/I
S. aureus 2	A,E,F0.94, B,D0.91, A,B,D,F0.84, B0.52 – 0.34, A0.52 – 0.26, CN/I	*E. cloacae* 22	A,B,F0.94, D0.91 - 0.47, A,B0.84, A,B0.52 - 0.26, C,D0.47, EN/I
S. aureus 4	A,B,C,D,E,F0.94, B,D0.91, A,B,D,F0.84, A0.60, A,B0.51, A,B0.49, A,B0.46, A,B0.45, A,B0.42, A,B0.40, A,B0.37, A,B,F0.34	*K. pneumoniae* 1 (*Enterobacteriaceae*)	A,B,C0.94, B,C0.84, D,E,FN/I
		K. pneumoniae 3	A0.57 – 0.10, B0.56 – 0.23, C,D,E,FN/I
		P. aeruginosa	A,B0.57 – 0.34, C,D,E,FN/I
		S. enterica 4C (*P. rettgeri*)	A,B,C0.91, A0.57 – 0.26, B0.57 – 0.34, D,E,FN/I
		S. aureus 5 (*Enterobacteriaceae*)	B0.94, B0.56 – 0.34, A0.57 – 0.26, C,D,E,FN/I

Key: A: EtOAc extract from ALR fermentation, SNF; B: EtOAc extract from ALR fermentation, cell mass; C: EtOAc extract from CSTR fermentation, SNF; D: EtOAc extract from CSTR fermentation, cell mass; E: EtOAc extract from 2L baffled flask fermentation, SNF; and F: EtOAc extract from 2 L baffled flask fermentation, cell mass. N/I: No inhibition

Day three to day 20 EtOAc extracts were pooled as similar bands were observed under short-wave length (UV_{254nm}). A comparative study of the bio-activity profiles of the compounds produced by CZA14[T] were performed between clinical ATCC control strains and environmental DRWH isolates. These results are presented in Tables 4.3 and 4.4, respectively. See Appendix H, Figures H1 – H25, for figure representation of information in the aforementioned Tables.

During the bioautography assay a number of active bands were observed against all of the test strains used in this study. Active bands with similar R_f values were detected from the various fermentation vessels used. More active compounds were observed during the bioautography assay than what was determined during the filter disk diffusion assay. Active bands were also more frequently observed in the crude extracts from the ALR.

4.4. DISCUSSION

Agar overlay assay

During bio-activity testing against a number of ATCC test strains using the agar overlay method with CZA14^T cultured on optimal media – 153 + glycerol, pH 6.0 at 30°C for 10 days; CZA14^T showed very weak bio-activity against the *Enterococcus* ATCC strains, *K. pneumoniae* ATCC 700603 and *S. enterica* ATCC 13314. Weak activity was exhibited against *B. cereus* ATCC 10876, *E. cloacae* ATCC BAA-1143, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and the *S. aureus* ATCC test strains. Poor bio-activity was displayed against the four yeast ATCC test strains with weak activity exhibited against *C. krusei* ATCC 34135 only.

Out of the twenty seven DRWH isolates received, fourteen strains were sensitive to compounds produced by strain CZA14^T and was used further in this study. No bio-activity was exhibited against the following DRWH isolates: *E .coli* Mi CCA, *E. cloacae* 19 and 23 isolates, *E. faecalis* C513, *S. enterica* 5J, *S. aureus* 3, 6 and 7 isolates, *K. pneumoniae* 2, 4, 5, 6, 7 and 8 isolates, and *P. aeruginosa*. These test strains, except *E. faecalis* C513 and *P. aeruginosa*, were not further used in this study. Regrowth around the CZA14^T colony was observed during agar overlay assay; these organisms are typically drug resistant and were thus included in this study. Very weak bio-activity was exhibited against *K. pneumoniae* 1 DRWH isolate. Weak bio-activity was displayed against *E. coli* Mi CCA, *E. cloacae* 11, 20 and 23 isolates, *S. aureus* 4, 5 and 6 isolates, and *K. pneumoniae* 3 DRWH system isolates. Moderate bio-activity was displayed against *B. cereus, S. aureus* 1 and 2 isolates, and strong bio-activity was displayed against *S. enterica* 4C DRWH system isolate.

On solid media, actinomycetes reproduce by sending up specialised aerial branches, which forms spores. Nutrient limitation causes the production of spores, melanin or diffusible pigment production due to stress; the reproductive phase is coordinated with the secretion of antibiotics which protect the colony against invading bacteria during aerial growth and sourcing

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of new nutrient sources. Diffusibility in the agar medium may, however, be limited, thereby resulting in the low levels of bio-activity detected in this study when the agar-overlay technique was used.

Identification of DRWH isolates

ATCC test strains used in this study are well-characterised in literature; however, isolates from environmental sources are not as well-characterised and are considered more virulent than clinical isolates as they are found in natural sources, where the probability of mutation and its ability to cause disease is much higher (Engering *et al*., 2013). Environmental strains isolated from DRWH systems used in this study were identified to genus level using universal F1 and R5 primers for 16S rRNA gene sequencing. The results are presented in the form of a neighbour-joining phylogenetic tree in Figure 4.2. Seven out of the fourteen DRWH isolates were confirmed for what it was previously identified as with the use of biochemical and phenotypic testing. The other seven species - *B. cereus, S. enterica* 4C, *K. pneumoniae* 3 were identified as *Ochrobactrum* spp., *Providencia rettgeri* DSM 4542[T] and *Raoultella ornithinolytica* JCM 6096[T] respectively; and *K. pneumoniae* 1, *E. coli* K4 CCA, *S. aureus* 1 and *S. aureus* 5 were identified as members of the family *Enterobacteriaceae*. These isolates are common waterborne pathogens. A study by Dobrowsky *et al*. (2014) (from which some of these samples were received) determined that of the bacteria isolated from DRWH systems situated in Kleinmond, South Africa, *Enterobacteriaceae* represented the largest family found in one of the sampling sessions. During two of the four sampling sessions, a number of *Providencia* spp., *Raoultella* spp. and *Ochrobactrum* spp., were also isolated. *S. aureus* 2 16S rRNA gene sequence show 100% similarity to *S. argenteus,* this is a newly described species that have near identical 16S rRNA gene sequences and are phenotypically similar to *S. aureus* (Tong *et al*., 2015). This can be seen by the arrangement of the phylogenetic tree (Figure 4.2), with *S. aureus* DSM 20231[T] (AMYL01000007) being grouped with the *S. aureus* 2 DRW isolate and *S. argenteus*. The *S. argenteus* strain has distinct differences – it demonstrates a lack of admixture, phylogentically distant at a whole-genome level, an average nucleotide identity of less than 95% and inferred DNA-DNA hybridisation of less than 70%. *K. pneumoniae* 3 shows 97% similarity to *R. ornithinolytica*, formally classified in the genus *Klebsiella*. *K. pneumoniae* 3 also shows similarity to two *K. pneumoniae* species and another species of *Klebsiella*. Further sequencing of the 16S rRNA gene could be performed to confirm the identity of this species. *E. cloacae* 11 shows 93% similarity to *Kosakonia cowanii* also known as *Enterobacter cowanii*. This is a novel species

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described by Inoue *et al*. (2000), with its closest neighbour being *E. cloacae* (which can also be seen by the arrangement of the phylogenetic tree in Figure 4.2).

After determining the antimicrobial bio-active profiles of CZA14[T], bioassays were performed on the crude extracted compounds from the various fermentation vessels to assess whether similar bio-activities will occur when CZA14[T] is cultured in liquid media.

Filter disk diffusion assay

Extra- and intracellularly produced compounds extracted as crude compounds from fermentation broth from 2 L baffled flasks, CSTR and ALR were used to test the efficacy of these crude extracts against a number of ATCC test strains and DRWH isolates using the filter disk diffusion assay. Similar compounds were produced during fermentation with these various fermentation vessels and crude compounds extracted at different sampling days from each fermentation vessel type were pooled together and used during bio-activity assays.

Filter disk diffusion assay using pooled crude extracts from 2 L baffled flasks:

Selected ATCC test strains and DRWH system isolates were used in the filter disk diffusion assay; selection was based on the strains which were inhibited by strain CZA14[T] when cultured on solid media. Activity was detected against *S. aureus* ATCC 29213 of compounds produced both extra- and intracellularly. Extracellularly produced compounds were active against *E. faecalis* ATCC 29212 and *B. cereus* DRWH system isolate which was re-identified as *Ochrobactrum* spp., after 16S rRNA gene sequencing. No activity was detected against *E. cloacae* 11 DRWH isolate. Regrowth was observed where a halo formed around the impregnated filter disk for all aforementioned strains except for the vancomycin-resistant *S. aureus* (VRSA) ATCC 29213. Bactericidal activity was exhibited by compounds produced both extra- and intracellularly by CZA14[T] against the *S. aureus* ATCC 29213 test strain. An average zone of inhibition of 10 mm (SD±1.4) was observed for intracellularly produced compounds while an average 13 mm (SD±9.2) zone of inhibition was observed for extracellularly produced compounds.

Filter disk diffusion assay using pooled crude extracts extracted from fermentation broth from CSTR:

Extra- and intracellularly produced compounds produced during fermentation in a CSTR were active against the following ATCC test strains: *B. cereus* ATCC 10876, *S. enterica* ATCC 13314, *P.*

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aeruginosa ATCC 27853, VRSA and *E. cloacae* ATCC BAA-1143. Only extracellularly produced compounds were active against *E. faecalis* ATCC 29212 and only intracellularly produced compounds were active against methicillin-resistant *S. aureus* ATCC 33591 (MRSA) and yeast ATCC strain *C. krusei* ATCC 34135. Poor bio-activity was detected against DRWH isolates. Extracellularly produced compounds were active against *Ochrobactrum* spp., *E. coli* K4 CCA (identified as a member of the family *Enterobacteriaceae* after 16S rRNA gene sequencing), *E. cloacae* 20 (*Enterobacteriaceae)*, *E. cloacae* 22 isolate, *S. aureus* 1 (*Enterobacteriaceae*) and 2 isolates. Intracellularly produced compounds were active against *E. coli* K4 CCA, *E. cloacae* 22, *S. enterica* 4C (identified as *P. rettgeri* strain DSM 4542^{T} after 16S rRNA gene sequencing) and *S. aureus* 5 (identified as a member of the family *Enterobacteriaceae* after 16S rRNA gene sequencing). In most cases regrowth was observed, this can be defined as bacteriostatic activity whereby the antimicrobial agent (in this case, the crude extract) prevented the growth of the test bacterium by keeping it in the stationary phase of growth. The biggest zone of inhibition was detected against the VRSA strain, with an average zone of inhibition 13.5 mm (SD±2.1) for each replicate of compounds produced intracellularly. These compounds exhibited bactericidal activity, meaning that it has the ability to kill the test bacterium.

Filter disk diffusion assay using pooled crude extracts extracted from fermentation broth from ALR:

Larger zones of clearing/inhibition were observed against ATCC test strains and DRWH isolates with compounds produced during fermentation in the ALR compared to bio-activity results of crude compounds produced during fermentation in 2 L baffled flasks and the CSTR used in this study, respectively. Extracellularly produced compounds were active against seven ATCC test strains and nine DRWH isolates (Figure 4.3 and 4.4). Regrowth was observed in most cases during the filter disk-diffusion assay. The most interesting is the 9 mm – 9.5 mm zone of inhibition against the VRSA strain and *E. faecalis* ATCC 29212. Another interesting result is bactericidal activity exhibited against the DRWH isolates - *E. cloacae* 20 and *E. faecalis* C513. Intracellularly produced compounds were active against ten ATCC test strains and ten DRWH isolates (Figure 4.3 and 4.4). The most interesting of these is the 9 mm – 10 mm zone of inhibition against VRE and *E. faecalis* C513 DRWH isolate. Other interesting bio-activity results are the average 10 mm (SD±0) zone of inhibition against MRSA. Bactericidal and bacteriostatic activity was exhibited, respectively. An average of 12 mm (SD±1.4) zone of inhibition was detected against *P. aeruginosa* DRWH isolate and crude compounds exhibited bactericidal

activity. An average zone of inhibition of 15 mm (SD±0) was detected against *S. aureus* 5 (which was identified as a member of the family *Enterobacteriaceae* after 16S rRNA gene sequencing) and these intracellularly produced crude compounds displayed bacteriostatic activity.

The commercial antibiotics used in this study fared well in inhibiting the growth of a number of ATCC test strains and DRWH isolates. Overall the filter disk-diffusion assay was successful in terms of eliminating the incidence of false-negative results as 0 mm zones of inhibition were detected for EtOAc control disks and sterile filter disk controls.

Filter disk diffusion assays as a stand-alone bioassay tend to be unreliable in detecting the inhibitory effects of compounds against test organisms as a number of factors can cause false-positive/negative results. False negative results with disk diffusion assays may be due to the rate at which the antimicrobial diffuses through the agar which is dependent on its chemical properties (i.e. polarity, etc.) as well as solubility properties of the antimicrobial in MH agar [in this study MH agar was used as it is considered the best medium for susceptibility testing as it shows acceptable batch-to-batch reproducibility; it provides satisfactory growth of most non-fastidious pathogenic bacteria and it is low in tetracycline, trimethoprim and sulphonamide inhibitors (Hudzick, 2013)]. The molecular weight of the produced antimicrobial also plays a role as larger molecules typically diffuse at a slower rate than those with a lower molecular weight. Other factors involved may be the concentration of the extracts tested and the depth of the agar which may cause a false positive result, to name a few. If the agar is too shallow it will result in larger zones of inhibition as antimicrobials diffuses in three-dimensions. Thus a second or even a third bioassay should be performed to validate the filter disk diffusion assay results. Bioautography assay was used as a second bioassay in this study.

Commercial antibiotics faired well against the ATCC test strains used in this study and produced larger zones of inhibition compared to the compounds produced by CZA14[T]. This could be due to the differences in concentration between the commercial antibiotics and compounds produced by CZA14[T]. The commercial antibiotics may be more targeted towards a certain group of organisms as they are pure compounds, compared to the crude CZA14[T] extracts. Activity with ampicillin against the MRSA strain and activity with vancomycin against the vancomycin resistant *Enterococcus* may be an anomaly as these antibiotics typically have no affect on these strains. This again may be attributed to poor drying of the compounds on the filter disk resulting in a false positive result. No inhibition was detected against the candida strain with

the commercial antibiotics, this was expected. Interestingly, crude intracellular compounds produced by CZA14[T] were active against the *C. krusei* ATCC test strain.

Bio-activity of compounds from the airlift reactor was thus compared to activity with commercial antibiotics. Vancomycin was active against the *Enterococcus* rainwater harvesting system isolate, suggesting that this strain may be a non-vancomycin resistant *Enterococcus* sp. Vancomycin was active against most of rainwater system isolates except, *Enterobacter* sp., strain 11 and *S. aureus* strain 2. No activity was detected against *S. aureus* strain 2 with the commercial antibiotics; however activity was detected against this strain with intracellular crude CZA14 extracts.

Bioautography assay

Extra- and intracellularly produced compounds extracted as crude compounds from fermentation broth from 2 L baffled flasks, CSTR and ALR were used to test the efficacy of these crude extracts against a number of ATCC test strains and DRWH isolates using the bioautography assay. Similar compounds were produced during fermentation with these various fermentation vessels and crude compounds extracted at different sampling days from each fermentation vessel type were pooled together and used during bio-activity assays.

Bioautography assay using pooled crude extracts from 2 L baffled flasks:

No inhibition was detected against the following ATCC test strains and DRWH isolates:

ATCC - *B. cereus* ATCC 10876, *S. enterica* ATCC 13314, *P. aeruginosa* ATCC 27853 and *K. pneumonia* ATCC 700603.

DRWH - *E. coli* K4 CCA (*Enterobacteriaceae*), *E. cloacae* 20 (*Enterobacteriaceae*), *E. faecalis* C513, *K. pneumonia* 1 (*Enterobacteriaceae*) and 3, *P. aeruginosa, S. enterica* 4C (*P. rettgeri* strain DSM 4542[T]) and *S. aureus* 5 (*Enterobacteriaceae*).

Extracellularly produced compounds were active against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212. These compounds were also active against a number of DRWH isolates namely, *E. cloacae* 11 and *S. aureus* isolates 2 and 4. Intracellularly produced compounds were active against *E. coli* ATCC 25922, *S. aureus* ATCC 29213, MRSA and *C. krusei* ATCC 34135. These intracellularly produced compounds were also active against *B. cereus* (*Ochrobactrum* spp.), *E. cloacae* 11 and 22 isolates and *S. aureus* 1 and 4 isolates, all isolated from DRWH systems.

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<u>Bioautography assay using pooled crude extracts extracted from fermentation broth from CSTR:</u>

No inhibition was observed against the following ATCC test strains and DRWH isolates:

ATCC - *S. enterica* ATCC 13314, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212.

DRWH - *B. cereus* (*Ochrobactrum* spp.,), *E. faecalis* C513, *K. pneumonia* 3, *P. aeruginosa* and *S. aureus* 5 (*Enterobacteriaceae*).

Extracellularly produced compounds were active against *B. cereus* ATCC 10876, *K. pneumonia* ATCC 700603, *S. aureus* ATCC 29213 and *E. cloacae* ATCC BAA-1143 ATCC test strains. These compounds were also active against a number of DRWH isolates such as *E. cloacae* 22, *K. pneumonia* 1 (*Enterobacteriaceae*), *S. enterica* 4C (*P. rettgeri* strain DSM 4542^T) and *S. aureus* 4. Intracellularly produced compounds were active against ATCC test strains: *B. cereus* ATCC 10876, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, MRSA, VRE, *E. cloacae* ATCC BAA-1143 and yeast strain, *C. krusei* ATCC 34135. These compounds were also active against DRWH isolates: *E. coli* K4 CCA (*Enterobacteriaceae*), *E. cloacae* 11 and 22, and *S. aureus* 1 (*Enterobacteriaceae*), 2 and 4.

<u>Bioautography assay using pooled crude extracts extracted from fermentation broth from ALR:</u>

More active compounds were produced during the culturing of CZA14^T in the ALR used in this study compared to the number of active compounds produced during culturing of CZA14^T in the 2 L baffled flasks and CSTR. Crude solvent extracts from the fermentation broth from the ALR was active against all of the ATCC test strains and DRWH isolates (Tables 4.3 and 4.4). These crude extracts were thus further accessed for the bio-active potential and partially purified. The results pertaining to the purification of these crude extracts are presented in Chapter 5 of this study.

Bioautography is a microbiological screening method that can be used to confirm results obtained with the filter disk diffusion assay. The method of bioautography used in this study is known as direct bioautography and is one of the bioautography methods most widely used as it is an inexpensive, simple and time-saving method that does not require sophisticated equipment and can easily be coupled with detection methods such as TLC. The method relies on a staining method using tetrazolium salts for visualisation of active compounds. Living organisms undergo dehydrogenase reactions which converts the tetrazolium salt into formazan

which is intensely coloured. This results in a cream coloured spot appearing around a purple background on the surface of the TLC plate which shows the presence of antimicrobial agents (Choma and Grzelak, 2011).

Common bands observed to be active against the ATCC clinical test strains (Table 4.3) had R_f values of 0.94, 0.91, 0.84, 0.62, 0.61, 0.57, 0.56, 0.55, 0.52, 0.47, and 0.26 (Appendix H). Bands active against the environmental DRWH isolates had bands with the same R_f values as those active against the ATCC test strains. The only interestingly different active bands observed were the 0.81 R_f value band active against *E. coli* K4 CCA (identified as a member of the family *Enterobacteriaceae*), *E. faecalis* C513 and *E. cloacae* 11. A band with an Rf value of 0.34 were active against *S. aureus* 1 (*Enterobacteriaceae*), 2 and 4 as well as the *P. aeruginosa* DRWH isolate and *S. enterica* 4C isolate (identified as *P. rettgeri*) (Appendix H). Active bands with R_f values of 0.60, 0.51, 0.49, 0.46, 0.45, 0.42, 0.40, and 0.37 were active against *S. aureus* 4 DRWH isolate (Appendix H)

Hospital acquired infections (HAIs) are commonly associated with Gram-positive pathogenic bacteria. These organisms are a key target for antimicrobial therapy. Gram-positives typically cause a spectrum of infections linked to/associated with skin structure and skin infections (SSSIs), endocarditis, bloodstream infections (BSIs), surgical site infections (SSIs), lower and upper respiratory tract infections, joint and bone infections, intestinal infections, diabetic foot infections, urinary tract infections (UTIs) and central nervous system infections. These organisms, as detailed in Chapter 1 and 2 of this study, are commonly associated with DRWH systems. Infections caused by these organisms may occur by consuming water polluted with Gram-positive bacteria, or due to contaminated artificial devices and central venous catheters associated with a hospital environment. The issue of antibacterial resistance is common amid Gram-positive bacterial pathogens, notably among enterococci and staphylococci. Staphylococcal resistance, especially MRSA are notorious examples of Gram-positive pathogens which are difficult-to-treat due to resistance to multiple classes of antibiotics. Members of this genus specifically are capable of global-scale diffusion causing various epidemics (EARS-Net, 2010). Multidrug resistant enterococci, especially *Enterococcus faecalis* are susceptible to the antibiotic daptomycin, which is not approved for the use in treating enterococci but is used often to treat severe infections caused by enterococci. A study by Palmer *et al*. (2011) described the resistance mechanisms of a multidrug resistant *E. facecalis* strain following the development of DapR during serial passaging. No observed mutations occurred in *yycE, rpoC,*

rpoB and *mprF* genes, however mutations were observed in *cls* (CL synthase) which arose during the development of the respective three lines of selection. It was suggested that enhanced functions were displayed by CL synthase enzymes as two of the three mutations led to substitutions of single amino acids and one of the three mutations led to a three amino acid in-frame deletion.

Gram-negative bacterial infections are an emerging threat to the immunocompromised and hospitalised patients, creating longer hospital stays or even mortality. The increasing crisis of Gram-negative bacteria with multidrug resistance has led to the use of an older polymyxin, colistin which is known to have nephrotoxicity and neurotoxicity (Rice, 2006; Kallel *et al*., 2006). However, resistance by several Gram-negative bacterial isolates to all available antibiotics as well as polymyxin have been reported (Falagas *et al*., 2008). Mechanisms of resistance are discussed in Chapters 1 and 2. 90 – 95% of all of the Gram-negative bacteria are pathogenic, whereas a number of Gram-positive bacteria are non-pathogenic. Gram-negatives are more resistant to antimicrobial agents whereas many Gram-positives are susceptible to antimicrobials. This could be as a result of the cell wall composition. Gram-negatives typically have cell walls that are 70 – 120 Armstrong thick. The cell wall is two layered with a high lipid content of 20 – 30% and a low Murein content of 10 – 20%. Gram-positives on the other hand are single layered with a cell wall 100 – 200 Armstrong thick. Low lipid content is observed in Gram-positives with a high Murein content of 70 – 80% (Silhavy *et al*., 2010). Most antimicrobials are targeted towards intracellular processes and their mechanism of action relies on its ability to penetrate the bacterial cell wall. Gram-negative bacteria provide formidable barriers which effect antimicrobial uptake into the bacterial cell. The protein and lipid content of the outer membrane play an important role on the sensitivity of bacteria to numerous types of antimicrobials; causing drug resistance involving modifications of these macromolecules (Delcour, 2009).

Systemic candidiasis results in high rates of worldwide morbidity and mortality in immunocompromised people. This common fungal infection imposes a great burden in industrialised country health-care systems as the antifungal resistance of *Candida* spp., is mostly unknown. Resistance to the commonly used antifungal drug, fluconazole, varies substantially by species and country. Some countries have already reported emerging resistance to echinocandins.

In order to fight against the crisis of resistance, new targets (such as, antimicrobial peptides) are required for the development of novel antimicrobials to treat infections caused by extensively drug resistance (XDR), pan-drug resistance (PDR) and the well-known multidrug resistance (MDR) bacteria. The most frequent MDR pathogenic bacteria currently are recognised as emerging threats of this century and result in infamous hospital acquired infections. These pathogenic microorganisms were dubbed 'ESKAPE' following their first initial and include *Enterococcus faecium/faecalis, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter* spp., *Pseudomonas aeruginosa* and *Enterobacter* spp. (Kanj and Kanafani, 2011).

Strain CZA14[T] produces antimicrobial compounds that inhibit the growth of the aforementioned pathogenic bacteria. Both clinical ATCC test strains and more virulent environmental DRWH isolates are inhibited by these antimicrobials. It's suggested that strain CZA14[T] produces antimicrobial peptides, also known as bacteriocins. Bacteriocins are typically extremely potent even at low concentrations. The bioactivities may be broad or narrow spectrum which are capable of targeting microorganisms from different genera or same species. Antimicrobial peptide mechanism of action occur by directly destabilising or damaging the bacterial, fungal or viral membrane of the organism or act on other targets of the microorganism (adapted from Durrell, 2013, honours thesis).

4.5. CONCLUSION

A comparison between the number of secondary metabolites produced by CZA14[T] in various fermentation vessels (2L baffled flasks, ALR and CSTR; Chapter 3) and the number of compounds active against the test strains used in this study (Chapter 4) determined using the bioautography assay is presented in Figure 4.5 and 4.6, respectively.
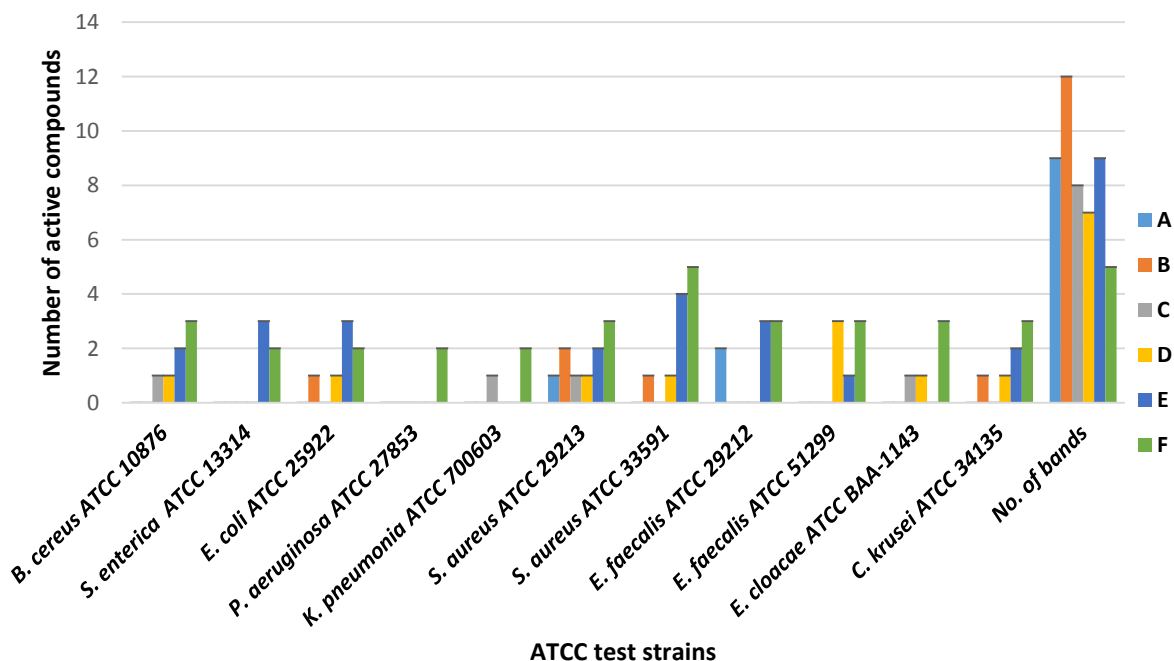
Figure 4.5: Number of compounds active against ATCC clinical test strains compared to the number of secondary metabolites produced during fermentation processes with CZA14[T] in various fermentation vessels.

Key: A: EtOAc extract from ALR fermentation, SNF; B: EtOAc extract from ALR fermentation, cell mass; C: EtOAc extract from CSTR fermentation, SNF; D: EtOAc extract from CSTR fermentation, cell mass; E: EtOAc extract from 2L baffled flask fermentation, SNF; and F: EtOAc extract from 2 L baffled flask fermentation, cell mass.
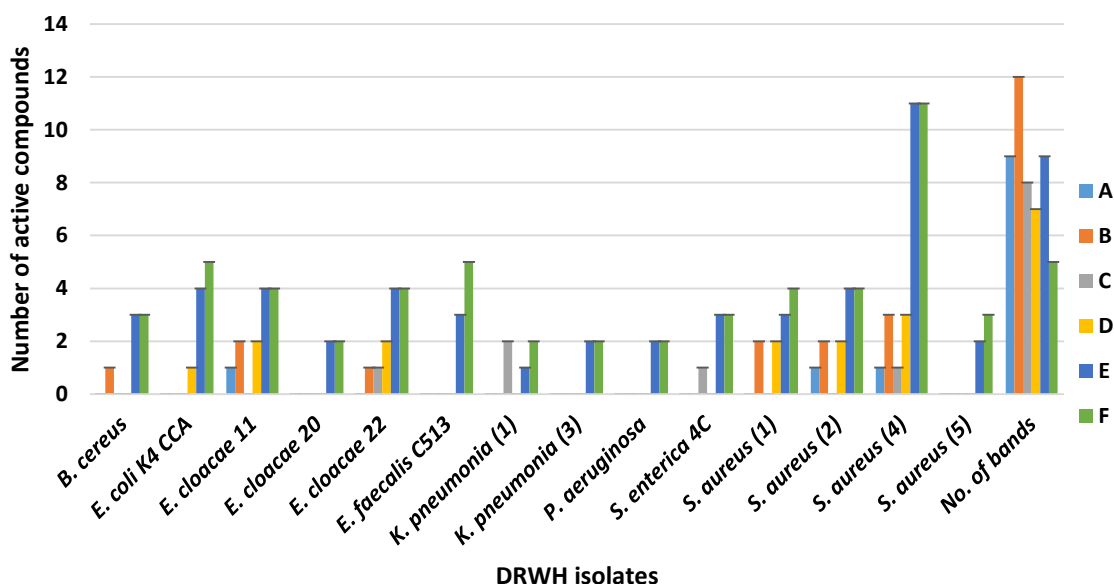
Figure 4.6: Number of compounds active against environmental DRWH isolates compared to the number of secondary metabolites produced during fermentation processes with CZA14[T] in various fermentation vessels.

Key: A: EtOAc extract from ALR fermentation, SNF; B: EtOAc extract from ALR fermentation, cell mass; C: EtOAc extract from CSTR fermentation, SNF; D: EtOAc extract from CSTR fermentation, cell mass; E: EtOAc extract from 2L baffled flask fermentation, SNF; and F: EtOAc extract from 2 L baffled flask fermentation, cell mass.

It was expected that antibacterial activity will be mostly against Gram-positive bacteria due to the classes of antimicrobials produced by actinomycetes which mainly target the bacterial cell wall of Gram-positive bacteria (Behal, 2000). Dominant antagonistic bio-activity was observed against Gram-positive ATCC clinical test strains and environmental DRWH system isolates during the bioautography assays. A number of compounds with the same R_f values were active against Gram-negative ATCC clinical test strains and environmental DRWH isolates and minor activity was detected against the yeast ATCC clinical test strain. The antimicrobial guided screening used in this study (overlays, filter disk assays and bioautography) showed that CZA14[T] possesses the ability to produce a number of different active antimicrobial compounds. A successful correlation between TLC bands and antimicrobial activity was performed, although standard filter disk assay showed less potential for bio-activity detection than what was found during bioautography. This may be attributed to poor diffusibility of the compounds or there are compounds binding to the filter disk into the agar medium, or there may be compounds binding to the filter disk. In addition, more bands were observed under UV light (TLC analysis) than what was active against the pathogenic strains used in this study.

These bands may have concentration/detection limits or may have other properties and other functionalities and most likely represent other SMs produced by strain CZA14[T]. It is important to note that the number of active compounds produced by CZA14[T] in the ALR may be more than estimated in Figures 4.5 and 4.6 as large zones of clearing was visible during bioautography assay and the exact band responsible for the activity could not be pin-pointed. Purification techniques were thus directed toward the purification of crude compounds extracted from the fermentation broth from the ALR and is further elaborated upon in the next chapter.

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CHAPTER 5:

The purification of selected compounds and the determination of their antimicrobial profiles
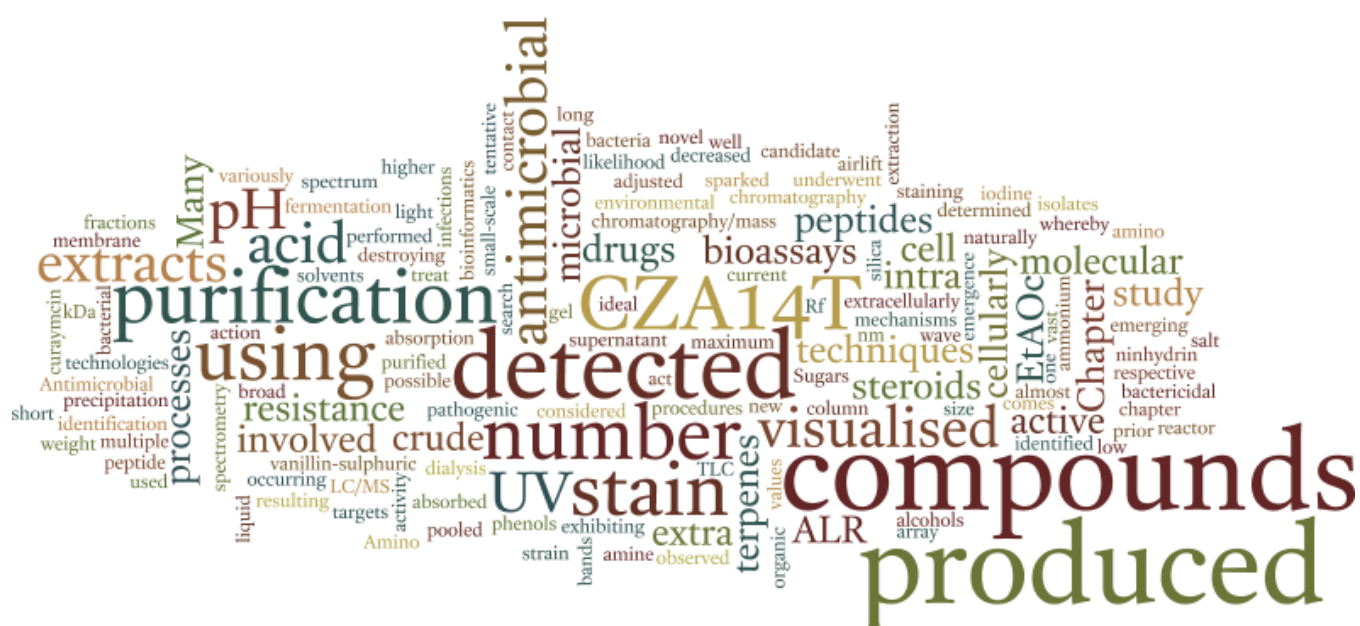
Abstract

The emergence of microbial resistance to current antimicrobial drugs have sparked the search for new drugs with novel mechanisms of action. Antimicrobial peptides are considered an ideal candidate to treat a vast array of microbial infections. These peptides are naturally occurring and they act by destroying the cell membrane of bacteria that it comes into contact with. They also have multiple cell targets resulting in the decreased likelihood of emerging resistance. In this study, a possible antimicrobial peptide was produced by strain CZA14^T. A low molecular weight compound (determined to be 6.7 kDa in size) and a number of other active compounds were produced by CZA14^T in the airlift reactor (ALR). These compounds exhibited a broad spectrum bactericidal activity against almost all of environmental domestic rainwater harvesting (DRWH) system isolates.

A number of purification processes were applied in the attempt to purify specific bio-active compounds. pH purification - whereby the pH of the supernatant filtrate from ALR fermentation was adjusted from pH 3.0 – 9.0 prior to extraction processes with various solvents. Other forms of purification techniques used in this study involved small-scale silica gel column chromatography, which was performed on pooled extra- and intra-cellularly produced ethyl acetate (EtAOc) crude extracts, as well as ammonium salt precipitation and dialysis on extracellularly produced compounds produced by CZA14^T in the ALR. A number of active bands with the same R_f values were detected during bioassays with the variously purified extracts. Many of these compounds were visualised under short- and long- wavelength UV light. Those that could not be visualised under UV were visualised using TLC staining techniques. Many of these compounds could be visualised using the universal iodine stain. Sugars, steroids and terpenes were detected with anisaldehyde-sulphuric acid stain. Amino acid, amine or amino groups were detected in one of the compounds when using the ninhydrin stain; and a number of higher alcohols, phenols, steroids and terpenes were detected with the vanillin-sulphuric acid stain. Bio-active antimicrobial compounds in liquid extract exhibited UV absorption between 280 – 570 nm. The tentative identification of curaymcin A, as identified by bioinformatics and molecular techniques (Chapter 6) were detected using liquid chromatography/mass spectrometry (LC/MS).

Key words: Purification, Antimicrobial peptides, compounds, Solvent extraction, ammonium sulphate precipitation, dialysis, silica gel chromatography

Introduction

Purification techniques should be applied to isolate one or more compounds from a complex mixture. During the purification process, 'non-active' and 'active' components of a complex mixture may be separated in order to separate the desired compound from all other metabolites in the mixture. The process of purification is important for the characterisation of the structure, function and interactions of a compound of interest. Separation of the desired compound may be achieved by exploiting the differences in physico-chemical properties, protein/compound size, and biological activity using preparative and/or analytical purification procedures (Darnell *et al.*, 1999).

A number of purification techniques were utilised during this study to fractionate crude extracts into individual components. Crude extracts can be partially purified using a number of solvent types with varying polarity. Another means can be by salt precipitation to remove all unwanted proteins from the sample prior to extraction. Bioassay-guided isolation was employed in this study whereby compounds in a mixture was separated using various analytical methods, with results collected from biological experimentation. The method involves the testing of the crude extract to determine its activity, after which the crude extracts are separated and the fractions are tested for bio-activity. Further fractionation of compounds of interest can be carried out while inactive fractions are discarded or set aside. This process of fractionation and biological testing is repeated until pure compound/s are recovered (Maryam *et al.*, 2012).

Chromatographic techniques are used to achieve fractionation of products. The term chromatography refers to the analysis of a particular product in which a mobile phase passes over a stationary phase so that the mixture of substances is separated into components. Thin-layer chromatography (TLC) is the primary chromatography method of separation, and involves isocratic chromatographic separation techniques wherein the stationary phase is made up of a thin layer which is applied to a solid support/substrate. In order for purification by liquid chromatography to be successful, the correct combination of operating conditions should be considered, such as the mobile phase and type of column packing, the diameter and length of the column, the flow rate of the column, sample size and elution temperature (Synder and Kirkland, 1979). Once an appropriate mobile phase is chosen, column chromatography is used

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to fractionate products. In this study a gradient mobile phase was used to elute products off a silica gel column. This is commonly performed by mixing different solvents to change the polarity conditions in the column. TLC and classical chromatography methods are time consuming and laborious (Moreda *et al.,* 2001), and requires the use of organic solvents in large quantities (Laczae *et al*., 2007). TLC have an advantage over classical column chromatography methods as the process is more rapid and the possibility of identifying the nature of the separated products by using specific reagents are much greater, but TLC also has a disadvantage over classical column chromatography methods as there is limited understanding of quantitative analysis of products given that the complete recovery of individual compounds are not sufficiently accurate (Moreda *et al*., 2001).

In this chapter, crude compounds extracted from fermentation broth after fermentation with CZA14^T in an airlift reactor were purified using various purification techniques such as: recovery of extracellularly produced compounds by adjusting the pH of the supernatant filtrate prior to extraction using various solvents, silica gel column chromatography, and salt precipitation using ammonium salt. This was performed to achieve objective four of this study. Methods employed to achieve this objective is presented in Figure 5.1.
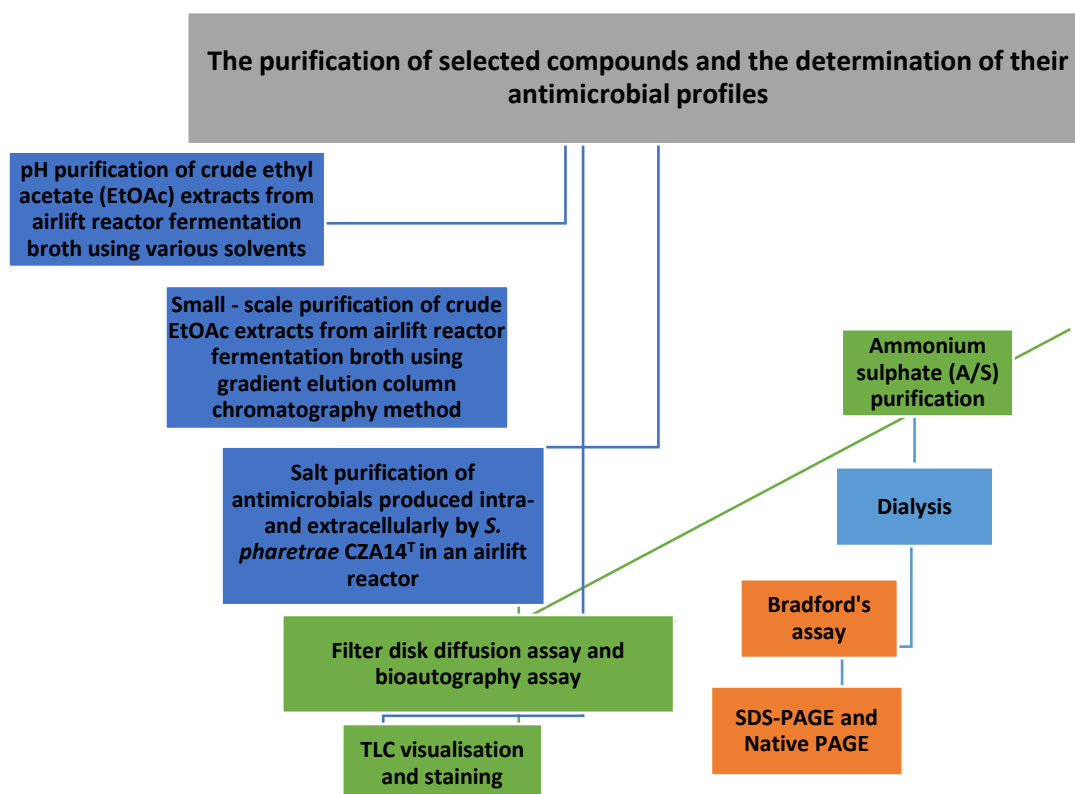
Figure 5.1: Flow-chart of methods used for the purification of selected compounds of crude extracts and the determination of their antimicrobial profiles.

Materials and Methods

5.1.   Materials

Most of the chemicals used in this study were purchased from Merck Millipore or Sigma-Aldrich unless otherwise stated. All solvents used were analytical grade.

5.2.   Methods

5.2.1. pH purification of extracellularly produced compounds by strain CZA14[T] in an airlift reactor

After fermentation in the airlift reactor (ALR) used in this study, the supernatant was separated from the mycelium by filtering it through 2X coffee filters followed by centrifuging the supernatant filtrate (SNF) at 10 000 rpm for 10 minutes. The SNF was separated from any remaining mycelia and the pH of the SNF was adjusted (by addition of 30 % HCl or 1M NaOH) to pH 3-9 in increments of 1 (the initial pH of the SNF was pH 8.28).

The pH-adjusted SNF was extracted overnight (O/N) through automatic shaking (160 rpm) at 37°C with various solvents such as n-butanol, chloroform, ethyl acetate, dichloromethane, benzene and petroleum ether at a 1:1 (v/v) ratio. The organic phase was removed, evaporated to dryness under a fume hood and reconstituted with the solvent it was extracted with to a 50 x concentrate. Silica gel G F$_{254}$ TLC plates were developed in a solvent system containing chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02 v/v/v/v) and allowed to dry O/N under a fume hood. The TLC containing separated crude extracts were bioassayed against domestic rainwater harvesting (DRWH) system isolate *Staphylococcus aureus* 4 as extra- and intra-cellular bio-activity was exhibited against this strain for almost all bands visualised under Ultra Violet (UV) light.

5.2.2. Silica gel column chromatography

Prior to small-scale purification of the crude EtOAc extracts, a 150 mL volume column with a diameter of 15 mm containing silica gel was used to purify the crude extracts using an isocratic elution containing a four component solvent system of chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02, v/v/v/v). The extra- and intra-cellular crude EtOAc extracts of samples day 0 - 20 were pooled at a 1:1 (v/v) ratio as bands with similar R$_f$ values were visible under shortwave 254 nm UV light. The crude extracts were applied to a packed silica gel 60 column. A

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150 mm length Pasteur pipette with a 4.5 mm diameter was used as a glass column for purification of the crude extracts. ~ 1 cm piece of cotton wool was placed into the lower end of the Pasteur pipette and ~ 1 cm level of sand was added to the top of the cotton wool. The acid-based silica gel (10 g) was deactivated for ten minutes using deionised water (dH$_2$O; 100 ml). Water was added to the column and once it reached one-third of column length the deactivated silica gel was added to the column and packed. The column was also gently flicked to encourage any bubbles to rise to the top; the column was filled to just below the indent of the pipette and the water was allowed to drain from the column. The column was equilibrated with a four component solvent system consisting of chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02, v/v/v/v) and sand was added to the surface of the silica gel to stabilise the column bed. Once the solvent reached ~ 5 mm above the sand, the 50 x concentrated crude extracts were applied to the column and 35 0.5 mL fractions were collected. The column was eluted with the abovementioned four component solvent system using a pipette bulb to apply pressure until all of the visible yellow coloured residual crude extract was eluted from the column. Two column volumes of 100% chloroform, 95: 5, 75: 25, 50: 50 v/v chloroform: EtOAc, EtOAc containing 85: 15 v/v EtOAc: methanol and 100% EtOAc were added to the column sequentially. All products were successfully eluted from the column except a dark red/brown coloured residual compound that stayed absorbed to the silica even after elution with dH$_2$O and absolute ethanol. The compound was removed along with some silica and will be freeze dried and tested for bio-activity in future studies.

5.2.3. Salt purification of bio-active proteins/peptides produced extracellularly by strain CZA14^T in an airlift reactor

5.2.3.1. Ammonium sulphate precipitation

The mycelium was separated from the supernatant by filtering the fermentation broth collected from the ALR after fermentation. Total proteins were slowly precipitated from the SNF using ammonium sulphate (A/S) at 80 % saturation (26.64 g per 50 ml volume of SNF; www.encorbio.com/protocols/AM-SO4.htm), stirring O/N at 4°C. The protein precipitate was obtained after centrifuging the SNF at 12 000 rpm for 10 minutes at 4°C. The supernatant was stored for further analysis and the pellet was resuspended in two pellet volumes of 20 mM Tris-HCl buffer, pH 7.5. Some of the sample was stored at 4°C for further analysis and the rest was dialysed.

5.2.3.2. Dialysis

Dialysis against 20 mM Tris-HCl buffer, pH 7.5 (at 4°C) was used to remove salts from the sample. Snake skin dialysis tubing was used and 30 mL of sample was added to the tubing. Buffer was changed every hour for the first 3 hours then dialysed overnight. Some of the sample was stored at 4°C while the rest was used in solvent extraction with EtOAc (2:3 v/v) O/N, shaking at 160 rpm at 37°C. The stored SNF was also extracted in the same manner to determine whether any bio-active compounds were still present and reconstituted to 50x concentrate with EtOAc.

5.2.3.3. Bradford's assay to quantify the total protein content in salt purified samples

The Bradford's protein assay (Kruger, N.J. 2010) was used to determine the total protein content of extracellular proteins produced by CZA14^T in an airlift reactor after salt precipitation, dialysis and solvent extraction. 1 mg/mL (bovine serum albumin) (BSA) (Sigma) stock was prepared by dissolving 1 mg BSA in 1 mL 0.15 M NaCl. A 1:10 dilution of the stock (with 0.15 M NaCl) was prepared to achieve a 100 μg/mL BSA working stock. Standards of known protein (BSA) concentrations were prepared as outlined in Table 5.1.

Table 5.1: Standards of known protein (BSA) concentrations used to construct a standard curve (refer to Appendix I) for determination of total protein content.

Standard	Vol 100 μg/mL BSA (μL)	Vol 0.15 M NaCl (μL)	[Protein] (μg/μL)
Blank	0	100	0
Standard 1	10	90	10
Standard 2	20	80	20
Standard 3	40	60	40
Standard 4	60	40	60
Standard 5	80	20	80
Standard 6	100	0	100

5.2.3.4. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

A 16% separating/resolving gel with 4% stacking gel (Appendix J) was used to achieve optimal separation of proteins using the BIO-RAD PROTEAN mini gel system. The separating gel was cast and allowed to set and the stacking gel was cast on top of the separating gel. The comb was placed into the stacking gel solution to create wells and the stacking gel was allowed to polymerize. Twenty μL of each of sample was mixed with 5x sample loading buffer containing dithriothreitol (DTT) at a ratio of 2:1 (Appendix J). The samples were boiled for 10 minutes and

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loaded onto the gel. Five µL of PageRuler pre-stained protein ladder (Fermentes) was loaded in wells on either side of the sample wells. Electropheresis buffer was prepared (Appendix J) and placed into the system. The gel was electrophoresed at 180 Volts (V) in electrophoresis buffer for 2–3 hours. The gel was then submerged in Page Blue (Coomassie) staining solution (Fermentes) and stained overnight on a rocking shaker. The gel was destained with a mixture of acetic acid, water and methanol (10:30:60, v/v/v) for ± 30 minutes. The molecular weight of the unknown proteins were extrapolated after constructing a standard curve using the molecular weights obtained during separation of the PageRuler pre-stained protein ladder.

5.2.4. Bioassays

The bioassays were performed as described in Chapter 4 (see sections 4.2.3 and 4.2.4).

5.2.5. Visualisation of compounds developed on TLC plates

5.2.5.1. Ultra Violet (UV) light

UV light was used to visualize bands which are UV-absorbent. Extracts are separated using TLC in a solvent system consisting of chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02, v/v/v/v) and visualised under short wavelength (254 nm) and long wavelength (365 nm) UV light. Organic molecules which absorb UV light will appear as a dark blue spot with a green fluorescent background under 254 nm wavelength and various coloured (e.g. blue, orange, pink) spots with a black background under 365 nm wavelength.

5.2.5.2. Iodine stain

A filter paper was placed at the bottom of a glass beaker, a few iodine crystals were added to the beaker and tightly closed with foil. The beaker containing the crystals was left at room temperature for 20 minutes to equilibrate and the developed TLC plate (see section 5.2.5.1) was placed in the chamber until light brown/yellow colour developed over the entire plate. Compounds that have an affinity for iodine will appear as dark brown spots on a lighter brown/yellow background.

5.2.5.3. *p*–anisaldehyde – sulphuric acid stain

0.5 mL of *p*–anisaldehyde was added to 50 mL glacial acetic acid and 1 mL 97% sulphuric acid was added to the solution. The stain reagent was sprayed on the developed TLC plate (see section 5.2.5.1) and heated to 110°C. Sugars produce blue, green and violet spots, whereas sugar phenylhydrazones produce yellow-green spots.

5.2.5.4.   Ninhydrin stain

1.5 g of ninhydrin was dissolved in 100 mL n-butanol and 0.3 mL acetic acid was added to the solution. The stain reagent was sprayed onto the developed TLC plate (see section 5.2.5.1) and heated to 110°C until reddish spots appeared for a positive result.

5.2.5.5.   Vanillin/sulphuric acid stain

1% (w/v) solution of vanillin was prepared in absolute ethanol and concentrated with sulphuric acid (0.5 g vanillin in 100mL sulphuric acid/ethanol (40:10, v/v). The stain reagent was sprayed onto the developed TLC plate (see section 5.2.5.1) and heated to 120°C until the formation of triarylmethane dyes cause a colour formation. Fluorescent spots on a dark background was visualised under 365 nm UV light.

5.2.6. Spectroscopic measurement

UV absorption maxima of extra- and intra-cellular compounds extracted as crude compounds using EtOAc, as well as extracellular compounds partially purified using A/S precipitation and dialysis, were measured with a UV/Visible spectrophotometer plate-reader over a 200 – 900 nm range (Molecular devices; SpectraMax M2). The solvent control and a plate control was taken into account during the scan.

5.3.   RESULTS

5.3.1. pH purification of extracellular compounds produced by strain CZA14^T in an airlift reactor

Optimal fermentation conditions (153 + glycerol media, pH 6.0 at 30°C) for maximum production of antimicrobials from strain CZA14^T was achieved using the airlift bioreactor. All purification studies were thus directed toward the use of fermentation broth from the airlift bioreactor. Antimicrobial compounds were extracted and partially purified by adjusting the pH of the supernatant prior to extraction of compounds using a number of different solvents. Crude solvent extracts were tested for inhibitory effects against *Staphylococcus aureus* ATCC 33591 and DRWH system isolate 4. These results are presented in Table 5.2 (Appendix K, Figures K1 – K18).

A number of similar compounds were extracted using different solvents at various pH values which was active against both the methicillin-resistant *S. aureus* ATCC 33591 test strain and the *S. aureus* 4 DRWH isolate.

5.3.2. Small - scale partial purification of crude extra- and intra-cellularly produced compounds

A yellow coloured residual crude extract was obtained from the extraction of metabolites from the mycelia (intracellular production) when using EtOAc and a dark red coloured residual crude extract was obtained from the extraction of metabolites from the SNF (extracellular production) (Figure 5.2).

Table 5.2: Bioautography assay on partially pH purified supernatant filtrate (SNF) using various solvents at a 1:1, v/v ratio

Test strain	Solvent	pH	R_f value of bio-active band
S. aureus ATCC 33591	Benzene	3-5	0.91
		6-7 and 9	0.94, 0.91
		8	N/I
	Chloroform	3 - 7	0.94, 0.91, 0.84
		8 - 9	I/C
	Dichloromethane	3 - 6	N/I
		7	0.91, 0.62
		8	0.91, 0.62, 0.57
		9	0.91, 0.84, 0.62, 0.57, 0.52
	Ethyl acetate	3 - 9	0.91, 0.62 – 0.34
	n-butanol	3 - 7	0.57, 0.55 – 0.32
		8 - 9	I/C
	Petroleum ether	pH 3	0.94, 0.62 – 0.36
		pH 4	0.94, 0.62, 0.57
		pH 5	0. 62, 0.57
		pH 6	0.62 – 0.36
		pH 7 - 9	0.91, 0.62 – 0.52
S. aureus 4	Benzene	pH 3 - 9	0.62 – 0.34
		pH 8	N/I
	Chloroform	pH 3 - 9	0.62, 0.57
		pH 5	N/I
	Dichloromethane	pH 3	0.57, 0.52 – 0.38
		pH 4 and 6	0.57, 0.52
		pH 5	0.57
		pH 7	0.57, 0.52 – 0.38
		pH 8-9	0.52
	Ethyl acetate	pH 3 - 9	0.62, 0.57 – 0.38
	n-butanol	pH 3 - 6	0.57, 0.52 – 0.38
		pH 7	0.57, 0.52
		pH 8 - 9	0.57
	Petroleum ether	pH 3	0.62, 0.57 – 0.26, 0.18 – 0
		pH 4 and 9	0.62, 0.57
		pH 5	0.62, 0.57 – 0.26, 0.14 – 0
		pH 6 and 8	0.62, 0.57 – 0.32
		pH 7	0.62, 0.57 – 0.42

Key: N/I: No inhibition; I/C: Inconclusive

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Figure 5.2: Red coloured (left) and yellow coloured (right) residual crude bio-active extracts.

Upon elution from the column with the four component solvent system of chloroform: EtOAc: formic acid: n-hexane (5: 4: 1: 0.02, v/v/v/v) only the yellow compounds were eluted. The red compounds were further separated into red and orange compounds and absorbed to the silica even after applying pressure with presurised dry air (Figure 5.3). The column was plugged and solvent system was left in contact with the bound compounds. Absolute ethanol was used to elute all the bound compounds and all the remaining compounds eluted from the column simultaneously. The purification was deemed unsuccessful and a small-scale purification method was developed using a gradient elution (Figure 5.4). Silica gel column chromatography was utilised for the preliminary purification of secondary metabolites (SMs) produced by strain CZA14[T]. 35 fractions were collected; fractions with similar R_f values were pooled and used during bioassay tests. Bioassay results are presented in Figure 5.5 (filter disk diffusion assay) and Table 5.3 (bioautography assay). Figures relating to this data are in Appendix L; Figures L1 – L12.
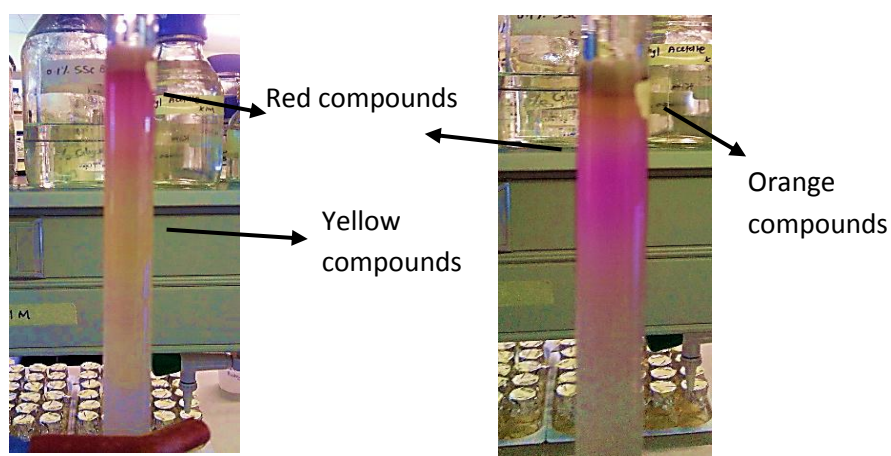
Figure 5.3: Pooled EtOAc crude extract from fermentation broth eluted from a 15 mm diameter silica gel column.

Figure 5.4: 4.5 mm diameter glass Pasteur pipette containing 10 % v/v water deactivated silica. Compounds were eluted with a gradient elution method.
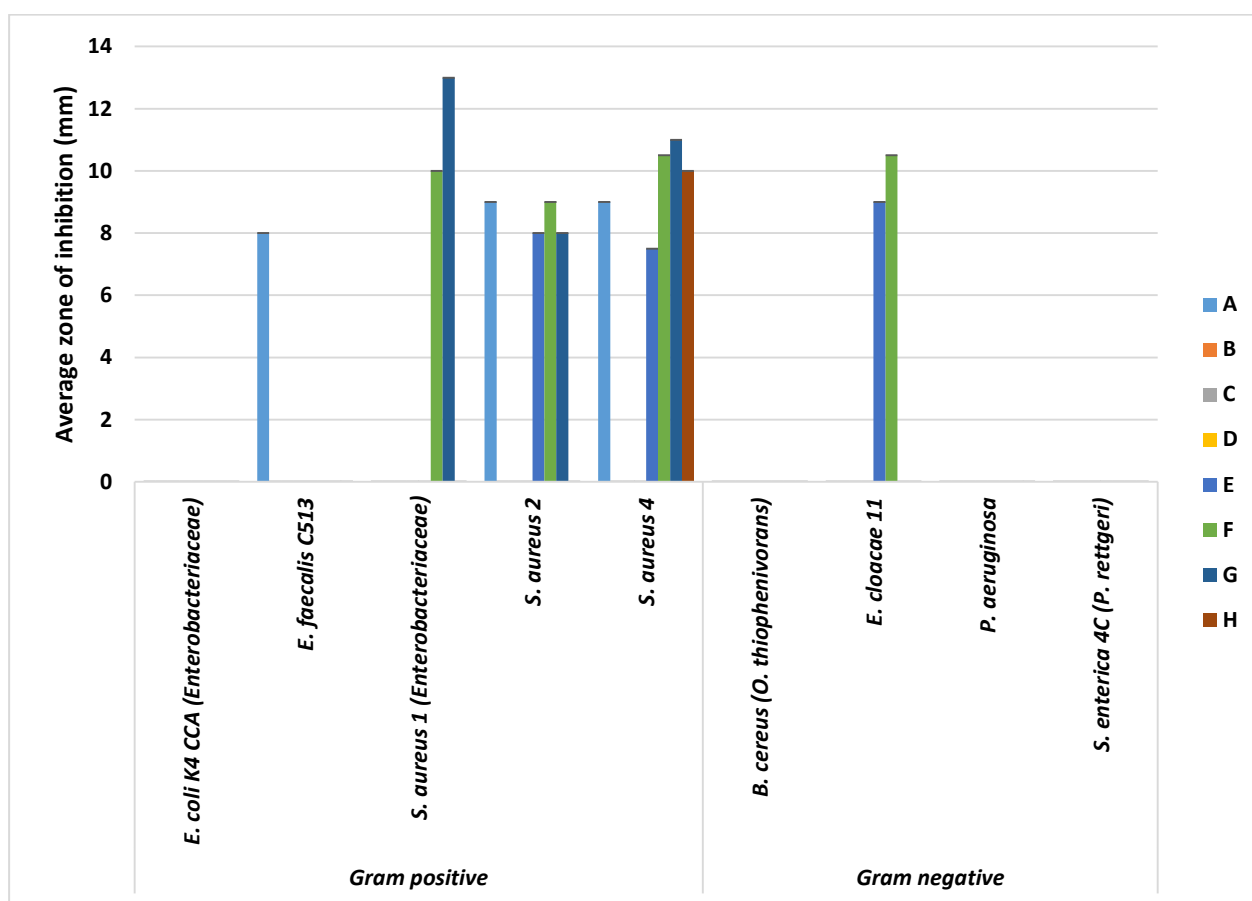
Figure 5.5: Filter disk assay against environmental DRWH system strains. Experiment (average of duplicate samples): A – Fraction A; B – Fraction B; C – Fraction C, D – Fraction D; E – Fraction E; F –

Fraction F; G – Fraction G; H – Fraction H. Controls – ampicillin; vancomycin; chloramphenicol; sterile filter disk; EtOAc control filter disk. 0 = No inhibition.

Poor diffusibility of the partially purified pooled extracts were displayed during the filter disk diffusion assay compared to the results achieved with the filter disk diffusion assay with the crude extracts from the ALR fermentation broth.

Table 5.3: R_f values of bands which exhibited inhibition against DRWH system isolates during bioautography assay

Test Strain	Zone of inhibition (R_f value)
B. cereus (*Ochrobactrum* spp.)	[A,B,C,D]0.96, [E,F,H]0.94, [A,E,F]0.91, [B]0.62 − 0.56, [C]0.62 − 0.46, [D]0.60 − 0.46, [E,F]0.56 − 0.42, [G]0.42 − 0.26, [H]0.40 − 0.22
E. coli K4 CCA (*Enterobacteriaceae*)	[A,B]0.94, [A,E,F]0.91, [E,F,G]0.84, [C,D]0.57, [D]0.57 − 0.36, [B,C,E,F]0.52, [E]0.52 − 0.38, [F]0.52 − 0.26, [G]0.38 − 0.16, [H]0.36 − 0.13
E. cloacae 11	[A,B]0.96, [A,E,F,H]0.94, [E,F]0.91, [C]0.57 − 0.47, [B,D,F]0.52, [D]0.52 − 0.42, [F]0.52 − 0.36, [E]0.50 − 0.38, [G]0.42 − 0.26
E. faecalis C513	[A,B,E,F]0.94, [A,E,F]0.91, [D]0.57 − 0.42, [H]0.57 − 0.26, [A,C,G]0.52, [G]0.52 − 0.36
K. pneumoniae 1 (Enterobacteriaceae)	[A,E,F,H]0.94, [A,B]0.91, [E,F]0.84, [D]0.56 − 0.40, [C,F]0.52, [F]0.52 − 0.24, [B,E]0.50, [E]0.50 − 0.36, [C]0.48, [G]0.40 − 0.24, [H]0.40 − 0.13
K. pneumoniae 3	[A]0.94, [A,E,F]0.91, [E,F]0.84, [C]0.57 − 0.45, [B,D]0.55, [D]0.55 − 0.42, [F]0.52 − 0.24, [E]0.50 − 0.24, [G]0.38 - 0.24, [H]0.36 − 0.13
Pseudomonas aeruginosa	[A,E,F]0.94, [B]0.91 − 0.50, [E,F]0.84, [C,D,F]0.50, [F]0.50 − 0.26, [E]0.45, [G]0.36 − 0.26, [H]0.36 − 0.16
S. enterica 4C (*P. rettgeri*)	[A]0.96, [A]0.91, [B,D]0.55, [C,D]0.52, [D,E]0.50, [F]0.48 − 0.36, [E]0.47, [E]0.45, [G,H]N/I
S. aureus 1 (*Enterobacteriaceae*)	[A]0.96, [A,B,E,F]0.91, [A,B,C,D,E,F,G]0.84, [A,B]0.72, [A]0.58, [A,B]0.57, [C]0.55, [D]0.52, [C,D,E,F]0.50, [E]0.50 − 0.40, [F]0.50 − 0.28, [C,D]0.45, [D]0.40, [G]0.38 − 0.20, [D]0.28, [E]0.16, [H]N/I
S. aureus 5 (*Enterobcateriaceae*)	[A,B]0.96 − [A,H]0.94, [E,F]0.91, [E,F]0.84, [D]0.58 − 0.40, [B,E]0.52, [E]0.52 − 0.50, [F]0.50 − 0.32, [C]0.42 − 0.46, [G]0.40 − 0.16

Key: (Table 5.3) *Experiment:* A – Fraction A; B – Fraction B; C – Fraction C, D – Fraction D; E – Fraction E; F – Fraction F; G – Fraction G; H – Fraction H.

A number of compounds with similar R_f values were found to be active after pH purification of the extracellularly produced compounds.

5.3.3. Salt purification of proteins produced extracellularly by strain CZA14[T] in an airlift reactor

Extracellularly (SNF) produced proteins were subjected to salt purification using A/S precipitation and dialysis. Figure 5.6 shows the colour change of the crude solvent extract from a brick-red colour to pink colour after A/S precipitation and dialysis.

Figure 5.6: Colour changes of EtOAc extraction during purification. A – Crude extracellular (Supernatant filtrate - SNF) EtOAc extract (brick red colour), B – EtOAc extract from SNF after ammonium sulphate precipitation (clear colour), C – EtOAc extract from pellet resuspended in 20 mM Tris-HCl after ammonium sulphate precipitation and dialysis (pink colour).

Figure 5.6 shows the effectiveness of the precipitation purification technique. Results pertaining to determining the molecular weight of the purified compound/s and bio-activity testing follow below.

5.3.3.1. Bradford's assay to quantify the total protein content in salt purified samples

The crude SNF was subjected to protein precipitation using an 80 % A/S saturation required to precipitate the total proteins produced by CZA14[T]. After A/S precipitation, dialysis was performed on the resuspended 80 % saturated pellet sample using 20 mM Tris-HCl buffer overnight. Table 5.4 shows the protein concentration of extracellularly produced proteins by CZA14[T] after A/S precipitation, dialysis and solvent extraction.

Table 5.4: Protein concentration of extracellularly produced proteins by *S. pharetrae* CZA14[T]

Sample	Starting volume (ml)	Protein concentration (mg/ml)
1	300	0.621
2	80	0.786
3	30	0.925
4	260	0.376
5	2	0.426
6	-	0.889
7	0.1	0
8	-	-0

Key: 1: Crude supernatant filtrate (SNF); 2: Pellet in 20 mM Tris-HCl after ammonium sulphate (A/S) precipitation (80 % saturation); 3: Dialysate; 4: Crude supernatant filtrate (SNF) after A/S precipitation; 5: Pellet in 20 mM Tris-HCl after A/S precipitation, dialysis, extraction with 2:3 v/v ethyl acetate (EtOAc) and reconstituted with 20 mM Tris-HCl to 50 x concentrate; 6: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc; 7: 50 x concentrated EtOAc extract extracted from crude supernatant after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc; 8: 20 mM Tris-HCl control.

5.3.3.2. Determining the molecular weight of salt precipitated proteins using sodium dodecyl sulphate–polyacrylamide gel electrophoresis assay

The molecular weight of the unknown protein of interest was determined using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) assay and the standards from the PAGE-ruler molecular weight marker. Results are presented in Table 5.5; Appendix M, Figures M1 and M2.

Table 5.5: Determination of the size of the unknown protein partially purified after A/S precipitation, dialysis and organic solvent extraction

	Length (mm)	R_f	Mw (kDa)	Log_{10} Mw
Gel length	50			
Standard	49	0.98	170	2.23
	47	0.94	130	2.11
	44.5	0.91	100	2.00
	42	0.89	70	1.85
	38.5	0.87	55	1.74
	35	0.83	40	1.60
	31.5	0.82	35	1.54

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	27	0.77	25	1.40
	18	0.57	15	1.18
	13.5	0.50	10	1.00
*Unknown	9	0.67	**6.7**	0.83

***Unknown:** Pellet in 20 mM Tris-HCl after A/S precipitation, dialysis, and extraction with 2:3 v/v ethyl acetate (EtOAc) and reconstituted with 20 mM Tris-HCl to 50 x concentrate

The size of the unknown protein/s of interest which was partially purified using A/S precipitation, dialysis and EtOAc extraction were determined using SDS-PAGE. The standards were used to plot a graph (Appendix M, Figure M2) and the size of the unknown protein/s were determined by extrapolating the data from the graph. The protein/s of interest was determined to be 6.7 kDa in size. Because the product is such a small size; this experiment needs to be repeased in future using a higher percentage separating gel.

5.3.4. Bioassays

Bioassays in terms of filter disk diffusion assays and bioautography assays were performed in duplicate against a number of DRWH system isolates. Results were averaged and are presented in Tables 5.6 and 5.7 (see Appendix M, Figure M3 for an example of a filter disk diffusion assay related to Table 5.6 and zones of inhibitions determinied during this study (section 5.7.4.1) and Appendix M, Figures M4–M8 (section 5.7.4.2) for bioautography assays related to Table 5.7).

Table 5.6: Filter disk diffusion assay against environmental DRWH isolates

Test strain	Controls	Zone of inhibition (mm) – experiment (Average from duplicate samples)	Zone of inhibition (mm) - controls
B. cereus (*Ochrobactrum* spp.)	1; 2; 3; 4; and 5	0	0; *19; *16; 0 and 0
E. coli K4 CCA (*Enterobacteriaceae*)	1; 2; 3; 4; and 5	*8	0; *24; *15; 0 and 0
E. cloacae 22	1; 2; 3; 4; and 5	*9	0; 0; *13; 0 and 0
E. faecalis C513	1; 2; 3; 4; and 5	*9	*12; *17; *0; 0 and 0
K. pneumoniae 3	1; 2; 3; 4; and 5	0	0; **25; 0; *15 and 0
P. aeruginosa	1; 2; 3; 4; and 5	0	0; 0; 0; 0 and 0
S. enterica 4C (*P. rettgeri*)	1; 2; 3; 4; and 5	**31.5	0; *15; 0; **14 and 0
S. aureus 1 (*Enterobacteriaceae*)	1; 2; 3; 4; and 5	*10	*10; *19; *9; 0 and 0

| S. aureus 4 | 1; 2; 3; 4; and 5 | *10 | *9; *19; *9; 0 and 0 |

Key: *Control* – 1 = ampicillin; 2 = vancomycin; 3 = chloramphenicol; 4 = sterile filter disk; and 5 = EtOAc control filter dis. #Filter disk assay was not performed against test strain. 0 = No inhibition. *Killed. **Regrowth*

Table 5.7: Bio-activity of partially purified extract against environmental DRWH isolates

Test strain	Bio-activity
B. cereus (*Ochrobactrum* spp.)	N/I
E. faecalis C513	A
K. pneumoniae 3	A
P. aeruginosa	A
S. aureus 1 (*Enterobacteriaceae*)	A
S. aureus 4	A
S. aureus 5 (*Enterobacteriaceae*)	A

Key: *N/I –* No Inhibition. **A –** Inhibition/active

Salt precipitated partially purified extracellularly produced crude extracts from the ALR exhibited bactericidal activity against all of the DRWH isolates tested against (Table 5.6) using the filter disk-diffusion assay except for *B. cereus* (*Ochrobactrum* spp.), *K. pneumoniae* 3 and *P. aeruginosa.* However, during the bioautography assay the compounds were active against all of the DRWH strains tested against (Table 5.7) except for *B. cereus*.

5.3.5. Staining of developed TLC plates containing EtOAc extracts partially purified during silica gel 60 column chromatography

After developing TLC plates it is necessary to visualise the compounds and determine their functional groups in order to characterise the compounds of interest. Results pertaining to the visualisation and staining of compounds of interest are presented in Table 5.8. The sample labelled as "PP" in the table is the 50x concentrated EtOAc extract from the pellet resuspended in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc. Fractions A–H are solvent fractions from the silica gel column chromatography technique.

Twelve compounds were visualised under short-wave UV light (254 nm), while seven compounds were visualised under long-wave UV light (365 nm) with three out of the seven compounds being of similar R_f value to those visualised under short-wave UV light. A number of

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compounds from fractions A-D showed compounds present after staining with iodine that were similar in R_f values to compounds visible under UV light. A number of different compounds in fractions E-H were detected using the iodine stain that was not visible under UV light. A number of compounds not visible under UV light in the 'PP' extract were visualised after the application of the universal iodine stain. Ten compounds were detected with R_f values ranging from 0.52-0.30. All silica purified fractions and 'PP' extract produced a positive result with the *p*–anisaldehyde – sulphuric acid stain. Fractions A-H rendered white/cream, brown and red coloured compounds with R_f values ranging from 0.98-0.16. Only one compound in fraction A from the silica gel 60 purified extracts were positive for the presence of free amino groups. This compound had an R_f value of 0.94. The vanillin/sulphuric acid stain produced a positive result for the presence of unsaturation by compounds detected in Fractions A, B, C and H of silica gel 60 purified samples.

Table 5.8: Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5: 4: 1: 0.02, v/v/v/v), stained using various TLC detection stains

Sample	UltraViolet Light (254nm)	UltraViolet Light (365nm)	Iodine stain	*p*–anisaldehyde – sulphuric acid stain		Ninhydrin stain	Vanillin/sulphuric acid stain	
Fraction A	0.98, 0.87, 0.74, 0.60, 0.58	0.94*	0.94, 0.87	0.94	Red	0.94	0.84	Green/brown
Fraction B	0.87, 0.74, 0.60, 0.58	0.94*, 0.78*, 0.74**, 0.61**, 0.58**	0.87, 0.60, 0.58	0.94 0.64 0.58	Faint red Faint red Brown	No bands	0.60 0.53	Brown/red Red
Fraction C	0.87, 0.58, 0.54, 0.50, 0.31	0.94*, 0.58**, 0.54**, 0.50**	0.54	0.56	Brown	No bands	0.53	Red
Fraction D	0.87, 0.31, 0.18	No bands	0.54, 0.50, 0.47	0.50	Grey/white	No bands	No bands	
Fraction E	0.87, 0.18	No bands	0.47, 0.45, 0.44	0.49	Grey/white	No bands	No bands	
Fraction F	0.88	No bands	0.47, 0.45, 0.44, 0.41, 0.40	0.98 0.50 – 0.26	Brown Grey/white	No bands	No bands	
Fraction G	0.90	No bands	0.90	0.98 0.87 0.36 – 0.24	Brown Red Grey/white	No bands	No bands	
Fraction H	0.91	No bands	0.91	0.87 0.33 – 0.16	Brown Grey/white	No bands	0.87	Green
#PP	No bands	No bands	0.52, 0.50, 0.47, 0.45, 0.42, 0.40, 0.37, 0.35, 0.32, 0.30	0.52 – 0.30	Grey/white	No bands	No bands	

*Orange fluorescent colour, **blue fluorescent colour

5.3.6. Spectroscopic measurement

The spectrometric measurement of antimicrobial compounds produced by CZA14[T] was performed by UV/Visible spectrometry (Figure 5.7). The extra- and intra-cellularly produced compounds extracted as crude compounds from fermentation broth from the ALR, as well as the partially purified extracellularly produced compounds ([#]PP) were analysed (Table 5.9).
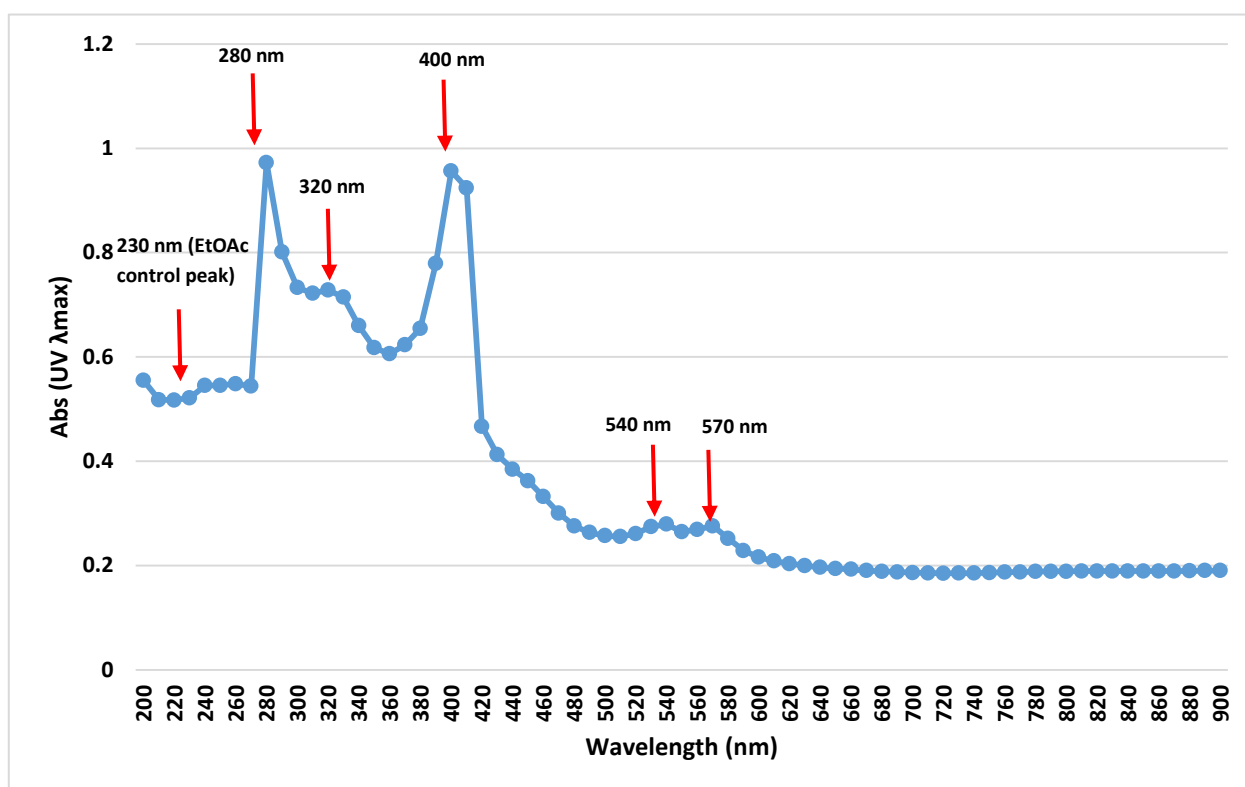
Figure 5.7: UV absorption spectrum of partially purified extracellularly produced compounds. Main peaks detected are indicated by red arrows.

UV scans of crude extracts from the ALR fermentation broth dissolved in EtOAc were performed. The absorbance maxima were obtained at 290 nm, 410 nm, 540 nm and 570 nm for extracellularly produced compounds and at 290 nm for intracellularly produced compounds (Table 5.9; Appendix N, Figures N1 and N2). UV scans of partially purified extracellularly produced compounds ([#]PP) showed absorbance maxima at 280 nm, 320 nm, 400 nm, 540 nm and 570 nm.

Table 5.9: UV maxima of compounds produced by *S. pharetrae* CZA14[T] in an ALR

Sample	UV max (nm)
Extracellularly produced crude compounds	290, 410, 540, and 570
Intracellularly produced crude compounds	290
Partially purified extracellularly produced compounds (A/S precipitation and dialysis) ([#]PP)	280, 320, 400, 540, and 570

5.4. DISCUSSION

Partial purification of compounds by changing the pH of extracellular fluid and extracting compounds using various solvents

Extracellularly produced antimicrobial compounds were extracted using different solvents, including benzene, chloroform, dichloromethane, EtOAc, n-butanol and petroleum ether. The partially purified extracellularly produced compounds were slightly soluble to soluble in all of the aforementioned solvents. The results with the crude EtOAc extracts presented in Chapter 4 saw compounds with R_f values in a range of 0.10 – 0.94, most of which were active against the test strains used in this study. In comparison to those results, similar compounds were extracted using different solvents from SNF adjusted to various pH values (Tables 5.2). These compounds were active against the methicillin-resistant ATCC *S. aureus* strain and the DRWH isolated *S. aureus*. The best extraction solvents for full extraction of antimicrobial compounds were EtOAc and petroleum ether. EtOAc was chosen over the other solvents for extraction purposes for current and future downstream processes due to it being inexpensive and due to its polarity and miscibility.

Preliminary small-scale purification of crude EtOAc extracts from airlift reactor fermentation broth using gradient elution column chromatography

Pooled extra- and intra-cellularly produced compounds extracted from ALR fermentation broth and mycelial mass was partially purified using silica gel 60 column chromatography. Partially purified compounds were bioassayed to ascertain the antimicrobial bio-active profile of these compounds against the DRWH system isolates used in this study and to compare activities to bio-active results obtained during bioassays with the crude extracts.

No inhibition was detected against *B. cereus*, *E. coli* K4 CCA, *P. aeruginosa* and *S. enterica* 4C when the filter disk diffusion assay (Figure 5.5) was used. Various factors such as the poor diffusibility of the compounds into the agar medium, could contribute to the lack of inhibition observed. Fractions E and F collected during the pdurification process were active against *E. cloacae* 22. Fraction A was active against *E. faecalis* C513, while fractions F and G were active against *K. pneumoniae* 3. Fractions A, E, F and G were active against *S. aureus* 2, and fractions A, E, F, G and H were active against *S. aureus* 4. The commercial antibiotics were active against the DRWH system isolates except for *P. aeruginosa*. No inhibition was detected in the EtOAc control and sterile filter disk control.

The bioautography assay showed that compounds present in all fractions were active against the DRWH system isolates with an exception to fractions G and H, which exhibited no bio-activity against *S. enterica* 4C and no bio-activity with fraction H against *S. aureus* 1 (Table 5.3).

All compounds in the crude extract active against the DRWH system isolates remained active after partial purification using silica gel column chromatography. Interestingly, the expected band with an R_f value of 0.81 active against *E. coli* K4 CCA, *E. cloacae* 11 and *E. faecalis* C513 during tests with the crude extracts showed no activity during the bioautography assays with the partially purified extracts. The functionality of the compound may have been lost during the purification process, it may not have eluted from the column or the concentration of the compound may be too low to exhibit an inhibitory effect. Another interesting observation was the inhibitory effect of a new active compound with an R_f value of 0.96, not previously observed during the bioautography assay with the crude extracts. This compound was active against *B. cereus*, *E. cloacae* 11, *S. enterica* 4C, *S. aureus* 1 and *S. aureus* 5. Concentrations of this compound may have increased after purification enabling it to have an inhibitory effect. Another theory could be due to a synergistic effect whereby compounds in the crude extract together causes bactericidal activity and separated exhibits no bio-activity. A number of growth promoters were observed throughout bioautography assays with partially purified extracts and (as well as crude extracts, Chapter 5) where purple bands were visualised on a developed TLC plate containing areas of enhanced growth of pathogenic bacteria stained with MTT (Appendix H and L).

Partial purification of antimicrobials produced extracellularly by strain CZA14[T] in an airlift reactor

It was determined with the Bradford's assay that after ammonium sulphate precipitation of the crude extract, the protein concentration of the 80% saturated pellet increased from 0.621 mg/mL to 0.786 mg/mL. After further purification methods using dialysis techniques, the protein concentration increased from 0.786 mg/mL to 0.925 mg/mL. The protein concentration decreased after extraction with EtOAc of salt purified pellet from 0.925 mg/mL to 0.426 mg/mL, indicating that some of the compounds reacting with the Bradford's reagent were extracted into the EtOAc. No protein was detected in the buffer used to constitute the EtOAc extracts after drying and no protein was detected in the crude supernatant extract after A/S precipitation, dialysis and solvent extraction with EtOAc.

Determining the molecular weight of partially purified proteins: sodium dodecyl sulphate–polyacrylamide gel electrophoresis

EtOAc extracted partially purified salt precipitated compounds were resolved on a denaturing SDS-PAGE to determine the molecular weight of the protein of interest. This was performed to ascertain whether an antimicrobial peptide was isolated during purification and extraction processes. Typically, antimicrobial peptides are low molecular weight compounds with a small size of < 10 kDa (Yount and Yeaman, 2004). A 6.7 kDa protein was isolated from the supernatant filtrate by salt precipitation, dialysis and EtOAc solvent extraction. The compound/s was labelled as "PP" and were further analysed to determine their bio-active profile and other characteristics using UV light, staining and spectrometric analysis.

Bioassays

Filter disk diffusion and bioautography assays on salt purified EtOAc extracts

After determining that the active compound/s were isolated by EtOAc extraction, the partially purified extract was subjected to filter disk diffusion assays and bioautography assays against a limited number of DRWH isolates due to the limited volume of extract available for testing purposes. During the filter disk diffusion assay, the partially purified compounds exhibited no inhibition against *B. cereus* (*Ochrobactrum* spp.), *K. pneumoniae* 3 and *P. aeruginosa*. Bactericidal activity was exhibited against *E. coli* K4 CCA, *E. cloacae* 22, *E. faecalis* C513, *S. aureus* 1 and 4 with an average zone of clearing of 8 mm, 9 mm, 9 mm, 10 mm and 10 mm,

respectively. Bacteriostatic activity was exhibited against *S. enterica* 4C with a large zone of inhibition of 31.5 mm (Table 5.6). No zone of inhibition was detected against *B. cereus* during bioautography assays. The partially purified extract was active against *E. faecalis* C513, *K. pneumoniae* 3, *P. aeruginosa*, *S. aureus* 1 and 4 and *S. aureus* 5 during the bioautography assays.

Visualisation of compounds separated on TLC plates (Appendix O, Figures O1 – O7)

After developing the TLC it is important to visualize the components within a reaction mixture. The main reason for doing so is because most organic compounds are colourless. Most chromophore-containing compounds can be visualised under short- and long-wave ultraviolet (UV) light (254 nm and 365 nm, respectively). Typical functional groups found in chromophore-containing compounds which may be visualised under UV light, are α, β- unsaturated carbonyls, aromatic groups, and any other compounds which contain extensively π-conjugated systems. For those compounds which do not absorb under UV light, permanent or semi-permanent TLC staining can be performed for visualisation of these compounds and determination of their functional groups (Kasal *et al*., 2010).

Fraction A contained compounds with R_f values of 0.98, 0.87, 0.74, 0.60 and 0.58 which absorbed under short-wave UV light (254 nm) and a compound with an R_f value of 0.94 was absorbed under long-wave UV light (365 nm) and fluoresced as an orange colour. Compounds 0.94 and 0.87 were detected with the universal iodine stain and compound 0.94 was detected using the anisaldehyde stain and ninhydrin stain. Compound 0.84 was not visible under UV but was detected using the vanillin/sulphuric acid stain. Compound 0.94 displayed broad range bio-activity against DRW isolates – *E. cloacae* 11, *E. faecalis* C513, *E. coli* K4 CCA, *K. pneumonia, K. pneumonia* 3, *S. aureus* 5 and *P. aeruginosa* (Table 5.5). A positive ninhydrin result (detection of amino acids, amines or amino-sugars) combined with the positive anisaldehyde result (detection of phenols, terpenes, steroids and sugars) could indicate the presence of an aminoglycoside antibiotic. CZA14[T] contain genes for the expression of an aminoglycoside which are situated on contig 54, cluster 9 of the CZA14[T] genome (Appendix P, Table P1). Compound 0.84 was active against *S. aureus* 1; this compound displayed a green/brown colour spot when stained with vanillin/sulphuric acid reagent. A green spot is indicative of phenols or higher alcohols. Typically, polyether antibiotics such as lysocellin give a bright yellow-green to green colour when stained with vanillin/sulphuric acid.

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Fraction B contained compounds with R_f values of 0.87, 0.74, 0.60 and 0.58 that was absorbed under 254 nm UV light. Compounds 0.94 and 0.78 fluoresced as orange compounds under 365 nm UV light and compounds 0.74, 0.61 and 0.58 fluoresced as blue compounds under 365 nm UV light. Compounds 0.87, 0.60 and 0.58 were iodine positive. Compounds 0.94, 0.64 and 0.58 were anisaldehyde positive and displayed red to brown colour spots and compounds 0.60 and 0.53 were vanillin positive and displayed as red spots. An anisaldehyde positive result and a vanillin/sulphuric acid positive result may indicate the presence of terpenes. Typically terpenes appear as red and blue colours with the vanillin/sulphuric acid stain. CZA14[T] have six biosynthetic gene clusters (BGC) which code for the expression of terpenes (Appendix P, Table P1). According to antiSMASH predictions, One of the BGCs coded for the terpene, albaflavenone (100 % similarity). This terpene is situated on contig 32, cluster 4 of the CZA14[T] genome. The compound with the same R_f value was also detected in Fraction C. Compound 0.60 was active against *B. cereus* and compound 0.53 may be active against *E. coli* K4 CCA and *E. cloacae* 11.

Fraction C contained compounds with R_f values of 0.87, 0.58, 0.54, 0.50 and 0.31 that absorbed under 254 nm UV light and; compound 0.94 which fluoresced as an orange compound and compounds 0.58, 0.54 and 0.50 which fluoresced as blue compounds under 365 nm UV light. Compound 0.54 was visible during the iodine stain. Compound 0.56 was active against *E. cloacae* 11 and *K. pneumoniae* 3, this compound appeared as a brown spot with the anisaldehyde stain – glucosamine compounds give brownish–red chromatographic spots. Compound 0.53 which was also detected in fraction B appeared as a red spot with the vanillin/sulphuric acid stain. As previously suggested, this compound may be a terpene.

Fraction D contains compounds with R_f values of 0.87, 0.31 and 0.18 which absorbs under 254 nm UV light. Compounds 0.54, 0.50 and 0.47 were detected using the iodine stain. Compound 0.50 was also detected with the anisaldehyde stain and appeared as a grey/white spot. This compound was active against *E. cloacae*, *P. aeruginosa*, *P. rettgeri* and *S. aureus* 1.

Fraction E contains compounds with R_f values of 0.87 and 0.18 which absorbs under 254 nm UV light. Compounds 0.47, 0.45 and 0.44 were detected using the iodine stain. Compound 0.49 appeared as a grey/white spot when stained with anisaldehyde. Compounds in fraction E were active against *Ochrobactrum* spp., *E. coli* K4 CCA, *E. cloacae* 11, *K. pneumoniae* 1, *K. pneumoniae* 3, *P. aeruginosa*, *S. aureus* 1 and 5.

Fraction F contains a compound 0.88 which absorbs at 254 nm. Compounds 0.47, 0.45, 0.44, 0.41 and 0.40 were detected with iodine. Compound 0.98 appeared as a brown spot on the developed TLC and compounds 0.50 – 0.26 appeared as grey/white spots after staining with anisaldehyde. Compounds in fraction F were active against *B. cereus*, *E. coli* K4 CCA, *E. cloacae* 11, *K. pneumoniae* 1, *K. pneumoniae* 3, *P. aeruginosa*, *P. rettgeri* and *S. aureus* 1 and 5.

Fraction G contained a compound with an R_f value of 0.90 which absorbed under 254 nm UV light. This compound was also detected using the iodine stain. Compound 0.98 appeared as a brown spot, compound 0.87 appeared as a red spot and compounds 0.36 – 0.24 appeared as a grey/white spot with the anisaldehyde stain. Compounds 0.36 – 0.24 were active against *B. cereus, E. coli* K4 CCA, *E. cloacae* 11, *E. faecalis* C513, *K. pneumoniae* 1, *K. pneumoniae* 3, *P. aeruginosa*, *S. aureus* 1 and 5.

Fraction H contained a compound with an R_f value of 0.91 which absorbed under 254 nm UV light. This compound was also detected using the iodine stain. Compound 0.87 appeared as a brown spot when stained with anisaldehyde and as a green spot when stained with vanillin/sulphuric acid. Compounds 0.33 – 0.16 were detected as a grey-white spot with anisaldehyde stain. These compounds were active against *B. cereus, E. coli* K4 CCA, *E. faecalis* C513, *K. pneumoniae* 1, *K. pneumoniae* 3 and *P. aeruginosa*.

A number of compounds were detected in the sample labelled "PP" when using the iodine stain and anisaldehyde stain which stained as grey/white spots. All these compounds were active against the DRWH isolates tested against in this study except for *B. cereus* (*Ochrobactrum* spp.). These compounds had R_f values of 0.52, 0.50, 0.47, 0.45, 0.42, 0.40, 0.37, 0.35, 0.32 and 0.30. These R_f values are very close to each other – it was suggested that these compounds could be isomers of each other. Isomers are two or more compounds that have the same molecular formula. However, these molecules possess a different arrangement causing a different chemical structure which in turn exhibits different properties when separated on a TLC plate.

Compounds in Fractions C–H and sample "PP" tested positive with anisaldehyde: spots coloured violet/brown, blue, red, grey or green may indicate the presence of terpenes, sugars and steroids. This could indicate a number of classes of antimicrobial compounds such as compounds produced by the polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) pathways. CZA14[T] contain a number of genes encoding for the production of antimicrobials from the aforementioned pathways (Appendix P, Table P1).

Spectroscopic measurement

Characteristics of antimicrobial compounds produced by CZA14[T] with UV spectroscopy analyses showed that SM compounds produced extracellularly and extracted as crude compounds exhibited a maximum UV absorption at 290, 410, 540 and 570 nm in EtOAc. Crude intracellularly produced active compounds exhibited a maximum UV absorption of 290 nm. These compounds exhibited broad-spectrum antibacterial and antifungal activity against the ATCC clinical test strains and DRWH isolates. Extracellularly produced crude compounds were purified using salt precipitation, dialysis and EtAOc solvent extraction. Active compounds exhibited a maximum UV absorption at 280, 320, 400, 540 and 570 nm. These compounds exhibited bio-activity against the DRWH isolates used in the current study.

5.5. CONCLUSION

A summary of the various physicochemical properties of the compounds produced by CZA14[T] in an airlift reactor is presented in Table 5.10

Table 5.10: Physicochemical properties of antimicrobial compounds produced by *S. pharetrae* CZA14[T]

Property	Extracellularly produced crude compounds	Intracellularly produced crude compounds	PP
Appearance	Deep red	Yellow	Pink
Solubility	Slightly soluble in benzene, chloroform, dichloromethane, n-butanol and petroleum ether. Soluble in EtOAc, water, methanol and DMSO	Soluble in EtOAc	Soluble in EtOAc and 20 mM Tris-HCl
Functional groups Iodine test	+		+
Ninhydrin test	+		-
Vanillin-sulphuric acid test	+		-
Anisaldehyde	+		+
UV λmax (nm)	290, 410, 540 and 570 nm	290 nm	280, 320, 400, 540 and 570 nm
Bio-active compounds: R_f values	Ranging between 0.96 – 0.13		0.52, 0.50, 0.47, 0.45, 0.42, 0.40, 0.37, 0.35, 0.32, 0.30
Active	*B. cereus* (*Ochrobactrum* spp.), *E. coli* K4 CCA		*E, faecalis* C513, *K.*

against DRWH isolates	(*Enterobacteriaceae*), *E. cloacae* 11, *E. faecalis* C513, *K. pneumoniae* 1 (*Enterobacteriaceae*)*, K. pneumoniae* 3, *P. aeruginosa*, *S. enterica* 4C (*P. rettgeri*), *S. aureus* 1 and *S. aureu*s 5 (*Enterobacteriaceae*)	*pneumoniae* 1 (*Enterobacteriaceae*), *P. aeruginosa*, *S .aureus* 1 (*Enterobacteriaceae*) and 4, *S. aureus* 5 (*Enterobacteriaceae*)

Evaluation of the crude and partially purified organic extracts identified a number of biologically active compounds with various physicochemical properties as presented in Table 5.10. After pH adjusted and partial purification of extracellularly produced compounds, a number of compounds with similar R_f values were detected. These compounds were active against methicillin-resistant *S. aureus* ATCC 33591 as well as the *S. aureus* 4 DRWH system isolate. Broad-range bio-activity was exhibited against all 14 of the DRWH system isolates tested against in this study by the organic extract which was preliminarily purified using small-scale silica gel 60 column chromatography. Extracellularly produced proteins were subjected to salt precipitation and these proteins were also active against the DRWH system isolates tested in this study. During the purification with ammonium sulphate salt, a 6.7 kDa protein was isolated. It was suggested that this protein could be a peptide antibiotic due to its small size and may be one of the compounds exhibiting activity against the DRWH system isolates (a way to detect if this protein is active is by direct bioassay, but this result was not clear and should be repeated – see Appendix Q, Figures Q1 and Q2). In Chapter 5, we see that the number of SM compounds produced (Chapter 3) were seriously underestimated as a number of these compounds were not visualised under UV light and could only be visualised using various stains and during bioautography assays.

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CHAPTER 6:

Molecular techniques to determine the biosynthetic potential of *Streptomyces pharetrae* CZA14[T]

Abstract

Microbial genome sequence analyses for access to the diversity of microbial biosynthetic pathways by the use of bioinformatics tools and molecular detection technologies have revealed a number of "cryptic" secondary metabolite biosynthetic gene clusters (smBGCs). This is valuable for the production of new natural products: 'genome mining' can be used to predict certain classes of antimicrobial agents and experiments for their expression can be designed. The CZA14$^{\text{T}}$ genome was 'mined' for the detection of its biosynthetic pathways using the online bioinformatics programme, antiSMASH, and molecular techniques were used in the discovery of smBGCs. AntiSMASH revealed eight out of the 55 smBGCs to be polyketide synthase based compounds (PKS). Using polymerase chain reaction (PCR) and fermentation (Chapter 4), one of the eight PKSs detected belonged to a Type-II PKS family containing ketosynthase (KSα) genes. AntiSMASH determined that CZA14$^{\text{T}}$ contained genes showing 100 % homology to curamycin A. This prediction was confirmed through the isolation of total RNA, its conversion to cDNA, and the use of the cDNA as a template for the PCR amplification of the KSα gene. Furthermore, a mass peak with the exact molecular weight as curamycin A was detected when partially purified extracts were analysed by liquid chromatography-mass spectrometry.

Key words: AntiSMASH, smBGCs, PCR, Type-II PKSs

Introduction

Full microbial genome sequencing is beneficial as it can be used to identify the genes which govern the biosynthesis of a natural product. These genes are often clustered together in so-called 'silent' or 'cryptic' pathways and more often than not, organisms contain more potential biosynthetic gene clusters for secondary metabolite (SM) production than the number of metabolites reported (Corre and Challis, 2009; Walsh and Fischbach, 2010; Wenzel and Muller, 2007). These 'silent' pathways hold much potential for researchers as it provides new opportunities for novel drug discovery, as putative natural products are often overlooked under standard detection and fermentation conditions (Walsh and Fischbach, 2010). Cryptic pathways are appealing not only because of their potential for novel natural product discovery, but also because most of the pathways contain genes coding for multi-modular and multi-domain PKS and NRPS – these enzymes are highly sought after as they are involved in the biosynthesis of many known therapeutic products (Walsh and Fischbach, 2010).

SM biosynthesis is regulated via specific sets of structural genes. These genes are typically clustered on the chromosome and organised in the order the biosynthetic reaction will occur. Genes regulating the expression of SMs are adjacent to the biosynthetic genes which control pathways specific for SM biosynthesis. The expression of genes of interest is dependent on environmental conditions which will either activate or suppress the secondary metabolite biosynthetic genes (SMBG) (van Wezel *et al*., 1997; Narberhaus, 1999; Vincente *et al*., 1999). In order to make the genomic screening more effective, molecular techniques in combination with a bioinformatics approach should be followed. Molecular detection methodology has become increasingly valuable in determining the biosynthetic potential of microorganisms and to determine the taxonomic diversity of microorganisms producing antibiotics (August *et al*., 1999; Dalboge and Lange, 1998). The molecular based technique, the polymerase chain reaction (PCR), may be used to amplify selected biosynthetic genes with the use of degenerate primers. This method, independent of culturing conditions, has proven to be reliable at indicating the potential of actinobacteria to produce certain classes of antimicrobial compounds or novel compounds (Seow *et al*., 1997; Morris *et al*., 1999).

In this part of the study, the genome sequence of *Streptomyces pharetrae* CZA14[T] was re-evaluated for the smBGCs present, through the use of the online bioinformatics tool antiSMASH. Degenerate primers for the amplification of Type I and Type II ketosynthases were used to detect the predicted polyketide gene clusters. The production of a polyketide was confirmed through the isolation of total RNA, conversion to cDNA, and the cDNA used as a template in PCR with the degenerate primers. The sequence information obtained was linked to the predicted gene cluster in the genome sequence. Figure 6.1 outline the methodology used for the results presented in this Chapter.

Determining the biosynthetic potential of *Streptomyces pharetrae* CZA14[T]

Genome mining using antiSMASH

Molecular biology methods

PCR screening for expression of biosynthetic gene cluster PKS-II

Solvent extraction after ammonium sulphate precipitation and dialysis (Chapter 5)

Link back information to antiSMASH results

Tentative identification: LC/MS analysis

Figure 6.1: Flow-chart of methods used for the determination of the biosynthetic potential of *Streptomyces pharetrae* CZA14[T].

Materials and Methods

6.1.   Materials

Most of the chemicals used in this study were purchased from Merck Millipore or Sigma-Aldrich unless otherwise stated.

6.2.   Methods

6.2.1.   Genome mining: bioinformatic analysis of *S. pharetrae* CZA14[T] genome

Prior to this study, DNA was isolated from *S. pharetrae* CZA14[T] by Dr M. Le Roes-Hill and the genome was sequenced by Dr B. Kirby at the University of the Western Cape, Next Generation Sequencing Facility. The genome was annotated by Dr M. Le Roes-Hill, Dr B. Kirby, Dr N. Khan, Dr P. Meyers, Dr J. Rohland and Dr G. Everest. For the prediction of biosynthetic gene clusters and final products, the bioinformatics tool antiSMASH (Antibiotics and Secondary Metabolite Anaylsis SHell) (Blin *et al*., 2013) (http://antismash.secondarymetabolites.org) was used. The genome sequence was submitted to the online server and the predicted biosynthetic gene clusters were evaluated:

(http://antismash.secondarymetabolites.org/upload/abef86c8-1752-4e11-8b3f-c29ac0e37aaf/index.html).

6.2.2.   Molecular biology methodology

Le Roes-Hill (2015; unpublished data), performed PCR on genomic DNA isolated from CZA14[T] and amplified Type-II polyketide synthases (PKS-II) genes to determine whether it contains the genes for the expression of a Type-II polyketide compound (Appendix R, Figure R1).

Molecular screening was performed to determine whether CZA14[T] was expressing genes for a Type-II PKS in the optimal media, fermentation vessel and conditions (153 + glycerol, ALR, 30°C over 7 – 15 days). Details concerning the expression of those biosynthetic genes are detailed below.

Total RNA isolation involved the growth of the CZA14[T] culture in an airlift reactor, followed by Gram stain to determine purity of cultures. Total RNA isolation was performed using the method described by the manufacturers of the Bioline kit used in this study. The RNA concentration was determined using a nanodrop, genomic DNA contaminants were determined

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by PCR with DNA controls and RNA samples using 16S rRNA primers. Pure RNA was converted to cDNA using reverse transcriptase (RT) polymerase chain reaction (PCR) and PKS products were amplified using PKS primers and the cDNA as template. The products were visualised under UV after separation on a 1% agarose gel.

6.2.2.1. Polymerase chain reaction (PCR) screening for the biosynthetic genes involved in the production of aromatic Type-II polyketide synthases (PKS-II)

Genomic DNA was used in a PCR amplification of a Type-II PKS (Wood *et al*., 2006). The expression of the biosynthetic gene clusters were determined by collecting 2 mL samples throughout the fermentation with the airlift reactor (ALR) used in this study (see Chapter 4, section 4.2.1) from day 0 – 12. Cells were harvested from 2 mL of the cell suspension by centrifuging at 12 000 rpm for 10 minutes and the supernatant discarded. RNA was isolated and purified according to the manufacturer's instructions using the Bioline isolate II RNA mini kit (catalogue no. BIO-52071).

To determine whether the RNA was free from contaminating genomic DNA (gDNA), universal 16S rRNA primers (16S-F: 5' – AGAGTTTGATCITGGCTCAG - 3', 16S-R: 5' – ACGGITACCTTGTTACGACTT – 3') were used to amplify any gDNA in the total RNA samples; gDNA from another actinomycete strain was used as a positive control. Conditions for amplification of the 16S rRNA gene were: 96°C for 2 minutes (initial denaturation), 30 cycles of 96°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 2 minutes (extension) and a final extension of 72°C for 5 minutes. Samples were separated on a 1% (w/v) 1 x tris-acetate-EDTA (TAE) agarose gel [100 volts (V) for 1 hour (hr) and viewed under UV (Appendix R, Figure R2)]. Upon determining whether the RNA isolated was pure, RNA was converted to cDNA by reverse transcription (RT) using RT-PCR according to the manufacturer's instructions using the Ambion RETROscript kit (catalogue no. AM1710); the conditions for cDNA synthesis were as follows: 44°C for 1 hour and 92°C for 10 minutes. 2 μg of total RNA was used as a template (experiments were performed in duplicate) and 2 controls (in duplicate) were used during the procedure (RT control 1: contained a positive RNA control with the addition of reverse transcriptase, and RT control 2: contained nuclease free dH_2O with the addition of reverse transcriptase).

Once RNA was successfully converted to cDNA. Samples were electrophoresed on a 1% (w/v) 1 x tris-borate-EDTA (TBE) agarose gel (100 V for 1 hr) and viewed under UV; Appendix R, Figure R3. 4 μL cDNA was used as the template with Thermo Fisher Scientific™ 2X dreamtaq PCR

master mix and aromatic type-II PKS primers (ARO-PKS-F: 5' – GGCAGCGGITTCGGCGGITTCCAG – 3', ARO-PKS-R: 5' – CGITGTTIACIGCGTAGAACCAGGCG – 3') in the amplification of the Type-II PKS biosynthetic gene in a 50 µL reaction. Gradient PCR was performed and the conditions for the amplification of the PKS-II gene were as follows: 96°C for 2 minutes (initial denaturation); 30 cycles of 96°C for 45 seconds (denaturation); 56°C Δ 9°C (annealing): gradient – 52.1°C, 53.3°C, 54.9°C, 56.2°C, 57.9°C, 59.4°C, 52.6°C, 54.1°C, 55.6°C, 57.1°C, 58.7°C, 59.7°C; 72°C for 2 minutes (extension); and 72°C for 5 minutes (final extension). Samples were separated on a 1% (w/v) 1 x tris-borate-EDTA (TBE) agarose gel (100 V for 1 hr) and viewed under UV. The PCR product was sequenced by Inqaba Biotec (http://www.inqababiotec.co.za/).

6.2.2.2. Analysis of sequenced amplified PCR products

Chromatograms received from Inqaba Biotec after sequencing were edited in Chromas (the Chromas Lite program (Technelysium) can be downloaded from: http://www.technelysium.com.au/chromas.html), exported as a FASTA format file and submitted to the basic local alignment search tool (BLAST) (using the BLASTn function) located on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A continuous sequence from the top hit (one with the highest identity) out of the sequences which produced a significant alignment during the BLASTn search was chosen and used to search for a similar/exact sequence(s) in the annotated CZA14^T genome. The contig information found in the annotated CZA14^T genome was then linked back to the antiSMASH information to determine the biosynthetic gene cluster responsible for the expression of the PKS-II.

6.2.3. Tentative identification of compounds via Liquid Chromatography/Mass Spectrometry (LC/MS)

A Dionex outlet 3000 high performance liquid chromatography (HPLC) coupled to an amazon SL ion trap mass spectrometer (MS) supplied by Bruker (http://www.bruker.com) was used to identify the exact mass of compounds present in the extracellularly (SNF) produced antimicrobials of strain CZA14^T that were subjected to partial purification using A/S precipitation and dialysis. The parameters used for the analysis, are summarised in Table 6.1.

Table 6.1: HPLC/UV/MS parameters used for the tentative identification of compounds produced by strain CZA14[T]

HPLC:	MS:
Gradient profile The gradient elution profile were as follow: 0 min: 2 % B; 0 – 70 min: 100 % B; 70 – 71 min: 2 % B; 71 – 80 min: 2 % B. *solvent A is 0.1 % formic acid and B is acetonitrile.	**Polarity** Negative and positive mode, respectively **Nebulizer Pressure** 2.4 Bar **Dry gas flow** 9 L/min **Dry gas temperature** 300 °C

6.3. RESULTS

6.3.1. Genome mining using antiSMASH

AntiSMASH was used to mine the genome of *Streptomyces pharetrae* CZA14[T] to determine its biosynthetic potential for SM production. Fifty-five biosynthetic gene clusters (Appendix P, Table P1) were detected using antiSMASH with eight being polyketide synthases (PKSs) which are of relevance to this study (Table 6.2).

Table 6.2: Polyketide synthase biosynthetic potential of *S. pharetrae* CZA14[T] determined by AntiSMASH

No.	Biosynthetic gene cluster	Contig/Cluster
1	Butyrolactone-PKS-II	96/14
2	PKS-I	116/16
3	PKS-II	149/18
4	PKS-II	196/24
5	PKS-III	221/27
6	PKS-I	336/35
7	Other KS (type IV) PKS	344/36
8	Other KS (type IV)-PKS-I	421/45

6.3.2. Polymerase chain reaction (PCR) screening for the biosynthetic genes involved in the production of aromatic Type-II polyketides (PKS-II)

Genes governing the expression of Type-II polyketide synthases (PKSs) were determined using aromatic PKS forward and reverse primers for PCR. The result is presented in Figure 6.2. Amplified PCR product obtained when cDNA was used (Figure 6.2) was submitted to Inqaba

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Biotec for sequencing and similarities of nucleic acid sequences of SM biosynthetic genes within and between species were determined (Table 6.3) and linked back to antiSMASH results (Figure 6.3).
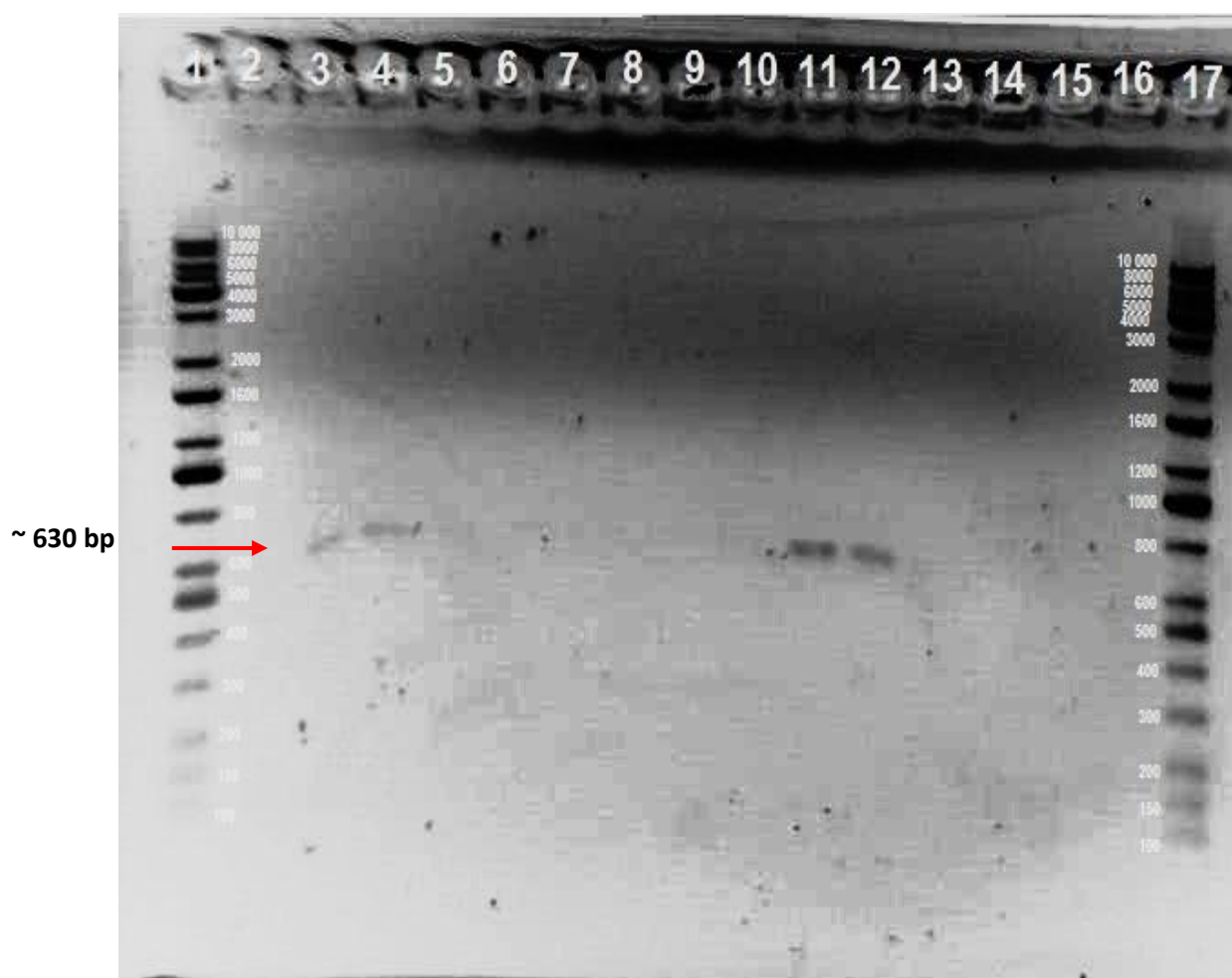
Figure 6.2: 1 % TBE agarose gel electrophoretogram of PCR amplification of type-II PKS gene of cDNA products isolated from airlift fermentation broth a various time periods for the genes involved in the synthesis of PKS-II. Lanes 1 and 16: 1kb Kapa Biosystems universal molecular weight marker, Lanes 3 and 4: cDNA of samples taken at day 0, Lanes 5 and 6: cDNA of samples taken at day 3; Lanes 7 and 8: cDNA of samples taken at day 6; Lanes 9 and 10: cDNA of samples taken at day 9; Lanes 11 and 12: cDNA of samples taken at day 12; Lane 13: RNA control; Lane 14: Control containing water (controls provided by manufacturer)

During PCR screening of the Type-II PKS gene, an approximate 630 bp fragment was amplified for samples taken on day 0 and day 12 of the fermentation with the airlift bioreactor. No amplification of the Type-II PKS gene took place at days 3, 6, and 9.

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Table 6.3: Continuous sequence from strain with highest identity during BLASTn search (*Streptomyces glaucescens* strain GLA) of ARO-PKS sequences found to be similar in annotated genome of CZA14[T]

Contig	Start	Stop	Strand	Function	Evidence code
196	4101	5342	+	Polyketide chain length factor WhiE – CLF	Spore pigment biosynthetic cluster in actinomycetes

Figure 6.3: Percentage similarity of homologous known gene clusters compared to the query sequence (CZA14[T]) determined by antiSMASH.

6.3.3. Analysis of sequenced amplified PCR products

The PCR screening for the biosynthetic genes involved in the biosynthesis of a type-II PKS antimicrobial agent was established on the basis of the amplification with specific primers.

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Sequencing data was analysed using the BLASTn function and the results were linked back to information found in the annotated CZA14[T] genome (Table 6.2) and antiSMASH results (Figure 6.3).

6.3.4. Tentative Identification via Liquid Chromatography/Mass Spectrometry (LC/MS)

The fragmentation profile of the partially purified compounds produced extracellularly by CZA14[T] ([#]PP) were elucidated using the ion trap in both positive and negative mode. The MS product-ion mass spectra of [#]PP is presented in Figure 6.4 along with the structural elucidation of the Type-II PKS, curamycin produced by CZA14[T] under optimal fermentation conditions with the ALR.
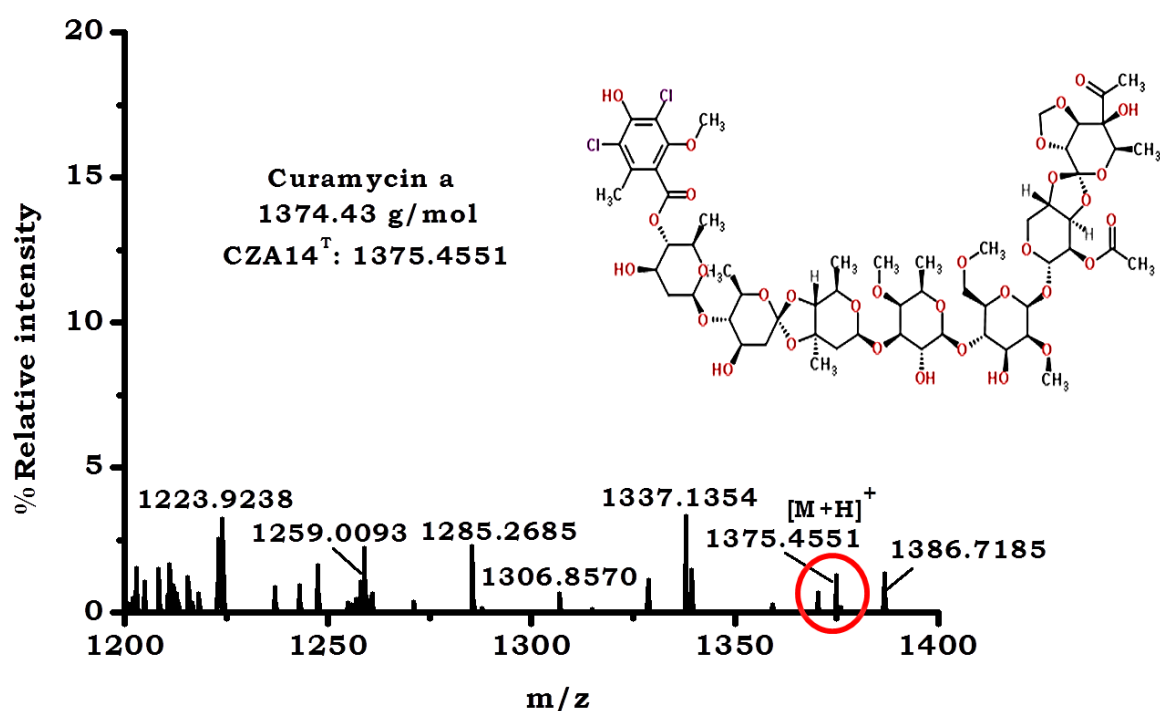
Figure 6.4: Chromatogram from LC/MS analysis of partially purified extract for the elucidation of antimicrobial agent curamycin A.

The compound, curamycin A, was tentatively identified through LC/MS analysis of the salt precipitated partially purified extracellularly produced compounds extracted as crude compounds from the ALR (named "PP"). The exact mass of the compound determine by the LC/MS used in this study was 1374.8 m/z (minus 1 from the mass determined in positive mode); curamycin A has an exact mass of 1374.4 g/mol.

6.4. DISCUSSION

Genome mining using antiSMASH

Identification of novel antibiotics has become a major issue even though the number of antibiotics ubiquitous in nature may truly be large. A number of these antibiotics have already been well researched and may not be entirely useable due to either lacking desired pharmalogical properties or displaying low, weak or selective toxicity towards bacteria (Peleaz, 2006). A number of microbiological approaches with the incorporation of genetic, structural and bioinformatics methods have been developed to deal with this issue and molecular screening has become invaluable to determine the chemical diversity of a microorganism via the detection of secondary metabolite biosynthetic genes.

Strain CZA14^T was chosen as an antimicrobial producer in this study as it showed weak antibiosis against *Mycobacterium aurum* A+, *Bacillus coagulans* ATCC 7050^T and *Acinetobacter calcoaceticus* strain C91 (le Roes and Meyers, 2005). It is a Gram-positive, aerobic actinomycete capable of producing spores, green aerial mycelium and yellow-blue substrate mycelium when cultured on ISP 4 (inorganic salts- starch agar) as well as ISP #2, and melanin which is associated with secondary metabolite activity (le Roes and Meyers, 2005). Le Roes and Meyers (2005) reported that the substrate mycelium colour is dependent on the medium CZA14^T is cultured in/on, but the colour of the aerial mycelial remains constant.

Actinomycetes have relatively large genomes compared to other bacteria and are the most prolific antibiotic producers with about 10% of all genes used during the production of secondary metabolites, such as polyketides and non-ribosomal peptides (Donadio *et al.*, 2007; Baltz, 2008).

Based on genome sequencing, certain microorganisms, especially actinomycetes, have been shown to contain 20 or more biosynthetic gene clusters (Davies, 2011). In this study antiSMASH was used to mine the genome of strain CZA14^T for biosynthetic gene clusters to determine its potential to produce antimicrobials. Fifty five biosynthetic gene clusters were detected (Appendix P, Table P1) with eight being PKSs (Table 6.1). This was accomplished by comparing individual genes in the CZA14^T genome against a collection of profile Hidden Markov Models (pHMMs) that was manually curated. Key biosynthetic enzymes of the NRPS and PKS classes detectable by antiSMASH are described by these pHMMs with the use of HMMer3 software

(Eddy, 2011). Furthermore, individual gene clusters encoding key enzymes are assigned to secondary metabolite-specific Clusters of Orthologous Groups 32 (smCOGs) and a core chemical structure of the putative biosynthetic compound based on the data within antiSMASH was generated and displayed as an output file (Blin *et al.*, 2013). AntiSMASH was chosen over other bioinformatics tools such as Clust Scanner (ClustScan), sequence analysis of polyketide synthases (SBSPKS) toolbox and NP.searcher, as the aforementioned bioinformatics tools are unfortunately largely limited to the analyses of NRPS and type I PKS clusters. Whereas, AntiSMASH identifies core genes as well as accessory genes for many other secondary metabolite platforms. AntiSMASH provides rapid and accurate detection of all known classes of secondary metabolite biosynthetic gene clusters (smBGCs), as well as predict PKS and NRPS product chemical structures and provide PKS and NRPS functional annotation with higher accuracy compared to existing techniques (Blin *et al.*, 2013).

Polyketides are generated by the assembly of large synthetases in a modular form. These modules consist of multiple domains each of which are able to catalyse a sequential reaction step (e.g. condensation, tailoring or building block activation) or recognizing a particular substrate (Walsh and Fischbach, 2010). Based on the fact that polyketides are templated, genomic loci that encode for biosynthetic pathways are easily identified by bioinformatics tools. This may provide clues to the properties and structure of natural products essential in the development of methods for their detection as well as their isolation.

Whilst there are a large number of NRPSs predicted by antiSMASH only eight PKSs were predicted (Table 6.1). γ-butyrolactones are produced by a large number of streptomycetes (Horinouchi and Beppu, 1992) and are involved in the initiation of secondary metabolism in several species. Type I, II, III and hybrid PKSs has been described in Chapter 2 and little is known about type IV/other KS (some parts of this paragraph was adapted from Durrell, 2013, honours thesis).

Using a combination of bioinformatics tools, antiSMASH and molecular techniques such as PCR; the genes governing the expression of curamycin A, a type-II PKS or a spore pigment was detected. Multi-enzyme type-II PKS, catalyzes the biosynthesis of an array of aromatic polyketide therapeutic agents (Shapiro, 1989). In this study, the primer pair ARO-PKS-F/ARO-PKS-R for detection of type-II PKS genes was used and amplified a fragment of approximately 630 bp for the ALR samples collected at days 0 and 12. The detection of amplified product at

day 0 could be a result of sporulation occurring in the ISP #2 pre-culture prior to inoculation of the ALR. According to Wood *et al*. (2007), depending on the bacterium tested, a fragment of 492 – 630 bp is expected. Furthermore, the amplification of an α-ketoacyl synthase (KSα) – β ketoacyl synthase (KSβ) fragment which is involved in genes for the synthesis of a spore-pigment is dependent on the primers used. Mono-functional proteins are involved in the synthesis of aromatic polyketides, these proteins contain catalytic activities. This is evident in Table 6.2, whereby the functional gene – polyketide chain length factor WhiE-CLF is involved in the biosynthesis of the type-II PKS by CZA14^T. This gene is found on the spore pigment cluster in actinomycetes. In CZA14^T, this gene is located on contig 196 (14 308 nucleotides (nt) in length), starting at 4101 nt and stops at 5342 nt on the positive strand of the CZA14^T dsDNA.

According to the antiSMASH data, 100% of CZA14^T genes show homology to a curamycin biosynthetic gene cluster as well as the spore pigment biosynthetic gene cluster from *Streptomyces glaucescens* strain GLA (Figure 6.3).

The antiSMASH and PCR screening of the Type-II PKS is indicative of CZA14^T's potential to possess genes which are necessary for the biosynthesis of that type of antimicrobial. It is only after purification and elucidation of the antimicrobials molecular structure which determines the identity of that antimicrobial. The optimal medium for maximum antimicrobial production by CZA14^T used in this study supported the production of curamycin A and is discussed in greater detail below.

Tentative Identification of curamycin A via Liquid Chromatography/Mass Spectrometry (LC/MS)

AntiSMASH analysis of the CZA14^T genome and PCR screening for Type-II PKS biosynthetic gene clusters from fermentation broth from the ALR in which CZA14^T was cultured, showed that CZA14^T possess the biosynthetic genes for the production of Type-II PKS and produces curamycin A when cultured in optimal production medium 153 + glycerol, pH 6.0 at 30°C. Extracellularly produced compounds partially purified using A/S precipitation, dialysis and EtOAc solvent extraction were subjected to LC/MS analysis to determine the production of curamycin A by CZA14^T. Curamycin A has a molecular formula of $C_{59}H_{84}Cl_2O_{32}$ and a molecular weight of 1376.18506 g/mol (http://pubchem.ncbi.nlm.nih.gov/compound/71587265#section=Top). This compound has an exact mass of 1374.432276 g/mol. The compound produced by CZA14^T was derived in the

positive mode only. A compound with an exact mass of 1375. 8 m/z was detected from the mass spectra presented in Figure 6.7. Positive ion mode data was adjusted accordingly by adding a Na atom and subtracting an electron producing a compound with an m/z of 1374. 8. The differences in exact mass by 0.4 m/z could be as a result of minor differences in the chemical and physicochemical properties of the compound or due to calibration inefficiencies of the LC/MS. Curamycin A is highly active against a number of Gram positive microorganisms. This PK antibiotic can inhibit the growth of *S. aureus*, *B. subtilis*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Neissera gonorrheae*, *Lactobacillus acidophilus*, *Streptococcus agalactiae*, *Streptococcus haemolyticus* and *Bacillus polymyxa* at concentrations as low as 0.5 mcg/mL. Curamycin does not inhibit the growth of Gram-negative bacteria *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Salmonella schottmulleri*, *P. aeruginosa* and *K. pneumoniae* even at concentrations as high as 50 mcg/mL. In this study, the sample labelled as "PP" containing the isolated compound, curamycin, was active against *K. pneumoniae* and *P. aeruginosa* DRWH system isolates during the bioautography assay. This suggests that more than one compound (including curamycin A) was isolated during solvent extraction and purification processes (Cataldi *et al.*, 1962).

6.5. CONCLUSION

AntiSMASH prediction software predicted a total of fifty-five biosynthetic gene clusters of which eight were PKSs. Predicting genes which govern the expression of compounds produced by *S. pharetrae* CZA14^T prior to fermentation studies showed to be advantageous as it aided in the design of media for the expression and identification of a Type-II PKS biosynthetic gene cluster. Along with molecular techniques, CZA14^T showed 100% homology to curamycin A via antiSMASH; this was confirmed by PCR. Although curamycin A was rediscovered and produced by CZA14^T in this study, CZA14^T possess a number of biosynthetic genes and the genetic information of strain CZA14^T has shown that these natural resources with regards to their metabolite production capabilities, have been seriously underestimated: the number of compounds produced under laboratory conditions (Chapter 3) are lower than the amount of biosynthetic gene clusters predicted by antiSMASH (Appendix A, Table 8.1). Salt purified compounds were tentatively identified using LC/MS analysis and a compound with an exact mass of 1376.18506 g/mol was detected. This compound was identified by mass as Curamycin A, a type II PK antimicrobial.

6.6.   REFERENCES

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CHAPTER 7:

Concluding remarks and recommendations

Conclusions

Pre-screening actinomycete genomes using bioinformatics tools such as antiSMASH and molecular techniques such as PCR prior to the commencement of fermentation studies provide a reliable indicator of the biosynthetic potential of the producing strain. CZA14^{T} contains a number of secondary metabolite biosynthetic gene clusters (smBGCs), some of which were expressed during this study. One of these were a Type-II polyketide (PK) named curamycin A as determined using PCR and LC/MS. The production of these compounds were favoured due to constituents in the liquid media. Antimicrobial bioassays used in this study successfully detected highly active products containing antibacterial and antifungal activity. Partially purified extracts were active against most of the domestic rainwater harvesting (DRWH) system isolates used in this study. Further antimicrobial assays such as minimal inhibitory concentrations (MICs) will be tested in future. This was not performed during this study due to low volume of extracts and time constraints. UV/Vis spectroscopic studies and various TLC staining methods were used in characterising crude and partially purified extracts. Further purification and characterisation will be performed on these compounds as they exhibit broad-spectrum activities against both American Type Culture Collection (ATCC) test strains and DRWH system isolates. Toxicity tests (e.g. in mouse or rat models) may be performed to ascertain whether these antimicrobials are safe to use in the treatment of infectious diseases caused by pathogenic bacteria found in DRWH systems and hospital settings where the clinical ATCC test strains were isolated.

In conclusion, linking back my results with objectives of this study:

Objective 1

Optimize medium-scale (2L – 5L) production of antimicrobials produced by *Streptomyces pharetrae* CZA14^{T}

Conclusion

The production of compounds of interest was favoured due to constituents in the liquid media as well as due to increased aeration in the airlift reactor (3 L).

Objective 2

Test the inhibitory efficacy of crude extracts against ATCC test strains and DRWH system isolates

Conclusion

Results obtained during bioassay screening of compounds produced by CZA14^T against rainwater harvesting system isolates were comparable to bio-activities detected against clinical ATCC isolates. However, more active compounds were produced during the culturing of CZA14^T in the airlift reactor compared to the number of active compounds produced during culturing of CZA14^T in the 2 L baffled flasks and continuous stirred tank reactor; this was confirmed by bioautography assays.

Objective 3

The partial purification of selected compounds and the determination of their antimicrobial profiles

Conclusion

Extracts partially purified by ammonium salt precipitation were active against all of the rainwater harvesting system isolates used in this study. Furthermore, exceeding the expectation of this study - it was found that predicting genes which govern the expression of compounds produced by CZA14^T prior to fermentation studies showed to be advantageous as it aided in the design of media for the expression and tentative identification by LC/MS of a Type-II PKS biosynthetic gene cluster and its product. Along with molecular techniques, CZA14^T showed 100% homology of its genes to curamycin A via antiSMASH; this was confirmed by PCR.

The bio-activities detected in this study may be a result of curamycin A along with other compounds extracted resulting in a synergistic effect. Although curamycin A was rediscovered and produced by CZA14^T in this study, CZA14^T possess a number of novel biosynthetic genes which may be explored in future.

What makes this project unique?

This project is unique as it is the first time CZA14^T, a novel *Streptomyces* species, is explored for its biosynthetic potential related to its ability to produce antimicrobials. There is also no other

research study that have focused on the determination of the bio-activity of antimicrobials produced by a *Streptomyces* species against pathogens isolated from rainwater harvesting systems.

Future work and Recommendations

- Genome mining has become increasingly inexpensive over the past decade – further analysis of the genome by other bioinformatics tools and molecular techniques (i.e. DNA probes for detection of 'silent' biosynthetic gene clusters) to aid in the discovery of novel natural products may be performed in future

- Other fermentation vessels, optimisation of currently used fermentation vessels in this study, and solid state fermentation may be explored to produce chemically different natural products

- Further purification of selected compounds using column chromatography methods (i.e. high performance liquid chromatography (HPLC) – preparative HPLC), precipitation and extraction methods may be performed

- The use of heterologous/homologous hosts to express gene clusters of interest containing silent phenotypes

- High-throughput techniques such as mass-spectroscopy and near-infrared spectroscopy to discover novel secondary metabolites

- Co-culturing of antibiotic-producing microorganisms with a target pathogen to accelerate evolution of the producer's ability to biosynthesize a chemical product that completely inhibits the growth of the target pathogen

Appendices

Appendix A

Flat-bottom flask vs baffled flask optimisation in 0.5 L flask volume
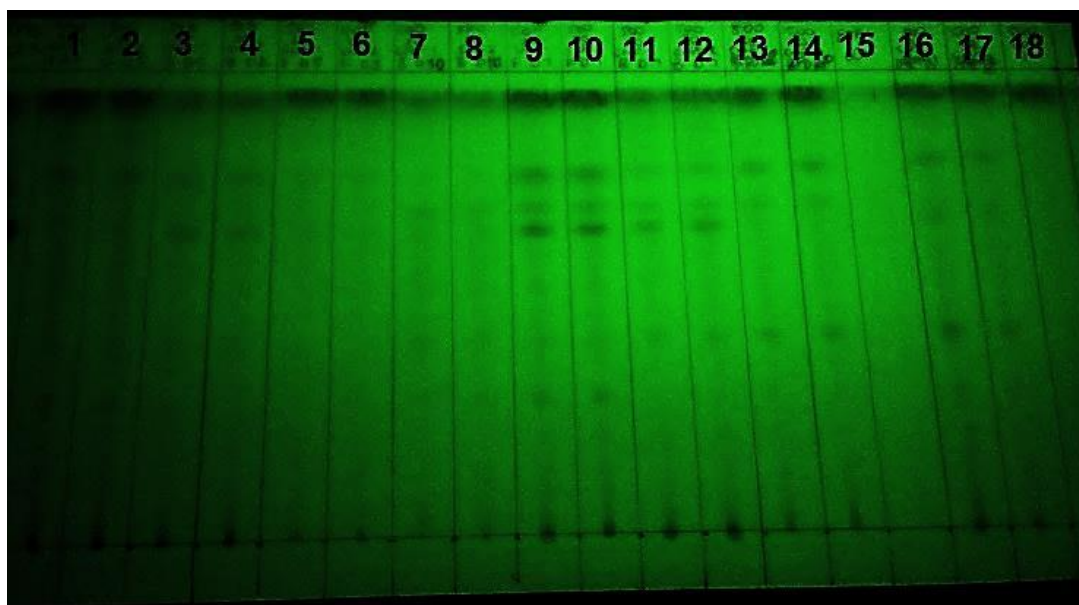
Figure A1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). 500 ml Flat-bottom flask vs baffled flask optimisation tests with *S. pharetrae* sp. strain CZA14[T] cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3, flat-bottom flask. Lane 3 and 4: EtOAc extracts – from SNF, day 3, baffled flask. Lane 5 and 6: EtOAc extracts – from SNF, day 5, flat-bottom flask. Lane 7 and 8: EtOAc extracts – from SNF, day 10, baffled flask. Lane 9 and 10: EtOAc extracts – from SNF, day 7, flat-bottom flask. Lane 11 and 12: EtOAc extracts – from SNF, day 7, baffled flask. Lane 13 and 14: EtOAc extracts – from SNF, day 10, flat-bottom flask. Lane 15: EtOAc control. Lane 16 and 17: EtOAc extracts – from SNF, day 3, baffled flask. Lane 18: 153 + glycerol media control.
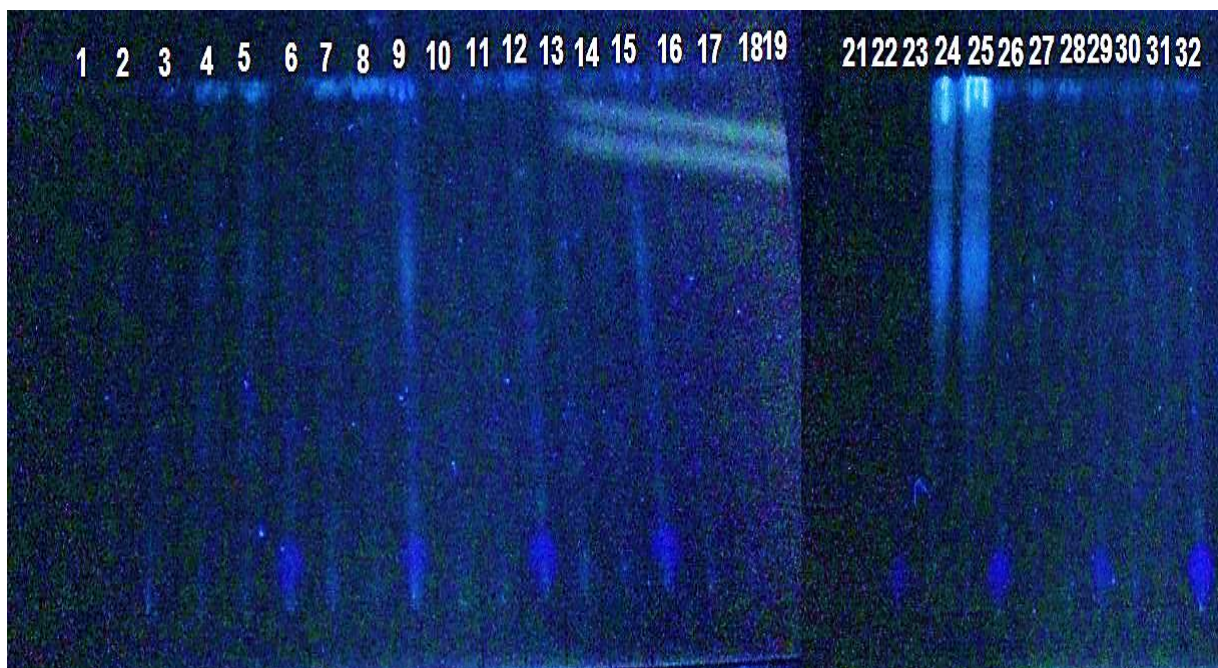
Figure A2: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). 500 ml Flat-bottom flask vs baffled flask optimisation tests with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3, flat-bottom flask. Lane 3 and 4: EtOAc extracts – from SNF, day 3, baffled flask. Lane 5 and 6: EtOAc extracts – from SNF,day 5, flat-bottom flask. Lane 7 and 8: EtOAc extracts – from SNF, day 10, baffled flask. Lane 9 and 10: EtOAc extracts – from SNF, day 7, flat-bottom flask. Lane 11 and 12: EtOAc extracts – from SNF, day 7, baffled flask. Lane 13 and 14: EtOAc extracts – from SNF, day 10, flat-bottom flask. Lane 15: EtOAc control. Lane 16 and 17: EtOAc extracts – from SNF, day 3, baffled flask. Lane 18: 153 + glycerol media control.
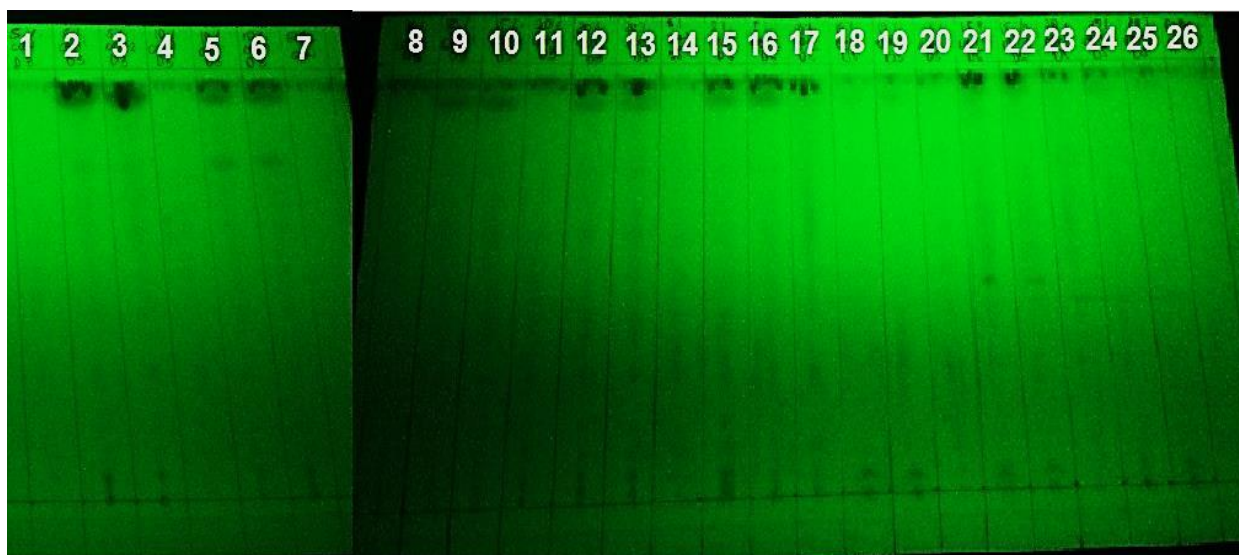
Figure A3: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Flat-bottom flask vs baffled flask optimisation tests with *S. pharetrae* sp. strain CZA14[T] cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from cell mass (cell), day 3, flat-bottom flask. Lane 3 and 4: EtOAc extracts – from cell, day 3, baffled flask. Lane 5 and 6: EtOAc extracts – from cell, day 5, flat-bottom flask. Lane 7 and 8: EtOAc extracts – from cell day 5, baffled flask. Lane 9 and 10: EtOAc extracts – from cell, day 7, flat-bottom flask. Lane 11 and 12: EtOAc extracts – from cell, day 7, baffled flask. Lane 13 and 14: EtOAc extracts – from cell, day 10, flat-bottom flask. Lane 15 and 16: EtOAc extracts – from cell, day 10, baffled flask. Lane 17: EtOAc control. Lane 18: 153 + glycerol control.

Figure A4: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Flat-bottom flask vs baffled flask optimisation tests with *S. pharetrae* sp. strain CZA14[T] cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from cell mass (cell), day 3, flat-bottom flask. Lane 3 and 4: EtOAc extracts – from cell, day 3, baffled flask. Lane 5 and 6: EtOAc extracts – from cell, day 5, flat-bottom flask. Lane 7 and 8: EtOAc extracts – from cell day 5, baffled flask. Lane 9 and 10: EtOAc extracts – from cell, day 7, flat-bottom flask. Lane 11 and 12: EtOAc extracts – from cell, day 7, baffled flask. Lane 13 and 14: EtOAc extracts – from cell, day 10, flat-bottom flask. Lane 15 and 16: EtOAc extracts – from cell, day 10, baffled flask. Lane 17: EtOAc control. Lane 18: 153 + glycerol control.

Appendix B

$$R_f = \frac{2.1}{2.8} = 0.75$$
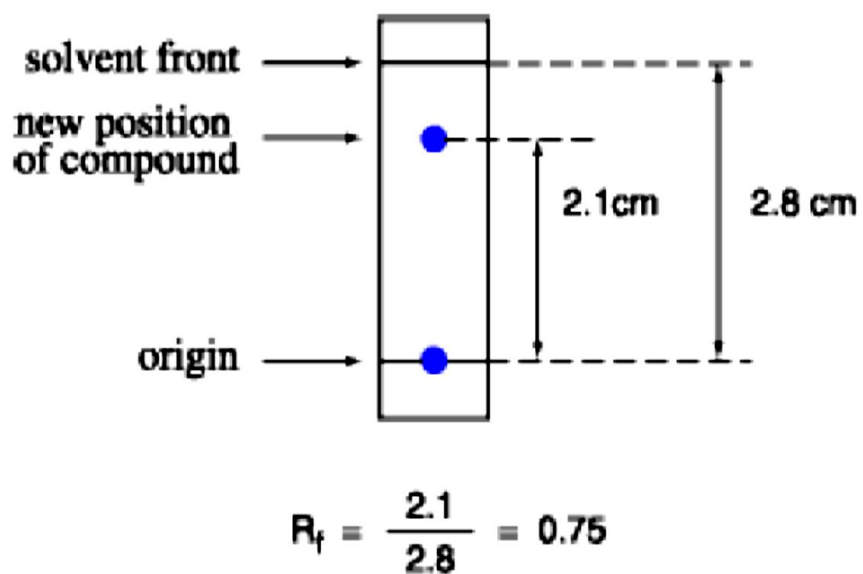
Figure B1: An example to demonstrate the way in which R_f values are calculated.

Appendix C

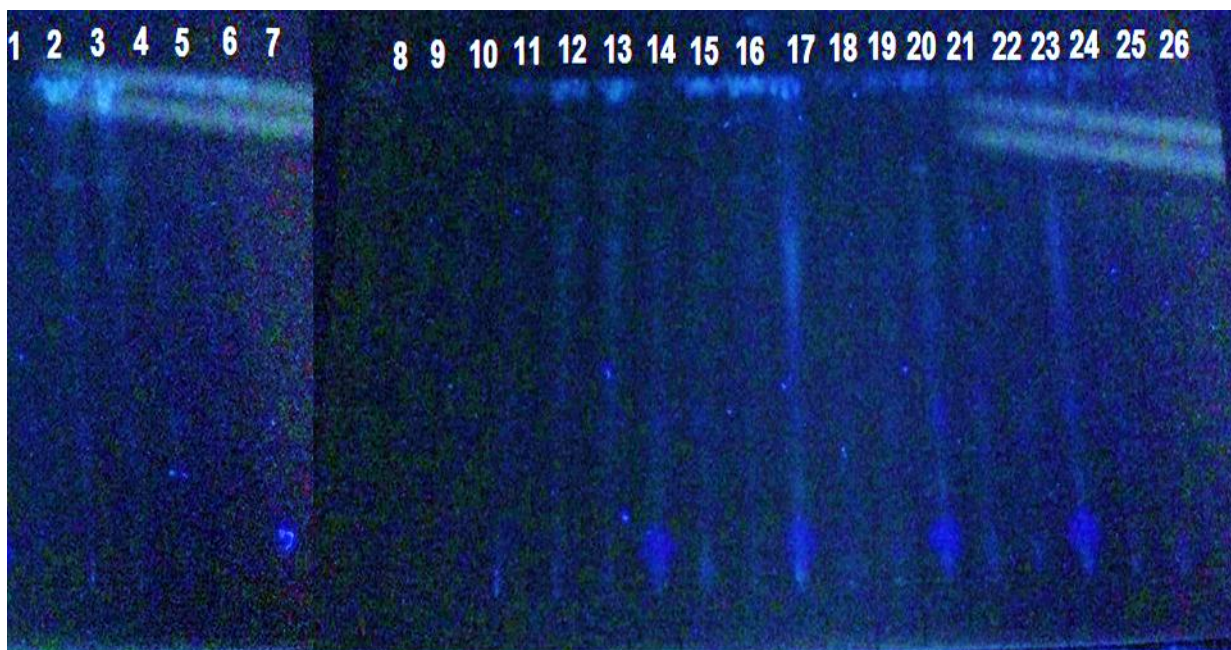**Optimisation of inoculum volume in 500 ml baffled flasks**
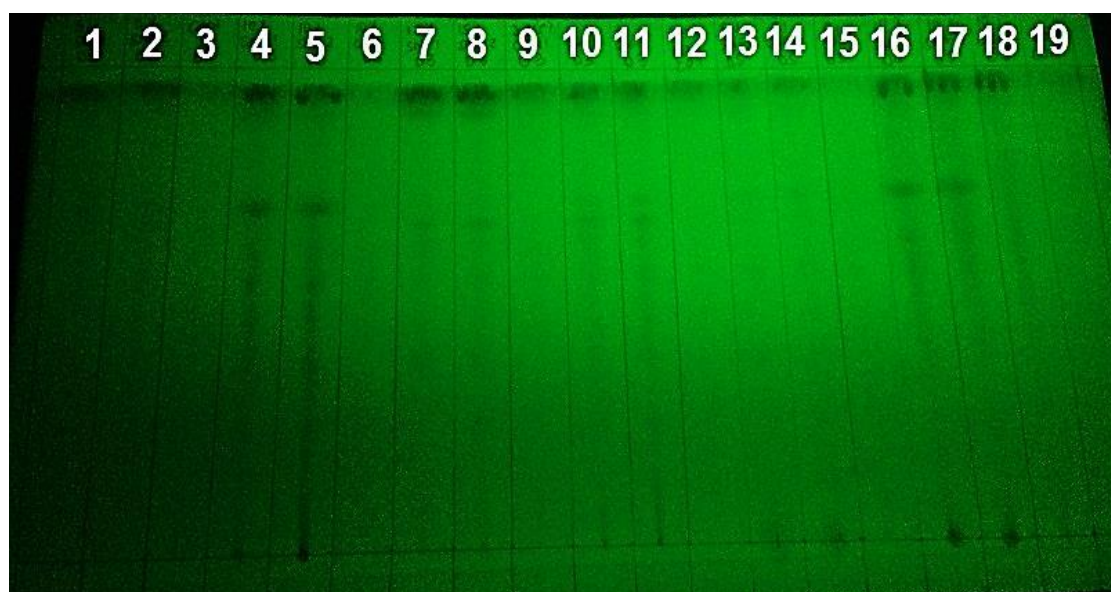
Figure C1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3, 5 % v/v inoculum vol. Lane 3: 153 + glycerol media control, day 3, 95 ml media vol; Lane 4 and 5: EtOAc extracts – from supernatant filtrate (SNF), day 3, 10 % v/v inoculum vol. Lane 6: 153 + glycerol media control, day 3, 90 ml media vol; Lane 7 and 8: EtOAc extracts – from supernatant filtrate (SNF), day 3, 15 % v/v inoculum vol. Lane 9: 153 + glycerol media control, day 3, 85 ml media vol; Lane 10 and 11: EtOAc extracts – from supernatant filtrate (SNF), day 3, 20 % v/v inoculum vol. Lane 12: 153 + glycerol media control, day 3, 80 ml media vol; Lane 13 and 14: EtOAc extracts – from supernatant filtrate (SNF), day 5, 5 % v/v inoculum vol. Lane 15: 153 + glycerol media control, day 5, 95 ml media vol; Lane 16 and 17: EtOAc extracts – from supernatant filtrate (SNF), day 5, 10 % v/v inoculum vol. Lane 18: 153 + glycerol media control, day 5, 90 ml media vol; Lane 19: EtOAc control.
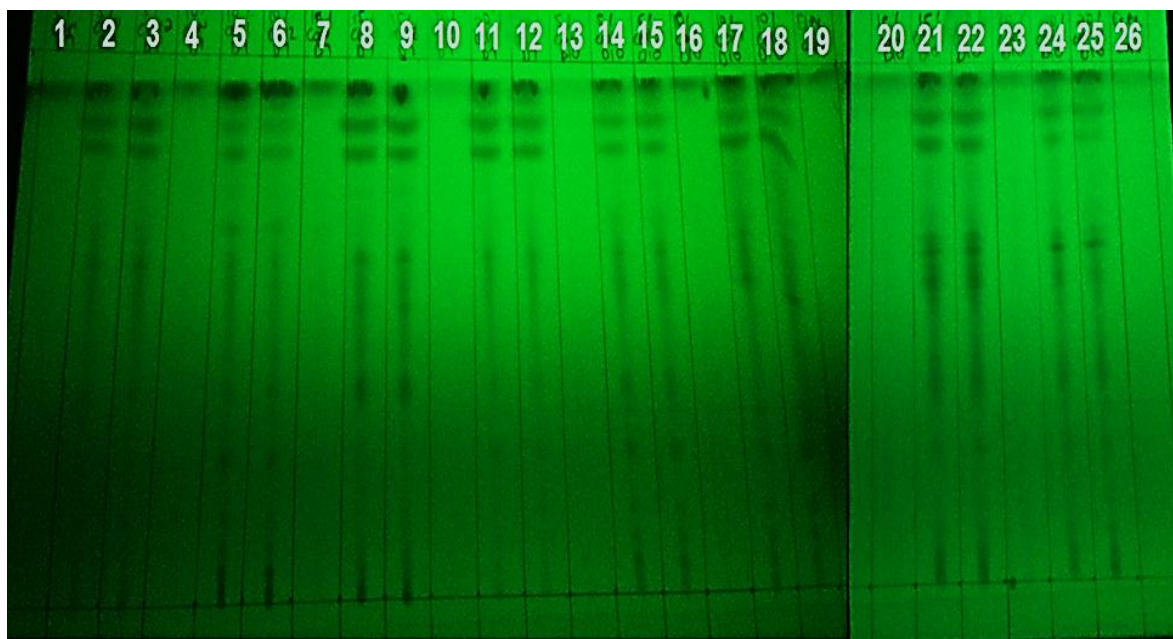
Figure C2: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3, 5 % v/v inoculum vol. Lane 3: 153 + glycerol media control, day 3, 95 ml media vol; Lane 4 and 5: EtOAc extracts – from supernatant filtrate (SNF), day 3, 10 % v/v inoculum vol. Lane 6: 153 + glycerol media control, day 3, 90 ml media vol; Lane 7 and 8: EtOAc extracts – from supernatant filtrate (SNF), day 3, 15 % v/v inoculum vol. Lane 9: 153 + glycerol media control, day 3, 85 ml media vol; Lane 10 and 11: EtOAc extracts – from supernatant filtrate (SNF), day 3, 20 % v/v inoculum vol. Lane 12: 153 + glycerol media control, day 3, 80 ml media vol; Lane 13 and 14: EtOAc extracts – from supernatant filtrate (SNF), day 5, 5 % v/v inoculum vol. Lane 15: 153 + glycerol media control, day 5, 95 ml media vol; Lane 16 and 17: EtOAc extracts – from supernatant filtrate (SNF), day 5, 10 % v/v inoculum vol. Lane 18: 153 + glycerol media control, day 5, 90 ml media vol; Lane 19: EtOAc control.
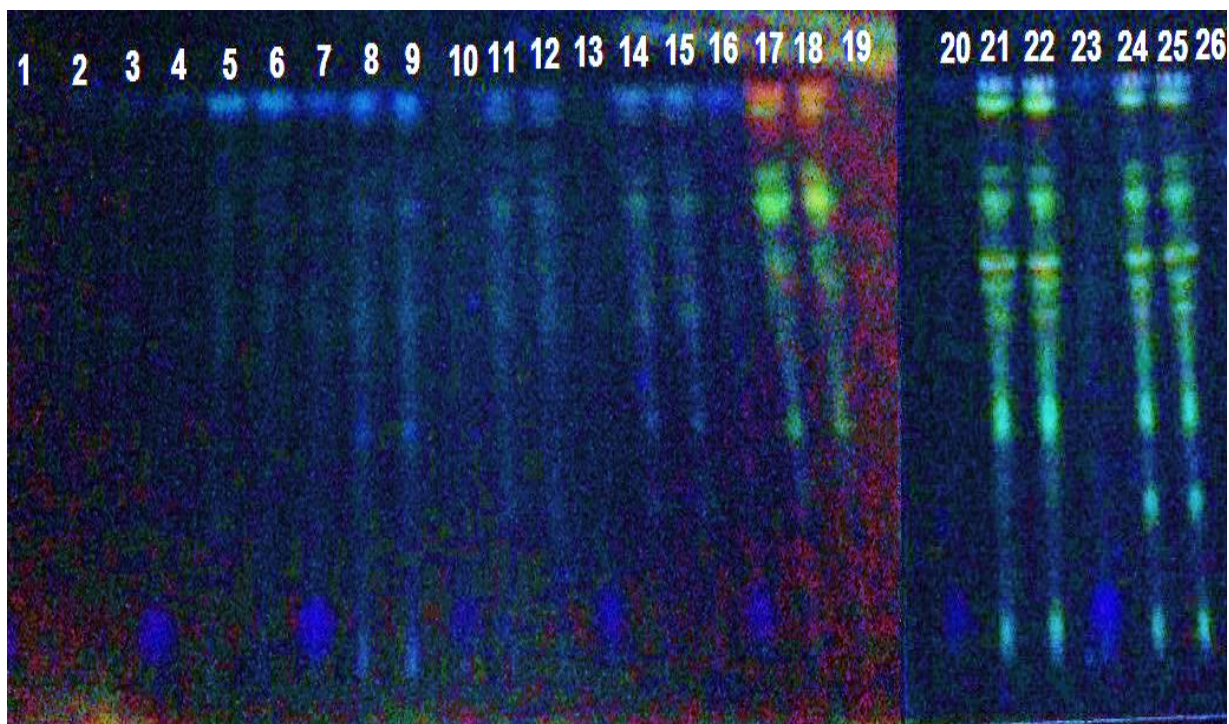
Figure C3: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with S. pharetrae sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 5, 15 % v/v inoculum vol. Lane 3: 153 + glycerol media control, day 5, 85 ml media vol; Lane 4 and 5: EtOAc extracts – from supernatant filtrate (SNF), day 5, 20 % v/v inoculum vol. Lane 6: 153 + glycerol media control, day 5, 80 ml media vol; Lane 7 and 8: EtOAc extracts – from supernatant filtrate (SNF), day 7, 5 % v/v inoculum vol. Lane 9: 153 + glycerol media control, day 7, 95 ml media vol; Lane 10 and 11: EtOAc extracts – from supernatant filtrate (SNF), day 7, 10 % v/v inoculum vol. Lane 12: 153 + glycerol media control, day 7, 90 ml media vol; Lane 13 and 14: EtOAc extracts – from supernatant filtrate (SNF), day 7, 15 % v/v inoculum vol. Lane 15: 153 + glycerol media control, day 7, 85 ml media vol; Lane 16 and 17: EtOAc extracts – from supernatant filtrate (SNF), day 7, 20 % v/v inoculum vol. Lane 18: 153 + glycerol media control, day 7, 80 ml media vol; Lane 19: EtOAc control; Lane 21 and 22: EtOAc extracts – from supernatant filtrate (SNF), day 10, 5 % v/v inoculum vol. Lane 23: 153 + glycerol media control, day 10, 95 ml media vol; Lane 24 and 55: EtOAc extracts – from supernatant filtrate (SNF), day 10, 10 % v/v inoculum vol. Lane 26: 153 + glycerol media control, day 10, 90 ml media vol; Lane 27 and 28: EtOAc extracts – from supernatant filtrate (SNF), day 10, 15 % v/v inoculum vol. Lane 29: 153 + glycerol media control, day 10, 85 ml media vol; Lane 30 and 31: EtOAc extracts – from supernatant filtrate (SNF), day 10, 20 % v/v inoculum vol. Lane 32: 153 + glycerol media control, day 10.
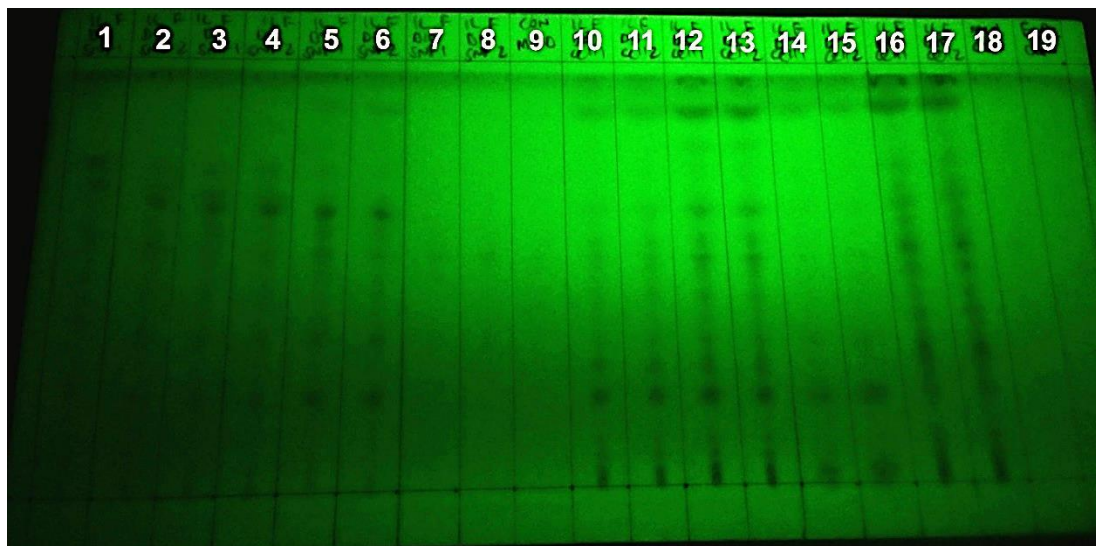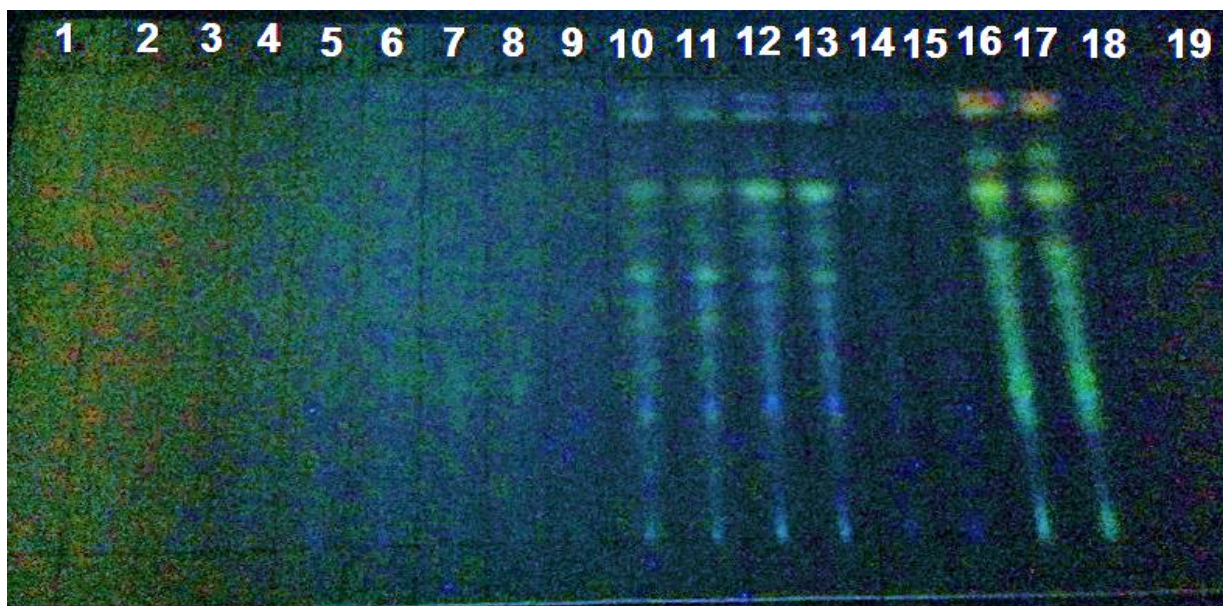
Figure C4: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 5, 15 % v/v inoculum vol. Lane 3: 153 + glycerol media control, day 5, 85 ml media vol; Lane 4 and 5: EtOAc extracts – from supernatant filtrate (SNF), day 5, 20 % v/v inoculum vol. Lane 6: 153 + glycerol media control, day 5, 80 ml media vol; Lane 7 and 8: EtOAc extracts – from supernatant filtrate (SNF), day 7, 5 % v/v inoculum vol. Lane 9: 153 + glycerol media control, day 7, 95 ml media vol; Lane 10 and 11: EtOAc extracts – from supernatant filtrate (SNF), day 7, 10 % v/v inoculum vol. Lane 12: 153 + glycerol media control, day 7, 90 ml media vol; Lane 13 and 14: EtOAc extracts – from supernatant filtrate (SNF), day 7, 15 % v/v inoculum vol. Lane 15: 153 + glycerol media control, day 7, 85 ml media vol; Lane 16 and 17: EtOAc extracts – from supernatant filtrate (SNF), day 7, 20 % v/v inoculum vol. Lane 18: 153 + glycerol media control, day 7, 80 ml media vol; Lane 19: EtOAc control; Lane 21 and 22: EtOAc extracts – from supernatant filtrate (SNF), day 10, 5 % v/v inoculum vol. Lane 23: 153 + glycerol media control, day 10, 95 ml media vol; Lane 24 and 25: EtOAc extracts – from supernatant filtrate (SNF), day 10, 10 % v/v inoculum vol. Lane 26: 153 + glycerol media control, day 10, 90 ml media vol; Lane 27 and 28: EtOAc extracts – from supernatant filtrate (SNF), day 10, 15 % v/v inoculum vol. Lane 29: 153 + glycerol media control, day 10, 85 ml media vol; Lane 30 and 31: EtOAc extracts – from supernatant filtrate (SNF), day 10, 20 % v/v inoculum vol. Lane 32: 153 + glycerol media control, day 10.

Figure C5: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1: 153 + glycerol media control, day 3, 95 ml media vol; Lane 2 and 3: EtOAc extracts – from cell mass (cell), day 3, 5 % v/v inoculum vol; Lane 4: 153 + glycerol media control, day 3, 90 ml media vol; Lane 5 and 6: EtOAc extracts – from cell mass (cell), day 3, 10 % v/v inoculum vol; Lane 7: EtOAc control; Lane 8: 153 + glycerol media control, day 3, 85 ml media vol; Lane 9 and 10: EtOAc extracts – from cell mass (cell), day 3, 15 % v/v inoculum vol; Lane 11: 153 + glycerol media control, day 3, 80 ml media vol; Lane 12 and 13: EtOAc extracts – from cell mass (cell), day 3, 20 % v/v inoculum vol. Lane 14: 153 + glycerol media control, day 5, 95 ml media vol; Lane 15 and 16: EtOAc extracts – from cell mass (cell), day 5, 5 % v/v inoculum vol. Lane 17: 153 + glycerol media control, day 5, 90 ml media vol; Lane 18 and 19: EtOAc extracts – from cell mass (cell), day 5, 10 % v/v inoculum vol; Lane 20: 153 + glycerol media control, day 5, 85 ml media vol; Lane 21 and 22: EtOAc extracts – from cell mass (cell), day 5, 15 % v/v inoculum vol; Lane 23: 153 + glycerol media control, day 5, 80 ml media vol; Lane 24 and 25: EtOAc extracts – from cell mass (cell), day 5, 20 % v/v inoculum vol; Lane 26: EtOAc control.
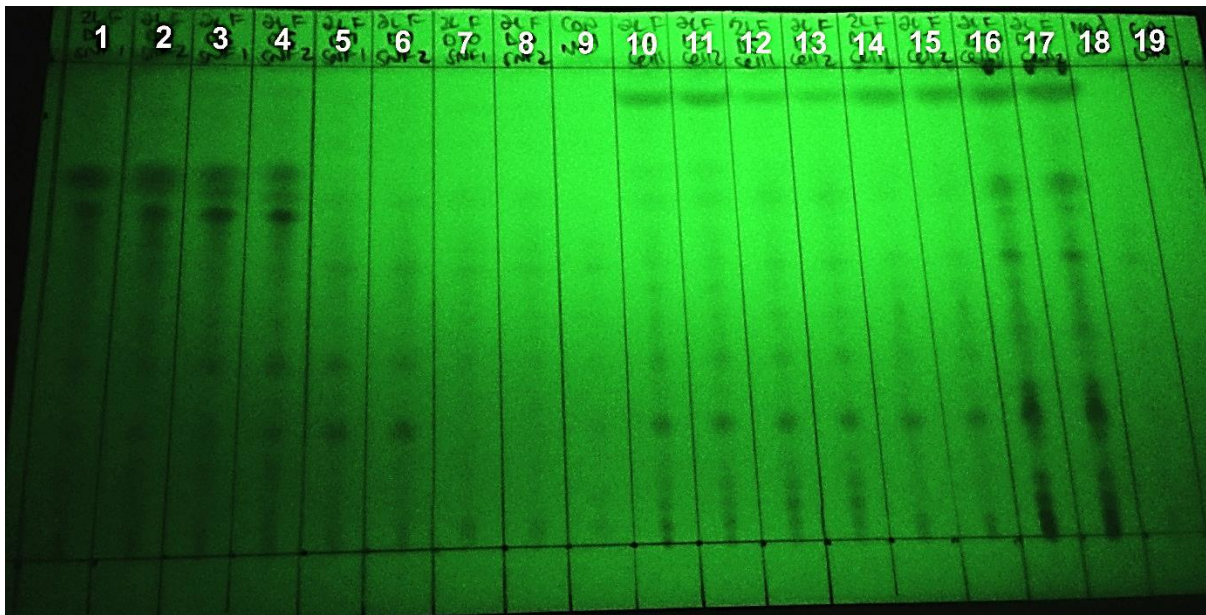
Figure C6: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with S. pharetrae sp. strain CZA14T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1: 153 + glycerol media control, day 3, 95 ml media vol; Lane 2 and 3: EtOAc extracts – from cell mass (cell), day 3, 5 % v/v inoculum vol; Lane 4: 153 + glycerol media control, day 3, 90 ml media vol; Lane 5 and 6: EtOAc extracts – from cell mass (cell), day 3, 10 % v/v inoculum vol; Lane 7: EtOAc control; Lane 8: 153 + glycerol media control, day 3, 85 ml media vol; Lane 9 and 10: EtOAc extracts – from cell mass (cell), day 3, 15 % v/v inoculum vol; Lane 11: 153 + glycerol media control, day 3, 80 ml media vol; Lane 12 and 13: EtOAc extracts – from cell mass (cell), day 3, 20 % v/v inoculum vol. Lane 14: 153 + glycerol media control, day 5, 95 ml media vol; Lane 15 and 16: EtOAc extracts – from cell mass (cell), day 5, 5 % v/v inoculum vol. Lane 17: 153 + glycerol media control, day 5, 90 ml media vol; Lane 18 and 19: EtOAc extracts – from cell mass (cell), day 5, 10 % v/v inoculum vol; Lane 20: 153 + glycerol media control, day 5, 85 ml media vol; Lane 21 and 22: EtOAc extracts – from cell mass (cell), day 5,15 % v/v inoculum vol; Lane 23: 153 + glycerol media control, day 5, 80 ml media vol; Lane 24 and 25: EtOAc extracts – from cell mass (cell), day 5, 20 % v/v inoculum vol; Lane 26: EtOAc control.
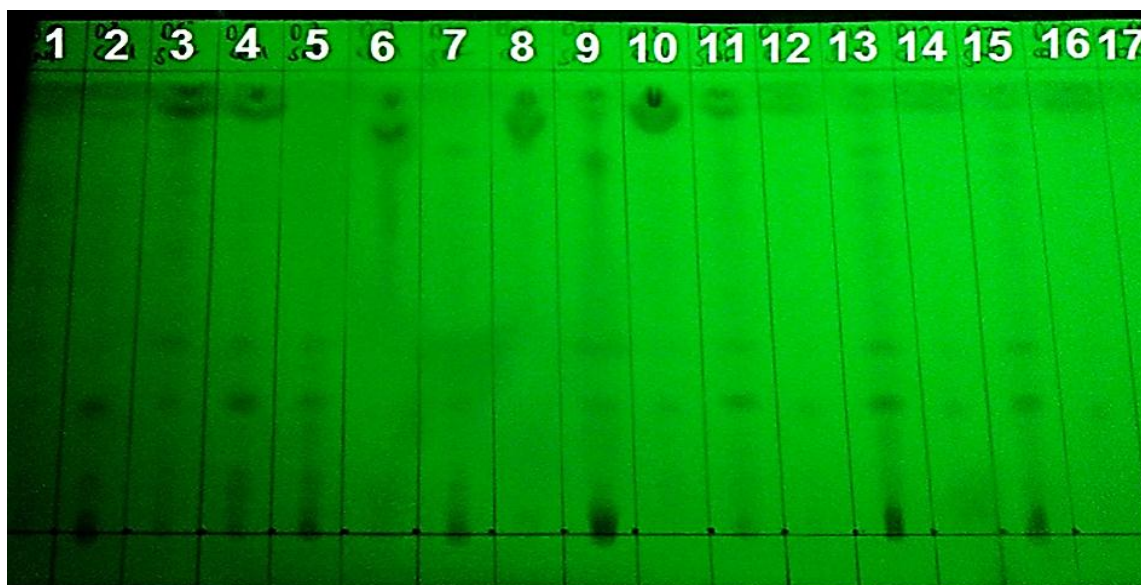
Figure C7: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with S. pharetrae sp. strain CZA14T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1: 153 + glycerol media control, day 7, 95 ml media vol; Lane 2 and 3: EtOAc extracts – from cell mass (cell), day 7, 5 % v/v inoculum vol; Lane 4: 153 + glycerol media control, day 7, 90 ml media vol; Lane 5 and 6: EtOAc extracts – from cell mass (cell), day 7, 10 % v/v inoculum vol; Lane 7: EtOAc control; Lane 8: 153 + glycerol media control, day 7, 85 ml media vol; Lane 9 and 10: EtOAc extracts – from cell mass (cell), day 7, 15 % v/v inoculum vol; Lane 11: 153 + glycerol media control, day 7, 80 ml media vol; Lane 12 and 13: EtOAc extracts – from cell mass (cell), day 7, 20 % v/v inoculum vol. Lane 14: 153 + glycerol media control, day 10, 95 ml media vol; Lane 15 and 16: EtOAc extracts – from cell mass (cell), day 10, 5 % v/v inoculum vol. Lane 17: 153 + glycerol media control, day 10, 90 ml media vol; Lane 18 and 19: EtOAc extracts – from cell mass (cell), day 10, 10 % v/v inoculum vol; Lane 20: 153 + glycerol media control, day 10, 85 ml media vol; Lane 21 and 22: EtOAc extracts – from cell mass (cell), day 10, 15 % v/v inoculum vol; Lane 23: 153 + glycerol media control, day 10, 80 ml media vol; Lane 24 and 25: EtOAc extracts – from cell mass (cell), day 10, 20 % v/v inoculum vol; Lane 26: EtOAc control.
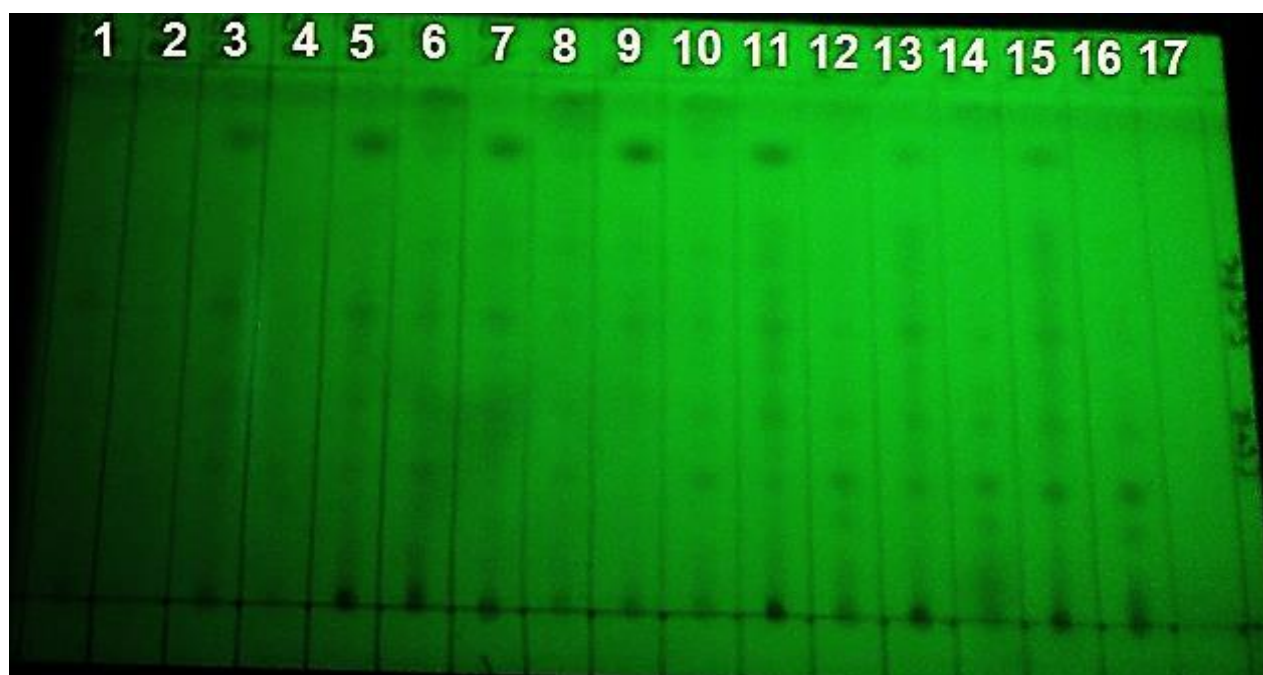
Figure C8: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Inoculum optimisation tests in 500 ml baffled flask with *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1: 153 + glycerol media control, day 7, 95 ml media vol; Lane 2 and 3: EtOAc extracts – from cell mass (cell), day 7, 5 % v/v inoculum vol; Lane 4: 153 + glycerol media control, day 7, 90 ml media vol; Lane 5 and 6: EtOAc extracts – from cell mass (cell), day 7, 10 % v/v inoculum vol; Lane 7: EtOAc control; Lane 8: 153 + glycerol media control, day 7, 85 ml media vol; Lane 9 and 10: EtOAc extracts – from cell mass (cell), day 7, 15 % v/v inoculum vol; Lane 11: 153 + glycerol media control, day 7, 80 ml media vol; Lane 12 and 13: EtOAc extracts – from cell mass (cell), day 7, 20 % v/v inoculum vol. Lane 14: 153 + glycerol media control, day 10, 95 ml media vol; Lane 15 and 16: EtOAc extracts – from cell mass (cell), day 10, 5 % v/v inoculum vol. Lane 17: 153 + glycerol media control, day 10, 90 ml media vol; Lane 18 and 19: EtOAc extracts – from cell mass (cell), day 10, 10 % v/v inoculum vol; Lane 20: 153 + glycerol media control, day 10, 85 ml media vol; Lane 21 and 22: EtOAc extracts – from cell mass (cell), day 10, 15 % v/v inoculum vol; Lane 23: 153 + glycerol media control, day 10, 80 ml media vol; Lane 24 and 25: EtOAc extracts – from cell mass (cell), day 10, 20 % v/v inoculum vol; Lane 26: EtOAc control.

Appendix D

1 litre and 2 litre baffled flask optimisation

Figure D1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Testing the effects of scale-up from 500 ml to 1 litre baffled flasks on antimicrobial production by *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3 in duplicate. Lanes 3 and 4: EtOAc extracts – from SNF, day 5 in duplicate. Lanes 5 and 6: EtOAc extracts – from SNF, day 7 in duplicate. Lanes 7 and 8: EtOAc extracts – from SNF, day 10 in duplicate. Lane 9: 153 + glycerol, pH 6.0 media control. Lanes 10 and 11: EtOAc extracts – from cell mass (cell), day 3 in duplicate. Lanes 12 and 13: EtOAc extracts – from cell, day 5 in duplicate. Lanes 14 and 15: EtOAc extracts – from cell, day 7 in duplicate. Lanes 16 and 17: EtOAc extracts – from cell, day 10 in duplicate. Lane 18: 153 + glycerol, pH 6.0 media control. Lane 19: EtOAc control.

Figure D2: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Testing the effects of scale-up from 500 ml to 1 litre baffled flasks on antimicrobial production by *S. pharetrae* sp. strain CZA14[T] cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3 in duplicate. Lanes 3 and 4: EtOAc extracts – from SNF, day 5 in duplicate. Lanes 5 and 6: EtOAc extracts – from SNF, day 7 in duplicate. Lanes 7 and 8: EtOAc extracts – from SNF, day 10 in duplicate. Lane 9: 153 + glycerol, pH 6.0 media control. Lanes 10 and 11: EtOAc extracts – from cell mass (cell), day 3 in duplicate. Lanes 12 and 13: EtOAc extracts – from cell, day 5 in duplicate. Lanes 14 and 15: EtOAc extracts – from cell, day 7 in duplicate. Lanes 16 and 17: EtOAc extracts – from cell, day 10 in duplicate. Lane 18: 153 + glycerol, pH 6.0 media control. Lane 19: EtOAc control.

Figure D3: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Testing the effects of scale-up from 1 litre to 2 litre baffled flasks on antimicrobial production by *S. pharetrae* sp. strain CZA14[T] cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days.. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3 in duplicate. Lanes 3 and 4: EtOAc extracts – from SNF, day 5 in duplicate. Lanes 5 and 6: EtOAc extracts – from SNF, day 7 in duplicate. Lanes 7 and 8: EtOAc extracts – from SNF, day 10 in duplicate. Lane 9: 153 + glycerol, pH 6.0 media control. Lanes 10 and 11: EtOAc extracts – from cell mass (cell), day 3 in duplicate. Lanes 12 and 13: EtOAc extracts – from cell, day 5 in duplicate. Lanes 14 and 15: EtOAc extracts – from cell, day 7 in duplicate. Lanes 16 and 17: EtOAc extracts – from cell, day 10 in duplicate. Lane 18: 153 + glycerol, pH 6.0 media control. Lane 19: EtOAc control.

Figure D4: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Testing the effects of scale-up from 1 litre to 2 litre baffled flasks on antimicrobial production by *S. pharetrae* sp. strain CZA14^T cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 3 – 10 days.. Lane 1 and 2: EtOAc extracts – from supernatant filtrate (SNF), day 3 in duplicate. Lanes 3 and 4: EtOAc extracts – from SNF, day 5 in duplicate. Lanes 5 and 6: EtOAc extracts – from SNF, day 7 in duplicate. Lanes 7 and 8: EtOAc extracts – from SNF, day 10 in duplicate. Lane 9: 153 + glycerol, pH 6.0 media control. Lanes 10 and 11: EtOAc extracts – from cell mass (cell), day 3 in duplicate. Lanes 12 and 13: EtOAc extracts – from cell, day 5 in duplicate. Lanes 14 and 15: EtOAc extracts – from cell, day 7 in duplicate. Lanes 16 and 17: EtOAc extracts – from cell, day 10 in duplicate. Lane 18: 153 + glycerol, pH 6.0 media control. Lane 19: EtOAc control.

Appendix E

Airlift reactor (ALR) fermentation optimisation

Figure E1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Antimicrobial production by *S. pharetrae* sp. strain CZA14^T in an airlift bioreactor cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 0 – 20 days. Lane 1: EtOAc extracts – from supernatant filtrate (SNF), day 3. Lanes 2: EtOAc extracts – from cell mass (cell), day 3. Lane 1: EtOAc extracts – from supernatant filtrate (SNF), day 3. Lanes 2: EtOAc extracts – from cell mass (cell), day 3. Lane 3: EtOAc extracts – from supernatant filtrate (SNF), day 5. Lanes 4: EtOAc extracts – from cell mass (cell), day 5. Lane 5: EtOAc extracts – from supernatant filtrate (SNF), day 7. Lanes 6: EtOAc extracts – from cell mass (cell), day 7. Lane 7: EtOAc extracts – from supernatant filtrate (SNF), day 10. Lanes 8: EtOAc extracts – from cell mass (cell), day 10. Lane 9: EtOAc extracts – from supernatant filtrate (SNF), day 13. Lanes 10: EtOAc extracts – from cell mass (cell), day 13. Lane 11: EtOAc extracts – from supernatant filtrate (SNF), day 15. Lanes 12: EtOAc extracts – from cell mass (cell), day 15. Lane 13: EtOAc extracts – from supernatant filtrate (SNF), day 17. Lanes 14: EtOAc extracts – from cell mass (cell), day 17. Lane 15: EtOAc extracts – from supernatant filtrate (SNF), day 20. Lanes 16: EtOAc extracts – from cell mass (cell), day 20. Lane 17: EtOAc control.

Appendix F

Continuous stirred tank reactor (CSTR) fermentation optimisation

Figure F1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Antimicrobial production by *S. pharetrae* sp. strain CZA14^T in a continuous stirred tank reactor (CSTR) cultured in 153 + glycerol, pH 6.0, 30 °C at 160 rpm for 0 – 20 days. Lane 1: EtOAc extracts – from supernatant filtrate (SNF), day 3. Lanes 2: EtOAc extracts – from cell mass (cell), day 3. Lane 1: EtOAc extracts – from supernatant filtrate (SNF), day 3. Lanes 2: EtOAc extracts – from cell mass (cell), day 3. Lane 3: EtOAc extracts – from supernatant filtrate (SNF), day 5. Lanes 4: EtOAc extracts – from cell mass (cell), day 5. Lane 5: EtOAc extracts – from supernatant filtrate (SNF), day 7. Lanes 6: EtOAc extracts – from cell mass (cell), day 7. Lane 7: EtOAc extracts – from supernatant filtrate (SNF), day 10. Lanes 8: EtOAc extracts – from cell mass (cell), day 10. Lane 9: EtOAc extracts – from supernatant filtrate (SNF), day 13. Lanes 10: EtOAc extracts – from cell mass (cell), day 13. Lane 11: EtOAc extracts – from supernatant filtrate (SNF), day 15. Lanes 12: EtOAc extracts – from cell mass (cell), day 15. Lane 13: EtOAc extracts – from supernatant filtrate (SNF), day 17. Lanes 14: EtOAc extracts – from cell mass (cell), day 17. Lane 15: EtOAc extracts – from supernatant filtrate (SNF), day 20. Lanes 16: EtOAc extracts – from cell mass (cell), day 20. Lane 17: EtOAc control.

Appendix G

DNA isolation of Domestic Rainwater Harvesting (DRWH) test strains for the identification of these strains to genus level using phylogenetic analysis

Figure G1: 1 % TAE agarose gel electrophoretogram of DNA extracted from DRWH isolates – Lanes 1 and 25: 1kb Kapa Biosystems universal molecular weight marker; Lanes 2-3: DNA isolated from *B. cereus*; Lanes 4-5: DNA isolated from *S. aureus* 1; Lanes 6-7: DNA isolated from *S. aureus* 2; Lanes 8-9: DNA isolated from *S. aureus* 4; Lanes 10-11: DNA isolated from *S. aureus* 5; Lanes 12-13: DNA isolated from *E. cloacae* 11; Lanes 14-15: DNA isolated from *E. cloacae* 20; Lanes 16-17: DNA isolated from *E. cloacae* 22; Lanes 18-19: DNA isolated from *E. faecalis* C513; Lanes 20-21: DNA isolated from *K. pneumonia* 1; Lanes 22-23: DNA isolated from *K. pneumonia* 3.

Figure G2: 1 % TAE agarose gel electrophoretogram of DNA extracted from DRWH isolates – Lanes 1 and 10: 1kb Kapa Biosystems universal molecular weight marker; Lanes 3-4: DNA isolated from *S. enterica* 4C; Lanes 5-6: DNA isolated from *P. aeruginosa*; Lanes 7-8: DNA isolated from *E. coli* K4 CCA.

Appendix H

Bioautography assay of crude extract from the SNF and cell mass extracted from fermentation broth from 2 L baffled flasks, CSTR and ALR
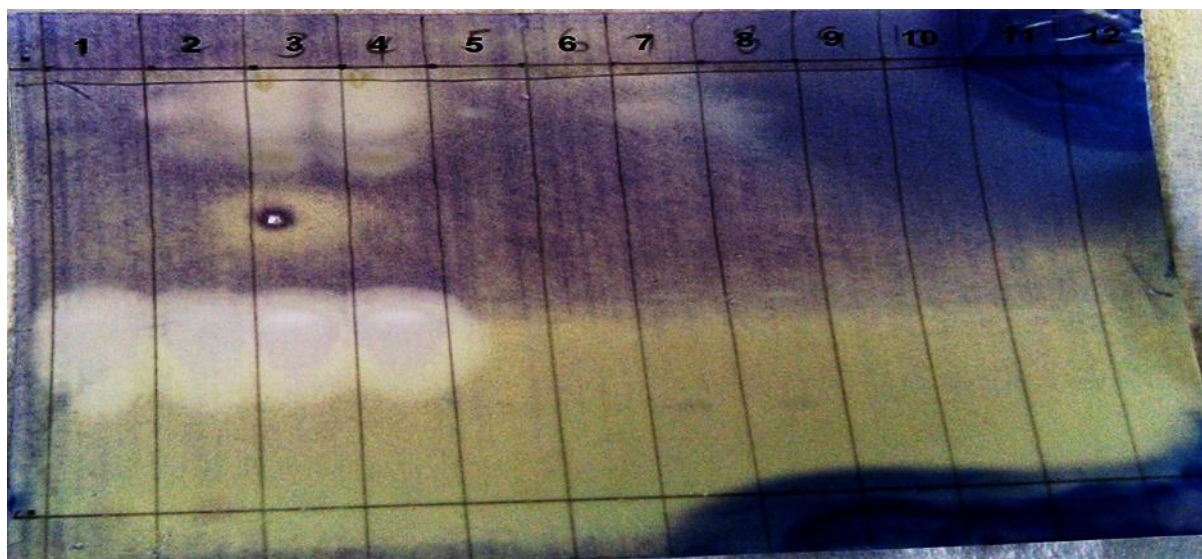
ATCC clinical control strains

Figure H1*: Bacillus cereus* ATCC 10876 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
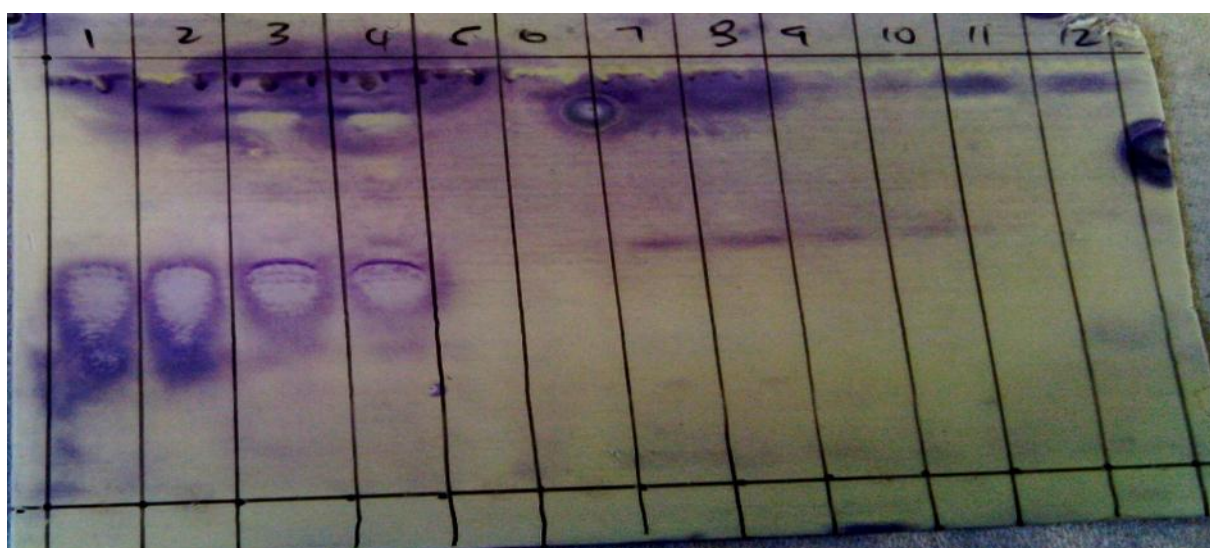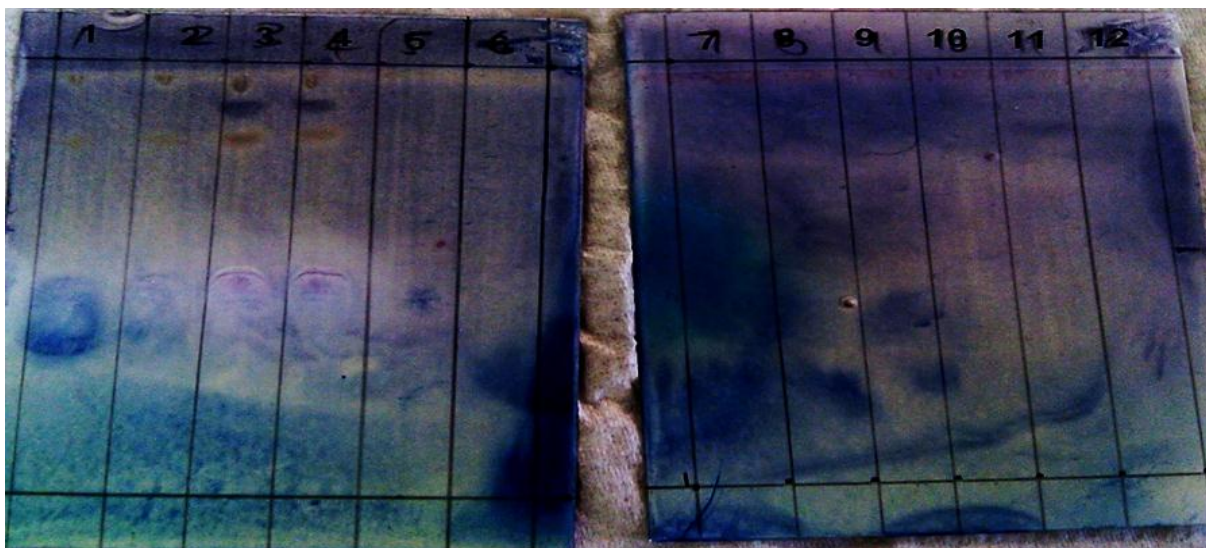
Figure H2: *Salmonella enterica* subsp. *arizonae* ATCC 13314 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
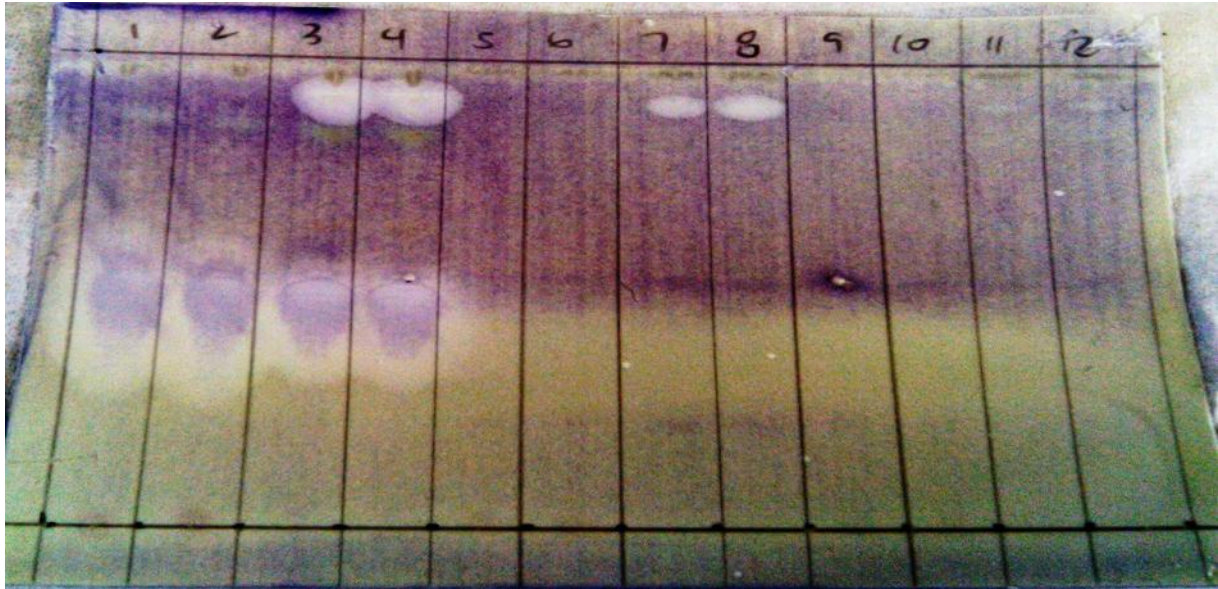
Figure H3: *Eschericia coli* ATCC 25922 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
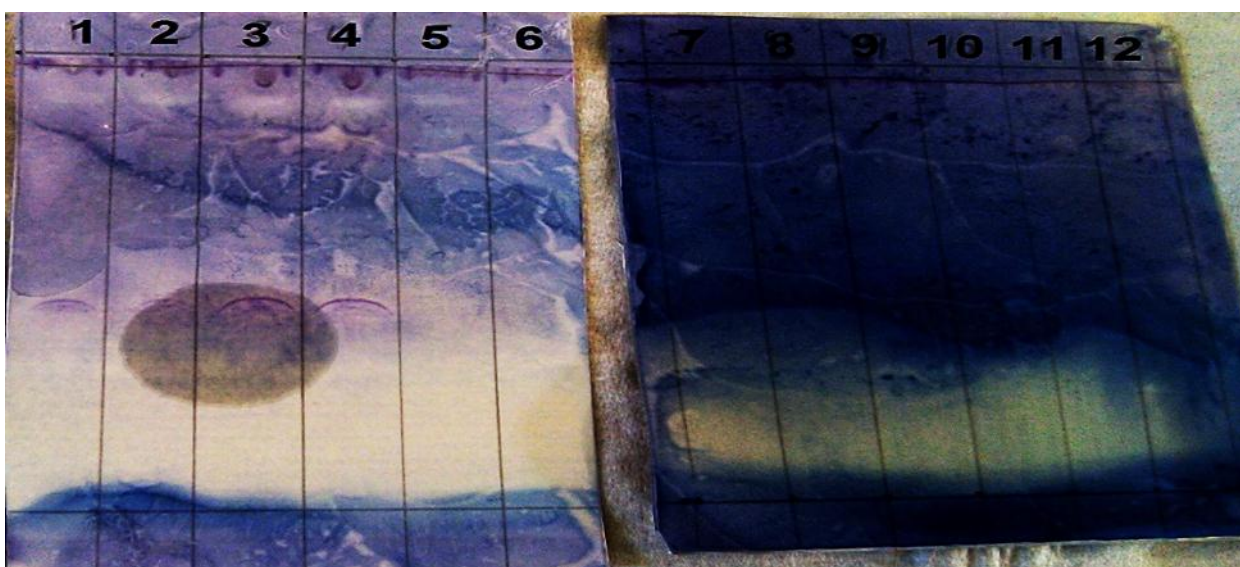

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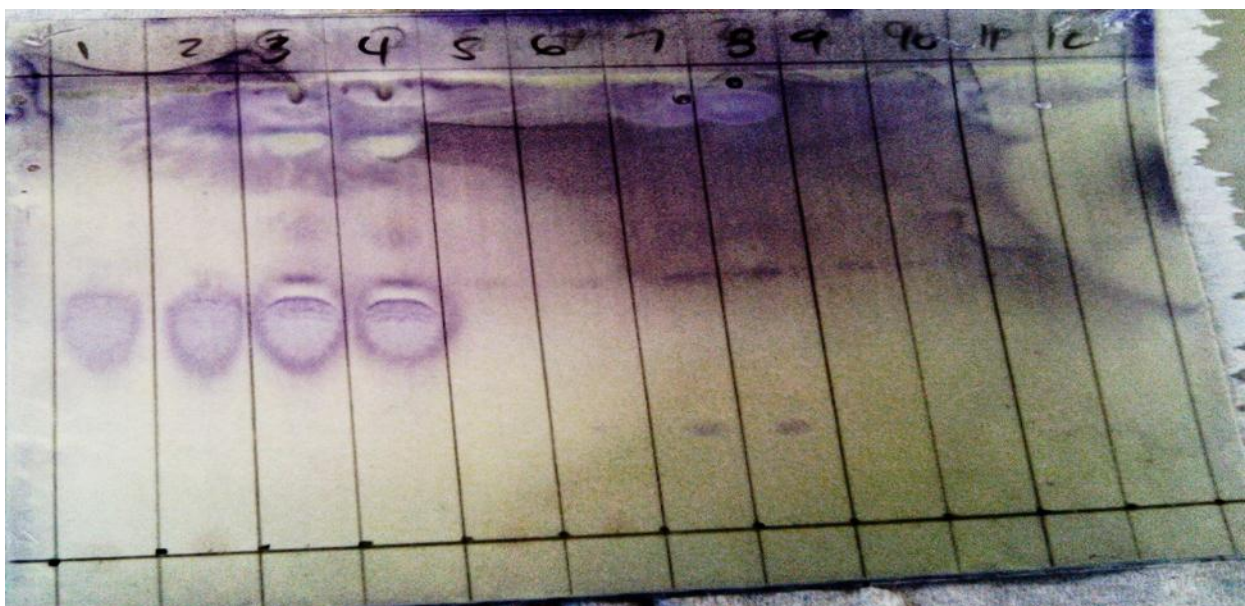
Figure H4: *Pseudomonas aeruginosa* ATCC 27853 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
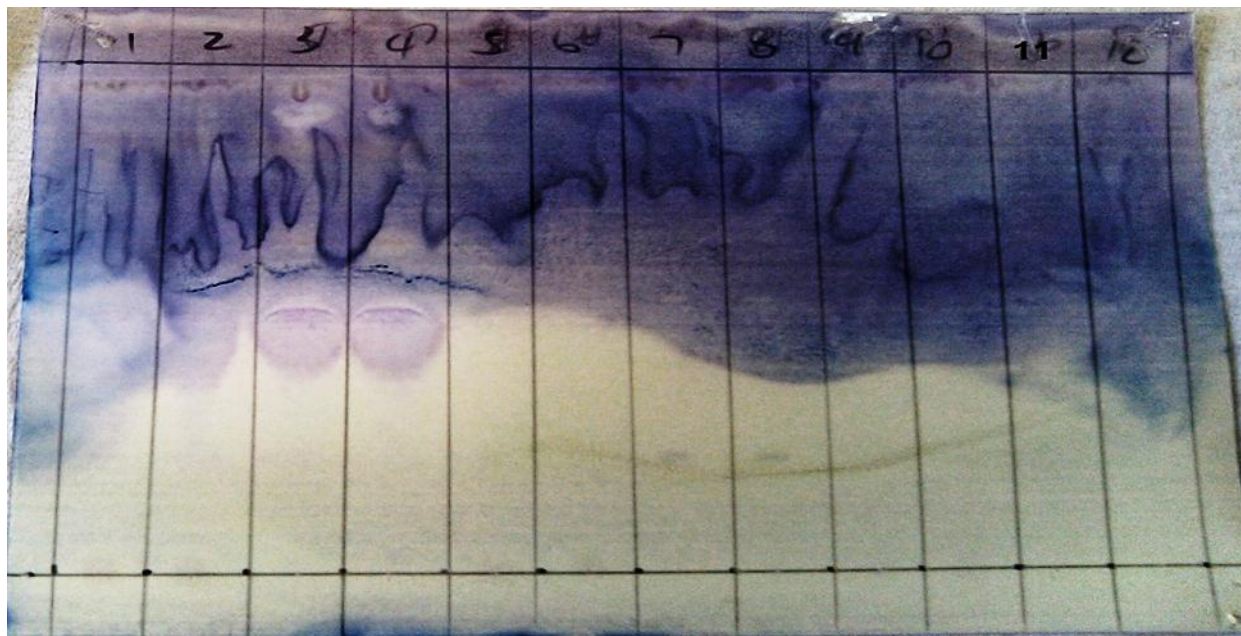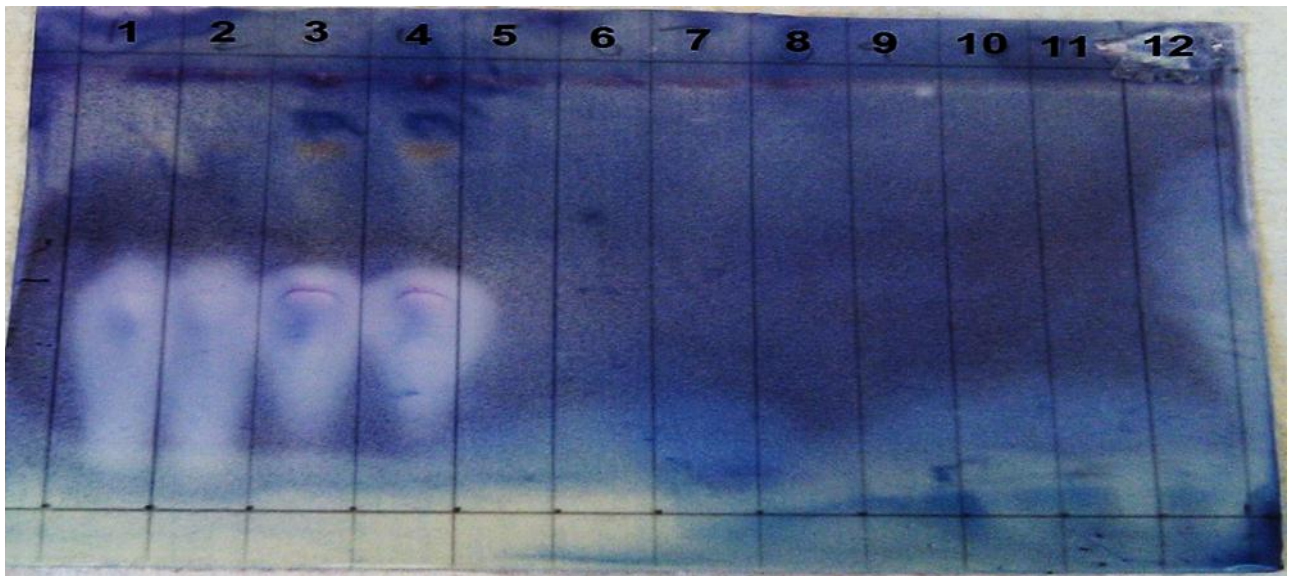

Figure H5*: Klebsiella pneumonia* subsp. *pneumonia* ATCC 700603 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
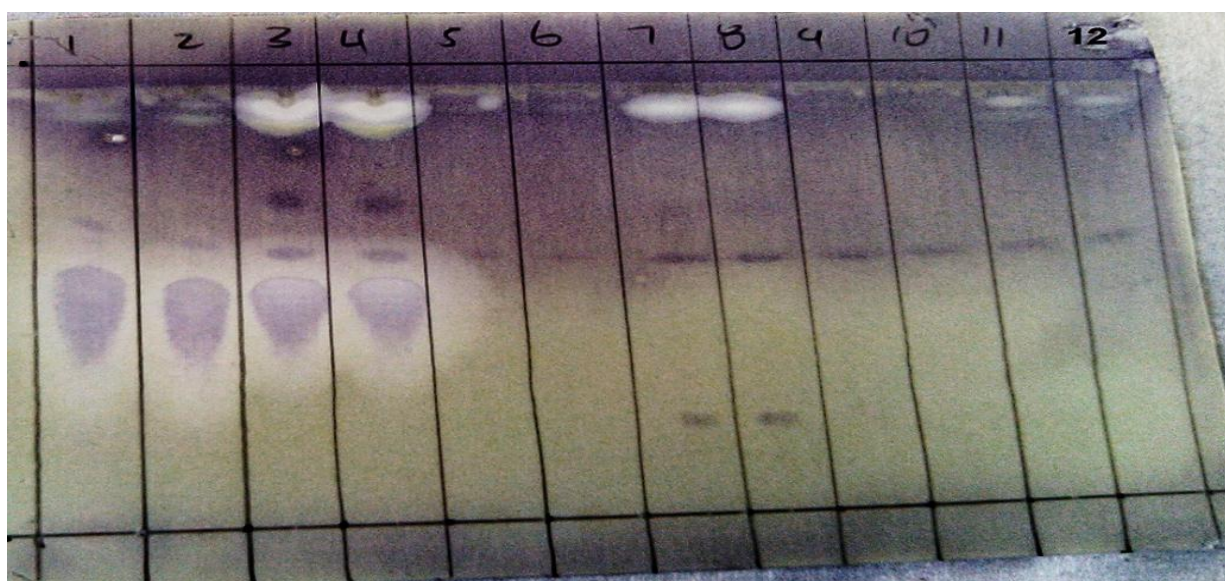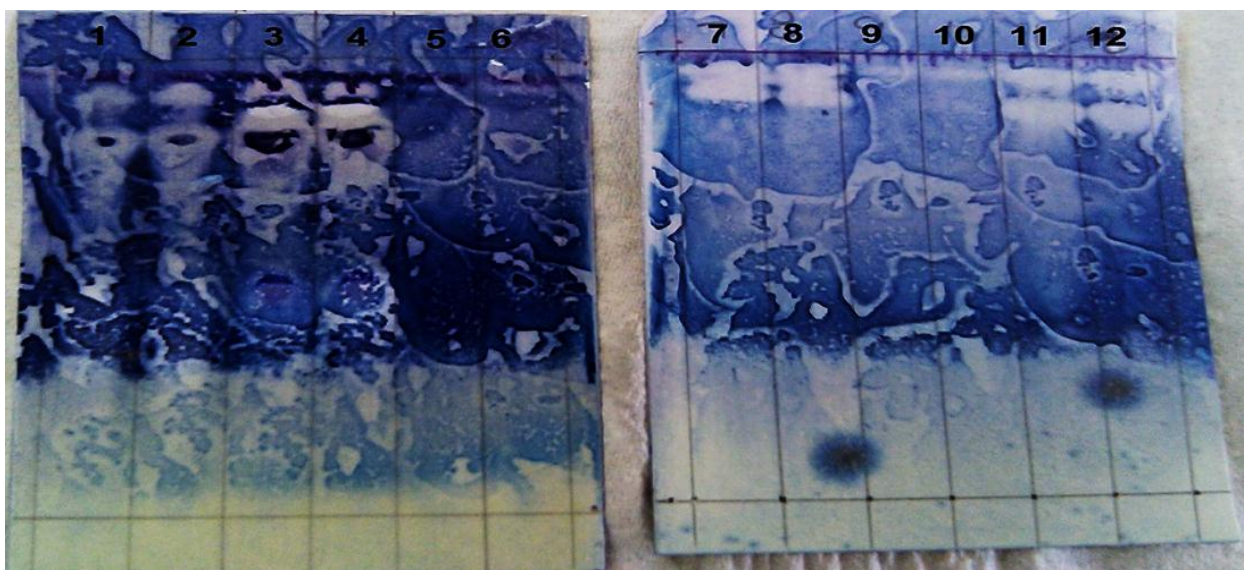
Figure H6: *Staphylococcus aureus* subsp. *aureus* ATCC 29213 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
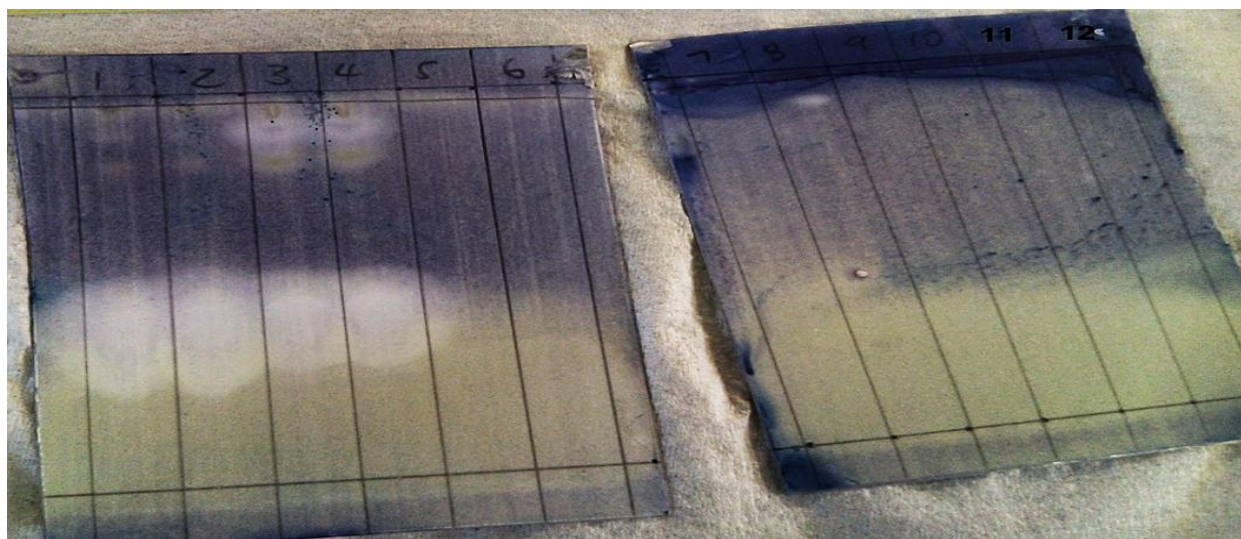
Figure H7: *Staphylococcus aureus* subsp. *aureus* ATCC 33591 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
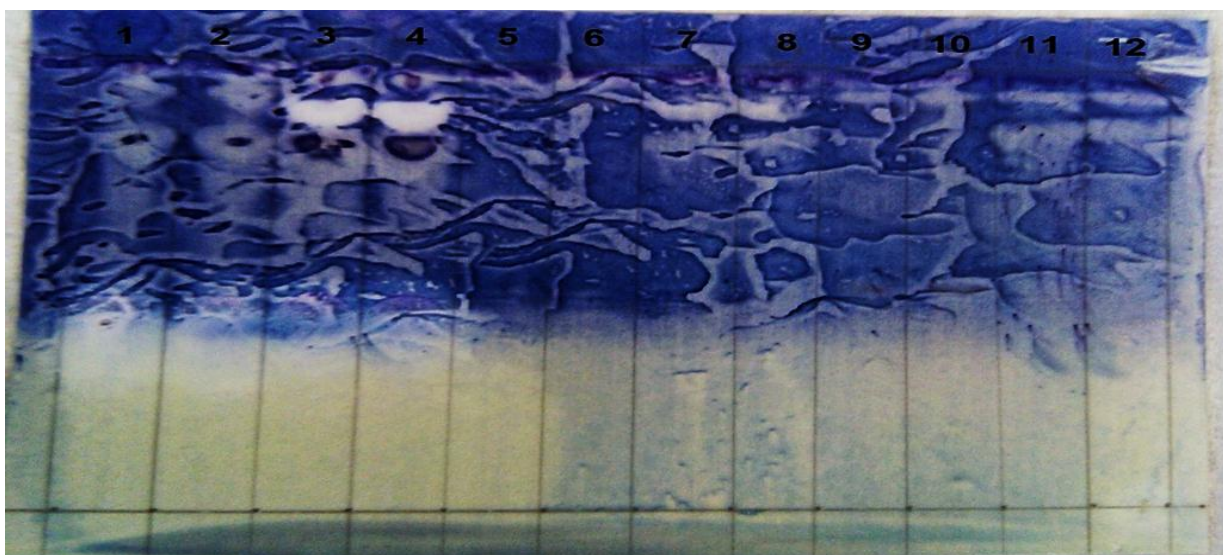
Figure H8: *Enterococcus faecalis* ATCC 29212 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
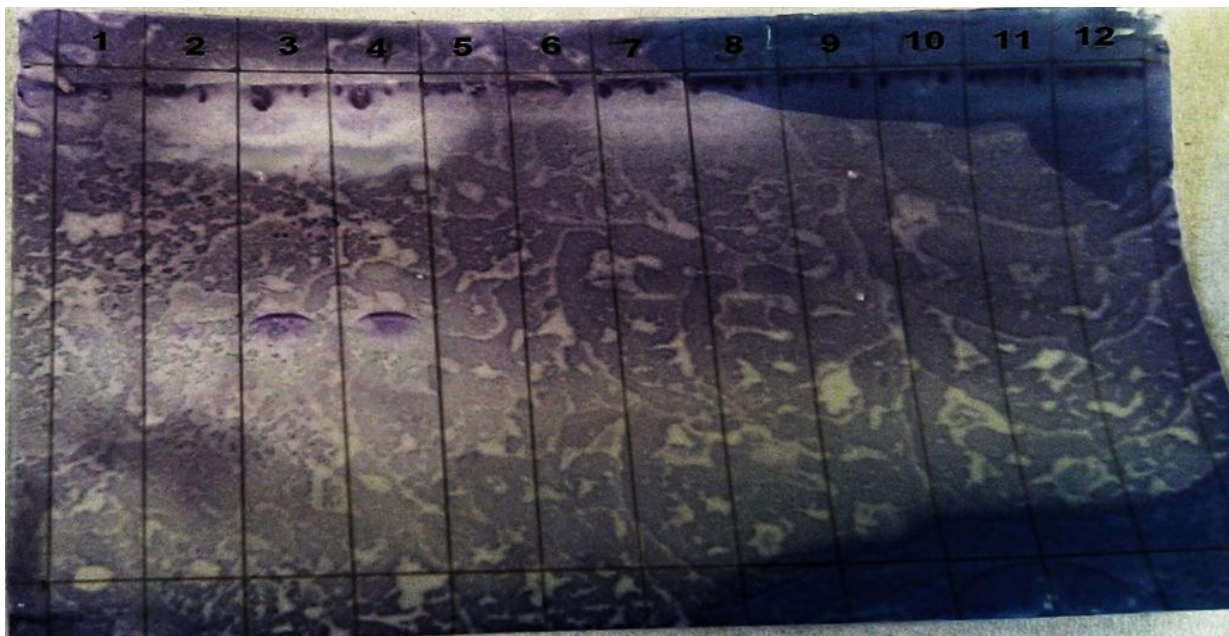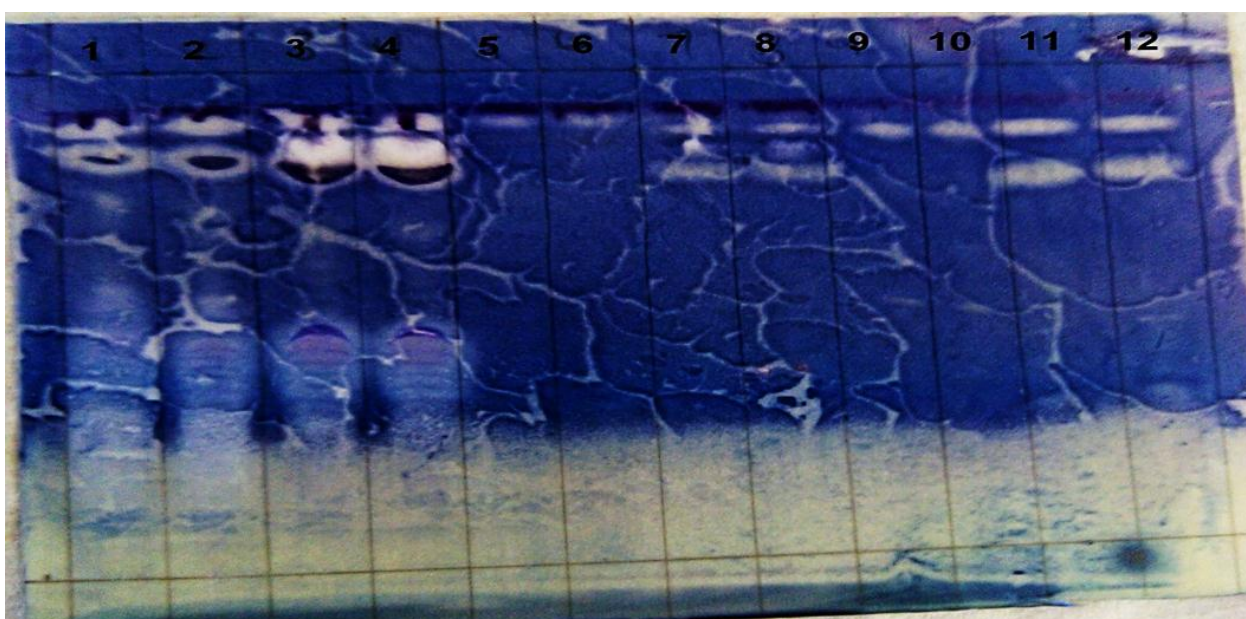

Figure H9: *Enterococcus faecalis* ATCC 51299 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
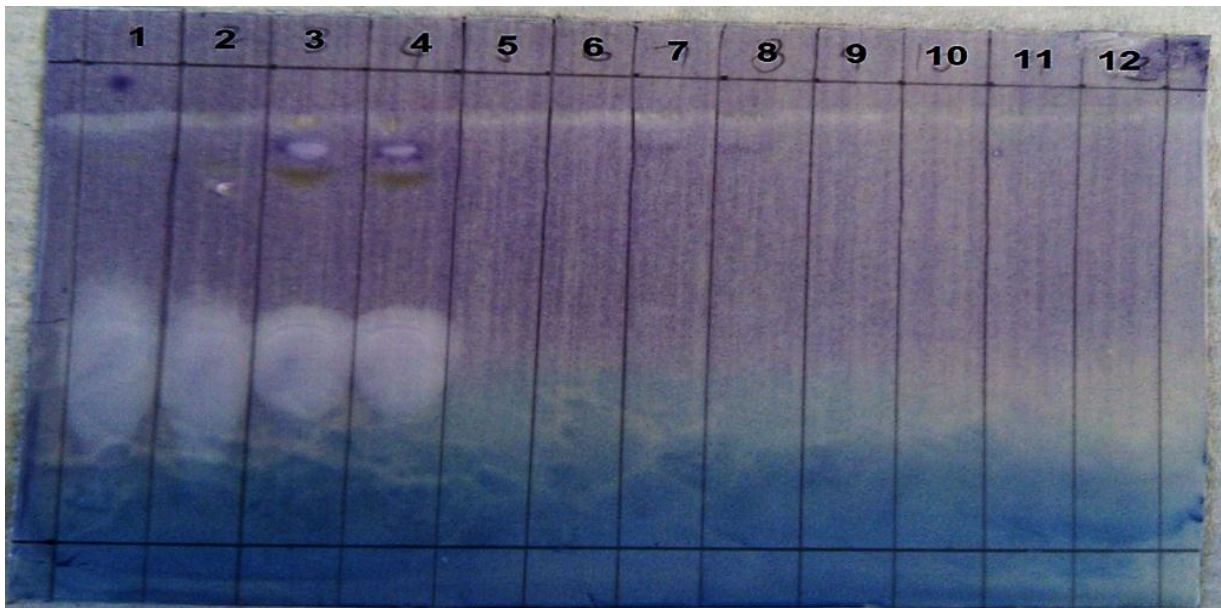
Figure H10: *Enterobacter cloacae* subsp. *cloacae* ATCC BAA-1143 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
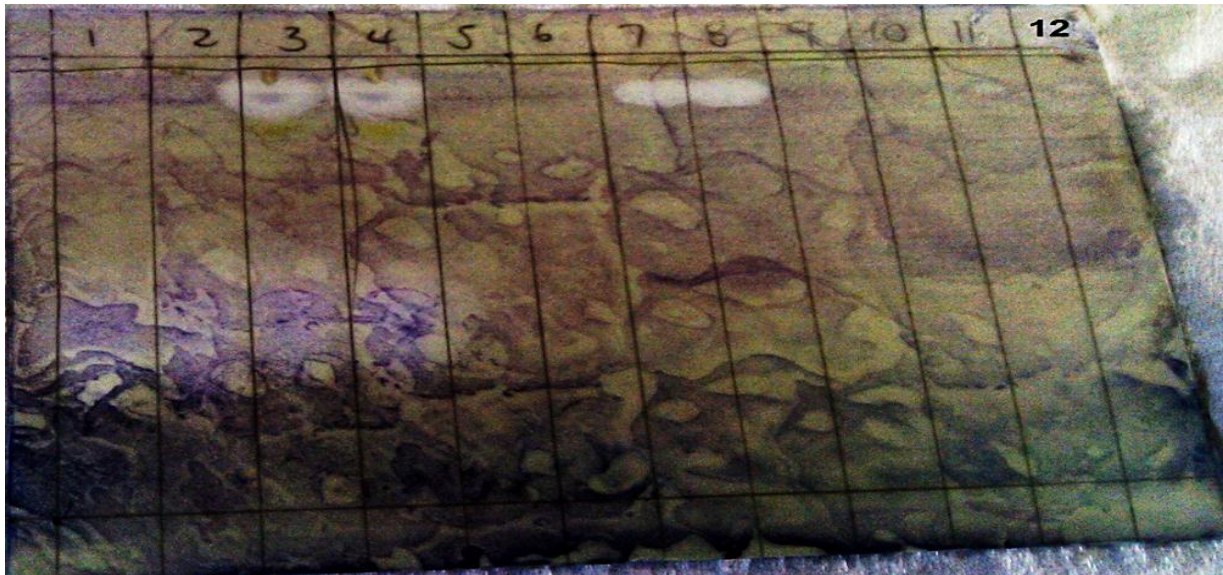
Figure H11: *Candida krusei* ATCC 34135 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
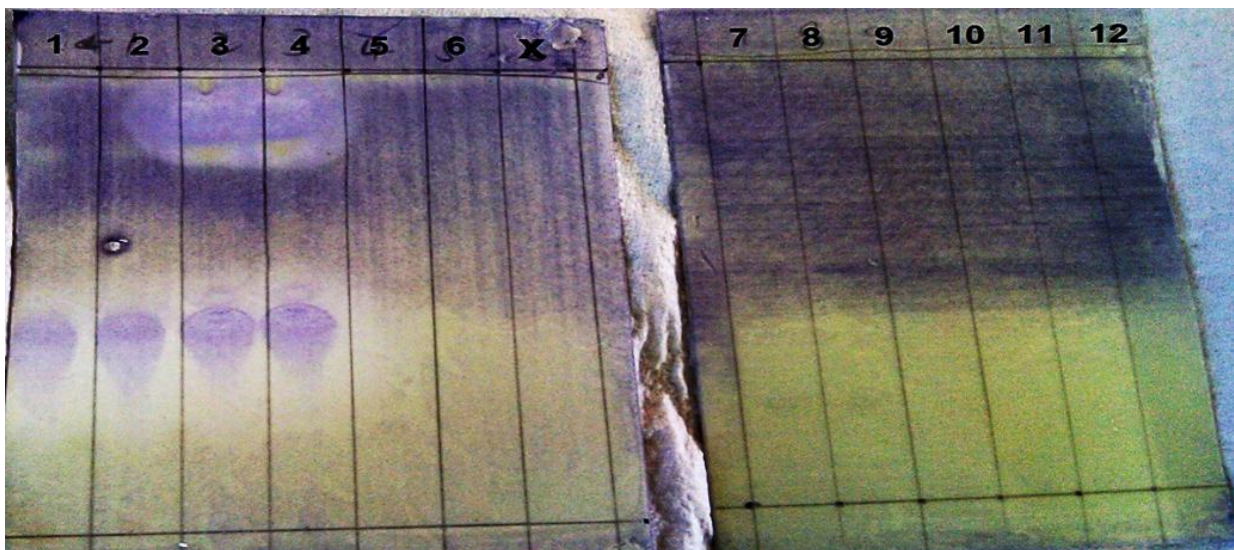
Environmental DRWH isolates
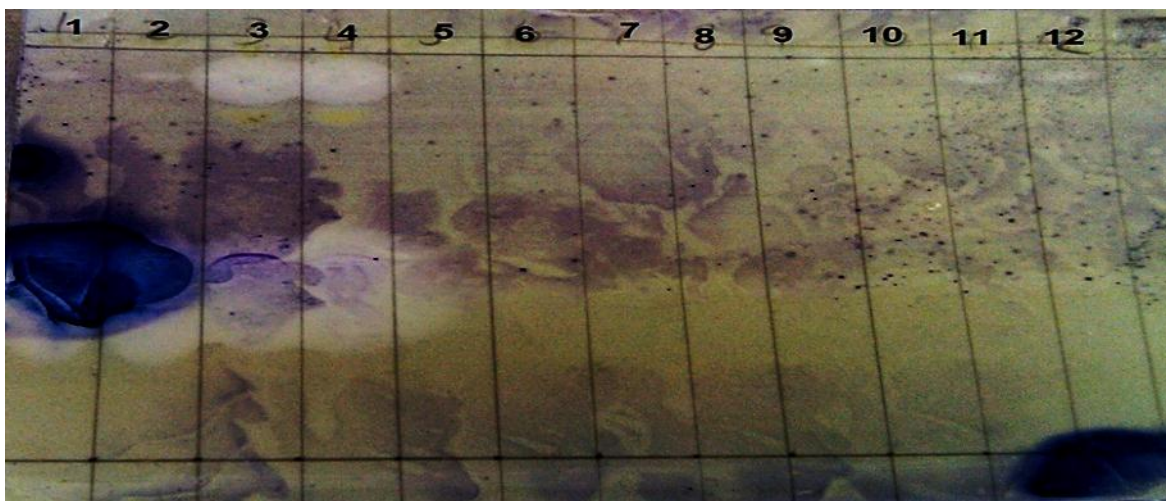
Figure H12: *Bacillus cereus* - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
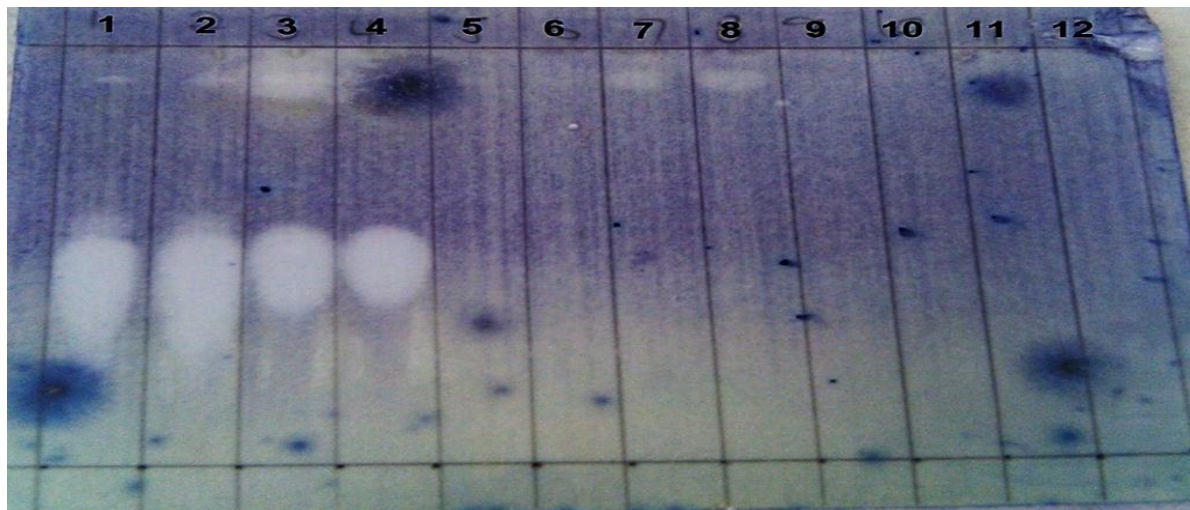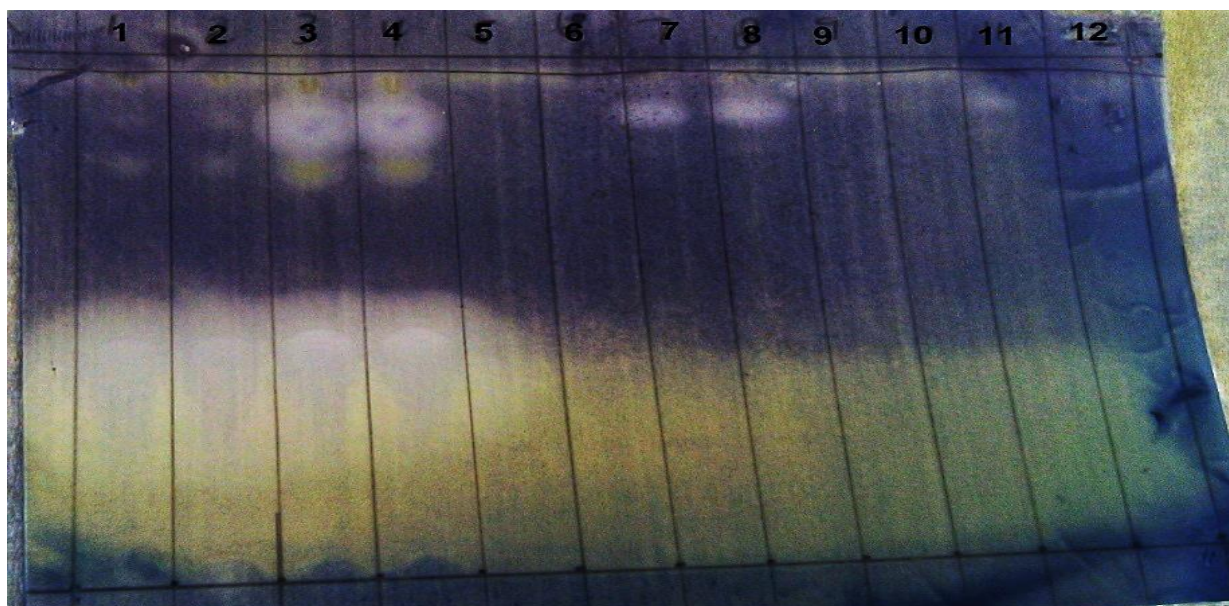

Figure H13: *Salmonella enterica* 4C - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
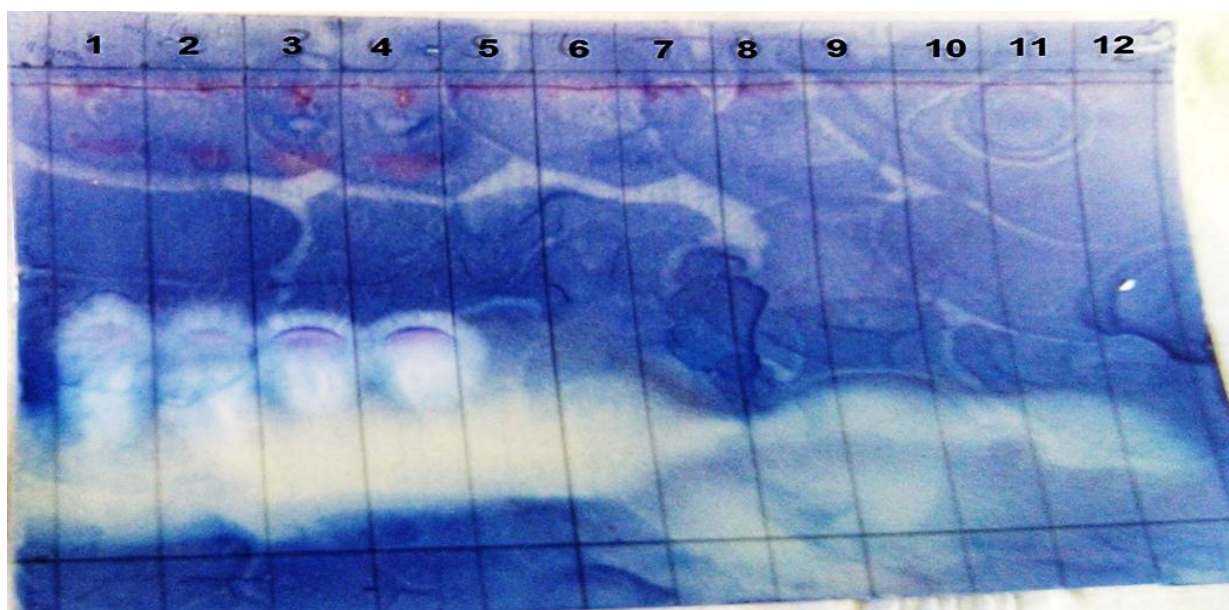

Figure H14: *Escherichia coli* K4 CCA - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H15: *Pseudomonas aeruginosa* - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.
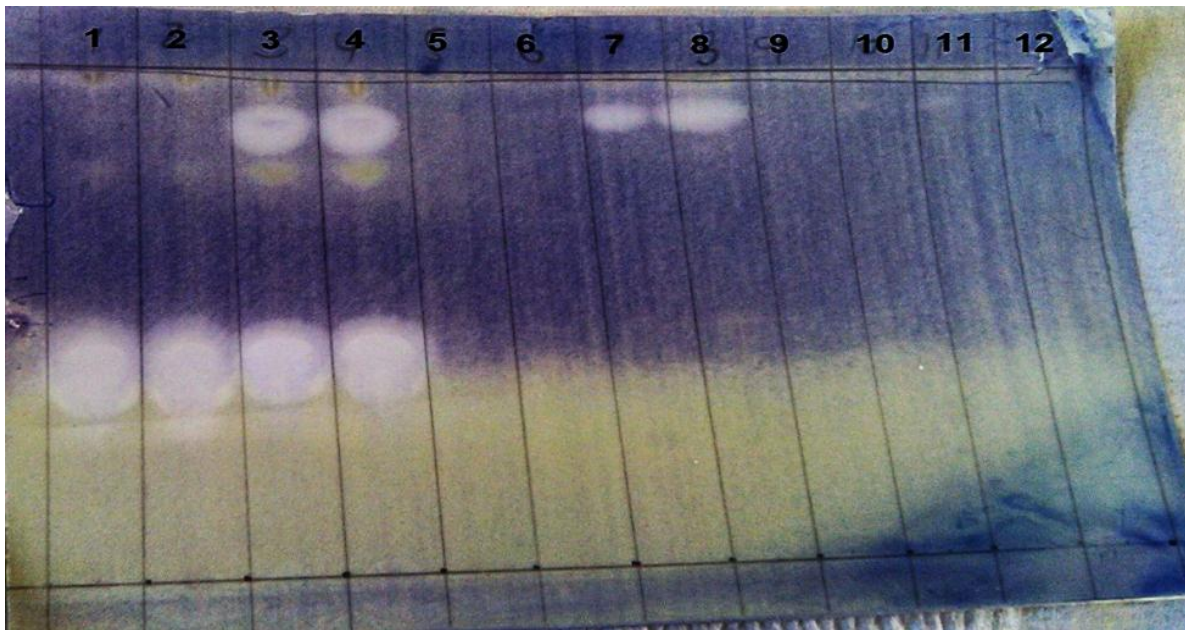

Figure H16: *Klebsiella pneumonia* 1 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H17: *Klebsiella pneumonia* 3 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H18: *Staphylococcus aureus* 1 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H19: *Staphylococcus aureus* 2 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H20: *Staphylococcus aureus* 4 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2L baffled flask fermentation, cell mass.

Figure H21: *Staphylococcus aureus* 5 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H22: *Enterococcus faecalis* C513 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H23: *Enterobacter cloacae* 11 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

Figure H24: *Enterobacter cloacae* 20 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2 L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2 L baffled flask fermentation, cell mass.

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Figure H25: *Enterobacter cloacae* 22 - Lanes 1-2: EtOAc extract from ALR fermentation, SNF; Lanes 3-4: EtOAc extract from ALR fermentation, cell mass; Lanes 5-6: EtOAc extract from CSTR fermentation, SNF; Lanes 7-8: EtOAc extract from CSTR fermentation, cell mass; Lanes 9-10: EtOAc extract from 2L baffled flask fermentation, SNF; and Lanes 11-12: EtOAc extract from 2L baffled flask fermentation, cell mass.

Appendix I

Partial purification of antimicrobials produced extracellularly by *Streptomyces pharetrae* sp. strain CZA14[T] in an airlift reactor

Bradford's assay to quantify the total protein content in partially purified samples
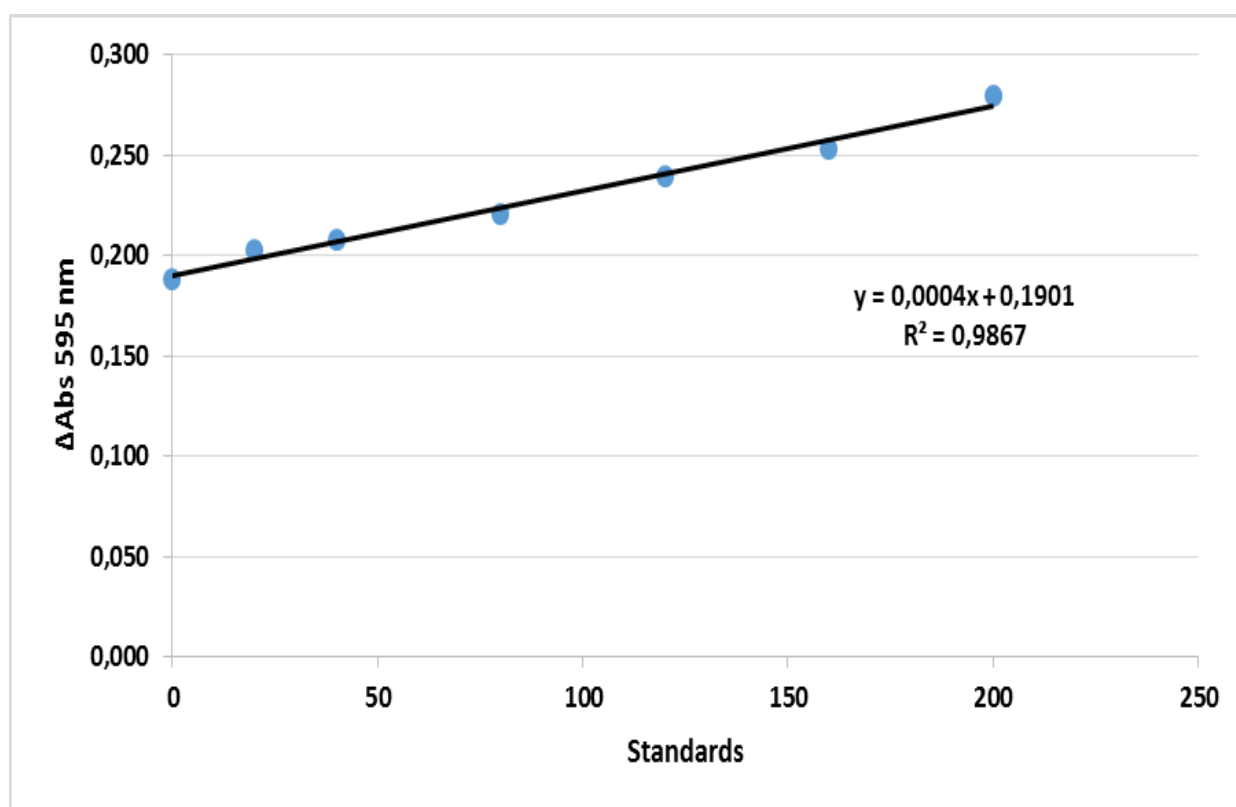
Figure I1: Standard curve of Abs_{620nm} vs protein concentration of standards constructed and used to determine protein concentration in the samples.

Appendix J

SDS – PAGE:

*prepared 10 % w/v SDS, 10 % w/v ammonium persulphate (AMPS)

Gel:

16 % resolving gel (10 mL volume):

2.1 mL dH2O; 5.3 mL 30 % acrylamide/bis-acrylamide (29.2g acrylamide and 0.8 g bis-acrylamide in 100 ml dH2O); 2.5 mL 1.5 M Tris-HCl, pH 8.8; 0.1 mL 10 % w/v SDS; 0.05 mL 10 % AMPS; and 0.005 mL TEMED

4 % stacking gel (10 mL volume):

6.1 mL dH2O; 1.3 mL 30 % acrylamide/ bis-acrylamide; 2.5 mL 0.5 M Tris-HCl, pH 6.8; 0.1 mL 10 % SDS; 0.05 mL 10 % AMPS; and 0.010 mL TEMED

Sample loading buffer:

3.55 mL dH$_2$O; 1.25 mL 0.5 M Tris-HCl, pH 6.8; 2.5 mL glycerol; 2.0 mL 0.5 % w/v SDS and 0.5 % w/v bromophenol blue. 0.05 mL dithriothreitol (DTT) was added to a 9.5 mL loading buffer prior to use.

10 x Electrode/running buffer:

(g/L): 30.3 g Tris; 144.0 g glycine; 10.0 g SDS. Dissolve and bring to volume using dH$_2$O. 10 x electrode buffer was diluted to 1 X concentration for the use during electrophoresis.

Native – PAGE:

10 % resolving gel (10 mL volume):

4.2 mL dH2O; 3.3 mL 30 % acrylamide/ bis-acrylamide; 2.5 ml 1.5 M Tris-HCl, pH 8.8, 0.05 mL 10 % AMPS; and 0.005 mL TEMED

4 % stacking gel (10 mL volume):

6.1 mL dH2O; 1.3 mL 30 % acrylamide/ bis-acrylamide; 2.5 mL 0.5 M Tris-HCl, pH 6.8; 0.05 mL 10 % AMPS; and 0.010 mL TEMED

Appendix K

Partial purification of supernatant filtrate (SNF) from airlift reactor fermentation broth using various solvents

Visualisation under short wave 254 nm ultraviolet light:

Figure K1: Developed TLC visualised under short wave 254 nm ultraviolet light. Benzene extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: Benzene solvent control.
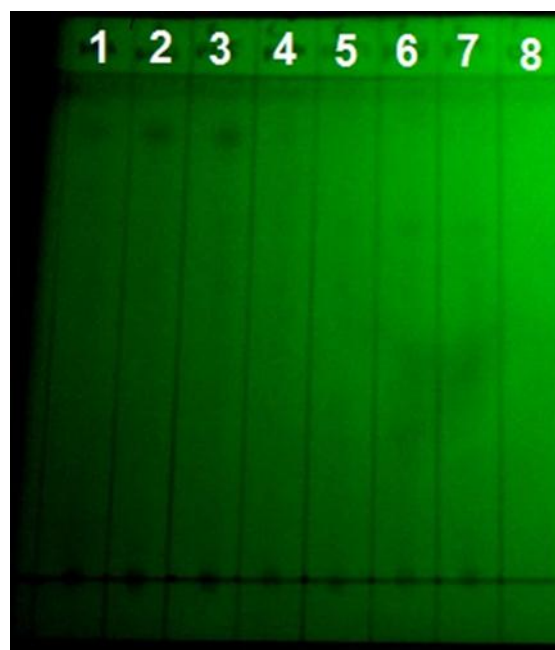
Figure K2: Developed TLC visualised under short wave 254 nm ultraviolet light. Chloroform extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: Chloroform solvent control.

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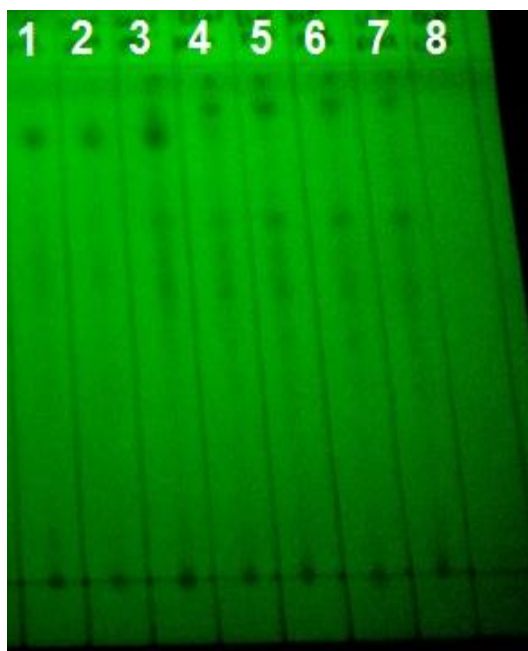
Figure K3: Developed TLC visualised under short wave 254 nm ultraviolet light. Dichloromethane extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: dichloromethane solvent control.
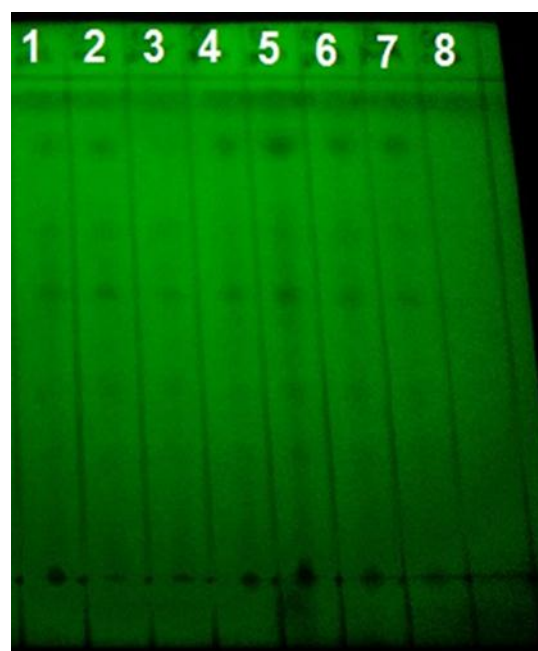
Figure K4: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: ethyl acetate solvent control.

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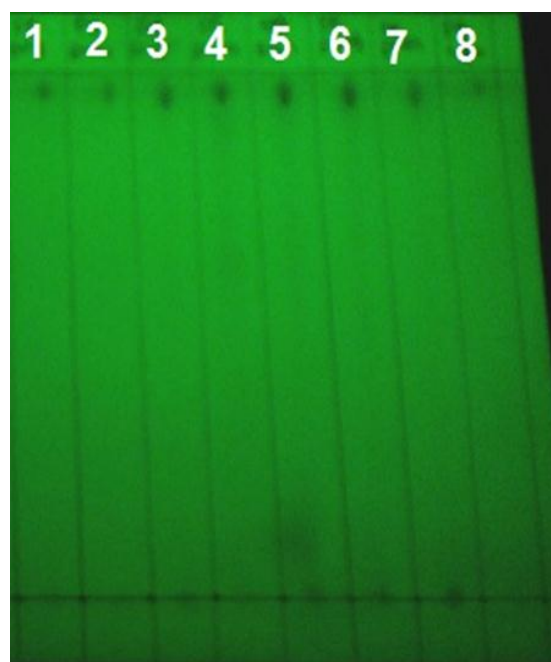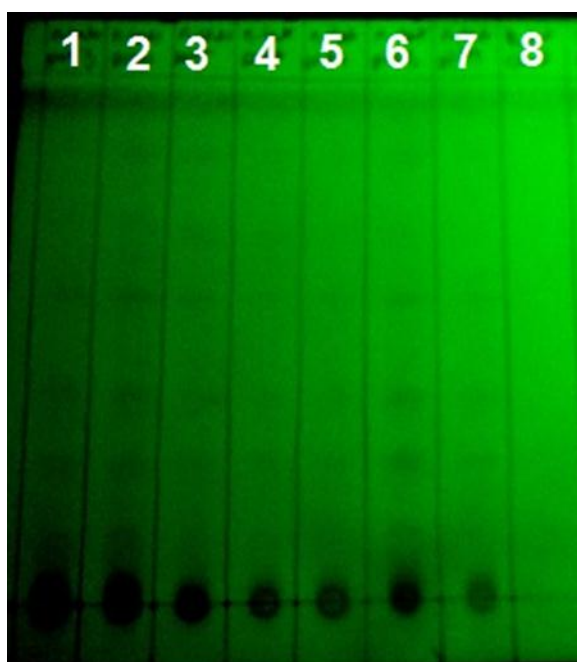
Figure K5: Developed TLC visualised under short wave 254 nm ultraviolet light. N-butanol extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: n-butanol solvent control.

Figure K6: Developed TLC visualised under short wave 254 nm ultraviolet light. Petroleum ether extracts separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: petroleum ether solvent control.

Bioautography assay on partially purified solvent extracts:

Figure K7: *S. aureus* ATCC 33591 – Benzene solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K8: *S. aureus 4* (DRW isolate) – Benzene solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K9: *S. aureus* ATCC 33591 – Chloroform solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K10: *S. aureus* 4 (DRW isolate) – Chloroform solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K11: *S. aureus* ATCC 33591 – Dichloromethane solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.
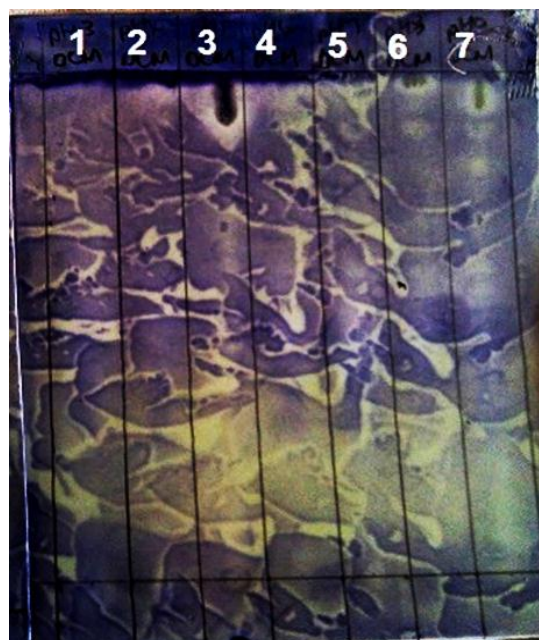
Figure K12: *S. aureus* 4 (DRW isolate) – Dichloromethane solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K13: *S. aureus* ATCC 33591 − Ethyl acetate solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9, Lane 8: solvent control.

Figure K14: *S. aureus* 4 (DRW isolate) − Ethyl acetate solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K15: *S. aureus* ATCC 33591 − n-butanol solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.
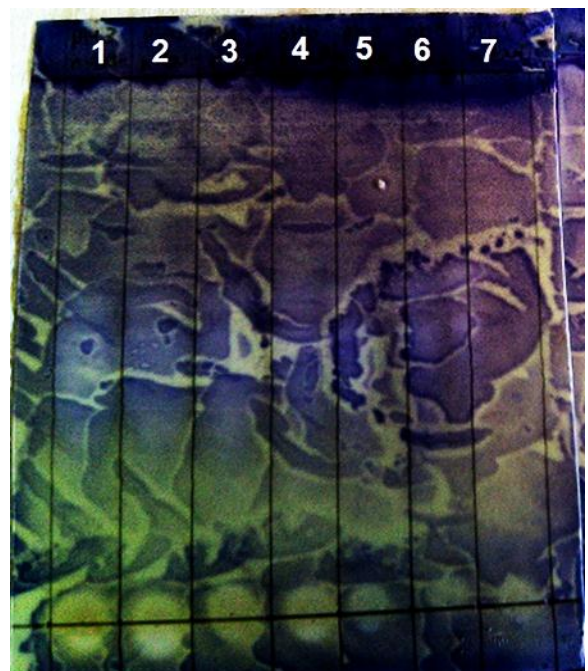
Figure K16: *S. aureus* 4 (DRW isolate) − n-butanol solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Figure K17: *S. aureus* ATCC 33591 – petroleum ether solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.
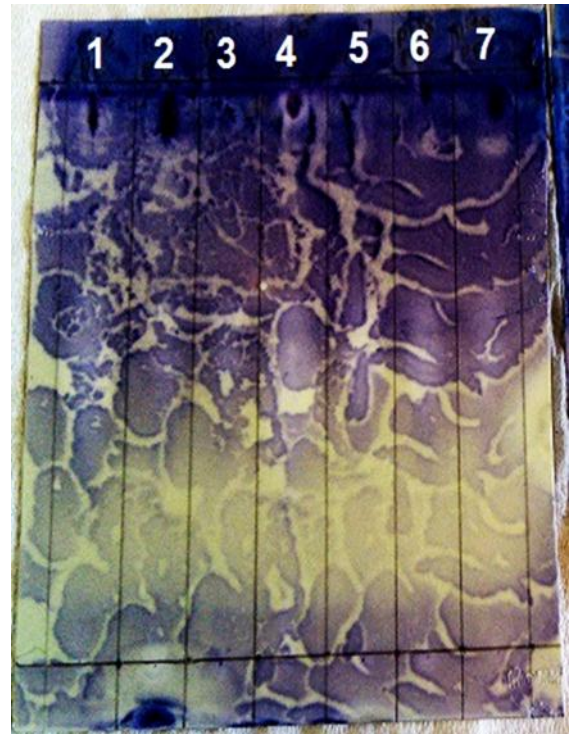
Figure K18: *S. aureus* 4 (DRW isolate) – petroleum ether solvent extracts: Lane 1: pH 3, Lane 2: pH 4, Lane 3: pH 5, Lane 4: pH 6, Lane 5: pH 7, Lane 6: pH 8, Lane 7: pH 9.

Appendix L

Small - scale purification of crude EtOAc extracts from airlift reactor fermentation broth using gradient elution column chromatography method

Figure L1: Bioautography assay of silica gel purified crude EtOAc extracts from airlift reactor fermentation broth against DRW isolate *S. aureus* 4. Lane 1 – 17: Fractions 1 – 17.
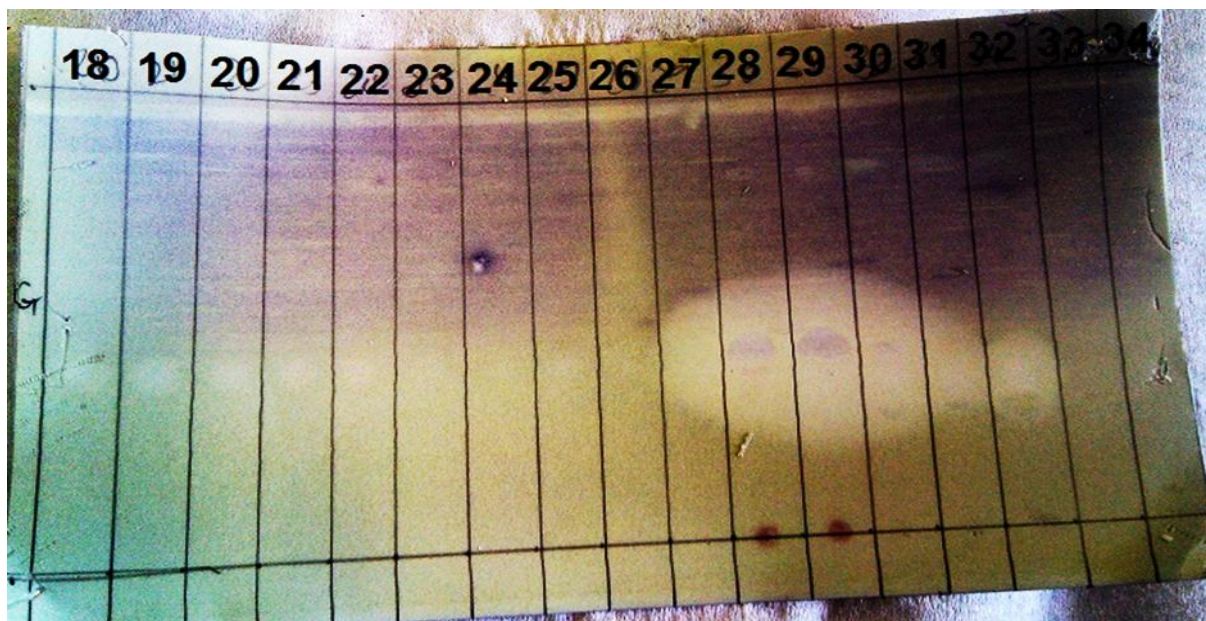
Figure L2: Bioautography assay of silica gel purified crude EtOAc extracts from airlift reactor fermentation broth against DRW isolate *S. aureus* 4. Lane 18 – 34: Fractions 18 – 34.

Figure L3: Bioautography assay against *B. cereus* (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.
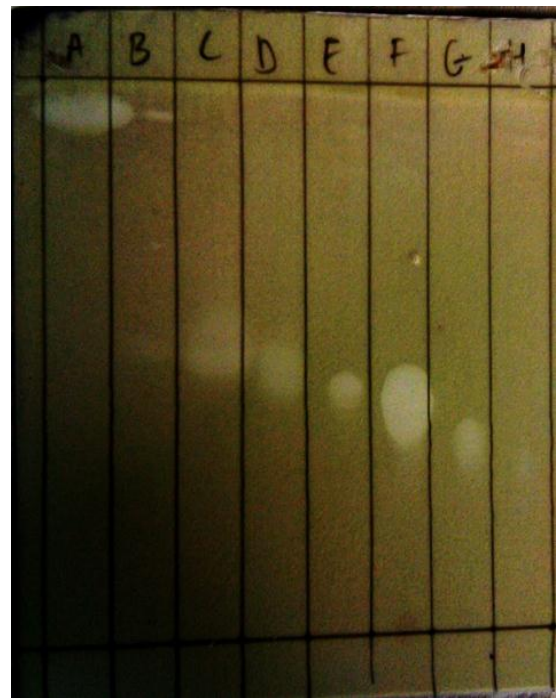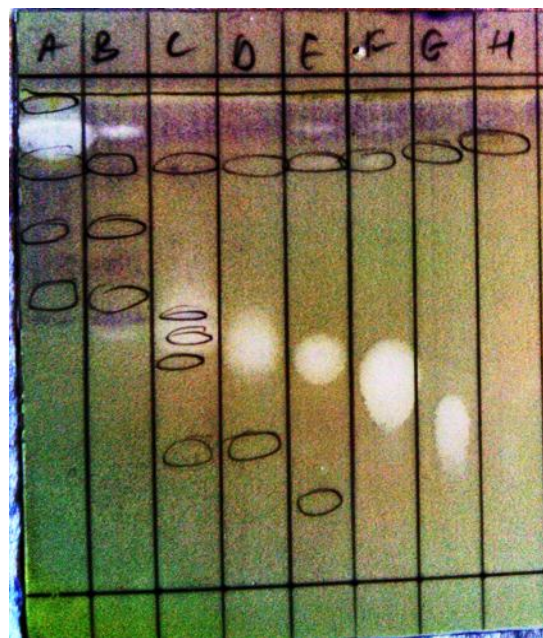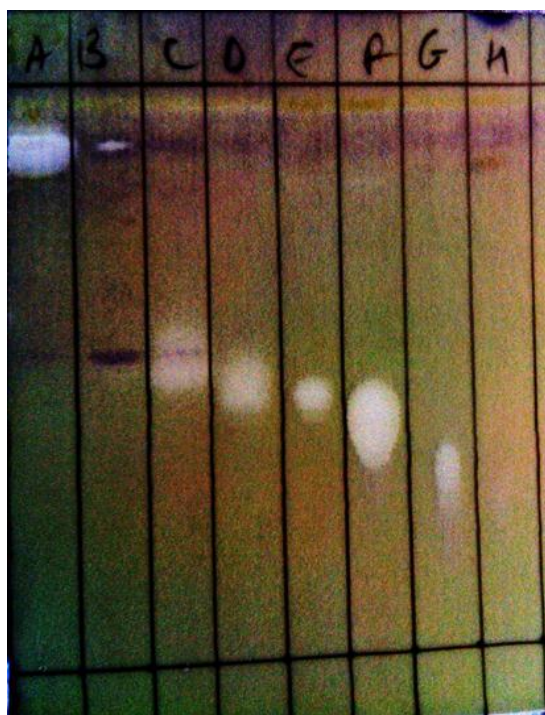

Figure L4: Bioautography assay against *E. cloacae* 11 (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.
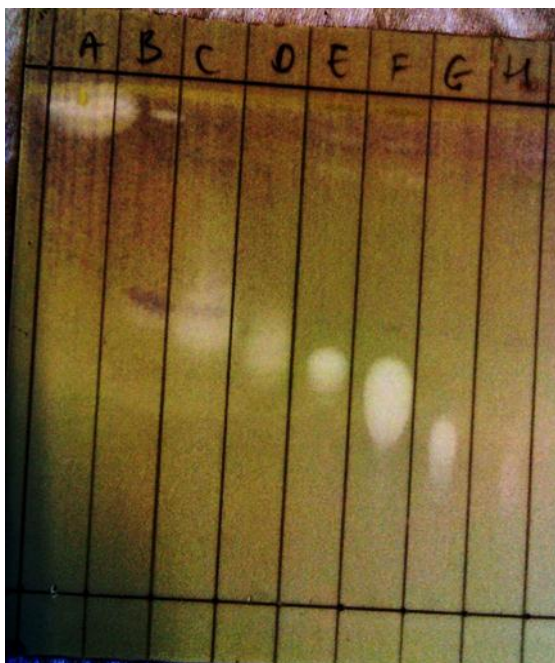
Figure L5: Bioautography assay against *E. coli* K4 CCA (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Figure L6: Bioautography assay against *E. faecalis* (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Figure L7: Bioautography assay against *K. pneumonia* 1 (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Figure L8: Bioautography assay against *K. pneumonia* 3 (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.
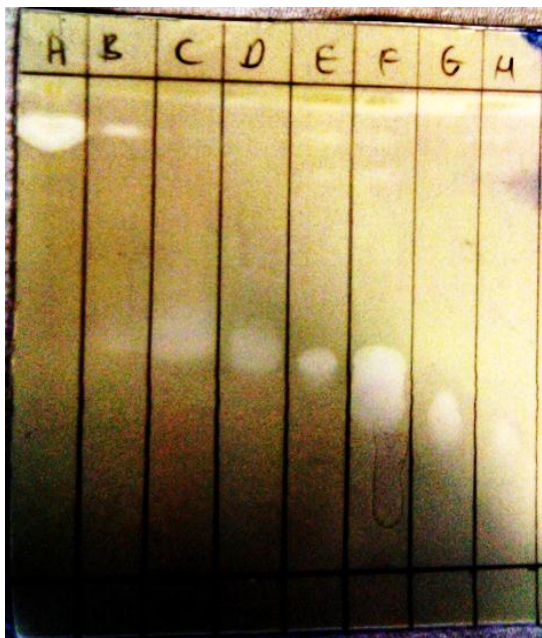
Figure L9: Bioautography assay against *P. aeruginosa* (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Figure L10: Bioautography assay against *S. aureus* 1 (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

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Figure L11: Bioautography assay against *S. aureus* 5 (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Figure L12: Bioautography assay against *S. enterica* 4C (DRW isolate) – Lane A: Fraction A; Lane B: Fraction B; Lane C: Fraction C; Lane D: Fraction D; Lane E: Fraction E; Lane F: Fraction F; Lane G: Fraction G; Lane H: Fraction H.

Appendix M

Determining the molecular weight of proteins partially purified using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) assay
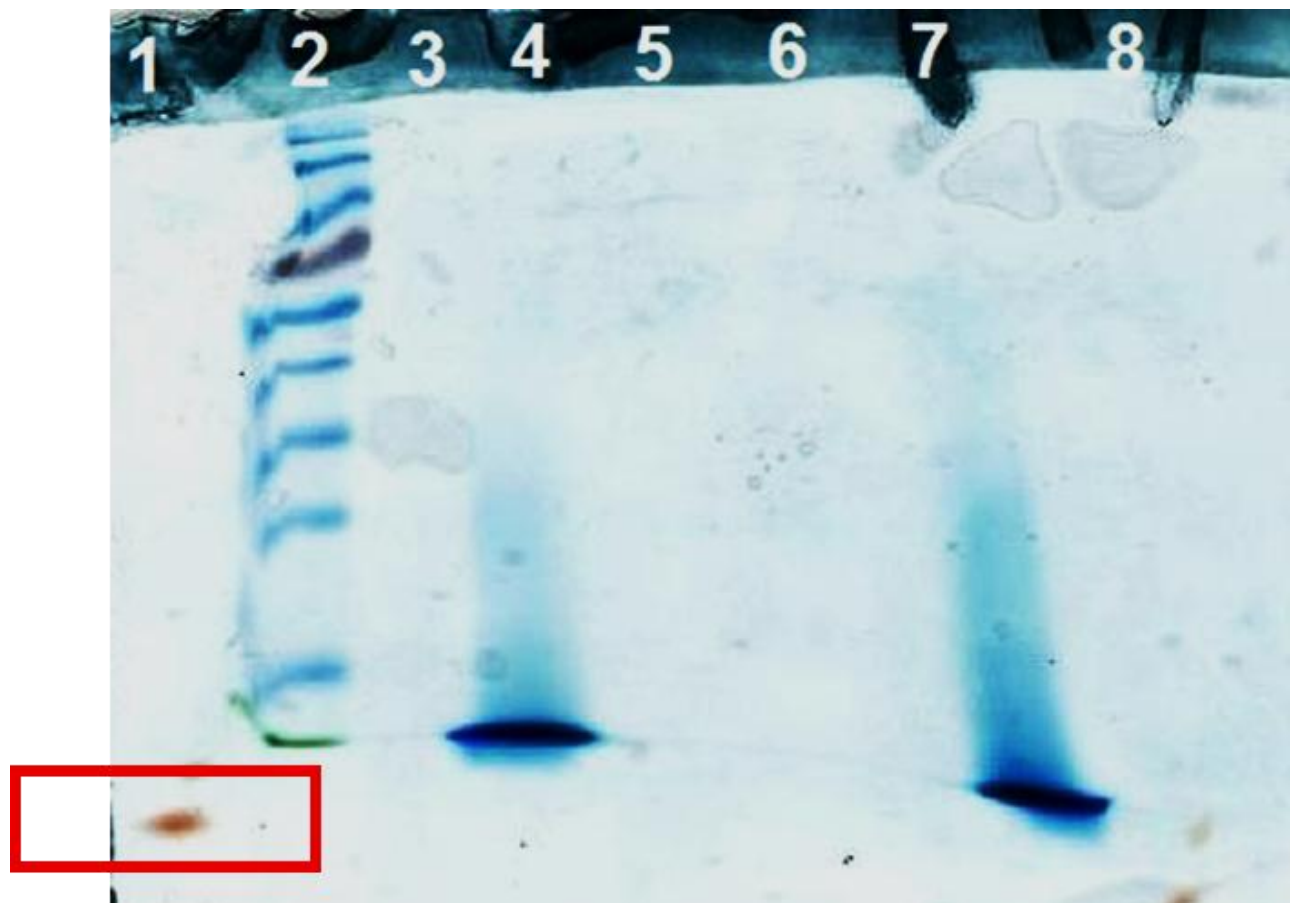
Figure M1: SDS-PAGE on partially purified extracellularly produced compounds by CZA14[T]. Lane 1: Unknown compound of interest - Pellet in 20 mM Tris-HCl after A/S precipitation, dialysis, and extraction with 2:3 v/v ethyl acetate (EtOAc) and reconstituted with 20 mM Tris-HCl to 50 x concentrate**;** Lane 4: Crude SNF after A/S precipitation; Lane 7: Dialysate.
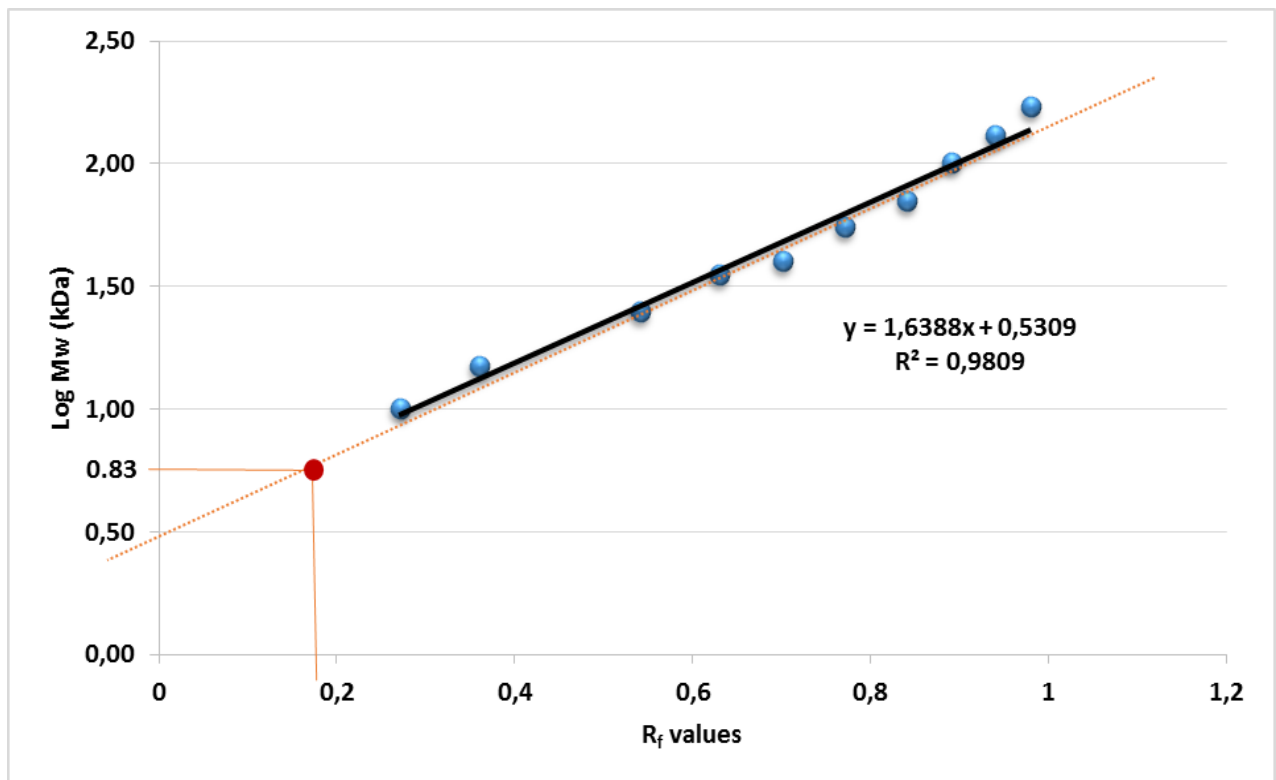
Figure M2: Line graph of standard curve of log Mw (kDa) of standards vs R_f values measured of standards. Extrapolation of graph to determine Mw of unknown compound of interest (red dot).

Bioassays

Filter disk diffusion assay on purified EtOAc extracts

Figure M3 Filter disk diffusion assay of 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc against DRWH isolate *S. enterica* 4C.

Bioautography assay to determine bio-active metabolites on purified EtOAc extracts

Figure M4: Bioautography assay against *S. aureus* 4.	**Figure M5:** Bioautography assay against *E. faecalis* C513	**Figure M6:** Bioautography assay against *K. pneumonia* 1	**Figure M7:** Bioautography assay against *P. aeruginosa*	**Figure M8:** Bioautography assay against *S. aureus* 1
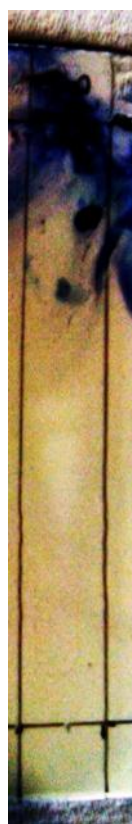
Appendix N

Spectroscopic measurements
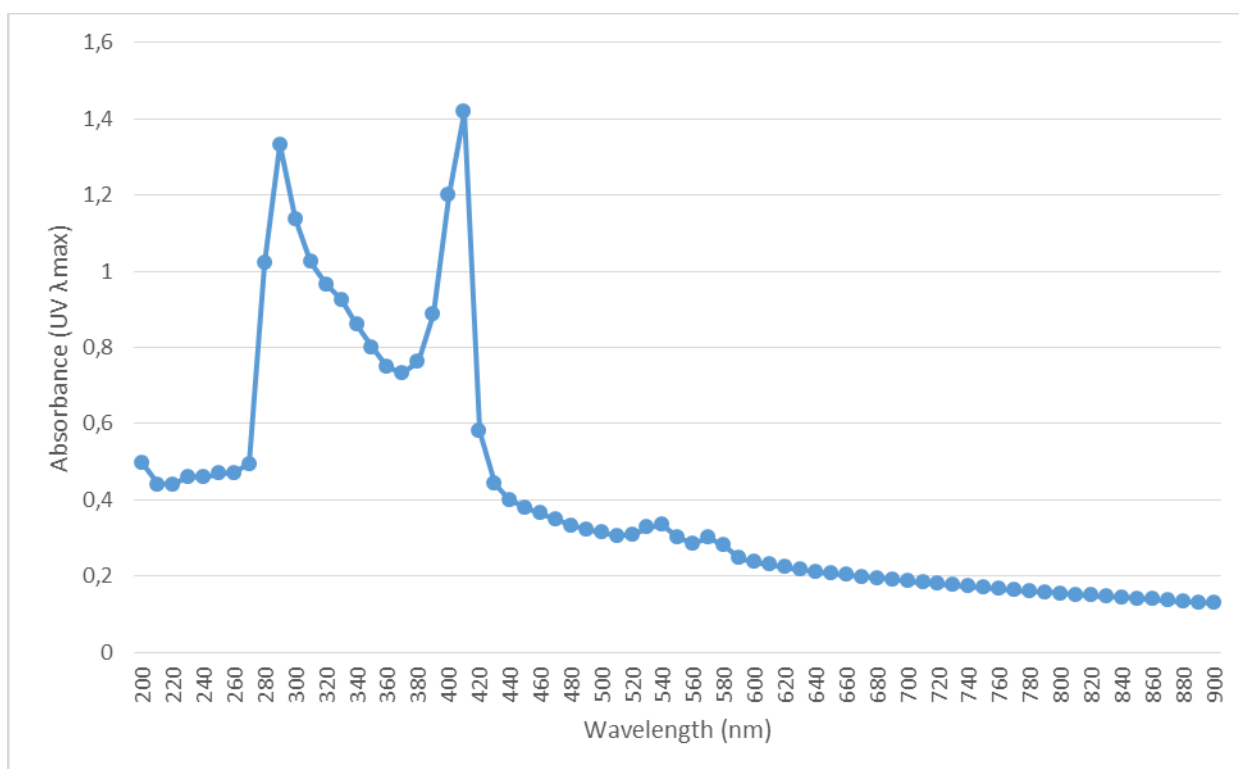
Figure N1: UV absorption spectrum of extracellularly produced crude compounds
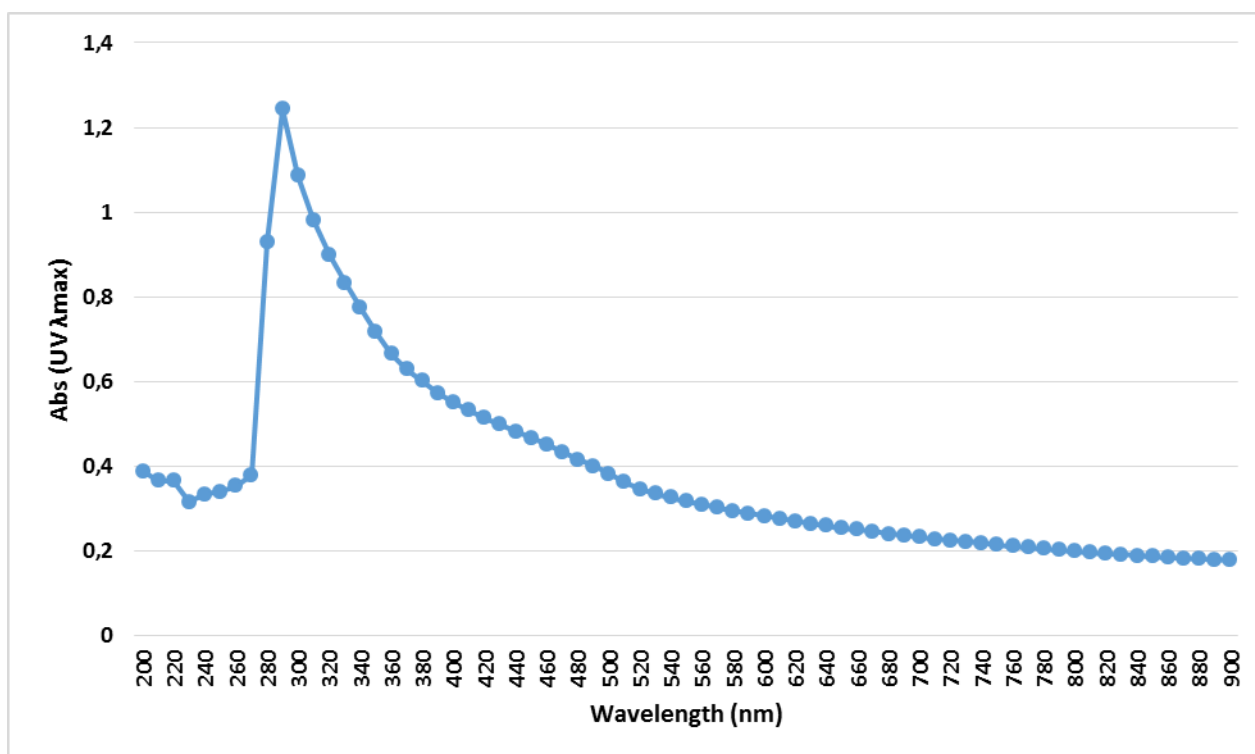
Figure N2: UV absorption spectrum of intracellularly produced crude compounds

Appendix O
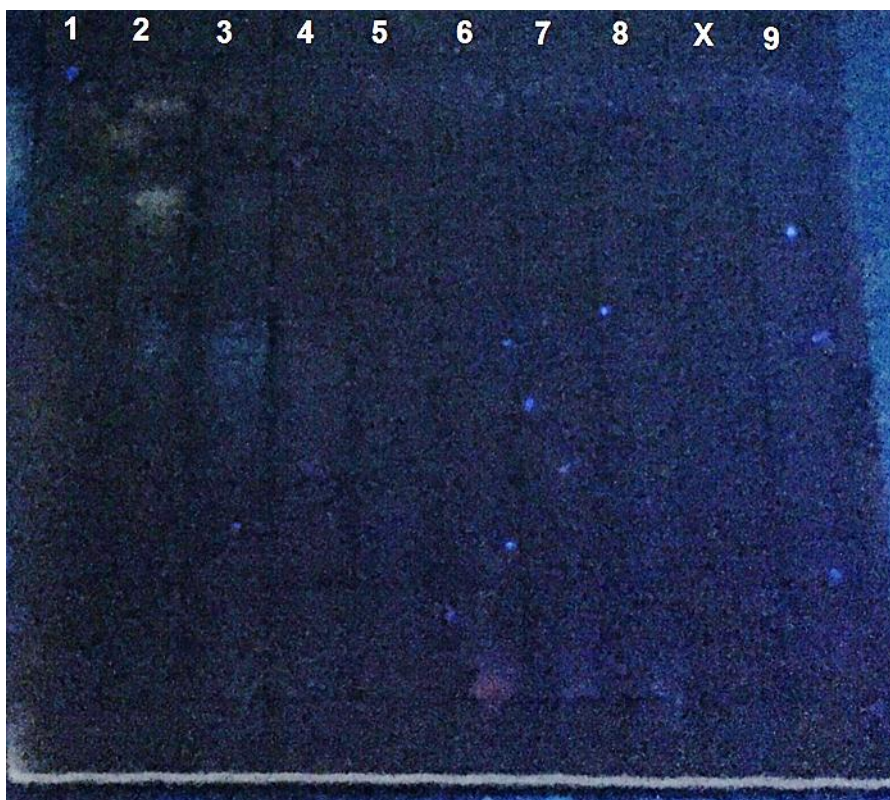
Ultra Violet (UV) light
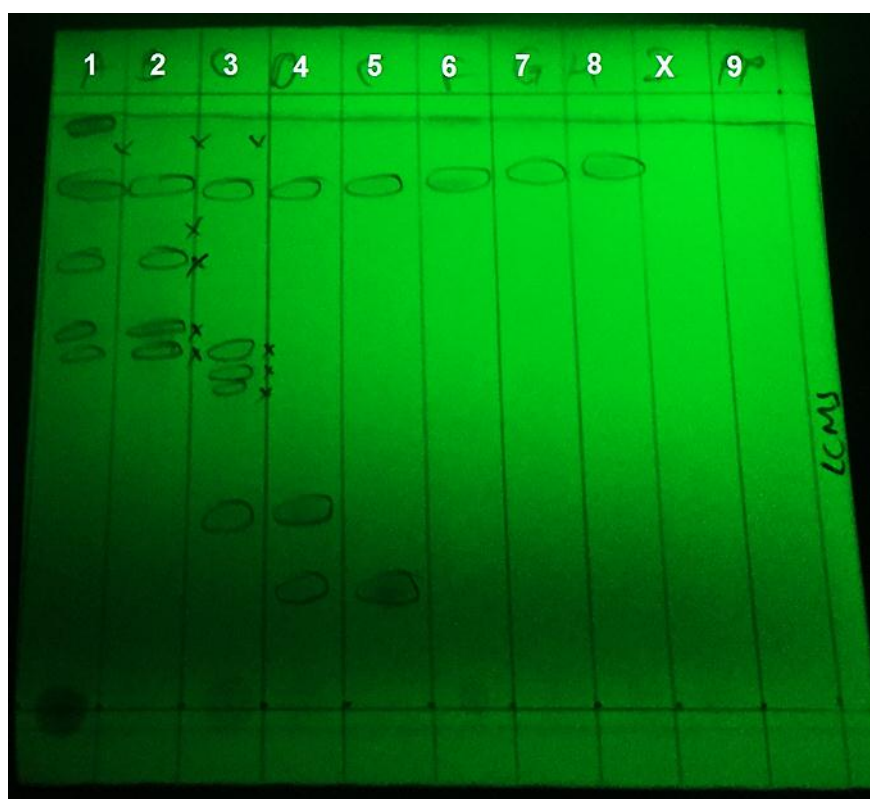
Figure O1: Developed TLC visualised under short wave 254 nm ultraviolet light. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane 1: fraction A; Lane 2: fraction B; Lane 3: fraction C; Lane 4: fraction D; Lane 5: fraction E; Lane 6: fraction F; Lane 7: fraction G; Lane 8: fraction H; Lane 9: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc

Figure O2: Developed TLC visualised under long wave 365 nm ultraviolet light. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane 1: fraction A; Lane 2: fraction B; Lane 3: fraction C; Lane 4: fraction D; Lane 5: fraction E; Lane 6: fraction F; Lane 7: fraction G; Lane 8: fraction H; Lane 9: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc
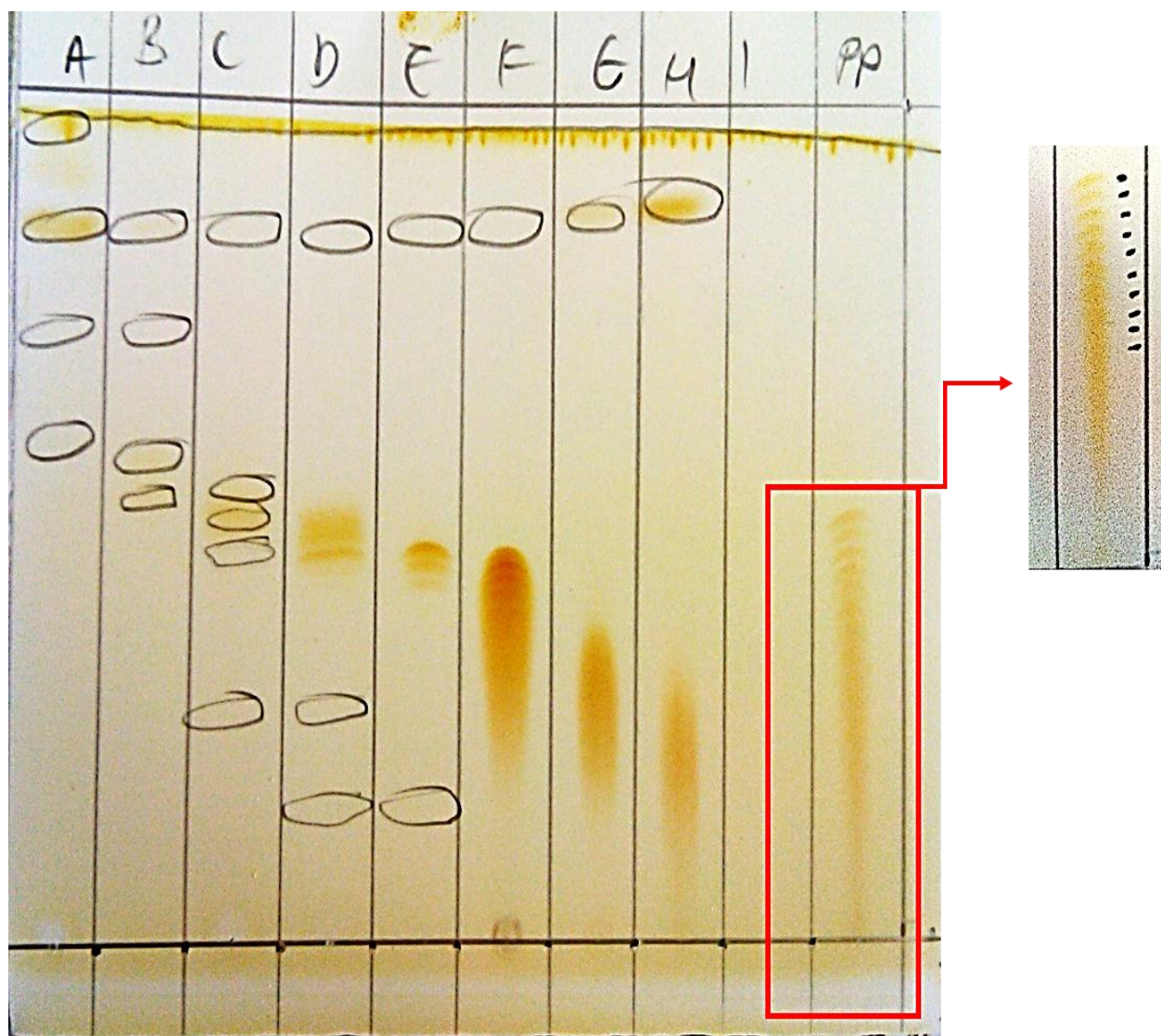
Iodine stain

Figure O3: Developed TLC visualised after staining with iodine. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane A: fraction A; Lane B: fraction B; Lane C: fraction C; Lane D: fraction D; Lane E: fraction E; Lane F: fraction F; Lane G: fraction G; Lane H: fraction H; Lane PP: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc.
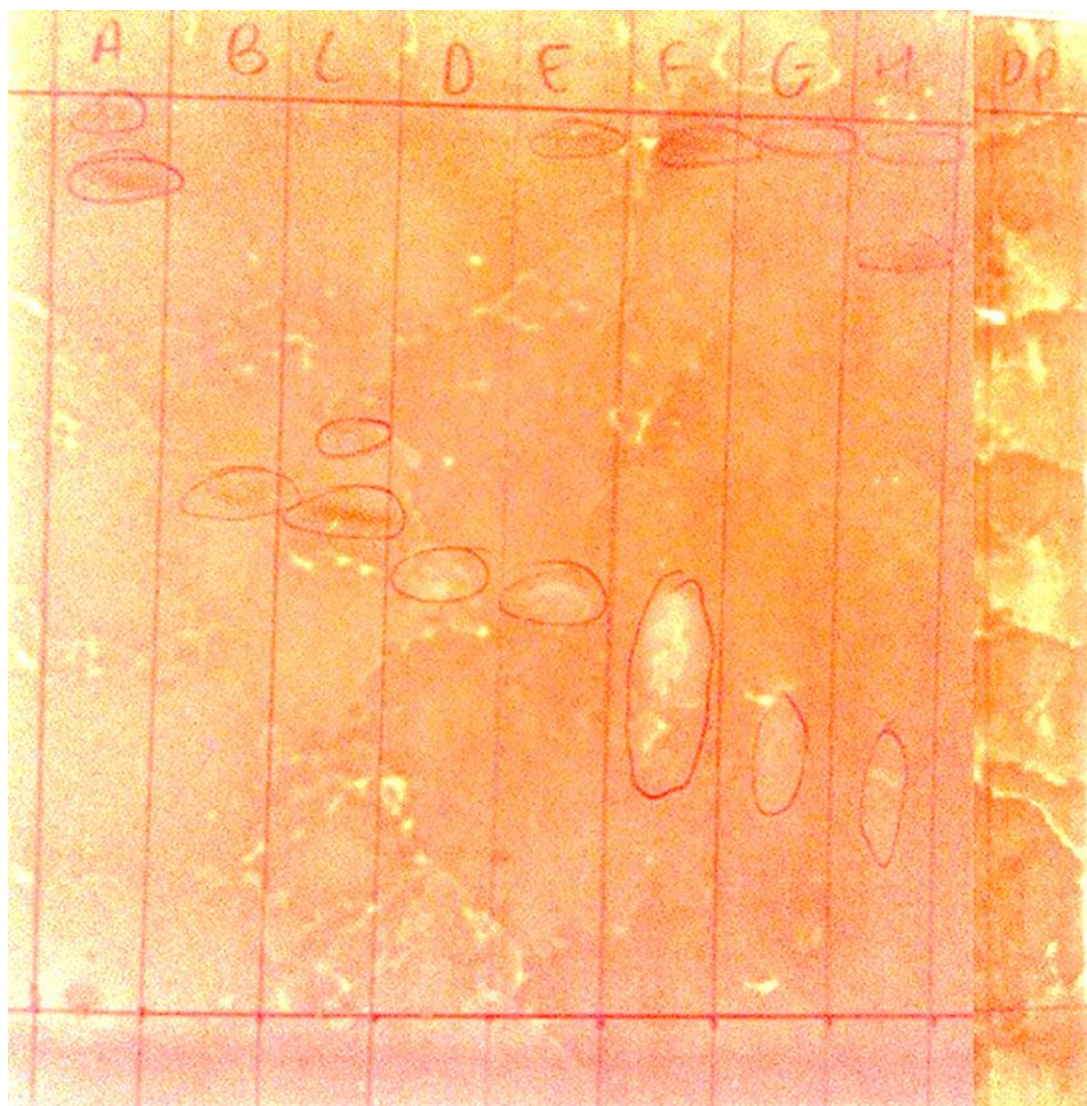
ρ – anisaldehyde – sulphuric acid stain

Figure O4: Developed TLC visualised after staining with ρ – anisaldehyde. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane A: fraction A; Lane B: fraction B; Lane C: fraction C; Lane D: fraction D; Lane E: fraction E; Lane F: fraction F; Lane G: fraction G; Lane H: fraction H; Lane PP: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc.
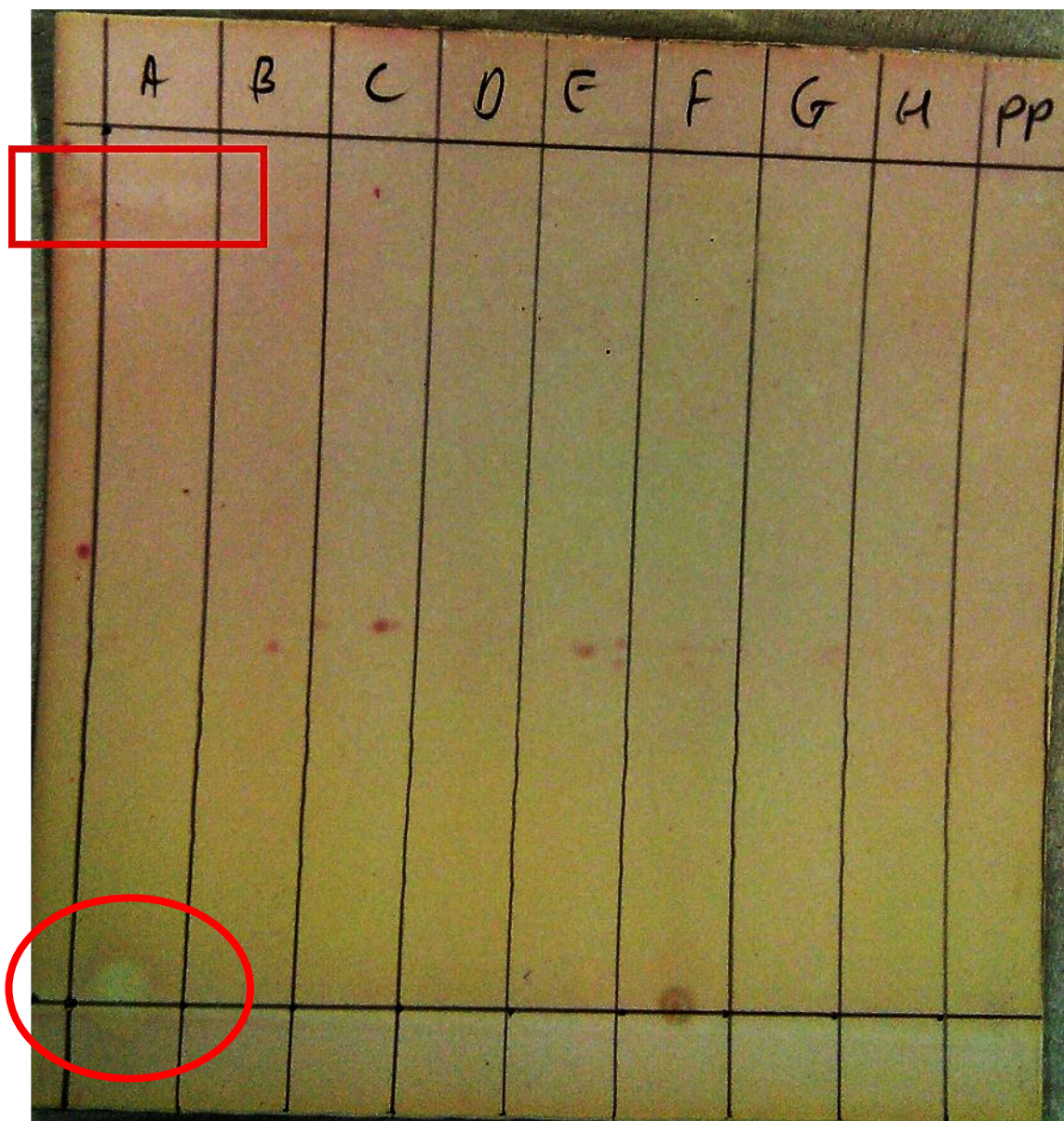
Ninhydrin stain

Figure O5: Developed TLC visualised after staining with ninhydrin. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane A: fraction A; Lane B: fraction B; Lane C: fraction C; Lane D: fraction D; Lane E: fraction E; Lane F: fraction F; Lane G: fraction G; Lane H: fraction H; Lane PP: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc.
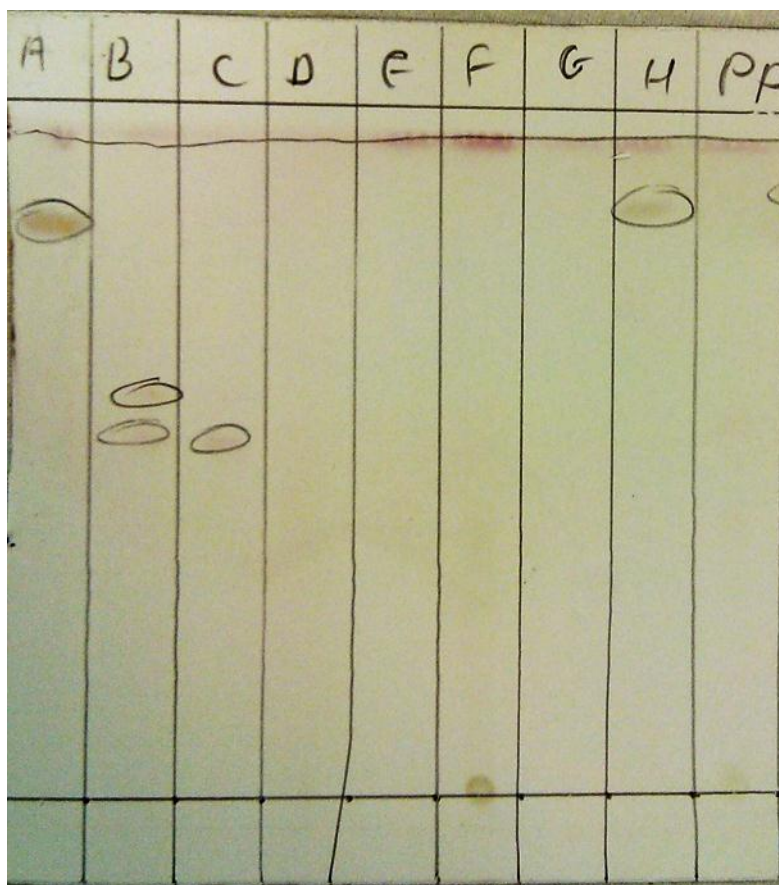
Vanillin-sulphuric acid stain

Figure O6: Developed TLC visualised after staining with vanillin-sulphuric acid. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane A: fraction A; Lane B: fraction B; Lane C: fraction C; Lane D: fraction D; Lane E: fraction E; Lane F: fraction F; Lane G: fraction G; Lane H: fraction H; Lane PP: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc.
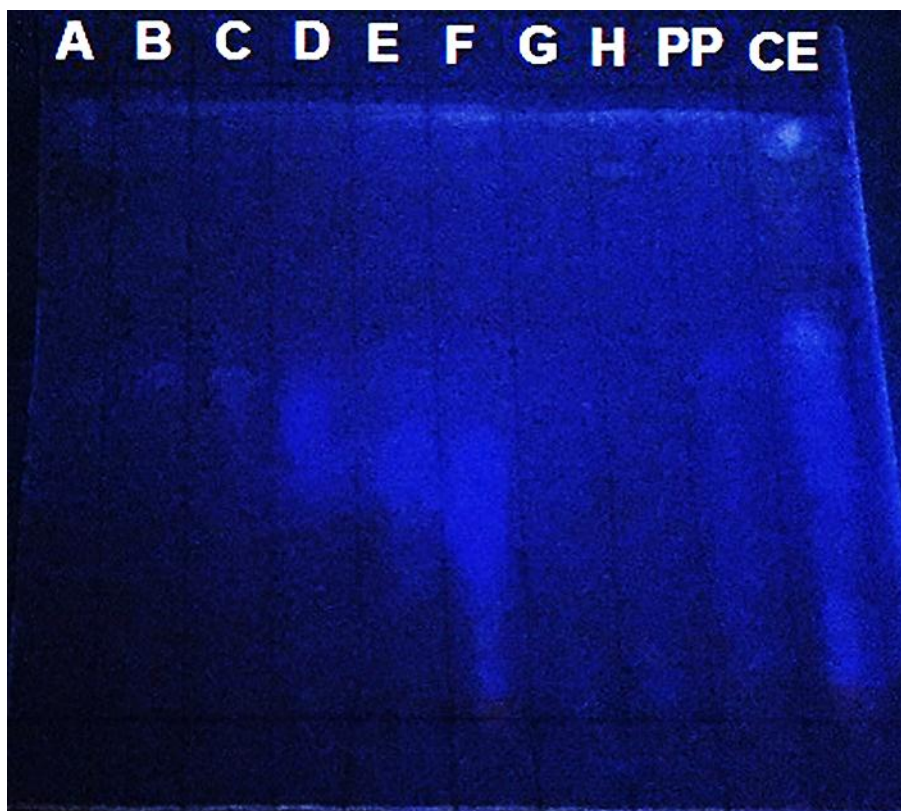
Figure O7: Developed TLC visualised after staining with vanillin-sulphuric acid under 365nm long-wave UV light. Ethyl acetate extracts were separated using a solvent system consisting of chloroform: formic acid: ethyl acetate: n-hexane (5:4:1:0.02 v/v/v/v). Partial purification of pooled crude extra- and intra- cellularly (SNF and cell mass, respectively) produced compounds using silica gel column chromatography. Lane A: fraction A; Lane B: fraction B; Lane C: fraction C; Lane D: fraction D; Lane E: fraction E; Lane F: fraction F; Lane G: fraction G; Lane H: fraction H; Lane PP: 50 x concentrated EtOAc extract extracted from pellet in 20 mM Tris-HCl after A/S precipitation, dialysis and extraction with 2:3 v/v EtOAc; Lane CE: crude extracellularly produced (SNF) EtOAc extract.

Appendix p

Table P1: Biosynthetic potential of *S. pharetrae* CZA14[T] determined by AntiSMASH

	Biosynthetic gene cluster	Contig/Cluster	Predicted products	% genes showing similarity		Biosynthetic gene cluster	Contig/Cluster	Predicted products	% genes showing similarity		Biosynthetic gene cluster	Contig/Cluster	Predicted products	% genes showing similarity
1	Siderophore	5/1	Desferrioxamine B	83	21	Terpene	180/21	Unknown	-	41	NRPS	400/41	Unknown	-
2	NRPS	11/2	Friulimicin	54	22	Terpene	184/22	Hopene	53	42	NRPS	408/42	Unknown	-
3	Lassopeptide	20/3	Borrelidin	4	23	NRPS	195/23	Zorbamycin	8	43	NRPS	413/43	Unknown	-
4	Terpene	32/4	Albaflavenone	100	24	PKS-II	196/24	Curamycin	100	44	NRPS	419/44	Unknown	-
5	NRPS	33/5	SF2575	4	25	Siderophore	201/25	Unknown	-	45	OtherKS-PKS-I	421/45	Unknown	-
6	Siderophore	36/6	Unknown	-	26	Terpene	211/26	Isorenieratene	42	46	Bacteriocin	435/46	Unknown	-
7	Bacteriocin	48/7	Unknown	-	27	PKS-III	221/27	Azinomycin B	6	47	NRPS	438/47	Unknown	-
8	Other	53/8	Unknown	-	28	Ectoine	222/28	Kosinostatin	18	48	NRPS	462/48	Unknown	-
9	Amglyccycl-NRPS	54/9	Unknown	-	29	NRPS	232/29	Quinomycin	11	49	NRPS	465/49	Unknown	-
10	Terpene	59/10	Unknown	-	30	Other	236/30	Unknown	-	50	Terpene	518/50	Carotenoid	27
11	Butyrolactone	62/11	Unknown	-	31	Melanin	269/31	Melanin	42	51	NRPS	523/51	Mannopeptimycin	7
12	Bacteriocin	65/12	Unknown	-	32	Bacteriocin	302/32	Unknown	-	52	NRPS	530/52	Unknown	-
13	Butyrolactone	88/13	Neocarzinostatin	4	33	NRPS	306/33	Antimycin	20	53	NRPS	534/53	Unknown	-
14	Butyrolactone-PKS-II	96/14	Kosinostatin	14	34	NRPS	328/34	Unknown	-	54	NRPS	542/54	Unknown	-
15	Ectoine	104/15	Ectoine	100	35	PKS-I	336/35	Antimycin	13	55	NRPS	556/55	Unknown	-
16	PKS-I	116/16	Divergolide	13	36	OtherKS	344/36	Unknown	-					
17	Melanin	138/17	Melanin	80	37	Lantipeptide	372/37	Unknown	-					
18	PKS-II	149/18	Xantholipin	12	38	NRPS	376/38	Althiomycin	33					
19	NRPS	159/19	SW-163	14	39	NRPS	389/39	Unknown						
20	Lassopeptide	172/20	Laspartomycin	20	40	NRPS	399/40							

Appendix Q

Direct bioassay of native-PAGE gels against DRWH isolate - *Staphylococcus aureus* 4

10 % resolving gel (see Appendix 5A) with a 4 % stacking gel was used during native-PAGE. Native-PAGE is a non-denaturing gel electrophoresis which involves the absence of SDS in the gel and dithriothreitol (DTT) in the loading buffer, no boiling of the sample is performed and no SDS is added to the electrode/running buffer as the aim of a native-PAGE assay is to retain the secondary structures of the proteins of interest. SDS-PAGE gels were also not included as SDS could have inhibitory effects against test strain which will render inconclusive results. The native-PAGE gel was directly assayed against DRWH isolate - *Staphylococcus aureus* 4 by placing the stained gel onto a petri dish containing Mueller Hinton agar (MHA) medium (g/L: 2 beef/meat extract, 17.5 casein hydrolysate, 1.5 starch and 17 bacteriological agar, pH 7.3) and overlaid with sterile 0.7 % nutrient broth sloppy agar inoculated with *S. aureus* 4. The number of µLs of test bacteria used per 6 ml sloppy agar overlay was such that when multiplying the number of µLs of bacterial culture used per sloppy agar by the OD_{600} of the broth culture approximate values of: 4 $OD_{600-\mu L}$ for *S. aureus* 4 was achieved. The sloppy agar was allowed to set, inverted, incubated overnight at optimal temperature (37 °C) and the zones of inhibition were recorded.

Bioassay was performed against DRWH isolate *S. aureus* 4 on the native-PAGE containing partially purified EtOAc and 20 mM Tris-HCl extracts, respectively. Lanes 1 and 2 contains the EtOAc extracted 20 mM Tris-HCl resuspended pellet after A/S precipitation and dialysis. The A/S (80 %) pellet resuspended in 20 mM Tris-HCl and dialysate was loaded into lanes 3 and 4, respectively (Figure 8.98). These samples were included in the assay to determine whether the active compound was in the EtOAc extract or in the partially purified 20 mM Tris-HCl samples. Results of the compound of interest and the inhibitory effects of the compound against *S. aureus* 4 are presented in Figures 8.98 and 8.99, respectively.
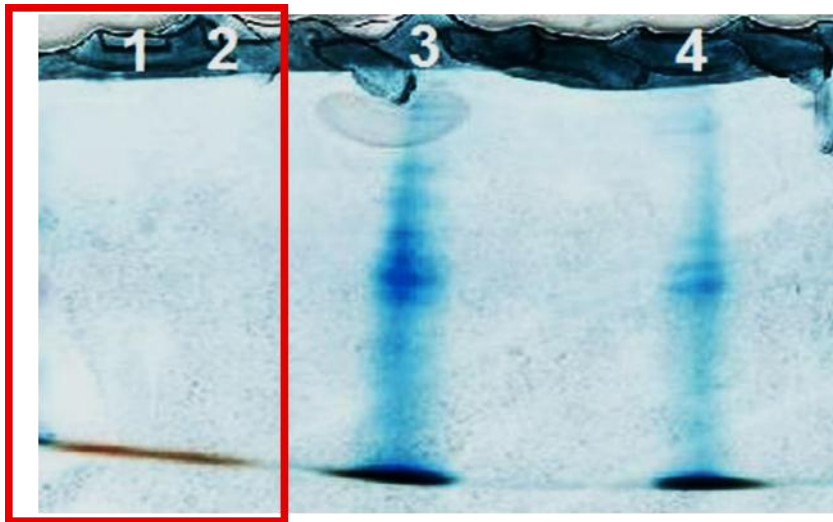
Figure Q1: Coomassie stained native-PAGE on CZA14^T partially purified samples. Lane 1 and 2 - Pellet in 20 mM Tris-HCl after A/S precipitation, dialysis, and extraction with 2:3 v/v ethyl acetate (EtOAc) and reconstituted with 20 mM Tris-HCl to 50 x concentrate. Lane 3 - A/S (80 %) pellet resuspended in 20 mM Tris-HCl. Lane 4 – dialysate.
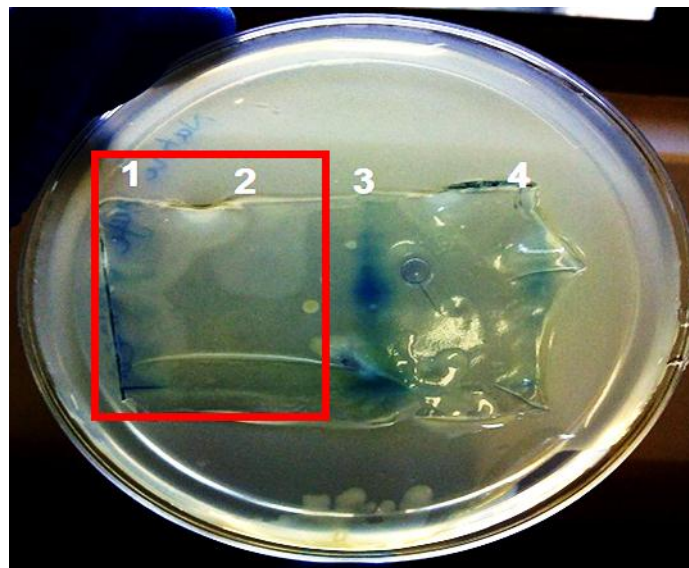

Figure Q2: Direct assay of Coomassie stained native-PAGE on CZA14^T partially purified samples against DRWH isolate S. aureus 4. . Lane 1 and 2 - Pellet in 20 mM Tris-HCl after A/S precipitation, dialysis, and extraction with 2:3 v/v ethyl acetate (EtOAc) and reconstituted with 20 mM Tris-HCl to 50 x concentrate. Lane 3 - A/S (80 %) pellet resuspended in 20 mM Tris-HCl. Lane 4 – dialysate.

The 6.7 kDa protein/s of interest were subjected to direct bioassay by overlaying the native-PAGE gel that contained the unknown protein with sloppy agar containing DRWH isolate *S. aureus* 4 to determine whether the compound of interest was active. A large zone of inhibition was detected for the first replicate around the entire area from the wells to where the compounds migrated within the gel during resolving via electrophoresis. However, a zone of inhibition only occurred around the wells of the gel for the duplicate sample. The sample may not have migrated sufficiently. No activity occurred against the 20 mM Tris-HCl control samples. The active compounds were thus only in the EtOAc solvent extracted compounds after salt precipitation and dialysis, reconstituted in 20 mM Tris-HCl. The test should be repeated as clear activity was only detected in one of the replicates.

Appendix R

Polymerase chain reaction (PCR) screening for the biosynthetic genes involved in the production of aromatic Type-II polyketide synthases (PKS-II)
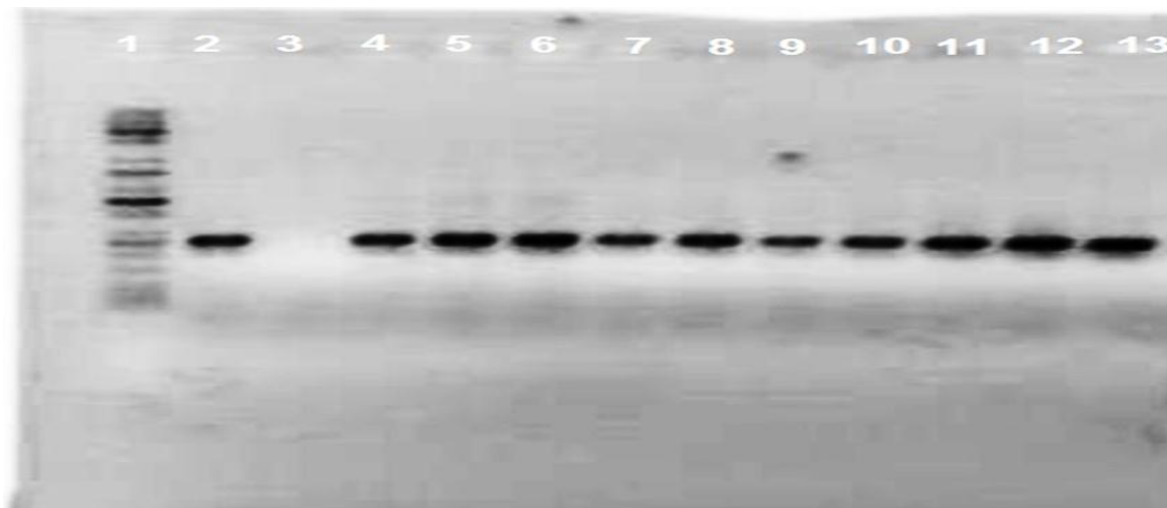

Figure R1: 1 % TBE agarose gel electrophoretogram of PCR amplification of type-II PKS gene of gDNA products isolated from *S. pharetrae* sp. strain CZA14[T]. Lane 1: 1kb Kapa Biosystems universal molecular weight marker, Lanes 2 - 13: Gradient PCR amplification of gDNA for the genes involved in the synthesis of type-II PKS.

Figure R2: 1 % TAE agarose gel electrophoretogram of PCR amplification of RNA and DNA samples with universal 16S rRNA primers. Lane 1 and 2: RNA extracted from samples taken at day 0; Lane 3 and 4: RNA extracted from samples taken at day 3; Lane 5 and 6: RNA extracted from samples taken at day 6; Lane 7 and 8: RNA extracted from samples taken at day 9; Lane 9 and 10: RNA extracted from samples taken at day 12; Lane 12: Control containing RNA control; Lane 13: Control containing water; Lane 15 and 16: DNA positive control isolated from *Streptomyces polyantibioticus* sp. nov., Lane 18: 1kb Kapa Biosystems universal molecular weight marker.
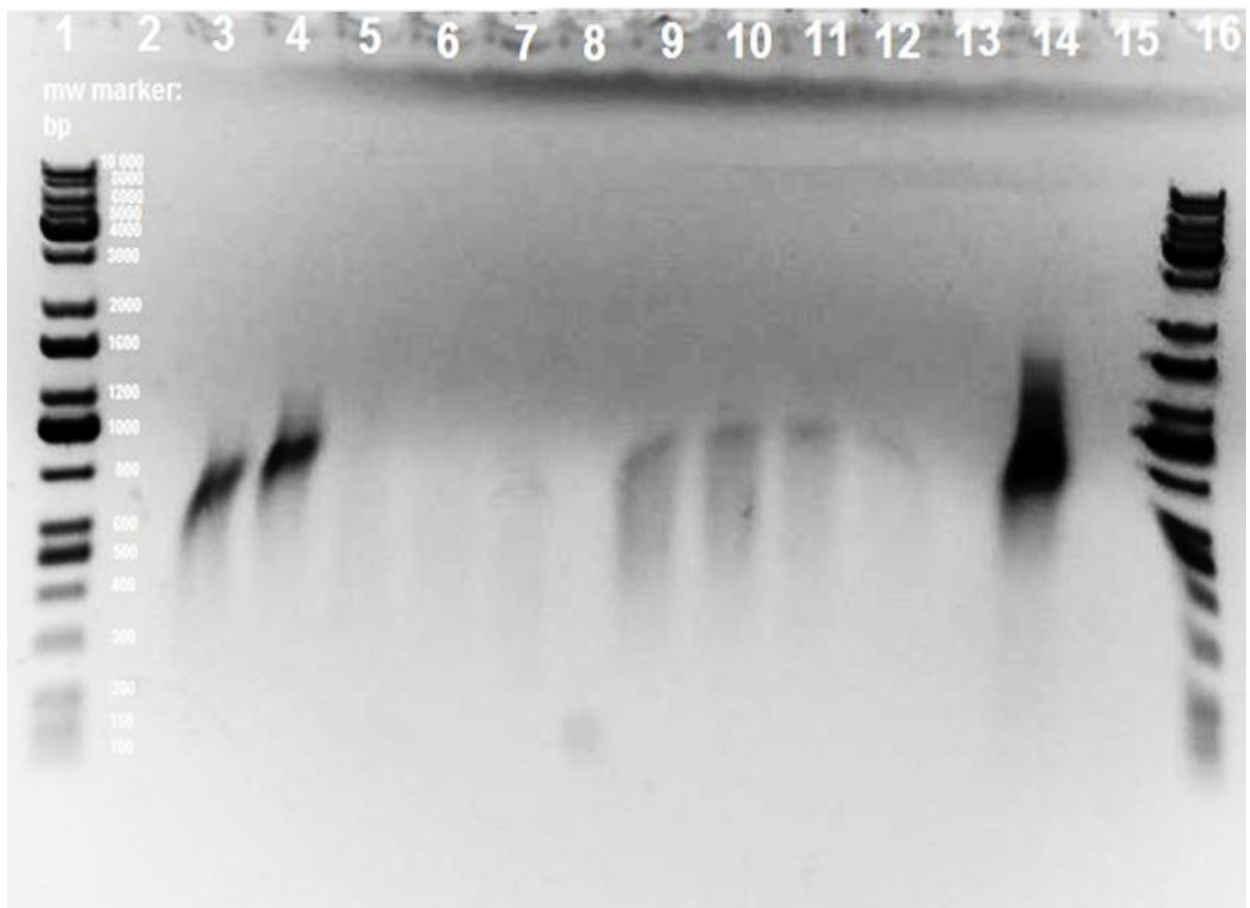
Figure R3: 1 % TBE agarose gel electrophoretogram of cDNA converted from RNA extracted from fermentation samples taken at different time periods from an airlift bioreactor. Lanes 1 and 16: 1kb Kapa Biosystems universal molecular weight marker, Lanes 3 and 4: cDNA of samples taken at day 0, Lanes 5 and 6: cDNA of samples taken at day 3; Lanes 7 and 8: cDNA of samples taken at day 6; Lanes 9 and 10: cDNA of samples taken at day 9; Lanes 11 and 12: cDNA of samples taken at day 12; Lane 13: Control containing RNA control; Lane 14: Control containing water.